

# A Pilot Study on Clinical Scores, Immune Cell Modulation, and Microbiota Composition in Allergic Patients with Rhinitis and Asthma Treated with a Probiotic Preparation

Enza Torre<sup>a</sup> Daniele Sola<sup>b</sup> Alice Caramaschi<sup>c, d</sup> Flavio Mignone<sup>d, e</sup> Elisa Bona<sup>c, d</sup>  
Silvia Fallarini<sup>a</sup>

<sup>a</sup>Department of Pharmaceutical Sciences, University of “Piemonte Orientale”, Novara, Italy; <sup>b</sup>S.C.D.U. Internal Medicine 1, A.O.U. “Maggiore della Carità”, Novara, Italy; <sup>c</sup>Department of Science and Innovation Technology (DiSIT), University of “Piemonte Orientale”, Vercelli, Italy; <sup>d</sup>UPO-CAAD Center for Translational Research on Autoimmune and Allergic Disease, Novara, Italy; <sup>e</sup>SmartSeq s.r.l., University of “Piemonte Orientale”, Alessandria, Italy

## Keywords

Asthma · Rhinitis · Microbioma · Probiotic · Immune cells

## Abstract

**Background:** Specific drugs and/or immunotherapies are widely used to treat allergies, but drug-induced adverse effects recently led to explore new additional strategies. We studied whether a probiotic preparation (iPROB<sup>®</sup>; Anallergo SpA, Florence, Italy) is effective in allergic patients and the mechanisms underlying clinical outcomes. **Methods:** Eligible patients ( $n = 28$ ), all suffering from allergic rhinitis with/without bronchial asthma, were consecutively recruited at the Allergology Medical Unit (Novara, Italy) and treated with this probiotic. From each patient, we collected blood and stool samples at the baseline, after 60 days of probiotic supplementation, and after 60 days from probiotic discontinuation. In each blood sample, the percentage of hematopoietic stem cells, eosinophils, and basophils was measured by FACS. To analyze stool microbiota composition, genomic DNA was extracted, bacterial 16S DNA libraries sequenced by Illumina platform (Miseq), and raw sequences processed. Generated data were statistically analyzed. **Results:** Probiotic-

ic-treated patients showed a significant decrease in Average Rhinitis Total Symptom Score ( $d = -10.5714$ ), and Visual Analog Scale ( $d = -2.00$ ) clinical indices, as well as important improvements in quality of life. In whole blood, a significant reduction in the percentage of activated eosinophils and basophils was determined, and this effect persisted after specific cell stimulation. Consistently, the serum levels of IL-4 and IL-5 decreased after probiotic treatment, suggesting a reduction in the Th2 cytokine profile. In addition, microbiome genomic analysis ( $n = 6$ ) showed an increase in microbiome biodiversity, which positively correlates with clinical and cellular data. **Conclusion:** Present study suggests that iPROB<sup>®</sup> preparation has clinical/biological properties to be a valid add-on supplementation in allergic patients with asthma and rhinitis.

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## Introduction

Allergic diseases are a group of immune-mediated disorders caused by inappropriate and/or exaggerated immune responses, characterized by a Th 2 cell polarization to

common harmless environmental and/or food antigens [1, 2]. The prevalence of allergic diseases has dramatically increased over the past few decades [3]. To date, a nearly 40% of the general population experiences at least one type of allergic diseases throughout life. Currently, air pollution [4, 5] and excessive hygiene [6] seem to be the most accredited etiopathogenetic hypothesis for rhinitis [7], bronchial asthma [8], and skin diseases [9]. The prevalence of allergic diseases is always increasing and it is estimated that it will be even greater in the future, making allergic diseases among the most impacting disorders on health systems [10]. Actually, allergic rhinitis, that symptomatically manifests as rhinorrhea, sneezing, and nasal congestion, affects about 10–30% of adult population [11]. This pathology represents the main risk factor for the development of bronchial asthma, a chronic inflammatory condition of the airways affecting about 12–15% of adults worldwide. These conditions affect quality of life and physical performance, and asthma, in some cases, can cause life-threatening respiratory crises. Typical treatments consist of a symptomatic drug therapy that includes mast cell stabilizers, antihistamines, epinephrine, or corticosteroids [12]. Allergen-specific immunotherapy, which consists in the administration of small quantities of allergens (or allergoids), typically associated with antihistamines and/or corticosteroids, can also be prescribed to selected patients for the induction of immune tolerance [13]. Nevertheless, allergic symptoms sometimes remain poorly managed, while drug-induced adverse effects can seriously affect the patient's quality of life. This points out that it is mandatory to look for new strategies that could help to obtain an optimal control of allergic diseases using the lowest possible number of medications [14].

Many evidences suggest that the manipulation of intestinal bacterial flora (microbiota) represents an add-on therapy for preventing medical interventions in allergic conditions. The correlation between the microbiota dysfunction (dysbiosis) and the development of allergic diseases has been suggested by many observations [15]. Numerous studies reported a low incidence of allergic diseases in children raised in rural environments, with older siblings, in contact with pets or farmer animals, exclusive breastfeeding for the first 4 months of life, and with reduced exposition to antibiotics [16]. These observations have led to the generation of the “hygiene hypothesis” that links the reduction of microbial exposure during infancy with consequent low microbiota diversity, to a reduction in immune system stimulation and a subsequent Th1/Th2 imbalance, predisposing to allergic diseases. Several animal and clinical studies have confirmed that the microbiota composition (together with bacterial met-

abolic products) can affect the development of allergic diseases by shaping immune system [16] and have suggested the possibility to obtain a better control of this disease by probiotic supplementation.

Probiotics are defined as live microorganisms that, apart from having protective intestinal effects, when administered in adequate amounts confer a health benefit modulating the immune system [17]. Probiotics which are capable of modifying intestinal microbiota, reducing pathogenic bacteria, and increasing the beneficial ones, are effective in increasing the epithelial barrier integrity and in modulating microbiota-related immunological processes [18]. Moreover, probiotics release during food fermentation a significant amount of short-chain fatty acids that have anti-inflammatory activities [3]. The reduction of pro-inflammatory responses can be also linked to a reduction in the nuclear factor-kappa-light-chain-enhancer of activated B-cell signaling, modulation of dendritic cell maturation toward an anti-inflammatory phenotype, and to a reduction in Th2 responses specifically involved in allergic diseases [19–22]. Other effects induced by probiotics, making them suitable for the modulation of allergic diseases, include the stimulation of mucosal IgA, as well as the control of lymphocyte responsiveness to oral and/or systemic antigen challenge [23].

Since the prevention from allergy development is currently one of the major clinical challenges, many studies have been conducted to evaluate the effects of various probiotic formulations with promising results [3]. Different authors have analyzed the effects of probiotic administration in infants and children with atopic dermatitis, showing a reduction in the scoring atopic dermatitis (Scorad) [24, 25], associated with a reduction in IL-6, IL-5, total IgE, and C-reactive protein levels [26]. An increase in IL-10, as well as in IgA, and serum levels were also measured, suggesting repressive immune responses [27, 28]. The bacterial strains mainly involved in these effects are *L. acidophilus*, *L. rhamnosus* GG, *B. animalis*, *L. paracasei*, *L. fermentum*, and *L. plantarum*, and the presence of these strains in a probiotic formulation was generally accompanied by Th2 response suppression and an improvement of clinical symptoms [29–31]. In food allergies, different studies reported that a combination of pre- plus postnatal administration of probiotics reduces the risk of food sensitization, while this risk does not change when probiotics are administered only pre- or post-birth. On the other end, there is a paucity of well-designed studies analyzing the tolerizing effect of probiotic in adult patients affected by food allergies, and this is probably due to the lack of an objective evaluation of a food challenge. Co-administra-

**Table 1.** Characteristics of the enrolled patients

| Median age <sup>a</sup> | Male/female, n | Mites allergy <sup>b</sup> , % | Asthma <sup>b</sup> , % | Stable antihistamines <sup>b</sup> , % | Systemic steroids <sup>b</sup> , % | Bronchodilators <sup>b</sup> , % |
|-------------------------|----------------|--------------------------------|-------------------------|----------------------------------------|------------------------------------|----------------------------------|
| 38                      | 11/17          | 100                            | 29                      | 100                                    | 7                                  | 22                               |

<sup>a</sup> Median age is expressed on average arithmetic. <sup>b</sup> Categorical variables are expressed as percentage over total number of enrolled patients.

tion of bacterial adjuvants together with oral immunotherapy has been suggested as a potential treatment for food allergies. Probiotics containing *L. rhamnosus* increase their effectiveness when co-administered with peanut oral immunotherapy or with hydrolyzed casein in milk allergic patients, in which an increase of fecal butyrate levels was found. Butyrate, indeed, seems to exert a pivotal role in immune tolerance induction through FOXP3<sup>+</sup> Treg generation, IL-10 production, a key cytokine in Treg regulation, B-cell differentiation, and promotion of IgA and IgG productions [32]. Conversely, clear evidences on the ability of probiotics in prevention/treatment of adult allergic patients with asthma and rhinitis are still lacking, and additional studies are needed to clarify the effectiveness and the safety of this treatment [33, 34].

