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"Diet, Microbiota and Epigenetics as target for innovative strategies against Food Allergy"

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TABLE OF CONTENTS

9. FIGURE LEGENDS

10. FIGURES

11. PUBLICATIONS IN THE LAST 3 YEARS

1. Introduction

1.1 The changing scenario of food allergy

Prevalence of allergies among children has become an increasing problem in the last few decades.[1] Food allergy (FA) is an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food.[2]

Studies have suggested that the epidemiology of FA has shown a notable increase in the severity of clinical manifestation, prevalence and risk of persistence into later ages, causing an increase in medical visits, hospital admissions, treatment, burden of care on families and healthcare system over the past twenty years.

Epidemiological data of the last decades showed a 7-fold increase in hospital admissions for a food severe allergic reactions in children in the UK, Italy, Usa and Australia over the last 10 years.[3,4] FA is one of the most common causes of anaphylaxis outside the hospital setting.[5,6] Milk, eggs, peanuts, fish, shellfish, wheat, soy, nuts and seeds and other foods have been identified as triggers of FA.[7,4,8]

In Australia Caraballo et al. have observed the highest prevalence of FA, reaching 10% of the infant population.[9,10] FA mostly affects children[11] but arising number of elderly subjects also have symptoms of FA.[12] Age-related gender differences are reported in FA.[13] For a long time, FA has been considered a pediatric disease because in the greater part of cases it begins in childhood and disappears with growth. Though, the current exponential growth of the adult and elderly population, especially in industrialized countries, and the environmental and lifestyle changes, have profoundly changed the epidemiology of FA with an increasing even in advanced age. A rising prevalence of FA has been shown in developing countries. Incidence rates similar to those of Western countries

have been reported in China and Africa. The economic growth of countries like China and the expansion of the phenomenon of globalization point to a future increase of FA prevalence.

1.2 Natural history of food allergy

Many subjects with FA naturally outgrow it over time; however, the natural course highly depends on the causative allergen. Cow's milk allergy (CMA), hen's egg and wheat allergy approximately resolve in 50% of children by the age of 5-10 years. Other FAs (including peanut, tree nut, fish) have low rates of resolution or are considered persistent.[14] In addition, many forms of FA, including CMA, may be associated with later development of other allergic diseases such as asthma, oculorhinitis, urticaria, and atopic dermatitis (the so called "Atopic March"),[15] as well as other diseases such as functional gastrointestinal disorders (FGIDs),[16] inflammatory bowel diseases (IBD),[17] and psychiatric disorders, such as attention deficit hyperactivity disorder (ADHD), autistic spectrum disorders (ASD) and obsessive-compulsive disorder (OCD).[18] The pathogenesis of these events is still largely unknown, but increasing evidence suggest that perturbation of gut microbiome, leading to alterations in immune system and gut-brain axis, could influence the occurrence of FA and FA-related conditions later in the life (Figure 1).

1.3 New insights in the pathogenesis of food allergy

FA derives from a breakdown of immune tolerance.[19] Induction and maintenance of tolerance to food antigens requires active generation of food antigens specific regulatory T cells (Tregs), which are influenced by the resident microbiome.[20,21] Current knowledge suggests that the epidemiology of FA might be influenced by genetics and genome-environment interactions leading to immune-system dysfunction, mediated at least in part by epigenetic mechanisms.[22,23]

There is mounting evidence that the alterations of gut microbiota composition (dysbiosis) early in life play a key role in early host immunological development and represents a critical factor underlying FA and occurrence of other allergic and not allergic diseases later in the life.[24,25,26,27,28,29]

Many epidemiologic data suggest a link between environmental factors able to influence gut microbiota composition and function, and the occurrence of FA (Figure 2). But these data only support the notion that several factors potentially influencing gut microbiota may be key risk modifiers for the development of FA. Unfortunately, data characterizing the microbiota of patients with FA are still preliminary. Table 1 summarizes main evidences on FA-associated gut microbiota features. Heterogeneity in study design, including sampling time points, methods used to characterize the gut microbiota, and different allergic phenotypes under study, make it difficult to establish a causal relation between specific bacterial taxa and development of FA. Despite these limitations at least 4 relevant observations on FA-associated gut microbiota can be raised:

- Dysbiosis precedes the FA onset;
- Microbial community structure early in life, in particular in the first 6 months of life, is more relevant in FA development;
- No specific bacterial taxa could be consistently associated with FA onset, with a broad range of microbes that could have positive or negative influence on tolerogenic mechanisms;
- Dysbiosis could influence not only the occurrence, but also the course of disease of FA. [27]

Many factors have been postulated to contribute to the onset of FA. Among the multiple immutable risk factors that could influence FA onset, there are the male sex, ethnicity (increased risk among Asian and black children compared with white children), and genetics (familial associations, HLA, and specific genes).[30,31,32,33,34] In addition, there are other risk factors that can be potentially addressed to reduce/prevent FA. These factors are related (mode of delivery, breast milk, use of acid-suppressive medications or antibiotics, use of antiseptic agents, rural environment, junk food-

based and/or low fibers/high fat diet, consumption of unpasteurized milk or fermented foods, exposure to pets) or unrelated (comorbid atopic dermatitis, vitamin D insufficiency, reduced consumption of omega-3-polyunsaturated fatty acids or antioxidants, timing and route of exposure to foods) to an influence on gut microbiota development and function. [24,35,25]

In the 1989 the idea that there might be a linking between the allergy epidemic and reduced microbial exposure was first proposed by David Strachan formulated the hygiene hypothesis, it suggested that a lower frequency of infections in early childhood could explain the rise in atopic diseases during the twentieth century:[36] later on this hypothesis was refined and it suggests that it is not the infections that protect against allergies; rather, the allergy epidemic is a result of a loss of sufficient interaction with health-promoting microbes which have been present through thousands of years of common evolution and the evolvement of the mammalian immune system. Von Hertzen and colleagues expanded the hygiene hypothesis in 2010 into the biodiversity hypothesis, which suggested that, in addition to microbes in the home, in food, in drinking water, and on domestic animals, microbes of the living environment, in general may play a key role in shaping the composition of microbial communities on the skin in the respiratory system and in the gut, with consequences for physiology and health.[37]

Even though the hygiene and biodiversity hypotheses and data from epidemiologic studies sustain that situation associated to differential microbial exposures are linked with allergy and FA risk, studies that have considered microbial diversity and composition in populations with and without FA provide direct evidence that GM differ in individuals with FA.

Several evidence indicates that the alterations of GM composition early in life has an important role in early host immunological development and represents a significant factor underlying FA and incidence of other allergic and not allergic diseases later in the life.[34,25,38,26] Azad and colleagues have shown elevated *Enterobacteriaceae/Bacteroidetes* ratio and a low GM richness in infant stool to be related with increased risk of food sensitization in the population-based Canadian Healthy

Infant Longitudinal Development (CHILD) birth cohort study. They have found food-sensitized infants to have decreased abundance of *Ruminococcaceae* at 1 year. Arrieta et al. have shown that microbial alpha diversity did not differentiate atopic children from control children in a larger subset of CHILD study. They have found that a reduction of 4 genera such as *Lachnospira, Faecalibacterium, Veillonella* and *Rothia* in the faecal microbiome at age 3 months was predictive of atopic disease later in life while Cait et al. have found that only *Faecalibacterium* and *Veillonella* were predictive of atopic disease.[28] In a recent work Roduit et al. found that children with the highest levels of butyrate and propionate (≥95th percentile) in feces at the age of one year had significantly less atopic sensitization and were less likely to have asthma between 3 and 6 years and children with the highest levels of butyrate were also less likely to have a reported diagnosis of food allergy or allergic rhinitis.[29] Goldberg and collegues demonstrated a link between IgE-mediated FA and the composition and metabolic activity of the gut microbiota, to characterize the gut microbiota composition and short-chain fatty acid (SCFAs) profiles associated with major food allergy groups, they recruited 233 patients with FA including milk, sesame, peanut, and tree nuts, and non-allergic controls. They found that the gut microbiota composition of allergic patients was significantly different compared to age-matched controls both in α-diversity and β-diversity. Distinct microbial signatures were observed for different type of FAs.

Prevotella copri (*P. copri*) was the most overrepresented species in non-allergic controls. SCFAs levels were significantly higher in the non-allergic compared to the FA groups, whereas *P. copri* significantly correlated with all three SCFAs.[39]

Experimental data from animal model suggest a connection between environmental factors able to influence GM composition and function, and the occurrence of FA. Treg was found reduced in mice treated with antibiotic or in germ free mice, with following inclination to allergy development.[40,41,42] Administration of defined *Clostridia*, or bacteria-derived SCFA to germ free mice induced a growth of Treg cells number, and reduced allergic response.[40,43,44] Several studies

in the animal model have shown the allergy-protective action of *Clostridia*, where a considerable protective effect consisting in regulation of innate lymphoid cell function, Foxp3+ Tregs, immunoglobulin (Ig)A and intestinal epithelial permeability was demonstrated.[20] A "humanized mice model" created with inoculation of microbiota-derived from human feces, resulted in an increase in Treg cells and a decrease of allergic symptoms.[45] The functional role of dysbiosis associated with FA was also exposed by the different capacity of the GM of allergen-sensitized mice to increase Th2 cells number and IgE responses and to promote allergic sensitization.[19]

A rising concept is the effect elicited by different dietary intervention in early life on modulation of the composition and diversity of GM. A modern study demonstrated that infants with non IgE-mediated CMA that consumed vegetable protein-based formulas (soy and rice based formula) showed low abundance of *Coriobacteriaceae*. In particular, those fed with rice formulas presented low abundances of representatives of *Coriobacteriaceae* and *Bifidobacteriaceae* in their fecal samples. Contrary, *Coriobacteriaceae*, and the genus *Collinsella*, the major lactose utilizers in the human gut, have been present in higher ratio in infants with non IgE-mediated CMA children that have consumed an extensively hydrolyzed formula. An encouraging correlation was found between *Coriobacteriaceae* and butyrate levels in non IgE-mediated CMA children that consumed an extensively hydrolyzed formula. This may be described by the stimulation of certain butyrate producers through cross-feeding mechanisms that are triggered by end fermentation metabolites, which are produced by *Coriobacteriaceae*. [46] We have demonstrated a GM dysbiosis in non-IgE-mediated CMA, driven by an enrichment of *Bacteroides* and *Alistipes* and these specific signatures coinciding with GM dysbiosis of IgE-mediated CMA children,[47] characterized by a progressive increase in Bacteroides from healthy to IgE-mediated CMA patients. The treatment with extensively hydrolysed casein formula (EHCF) plus the probiotic L. rhamnosus GG (LGG) positively infuenced gut dysbiosis composition in non-IgE-mediated CMA children, to restore the Bacteroides sub-genus composition and structure, which exhibited diversity similar to that shown

by the healthy controls.[47] Fazlollahi et al. in a study of 141 children with a reliable diagnosis of egg allergy and healthy control genera from the *Lachnospiraceae, Streptococcaceae* and *Leuconostocaceae* families were found to be differentially abundant in GM of children with egg allergy versus control children.[48]

Bunyavanich et al. studied 226 infants with milk allergy to examine the relationship between the infant GM and the clinical course of FA. They found that taxa from the *Firmicutes* phylum, including *Clostridia*, were enriched in the GM of infants with milk allergy. Moreover, Tanaka et al. have shown evidence that the GM at age of 2 months is associated with the development of self-reported FA by age 2 years. They found that the genera *Leuconostoc, Weissella and Veillonella* were underrepresented in the fecal samples of children with egg, cow's milk, soy and /or wheat allergy compared to children without FA while Savage et al. have found among 225 children a lower relative abundance of *Haemophilus, Dialister, Dorea* and *Clostridium* in stool sample was associated with sensitization to at least 1 food allergen among milk, egg, peanut, soy and wheat. [49,34]

There are many factors that could modulate GM-immune system axis composition influencing the occurrence of FA: animal exposure, mode of delivery, birth order and family size, antibiotic exposure, diet and environmental exposures. Recent studies suggest that the protective association between farming and the development of allergic disease may be due to differences in microbial exposure. Ege et al. found that the frequency of asthma was inversely associated with the diversity of microbial exposure even after controlling for farming status. Several studies have shown that infants who develop allergic disease later in life tended to have less *Bacteroides*, *Bifidobacteria* and *Enterococci*, but more *Clostridiae* comprising their microbiome early in life.[50,51,52] It has been shown that a diverse microbial exposure early in life modifies both the innate and adaptative immune system resulting in a significantly decreased risk of allergic disease.[53,54]

Babies born by cesarean section and those born through vaginal delivery show differences in immune responsiveness. Caesarean section could be change the GM composition. The baby is

delivered through a sterile surface instead of the birth canal where colonization by maternal microbiota would typically occur.[55] Delivery by Caesarean-section has been shown to delay the development of the GM and shape its colonization to patterns similar to the maternal skin.[56] Recent evidences have shown that children born by Caesarean-section had decreased microbial diversity and reduced Th1 responses during the first two years of life.[57]

Other studies have shown a link between Caesarean-section delivery and the development of asthma, allergic rhinitis and eczema. [52,58,59]

Specically, the GM of children born by Caesarean-section showed a reduced abundance of *Bacteroides, Bifidobacteria*, and *E. coli* but increased amounts of *Enterobacter, Enterococcus, Klebsiella* and *Clostridia.*[57,52,60,61] An infant delivered vaginally has colonization representative of the mother's vaginal tract, including *Lactobacillus, Prevotella, or Sneathia spp.*[56] Infants born via cesarean section have colonization more consistent with maternal skin and oral microbes, such as *Enterobacter hormaechei/E. cancerogenus, Haemophilus parainfluenzae/H. aegyptius/H. influenza/H. haemolyticus, Staphylococcus saprophyticus/S. lugdunensis/S. aureus, Streptococcus australis, and Veillonella dispar/V. parvula.*[62] An infant delivered by C- section misses out on contact with the microbes in the mother's vaginal canal, and one study shows that these infants have a significantly lower abundance of *Bacteroides* over time, regardless of feeding mode.[56] Infants born via C-section bypass microbes found in mom's stool as well, including *Escherichia/Shigella, Bifidobacterium longum, Enterococcus faecalis, Bacteroides fragilis, B. thetaiomaomicron, and Bilophila wadsworthia*, which have increased prevalence in the intestinal microbiomes of infants born vaginally.[62] Moreover, children born via C-section tended to have a higher need for antibiotics due to respiratory infections in the first year of life.[63] This was attributed to differences in acquisition of bacteria. In children born via C- section the gut microbiota was less stable with delay to *Bifidobacterium* emergence and higher pathogen abundance, from the genera *Klebsiella* and *Enterococcus.* These taxa are specifically noted to be associated with higher incidence of

respiratory infections within the first year of life.[63] A review of samples from nearly 600 infants in the UK by Shao et al. suggests that this could be due to the fact that these children lack commensal maternal *Bacteroides* strains of GM and are instead dominated with opportunistic pathogens (including *Enterococcus, Enterobacter* and *Klebsiella* species) associated with hospital environments.[64] Strachan showed with his hypothesis that infants with higher numbers of siblings had a decreased incidence of allergic disease. Since then, several studies have reproduced the inverse relationship between siblings' number and asthma, allergic rhinitis and eczema.[65] This association was initially due to the increased exposure to infections during childhood. Penders and colleagues have shown that birth order and family size may mediate their protective effect through alterations in the GM.[61] It has been showed that infants with an increased number of older siblings had decreased colonization rates of *Clostridia* and increased rates of *Bacteroides* and *Lactobacillus*. Penders and his collegues showed that colonization with Clostridia was linked with an increased risk of developing atopic dermatitis.[61] An Australian birth cohort study of 5276 children, 453 with reliable diagnosis of egg allergy, found that having older siblings and dog ownership were each associated with decreased egg allergy risk.[66] Use of antibiotic in early life could influence the GM. Several studies on full term infants receiving perinatal antibiotics showed that antibiotic treatment is associated with less bacterial diversity along with higher proportions of *Proteobacteria* and *Enterobacteriaceae* and lower proportions of *Bifidobacterium* and *Lactobacillus*.[67,49]

The Prevention of Allergy-Risk Factors for sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) and The Multidisciplanary Study to identify the Genetic and Environmental Causes of Asthma in the European Community (GABRIEL) studies estabilished that farm living is very important to the exposition to a wider range of microbes and this exposure explains much of the inverse relation between asthma and hay fever and growing up on a farm.[53] Lynch et al. in the Urban Environment and Childhood Asthma (URECA) study examined innercity homes with low neighborhood SES across different cities and assessed the house dust content.[68]

They showed that higher exposure to specific *Bacteroidetes* and *Firmicutes* together with high levels of pests and pests allergen conferred protection against the development of allergies. Diet is another area that has been shown to have significant effect on the GM.[69] One specific dietary that has been studied to the effect of GM composition and the development of allergic disease is breast-feeding and formula. It has been confirmed that breast milk may contain small oligosaccharides that promote the colonization of positive bacteria such as *Bifidobacteria.*[70] Several studies over the last 30 years have only shown minor differences in the GM between breast and formula-fed infants.[60] Fed-infants have higher amounts of *Clostrium difficile* that compose their GM.[71,72] Several studies have shown that *Bacteroides, Enterococci* and *Enterobacteriaceae* may be more common in the GM of formula fed infant, where staphylococci tend to be more prevalent in breast fed infants.[60] It is known that westernized countries have a higher prevalence of allergic disease,[73] and modern western diets have been associated with differences in the GM.[69] Evidences have shown that differences in consumption of animal fat, carbohydrates, and fiber can produce changes in GM that can have important effects on the immune system.[74,75] Advances in metagenomics and metabolomics implicate diet and gut microbiota (the diet-gut microbota axis) as key modulators of the maturation of the immune system. Findings from a recent systematic review further support the relationship between maternal diet during pregnancy and lactation and allergic sensitization to food during childhood.[76] Diet during the first 1000 days of life, from conception up to the first 24 months of age, may influence the risk of developing FA.[77,78,79] A study examining the influence of dietary patterns on the development of FA at the age of 24 months suggests that a healthy child diet with high levels of fruits, vegetables, and home-made foods is associated with less FA.[80]

The role of maternal and infant diet in the development of food allergy has been a major focus of research throughout this period. Allergen exposure can potentially occur in utero could impact on food allergy development. Early introduction of peanut has been shown to be protective against the development of peanut allergy in high-risk children with the body of evidence suggesting the same

is true for egg allergy.[81] One cohort reported that the delayed introduction of rice/wheat cereal (>6 months of age) was associated with a lower risk of food allergy.[82] In addition to allergenic food introduction, postnatal vitamin D status and prebiotic and/or probiotic supplementation has been suggested to be associated with the development of food allergy. Zhang et al. recently performed a meta-analysis of randomized controlled trials about probiotic supplementation during pregnancy and/ or infancy and their effects on atopy in children.[83] They found that administering probiotics prenatally to pregnant mothers and postnatally to the child both could reduce the risk of food sensitization. Based on a review of the literature, the World Allergy Organisation guideline panel suggests using prebiotic supplementation in infants who are not exclusively breastfed, but not for exclusively breastfed infants.[84]

Several studies reported that nutrients impact the gut microbiota and the bacterial metabolites production.[85] The Mediterranean diet (MD) is highly regarded as a healthy balanced diet. It is characterized by high consumption of assorted fruits, vegetables, cereals, legumes, olive oil, and nuts; moderate consumption of fish, poultry, and red wine; and a lower intake of dairy products, red meat, processed meat and sweets. It has been demonstrated that adherence to MD during pregnancy and early life has a protective effect on allergic disease in children.[86] These effects could derive from the high intake of non-digestible dietary carbohydrates (NDC), the beneficial fatty acid profile that is rich in omega-3, the high levels of polyphenols and other antioxidants.[87] Non-digestible dietary carbohydrates represent the primary nutrient source for the gut bacteria and their fermentation leads to the production of short chain fatty acids (SCFA).[88] It has been demonstrated that reduced availability of NDC lowered the concentration of fiber-degrading bacteria and increased mucin degrading bacteria.[89] De Filippis et al. observed a significant association between degree of adherence to the MD and increased levels of SCFAs, *Prevotella* bacteria, and other *Firmicutes*.[90]

The immunomodulatory mechanisms stimulated by SCFAs represent one of the strongest connections between diet, gut microbiota, and allergic diseases.[91] SCFAs are 2-carbon to 5-carbon weak acids, including acetate, propionate, butyrate and valerate.[92] SCFAs-producing bacteria represent a functional group, including *Bacteroidetes* phylum that are good producers of acetate and propionate, whereas *Faecalibacterium prausnitzii*, which belongs to the *Clostridium leptum* cluster (or clostridial cluster IV), and *Eubacterium rectale/Roseburia spp*., which belong to the *Clostridium coccoides* cluster (or clostridial cluster XIVa) of Firmicutes bacteria are efficient butyrate producers.[93] SCFAs are major energy source for colonocytes and influence gene expression necessary for the expression of epithelial barrier-forming molecules and mucin production defense functions and regulation of immune cells, such as macrophages, neutrophils, DCs, T and B-cells.[94,95,96,97,98,99,100] They are absorbed by colonocytes and other cells via transporters (SLC16a1 and SLC5a8), via simple diffusion or through G-protein coupled receptors (GPCRs), such as GPR43, GPR41, GPR109A and Olfr78.[101,102,103,104] GPR43 and GPR41 are highly expressed by intestinal epithelial cells.[105] Neutrophils, macrophages and DCs, express GPR43 and GPR109A, but T- and B-cells do not express these SCFA receptors. [101,102,106,107,108]

Among SCFAs, butyrate exerts a pivotal role in immune tolerance induction. It is able to regulate DCs, reducing pro-inflammatory cytokines and chemokines production and enhancing retinoic acid (RA) expression and subsequent generation of RA-regulated tolerogenic DCs.[109] It has been found that SCFA individually or in combination (SCFA mix) are able to increase colonic Treg frequency and number and that this effect coincides with increased luminal SCFA. Furthermore, SCFA are able to increase also CD4+ T cell frequency and number but did not alter colonic Th1 or Th17 cell numbers.[110] In vitro treatment of colonic Tregs from germ free mice with propionate significantly increased FoxP3 and IL-10 expression, a key cytokine in Treg-mediated suppression suggesting that SCFA specifically induce FoxP3+ IL-10-producing Tregs.[110] In a mouse model, it has been demonstrated that butyrate, facilitated generation of colonic Treg cells, acting as to HDAC-

inhibitory and enhance acetylation of the Foxp3 locus, induced a decrease of proinflammatory cytokine expression within DCs to stimulate colonic Tregs.[111] The mechanisms are multiple and involve a strong epigenetic regulation of gene expression through the inhibition of histone deacetylase (HDAC). Butyrate promoting B-cell differentiation and increasing IgA and IgG production through their HDAC inhibitory activity.[112] It has been demonstrated that butyrate suppresses CD4+ T-cell proliferation and increase colonic FoxP3+ Tregs, potentially through their HDAC inhibitor activity.[110,111,113] The inhibition of HDAC 9 and 6 increases FoxP3 gene expression, as well as the production and suppressive function of Tregs.[114] Butyrate deficiency has been observed in allergic patients.[115] Bacteria-produced SCFAs have been implicated in the regulation of both the proportions and functional capabilities of Tregs, which, in some studies, has been specifically attributed to butyrate production by spore-forming *Clostridiales.* It has been observed an enrichment of taxa from the *Clostridia* class and Firmicutes phylum in human subjects with faster CMA resolution.[115] Altogether these evidences suggest the potential of a "post-biotic" approach, based on the use of butyrate against FA. In this light, data from our laboratory showed that oral butyrate induces a dramatic inhibition of acute allergic skin response, anaphylactic symptom score, body temperature decrease, intestinal permeability increase, anti-β lactoglobulin (BLG) IgE, IL-4 and IL-10 production in a murine model of CMA, suggesting a protective role of butyrate against FA.[116] We evaluated the direct effects of butyrate on peripheral blood mononuclear cells (PBMCs) from children affected by challenge-proven IgE-mediated CMA. PBMCs were stimulated with BLG in the presence or absence of butyrate. Preliminary results showed that butyrate stimulates IL-10 and IFN-γ production and decreases DNA methylation rate of these two cytokines. Same effective butyrate dose induces FoxP3 promoter region demethylation and HDAC6/HDAC9 expression down-regulation.[116,117] Additional potential mechanisms by which diet could exert pro-tolerogenic effects in the gut are related to the production of immunoregulatory metabolites, which interact with the host immune cells to promote non-responsiveness to innocuous luminal antigens.[118] Tryptophan is essential amino acid which cannot be synthesized independently

by humans, thus, need to be ingested with the diet. A part of tryptophan is utilized to synthesize protein, the other portion is catabolized to produce variety bioactive compounds, such as kynurenine, serotonin, melatonin. Tryptophan absorbed by intestinal epithelial cells directly activates the mTOR pathway by intracellular tryptophan receptors through a PI3K/AKT-independent mechanism. [119,120] As we known, mTOR plays an important role in connecting metabolism and immune system. During an inflammatory process, tryptophan is metabolized through the kynurenine (Kyn) pathway. Kyn is an active metabolite and its biological activity is mediated by aryl hydrocarbon receptor (AhR).[121] The bond of Kyn to AhR receptor;[122] lead to the inhibition of dendritic cells (DCs) maturation;[123] and the proliferation of Th17 cells and Treg, increasing IL-22 and IL-10 production. [124,125] Indole, indole 3-propionic acid (IPA) and indole-3-aldehyde (I3A) are produced by catabolism of tryptophan through intestinal commensal bacteria. A study demonstrated that *Peptostreptococcus anaerobius* CC14N and three strains of Clostridium cadaveris utilizing tryptophan to produce IPA. Another study suggested that IPA could promote the intestinal barrier function via PXR and TLR4 pathway. Tryptophan can be also catabolized by lactobacilli to I3A. This metabolite protects gut mucosa against inflammation through AhR recognition.[126] It has been demonstrated that indole-3-carbinole (I3C), an AhR ligand, was able to boost oral tolerance in ovalbumin (OVA)-sensitized mouse model. Mice fed I3C showed lower titers of anti-OVA IgG1 antibodies and higher.[127]

