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Diet, Microbiota and Epigenetics as target for innovative strategies against food allergy: deciphering the protective mechanism of butyrate as crucial human milk effector

TUTOR

Prof. Roberto Berni Canani

PH.D. STUDENT

Dr. Lorella Paparo

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1. Introduction

1.1 The changing scenario of food allergy

Food allergy (FA) is an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food (1). It is one of the most common allergic disorders in the pediatric age, and it has been recognized as a global health problem, particularly in industrialized countries. Studies have suggested that the epidemiology of FA has changed during the last two decades, with a dramatic rise in the prevalence, severity of clinical manifestations, and risk of persistence into later ages, leading to an increase in hospital admissions, medical visits, treatments, burden of care on families and to an important economic impact, with significant direct costs for the families and healthcare system (2-4). In Europe, around 7 million of people suffer from challenge-proven FA. If this prevalence is projected onto the world's population of 7 billion, it translates into 63 million-1.16 billion of potential food-allergic people, with a greater incidence in children (5-8%) than in adults (1-2%) (5). Furthermore, about 8% of people suffering from FA are exposed to the risk of potentially life-threatening allergic reaction resulting in death, mainly amongst children aged 0–14 years. According to the most recent epidemiological data, time trend analysis showed up to 7-fold increase in hospital admissions for food severe allergic reactions in children in the UK, USA, Italy and Australia over the last 10 years (6-10). Although more than 170 foods have been identified as triggers of FA, there is rather short list of foods account for most of the more serious disease burden, namely peanut, tree nuts, fish, shellfish, egg, milk, wheat, soy, and seeds, with national and geographical variations concerning the most common FA (1,11-16).

1.2 New insights in the pathogenesis of food allergy

FA derives from a breakdown of immune tolerance (17). 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 (18,19). 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 (20,21). 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) (22-26). 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 (27-30).

1.3 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 (31). 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") (32), as well as other diseases such as functional gastrointestinal disorders (FGIDs) (33), inflammatory bowel diseases (IBD) (34), and psychiatric disorders, such as attention deficit hyperactivity disorder (ADHD), autistic spectrum disorders (ASD)

and obsessive-compulsive disorder (OCD) (35). The pathogenesis of these events is still largely unknown, but increasing evidence suggest that perturbation of gut microbiota, 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).

1.4 Gut microbiota features in food allergy: investigating the metagenomic and metabolomics features

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 (36) 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 (37). 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 (38). 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 (39). 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 (40). 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 (41,42). 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 (43,44). 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 Evidence on gut microbiota dysbiosis in food allergy

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 (28,30,45,46). 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 disease course of FA (46).

1.6 Targeting gut microbiota in food allergy: the importance of the diet-gut microbiota axis

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 (47). 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 (48-50). A recent 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 (51).

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 (52). One cohort reported that the delayed introduction of rice/wheat cereal (>6 months of age) was associated with a lower risk of food allergy (53). 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. (54) recently performed a meta-analysis of randomized controlled trials about probiotic supplementation during pregnancy and/ or infancy and their effects on atopy in children. 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 (55).

Several studies reported that nutrients impact the gut microbiota and the bacterial metabolites production (56,57). 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 (58). 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 (59). 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) (60). It has been demonstrated that reduced availability of NDC lowered the concentration of fiber-degrading bacteria and increased mucin degrading bacteria (61). 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* (62).

The immunomodulatory mechanisms stimulated by SCFAs represent one of the strongest connections between diet, gut microbiota, and allergic diseases (63). SCFAs are 2-carbon to 5-carbon weak acids, including acetate, propionate, butyrate and valerate (64). 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 (65). 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 (66-72). 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 (73-76). GPR43 and GPR41 are highly expressed by intestinal epithelial cells (77). Neutrophils, macrophages and DCs, express GPR43 and GPR109A, but T- and B-cells do not express these SCFA receptors (78-82).

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 (83).

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 coincide 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 (84). *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 (85). In a mouse model, it has been demonstrate 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 (86).

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 (87). It has been demonstrated that butyrate suppresses CD4+ T-cell proliferation and increase colonic $FoxP3^+$ Tregs, potentially through their HDAC inhibitor activity (88-90). The inhibition of HDAC 9 and 6 increases FoxP3 gene expression, as well as the production and suppressive function of Tregs (91).

Butyrate deficiency has been observed in allergic patients (92). 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 (92). 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 (93). 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 (93,94).

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 (95). 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 (96,97). 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) (98). The bond of Kyn to AhR receptor (99) lead to the inhibition of dendritic cells (DCs) maturation (100) and the proliferation of Th17 cells and Treg, increasing IL-22 and IL-10 production (101,102). 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 (103).

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 number of $CD103^{+}MHC\text{-}II^{+}$ tolerogenic DCs compared to normal chow-fed control mice (104).

1.7 The role of breastfeeding in food allergy

Breastfeeding has important health benefits for infants and represents the gold standard of nutrition for infants (105). However, the role of human milk (HM) in the primary prevention of FA (10) 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 (106). 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 (107). HM is a complex living nutritional fluid that contains antibodies, enzymes, and hormones, all of which have health benefits (108). It has been demonstrated a pivotal role of breast-milk antibodies for the development immature immune system of a newborn (109). 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 (109,110). 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 (111). 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 (112). 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 (113). Milk lipids is the main source to supply energy for newborns such that it comprises ~50% of the energy that they need (114). 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 (115-117). 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 (118). However, butyrate levels in HM are not well established.

1.8 Engineering gut microbiota with probiotics in food allergy

Immune tolerance is a major therapeutic target in FA. Evidence support the concept that probiotics, defined as live microorganisms which when ingested in adequate amounts confer a beneficial effect on the host (119), could act at different level on immune tolerance network in FA: modulating gut microbiota composition and function (increased production of butyrate) (43); interacting with enterocytes with subsequent modulation of non-immune (gut permeability and mucus thickness) (120-123) and immune tolerogenic mechanisms (stimulation of sIgA and β-defensins production) (124); modulation of cytokines response by immune cells (125-128). Main pre-clinical evidence on the probiotics activity against FA are summarized in Table 2. In the last decades, a number of experimental investigations have

been developed in order to characterize those organisms could be used to modulate the gut microbiota for FA. Stimulation of human peripheral blood mononuclear cells (PBMCs) with selected probiotic strains is a commonly used experimental tool for the investigation of the effect of these microorganisms on immune cells. The incubation of PBMCs with lactic acid bacteria (LAB) strains (such as *L.plantarum* and *B.adolescentis*) resulted in an increased production of the regulatory cytokine IL-10 by monocytes and dendritic cells, and to an enhance of IFN-γ production by T cells (129-131). The addition of probiotics mixture (*L.acidophilus* W55, *L.casei* W56, *L.salivarius* W57, *L.lactis* W58, *B infantis* W52, *B.lactis* W18 and *B.longum* W51) to PBMCs from children with FA resulted in increased T cell proliferation with enhanced production of Th1 and regulatory cytokines. An increase in T and B cells proliferation and a reduction in IgE production were also observed in PBMCs from children with FA treated for 3 months with the same probiotic mixture (132). Using a 3D co-culture model of intestinal epithelial cells and PBMCs as an *in vitro* model of the intestinal mucosal immune system, Ghadimi et al demonstrated that the probiotics *B.breve* and *L.rhamnosus* GG (LGG) inhibit activation of inflammatory IL-23 and IL-17 cytokines, thereby reducing histone acetylation and simultaneously enhancing DNA methylation (133). The limitation of studying the effect of probiotics *in vitro* lies in the extrapolation of the results to *in vivo* benefits. For that reason, another commonly used experimental tool in this area is based on the use of animal model of FA. Differential effects in relation to molecular action of oral administration of three LAB strains (*B. coagulans* 09.712, *L.plantarum* 08.923 and *B.infantis* 11.322) in alleviating Th2-driven intestinal inflammation and other symptoms associated with food-induced anaphylaxis were demonstrated in a murine model induced by a major shrimp allergen, ST. In particular, oral supplementation with *B.coagulans* 09.712 and *L.plantarum* 08.923 significantly ameliorates anaphylaxis symptoms and increases the population of $CD4^+$ $CD25^+FoxP3^+$ T cells in ST-sensitized mice through mTORC inhibition,

FoxP3 up-regulation and GATA-3 down-regulation (134). Zang et al investigated the preventive and therapeutic effects of oral *C.butyricum* on anaphylactic symptoms in FA mice model induced by a β -lactoglobulin (BLG) as allergen, a well-established model of CMA. The authors observed that the oral treatment with *C.butyricum* significantly ameliorated anaphylaxis symptoms and increased sIgA and $CD4^+$ $CD25^+$ $FoxP3^+$Treg cell in spleen from BLG-sensitized mice (135). Neonatal monocolonization of germ-free mice by *L.casei* BL23 modulated the allergic sensitization to cow's milk proteins, developed higher IgG responses against caseins, elicited by *L.casei* that was able to hydrolyze insoluble caseins into soluble immunogenic peptides (136). Using OVA-sensitized murine model, it was demonstrated that oral administration of *B.infantis* ameliorated allergic symptoms, reducing OVA specific-IgE, and -IgG1 levels in the serum, and Th2 cytokines release in spleen. Moreover, gut microbiota analysis showed that the probiotics-mediated protection was conferred by up-regulation of the relative abundance of *Coprococcus* and *Rikenella* at genus level (137). Similar results was obtained by others that observed a decrease of concentrations of IgE, IL-4 and IL-13 following administration of *B.infantis* CGMCC313-2 in BLG-sensitized mice (138). Oral administration of VSL#3 (a mixture of *Streptococcus thermophilus* BT01, *B. breve* BB02, *B.longum* BL03, *B.infantis* BI04, *L.acidophilus* BA05, *L.plantarum* BP06, *L.paracasei* BP07, *L.delbrueckii* subsp. *bulgaricus* BD08) to sensitized mice significantly reduces Th2 immune responses and protects against anaphylactic reactions in a mouse model of FA. Also the incubation of mouse spleen cells from sensitized mice with probiotic mixture reduced allergen-stimulated IL-5 and IL-13 production and increased of IFN-γ and IL-10 production (139). An immunoregulatory action by LGG has been demonstrated in a murine model of CMA. LGG administration was able to suppress of Th2 responses such as reduced hypersensitivity scores and lowered serum CMP-specific IgG1 while promoting Th1 responses by causing elevated IFN-γ and CMP-specific IgG2a levels (140). Similar results

have been reported by our group in a BLG-sensitized mice model, in which we found that the administration of LGG added to the extensively hydrolyzed casein formula (EHCF) elicited a significantly reduction of allergic reaction, and of IL-4, IL-5, IL-13 and specific IgE production (141). Clinical studies have investigated the efficacy of selected probiotic strains against FA. The effect appears strain-specific and more evident in the pediatric age. In a randomized double-blind placebo-controlled trial, it has been demonstrated that the administration of *L.casei* CRL431 and *B.lactis* BB12 added to hypoallergenic formula for 12 months did not affect the acquisition of immune tolerance to cow's milk proteins in infants with CMA (142). Whereas, using a similar study design we have demonstrated that the addition of the probiotic LGG to EHCF is able to accelerate immune tolerance acquisition in infants with CMA. Infants (aged 1–12 months), consecutively referred for suspected CMA (IgE- or non-IgE-mediated), but still receiving cow's milk proteins, were invited to participate in the study. Subjects were randomly allocated to one of the two groups of dietary interventions: control group, received an EHCF; and active group, received an EHCF containing LGG (at least 1.4×10^7 CFU/100 mL). After 12 months, the double-blind placebo-controlled food challenge was negative in 15 of 28 control infants (53.6%) and in 22 of 27 infants receiving EHCF with LGG (81.5%, $p = 0.027$) (143). The results were confirmed in a subsequent trial, when the effect of 5 different dietary strategies was investigated: EHCF, EHCF + LGG, partially hydrolyzed rice formula, soy formula or amino acid-based formula, in children affected by IgE- or non-IgE-mediated CMA. After the treatment period of 12 months, the proportion of children acquiring immune tolerance to cow's milk proteins was significantly higher in the group receiving EHCF+ LGG (78.9%) than in other groups (144). At the 3-year follow-up of another pediatric cohort, a further confirmation of a greater rate of resolution of IgE-mediated CMA as well as a lower incidence of other atopic manifestations was described after treatment with EHCF+LGG (145). These effects could derive at least in

part by a modulation elicited by selected LGG components on immune functions through different pathways including enterocytes, monocytes, mast cells, DCs and Tregs (146-149), and by an expansion of butyrate-producing gut microbiota (43). Accordingly, studies in infants with eczema and/or CMA who received EHCF supplemented with LGG showed benefits in decreasing gastrointestinal symptoms and inflammation (107,150). Probiotics has been also proposed to reinforce the effectiveness of immunotherapy (151). Oral food immunotherapy (OIT) is currently the most investigated approach for persistent FA and it is based on the concept that repeated oral/intestinal exposures to antigens normally leads to tolerance. Tang et al. performed a randomized double-blind placebo-controlled trial with the probiotic *L.rhamnosus* CGMCC 1.3724 and peanut OIT (PPOIT) in 62 children with peanut allergy. Subjects received a fixed dose of probiotic (or placebo) together with peanut OIT (or placebo) once daily for a total of 18 months. Sustained unresponsiveness (SU), determined by DBPCFC conducted 2 to 5 weeks after discontinuation of treatment, was achieved in 82.1% of patients receiving PPOIT compared with 3.6% of those receiving placebo, the highest rate of SU reported for any food immunotherapy treatment evaluated in a randomized controlled study to date. PPOIT also induced high rates of desensitization (90%) and was associated with reduced peanut skin test reactivity, decreased peanut-specific IgE, and increased peanut-specific IgG4 levels. PPOIT was well tolerated with no participants withdrawing because of adverse reactions (6 participants withdrew for reasons unrelated to PPOIT treatment); this is in stark contrast to OIT whereby 10% to 30% of participants withdraw because of adverse reactions. At approximately 4 years after the study ended, 67% were still consuming peanut, and only 58% of the 12 participants who stopped peanut ingestion for 8 weeks demonstrated sustained unresponsiveness. Importantly, no OIT control group was evaluated to determine if the probiotic itself had any effect on SU (152). Further studies comparing peanut OIT with probiotic with peanut OIT with placebo will be required to evaluate this strategy further.

1.9 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 (153,154). 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 (156-159). The best-characterized post-translational histone modifications include phosphorylation, ubiquitination, acetylation and methylation, the last two of which are the most extensively studied (160,161). 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 (162,163). DNA methylation and histone modifications mutually interact (164). MicroRNAs (miRNAs) represent post-transcriptional control elements, important epigenetic regulators of gene expression (165;166). 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 (165-170).