In this context, we examined the impact of add-on probiotic supplementation (iPROB<sup>®</sup> produced by PROBIOTICAL S.p.A., Novara, Italy, for ANALLERGO S.p.A., Florence, Italy) on the main clinical scores (Visual Analog Scale [VAS] about allergy; Average Rhinoconjunctivitis Total Symptom Score [ARTSS] and RhinAsthma Patient Perspective [RAPP]) of allergic patients, as well as the frequency/activation status of cells typically involved in allergic responses, such as hematopoietic stem cells (HSCs), eosinophils, and basophils. Finally, the microbiome composition was studied in order to evaluate the bacterial-community modulation after probiotic intake and assess its correlation with clinical conditions of enrolled subjects. This pilot study suggests that iPROB<sup>®</sup> preparation has positive effects on allergic patients with asthma and rhinitis.

## Materials and Methods

### Protocol of the Study

Twenty-eight Caucasian patients (median age 38, male/female 40/60%), who met the inclusion criteria (prevalence of allergic bronchial asthma 29%, in accordance with the classification criteria included in the Global Initiative for Asthma (GINA) guidelines, all suffering from allergic rhinitis, in accordance with the classification criteria included in the Allergic Rhinitis and its Impact on

Asthma (ARIA) guidelines, polysensitivity 92%, stable disease, and nonsmoking), all allergic to dust mites and having grade 2 ARIA severity criteria (allergy symptoms that alter daily activities or sleep quality), were enrolled in the study at the Allergology Unit of “Ospedale Maggiore della Carità,” Novara, Italy. The characteristics of the population are summarized in Table 1. All patients were submitted to medical examination; prick test [35], ARTSS [36]; RAPP [37]; and VAS [38], clinical indices were also evaluated. All enrolled patients received oral iPROB<sup>®</sup> (*Lactobacillus acidophilus* [LA02 DSM 21717] not <100 million, *Lactobacillus delbrueckii subsp. bulgaricus* [LDB01 DSM 16606] not <5 million, *Lactobacillus rhamnosus* [LR04 DSM 16605] not <1 million, *Streptococcus thermophilus* [FP04 DSM 18616] not <2 billion per dose), free of charge, every day for 60 days. For each patient, a blood sample (10 mL) was taken at T0 (before the beginning of probiotic treatment), at T1 (at the end of probiotic supplementation for 60 days), and at T2 (60 days after probiotic discontinuation) and analyzed at the Immunopharmacology Laboratory of Department of Pharmaceutical Sciences of the “Università del Piemonte Orientale,” Novara, Italy. Stool samples were collected from patients at T0, T1, and T2 times and stored at –80°C before the microbiome analysis at the Center on Autoimmune and Allergic Diseases (CAAD) Microbiology Laboratory of the “Università del Piemonte Orientale.”

### Flow Cytometry Analysis

To identify the selected cell populations, 100 µL of whole blood samples were treated with FC blocking buffer (BD Biosciences, Milan, Italy) for 10 min at room temperature and labeled with anti-human CD34, CD45, CCR3, CD125, and CD203c for 20 min in the dark at room temperature. After antibodies incubation, red blood cells were lysed by lysis solution (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA; pH 7.2–7.4). Furthermore, the percentage of HSCs (CD45<sup>+</sup>, CD34<sup>+</sup>, CD125<sup>+</sup>, and CD203c<sup>+</sup>), eosinophils (CD45<sup>+</sup>, CCR3<sup>+</sup>, and CD125<sup>+</sup>), and basophils (CD45<sup>+</sup>, CCR3<sup>+</sup>, and CD203c<sup>+</sup>) were defined by flow cytometric analysis (Attune NxT; Thermo Fisher Scientific, Waltham, MA, USA). To evaluate the activation status of eosinophils and basophils, 50 µL of whole blood samples were activated by specific stimuli (50 ng/mL IL-5 for 2 h or 10 µg/mL anti-Fc3RIα for 30 min for eosinophils and basophils, respectively).

Thereafter, cells were stained with anti-human CD45, CCR3, CD125, and CD69 (eosinophil markers) and CD45, CCR3, CD203c, and CD63 (basophil markers), red blood cells lysed by lysis solution, and cell populations analyzed by a flow cytometer. Unactivated blood samples were always used as controls. The program used for computational analysis was FlowJo X 10.7, while to identify and analyze the cell populations of interest, we used a gating strategy previously used in our laboratories [39].

