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Title

Butyrate as bioactive human milk protective component against food allergy

Short title: The gut metabolite butyrate against food allergy

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Abstract

Background: Food allergy (FA) is a growing health problem worldwide. Effective strategies are advocated to limit the disease burden. Human milk (HM) is considered a protective factor for FA, but its mechanisms remain unclear. Butyrate is a gut microbiota-derived metabolite able to exert several immunomodulatory functions. **Objectives:** To define the butyrate concentration in HM. To see whether the butyrate concentration detected in HM is able to modulate the mechanisms of immune tolerance. **Methods:** HM butyrate concentration from 109 healthy women was assessed by GS-MS. The effect of HM butyrate on tolerogenic mechanisms was assessed *in vivo* and *in vitro* models. **Results:** The median butyrate concentration in mature HM was 0.75 mM. This butyrate concentration was responsible for the maximum modulatory effects observed in all experimental models evaluated in this study. Data from mouse model show that in basal condition, butyrate up-regulated the expression of several biomarkers of gut barrier integrity, and of tolerogenic cytokines. Pre-treatment with butyrate significantly reduced allergic response in three models of FA, with a stimulation of tolerogenic cytokines, inhibition of Th2 cytokines production and a modulation of oxidative stress. Data from human cells models show that butyrate stimulated human beta defensin-3, mucus components and tight junctions expression in human enterocytes, and IL-10, IFN-γ and FoxP3 expression through epigenetic mechanisms in PBMCs from FA children. Furthermore, it promoted the precursors of M2 macrophages, DCs and regulatory T cells. **Conclusion:** The study's findings suggest the importance of butyrate as a pivotal HM compound able to protect against FA.

Keywords: breast milk, immune tolerance, short chain fatty acids, tolerogenic mechanism

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Introduction

Food allergy (FA) is a potentially deadly immune reaction triggered by the ingestion of food protein antigens (1). Over the past two decades, the prevalence and severity of childhood FA has increased with significant direct medical costs for the health care systems of industrialized countries and even greater costs for families of food-allergic children (2). In light of this changing FA scenario, effective preventive and therapeutic strategies are of substantial clinical and socioeconomic value. Studies support the hypothesis that altered microbial exposure and colonization in early life influence the occurrence of FA (3,4).

Differential gut microbiota structures confer protection or susceptibility to FA by modulating the tolerogenic mechanisms (4). Several protective functional effects of gut microbiota against FA derive from the action of its metabolites (5). Growing evidence demonstrates that gut microbial metabolites, mainly short-chain fatty acids (SCFAs) exert immunomodulatory functions that are beneficial to the host and play a pivotal role in shaping the immune system (5,6). Among the SCFAs, butyrate has emerged since the discovery of its role in driving T regulatory cells (Tregs) differentiation and maintenance of gut homeostasis (7-11). After absorption into enterocytes, butyrate enters the circulation and modulates immune functions in peripheral tissues (12). Via the inhibition of histone deacetylases (HDAC) and activation of G protein coupled receptors (GPCR) on epithelial and immune cells butyrate influences gene expression and inflammatory responses (13).

Despite no conclusions can be made about the role of breastfeeding in either preventing or delaying the onset of FA, it has been thought that immunologic components of human milk

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(HM) may modify induction of immune tolerance and decrease the risk of allergic diseases (14,15).

Human milk is a complex nutritional fluid that contains several compounds able to provide health benefits (15). Accumulated data suggest that a wide range of bioactive factors such as the macro- and micronutrients, microbial components, and metabolites present in HM can influence infant immune system maturation (16). Considering its pivotal role in immune tolerance, it could be relevant to define the butyrate content in HM and its potential preventive role against FA. A preliminary study examined HM as a potential source of butyrate for neonates with a different content than infant formula (17), but the butyrate levels in HM remain poorly defined.

The aims of this study were to evaluate the concentration of butyrate in HM and to determine if this concentration is able to effectively modulate the main tolerogenic mechanisms involved in the protection against FA.

Materials and Methods

1. Study design

This study was designed to evaluate butyrate concentration in HM (HM butyrate) and investigate their potential to exert a protective role against FA. First, we defined HM butyrate concentrations in a cohort of healthy lactating women. Subsequently, we explored the tolerogenic effects of HM butyrate in different animal models of FA and in human enterocytes. Finally, we assessed the tolerogenic action in peripheral blood mononuclear cells (PBMCs) from healthy donors and FA children. The study was approved by the Ethics Committee of the University of Naples "Federico II", Naples, Italy.

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Healthy mothers (aged 21-42 years) who participated in this study were enrolled after full-term singleton pregnancy at the Betania Evangelical Hospital (Naples, Italy), and written informed consent was obtained from all of the participants. The use of antibiotics or proton-pump inhibitors or pre-/pro-/symbiotics in the previous 6 months; and the presence of infections, allergic diseases, inflammatory bowel diseases, autoimmunity, celiac disease, food intolerances, genetic and metabolic disorders, malformations of the gastrointestinal (GI) tract, and a history of surgical procedures involving the GI tract were excluded in all of the enrolled subjects.

HM samples were obtained at 3 days (colostrum), and monthly for the first 5 months post-partum. HM samples (2 mL) were collected at the beginning of lactation by either manual or electric breast pump into sterile tubes, as previously described (18). Samples were immediately frozen and then stored at −80°C until analysis. For each enrolled subject, anamnestic, demographic, clinical and laboratory data were recorded in a data collection sheet. The sampling procedures applied ensured that breastfeeding had been well established and that the baby was thriving.

2. Determination of butyrate concentration in human milk samples

Butyrate extraction from HM was conducted as previously described (19). All samples from -80°C were defrosted at 4°C for 12h, then invert 10 times to mix at RT. 1 mL of each sample was acidified with 40µl of H_3PO_4 85%(w/v), vortexed for 5 min and sonicated for 10 min at 40 KHz immersed in an ice bath (Branson 2800 ultrasonic). 1 mL of ethyl acetate was added, vortexed for 10 min and centrifuged (12000 RPM for 45 min). Finally, it was taken from the supernatant (organic phase, of about 1mL) with a Pasteur pipette and placed in a new glass tube for gas-chromatography mass spectrometry (GC-MS) analysis. The GC column was an Agilent 122-7032ui (DB-WAX-U, Agilent Technologies, Santa Clara, California, USA) of 30 m, internal

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diameter of 0.25 mm, and film thickness of 0.25 μm. The GC was programmed to achieve the following run parameters: initial temperature of 50°C, hold of 1 min, ramp of 10°C min^{-1} up to a final temperature of 180°C, total run time of 20 min, gas flow of 70 ml min^{-1} splitless to maintain 12.67 p.s.i. column head pressure, and septum purge of 2.0 ml min^{-1}. Helium was the carrier gas (1.5ml min^{-1} costant). Parameters of mass spectrometer were: source at 230°C and MS Quad at 150°C. The GraphPad PRISM 5 program was used to determine the concentration in mM. The data were inserted in the "XY" form in which in the "X" frame the values of the straight concentration-response were reported, while in the "Y" box the values of the area under the curve (AUC) related to the peaks obtained from the mass gas were reported. The AUC values of the single samples (obtained from the mass gas) were interpolated with the line X (concentration-response) to determine the corresponding mM.

3. Animal models

For all experiments three-week-old female C3H/HeJ mice, were purchased from Charles River Laboratories (Calco, Lecco, Italy). The mice were housed in an animal facility under a 12L:12D lighting cycle, with a 20°-24ºC range of ambient temperature and 40-70% relative humidity. The mice were acclimated to their environment for 1 week before the experiments. Each experiment was littermate controlled and the mice were co-housed throughout. The mice were fed a commercially available standard diet (Teklad Rodent Diet 8604, Harlan, Madison, WI). All of the procedures involving the animals were conducted in adherence to the NIH Guide for the Care and Use of Laboratory Animals and 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 approved by the Institutional Committee on the Ethics of Animal

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Experiments (CSV) of the University of Naples Federico II and by the Italian Ministry of Health (protocol no. 2012-0024683).

Evaluation of butyrate effects in basal condition on gut barrier and on cytokines response

The animals were divided in two groups: (1) control mice (n = 4), receiving water as vehicle; (2) treated mice (n = 4), receiving butyrate once daily (30 mg/kg/os). After 14 days mice were sacrificed, and ileum, jejunum and colon, spleen and mesenteric lymph nodes (MLN) were removed and stored at -80°C.

Measurement of gut permeability in vivo

To investigate intestinal permeability, we used 4,000 Da FITC-labeled dextran as previously described (26). FITC-dextran concentration was determined by spectrophotometry (HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, Massachusetts, USA).

Gut barrier biomarkers and cytokines response

Total cellular RNA was extracted from ileum, jejunum and colon, spleen and MLN 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. Quantitative real-time PCR (qRT-PCR) analysis was performed with the TaqMan gene expression assay kit (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. The TaqMan probe for IL-22, a marker of intestinal permeability, zonula occludens 1 (ZO-1) and occludin, proteins involved in the regulation of barrier integrity, mucin 2 (Muc2), protein involved in the regulation of mucus thickness, the cytokines Th2 (IL-4, IL-5, IL-13) Th1 (IFN-γ) and IL-10 were inventoried and tested by Applied Biosystems manufacturing facility (QC). 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

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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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to normalize the level of mRNA expression.

