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TITLE

Tolerogenic effect elicited by protein fraction derived from different formulas for dietary treatment of cow's milk allergy in human cells

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Abbreviations

AAF	amino acid-based formula
AMs	allergic manifestations
AP1	activator protein-1
ARD	absolute risk difference
BLG	β-lactoglobulin
BSA	bovine serum albumin
CCA	complete case analysis
CI	confidence interval
CMA	cow's milk allergy
CMPs	cow's milk proteins
DCs	dendritic cells
EHCF	extensively hydrolyzed casein formula
EHF	extensively hydrolyzed formula
EHWF	extensively hydrolyzed whey formula
FOXP3	forkhead box P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HRF	hydrolyzed rice formula
IFN-γ	interferon-γ
IL	interleukin
ILC2	type 2 innate lymphoid cells
LGG	*Lactobacillus rhamnosus* GG

miRNAs	microRNAs
Muc5AC	mucin 5AC
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kB
OR	odds ratio
PBMCs	peripheral mononuclear blood cells
SCFA	short chain fatty acid
SD	standard deviations
SF	soy formula
TEER	transepithelial electric resistance
Th	T helper
TLR	T like receptor
Tregs	T regulatory cells
TSDR	Treg specific demethylation region
TSLP	thymic stromal lymphopoietin
ZO-1	zonula occludens-1

Abstract

Several formulas are available for the dietary treatment of cow's milk allergy (CMA). Clinical data suggest potential different effect on immune tolerance elicited by these formulas. We aimed to comparatively evaluate the tolerogenic effect elicited by the protein fraction of different formulas available for the dietary treatment of CMA. Five formulas were compared: extensively hydrolyzed whey formula (EHWF), extensively hydrolyzed casein formula (EHCF), hydrolyzed rice formula (HRF), soy formula (SF), and amino acid-based formula (AAF). Formulas were reconstituted in water according to manufacturer's instructions and subjected to an *in vitro* infant gut simulated digestion using a sequential gastric and duodenal static model. Protein fraction was then purified and used for the experiments on non-immune and immune components of tolerance network in human enterocytes and in peripheral mononuclear blood cells (PBMCs). We assessed epithelial layer permeability and tight junction proteins (occludin and zonula occludens 1, ZO-1), mucin 5AC (Muc5AC), IL-33 and thymic stromal lymphopoietin (TSLP) in human enterocytes. In addition, Th1/Th2 cytokines response and T regulatory cells (Tregs) activation were investigated in PBMCs from IgE-mediated CMA infants. EHCF-derived protein fraction positively modulated the expression of gut barrier components (Muc5AC, occludin and ZO-1) in human enterocytes, while SF was able to stimulate only the expression of occludin. EHWF and HRF protein fractions elicited a significant increase in TSLP production, while IL-33 release was significantly increased by HRF and SF protein fractions in human enterocytes. Only EHCF-derived protein fraction elicited an increase in the production of the tolerogenic cytokines (IL-10, IFN-γ) and

in the number of activated $CD4^{+}FoxP3^{+}$Tregs, through NFAT, AP1 and NF-kB1 pathway. The effect paralleled with an up-regulation of *FOXP3* demethylation rate. Protein fraction from all study formulas was unable to induce Th2 cytokines production. The results suggest a different regulatory action on tolerogenic mechanisms elicited by protein fraction from different formulas commonly used for CMA management. EHCF-derived protein fraction was able to elicit tolerogenic effect through at least in part an epigenetic modulation of *FOXP3* gene. These results could explain the different clinical effects observed using EHCF on immune tolerance acquisition in CMA patients and on allergy prevention in children at risk for atopy (see Graphical abstract in Figure 1).

1. Introduction

Cow's milk allergy (CMA) is an important problem worldwide with lifelong implications for health. With an estimated prevalence up to 3%, CMA is one of the most common food allergies and one of the main causes of food-induced anaphylaxis in the pediatric age (1-6). Cow's milk allergy derives from a breakdown of immune tolerance mechanisms that leads to an abnormal immune-mediated response to cow's milk proteins (CMPs) (7). This pathological condition entails significant costs for the health care systems as well as for patients' families, and it emerged as one of the most expensive allergic diseases (8,9). Whatever the clinical pattern of CMA, the mainstay of treatment is the elimination diet for CMPs. The latest treatment guidelines recommend the continuation of breastfeeding and the elimination of CMPs from the maternal diet in exclusively breast-fed infants with CMA. For infants that cannot be breastfed, different special formulas adapted to CMA dietary management are currently available on the market. Special formulas for CMA treatment must have a safe allergological and nutritional profile. The most used are the following: extensively hydrolyzed whey (EHWF) or casein formula (EHCF), soy formula (SF), hydrolyzed rice formula (HRF) or amino acid-based formula (AAF) (10). Extensively hydrolyzed cow's milk formulas with documented hypoallergenicity are being recommended as a first-choice formula for infants and young children with CMA (11,12).

Special formulas emerged as the primary cost driver for CMA management (13). It has been estimated 2.5 to 6 times higher cost to feed a child with CMA (10). Thus, options to accelerate the process of immune tolerance acquisition would be very welcomed by patients' families and health care systems.

Special formulas are traditionally considered useful for symptoms' resolution, but not for the immune tolerance acquisition. Special formulas available for CMA treatment differ mainly regarding the protein fraction features, such as source (cow's milk, soy, or rice), degree and procedure of hydrolysis (14). It has been suggested that selected milk protein hydrolysates may be able not only to avoid allergic symptoms in CMA children but might also have immune-modulating properties like the induction of T cell tolerance and the prevention of allergic sensitization (7). Increasing data suggest a potential different impact on immune tolerance acquisition induced by different formulas available for CMA management (3,5,15-21). There is also evidence that selected cow's milk proteins-based hydrolyzed infant formulas may have a long-lasting preventive effect on the development of allergy in children at risk for atopy (22-26). This evidence points out the importance to better define the potential tolerogenic effects elicited by the protein fraction from formulas commonly used for CMA treatment.

2. The formula choice in CMA

The first objective in the treatment of CMA is symptoms' resolution. The current standard of care for CMA is strict dietary avoidance of CMPs, with use of substitute formulas in non-breastfed subjects (10,11). The formulas considered effective in the dietary management of CMA include: EHWF, EHCF, SF, HRF, AAF. These formulas resolve allergic symptoms due to the lack of IgE binding epitopes (27).

The second objective in the treatment of CMA is immune tolerance acquisition. Many

children acquire immune tolerance to CMPs within the first 5 years of life. However, recent studies suggest that the natural history of food allergy is changing, with an increasing persistence until later ages (1). In a prospective multicenter study, we demonstrated that the selection of a particular type of formula influences the rate of immune tolerance acquisition in children with CMA. Otherwise healthy children (aged 1-12 months) diagnosed with CMA were prospectively evaluated. The study population was divided into 5 groups based upon the formula used for management: (1) EHCF (n=55); (2) EHCF+*Lactobacillus rhamnosus* GG (LGG) (n=71); (3) HRF (n=46); (4) SF (n=55); and (5) AAF (n=33). A food challenge was performed after 12 months to assess immune tolerance acquisition. Two hundred sixty children were evaluated (167 males, 64.2%; age 5.92 months, 95% CI 5.48-6.37; IgE-mediated CMA 111, 42.7%). The rate of children acquiring immune tolerance after 12 months was significantly higher ($p<0.05$) in the groups receiving EHCF (43.6%) or EHCF+LGG (78.9%) compared with the other groups: HRF (32.6%), SF (23.6%), and AAF (18.2%). Binary regression analysis coefficient (B) revealed that the rate of patients acquiring immune tolerance at the end of the study was influenced by 2 factors: (1) IgE-mediated mechanism (B −2.05, OR 0.12, 95% CI 0.06-0.26; $p<0.001$); and (2) formula choice, such that those receiving either EHCF (B 1.48, OR 4.41, 95% CI 1.44-13.48; P=0.009) or EHCF+LGG (B 3.35, OR 28.62, 95% CI 8.72-93.93; $p<0.001$) (16).

Cow’s milk allergy is often the first manifestation of the so-called "atopic march", characterized by the appearance of other allergic manifestations (AMs) in later years, such as allergic rhinitis and asthma. Thus, a potential third objective in the treatment of a child with CMA could be the prevention of the atopic march (28).

Our study group performed a randomized controlled trial to test the potential of a formula-based dietary intervention on AMs prevention in CMA pediatric patients (5). In a prospective trial, a total of 220 infants with IgE-mediated CMA (67% males, median age 5.0 months) were randomized into two dietary groups: 110 subjects were placed on EHCF-based diet, and 110 children were placed on EHCF+LGG-based diet. Patients were followed up for 36 months. In the complete case analysis (CCA), the absolute risk difference (ARD) for the occurrence of at least one atopic manifestation over 36 months was −0.23 (95% CI −0.36 to −0.10, $p<0.001$). Even under the worst-case scenario, a difference in favor of EHCF+LGG was still detected. Using the CCA estimate of the ARD, the number needed to treat was 4 (95% CI 3-10) (5). These findings are consistent with those of recent studies revealing that the first-line approach with EHCF+LGG for CMA infants may slow down the AMs, compared to infants treated with other formulas. A retrospective observational study on 211 subjects with CMA was conducted for new score validation for the risk of developing AMs, using selected clinical and laboratory data (21). The authors found that the type of substitutive formula for CMA treatment may influence the natural history of these children. They divided the patients into five groups, based on formula composition: vegetable-based formulas (rice or soy), high-grade extensively hydrolyzed formula (EHF) for those in which >95% of peptides were 1,000 kDa, high-grade EHF plus LGG (EHF+LGG), low-grade EHF for those with a higher proportion of peptides (>1,000 kDa), or AAF. Authors found that the risk of AMs occurrence decreased in those treated with high-grade EHF (OR 0.42; 95% CI 0.20-0.87, p=0.02), and these results were stronger in patients treated with high-grade EHF+LGG (OR 0.30; 95% CI 0.09-0.98, p=0.048). The authors concluded that the first-line approach with EHF

