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Safety and tolerability of the probiotic formula I-PROB

Mastrandrea F[1], Cadario G[2], Coradduzza G[1], Casino G[3], Cesinaro Di Rocco P[4], Condoluci M[5], Cortellini G[6], D'Ippolito G M[7], Di Rienzo V[8], Foscherini P[1], Gargano D[9], Greco G[10], Lanza C[11], Marengo F[2], Minardi A[1], Montera MC[12], Pinter E[13], Romano C[14], Scarcia G[1], Spina F[15], Ventura MT[16], Massolo A[17]

[1]Allergy and Clin Immunol C. S., H. SS Annunziata, ASL TA, Taranto, Italy; [2]Allergy and Clin Immunol C. S., A. O. Città della Salute e della Scienza di Torino, P. H. Molinette, Turin, Italy; [3]Allergy U.O., H. Piedimonte Matese, ASL1, Caserta, Italy; [4]Allergy Ambulatory, H. S Spirito, Pescara, Italy; [5]Allergy Specialty Ambulatory, ASP Vibo Valentia, Italy; [6]Internal Medicine and Rheumatology C. S., H. Infermi, Rimini, Italy; [7]Allergy Specialty Ambulatory, H. Francavilla Fontana, ASL BR1, Francavilla Fontana, Brindisi, Italy; [8]Allergy Specialty Ambulatory, ASL FR4, Frosinone, Italy; [9]O.U. Allergy and Clinical Immunology, H. S.G. Moscati, Avellino, Italy; [10]Allergy Specialty Ambulatory, ASP5, Crotone, Italy; [11]PLS, Trebisacce, ASP CS, Italy; [12]O. U. Allergy and Clinical Immunology, H. Mercato San Severino, ASL SA, Salerno, Italy; [13]Dpt. Clinical Medicine, University of Rome La Sapienza, Rome, Italy; [14]Allergy A., ASL NA3sud, Distr.59 Napoli, Italy, ; [15]H. Rossano, ASP Cosenza, Cosenza, Italy; [16]Dpt. Internal Medicine, Immunology and Infectious Diseases, University of Bari, Bari, Italy; [17]Dpt. Ecosystem and Public Health, University of Calgary, Calgary, AB, Canada

Abstract

Background: probiotics are widely studied for the prevention and treatment of allergic diseases even if the heterogeneity of results does not allow firm conclusions yet.

Objective: to evaluate safety and tolerability of the multi-strain probiotical formula *I-Prob* (primary end-point) and explore favorable immunological effects potential (secondary end-point).

Methods: one hundred and thirty patients (69F-61M, aged 3-82 years, mean age 31), with allergic rhinitis and/or asthma, atopic dermatitis and irritable bowel syndrome (ARIA, GINA, PRACTALL and Rome III classifications) entered consecutively into the study.

They received standard drug treatment plus one sachet a day, for 60 days, of a mixture of *Lactobacillus acidophilus* LA02, *L. delbrueckii subs. bulgaricus* LDB01, *L. rhamnosus* and *Streptococcus thermophilus*. No serious Adverse Event (AE) occurred. Three mild local reactions (2.3 %; 3/129) have been reported (diarrhoea within the first week, spontaneously resolved with the discontinuation of treatment).

Results from the *ex-vivo* investigation were encouraging since, in the 22 subjects studied, values of circulating $CD34^+$ hematopoietic precursor and stem cells measured before (t^0) and after treatment (t^1) reduced significantly (p 0.002); less marked but yet significant (p 0.048) was the reduction of the ratio Th2/Th1 after treatment.

Conclusion

Data indicate that the *add-on treatment* with the investigated probiotic formula is accompanied by an optimal safety and tolerability profile. Observed reduction of $CD34^+$ peripheral cells traffic as well as Th2/Th1 ratio reduction, taken together, may suggest differentiation effects on the recently identified lymphocyte lineages belonging to the innate immunity.

Key Words: Adverse reaction, allergy, asthma, atopic dermatitis, $CD34^+$ cell, hematopoietic precursors, inflammation, innate lymphoid cell, I-Prob, irritable bowel syndrome, intestinal stem cell, probiotic, rhinitis, safety, stem cell, tolerability.