Considering their biological importance, miRNAs have been involved in multiple human pathologies (167). These include also allergic diseases, in which the role of miRNAs has been rather extensively studied (171-175). 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 (176) and in therapeutic applications as one of the possible antisense approaches (177). Connections between miRNAs and DNA methylation/histone modifications have also been described (165,167,169,170, 178). Several studies provide direct evidence linking epigenetics and FA (179-184). Martino et al. investigated whether variation in DNA methylation underscores the suboptimal neonatal CD4+ T-cell gene expression associated with the development of FA (179), including impaired T-cell expansion and reduced IFN- γ production (185-188). 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 (179). 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. (189). 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 (180). 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 (190). Preliminary cross-sectional studies have suggested that Th1/Th2 cytokine genes DNA methylation pattern and selected miRNAs expression change significantly during CMA disease course (182-184). In particular, we demonstrated a potential role of miR-193a-5p in regulation of Th2 response in children with IgE-mediated CMA (184). 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 (183). Dietary factors exert a pivotal role in the regulation of epigenetic mechanisms (191). 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 (182-184).

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 (192-193). Thus, epigenetics may be the missing piece to understanding environmental–genetic interactions and FA risk.

2. Aim

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

3. Materials and Methods

3.1 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.

3.2 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.

3.3 Peripheral blood mononuclear cells stimulation and measurement of IL-4, IL-5, IL-13, IL-10, and IFN-γ culture media concentration

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 (183). PBMCs 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 CD4+T cells 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.

3.4 DNA and RNA extraction from CD4+ T-cells

CD4+ T-cells were obtained by negative selection using the CD4+ T-Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) from stimulated PBMCs. 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 $2x10^6$ 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 $2x10^5$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.

3.5 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 (182,183). High-resolution melting real-time PCR for methylation analysis was performed as described previously (182,183). 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).

3.6 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.

3.7 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% CO2. 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.

3.8 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 ($3x10^7$ 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.

3.9 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).

3.10 Food allergen sensitization and challenge

The experimental design is reported in Figure 4. 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 [16]: 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.

3.11 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).

3.12 Spleen culture and cytokine measurement

Single-cell suspensions were prepared from spleens harvested 24 h after challenge. Cells were plated at 2×10^5cells 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).

3.13 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 KH2PO4 (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).

3.14 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 $MnCl_2$, 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).

3.15 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.

4. Results

4.1 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 (194). 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.

4.2 Effects of butyrate on $CD4^+$ T cells

To investigate immunological effects of butyrate, we performed time-course and dose response experiments on PBMCs 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). PBMCs 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 6). 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 6).

4.3. 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 7). 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 (195). As shown in Figure 7, 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 7).

4.4 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 8).

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 8).

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 8).

4.5 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 (196). 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 9).

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 (197). 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) (198). 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 (199). Acetylation of Foxp3 is an important posttranslational mechanism that affects Foxp3 abundance, because it protects Foxp3 proteasomal degradation (200). This is a signature mechanism of action of several HDACs in Tregs (201-205).

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 (206). These cytokines promote Th2-type allergic response by activation of ILC2s, mast cells, basophils, and DCs (207). Activation of ILC2s stimulates production of IL-4–, IL-5–, and IL-13–promoting Th2-type allergic responses (208). 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 (209).

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 (210). 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 (211). The mucus layer does not merely form a nonspecific physical barrier, but also constrains the immunogenicity of gut antigens by delivering tolerogenic signals (212). In the colon, the mucus layer is directly in contact with the butyrate produced by gut microbiota, representing the major energy source for colonocytes (213).

Moreover, butyrate stimulates the HBD-3 synthesis, an innate immune peptide, involved in induction of FoxP3+ T cells (195).

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 (214-217). The involvement of liver in FA is emerging (218). 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 (219). 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 (220).

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 (221). 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 (222). 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 (223). 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 (224). 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 (225). 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.

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 10).

7. References

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8. Tables and Figures

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*	*226*
Thompson-Chagoyan OC et al. 2010 (n=46:FA)	↑	N.R.	*Bacterial culture*	↑ *Lactobacilli* ↓ *Bifidobacteria*	*227*
Thompson-Chagoyan OC et al. 2011 (n=46:FA)	N.R.	N.R.	*Bacterial culture*	↑ *C.coccoides, Atophium cluster*	*228*
Nakayama J et al. 2011 (n=11: FA)	=	=	16s rRNA sequencing	↑ *Bacteroides,Propionibacterium , Klebsiella* ↓ *Acinobacterium, Clostridium*	*229*
Ling Z et al. 2014 (n=34: FA)	↓	=	16s rRNA sequencing	↑ *Bacteroidetes, Proteobacteria, Actinobacteria* ↓ *Firmicutes*	*230*
Azad MB et al. 2015 (n=12: FS)	↓	= ↓	16s rRNA sequencing	↓ *Enterobacteriaceae, Bacteroidaceae*	*45*
Chen CC et al. 2015 (n=23: FS)	N.R.		16s rRNA sequencing	↑ *Firmicutes, Proteobacteria, Actinobacteria* ↓ *Veillonella*	*231*
Berni Canani et al. 2016 (n=39; FA)	↑	N.R.	16s rRNA sequencing	↑ *Ruminococcaceae, Lachnospiraceae* ↓ *Bifidobacteriaceae, Streptococcaceae, Enterobacteriaceae*	43
Bunyavanich S.et al.2016 (n=226; FA)	↑	N.R.	16s rRNA sequencing	↑ *Bacteroidetes, Enterobacter*	*46*
Inoue R et al. 2017 (n=4: FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Lachnospira, Veillionella, Suterella* ↓ *Dorea, Akkermansia*	*232*
Kourosh A. et al. 2018 (n=68; FA)	↑	N.R.	16s rRNA sequencing	↑ *Oscillobacter valericigenes, Lachnocrostidium bolteae, Faecalibacterium sp.*	*233*
Fazlollahi M. et al.2018 (n=141; FA)	N.R.	N.R.	16s rRNA sequencing	↑ *Lachnospiraceae, Streptococcaceae, Leuconostocaceae*	*234*

Abbreviations: FA= food allergy; FS= sensitization to food antigens; OTUs = operational taxonomic units;

N.R.= not reported

Table 2. Main preclinical evidences on the probiotics role against food allergy

Biological effects	Bacterial strains	References
Intestinal Barrier Maturation	*B. lactis/bifidum*; *L. rhamnosus GG*	123,125, 235,
Th1/Th2 response balance: Th1 stimulation	*B.lactis/bifidum/ infantis*; *L. acidophilus/reuteri; L.rhamnosus GG*	127,128,236
Th1/Th2 response balance: Th2 suppression	*B. bifidum/infantis/longum;L.actobacillus acidophilus/reuteri; L.rhamnosus GG*	127,129, 130,132,133
Immune system regulation: Tregs development	*B.bifidum/infantis/lactis;L.acidophilus/reuteri/casei;L. rhamnosus GG*	235,236
Increase in B and T cell proliferation with enhanced production of Th1 and regulatory cytokines	*L.acidophilus;L. casei;L.salivarius;L.lactis;B. infantis; B. lactis; B. longum*	133
Immune system regulation: tolerogenic DCs development	*B.bifidum;L.reuteri/casei*; *L.rhamnosus GG*	237, 238
Suppression of IgE production	*B. bifidum/longum*; *B.lactis Bb-12*; *L.acidophilus*; *L.rhamnosus GG*	239,240
Epigenetic modulation of Th1/Th2 genes expression	*B.breve;L. rhamnosus GG*	134
Increase in the production of the regulatory cytokine IL-10 by monocytes and dendritic cells; enhance of IFN-γ production by T cells	*L. plantarum;B. adolescentis*	130,131, 132
Increase in the population of $CD4^+FoxP3^+$ T cells, up-regulation of FoxP3 and down-regulation of GATA-3	*L.plantarum;B. coagulans*	135
Reduction of allergic reaction; reduction of IL-4, IL-5, IL-13 and specific IgE production	*L. rhamnosus GG*	140
Improvement of anaphylaxis symptoms and increase of sIgA and CD4+ CD25+ FoxP3Treg cell	*C.butyricum*	136

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 structure of the gut microbiota-immune system axis

Within the gut microbiota-immune system axis the cross talk between microbes and the immune system may occur directly through microbial components or indirectly through the action of metabolites, such as SCFAs. A positive modulation of this axis can counteract the pathogenesis of FA by promoting epithelial integrity, gut permeability, mucus production, $CD103^+$ tolerogenic DCs, Treg differentiation, cytokines production and sIgA release from plasma cells.

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

PBMCs 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. A significant inhibition of IL-4, IL-5 and IL-13 production was observed with 0.75 mM butyrate. Butyrate stimulated, at the some dose, IL-10 and IFN-γ production and FoxP3 expression, through a demethylation of respective gene. 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 7. Immune and non immune effects and HDAC activity of butyrate effects on human enterocytes.

Dose-response direct effects of butyrate on HBD-3, Muc5AC, Muc2 and tight junctions expression levels on HT-29 cells. 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. *$p<0.05$; **$p<0.001$*vs* untreated cells.

Figure 8. 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, serum MCP-1 and sIgE levels.

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. Butyrate stimulated IL-10 and IFN-γ **(E)** production and inhibited IL-4 and IL-13 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. *$p<0.05$; **$p<0.01$; ***$p<0.001$

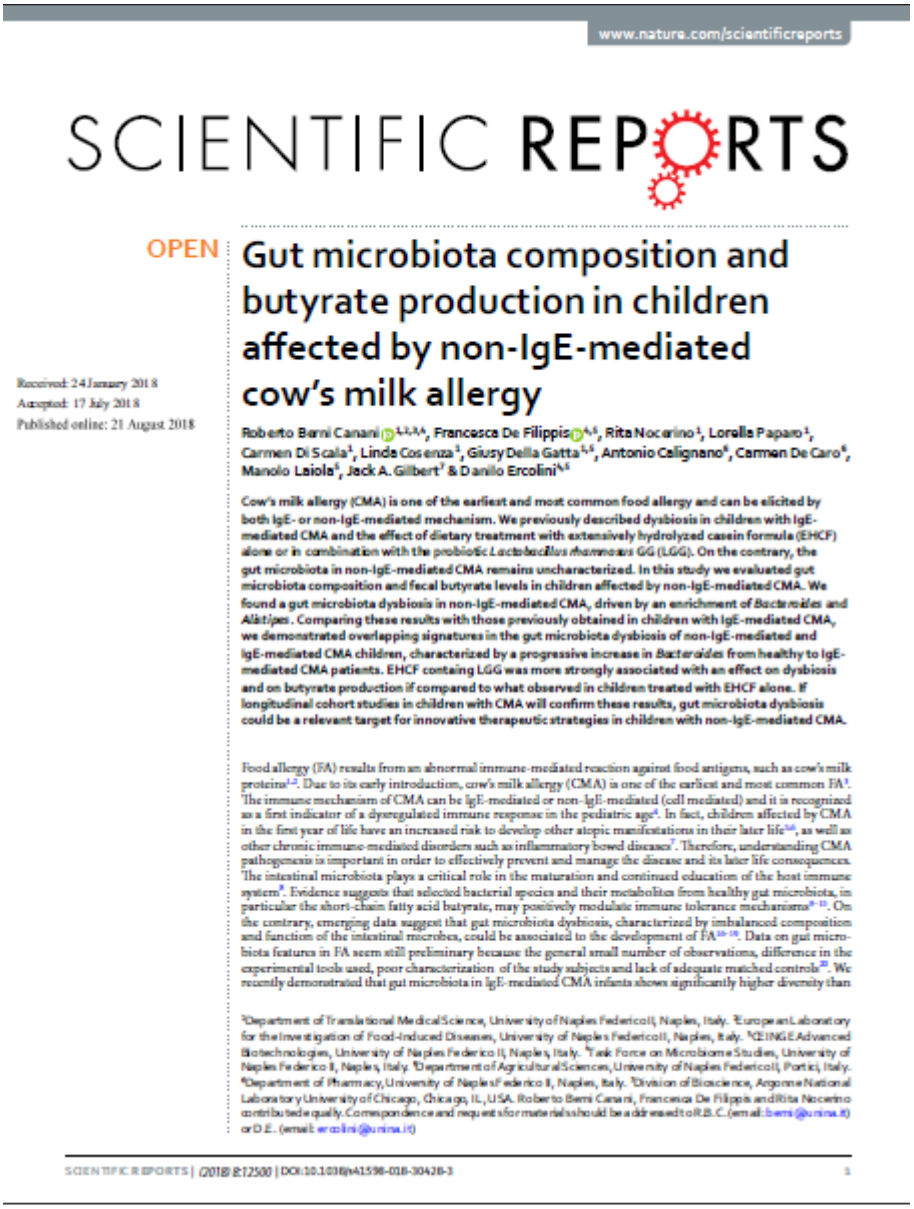
Figure 9. Evidence of hepatic mitochondrial dysfunction in mice sensitized to food allergens.

The mitochondrial H_2O_2 yield and 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 10. 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.

9. Publications in the last 3 years



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SCIENTIFIC REPORTS

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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[4,5], Rita Nocerino[1], Lorella Paparo[1], Carmen Di Scala[1], Linda Cosenza[1], Giusy Della Gatta[1,5], Antonio Calignano[6], Carmen De Caro[6], Manolo 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[3]. 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[4]. 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[5,6], as well as other chronic immune-mediated disorders such as inflammatory bowel disease[7]. 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[8]. 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[9–11]. 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[10–19]. 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[20]. We recently demonstrated that gut microbiota in IgE-mediated CMA infants shows significantly higher diversity than

[1]Department of Translational Medical Science, University of Naples Federico II, Naples, Italy. [2]European Laboratory for the Investigation of Food-Induced Diseases, University of Naples Federico II, Naples, Italy. [3]CEINGE Advanced Biotechnologies, University of Naples Federico II, Naples, Italy. [4]Task Force on Microbiome Studies, University of Naples Federico II, Naples, Italy. [5]Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy. [6]Department of Pharmacy, University of Naples Federico II, Naples, Italy. [7]Division of Bioscience, Argonne National Laboratory University of Chicago, Chicago, IL, USA. Roberto Berni Canani, Francesca De Filippis and Rita Nocerino contributed equally. Correspondence and request for materials should be addressed to R.B.C. (email: berni@unina.it) or D.E. (email: ercolini@unina.it)

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Article

Hepatic Mitochondrial Dysfunction and Immune Response in a Murine Model of Peanut Allergy

Giovanna Trinchese [1,†], Lorella Paparo [2,†], Rosita Aitoro [2,†], Carmela Fierro [2], Michela Varchetta [2], Rita Nocerino [2], Maria Pina Mollica [1] and Roberto Berni Canani [2,3,4,5,*]

1 Department of Biology, University of Naples "Federico II", 80126 Naples, Italy; giovanna.trinchese@unina.it (G.T.); mariapia.mollica@unina.it (M.P.M.)
2 Department of Translational Medical Science—Pediatric Section, University of Naples "Federico II", 80131 Naples, Italy; paparolorella@gmail.com (L.P.); aitoro.rosita@gmail.com (R.A.); carmelafierro08@gmail.com (C.F.); mic.varchetta@studenti.unina.it (M.V.); ritanocerino@alice.it (R.N.)
3 European Laboratory for the Investigation of Food-Induced Diseases (ELFID), University of Naples "Federico II", 80131 Naples, Italy
4 CEINGE Advanced Biotechnologies, University of Naples "Federico II", 80131 Naples, Italy
5 Task Force on Microbiome Studies, University of Naples "Federico II", 80131 Naples, Italy
* Correspondence: berni@unina.it; Tel.: +39-081-7462680
† These Authors contributed equally to the work.