may be beneficial to prevent the occurrence of AMs, and LGG implementation strengthened this trend. They supposed that the hypoallergenic composition of this high-grade EHF and the gut microbiome may have helped to positively influence the immune tolerance network, decreasing the risk of developing AMs (21). Similarly, in a recent retrospective cohort study of 940 infants with CMA, a binary logistic regression analysis showed that infants fed with EHWF had a significantly higher relative risk at 24 months of atopic dermatitis (OR: 3.438; 95% CI: 1.975-5.985; $p<0.001$) and asthma (OR: 2.651; 95% CI: 1.242-5.660; $p<0.02$) compared with those fed with EHCF+LGG. The authors concluded that the first-line therapeutic approach for newly diagnosed CMA children with EHCF+LGG, reduces the development of other allergic diseases later in life (20). Recently, in a 36-month prospective cohort study the occurrence of other AMs (eczema, urticaria, asthma, and rhinoconjunctivitis) and the time of immune tolerance acquisition were comparatively evaluated in IgE-mediated CMA children treated with: EHCF+LGG, RHF, SF, EHWF or AAF. 365 subjects were enrolled into the study, 73 per formula cohort. The incidence of AMs in the RHF, SF, EHWF and AAF cohorts *vs.* the EHCF+LGG cohort was always significantly higher. Moreover, the 36-month immune tolerance acquisition rate was higher in the EHCF+LGG cohort (29). Therefore, the choice of formula for CMA treatment influences not only the time of immune tolerance acquisition but also the occurrence of other AMs.

3. Potential mechanisms of action of infant formulas in CMA

It has been suggested that selected milk protein hydrolysates used for CMA management may be able to not only avoid allergic symptoms in CMA infants due to the breakdown of IgE antigens but also play a role in immune system modulation, inducing tolerance and preventing allergic sensitization (7,30-32). These peptides can interact with Toll like receptors (TLR)2 and TLR4, modulating cytokine release by epithelial and immune cells (33). It has also been demonstrated that specific peptides from casein hydrolysates, driving T cell switching from Th2 to Th1 or to T regulatory cells (Tregs) subtype, could exert a protective effect for food allergy (30,34). Animal studies have demonstrated that these peptides can suppress Th2 response through an IL-10 up regulation and IL-2 down regulation (7). Moreover, the production of the tolerogenic cytokine IL-10 was higher in Jurkat T cells that underwent a casein hydrolysate stimulus (32). Preliminary data by our group suggest that formula choice is able to induce immune system modulation through epigenetic mechanisms in CMA infants (35-37); specifically, evidence suggests that EHCF+LGG is able to modulate gut microbiome, raising the abundance of selected genera (*Roseburia, Coprococcus, and Blautia*) with increased production of butyrate (38). Butyrate is a short chain fatty acid (SCFA) with a pivotal role in immune tolerance to food antigens. Butyrate contributes to protection against the development of food allergy through multiple tolerogenic mechanisms, as demonstrated in preclinical and clinical studies (39-41).

A significant difference in DNA methylation of Th2 and Th1 cytokine (IL-4, IL-5, IL-10, and IFN-γ) genes and of *FOXP3,* the transcription factor that modulates the fate of Tregs, was observed in infants treated with EHCF+LGG who develop immune

tolerance compared to children who received other formulas (35,36). A DNA methylation status of all allergy-related genes in infants treated with EHCF+LGG was closer to that observed in healthy children. Analyzing the potential factors able to modulate DNA methylation status in tolerant children, the authors found that the variable that greatly influenced the DNA methylation status was EHCF+LGG formula use (35,36). A longitudinal study, the EPICMA trial, compared the DNA methylation of *FOXP3*, Th1/Th2 cytokine genes, and allergy-related microRNAs (miRNAs) profile in IgE-mediated CMA infants taking EHCF+LGG compared to SF. This study demonstrated that treatment with EHCF+LGG is characterized by a more pronounced effect on *FOXP3* demethylation compared to SF and by a higher methylation status of IL-4 and IL-5 and a lower methylation status of IL-10 and IFN-γ (37). Moreover, children treated with EHCF+LGG showed a selected miRNA expression toward a Th1-oriented response, leading to the activation of immune tolerance mechanisms (37). However, the impact of diet on epigenetic mechanisms may not only be direct but also mediated by the gut microbiome and its metabolites in a complex interplay between diet and gut microbiome (42). Altogether, these data highlight the relevance of "immunenutrition management" able to reduce disease duration and to protect against the occurrence of other AMs the CMA children.

4. Aim of the study

This study aimed to comparatively evaluate the tolerogenic effect elicited by the protein fraction derived from different special formulas available for the dietary treatment of CMA.

5. Materials and methods

5.1 Study design

The study was designed as comparative blinded evaluation of the effects elicited by protein fraction from different formulas on main non-immune and immune components of the tolerance network in human enterocytes and PBMCs. In total, five different commercially available formulas were analyzed: EHCF, EHWF, HRF, SF, and AAF.

To simulate what happens into the infant gut lumen after oral administration, each formula was subjected to an *in vitro* simulated infant gut digestion. We assessed, in a blinded manner, gut barrier permeability, tight-junction proteins involved in the regulation of gut barrier integrity (occludin and zonula occludens-1, ZO-1), mucin 5AC (Muc5AC) protein involved in the regulation of gut barrier mucus thickness, and the epithelial cell-derived danger signal mediators, IL-33 and thymic stromal lymphopoietin (TSLP) in human enterocytes. Whereas, the Th1/Th2 cytokines response and Tregs activation were investigated in PBMCs from IgE-mediated CMA infants. Only after the analysis was completed, the formula identity was disclosed.

5.2 Study formulas protein fraction preparation

In the Table 1 were reported the main features of the study formulas. The commercially available study formulas were reconstituted in tap water according to the manufacturer's instructions. Protein content in the formulas was determined by measuring protein nitrogen as previously described (43). Aliquots corresponding to 500 mg of protein fraction were subjected to an *in vitro* simulated infant gut digestion

using a harmonized sequential gastric and duodenal static model, as previously described (44). Simulated digestion was performed in disposable sterile plastic tubes. Compared with the adult model, the substrate-to-enzyme ratio of pepsin (gastric phase) and pancreatin (duodenal phase) was reduced eightfold and tenfold, respectively, to mirror those of the infant digestion process (45). Similarly, gastric phospholipids and bile salts were tenfold reduced. Formulas were incubated 2 hours for both the simulated gastric and duodenal phases. Immediately after duodenal incubation, the digests were centrifuged (3000 g, 15 min, 4°) and the floating lipid layer was removed. The resulting protein digests were purified using C18 reversed phase pre-packed cartridges (Sep-Pak, Waters, Milford, MA, USA). Protein fractions were recovered in 70% acetonitrile/0.1% trifluoroacetic acid and finally vacuum-dried. The protein fraction purification steps were carried out with toxin-free disposable devices. To limit the bias potentially induced by LPS contamination we assessed in each study formula-derived protein fraction the LPS content as previously described (14). To remove also traces of LPS contamination a two-phase detergent-based (Triton X-114) extraction was also performed, as previously described (46). To evaluate possible inter-batches variability, three commercially available batches from each study formula were digested *in vitro* and analyzed by nanoflow HPLC coupled with high resolution mass spectrometry (Orbitrap technology).

The protein fraction purified from three different batches of the EHCF were identified from the merged replicate analyses using the Andromeda search engine of MaxQuant open source bioinformatic suite (version 1.6.2.10). Relative number of peptides was inferred by the signal ion count. Analysis and software-assisted identification workflows were performed as previously described (47). Peptide maps were

visualized using the Peptigram web application (http://bioware.ucd.ie/peptigram/).

5.3 Human enterocyte cell lines

Caco-2 cells were obtained from the American Type Tissue Culture Collection (ATCC® HTB-37, Teddington, UK). NCM460 cells were purchased from INCELL Corporation (San Antonio, TX, USA). Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Berlin, Germany) with a high glucose concentration (4.5 g/L) and L-Glutamine, supplemented with 10% fetal bovine serum (FBS, Gibco) 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco), 1% penicillin/streptomycin (Gibco). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2 days.

5.4 Blood sampling and isolation of peripheral mononuclear blood cells

Peripheral blood samples were obtained from six IgE-mediated, challenge proven, CMA infants. Main demographic and clinical features of the study subjects are depicted in Table 2. Blood samples were analyzed in an anonymized manner with the permission of the Ethics Committee of the University Federico II of Naples. Informed written consent was obtained from parents/tutors of each patient. PBMCs were isolated from heparinized peripheral blood (8 ml) by Ficoll density gradient centrifugation (Ficoll Histopaque-1077, Sigma, St. Louis, Missouri, USA). Briefly, cells were stratified on 3 mL of Ficoll and centrifuged 15 minutes at 2000 rpm at room temperature. After centrifugation, the opaque interface containing mononuclear cells was carefully aspirated with a Pasteur pipette and cells were washed with 10 mL of

PBS and centrifuged 10 minutes at 1200 rpm at room temperature. After centrifugation, the upper layer was discarded and PBMCs (2 x 10^5 cells/well) were cultured in duplicates in 96-well plates in 200 µL culture medium (RPMI 1640, Gibco) containing 10% FBS (Gibco), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco), and 1% penicillin/streptomycin (Gibco).

5.5 Human enterocytes stimulation protocol

Caco-2 and NCM460 cells were stimulated after 15 days post-confluence cultured in six-well plates. The epithelial monolayer was stimulated with digested protein fraction derived from different formulas (EHCF, EHWF, HRF, SF, AAF) or with β-lactoglobulin (BLG) or bovine serum albumin (BSA), as control, at 25 µg/mL for 48 hours. Afterward, the supernatants were harvested and stored at −20°C for further use. Cells with only medium were also used as negative control. BLG and BSA were purchased from Sigma (Sigma-Aldrich, Milan, Italy). To remove endotoxin from BSA and BLG, a two-phase detergent-based (Triton X-114) extraction was performed, as previously described (46). PBMCs were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 days, and stimulated with 25 µg/mL of digested protein fraction derived from the study formulas (EHCF, EHWF, HRF, SF, AAF) or with BLG or BSA, as control. Cells with only medium were also used as negative control. After incubation period, culture supernatants were collected to assess Tregs and Th1 and Th2 cytokines.