Lymphocytes isolation and Tregs analysis by flow cytometry

To enrich mononuclear cell population and remove dead cells and red blood cell from mice spleens and MLN, we used a gradient method by a high-density with Ficoll solution as previously described (20-22). The mononuclear cells, floating on top of the high-density solution were collected and used for FACS analysis. MLN were isolated from colon as previously described (23). Colon was removed, cleared of mesenteric fat, flushed with PBS to remove feces, opened longitudinally and cut into small pieces. The small pieces were transferred into PBS containing 5mM EDTA (ThermoFisher, Waltham, Massachusetts, USA) and incubated at 37°C for 20 min in a shaking incubator. Following this the EDTA solution were removed and incubated in RPMI 1640 supplemented with 4% fetal calf serum (FCS; Lonza, Visp, Switzerland), 0.5 mg/ml collagenase D (Roche, Penzberg, Germany), 0.5 mg/ml dispase (Roche) and 40 µg/ml DNAse (Sigma) at 37°C for 40 min in a shaking incubator. The digested tissue was passed through a cell strainer, washed with RPMI/4% FCS and resuspended in 40% Percoll (GE Healthcare, Chicago, Illinois, USA) and overlaid onto 80% Percoll. Percoll gradient was centrifuged at 1200 xg for 35 min. Cells were collected at the interface of the Percoll layers, washed with RPMI/4% FCS and used immediately for FACS analysis. Tregs from different tissue were identified as CD4+/CD25+/FoxP3+ cells by flow cytometry analysis. The staining

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was performed using Mouse Regulatory T cells staining Kit (eBioscience, Santa Clara, California, USA) and the results analyzed by BD CANTO II flow cytometer and DIVA software (BD, Franklin Lakes, New Jersey, USA).

Food allergen sensitization and challenge

The experimental design is shown in Fig.3A. Briefly, two weeks prior to sensitization, the mice were administered 30 mg/kg/day of sodium butyrate (Sigma-Aldrich) by oral gavage and continued throughout the study. The control animals received PBS only. After 14 days, the mice were sensitized orally using a blunt needle. On days 0, 7, 14, 21, and 28 they were administered 20 mg of BLG (Sigma-Aldrich), 1 mg of OVA (Sigma-Aldrich), or 12 mg of PN (kindly provided by the Prof. C. Nagler, University of Chicago, US) mixed with 10 μg cholera toxin (CT) as adjuvant. The control mice received CT only. One week after the final sensitization, two doses of 50 mg BLG, 5 mg OVA, or 36 mg PN each were administered 30 min apart via intragastric gavage. Core body temperature was measured prior to allergen challenge and every 5 min after the first challenge until at least 30 min 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 h after oral challenge: 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 h after the second challenge to measure the mouse mast cell protease-1 (mMCP-1) levels. The spleen and serum were collected 24 h after challenge for splenocyte culture and antibody measurements.

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ELISAs

mMCP-1 was quantified in the serum collected 1 h after challenge according to the manufacturer's protocol (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA). BLG/PN-specific ELISAs were performed using protocols modified from (24). Briefly, plates were coated overnight at 4˚C with 100μg/mL BLG/PN in 100 mM carbonate-bicarbonate buffer (pH 9.6). Plates were blocked for 2 h 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. Secondary antibody (biotin-anti-IgE, BD Pharmingen, San Jose, CA, USA) was added at a 1:500 dilution overnight at 4°C. On the third day, the plate was incubated with streptavidin-HRP (Thermo Fisher) for 1 h at room temperature and developed with TMB (Sigma-Aldrich) 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).

Spleen culture and cytokines measurement

Single-cell suspensions were prepared from spleens harvested 24 h after challenge. Cells were plated at 2×10^5cells/ well with the 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, the plates were frozen at −20°C. Supernatant IL-4, IL-13, IL-10, and INF-γ concentrations were measured using commercially ELISA kits (eBioscience).

Preparation of isolated mitochondria and polarographic measurement of respiration

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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). The protocol was performed as previously described (33).

Determination of mitochondrial enzymatic activities and H_2O_2 release

For the determination of aconitase specific activity we used protocols previously described (33). The level of aconitase activity measured equals active aconitase (basal level). Aconitase inhibited by ROS in vivo was reactivated so that total activity could be measured by incubating mitochondrial extracts in a medium containing 50 mM dithiothreitol, 0.2 mM Na_2S, and 0.2 mM ferrous ammonium sulphate (26). The rate of mitochondrial H_2O_2 release was measured at 30°C following the protocol previously described (33). Determinations were carried out spectrophotometrically (550 nm) at 25°C, by monitoring the decrease in the reduction rate of cytochrome c by superoxide radicals, generated by the xanthine–xanthine oxidase system. One unit of SOD activity is defined as the concentration of enzyme that inhibits cytochrome c reduction by 50% in the presence of xanthine + xanthine oxidase (28).

4. Human enterocyte cell lines

Human enterocyte cell lines Caco-2 and HT-29 were used (American Type Culture Collection, Manassas, VA, USA). Caco-2 and HT29 cells were separately grown as previously described (30). For the following experiments, monocultures of Caco-2 and HT-29 cells were seeded on the apical chamber of Transwell inserts with different proportions 1:0 and grown in 12-well Transwell plates with a final density of $1x10^5$ cells/cm2 in each insert to obtain a co-colture, as previously described (29). Cells were maintained in the same conditions and allowed to grow for

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15 days with medium changes every other day. All the experiments were performed in triplicate and repeated twice.

Tolerogenic biomarkers analysis in human enterocytes

At full confluence (15-21 days), Caco-2 /HT-29 cells were stimulated for 48h with or without butyrate (Sigma-Aldrich) 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 beta-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. ZO-1, occludin, Muc2 and mucin 5AC (Muc5AC) and FoxP3 gene expression analysis were performed as described above. The measurement of the mucus thickness was performed as previously described (30).

HDAC activity assay

To assess HDAC activity, human cellular models were treated with 10 nmol/L TSA (Sigma-Aldrich) and with butyrate (Sigma-Aldrich) at different doses (0.1; 0.5; 0.75; 1; and 2 mM) for 48 hours. The 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, USA), according to the manufacturer's instructions.

5. Peripheral blood mononuclear cells stimulation

Four otherwise 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 Department of

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Translational Medical Science at the University of Naples "Federico II" because of minimal surgical procedure, and twelve children with challenge-proven IgE-mediated FA (4 with cow's milk allergy, 4 with peanut allergy, 4 with hen egg allergy; all Caucasian male, age 24 months) were recruited. The concomitant presence of other conditions was excluded in all FA subjects by the result of a full anamnestic and clinical evaluation performed at the Center by experienced pediatricians and pediatric allergists. The main features of the study population are reported in Table S1. Patients and control subjects donated a venous blood samples in heparin tubes (12 ml), after written informed consents collected by the parents/tutors. PBMCs were isolated using the Ficoll-Hystopaque (Sigma-Aldrich) method, as described previously (26-28). PBMCs were seeded at 2x105/ml and grown in RPMI medium 1640 (Gibco, Berlin, Germany) supplemented with 10% FCS (Lonza) 1% L-glutamine (Lonza) and 1% penicillin/streptomycin (Lonza). Cells were stimulated in the presence of or in the absence of butyrate (Sigma) at the dose of 0.75mM or with specific allergen: we used OVA for egg allergy, BLG for cow'milk allergy and PN for peanut allergy (100 μg/ml). Cells were cultured at 37°C in a water-saturated atmosphere consisting of 95% air and 5%CO_2 for 24h. After stimulation, the supernatants were collected and the cells were washed and harvested for CD4+T cells isolation.

Cell viability assay and apoptosis rate evaluation

PBMCs viability assays were assessed using MTT (the bromide salt of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium) (Sigma), as previously described (30). PHA (10ug/ml) was used as a positive control. Cell apoptosis rates were measured using Annexin V Apoptosis Detection Kit FITC (Invitrogen, Carlsbad, California, US) according to the manufacture's protocol. In brief, after washing the collected cells with PBS were incubated with 1×Annexin V binding buffer. Then 5×10^5cells were stained with Annexin V-FITC for 10 min at room temperature in the dark

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and before reading to flow cytometer was added Propidium Iodide 5 μg/ml. Each sample were acquired and analysed using BD CANTO II flow cytometer and DIVA software (BD).

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

The concentrations of IL-4 and IL-10 were measured in supernatants with a Human IL-4 and IL-10 Enzyme immunoassay kit (Boster Biological Technology, Ltd., Pleasanton, CA, USA) according to the type of stimulation and stimulant. Human IL-5, IL-13 and IFN-γ ELISA (BioVendor, Brno, Czech Republic) were used to detect the IL-5, IL-13 and IFN-γ concentrations. Absorbance was read at 450 nm.

Isolation of naïve CD4+ T-cells from PBMCs for flow cytometry and epigenetic analyses

Naïve 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 labelled with a cocktail of biotin-conjugated monoclonal antibodies (MicroBead Cocktail, Miltenyi Biotec) and the magnetically labeled non-target T cells were retained 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 re-suspended at $2x10^6$ cells/ml in RPMI-1640 culture medium (Gibco) supplemented with 10% FBS, 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 2x105cells/ml in 24-well plate (Nunc, Roskilde, Denmark). Naïve CD4+ T-cells obtained were processed for flow cytometry assays and for DNA/RNA extraction. All experiments were performed in triplicate and repeated twice.

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DNA methylation analysis

One microgram of the DNA extracted from the naïve CD4+ T cells was modified with sodium bisulphite to convert all of the unmethylated but not the methylated cytosines to uracil. The bisulphite conversion was carried out using the EZ DNA Methylation Gold Kit (Zymo Research Co., Irvine, CA, USA) according to the manufacturer's instructions. The converted DNA was stored at -20°C until use. Fully methylated and fully unmethylated DNA (Merck Millipore, Darmstadt, Germany) were used as the controls for the optimization of the assay conditions and to calculate the percent of methylation (0% to 100%). The primers used for the DNA methylation analysis of IL-4, IL-5, IL-10, IFN-γ, and FoxP3 TSDR were previously reported elsewhere (29,30). High-resolution melting real-time PCR for methylation analysis was performed as described previously (25,26). Real-time PCR was performed with the LightCycler 480 instrument (Roche). The amplification programme was described in previous studies (67-69). The results of the methylation analysis were confirmed by direct sequencing (using the Sanger method modified as follows: ddNTPs labelled with four different fluorophores) and analyzed via capillary electrophoresis (the analytical specificity and sensitivity of the test was > 99%).