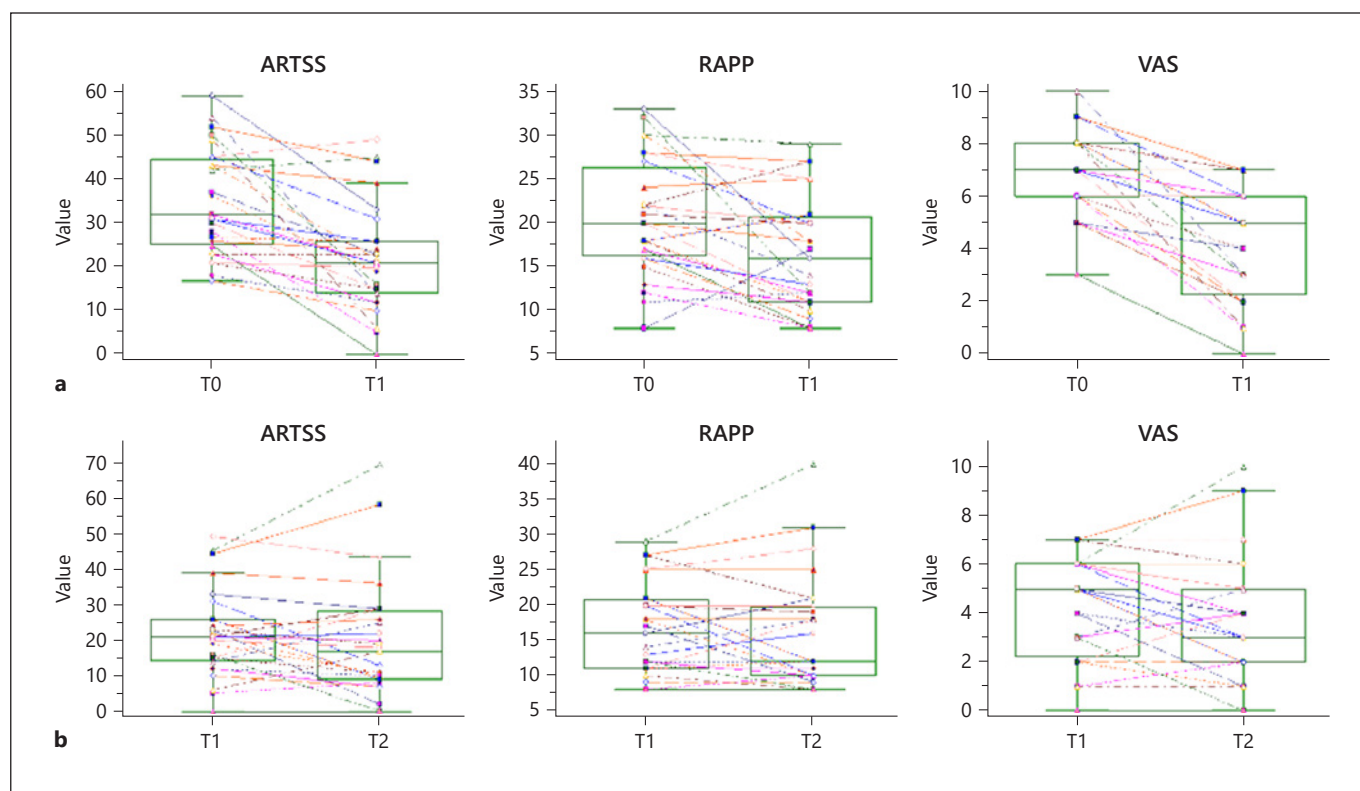


**Fig. 1. a** Wilcoxon test graphs comparing the 3 clinimetric scores (ARTSS, RAPP, and VAS) to T0 (before the start of probiotic treatment) and T1 (at the end of treatment) ( $p \leq 0.05$ ). **b** The same comparisons between T1 and T2 (60 days after the probiotic discontinuation). ARTSS, Average Rhinitis Total Symptom Score; RAPP, RhinAsthma Patient Perspective; VAS, Visual Analog Scale.

#### IL-4 and IL-5 Levels in Serum Samples

To evaluate the cytokine profiles, 5 mL of whole blood samples were centrifuged at 1,600 rpm for 30 min, serum collected and stored under liquid nitrogen ( $-210^{\circ}\text{C}$ ) until analyzed. In each patient, the IL-4 and IL-5 serum levels (Th2 profile) were measured using ELISA kit, and the experimental procedure was conducted strictly according to the manufacturer's instructions (BioLegend®, San Diego, CA, USA).

#### Microbiome Analysis in Stool Samples

Genomic DNA was extracted using a kit (QIAasympy PowerFecal Pro DNA kit) provided by Qiagen (Milan, Italy) from 0.25 g of stool following the manufacturer's instructions. Obtained DNA was quantified following a standard protocol (Invitrogen Qubit 4 User Manual) provided by Thermo Fisher (Monza, Italy). The preparation of the bacterial 16S DNA libraries was performed using the "Microbiota solution B" kit (amplifying the hypervariable regions V3–V4–V6) provided by Arrow Diagnostics srl (Genoa, Italy). Sequencing analysis was performed by MiSeq platform using MiSeq Reagent Nano Kit v2 (500-cycles). As internal standard was used Phix, a standard provided by Illumina Inc. (San Diego, CA, USA). Raw sequences were processed by MicrobAT software (SmartSeq srl, Novara, Italy) based on the Ribosomal Database Project (RDP) database. Statistical analysis regarding variations within the bacterial communities was performed using MicrobiomeAnalyst software.

In particular, heat tree analysis was performed using R package metacoder package, according to Foster et al. [40]. Alpha diversity was characterized via the total number of species (observed); the abundances of the species (evenness) (Shannon and Simpson indices) were measured to describe the diversity of the considered community using the phyloseq package [41]. Beta diversity analysis method was used to compare the diversity between the considered microbial communities using the phyloseq package [41]. Each sample gets compared to every other sample generating a distance or dissimilarity matrix. The parameter considered was nonphylogenetic Bray-Curtis distance. The statistical significance of the clustering pattern in ordination plots was evaluated using Permutational one-way analysis of variance (PERMANOVA).

Finally, Linear Discriminant Analysis Effect Size method was applied [42] in order to identify a specific signature. The default  $p$  value cutoff was 0.05.

Moreover, the results of the immunological analysis (variables) were combined to the composition of bacterial communities to perform the principal component analysis. Principal component analysis was performed using all the considered clinical parameters. This analysis was performed using R (v. 3.5.1) R Core Team [43], FactoMineR [44], and Factorextra [45] packages.

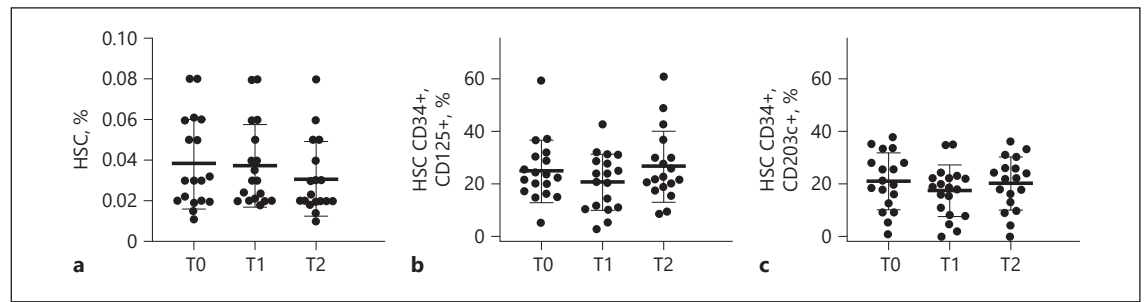


**Fig. 2.** **a** Percentage of HSCs in whole blood at T0 (baseline), T1 (after 60 days of probiotic supplementation), and T2 (after 60 days of probiotic discontinuation). **b** Percentage of eosinophil progenitor HSCs (CD125<sup>+</sup>) at the three times considered. **c** Percentage of basophil progenitor HSCs (CD203c<sup>+</sup>) at the three times considered. HSCs, hematopoietic stem cells.

#### Statistical Analysis

Flow cytometric and ELISA data were statistically analyzed using GraphPad Prism 7.00 and MedCalc 19.0.5. The one-way ANOVA was used for statistical analysis, with the level of significance set at  $p < 0.05$ . For the comparisons of the clinimetric scores, a Shapiro-Wilk data distribution test was first performed which rejected the hypothesis of a normal distribution; therefore, the Wilcoxon test (nonparametric) was preferred to the student's  $t$  test for paired data.

## Results

#### Clinical Data

Data obtained from the enrolled patients clearly suggested that iPROB<sup>®</sup> supplementation in these patients can significantly improve their clinical status and reduce the perception of disease by improving their quality of life. Specifically, we measured clinical improvement and quality of life, with scores suitable for this purpose, previously validated by other clinical studies. In this study, a statistical comparison was made for paired data by analyzing ARTSS, VAS, and RAPP. These scores allow translating the clinical trend and the state of health perceived by patients into a number.

As we expected, the probiotic treatment induced a measurable clinical efficacy with a significant reduction in the ARTSS clinical index T0 versus T1 ( $d = -11.25$ ,  $p < 0.001$ ), RAPP T0 versus T1 ( $d = -4.00$ ,  $p < 0.02$ ), and VAS T0 versus T1 ( $d = -2.50$ ,  $p < 0.001$ ) (shown in Fig. 1a). The beneficial effects obtained by probiotic administration persist at T2 (60 days after the end of therapy) (see: ARTSS T1 vs. T2, RAPP T1 vs. T2, and VAS T1 vs. T2) (shown in Fig. 1b).

#### HSCs Percentage in Whole Blood

Following probiotic supplementation, the HSCs percentage in whole blood samples remains stable at all times considered (T0–T2): no significant modification on the

percentage of this cell population was, indeed, measured (shown in Fig. 2a). On the contrary, a reduction in the percentage of HSCs in the way of differentiation to eosinophils (CD34<sup>+</sup>, CD125<sup>+</sup>) (shown in Fig. 2b) or basophils (CD34<sup>+</sup>, CD203c<sup>+</sup>) (shown in Fig. 2c) was determined at the end of probiotic supplementation (T1). This reduction appears to return to the T0 level (baseline) 60 days after probiotic discontinuation (T2). Despite not reaching statistical significance, probably for the low number of enrolled patients, these results suggest that this probiotic preparation is able to modulate the differentiation of HSCs toward important effectors in human allergic diseases (eosinophils and basophils).