5.6 Transepithelial electrical resistance

To evaluate the monolayer integrity by transepithelial electrical resistance (TEER), 2×10^6 Caco-2 and NCM460 cells per well were seeded on polycarbonate 6-well Transwell® membranes (Corning, Life Science, Kennebunk, USA). The TEER was measured every 24 hours for a total of 72 hours, using an epithelial Volt-Ohm Meter (Millicel-ERS-2, Millipore, Billerica, MA, USA). The measured resistance value was multiplied by the area of the filter to obtain an absolute value of TEER, expressed as $\Omega\ cm^2$ and the TEER values were measured as follows: TEER = (measured resistance value−blank value) × single cell layer surface area (cm^2).

5.7 Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Gibco BRL, Paisley, UK) and reverse transcribed 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 −80°C until use. Quantitative real-time PCR (qRT-PCR) analysis was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Vilnius, Lithuania) to evaluate the gene expression of mucin5AC (Muc5AC; Hs01365616_m1), and tight junction proteins occluding (Hs05465837_g1) and ZO-1 (Hs01551871_m1), CD137 (Hs00155512_m1), NFAT5 (Hs00232437_m1), AP1 (Hs99999141_s1) and NF-kB1 (Hs00765730_m1). The TaqMan probes for these genes were inventoried and tested by Applied Biosystems manufacturing facility (QC). The amplification protocol was 40 cycles of 15s of denaturation at 95°C, 60s of annealing at 60°C, and 60s of elongation at 60°C in a

Light Cycler 7900HT (Applied Biosystems, Grand Island, NY, USA). Data were analyzed using the comparative threshold cycle method. We used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to normalize the level of mRNA expression.

5.8 Assessment of IL-33 and TSLP

The epithelial cell-derived danger signal mediators, IL-33 and TSLP, were assessed in Caco-2 cells culture media, using commercially available ELISA kits specific for human IL-33 (BioVendor Research and Diagnostic Products, Karasek, Brno, Czech Republic; detection limit of 0.2 pg/mL) and for human TSLP (Elabscience Biotechnology Inc. Wuhan, Hubei; detection limit of 18.75 pg/mL), respectively. The ELISAs were conducted according to the manufacturer's recommendations.

5.9 Assessment of Th1/Th2 cytokines in PBMCs culture supernatant

The concentrations of IL-4 and IL-10 were measured with a Human IL-4/IL-10 Enzyme immunoassay kit (Boster Biological Technology, Ltd., Fremont, CA, USA). The IL-5, IFN-γ and IL-13 concentrations were measured using the human ELISA assay kit (BioVendor). The minimum detection concentrations were 1.5 pg/mL for IL-4, 7.8 pg/mL for IL-5, 0.5 pg/mL for IL-10, 0.99 pg/mL for IFN-γ and 0.7 pg/mL for IL-13. The ELISAs were conducted according to the manufacturer's recommendations.

5.10 Treg population analysis by flow cytometry and cell sorting

Tregs were identified as $CD4^+/CD25^+/FoxP3^+$ cells by flow cytometry analysis. The staining was performed using Treg detection human kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and the results analyzed by BD CANTO II flow cytometer and DIVA software (Becton-Dickinson, Franklin Lakes, New Jersey, USA). A total of 100,000 events were acquired for analysis, after gating of lymphocytes based on the FSC/SSC dot plot. For sorting, labeled cells were sorted using a FACs Aria I sorter (BD Biosciences). For Treg isolation, a $CD4+CD25^{veryhi}$ gate was used and sorted cells were collected in media (RPMI/20% FBS), washed once and suspended in culture media.

5.11 DNA methylation analysis

DNA was extracted from sorted Tregs from stimulated PBMCs, using the DNA Extraction Kit (GE Healthcare). One microgram of extracted DNA 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 −70°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 IFN-γ, IL-10 and *FOXP3* in Treg-specific-demethylation-region (TSDR) are reported elsewhere (36). High-resolution melting real-time PCR for methylation analysis was performed as

described previously (48). 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%).

5.12 Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether variables was normally distributed. Descriptive statistics were reported as means and standard deviations (SD) for continuous variables. 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 data were collected in a dedicated database and analyzed by a statistician using GraphPad Prism 7 (La Jolla, CA, USA).

6. Results

6.1 Study formulas protein fraction evaluation

Potential batch-to-batch variability of the protein fraction for the study formulas was investigated. No relevant batch-to-batch protein fraction variations were observed for the five study formulas (Fig.2A-B). Protein fraction from EHCF and EHCF digests resulted in 61 and 40 casein-derived peptides, which primarily originated from β-casein and to a lesser extent from α_{s1}-casein and α_{s2}-casein, respectively (Table 3). The patterns of peptides in EHCF and EHCF digests were substantially unmodified among three different sample batches, as confirmed by comparative analysis of the

chromatograms (Fig.2A). Apart from the 13 amino acids-long α_{s1}-casein f(174-176), no peptide longer than 10 amino acid residues was detected in EHCF. The peptide maps of casein fragments in EHCF and EHCF digests were visualized using Peptigram plots, where the green shade intensity indicates the peptide amount (Fig.3). A detailed map of the β-casein-derived sequences showed that peptide fragments originated from the β-casomorphin region are among the most abundant in EHCF and for the greatest part they survived the simulated infant gastroduodenal digestion. However, as showed in Fig.4, we confirmed the absence of oligopeptides in AAF at detectable amount.

6.2 Gut barrier integrity

Protein fraction from different study formulas did not affect intestinal epithelial permeability, as demonstrated by TEER measurement up to 72 hours of incubation in Caco-2 cells (Fig.5).

Occludin expression was significantly up-regulated by protein fraction from EHCF and SF (Fig.6A), whereas only EHCF-derived protein fraction induced an increased expression of ZO-1 (Fig.6B). Similarly, only EHCF digests upregulated the expression of Muc5AC in Caco-2 cells (Fig.6C).

Experiments performed with NCM460 cell line confirmed what observed in Caco-2 cell line: the protein fraction from different study formula was unable to modulate TEER (Fig.7A). EHCF-derived protein fraction upregulated occludin (Fig.7B), ZO-1 (Fig.7C), while SF was able to stimulate only the expression of occludin (Fig.7B).

6.3 Epithelial cell-derived danger signal mediators

Protein fraction derived from EHWF and HRF elicited an increase in TSLP production in Caco-2 cells (Fig.8A). IL-33 release was increased by protein fraction from HRF and SF (Fig.8B). No modulation of these two cytokines was observed exposing human enterocytes to the protein fraction from the other study formulas.

6.4 Th1 and Th2 cytokines response in PBMCs from CMA infants

All protein fractions from different study formulas were unable to modulate IL-4, IL-5 and IL-13 production in PBMCs from IgE-mediated CMA infants (Fig.9A-C). Conversely, only EHCF-derived protein fraction was able to significantly up-regulate the IFN-γ and IL-10 production (Fig.9D-E). IL-10 and IFN-γ data were further evaluated according to casein sensitization status of CMA patients and no differences were observed comparing PBMCs response in patients with or without casein sensitization. No modulation in DNA methylation status of IFN-γ and IL-10 genes was observed for EHCF, and for other study formulas.

6.5 Tregs activation in PBMCs from CMA infants

Only EHCF-derived protein fraction was able to significantly increase $CD4^+/CD25^{+/}FoxP3^+$ Tregs number and up-regulated CD137 expression, a marker of activated Tregs (Fig.10A-B). The effect paralleled with an up-regulation of *FOXP3* demethylation rate in TSDR (Fig.10B). Protein fractions from other study formulas were unable to modulate $CD4^+/CD25^+/FoxP3^+$ Tregs and its demethylation status in PBMCs from CMA infants (Fig.10A-C). In addition, we found that only EHCF-

derived protein fraction significantly increased *NFAT, AP1 and NF-kB1* expression in Tregs from CMA children, further confirming a specific activation of Tregs through this intracellular pathway (Fig.11).

7. Discussion and conclusions

Our findings demonstrate a different modulation on tolerogenic mechanisms elicited by the protein fraction derived from the formulas commonly used for CMA management.

The gut barrier-related non-immune mechanisms, such as epithelial permeability and mucus thickness, are considered relevant in preserving immune tolerance (49). Loss of gut barrier integrity increases antigen uptake and promotes Th2-type allergic response by activation of type 2 innate lymphoid cells (ILC2s), mast cells, basophils, and dendritic cells (DCs) (50). We found that EHCF-derived protein fraction could improve gut barrier integrity increasing occludin, ZO-1 and Muc5AC expression in human enterocytes. These data are well in line with previous observation reporting the up regulation of Muc5AC expression after stimulation with casein hydrolysate in human enterocytes (51). Of note, we observed that also SF-derived protein fraction stimulated occludin expression. Similarly, it has been previously demonstrated that peptides derived from fermented soybean improved epithelial barrier function, enhancing occludin expression (52). The other study formulas (EHWF, HRF, and AAF) were unable to modulate these components of gut barrier.

Epithelium-derived cytokines, including TSLP and IL-33, have a pivotal role in the development of allergic response at gut barrier surface and have been linked to the

pathogenesis of type 2 inflammatory diseases, including food allergy and asthma (50). We found that protein fraction derived from EHWF, HRF and SF elicited an increase in TSLP and/or IL-33 production. In contrast, the protein fractions from EHCF and AAF were unable to modulate the expression of such cytokines.