1.4 The Gut Microbiome: investigating the metagenomic and metabolomics features

The human body is characterized by the presence of 10-100 trillion of symbiotic microbial cells and the term ‘microbiota’ refers to a collection of all taxa constituting microbial communities, most of them located in the human gut.[128] Similarly, the term microbiome is used to indicate the whole community of living microorganisms living in a specific matrix along with its potential genome activities that might influence the metabolic capabilities and functioning of such micro-

environment. It was estimated that the genes of our microbiome outnumber 100 times the number of the genes represented in our genomes. [129,130] In particular, each individual has a unique and a specific gut microbial composition as a peculiar fingerprint and it has been agreed that the development of several types of diseases in humans might be linked to gut microbiome perturbation, such condition known as dysbiosis. In fact, the gut microbiota is in symbiosis with the human host, a mutual association established due to a long story of coevolution and the homeostasis of such strictly anaerobic ecosystem is necessary for the maintenance of human health.[131] Overall, the human microbiome is recognized as our second genome and the understanding of its composition and modulation could be used as therapeutic target for personalized microbiome-directed interventions in order to prevent the onset of human diseases. In particular, the gut microbiota is made of hundreds of species and while its composition could vary in the early stages of life, it has been observed an overall microbial stability during adulthood.[132] The composition of GM may slightly chande based on endogenous and exogenous factors. Among others, antibiotics, diet and/or food supplements as well as pathological conditions are able to provisionally modulate the microbial structure. Therefore, the resilience, or rather the gut ability to resist to perturbations depends on the responsive capabilities of the core taxa. Such condition plays a role in maintaining gut homeostasis, its normal composition and functioning, affecting in turn the microbiota-host interaction.[133] It was demonstrated that under the influence of diet, drugs and other factors in a given individual it was possible to observe shifts and movements of GM composition through the space of these three configurations, even though each enterotype had a specific ecological stability and a lower propensity to switch to another one.[134] However, this paradigm was overtaken since among individuals considerable variations in GM composition were described.[135,136] Despite being core taxa contributing to enterotype clustering, it has been shown a large variance in terms of relative abundances.[135] Morevover, species- and strain-level variations were neglected and several studies highlithted the important contribution of such taxa to functional differences between individuals in clinical contexts.[137,138] In addition, recent meta-analyses made progress in

understanding the strain-level diversity within the human gut microbiome and gene-level variation across strains was found to be related to gut microbiome diversity in human health and disease.[139,140,141] Despite less prevalent species in wellstudied populations are newly identified, the bacterial diversity remains uncultured and the complete bacterial and functional repertoire of the human gut microbiome remains still to be undefined.[139] However, the identification of the complex gut ecosystem may help us understanding human health and disease status.

The knowledge and awareness of the role the gut microbiota and metabolites in the balance between health and disease is rapidly increasing. This is mainly due to the advance in technology and the availability we currently have of high sensitivity means to study microbial communities in any type of ecosystem. It is important for the clinicians and researchers dedicated to the FA field to know potential and limits of these technologies to better understand the value and significance of the findings reported in literature. Thanks to the power of genome DNA sequencing, we have learned much about the composition of gut microbial communities. In addition, the potential of transcriptomics, proteomics and metabolomics are enlarging our understanding of the gut microbiota role in human health. Until the 1990s, knowledge of the gut microbiota was limited because the only technique used to study and characterize the composition of gut microbiota was bacteriological culture. Since the 1990s, there were advances in culture-independent techniques. These new techniques are fast, facilitate high throughput, identify organisms that are uncultured to date and enable enumeration of organisms present in the gut microbiota. In the last decade, the composition of the gut microbiota was described by next generation sequencing of 16S ribosomal RNA genes. Lately, it is widening the amount of information that can be retrieved by studying metagenomes from human samples, with the capability to infer the abundance of genes and potential metabolic pathways that characterize a microbial community. It is possible to describe the taxonomic composition of the microbiota and also to study the potential functions in a given system. Such methodological background is fundamental to investigate associations between

microbiota structure and health as well as other environmental factors [136] and also to observe the changes of the gut microbiota in response to disease or perturbations in diet or lifestyle. An advanced technique to investigate gut microbiota at deep level is shotgun sequencing that represents a massive parallel sequencing of the whole genome. This is done by massive parallel sequencing of the mixed DNA sample. Shotgun sequencing involves random fragmentation of DNA, sequencing of DNA fragments and reconstruction of overlapping sequences to assemble them into a continuous sequence. [142] Metabolomics represents one of the meta-omic approach to study gut microbiota function. Metabolomics uses high throughput techniques to characterize and quantify small molecules in several biological samples such as feces, urine, plasma, serum, saliva.[143] The use of metabolomics is considered a powerful top-down systems biology approach, and it is essential to reveal the genetic-environment-health relationship, as well as the clinical biomarkers of diseases.[144] Currently, the rapid development of several analytical platform, including Gas Chromatography Mass Spectrometry (GC- MS), liquid chromatography (LC), high pressure LC (HPLC), ultra pressure LC (UPLC), Fourier transform infrared spectroscopy (FTIR), ion cyclotrone resonance-FT (ICR-FT), capillary electrophoresis (CE) coupled to mass spectrometry (MS), and nuclear and proton nuclear magnetic resonance spectroscopy (NMR-1H-NMR), allowed to separate, detect, characterize and quantify metabolites and their metabolic pathways.[145] (Table 2) What is needed is a transition from descriptive research to understanding the ways the microbiome interacts with the host and plays a role in health and disease. In this frame, controlled clinical interventions are of utmost importance to establish microbiota causative involvement and are the basis to implement approaches of personalized medicine. [130,146] The study of the relationship between microbiota and FA may start from association and be translated to causation and clinical practice with appropriate advances in knowledge. An initial wide screening of microbial diversity in gut microbiota of patients with a sure diagnosis of FA, including a well-matched control population, may identify useful signatures in the microbiota that are specific for certain types of FA.[147,38] If the wide screening included cohorts of patients with different dietary style or ethnicity, the common

microbial signatures would be even stronger and provide a solid indication of the microbial biomarkers of FA. A further mapping of the genomic features associated to FA maybe inferred by metagenomics and metabolomics, which may inform on the functional microbial signatures that can be recognized in FA patients. Biomarkers strains or defined microbial systems may be tested in gnotobiotic or humanized animal models to observe the development of the disease, and beneficial vs detrimental microbial metabolites can be recognized and used as final target of microbiome-targeted personalized interventions. The identification of bacterial metabolites, that affect positively the immune tolerance network, may be an interesting strategy against FA using a post-biotic approach.

1.5 The role of breastfeeding in food allergy

Breastfeeding has important health benefits for infants and represents the gold standard of nutrition for infants. [148] However, the role of human milk (HM) in the primary prevention of FA [149] are still undefined. One birth cohort from Taiwan reported exclusive or partial breastfeeding for 6 months was associated with reduced milk sensitization at ages 1 and 1.5 years compared with formula feeding. [150] An influence of breast milk on the development of disease in offspring may also be explained by the composition of breast milk. Mothers' diet during lactation can alter the composition of breast milk, which might have an impact on allergic outcomes. A small cohort study (FARMFLORA) in Sweden showed mothers consuming more margarine, margarine and oils and low-fat milk during pregnancy and were more likely to have pediatrician-confirmed allergic children. [151] *(*HM is a complex living nutritional fluid that contains antibodies, enzymes, and hormones, all of which have health benefits. *[152]* It has been demonstrated a pivotal role of breast-milk antibodies for the development immature immune system of a newborn. [153] Breast-milk antibodies that transfer in the newborn's gut can help to determine the composition of microbes that colonize the intestine, and thereby prevent excessive immune responses to non-pathogenic

microbes. [153] Yet despite this maternally provided immune protection, an infant still faces an extremely sensitive period in early life when the progressive microbial colonization of internal and external body surfaces occurs concurrently with the development and maturation of the immune system. [154] In addition to passive immunity (from bioactive components such as secretory IgA and IgG), human milk also contains factors that actively stimulate the infant immune system. [155] Accumulated data suggests that a wide range of bioactive factors: such as proteins, polyunsaturated fatty acids, oligosaccharides, microbial content, metabolites, lipids and micronutrients present in HM can influence the infant's gut immune maturation. [156] Milk lipids is the main source to supply energy for newborns such that it comprises ~50% of the energy that they need. [157] Milk lipids provide essential nutrients, which are needed, as structural elements, for all cell membranes and, as integral constituents of neural tissues, for rapidly growing infants. [158, 159] Among fatty acids, human milk has been examined as a potential source of the major SCFAs butyrate for neonates, with a different content of SCFAs than infant formula. [160] However, butyrate levels in HM are not well established.

1.6 Epigenetics regulation of food allergy

Epigenetic modifications are biochemical changes of the chromatin, in other words, DNA or histones, that are functionally relevant, but do not affect the nucleotide sequence of the genome. DNA methylation, a covalent addition of a methyl group, occurs at the cytosine nucleotide belonging to CpG dinucleotide (called 'CpG site'), which is a DNA sequence where a cytosine nucleotide (C) is directly followed by a guanine nucleotide (G). CpG sites frequently cluster to form 'CpG islands', typically located in the elements of a gene regulatory element with impact on its transcription, for example, promoters or enhancers. [161,162] High DNA methylation levels in the CpG island of a promoter are usually associated with lower gene expression up to full gene silencing. The reaction of DNA methylation is catalyzed by DNA methyltransferases (DNMTs), including

DNMT1 and DNMT3A and DNMT3B. [163,164,165,166] The best-characterized post-translational histone modifications include phosphorylation, ubiquitination, acetylation and methylation, the last two of which are the most extensively studied. [167,168] Histone acetylation occurs at the lysine residues and it is catalyzed by histone acetyltransferases, while the opposite reaction by histone deacetylases (HDACs). Histone acetylation independent of the position of the lysine amino acid generally correlates with potentially active genes or gene regulatory elements. [169, 170]

DNA methylation and histone modifications mutually interact. [171] MicroRNAs (miRNAs) represent post-transcriptional control elements, important epigenetic regulators of gene expression. [172, 173] These approximately 22-nt noncoding RNA molecules are highly abundant, with more than 2500 mature miRNA molecules characterized in humans. To exert its function, mature miRNAs become incorporated into the RNA-induced silencing complex. The RNA-induced silencing complex is in turn guided by miRNAs to specifically target mRNAs. This leads to the cleavage or degradation of the bound mRNA molecule or suppression of its translation by reducing the speed of the ribosomal machinery. [172,173, 174,175,176,177]

Considering their biological importance, miRNAs have been involved in multiple human pathologies. [174] These include also allergic diseases, in which the role of miRNAs has been rather extensively studied. [178,179,180, 181,182] It is also worth mentioning that the mechanism of RNA-mediated silencing of gene expression has been utilized in biomedical research as a powerful laboratory tool [183] and in therapeutic applications as one of the possible antisense approaches.[184] Connections between miRNAs and DNA methylation/histone modifications have also been described. [172,174, *185,186,187*] Several studies provide direct evidence linking epigenetics and FA.[188,189,190,191,192,193]

Martino et al. investigated whether variation in DNA methylation underscores the suboptimal neonatal CD4+ T-cell gene expression associated with the development of FA [194] including impaired T-cell expansion and reduced IFN- γ production. [195,196,197,194] In a follow-up study, it was

examined the genome-wide DNA methylation profiles in CD4+ T-cells from 12 children with FA and from 12 non-allergic controls at birth and again at 12 months. The authors found that the dysregulation of DNA methylation at MAPK signaling-associated genes during early CD4+ T-cell development may contribute to T lymphocyte responses in early childhood associated with the development of FA.[194] Further linking epigenetics and FA, the first genome-wide association study (GWAS) of FA in 2759 US participants revealed the important role of differential DNA methylation in mediating identified genetic risk factors for peanut allergies.[198] Syed et al demonstrated that subjects who acquired immune tolerance to peanuts after 3 months of immunotherapy had higher numbers of Tregs with higher levels of FoxP3 demethylation, compared to non-tolerant and healthy subjects. [199] Recent study demonstrated that naive CD4+T cells from children with FA exhibit an intrinsic molecular defect during the early state of priming and depressed capacity for proliferation, which is related to epigenetic changes in metabolic (RPTOR, PIK3D, MAPK1, FOXO1) and immunological genes (IL1R, IL18RAP, CD82). Furthermore, the authors found that children who fail to resolve FA in later childhood exhibit cumulative increases in epigenetic disruption at T cell activation genes and a decrease of CD4+ T cells proliferative responses compared to children who resolved FA. [200] Preliminary cross-sectional studies have suggested that Th1/Th2 cytokine genes DNA methylation pattern and selected miRNAs expression change significantly during CMA disease course. [191,192, 193] In particular, we demonstrated a potential role of miR-193a-5p in regulation of Th2 response in children with IgE-mediated CMA.[193] Different demethylation rates in Treg-specific-demethylation region (TSDR) of FoxP3 have been also demonstrated comparing CMA children with active disease with those with recent evidence of immune tolerance acquisition. [192] Dietary factors exert a pivotal role in the regulation of epigenetic mechanisms. [22] Previous observations suggest that formula choice for CMA treatment could influence these mechanisms. Specifically, we observed a significant difference in DNA demethylation rate in TSDR of FoxP3, and in the promoter region of T helper (Th)1/Th2 cytokine genes in children who acquired immune tolerance after treatment with extensively hydrolyzed

casein formula containing the probiotic Lactobacillus rhamnosus GG (EHCF+LGG) compared to subjects who received other formulas. [191,192,193]

The study of epigenetics in FA is a promising avenue that may lead to a better understanding of the mechanisms underlying FA etiology. It has been shown that both genetics and environmental factors can alter epigenetic profile. [201,202]Thus, epigenetics may be the missing piece to understanding environmental–genetic interactions and FA risk.

In the first year of PhD, I studied the potential protective role of human milk against FA. We identified a compound butyrate that could regulate a wind range of immune and non immune mechanism, preveting FA.

In the second and in the third year of PhD, I focused on the characterization of GM composition and function of FA children to identify a possible target of intervention for this condition.

2. Aims

Aim #1

The aim of our study was to evaluate butyrate concentrations in HM and to see whether these butyrate concentrations can exert protective actions against FA, exploring several immune and non-immune tolerogenic mechanisms in different experimental models:

1. CD4+ T cells from healthy controls and children with IgE-mediated FA

2. human enterocytes cell lines, HT-29 and Caco-2

3. mouse model of FA

Aims #2

The primary aim was the comparative evaluation of gut microbiome features in children affected by IgE-mediated allergies and in healthy controls.

The secondary aims were:

- the comparative evaluation of gut microbiome features in children affected by IgE-mediated FA or respiratory allergies and in healthy controls;

- the comparative evaluation of gut microbiome features in children affected by IgE-mediated food allergies induced by different food allergens;

- the evaluation of the influence elicited by gut microbiome on immune tolerance acquisition timing in IgE-mediated FA children.

3. Methods

1# Methods

1.1 Donors and collection of human milk samples

Mothers who participated in the study were enrolled after full-term, singleton births, with all mothers intending to breastfeed infants for at least 75% of feedings for 3 months from the Villa Betania Evangelic Hospital (Naples, Italy) in accordance with the Research Ethics Committee of the University of Naples "Federico II". All donors were healthy, aged 21-42 years and HM samples were donated at 3 days (colostrum) and during the first 5 months post-partum. Written informed consent was obtained from all participants. HM samples were collected by either manual or electric breast pump expression into 2 mL a sterile milk tubes. Samples were immediately frozen and then stored at −80°C. For each enrolled subject, anamnestic, demographic, clinical and laboratory data were recorded in a data collection sheet. 3-days dietary diary before the collection of HM samples was obtained. The sampling procedures applied ensured that breastfeeding had been well established and that the baby was thriving.

1.2 Determination of butyrate concentration in human milk samples

Butyrate extraction from HM was performed as previously described *(Clinica Chimica Acta, 78 (1977) 243-250 Pretreatment methods prior to gaschromatographic analysis of volatile fatty acids from faecal samples J.B. Zijlstra, J.Beukema, B.G.Wolthers, B.M.Byrne, A.Groen, J.D.Anker)*. 0.5 ml of HM were acidified with 20 µl of 85% (w/v) ortophosphoric acid and 0.5 ml of ethyl acetate, mixed, centrifuged (12,000 x g) for 1 h and extracted in duplicate. About 0.5 ml of extract containing 3mmol/L of 2-ethylbutyric acid (internal standard) was transferred into a 2 ml glass vial and loaded onto an Agilent Technologies (Santa Clara, CA, USA) 7890 gas chromatograph (GC) system with automatic loader/injector. The GC column was an Agilent J&W DB-FFAP (Agilent

Technologies) of 30 m, internal diameter 0.25 mm and film thickness 0.25 μm. The GC was programmed to achieve the following run parameters: initial temperature 90°C, hold 0.5 min, ramp of 20°C min−1 up to a final temperature of 190°C, total run time 8.0 min, gas flow 7.7 ml min−1 split less to maintain 3.26 p.s.i. column head pressure, septum purge 2.0 ml min−1. Detection was achieved using a flame ionization detector. Peaks were identified using a mixed external standard and quantified by peak height/internal standard ratio.

1.3 DNA and RNA extraction from CD4+ T-cells isolated from peripheral blood mononuclear cells

Two healthy children (Caucasian male, age 24 months with negative clinical history for any allergic conditions and not at risk for atopic disorders), referred to the Pediatric Department of the University of Naples "Federico II" because of minimal surgical procedure, and six children with challenge-proven FA (2 cow's milk allergy, 2 peanut allergy, 2 egg allergy; all Caucasian male, age 24 months) were recruited. Patients and control subjects donated a venous blood samples in heparin tubes (8 ml), after written informed consents. Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Paque (Sigma-Aldrich, St. Louis, MO, USA) method, as described previously. [192] CD4+ T-cells were obtained by negative selection using the CD4+ T-Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Non-target cells were labeled with a cocktail of biotin-conjugated monoclonal antibodies (MicroBead Cocktail, Miltenyic Biotec) and the magnetically labeled non-target T cells were retaining on a column in the magnetic field of a separator (Miltenyi Biotec). This protocol produces >95% pure CD4+ T cells, as tested by fluorescence-activated cell sorting analysis. Cells were resuspended at 2x106 cells/ml in RPMI-1640 cultur medium (Gibco) supplemented with 10% fetal bovine serum, penicillin/streptomycin (1%) (Lonza), L-glutamine (1%), sodium pyruvate (1%) (Lonza) and NEAA (1%) (Lonza). Cells were cultured at 37°C in complete medium at concentrations of 2x106 cells/ml in 24-well plate

(Nunc, Roskilde, Denmark). CD4+ T-cells obtained were processed for DNA and RNA extraction. All experiments were performed in triplicate and repeated twice.

1.4 Measurement of IL-4, IL-5, IL-13, IL-10, and IFN-γ culture media concentration

CD4+ T cells were stimulated with beta-lactoglobulin (BLG;100µg/ml), peanut extract (PN;100µg/ml) or ovalbumin (OVA;100µg/ml) in the presence or absence sodium butyrate (Sigma-Aldrich, Darmstadt, Germany) at dose of 0.75mM for 24h. After stimulation, the supernatants were collected and the cells were washed and harvested for total DNA and RNA isolation. The concentrations of IL-4 and IL-10 were measured in supernatants with a Human IL4/IL10 Enzyme immunoassay kit (Boster Biological Technology, Ltd., Fremont, CA, USA) according to the type of stimulation and stimulant. Human IL-5, IL-13 and IFN-γ ELISA, High Sensitivity (BioVendor, Brno, Czech Republic) were used to detect the IL-5, IL-13 and IFN-γ concentrations. Absorbance was read at 450 nm. The minimum detection concentrations were 15.6 pg/ml for IL-4, 7.8 pg/ml for IL-5, and IL-10, 1.6 pg/ml for IL-13, and 0.78 pg/ml for IFN-γ.

1.5 DNA methylation analysis

One microgram of DNA, extracted from CD4+T cells, was modified with sodium bisulfite to convert all unmethylated, but not methylated-cytosines to uracil. Bisulfite conversion was carried out using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA, USA), according to the manufacturer's instructions. The converted DNA was stored at −20°C until used. Fully methylated and fully unmethylated DNA (Merck Millipore, Darmstadt, Germany) were used as controls for the optimization of the assay conditions and to calculate the percent of methylation (0% to 100%). The primers used for DNA methylation analysis of IL-4, IL-5, IL-13, IL-10, IFN-gamma

and FoxP3 TSDR are reported elsewhere. [191,192] High-resolution melting real-time PCR for methylation analysis was performed as described previously. [191,192]

The results of methylation analysis were verified by direct sequencing (using the Sanger method modified as follows: ddNTPs labeled with four different fluorophores) and analyzed by capillary electrophoresis (the analytical specificity and sensitivity of the test was >99 %). Real-time PCR was performed with the LightCycler® 480 instrument (Roche Applied Science, Penzberg, Germany) using 96-well plates (Roche Applied Science).

1.6 Human enterocytes cell line

Human enterocytes cell line Caco-2 and HT-29 were used (American Type Culture Collection, Teddington, Middlesex TW11 0LY, UK). Caco-2 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; Gibco, Berlin, Germany) supplemented with 10% fetal calf serum (Lonza, Visp, Switzerland), 1% L-glutamine (Lonza), 1% nonessential amino acids, and 1% penicillin/streptomycin (Lonza). HT-29 cells were grown to confluence in RPMI medium 1640 (Gibco, Berlin, Germany) supplemented with 10% fetal calf serum (Lonza, Visp, Switzerland) 1% L-glutamine (Lonza) and 1% penicillin/streptomycin (Lonza). Cells were cultured at 37°C in a water-saturated atmosphere consisting of 95% air and 5% CO2, and the medium was changed every 2 days. All experiments were performed in triplicate and repeated twice.

1.7 Immune and non-immune biomarkers analysis on human enterocytes

At full confluence (15 days), when a human enterocytes monolayer was obtained from Caco-2 and HT-29 in six-well plates (Falcon, Heidelberg, Germany), cells were stimulated for 48h with or without sodium butyrate at different doses (0.1; 0.5; 0.75; 1; 2 mM). Afterward, the supernatants were harvested and stored at −80°C for further use. Cells were used for RNA extraction to perform

real-time PCR experiments. The concentration of β-defensin 3 (HBD-3) in the supernatants was measured using a commercially available ELISA kit specific for human HBD-3 (MyBioSource, San Diego, CA, USA) with a detection limit of 11.3 pg/ml. The ELISA was conducted according to the manufacturer's recommendations. For ZO-1, Occludin, Muc2 and Muc5AC and FoxP3 expression analysis, total cellular RNA was extracted from cells with TRIzol reagent (Gibco BRL, Paisley, Scotland). RNA (1 μg) was reverse transcribed at 37°C in cDNA with a High-Capacity RNA-to-cDNA™ Kit (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was stored at −20°C until use. Quantitative real-time PCR (qRT-PCR) analysis was performed with the TaqMan miRNA assay kit and the TaqMan gene expression assay kit, respectively (both from Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. Samples were run in duplicate at 95°C for 15 seconds and 60°C for 1 minute using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). After a hot start, the amplification protocol was 40 cycles of 30 sec of denaturation at 95°C, 30 sec of annealing at 60°C, and 1 min of elongation at 72°C. Data analysis was performed with the comparative threshold cycle (CT) method. We used the GAPDH gene to normalize the level of mRNA expression.