Generation and treatment of monocyte-derived dendritic cells (Mo-DC)

To generate DCs, CD14+ monocytes were positively selected from PBMCs of healthy donors and IgE-mediated FA patients by immunomagnetic procedure (MiltenyBiotec). Immature DC were then obtained by culturing CD14+ cells at 106 cells/ml in RPMI 1640 (Invitrogen), 10% FCS (Lonza), 50 ng/ml GM-CSF and 1000 U/ml IL-4 with or without sodium butyrate (Sigma-Aldrich) as previously described (31).

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Antibodies and flow cytometry

Single-cell suspensions from PBMCs were stained with mAb against human CD14 (HCD14; Biolegend), human CD206 (DCN228; Miltenyi Biotec), human/mouse CD11b (M1/70.15.11.5; Miltenyi Biotec), human CD1a (HI149; Miltenyi Biotec), human CD1a (HI149; Miltenyi Biotec), human CD86 (REA968; Miltenyi Biotec), human CCR7 (REA108; Miltenyi Biotec), human CD45 RA (T6D11; Miltenyi Biotec), CD4 (A161A1; Biolegend), human CD103 (REA803; Miltenyi Biotec). After 20 min incubation at 4°C in the dark, cells were washed and resuspended in PBS for the FACS analysis. For each test, cells were analyzed using FACS Verse Flow Cytometer and DIVA software (BD Biosciences).

Establishment of DC-T cells co-culture

To induce Tregs differentiation in *in vitro*, butyrate-DC cells and naïve CD4+ T cells were seeded into a round-bottomed 96-well plate in 200 µl medium, then co-cultured further in 5% CO2 at 37°C for 4 days. On day 5, cells were harvested and analyzed by FACS Verse Flow Cytometer for the definition of the most peculiar subsets among CD4+ T cell compartment including effector memory (T_{EM}; CD45RA−CCR7+), effector memory RA (T_{EMRA}; CD45RA+CCR7−), naïve (T_N; CD45RA+CCR7+) and central memory (T_{CM}; CD45RA-CCR7+) T cells and Tregs. Specifically, staining and enumeration of CD4+CD25+CD127 dim/^{n}eg Tregs were performed by using the Treg Detection Kit (CD4/CD25/CD127) (Miltenyi Biotec), according to the manufacturer's instructions. In a new set of experiments, naïve CD4+ T cells were further exposed to the 0.75 mM of butyrate alone for 5 days and then stained with the Treg Detection Kit (CD4/CD25/CD127) (Miltenyi Biotec).

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Cell culture and pre-treatment of immune cells from IgE-mediated FA patients

Pre-stimulated PBMCs, with 0.75 mM butyrate, were cultured in complete medium, with or without specific allergen (OVA for egg allergy, BLG for cow'milk allergy and PN for peanut allergy; 100µg/ml) for 24h. Then CD14+ gated-monocytes were assayed for the surface expression of CD86 or alternatively CD206 activation marker. At the end of the 7 days culture, Mo-DC generated in presence of butyrate were analyzed by flow cytometry for detected CD1a or alternatively CD103+ expression. The polarization of naïve CD4+ T cells into different subtypes was evaluated at the end of the 5 days culture in presence of 0.75 mM of butyrate or after immature or 24h specific allergen (BLG, OVA and PN)-stimulated butyrate DC-co-culture as previously described (32). All cell cultures were conducted at 37°C in humidified 5% CO_2 atmosphere.

6. Statistical Analysis

The Kolmogorov-Smirnov test was used to determine whether the variables were normally distributed. For the continuous variables, the groups were compared using the equality of means test. For each type of assay for the flow cytometric analysis, the data obtained from multiple experiments were calculated as mean±SD and analyzed using the two-tailed Student's t-test for the independent groups or ANOVA test. The level of significance for all of the statistical tests was two-sided, $p < 0.05$. All of the data were collected in a dedicated database and analyzed by a statistician using SPSS software (SPSS Inc, version 23.0, Chicago, IL, USA) and GraphPad Prism 7.0 (La Jolla, CA, USA).

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Results

1. *Butyrate concentration in human milk*

A total of 109 healthy lactating women (all Caucasian, mean age 31.6 ±6.0 SD) consented to participate in this study and provided HM samples during the first 5 months of lactation. Butyrate concentration was significantly higher in mature HM than in colostrum (Fig. 1, panel

A) and remained stable until the fifth month of lactation (Fig.1, panel B). Total butyrate concentration observed in HM samples from healthy women ranged from 0.15 to 1.93 mM (IQR 0.43) in colostrum and 0.16 to 1.97 mM (IQR 0.43) in mature milk. The median butyrate concentration in mature HM was 0.75 mM, suggesting that a fully breastfed infant could receive a daily dose of butyrate of approximately 30 mg/kg body weight. The same dose was adopted in the animal model.

2. *Evaluation of butyrate effects in basal condition on gut barrier and on cytokines response in the animal model*

To explore the regulatory role elicited by butyrate, at a median HM concentration (0.75mM) on gut barrier we focused on gut permeability, IL-22, occludin, ZO-1 and Muc2, as biomarkers of gut barrier integrity (Fig.2). As showed in Fig.2, butyrate was able to reduce the gut permeability and to modulate all these biomarkers throughout the intestinal tract with differences related to the intestinal segment evaluated. The regulatory action elicited by ~~HM~~ butyrate on gut barrier paralleled a significant regulation of cytokines expression in MLN and in spleen, consisting in a significant reduction of IL-4, IL-5 and IL-13 and a significant increase of IFN-γ and IL-10 expression (Fig.2, panels A-I). Finally, butyrate induced a significant increase of CD4+/CD25+/FoxP3+ cells (Tregs) in spleen, MLN and colon (Fig.3, panels L-M).

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3. *Preventive butyrate action against FA in the animal model*

To determine whether the estimated daily dose of butyrate (0.75mM) received by a breastfed infant could elicit a protective action against the onset of FA, we adopted three different animal models resembling the most common forms of FA in childhood: cow's milk allergy (CMA), hen's egg allergy, and peanut allergy. Mice sensitized with β-lactoglobulin (BLG), ovalbumin (OVA) or with peanut extract (PN) showed a significantly higher anaphylactic symptom score and body temperature decrease compared to the control animals. Exposing the animals to an oral daily dose of 30 mg/kg of body weight of butyrate resulted in a significant inhibition of all of these parameters of allergic response (Fig. 3, panel A). 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. mMCP-1 serum levels were significantly increased in BLG-, OVA-, and PN-sensitized mice compared to the control animals. However, butyrate caused a significant reduction in mMCP-1 concentrations in the sensitized mice (Fig. 3, panel B). According to these data, butyrate administration also caused a reduction in serum-specific IgE concentrations (Fig. 3, panels C-E).

To investigate the mechanisms underlying the effect of butyrate in reducing allergic response, the splenocyte cytokines production in BLG-, OVA-, and PN-sensitized mice was evaluated. It was evident that there was a significant decrease in the Th2 cytokines (IL-4 and IL-13) (Fig. 3, panels F and G) and a significant increase in the IFN-γ and IL-10 production compared to control animals after butyrate administration (Fig. 3, panels H and I).

Recent evidence demonstrated that hepatic mitochondrial dysfunction and the consequent excessive generation of ROS are related to Th2 response in FA (33). Oral butyrate (0.75 mM)

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significantly modulated the oxidative stress in all three FA mouse models, reducing H_2O_2 release and superoxide dismutase activity (SOD) and reactivating the aconitase enzyme (Fig. 3, panels L-N).

3. Direct effects of butyrate on tolerogenic mechanisms in human enterocytes

Intestinal permeability and mucous layer exert a pivotal role in oral tolerance (34). Dose response and time course experiments showed that HM butyrate stimulates the expression of tight junction (TJ) proteins, ZO-1 and occludin in human enterocytes. Interestingly, the maximal effective dose was 0.75 mM, equal to median concentration detected in mature HM (Fig.4, panels A and B). Similar results were observed for Muc2 and Muc5AC expression (Fig.4, panels C and D). Accordingly, 0.75 mM butyrate was also the maximal effective dose for the regulation of mucus layer thickness in human enterocyte (from 0.0 to 5.0±2 μm; basal *vs* butyrate, $p<0.05$). It has been demonstrated that HBD-3, an innate immunity peptide produced by human enterocytes, is involved in tolerogenic mechanisms through FoxP3+ T cells induction (35). We found that 0.75 mM butyrate was the maximal effective dose for the production of the HBD-3 by human enterocytes (Fig.4, panel E).

Butyrate exhibits also HDAC inhibitory activity, in fact it induces histone acetylation in multiple cell types, and through HDAC inhibition, elicits its stimulatory action on mRNA expression (36,37). To assess whether butyrate acts as an HDAC inhibitor in human enterocytes, we analyzed its effect compared to trichostatin A (TSA), a well-established HDAC inhibitor. Butyrate treatment significantly reduced HDAC activity in a dose-dependent manner in human enterocytes, with the maximal effect observable at 0.75 mM (Fig. 4, panel F).

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4. Evaluation of direct immunoregulatory action of butyrate on human peripheral blood mononuclear cells

To assess the efficacy of butyrate (0.75mM) to drive tolerogenic mechanisms, we tested its effect on PBMCs derived from healthy donors or children affected by FA.

To test the system, we performed a proliferation test to evaluate if butyrate affects cell viability. Our data showed that butyrate, at this concentration, significantly increased the percentage of cells viability (Fig.S1, panel A). Detection of apoptosis demonstrated that the mortality cells rate after treatment with butyrate was comparable to the control (Fig. S1, panel B).

From time-course and dose-response experiments on PBMCs from healthy children to define the stimulation time and the useful dose of butyrate, we detected IL-10 production. We found that IL-10 release increased at a minimum dose of 0.5 mM of butyrate with a maximal effect at 0.75 mM after 24 h of incubation. No modulation was observed for the production of IL-4, IL-5, IL- 13, and IFN-γ (data not shown).