To further investigate the immunomodulatory effect elicited by protein fraction from different study formulas, Th1 and Th2 cytokines production was measured in PBMCs from IgE-mediated CMA infants. It has been already demonstrated that allergen specific stimulation induced an immune reactivity in PBMCs from IgE-mediated CMA pediatric patients (53). All protein fractions from the different study formulas were unable to elicit Th2 cytokines response. But, only EHCF-derived protein fraction significantly enhanced the production of the Th1 cytokine IFN-γ and of the key mediator of immune tolerance IL-10. Similarly, it has been demonstrated that casein hydrolysates could induce a significant increase of IL-10 levels in rats (54), but the results were not confirmed by others (14).

Clinical trials demonstrated that EHCF could accelerate immune tolerance acquisition in CMA children if compared with other dietary strategies through, at least in part, an epigenetic modulation of *FOXP3* gene (16,36). $CD4^+/CD25^+/FoxP3^+$ Tregs are central in the maintenance of immune homeostasis and tolerance. It has been demonstrated that the enzymatic digest of milk caseins was able to induce immune tolerance in the mouse model (55). We found that only EHCF-derived protein fraction elicited a significant activation of $CD4^+/CD25^+/FoxP3^+$ Tregs, through DNA demethylation of the *FOXP3* transcription factor. This mechanism could be related to the activation of the transcription factor complex NFAT/NF-kB/AP1 on T cells partially methylated in *FOXP3* promoter (56,57). Other study formulas (EHWF, HRF,

SF and AAF) were unable to modulate such mechanism.

Amino acid-based formulas, consisting of essential and nonessential amino acids, are currently recommended for infants who do not tolerate extensively hydrolyzed formulas or with multiple and/or extremely severe or life-threatening food allergies (58). Despite AAF is considered the safer dietary strategy for severe CMA infants, experimental and clinical data suggest that this formula is unable to promote tolerogenic effects, substantially due to the absence of peptides (16,58). Indeed, using nanoflow-HPLC-MS/MS and HPLC-UV approach, we confirmed the absence of oligopeptides in AAF at detectable amount. Lower lymphocytes count in small intestine lamina propria, decreased Payer's patches size, lower number of $CD4^+$ $FoxP3^+$ Treg cells, with higher level of specific IgE against dietary antigens and higher severity of AMs have been previously associated with the use of AAF (59). The results of the present study, showing absence of effects on gut barrier components, Th1/Th2 cytokines response and Tregs activation, are well in line with these previous findings. A study limitation is due to the fact that we didn't identify which specific EHCF-derived peptides or amino acid sequences were responsible for the tolerogenic effects. However, we performed a characterization of EHCF-derived peptides and we detected the presence of several peptides with a previously described immunomodulatory action. A relatively high number of peptides in the EHCF-derived protein fraction derived from the β-casomorphin domain of β-casein, which is considered a "strategic" sequence because it can release potentially potent bioactive peptides (60,61). β-casomorphins and related peptides detected in EHCF may also prevent the uptake of luminal antigens, as they stimulate intestinal mucin production (67). Additional EHCF-derived peptides were β-casein f(134-138) (sequence: HLPLP), and β-casein f

(177-183) (sequence: AVPYPQR) (44). The peptides from both these regions have been found to inhibit ACE and may act on the immune system by preventing the breakdown of bioactive bradykinin, which importantly contributes to immunoregulation by inducing maturation of DCs by driving Th1 oriented-response (68). Noteworthy, several EHCF-derived peptides shared the common amino acid motif PFP (Pro-Phe-Pro), deriving from three different positions within β-casein, namely 61-63, 110-112 and 204-206. The amino acid motif PFP has been previously indicated as the possible determinant of the immunoregulatory activity of β-casein regions (69,70). Another limitation of the study could be related to the lack of identification of human cell receptors involved in the tolerogenic response. Cow's milk protein hydrolysates binding on TLRs has been demonstrated in epithelial cells (7), but more research is needed to evaluate the direct interaction with TLRs on immune cells. Lastly, we didn't explore the effect of other components of commercially available formulas for CMA treatment potentially able to elicit tolerogenic effects, such as polyunsaturated fatty acids or vitamin D (71). It should be considered that these components occur at very similar concentration among different commercially available formulas. For that reasons, we focused only on the protein fraction, which largely differ among the commercially available formulas for CMA treatment. Thus, a strength of our study is that we investigated the effect deriving by the direct interaction of purified protein fractions with human cells. This could have reduced the bias of previous similar observation exploring the effects of whole infant formulas on human cells (14).

In conclusion, our results suggest a different regulatory action on immune and non-immune tolerogenic mechanisms elicited by protein fraction from different formulas

for CMA management. In particular, we found that EHCF-derived protein fraction could activate several tolerogenic mechanisms through, at least in part, an epigenetic regulation of gene expression. These results could explain the beneficial effects observed on immune tolerance acquisition in CMA patients, and on allergy prevention in children at risk for atopy (3-5,16). The precise identification of EHCF peptides responsible for these effects, together with a better definition of the tolerogenic mechanisms elicited by such peptides, could provide pivotal information and could inspire the composition of next generation hypoallergenic formulas for the prevention and treatment of CMA.

Data Availability

HPLC-MS data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE (ref)repository and can be accessed with identifier PXD023355 (51).

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9. Tables and figures

Table 1. Main features of the study formulas.

Product	Manufacturer	Source	Condition	Protein content (g/100 g)	Endotoxin content (EU in 100 µg protein)
Nutramigen	Mead Johnson Nutrition	C	EH	14.0	0.036
Alfarè	Nestlè	W	EH	14.8	0.039
Blemil	Ordesa	R	PH	12.0	0.034
Sinelac	Humana	S	FP	12.6	0.035
Neocate	Nutricia	AA	AA	12.3	0.041

C, casein; EH, extensively hydrolyzed; W, whey; R, rice; S, soy; PH, partially hydrolyzed; FP, full proteins; AA, amino acids.

Table 2. Baseline features of the study population.

	Subjects with IgE-mediated CMA
N.	6
Male, n (%)	2 (33.3)
Born at term, n (%)	6 (100)
Caesarean delivery, (%)	2 (33.3)
Breastfed for at least 2 months, n (%)	6 (100)
Ethnicity, Caucasian, n (%)	6 (100)
Mean age at enrollment, months (±SD)	5.2 (0.9)
Body weight, mean (kg) (±SD)	6.8 (1.1)
Length, mean (cm) (±SD)	63.4 (3.9)
Head circumference (cm) (±SD)	42.7 (2.1)
Positive prick by prick test for fresh cow's milk	6 (100)
Positive skin prick test for α-lactalbumin	5 (83.3)
Positive skin test positive for β-lactoglobulin	6 (100)
Positive skin prick test positive for casein	3 (50)
Total serum IgE, kU/l (±SD)	236.3 (102.1)
Specific IgE for α-lactalbumin, kU/l (±SD)	6.5 (1.1)
Specific IgE for β-lactoglobulin, kU/l (±SD)	35.3 (8.9)
Specific IgE for casein, kU/l (±SD)	4.8 (6)
Gastrointestinal symptoms at CMA onset, n (%)	4 (66.6)
Cutaneous symptoms at CMA onset, n (%)	2 (33.3)
Respiratory symptoms at CMA onset, n (%)	1 (16.7)

CMA: cow's milk allergy; SD: standard deviation

Table 3. List of casein-derived peptides identified in EHCF and EHCF_GD by nanoflow-HPLC-MS/MS.

EHCF peptides	**EHCF_GD digests peptides**	**position within the protein**		**position without pro-peptide**	
β-casein		**start**	**end**	**start**	**end**
EPVLGPV		210	216	195	201
FPGP	FPGP	77	80	62	65
FPGPI	FPGPI	77	81	62	66
	FPGPIP	77	82	62	67
FPGPIPN	FPGPIPN	77	83	62	68
FPK	FPK	126	128	111	113
FPP		172	174	157	159
FPPQ	FPPQ	172	175	157	160
HLPLP	HLPLP	149	153	134	138
HQPHQPLPP	HQPHQPLPP	160	168	145	153
HQPHQPLPPT	HQPHQPLPPT	160	169	145	154
KEMPFPK		122	128	107	113
LPLL		152	155	137	140
LPLP	LPLP	150	153	135	138
LPLPL		150	154	135	139
LPQ	LPQ	85	87	70	72
LPVPQ	LPVPQ	186	190	171	175
LPVPQK		186	191	171	176
MPFP	MPFP	124	127	109	112
PFP	PFP	76	78	61	63
PFPGP		76	80	61	65
PFPGPI	PFPGPI	76	81	61	66

PFPGPIP	PFPGPIP	76	82	61	67
PFPGPIPN	PFPGPIPN	76	83	61	68
PFPGPIPNS		76	84	61	69
PGPIP		78	82	63	67
PHQPLPPT	PHQPLPPT	162	169	147	154
PLP	PLP	151	153	136	138
PVP		187	189	172	174
PVPQ		187	190	172	175
PVPQK		187	191	172	176
PVVVP	PVVVP	96	100	81	85
PVVVPP	PVVVPP	96	101	81	86
PYP		194	196	179	181
PYPQ		194	197	179	182
	RGPFP	217	221	202	206
TPVVVP		95	100	80	85
TPVVVPP		95	101	80	86
VLPVP		185	189	170	174
VLPVPQ	VLPVPQ	185	190	170	175
VPQ	VPQ	188	190	173	175
VPYP	VPYP	193	196	178	181
VPYPQ	VPYPQ	193	197	178	182
	VYPFPGPI	74	81	59	66
YPFP	YPFP	75	78	60	63
YPFPGP	YPFPGP	75	80	60	65
YPFPGPI	YPFPGPI	75	81	60	66
YPFPGPIP	YPFPGPIP	75	82	60	67
YPFPGPIPN	YPFPGPIPN	75	83	60	68

YPQ		195	197	180	182
YPVEP	YPVEP	129	133	114	118
YPVEPF		129	134	114	119
as1-casein		**start**	**end**	**start**	**end**
APFP	APFP	41	44	26	29
APFPE		41	45	26	30
DIPNP		196	200	181	185
	EEIVPN	84	89	69	74
IPNPI	IPNPI	197	201	182	186
IPNPIG	IPNPIG	197	202	182	187
NPI	NPI	199	201	184	186
SDIPNPI		195	201	180	186
TDAPSFSDIPNPI	TDAPSFSDIPNPI	189	201	174	186
as2-casein		**start**	**end**	**start**	**end**
QKFPQ		105	109	90	94
VPITP	VPITP	132	136	117	121
VPITPT	VPITPT	132	137	117	122

Figure 1. Graphical abstract.