For measurement of the mucus thickness, human enterocytes cell lines were seeded onto polycarbonate membranes (0.4 μm pore size) from TranswellTM (Corning Inc., New York, US) at 37°C in an atmosphere with 95% humidity and 5% CO_2. At full confluence (14 days), the cells were stimulated for 24, 48 and 72 h with or without butyrate. A pair of membranes cultured under the same conditions were embedded immediately in an optimal cutting temperature compound (BioOptica, Milan, Italy) to avoid mucus damage during processing. Five-micrometre frozen sections of each sample were cut, and after rinsing in 3% acetic acid (Merck, Germany), the cells were stained with 1% Alcian Blue (Merck, Germany) and oxidized in 1% periodic acid (Merck, Germany). Finally, sections were counterstained with Mayer's haematoxylin and mounted with

Aquamount (BDH, Poole, UK). Measurement of the mucus thickness was performed in the middle of the membrane using the ruler tool provided by the Zeiss Axio Observer/ApoTome.

1.8 HDAC activity assay

To assess HDAC activity, HT-29 cells were treated with 10 nmol/L trichostatin A (TSA, Sigma-Aldrich) and with butyrate (0.1; 0.5; 0.75; 1; 2 mM) for 48 hours. HT-29 cells nuclear extracts (3x107 cells/well in 10 mL of culture medium) were prepared by using the Nuclei EZ Prep kit (Sigma-Aldrich) and quantified by with the Micro BCA Protein Assay Kit. HDAC activity was measured with the EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek, Farmingdale, NY), according to the manufacturer's instructions.

1.9 Food allergy animal model

For all experiments three week-old female C3H/HeJ mice were purchased from Charles River Laboratories (Calco, Lecco, Italy). Mice were housed in the animal facility under a 12L:12D lighting cycle, 20-24ºC range of ambient temperature and 40-70% of relative humidity. The mice were acclimated to their environment for 1 week before experiments. Each experiment was littermate controlled and mice were cohoused throughout manipulations. All procedures involving the animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of the Italian Ministry of Health and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC) and they were approved by the Institutional Committee on the Ethics of Animal Experiments (CSV) of the University of Naples "Federico II" and by the Ministero della Salute (protocol no.2012-0024683).

1.10 Food allergen sensitization and challenge

The experimental design is reported in Figure 3. Briefly, two weeks prior to sensitization, mice were given 30 mg/kg/day of butyrate by oral gavage and continued during the whole study. The control animal received only PBS. After 14 days, mice were sensitized orally using a blunt needle on day 0, 7, 14, 21, 28 with 20 mg of β-lactoglobulin (BLG) (Sigma-Aldrich, Steinheim, Germany) or 1 mg of ovalbumin (OVA) (Sigma-Aldrich, Steinheim, Germany) or 12 mg of peanut extract (kindly provided by Prof. Nagler) mixed with 10 µg cholera toxin (CT) as adjuvant. The control mice receive CT only.

One week after the final sensitization, two doses of 50 mg BLG or 5 mg OVA or 36 mg PN each were administered via intragastric gavage 30 minutes apart. Core body temperature was measured prior to allergen challenge and every 5 minutes after the first challenge until at least 30 minutes after the second challenge using a rectal probe (Mitutoyo, Lainate, Italy). Anaphylaxis symptoms were scored by an investigator blind to the study group assignment 1 hour after oral challenge, as previously described *[National Academies of Sciences, Engineering and Medicine. Finding a path to safety in food allergy: assessment of global burden, causes, prevention, management, and public policy (2016). Washington (DC) National Academies of Sciences, Engineering and Medicine.*]: 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding and puffiness around the eyes and mouth; 4 = no activity after prodding, labored respiration, and cyanosis around the mouth and the tail; and 5 = death. Serum was collected 1 hour after the second challenge to measure mMCP-1 levels. Spleen and serum were collected 24 hours after challenge for splenocyte culture and antibody measurements.

1.11 ELISAs

mMCP-1 was quantified in serum collected 1 hour after challenge according to the manufacturer's protocol (eBioscience). BLG/PN-specific ELISAs were performed using protocols modified from ref X. Briefly, plates were coated overnight at 4˚C with 100µg/mL BLG/PN in 100mM carbonate-bicarbonate buffer (pH 9.6). Plates were blocked for 2 hours at room temperature with 3% BSA. Samples were added in 1% BSA and incubated overnight at 4˚C. Assays were standardized with BLG/PN-specific antibodies (IgE) purified from mice immunized with BLG+alum or PN+alum on a CNBr-Sepharose affinity column(X). Secondary antibody (biotin-anti-IgE, BD Pharmingen) was added at a 1:500 dilution overnight at 4˚C. On the third day, the plate was incubated with streptavidin-HRP (ThermoFisher) for 1 hour at room temperature and developed with TMB (Sigma) and 15 min were allowed for the development of colorimetric reactions. Absorbance were read at a wavelength of 450 nm in a microplate reader. OVA-specific IgE were measured using commercially ELISA kit (eBioscience).

1.12 Spleen culture and cytokine measurement

Single-cell suspensions were prepared from spleens harvested 24 h after challenge. Cells were plated at 2×105cells per well with media alone, 1 µg/mL anti-CD3 (clone 2C11), or 200 µg/mL BLG/OVA/PN and incubated at 37 °C for 72 h. After 72 h, plates were frozen at −20 °C. IL-4, IL-13, IL-10, and INF-γ concentrations in supernatants were measured using commercially ELISA kit (eBioscience).

1.13 Preparation of isolated mitochondria and polarographic measurement of respiration

After removal, the livers were finely minced and washed in a medium containing 220 mM-mannitol, 70 mM sucrose, 20 mM -N'-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid

(HEPES) (pH 7.4), 1 mM-EDTA, and 0•1 % (w/v) fatty-acid-free bovine serum albumin (BSA). Tissue fragments were homogenised with the above medium (1:4, w/v) in a Potter Elvehjem homogeniser (Heidolph, Kelheim, Germany) set at 500 rpm (4 strokes/min). The homogenate was centrifuged at 1000 gav for 10 min and the resulting supernatant fraction was again centrifuged at 3000 gav for 10 min. The mitochondrial pellet was washed twice and finally re-suspended in a medium containing 80 mM-KCl, 50 mM-HEPES (pH 7.0), 5 mM KH2PO4, and 0.1% (w/v) fatty-acid-free BSA. The protein content of the mitochondrial suspension was determined by the method of Hartree (1972) using BSA as the protein standard. Isolated mitochondria were then used for the determination of respiratory parameters. Mitochondrial O2 consumption was estimated by a Clark type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA), maintained in a water-jacketed chamber at 30°C. Hepatic mitochondria (0.5 mg protein) were incubated in a medium (3 ml) containing 80 mM KCl, 50 mM HEPES, 1 mM EGTA, 5 mM KH_2PO_4 (pH 7.0), and 0.1% (w/v) fatty-acid-free BSA. The substrates used for liver respiration were 10 mM succinate + 3.75 µM-rotenone or 40 µM-palmitoyl-carnitine + 2.5 mM-malate for the determination of fatty acid oxidation rate. State 3 measurements were performed in the presence of 0.6 mM ADP. State 4 respiration was measured in the absence of ADP in isolated mitochondria. The ratio between state 3 and 4, called the respiratory control ratio, was calculated according to Estabrook (1967).

1.14 Determination of mitochondrial enzymatic activities and H_2O_2 release

Total activity of the Carnitine Palmitoyl-CoA Transferase (CPT) was followed spectrophotometrically as CoA-sH production by the use of 5,5'-dithiobis (nitrobenzoic acid) (DTNB) and as substrate palmitoyl CoA. The medium consisted of 50 mM KCl, 10 mM Hepes (pH 7.4), 0.025% Triton X-100, 0.3 mM DTNB, and 10-100 pg of mitochondrial protein in a final volume of 1.0 ml. Two cuvettes were used, both containing the same medium as that used in the mitochondrial samples. In addition, the sample cuvette contained 1 mM L-carnitine. The reaction

was started by simultaneous addition of acyl-CoA to both cuvettes, and the change in absorbance difference between the two cuvettes was followed at 412 nm. Enzyme activity was calculated from E412 = 13,6OO/(M X cm). The temperature was thermostated to 25°C. (Alexson 1988). Mitochondria (40–60 μg) were solubilized in 1% Triton X-100. Aconitase specific activity was measured in a medium containing 30 mmol/l sodium citrate, 0.6 mmol/l MnCl2, 0.2 mmol/l NADP, 50 mmol/l Tris-HCl, pH 7.4, and 2 units isocitrate dehydrogenase, and the formation of NADPH was followed spectrophotometrically (340 nm) at 25°C. (Hausladen, 1996). The rate of mitochondrial H_2O_2 release was measured at 30°C following the linear increase in fluorescence (exicitation at 320nm, emission at 400 nm) due to oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase in a computer controlled Jasco fluorometer equipped with a thermostatically controlled cell-holder. The reaction mixture consisted of 0.4 mg of mitochondrial proteins and succinate (0.6M) added at the end to stat the reaction in the same incubation buffer used for measurements of O_2 consumption. Known concentrations of H_2O_2 were used to establish the standard concentration curve (Barja, 1998).

1.15 Statistical Analysis

The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. To evaluate the differences among continuous variables, the independent sample t-test was performed. The level of significance for all statistical tests was 2-sided, $p < 0.05$. All analyses were conducted by a statistician, using SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 5.0.

#2 Methods

2.1 Ethics

The study was conducted in accordance with the Helsinki Declaration (Fortaleza revision, 2013), the Good Clinical Practice Standards (CPMP/ICH/135/95), the Italian Decree-Law 196/2003 regarding personal data, and the European regulations on this subject. The study protocol, the subject information sheet and the informed consent form were reviewed and approved by the Ethics Committee of our Institution. The study was registered in the Clinical Trials Protocol Registration System at ClinicalTrials.gov with the identifier NCT04750980.

2.2 Study subjects

Children (age range 48-84 months) with a sure diagnosis of IgE-mediated FA and RA, visiting our tertiary Center for Pediatric Allergy (www.allergologiapediatrica.eu), were considered for the study.

The exclusion criteria were: age at enrollment <48 months or >84 months; history of non IgE-mediated allergy; eosinophilic disorders of the gastrointestinal tract; chronic systemic diseases; congenital cardiac defects; acute or chronic infections; autoimmune diseases; immunodeficiencies; chronic inflammatory bowel diseases; celiac disease; cystic fibrosis or other forms of primary pancreatic insufficiency; genetic and metabolic diseases; food intolerances; malignancy; chronic pulmonary diseases; malformations of the respiratory tract or of the gastrointestinal tract; pre-, pro- or sinbiotic use in the previous 3 months; antibiotics or gastric acidity inhibitors use in the previous 3 months.

Written informed consent was obtained from the parents/caregiver of each subject.

During the same study period, consecutive age-matched healthy children, with negative history for any allergic condition and not at risk for allergy, visiting our Department because of minimal

surgical procedures or vaccination program were also enrolled. The same exclusion criteria were adopted for this study population.

Anamnestic, demographic, anthropometric and clinical data from each subject were recorded in a dedicated database.

For all study subjects two stool samples (3 g/each) were collected and stored at −80°C until analyses according to the Human Microbiome Project procedures. [203]

All allergic patients were followed at the Center for at least 36 months after the enrollment. In children with FA the possible acquisition of immune tolerance was assessed yearly by the results of skin prick tests, serum specific IgE levels and oral food challenge performed as previously described. [204] Similarly, healthy controls were followed by the physicians involved in the study for the possible occurrence of any allergic conditions for 36 months.

2.3 Metagenome sequencing

DNA extraction from faecal samples was carried out following the Standard Operating Procedure (SOP) 07 developed by the International Human Microbiome Standard Consortium (www.Microbiome-standards.org). Briefly, 3 ml of STE Buffer (100mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA) was added to faecal samples (2g) and then, they were vortexed and centrifuged the samples for 1 minute at 10 000 x g. For each sample, a volume of 600 µl of fecal slurry was transferred into 2ml screw cap tubes containing 0.1mm silica balls. 250 µl of Guanidine Thiocyanate 4M (protein denaturant) and 500 µl of 5% N-Lauroylsarcosin (ionic surfactant) were added. The samples were incubated under stirring (700 rpm) for 1 hour at 70 ° C and then centrifuged for 1 minute at 14 000 x g. The supernatant was transfered to 2 ml eppendorf containing 15 mg of Polyvinylpyrrolidone (polymer used to remove phenolic and faecal contaminants). After vortexing, the samples were centrifuged at 4 ° C for 3 minutes at 15 000 x g and transfered 500 µl

of supernatant to new eppendorfs. 8 µl of RNase (final concentration of 10 mg / ml) was added and the samples were incubated under stirring (700 rpm) for 20 minutes at 37 ° C. The DNA was purified using silica membranes (NucleoSpin gDNA Clean-up Kit, Macherey-Nagel™ 740230.250, Waltham, Massachusetts, USA), according to the manifacture's protocol. DNA libraries were sequenced on Illumina NovaSeq platform leading to 2x150bp, paired-end reads.

2.4 Metagenome analyses

Reads filtering, taxonomic and functional analyses

Human reads were removed using the Human Removal pipeline developed within the Human Microbiome Project by using the Best Match Tagger (BMtagger; https://hmpdacc.org/hmp/doc/HumanSequenceRemoval_SOP.pdf). Then non-human reads were quality-filtered using PRINSEQ:[205)] reads with bases having a Phred score < 15 were trimmed and those < 75bp were discarded. Number of reads for each sample is reported in Table S1. High-quality reads were imported in MetaPh1An3 [206] to obtain species-level, quantitative taxonomic profiles. Functional profiling was obtained using HUMAnN3. [207]

Assembly-free strain level analysis

PanPh1An3 [206] was applied on high-quality reads using default parameters, generating a presence/absence gene-family profiles for the top 11 most abundant species. Jaccard distance between each couple of samples was computed using (dist.binary R function) and Classical Multidimensional Scaling (MDS, cmdscale R function) was carried out on Jaccard distance matrix.

Assembly and genome reconstruction from metagenomics reads

High- quality reads were assembled independently using MEGAHIT (ref) and contigs >1,000 bp were used to predict genes by using MetaGeneMark.[208] Predicted genes were aligned (using BlastX;

ref) against Ruminococcus gnavus genes coding for an inflammatory polysaccharide (as reported by Henke et al. [209]). An e-value cutoff of 1e-5 was applied, and a hit was required to display > 90% of identify over at least 50% of the query length. Assembly results are reported in Table S1.

Contigs (< 1,000 bp) were also binned using MetaBat2, and Metagenome Assembled Genomes (MAG) quality was estimated with CheckM (ref). Only MAGs with < 50% completeness and < 5% contamination were retained for further analyses. MAGs bined in this study were clustered to genomic database including 107,442 high-quality MAGs previously reconstructed from human metagenomes 140 and 185,939 isolate genomes downloaded from NCBI RefSeq on May 2020. Pairwise genetic distances between genomes were calculated using Mash (version 2.0; option "-s 10000" for sketching). [210] A Mash distance <5 % from any of the database genomes was considered to place the MAG within the relative Species- level Genome Bin (SGB). RAxML (Statamatakis, 2014) was used to generate species-specific phylogenetic trees, which were visualized in iTOL 5.5.1. [211]

2.5 Fecal short-chain fatty acids determination

One gram of feces was diluted with saline buffer, vortexed and centrifuged (12,000 × g) for 10 min in 2 ml tubes. The supernatant was filtered (0.45 μm) and stored at −80 °C until analysis. Frozen fecal extracts from -80°C were defrosted at 4°C for 12h, then invert 10 times to mix at RT. 1ml of each sample was acidified with 40μl of H3PO4 85%(w/v), vortexed for 5 min and sonicated for 10 min at 40 KHz immersed in an ice bath (Branson 2800 ultrasonic). 1 mL of ethyl acetate was added, vortexed for 10 min and centrifuged (12000 RPM for 45 min). Finally, it was taken from the supernatant (organic phase, of about 1mL) with a Pasteur pipette and placed in a new glass tube for gas-chromatography mass spectrometry (GC-MS) analysis. The GC column was an Agilent 122-7032ui (DB-WAX-U, Agilent Technologies, Santa Clara, California, USA) of 30 m, internal diameter of 0.25 mm, and film thickness of 0.25 μm. The GC was programmed to achieve the

following run parameters: initial temperature of 50°C, hold of 1 min, ramp of 10°C min-1 up to a final temperature of 180°C, total run time of 20 min, gas flow of 70 ml min-1 splitless to maintain 12.67 p.s.i. column head pressure, and septum purge of 2.0 ml min-1. Helium was the carrier gas (1.5ml min-1 costant). Parameters of mass spectrometer were: source at 230°C and MS Quad at 150°C. The GraphPad PRISM 5 program was used to determine the concentration in mM. The data were inserted in the "XY" form in which in the "X" frame the values of the straight concentration-response were reported, while in the "Y" box the values of the area under the curve (AUC) related to the peaks obtained from the mass gas were reported. The AUC values of the single samples (obtained from the mass gas) were interpolated with the line X (concentration-response) to determine the corresponding mM.

2.6 Statistical Analyses

Comparisons of taxa or gene abundance between groups were carried out using pairwise Wilcoxon tests. Statistical significance of pangene prevalence was verified through Fisher's test with multiple-hypothesis testing corrections via the false discovery rate (FDR).

Machine learning-based classification analysis was done using the MetAML package [212] and by consering random forests (RFs) as back-end classifier for all the experiments. Results were through a five-fold cross-validation and averaged on 20 independent runs.

The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. Descriptive statistics were reported as the means and standard deviations for continuous variables, and discrete variables were reported as the number and proportion of subjects with the characteristic of interest. The level of significance for all statistical tests was 2-sided, $p<0.05$. All data were collected in a dedicated database and analysed by a statistician using SPSS for Windows (SPSS Inc, version 23.0, Chicago, IL).

4. Results

Results #1

1.1 Butyrate concentration in human milk

100 healthy lactating women were consented to participate in the study and provided HM samples during the first 5 months of lactation. Butyrate concentrations in colostrum and in mature HM are reported in Figure 5. Butyrate concentration resulted significantly higher in mature HM than in colostrum starting from the 0.5 to 0.75 mM in the 2 ° month of lactation, and remain stable until the 5° month of lactation. This result was in line with previous study in which HM has been examined as a potential source of butyrate for neonates. [213] Median butyrate concentration in mature HM was 0.75 mM. This means that a breastfed infant could receive a daily dose of butyrate of about 30 mg/Kg body weight.

1.2 Effects of butyrate on CD4+ T cells

To investigate immunological effects of butyrate, we performed time-course and dose response experiments on CD4+T cells from healthy children. Butyrate was able to stimulate IL-10 production, with a maximal effect at 0.75 mM after 24 hours of incubation. No modulation was observed for IL-4, IL-5, IL-13 and IFN-γ production (data not shown). CD4+ T cells from IgE-mediated FA patients, stimulated with BLG, OVA, PN in absence of butyrate, resulted in a significant increase in Th2 cytokines production: IL-4, IL-5 and IL-13. Co-incubating the cells with 0.75 mM, butyrate induced a significant inhibition of these effects (Figure 4). This effect was independent on a methylation of the promoter region of the IL-4, IL-5 and IL-13 genes. Instead, butyrate stimulated the tolerogenic cytokines IL-10 and IFN-γ production through a demethylation of respective genes at 0.75 mM dose. This effect paralleled with a modulation of TSDR FoxP3 demethylation and its expression (Figure 4).

1.3. Effects of butyrate on human enterocytes

The direct regulatory action elicited by butyrate on human enterocytes in the regulation of non-immune tolerogenic mechanisms is depicted in Figure 5. Butyrate stimulated the expression of tight junction (TJ) proteins, ZO-1 and occludin in Caco-2 cells. The maximal effective dose was 0.75 mM. The same butyrate dose was also able to up-regulate Muc2 and Muc5AC expression, in HT-29 and Caco-2 cells, respectively (Figure 6). Accordingly, 0.75 mM butyrate was able to increase the enterocyte mucus layer thickness (from 0.0 to 5±2 μm, NT vs 0.75 mM, $p<0.05$). We also investigated the butyrate effect on the innate immune peptide synthesis, HBD-3, that is involved in Foxp3+ T cells induction. [214]As shown in Figure 6, butyrate elicited a significant effect on HBD-3 synthesis by human enterocytes with maximum effective dose of 0.75 mM.

To determine whether butyrate acts as an HDAC inhibitor in HT-29 cells, we analyzed the HDAC activity in comparison with TSA, a well-established HDAC inhibitor. We observed that HDAC activity was reduced in a dose-dependent manner in HT-29 cells treated with butyrate (Figure 6).

1.4 Effects of butyrate in FA animal model

BLG-, OVA- and PN- sensitized mice showed a significantly higher anaphylactic symptoms score and body temperature decrease, compared to control animals. Exposing the animal to oral butyrate caused a significant inhibition of these parameters of allergic response (Figure 7).

To determine whether the butyrate administration was effective in reducing local mucosal mast cell degranulation in sensitized mice, mMCP-1 serum concentrations were measured after oral challenge. Serum mMCP-1 concentration was significantly increased in BLG-, OVA- and PN-sensitized mice compared to the control animals. Whereas, oral butyrate caused a significant reduction in mMCP-1 concentration compared to control mice. According with these data, butyrate

administration also caused a reduction in specific IgE concentrations compared to control group (Figure 7).

To study the mechanism underlying the effect of butyrate in reducing allergic response, splenocytes cytokines production was investigated. BLG-, OVA- and PN- sensitized mice showed a significant increase in Th2 cytokines production (IL-4 and IL-13) and a significant decrease in Th1 cytokines production (IL-10 and IFN-gamma) compared to control animals. These effects were significantly inhibited by butyrate administration (Figure 7).

1.5 Effects of butyrate on hepatic mitochondrial oxidative capacity and hepatic oxidative stress

Pivotal role for hepatic mitochondrial dysfunction, and consequent excessive generation of ROS, in a murine model of FA has been recently demonstrated.[215] H_2O_2 yield and ROS-induced damage on aconitase activity were measured in hepatic mitochondria. The increased ROS production was proven by the higher mitochondrial H_2O_2 yield and the lower basal/total aconitase ratio in all sensitized animals when compared to the control group. Butyrate administration efficiently modulated the oxidative stress as demonstrated by the lower H_2O_2 release and by the reactivation of the aconitase enzyme in all treated groups when compared to control animals (Figure 8).

Results #2

2.1 Study subjects

From June 2017 to June 2020, 90 subjects with a sure diagnosis of IgE-mediated allergy and 30 age-matched healthy controls (HC) were evaluated for the study. All patients accepted to participate and stool samples were collected from each child. Six stool samples failed in sequencing procedures, then metagenomics analysis was performed on 85 subjects: 30 with respiratory allergies (RA) (15 with allergic asthma and 15 with oculorhinitis), 55 with FA, and 29 HC. Among FA children, 22 resulted allergic to one allergen (11 cow's milk; 6 hen's egg; 3 nuts; 2 peach), while the other 33 resulted allergic to ≥ 2 food allergens (18 cow's milk and hen's egg; 2 cow's milk and food other than hen's egg; 8 hen's egg and food other than milk; 5 food allergens different from milk and hen's egg).

Baseline main demographic and clinical characteristics were reported in Table 3.

At 36-month of follow up, 17 out of 55 (30.9%) children with FA acquired immune tolerance. All healthy controls remained free from any allergic disease during the 36-month follow-up.

2.2 Specific microbial signatures are associated with the allergy state

We didn't find significant differences in the overall GM taxonomic composition according to the disease status (CT vs ALLERGY or CT vs FA/RA) by MANOVA computed on Jaccard distance matrix ($p>0.05$). Therefore, no specific clustering of the subjects was observed in PCoA plots based on Jaccard distance matrix (Figure 9A-9B). In addition, microbial diversity indices were not different in FA compared with CT, while higher diversity was observed in RA (Figure 10). Firstly, we evaluated the hypothesis that the allergic state (FA or RA) could be associated with specific signatures in the GM (Figure 11A). Allergic children showed significantly higher abundance of *Ruminococcus gnavus*, *Faecalibacterium prausnitzii*, *Dialister invisus*, *Anaerostipes hadrus*,

several *Blautia* and *Parabacteroides* species compared with healthy controls (Wilcoxon test, $p<0.05$). On the contrary, their GM was depleted of *Bifidobacterium longum*, *Bacteroides dorei*, *B. vulgatus* and some fibre-degrading taxa (e.g., *Roseburia CAG_471*, *Ruminococcus bromii*; Figure 11A). However, when evaluating the differences associated with the type of allergy, we also identified some allergy-specific signatures. Children with FA showed a microbial pattern characterized by decreased abundance of *B.vulgatus*, and higher levels of *Blautia wexlerae* compared with RA ($p<0.05$; Figure 11B). On the contrary, *Anaerostipes hadrus* and *Prevotella copri* were higher in subjects with RA compared with both CT and paiernts with FA (Figure 11B). We also evaluated differences in the GM in FA children showing sensitization to multiple allergens (N=33) compared with those having a single allergy (N=22), but we did not find significant differences associated with the number of FAs. Interestingly, fecal levels of butyrate and propionate were consistently higher in CT compared with both allergic groups (Figure 12).

