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Intestinal Microbiota in IgE-mediated Cow's Milk Allergy: microbial dysbiosis and possible modulation through probiotics

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INTRODUCTION

Epidemiology of food allergy in pediatrics

In the last 50 years, and particularly since 1990, there has been a significant increase in cases of asthma, eczema and allergic rhinitis (1). This global epidemic of pathologies with atopic etiopathogenesis has caused an increase in public spending for affected individuals, their families and health resources (2–4). Recent studies confirm the emergence of a further epidemic "wave", with particular involvement of food allergies (5–7). To confirm these data, a recent meta-analysis of 42 studies states that in Europe the prevalence of food allergy varies from 0.1 to 6%. In particular, the prevalence of food allergy proven by oral food challenge (OFC) for cow's milk proteins, egg, wheat, soy, peanuts, nuts, fish and shellfish is respectively: 0.6%, 0, 2%, 0.1%, 0.3%, 0.2%, 0.5%, 0.1%, and 0.1%. In the same study, however, a profound discrepancy emerges (up to 15 times) between the prevalence of self-reported food allergy cases and those proven by OFC (6).

The issue of self-reported food allergy is investigated by Branum et al. that have described the dramatic increase among American children in the period 1997-2007 with severe repercussions on the use of health resources: the number of outpatient visits due to suspected food allergy and the total number of hospital discharges codified as related disorders is in fact tripled food allergy goes from an average of 2600 to 9500 per year (8).

It is also very interesting to note the significant epidemiological differences that exist at the geographical level.

The most common foods which induce IgE mediated allergy in Japan are egg (38.2%), cow's milk (15.9%), wheat (8%), shellfish (6%), fruit (5%), fish (4%), peanut (3%) and fish eggs (3%). The distribution in United States is different: peanut (25.2%), cow's milk (21.1%), crustaceans (17.2%), hazelnut (13.1%), egg (9.8%), fish (6.2%), strawberry (5.3%) and wheat (5.0%). In European patients, the foods most involved in decreasing order of frequency are cow milk, wheat, egg, fish, soy, hazelnut, crustaceans, and peanut (6, 9-11).

This data therefore places cow's milk at the center of the objectives of modern Italian research for the resolution of a constantly increasing problem.

Epidemiology allergy to cow's milk proteins

The real prevalence of cow’s milk proteins allergy (CMA) in children is difficult to determine due to the profound heterogeneity in clinical presentation and the not always correct application of diagnostic criteria (6). Previously, several population studies have defined the presence of CMA through the presence of suggestive anamnestic data, the positivity of specific sensitization tests and / or oral food challenge (12 - 16). Through the application of similar criteria, there are numerous cases of CMA characterized by blurred IgE-mediated reactions that escape the real diagnosis. To all this, is added the lack of numerous data from Eastern and Southern Europe.

In order to evaluate the cumulative incidence, prevalence patterns and natural history of food allergy in early childhood, the EuroPrevall study was carried out. This cohort study used a double-blind placebo oral challenge test and used a standardized method to describe the diagnosis and follow-up of allergy in European countries with different climatic conditions in the most reliable way.
(17, 18).

This research described an incidence of CMA of 0.54% in the first two years of life (0-1.3%; the lowest incidence in southern and eastern countries).

The authors speculate on the variability of the incidence assuming possible influences of the maternal diet and of nutritional, epigenetic and environmental factors.

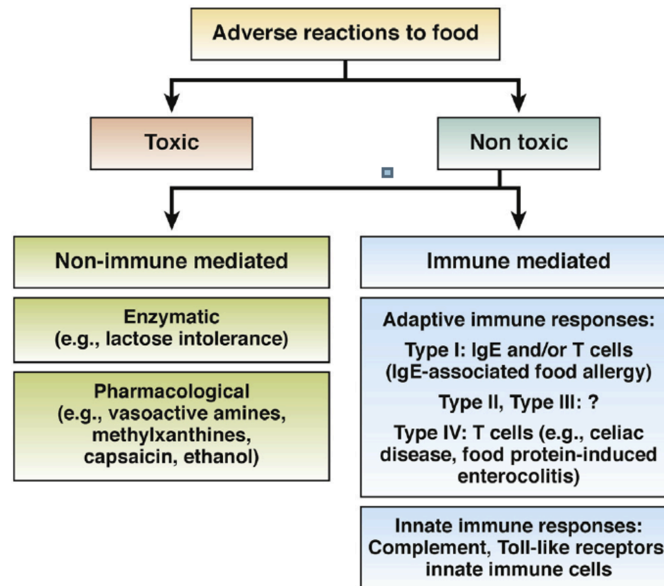
In this study, allergy was expressed in 84% of cases with dermatological manifestations, in 20% with respiratory and in 18% with gastroenterological symptoms. During follow-up, 69% of patients achieved tolerance to cow's milk proteins.

In this study, Italy showed an average incidence of IgE-mediated CMA of 0.29%.

Physiopathology of allergy to cow's milk proteins

The term "allergy" was coined in 1906 by the Australian pediatrician Clemens von Piquet who described cases of serum diseases treated with solutions containing antibodies. This terminology

appears today always used with less appropriateness by patients and sometimes by the clinicians themselves who lose themselves in determining the food causal agent of non-specific disorders (19). Valenta R et al. in order to schematize the types of adverse reactions to food, carry out the following scheme (20):



Reactions to foods are therefore divided into toxic or non-toxic reactions.

Non-toxic reactions can in turn be classified into:

a) Non-immune-mediated reactions: the most frequent reactions and generally caused by enzymatic defects.

Example: Lactose intolerance.

b) Immuno-mediated reactions

In general, according to Coombs and Gell, there are 4 types of immune-mediated hypersensitivity reactions (21). The most common immune-mediated adverse reaction to foods (type I reactions) is the form which involves the production of IgE antibodies directed against protein fractions contained in the food itself: this reaction can be accompanied by cell-mediated inflammation by T lymphocytes and eosinophils and can be identified by the presence of IgE in the patient's blood against specific food.

Type II and III reactions are also defined respectively: cytotoxic, if the antibodies interact with the antigens present on the cell surface, directly damaging the cells and making them susceptible to phagocytosis; mediated by immunocomplexes, where the antigen-antibody bond forms a complex that activates the complement system. There is not enough scientific evidence to show that these types of reactions can occur due to food.

In type IV or cell-mediated reactions, tissue damage is produced by activation of cells in the immune system (T lymphocytes). This type of reaction in the context of food-related diseases is described in the pathogenesis of Celiac Disease and in food-induced protein (FPIE) Enterocolitis.

Some studies have shown that some food proteins are able to induce direct activation of elements of innate immunity. For example, trypsin and amylase inhibitors contained in wheat and some oligosaccharides present in milk are able to activate Toll-like receptor 4 and other food allergens are able to directly stimulate the action of innate immunity (22-25).

IgE-mediated food allergies can constitute a predisposing substrate for serious and dangerous reactions for the survival of the individual. They are the only hypersensitivity reactions that can really be defined as "food allergies" and manifest themselves through immediate and sensational reactions after ingesting the food.

Their correct diagnosis can therefore have important repercussions on patients' health.

In general, milk, egg and wheat allergies tend to be overcome by the individual after evolutionary times, while those with fish, nuts and peanuts tend to persist.

To be able to verify a real allergic reaction to a food it is necessary that the phenomenon of "allergic sensitization", or a first contact with the allergen itself that causes the induction of an allergic immune response, must occur.

Two possible ways of raising awareness have been identified:

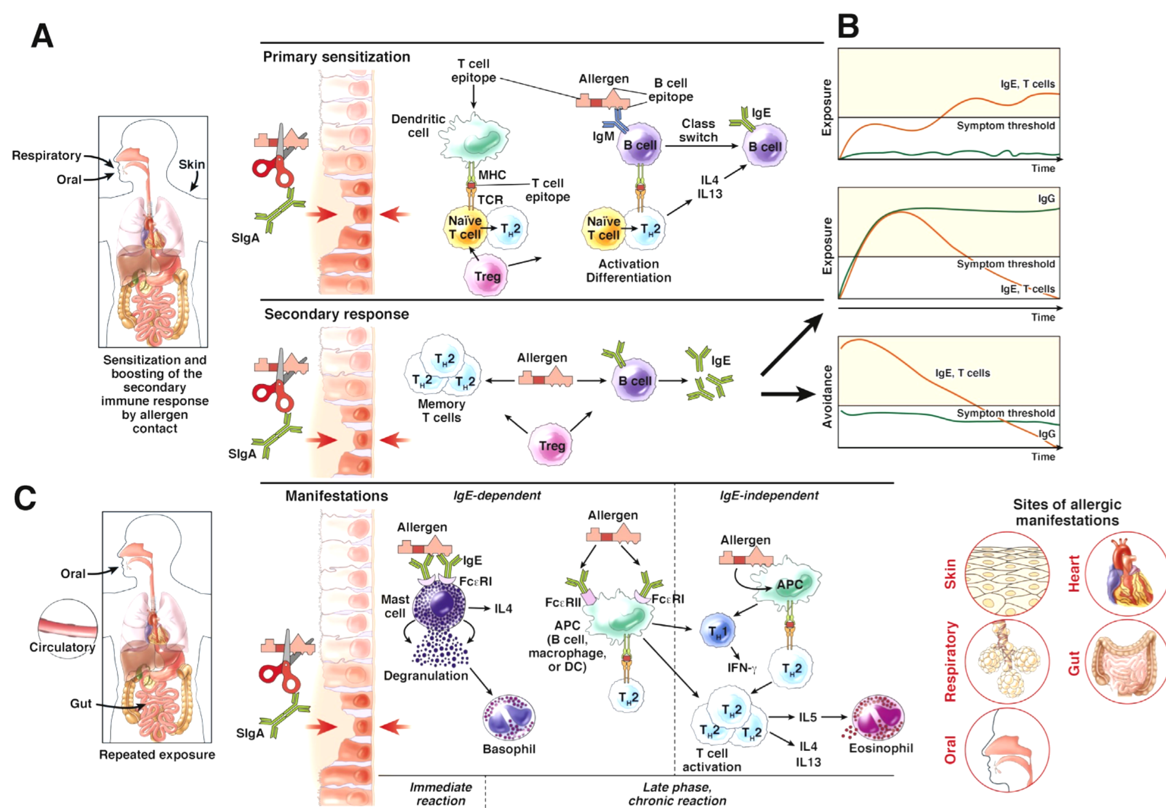
1) Class 1 food allergens (for example, milk, egg or peanuts) are oral allergens that cause sensitization through the passage of the allergen in the digestive tract and direct contact with the mucosa;

2) Class 2 allergens, represented by aeroallergens

which cause sensitization through transit in the respiratory tract. Immune responses against these allergens can cause cross-reactions against food allergens (for example, Bet v1 birch pollen allergen can induce a reaction against the protein fraction of the apple allergen, Mal d1).

Recently, other awareness models have also been proposed, including the "contact" model.

The latter gave suggestive cues on the possible intestinal mucosal production of specific IgE after simple epicutaneous contact with the allergen, but there are still no definitive confirmations.



Once the sensitization process has taken place, the next contact with the allergen produces IgE able to bind with high affinity to the FcεRI receptor present on the surface of mast cells and basophils. This link activates these cells and induces their degranulation with the release of inflammatory mediators (in particular histamine) and the synthesis of other pro-inflammatory molecules (leukotrienes), proteases (for example the tryptase), cytokines (IL-4 and IL -5) and chemotactic molecules. The activation of mast cells and basophils takes place in a few minutes after ingestion of the allergen and since the latter, after its processing, reaches the systemic circulation, local reactions

(mouth, esophagus, stomach and intestine) may occur, but also systemic. The type of reaction depends on the stability of the allergen after the digestion of the food that contains it, the amount of allergen taken and the integrity of the epithelial barrier itself.

The immediate inflammatory process can be very intense and can result through the release of vasoactive mediators, even cardiovascular collapse and shock.

In addition to immediate reactions, late allergic reactions are described.

In fact, several hours after contact with the allergen there is a noticeable release of basophils and eosinophils (26). The consequences of this phenomenon are not yet fully understood. Some studies describe its involvement in the pathogenesis of eosinophilic esophagitis and more generally in eosinophilic enteritis. In addition to the activity of basophils and eosinophils, the activation of the previously described type IV hypersensitivity mechanisms is described in the following 24-48 hours, which can be involved even without IgE-mediated mechanisms.

It is therefore possible to classify the type of symptomatology based on its characteristics:

Organ system	Clinical manifestations	Immunopathology	Features	Age and natural course
Skin	Urticaria, angioedema Flush, pruritus Oral allergy syndrome (local itching and tingling and/or edema of lips, tongue, palate and pharynx)	IgE-mediated mast cell/basophil degranulation	Acute onset after food ingestion (minutes-hours)	In infants and adults, may resolve with age Appears mainly in adults with established pollen allergies, long-lived, boosted by pollen contact
	Contact urticaria		After direct skin contact	In infants and adults
	Atopic dermatitis Protein contact dermatitis	T cell-mediated (with or without involvement of IgE)	Delayed type reaction > 24 hours after food ingestion	In infants and adults
Respiratory tract	Laryngeal and/or pharyngeal edema Hoarseness, cough	IgE-mediated mast cell/basophil degranulation	Acute onset after food ingestion (minutes-hours)	In infants and adults
	Rhinoconjunctivitis Bronchial asthma	T cell-mediated (with or without involvement of IgE)	Delayed type reaction > 24 hours after food ingestion	In infants and adults Baker's asthma in adults
Gastrointestinal tract	Colitis Diarrhea Gastroenteritis Anorexia Nausea, vomiting Abdominal pain Flatulence Abdominal distension	IgE-mediated mast cell/basophil degranulation	Acute onset after food ingestion (minutes-hours)	In infants and adults
	Gastroenteritis	T cell-mediated (with or without involvement of IgE)	Delayed type reaction > 24 hours after food ingestion, increased pro-inflammatory cytokine responses	In infants and adults, may resolve with age
	Eosinophilic gastroenteritis Eosinophilic esophagitis	Eosinophilic gastroenteritis	Eosinophil-activation by cytokines	In infants and adults
Cardiovascular system	Tachycardia Hypotension Vascular collapse Anaphylactic shock Cardiac dysrhythmia	IgE-mediated mast cell/basophil degranulation	Acute onset after food ingestion (minutes-hours)	In infants and adults
Nervous system	Irritability Anxiety Confusion Loss of consciousness	IgE-mediated mast cell/basophil degranulation? and/or T cell-mediated (with or without involvement of IgE)?	Acute onset after food ingestion (minutes-hours) and/or Delayed type reaction > 24 hours after food ingestion	In infants and adults

Diagnosis of cow's milk proteins IgE mediated allergy

According to the most recent guidelines of British Society for Allergy and Clinical Immunology (BSACI) allergy to cow's milk proteins develops mainly before 12 months of age and has a very variable prognosis (27).

The IgE symptomatic reactions mediated to the protein antigens of cow's milk are realized within 1 hour from the intake and can be summarized according to the following table:

Cutaneous
- Pruritus without skin lesions
- Urticaria
- Angio-oedema
- Atopic eczema exacerbation

Gastrointestinal
- Vomiting
- Diarrhoea
- Bloody stools
- Gastro-oesophageal reflux
- Abdominal pain

Respiratory
- Upper respiratory
  - Rhinitis
  - Nasal congestion
- Lower respiratory
  - Wheeze
  - Cough
  - Stridor
  - Difficulty breathing

Cardiovascular
- Anaphylaxis
- Hypotony
- Hypotension/shock
- Prostration

General
- Anaphylaxis
- Irritability
- Failure to thrive

Non-IgE mediated reactions occur instead within 72 hours of food intake and are generally represented by: gastro-oesophageal reflux, diarrhea, constipation and eczema.

In order to make a correct diagnosis of allergy to IgE-mediated cow's milk proteins, Skin Prick Tests (SPT) are performed following the discovery of suggestive clinical symptoms.

These consist in the application on the skin surface of some drops of purified allergen, and in the subsequent scratching of the skin with a disposable lancet in order to let the allergen penetrate through the more superficial layers of the skin. After waiting for about 15-20 minutes, the skin reaction obtained at each previously positioned allergen is evaluated. The reaction at the level of the individual

allergens tested is compared with a positive control (usually consisting of histamine) and a negative control (usually consisting of glycerine or saline solution).

In the event of a positive reaction it is possible to observe the appearance of a circumscribed area of skin edema (wheal), intensely itchy, surrounded by an erythema halo. The diameter of the wheal is measured by the clinician who performs the examination in order to determine the diagnostic value.

Although several guidelines propose diagnostic protocol, the double-blind randomized oral challenge test remains the gold standard of diagnosis:

The use of standardized and universally recognized diagnostic protocols allows specialists to significantly reduce diagnostic errors and to exploit diagnostic tools in an integrated manner.

Today the most recent guidelines for CMA diagnosis are those from European Academy of Allergy and Clinical Immunology and "The Diagnosis and Rationale for Action Against Cow's Milk Allergy (DRACMA)", issued by World Allergy Organization (WAO) using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology (28,29).

However, many aspects need to be clarified on the pathogenesis of food allergy and in particular of allergy to cow's milk proteins.

Microbiota

Microbiota are "ecological communities of commensal, symbiotic and pathogenic microorganisms" (30) found in and on all multicellular organisms studied to date from plants to animals. Microbiota includes bacteria, archaea, protists, fungi and viruses. Microbiota have been found to be crucial for immunologic, hormonal and metabolic homeostasis of their host.