Introduction

The epidemiology of infectious diseases is known to be inversely related to the incidence of Immune Mediated Inflammatory Disorders (IMID) (1). To explain this phenomenon, the "Hygiene hypothesis" has been proposed and recently updated (2); it relates the pandemic increase of allergy and autoimmunity to the reduced microbial exposure, proper of Western' societies lifestyle.

Non-pathogenic bacteria thus have received increasing attention as adjuvant potential for the prevention and treatment of allergy.

Promising results have been reported with probiotics in several human inflammatory diseases but the heterogeneity of studies, strains, duration of administration, doses and patients does not allow comparisons and definitive conclusions.

The present multicentre study has been designed to evaluate safety and tolerability (primary end-point) of the new probiotical formula named *I-Prob*, in a sample of population with a wide range of age and different atopic or atopy-associated inflammatory diseases.

A small sample has been further investigated to explore the capability potential of inducing favorable immunological modification (secondary end-point) on the increased peripheral traffic of Hematopoietic precursor and stem cells (3-11) and on the T-lymphocyte helper 2 and 1 subpopulation polarization.

Study design

The study was pragmatic, observational, prospective for safety and tolerability (primary endpoint), explanatory (12) for the immunological purposes (secondary end-point).

Patients and methods

Subjects were eligible when suffering from allergic Rhinitis (R), Asthma (A), Atopic Dermatitis (AD), food intolerance (Irritable Bowel Syndrome; IBS) with allergen-specific IgE (extrinsic form), or in few cases negative for IgE but with no other identifiable causes of symptoms (intrinsic form).

Diagnosis and severity of allergic rhinitis and asthma were made according to ARIA (13) and GINA criteria (14) as well as atopic dermatitis was assessed using a four-step severity score developed to parallel ARIA and GINA criteria, rating the disease course as intermittent or persistent (15, 16) and taking into account PRACTALL Consensus Report recommendations (17). IBS was defined by Rome III diagnostic criteria (18), and graded on the analogy of other pathologies (four step severity score). A total of 130 patients were enrolled; 65 suffering from a single pathology (31 with Rhinitis alone, 10 with Asthma, 11 with IBS and 13 with Atopic Dermatitis) and 65 with different association of mentioned pathologies.

Skin tests and IgE measurement

Skin prick tests were performed with commercial extracts of Grasses, *Parietaria judaica*, Olive, Mugwort, *Cupressus spp.*, *Plantago lanceolata*, *Alternaria spp.*, *Dermatophagoides pteronyssinus* and *D. farinae* . The skin test response was examined after 15 and 30 minutes and expressed according to a scale of 0 to 4, where 0 indicated a wheal similar to the wheal elicited by the negative control, and 3 a wheal similar to that elicited by 0.1% histamine.

When necessary, serum concentrations of IgE-specific to the above listed aeroallergens and to milk, egg, fish, casein, wheat, peanut, soy and profilin (Bet v 2) were evaluated by the commercial method *ImmunoCAP*, Phadia.

Subjects and *personal diary*

Hundred and thirty patients, 69 females and 61 males ($X^2 = 0.492$, $df = 1$, $p_{Exact} = 0.539$), aged 3 to 82 years (mean ± SE = 31±1.551), entered consecutively into the study.

Sixty-five suffered from a single pathology (31 with Rhinitis alone, 10 with Asthma, 11 with IBS and 13 with Atopic Dermatitis) and sixty-five with different association of mentioned *diseases*.

They received the best standard treatment according to disease-specific guidelines, plus *I-Prob* for 60 days (add-on therapy). IBS patients received no drugs except a last-generation, low-dose, anti-Histamine used as down-modulator of the vascular adhesion molecules VCAM-1 and ICAM-1 expression (*Ebastine* c. mg 10/die).

At the first diagnosis (t_0) and at the end of the treatment (t_1) a score of the severity of symptoms (Symptom Severity Score, SSS) built on a scale 0-4 (e.g. rhinitis 0 =absent; 1=mild intermittent; 2 =mild persistent; 3= moderate- severe intermittent; 4= moderate- severe persistent), according to the above mentioned validated classifications, has been assigned and registered by the physician in each patient's diary.

Subjects were asked to report, in the appropriate section of their diary, any adverse reactions or unwanted effects appeared throughout the whole period of the study and related, even potentially, to the treatment. Adverse Events (AE) were detailed and recorded as: None (N), None Severe (NS: mild local) and Severe (S: severe local or systemic).