We used a machine-learning based classification approach (see Methods) to evaluate capabilities of the GM in terms of species-level relative abundances to discriminate among different conditions. We observed a moderate (area under the curve AUC = 0.64) but significant ($p < 0.01$ by computing the statistical test against the null hypothesis of equal AUC for classification of true and shuffled labels) discrimination between healthy and allergic children irrespective of the allergy type. Moreover, we found a high discrimination (AUC = 0.79) when comparing FA and RA, supporting the finding that different microbial taxa are associated with different allergy types.

Finally, we found that specific GM features at baseline (FDR $q<0.1$) were associated with the acquisition of immune tolerance, suggesting a possible influence of the microbiome on the natural history of FA (Figure 13). Children with cow's milk allergy who developed immune tolerance (T) showed higher abundance of *B.longum*, *Lachnospira pectinoschiza* and *Anaerostipes hadrus* at diagnosis, as well as lower levels of *Ruthenibacterium lactatiformans* and *Clostridium leptum* if compared with children who not acquired immune tolerance (NT), while the baseline fecal level of

butyrate and propionate were similar into the two groups. Consistently, the machine learning-based classification showed a high discrimination between T and NT (AUC=0.74; Figure 13), that was not affected by the addition of SCFAs concentration to the model.

We used HUMAnN3 to define the functional potential of the gut metagenome and found that the GM of allergic children was characterized by higher inflammatory potential. Indeed, genes involved in the biosynthesis of the bacterial lipopolysaccharide (LPS; UniRef_A0A395J976 and UPI000F05499B) were more abundant in FA and RA compared with CT ($p<0.05$, Figure 14). Moreover, genes coding for urease (E.C. 3.5.1.5) were also enriched in allergic children, showing higher microbial potential for urea degradation with consequent ammonia production ($p<0.05$, Figure 14). On the contrary, some genes involved in complex polysaccharides degradation (E.C. 3.2.1.31, E.C. 3.2.1.15) were depleted only in FA compared with both CT and RA ($p<0.05$; Figure 14).

2.3 Allergic children harbor different functional types of *R.gnavus* and *B.longum*

We explored the possibility that a selection at strain level occurs in the GM of allergic children. Firstly, we used a mapping-based approach to define the pangenome of the 10 most abundant species. We carried out this analysis on 11 species (*Bifidobacterium bifidum*, *B.breve*, *B. adolescentis*, *B.longum*, *Bacteroides vulgatus*, *B.fragilis*, *B.uniformis*, *Eubacterium rectale*, *Akkermansia muciniphila*, *Ruminococcus gnavus*, *R.bromii*) that were selected as the most abundant and present at >2% abundance in at least 80% of the subjects. Among the taxa investigated, we identified differences associated with the allergic state in the pangenome of *B.bifidum* and *R.gnavus*. In particular, 76 and 155 pangenes of *B.bifidum* and *R.gnavus* respectively, occurred differently in CT and allergic children (either FA or RA; Table S2). *B.bifidum* pangenome discriminates healthy from allergic children, regardless the type of allergy (FA or RA; Figure 15A-B). On the contrary, when considering *R.gnavus* pangenome, we observed that CT clustered apart

from allergic children, who also separated according to the type of allergy (Figure 16A-B), suggesting the presence of different *R.gnavus* strains. Among *R.gnavus* genes that were enriched in healthy children, we identified several genes involved in complex polysaccharides degradation (e.g., acetylxylan esterase, alpha-L-fucosidase, beta-xylosidase; Figure 16C). Conversely, allergy-associated strains were characterized by higher potential to adhere to the gut epithelium, having higher prevalence of genes related with pilin and anchoring factors (Figure 16C and Table S2). We further explored the role of *R.gnavus* specifically looking for the presence of genes related with the biosynthesis of a pro-inflammatory polysaccharide[154] and we identified a significantly higher number of hits in FA and RA compared with healthy children (Figure 16D), highlighting the presence of a potential mechanism leading to inflammation in allergic children. The difference was still significant even normalizing the number of hits for the number of predicted genes in each sample.

2.4 MAGs reconstruction highlights influence of the delivery mode on sub-species diversity

To further explore the effect of allergy on sub-species diversity of the GM, we also reconstructed Metagenome Assembled Genomes (MAGs). We binned a total of 3,357 MAGs from the 117 samples that were clustered into 470 SGBs and taxonomically assigned as reported in Table S3. We further analysed newly reconstructed MAGs to explore the influence of other metadata (i.e., delivery mode and breastfeeding) on sub-species diversity. Interestingly, we identified specific strain-level signatures associated with vaginal or C-section delivery in two species (*Blautia wexlerae* and *Bacteroides vulgatus*). For both, we could identify two putative sub-species based on phylogenetic diversity (Figure 17A-B). In the case of *Bl. wexlerae*, one sub-species was almost exclusively (11 out of 12 MAGs) found in C-section delivered children (Figure 17A). For *B. vulgatus*, we identified one sub-species found both in C-section and vaginal delivered children,

while another was exclusive of vaginal delivery (Figure 17B). In both cases, no association with allergy was found.

5. Discussion

The results of our study demonstrate that HM butyrate stimulates a wide range of immune and non-immune tolerogenic mechanisms able to protect against FA. Immune tolerance is a state of active non-responsiveness to ingested soluble antigens mediated by gut-associated intestinal lymphoid tissue. Inducible FoxP3+ CD4+ Treg cells are central to the maintenance of immune homeostasis and tolerance throughout the body, particularly in the gut. Other evidence suggests also a role for the complex interaction between gut microbiota and immune and non-immune cells in shaping immune tolerance. The presence of both diet-and microbe-induced populations of Treg cells is required for oral tolerance to food antigens.[216] In our study, we observed that butyrate is able to modulate these mechanisms of oral tolerance.

We evaluated the direct effects of butyrate on CD4+ T cells isolated from PBMCs from children affected by challenge-proven IgE-mediated FA. CD4+ T cells were stimulated in vitro with different allergens, BLG, PN and OVA, in the presence or absence of butyrate. Butyrate stimulated, in a dose-dependent manner, tolerogenic cytokines, such as IL-10 production, through a demethylation of respective gene. We found also that butyrate induced a FoxP3 demethylation and a concomitantly increase of its expression. The mechanisms of action of butyrate are multiple, many of these involve an epigenetic regulation of gene expression through the inhibition of histone deacetylase (HDAC). [217] It has been demonstrated that the inhibition of HDAC 6 and 9 is responsible for the increase in FoxP3 gene expression and increase of Tregs. [218] Acetylation of Foxp3 is an important posttranslational mechanism that affects Foxp3 abundance, because it protects Foxp3 proteasomal degradation. [219] This is a signature mechanism of action of several HDACs in Tregs. [220,221, 222,223,224]

We also explore the direct effects of butyrate on human enterocytes. Our data suggest that SCFA butyrate contribute to mucosal homeostasis through the induction of Treg cells and the regulation of epithelial barrier integrity. Loss of epithelial integrity in the gut increases antigen uptake and

promotes secretion of epithelial-derived cytokines IL-33, thymic stromal lymphopoietin, and IL-25.[225] These cytokines promote Th2-type allergic response by activation of ILC2s, mast cells, basophils, and DCs. [226] Activation of ILC2s stimulates production of IL-4–, IL-5–, and IL-13–promoting Th2-type allergic responses. [227] Overall, the state of the epithelial barrier is thought to be important for sensitization to food antigens.

The positive modulation of gut mucosa integrity by butyrate is supported by the up-regulated expression of TJ proteins, which in turn are involved in the tuning of epithelial permeability. These results are in line with previous observations demonstrating that butyrate is able to maintain epithelium barrier integrity through an increase of ZO-1 and occludin expression. [228]

A significant increase of Muc2, Muc5AC expression and mucus layer thickness was also observed after stimulation of human enterocytes with butyrate. Similarly, Gaudier et al. demonstrated that butyrate differently stimulates the expression of various mucin genes in the colon, with maximum effects on Muc2 expression. [229] The mucus layer, covering the gastrointestinal mucosa, is considered as the first line of defense against mechanical, chemical, or microbiological aggressions arising from the luminal contents. [230] The mucus layer does not merely form a nonspecific physical barrier, but also constrains the immunogenicity of gut antigens by delivering tolerogenic signals. [231] In the colon, the mucus layer is directly in contact with the butyrate produced by gut microbiota, representing the major energy source for colonocytes. [232]

Moreover, butyrate stimulates the HBD-3 synthesis, an innate immune peptide, involved in induction of FoxP3+ T cells. [233]

To confirm the butyrate immune and non immune effects demonstrated in vitro, we performed an in vivo model of FA. In a murine model of FA, the results of anaphylactic symptom score and body temperature decrease demonstrated that butyrate administration inhibits allergic response. Accordingly, butyrate administration caused a significant reduction in mMCP-1 concentrations,

which reflects a reduction in mast cell degranulation, and a reduction in sIgE concentrations compared to control group.

To study the mechanism underlying the effects of butyrate administration in reducing allergic response, cytokine production by T-cells in the spleen was studied and a modulation of Th1/Th2 cytokine expression was observed. We found an enhancement in IL-10 expression, a major immune-regulatory cytokine that maintains mucosal homeostasis and limits excessive immune responses against dietary and bacterial antigens present in the intestinal lumen.

We found liver mitochondrial dysfunction in FA sensitized mice. After sensitization with three antigens, a significant decrease in mitochondrial state 3 respiration rates was observed in PN and BLG sensitized animals when compared to control animals, while butyrate administration improved mitochondrial respiration rates in treated animals. Instead, butyrate administration efficiently modulated the oxidative stress as demonstrated by the lower H_2O_2 release and by the reactivation of the aconitase enzyme in all treated mice groups. Mitochondrial dysfunction and elevated ROS have been reported in allergic disease. [234,235,236,237] The involvement of liver in FA is emerging.[238] Studies in a murine model suggest that the liver could act as a source of helper CD4+ T cells and could play an important role in the IgE response to dietary antigens. [239] As demonstrated in the airway epithelium of asthma animal model, mitochondrial dysfunction precedes allergic inflammation, where prior to antigen exposure, mitochondrial dysfunction exacerbates allergen-induced accumulation of eosinophils, mucin levels, and airway hyperresponsiveness. [234]

Interestingly, the most effective butyrate dose is similar to mean butyrate concentration in HM. Data generated from Global Exploration of Human Milk (GEHM) study suggests that butyrate concentration in HM through the first year of lactation are consistent across regions, but that factors such as maternal diet or microbiome could influence overall levels given butyrate's association with individual donor. [240] From the analysis of 3-days dietary diary of the lactating mothers, we observed a significant correlation between their daily fiber intake and HM butyrate concentration. As more

dietary fibers are ingested, SCFAs production increases. [241] New evidence show that maternal SCFA levels during pregnancy can directly influence the health of infants. Thorburn et al. observed that when a high-fiber diet was consumed during pregnancy, maternal serum acetate levels were higher. [242] Lower serum levels of acetate during pregnancy were associated with wheeze in infants. In a follow-up murine model experiment, feeding dams acetate during pregnancy and the immediate postpartum period reduced the development of allergic airway inflammation in offspring. In the field of FA, we know that an infant diet consisting of high levels of fruits, vegetables, and home-prepared foods is associated with less FA by the age of 2 years. [243] The finding that high fiber diet protects mice against peanut allergy through diverse cellular pathways supports these data. Tan et al. find that this effect involves reshaping of the gut microbiota as well as increased levels of SCFAs and activity of their receptors. High fiber feeding also increased tolerogenic CD103+ DCs activity, leading to increased Treg cells differentiation. [244] The results from our work suggest that the dietary fiber intake of the lactating mothers could influence the HM butyrate concentration, with multiple protective effects against FA. This could be relevant for future research, because all the observed effects were dose dependent. Our data open the possibility of using butyrate as a supplement in the formulas of non-breastfed children for preventing FA in early life. A limitation of our study is that we are unable to determine where the butyrate present in HM derives. Considering that there are bacterial species in the HM, we cannot rule out that it derives from these ones or from maternal intestinal microbiota. Another limitation of our study is that HM contains a wide range of bioactive factors in addition to butyrate: such as proteins, polyunsaturated fatty acids, oligosaccharides, microbial content, metabolites, and micronutrients that can influence the infant's gut immune maturation.

Gut dysbiosis refers to an unbalance in the composition and activity of the GM. Dysbiosis was previously associated with different disease conditions, although a cause-effect mechanism remained largely undefined.[130] The link between GM and allergic diseases was explored in several

studies and microbial signatures specific of the different allergies were identified, although a general agreement does not exist.[245] This is the first study employing a shotgun metagenomics approach to the study of GM composition and functional potential in children with IgE-mediated allergy. We identified common features in the GM of allergic children, regardless the type of allergy (food or respiratory). Indeed, we found an increase in *Firmicutes* and a decrease in *Bacteroidetes* taxa in allergic children, as previously reported.[245] In agreement with previous findings, the GM in FA and RA was characterized by higher abundance of *F.prausnitzii*, *R.gnavus*, *Blautia wexlerae*, *Anaerostipes hadrus*, as well as lower levels of *B.longum*, *B.dorei*, *B.vulgatus* and several fibre-degrading species compared with healthy controls.[147,246,247,28,248] In addition, we didn't find differences related to the co-occurrence of multiple FAs, which is in agreement with previous findings.[39] However, comparison with previous data is difficult, since existing studies are all based on lower resolution techniques (e.g., 16S rRNA sequencing) and often achieved taxonomic identification at genus level or even above. Althought the microbiome in FA is a widely researched subjected, we need still others studies. Bao et al. collected fecal sample from a cohort of twins concordant or discordant for FA to identify bacterial signatures and metabolic pathways that may influence the expression of this disease.[249] A bacterial signature of 64 operational taxonomic units (OTUs) distinguished healthy from allergic twins: the OTUs enriched in the healthy twins were largely taxa from the *Clostridia* class. Bao and collegues detected significant enrichment in distingt metabolite pathways in each group. The enrichment of diacylglycerol in healthy twins is of particular interest for its potential as a readily measurable fecal biomarker of health. In addition, an integrated microbial-metabolomic analysis identified a significant association between healthy twins and *Phascolarctobacterium faecium* and *R. bromi*.

Moreover, in this study, we firstly highlighted that the GM dysbiosis at taxonomic level is also reflected in an altered functional potential. The GM of allergic children showed higher levels of ureases and genes related with LPS biosynthesis. LPS stimulates the production of pro-

inflammatory cytokine, thus activating the inflammatory cascades[250] and it was previously associated with the onset of allergic rhinosinusitis.[251] Ureases are involved in urea degradation and ammonia production. An increased potential for urea degradation was suggested to promote gut microbiota dysbiosis and to exacerbate colitis in mice.[252] Conversely, allergy GM showed lower potential for complex fibre degradation, explaining the lower concentration of the SCFAs butyrate and propionate found in allergic subjects compared with healthy controls, both in this study and in previous reports. [47,39] Therefore, the metagenome of allergic diseases is defined by an overall higher pro-inflammatory potential compared with healthy children, with an increased production of pro-ainflammatory molecules, and a decreased biosynthesis of anti-inflammatory SCFAs.

In addition, we identified specific signatures at sub-species levels linked with the allergic disease, suggesting the presence of a strain-level adaptation to the pro-inflammatory environment typical of the allergic condition. Indeed, we recognized a strain diversity linked to allergy in *B. longum* and *R. gnavus*. In particular, *R. gnavus* strains associated with allergy showed an enriched ability to adhere to the gut epithelium and colonize the gut environment, that may contribute to a pathogenic mechanism, [253,254] as well as a depletion of genes involved in complex polysaccharides break-down, negatively contributing to the reduced concentration of SCFAs found in allergic children. These results supported a previous hypothesis of a determinant role of *R. gnavus* in the development of allergy in the pediatric age,[246] but firstly highlighted that this association may be strain-dependent. Indeed, recent findings suggest that high variability at strain level exists in the GM and that different strains may be differently linked with health or diseases [255, 256, 257] *R. gnavus* abundance increased with the consumption of an unhealthy diet rich in fat and animal products.[255, 258] In addition, it was previously associated with inflammatory bowel diseases (IBD)[259,260] and a recent study proposed a mechanism mediated by the production of an inflammatory polysaccharide. [209] Accordingly, we identified a higher number of genes involved in the production of this polysaccharide [209] in the gut metagenome of allergic children. In addition, Hall et al. identified

disease-specific clades of *R. gnavus* associated with IBD, [260] characterized by higher adhesion potential to the gut epithelium, consistently with our results.

Finally, we identified specific microbial signatures that may be involved in the resolution of FA after 36 months of exclusion diet. In a previous study, FA resolution at 8 years was linked with increased baseline abundance of *Clostridia.*[27] Thanks to a higher resolution, we identified higher abundance of *Lachnospira pectinoschiza* and *Anaerostipes hadrus* (both *Clostridia* class), as well as of *B. longum.* Indeed, gut microbiome composition could predict the development of immune tolerance in a Random Forest classification model. This may suggest a possible implication of the GM in the immune tolerance acquisition pathways.

6. Conclusion

Gut microbiota could be a promising target for innovative preventive and therapeutic strategies against FA. Studies are promising, but more data are needed to better define the potential of modulating the diet-gut microbiota–immune system axis to fight FA. We are approaching a new era where we can regulate immune system development and function through dietary intervention and measure the clinical impact through gut microbes and their metabolites. Given the current gaps in the investigational approaches, and data analysis and interpretation, we need more scientific evidence that can be translated in clinical practice. Understanding how nutrients and metabolites, or probiotics could influence gut bacteria communities and the immune system will contribute to build up a precision medicine approach for FA care (Figure 18).

In summary, using high-resolution metagenomics, we highlighted GM dysbiosis in allergic children and strain-level adaptation in allergy, with R. gnavus emerging as likely involved in the allergic disease. We also suggest that the production of pro-inflammatory molecules and the reduced ability to catabolize complex polysaccharides may be associated with the increased inflammation typical of allergic conditions. These findings support the importance of the GM in the onset of allergic diseases and may open new cues in the development of innovative therapeutic strategies based on microbiome manipulation for allergy treatment.

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8. Tables

Table 1: Main gut microbiota features in food allergy

	OTUs	DIVERSITY	TECHNOLOGY	MAIN FEATURES	REFERENCES
Byorkesten et al. 1999 (n=62; FA)	N.R.	N.R.	*Bacterial culture*	↑ *Coliforms, S. Aureus* ↓ *Lactobacilli, Bifidobacteria*	*261*
Thompson-Chagoyan OC et al. 2010 (n=46:FA)	↑	N.R.	*Bacterial culture*	↑ *Lactobacilli* ↓ *Bifidobacteria*	*262*
Thompson-Chagoyan OC et al. 2011 (n=46:FA)	N.R.	N.R.	*Bacterial culture*	↑ *C.coccoides, Atophium cluster*	*263*
Nakayama J et al. 2011 (n=11: FA)	=	=	16s rRNA sequencing	↑ *Bacteroides,Propionibacterium , Klebsiella* ↓ *Acinobacterium, Clostridium*	*264*
Ling Z et al. 2014 (n=34: FA)	↓	=	16s rRNA sequencing	↑ *Bacteroidetes, Proteobacteria, Actinobacteria* ↓ *Firmicutes*	*265*
Azad MB et al. 2015 (n=12: FS)	↓	=	16s rRNA sequencing	↓ *Enterobacteriaceae, Bacteroidaceae*	*26*
Chen CC et al. 2015 (n=23: FS)	N.R.	↓	16s rRNA sequencing	↑ *Firmicutes, Proteobacteria, Actinobacteria* ↓ *Veillonella*	*266*
Berni Canani et al. 2016 (n=39; FA)	↑	N.R.	16s rRNA sequencing	↑ *Ruminococcaceae, Lachnospiraceae* ↓ *Bifidobacteriaceae, Streptococcaceae, Enterobacteriaceae*	147
Bunyavanich S.et al.2016 (n=226; FA)	↑	N.R.	16s rRNA sequencing	↑ *Bacteroidetes, Enterobacter*	*27*
Inoue R et al. 2017 (n=4: FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Lachnospira, Veillionella, Suterella* ↓ *Dorea, Akkermansia*	*267*
Kourosh A. et al. 2018 (n=68; FA)	↑	N.R.	16s rRNA sequencing	↑ *Oscillobacter valericigenes, Lachnocrostidium bolteae, Faecalibacterium sp.*	*268*
Fazlollahi M. et al.2018 (n=141; FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Lachnospiraceae, Streptococcaceae, Leuconostocaceae*	*48*
Dong et al. 2018 (n=60; FA	N.R.	N.R.	16s rRNA sequencing	↑ *Lactobacillaceae* ↓ *Bifidobacteriaceae, Ruminococcaceae*	*269*
Berni Canani R. et al. 2018 (n=46; FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Bacteroides, Alistipes*	*47*
Diaz M. et al. 2018 (n=27; FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Coriobacteriaceae*	*46*
Abdel Gadir A. et al. 2019 (n=56; FA)	↑	N.R.	16s rRNA sequencing	↓ *Clostridiales species, Bacteroidales*	*270*
Cait A. et al. 2019 (n=105; FS)	N.R.	N.R.	Shotgun metagenomic analysis	↑ *F. prausnitzii* ↓ *Roseburia, Ruminococcus bromii, Bacteroides dorei*	*28*
Feehley et al. 2019 (n=24; CMA)	↓	N.R.	16s rRNA sequencing	↑ *Parabacteroides, Blautia, Enterococcus*	*247*
Galazzo G. et al 2020 (n=440; FS)	N.R.	N.R.	16s rRNA sequencing	↓ *Faecalibacterium; Dialister*	*248*
Goldberg et al. 2020 (n=233; FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Collinsella aerofaciens, Dorea formicigenerans, unclassified Methanobrevibacter), Blautia obeum, Coprococcus catus* ↓ *P. Copri, Bifidobacterium adolescentis*	*39*
Bao R. et al. 2021 (n=36; FA)	↓	N.R.	16s rRNA sequencing	↓ *Phascolarctobacterium faecium, Ruminococcus bromii.*	*249*
Mennini M. et al. 2021 (n=26; FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Haemophilus, Klebsiella, Prevotella, Actinobacillus and Streptococcus*	*271*

Abbreviations: FA= food allergy; FS= sensitization to food antigens; OTUs = operational taxonomic units; N.R.= not reported

Table 2. Main Tools for the study of gut microbiota

Technique	Description	Advantages	Disadvantages
Culture	Isolation of bacteria on selective media	Cheap, semi-quantitative	Labour intensive, <30% of gut microbiota have been cultured to date
Qpcr	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluoresces when it binds to double-stranded DNA	Fast,quantitative, Phylogenetic identification	PCR bias, unable to identify unknown species
DGGE/TGGE	Gel separation of 16S rRNA amplicons using denaturant/ temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments separated by gel electrophoresis	Fast, cheap, semi-quantitative	No phylogenetic identification, PCR bias, low resolution
Fish	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	Phylogenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences—unable to identify unknown species
DNA microarrays	Fluorescently labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser	Fast, Phylogenetic identification, semi-quantitative	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, laborious, expensive, cloning bias
Gas Chromatography Mass Spectrometry (GC-MS)	Thermally stable and volatile compounds are separated by GC and then eluting metabolites are detected by electron-impact (EI) mass spectrometers.	High efficiency, reproducibility and sensitivity	It can only be performed for volatile compounds
Liquid Chromatography Mass Spectrometry (LC)	Allows to separate compounds with little effort in a few pre-analytics steps (compared to GC-MS). The metabolite separation obtained with LC is followed by electrospray ionization (ESI) or atmospheric chemical ionization under pressure (APCI)	Lower temperatures of analysis, and it does not require sample volatility. Sensitivity, specificity, resolving power, and capability to extract additional information about metabolites from their retention time (RT) domain.	
Capillary Electrophoresis Mass Spectrometr (CE)	Offers high-analyte resolution and detect a wider spectrum of (polar) compounds compared to HPLC.	High resolution	It is properly applicable only to charged analytes
Fourier Transform Infrared Spectroscopy (FTIR)	Allows rapid, non-destructive and high-throughput determination of different sample types. This technique allows detecting different molecules, such as lipids and fatty acids (FAs), proteins, peptides, carbohydrates, polysaccharides, nucleic acids.	Ultra-high mass resolution able to distinguish slight variations in a wide number of mass signals,and allowing to obtain the structural identification of new biomarkers	Not high sensitivity and selectivity
Nuclear Magnetic Resonance Spectroscopy (NMR)	It uses the intramolecular magnetic field around atoms in molecules to change the resonance frequency, thus allowing access to details of molecules' electronic structure and obtaining information about their dynamics, reaction state, and chemical environment.	Useful to determine metabolic fingerprints leading to the identification and quantification of compounds in a non-targeted large-scale, in a non-destructive way, and with a high reproducibility	It is a relatively insensitive technique, and can only detect metabolites in high concentrations
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons for example, 454 Pyrosequencing® (Roche Diagnostics GMBH Ltd, Mannheim, Germany) (amplicon immobilized on beads, amplified by emulsion PCR, addition of luciferase results in a chemoluminescent signal)	Fast, Phylogenetic identification, quantitative, identification of unknown bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome (e.g. 454 pyrosequencing® or Illumina®, San Diego, CA, USA)	Phylogenetic identification, quantitative	Expensive, analysis of data is computationally intense

Abbreviations: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence in situ hybridization; qPCR, quantitative PCR; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism.