We analyzed the effect on PBMCs from children affected by IgE-mediated CMA, hen's egg allergy, or peanut allergy. Stimulation with BLG, OVA, or PN resulted in a significant increase in Th2 cytokines production: IL-4, IL-5, and IL-13, and the unchanged expression of IL-10, IFN-γ and FoxP3, respectively. Conversely, the treatment with 0.75 mM of butyrate or its co-incubation with the specific allergen caused an inhibition in Th2 cytokines production and a significant up-regulation of IL-10 and IFN- γ production (Fig. 5, panels A-E).

As shown in Fig.5 (panels G-I) the treatment with 0.75 mM of butyrate alone and in presence of specific allergen induced the production of IL-10 and IFN-γ, through a demethylation of respective gene promoters. This effect paralleled with a modulation of FoxP3 demethylation and its expression (Fig.5, panels F and I). We demonstrated that the effect of HM butyrate on gene methylation was *via* HDAC inhibitor (Fig. 5, panel L).

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Subsequently, we stimulated PBMCs from healthy children and IgE-mediated CMA patients, with BLG in the absence, or in the presence of 0.75 mM of butyrate to verify whether this SCFA could shift the pro-inflammatory (M1 state) phenotype of FA monocytes toward an anti-inflammatory, regulatory and protective response state (M2 state). Specifically, polarization is a candidate biomarker of disease-related inflammatory status, the M1/M2 profile was assessed measuring CD86/CD206 ratio as a marker of the relative proportion of M1 versus M2 cells in gated $CD14^+$ monocytes from FA subjects stimulated with specific allergen, in absence or presence of butyrate. Interestingly, flow cytometry analysis of CD86/CD206, indicated that HM butyrate increased M1 monocytes (Fig. 6, panels A-B), similar results were observed in children affected by IgE-mediated hen's egg allergy or peanut allergy (data not shown).

We subsequently investigated HM butyrate's ability to affect the differentiation of iMoDCs from IgE-mediated FA patients. We generated iMoDCs and treated them with butyrate immediately before incubation with GM-CSF and IL-4. After 7 days of incubation, the expression of CD1a decreased (Fig. 6, panel C) in the butyrate-treated cells compared to the untreated cells. However, although butyrate (0.75 mM) partially inhibited the differentiation process and reduced the yield of conventional CD1a+ iDCs, there was a high expression of CD86, a DC maturation marker, in the cells from patients with IgE-mediated FA (Fig. 6, panel D). Conversely, iDCs from both IgE-mediated FA patients and healthy subjects, generated in the presence of butyrate, expressed higher levels of CD103 and CD11b on their surfaces, indicating the tendency of butyrate-exposed DCs to acquire a tolerogenic phenotype (Fig. 6, panels E-G). Starting from the evidence that CD103+CD11b+ DCs were able to induce the differentiation of CD4+ naïve T cells toward a tolerogenic phenotype *in vitro* and *in vivo*, we next assessed the function of butyrate on Tregs differentiation. As first, butyrate-exposed DCs from both the healthy and

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CMA subjects were tested for their ability to induce the differentiation of naïve CD4 +T cells after 7 days of co-culture. In this condition, a comparable percentage of CD25+CD127-/dim Tregs was found in the untreated and butyrate-treated groups both in the presence and absence of BLG stimulus, indicating that butyrate-DC are inefficient to induce the differentiation of Tregs *in vitro* (Fig. 6, panel H), and suggesting the potential role of a peculiar chemokines milieu obtainable only in the *in vivo* setting. Conversely, when directly added as a stimulus to T cell culture, butyrate itself displayed efficient regulation of the differentiation of naïve CD4+ T cells to acquire a regulatory phenotype in healthy controls and to a greater extent in FA patients. Specifically, the stimulation with 0.75 mM of butyrate of CD4+ naïve T cells elicited an increase of the number of CD25+CD127-/dim Tregs in IgE-mediated FA patients (Fig. 7, panels A-B). Finally, we investigated if butyrate-DC could affect the generation of other additional subsets of CD4+ T cells. A reduction in the mean percentages of effector memory T cells (T_{EM}; CD45RA-CCR7) and effector memory RA T cells (T_{EMRA}; CD45RA+CCR7-) after autologous T:DC co-culture was observed in IgE-mediated FA patients compared to healthy controls (Fig. 7, panel C). A further decrease was observed after allergen stimulation in IgE-mediated FA patients compared to healthy controls (Fig. 7, panels D-E). The administration of 0.75 mM butyrate restored the compartment of both T_{EM} and T_{EMRA} of allergen-stimulated CD4+ naïve T cells from IgE-mediated FA patients compared to healthy controls (Fig. 7, panels D-E).

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Discussion

The actual picture emerging from the literature suggests a crucial role for dietary fiber-fermenting bacteria that, through the production of butyrate, modulate gut barrier function and support the establishment of a tolerogenic environment by inducing Tregs as well inhibiting pro-inflammatory DC functions (38).

In a large cohort of healthy lactating women, we demonstrated that butyrate is present in HM at an effective concentration that is able to modulate a wide range of tolerogenic mechanisms. Data on the concentration of butyrate in HM are conflicting. Discrepancies in butyrate values in HM, as well as in other biological samples, could derive not only from the analytical method used (40), but also by other factors including the extraction method, the features of the study population and the breast milk sampling and storing procedures. However, the HM butyrate levels observed in our study are well in line with those reported by recent studies where the samples were analyzed by two different techniques, ^{1}H-nuclear magnetic resonance spectroscopy (NMR) and GC-MS (40,41).

Our findings highlight the role of butyrate as a pivotal HM compound that exerts a wide modulation of immune tolerance mechanisms. Data from animal model experiments demonstrate that butyrate at 0.75mM could drive a non-specific protection against the most common forms of FA. A positive modulation of anaphylactic symptom score, body temperature and Th1/Th2 cytokines expression was demonstrated in animals receiving butyrate. We found an enhancement in IL-10 expression, a major immune-regulatory cytokine that maintains mucosal homeostasis and limits excessive immune responses against dietary antigens (42). In this light, recent evidence suggest that butyrate, but not acetate or propionate, is able to modulate Th2 cytokines production and to ameliorate type 2 innate lymphoid cells (ILC2)-driven airway hyper-reactivity

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and inflammation in murine model of asthma (43). In addition, it has been demonstrated that high fiber consumption increased SCFAs level and led to an increase in tolerogenic CD103+ DCs activity and Tregs differentiation in peanut allergy mouse model (44). Emerging data show that intraluminal butyrate is rapidly absorbed along the gastrointestinal tract (*Schmitt MG. et al. Gastroenterology 1976;70:211. Guilloteau P et al. Nutrition Research Reviews 2010;23:366. Jiminez JA, et al. mSphere 2017;2:e00243; Ruppin H et al Gastroenterology 1980;78:1500-07; Bergman EN. Physiological Review 1990;70:567-90*). Thus, it is possible to speculate that butyrate could modulate the immune tolerance network both in the gastrointestinal tract and at systemic level. In addition, it has been demonstrated that increasing intraluminal butyrate through dietary supplementation could exert a beneficial effect on gut microbiota increasing the abundance of butyrate-producing bacteria (*Jiminez JA, et al. mSphere 2017;2:e00243. Zhou D,et al. World J Gastroenterol. 2017 7;23(1):60-75. Wu W, et al. PLoS ONE 2018;13(5): e0197762*). The involvement of the liver in FA is emerging (45). Studies have suggested that the liver could act as a source of helper CD4+ T cells and play a role in IgE response to dietary antigens (46). Mitochondrial dysfunction and elevated ROS have been reported in allergic diseases 6(47-50). We recently reported a significant correlation between hepatic mitochondrial dysfunction and specific IgE, IL-5 and IL-13 mRNA expression in the peanut allergy animal model (33). Here, we demonstrated that 0.75mM of butyrate is able to significantly prevent mitochondrial dysfunction in all the three FA models, reducing H_2O_2 release and SOD activity, and reactivating the aconitase enzyme.

Food allergy depends by a breakdown of the immune tolerance network (51). This network is composed by immune and non-immune mechanisms (5). Among non-immune mechanisms, epithelial mucus thickness and permeability are considered very relevant in preserving immune

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tolerance (52). Loss of epithelial integrity in the gut increases antigen uptake and promotes Th2-type allergic response by activation of ILC2s, mast cells, basophils, and DCs (53). We found that HM median concentration of butyrate is able to up-regulate TJ proteins expression. A significant increase of Muc2, Muc5AC expression and mucus layer thickness was also observed after stimulation of human enterocytes with 0.75 mM of butyrate. Similarly, it has been demonstrated that butyrate differently stimulates the expression of various mucin genes in the colon, with maximum effects on Muc2 expression (54). The mucus layer does not merely form a non-specific physical barrier, but also constrains the immunogenicity of gut antigens by delivering tolerogenic signals (34). Interestingly, it has been shown that Muc2 enhances IL-10 secretion in $CD1c^+$ DCs and increases DC transcription and secretion of transforming growth factor-β1 (TGF-β1), a SMAD-signaling cytokine that helps the induction of Tregs by tolerogenic $CD103^+$ DCs, stimulating immune tolerance (34). Finally, Muc2–/– mice restored oral tolerance when gavaged with OVA in the presence of exogen Muc2 (55).

Among immune mechanisms, we found that butyrate is able also to stimulate the immune tolerance, enhancing the HBD-3 production by human enterocytes, involved in induction of $FoxP3^+$ T cells (35).

According to previous findings (13,56), we demonstrated that ~~HM~~ butyrate, at a median HM concentration, inhibits HDAC activity. It has been demonstrated that this action leads to transcriptional activation, with increased access of basal transcription factors and RNA polymerase II to the promoter region (57). Whether the effect of butyrate is due to direct acetylation of permeability and mucus-related genes or indirectly through transcriptional activation/repression of other targets, that could enhance the expression of these genes, warrants further investigation.