The synonymous term microbiome describes either the collective genomes of the microorganisms that reside in an environmental niche or the microorganisms themselves (31-33).

The microbiome and host emerged during evolution as a synergistic unit from epigenetics and genetic characteristics, sometimes collectively referred to as a holobiont (34, 35).

Definition of dysbiosis

Our mucosal surfaces are colonized by complex communities of micro-organisms (microbiota), which are uniquely adapted to the different environmental niches in the human body.

This different microbiota is composed of distinct and specialized consortia of micro-organisms which are characteristic of the respective niche, for example, the mouth, the gastrointestinal or the genito-urinary tract. The microbiota on our mucosal surfaces and other anatomical locations in the body comprise the human microbiome.

An unfavorable alteration of the microbiota composition is called dysbiosis and it has become increasingly evident that this phenomenon, characterized by alterations to the normally balanced microbial populations at different sites of the human body may have profound effects of perturbation of this to the development of disease has been recognized for decades.

The host-associated microbiota changes throughout normal human development.

For example, the intestinal microbiota at birth is initially colonized by *Enterobacteria*. However, in the following days, strict anaerobes dominate the microbiota community. During the first month, bifidobacterial species predominate but then, following the introduction of solid foods, clostridial species expand. By 2-3 years, the microbiota consists mainly in Bacteroidacae, Lachnospiracae and Ruminococcacae, which then remain relatively stable to adulthood (36, 37).

Dietary fiber ingestion positively correlated with next-day abundance of clusters of bacteria representing over 15% of the total community.

Dysbiosis is now recognized as a change in the microbiota at a given site in the body, crucially accompanied by a breakdown of host-microbial mutualism.

It is characterized by:

1) Reduction in the overall microbial diversity of the corresponding symbiotic community: a reduction of taxonomic diversity and species membership of the microbiota has been observed in multiple studies of the human gut microbiota in disease as well as in animal infection models.

2) Depletion of obligate anaerobic bacteria such as Bacteroides and Ruminococcus spp. And conversely increase in facultative anaerobes, including the family of Enterobacteriaceae.
3) Reduced metabolic capacity, exemplified by a decline in short-chain fatty acid production, which may contribute to the impairment of host defenses and thereby promote the stability of a dysbiotic community.
4) Preferential loss of organisms considered beneficial to human health and a corresponding increase in pathobionts, members of the normal commensal microbiota with the potential to cause pathology.

Changes in the microbial population structure in dysbiosis are intimately correlated with disease. Correlations, however, do not equate to causality. It is feasible to argue that alterations to the inflammatory status of a site, triggered perhaps by an autoimmune reaction, will lead to the selection of those members of the microbial community best adapted to survive the increased inflammatory pressure and/or take maximal advantage of the changed nutritional environment presented by such an alteration. Bacteria less well adapted to the injurious properties of the innate response or ill equipped to compete metabolically in this new environment will diminish in their overall proportions in the population. In this scenario, the dysbiosis seems consequential, not causative (38, 39).

Importance of Microbial Exposure for the Development of Immune Tolerance

Immune tolerance is the state of unresponsiveness of the immune system to substances or tissues that have the potential to induce an immune response. Tolerance is achieved through both central tolerance and peripheral tolerance mechanisms (40). The exact mechanisms involved in the development of immune tolerance have not been not fully defined (41). Current evidence suggests that the gut microbiota and its metabolites (mainly short chain fatty acids), together with to exposure to dietary factors in early life, critically influence the establishment of immune tolerance to food antigens (42). Germ-free mice are unable to achieve immune tolerance to food antigens (43). During the early stage of post-natal life, development of the gut microbiota parallels maturation of the immune system (44). During vaginal delivery, infants receive their first bacterial inoculum from the

maternal vaginal tract, skin tissue, and often fecal matter, exposing the immature immune system of newborns to a significant bacterial load (38). Maturation of a healthy gut microbiota in early life allows for a change in the Th2/Th1 balance, favoring a Th1 cell response (45), while dysbiosis alters host-microbiota homeostasis, favoring a shift in the Th1/Th2 cytokine balance toward a Th2 response (46). Gut microbes induce the activation of Tregs which are depleted in germ-free mice (47). Microbiota-induced Tregs express the nuclear hormone receptor RORγt and differentiate along a pathway that also leads to Th17 cells; while in the absence of RORγt in Tregs, there is an expansion of GATA-3-expressing Tregs, as well as conventional Th2 cells, and Th2-associated pathology is exacerbated (48). Moreover, it has been demonstrated that under normal physiological conditions, macromolecules obtained via the diet induce Treg cell development in the small intestinal lamina propria, which is essential for suppressing the default strong immune response to dietary antigens. The presence of both diet- and microbe-induced populations of Treg cells may be required to induce complete tolerance to food antigens (49).

It has been speculated that microbiota can activate MyD88 signaling in the lamina propria and follicular dendritic cells (DCs) (50). Mucosal plasma cells, upon induction by DCs, produce secretory IgA (sIgA). The sIgA system is considered important in the pathogenesis of FA. Delayed development of IgA-producing cells or insufficient sIgA-dependent function at the intestinal surface barrier appears to contribute substantially to FA (48). This agrees with previous study of minor dysregulations of both innate and adaptive immunity (particularly low levels of IgA) in children with multiple FAs (51). Furthermore, the gut microbiota stimulates DCs in the Peyer's patches to secrete transforming growth factor (TGF)-β, C-X-C motif chemokine ligand 13, and B-cell activating protein, which leads to IgA production and class switching (52).

Accordingly, it has been recently demonstrated that dietary elements, including fibers and vitamin A, are essential for the tolerogenic function of CD103+ DCs and maintenance of mucosal homeostasis, including IgA production and epithelial barrier function (53).Moreover, in experimental studies,

some mice are protected from the development of FA (non-responders) compared with animals showing marked systemic FA symptoms after immunizations (54, 55). This differential immune response is associated with a distinct microbiota composition in mice with a non-responding phenotype (56). Recent findings have also suggested that neonatal gut microbiome dysbiosis promotes $CD4^{+}$ T cell dysfunction associated with allergy (57) and supports age-sensitive interactions with microbiota (58). Early-life may be a key "window of opportunity" for intervention given the age-dependent association of the gut microbiome and FA outcomes (59).The microbiota also promotes B cell receptor editing within the lamina propria upon colonization (60). Regulatory B (Breg) cells are characterized by their immunosuppressive capacity, which is often mediated by interleukin (IL)-10 secretion, but also IL-35 and TGF-β production (61). An additional immunoregulatory role is the up-regulation of IgG4 antibodies during differentiation to plasma cells. Several studies have demonstrated a potential role for Breg in the induction and maintenance of the tolerance mechanism (62-64). Several types of Bregs with distinct phenotypic characteristics and mechanisms of suppression have been described (62-64); therefore, additional studies are necessary to understand the effective role of Bregs in oral tolerance.In addition, there is a body of data reporting the activation of non-immune pathways in food oral tolerance. Data suggest that a healthy gut microbiota may protect against allergic sensitization by affecting enterocyte function and regulating its barrier-protective properties. Similarly, innate lymphoid cells (ILCs) that are abundant in mucosal and barrier sites are involved in these defence mechanisms (65). While several subsets of ILCs have been identified, particular attention has been given to ILC3 and its interactions with the microbiota. Among other factors, these cells produce IL-22, a cytokine of central importance in maintaining tissue immunity and physiology via its pleiotropic action in promoting antimicrobial peptide production, enhancing epithelial regeneration, increasing mucus production, and regulating intestinal permeability (66). How the microbiota affects the turnover of ILC3 remains unclear, but recent evidence supports that defined commensals preferentially impact this subset. Particularly, Clostridia-induced IL-22 has been demonstrated to be an innate mechanism by which the microbiota can regulate

the permeability of the epithelial barrier and contribute to protection against food allergen sensitization (43). In contrast, gut microbiota dysbiosis induces alterations in intestinal epithelial function resulting in aberrant Th2 responses toward allergic, rather than tolerogenic, responses (67).

Gut Microbiota in food allergy

Epidemiological studies have established a correlation between factors that disrupt the microbiota during childhood and immune and metabolic conditions later in life. Several factors responsible for dysbiosis have been associated with the occurrence of food allergy (FA), such as caesarean delivery (68), lack of breast milk (69), drug use (mainly antibiotics and gastric acidity inhibitors) (70), antiseptic agent use, and low fiber/high fat diet (71). Emerging data from human studies link the use of antimicrobial agents to the increasing prevalence of FA. Neonatal antibiotic treatment reduced microbial diversity and bacterial load in both fecal and ileal samples and enhanced food allergen sensitization (43). Even low-dose early-life antibiotic exposure can lead to long-lasting effects on metabolic and immune responsiveness (72). Maternal use of antibiotics before and during pregnancy, as well as antibiotic courses during the first months of life, are associated with an increased risk of cow's milk allergy in infants (73).

Data characterizing the microbiota of patients with FA are still preliminary because of multiple environmental stimuli that profoundly influence the composition of the gut microbiota (74). Some studies have failed to identify differences in infant microbiota according to later allergic status or have found different changes in gut microbiota depending on the cases and groups of subjects. Although compelling evidence for the association of gut microbiota dysbiosis with FA is emerging, heterogeneities in study design, including sampling time points, methods used to characterize the microbiota, and different allergic phenotypes under study, make it difficult to establish a clear correlation between specific bacterial taxa and allergy development. To better identify microbiota changes associated with the emergence of FA, well-phenotyped birth cohorts are needed with long-term follow up.

First studies using bacterial cultures showed that infants allergic to cow's milk had higher total bacteria and anaerobic counts (75). There was no association between culturable bacteria and food sensitization by 18 months of age in three cohorts of European infants (76). Kendler et al. found no association between culturable gut bacteria and sensitization to food including milk, egg, peanut, and hazelnut (77). Pyrosequencing technology can identify approximately 80% more bacteria in the gut than those identified by conventional culture-based methods, revealing the high complexity and diversity of the gut microbiota. Recent evidence suggests that gut dysbiosis precedes FA and influences during early life affected the subsequent development of allergic disease (78). Nakayama et al. profiled the fecal bacteria compositions of allergic and non-allergic infants and correlated changes in gut microbiota composition with allergy development in later years (79). They found that in the allergic group, the genus *Bacteroides* at 1 month and genera *Propionibacterium* and *Klebsiella* at 2 months were more abundant, while the genera *Acinetobacter* and *Clostridium* at 1 month were less abundant than in the non-allergic group (80). Additionally, the relative abundance of total *Proteobacteria*, excluding genus *Klebsiella*, was significantly lower in the allergic than in the non-allergic group at the age of 1 month. Allergic infants with high colonization of *Bacteroides* and/or *Klebsiella* showed less colonization of *Clostridium* within the major phylotypes, suggesting antagonism between these bacterial groups in the gut. *Bacteroides* are sensitive to short-chain fatty acids (SCFAs), particularly under low pH conditions (81), suggesting that the observed antagonism is attributable to an SCFA produced by *Clostridium* (82). Azad et al. found that an increased *Enterobacteriaceae/Bacteroidaceae* ratio and low *Ruminococcaceae* (*Clostridia* class) abundance, in the context of low gut microbiota richness in early infancy, are associated with subsequent food sensitization, suggesting that early gut dysbiosis contributes to subsequent development of FA (83). A low level of microbial diversity with reduced *Clostridiales*, and increased *Bacteroidales* have been also observed in the gut microbiota of allergic patients (84).

Cross-sectional studies comparing the intestinal microbial composition of food allergies in healthy subjects have also been performed. Fecal microbial composition was assessed using 16 S rRNA sequencing to determine the differences between children with FA (n = 17 with IgE-mediated FA, n = 17 with non-IgE-mediated FA) and healthy controls (n = 45) (85). There was no difference in microbial diversity between groups. Subjects with IgE-mediated FA showed increased levels of *Clostridium sensu stricto* and *Anaerobacter* (*Clostridia* class) and decreased levels of *Bacteroides* and *Clostridium* XVIII. Levels of *C. sensu stricto* were also correlated with the levels of IgE (86). Chen et al. recently showed that children with food sensitization in early life have an altered fecal microbiota and lower microbiota diversity compared to healthy controls. Children with food sensitization showed significantly decreased numbers of *Bacteroidetes*and a significantly increased number of *Firmicutes* compared to healthy children. The most differentially abundant taxa in children with food sensitization were characterized by increased abundances of *Clostridium* IV and *Subdoligranulum* (*Clostridia* class) and decreased abundances of *Bacteroides* and *Veillonella* (*Clostridia* class) (86). Recently, enriched taxa from the *Clostridia* class and *Firmicutes* phylum were observed in children with a more favourable CMA disease course (87). Accordingly, a low abundance of some sub-taxa belonging to *Clostridia* may be associated with the development of FA. The *Clostridia*class has become one of the largest genera of bacteria, and presently contains more than 100 species. Some *Clostridia* groups possess pathogenic species; however, most *Clostridia* have a commensal relationship with the host (88).In agreement with this view, a pivotal study by Atarashi et al. showed that the spore-forming component of gut microbiota, particularly clusters IV and XIVa of the genus *Clostridium*, promoted Tregs accumulation in the colonic mucosa. Colonization of mice by a defined mix of *Clostridium*strains provided an environment rich in TGF-β and affected the number and function of colonic Tregs expressing the Foxp3 transcription factor (Foxp3+ Tregs) (89). Foxp3+ Tregs play a critical role in oral tolerance (90). In a subsequent study, Atarashi et al. isolated 17 strains within *Clostridia* clusters XIVa, IV and

XVIII from a human fecal sample and demonstrated that these strains affect Tregs differentiation, accumulation and function in mouse colon (91).

Many bacterial metabolites are an important communication tools between the host immune system and commensal microbiota, establishing a broad basis for mutualism (92). Among these, SCFAs are among the most abundant, and play a critical role in mucosal integrity, and local and systemic metabolic function, and stimulate regulatory immune responses (93-95). *Clostridia* species belonging to cluster IV and XIVa are prominent source of SCFAs in the colon. SCFAs have been implicated in the regulation of both the proportions and functional capabilities of colonic Tregs (92), which, in some studies, has been specifically attributed to butyrate production by spore-forming *Clostridiales* (93). Moreover, SCFAs can increase epithelial barrier functions, as measured by fluorescein isothiocyanate-dextran permeability assay, in a GPR43-dependent manner (53) or through the stabilization of hypoxia-inducible factor-alpha, particularly by butyrate (94). Therefore, SCFAs can promote the barrier functions of the intestine, suggesting another protective role of butyrate against FA. In FA children compared to healthy subjects, different levels of fecal SCFAs, particularly butyrate, have been described (95-97). As recently demonstrated, dysbiosis in *Faecalibacterium prausnitzii* is associated with AD, but it was shown that the presence of subspecies is more associated with AD than with the species overall (98). Dysbiosis results in the suppression of high-butyrate-producer subspecies, leading to a reduction in overall butyrate production. Thus, different types of dysbiosis may share the same metabolic features leading to similar effects in term of SCFAs or of other metabolites levels that could facilitate the occurrence of FA. Interestingly, substantial correlations exist between the 16S rRNA profile, predicted metagenome, and metabolome of neonatal fecal samples, indicating a deterministic relationship between the bacterial community composition and metabolic microenvironment of the neonatal gut (57).

It is also crucial that studies move beyond cataloguing of bacteria and toward functional characterization and mechanistic understanding. Meta-transcriptomic studies will provide information regarding not only which bacteria and bacterial genes are present in a sample, but also the transcriptional activity of the community (99). Metabolomics can reveal how bacterial metabolites facilitate interactions with the host and how they may influence the health state of the host (100, 101). Fine-level characterization of bacterial species can help reveal the function of the microbiome, which is affected by interactions among closely related bacteria that may compete for the same niche but have distinct activities. Together, these studies will provide a high-resolution picture of bacteria-host interactions that can lead to disease. Moreover, studies on germ-free mice may enable more precise determination of how microbial imbalances result in disease.

Probiotics in pediatrics

Interest in probiotic research and its potential benefits in paediatric nutrition are relatively recent but significantly increasing. According to a recent bibliometric analysis, the total number of documents published on probiotics in paediatrics over the period 1994-2014 was 2817. Research activity on probiotics in paediatrics showed a 90-fold increase during the study period. Approximately 22% of published articles originated from USA and has the greatest share.

The top ten cited articles over the past two decades revealed that most of the hot articles focused on the role of probiotics in treatment of allergy and diarrhoea in children. Microbiology, immunology and potential mechanism of benefits of probiotics in children were also topics of interest in this area (102).

Timing and duration of treatment, the optimal probiotic strains, and factors that may alter the composition and function of the microbiome are still in need of further research. Other treatments such as prebiotics, faecal microbial transplantation, and antibiotics have limited evidence. Future translational work, *in vitro* models, long-term and follow-up studies, and guidelines for the composition and viability of probiotic and microbial therapies need to be developed. Overall, while manipulating the microbiome with probiotics early in life can help prevent or reduce the severity of

some childhood diseases, further research is needed to elucidate biological mechanisms and determine optimal treatments.