Table 3. Demographic and clinical characteristics of the population

	Patients with respiratory allergy	Patients with food allergy	Healthy controls
N.	30	55	29
Male, n (%)	21 (70)	34 (61.8)	15 (51.7)
Spontaneous delivery, n (%)	16 (53.3)	32 (58.2)	16 (55.2)
Born at term, n (%)	30 (100)	55 (100)	29 (100)
Birth weight, gr (mean, SD)	3207 (372.8)	3241.8 (453.4)	3145.9 (546.3)
Age at diagnosis, months (mean, SD)	57.8 (10.9)	14 (15.1)	-
Age at enrollment, months (mean, SD)	57.8 (10.9)	57.4 (11)	62.1 (10.1)
Breastfeeding for at least 4 weeks, n (%)	15 (50)	41 (74.5)	20 (69)
Duration of breastfeeding, months (mean, SD)	7.4 (6.2)	8.6 (7.4)	9.5 (9)
Weaning age, months (mean, SD)	5.3 (0.8)	5.3 (1.2)	5.1 (0.7)
Familial allergy risk, n (%)	19 (63.3)	40 (72.7)	0 (0)
Gastrointestinal symptoms , n (%)	0 (0)	30 (54.5)	-
Vomiting, n (%)	0 (0)	20 (36.4)	-
Diarrhea, n (%)	0 (0)	16 (29.1)	-
Cutaneous symptoms, n (%)	0 (0)	30 (54.5)	-
Urticaria, n (%)	0 (0)	30 (54.5)	-
Respiratory symptoms, n (%)	30 (100)	0 (0)	-
Asthma, n (%)	15 (50)	0 (0)	-
Oculorhinitis, n (%)	15 (50)	0 (0)	-

9. Figure Legends

Figure 1. The food allergy pyramid

Children with FA present an increased risk to develop later in the life other conditions such as allergic disorders (atopic march), inflammatory bowel diseases (IBD), functional gastrointestinal disorders (FGIDs), and neuropsychiatric disorders. Several genetic factors are implicated in the pathogenesis of these conditions, but recent evidence suggest the pivotal role of gut microbiota dysbiosis (induced by environmental factors). Emerging evidence support the hypothesis of dysbiosis as the first hit in the development of alterations in intestinal barrier and immune system function (responsible for the occurrence of FA and atopic march) and dysregulation of the brain-gut endocrine-immune system axis (responsible for the occurrence of FGIDs, IBD and neuropsychiatric disorders), at least in part through an activation of epigenetic mechanisms.

Figure 2. Gut microbiota as a target of intervention against food allergy

Several genetic, environmental and dietary factors could modulate the gut microbiota-immune system axis influencing the occurrence of FA. For instance, increased family size, exposure to pets and/or rural environment, healthy diet (full of fibers, fermented foods, antioxidants, omega-3), breastfeeding and use of probiotics are associated with protection to FA. Conversely, C-section, prenatal and early-life exposure to antibiotics/gastric acidity inhibitors/antiseptic agents, unhealthy diet (low fibers/high saturated fats and junk foods) may increase the risk for the development of FA. All these environmental factors act mainly on a modulation of gut microbiota composition and function which in turn could be responsible for the epigenetic regulation of genes involved in immune tolerance.

Figure 3. The experimental design of food allergy mouse model

Four-weeks-old female C3H/HeJ mice were used in FA animal model. Two weeks before first sensitization, oral gavage with 30 mg/kg/d of butyrate was started and continued during the whole study. Mice were sensitized orally on day 0, 7, 14, 21, 28 with 20 mg of BLG or 1 mg of OVA or 12 mg of PE mixed with 10 µg cholera toxin (CT) as adjuvant. Control mice receive CT only. On day 35 mice were challenged by gavage with BLG (50mg) or OVA (5mg) or PE (36mg). Anaphylaxis score and rectal temperature were assessed for 1 h after challenge and blood samples were collected to measure mMCP-1 and sIgE. After 24h, mice were sacrificed; colon, ileum and spleen were collected.

Figure 4 Modulation of Th1 /Th2 response and FoxP3 expression in CD4+ T cells from children affected by food allergy stimulated with butyrate

(A) CD4+ T cells from children with IgE-mediated FA were stimulated with BLG, OVA,PN (100 µl/well) and butyrate (0.75 mM) for 24h and a significant increase of IL-4,IL-5, IL-13 production were observed.

(B) A significant inhibition of IL-4, IL-5 and IL-13 production was observed with 0.75 mM butyrate.

(C) Butyrate stimulated, at the some dose, IL-10 and IFN-γ production and FoxP3 expression, through a demethylation of respective gene (D). Data were analyzed with independent sample t-test. NT= untreated cells; BLG= β-lactoglobulin; OVA= ovalbumin; PN = peanut extract. *$p<0.05$; **$p<0.01$.

Figure 5. Butyrate breast milk concentrations during first 5 months of lactaction.

(A) butyrate concentrations increase in mature HM compared to colostrum.

(B) resulted significantly higher in mature HM than in colostrum starting from the 0.5 to 0.75 mM in the 2 ° month of lactation, and remain stable until the 5° month of lactation. Data were analyzed with independent sample t-test. *p<0.05.

Figure 6. Immune and non immune effects and HDAC activity of butyrate effects on human enterocytes.

Dose-response direct effects of butyrate on HBD-3 (A), Muc5AC (B), Muc2 (C) and tight junctions expression levels (D) on HT-29 cells.

(E) Butyrate acts as an HDAC inhibitor in a dose-dependent manner.

Data represent the mean (± standard deviation; represented by vertical bars) of two independent experiments, each performed in triplicate; Data were analyzed with independent sample t-test. HBD-3= β-defensin 3; Muc5AC=mucin 5AC; Muc2=mucin 2; ZO-1= zonula occludens 1; GUS = glucuronidase; TSA=trichostatin A. *p<0.05; **p<0.001vs untreated cells.

Figure 7. Evidence on effective sensitization to food allergy in the animal model and Th2 and Th1 response in spleen from mice sensitized to food allergens.

Pre-treatment with 30 mg/kg/day of butyrate in a BLG-, OVA-, PN- sensitized mice significantly reduced anaphylactic score (A), serum MCP-1 (B) and sIgE levels (C).

Cells from spleen were stimulated 1 μg/mL anti-CD3 (clone 2C11), or 200 μg/mL BLG/OVA/PN and incubated at 37 °C for 72 h. (D) Butyrate stimulated IL-10 and IFN-γ (E) production and

inhibited IL-4 (F) and IL-13 (G) production in BLG-, OVA-, PN- sensitized mice. Data are reported as means ± SEM from duplicate analyses. Data were analyzed with independent sample t-test. CTRL= control mice; BLG= mice sensitized with β-lactoglobulin; OVA= mice sensitized with ovalbumin; PN = mice sensitized with peanut extract. *p<0.05; **p<0.01; ***p<0.001

Figure 8. Evidence of hepatic mitochondrial dysfunction in mice sensitized to food allergens.

(A) The mitochondrial H2O2 yield and (B) the basal activity of the aconitase were reported. Data are reported as means ± SEM from duplicate analyses. Data were analyzed with independent sample t-test. Control = control mice; BLG= mice sensitized with β-lactoglobulin; OVA= mice sensitized with ovalbumin; PN = mice sensitized with peanut extract. * p<0.05 vs control;# p<0.05 vs sensitized group.

Figure 9. Overall gut microbiome composition does not discriminate allergic and healthy children

Principal coordinates analysis based on the gut microbiome composition (as obtained by MetaPhlAn3). Allergic children are included in a unique group (A) or separated according to the type of allergy (B).

Figure 10. Respiratory allergy leads to higher gut microbial diversity

Box plots showing Shannon (A, C) and Simpson (B, D) diversity index in allergic and healthy children. Allergic children are included in a unique group (C, D) or separated according to the type of allergy (A, B). The significance was tested by applying pair-wise Wilcoxon test. CT, healthy controls; ALLERGY, allergic children; FA, food allergy; RA, respiratory allergy.

Figure 11. Microbial signatures in allergic children gut microbiome

Heatplot reporting the average relative abundance of microbial taxa significantly different between healthy (CT) and allergic children (ALLERGY) (A) or healthy children (CT) and children with food (FA) or respiratory (RA) allergies (B), as defined by Wilcoxon test (FDR $q<0.1$). In panel B, * indicates significance between CT and FA; # indicates significance between CT and RA.

Figure 12. Short-chain fatty acids are depleted in the gut of allergic children

Box plots showing the concentration of butyrate (A, C) and propionate (B, D) in faecal samples of allergic and healthy children. Allergic children are included in a unique group (C, D) or separated according to the type of allergy (A, B). The significance was tested by applying pair-wise Wilcoxon test. CT, healthy controls; ALLERGY, allergic children; FA, food allergy; RA, respiratory allergy.

Figure 13. Gut microbiome features may predict the development of oral tolerance

Heatplot reporting the average relative abundance of microbial taxa significantly different between food allergy children developing (T) or not (NT) oral tolerance upon 36 months of exclusion diet, as defined by Wilcoxon test (FDR $q<0.1$). Taxa are ordered according to the feature importance score from Random Forest classification model, indicated in the side colored bar.

Figure 14. Gut microbiome of allergic children shows a higher inflammatory potential and a reduced ability to degrade complex polysaccharides

Box plots showing the relative abundance of microbial genes involved in lipopolysaccharide biosynthesis (A, B), urea (C) and fibre degradation (D, E) in healthy (CT), food (FA) and respiratory (RA) allergic children.

Figure 15. Allergic children harbor a different B. bifidum pangenome

A: Presence and absence of 76 *B. bifidum* genes significantly different between healthy (CT), food (FA) and respiratory (RA) allergic children (blue, present; gray, absent). The significance was tested by applying paired chi-squared tests; B: Principal coordinates analysis based on presence/absence of the 76 significant genes. The complete list of the 76 significant genes and their prevalence is reported in Supplementary Table S1B.

Figure 16. Allergic children harbor a different R. gnavus pangenome

A: Presence and absence of 155 *R. gnavus* genes significantly different between healthy (CT), food (FA) and respiratory (RA) allergic children (blue, present; gray, absent). The significance was tested by applying paired chi-squared tests; B: Principal coordinates analysis based on presence/absence of the 155 significant genes; C: Heatplot showing the prevalence (%) of selected significant genes in the three children groups (CT, healthy controls; FA, food allergy; RA, respiratory allergy). The complete list of the 155 significant genes and their prevalence is reported in Supplementary Table S1A; D: Box plots showing the number of hits (> 90% identity over 50% of query length) against *R. gnavus* genes involved in the production of a pro-inflammatory polysaccharide.

Figure 17. Delivery mode selects different Blautia wexlerae and Bacteroides vulgatus strains, independently from the health status

Phylogenetic tree of the *Blautia wexlerae* (A) and *Bacteroides vulgatus* (B) Metagenome-Assembled Genomes (MAGs) retrieved from the children analysed. Outer ring is colored according to the delivery mode (pink, C-section; green, vaginal delivery).

Figure 18. Toward a gut microbiota-based precision medicine against food allergy

We are approaching an era where the metagenomic and metabolomic evaluation of gut microbiota in children at risk for FA will drive personalized intervention to preserve or restore an "eubiosis" state based on nutritional counseling and educational programs.

10. Figures

Figure 1. The food allergy pyramid

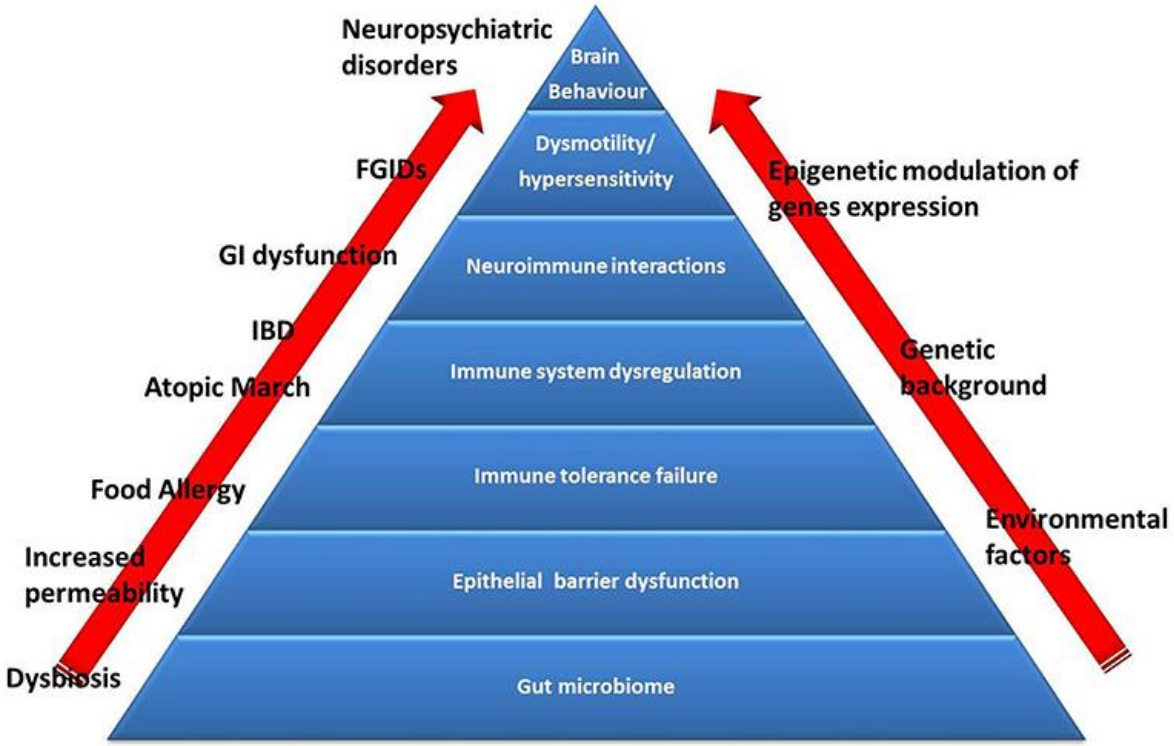


Figure 2. Gut microbiota as a target of intervention against food allergy

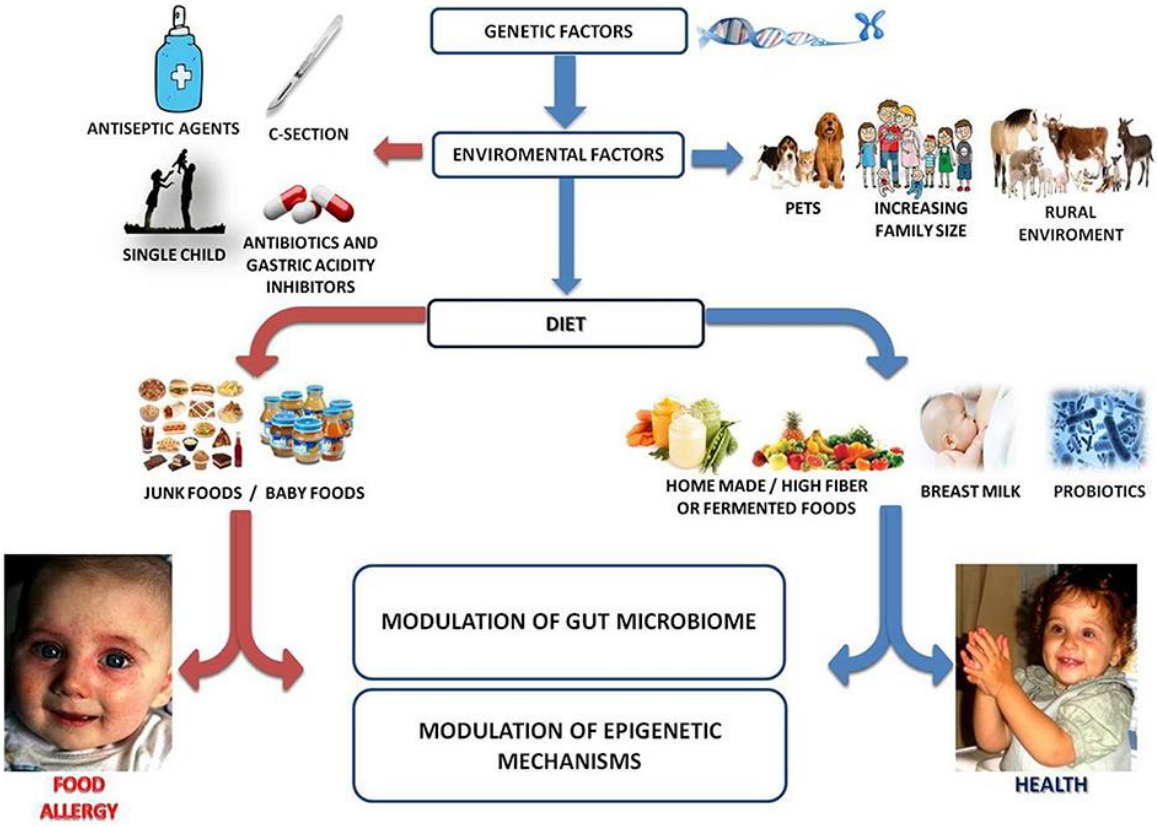
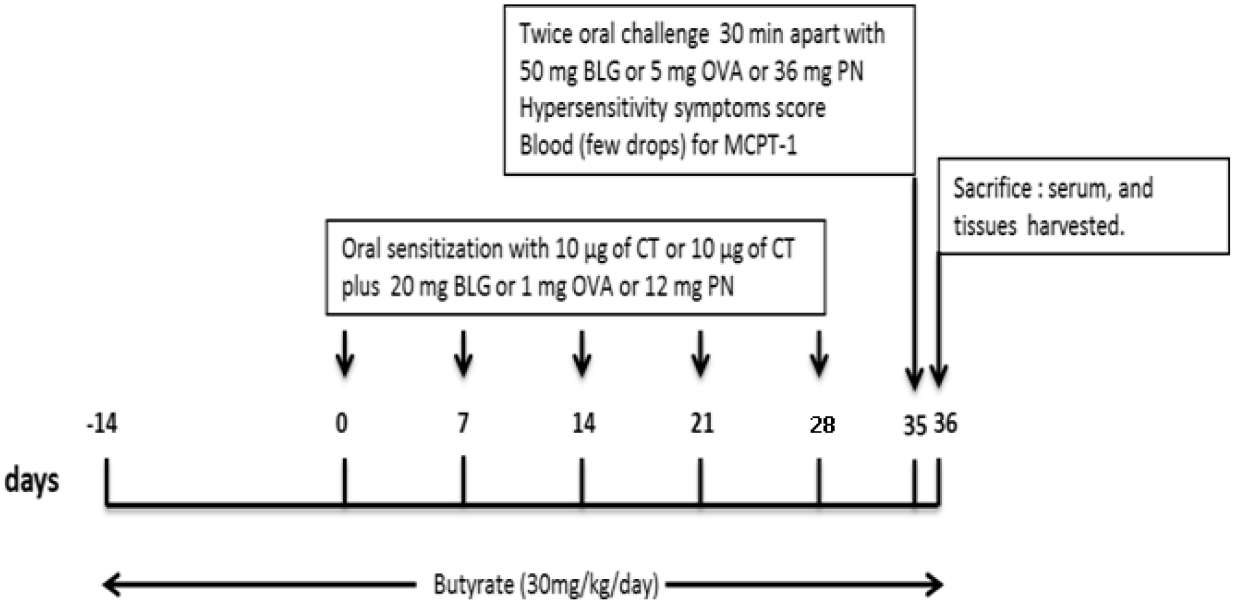


Figure 3. The experimental design of food allergy mouse model



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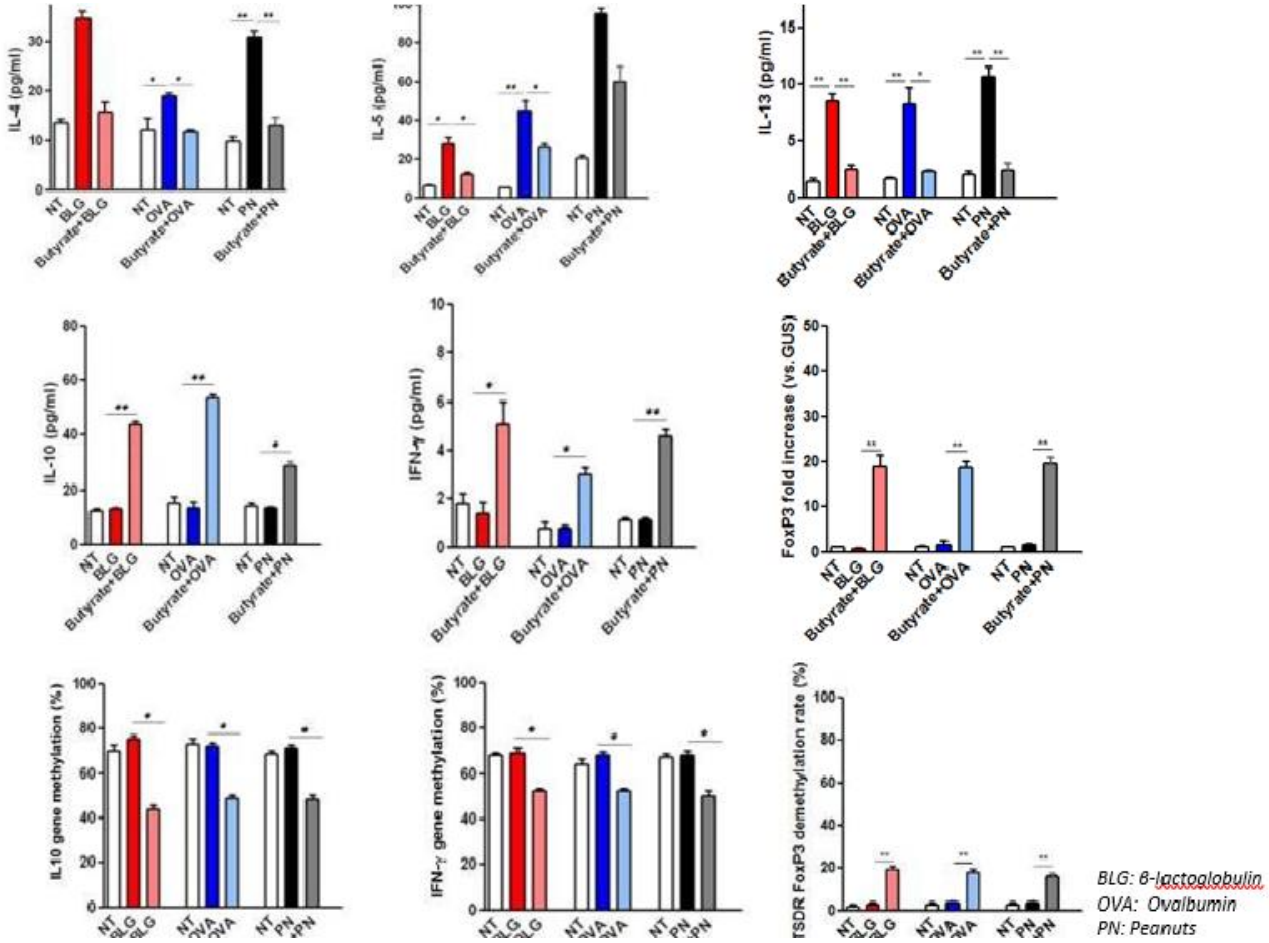


Figure 5. Butyrate breast milk concentrations during first 5 months of lactaction.