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Abstract: Background: Evidence suggests a relevant role for liver and mitochondrial dysfunction in allergic disease. However, the role of hepatic mitochondrial function in food allergy is largely unknown. We aimed to investigate hepatic mitochondrial dysfunction in a murine model of peanut allergy. Methods: Three-week-old C3H/HeOuJ mice were sensitized by the oral route with peanut-extract (PNT). We investigated: 1. the occurrence of effective sensitization to PNT by analysing acute allergic skin response, anaphylactic symptoms score, body temperature, serum mucosal mast cell protease-1 (mMCP-1) and anti-PNT immunoglobulin E (I_gE) levels; 2. hepatic involvement by analysing interleukin (IL)-4, IL-5, IL-13, IL-10 and IFN-γ mRNA expression; 3. hepatic mitochondrial oxidation rates and efficiency by polarography, and hydrogen peroxide (H_2O_2) yield, aconitase and superoxide dysmutase activities by spectrophotometry. Results: Sensitization to PNT was demonstrated by acute allergic skin response, anaphylactic symptoms score, body temperature decrease, serum mMCP-1 and anti-peanut I_gE levels. Liver involvement was demonstrated by a significant increase of hepatic Th2 cytokines (IL-4, IL-5 and IL-13) mRNA expression. Mitochondrial dysfunction was demonstrated by lower state 3 respiration rate in the presence of succinate, decreased fatty acid oxidation in the presence of palmitoyl-carnitine, increased yield of ROS proven by the inactivation of aconitase enzyme and higher H_2O_2 mitochondrial release. Conclusions: We provide evidence of hepatic mitochondrial dysfunction in a murine model of peanut allergy. These data could open the way to the identification of new mitochondrial targets for innovative preventive and therapeutic strategies against food allergy.

Keywords: food allergy; mitochondrial function; oxidative stress; Th2 cytokines

1. Introduction

Food allergy (FA), defined as an adverse immune response to food proteins, is a major public health issue in Western countries due to its increasing prevalence and severity as well as the negative impact on quality of life and medical care costs [1]. Peanut allergy (PA) is one of the most common types of FA [2]. The prevalence of PA among children in Western countries has doubled in the past 10 years, reaching rates of 1.4–3.0% [3]. In contrast to other FA, such as cow milk allergy, the majority

Direct effects of fermented cow's milk product with *Lactobacillus paracasei* CBA L74 on human enterocytes

L. Paparo[1], R. Aitoro[1], R. Nocerino[1], C. Fierro[1], C. Bruno[1] and R. Berni Canani[1,2,3*]

[1]*Department of Translational Medical Science, University of Naples 'Federico II', via S. Pansini 5, 80131 Naples, Italy;* [2]*European Laboratory for the Investigation of Food-Induced Diseases, University of Naples 'Federico II', via S. Pansini 5, 80131 Naples, Italy;* [3]*CEINGE Advanced Biotechnologies, University of Naples 'Federico II', via S. Pansini 5, 80131 Naples, Italy; berni@unina.it*

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RESEARCH ARTICLE

Abstract

Cow's milk fermented with *Lactobacillus paracasei* CBA L74 (FM-CBAL74) exerts a preventive effect against infectious diseases in children. We evaluated if this effect is at least in part related to a direct modulation of non-immune and immune defence mechanisms in human enterocytes. Human enterocytes (Caco-2) were stimulated for 48 h with FM-CBAL74 at different concentrations. Cell growth was assessed by colorimetric assay; cell differentiation (assessed by lactase expression), tight junction proteins (zonula occludens1 and occludin), mucin 2, and toll-like receptor (TRL) pathways were analysed by real-time PCR; innate immunity peptide synthesis, beta-defensin-2 (HBD-2) and cathelicidin (LL-37) were evaluated by ELISA. Mucus layer thickness was analysed by histochemistry. FMCBA L74 stimulated cell growth and differentiation, tight junction proteins and mucin 2 expression, and mucus layer thickness in a dose-dependent fashion. A significant stimulation of HBD-2 and LL-37 synthesis, associated with a modulation of TLR pathway, was also observed. FM-CBAL74 regulates non-immune and immune defence mechanisms through a direct interaction with the enterocytes. These effects could be involved in the preventive action against infectious diseases demonstrated by this fermented product in children.

Keywords: infectious diseases, fermented foods, innate immunity, gut mucosa, intestinal permeability

1. Introduction

Common infectious diseases (CIDs) affecting the respiratory and gastrointestinal tract are a common problem for young children attending day-care centres or schools (Maldonado *et al.*, 2012). These conditions are exacerbated by a general immaturity of the immune system and of respiratory and gastrointestinal tract functions (Maldonado *et al.*, 2012). Evidence suggests that functional foods, derived from the fermentation of cow's milk with probiotics, could be an effective dietary strategy to prevent infectious diseases in children, but data are still conflicting (Agostoni *et al.*, 2007; Brunser *et al.*, 1989; Campeotto *et al.*, 2011; Merenstein *et al.*, 2010; Mullié *et al.*, 2004; Nagata *et al.*, 2011; Thibault *et al.*, 2004). Differences in the experimental design, study populations, and in bacterial strains used in the preparation of the fermented products could be responsible for these discrepancies (Agostoni *et al.*, 2007; Brunser *et al.*, 1989; Campeotto *et al.*, 2011; Merenstein *et al.*, 2010; Mullié *et al.*, 2004; Nagata *et al.*, 2011; Thibault *et al.*, 2004). Another limitation derives from the still largely undefined mechanisms of action elicited by fermented foods against infectious diseases (Shafi *et al.*, 2014). The protective properties of fermented foods could be strain specific and dose-dependent. They could also derived from the activity of the probiotics (Hill *et al.*, 2014) and/or of food components produced during the fermentation process (Tamang *et al.*, 2016). In a double-blind, randomised, placebo-controlled trial, we found that fermented cow's milk product with *Lactobacillus paracasei* CBA L74 (FM-CBAL74) efficiently protects schooled children against CIDs and that this protective effect is associated with a significant stimulation of both innate (α- and β-defensins and cathelicidin (LL-37)) and acquired (secretory immunoglobulin A) immunity (Nocerino *et al.*, 2017).

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ORIGINAL ARTICLE
Experimental Allergy and Immunology

WILEY Allergy

Altered miR-193a-5p expression in children with cow's milk allergy

V. D'Argenio[1,2] | V. Del Monaco[1] | L. Paparo[3] | F. D. E. De Palma[1] | R. Nocerino[3] | F. D'Alessio[1] | F. Visconte[1,2] | V. Discepolo[1,3] | L. Del Vecchio[1,2] | F. Salvatore[1,2,4] | R. Berni Canani[1,3,5]

[1]CEINGE-Biotecnologie Avanzate s.c.a r.l., Naples, Italy
[2]Department of Molecular Medicine and Medical Biotechnologies, University of Naples Federico II, Naples, Italy
[3]Department of Translational Medical Science, University of Naples Federico II, Naples, Italy
[4]IRCCS-Fondazione SDN, Naples, Italy
[5]European Laboratory for the Investigation of Food-Induced Diseases, University of Naples Federico II, Naples, Italy

Correspondence
Francesco Salvatore, CEINGE-Biotecnologie Avanzate s.c.a r.l., Naples, Italy.
Email: salvator@unina.it
and
Roberto Berni Canani, Department of Translational Medical Science, University of Naples Federico II, Naples, Italy.
Email: berni@unina.it

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Abstract

Background: Cow's milk allergy (CMA) is one of the most common food allergies in children. Epigenetic mechanisms have been suggested to play a role in CMA pathogenesis. We have shown that DNA methylation of Th1/Th2 cytokine genes and FoxP3 affects CMA disease course. Preliminary evidence suggests that also the miRNome could be implicated in the pathogenesis of allergy. Main study outcome was to comparatively evaluate miRNome in children with CMA and in healthy controls.

Methods: Peripheral blood mononuclear cells were obtained from children aged 4-18 months: 10 CMA patients, 9 CMA patients who outgrew CMA, and 11 healthy controls. Small RNA libraries were sequenced using a next-generation sequencing-based approach. Functional assessment of IL-4 expression was also performed.

Results: Among the miRNAs differently expressed, 2 were upregulated and 14 were downregulated in children with active CMA compared to healthy controls. miR-193a-5p resulted the most downregulated miRNA in children with active CMA compared to healthy controls. The predicted targets of miR-193a-5p resulted upregulated in CMA patients compared to healthy controls. Peripheral blood $CD4^+$ T cells transfected with a miR193a-5 inhibitor showed a significant upregulation of IL-4 mRNA and its protein expression. Children who outgrew CMA showed miRNA-193a-5p level, and its related targets expression, similar to that observed in healthy controls.

Conclusions: Our results suggest that miR-193a-5p is a post-transcriptional regulator of IL-4 expression and could have a role in IgE-mediated CMA. This miRNA could be a novel diagnostic and therapeutic target for this common form of food allergy in childhood.

KEYWORDS
epigenetics, food allergy, IL-4, miRNome

Abbreviations: CMA, cow's milk allergy; DBPCFC, double-blind placebo-controlled food challenge; EHCF + LGG, extensively hydrolyzed casein formula containing the probiotic *Lactobacillus rhamnosus* GG; EoE, eosinophilic esophagitis; IgE, immunoglobulin E; IL-10, interleukin-10; IL-4, interleukin-4; IL-5, interleukin-5; INF-γ, interferon-γ; miRNA, microRNA; miRNome, full spectrum of miRNAs expressed in a specific genome; NGS, next-generation sequencing; PBMCs, peripheral blood mononuclear cells; Th1, Type 1 helper; Th2, Type 2 helper; Th, T helper.

D'Argenio, Del Monaco, and Paparo equally contributors.

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MICROBIAL ECOLOGY

Specific Signatures of the Gut Microbiota and Increased Levels of Butyrate in Children Treated with Fermented Cow's Milk Containing Heat-Killed *Lactobacillus paracasei* CBA L74

Roberto Berni Canani,[a,b,c,d] Francesca De Filippis,[d,e] Rita Nocerino,[a] Manolo Laiola,[e] Lorella Paparo,[a] Antonio Calignano,[f] Carmen De Caro,[f] Lorena Coretti,[g] Lorenzo Chiariotti,[d,g] Jack A. Gilbert,[h] Danilo Ercolini[d,e]

Department of Translational Medical Science, University of Naples Federico II, Naples, Italy[a]; European Laboratory for the Investigation of Food-Induced Diseases, University of Naples Federico II, Naples, Italy[b]; CEINGE Advanced Biotechnologies, University of Naples Federico II, Naples, Italy[c]; Task Force on Microbiome Studies, University of Naples Federico II, Naples, Italy[d]; Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy[e]; Department of Pharmacy, University of Naples Federico II, Naples, Italy[f]; Istituto di Endocrinologia ed Oncologia Sperimentale, CNR, and Dipartimento di Medicina Molecolare e Biotecnologie Mediche, University of Naples Federico II, Naples, Italy[g]; The Microbiome Center, Department of Surgery, University of Chicago, Chicago, Illinois, USA[h]

ABSTRACT We recently demonstrated that cow's milk fermented with the probiotic *Lactobacillus paracasei* CBA L74 (FM-CBAL74) reduces the incidence of respiratory and gastrointestinal tract infections in young children attending school. This effect apparently derives from a complex regulation of non-immune and immune protective mechanisms. We investigated whether FM-CBAL74 could regulate gut microbiota composition and butyrate production. We randomly selected 20 healthy children (12 to 48 months) from the previous randomized controlled trial, before (t0) and after 3 months (t3) of dietary treatment with FM-CBAL74 (FM) or placebo (PL). Fecal microbiota was profiled using 16S rRNA gene amplicon sequencing, and the fecal butyrate concentration was also measured. Microbial alpha and beta diversities were not significantly different between groups prior to treatment. FM-CBAL74 but not PL treatment increased the relative abundance of *Lactobacillus*. Individual *Blautia*, *Roseburia*, and *Faecalibacterium* oligotypes were associated with FM-CBAL74 treatment and demonstrated correlative associations with immune biomarkers. Accordingly, PICRUSt analysis predicted an increase in the proportion of genes involved in butyrate production pathways, consistent with an increase in fecal butyrate observed only in the FM group. Dietary supplementation with FM-CBAL74 induces specific signatures in gut microbiota composition and stimulates butyrate production. These effects are associated with changes in innate and acquired immunity.

IMPORTANCE The use of a fermented milk product containing the heat-killed probiotic strain *Lactobacillus paracasei* CBAL74 induces changes in the gut microbiota, promoting the development of butyrate producers. These changes in the gut microbiota composition correlate with increased levels of innate and acquired immunity biomarkers.

KEYWORDS gut microbiota, immune system, fecal butyrate

Common infectious diseases, affecting the respiratory and gastrointestinal tracts, are an important problem for young children attending preschool or day care centers (1). Young children are especially prone to infection, and this susceptibility is thought to be driven by immaturity in organ function, immune response, and also potentially in

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Address correspondence to Roberto Berni Canani, bernie@unina.it, or Danilo Ercolini, ercolini@unina.it.

R.B.C. and F.D.F. contributed equally to this article.

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Review

Gut Microbiota as a Target for Preventive and Therapeutic Intervention against Food Allergy

Rosita Altoro [1], Lorella Paparo [1], Antonio Amoroso [1], Margherita Di Costanzo [1], Linda Cosenza [1], Viviana Granata [1], Carmen Di Scala [1], Rita Nocerino [1], Giovanna Trinchese [1], Mariangela Montella [1], Danilo Ercolini [2,3] and Roberto Berni Canani [1,3,4,5,*]

1 Department of Translational Medical Science-Pediatric Section, University of Naples "Federico II", 80131 Naples, Italy; altoro.rosita@gmail.com (R.A.); paparolorella@gmail.com (L.P.); antonioamoroso87@gmail.com (A.A.); mara.dicostanzo@live.it (M.D.C.); lindacosenza@libero.it (L.C.); vivianagranata@gmail.com (V.G.); carmendiscala@gmail.com (C.D.S.); ritanocerino@alice.it (R.N.); giovanna.trinchese@unina.it (G.T.); mariangelamontella@libero.it (M.M.)

2 Department of Agricultural Sciences, Division of Microbiology, University of Naples "Federico II", 80055 Portici, Italy; ercolini@unina.it

3 Task Force on Microbiome Studies, University of Naples "Federico II", 80131 Naples, Italy

4 European Laboratory for the Investigation of Food Induced Diseases, University of Naples "Federico II", 80131 Naples, Italy

5 CEINGE Advanced Biotechnologies, University of Naples "Federico II", 80131 Naples, Italy

* Correspondence: berni@unina.it; Tel.: +39-081-746-2680

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Abstract: The gut microbiota plays a pivotal role in immune system development and function. Modification in the gut microbiota composition (dysbiosis) early in life is a critical factor affecting the development of food allergy. Many environmental factors including caesarean delivery, lack of breast milk, drugs, antiseptic agents, and a low-fiber/high-fat diet can induce gut microbiota dysbiosis, and have been associated with the occurrence of food allergy. New technologies and experimental tools have provided information regarding the importance of select bacteria on immune tolerance mechanisms. Short-chain fatty acids are crucial metabolic products of gut microbiota responsible for many protective effects against food allergy. These compounds are involved in epigenetic regulation of the immune system. These evidences provide a foundation for developing innovative strategies to prevent and treat food allergy. Here, we present an overview on the potential role of gut microbiota as the target of intervention against food allergy.