In 2001, the FAO/WHO defined probiotics as ''live microorganisms that, when administered in adequate amounts, confer a health benefit on the host''. This definition has been widely adopted by regulatory agencies such as Codex alimentarius, and the European Food Safety Authority (EFSA), scientists, industry, and consumers. The definition translates into scientific terms that a specific probiotic strain should have be investigated in properly controlled studies to confer a specific benefit before a claim with respect to this benefit can be made. If this is not fulfilled the only allowed claim would be ''contains probiotics''. Studies using probiotics or prebiotics have generally been designed as exploratory and were not sufficiently designed to fulfil the criteria for substantiation of a health claim under the current claims regulation by EFSA (103).

Selected probiotics, such as *Lactobacillus rhamnosus* GG (LGG), were found to lower the risk of atopic dermatitis when used by women during the last trimester of pregnancy, by breastfeeding mothers, or when given to infants (104). Studies examining the efficacy of currently available probiotics in treating FA have yielded conflicting results. It was recently demonstrated that oral immunotherapy supplemented with the probiotic *L. rhamnosus* CGMCC 1.3724 led to peanut unresponsiveness in 82% of allergic children (105). In one randomized, double-blind, placebo-controlled study of infants with challenge-proven CMA, administration of *Lactobacillus casei* CRL431 and *Bifidobacterium lactis* Bb12 for 12 months did not affect the acquisition of tolerance to cow's milk (106). In contrast, we demonstrated in different studies that an extensively hydrolyzed casein formula (EHCF) containing LGG accelerated the development of tolerance acquisition in infants with CMA and reduced the incidence of other allergic manifestations (107-109). When we compared the fecal microbiota of infants receiving this tolerance-inducing probiotic-supplemented formula to that obtained from infants receiving EHCF alone, we found significant positive correlations between the abundance of butyrate-producing genera, and an increase concentration of fecal butyrate (96). The mechanisms of action of butyrate are multiple, but many of

these involve epigenetic regulation of gene expression by inhibiting histone deacetylase (HDAC). Inhibition of HDAC 9 and 6 increased FoxP3 gene expression, and the production and suppressive function of Tregs (110). We demonstrated that the use of EHCF+LGG induces stronger epigenetic regulation of Th1 and Th2 cytokines genes as revealed by the significantly different levels of promoter region methylation (111). Similar results were obtained by examining the FoxP3 Treg-specific demethylated region (TSDR) methylation profile. FoxP3 TSDR demethylation and expression were significantly higher in children treated with EHCF+LGG compared to in children treated with other dietary strategies (112). These results strongly suggest that acting on gut microbiota composition and function can have long-term protective effects in children with FA.

Probiotics in allergy prevention and treatment

To recommend probiotics for allergy prevention or treatment it needs to be proven that a specific probiotic bacterium or a mixture of probiotic strains given to the pregnant or breastfeeding mother or directly to the infant or young child reduces the risk of later allergies or the symptoms of allergy as well.

Evidence on animal models show the potential benefits of Bifidobacteria species in the prevention and treatment of allergies.

It was demonstrated that the oral administration of Bifidobacteria TMC3115, during early life could reduce the risk of IgE-mediated allergies in adult host animals provoking modifications of intestinal microbiota, Small Chain Fatty Acids metabolism and anti-inflammatory cytokine IL-10 production at both neonatal and adult stages (113)

Furthermore, dietary intervention with short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) and Bifidobacterium breve M-16V (Bb) (GF/Bb) suppresses food allergic symptoms in mice, potentially via intestinal epithelial cell (IEC)-derived galectin-9. Furthermore, in vitro studies showed galacto- and fructo-oligosaccharides (GF) to enhance the immunomodulatory capacity of a TLR9 ligand representing bacterial CpG DNA when exposed to IEC (114).

In addition, oral administration of *Bifidobacterium infantis* CGMCC313-2 during or after allergen sensitization suppressed allergic inflammation in lung and intestinal tissues inhibiting the secretion of allergen-induced IgE, IL-4 and IL-13, and attenuates allergic inflammation, while the proportion of infiltrating inflammatory cells was significantly decreased in the BALF of allergic asthma mice. In other terms, *B. infantis* CGMCC313-2 (115).

Another recent evidence affirmed that prior exposure to BLG-peptides and a FF/Bb-enriched diet is a promising approach for protecting the intestinal Th1/Th2 balance and reducing the allergic response to whole whey protein. Therefore, it might have implications for developing successful nutritional strategies for CMA prevention (116).

The ability of some probiotic strains to modulate mouse models on the balance between Th1 and Th2 is further confirmed by another source: a reduction in mast cell degranulation (mMCP-1) and changes in regulatory and effector T-cell subsets and increases in the SCFA propionic acid. These results suggest immune modulatory benefits of dietary intervention with a unique combination of scFOSlcFOS + Bifidobacterium breve in established food allergy. Whether these effects translate to human applications is subject for ongoing clinical studies (117).

It is known that *Bifidobacterium longum* predominate in the fecal microbiota of breast-fed infants because of their ability to metabolize human milk oligosaccharides (HMOs), which has a profound positive effect on the short- and long-term well-being of the individual. In contrast, formula-fed infants are dominated by alternative species such as *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*, which are commonly found in adults (118). Earlier studies suggested that bifidobacteria inhabiting the infant intestine are superseded by other strains after weaning until adulthood (119). However, recent studies have shown that strains of *Bifidobacterium longum ssp. longum* persist in the gut microbiota over time, as strains isolated from the same babies at one and 2 (120).

Regardless of the strain, the most important characteristic for the probiotic to be effective is the capability to colonize the ecosystem in which it is introduced and that its survival in the microbiota

is sufficiently durable to bring about the effects demonstrated in literature above all on animal models.

AIM OF THE STUDY

Our hypothesis was that the intestinal microbiota of infants suffering from CMA was different from that of healthy subjects and from that of subjects sensitized to cow's milk proteins, but not allergic.

The aim of the study then was:

1) To evaluate intestinal microbiota composition of IgE mediated CMA infants compared to that of sensitized but not allergic infants and healthy controls;

2) To evaluate the baseline presence of *Bifidobacterium longum BB536*, *Bifidobacterium breve M-16V* and *Bifidobacterium infantis M-63* in stools of infants with CMA compared to that of sensitized but not allergic and healthy controls;

3) To evaluate the presence of the same strains in CMA patients during administration of multi-strain probiotics containing *Bifidobacterium longum BB536*, *Bifidobacterium breve M-16V* and *Bifidobacterium infantis M-63* and after 60 days from the discontinuation.

4) To evaluate intestinal microbiota composition of children with CMA during and after administration of probiotics mixture.

MATERIALS AND METHODS

Study design and sample collection

The study was a prospective multicentric study.

Patients were consecutively enrolled at Pediatric Gastroenterology and Liver Unit of Sapienza University of Rome and at the Allergy Division of Bambino Gesù Children's Hospital in Rome since January 2016 to December 2018.

- Inclusion criteria: all 10-15 months' children evaluated for suspected IgE mediated CMA.
- Exclusion criteria: gastrointestinal disease in the last 30 days, metabolic or congenital disorders.

Intake of antibiotics, probiotics, proton pump inhibitors, fermented milks or other functional foods in the 2 weeks prior to the start of the study.

For each patient, informed consent was obtained, and then anamnestic data were recorded: type of birth and duration of breastfeeding.

For each patient, total IgE, Skin Prick Tests and specific IgE for cow milk (alpha-lactalbumin, beta-lactoglobulin and casein) protein were evaluated.

In positive patients, double-blind placebo- controlled food challenge (DBPCFC) were then performed.

Based on the results of in vitro and in vivo exams and DBPCFC, all enrolled patients were then divided in 3 groups:

- **Group 1:** patients with positivity for specific IgE against cow milk proteins and DBPCFC confirmed IgE mediated cow milk allergy.
- **Group 2:** patients showing positivity for specific IgE against cow milk proteins but tolerant to cow milk proteints at DBPCFC.
- **Group 3:** age and sex matched healthy children.

For each patient, a stool sample was collected at enrolment (T_0).

In Group 1 probiotic mixture (TRIBIF® composed of *B. breve* M-16V, *B. longum* subsp. *longum* BB536 and *B. longum* subsp. *infantis* M-63) was then administered twice per day (3.5×10^9 UFC/dose) for 30 consecutive days and stool samples were collected during clinical visits following this schedule:

T_1 (7 days from probiotic intake)

T_2 (15 days from probiotic intake)

T_3 (30 days from probiotic intake)

T_4 (after 30 day from probiotic discontinuation)

T_5 (after 60 day from probiotic discontinuation)

All samples were stored at -80°C at the Human Microbiome Unit of Bambino Gesù Children's Hospital in Rome until DNA extraction.

This study was approved by the OPBG Ethics Committee (protocol number 787_OPBG_2014) and was designed in according to Declaration of Helsinki.

The study was registered on Clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT03639337).

B. breve M-16V, B. longum subsp. longum BB536 and B. longum subsp. infantis M-63 from the probiotics

B. breve M-16V, *B. longum* subsp. *longum* BB536 and *B. longum* subsp. *infantis* M-63 were cultured on Columbia agar + 5% sheep blood medium (COS, Biomerieux Marcy l'Etoile, France) at 37°C for 24 h under anaerobic conditions. Single colonies were isolated and purified on new COS plates based on their morphology. Bacterial identification was confirmed using a matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) biotyper and a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) as described.

Bacterial DNA extraction from colony and 16S ribosomal locus amplification

DNA was extracted from bacterial colonies using the EZ1 DNA Tissue Kit and automatic extractor biorobot EZ1 according to the manufacturer's instructions (Qiagen, Hilden, Germany). The entire ITS (internal transcription spacer) locus (1430 bp) was amplified from the extracted DNA using universal primers (FW: 5'- GGT GTG AAA GTC CAT CGC T-3'; RV: 5'-GTC TGC CAA GGC ATC CAC CA-3'). PCR was performed with a reaction mixture containing 5 µl 10X Buffer, 2 µl 2.5 mM $MgCl_2$, 1 µl each primer (10 µmol/L), 2 µl dNTPs (10 mmol/L), 1 µl Taq DNA polymerase (5 U/µl) (KAPA Taq PCR kit, KAPA Biosystems, Boston, USA), 5 µl DNA template (10 ng/µl) and molecular-grade H_2O to a final reaction volume of 50 µl. The amplification protocol consisted of one cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 56.5°C for 1 min, and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The resulting amplicons were purified using centrifugal filter units (Amicon Ultra-0.5 mL

Centrifugal filters 30 K, Sigma-Aldrich, MO, USA) and quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, DE, USA).

Bacterial DNA cloning and species-specific primer and probe design

The purified PCR products were sequenced and cloned into the PGEM vector following the instructions provided by the pGEM®-T Easy Vector System kit (Promega, Italy) using *Escherichia coli* competent cells as a host. The obtained plasmids, pGEM-BB (pGEM+*B. breve*), pGEM-BL (pGEM+*B. longum* subsp. *longum*), pGEM-BI (pGEM+*B. longum* subsp. *infantis*), were extracted (Plasmid Miniprep Kit, Promega, Italy), quantified using the NanoDrop ND-1000 spectrophotometer and diluted. The dilutions, which ranged from 10^6 to 10^1 vector copy numbers, were used as standards in the quantitative RT-PCR (qRT-PCR) assays. The cloned fragments of ITS rDNA in the pGEM-vectors were amplified and sequenced with an automated sequence analyser (Genetic Analyser 3500, Applied Biosystems, CA, USA) using a 50-cm capillary array and a POP-7 polymer (Applied Biosystems) and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, version 3.1) according to the manufacturer's instructions. All electropherograms were manually edited for base ambiguity. The obtained FASTA sequences were aligned using CLUSTAL-W software (http://www.ebi.ac.uk/clustalw/) and used for the design of species-specific primers and TaqMan probes (Roche Diagnostics, Mannheim, Germany).

Bacterial DNA extraction from stool samples

Frozen stool samples were thawed at room temperature, and DNA was manually extracted using the QIAmp Fast DNA Stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was quantified using the NanoDrop ND-1000. Comparable amounts of DNA (80 ng) from each sample were used in the qRT-PCR assays.

qRT-PCR

Quantification of *B. breve*, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* in faecal samples was carried out by qRT-PCR using the Light Cycler 480 platform (Roche Diagnostics, Mannheim, Germany). The assays were performed with a 20 µl PCR amplification mixture containing: 10 µl

LightCycler 480 Probe Master mix (Roche Diagnostics), 2 µl primers and probes (optimized concentrations, 0.5 µM and 0.1 µM, respectively), 3 µl molecular-grade H_2O and 5 µl DNA template. Each sample was tested in duplicate to ensure data reproducibility. The RT-PCR temperature profile consisted of an initial denaturation at 95°C for 10 min, 45 amplification cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 1 sec followed by a final cooling step at 40°C for 30 sec. Absolute quantification was performed using the "second derivative maximum method".

Primers and probes designed for *B. breve*, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* for the Real Time assay.

Species specificity	Primer or probe	Sequence	Product size
B. breve	Fw_Bbreve	5'-gTg gTg gCT TgA gAA CTg gAT Ag 3'	117 bp
	Rv_Bbreve	5'-CAA AAC gAT CgA AAC AAA CAC TAA A-3'	
	P_Bbreve	5'-FAM-CGC ACC CAC CGC A-BHQ-3'	
B. longum subsp. *longum*	Fw_*Blongum* subsp. *longum*	5'-Tgg AAg ACg TCg TTg gCT TT-3'	109 bp
	Rv_ *Blongum* subsp. *longum*	5'-ATC gCg CCA ggC AAA A-3'	
	P_ *Blongum* subsp. *longum*	5'-FAM-CGC ACC CAC CGC A-BHQ-3'	
B. longum subsp. *infantis*	Fw_*Blongum* subsp. *infantis*	5'-CTC CTT TCT ACg gAg AAT ACA ggA T-3'	113 bp
	Rv_ *Blongum* subsp. *infantis*	5'-gAA CCg AAA gCC CAT gAT CT-3'	
	P_ *Blongum* subsp. *infantis*	5'-CgC gAT ggT CgC gTg Tgg A-3'	

16S rRNA targeted-metagenomics of fecal microbiota

DNA from stool samples was manually extracted using QIAmp Fast DNA Stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For each sample, the variable region V3-V4 from the 16S rRNA gene (~460 bp) was PCR amplified using the primers 16S_F 5′-(TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG)-3′ and 16S_R 5′-(GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C)-3′ as described in the MiSeq rRNA Amplicon Sequencing protocol (Illumina, San Diego, CA). The first PCR reaction was set up using Fast Start Hifi Taq (Roche Diagnostics, Mannheim, Germany) with the following conditions: initial denaturation at 95 °C for 3 min, 32 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. DNA amplicons were cleaned-up by 20 ul of KAPA Pure Beads (Roche Diagnostics, Mannheim, Germany). Indexed libraries were prepared by a second amplification step using Nextera technology. The final library was cleaned-up using 50 µl of AMPure XP beads, quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA) and diluted in equimolar concentrations (4 nM). Samples were pooled together, denatured and diluted to 7 pM before the sequencing on an Illumina MiSeqTM platform (Illumina, San Diego, CA, United States) according to the manufacturer's specifications to generate paired-end reads of 300 base-length.

Biocomputational and statistical analyses

Illumina Miseq reads were first analyzed for their quality, length and chimera presence using the Qiime v1.8 pipeline. Then, sequences were organized into Operational Taxonomic Units (OTUs) with a 97% of clustering threshold of pairwise identity. PyNAST v.0.1 program was used to carry out a multiple sequence alignment against Greengenes 13_08 database with a 97% similarity for bacterial sequences (121). The OTUs multiple sequence alignment was used as well to build a phylogenetic

tree (122). Alpha and beta diversity were performed by phyloseq of R package (123), while OTUs relative abundances during the time course, was assessed by Kruskal-Wallis test, corrected for FDR. PERMANOVA test was applied on beta diversity metrics with 999 permutations to compare samples at different time points.

RESULTS

The study was proposed to 63 children.

Twenty-three were excluded by the enrolment: in 1 child celiac disease was diagnosed; 4 patients had assumed antibiotic therapy in the previous 2 weeks; 3 were following therapy with proton pomp inhibitor because of oesophageal reflux disease; 11 were just taking different probiotic strain and 4 were fed with fermented milk.

A total of 40 children were then enrolled (median age 12.8 months; 19 females).

According to DRACMA guidelines for diagnosis of IgE mediated CMA (29), the patients underwent Skin Prick Test, serological evaluation and DBPCFC.

They were then divided into 3 groups:

Group 1: 14 infants (6 females) showing positivity for specific IgE against at least one cow milk proteins and for DBPCFC.

Group 2: 12 infants (5 females) showing positivity for specific IgE against at least one cow milk proteins but negative to DBPCFC.

Group 3: 14 age and sex matched healthy infants (8 females).

In the table demographic and clinical characteristics are reported.