#### Eosinophils and Basophils Percentage in the Whole Blood

No differences were determined in the percentage of eosinophils and basophils at the three times considered (T0–T2) (shown in Fig. 3a, d). After 60 days of probiotic supplementation (T1), a significant reduction in both percentage of activated eosinophils ( $p < 0.0001$ ) and basophils ( $p < 0.0005$ ) was measured (shown in Fig. 3b, e). These effects were confirmed in cells activated by specific stimuli (50 ng/mL IL-5 for eosinophils and 10  $\mu$ g/mL anti-Fc $\epsilon$ RI $\alpha$  for basophils) ( $p < 0.05$ ) (shown in Fig. 3c, f). After 60 days of probiotic discontinuation (T2), the cell population number returned to the baseline levels in both unstimulated and stimulated cells. Flow cytometric representative dot plots of a sample are shown in Figure 4.

#### Th2 Profile

The above-described inhibitory effects of probiotic supplementation for 60 days on the percentage number of blood cells involved in allergic diseases functionally correlates with a decrease in the serum levels of IL-4 ( $p <$

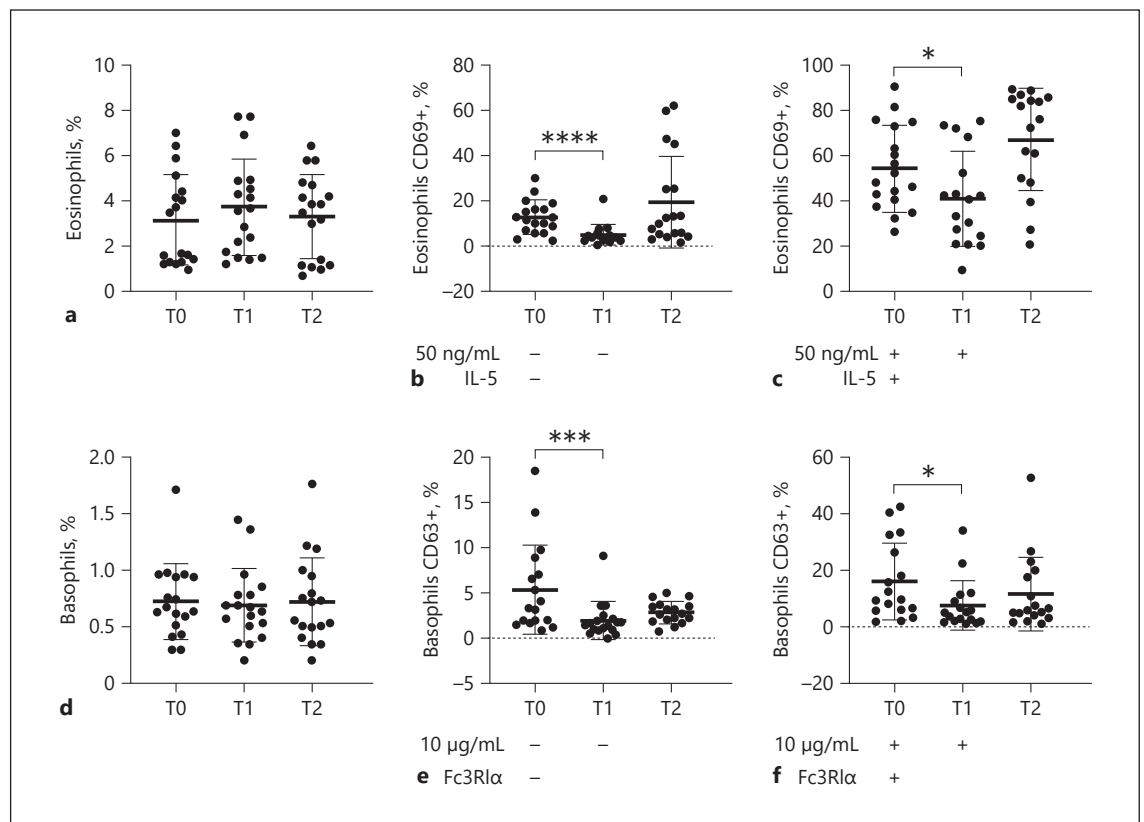


**Fig. 3.** **a, d** Percentage of eosinophils and basophils in whole blood at T0–T2; effect of probiotic supplementation in the percentage of unstimulated (**b, e**) and stimulated cells (**c, f**) ( $p \leq 0.05$ ).

0.005) and IL-5 ( $p < 0.01$ ), essential cytokines in regulating the allergic phenotype, the IgE response and/or the inflammatory cell-mediated functions. These data suggest that the probiotic supplementation induced a decrease in the plasma Th2 cytokine profile in allergic patients. Consistently with the cellular results, the IL-4 and IL-5 serum concentrations returned to the basal levels at T2 (shown in Fig. 5).

### Microbiota Biodiversity

#### Community Overview

The taxonomic community composition through direct quantitative comparison of abundances at phylum level showed that comparing the microbiome profile at T0, T1, and T2, the most abundant phyla were represented by *Bacteroides* and *Firmicutes* followed by *Proteobacteria*, *Actinobacteria*, and a proportion of unclassified bacteria in all the enrolled subjects. More in detail, the relative abundance of *Bacteroides* decreased after 60 days of probiotic treatment compared with the microbiome composition at baseline, but this abundance variation

does not last over time (43%–T0, 39%–T1, and 43%–T2). The *Firmicutes* phylum reports a particular and interesting behavior: after treatment, a significant increase, which remains stable even 60 days after the end of treatment (25%–T0, 38%–T1, 36%–T2), was observed. *Proteobacteria* abundance level at baseline was 2%, after 60 days of supplementation reaches 4%, and 3% at T2 sampling time. Finally, *Actinobacteria* relative abundance remained about 2% during the duration of the entire study.

#### Community Profiling

The supplementation with the probiotic determined an increase in the biodiversity of the intestinal microbiome of the treated patients (shown in Fig. 6a). Beta diversity shows a trend of difference (not statistically significant) in the population associated with the first sampling (T0) compared to that associated with the third sampling (T2) (Shown in Fig. 6b).

Principal Coordinates Analysis (PCoA), performed on the recorded species (shown in Fig. 6b), shows that the first axis explains 31% of the differences and the second