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Immune system cells exert a pivotal role in regulating a state of active non-responsiveness to food antigens (58). For this reason, we explored the direct effects of 0.75mM of butyrate on PBMCs from FA children. In agreement with previous findings, we found that butyrate inhibits Th2 cytokines production (44). This was not due to a change in the methylation status of the genes, but most likely through the modulation of the transcriptional activation/repression of other genes that can alter the expression of Th2-related genes (44). In contrast, we observed that HM butyrate concentration stimulated tolerogenic cytokines production and FoxP3 expression through a demethylation of the respective genes. It has been demonstrated that the inhibition of HDAC 6 and 9 is responsible for the increase in FoxP3 gene expression and the increase in Tregs (59). Acetylation of FoxP3 is an important post-translational mechanism that affects FoxP3 abundance, because it protects FoxP3 proteasomal degradation (60). This is a signature mechanism of action of several HDACs in Tregs (61-63). Studies have suggested a direct effect of butyrate as HDACs inhibitor in the activation of Tregs (7,9). Treatment of naïve T cells with butyrate enhances histone H3 acetylation in the conserved non-coding sequence regions of the FoxP3 locus (TSDR), suggesting a possible mechanism for how butyrate regulates the differentiation of Tregs (8). The switch toward a tolerogenic response by butyrate may be partly mediated by its action also on myeloid compartment because mono-macrophages exhibit a sliding scale of functional heterogeneity ranging from pro-inflammatory M1 state to anti-inflammatory M2 state (64). Flow cytometry analysis of PBMCs from IgE-mediated FA patients demonstrated that butyrate at median concentration found in HM increases the M1/M2 ratio of peripheral monocytes. In the context of allergy, recent studies highlighted that the inflammatory monocytes can differentiate into anti-inflammatory M2-type macrophages, which in turn dampen allergic inflammation (64). FoxP3+ CD4+ Tregs are central in the maintenance of immune

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homeostasis and tolerance throughout the body, particularly in the gut. We found that butyrate, at a median HM concentration, elicited an increase in the number of tolerogenic CD103+CD11b+ DCs and CD25+CD127-/dim Tregs, as previously suggested by others (7,9,65,66).

Following antigen encounter and subsequent resolution of the immune response, a single CD4+ naïve T cell is able to generate also multiple subsets of memory T cells with different phenotypic and functional properties. CD45RA and CCR7 markers allows the definition of most peculiar subsets among CD4+ T cell compartment including CD45RA−CCR7 T_{EM} CD45RA+CCR7−T_{EMRA}, naïve (T_N; CD45RA+CCR7+) and central memory (T_{CM}; CD45RA-CCR7+) T cells (67-70). We demonstrated that butyrate was able to restore T_{EM} and T_{EMRA} of IgE-mediated FA patients. As effector memory T cells have been observed to play a potential role in immune diseases (67,71,72), the fine tuning of CD4+ T cells differentiation by butyrate may be a new intriguing mechanism of its protective action.

This work had strengths and limitations. We determined HM butyrate concentration in a large cohort of healthy lactating women, and we provided evidence about multiple regulatory actions on tolerogenic mechanisms elicited by butyrate HM median concentration. The study limitations are related to the fact that we did not explore the effect of butyrate against other forms of FA or investigate the source of HM butyrate. The origin of HM butyrate remains to be elucidated. Recent evidence suggest a pivotal contribution by mammalian gland /HM microbiota in butyrate production deriving from metabolization of human milk oligosaccarides (73-78). Another limitation of our study is that HM contains a wide range of bioactive factors, in addition to butyrate, that are able to shape immune system function (79), and we did not evaluate if the effect of butyrate could be additive to other HM biofactors.

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In conclusion, a powerful HM compound has been defined. Altogether the results obtained in this study could explain the actual conflicting data on the efficacy of HM in preventing FA: the efficacy of HM could be based on the content of selected immunoregulatory compounds, such as butyrate. Increasing the concentration of such compound in HM, by modulating the maternal diet and lifestyle factors, could be an effective strategy to increase the protective role of HM against FA. At the same time, our data reveal the possibility of using butyrate as a supplement in the formulas for non-breastfed infants to protect from FA in early life.

Authorship

RBC, LP, RN, designed the study, coordinated the research team, and wrote the first draft of this report. CDC, MDC, AP, AA, LV, and GDG were responsible for the study subjects and evaluated their health status. LP, EC, CDC, RR, GT, RA, FM, AA, and CB conducted the laboratory experiments. LP, RN, GT, and EC performed the statistical analysis and data interpretation. All of the authors revised and approved the final version of this article.

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However, the Italian Ministry of Health, the Italian Ministry of the University and Scientific Research, the Cariplo Foundation, and the Umberto Veronesi Foundation had no influence on (1) the study design; (2) the collection, analysis, and interpretation of the data; (3) the writing of the manuscript; or (4) the decision to submit the manuscript for publication.
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Conflict of Interest Statement

The authors have no other conflict of interests that are directly relevant to the content of this manuscript, which remains their sole responsibility.

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Figure Legends

Figure 1. Butyrate concentrations in human milk

(A) Individual dot plot of butyrate concentrations observed in the colostrum and in the mature human milk samples collected by 109 lactating healthy women, * $p<0.001$.
(B) Longitudinal results of butyrate concentration during the first 5 months of lactation. Data were analyzed using repeated measures ANOVA test ($p<0.05$).

Figure 2. Effects of HM butyrate in basal condition on gut barrier and on cytokines response.

Four-weeks-old female C3H/HeJ mice were divided in two groups: (1) control mice (n = 4), receiving water as vehicle; (2) treated mice (n = 4), receiving butyrate once daily (30 mg/kg/os). After 14 days all mice were sacrificed, and intestine, in particular ileum, jejunum and colon, spleen and mesenteric lymph nodes (MLN) were removed. **(A-E)** Oral butyrate treatment elicited a significant reduction of gut permeability, measured by plasma FITC-dextran quantification, and increase of IL-22, occludin, ZO-1 and Muc2 expression. **(F-L)** Oral butyrate treatment induced a significant reduction of IL-4, IL-5 and IL-13 and a significant increase of IFN-γ and IL-10 expression in MLN and spleen. **(M-N)** CD4+/CD25+/FoxP3+ cells number were increased after butyrate treatment in mouse spleen MLN and colon.
Data are representative of at least 2 independent experiments, reported as median with range and analyzed using the unpaired t-test. *$p<0.05$.

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Figure 3. Preventive HM butyrate action against FA in the animal model

(A) The experimental design of the food allergy mouse model. Four-week-old female C3H/HeJ mice (n = 4 in each group) were used in the FA animal model. Two weeks before first sensitization, oral gavage with 30 mg/kg/d of butyrate was started and continued throughout the study. The mice were sensitized orally on days 0, 7, 14, 21, and 28, with 20 mg of BLG, 1 mg of OVA, or 12 mg of PN mixed with 10 μg cholera toxin (CT) as adjuvant. The control mice receive CT only. On day 35, the mice were challenged by gavage with BLG (50 mg), OVA (5 mg), or PN (36 mg). Anaphylaxis scores and rectal temperatures were assessed for 1 h after challenge and blood samples were collected to measure mMCP-1 and sIgE. After 24 h, the mice were sacrificed, and their serum and tissues were collected. Two independent experiments were performed. Oral butyrate treatment significantly reduced anaphylactic score **(B)**, serum MCP-1 **(C-)** and sIgE levels against BLG **(D)**, OVA **(E)**, PN **(F)**. Splenocytes from FA-sensitized mice were stimulated 1 μg/mL of anti-CD3 (clone 2C11), or 200 μg/mL BLG/OVA/PN and incubated at 37 °C for 72 h. Butyrate inhibited IL-4 **(G)** and IL-13 **(H)** production and stimulated IL-10 **(I)** and IFN-γ **(L)** production in BLG-, OVA-, PN- sensitized mice. H_2O_2 yield **(M)**, SOD **(N)** and ROS-induced damage on aconitase activity **(O)** were measured in hepatic mitochondria. The mitochondrial ROS-induced dysfunction was proven by the higher mitochondrial H_2O_2 yield, higher SOD activity and lower basal/total aconitase ratio observed in all FA-sensitized animals compared to the control group. Butyrate administration positively modulates the oxidative stress as demonstrated by the lower H_2O_2 release, the decreased SOD activity and by the reactivation of the aconitase enzyme in all treated groups compared to control animals.
Data are representative of at least 2 independent experiments, reported as median with range and analyzed using the unpaired t-test.

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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 4. Direct effect of butyrate on non-immune and immune tolerogenic mechanisms in human enterocytes

Dose-response effects of butyrate (from 0.1 to 2 mM) after 48h of stimulation in Caco-2/HT-29 cells. ZO-1 **(A),** occludin **(B),** Muc2 **(C)**, Muc5A **(D),** HBD-3 **(E)** resulted overexpressed after the stimulation with butyrate. Butyrate treatment significantly reduced HDAC activity in a dose-dependent manner in Caco-2/HT-29 cells **(F)**.
Data represent the median with range of 2 independent experiments, each performed in triplicate. Data were analyzed using the unpaired t-test.
ZO-1= zonula occludens 1; Muc2=mucin 2; Muc5AC=mucin 5AC; HBD-3= β-defensin 3; TSA=trichostatin A.
*p<0.05 NT vs all doses; #p<0.05 0.75 vs 0.5; °p<0.05 0.75 vs 1.