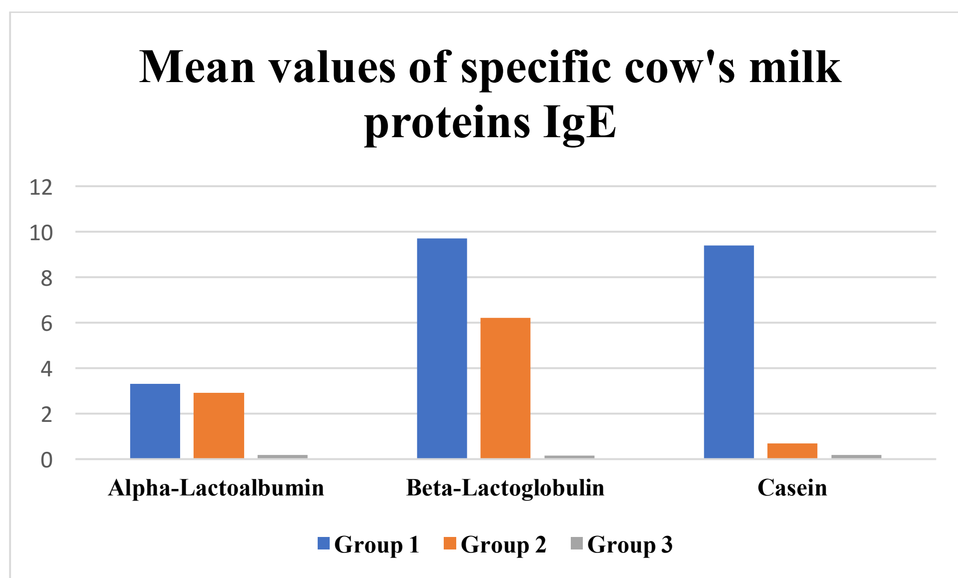
	Group 1	Group 2	Group 3	p
Mean age +/- SD	12.86 +/-1.68	13.36 +/- 1,01	12.6 +/- 1.53	0.08
Male / Female	8 / 6	7 / 5	6 / 8	0.7
Cesarean section	21.4%	25%	43%	0.6
Mean breastfeeding duration (months)	7.2	7.5	8	0.44
Mean diameter Prick by Prick with Cow Milk (mm)	5	1.25	0	0.09
Mean diameter Skin Prick Test with Alpha-Laactoalbumin (mm)	4.4	1	0	**0.04**
Mean diameter Skin Prick Test with Beta-Lactoglobulin (mm)	3.8	0.85	0	0.2
Mean diameter Skin Prick Test with Casein (mm)	6.4	1	0	**0.039**
Total IgE (kU/L)	228.2	51.2	33.2	**<0.01**
Specific IgE for Cow milk proteins	23.8	0.8	0	**0.053**
Specific IgE for Alpha-Lactoalbumin	3.5	0.1	0	0.14
Specific IgE for Beta-Lactoglobulin	9.0	0.5	0	0.26
Specific IgE for Casein	9.4	0.4	0	0.18

Three out of 14 (21.4%) in Group 1, 3/12 (25%) in Group 2 and 6/14 (43%) in Group 3 were delivered by caesarean section (p=0.6).

Ten out of 14 in Group 1 (71.4%), 9/12 in Group 2 (75%) and 10/14 in Group 3 (71.4%) were breastfed till weaning.

Prick by prick for fresh cow milk in 11/14, 6/12, 0/14 and specific IgE for cow milk were positive in 13/14, 9/12, 0/14 in Group 1, 2 and 3 respectively.

In the Graph details on sensitization status are provided.



After DBPCFC, all Group 1 infants followed cow milk protein's free diet and regularly took the probiotic mixture TRIBIF® (*B. breve M-16V, B. longum subsp. longum BB536 and B. longum subsp. infantis M-63*) twice per day (3.5×109 UFC/dose) for 30 days without any relevant symptoms.

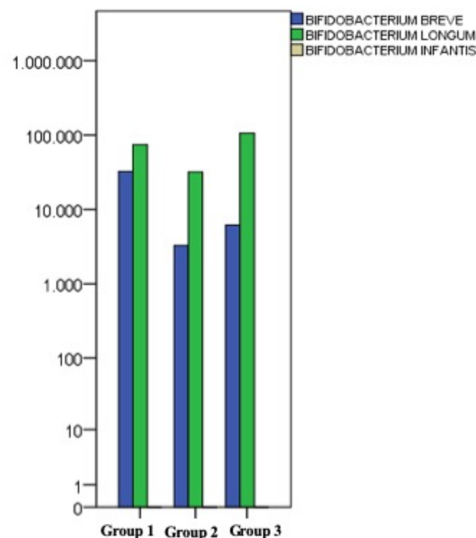
One stool sample for each patient was collected at T1 (7 days from probiotic intake), T2 (15 days from probiotic intake); T3 (30 days from probiotic intake), T4 (after 30 day from probiotic discontinuation) and T5 (after 60 day from probiotic discontinuation).

RT-PCR analysis

RT-PCR was performed on patient faecal samples (33 T_0, 12 T_1, 12 T_2, 11 $T_{3,}$ 12 T_4 and 11 T_5) using primers and probes specific for *B. breve*, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*.

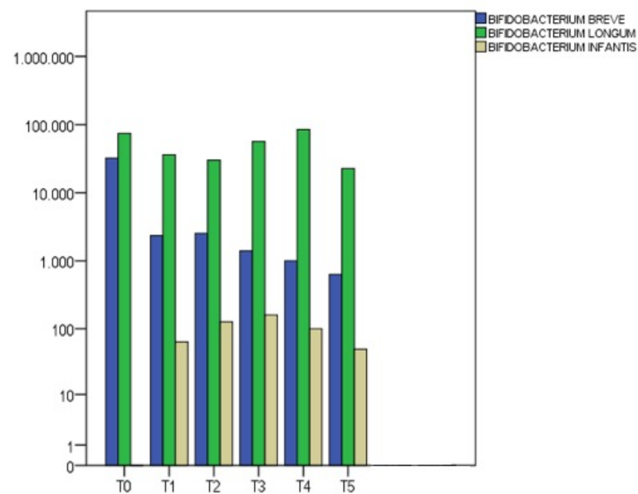
At baseline, *B. breve* and *B. longum* subsp. *longum* were present in the gut microbiota of patients in the order of 10^4-10^5 cells/ul in the three Groups without significant differences.

In the Graph, median values differences between *B. breve*, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* levels at T0.



In Group 1, TRIBIF® intake did not cause a significant increase of *B. breve* and *B. longum* subsp. *longum* in faecal samples.

In the following graph, median values differences between *B. breve*, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* levels at each point of the time-course in Group 1.

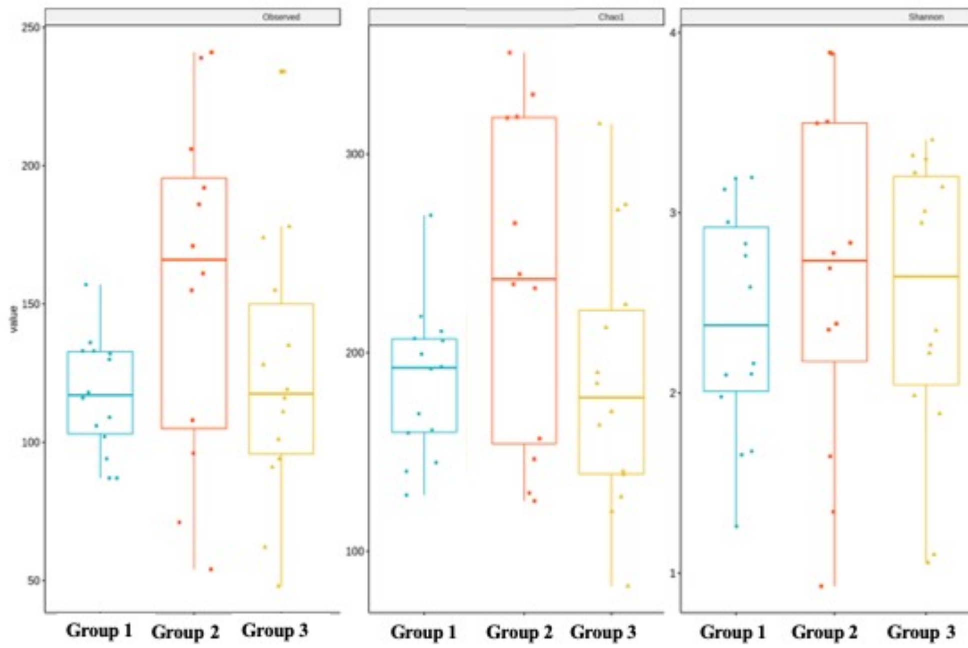


For *B. longum* subsp. *infantis* we observed a peculiar trend in its concentration values during the time-course. At baseline, its median concentration value was 0 in all the three groups, indicating a total absence of this species in infants gut microbiota. During probiotic intervention, its amount significantly increased from T_1 (7 days from first day of therapy) to T_3 (after 30 days of therapy), while it started to decrease from point T_4 (after 30 day from probiotic discontinuation).

Ecological analysis

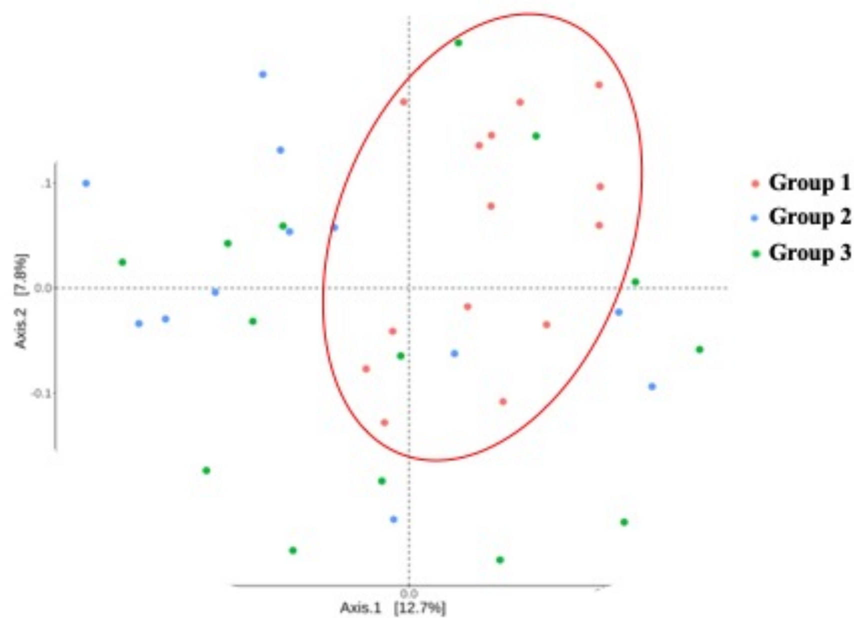
At baseline, alpha diversity was calculated using Observed, Chao1 and Shannon indices in order to evaluate OTUs *evenness, rarity* and *richness* within the Group 1, Group 2 and Group 3. Even if no statistically significant differences were found, Group 2 showed a higher number of total OTUs (Observed index) including rare OTUs (Chao 1 index), while the richness (Shannon index) of gut microbiota resulted comparable between the 3 groups.

In the figure boxplots representing alpha-diversity indices Observed, Chao1 and Shannon indexes among groups. Boxes represent the median, 25th and 75th percentiles calculated for Group 1 (blue), Group 2 (red), and Group 3 (yellow) groups.



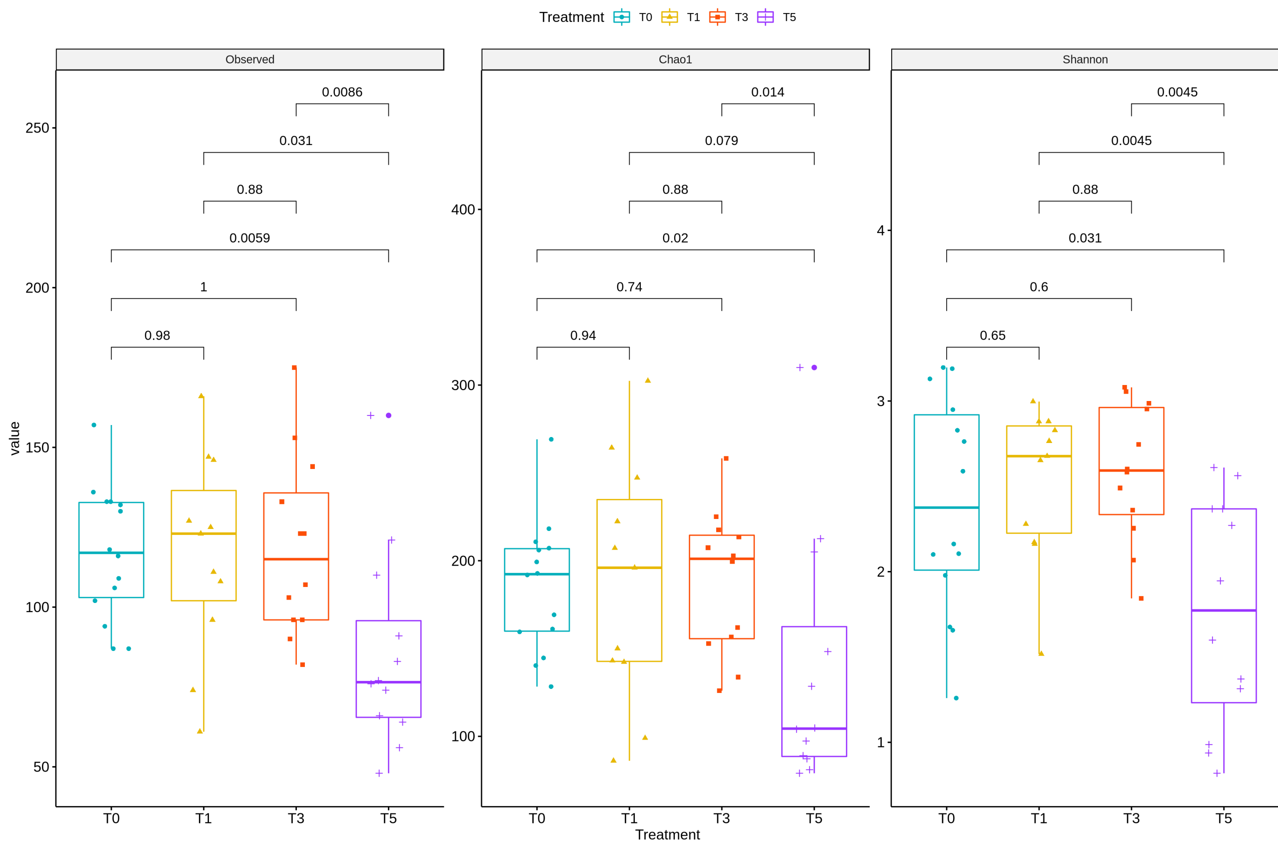
To evaluate how OTUs were differentially distributed in the 3 groups in terms of phylogenetic relatedness (*i.e.*, unweighted UniFrac algorithm), differences in beta diversity were calculated. Group 1 was cleared separated from the other groups, which was verified using the PERMANOVA test (p=0.019).

Beta-diversity analysis applied for ALLERGIC, Healthy and NCA samples. The plots show the first two principal axes for PCoA using unweighted UniFrac algorithm. P-values obtained by PERMANOVA analyses are reported.



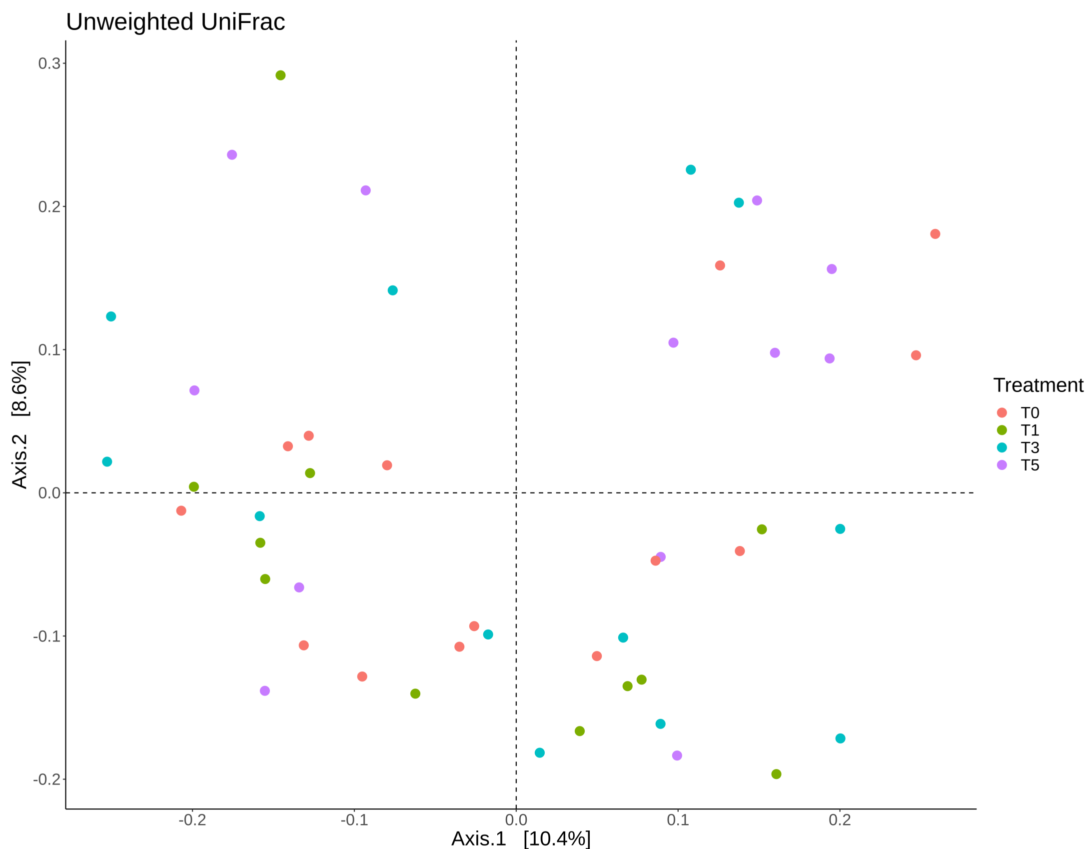
In Group 1, after probiotics administration there was no statistically significant changes in alpha diversity measures, but at T_5, when the probiotic intake was interrupted for 60 days, there was a significant strong decrease of all the alpha diversity indices values.