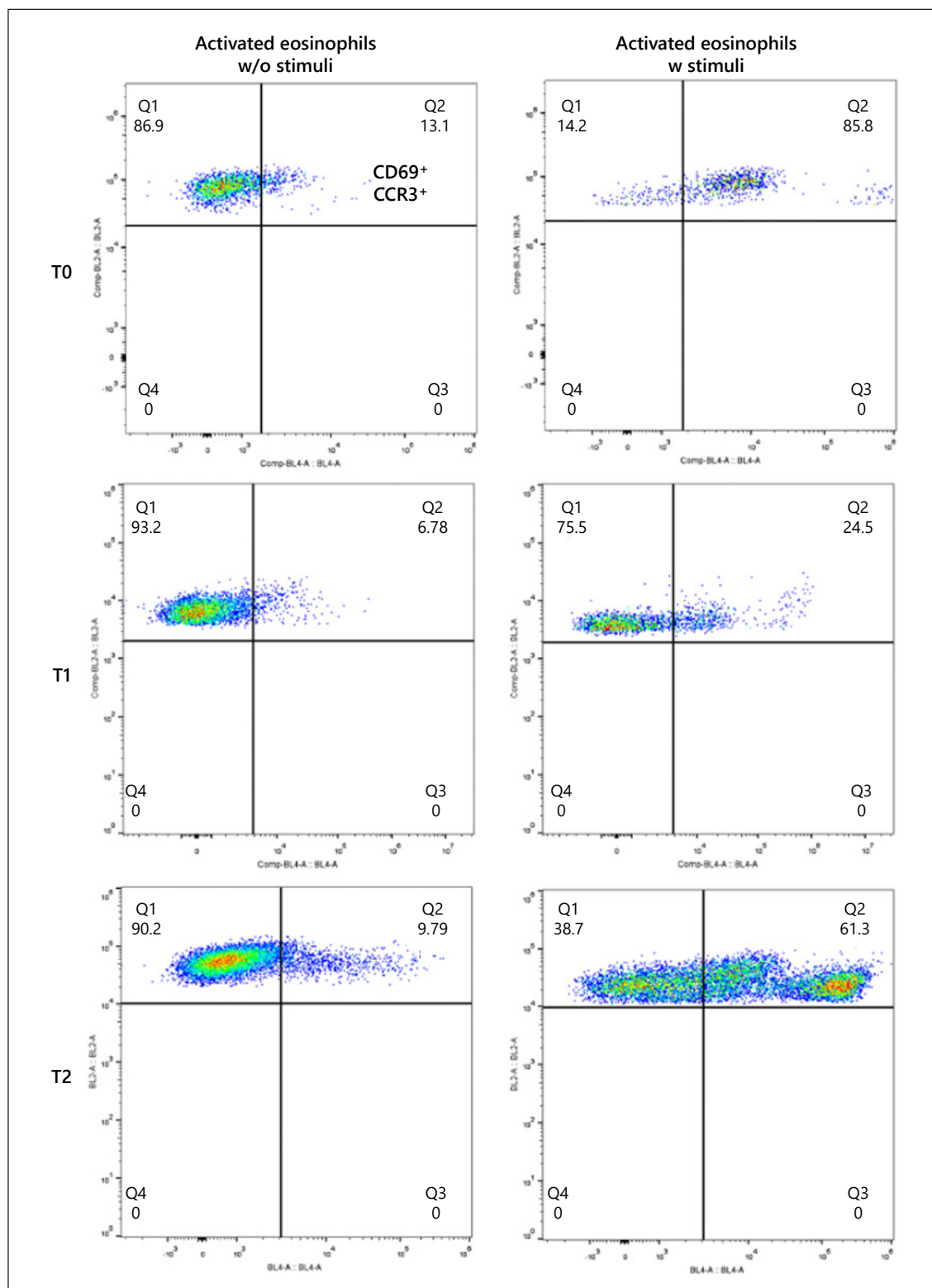
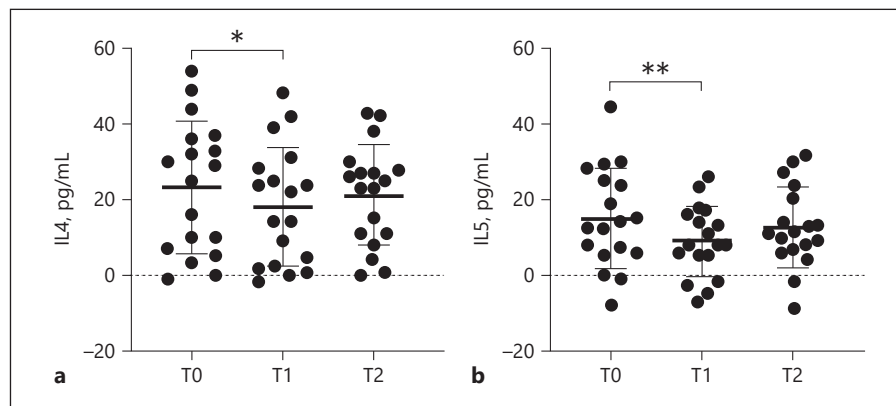


**Fig. 4.** Representative flow cytometric analysis of activated eosinophils in allergic patients. The percentage of activated eosinophils is shown in the upper right-hand corner. The percentage of these cells decreases after 60 days of probiotic supplementation (T1) either in the absence or presence of specific stimuli (50 ng/mL IL-5 for 2 h). After 60 days of probiotic discontinuation (T2), the number of cell populations returns to basal levels in both cases.

**Fig. 5.** Serum levels of IL-4 (a) and IL-5 (b) measured in patients (T0–T2). Both IL-4 and IL-5 levels decrease after 60 days (T1) of probiotic supplementation. At T2 (60 days after probiotic discontinuation), the cytokine levels returned to baseline ( $p \leq 0.05$ ).



one 18%. The overall composition of the gut microbiota, considered at species level, was not significantly affected by the probiotic intake as determined by nonparametric multivariate analysis of variance testing (PERMANOVA;  $p < 0.177$  for all factors). This grouping was also confirmed by the analysis of similarity test, which evaluates significance of sample grouping ( $R = 0.010873$ ,  $p < 0.41$ ). Although the Beta diversity analysis (PERMANOVA and analysis of similarity) did not reveal statistically significant differences, a trend highlighted a different composition of the populations associated with the subjects after probiotic supplementation. Thereafter, heat tree method demonstrated the difference in the abundance of the taxa composition in each subject category.

Figure 7 shows increased microbiota diversity in patients after probiotic supplementation. This greater biodiversity was also maintained at T2. In particular, a decrease in Bacteroidetes starting from T1, a decrease in *Akkermansia muciniphila*, and an increase in anaerobes belonging to the Clostridiales (Firmicutes) group were observed.

After iPROB® intake, a higher presence of the following different taxa was observed: *Lachnospiraceae incertae sedis* ( $p$  value = 0.0079), *Blautia* ( $p$  value = 0.0079), *Clostridium* XIVa ( $p$  value = 0.019), unclassified (this term means that a taxa cannot be included in a specific genus, but that it surely belongs to that genus), *Fecalibacterium* ( $p$  value = 0.0079), unclassified *Lachnospiraceae incertae sedis* ( $p$  value = 0.0013), unclassified *Blautia* ( $p$  value = 0.002), unclassified *Ruminococcus* ( $p$  value = 0.0293), *Lachnospira pectinoschiza* ( $p$  value = 0.010), and *Clostridium clostridioforme* ( $p$  value = 0.023). While the following taxa showed a lower presence: *Bacteroides vulgatus* ( $p$  value = 0.0168), *Fecalibacterium prausnitzii* ( $p$  value = 0.028), and *Bacteroides* sp. AR29 ( $p$  value = 0.044).

Sixty days after the end of the probiotic intake, the increase observed at T1 was confirmed for the following taxa: *Lachnospiraceae incertae sedis* ( $p$  value = 0.018), *Clostridium* XIVa ( $p$  value = 0.0108), *Coprococcus* ( $p$  value = 0.003), unclassified *Fecalibacterium* ( $p$  value = 0.018), unclassified *Lachnospiraceae incertae sedis* ( $p$  value = 0.006), unclassified *Blautia* ( $p$  value = 0.045), unclassified *Clostridium* XIVa ( $p$  value = 0.0186), *Lachnospira pectinoschiza* ( $p$  value = 0.009), and *Clostridium clostridioforme* ( $p$  value = 0.033). Moreover, unclassified *Coriobacteriaceae* ( $p$  value = 0.029), unclassified *Roseburia* ( $p$  value = 0.029), and unclassified *Dorea* ( $p$  value = 0.045) were higher at the end of treatment. Finally, the reduction of the Bacteroides group was confirmed: *Bacteroides* sp. ( $p$  value = 0.015) and *B. vulgatus* ( $p$  value = 0.018).

Multivariate analysis showed that the microbiome modifications positively correlate with the analysis of the different components of the immune system (eosinophil and basophil populations with/without stimuli) (shown in Fig. 8a). Figure 8b shows the relative contribution of each variable to the system variability and confirmed the observed results. The significant species described above, which are modulated in the enrolled subjects after probiotic supplementation, were also confirmed to be the ones responsible for the variability of the system. Moreover, the graph describes the involvement of the immune system components as determinant in the explanation of the whole variability of the described system.