Figure 5. Evaluation of direct immunoregulatory action of HM butyrate on human peripheral blood mononuclear cells (PBMCs): Th2/Th1 and IL-10 response and evaluation of FoxP3 expression

PBMCs from children with IgE-mediated FA were stimulated with butyrate HM concentration (0.75 mM) or with BLG, OVA,PN (100µl/well) in presence or in absence of butyrate HM median concentration for 24h. BLG, OVA, PN induced a significant increase of IL-4 **(A)**, IL-5

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(B), IL-13 **(C)** production, but the presence of 0.75 mM butyrate significantly reduced the release of these Th2 cytokines. Butyrate alone or in presence of specific allergen stimulated, at the same dose, IL-10 **(D)** and IFN-γ **(E)** production and FoxP3 expression **(F)**, through a demethylation of respective gene **(G, H, I)** in CD4+T cells purified from stimulated PBMCs from children with IgE-mediated FA. **(L)** Butyrate treatment significantly reduced HDAC activity in a dose-dependent manner in PBMCs.
Data represent the median with range of 2 independent experiments, each performed in triplicate. Data were analyzed using the unpaired t-test.
NT= untreated cells; BLG= β-lactoglobulin; OVA=ovalbumin; PN = peanut extracts.

*p<0.05; **p<0.01.

Figure 6. Evaluation of direct immunoregulatory action of HM butyrate on human peripheral blood mononuclear cells (PBMCs): modulation of mono-dendritic cells expression.
PBMCs from children with IgE-mediated FA were stimulated with butyrate HM concentration (0.75 mM) or with specific allergen (BLG, OVA, PN) (100µl/well of each allergen) in presence or in absence of butyrate for 48h **(A-B)**. CD45hi CD14+ monocytes were stained with flow cytometric markers CD206+ or CD86+ to discern the CD206 M2 type from CD86 M1 type of total monocytes. A representative dot plot panel (left panel) is presented. The graph on the right report the mean ± SD of ratios of M1 versus M2 peripheral monocytes (CD86 /CD206 ratio) from different FA patients (ANOVA; ***p < 0.001 *vs* CTRL) **(B).**
Purified CD14+ monocytes from IgE-mediated FA were cultured with regular medium containing GM-CSF (50 ng/ml) and IL-4 (1000 U/ml), with or without butyrate HM median

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concentration (0.75 mM). After 7 days, cells were collected, stained with the reported antibodies, and analyzed by flow cytometry **(C-E)**. Representative example of dot plots of iDCs generated in the presence or in the absence of butyrate **(C)**. The bars graph reports mean and standard deviation of % of CD14-CD1a+ from independent experiments using different IgE-mediated FA. (ANOVA; ***p< 0.001 vs CTRL) **(D)**. Immature DC were collected and exposed to LPS activating stimulus (1ug/ml). After 24h, cells were stained for CD86 maturation marker. Bar graphs report the percentage of positive cells for the indicated markers in gated viable CD14-CD1a+ cells (ANOVA; ***p<0.001 vs CTRL) **(E).**

Phenotypical characterization of iDCs generated in the presence or in the absence of 0.75 mM of butyrate for 7 days in healthy subjects (left panel) and in IgE-mediated FA (right panel) Bars graph reports mean and standard deviation of % of CD11b+CD103+ from independent experiments using IgE-mediated FA. (ANOVA; ***p < 0.001 vs CTRL) **(F-G).** Butyrate-DC had no effect on regulatory cell differentiation. Naïve CD4+T cells from IgE-mediated FA were cultured with immature or mature DCs generated in presence of butyrate at 1: 10 DC:T ratio. After being cultured for 5 days, cells were collected. CD4+CD25+CD127 dim/^{n}eg Tregs were identified and enumerated with a flow cytometer **(H).** The bar graphs report the percentage of positive cells for the indicated markers.

Figure 7. Evaluation of direct immunoregulatory action of HM butyrate on human peripheral blood mononuclear cells (PBMCs): effect on CD4+ T cell subsets

Butyrate directly affects the Tregs generation. Naïve CD4+T cells were collected from healthy controls and from IgE-mediated FA children, and were maintained in culture with or without 0.75 mM of butyrate. After 5 days, cells were collected. CD4+CD25+CD127 dim/neg Tregs

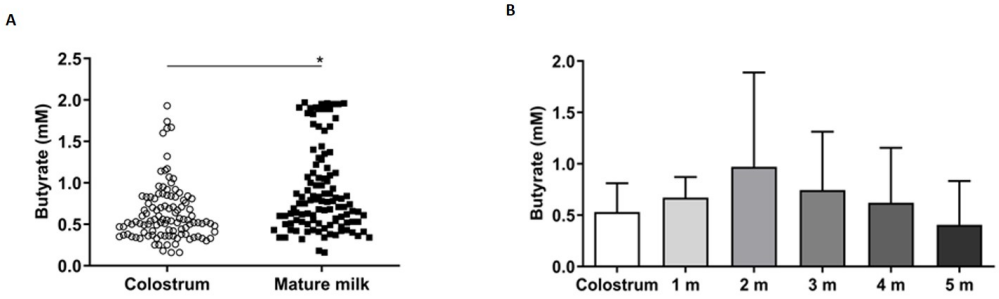
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were identified and enumerated with a flow cytometer. The bar graphs on the right report the percentage of positive cells for the indicated markers (ANOVA; *p< 0.05 vs CTRL) **(A-B).** Butyrate-DC had effect on TCD4+ cell differentiation. Naïve CD4+T cells from IgE-mediated FA patients and healthy controls were cultured with DCs generated in presence of butyrate at 1 : 10 DC:T ratio. After 5 days, cells were collected and stained with the indicated markers prior to FACS analysis. CD45RA and CCR7 markers allows the definition of most peculiar subsets among CD4+ T cell compartment including effector memory (T_{EM}; CD45RA−CCR7), effector memory RA (T_{EMRA}; CD45RA+CCR7−), naïve (T_N; CD45RA+CCR7+) and central memory (T_{CM}; CD45RA-CCR7+) T cells **(C-E).** Representative example of dot plots of TCD4+ cells from control and IgE-mediated FA subjects after autologous T:DC co-colture **(D-E).**The bar graphs report the percentage of positive cells for the indicated markers in CD4+ gated viable cells (ANOVA; *p<0.05, **p<0.01, ***p< 0.001 vs CTRL).