Boxplots representing alpha-diversity indices Observed, Chao1 and Shannon indexes during time-course of ALLERGIC group. Boxes represent the median, 25th and 75th percentiles calculated for ALLERGIC group at T0 (blue), T1 (yellow), T3 (red) and T5 (purple). p-values are reported.



However, there were no clearly defined clusters for the patients stratified into T0 and T2–T5 groups (PERMANOVA = 0.829).

Beta-diversity analysis applied for all the time points of Group 1. The plots show the first two principal axes for PCoA using unweighted UniFrac algorithm. P-values obtained by PERMANOVA analyses are reported.



Gut microbiota profiling

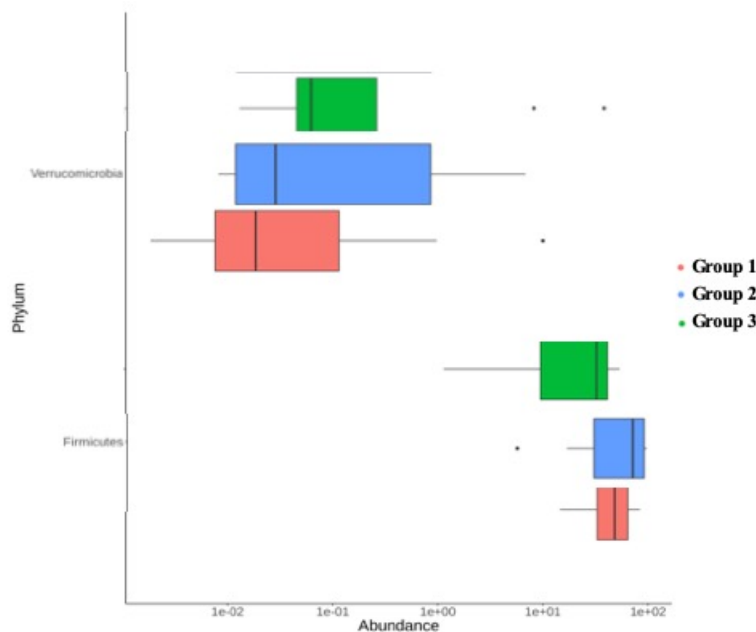
To detect differences in OTU composition among groups, we compared time point T0 of the Group 1 (ALLERGIC group) (i.e., before the probiotic intake) to the Group 2 (NCA) and Group 3 (HEALTHY groups).

OTU distribution was investigated at the phyla and genus levels.

At phylum level, Kruskal–Wallis test highlighted that Verrucomicrobia were higher in HEALTHY group and gradually decreased from NCA to ALLERGIC group (pFDR<0.05). On the contrary, Firmicutes resulted more abundant in NCA group and lower in HEALTHY subjects, while the

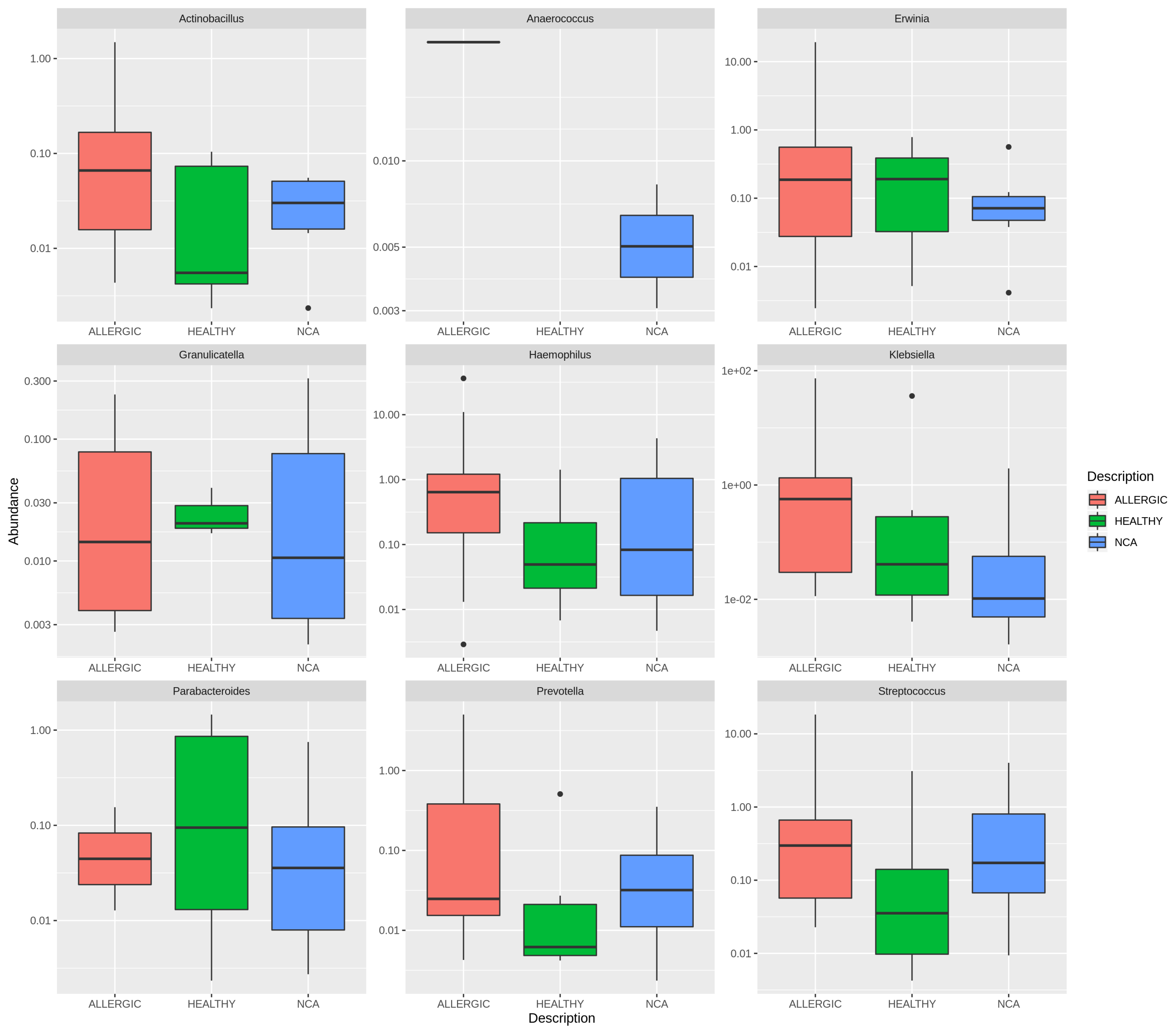
ALLERGIC group showed an intermediate level of abundance between the other two groups (pFDR<0.05).

Kruskal Wallis test-based OTU distribution of the gut microbiota in the 3 groups. The bar graphs represent the average distribution of the OTUs at phylum level. Only statistically significant OTUs (p(FDR)<0.05) are plotted.



At genus level, *Haemophilus*, *Actinobacillus*, *Prevotella* and *Streptococcus* resulted associated to allergy, with a significant increase ($p<0.05$) in Group 1 and less in Group 2 compared to Group 3 subjects. Also *Klebsiella* showed a higher abundance in Group 1 but not in Group 2 compared to Group 3. *Parabacteroides* and *Granulicatella* were instead more abundant in Group 3.

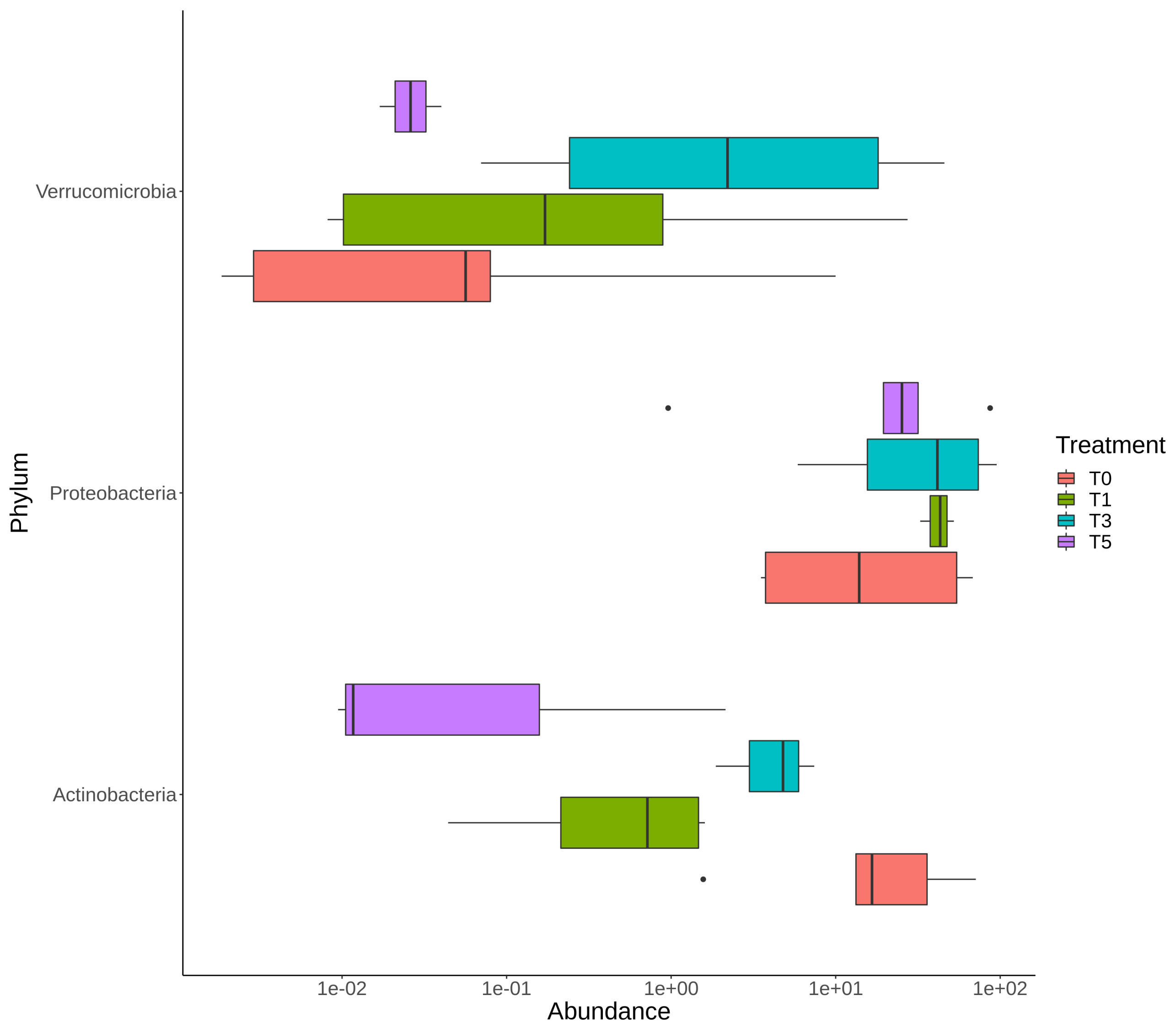
Kruskal Wallis test-based OTU distribution of the gut microbiota in the 3 groups. The bar graphs represent the average distribution of the OTUs at genus level. Only statistically significant OTUs (p(FDR)<0.05) are plotted.



To evaluate the influence of probiotic intake on gut microbiota modulation, we tested the OTU distribution during the time-course in Group 1. At phylum level, there was an increase of Verrucomicrobia during probiotic intake with a peak of abundance at T3. At T5, after 60 days since probiotic interruption, there was a rapid decrease of Verrucomicrobia abundance. Proteobacteria showed a gradually increase during probiotic intake and this increment was maintained also at T5.

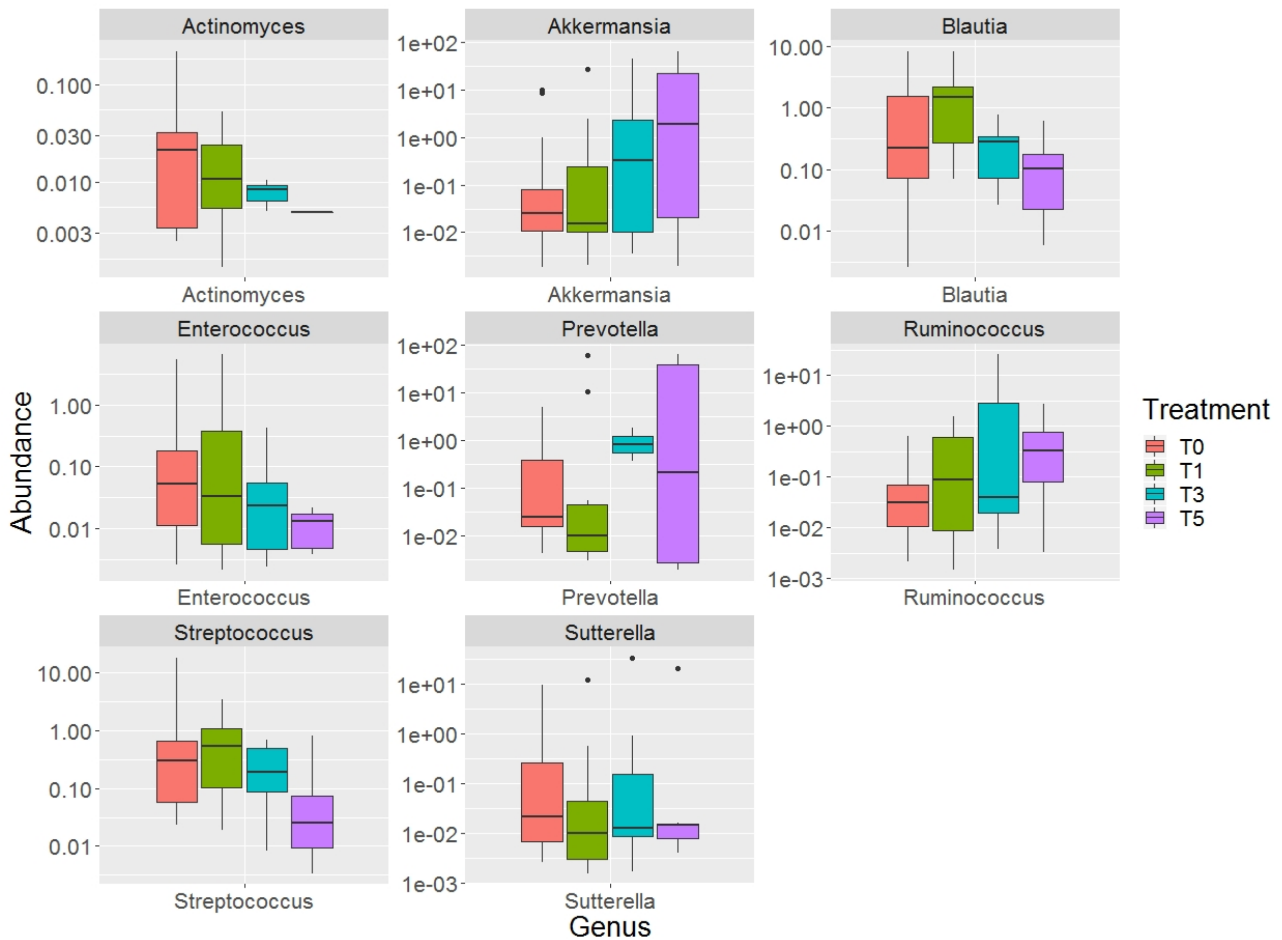
On the contrary, Actinobacteria decreased during the time-course, even if there was an increase form point T1 to point T3.

Kruskal Wallis test-based OTU distribution of the gut microbiota during the time-course of the ALLERGIC group. The bar graphs represent the average distribution of the OTUs at phylum level. Only statistically significant OTUs (p(FDR)<0.05) are plotted.



At genus level, probiotic intake determined an increase of *Akkermansia*, *Prevotella* and *Ruminococcus* that was maintained also at T5. *Blautia* increased during all the period of probiotic intake, until T3 point, while at T5 it started to decrease and showed a level of abundances lower of that observed at T0. *Actinomyces*, *Enterococcus*, *Streptococcus* and *Sutterella* resulted instead diminished after probiotic intervention.

Kruskal Wallis test-based OTU distribution of the gut microbiota during the time-course of the ALLERGIC group. The bar graphs represent the average distribution of the OTUs at phylum level. Only statistically significant OTUs (p(FDR)<0.05) are plotted.



DISCUSSION

Our hypothesis was that the intestinal microbiota of infants suffering from CMA was different from that of healthy subjects and from that of subjects sensitized to cow's milk proteins, but not allergic.

Several studies focused on the comparison of the intestinal microbiota composition between heathy individuals and allergic children, but results are contradictory (96; 124-126).

The Bifidobacterium spp. are assumed to be beneficial for human health due to their several effects such as vitamin production, immune system stimulation, inhibition of potentially pathogen bacteria,

improvement of food ingredients digestion (127, 128) and their decreased abundance have been related also to many different diseases (129-131).