Keywords: cow's milk allergy; diet; immune tolerance; dysbiosis; probiotics; short chain fatty acids; butyrate

1. Introduction

During the last several decades, a changing patterns in the epidemiology of food allergy [FA] have been observed, with an increased prevalence, severity of clinical manifestations, and risk of persistence until later ages [1]. Atopic family history, ethnicity, atopic dermatitis (AD), and related genetic polymorphisms have been associated with FA development [2]. Although genetic factors may predispose individuals to the development of FA among selected individuals, they cannot explain the changes in epidemiology over this short time frame, suggesting that environmental factors promote FA [3]. FA develops following loss of immune tolerance, which results in allergic sensitization and subsequent disease manifestation and progression.

The initial exposure to food allergens occurs predominantly via the gastrointestinal tract or skin. An impaired skin barrier could lead to increased transcutaneous passage of antigens and subsequent

nutrients

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Article

Preventive Effect of Cow's Milk Fermented with *Lactobacillus paracasei* CBA L74 on Common Infectious Diseases in Children: A Multicenter Randomized Controlled Trial

Giovanni Corsello [1], Maurizio Carta [1], Roberto Martinello [2], Marina Picca [3], Giulio De Marco [4], Maria Micillo [4], Dante Ferrara [5], Patrizia Vigneri [6], Gaetano Cecere [4], Pasqualina Ferri [4], Paola Roggero [7], Giorgio Bedogni [8], Fabio Mosca [7], Lorella Paparo [4], Rita Nocerino [4] and Roberto Berni Canani [4,9,10,*]

1 Operative Unit of Pediatrics and Neonatal Intensive Therapy, Mother and Child Department, University of Palermo, 90121 Palermo, Italy; giocors@alice.it (G.C.); cvbm@unin.it (M.C.)
2 Federazione Italiana Medici Pediatri Lombardia, 46100 Mantova, Italy; hethi@unin.it
3 Pediatric Society of Primary Health Care (SICuPP), 20135 Milan, Italy; dafnfhm@unin.it
4 Department of Translational Medical Science—Pediatric Section, University of Naples "Federico II" Via S. Pansini, 5, 80131 Naples, Italy; gmtm@unin.it (G.D.M.); csvbu@unin.it (M.M.); ctnim@unin.it (G.C.); ryhm@unin.it (P.F.); paparolorella@gmail.com (L.P.); ritanocerino@alice.it (R.N.)
5 Department of Sciences for Health Promotion and Mother and Child Care, University of Palermo, 90121 Palermo, Italy; ulvbu@unin.it
6 Family Pediatrician, 90133 Palermo, Italy; vignerip@gmail.com
7 Department of Clinical Science and Community Health, Neonatal Intensive Care Unit, Fondazione I.R.C.C.S. Cà Granda Ospedale Maggiore Policlinico, University of Milan, 20143 Milan, Italy; sdivedi@unin.it (P.R.); fabio.mosca@mangiagalli.it (F.M.)
8 Clinical Epidemiology Unit, Liver Research Center, Basovizza, 34012 Trieste, Italy; giorgiobedogni@gmail.com
9 European Laboratory for the Investigation of Food-Induced Diseases, University of Naples "Federico II", 80131 Naples, Italy
10 CEINGE Advanced Biotechnologies, University of Naples "Federico II", 80131 Naples, Italy
* Correspondence: berni@unina.it; Tel.: +39-081-7462680

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Abstract: Background: Fermented foods have been proposed to prevent common infectious diseases (CIDs) in children attending day care or preschool. Objectives: To investigate the efficacy of dietary supplementation with cow's skim milk fermented with the probiotic *Lactobacillus paracasei* CBA L74 in reducing CIDs in children attending day care or preschool. **Methods:** Multicenter, randomized, double-blind, placebo-controlled trial on healthy children (aged 12–48 months) consuming daily 7 grams of cow's skim milk fermented with *L. paracasei* CBA L74 (group A), or placebo (maltodextrins group B) attending day care or preschool during the winter season. The main outcome was the proportion of children who experienced ≥1 episode of CID during a 3-month follow-up. Fecal biomarkers of innate (α- and β-defensins, cathelicidin) and acquired immunity (secretory IgA) were also monitored. **Results:** A total of 126 children (71 males, 56%) with a mean (SD) age of 33 (9) months completed the study, 66 in group A and 60 in group B. At intention to treat analysis, the proportion of children presenting ≥1 CID was 60% in group A vs. 83% in group B, corresponding to an absolute risk difference (ARD) of −23% (95% CI: −37% to −9%, $p < 0.01$). At per-protocol-analysis (PPA), the proportion of children presenting ≥1 CID was 18% in group A vs. 40% in group B, corresponding to an absolute risk difference (ARD) of −22% (95% CI: −37% to −6%, $p < 0.01$). PPA showed that the proportion of children presenting ≥1 acute gastroenteritis (AGE) was significantly lower in group A (18% vs. 40%, $p < 0.05$). The ARD for the occurrence of ≥1 AGE was −22% (95% CI: −37% to −6%, $p < 0.01$) in group A. Similar findings were obtained at PPA regarding the proportion of

ORIGINAL ARTICLE FOOD

Extensively hydrolyzed casein formula alone or with *L. rhamnosus* GG reduces β-lactoglobulin sensitization in mice

Rosita Aitoro[1], Raffaele Simeoli[2], Antonio Amoroso[1], Lorella Paparo[1], Rita Nocerino[1], Claudio Pirozzi[2], Margherita di Costanzo[1], Rosaria Meli[2], Carmen De Caro[2], Gianluca Picariello[3], Gianfranco Mamone[3], Antonio Calignano[2], Cathryn R. Nagler[4] & Roberto Berni Canani[1,5,6]

Department of [1]Translational Medical Science, [2]Pharmacy, University of Naples "Federico II", Naples, Italy; [3]Institute of Food Sciences, CNR, Avellino, Italy; [4]Committee on Immunology and Department of Pathology, University of Chicago, Chicago, IL, USA; [5]European Laboratory for The Investigation of Food Induced Diseases; [6]CEINGE Advanced Biotechnologies, University of Naples "Federico II", Naples, Italy

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Keywords
bovine casein; food allergy; hypoallergenic formula; intestinal permeability; probiotics

Correspondence
Roberto Berni Canani, Pediatric Allergy Program - Department of Translational Medical Science, European Laboratory for The Investigation of Food Induced Diseases, and CEINGE Advanced Biotechnologies, University of Naples "Federico II", Via Sergio Pansini, 5, Naples 80131, Italy
Tel.: +390817462680
Fax: +390817462375–4228
E-mail: bernir@unina.it

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Abstract

Background: Extensively hydrolyzed casein formula (EHCF) has been proposed for the prevention and is commonly used for the treatment of cow's milk allergy (CMA). The addition of the probiotic *Lactobacillus rhamnosus* GG (LGG) to EHCF may induce faster acquisition of tolerance to cow's milk. The mechanisms underlying this effect are largely unexplored. We investigated the effects of EHCF alone or in combination with LGG on β-lactoglobulin (BLG) sensitization in mice.

Methods: Three-week-old C3H/HeOuJ mice were sensitized by oral administration of BLG using cholera toxin as adjuvant at weekly intervals for 5 weeks (sensitization period). Two experimental phases were conducted: (i) EHCF or EHCF+LGG given daily, starting 2 weeks before the sensitization period and then given daily for 5 weeks and (ii) EHCF or EHCF+LGG given daily for 4 weeks, starting 1 week after the sensitization period. Diet free of cow's milk protein was used as control. Acute allergic skin response, anaphylactic symptom score, body temperature, intestinal permeability, anti-BLG serum IgE, and interleukin (IL)-4, IL-5, IL-10, IL-13, IFN-γ mRNA expression were analyzed. Peptide fractions of EHCF were characterized by reversed-phase (RP)-HPLC, MALDI-TOF mass spectrometry, and nano-HPLC/ESI-MS/MS.

Results: Extensively hydrolyzed casein formula administration before or after BLG-induced sensitization significantly reduced acute allergic skin reaction, anaphylactic symptom score, body temperature decrease, intestinal permeability increase, IL-4, IL-5, IL-13, and anti-BLG IgE production. EHCF increased expression of IFN-γ and IL-10. Many of these effects were significantly enhanced by LGG supplementation. The peptide panels were similar between the two study formulas and contained sequences that could have immunoregulatory activities.

Conclusions: The data support dietary intervention with EHCF for CMA prevention and treatment through a favorable immunomodulatory action. The observed effects are significantly enhanced by LGG supplementation.

Extensively hydrolyzed casein formula (EHCF) has been proposed for CMA prevention in at-risk infants (1), and it is commonly used as a first-line option for CMA treatment (2, 3). The efficacy of extensively hydrolyzed formulas on food allergy prevention is still controversial (4). Conversely, in a prospective study of infants with confirmed CMA, treatment with EHCF induced faster tolerance acquisition compared with other available formulas (hydrolyzed rice formula, soy formula and amino acid-based formula) (5). This effect was potentiated by the addition of the probiotic *Lactobacillus rhamnosus* GG (LGG) (5–7). Apart from being hypoallergenic, little is known regarding a possible immunomodulatory role of the casein hydrolysis-derived peptides against CMA, whether alone or in combination with LGG. Immunomodulatory effects of EHCF have been demonstrated in a rat model of type 1 diabetes (8), suggesting possible similar effects against other

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Clinical Epigenetics

SHORT REPORT Open Access

Epigenetic features of *FoxP3* in children with cow's milk allergy

Lorella Paparo[1], Rita Nocerino[1], Linda Cosenza[1], Rosita Aitoro[1], Valeria D'Argenio[2,3], Valentina Del Monaco[2,3], Carmen Di Scala[1], Antonio Amoroso[1], Margherita Di Costanzo[1], Francesco Salvatore[2,3,4] and Roberto Berni Canani[1,2,5*]

Abstract

Background: DNA methylation of the Th1 and Th2 cytokine genes is altered during cow's milk allergy (CMA). Forkhead box transcription factor 3 (*FoxP3*) is essential for the development and function of regulatory T cells (Tregs) and is involved in oral tolerance acquisition. We assessed whether tolerance acquisition in children with IgE-mediated CMA is associated with DNA demethylation of the Treg-specific demethylated region (TSDR) of *FoxP3*.

Results: Forty children (aged 3–18 months) were enrolled: 10 children with active IgE-mediated CMA (group 1), 10 children who outgrew CMA after dietary treatment with an extensively hydrolyzed casein formula containing the probiotic *Lactobacillus rhamnosus* GG (group 2), 10 children who outgrew CMA after treatment with other formulas (group 3), and 10 healthy controls (group 4). *FoxP3* TSDR demethylation and expression were measured in mononuclear cells purified from peripheral blood of the four groups of children. *FoxP3* TSDR demethylation was significantly lower in children with active IgE-mediated CMA than in either children who outgrew CMA or in healthy children. Formula selection influenced the *FoxP3* TSDR demethylation profile. The *FoxP3* TSDR demethylation rate and expression level were correlated.

Conclusions: Tolerance acquisition in children with IgE-mediated CMA involves epigenetic regulation of the *FoxP3* gene. This feature could be a new target for preventive and therapeutic strategies against CMA.

Keywords: Food allergy, Extensively hydrolyzed casein formula, Oral tolerance, *Lactobacillus rhamnosus* GG

Background

Epigenetic mechanisms have been implicated in the pathogenesis of food allergy [1]. We previously demonstrated that tolerance acquisition in children with IgE-mediated cow's milk allergy (CMA) is driven by epigenetic modulation of the Th1 and Th2 cytokine genes [2]. A regulatory T cell (Treg) suppressive phenotype, characterized by stable expression of the transcription factor "Forkhead box Protein 3" (*FoxP3*), plays a pivotal role in food tolerance [3–7]. *FoxP3* messenger RNA (mRNA) expression is lower in children with atopic asthma or IgE-mediated food allergy than in healthy children [8]. *FoxP3* stable expression requires full CpG demethylation of its transcriptional regulatory regions [9, 10], and, moreover, hypermethylation of the *FoxP3* gene has been associated with reduced Treg function and allergy [7, 11].

DNA methylation is a biologically and chemically stable epigenetic modification that locks in long-term gene expression patterns [12, 13]. The demethylation status of *FoxP3* at a highly conserved region within the Treg-specific demethylated region (TSDR), a CpG-rich region, located on the 2nd conserved non-coding sequence of *FoxP3* (CNS2), is restricted to Tregs [14, 15]. Transcriptional activity of the TSDR is essentially determined by its methylation status: it is completely inactive in its methylated state, but when the TSDR is demethylated, transcription factors such as Ets-1 and Creb can bind to the TSDR [16] TSDR demethylated and open chromatin conformation in the *Foxp3* locus leads to stable phenotype differentiated Foxp3+ Treg [17, 18]. *FoxP3* TSDR demethylation in peripheral blood mononuclear

* Correspondence: berni@unina.it
[1]Department of Translational Medical Science, University of Naples "Federico II", Via S. Pansini, 5 80131 Naples, Italy
[2]CEINGE-Biotecnologie Avanzate s.c.a.r.l, Via Gaetano Salvatore 486, 80131 Naples, Italy
Full list of author information is available at the end of the article

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Randomized control trials

Cow's milk and rice fermented with *Lactobacillus paracasei* CBA L74 prevent infectious diseases in children: A randomized controlled trial

Rita Nocerino [a], Lorella Paparo [a], Gianluca Terrin [b], Vincenza Pezzella [a], Antonio Amoroso [a], Linda Cosenza [a], Gaetano Cecere [a], Giulio De Marco [a], Maria Micillo [a], Fabio Albano [a], Rosa Nugnes [a], Pasqualina Ferri [a], Giuseppe Ciccarelli [a], Giuliana Giaccio [a], Raffaella Spadaro [a], Ylenia Maddalena [a], Francesco Berni Canani [c], Roberto Berni Canani [a, d, e, *]

[a] Department of Translational Medical Science, University of Naples "Federico II", Naples, Italy
[b] Department of Gynecology-Obstetrics and Perinatal Medicine, University of Rome "La Sapienza", Rome, Italy
[c] ENT Unit, Ospedale Spirito Santo di Pescara, Pescara, Italy
[d] European Laboratory for the Investigation of Food Induced Diseases (ELFID), University of Naples "Federico II", Naples, Italy
[e] CEINGE – Advanced Biotechnologies, University of Naples "Federico II", Naples, Italy

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SUMMARY

Background & aim: Fermented foods have been proposed for the prevention of infectious diseases. We evaluated the efficacy of fermented foods in reducing common infectious diseases (CIDs) in children attending daycare.