### Signature

Specific microbial signature was outlined after 60 days of probiotic intake (Table 2). The signature was composed by: unclassified *Lachnospiraceae incertae sedis*, *Lachnospira pectinoschiza*, unclassified *Coprococcus*, un-

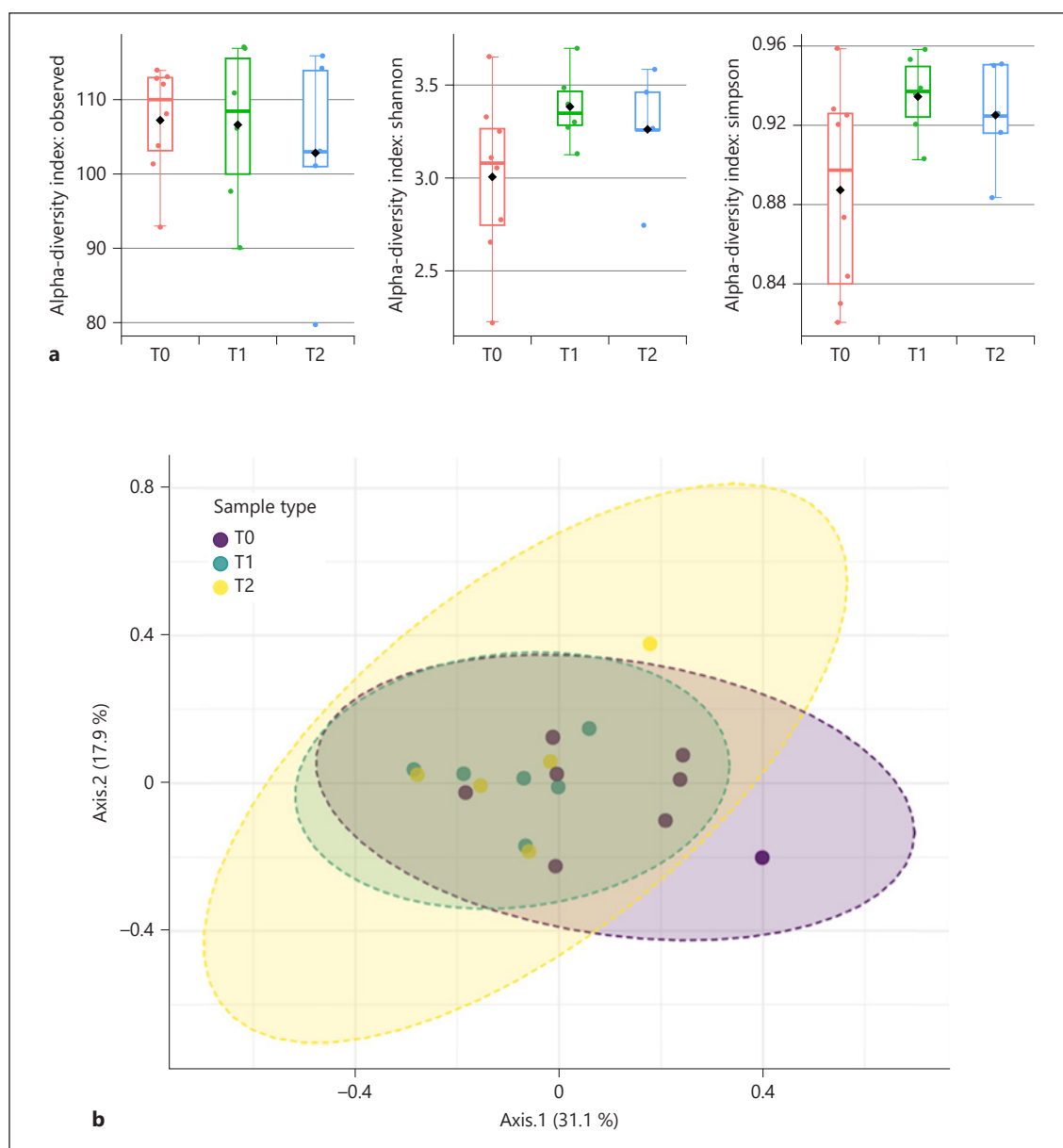


**Fig. 6. a** Alpha diversity measured using Observed, Shannon and Simpson at species level represented as boxplot. Each boxplot represents the different distribution of a group present within Sample-Type class: Observed species (Statistical significance:  $p$  value: 0.9264; [Kruskal-Wallis] statistic: 0.1529); Shannon index (Statistical significance:  $p$  value: 0.12943; [Kruskal-Wallis] statistic: 4.0892); Simpson index (Statistical significance:  $p$  value: 0.1861; [Kruskal-Wallis] statistic: 3.3629). Beta diversity. 2-D PCoA plot

using bray distance. **b** Comparison by PCoA of the distance (with the Bray-Curtis distance-based method) of the different samples (T0–T2). Statistical significance was performed using (PERMANOVA)  $F$  value: 1.2998;  $R^2$ : 0.13977;  $p$  value <0.177. Beta diversity analysis was performed using the phyloseq package of MicrobiomeAnalyst. PCoA, principal coordinates analysis; PERMANOVA, Permutational ANOVA.

classified *Fecalibacterium*, *Clostridium clostridioforme*, unclassified *Clostridium* XIVa, unclassified *Blautia*, and unclassified *Coriobacteriaceae* that were more abundant after probiotic intake while *Bacteroides caccae*, *Akker-*

*mansia muciniphila*, *Butyrivibrio fibrisolvens*, and *B. vulgatus* were inhibited. Table 2 reports that the signature outlined after iPROB intake was maintained after 60 days from treatment discontinuation.