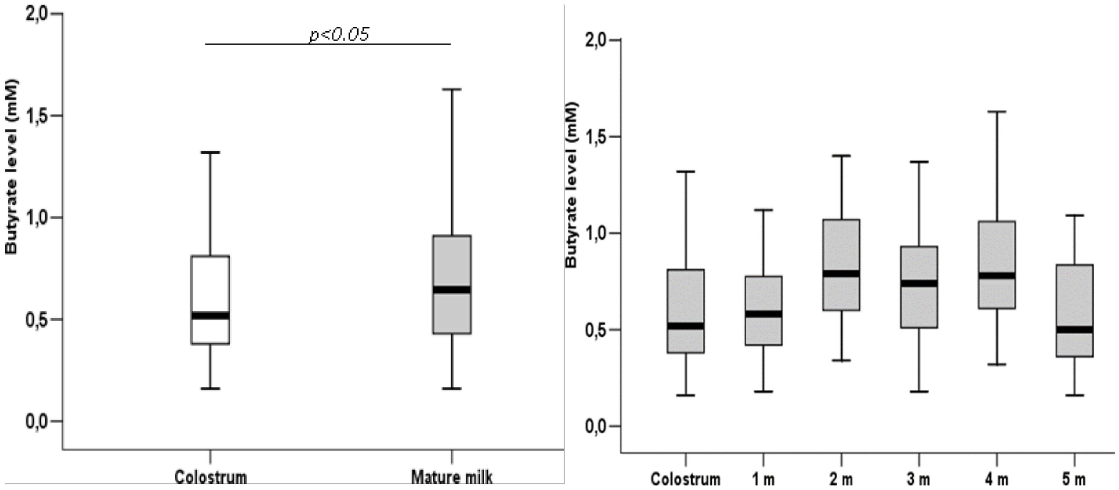


Figure 6. Immune and non immune effects and HDAC activity of butyrate effects on human enterocytes.

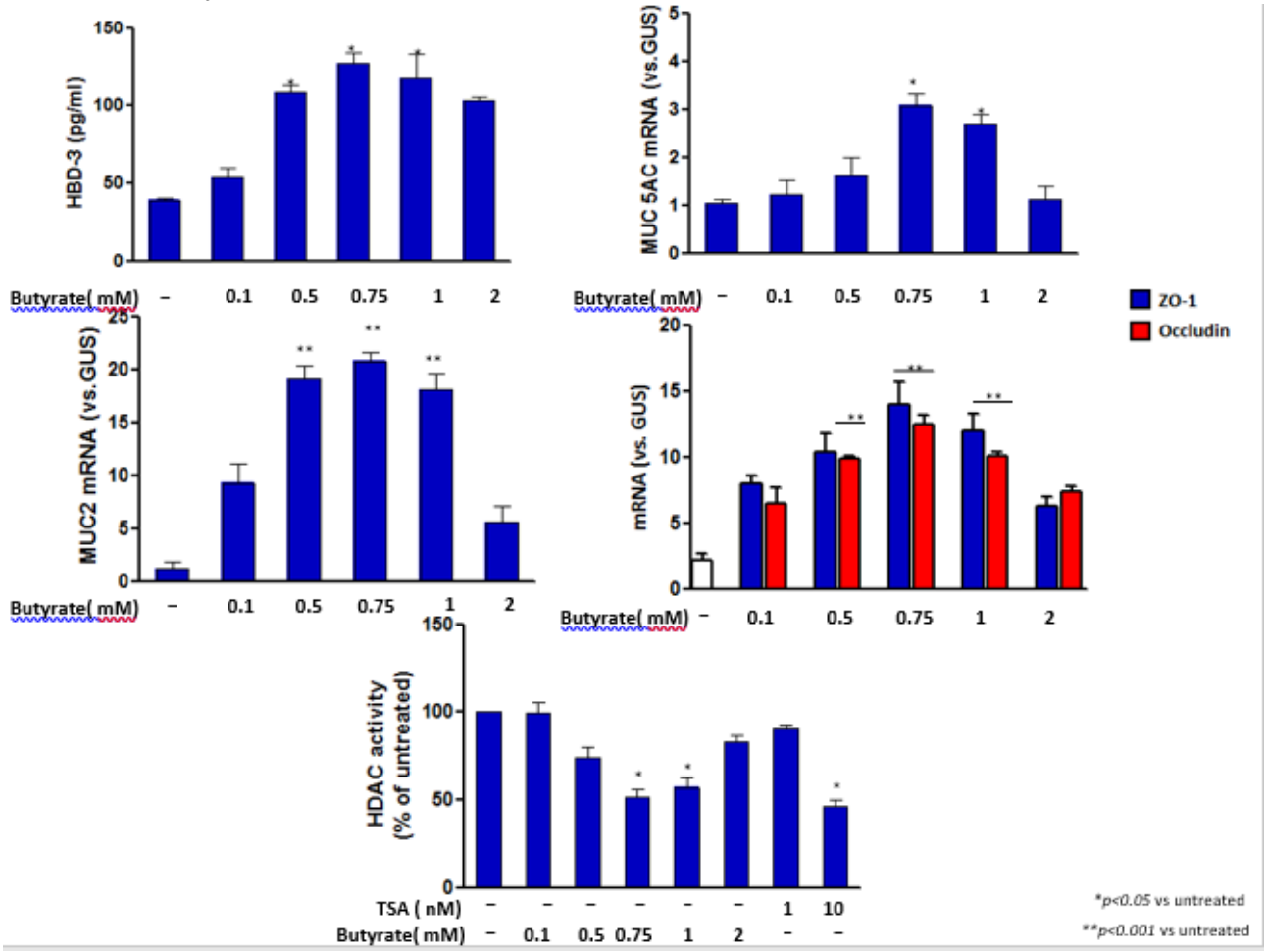


Figure 7. Evidence on effective sensitization to food allergy in the animal model and Th2 and Th1 response in spleen from mice sensitized to food allergens.

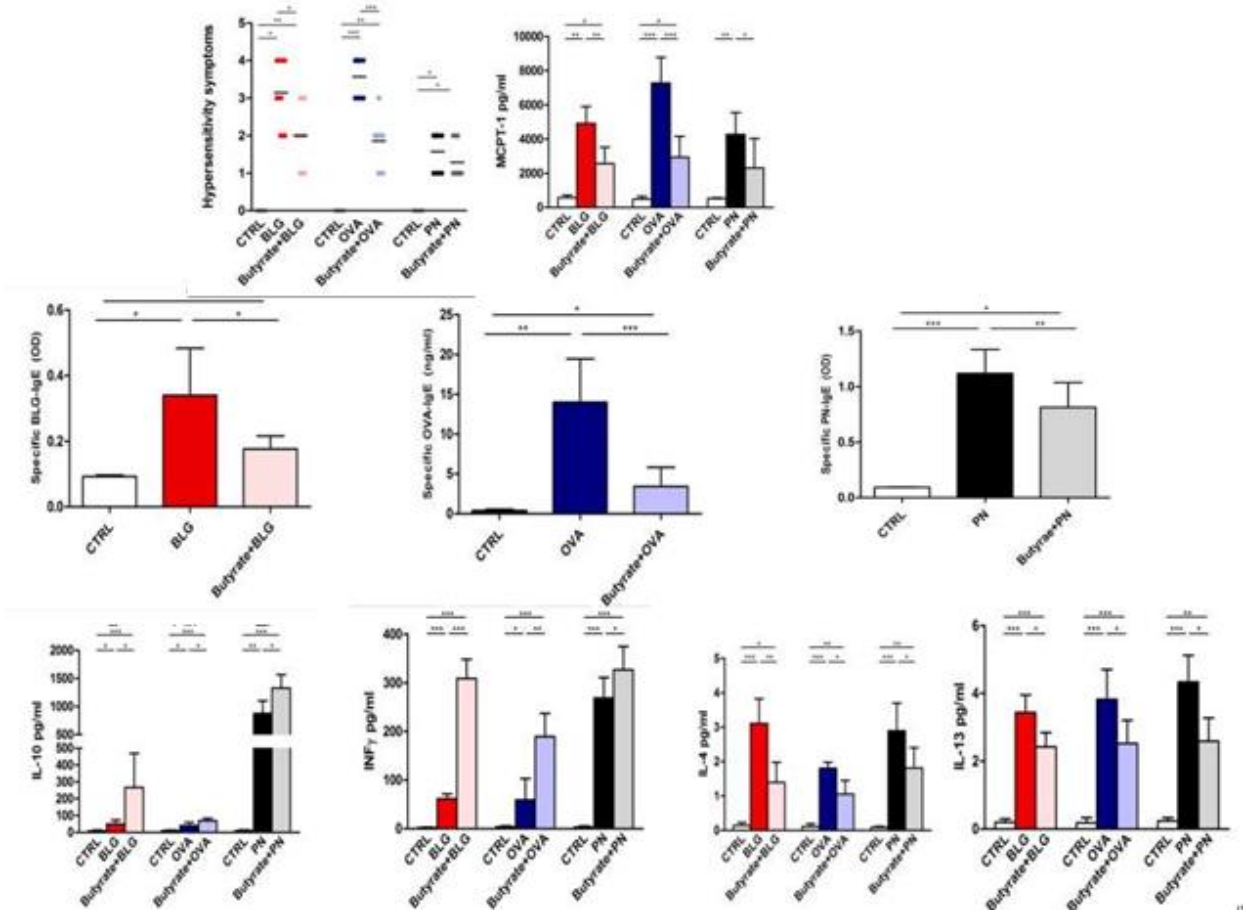
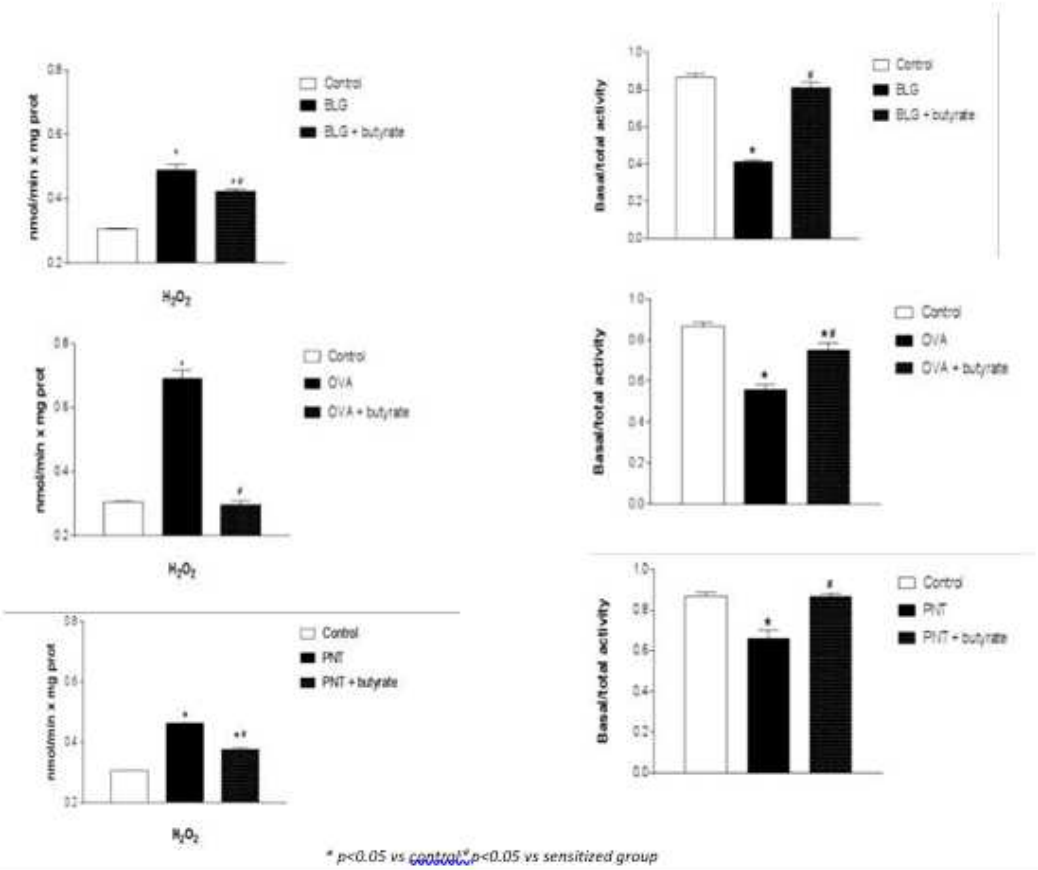


Figure 8. Evidence of hepatic mitochondrial dysfunction in mice sensitized to food allergens.



* p<0.05 vs control; # p<0.05 vs sensitized group

Figure 9. Overall gut microbiome composition does not discriminate allergic and healthy children

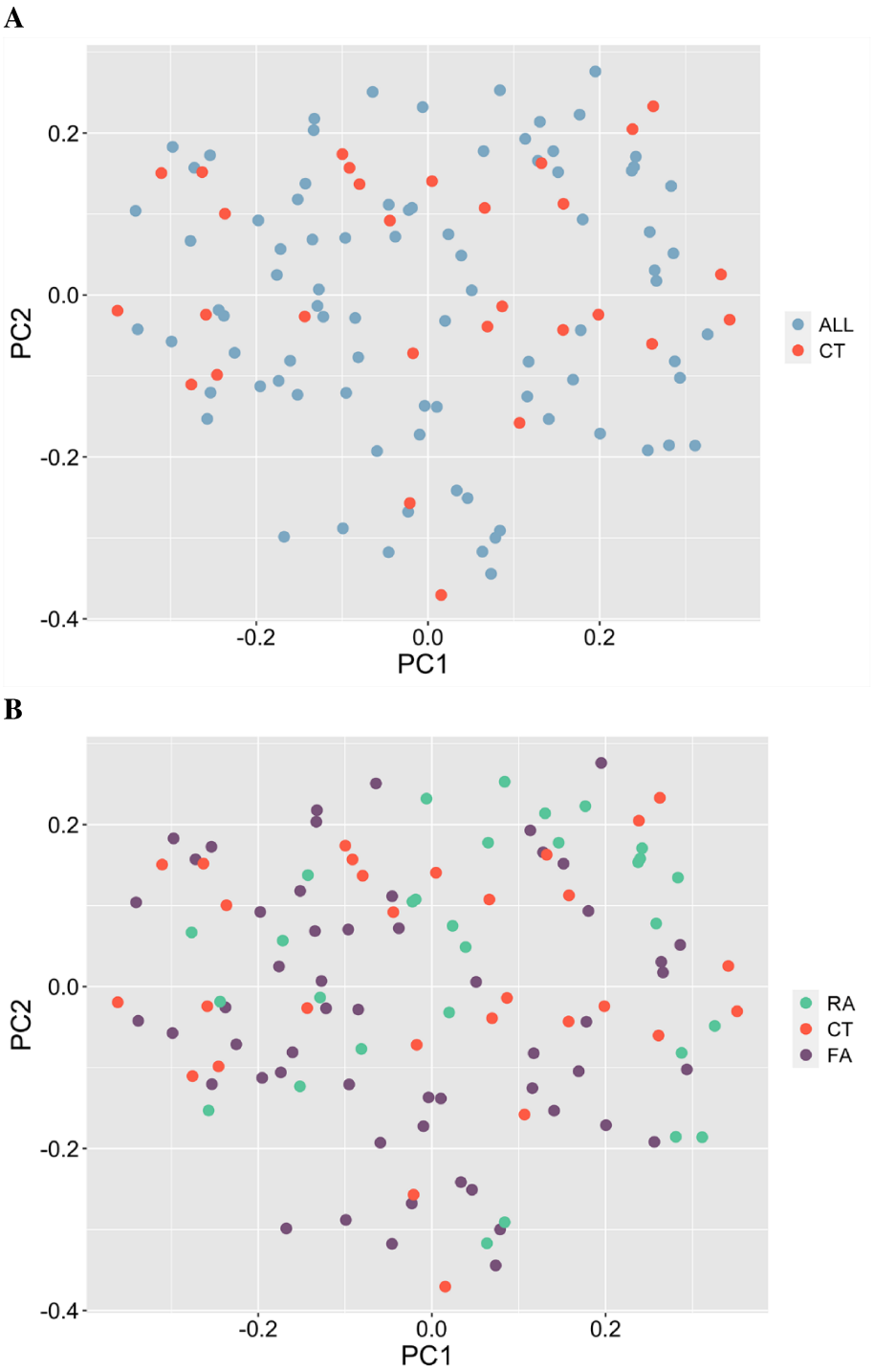


Figure 10. Respiratory allergy leads to higher gut microbial diversity

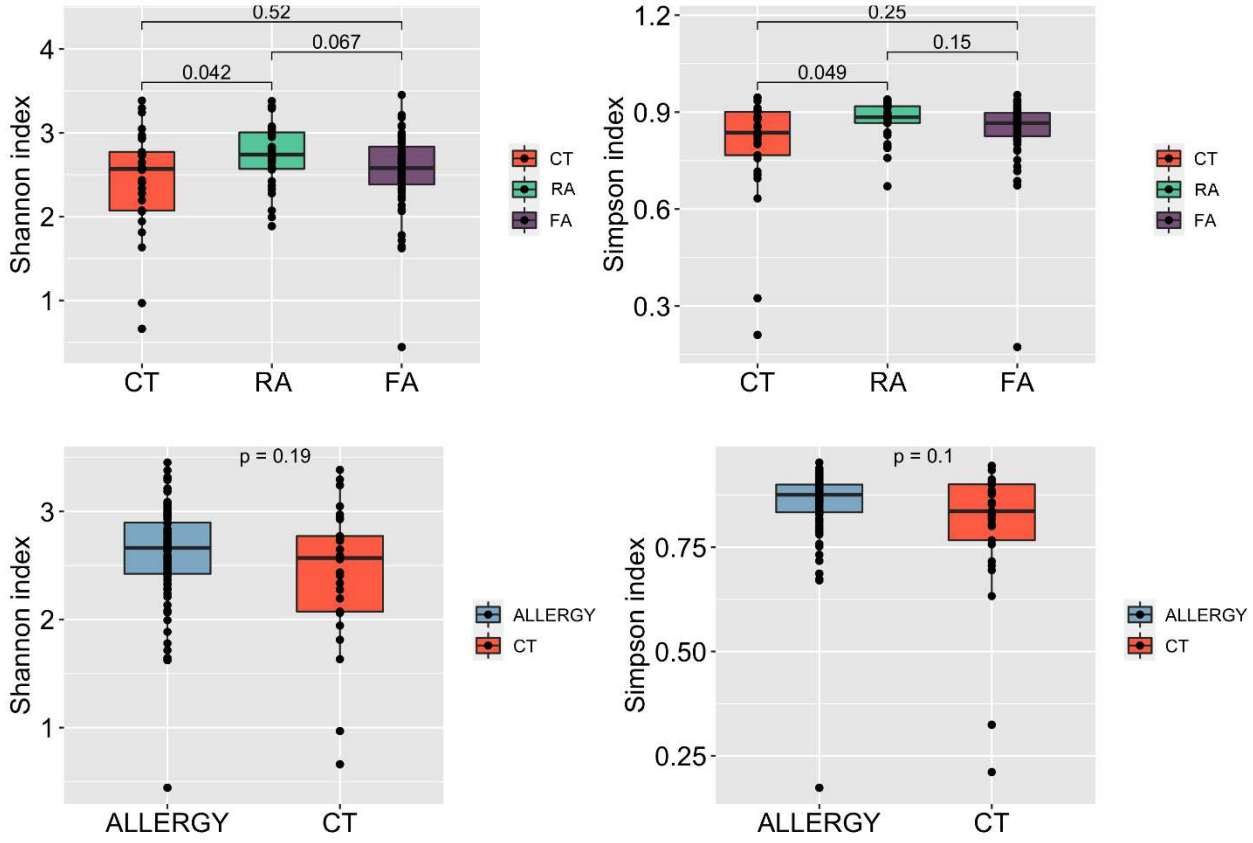


Figure 11. Microbial signatures in allergic children gut microbiome
11A

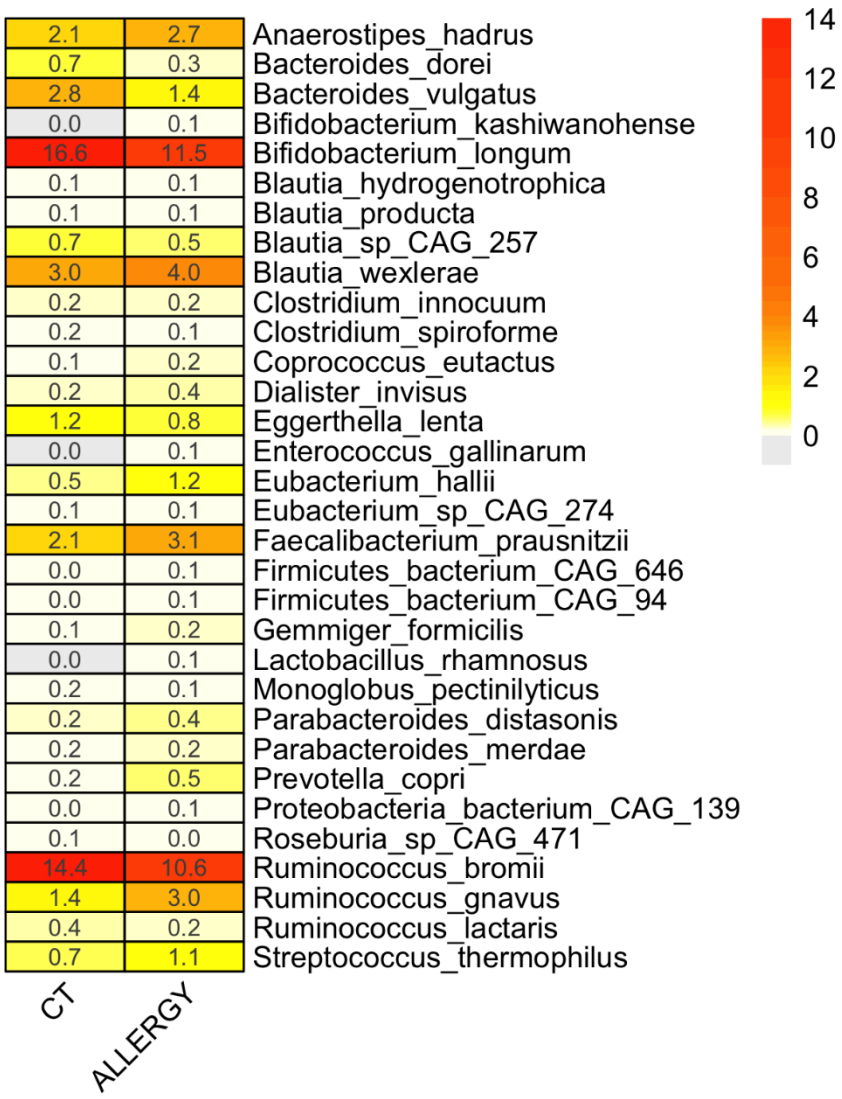


Figure 11B

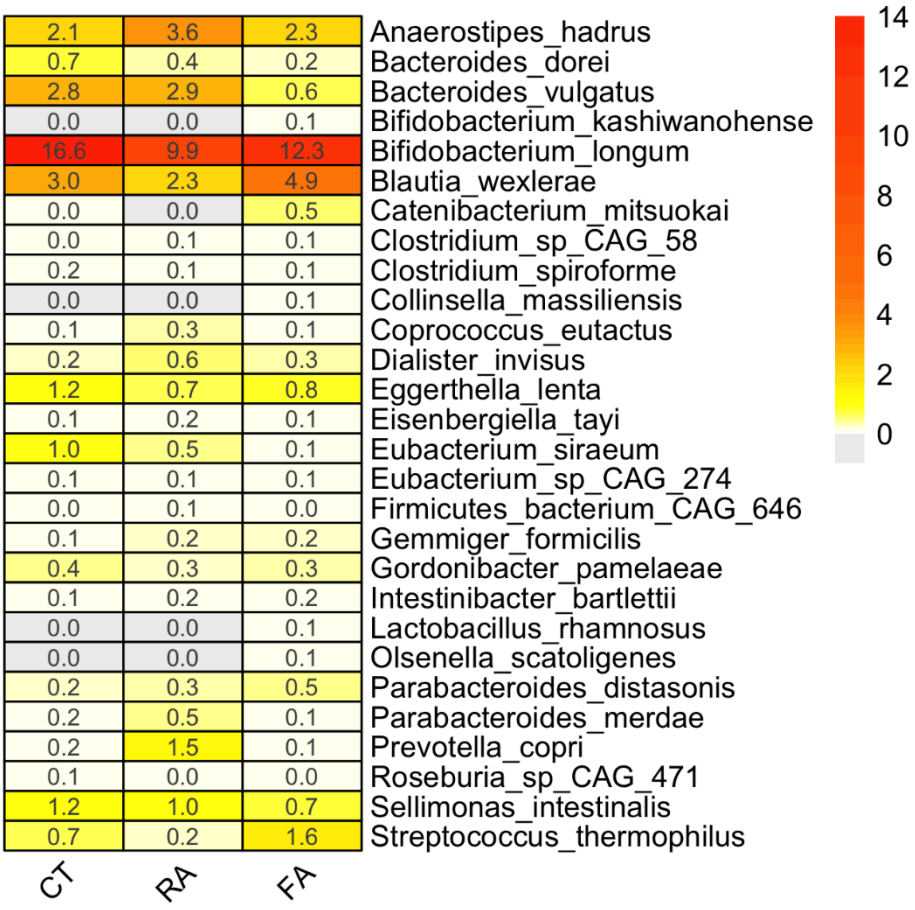

2.1 3.6 2.3 Anaerostipes_hadrus
0.7 0.4 0.2 Bacteroides_dorei
2.8 2.9 0.6 Bacteroides_vulgatus
0.0 0.0 0.1 Bifidobacterium_kashiwanohense
16.6 9.9 12.3 Bifidobacterium_longum
3.0 2.3 4.9 Blautia_wexlerae
0.0 0.0 0.5 Catenibacterium_mitsuokai
0.0 0.1 0.1 Clostridium_sp_CAG_58
0.2 0.1 0.1 Clostridium_spiroforme
0.0 0.0 0.1 Collinsella_massiliensis
0.1 0.3 0.1 Coprococcus_eutactus
0.2 0.6 0.3 Dialister_invisus
1.2 0.7 0.8 Eggerthella_lenta
0.1 0.2 0.1 Eisenbergiella_tayi
1.0 0.5 0.1 Eubacterium_siraeum
0.1 0.1 0.1 Eubacterium_sp_CAG_274
0.0 0.1 0.0 Firmicutes_bacterium_CAG_646
0.1 0.2 0.2 Gemmiger_formicilis
0.4 0.3 0.3 Gordonibacter_pamelaeae
0.1 0.2 0.2 Intestinibacter_bartlettii
0.0 0.0 0.1 Lactobacillus_rhamnosus
0.0 0.0 0.1 Olsenella_scatoligenes
0.2 0.3 0.5 Parabacteroides_distasonis
0.2 0.5 0.1 Parabacteroides_merdae
0.2 1.5 0.1 Prevotella_copri
0.1 0.0 0.0 Roseburia_sp_CAG_471
1.2 1.0 0.7 Sellimonas_intestinalis
0.7 0.2 1.6 Streptococcus_thermophilus
CT
RA
FA
14
12
10
8
6
4
2
0

Figure 12. Short-chain fatty acids are depleted in the gut of allergic children.