Methods: Prospective randomized, double-blind, placebo-controlled trial (registered under Clinical Trials.gov identifier NCT01909128) on healthy children (aged 12–48 months) consuming daily cow's milk (group A) or rice (group B) fermented with *Lactobacillus paracasei* CBA L74, or placebo (group C) for three months during the winter season. The main study outcome was the proportion of children who experienced at least one CID. All CIDs were diagnosed by family pediatricians. Fecal concentrations of innate (α- and β-defensins and cathelicidin LL-37) and acquired immunity biomarkers (secretory IgA) were also evaluated.

Results: 377 children (193 males, 51%) with a mean (SD) age of 32 (10) months completed the study: 137 in group A, 118 in group B and 122 in group C. Intention-to-treat analysis showed that the proportion of children who experienced at least one CID was lower in group A (51.8%) and B (65.9%) compared to group C (80.3%). Per-protocol analysis showed that the proportion of children presenting upper respiratory tract infections was lower in group A (48.2%) and group B (58.5%) compared with group C (70.5%). The proportion of children presenting acute gastroenteritis was also lower in group A (13.1%) and group B (19.5%) compared with group C (31.1%). A net increase of all fecal biomarkers of innate and acquired immunity was observed for groups A and B compared to group C. Moreover, there was a negative association between fecal biomarkers and the occurrence of CID.

Conclusion: Dietary supplementation with cow's milk or rice fermented with *L. paracasei* CBA L74 prevents CIDs in children attending daycare possibly by means of a stimulation of innate and acquired immunity.

1. Introduction

Daycare centers and schools are ideal places for the occurrence and transmission of common infectious diseases (CIDs) affecting respiratory and gastrointestinal tract in young children, often resulting in many missed days of both daycare and parental work [1]. Young children attending daycare centers and schools have a 1.5–3.0 times higher risk of respiratory and gastrointestinal tract

* Corresponding author. Department of Translational Medical Science – Pediatric Section, European Laboratory for the Investigation of Food Induced Diseases, CEINGE – Advanced Biotechnologies, University of Naples "Federico II", Via S. Pansini n.5, 80131 Naples, Italy. Tel.: +39 081 7462680.
E-mail address: bernik@unina.it (R. Berni Canani).

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Antibody-independent identification of bovine milk-derived peptides in breast-milk

Gianluca Picariello[1,*], **Francesco Addeo**[1,2], **Pasquale Ferranti**[1,2], **Rita Nocerino**[3], **Lorella Paparo**[3], **Annalisa Passariello**[3], **David C. Dallas**[4], **Randall C. Robinson**[5], **Daniela Barile**[5], and **Roberto Berni Canani**[3,*]

[1] Institute of Food Sciences, National Research Council (CNR), Avellino, Italy

[2] Department of Agriculture, University of Naples "Federico II", Portici (Naples), Italy

[3] Department of Translational Medical Science, University of Naples "Federico II", Italy

[4] Nutrition Program, School of Biological and Population Health Sciences, College of Public Health and Human Sciences, Oregon State University, Corvallis, USA

[5] Department of Food Science and Technology, University of California, Davis, USA

Abstract

Exclusively breast-fed infants can exhibit clear signs of IgE or non IgE-mediated cow's milk allergy. However, the definite characterization of dietary cow's milk proteins (CMP) that survive the maternal digestive tract to be absorbed into the bloodstream and secreted into breast milk remains missing. Herein, we aimed at assessing possible CMP-derived peptides in breast milk. Using high performance liquid chromatography (HPLC)-high resolution mass spectrometry (MS), we compared the peptide fraction of breast milk from 12 donors, among which 6 drank a cup of milk daily and 6 were on strict diary-free diet.

We identified two bovine β-lactoglobulin (β-Lg, 2 out 6 samples) and one α_{s1}-casein (1 out 6 samples) fragments in breast milk from mothers receiving a cup of bovine milk daily. These CMP-derived fragments, namely β-Lg (f42-54), (f42-57) and α_{s1}-casein (f180-197), were absent in milk from mothers on dairy-free diet. In contrast, neither intact nor hydrolyzed β-Lg was detected by Western blot and competitive ELISA in any breast milk sample. Eight additional bovine milk-derived peptides identified by software-assisted MS were most likely false positive. The results of this study demonstrate that CMP-derived peptides rather than intact CMP may sensitize or elicit allergic responses in the neonate through mother's milk. Immunologically active peptides from the maternal diet could be involved in priming the newborn's immune system, driving tolerogenic response.

Graphical abstract

*Corresponding authors: Gianluca Picariello, Institute of Food Sciences, National Research Council (CNR), Via Roma 64, Avellino, Italy, Tel. +39 0825 299521; Fax +39 0825 781585, picariello@isa.cnr.it, Roberto Berni Canani, Department of Translational Medical Science, Pediatric Section, European Laboratory for the Investigation of Food Induced Diseases, University of Naples 'Federico II', Via Pansini, 80131 Naples, Italy, Tel. + 39 081 7462680, berni@unina.it.

The authors have declared no conflict of interest.

Chapter 14

Food Allergies: Novel Mechanisms and Therapeutic Perspectives

Margherita Di Costanzo, Lorella Paparo, Linda Cosenza, Carmen Di Scala, Rita Nocerino, Rosita Aitoro, and Roberto Berni Canani

Abstract

Childhood food allergy (FA) rates have rapidly increased with significant direct medical costs for the health care system and even larger costs for the families with a food-allergic child. The possible causes of food allergy become the target of intense scrutiny in recent years. Increasing evidence underline the importance in early life of gut microbiome in the development of allergic diseases. There are a range of factors in the modern environment that may be associated with changes to both the gut microbiome and risk of FA, such as mode of delivery, antibiotic exposure, infant feeding practices, farming environment, and country of origin. Knowledge of the relationship between early life gut microbiome and allergic diseases may facilitate development of novel preventive and treatment strategies. Based on our current knowledge, there are no currently available approved therapies for food allergy. More studies are needed to evaluate the safety and efficacy of allergen-specific and allergen-nonspecific approaches, as well as combination approaches.

Key words Immunotherapy, Probiotics, Intestinal microflora, Immune system, Tolerance acquisition

1 Introduction

Food allergy (FA) is a major health issue in Western countries with a substantial effect on quality of life of both patients and their relatives. On the basis of numerous studies, food allergy likely affects nearly 5 % of adults and 8 % of children, with growing evidence of an increase in prevalence [1]. Although any food can provoke a reaction, relatively few foods are responsible for the vast majority of significant food induced allergic reactions: cow's milk (2.2 %), peanuts (1.8 %), and tree nuts (1.7 %) are the most common allergens in children, and shellfish (1.9 %), fruits (1.6 %), and vegetables (1.3 %) are the most common allergens in adults. Recent publications focusing on peanut allergy indicated increases with a doubling (UK) or tripling (USA) in diagnoses [2, 3]. In general, childhood FA to milk, egg, wheat, or soy typically resolves during childhood, whereas allergies to peanut, tree nuts, fish, and shellfish

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OPEN

ORIGINAL ARTICLE

Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants

Roberto Berni Canani[1,8], Naseer Sangwan[2,8], Andrew T Stefka[3,8], Rita Nocerino[1], Lorella Paparo[1], Rosita Aitoro[1], Antonio Calignano[4], Aly A Khan[5], Jack A Gilbert[2,6,7] and Cathryn R Nagler[3]

[1]*Department of Translational Medical Science, Section of Pediatrics, European Laboratory for the Investigation of Food-Induced Diseases, University of Naples, Federico II, Naples, Italy;* [2]*Institute for Genomics and Systems Biology, Department of Biosciences, Argonne National Laboratory, Argonne, IL, USA;* [3]*Committee on Immunology and Department of Pathology, University of Chicago, Chicago, IL, USA;* [4]*Department of Pharmacy, University of Naples Federico II, Naples, Italy;* [5]*Toyota Technological Institute at Chicago, Chicago, IL, USA;* [6]*Department of Surgery, University of Chicago, Chicago, IL, USA and* [7]*Department of Ecology and Evolution, University of Chicago, Chicago, IL, USA*

Dietary intervention with extensively hydrolyzed casein formula supplemented with *Lactobacillus rhamnosus* GG (EHCF+LGG) accelerates tolerance acquisition in infants with cow's milk allergy (CMA). We examined whether this effect is attributable, at least in part, to an influence on the gut microbiota. Fecal samples from healthy controls ($n=20$) and from CMA infants ($n=19$) before and after treatment with EHCF with ($n=12$) and without ($n=7$) supplementation with LGG were compared by 16S rRNA-based operational taxonomic unit clustering and oligotyping. Differential feature selection and generalized linear model fitting revealed that the CMA infants have a diverse gut microbial community structure dominated by Lachnospiraceae (20.5 ± 9.7%) and Ruminococcaceae (16.2 ± 9.1%). *Blautia*, *Roseburia* and *Coprococcus* were significantly enriched following treatment with EHCF and LGG, but only one genus, *Oscillospira*, was significantly different between infants that became tolerant and those that remained allergic. However, most tolerant infants showed a significant increase in fecal butyrate levels, and those taxa that were significantly enriched in these samples, *Blautia* and *Roseburia*, exhibited specific strain-level demarcations between tolerant and allergic infants. Our data suggest that EHCF+LGG promotes tolerance in infants with CMA, in part, by influencing the strain-level bacterial community structure of the infant gut.
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Introduction

The prevalence of allergic responses to food has been experiencing an unprecedented increase in developed societies, rising by as much as 20% in a recent 10-year period (Branum and Lukacs, 2009; Osborne *et al*., 2011; Wang and Sampson, 2011; Prescott *et al*., 2013). Genetic variation alone cannot account for a dramatic increase in disease prevalence over such a short time frame. Emerging evidence suggests that twenty-first century environmental interventions, including widespread antibiotic use, consumption of a high-fat/low fiber diet, elimination of previously common enteropathogens (including *Helicobacter pylori* and helminthic parasites), reduced exposure to infectious disease, Caesarean birth, and formula feeding, may have perturbed the mutually beneficial interactions established over millions of years of co-evolution with the bacteria that comprise our commensal microbiota (Cho and Blaser, 2012). This dysbiosis can predispose genetically susceptible individuals to allergic disease (reviewed in ref. Feehley *et al*., 2012). Cow's milk allergy (CMA) is one of the most common food allergies of infancy and early childhood with an estimated prevalence of 2–3% worldwide (Sicherer, 2011). We have demonstrated that dietary management with a formula containing an extensively hydrolyzed form of the cow's milk protein casein (EHCF), supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG), results in a higher rate of tolerance acquisition in infants with CMA than in those treated with

Correspondence: JA Gilbert, Department of Surgery, University of Chicago, 5841 South Maryland Avenue, Chicago, IL, 60637, USA.
E-mail: gilbertjacob@uchicago.edu
or CR Nagler, Department of Pathology, University of Chicago, 924 East 57th Street R120, Chicago, IL 60637.
E-mail: cnagler@bsd.uchicago.edu
[8]These authors contributed equally to this work.
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CLINICAL EPIGENETICS

RESEARCH Open Access

Differences in DNA methylation profile of Th1 and Th2 cytokine genes are associated with tolerance acquisition in children with IgE-mediated cow's milk allergy

Roberto Berni Canani[1,2,4*], Lorella Paparo[1], Rita Nocerino[1,4], Linda Cosenza[1], Vincenza Pezzella[1], Margherita Di Costanzo[1], Mario Capasso[3,4], Valentina Del Monaco[3,4], Valeria D'Argenio[3,4], Luigi Greco[1,2] and Francesco Salvatore[3,4]

Abstract

Background: Epigenetic changes in DNA methylation could regulate the expression of several allergy-related genes. We investigated whether tolerance acquisition in children with immunoglobulin E (IgE)-mediated cow's milk allergy (CMA) is characterized by a specific DNA methylation profile of Th2 (IL-4, IL-5) and Th1 (IL-10, IFN-γ)-associated cytokine genes.

Results: DNA methylation of CpGs in the promoting regions of genes from peripheral blood mononuclear cells and serum level of IL-4, IL-5, IL-10 and INF-γ were assessed in children with active IgE-mediated CMA (group 1), in children who acquired tolerance to cow's milk proteins (group 2) and in healthy children (group 3). Forty children (24 boys, aged 3 to 18 months) were enrolled: 10 in group 1, 20 in group 2, and 10 in the control group. The DNA methylation profiles clearly separated active CMA patients from healthy controls. We observed an opposite pattern comparing subjects with active IgE-mediated CMA with healthy controls and group 2 children who outgrew CMA. The IL-4 and IL-5 DNA methylation was significantly lower, and IL-10 and INF-γ DNA methylation was higher in active IgE-mediated CMA patients. Gene promoter DNA methylation rates of all cytokines and respective serum levels were strongly correlated. Formula selection significantly influenced cytokine DNA methylation profiles in group 2.

Conclusions: Tolerance acquisition in children with IgE-mediated CMA is characterized by a distinct Th1 and Th2 cytokine gene DNA methylation pattern. These results suggest that DNA methylation may be a target for CMA prevention and treatment.

Keywords: Epigenetics, Interleukin-4, Interleukin-5, Interleukin-10, Interferon-γ, Food allergy, Extensively hydrolyzed casein formula, Lactobacillus rhamnosus GG, Hypoallergenic formulae

Background

Cow's milk allergy (CMA) is one of the most common food allergies (FA) in early childhood, with an estimated incidence ranging between 2% and 3% [1]. Evidence suggests that the natural history of CMA is changing, with an increasing persistence until later ages [2]. It has been demonstrated that immunoglobulin E (IgE)-mediated CMA could be the first manifestation of the so-called 'atopic march', characterized by the occurrence of other allergic disorders in the years after the onset of CMA [3]. The pathogenetic basis of IgE-mediated CMA is not completely understood. Critical to the development of IgE-mediated chronic allergic inflammation is an increased production of Th2 cytokines, such as interleukins IL-4 and IL-5, which are not adequately counter-regulated by Th1 cytokines, such as interferon gamma (IFN-γ) and IL-10 [4]. We previously found an opposite response profile to these cytokines in freshly isolated peripheral blood mononuclear cells (PBMCs) between children with active

* Correspondence: bernic@unina.it
[1]Department of Translational Medical Science, University of Naples Federico II, Via S. Pansini, 5 80131 Naples, Italy
[2]European Laboratory for the Investigation of Food-Induced Diseases, University of Naples Federico II, Via S. Pansini, 5 80131 Naples, Italy
Full list of author information is available at the end of the article

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SPECIAL ISSUE: Probiotics and probiotics in paediatrics

Bugs for atopy: the *Lactobacillus rhamnosus* GG strategy for food allergy prevention and treatment in children

L. Cosenza[1], R. Nocerino[1], C. Di Scala[1], M. di Costanzo[1], A. Amoroso[1], L. Leone[1], L. Paparo[1], C. Perzella[1], R. Aitoro[1] and R. Berni Canani[1,2,3]*

[1]Department of Translational Medical Science, University of Naples 'Federico II', Via Sergio Pansini 5, 80131 Naples, Italy; [2]European Laboratory for The Investigation of Food Induced Diseases, University of Naples 'Federico II', Via Sergio Pansini 5, 80131 Naples, Italy; [3]CEINGE Advanced Biotechnologies, University of Naples 'Federico II', Via Sergio Pansini 5, 80131 Naples, Italy; berni@unina.it

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REVIEW ARTICLE

Abstract

Food allergy (FA) is a major health issue for children living in Western countries. At this time the only proven treatment for FA is elimination of offender antigen from the diet. It is becoming clear that the development of gut microbiota exerts a profound influence on immune system maturation and tolerance acquisition. Increasing evidence suggests that perturbations in gut microbiota composition of infants are implicated in the pathogenesis of FA. These findings have unveiled new strategies to prevent and treat FA using probiotics bacteria or bacterial substance to limit T-helper (Th)/Th2 bias, which changes during the disease course. Selected probiotics administered during infancy may have a role in the prevention and treatment of FA. *Lactobacillus rhamnosus* GG (LGG) is the most studied probiotic in this field. Administration of LGG in early life have a role in FA prevention. Preliminary evidence shows that LGG accelerates oral tolerance acquisition in cow's milk allergic infants. We are understanding the mechanisms elicited by LGG and metabolites in influencing food allergen sensitization. A deeper definition of these mechanisms is opening the way to new immunotherapeutics for children affected by FA that can efficiently limit the disease burden.