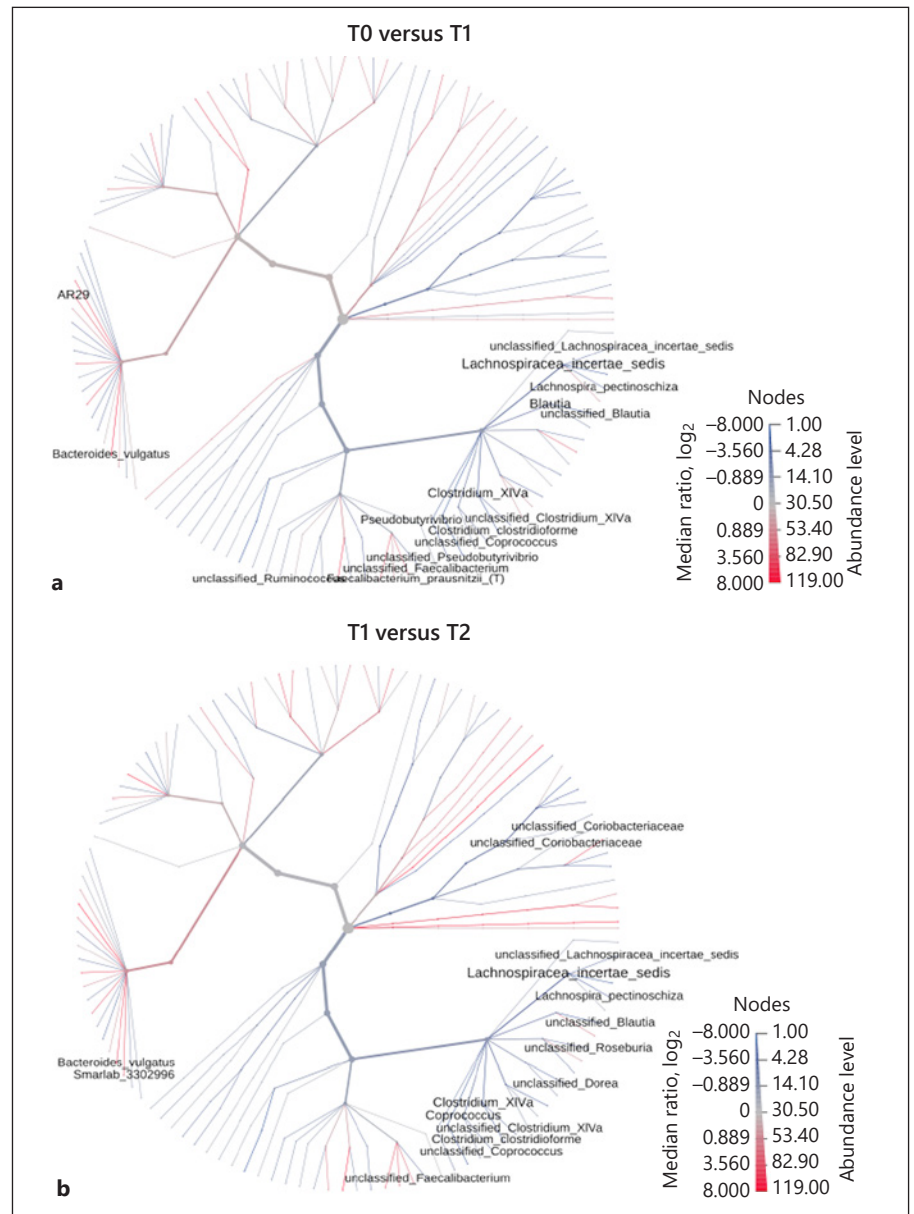


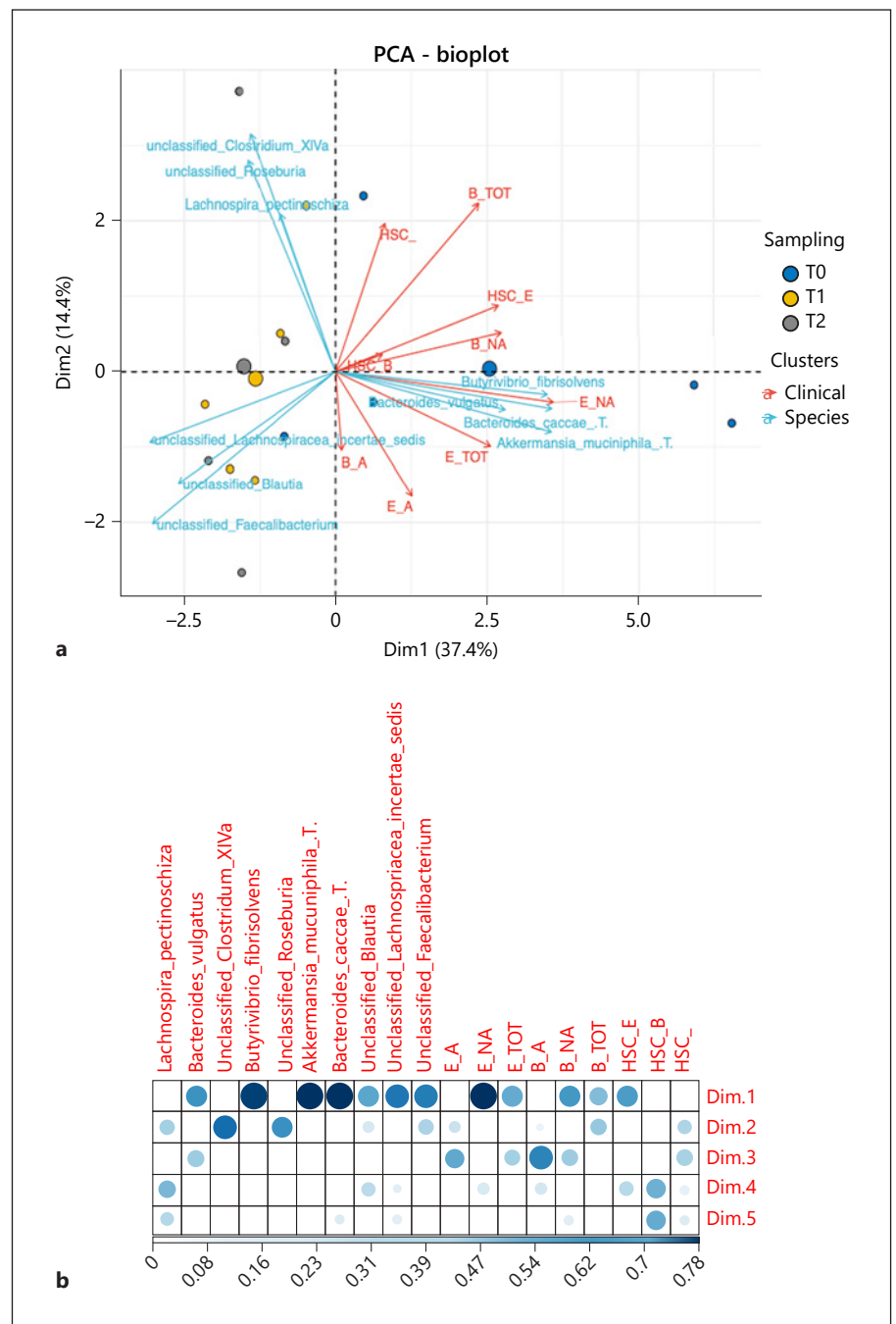
**Fig. 7. a, b** Heat tree of factor time. Heat trees report the effect of the treatment on hierarchical structure of taxonomic classifications (median abundance, nonparameter Wilcoxon Rank Sum test). Heat tree analysis was performed using R metacoder package of MicrobiomeAnalyst.

## Discussion/Conclusion

In the last few years, the role of host-associated microbial communities (microbiota) in balancing immunological tolerance and inflammation has been deeply studied [46, 47]. Many research groups focused to understand how microbiota functions and the consequences of microbiota imbalance. Dysbiosis of gut microbiota has been associated to many human diseases, including allergies [48]. The administration of specific beneficial live microorganisms (probiotics) can positively act on health status

[17], and properties of probiotics in restoring immune functions and in the prevention/treatment of allergic responses in humans have been widely explored [49]. To date, results are still controversial: according to some evidences, probiotics are not effective in the prevention/improvement of this disease [50], while other data show that probiotics can induce positive effects in allergic patients [51].

By the present study, we aimed to add our contributions, based on clinical, cellular, and microbiological observations, fully performed in our University Hospi-



tal and Laboratories (Novara, Italy). The final goal was to determine if the supplementation of this probiotic preparation for 60 days is effective in reducing allergic symptoms in patients, already affected by rhinitis and asthma. Our results, even if preliminary, clearly suggest that the probiotic preparation induces multifactorial beneficial effects on patients with asthma and allergic rhinitis.

We checked the clinical data, considering some of the most important allergic parameters, which indicated the severity of the disease, in enrolled patients at the baseline, after the probiotic supplementation and after 60 days from the suspension. The aim was to determine if the probiotic would lead to an improvement in the life conditions of these patients and if the benefit could last over time. What bodes well for the use of this probiotics is

**Table 2.** Linear discriminant analysis

| Species                                            | Phylum          | Order              | LDA score | Fold change, % |       | <i>p</i> value |
|----------------------------------------------------|-----------------|--------------------|-----------|----------------|-------|----------------|
|                                                    |                 |                    |           | T1             | T2    |                |
| <i>Akkermansia muciniphila</i>                     | Verrucomicrobia | Verrucomicrobiales | 4.46      | 12             | 0     | 0.047489       |
| <i>Bacteroides caccae</i>                          | Bacteroidetes   | Bacteroidales      | 4.50      | 0              | 0     | 0.0042795      |
| <i>Butyrivibrio fibrisolvens</i>                   | Firmicutes      | Clostridiales      | 4.09      | 0              | 0     | 0.013697       |
| <i>Bacteroides vulgatus</i>                        | Bacteroidetes   | Bacteroidales      | 5.19      | 2              | 1     | 0.014407       |
| <i>Unclassified Lachnospiraceae incertae sedis</i> | Firmicutes      | Clostridiales      | 4.81      | 432            | 434   | 0.0030391      |
| <i>Lachnospira pectinoschiza</i>                   | Firmicutes      | Clostridiales      | 4.11      | 940            | 1,223 | 0.0064258      |
| <i>Unclassified Coprococcus</i>                    | Firmicutes      | Clostridiales      | 3.98      | 445            | 620   | 0.007502       |
| <i>Unclassified Faecalibacterium</i>               | Firmicutes      | Clostridiales      | 5.35      | 322            | 305   | 0.012047       |
| <i>Clostridium clostridioforme</i>                 | Firmicutes      | Clostridiales      | 4.00      | 511            | 356   | 0.0231         |
| <i>Unclassified Clostridium_XIVa</i>               | Firmicutes      | Clostridiales      | 4.35      | 286            | 592   | 0.023177       |
| <i>Unclassified Blautia</i>                        | Firmicutes      | Clostridiales      | 4.64      | 367            | 347   | 0.024275       |
| <i>Unclassified Coriobacteriaceae</i>              | Actinobacteria  | Coriobacteriales   | 4.01      | 235            | 234   | 0.04647        |