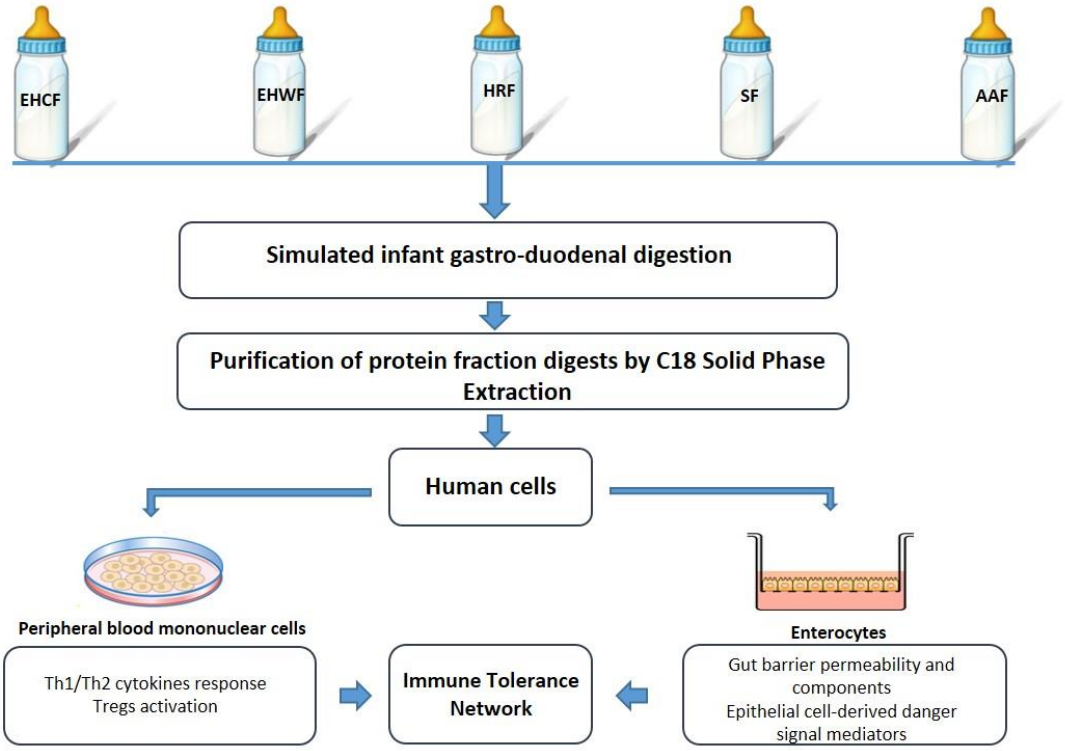
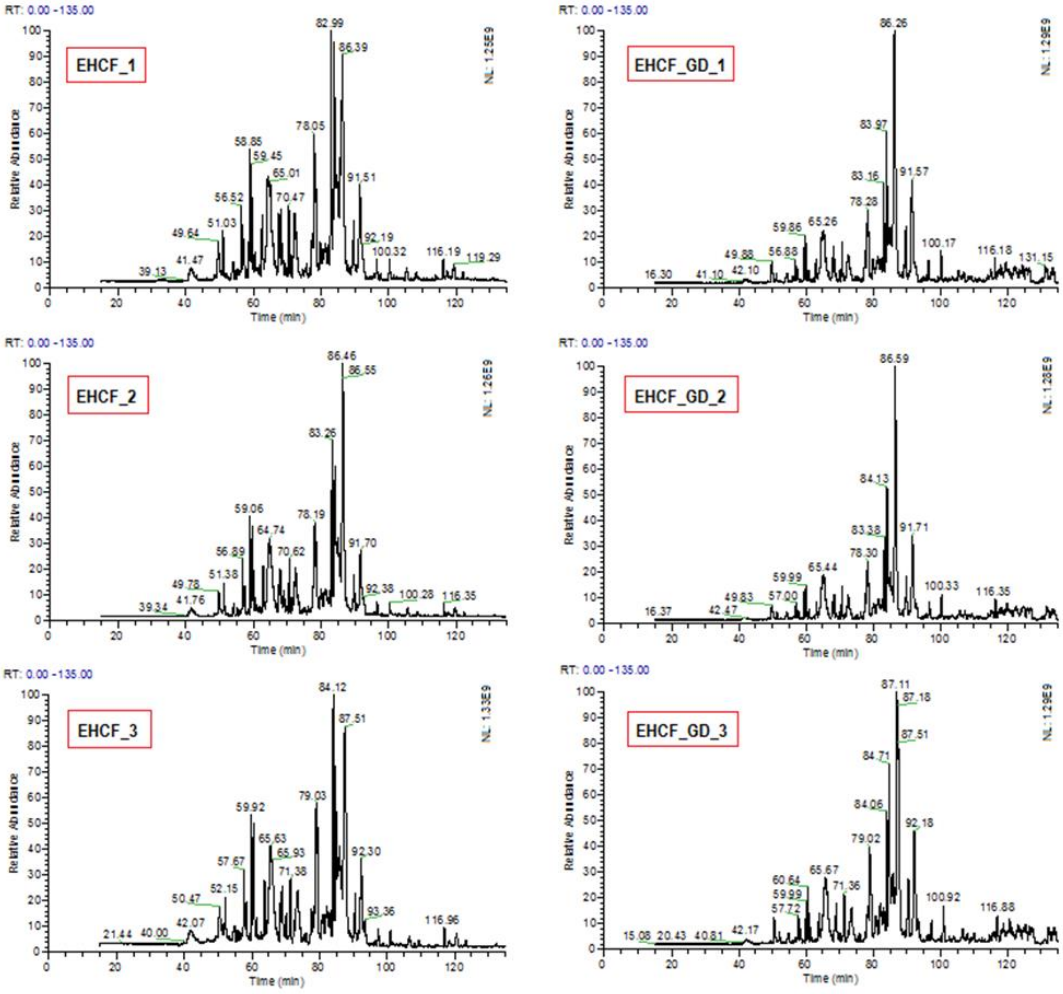
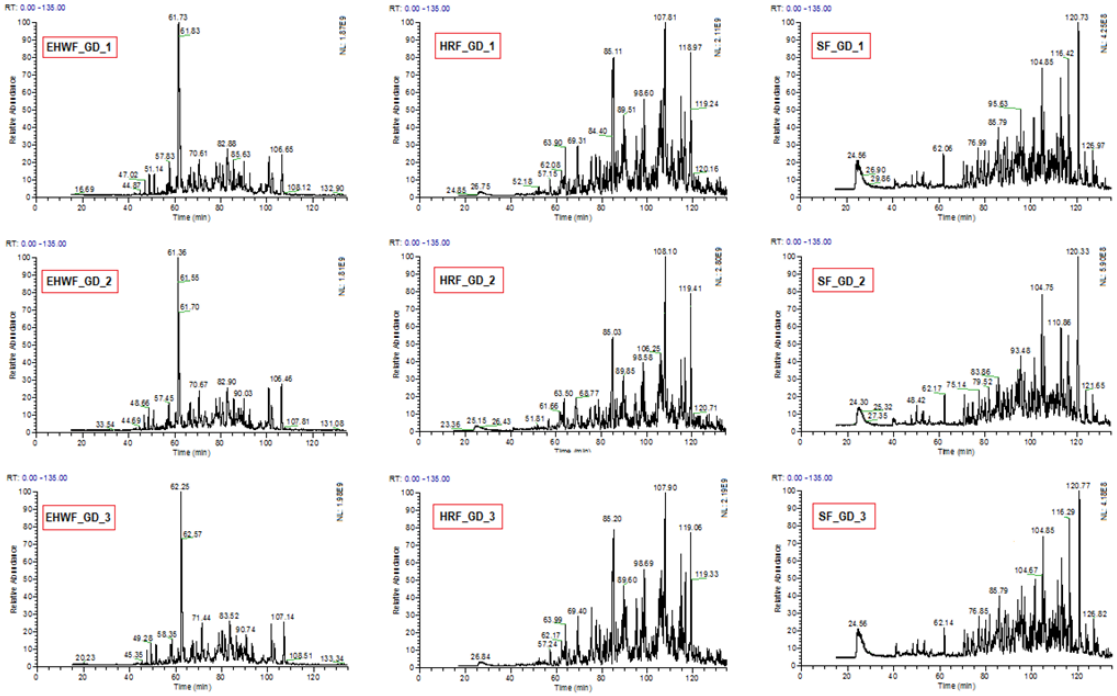


Figure 2A.