Keywords: gut microbiota, short chain fatty acids, butyrate, eczema, cow's milk allergy, oral tolerance

1. Introduction

Food allergy (FA) is an increasing public health problem (Berin and Sampson, 2013). Cow's milk allergy (CMA) is one of the most common FA in early childhood, with an estimated prevalence ranging between 2 and 3% in infants (Apps and Beattie, 2009). During the last decade, we observed a changing pattern in FA with increased prevalence, severity of clinical manifestations and risk of persistence until later ages. The Centers for Disease Control and Prevention documented an 18% increase among children in the USA between 1997 and 2007. In the same country, FA accounts for about 30,000 emergency room visits and 150 deaths per year (http://foodallergy.org). Similarly, in Italy we observed that the number of hospital admissions for food-induced anaphylaxis doubled in only 5 years, and that cow milk proteins were the leading food allergens (Berni Canani *et al.*, 2012a). There is evidence that resolution rates have slowed for allergies that have been commonly outgrown, such as those to milk, egg, wheat and soy. For example, Ekrur *et al.* (2012) in a population-based study reported that only 57.4% of CMA children resolved their allergy at 5 years of age. FA has deleterious effects on family economics, social interactions, school and work attendance, and health-related quality of life; it can be costly in terms of medical visits and treatments (Sicherer and Sampson, 2014). Given the morbidity resulting from FA, there is considerable interest in generating efficient approaches that may stimulate oral tolerance acquisition and maintenance. Rising disease prevalence over a short period of time cannot be explained by genetic variation alone, renewing interest in the role of the environment in

Figure 1

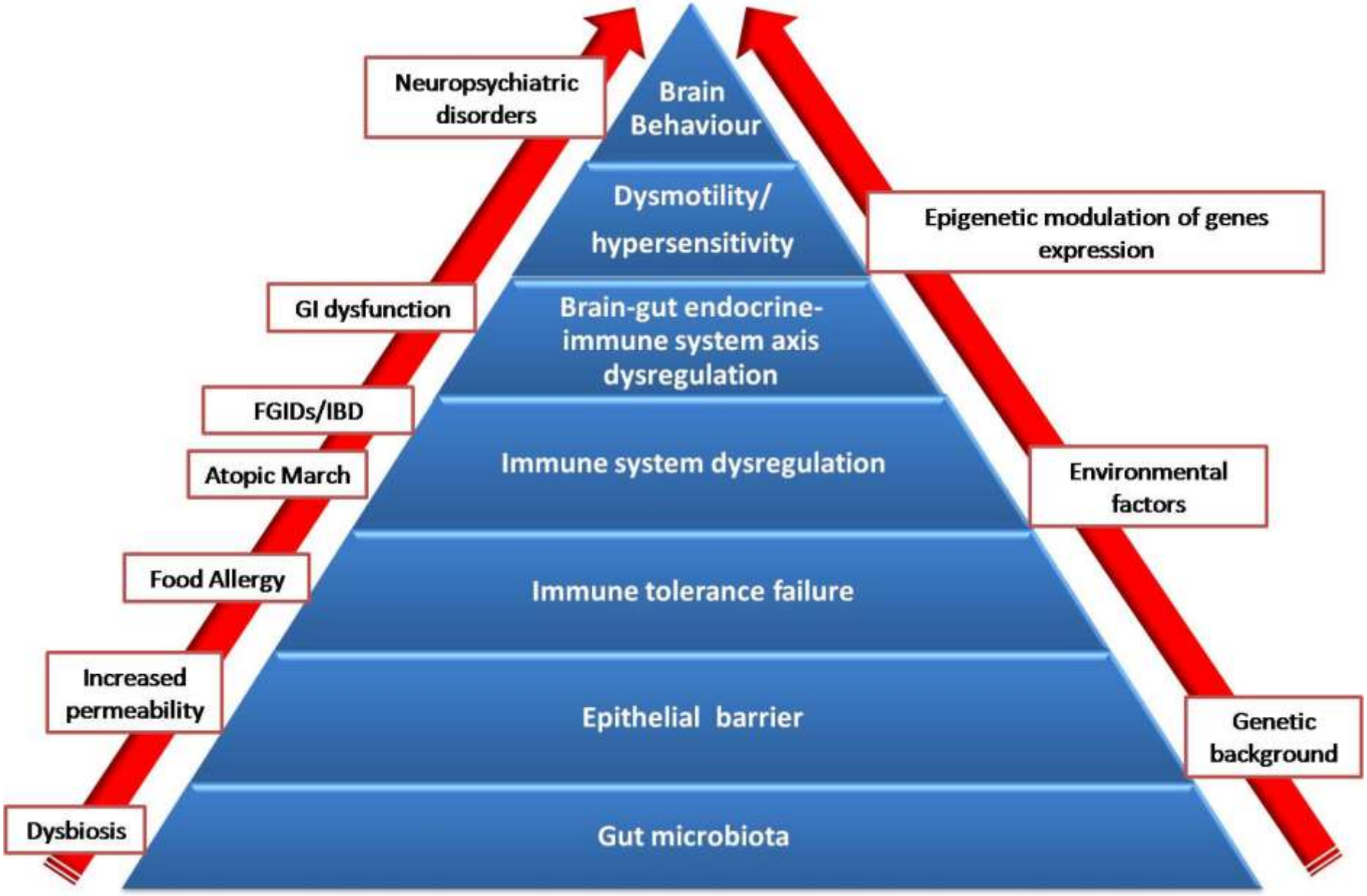

Neuropsychiatric disorders
Brain Behaviour
Dysmotility/ hypersensitivity
Epigenetic modulation of genes expression
GI dysfunction
Brain-gut endocrine-immune system axis dysregulation
FGIDs/IBD
Atopic March
Immune system dysregulation
Environmental factors
Food Allergy
Immune tolerance failure
Increased permeability
Epithelial barrier
Genetic background
Dysbiosis
Gut microbiota

Figure 2

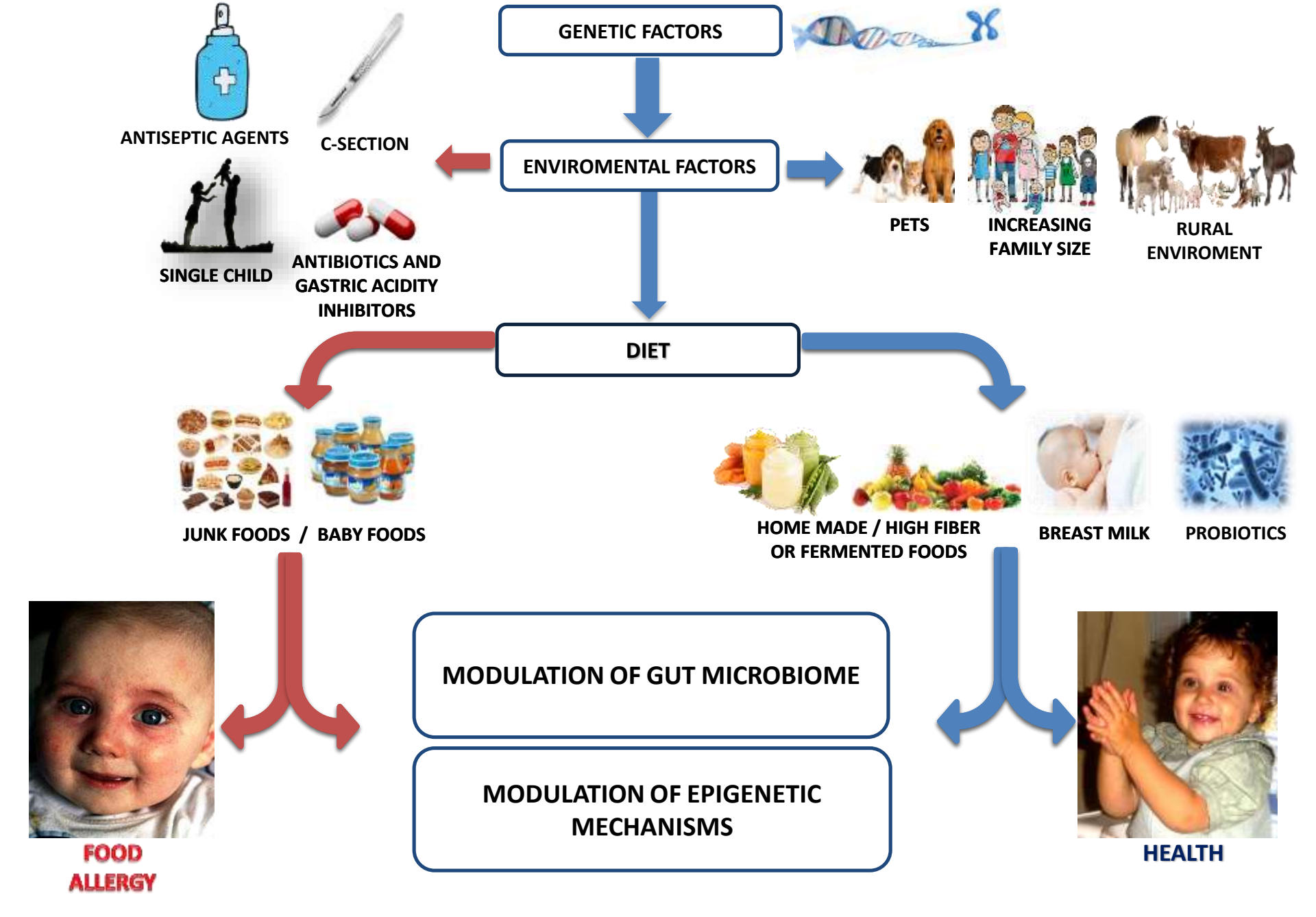

GENETIC FACTORS
ENVIROMENTAL FACTORS
DIET
ANTISEPTIC AGENTS
C-SECTION
SINGLE CHILD
ANTIBIOTICS AND GASTRIC ACIDITY INHIBITORS
PETS
INCREASING FAMILY SIZE
RURAL ENVIROMENT
JUNK FOODS / BABY FOODS
HOME MADE / HIGH FIBER OR FERMENTED FOODS
BREAST MILK
PROBIOTICS
MODULATION OF GUT MICROBIOME
MODULATION OF EPIGENETIC MECHANISMS
FOOD ALLERGY
HEALTH

Figure 3

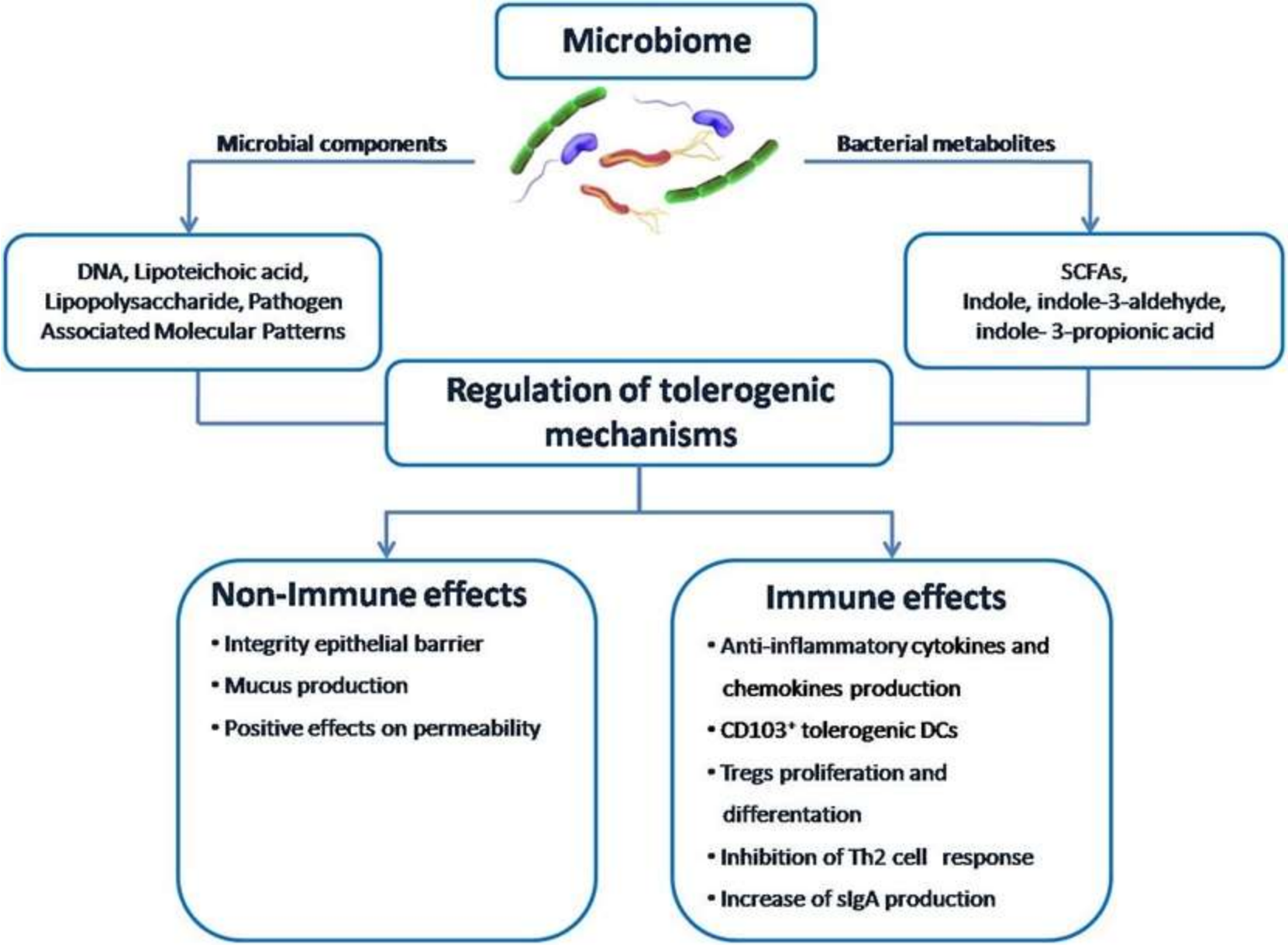

Microbiome
Microbial components
Bacterial metabolites
DNA, Lipoteichoic acid, Lipopolysaccharide, Pathogen Associated Molecular Patterns
SCFAs, Indole, indole-3-aldehyde, indole- 3-propionic acid
Regulation of tolerogenic mechanisms
Non-Immune effects
• Integrity epithelial barrier
• Mucus production
• Positive effects on permeability
Immune effects
• Anti-inflammatory cytokines and chemokines production
• CD103+ tolerogenic DCs
• Tregs proliferation and differentation
• Inhibition of Th2 cell response
• Increase of sIgA production