The modulation of gut microbiota is perhaps an ancestral, innate concept for human beings. At this time, the restoration of gut microbiota impairment is a well-established concept in mainstream medicine, and several therapeutic approaches have been developed in this regard. Antibiotics, prebiotics and probiotics are the best known and commercially available options to overcome gastrointestinal dysbiosis.

In a recent position paper ESPGHAN Working Group for Probiotics and Prebiotics provides evidence on the inadequate quality of commercial probiotic products, regarding microorganism specification, their numbers, functional properties, and the presence of contaminating microorganisms. In this document more stringent quality control procedures are suggested, which should be mandatory for products prescribed for specific clinical situations, and for use in vulnerable populations such as infants and children (132).

The present study demonstrates the ability of a specific Bifidobacteria mixture to colonize and persist within the microbiota of children affected by cow's milk proteins IgE mediated allergy.

Previously it was demonstrated that children treated with the same probiotic mixture achieved a significant improvement of symptoms and QoL in allergic rhinitis and intermittent asthma (133) but no data were provided about the real presence of probiotics in patients' microbiota.

Few studies demonstrate the real ability of probiotics to colonize the intestine. This aspect does not allow clinicians to choose the probiotic strain sure that it will persist in the intestinal mucosa.

Colonization of the human gut is influenced by various factors such as the method of delivery (i.e., vaginal or cesarean), type of feeding (breast-fed or formula-fed), exposure to antibiotics, frequency, and nature of diseases and hygiene conditions (134).

Bifidobacteria dominate the total gut bacterial population in healthy breast-fed infants (135-137), although this dominance decreases following weaning (138).

At baseline, *B. breve* and *B. longum* subsp. *longum* were present in the gut microbiota of patients in the order of 10^4-10^5 cells/ul without significant differences in the three Groups.

This could be explained because our subjects were all of the same strict age range and without any significant differences for delivery method, breastfeeding and use of probiotics, antibiotics and protonic pump inhibitor.

On the contrary, *B. longum* subsp. *infantis* was totally absent at baseline in all the three groups. During probiotic intervention, its amount increased significantly during 30 days of therapy, while it started to decrease after 30 days from probiotic discontinuation.

It was demonstrated that during early postnatal development, changes in the composition of intestinal antimicrobial peptides (Defensins) provoke mutations in microbiota composition (139).

These mutations can cause increase of Gram (-) bacteria, repression of TLR signalling and increase of Nuclear factor kB inhibitor.

Consequently, we observe an increase in CD103+ dendritic cells and CX3CR1+ macrophages home to the gut mucosa. In this way, regulatory T (Treg) cells populate intestinal mucosa. CD103+ migratory dendritic cells carry antigen from the gut to Mesenteric Lymph nodes where they promote the induction of Treg.

In previous studies, it was demonstrated that *Bifidobacterium infantis* markedly induce FOXP3+T reg cells in this phase. After postnatal period, members of the Clostridium cluster IV and XIVa might take over the role of *B. infantis* (140).

After weaning, the composition of the bifidobacterial population changes toward species capable of adapting to the metabolism of plant-derived sugars. For example, *B. longum* subsp. *longum* and *B. adolescentis* can utilize such diet-derived carbohydrates, while *B. bifidum* may shift its HMO-metabolic abilities toward mucin degradation (141-145).

We can speculate that we did not find *B. longum subsp. infantis* in faceal samples of our population because of the age range (10-15 months) and its presence after administration represents the real proof of the colonization capacity of probiotics mixture.

One outcome from the fermentation of prebiotics by the gut microbiota is the production of short chain fatty acids (SCFAs), such as acetate, butyrate and propionate (146). Both Bifidobacteria and Lactobacilli produce acetate (and lactate), thus contributing to the SCFA-mediated health effects of prebiotics, although these two microorganisms do not produce butyrate and/or propionate (147, 148). Furthermore, a recent study has demonstrated that acetate produced by *B. longum* NCC2705 acts as an essential co-substrate for butyrate production and growth by *Eubacterium rectale* ATCC 33656 (149).

We could then imagine the possible increase of SCFA production after probiotics mixture administration.

From basal microbiota comparison, we demonstrated that allergic patients clustered in Beta-diversity analysis (PERMANOVA test (p=0.019)) and showed a peculiar phylogenetic relatedness.

In a previous study no differences in microbial diversity (according to the Shannon index) between the children with and without food allergy was find out (150).

That population showed a wide age range (0-18 years) that makes impossible to make a comparison with our study.

In our allergic patients, probiotics administration did not provoke statistically significant changes in microbiota alpha diversity measures, but after 60 days from probiotics discontinuation, there was a significant strong decrease of all the alpha diversity indices values.

This evidence was before demonstrated for other kind of probiotics: a transient increase in community evenness and diversity of the distal intestinal microbiome in animals treated with *L. reuteri* compared with that of vehicle-treated animals (151).

The diversity in microbial communities was shown to be associated with increased ecological stability (152).

From microbiota profiling, it is worth noting that at phylum level, Kruskal–Wallis test highlighted that Verrucomicrobia were higher in healthy group and gradually decreased from Group 2 to Group 3 (pFDR<0.05).

This phylum is a member of the PVC (Planctomycetes-Verrucomicrobia-Chlamydiae) superphylum which includes phylogenetically related bacteria with unusual characteristics such as the existence of a complex and dynamic endomembrane system that, in some aspects, makes them closer to eukaryotic cells. It includes a small number of genera isolated from fresh water, soil and animal feces; *Akkermansia muciniphila* is the main member of this phylum identified in humans (153).

In our population Firmicutes resulted more abundant in Group 2 and lower in healthy subjects, while Group 1 showed an intermediate level of abundance between the other two groups (pFDR<0.05). Chen et al. recently showed that children with food sensitization in early life have an altered fecal microbiota and lower microbiota diversity compared to healthy controls. Children with food sensitization showed significantly decreased numbers of Bacteroidetes and a significantly increased number of Firmicutes compared to healthy children (154). This evidence confirms our results for Group 2.

At genus level, *Haemophilus*, *Actinobacillus*, *Prevotella* and *Streptococcus* resulted associated to allergy, with a significant increase (p<0.05) in Group 1 and less in Group 2 compared to Group 3 subjects. *Klebsiella* showed a higher abundance in Group 1 but not in Group 2 compared to Group 3. *Parabacteroides* and *Granulicatella* were instead more abundant in Group 3.

In a previous study, *Prevotella copri* has been indicated to possess a number of enzymes and gene clusters essential for fermentation and utilization of complex polysaccharides (155).

Furthermore, *Prevotella* has been associated with proinflammatory function. Treatment mice with *Prevotella copri* exacerbate colitis induced by dextran sulfate sodium (156).

Nakayama et al. found that in the allergic group, the genus Bacteroides at 1 month and genera *Propionibacterium* and *Klebsiella* at 2 months were more abundant, while the genera *Acinetobacter*

and *Clostridium* at 1 month were less abundant than in the non-allergic group (157). Additionally, the relative abundance of total *Proteobacteria*, excluding genus *Klebsiella*, was significantly lower in the allergic than in the non-allergic group at the age of 1 month. Allergic infants with high colonization of *Bacteroides* and/or *Klebsiella* showed less colonization of *Clostridium* within the major phylotypes, suggesting antagonism between these bacterial groups in the gut.

It is interesting that Wang L et al. linked asthma, another allergic condition, with decreased abundance of sputum Granulicatella (158) and that Chen CC et al. demonstrated the decrease of Parabacteroidetes in allergic children (84).

At phylum level, there was an increase of Verrucomicrobia during probiotic intake with a peak of abundance at T3. At T5, after 60 days since probiotic interruption, there was a rapid decrease of Verrucomicrobia abundance. Proteobacteria showed a gradually increase during probiotic intake and this increment was maintained also at T5.

On the contrary, Actinobacteria decreased during the time-course, even if there was an increase form point T1 to point T3.

Tanabe H et al. showed that the diversity of Proteobacteria and the relative abundance of Actinobacteria in maternal feces were negatively associated with dermatitis of early infancy, which may be associated with the risk of allergy development in infancy (159).

In our study at genus level, probiotic intake determined an increase of *Akkermansia*, *Prevotella* and *Ruminococcus* that was maintained also at T5. *Actinomyces*, *Enterococcus*, *Streptococcus* and *Sutterella* resulted instead diminished after probiotic intervention.

The bacterium *Akkermansia muciniphila* may represent 3–5% of the microbial composition in the healthy human intestinal tract and have a crucial role in the regulation of the gut barrier and other homeostatic and metabolic functions (160-162).

Studies have identified a loss in abundance of *A. muciniphila* in patients with obesity and type 2 diabetes (T2D) (163, 164). However, the precise physiological mechanisms affected by this bacterium during metabolic disorders and intestinal permeability regulation remain unclear.

Nevertheless, the role of *Bifidobacterium, Akkermansia*, and *Faecalibacterium* is reaffirmed again in a birth-cohort of Estonian and Finnish children followed from 3 to 36 months of age. The authors demonstrated that the composition of neonatal intestinal microbiota modulates the maturation of Treg population during the first year of life, and the risk of allergic diseases later in life. An earlier study in a US cohort identified a specific neonatal gut microbiota composition, which is characterized by a lower relative abundance of *Bifidobacterium, Akkermansia*, and *Faecalibacterium*, and a higher relative abundance of particular fungi, as a risk of atopy and asthma in a follow-up up to 4 years of age (57).

In our samples *Blautia* increased during all the period of probiotic intake, until T3 point, while at T5 it started to decrease and showed a level of abundances lower of that observed at T0.

Berni Canani et al. showed that bacterial families characteristic of the healthy infant gut (notably, Enterobactericeae and Bifidobacteriaceae) were significantly less abundant in the CMA gut and were replaced by an increase in Lachnospiraceae and Ruminococcaceae, representing an emergence of Firmicutes (particularly, Clostridiales). *Blautia, Roseburia* and *Coprococcus* were significantly enriched following treatment with hydrolyzed formula and Lactobacillus rhamnosus GG, but only one genus, *Oscillospira*, was significantly different between infants that became tolerant and those that remained allergic. However, most tolerant infants showed a significant increase in fecal butyrate levels, and those taxa that were significantly enriched in these samples, *Blautia* and *Roseburia*, exhibited specific strain-level demarcations between tolerant and allergic infants (96).

A weakness of our study can be represented by the low sample size.

Our choice to select a narrow age range (10-15 months) was dictated by the intent to reduce interindividual variability due to age and therefore make data more easily comparable.

It is demonstrated that during vaginal delivery, infants receive their first bacterial inoculum from the maternal vaginal tract, skin tissue, and often fecal matter, exposing the immature immune system of newborns to a significant bacterial load (38).

Our population doesn't show significant differences for the delivery modality and breastfeeding lasting in the three groups.

The aim of our study did not involve the possible beneficial aspects of Bifidobacteria administration on the allergy, but it was only to verify the efficacy in colonization and the persistence of the selected probiotic mixture. In these terms, we observed a clear probiotic effect only for *B. longum subsp. infantis*, that could survive during the transit in the gastrointestinal tract and to persist in the gut microbiota for all the time-course taken into exam. We don't have data about long term persistence of probiotics in gut mucosa.

Regardless of the strain, the most important characteristic for the probiotic to be effective is the capability to colonize the ecosystem in which it is introduced and that its survival in the microbiota is sufficiently durable to bring about the effects demonstrated in literature above all on animal models. Today a critical review of the very large literature related to probiotics is needed.

In the market, there is an increasing tendency to work with products with a high number of different strains. To the producers, more strains imply more chances of success; it can mean a broader spectrum of efficacy, and there is often the hope that there are at least additive and, potentially, even synergistic effects. A recent review did not find convincing evidence that these assumptions are valid. There is, however, also no strong evidence that the assumptions are incorrect and/or that there is antagonistic activity between strains in a combination. To answer these questions, structured research must be conducted. It is also important in such research to take into consideration the doses, as a combination product will have a higher total dose (165).

It is no longer sufficient to study the effects of these products afterwards by transposing them to the clinic. In our study we can't verify if the demonstrated changes in microbiota of allergic infants

provoke an improvement in the state of allergy. The guidelines suggest the verification of the state of allergy after at least 6 months from the last positive DBPFC.

Furthermore, to confirm that our results are related to the administration of probiotics, it would be necessary the perform microbiota evaluation in Group 2 and Group 3 all over the same time period.

It appears necessary to study the microbiological properties of each strain to realize plausible use hypotheses and subsequently verify their real colonization capacity.

More information could come from using the shotgun method instead of the 16s RNA method. In that way we could also obtain a functional analysis of the microbiota, coming to understand the actual functional changes of the same during the administration of probiotics.

In conclusions, early infancy is a window during which gut microbiota may shape food allergy outcomes in childhood and probiotics could be a rational way to modulate it.

Nevertheless, an “a priori” study through shotgun method of the microbiota of food allergy patients, associated with metabolomics studies could allow us to understand the real defects of intestinal microbiota in allergic children compared to healthy subjects.

Only then, by exploiting modern culturomics techniques, will it be possible to select specific strains for pathology and individual.

Therefore, through a translational approach, the goal of precision medicine in the intestinal microbiota will be achieved.

References

1. Pasqui F, Poli C, Colecchia A, Marasco G, Festi D. Adverse Food Reaction and Functional Gastrointestinal Disorders: Role of the Dietetic Approach. J Gastrointestin Liver Dis. 2015;24:319-27.

2. Valenta R, Hochwallner H, Linhart B, Pahr S. Food allergies: the basics. Gastroenterology. 2015;148:1120-31.e4.

3. Gell PGH, Coombs RRA, eds. Clinical Aspects of Immunology. 1st ed. Oxford, England: Blackwell; 1963. Section IV, Chapter 1

4. Charlesworth, E.N., Hood, A.F., Soter, N.A. et al, Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. J Clin Invest. 1989;83:1519–1526.

5. Asher M, Montefort S, Bj€orkst_en B, Lai C, Strachan D, Weiland S et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC phases one and three repeat multicountry cross-sectional surveys. Lancet 2006;368:733–743.

6. Lewis-Jones S. Quality of life and childhood atopic dermatitis: the misery of living with childhood eczema. Int J Clin Pract 2006;60:984–992.

7. Meltzer E. Quality of life in adults and children with allergic rhinitis. J Allergy Clin Immunol 2001;108:S45–S53.

8. Guilbert T, Garris C, Jhingran P, Bonafede M, Tomaszewski K, Bonus T et al. Asthma that is not well-controlled is associated with increased healthcare utilization and decreased quality of life. J Asthma 2011;48:126–132.

9. Prescott S, Allen K. Food allergy: riding the second wave of the allergy epidemic. Pediatr Allergy Immunol 2011;22:155–160.

10. Nwaru B, Hickstein L, Panesar S, Muraro A, Werfel T, Cardona V et al. The epidemiology of food allergy in Europe: a systematic review and meta-analysis. Allergy 2014;69:62–75.

11. Nwaru B, Hickstein L, Panesar S, Roberts G, Muraro A, Sheikh A. Prevalence of common food allergies in Europe: a systematic review and meta-analysis. Allergy 2014;69:992–1007.

12. Urisu A, Ebisawa M, Ito K, Aihara Y, Ito S, Mayumi M, Kohno Y, Kondo N; Committee for Japanese Pediatric Guideline for Food Allergy; Japanese Society of Pediatric Allergy and Clinical Immunology; Japanese Society of Allergology. Japanese guideline for food allergy 2014. Allergol Int 2014;63:399e419.

13. Ebisawa M, Nishima S, Ohnishi H, Kondo N. Pediatric allergy and immunology in Japan. Pediatr Allergy Immunol 2013;24:704e14.

14. Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, Holl JL. The prevalence, severity, and distribution of childhood food allergy in the United States. Pediatrics 2011;128:e9e17.

15. Host A, Halken S, Jacobsen HP, Christensen AE, Herskind AM, Plesner K. Clinical course of cow's milk protein allergy/intolerance and atopic diseases in childhood. Pediatr Allergy Immunol 2002;13(Suppl 15):23–28.

16. Saarinen KM, Vaarala O, Klemetti P, Savilahti E. Transforming growth factor-beta1 in mothers' colostrum and immune responses to cows' milk proteins in infants with cows' milk allergy. J Allergy Clin Immunol 1999;104:1093–1098.

17. Kvenshagen B, Halvorsen R, Jacobsen M. Adverse reactions to milk in infants. Acta Paediatr 2008;97:196–200.

18. Venter C, Pereira B, Grundy J, Clayton CB, Roberts G, Higgins B, Dean T. Incidence of parentally reported and clinically diagnosed food hypersensitivity in the first year of life. J Allergy Clin Immunol 2006;117:1118–1124.

19. Schrander JJ, van den Bogart JP, Forget PP, Schrander-Stumpel CT, Kuijten RH, Kester AD. Cow's milk protein intolerance in infants under 1 year of age: a prospective epidemiological study. Eur J Pediatr 1993;152:640–644.

20. Mills EN, Mackie AR, Burney P, Beyer K, Frewer L, Madsen C, Botjes E, Crevel RW, van Ree R. The prevalence, cost and basis of food allergy across Europe. Allergy 2007;62:717–722.