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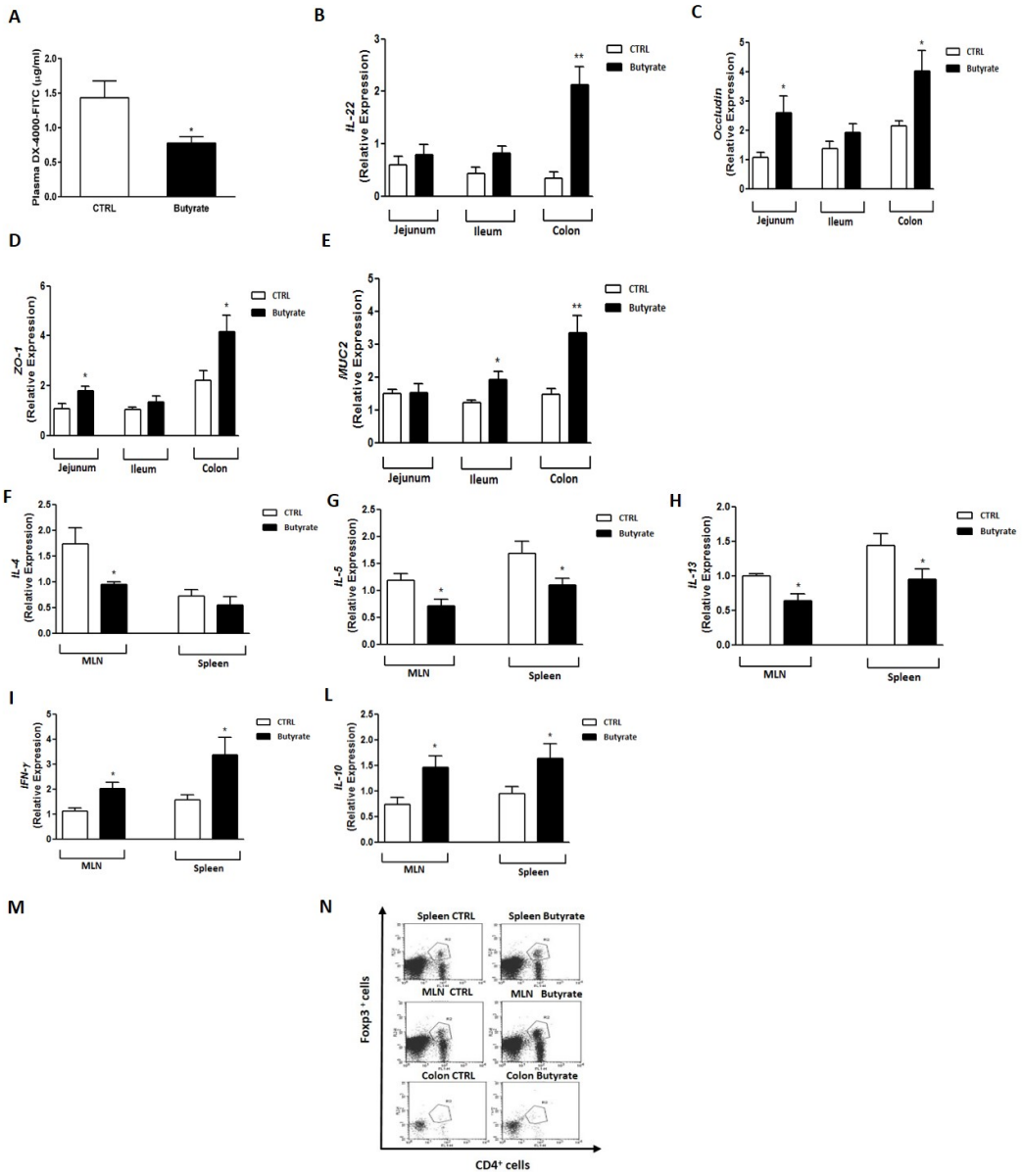
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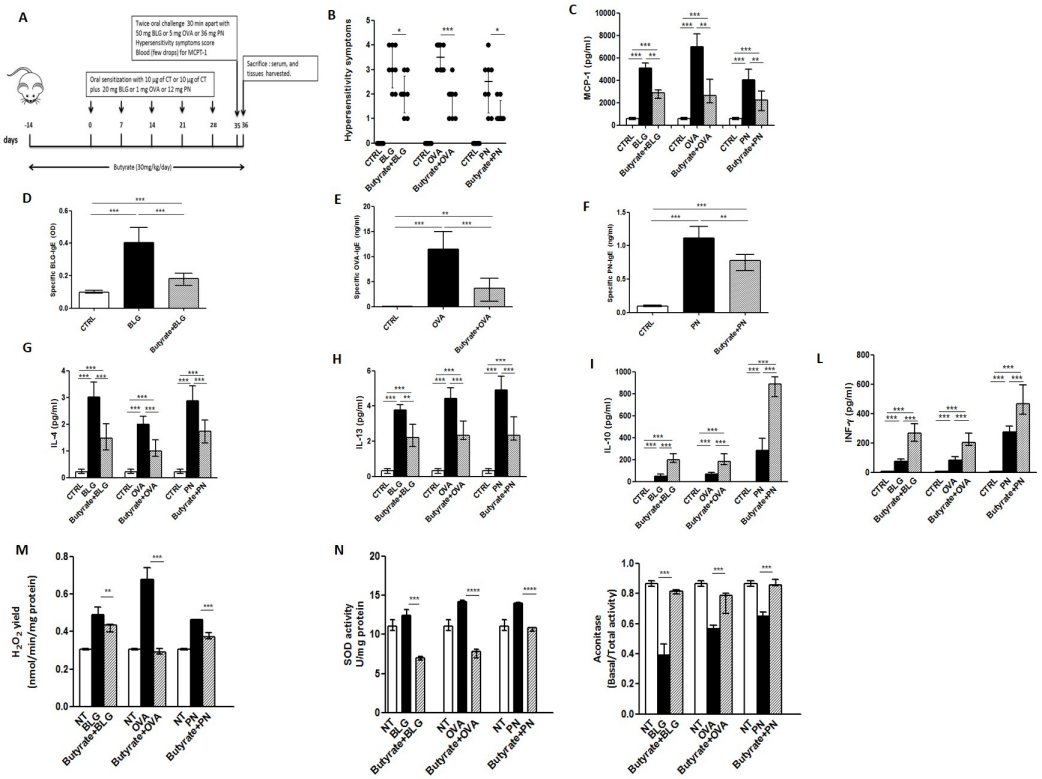
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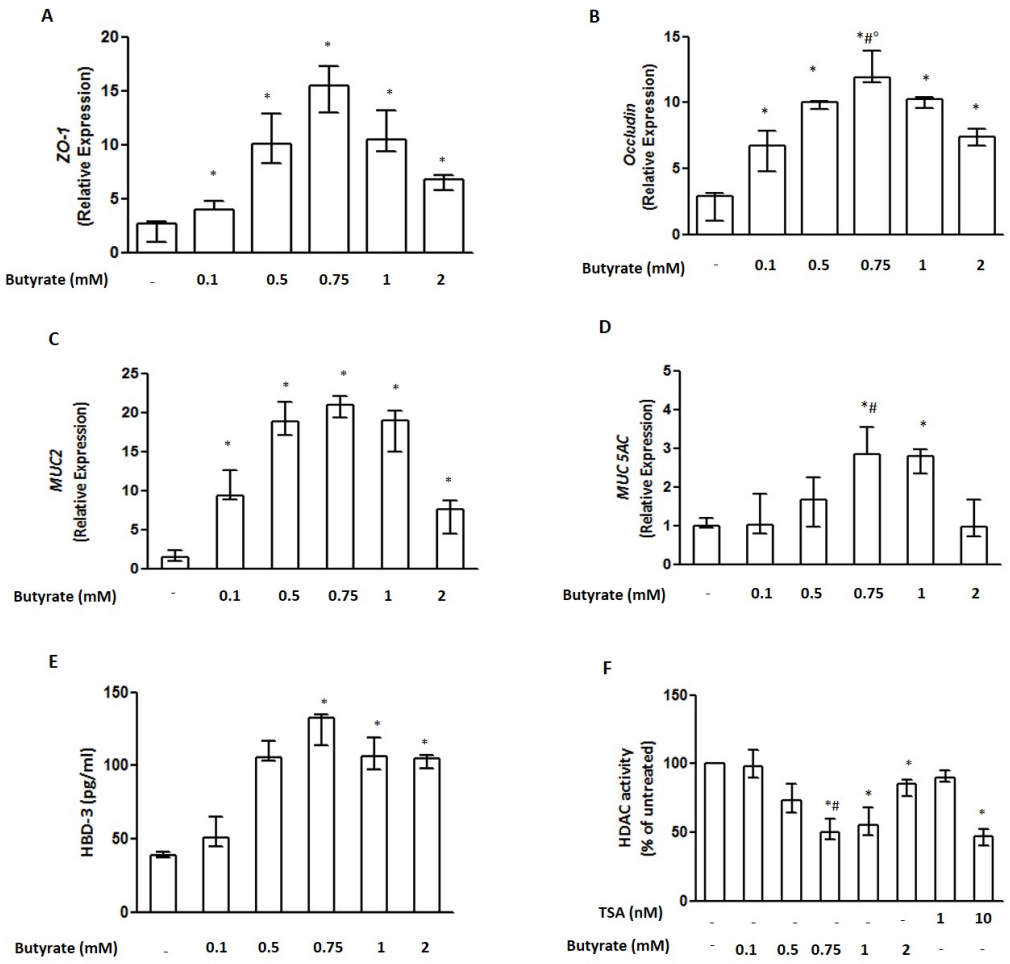
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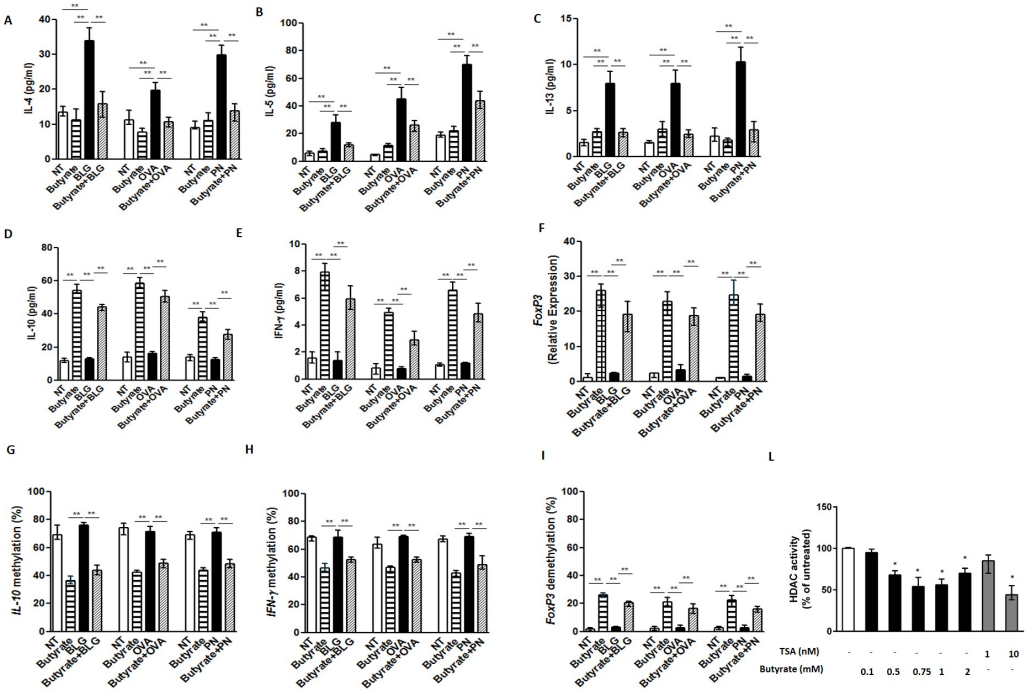
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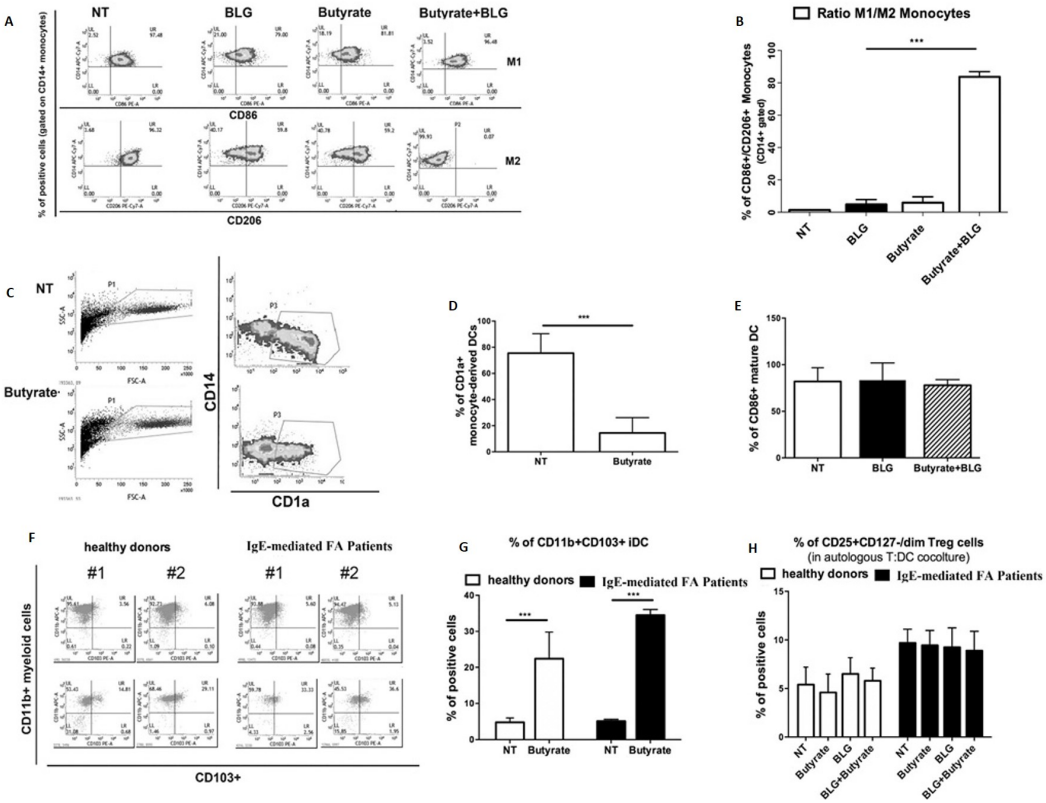
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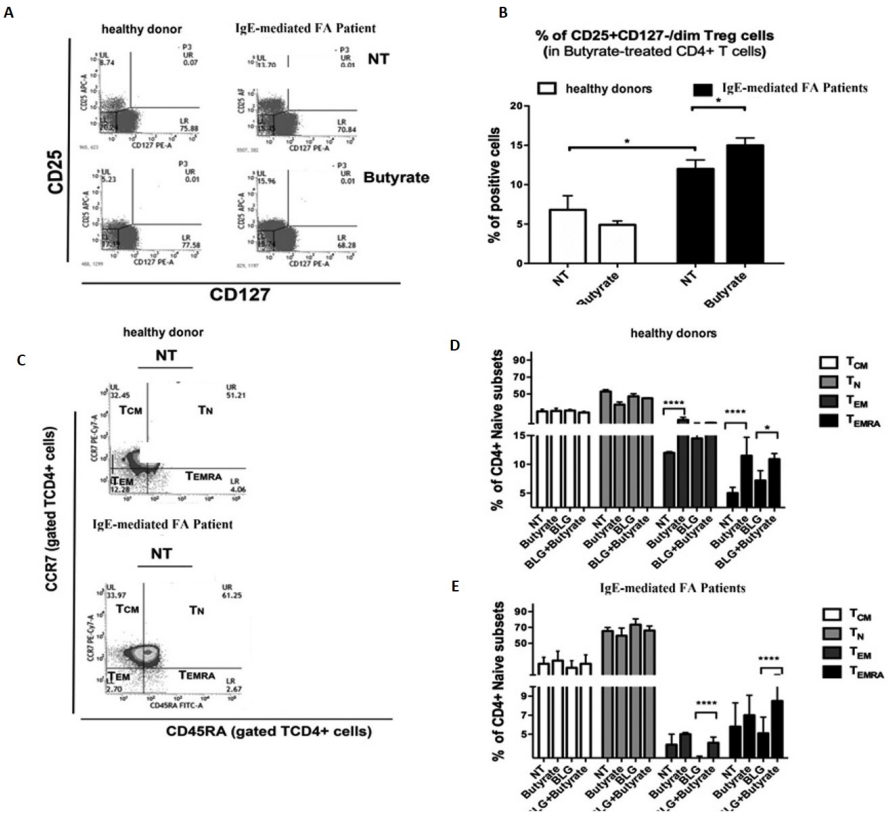
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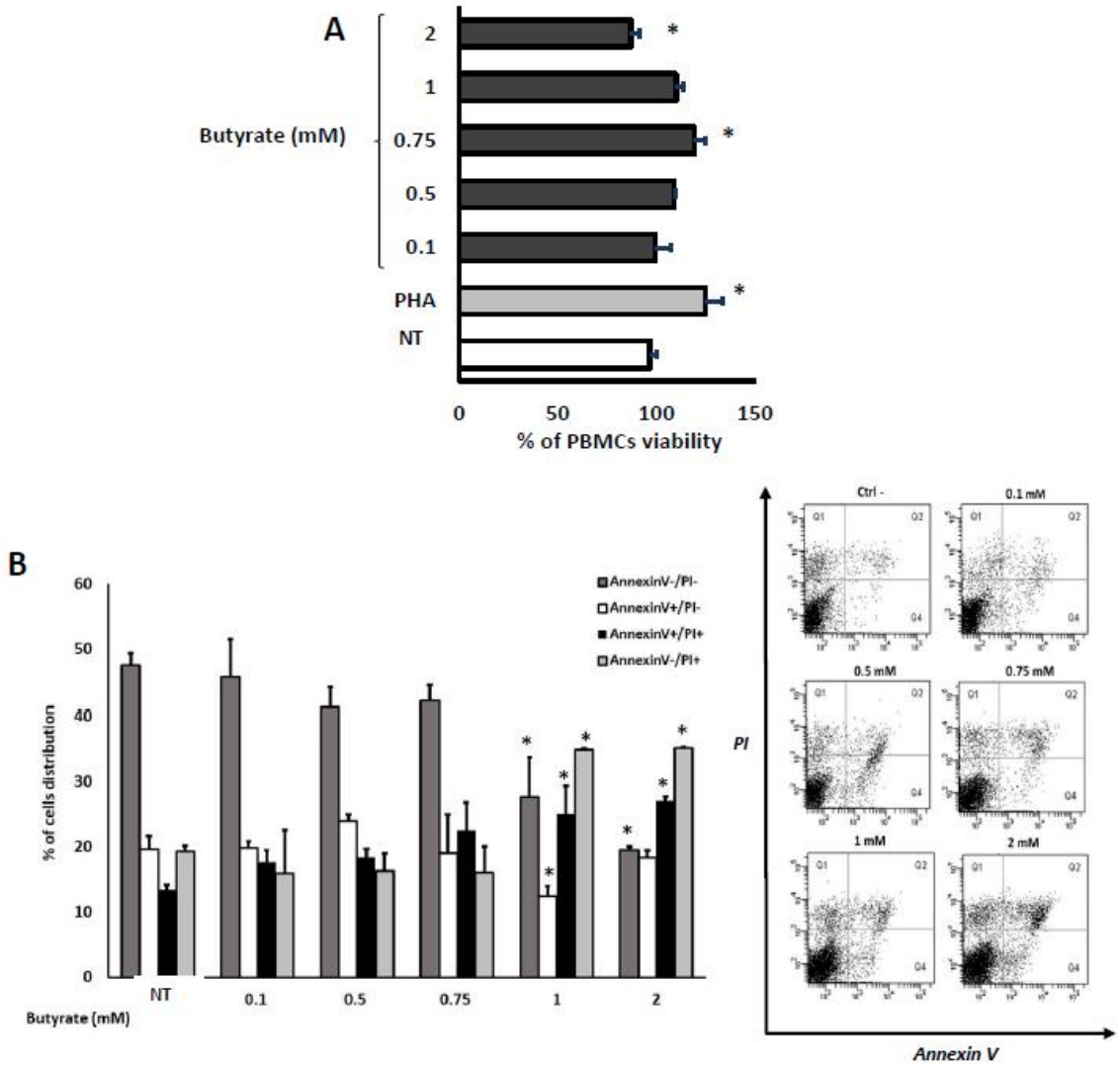
Butyrate as bioactive human milk protective component against food allergy