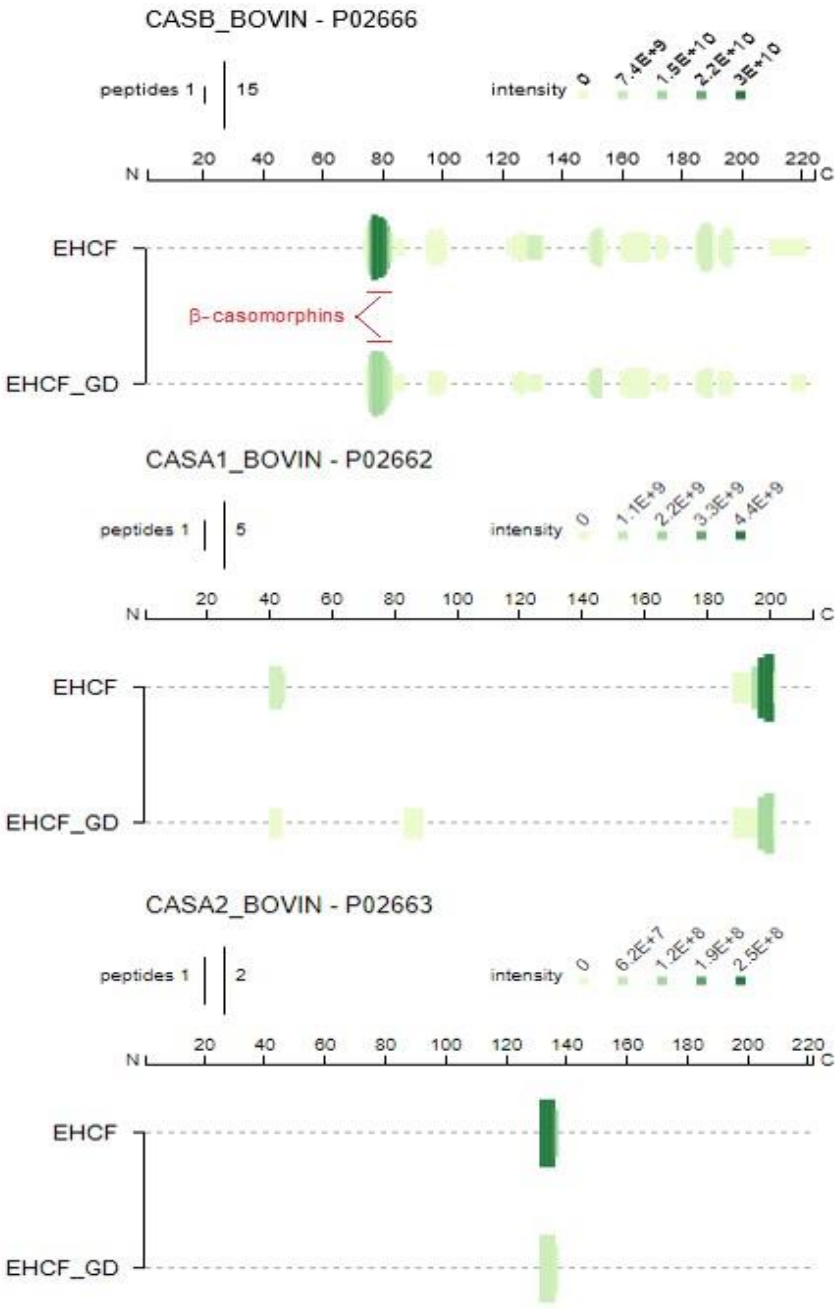
Total ion current (TIC) nanoflow-HPLC-MS/MS chromatograms of peptides purified from three batches of EHCF (left panels) and from the corresponding EHCF gastroduodenal digests (EHCF_GD, right panels). The strict similarity of samples purified from the three different batches supports the substantial equivalence of the extracts used in triplicate biological assays.

Figure 2B.



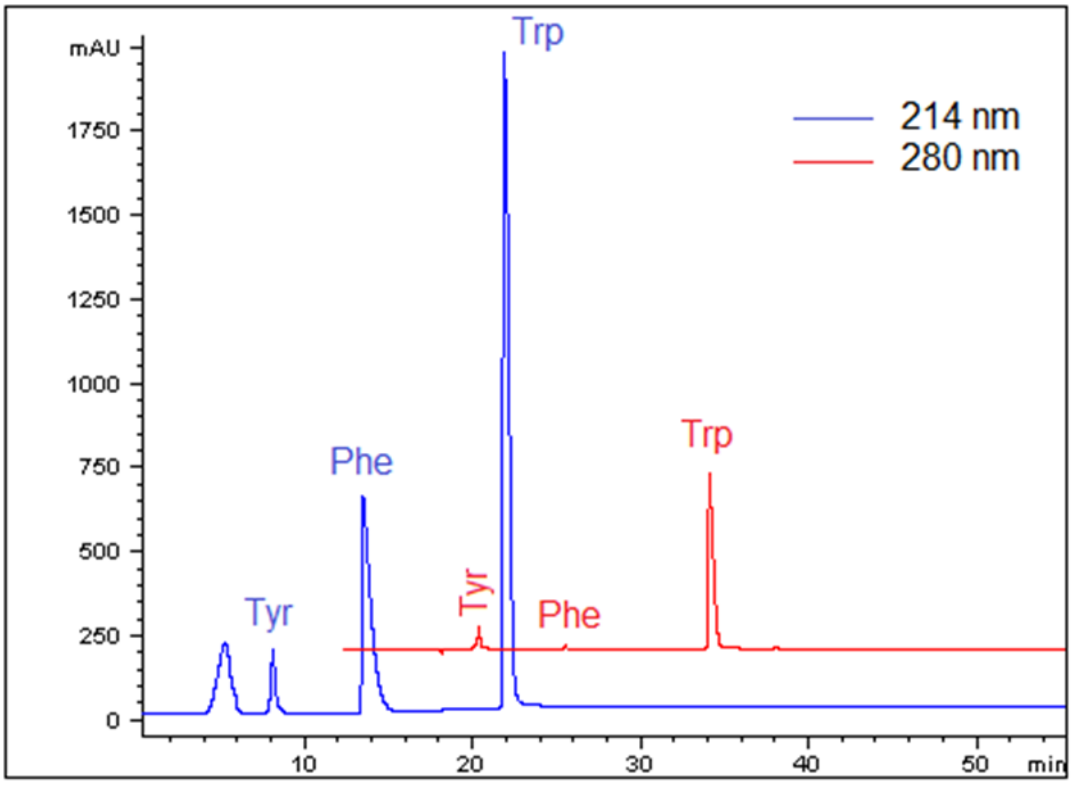
Total ion current (TIC) nanoflow-HPLC-MS/MS chromatograms of three different preparations of *in vitro* gastroduodenal digests (infant model) from extensively hydrolyzed whey (EHWF), hydrolized rice (HRF) and soy-based (SF) formulas from three batches. The comparative analysis demonstrated very low inter-batches variability.

Figure 3. Schematic comparison (Peptigrams) of the peptide entries identified in EHCF and EHCF *in vitro* digested peptides.



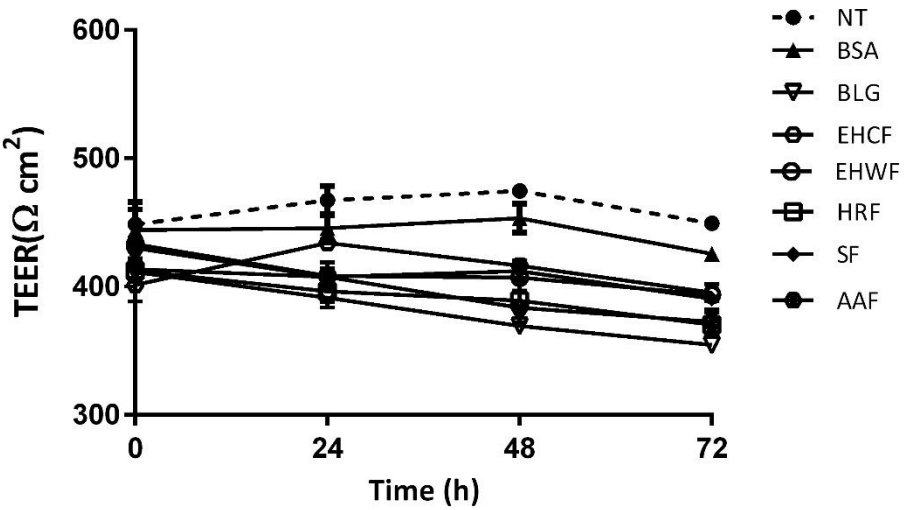
Maps have been visualized using the open source tool Peptigram (http://bioware.ucd.ie/peptigram/).

Figure 4. HPLC-UV analysis of amino acid-based formula.



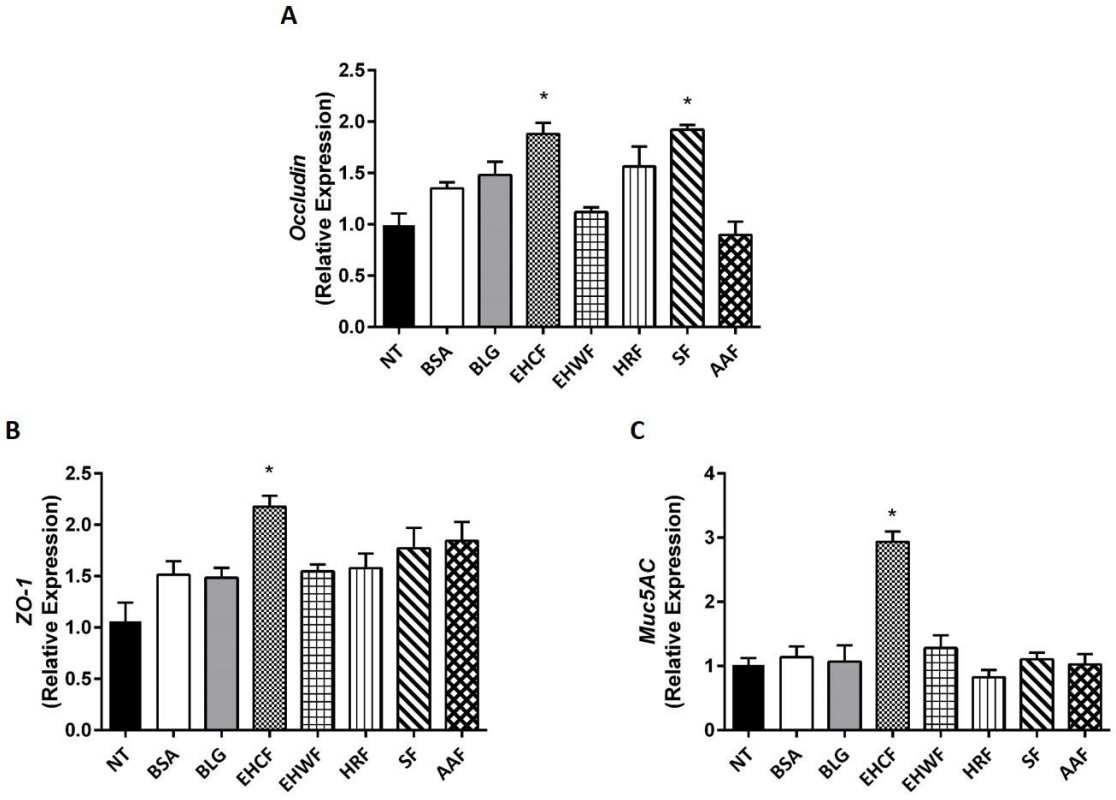
Chromatograms were recorded at 214 and 280 nm, corresponding to the UV absorption bands of peptide bonds and aromatic rings. The analysis confirmed the absence of oligopeptides, also including di-/tri-peptides, at detectable amount.