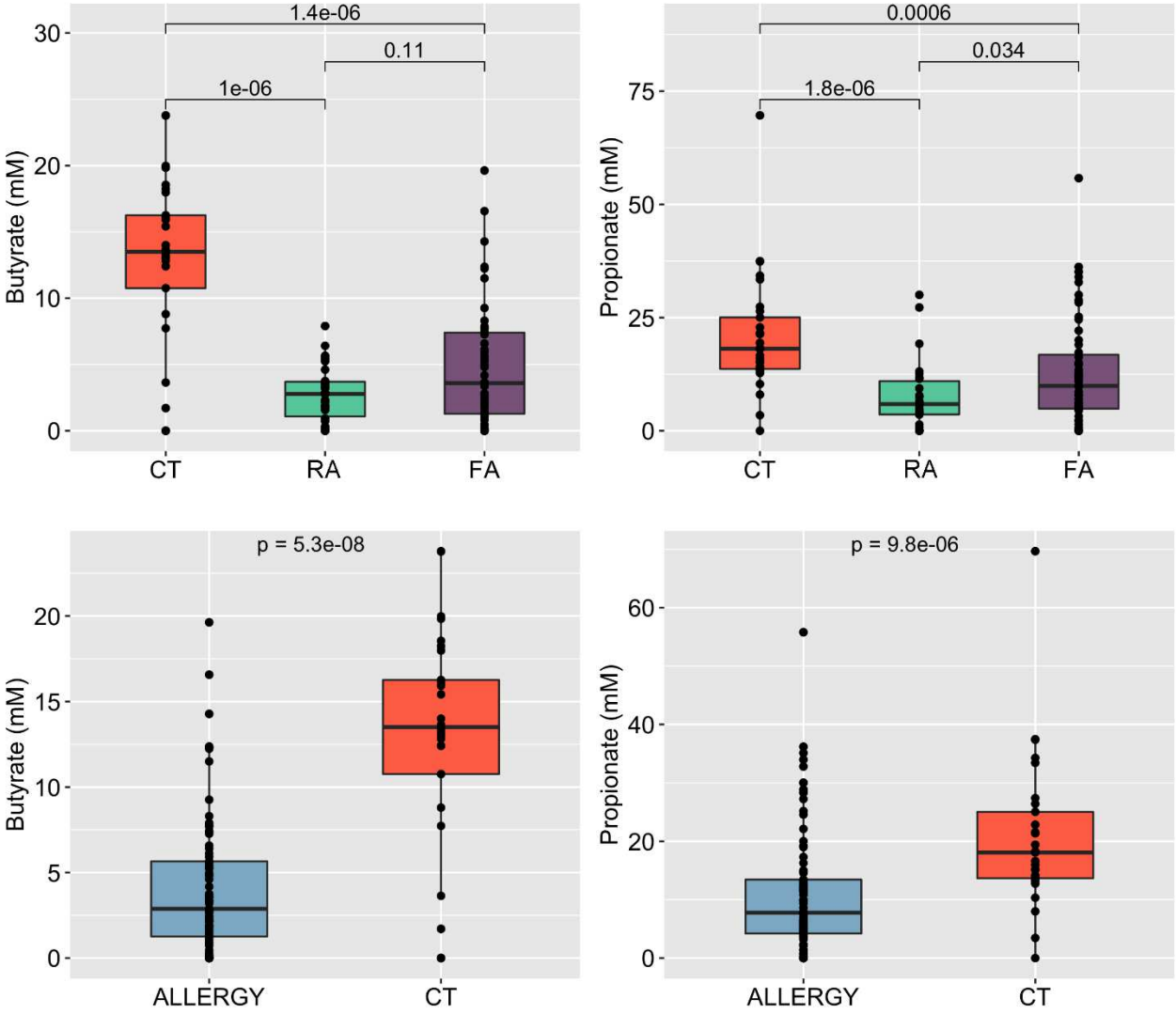


Figure 13. Gut microbiome features may predict the development of oral tolerance

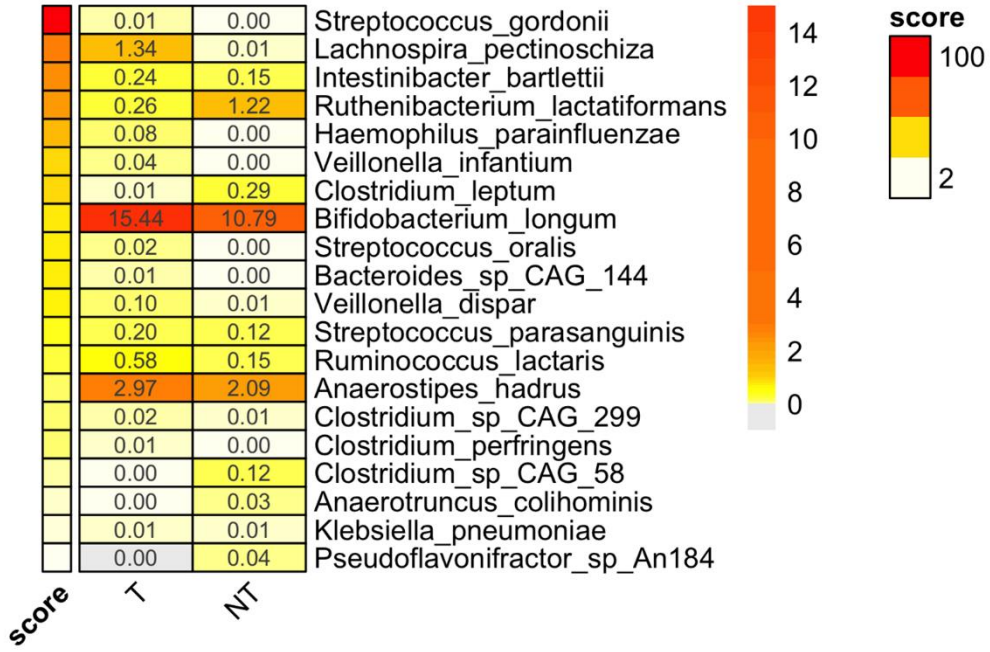
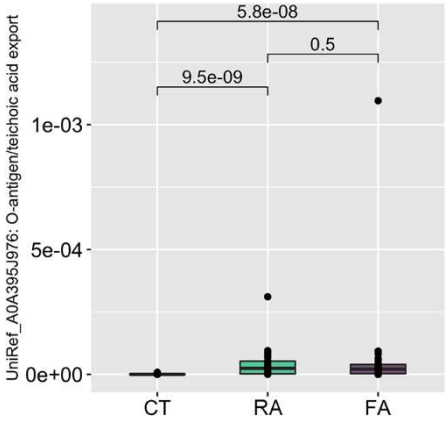
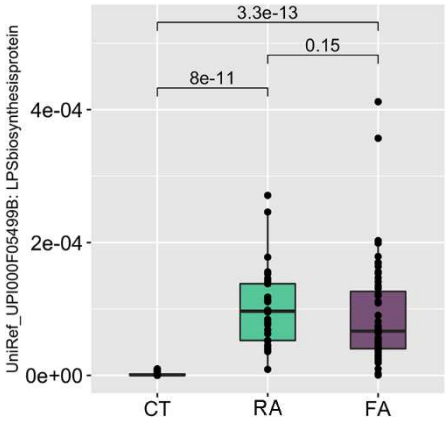


Figure 14. Gut microbiome of allergic children shows a higher inflammatory potential and a reduced ability to degrade complex polysaccharides

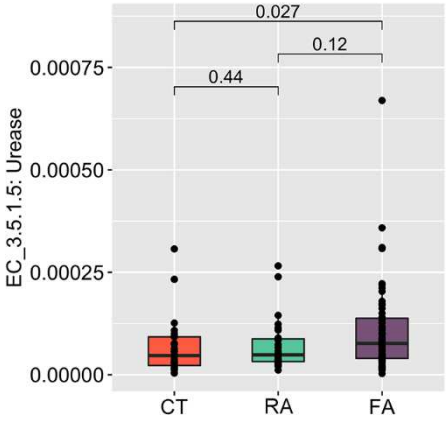
14A



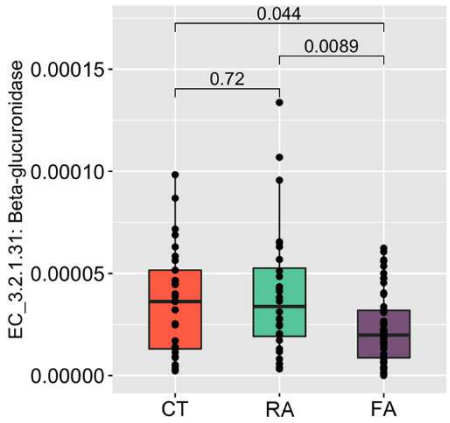
14B



14C



14D


0.044
0.0089
0.72
EC_3.2.1.31: Beta-glucuronidase
0.00015
0.00010
0.00005
0.00000
CT
RA
FA

14E

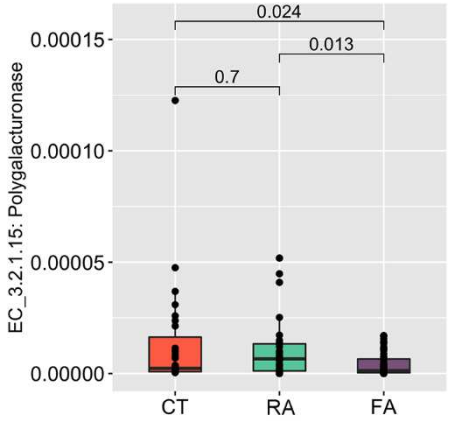
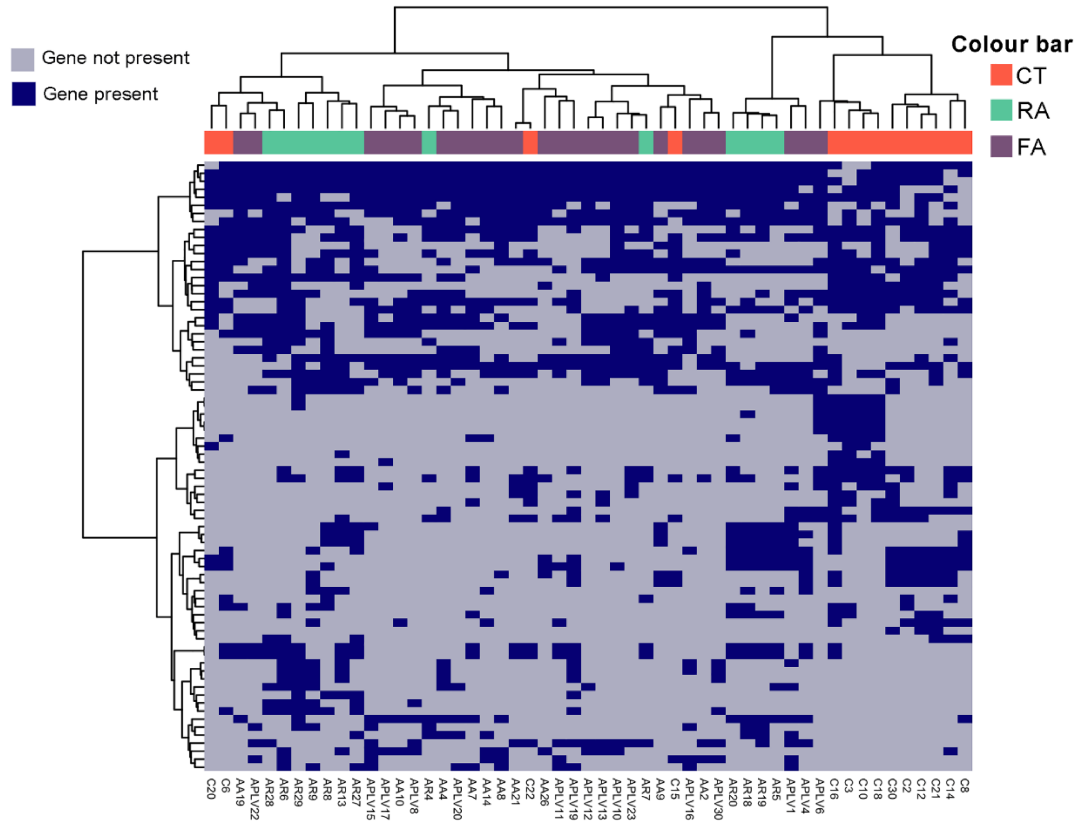

0.024
0.013
0.7
EC_3.2.1.15: Polygalacturonase
0.00015
0.00010
0.00005
0.00000
CT
RA
FA

Figure 15. Allergic children harbor a different *B. bifidum* pangenome

15A



15B

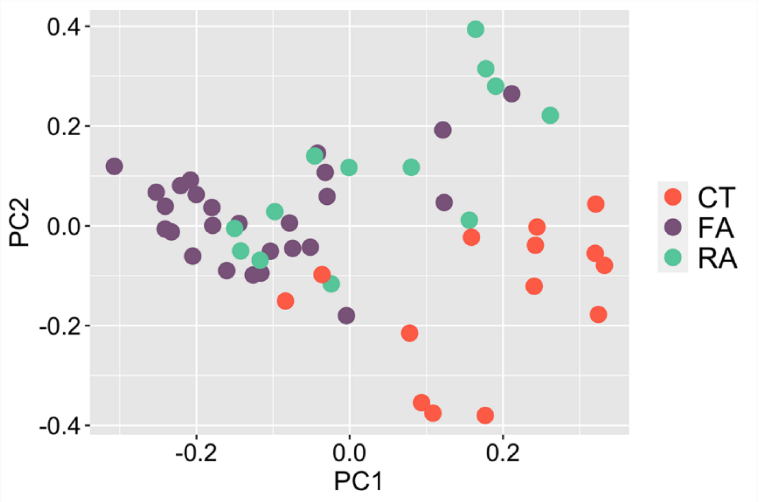
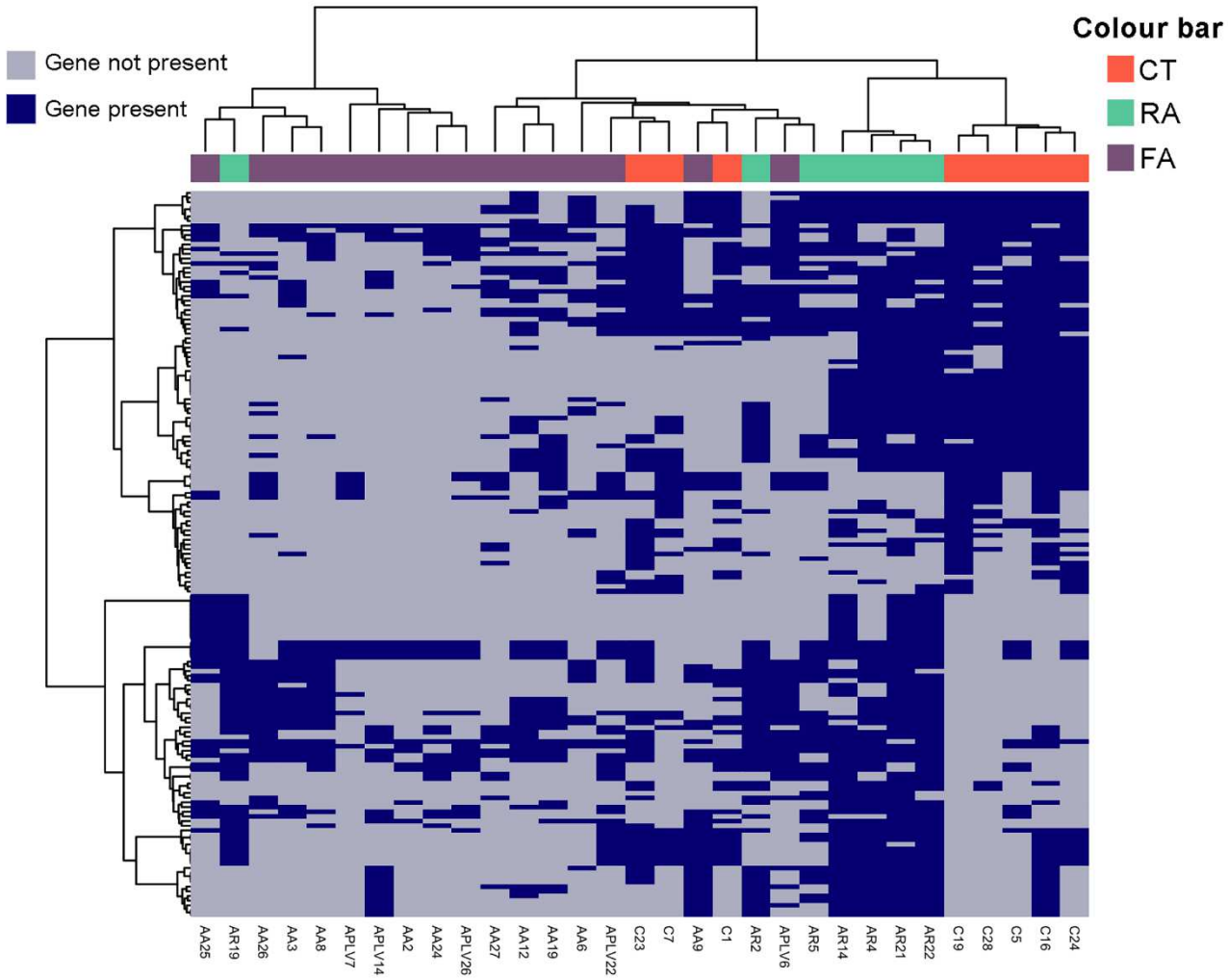
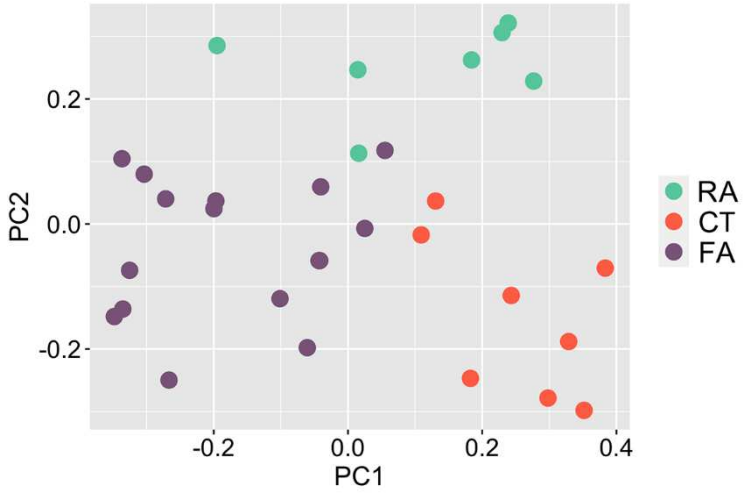


Figure 16. Allergic children harbor a different *R. gnavus* pangenome

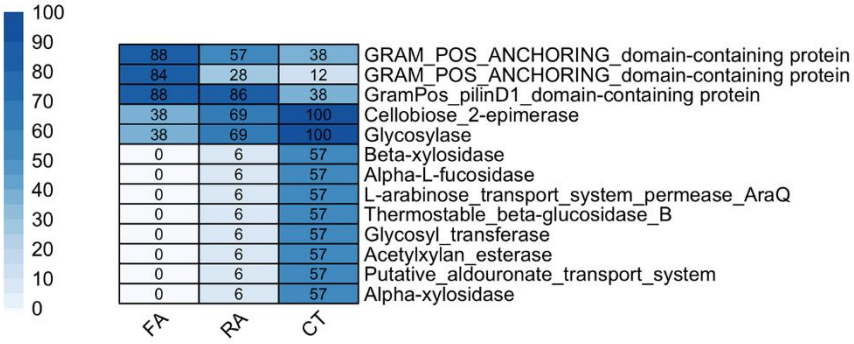
16A



16B



16C


100
90
80
70
60
50
40
30
20
10
0
88 57 38 GRAM_POS_ANCHORING_domain-containing protein
84 28 12 GRAM_POS_ANCHORING_domain-containing protein
88 86 38 GramPos_pilinD1_domain-containing protein
38 69 100 Cellobiose_2-epimerase
38 69 100 Glycosylase
0 6 57 Beta-xylosidase
0 6 57 Alpha-L-fucosidase
0 6 57 L-arabinose_transport_system_permease_AraQ
0 6 57 Thermostable_beta-glucosidase_B
0 6 57 Glycosyl_transferase
0 6 57 Acetylxylan_esterase
0 6 57 Putative_aldouronate_transport_system
0 6 57 Alpha-xylosidase
FA
RA
CT