Figure 4

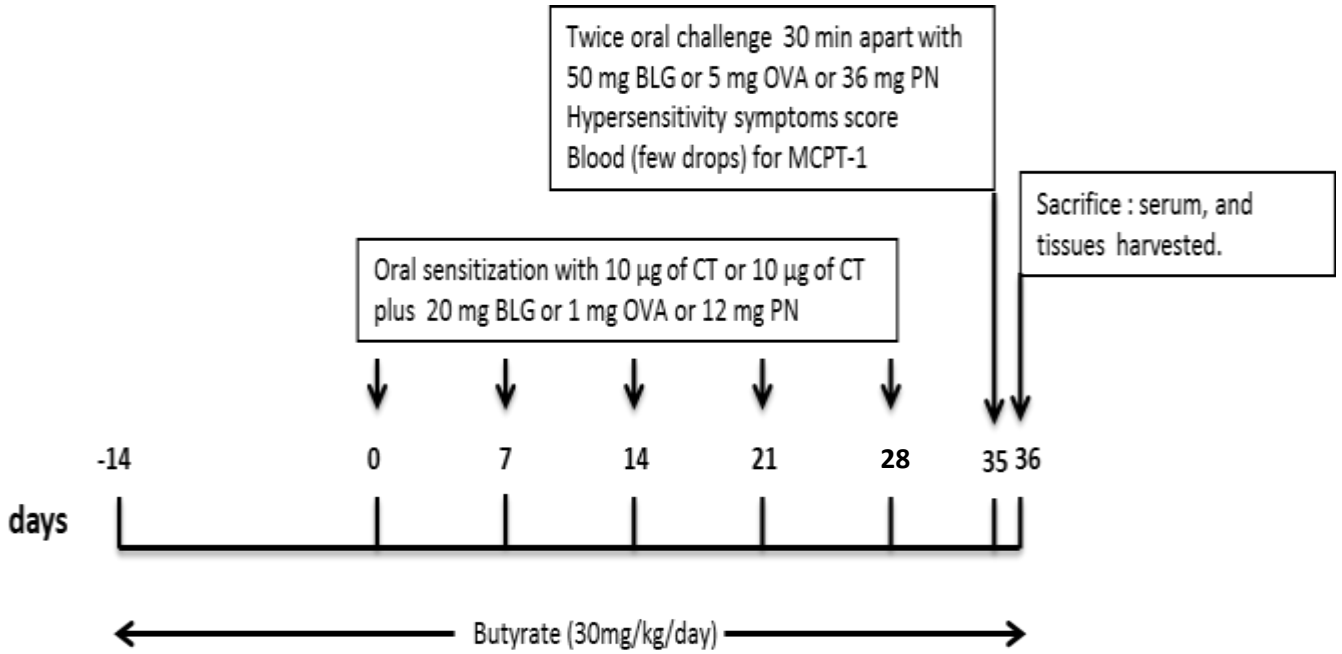
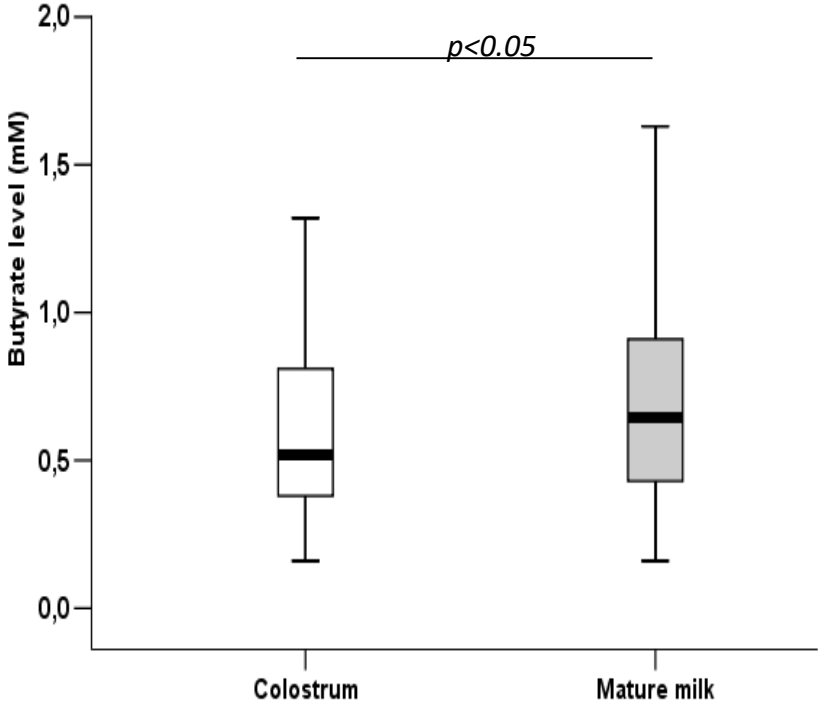

Twice oral challenge 30 min apart with
50 mg BLG or 5 mg OVA or 36 mg PN
Hypersensitivity symptoms score
Blood (few drops) for MCPT-1
Sacrifice : serum, and
tissues harvested.
Oral sensitization with 10 µg of CT or 10 µg of CT
plus 20 mg BLG or 1 mg OVA or 12 mg PN
-14
0
7
14
21
28
35
36
days
Butyrate (30mg/kg/day)

Figure 5


p<0.05
Butyrate level (mM)
2,0
1,5
1,0
0,5
0,0
Colostrum
Mature milk

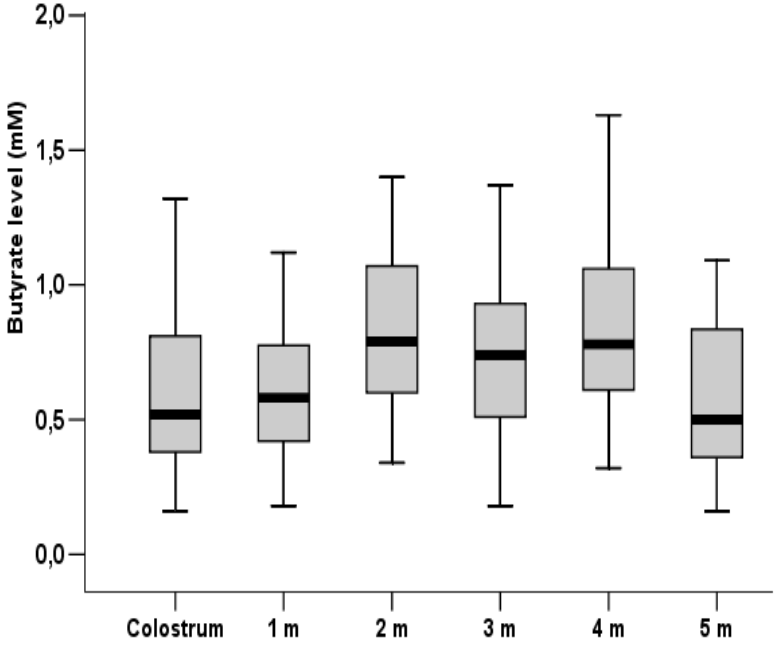
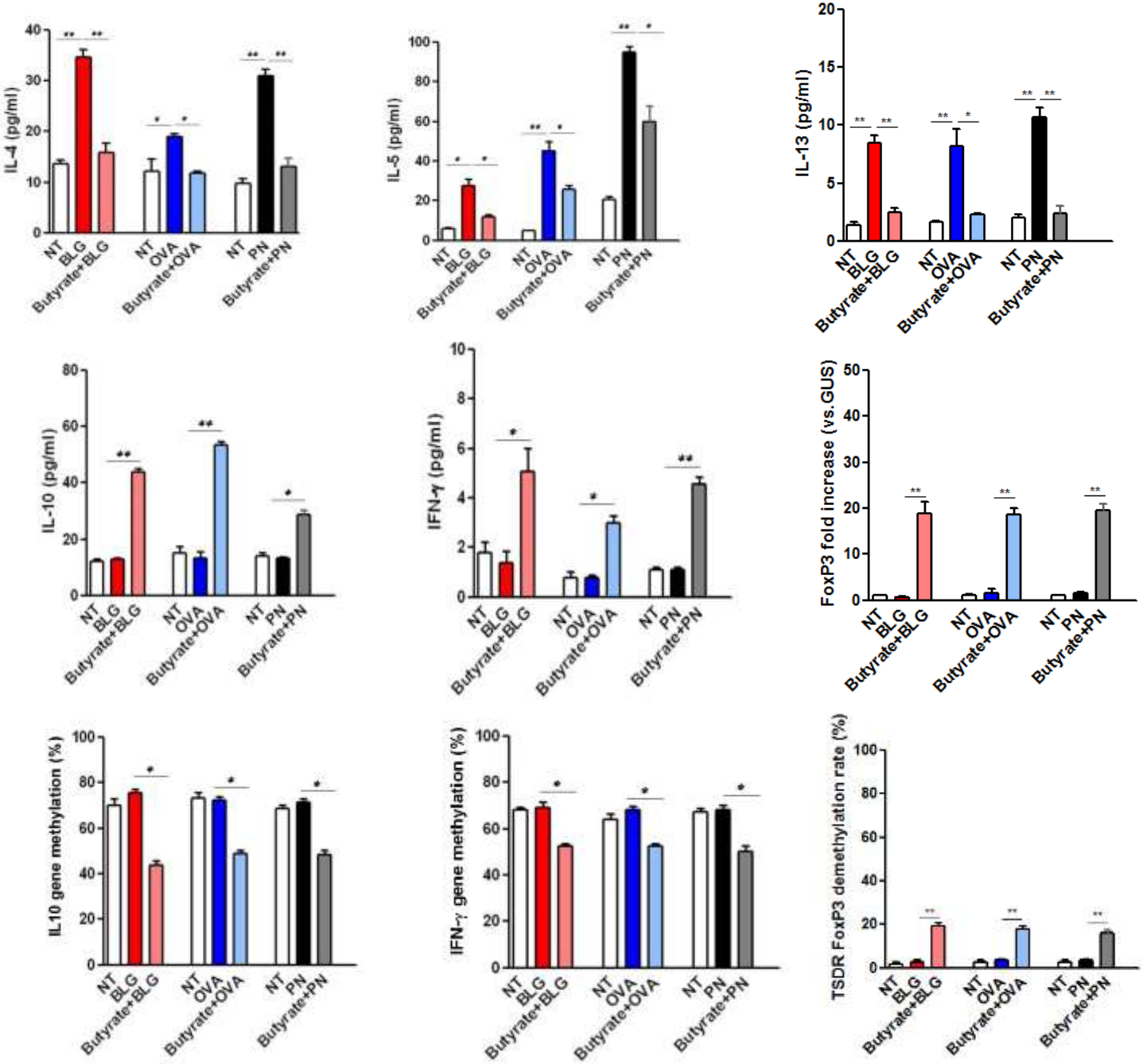
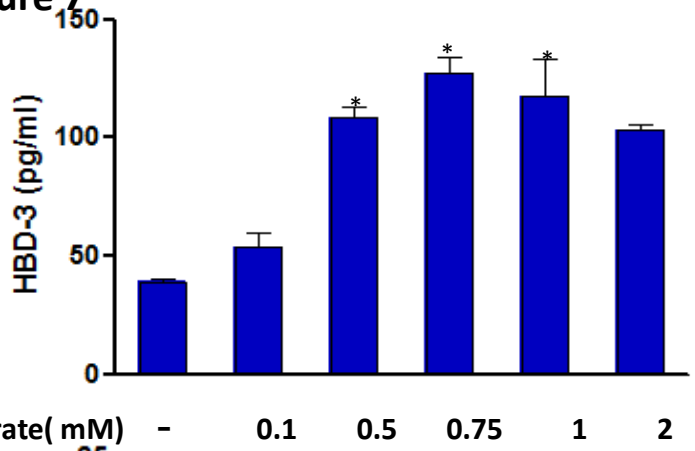

Butyrate level (mM)
2,0
1,5
1,0
0,5
0,0
Colostrum
1 m
2 m
3 m
4 m
5 m

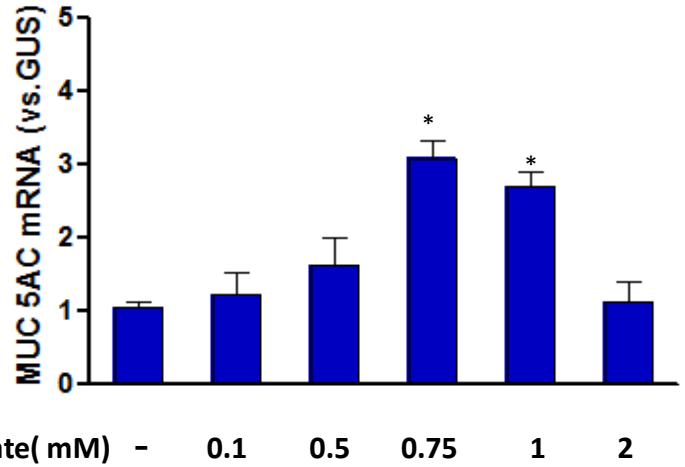
Figure 6

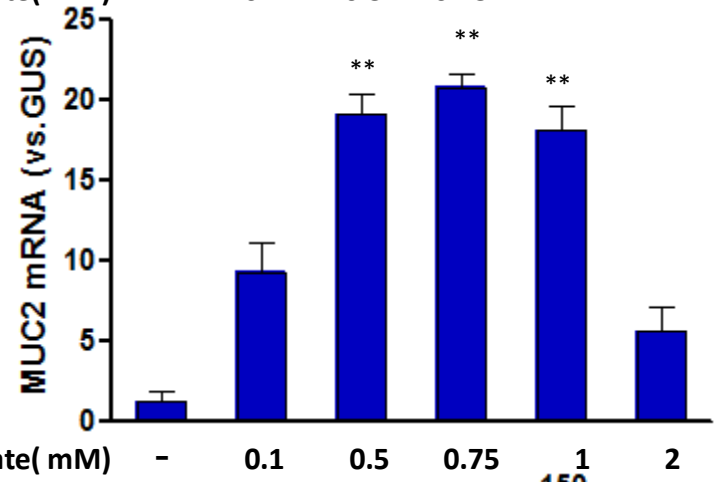


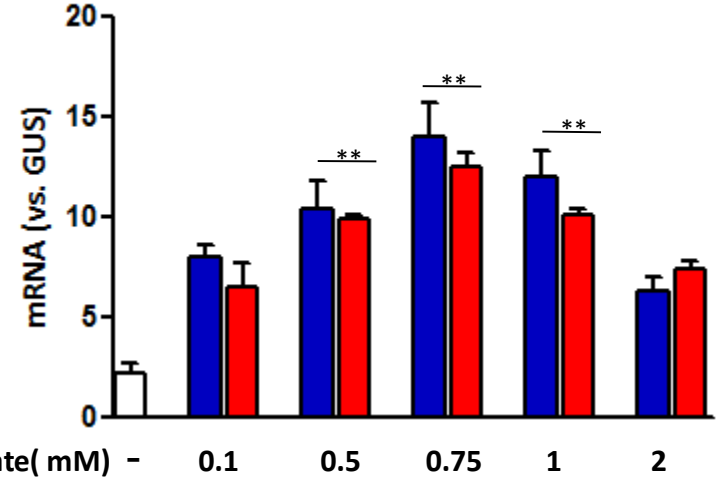
BLG: β-lactoglobulin
OVA: Ovalbumin
PN: Peanuts
**p<0.05; **p<0.01*