Figure 5. Effect of protein fractions derived from study formulas on transepithelial electric resistance (TEER) as biomarker of intestinal epithelial permeability in Caco-2 cells.



Protein fraction (25 µg/ml) from different study formulas did not affect intestinal epithelial permeability, as demonstrated by TEER measurement up to 72 hours of incubation. The TEER values were measured as follows: TEER = (measured resistance value−blank value) × single cell layer surface area (cm^2). Data represent the means with SD of 3 independent experiments, each performed in triplicate.

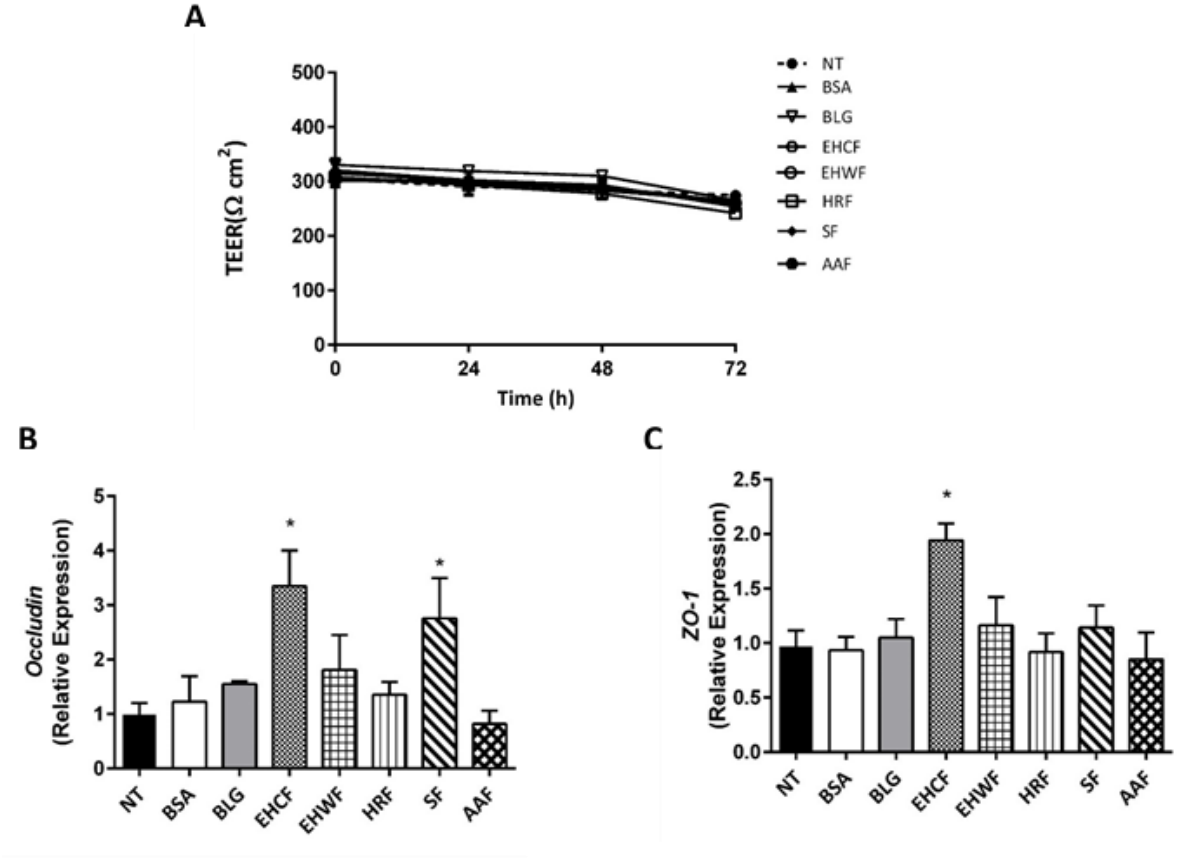
Figure 6. Modulation of gut barrier components (occludin, ZO- 1 and Muc5AC) elicited by the study formulas-derived protein fraction in human enterocytes (Caco-2 cells).



The 48h incubation of Caco-2 cells with EHCF-derived protein fraction (25 µg/ml) stimulated the production of all three components of gut barrier that were investigated in Caco-2 cells monolayer: Occludin **(A)**, Zonula occludens 1 (ZO-1) **(B)** and Muc5AC **(C)**. SF was able to stimulate the expression of occludin only **(A)**. The other three study formulas were unable to modulate the three gut barrier components.

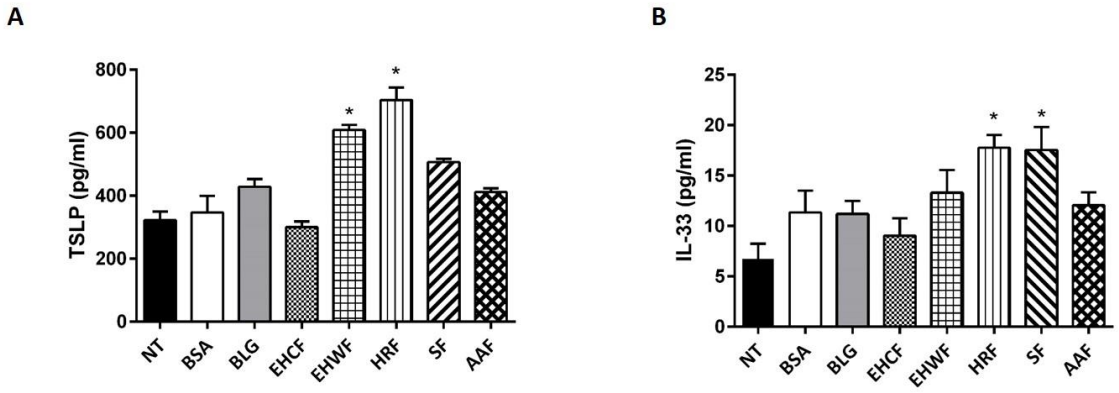
Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using the paired t-test. *p<0.05 vs untreated cells (NT)

Figure 7. Effect of protein fractions derived from study formulas on transepithelial electric resistance (TEER) and tight junction proteins (occludin and ZO-1) as biomarkers of intestinal epithelial permeability in normal human colon epithelial cell line (NCM460).



Protein fraction (25 µg/ml) from different study formulas did not affect intestinal epithelial permeability in NCM460 cell line, as demonstrated by TEER measurement up to 72 hours of incubation **(A)**. The TEER values were measured as follows: TEER = (measured resistance value−blank value) × single cell layer surface area (cm^2). The 48h incubation with EHCF-derived protein fraction (25 µg/ml) stimulated Occludin **(B)** and Zonula occludens 1 (ZO-1) **(C)** expression. SF was able to stimulate the expression of occludin only **(B)**. The other three study formulas were unable to modulate the tight junction proteins expression. Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using the paired t-test. *p<0.05 *vs* untreated cells (NT).

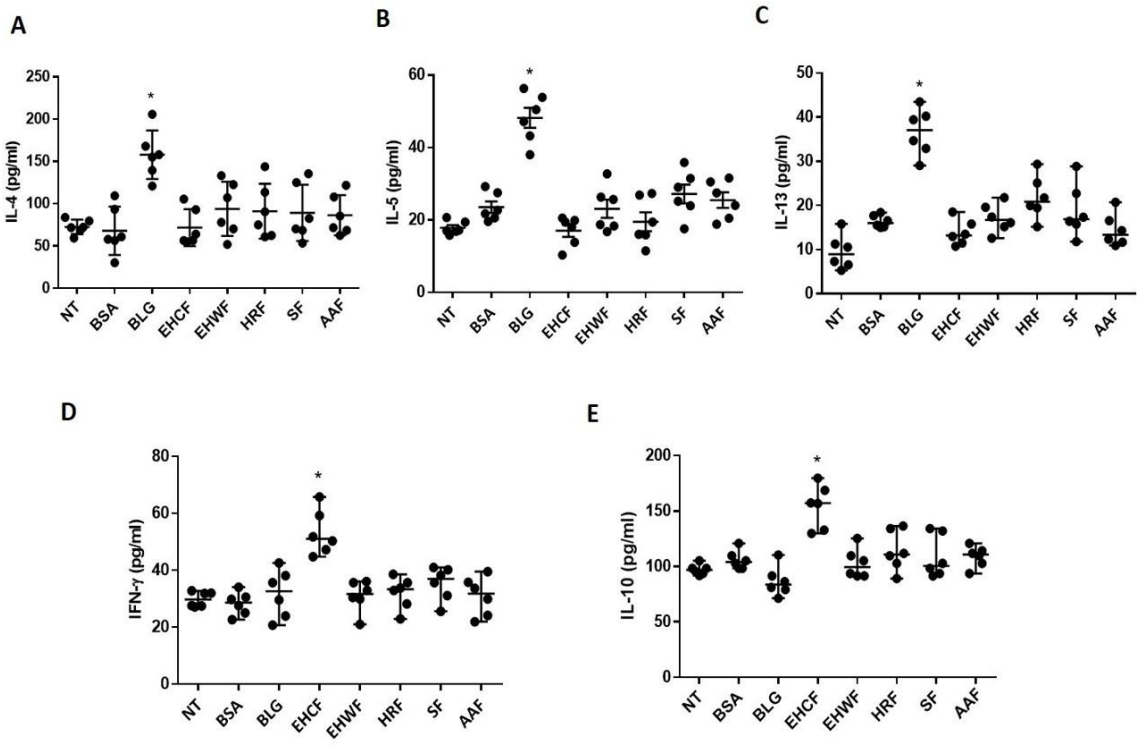
Figure 8. Modulation of the danger signal mediators thymic stromal lymphopoietin and interleukin 33 in human enterocytes (Caco-2 cells).