16D

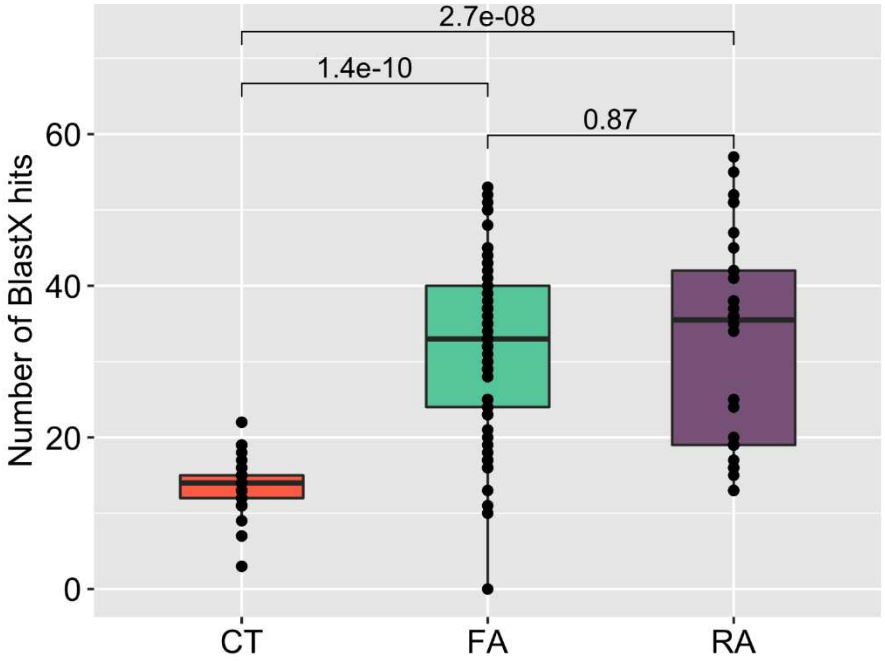
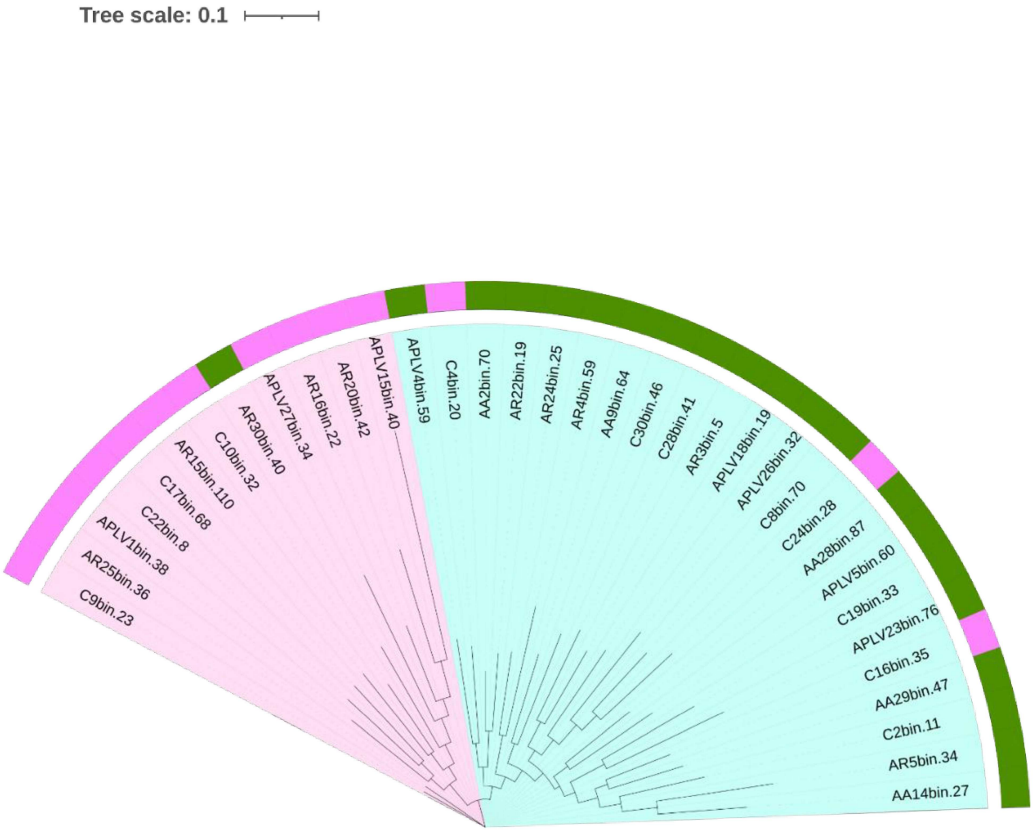

2.7e-08
1.4e-10
0.87
Number of BlastX hits
60
40
20
0
CT
FA
RA

Figure 17. Delivery mode selects different *Blautia wexlerae* and *Bacteroides vulgatus* strains, independently from the health status

17A



17B

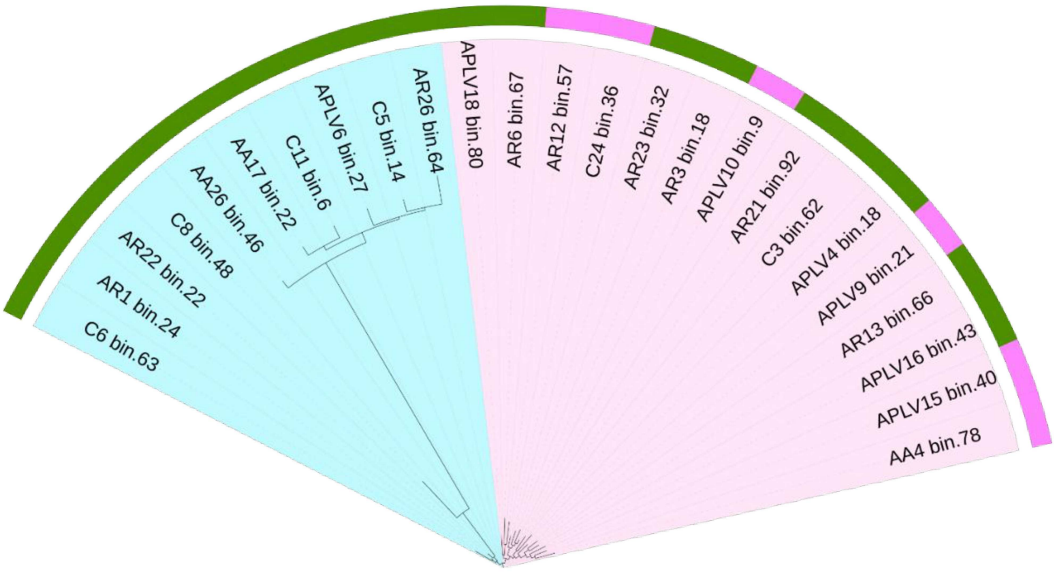
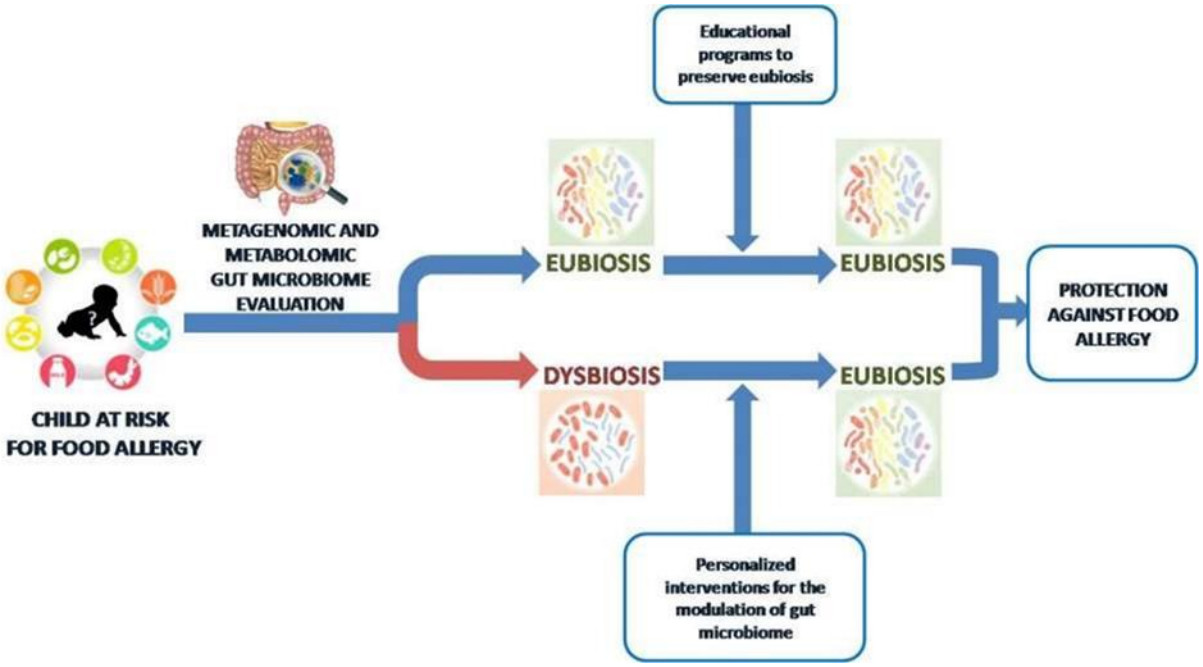
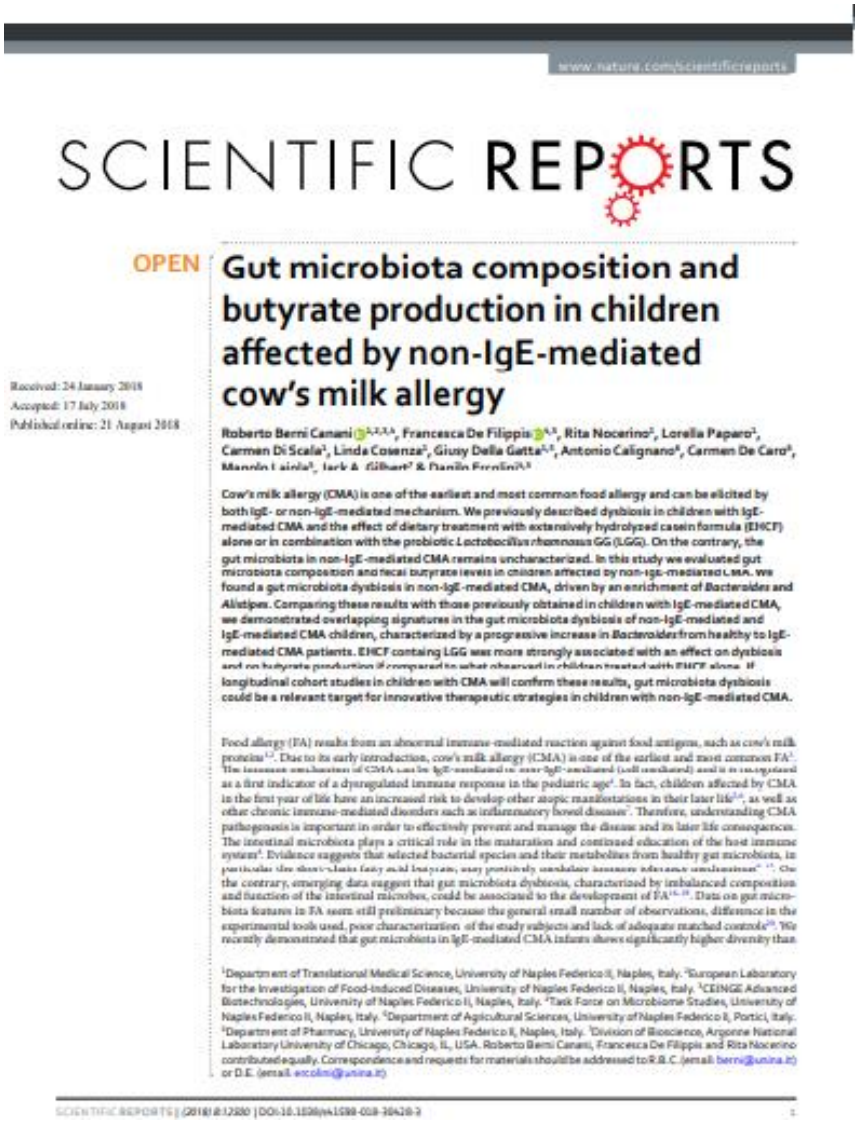


Figure 18. Toward a gut microbiota-based precision medicine against food allergy



11. Publications in the last 3 years



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SCIENTIFIC REPORTS

OPEN

Gut microbiota composition and butyrate production in children affected by non-IgE-mediated cow's milk allergy

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Roberto Berni Canani[1,2,3,4], Francesca De Filippis[1,4,5], Rita Nocerino[2], Lorella Paparo[2], Carmen Di Scala[2], Linda Cosenza[2], Giusy Della Gatta[4,5], Antonio Calignano[6], Carmen De Caro[3], Mayrdo Laiola[5], Jack A. Gilbert[7] & Danilo Ercolini[4,5]

Cow's milk allergy (CMA) is one of the earliest and most common food allergy and can be elicited by both IgE- or non-IgE-mediated mechanism. We previously described dysbiosis in children with IgE-mediated CMA and the effect of dietary treatment with extensively hydrolyzed casein formula (EHCF) alone or in combination with the probiotic *Lactobacillus rhamnosus* GG (LGG). On the contrary, the gut microbiota in non-IgE-mediated CMA remains uncharacterized. In this study we evaluated gut microbiota composition and fecal butyrate levels in children affected by non-IgE-mediated CMA. We found a gut microbiota dysbiosis in non-IgE-mediated CMA, driven by an enrichment of *Bacteroides* and *Alistipes*. Comparing these results with those previously obtained in children with IgE-mediated CMA, we demonstrated overlapping signatures in the gut microbiota dysbiosis of non-IgE-mediated and IgE-mediated CMA children, characterized by a progressive increase in *Bacteroides* from healthy to IgE-mediated CMA patients. EHCF containg LGG was more strongly associated with an effect on dysbiosis and on butyrate production if compared to what observed in children treated with EHCF alone. If longitudinal cohort studies in children with CMA will confirm these results, gut microbiota dysbiosis could be a relevant target for innovative therapeutic strategies in children with non-IgE-mediated CMA.

Food allergy (FA) results from an abnormal immune-mediated reaction against food antigens, such as cow's milk proteins[1,2]. Due to its early introduction, cow's milk allergy (CMA) is one of the earliest and most common FA[1]. The immune mechanism of CMA can be IgE-mediated or non-IgE-mediated (cell-mediated) and it is recognized as a first indicator of a dysregulated immune response in the pediatric age[3]. In fact, children affected by CMA in the first year of life have an increased risk to develop other atopic manifestations in their later life[3,4], as well as other chronic immune-mediated disorders such as inflammatory bowel diseases[5]. Therefore, understanding CMA pathogenesis is important in order to effectively prevent and manage the disease and its later life consequences. The intestinal microbiota plays a critical role in the maturation and continued education of the host immune system[6]. Evidence suggests that selected bacterial species and their metabolites from healthy gut microbiota, in particular the short-chain fatty acid butyrate, may positively modulate immune tolerance mechanisms[7–11]. On the contrary, emerging data suggest that gut microbiota dysbiosis, characterized by imbalanced composition and function of the intestinal microbes, could be associated to the development of FA[12–14]. Data on gut microbiota features in FA seem still preliminary because the general small number of observations, difference in the experimental tools used, poor characterization of the study subjects and lack of adequate matched controls[15]. We recently demonstrated that gut microbiota in IgE-mediated CMA infants shows significantly higher diversity than

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REVIEW
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Gut Microbiome as Target for Innovative Strategies Against Food Allergy

Roberto Berni Canani[1,2,3,4], Loralla Paparo[1,2], Rita Nocerino[1,2], Carmen Di Scala[1,2], Giuay Della Gatta[1,2], Yllenia Maddalena[1], Aniello Buono[1,2], Cristina Bruno[1,2], Luana Voto[1] and Danilo Ercolini[4,5]

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The dramatic increase in food allergy prevalence and severity globally requires effective strategies. Food allergy derives from a defect in immune tolerance mechanisms. Immune tolerance is modulated by gut microbiota function and structure, and microbiome alterations (dysbiosis) have a pivotal role in the development of food allergy. Environmental factors, including a low-fiber/high-fat diet, cesarean delivery, antiseptic agents, lack of breastfeeding, and drugs can induce gut microbiome dysbiosis, and have been associated with food allergy. New experimental tools and technologies have provided information regarding the role of metabolites generated from dietary nutrients and selected probiotic strains that could act on immune tolerance mechanisms. The mechanisms are multiple and still not completely defined. Increasing evidence has provided useful information on optimal bacterial species/strains, dosage, and timing for intervention. The increased knowledge of the crucial role played by nutrients and gut microbiota-derived metabolites is opening the way to a post-biotic approach in the stimulation of immune tolerance through epigenetic regulation. This review focused on the potential role of gut microbiome as the target for innovative strategies against food allergy.

Keywords: immune tolerance, gut microbiota, mediterranean diet, dysbiosis, probiotics, gut microbiota metabolites, short chain fatty acids, butyrate

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INTRODUCTION

The Changing Scenario of Food Allergy

Food allergy (FA) is one of the most common allergic disorders in the pediatric age, and it has been considered as a global health problem, particularly in industrialized world (1). During the last two decades, studies have suggested that the epidemiology of FA has shown a dramatic increase in the prevalence, severity of clinical manifestations and risk of persistence into later ages, leading to an increase in medical visits, hospital admissions, treatments, burden of care on families, and economic impact, with an increase of costs for the families and healthcare system (2–6). According to the most recent epidemiological data, time trend analysis showed up to a 7-fold increase in hospital admissions for food severe allergic reactions in children in the

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Targeting Food Allergy with Probiotics

Lorella Paparo, Rita Nocerino, Carmen Di Scala, Giusy Della Gatta, Margherita Di Costanzo, Aniello Bucino, Cristina Bruno, and Roberto Berni Canani

Abstract

The dramatic increase in food allergy prevalence and severity globally is demanding effective strategies. Food allergy derives from a defect in immune tolerance mechanisms. Immune tolerance is modulated by gut microbiota composition and function, and gut microbiota dysbiosis has been associated with the development of food allergy. Selected probiotic strains could act on immune tolerance mechanisms. The mechanisms are multiple and still not completely defined. Increasing evidence is providing useful information on the choice of optimal bacterial species/strains, dosage, and timing for intervention. The increased knowledge on the crucial role played by gut microbiota-derived metabolites, such as butyrate, is also opening the way to a post-biotic approach in the stimulation of immune tolerance.

Keywords

Butyrate · Cow's milk allergy · Gut microbiota · Immune tolerance · Post-biotics · Probiotics

Authors Lorella Paparo, Rita Nocerino, Carmen Di Scala, Giusy Della Gatta and Margherita Di Costanzo have been equally contributed to this chapter.

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e-mail: bernicanani@unina.it

Abbreviations

BLG	β-lactoglobulin
CMA	cow's milk allergy
EHCF	extensively hydrolyzed casein formula
FA	food allergy
LAB	lactic acid bacteria
LGG	*Lactobacillus rhamnosus* GG
OIT	oral food immunotherapy
OVA	ovalbumin
PBMCs	peripheral blood mononuclear cells

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The therapeutic efficacy of *Bifidobacterium animalis* subsp. *lactis* BB-12® in infant colic: A randomised, double blind, placebo-controlled trial

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Summary

Background: The pathogenesis of infant colic is poorly defined. Gut microbiota seems to be involved, supporting the potential therapeutic role of probiotics.

Aims: To assess the rate of infants with a reduction of ≥50% of mean daily crying duration after 28 days of intervention with the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12® (BB-12). Secondary outcomes were daily number of crying episodes, sleeping time, number of bowel movements and stool consistency.

Methods: Randomised controlled trial (RCT) on otherwise healthy exclusively breast-fed infants with infant colic randomly allocated to receive BB-12 (1×10^9 CFU/day) or placebo for 28 days. Gut microbiota structure and butyrate, beta-defensin-2 (HBD-2), cathelicidin (LL-37), secretory IgA (sIgA) and faecal calprotectin levels were assessed.

Results: Eighty infants were randomised, 40/group. The rate of infants with reduction of ≥50% of mean daily crying duration was higher in infants treated with BB-12, starting from the end of 2nd week. No infant relapsed when treatment was stopped. The mean number of crying episodes decreased in both groups, but with a higher effect in BB-12 group (−4.7 ± 3.4 vs −2.3 ± 2.2, $P = 0.05$). Mean daily stool frequency decreased in both groups but the effect was significantly higher in the BB-12 group. Stool consistency was similar between the two groups. An increase in *Bifidobacterium* abundance (with significant correlation with crying time reduction), butyrate and HBD-2, LL-37, sIgA levels associated with a decrease in faecal calprotectin level were observed in the BB-12 group.

Conclusions: Supplementation with BB-12 is effective in managing infant colic. The effect could derive from immune and non-immune mechanisms associated with a modulation of gut microbiota structure and function.

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Dietary Treatment with Extensively Hydrolyzed Casein Formula Containing the Probiotic *Lactobacillus rhamnosus* GG Prevents the Occurrence of Functional Gastrointestinal Disorders in Children with Cow's Milk Allergy

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Objective To investigate whether the addition of the probiotic *Lactobacillus rhamnosus* GG (LGG) to the extensively hydrolyzed casein formula (EHCF) for cow's milk allergy (CMA) treatment could reduce the occurrence of functional gastrointestinal disorders (FGIDs).

Study design This cohort study included children with a positive history for CMA in the first year of life who were treated with EHCF alone or in combination with LGG and had evidence of immune tolerance acquisition to cow's milk for at least 12 months. FGID was diagnosed according to the Rome III diagnostic criteria by investigators unaware of previous treatment. A cohort of consecutive healthy children was also evaluated as a control population.

Results A total of 330 subjects were included, 110 per cohort (EHCF, EHCF+LGG, and healthy controls). The rate of subjects with ≥1 FGID was significantly lower in the EHCF+LGG cohort compared with the EHCF cohort (40% vs 16.4%; $P < .05$). In the EHCF+LGG cohort, a lower incidence was observed for all components of the main study outcome. The prevalence of FGIDs in the healthy cohort was lower than that in the EHCF cohort and similar to that in the EHCF+LGG cohort. The incidence rate ratio of FGIDs for the EHCF+LGG cohort vs the EHCF cohort (0.40; 95% CI, 0.25-0.65; $P < .001$) was unmodified after correction for age at CMA diagnosis, breastfeeding, weaning time, and presence of a first-degree relative with an FGID.

Conclusions These results confirm the increased risk for developing FGIDs in children with CMA and suggest that EHCF+LGG could reduce this risk. (*J Pediatr 2019;213:137-42*).

Functional gastrointestinal disorders (FGIDs), defined as a variable combination of chronic or recurrent gastrointestinal symptoms that cannot be explained in terms of structural or biochemical abnormalities, are a very common problem in childhood.[1] FGIDs are the result of any combination of motility disturbance, visceral hypersensitivity, altered mucosal and immune function, altered gut microbiota, and altered central nervous system processing.[2] There is increasing evidence suggesting that FGIDs can result from immune system dysregulation and gut microbiota dysbiosis.[3,4]

Cow's milk allergy (CMA) is a common food allergy in early childhood, with an estimated prevalence of 2%-3%.[5] CMA usually occurs in the first months of life and is associated with gastrointestinal inflammation and gut dysbiosis.[6] There is an emerging evidence pointing to CMA as a predisposing condition in patients with FGIDs.[7-10] Genome-wide association studies have identified single nucleotide polymorphisms common to allergies that confer a risk for FGIDs.[11-13] This evidence indicates the importance of effective strategies to prevent FGIDs in children with CMA.

Dietary intervention with extensively hydrolyzed casein formula (EHCF) supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) has shown benefits in decreasing inflammation and gastrointestinal symptoms in children with CMA,[14] as well as in reducing the duration of disease and the occurrence of other allergic manifestations later in life.[15-17] Multiple mechanisms might be responsible for these clinical effects, including a positive effect on gut dysbiosis and epigenetic regulation of immune and nonimmune gene expression.[18-20] *L. rhamnosus* GG also has been proposed for the treatment of pediatric FGIDs.[8]

CFU	Colony-forming units
CMA	Cow's milk allergy
EHCF	Extensively hydrolyzed casein formula
FGID	Functional gastrointestinal disorder
IRR	Incidence rate ratio
LGG	*Lactobacillus rhamnosus* GG
PWRM	Poisson working regression model
QPGS-RIII	Rome III diagnostic criteria

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Editorial: interventions in infantile colic – can efficacy be attributed to treatment or to time? Authors' reply

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Butyrate as bioactive human milk protective component against food allergy

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Abstract

Background: Food allergy (FA) is a growing health problem worldwide. Effective strategies are advocated to limit the disease burden. Human milk (HM) could be considered as a protective factor against FA, but its mechanisms remain unclear. Butyrate is a gut microbiota-derived metabolite able to exert several immunomodulatory functions. We aimed to define the butyrate concentration in HM, and to see whether the butyrate concentration detected in HM is able to modulate the mechanisms of immune tolerance.

Methods: HM butyrate concentration from 109 healthy women was assessed by GS-MS. The effect of HM butyrate on tolerogenic mechanisms was assessed in in vivo and in vitro models.

Results: The median butyrate concentration in mature HM was 0.75 mM. This butyrate concentration was responsible for the maximum modulatory effects observed in all experimental models evaluated in this study. Data from mouse model show that in basal condition, butyrate up-regulated the expression of several biomarkers of gut barrier integrity, and of tolerogenic cytokines. Pretreatment with butyrate significantly reduced allergic response in three animal models of FA, with a stimulation of tolerogenic cytokines, inhibition of Th2 cytokines production and a modulation of oxidative stress. Data from human cell models show that butyrate stimulated human beta defensin-3, mucus components and tight junctions expression in human enterocytes, and IL-10, IFN-γ and FoxP3 expression through epigenetic mechanisms in PBMCs from FA children. Furthermore, it promoted the precursors of M2 macrophages, DCs and regulatory T cells.

Conclusion: The study's findings suggest the importance of butyrate as a pivotal HM component able to protect against FA.

Lorella Paparo and Rita Nocerino equally contributed to this paper.