Probiotical formula *I – Prob*

Each patient received one sachet a day for sixty days of the probiotic bacteria *I-Prob* (Anallergo, Florence, Italy), away from meals. A single sachet containing a mixture of *Lactobacillus acidophilus* LA02 (100 x10^6 at least), *L. delbrueckii subs. bulgaricus* LDB01 (5 x 10^6), *L. rhamnosus* (1x 10^6) and *Streptococcus thermophilus* (2 x 10^9). When IBS was severe persistent, first ten sachets of *I-PROB* have been administered on alternate days, depending on physician's judgment.

Explanatory section

Peripheral Blood CD34$^+$ cells and Lymphocyte sub-sets evaluation

A sample of 22 patients (mean age = 31.9 ± 4.353, range 3 to 69), 12 females and 10 males (X^2 = 0.492, *df* = 1, p_{Exact} = 0.539), underwent further investigation with respect to values of peripheral blood traffic of CD34$^+$ Hematopoietic Precursor and Stem Cell (HPC) as well as T-lymphocyte helper sub-population polarization, before (t_0) and after the treatment (t_1) with *I-prob* (Pre-test post-test study) (19). Cells have been enumerated by means of standardized flow cytometry techniques, elsewhere detailed and discussed (6, 7).

Briefly, peripheral blood venous samples were collected in ethylendiaminetetraacetic acid (EDTA) tubes. Three aliquots of 50 ml of whole blood were incubated, at 4°C for 25 min. in the dark, one with 15 ml of phycoerythrin (PE)-conjugated murine monoclonal antibody (MAB) specific to CD34 molecule, one with PE-conjugated MABs specific to unrelated molecules to human leukocyte antigens, and one as unlabeled control. Monoclonal antibodies were from Becton Dickinson (BD), Milan, Italy. PE anti-CD34 MAB was the Anti-HPCA2, Clone 8G12 (BD), suitable for routine use. Then, 2 ml of red blood cell lysis buffer (BD, Italy) were added to each tube and incubate at room temperature for 10 min, in the dark. After lysis cells were washed twice with 2 ml of cold phosphate buffered salt solution (PBS) (BD, Italy) at 1, 200 revolution per minute (rpm) (300 g) for 7 min at 4°C and then resuspended in 500 ml of PBS. Cells were analyzed using a FACSCalibur flow cytometer equipped with Cell Quest software. Three data parameters were acquired and stored in list mode files: linear forward scatter (FSC) (vertical axis), linear side-angle scatter (SSC) (horizontal axis) and log PE fluorescence, by gating whole viable cells. Since aim was to enumerate both CD34^{bright} and CD34^{dim} cells, the setting of the fluorescence analysis region 2 (R2) has been fixed with the lower limit at 10^2 avoiding any compensation. For each measurement 10, 000 events were acquired. Results have been expressed as percentage of positive cells.

T-helper-cell polarization study was performed using the following MABs. The monoclonal antibody to CXCR4/CD184, receptor up-regulated by IL4 (20, 21) and down-regulated by IFNg (21), has been used to evaluate a TH2 skewed polarization (20-24) when the monoclonal to the CXCR3/183, receptor expressed only on the surface of cells contacted by IFNg, has been employed to mark cells expressing a TH1 profile (24-26). Mouse anti-human Monoclonal Antibodies were the following: CD2 FITC, CD3 PerCP (*Peridinin-Chlorophyll-Protein*), CD4 FITC or PerCP , all purchased by BD.

CD184 PE, clone 12G5, was a mouse IgG2a specific for CXCR4 and CD183 PE, clone 1C6/CXR3, was a mouse IgG1 specific for CXCR3; both monoclonals were supplied by Pharmingen. Cells were processed and analysed by current unmodified three-colour-immuno-fluorescence techniques and results expressed as percentage of positive cells.

Statistics

For investigational purposes we stratified patients in three different age classes: 1) less than 18 yrs, 2) larger or equals to 18 yrs and less than 60 yrs, 3) larger or equals to 60 yrs. Values of CD34+ and Th2/Th1 before and after the treatment have been tested with *t* test for paired samples (27), and then by Factorial ANOVA, using the gender, the presence of more than 1 pathology and the age classes as controlling factors.

SS scores before and after, being an ordinal variable, have been tested by a Wilcoxon non-parametric test for paired samples, while the differences in the variation of SS scores in different sexes, between age classes and between patients with one or more pathologies have been tested by Mann-Whitney non-parametric test for independent samples. Normality was tested by one-sample Kolmogorov-Smirnov test (27).