21. Keil T, McBride D, Grimshaw K, Niggemann B, Xepapadaki P, Zannikos K, Sigurdardottir ST, Clausen M, Reche M, Pascual C, Stanczyk AP, Kowalski ML, Dubakiene R, Drasutiene G, Roberts

G, Schoemaker AF, Sprikkelman AB, Fiocchi A, Martelli A, Dufour S, Hourihane J, Kulig M, Wjst M, Yazdanbakhsh M, Szépfalusi Z, van Ree R, Willich SN, Wahn U, Mills EN, Beyer K. The multinational birth cohort of EuroPrevall: background, aims and methods. Allergy 2010;65:482–490.

22. Luyt D, Ball H, Makwana N, Green MR, Bravin K, Nasser SM, Clark AT; Standards of Care Committee (SOCC) of the British Society for Allergy and Clinical Immunology (BSACI). BSACI guideline for the diagnosis and management of cow's milk allergy. Clin Exp Allergy. 2014:642-72.

23. Tsai HJ1, Kumar R, Pongracic J, Liu X, Story R, Yu Y, Caruso D, Costello J, Schroeder A, Fang Y, Demirtas H, Meyer KE, O'Gorman MR, Wang X. Familial aggregation of food allergy and sensitization to food allergens: a family-based study. Clin Exp Allergy. 2009;39:101-9.

24. Hong X, Tsai HJ, Wang X. Genetics of food allergy. Curr Opin Pediatr 2009;21: 770-6.

25. Tan TH, Ellis JA, Saffery R, Allen KJ. The role of genetics and environment in the rise of childhood food allergy. Clin Exp Allergy 2012;42:20-9.

26. Chen TK, Lee JH, Yu HH, et al. Association between human IL-10 gene polymorphisms and serum IL-10 level in patients with food allergy. J Formos Med Assoc 2012;111:686-92.

27. Luyt D, Ball H, Makwana N, Green MR, Bravin K, Nasser SM, Clark AT; Standards of Care Committee (SOCC) of the British Society for Allergy and Clinical Immunology (BSACI). BSACI guideline for the diagnosis and management of cow's milk allergy. Clin Exp Allergy. 2014;44(5):642-72.

28. Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Beyer K, Bindslev-Jensen C, Cardona V, Dubois A, duToit G, Eigenmann P, Fernandez Rivas M, Halken S, Hickstein L, Høst A, Knol E, Lack G, Marchisotto MJ, Niggemann B, Nwaru BI, Papadopoulos NG, Poulsen LK, Santos AF, Skypala I, Schoepfer A, Van Ree R, Venter C, Worm M, Vlieg-Boerstra B, Panesar S, de Silva D, Soares-Weiser K, Sheikh A, Ballmer-Weber BK, Nilsson C, de Jong NW, Akdis CA; EAACI Food Allergy and Anaphylaxis Guidelines Group. EAACI food allergy and anaphylaxis guidelines: diagnosis and management of food allergy. Allergy. 2014 Aug;69(8):1008-25.

29. Fiocchi A, Brozek J, Schünemann H, Bahna SL, von Berg A, Beyer K, Bozzola M, Bradsher J,

Compalati E, Ebisawa M, Guzman MA, Li H, Heine RG, Keith P, Lack G, Landi M, Martelli A, Rancé F, Sampson H, Stein A, Terracciano L, Vieths S. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. World Allergy Organ J. 2010 Apr;3(4):57-161.

30. NIH HMP Working Group, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, Deal C, Baker CC, Di Francesco V, Howcroft TK, Karp RW, Lunsford RD, Wellington CR, Belachew T, Wright M, Giblin C, David H, Mills M, Salomon R, Mullins C, Akolkar B, Begg L, Davis C, Grandison L, Humble M, Khalsa J, Little AR, Peavy H, Pontzer C, Portnoy M, Sayre MH, Starke-Reed P, Zakhari S, Read J, Watson B, Guyer M. The NIH Human Microbiome Project. Genome Res. 2009 Dec;19(12):2317-23.

31. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005 Mar 25;307(5717):1915-20.

32. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007 Oct 18;449(7164):804-10.

33. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006 Feb 24;124(4):837-48.

34. Salvucci E. Microbiome, holobiont and the net of life. Crit Rev Microbiol. 2016 May;42(3):485-94.

35. Guerrero R, Margulis L, Berlanga M. Symbiogenesis: the holobiont as a unit of evolution. Int Microbiol. 2013 Sep;16(3):133-43.

36. Frank DN, Zhu W, Sartor RB, Li E. Investigating the biological and clinical significance of human dysbioses. Trends Microbiol. 2011 Sep;19(9):427-34.

37. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial community variation in human body habitats across space and time. Science. 2009 Dec 18;326(5960):1694-7.

38. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG,

Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI. Human gut microbiome viewed across age and geography. Nature. 2012 May 9;486(7402):222-7.

39. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, Knights D, Gajer P, Ravel J, Fierer N, Gordon JI, Knight R. Moving pictures of the human microbiome. Genome Biol. 2011;12(5):R50.

40. Eberl G. Immunity by equilibrium. Nat Rev Immunol. 2016 Aug;16(8):524-32.

41. Pabst O, Mowat AM. Oral tolerance to food protein. Mucosal Immunol. 2012 May;5(3):232-9.

42. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, Lee JY, Lee M, Surh CD. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science. 2016 Feb 19;351(6275):858-63.

43. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, Tjota MY, Seo GY, Cao S, Theriault BR, Antonopoulos DA, Zhou L, Chang EB, Fu YX, Nagler CR. Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A. 2014 Sep 9;111(36):13145-50.

44. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature. 2012 Sep 13;489(7415):231-41.

45. Tulic MK, Hodder M, Forsberg A, McCarthy S, Richman T, D'Vaz N, van den Biggelaar AH, Thornton CA, Prescott SL. Differences in innate immune function between allergic and nonallergic children: new insights into immune ontogeny. J Allergy Clin Immunol. 2011 Feb;127(2):470-478.e1.

46. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005 Jul 15;122(1):107-18.

47. Berni Canani R, Gilbert JA, Nagler CR. The role of the commensal microbiota in the regulation of tolerance to dietary allergens. Curr Opin Allergy Clin Immunol. 2015 Jun;15(3):243-9.

48. Ohnmacht C, Park JH, Cording S, Wing JB, Atarashi K, Obata Y, Gaboriau-Routhiau V, Marques R, Dulauroy S, Fedoseeva M, Busslinger M, Cerf-Bensussan N, Boneca IG, Voehringer D, Hase K, Honda K, Sakaguchi S, Eberl G. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2

immunity through RORγt⁺ T cells. Science. 2015 Aug 28;349(6251):989-93.

49. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, Lee JY, Lee M, Surh CD. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science. 2016 Feb 19;351(6275):858-63.

50. Wang S, Villablanca EJ, De Calisto J, Gomes DC, Nguyen DD, Mizoguchi E, Kagan JC, Reinecker HC, Hacohen N, Nagler C, Xavier RJ, Rossi-Bergmann B, Chen YB, Blomhoff R, Snapper SB, Mora JR. MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting properties. J Immunol. 2011 Jul 1;187(1):141-50.

51. Latcham F, Merino F, Lang A, Garvey J, Thomson MA, Walker-Smith JA, Davies SE, Phillips AD, Murch SH. A consistent pattern of minor immunodeficiency and subtle enteropathy in children with multiple food allergy. J Pediatr. 2003 Jul;143(1):39-47.

52. uzuki K, Maruya M, Kawamoto S, Sitnik K, Kitamura H, Agace WW, Fagarasan S. The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut. Immunity. 2010 Jul 23;33(1):71-83.

53. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, Mackay CR. Dietary Fiber and Bacterial SCFA Enhance Oral Tolerance and Protect against Food Allergy through Diverse Cellular Pathways. Cell Rep. 2016 Jun 21;15(12):2809-24.

54. Diesner SC, Knittelfelder R, Krishnamurthy D, Pali-Schöll I, Gajdzik L, Jensen-Jarolim E, Untersmayr E. Dose-dependent food allergy induction against ovalbumin under acid-suppression: a murine food allergy model. Immunol Lett. 2008 Nov 16;121(1):45-51.

55. Untersmayr E, Diesner SC, Oostingh GJ, Selzle K, Pfaller T, Schultz C, Zhang Y, Krishnamurthy D, Starkl P, Knittelfelder R, Förster-Waldl E, Pollak A, Scheiner O, Pöschl U, Jensen-Jarolim E, Duschl A. Nitration of the egg-allergen ovalbumin enhances protein allergenicity but reduces the risk for oral sensitization in a murine model of food allergy. PLoS One. 2010 Dec 2;5(12):e14210.

56. Diesner SC, Bergmayr C, Pfitzner B, Assmann V, Krishnamurthy D, Starkl P, Endesfelder D, Rothballer M, Welzl G, Rattei T, Eiwegger T, Szépfalusi Z, Fehrenbach H, Jensen-Jarolim E,

Hartmann A, Pali-Schöll I, Untersmayr E. A distinct microbiota composition is associated with protection from food allergy in an oral mouse immunization model. Clin Immunol. 2016 Dec;173:10-18.

57. Fujimura KE, Sitarik AR, Havstad S, Lin DL, Levan S, Fadrosh D, Panzer AR, LaMere B, Rackaityte E, Lukacs NW, Wegienka G, Boushey HA, Ownby DR, Zoratti EM, Levin AM, Johnson CC, Lynch SV. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med. 2016 Oct;22(10):1187-1191.

58. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN, Siebert R, Baron RM, Kasper DL, Blumberg RS. Microbial exposure during early life has persistent effects on natural killer T cell function. Science. 2012 Apr 27;336(6080):489-93.

59. Huang YJ, Marsland BJ, Bunyavanich S, O'Mahony L, Leung DY, Muraro A, Fleisher TA. The microbiome in allergic disease: Current understanding and future opportunities-2017 PRACTALL document of the American Academy of Allergy, Asthma & Immunology and the European Academy of Allergy and Clinical Immunology. J Allergy Clin Immunol. 2017 Apr;139(4):1099-1110.

60. Wesemann DR, Portuguese AJ, Meyers RM, Gallagher MP, Cluff-Jones K, Magee JM, Panchakshari RA, Rodig SJ, Kepler TB, Alt FW. Microbial colonization influences early B-lineage development in the gut lamina propria. Nature. 2013 Sep 5;501(7465):112-5.

61. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. Immunity. 2015 Apr 21;42(4):607-12.

62. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Söllner S, Akdis DG, Rückert B, Akdis CA, Akdis M. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. J Allergy Clin Immunol. 2013 Apr;131(4):1204-12.

63. Liu ZQ, Wu Y, Song JP, Liu X, Liu Z, Zheng PY, Yang PC. Tolerogenic CX3CR1+ B cells suppress food allergy-induced intestinal inflammation in mice. Allergy. 2013 Oct;68(10):1241-8.

64. Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine

model. J Allergy Clin Immunol. 2010 May;125(5):1114-1124.e8.

65. Tait Wojno ED, Artis D. Emerging concepts and future challenges in innate lymphoid cell biology. J Exp Med. 2016 Oct 17;213(11):2229-2248.

66. Eyerich K, Dimartino V, Cavani A. IL-17 and IL-22 in immunity: Driving protection and pathology. Eur J Immunol. 2017 Apr;47(4):607-614.

67. Plunkett CH, Nagler CR. The Influence of the Microbiome on Allergic Sensitization to Food. J Immunol. 2017 Jan 15;198(2):581-589.

68. Papathoma E, Triga M, Fouzas S, Dimitriou G. Cesarean section delivery and development of food allergy and atopic dermatitis in early childhood. Pediatr Allergy Immunol. 2016 Jun;27(4):419-24.

69. Muraro A, Halken S, Arshad SH, Beyer K, Dubois AE, Du Toit G, Eigenmann PA, Grimshaw KE, Hoest A, Lack G, O'Mahony L, Papadopoulos NG, Panesar S, Prescott S, Roberts G, de Silva D, Venter C, Verhasselt V, Akdis AC, Sheikh A; EAACI Food Allergy and Anaphylaxis Guidelines Group. EAACI food allergy and anaphylaxis guidelines. Primary prevention of food allergy. Allergy. 2014 May;69(5):590-601.

70. Trikha A, Baillargeon JG, Kuo YF, Tan A, Pierson K, Sharma G, Wilkinson G, Bonds RS. Development of food allergies in patients with gastroesophageal reflux disease treated with gastric acid suppressive medications. Pediatr Allergy Immunol. 2013 Sep;24(6):582-8.

71. Grimshaw KE, Maskell J, Oliver EM, Morris RC, Foote KD, Mills EN, Margetts BM, Roberts G. Diet and food allergy development during infancy: birth cohort study findings using prospective food diary data. J Allergy Clin Immunol. 2014 Feb;133(2):511-9.

72. Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, Kim SG, Li H, Gao Z, Mahana D, Zárate Rodriguez JG, Rogers AB, Robine N, Loke P, Blaser MJ. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell. 2014 Aug 14;158(4):705-721.

73. Metsälä J, Lundqvist A, Virta LJ, Kaila M, Gissler M, Virtanen SM. Mother's and offspring's use of antibiotics and infant allergy to cow's milk. Epidemiology. 2013 Mar;24(2):303-9.

74. Marrs T, Bruce KD, Logan K, Rivett DW, Perkin MR, Lack G, Flohr C. Is there an association between microbial exposure and food allergy? A systematic review. Pediatr Allergy Immunol. 2013 Jun;24(4):311-320.e8.

75. Thompson-Chagoyan OC, Vieites JM, Maldonado J, Edwards C, Gil A. Changes in faecal microbiota of infants with cow's milk protein allergy--a Spanish prospective case-control 6-month follow-up study. Pediatr Allergy Immunol. 2010 Mar;21(2 Pt 2):e394-400.

76. Adlerberth I, Strachan DP, Matricardi PM, Ahrné S, Orfei L, Aberg N, Perkin MR, Tripodi S, Hesselmar B, Saalman R, Coates AR, Bonanno CL, Panetta V, Wold AE. Gut microbiota and development of atopic eczema in 3 European birth cohorts. J Allergy Clin Immunol. 2007 Aug;120(2):343-50.

77. Kendler M, Uter W, Rueffer A, Shimshoni R, Jecht E. Comparison of fecal microflora in children with atopic eczema/dermatitis syndrome according to IgE sensitization to food. Pediatr Allergy Immunol. 2006 Mar;17(2):141-7.

78. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, Kuzeljevic B, Gold MJ, Britton HM, Lefebvre DL, Subbarao P, Mandhane P, Becker A, McNagny KM, Sears MR, Kollmann T; CHILD Study Investigators, Mohn WW, Turvey SE, Finlay BB. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med. 2015 Sep 30;7(307):307ra152.

79. Nakayama J, Kobayashi T, Tanaka S, Korenori Y, Tateyama A, Sakamoto N, Kiyohara C, Shirakawa T, Sonomoto K. Aberrant structures of fecal bacterial community in allergic infants profiled by 16S rRNA gene pyrosequencing. FEMS Immunol Med Microbiol. 2011 Dec;63(3):397-406.

80. Duncan SH, Louis P, Thomson JM, Flint HJ. The role of pH in determining the species composition of the human colonic microbiota. Environ Microbiol. 2009 Aug;11(8):2112-22.

81. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, Mandhane PJ, Turvey SE, Subbarao P, Becker AB, Scott JA, Kozyrskyj AL; CHILD Study Investigators. Infant gut microbiota and food sensitization: associations in the first year of life. Clin Exp Allergy. 2015 Mar;45(3):632-43.

82. Hua X, Goedert JJ, Pu A, Yu G, Shi J. Allergy associations with the adult fecal microbiota: Analysis of the American Gut Project. EBioMedicine. 2015 Nov 27;3:172-179.

83. Ling Z, Li Z, Liu X, Cheng Y, Luo Y, Tong X, Yuan L, Wang Y, Sun J, Li L, Xiang C. Altered fecal microbiota composition associated with food allergy in infants. Appl Environ Microbiol. 2014 Apr;80(8):2546-54.

84. Chen CC, Chen KJ, Kong MS, Chang HJ, Huang JL. Alterations in the gut microbiotas of children with food sensitization in early life. Pediatr Allergy Immunol. 2016 May;27(3):254-62.

85. Bunyavanich S, Shen N, Grishin A, Wood R, Burks W, Dawson P, Jones SM, Leung DYM, Sampson H, Sicherer S, Clemente JC. Early-life gut microbiome composition and milk allergy resolution. J Allergy Clin Immunol. 2016 Oct;138(4):1122-1130.

86. Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JA. The phylogeny of the genus Clostridium: proposal offive new genera and eleven new species combinations. Int J Syst Bacteriol. 1994 Oct;44(4):812-26.

87. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, Honda K. Induction of colonic regulatory T cells by indigenous Clostridium species. Science. 2011 Jan 21;331(6015):337-41.

88. Xie X, Stubbington MJ, Nissen JK, Andersen KG, Hebenstreit D, Teichmann SA, Betz AG. The Regulatory T Cell Lineage Factor Foxp3 Regulates Gene Expression through Several Distinct Mechanisms Mostly Independent of Direct DNA Binding. PLoS Genet. 2015 Jun 24;11(6):e1005251.

89. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi

S, Taniguchi T, Morita H, Hattori M, Honda K. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013 Aug 8;500(7461):232-6.

90. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013 Dec 19;504(7480):451-5.

91. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013 Dec 19;504(7480):446-50.

92. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013 Aug 2;341(6145):569-73.

93. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol. 2011 Jan;12(1):5-9.

94. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, Weir TL, Ehrentraut SF, Pickel C, Kuhn KA, Lanis JM, Nguyen V, Taylor CT, Colgan SP. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe. 2015 May 13;17(5):662-71.

95. Sandin A, Bråbäck L, Norin E, Björkstén B. Faecal short chain fatty acid pattern and allergy in early childhood. Acta Paediatr. 2009 May;98(5):823-7.

96. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, Calignano A, Khan AA, Gilbert JA, Nagler CR. Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J. 2016 Mar;10(3):742-50.

97. Geuking MB, McCoy KD, Macpherson AJ. Metabolites from intestinal microbes shape Treg. Cell Res. 2013 Dec;23(12):1339-40.

98. Song H, Yoo Y, Hwang J, Na YC, Kim HS. Faecalibacterium prausnitzii subspecies-level dysbiosis in the human gut microbiome underlying atopic dermatitis. J Allergy Clin Immunol. 2016 Mar;137(3):852-60.

99. Franzosa EA, Morgan XC, Segata N, Waldron L, Reyes J, Earl AM, Giannoukos G, Boylan MR, Ciulla D, Gevers D, Izard J, Garrett WS, Chan AT, Huttenhower C.
Relating the metatranscriptome and metagenome of the human gut. Proc Natl Acad Sci U S A. 2014 Jun 3;111(22):E2329-38.

100. Sellitto M, Bai G, Serena G, Fricke WF, Sturgeon C, Gajer P, White JR, Koenig SS, Sakamoto J, Boothe D, Gicquelais R, Kryszak D, Puppa E, Catassi C, Ravel J, Fasano A. Proof of concept of microbiome-metabolome analysis and delayed gluten exposure on celiac disease autoimmunity in genetically at-risk infants. PLoS One. 2012;7(3):e33387.

101. Stewart CJ, Skeath T, Nelson A, Fernstad SJ, Marrs EC, Perry JD, Cummings SP, Berrington JE, Embleton ND. Preterm gut microbiota and metabolome following discharge from intensive care. Sci Rep. 2015 Nov 24;5:17141.

102. Sweileh WM, Shraim NY, Al-Jabi SW, Sawalha AF, Rahhal B, Khayyat RA, Zyoud SH. Assessing worldwide research activity on probiotics in pediatrics using Scopus database: 1994-2014. World Allergy Organ J. 2016 Jul 25;9:25

103. van Loveren H, Sanz Y, Salminen S. Health claims in Europe: probiotics and prebiotics as case examples. Annu Rev Food Sci Technol. 2012;3:247-61

104. Elazab N, Mendy A, Gasana J, Vieira ER, Quizon A, Forno E. Probiotic administration in early life, atopy, and asthma: a meta-analysis of clinical trials. Pediatrics. 2013 Sep;132(3):e666-76.

105. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. J Allergy Clin Immunol. 2015 Mar;135(3):737-44.e8.

106. Hol J, van Leer EH, Elink Schuurman BE, de Ruiter LF, Samsom JN, Hop W, Neijens HJ, de Jongste JC, Nieuwenhuis EE; Cow's Milk Allergy Modified by Elimination and Lactobacilli study group. The acquisition of tolerance toward cow's milk through probiotic supplementation: a randomized, controlled trial. J Allergy Clin Immunol. 2008 Jun;121(6):1448-54.

107. Berni Canani R, Nocerino R, Terrin G, Coruzzo A, Cosenza L, Leone L, Troncone R. Effect of Lactobacillus GG on tolerance acquisition in infants with cow's milk allergy: a randomized trial. J Allergy Clin Immunol. 2012 Feb;129(2):580-2, 582.e1-5.

108. Berni Canani R, Nocerino R, Terrin G, Frediani T, Lucarelli S, Cosenza L, Passariello A, Leone L, Granata V, Di Costanzo M, Pezzella V, Troncone R. Formula selection for management of children with cow's milk allergy influences the rate of acquisition of tolerance: a prospective multicenter study. J Pediatr. 2013 Sep;163(3):771-7.e1.

109. Berni Canani R, Di Costanzo M, Bedogni G, Amoroso A, Cosenza L, Di Scala C, Granata V, Nocerino R. Extensively hydrolyzed casein formula containing Lactobacillus rhamnosus GG reduces the occurrence of other allergic manifestations in children with cow's milk allergy: 3-year randomized controlled trial. J Allergy Clin Immunol. 2017 Jun;139(6):1906-1913.e4.

110. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, Hancock WW. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med. 2007 Nov;13(11):1299-307.

111. Berni Canani R, Paparo L, Nocerino R, Cosenza L, Pezzella V, Di Costanzo M, Capasso M, Del Monaco V, D'Argenio V, Greco L, Salvatore F. Differences in DNA methylation profile of Th1 and Th2 cytokine genes are associated with tolerance acquisition in children with IgE-mediated cow's milk allergy. Clin Epigenetics. 2015 Mar 31;7:38.

112. Paparo L, Nocerino R, Cosenza L, Aitoro R, D'Argenio V, Del Monaco V, Di Scala C, Amoroso A, Di Costanzo M, Salvatore F, Berni Canani R. Epigenetic features of FoxP3 in children with cow's milk allergy. Clin Epigenetics. 2016 Aug 12;8:86.

113. Cheng RY, Yao JR, Wan Q, Guo JW, Pu FF, Shi L, Hu W, Yang YH, Li L, Li M, He F. Oral administration of Bifidobacterium bifidum TMC3115 to neonatal mice may alleviate IgE-mediated allergic risk in adulthood. Benef Microbes. 2018 Jun 11:1-14

114. de Kivit S, Kostadinova AI, Kerperien J, Morgan ME, Muruzabal VA, Hofman GA, Knippels LMJ, Kraneveld AD, Garssen J, Willemsen LEM. Dietary, nondigestible oligosaccharides and Bifidobacterium breve M-16V suppress allergic inflammation in intestine via targeting dendritic cell maturation. J Leukoc Biol. 2017 Jul;102(1):105-115

115. Liu MY, Yang ZY, Dai WK, Huang JQ, Li YH, Zhang J, Qiu CZ, Wei C, Zhou Q, Sun X, Feng X, Li DF, Wang HP, Zheng YJ. Protective effect of Bifidobacterium infantis CGMCC313-2 on ovalbumin-induced airway asthma and β-lactoglobulin-induced intestinal food allergy mouse models. World J Gastroenterol. 2017 Mar 28;23(12):2149-2158

116. Kostadinova AI, Meulenbroek LA, van Esch BC, Hofman GA, Garssen J, Willemsen LE, Knippels LM. A Specific Mixture of Fructo-Oligosaccharides and Bifidobacterium breve M-16V Facilitates Partial Non-Responsiveness to Whey Protein in Mice Orally Exposed to β-Lactoglobulin-Derived Peptides. Front Immunol. 2017 Jan 12;7:673

117. van Esch BC, Abbring S, Diks MA, Dingjan GM, Harthoorn LF, Vos AP, Garssen J. Post-sensitization administration of non-digestible oligosaccharides and Bifidobacterium breve M-16V reduces allergic symptoms in mice. Immun Inflamm Dis. 2016 Mar 24;4(2):155-165

118. Garrido D, Ruiz-Moyano S, Mills DA. Release and utilization of N-acetyl-D-glucosamine from human milk oligosaccharides by Bifidobacterium longum subsp. infantis. Anaerobe. 2012 Aug;18(4):430-5.

119. Tannock GW. The bowel microbiota and inflammatory bowel diseases. Int J Inflam. 2010 Aug 5;2010:954051.

120. Arboleya S, Stanton C, Ryan CA, Dempsey E, Ross PR. Bosom Buddies: The Symbiotic Relationship Between Infants and Bifidobacterium longum ssp. longum and ssp. infantis. Genetic and Probiotic Features. Annu Rev Food Sci Technol. 2016;7:1-21

121. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335-6.

122. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006 Jul;72(7):5069-72.

123. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013 Apr 22;8(4):e61217.

124. Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri Y, Kumemura M. Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. J Allergy Clin Immunol. 2003 Mar;111(3):587-91.

125. Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, Masetti R, Ricci G, Pession A, Brigidi P. Unbalance of intestinal microbiota in atopic children. BMC Microbiol. 2012 Jun 6;12:95.

126. Nylund L, Satokari R, Nikkilä J, Rajilić-Stojanović M, Kalliomäki M, Isolauri E, Salminen S, de Vos WM. Microarray analysis reveals marked intestinal microbiota aberrancy in infants having eczema compared to healthy children in at-risk for atopic disease. BMC Microbiol. 2013 Jan 23;13:12.

127. Parvez S, Malik KA, Ah Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. J Appl Microbiol. 2006 Jun;100(6):1171-85.

128. Vitali B, Ndagijimana M, Cruciani F, Carnevali P, Candela M, Guerzoni ME, Brigidi P. Impact of a synbiotic food on the gut microbial ecology and metabolic profiles. BMC Microbiol. 2010 Jan 7;10:4.

129. Kerckhoffs AP, Samsom M, van der Rest ME, de Vogel J, Knol J, Ben-Amor K, Akkermans LM. Lower Bifidobacteria counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients. World J Gastroenterol. 2009 Jun 21;15(23):2887-92.

130. Wang IK, Lai HC, Yu CJ, Liang CC, Chang CT, Kuo HL, Yang YF, Lin CC, Lin HH, Liu YL, Chang YC, Wu YY, Chen CH, Li CY, Chuang FR, Huang CC, Lin CH, Lin HC.
Real-time PCR analysis of the intestinal microbiotas in peritoneal dialysis patients. Appl Environ Microbiol. 2012 Feb;78(4):1107-12.

131. Rajilić-Stojanović M, Shanahan F, Guarner F, de Vos WM. Phylogenetic analysis of dysbiosis in ulcerative colitis during remission. Inflamm Bowel Dis. 2013 Mar;19(3):481-8.

132. Kolaček S, Hojsak I, Berni Canani R, Guarino A, Indrio F, Orel R, Pot B, Shamir R, Szajewska H, Vandenplas Y, van Goudoever J, Weizman Z; ESPGHAN Working Group for Probiotics and Prebiotics. Commercial Probiotic Products: A Call for Improved Quality Control. A Position Paper by the ESPGHAN Working Group for Probiotics and Prebiotics. J Pediatr Gastroenterol Nutr. 2017 Jul;65(1):117-124.

133. Miraglia Del Giudice M, Indolfi C, Capasso M, Maiello N, Decimo F, Ciprandi G. Bifidobacterium mixture (B longum BB536, B infantis M-63, B breve M-16V) treatment in children with seasonal allergic rhinitis and intermittent asthma. Ital J Pediatr. 2017 Mar 7;43(1):25.

134. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl. 2003 Sep;91(441):48-55.

135. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG,Welling GW. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr. 2000 Jan;30(1):61-7.

136. Favier CF, Vaughan EE, De Vos WM, Akkermans AD. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol. 2002 Jan;68(1):219-26.

137. Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D. Getting better with bifidobacteria. J Appl Microbiol. 2005;98(6):1303-15.

138. Ventura M, van Sinderen D, Fitzgerald GF, Zink R. Insights into the taxonomy, genetics and physiology of bifidobacteria. Antonie Van Leeuwenhoek. 2004 Oct;86(3):205-23.

139. Kai-Larsen Y, Bergsson G, Gudmundsson GH, Printz G, Jörnvall H, Marchini G, Agerberth B. Antimicrobial components of the neonatal gut affected upon colonization. Pediatr Res. 2007 May;61(5 Pt 1):530-6.

140. Renz H, Brandtzaeg P, Hornef M. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat Rev Immunol. 2011 Dec 9;12(1):9-23.

141. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A. 2002 Oct 29;99(22):14422-7. Epub 2002 Oct 15. Erratum in: Proc Natl Acad Sci U S A. 2005 Jun 28;102(26):9430.

142. Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sánchez B, Bidossi A, Ferrarini A, Giubellini V, Delledonne M, Henrissat B, Coutinho P, Oggioni M, Fitzgerald GF, Mills D, Margolles A, Kelly D, van Sinderen D, Ventura M. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A. 2010 Nov 9;107(45):19514-9.

143. Sela DA. Bifidobacterial utilization of human milk oligosaccharides. Int J Food Microbiol. 2011 Sep 1;149(1):58-64.

144. Duranti S, Milani C, Lugli GA, Turroni F, Mancabelli L, Sanchez B, Ferrario C,Viappiani A, Mangifesta M, Mancino W, Gueimonde M, Margolles A, van Sinderen D, Ventura M. Insights from genomes of representatives of the human gut commensal Bifidobacterium bifidum. Environ Microbiol. 2015 Jul;17(7):2515-31.

145. Egan M, Motherway MO, Kilcoyne M, Kane M, Joshi L, Ventura M, van Sinderen D. Cross-feeding by Bifidobacterium breve UCC2003 during co-cultivation with Bifidobacterium bifidum PRL2010 in a mucin-based medium. BMC Microbiol. 2014 Nov 25;14:282.

146. Broekaert WF, Courtin CM, Verbeke K, Van de Wiele T, Verstraete W, Delcour JA. Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Crit Rev Food Sci Nutr. 2011 Feb;51(2):178-94.

147. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 2011 Jan 27;469(7331):543-7.

148. Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a more comprehensive concept for prebiotics. Nat Rev Gastroenterol Hepatol. 2015 May;12(5):303-10.

149. Rivière A, Gagnon M, Weckx S, Roy D, De Vuyst L. Mutual Cross-Feeding Interactions between Bifidobacterium longum subsp. longum NCC2705 and Eubacterium rectale ATCC 33656 Explain the Bifidogenic and Butyrogenic Effects of Arabinoxylan Oligosaccharides. Appl Environ Microbiol. 2015 Nov;81(22):7767-81.

150. Fieten KB, Totté JEE, Levin E, Reyman M, Meijer Y, Knulst A, Schuren F, Pasmans SGMA. Fecal Microbiome and Food Allergy in Pediatric Atopic Dermatitis: A Cross-Sectional Pilot Study. Int Arch Allergy Immunol. 2018;175(1-2):77-84

151. Preidis GA, Saulnier DM, Blutt SE, Mistretta TA, Riehle KP, Major AM, Venable SF, Finegold MJ, Petrosino JF, Conner ME, Versalovic J. Probiotics stimulate enterocyte migration and microbial diversity in the neonatal mouse intestine. FASEB J. 2012 May;26(5):1960-9.

152. Eisenhauer N, Scheu S, Jousset A. Bacterial diversity stabilizes community productivity. PLoS One. 2012;7(3):e34517.

153. Fujio-Vejar S, Vasquez Y, Morales P, Magne F, Vera-Wolf P, Ugalde JA, Navarrete P, Gotteland M. The Gut Microbiota of Healthy Chilean Subjects Reveals a High Abundance of the Phylum Verrucomicrobia. Front Microbiol. 2017 Jun 30;8:1221

154. Chen CC, Chen KJ, Kong MS, Chang HJ, Huang JL. Alterations in the gut microbiotas of children with food sensitization in early life. Pediatr Allergy Immunol. 2016 May;27(3):254-62.

155. Dodd D, Mackie RI, Cann IK. Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. Mol Microbiol. 2011 Jan;79(2):292-304.

157. Nakayama J, Kobayashi T, Tanaka S, Korenori Y, Tateyama A, Sakamoto N, Kiyohara C, Shirakawa T, Sonomoto K. Aberrant structures of fecal bacterial community in allergic infants profiled by 16S rRNA gene pyrosequencing. FEMS Immunol Med Microbiol. 2011 Dec;63(3):397-406.

158. Wang L, de Ángel Solá D, Mao Y, Bielecki P, Zhu Y, Sun Z, Shan L, Flavell RA, Bazzy-Asaad A, DeWan A. Family-based study reveals decreased abundance of sputum Granulicatella in asthmatics. Allergy. 2018 Sep;73(9):1918-1921.

159. Tanabe H, Sakurai K, Kato T, Kawasaki Y, Nakano T, Yamaide F, Taguchi-Atarashi N, Watanabe M, Ochiai S, Ohno H, Fukuoka H, Shimojo N, Mori C. Association of the maternal microbiome in Japanese pregnant women with the cumulative prevalence of dermatitis in early infancy: A pilot study from the Chiba study of Mother and Child Health birth cohort. World Allergy Organ J. 2019 Nov 1;12(10):100065.

160. Belzer C, de Vos WM. Microbes inside--from diversity to function: the case of Akkermansia. ISME J. 2012 Aug;6(8):1449-58.

161. Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol. 2004 Sep;54(Pt 5):1469-76.

162. Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S. Intestinal integrity and Akkermansia muciniphila, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol. 2007 Dec;73(23):7767-70.

163. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, Guiot Y, Derrien M, Muccioli GG, Delzenne NM, de Vos WM, Cani PD. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A. 2013 May 28;110(22):9066-71.

164. Derrien M, Van Baarlen P, Hooiveld G, Norin E, Müller M, de Vos WM. Modulation of Mucosal Immune Response, Tolerance, and Proliferation in Mice Colonized by the Mucin Degrader Akkermansia muciniphila. Front Microbiol. 2011 Aug 1;2:166.

165. Ouwehand AC, Invernici MM, Furlaneto FAC, Messora MR. Effectiveness of Multistrain Versus Single-strain Probiotics: Current Status and Recommendations for the Future. J Clin Gastroenterol. 2018 May 4