Lorella Paparo, Rita Nocerino, Elena Ciaglia, Carmen Di Scala, Carmen De Caro, Roberto Russo, Giovanna Trinchese, Rosita Aitoro, Antonio Amoroso, Cristina Bruno, Margherita Di Costanzo, Annalisa Passariello, Francesco Messina, Annalisa Agangi, Marcello Napolitano, Luana Voto, Giusy Della Gatta, Laura Pisapia, Francesco Montella, Maria Pina Mollica, Antonio Calignano, Annibale Puca, and Roberto Berni Canani.

Table S1. The main clinical and demographic characteristics of study population.

	Subjects with IgE-mediated food allergy to:			
	Cow's milk	**Egg**	**Peanut**	**Healthy subjects**
N.	4	4	4	4
Male, n (%)	4 (100)	4 (100)	4 (100)	4 (100)
Age, months (SD)	22 (1.8)	22.7 (0.9)	22.5 (1.3)	22.7 (1.2)
Spontaneous delivery, n (%)	2 (50)	3 (75)	2 (50)	2 (50)
Breastfed for ≤8 weeks, n (%)	4 (100)	4 (100)	4 (100)	4 (100)
Age at weaning, month (SD)	5 (0.8)	5.2 (0.9)	5.2 (0.5)	5 (0.8)
Familial allergy risk, n (%)	3 (75)	2 (50)	3 (75)	0
Gastrointestinal symptoms at FA onset, n (%)	4 (100)	4 (100)	4 (100)	-
Cutaneous symptoms at FA onset, n (%)	3 (75)	2 (50)	3 (75)	-
Respiratory symptoms at FA onset, n (%)	1 (25)	1 (25)	1 (25)	-
Positive total serum IgE, n (%)	4 (100)	4 (100)	4 (100)	0
***Total serum IgE, kU/l (SD)**	145.2 (57.9)	122.1 (41.6)	168.6 (48.9)	0.16 (0.06)

**Reference value < 0.35 kU/l*



Supplementary Figure S1. Effects of butyrate on peripheral mononuclear blood cells (PBMCs) viability.

(A) MTT assay demonstrated that 0.75 mM butyrate stimulation significantly increases the percentage of PBMCs viability after 24h of treatment **(B)** PBMCs apoptosis rate after treatment with different doses of butyrate (from 0.1 to 2 mM). The death cells rate after treatment with 0.75 mM butyrate was comparable to the control after 24h of incubation.

Data represent the median with range of 2 independent experiments, each performed in triplicate. Data were analyzed using the unpaired t-test.

NT= untreated cells; PHA=phytohaemagglutinin *$p<0.05$;