The probability levels for Wilcoxon, Mann-Whitney and one-sample Kolmogorov-Smirnov tests were computed using a complete randomization method (permutation or exact test; p_{Exact}) or by a Monte Carlo simulations based on a 100.000 sampled tables (p_{MC}) when computation by the permutation method was not possible (28). All the statistical analyses have been computed using the Statistical Package for Social Sciences ver. 15.00 (SPSS®) (29).

Results

The severity of symptom score before (t_0) and after *I-Prob* assumption (t_1) decreased significantly (Wilcoxon *z* = -9.938, p_{Exact} < 0.001) from a median = 3 (25^{th}perc = 2, 75^{th}perc = 3) at t_0 to a median = 1 (25^{th}perc = 2, 75^{th}perc = 3) at t_1. Owing to the bias generated by the lack of placebo-controlled group, such results have to be considered with all the limits of the observational outcome (Fig.1).

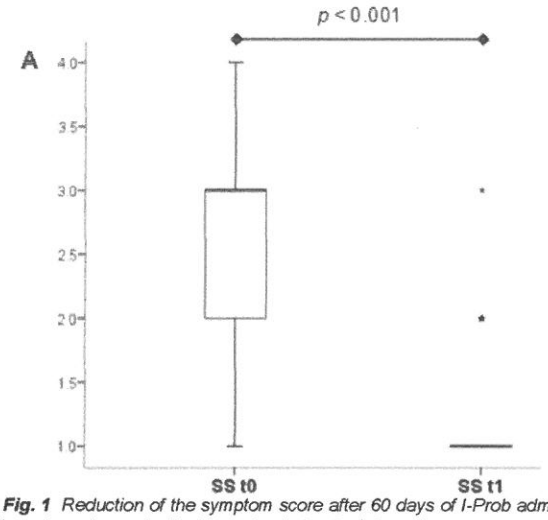

Fig. 1 *Reduction of the symptom score after 60 days of I-Prob administered as add-on treatment in a sample of 129 subjects with asthma, rhinitis, atopic dermatitis and irritable bowel syndrome (patients received standard drug therapy as recommended by each disease-specific guidelines plus one sachet a day of the investigated probiotic formula). Results are potentially flawed by the lack of a placebo-controlled group.*

Safety and tolerability

No serious adverse event, even potentially related to the treatment under investigation, appeared at any time of the study.

Only three mild local reactions (2.3 %; 3/129; cases # 34, 101 and 108) have been reported, namely diarrhoea always appeared within the first week of treatment. The discontinuation of *I-Prob* assumption resolved the events, and no other intervention was necessary.

Subjects # 101 and 108 suffered from atopic dermatitis alone, without overt signs of IBS.

All patients who had experienced the mentioned adverse events, could enter the study again after an induction period consisting in the intake of one sachet on alternate days for 20 days. Only one subject (# 60) dropped-out the study due to personal reasons, unrelated to health conditions.

All patients, included those that experienced the three mild local AEs, agreed with an optimal profile of tolerability of *I-Prob* assumption.

Explanatory section

The highest values of circulating HPC CD34$^+$ (normal values < 0.05 %) (6, 7) have been observed in patients suffering from Irritable Bowel Syndrome (cases # 21 and 118) and respiratory allergy as Asthma (# 56) and Rhinitis (# 50) that displayed high values of SSS before starting treatment.

The HPC CD34$^+$ values after treatment showed a significant decrease from a mean of 0. 22 ± 0. 036 at t_0 to a mean of 0. 11 ± 0. 014 at t_1 (t = 3.542, df = 20, p = 0.002; Fig. 2A).

When analyzed by means of factorial ANOVA, the significant decrease was detectable mainly in the sub-group of patients suffering from a single pathology (Fig. 3A) (variation*Poly_pathology, F = 10.446, df = 1, p = 0.008).