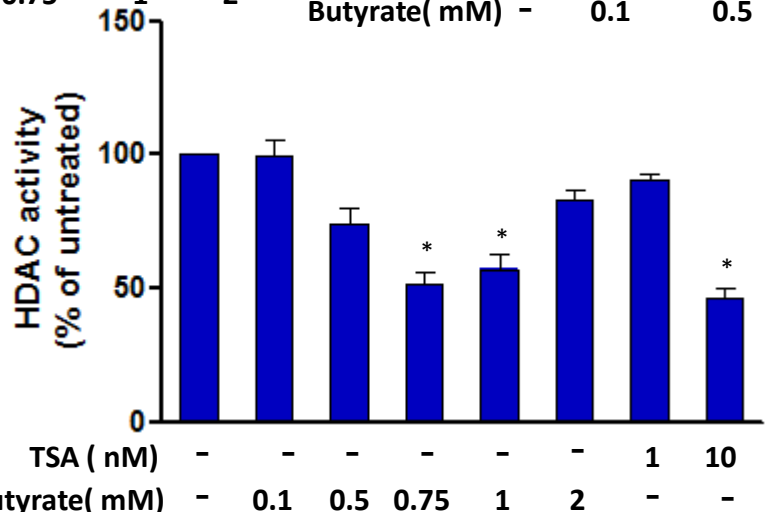
Figure 7











*p<0.05 vs untreated

**p<0.001 vs untreated

Figure 8

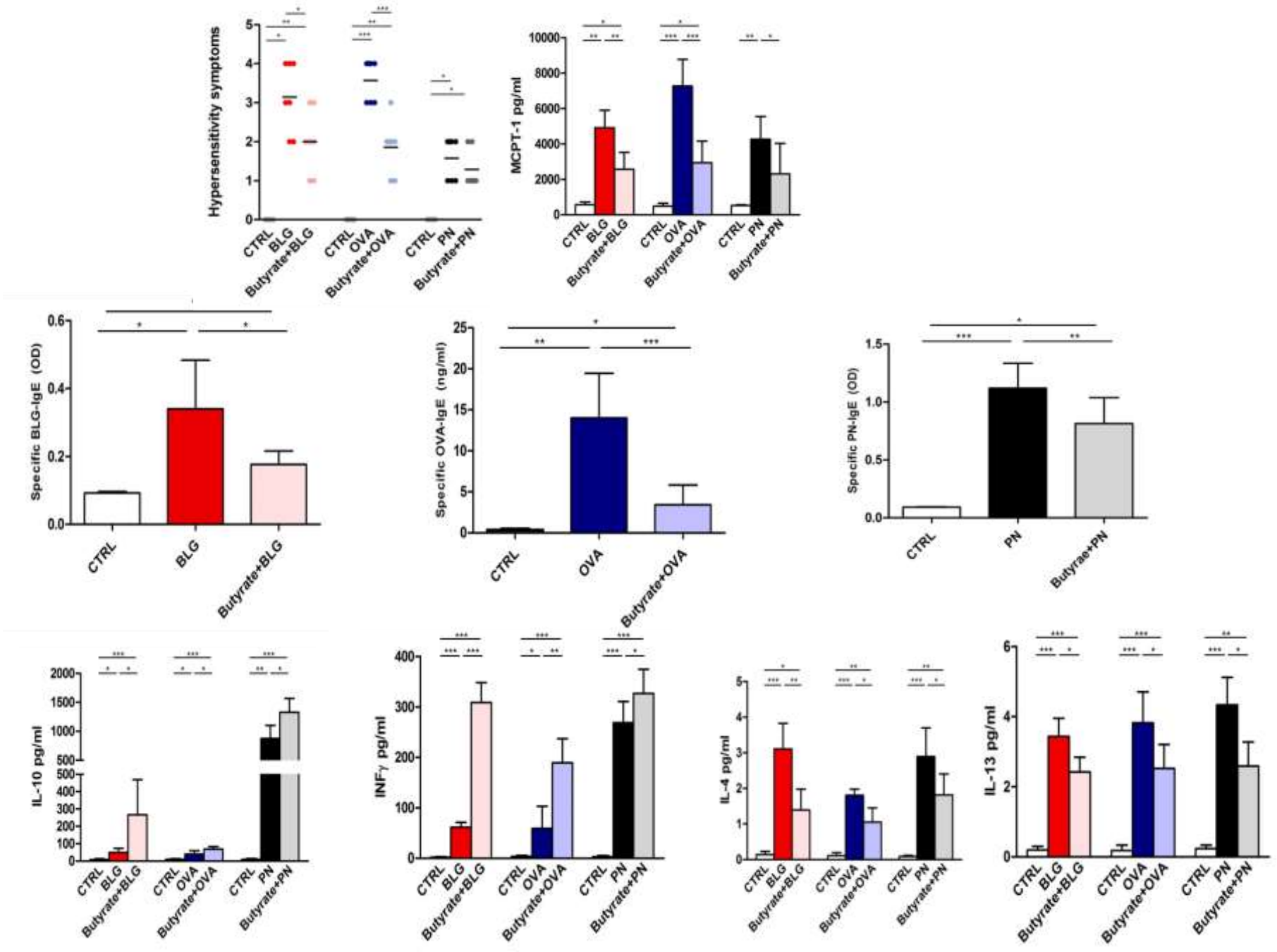
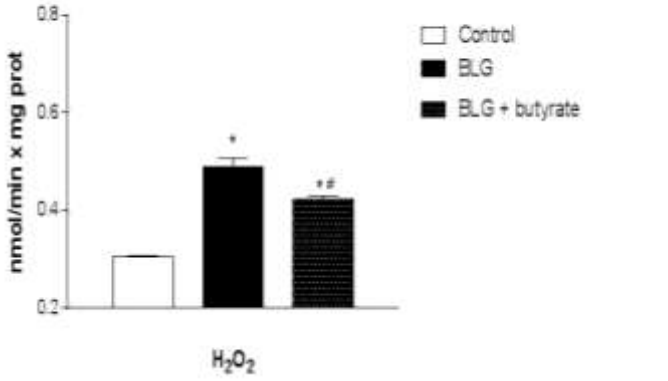
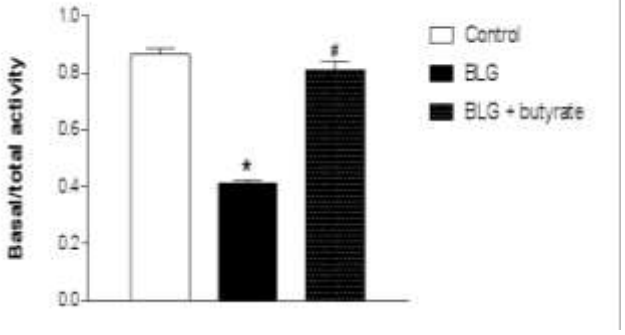
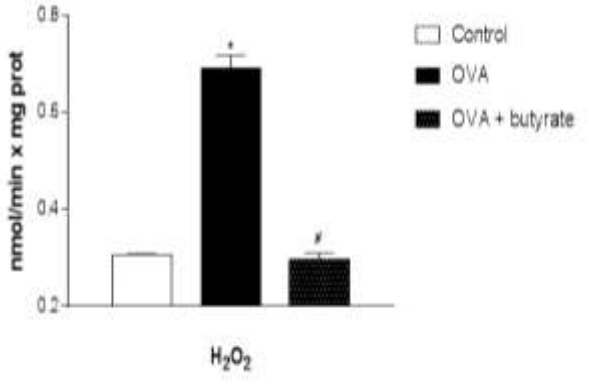
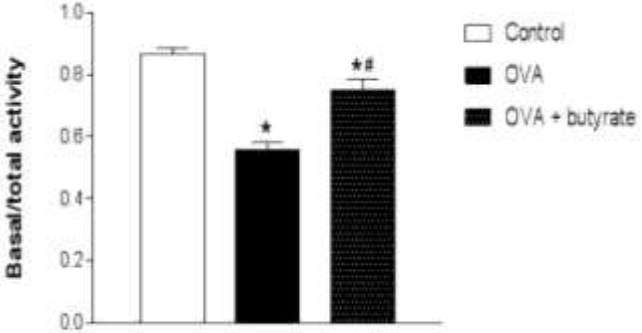

Hypersensitivity symptoms
MCPT-1 pg/ml
Specific BLG-IgE (OD)
Specific OVA-IgE (ng/ml)
Specific PN-IgE (OD)
IL-10 pg/ml
INFγ pg/ml
IL-4 pg/ml
IL-13 pg/ml
CTRL
BLG
Butyrate+BLG
OVA
Butyrate+OVA
PN
Butyrate+PN

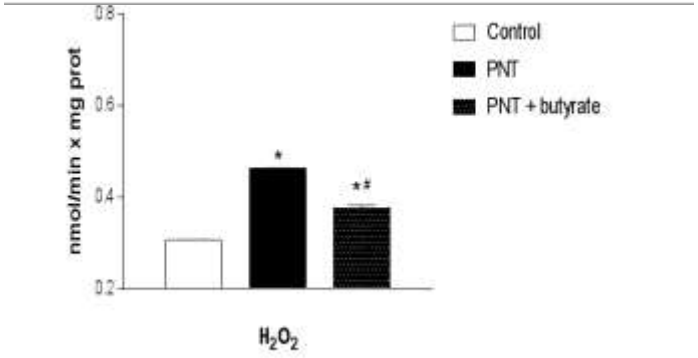
Figure 9

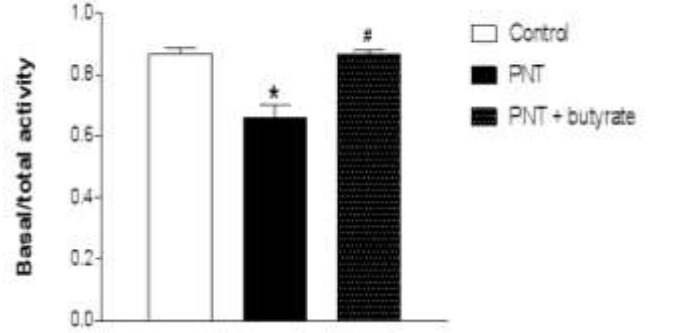






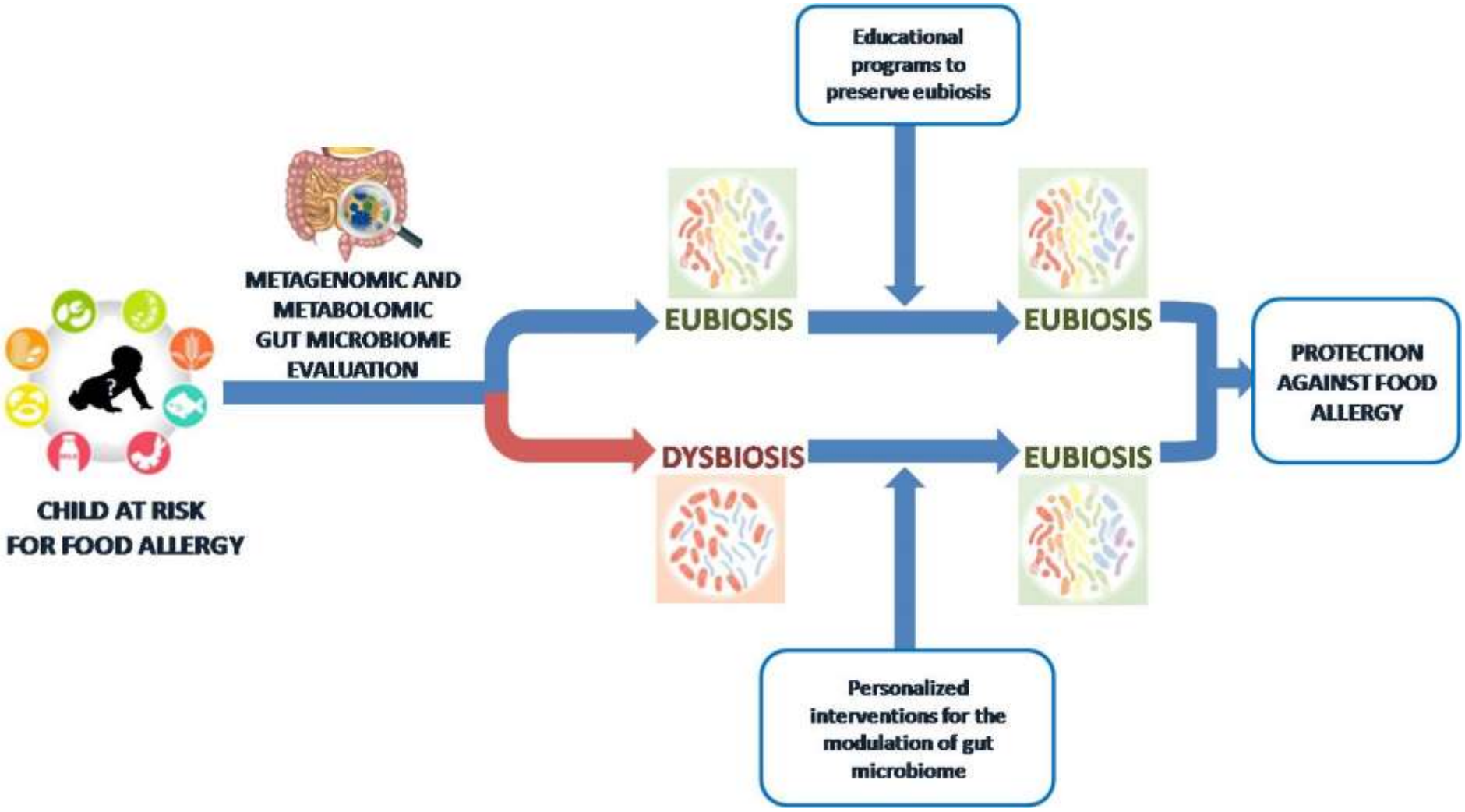






** p<0.05 vs control;# p<0.05 vs sensitized group*

Figure 10


Educational programs to preserve eubiosis
METAGENOMIC AND METABOLOMIC GUT MICROBIOME EVALUATION
EUBIOSIS
EUBIOSIS
PROTECTION AGAINST FOOD ALLERGY
DYSBIOSIS
EUBIOSIS
CHILD AT RISK FOR FOOD ALLERGY
Personalized interventions for the modulation of gut microbiome