The 48h incubation of Caco-2 cells with EHRF-derived protein fraction (25 µg/ml) resulted in a stimulation of both biomarkers of gut epithelial cells danger signal mediators production: thymic stromal lymphopoietin (TSLP) **(A)** and interleukin (IL)-33 **(B)**. TSLP production resulted also increased upon stimulation with 25 µg/ml EHWF **(A)**, whereas IL-33 production resulted increased stimulating Caco-2 cells with 25 µg/ml SF **(B)**. EHCF and AAF-derived protein fractions were unable to modulate the two biomarkers.

Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using the paired t-test. *$p<0.05$ vs untreated cells (NT).

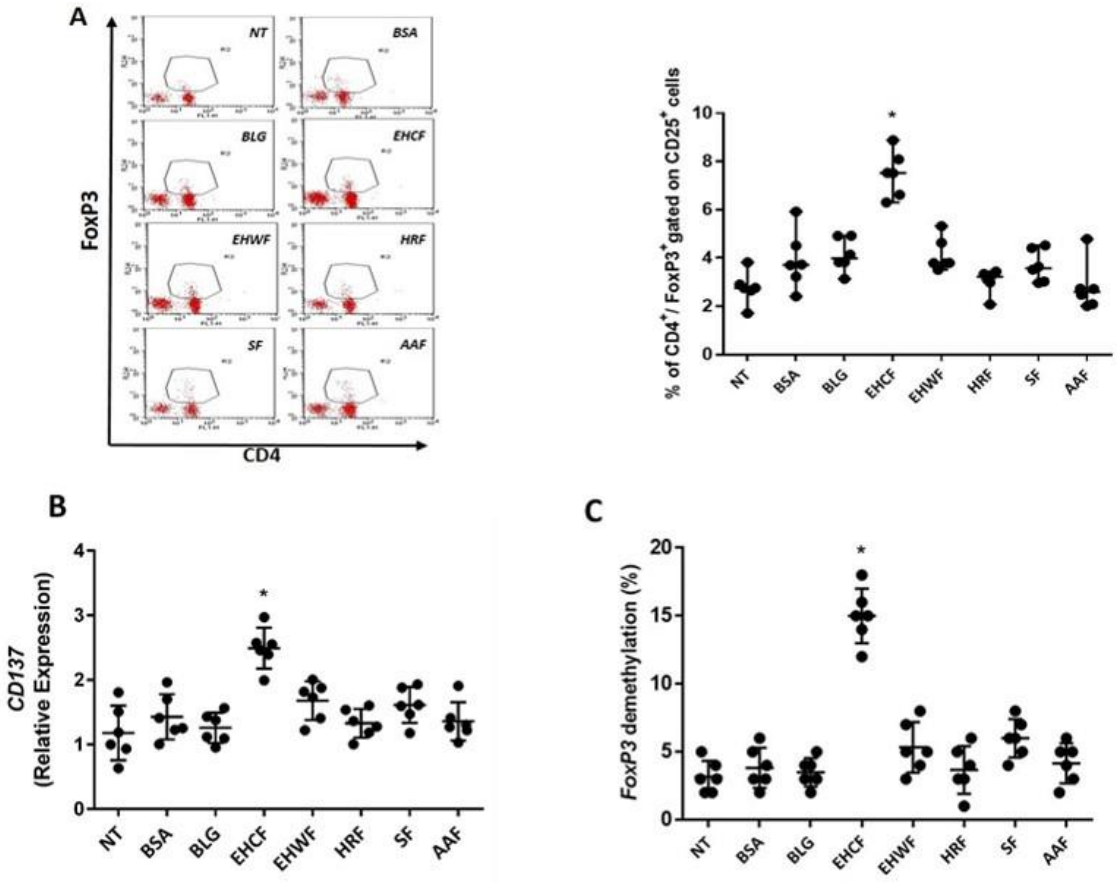
Figure 9. Modulation of Th2/Th1 cytokines production in peripheral mononuclear blood cells from infants affected by cow's milk allergy.



Exposing peripheral mononuclear blood cells (PBMCs) collected from six infants affected by IgE-mediated cow's milk allergy (CMA) for 5 days with 25 μg/ml β-lactoglobulin (BLG) resulted in a significant increase of all Th2 cytokines production: IL-4 **(A)**, IL-5 **(B)**, IL-13 **(C)**. The protein fraction derived from the study formulas were unable to increase the production of Th2 cytokines. Whereas, only EHCF-derived protein fraction was able to increase IL-10 **(D)** and IFN-γ **(E)** production.

Each data point represents the single patient response. Horizontal bars represent the means with SD obtained within each group. Data were analyzed using the paired t-test. *$p<0.05$ vs untreated cells (NT).

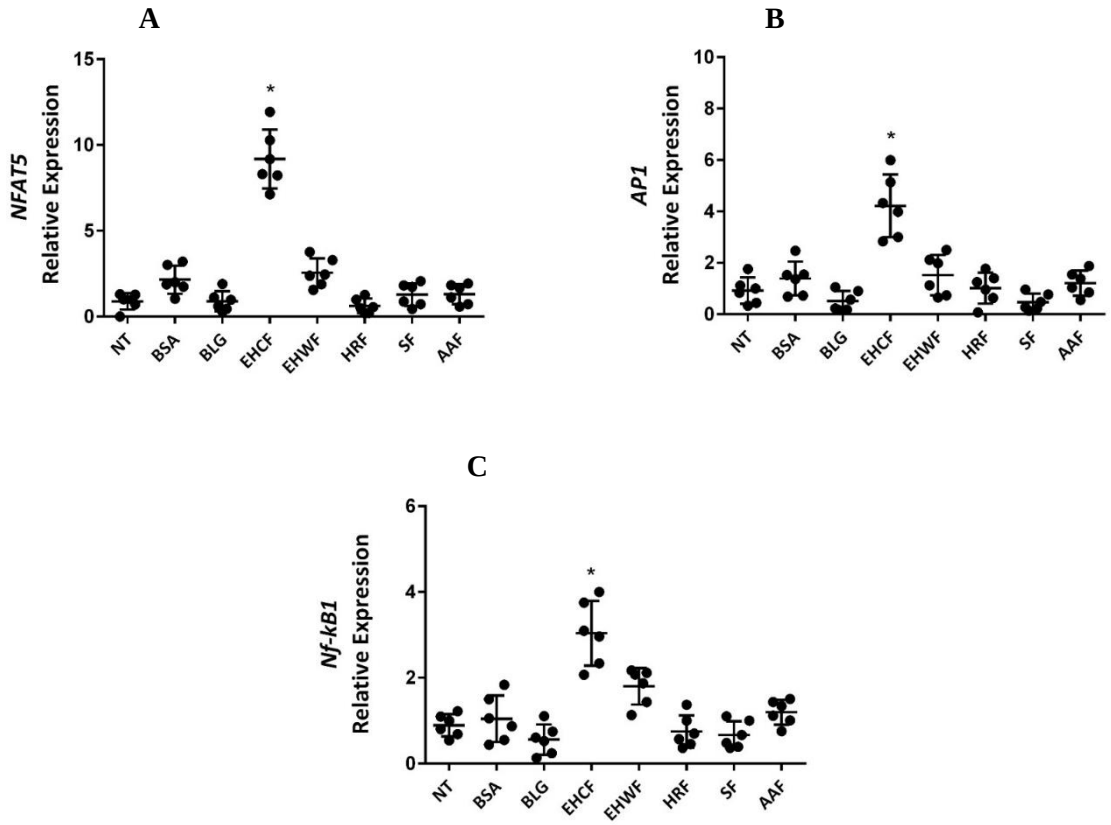
Figure 10. Regulatory T cells activation in peripheral mononuclear blood cells from infants affected by cow's milk allergy.



Exposing peripheral mononuclear blood cells (PBMCs) collected from six infants affected by IgE-mediated cow's milk allergy (CMA) for 5 days with 25 µg/ml EHCF-derived protein fraction resulted in a stimulation of $CD4^+/CD25^+/FoxP3^+$ cells number **(A)**. An upregulation of CD137 expression, a marker of Tregs activation, was observed in PBMCs stimulated only EHCF-derived protein fraction **(B)**. The effect paralleled with an up regulation of *FOXP3* demethylation rate in Treg-specific-demethylation-region (TSDR) **(C)**. All other study formula-derived protein fractions were unable to modulate regulatory T cells activation.

Each data point represents the single patient response. Horizontal bars represent the means with SD obtained within each group. Data were analyzed using the paired t-test. *$p<0.05$ vs untreated cells (NT).

Figure 11. **Effect of study formulas-derived protein fraction on the transcription factor complex *NFAT5, AP1* and *NF-kB1* expression.**



Exposing peripheral mononuclear blood cells (PBMCs) collected from six infants affected by IgE-mediated cow's milk allergy for 5 days with 25 μg/ml EHCF-derived protein fraction resulted in increase of the transcription factor complex *NFAT5* **(A)**, *AP1* **(B)**, *NF-kB1* **(C)** expression.

Each data point represents the single patient response. Horizontal bars represent the means with SD obtained within each group. Data were analyzed using the paired t-test. **p*<0.05 *vs* untreated cells (NT).