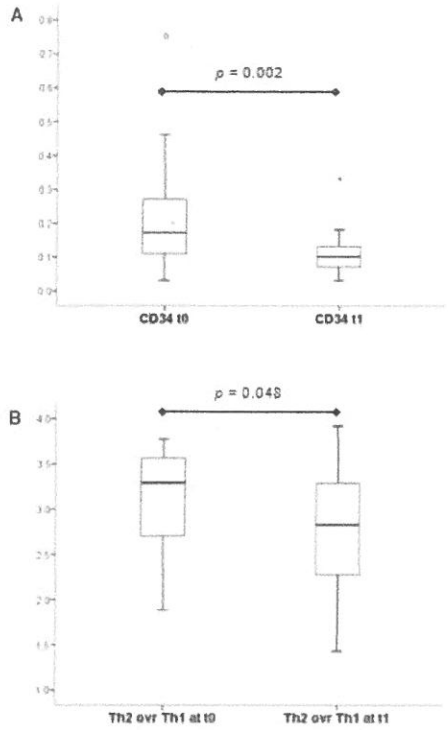

Fig. 2 *Reduction of the CD34$^+$ HPC values (A) and of Th2/Th1-ratio (B) in peripheral blood of a sample of 22 subjects with asthma, rhinitis, atopic dermatitis and irritable bowel syndrome after 60 days of assumption of I-Prob administered as add-on treatment.*

Furthermore, the qualitative evaluation of the CD34$^+$ situation for age classes and number of pathologies revealed that the paediatric patients did not show any consistent decrement of the CD34$^+$ since their values were already low at baseline (Fig. 3B).

When the pediatric patients were excluded from the analyses, the reduction of HPC CD34$^+$ values after treatment was associated with Severity Score reduction (n=14; CD34+ *vs.* SS, $r_S = 0.728$, $p = 0.003$).

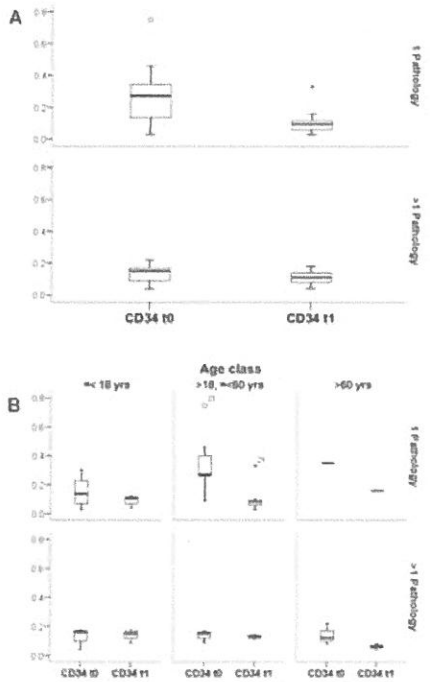

Fig. 3 *Variations of CD34$^+$ in a sub-sample of 22 patients before and after (t^0 and t^1) the treatment with probiotic bacteria I-Prob, grouped per number of pathologies (A), and then per age classes and number of pathologies (B).*

In figures are reported the medians (thick line), the 2nd and 3rd quartiles (inferior and superior box ends, respectively), along with the extreme values (bars), the outliers (circles, cases with values that are between 1.5 and 3 box lengths from either end of the box) and the most extreme cases (asterisks, cases with values more than 3 box lengths from either end of the box).

The numbers in B refer to a specific patient (#21) that presented an IBS (see text).

Student's t test results for paired samples are reported as well.

The Th_2/Th_1 ratio showed a slight but significant reduction after the treatment ($t = 2.147$, df = 16, $p = 0.048$; Fig. 2B). No significant effect of either gender, presence of more than one pathology or age on the Th_2/Th_1 ratio variation was detected.

Discussion

Among the 129 subjects who completed the study, only three experienced a mild local adverse reaction namely diarrhoea, and in all cases within the first week of *I-Prob* assumption. Reactions resolved spontaneously with the discontinuation of therapy that could be resumed and completed under a slacken down regimen of administration (one sachet of *I-Prob* on alternate days for 20 days). *I-Prob* treatment thus was safe and optimally tolerated by patients.

Cases # 101 and 108, who presented AEs, suffered from atopic dermatitis but did not refer overt signs or symptoms of IBS at the recruitment, thus confirming that, even when

allergy appears to involve only one organ or apparatus, it actually remains a systemic disease as to pathogenesis.
Therefore a sub-clinical inflammation involving other districts should be suspected, as suggested by several lines of evidence (5, 6, 10, 11). About 60-80% of subjects with AD without other symptoms, in fact, showed to be positive to bronchial provocation tests (30, 31) and a number of patients presented an increased permeability of the gut mucosa (32, 33).
Furthermore, in atopic dermatitis, a significant increase not only of CTACK/CCL27, the skin-specific chemokine regulating lymphocyte traffic towards the cutaneous microenvironment, but also of the muco-epithelial specific chemokines TECK/CCL25 and MEC/CCL28 (34, 35), has been reported.
A recent study confirms the link emerged between atopy and Irritable Bowel Syndrome.
The likelihood of association of IBS is significantly high in seasonal allergic rhinitis but the highest in allergic eczema (36). Association data in the investigated population were confluent since, among the 66 multi-symptomatic subjects, 34 (51.5%) presented the classic forms of atopy (Asthma, Rhinitis and Atopic Dermatitis) associated with IBS.
Notably, the extreme value of circulating $CD34^+$ cells (0.75%) has been measured in peripheral blood of patient # 21 who suffered from a severe persistent irritable bowel syndrome, without any other symptoms.
Thus a sub-clinical IBS should be regarded with attention as a feature to be possibly associated with atopic diseases, particularly atopic dermatitis.
Owing to the above mentioned, even if probiotics belong to the category of products regarded as GRAS (generally regarded as safe) because of their history of safety and the lack of pathogenicity reports, the possibility that a translocation might occur from gut environment to other sites, is still a matter of debate (37, 38).
Thus it is conceivable that conditions of gut inflammation, enhancing the epithelial permeability, may increase the remote translocation risk, suggesting an induction period with reduced doses of probiotic, as a caution.
Outcomes from the explanatory section rise significant interest when discussed in the new and fast moving chapter of the physiology of the *innate immunity*.
In fact, if an abnormally increased peripheral traffic of low-differentiated cells marked by the surface antigen CD34 (Hemopoietic Precursor and Stem Cells) has been identified as a feature of allergic inflammation for the last twenty years, to date HPSCs are also involved in mechanisms of peripheral immunosurveillance (39) as well as in the replenishment of innate immune system (40, 41).
Furthermore the phenotype $CD34^+$ Lin^-, proper to Multipotent Precursors (MPP) marks and is shared by the "innate helper cells" or "innate lymphoid cells" (42).
Our data showing a low peripheral movement of $CD34^+$ HPSC among pediatric subjects differently from adult subjects (Fig. 3B), should be explained since in youth cellular prevalent immunity is depending on Thymus.
During adult life, on the contrary, after the "*Thymic involution*", it appears conceivable that lineages belonging to the "innate branch" of immunity such as natural killer and innate lymphoid cells are mainly used as effector cells (43, 44).
The significant reduction of the $CD34^+$ circulating cells observed in mono-symptomatic subjects aged more than 18 years, after the treatment, may be discussed under the same perspective.
Further to the classic central and "canonical" Hematopoiesis, occurring at the bone marrow "*Niches*", it is now emerging, supported by a large body of evidence, a new concept for a peripheral "*non-canonical*" Hematopoiesis, closely related to the innate immune-response.
Briefly, when the peripheral effector cells with immunosurveillance function, which are hematopoietic progenitor and stem cells (39, 45), are stimulated by pathogens through *Pattern Recognition Receptors (PRRs), they first proliferate, i. e. expand, and then rapidly differentiate.*
Thus, once absolved "in situ" the defensive primary function against the invading pathogens, the survived cells further contribute to replenish the cellular compartment of the innate immunity (40, 41, 46, 47).
Such an emerging "*peripheral immunopoiesis*" appears flawed in Western countries by the excessively hygienic lifestyle; the observed movement of $CD34^+$ HPSC could represent a bone-marrow attempt to restore such an impaired mechanism.
The reduction observed after the *I-Prob* treatment may be related to mechanisms involving the Intestinal Stem Cells (ISC) (48-50) residing in the *Lieberkuhn cryptopatches (51, 52).* $CD34^+$ precursor and stem cells, in fact, express Toll Like Receptors (53, 54) as well as Intestinal Stem Cells (55); a bacterial engagement of ISC PPRs may thus induce somewhat like a replacing "peripheral immunopoiesis" using peripheral stem cell niches.
Concluding, the outcomes of the present study indicate that the investigated probiotical formula is associated with an optimal profile of safety and tolerability.
Experimental data suggest for further investigations on the beneficial effect potential involving the innate immunity ontogeny.

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