Important features identified by LEfSe at species level. Features are significant based on their adjusted *p* value ( $\leq 0.05$ ). LDA, Linear Discriminant Analysis; LEfSe, Linear Discriminant Analysis Effect Size.

strictly the clinical results; data show significant improvement in quality of life with a decrease in symptomatic event number during treatment period. Interestingly, the effectiveness of this probiotic preparation appears to be independent on the specific drug therapy taken by each patient. Moreover, since this effect is attributable to intestinal repopulation by the new microbiota, it is not surprising that the efficacy lasts over time. None of the enrolled patients were treated with antibacterial drugs during the observation of this study.

In parallel, several cellular analyses were performed on blood samples from the same patients to evaluate the effects of probiotic supplementation on those cell populations that are mainly involved in allergy responses. We, first, considered the HSC population because these cells are progenitors of those playing crucial roles in both IgE-dependent and -independent allergic inflammation. Previously, Mastrandrea et al. [52] showed a significant decrease in HSCs in patients treated with the same probiotic preparation. Unfortunately, our study did not confirm this result at all times considered (T0–T2). One possible explanation could rely on the low relative abundance of HSC population in the blood samples [52]. The technical procedures for their identification from other cells in a heterogeneous biological sample are quite difficult and, consequently, subjected to high variability among laboratories. However, when we considered specific antigenic markers of HSCs toward eosinophil or basophil differentiation, a reduction in the percentages of both cell populations was measured. Our results did not

reach the statistical significance, probably for the small number of enrolled patients, but we are confident to achieve the statistical significance by the continuation of this study.

Blood eosinophils are associated with allergic asthma exacerbation frequency [53, 54], and activated basophils release histamine and promote inflammation [55]. The precise role of these cells in the pathogenesis/progression of allergic asthma and rhinitis remains poorly understood, and several animal models were used to investigate their potential roles in the development/propagation of allergic airway inflammation. For example, Hill et al. [56], using a murine model of house dust mite-induced airway inflammation, found that basophils play a direct role in promoting optimal Th2 cytokine responses [57]. We, consequently, evaluated the percentage number and the activation status of both eosinophils and basophils in whole blood taken from allergic-enrolled patients before and after probiotic supplementation (T0–T2). As showed in Figure 3, a significant decrease in the percentage of these cell populations was measured either in unstimulated or stimulated cells. The maximum reduction resulted in activated cells after 60 days of probiotic administration, suggesting a possible role of this probiotic in minimizing symptoms and preventing allergy exacerbations. Despite the maintenance of significant improvement in quality of life at T2, the level of activated eosinophils returns to the basal (T0) values. Even if a correlation between allergic diseases and number of circulating CD69<sup>+</sup> eosinophils exists, no correlation has been so far de-

scribed between the number of circulating eosinophils and the lung resident eosinophils. This could suggest that the increase in circulating activated eosinophils, we observed at T2, does not correspond to an increase in lung homed CD69<sup>+</sup> eosinophils, which are related to the symptoms worsening. Potentially, probiotics could promote Th1 cytokine pathway and restore Treg cells, leading to a modification of Th1/Th2 ratio, which was unbalanced toward Th2 profile in allergy [58].

Consistently to literature data, showing that probiotic administration modulates the Th1/Th2 balance, suppressing Th2 with shifts to Th1 responses, and prevent allergies [3], we also evaluated IL-4 and IL-5 serum levels in the same patients. In fact, in response to an allergen, basophils produce IL-4 upon cross-linking of their FcεRI receptors [59], and IL-5 represents a cytokine with a central role in the allergic reaction [60]. An increase in the blood levels of these cytokines stimulates Th2 pathway, and consequently, worsens allergic reactions. After probiotic supplementation, we observed a significant decrease in IL-4 and IL-5 serum levels, confirming previously shown clinical and cellular data.

Furthermore, we wanted to investigate whether iPROB<sup>®</sup> could interact, and even modify, intestinal microbiota. The results we obtained, even if preliminary, are very interesting. First of all, the administration of the probiotic induced, in the treated patients, a modification of the Firmicutes/Bacteroidetes ratio. In fact, we observed a significant decrease in the Bacteroidetes phylum and a significant increase in the species belonging to the phylum Firmicutes (order of *Clostridiales*). Similar microbial identifications were obtained in the large-scale “American Gut Project,” which comprised 1,879 participants. This study revealed the same microbial diversity modulation in participants with drug, food, and seasonal allergies [61]. The genus *Bacteroides* are Gram negative anaerobic bacteria, which can use simple sugars, even if their main source is glycans. They are predominantly associated with a diet high in fats and proteins. In particular, *Bacteroides caccae*, first isolated from human feces in the 1980s, is an intestinal, opportunistic pathogenic bacterium. It can invade the mucosa of the intestine and cause various abdominal suppurative infections [62]. In this context, the induced reduction of the described species could be considered as a positive effect of the treatment. In probiotic-treated patients, even after discontinuation of probiotic supplementation, we observed a significant increase in Firmicutes, particularly in *Lachnospiraceae*, *Clostridium*, *Blautia*, *Roseburia*, and *Faecalibacterium*. All these genera are important mem-

bers of the *Clostridium* cluster, essential in maintaining intestinal homeostasis. Literature data show that *Faecalibacterium* increases in patients, suffering of seasonal allergies, treated with a probiotic containing *Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum* G9-1, and *Bifidobacterium longum* MM-2. This enhancement is associated to an improvement of symptoms-related aspects of allergy [63].

*Lachnospiraceae* and *Clostridium clostridioforme* were anaerobic, spore-forming bacteria, able to ferment vegetable polysaccharides with short-chain fatty acids (SCFAs). They produce also butyrate, which, together with SCFAs, act as intestinal inflammation reducers [40–42]. *Clostridium clostridioforme* whole genome sequences revealed pathways associated to butyrate synthesis, production that plays a key role in colonic health. The locus composition and arrangement were similar to those present in *Faecalibacterium prausnitzii*, a major butyrate producer of the human large intestine [64]. Butyrate and SCFAs, produced by commensal microbiota following fermentation of dietary fibers, can expand and differentiate intestinal Treg cells. Butyrate, in particular, suppresses the onset of colon cancer and reduces allergic responses in mice [65, 66]. Of great importance, the effectiveness of this probiotic preparation in modulating the composition of microbial communities in the enrolled patients was stable after 60 days from probiotic discontinuation. The metagenome analysis we performed revealed a close relationship between significant upregulation of some taxa (as previously explained in detail) and modulation of immune cell system, suggesting that both mechanisms could underlie the clinically observed long-lasting reduction of the allergy symptomatology.

In conclusion, present study clearly demonstrates that iPROB<sup>®</sup> preparation induces positive effects in allergic patients with asthma and rhinitis. We are aware that our results are preliminary observations, which need a confirmation through future studies lasting a long time and enrolling a wide number of allergic patients. In our opinion, however, data here shown represent a valid assumption because they rely on multifactorial determinations (clinical, cellular, and microbiological), performed by independent research groups. In addition, each blood and fecal analyzed sample derives from same allergic patients. All results we have obtained are consistent among the different evaluations, and the clinical data positively correlate with both cellular and microbiological determinations. In conclusion, the probiotic preparation we have studied has clinical and biological properties to be a valid add-on supplementation in allergic patients.

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## Statement of Ethics

The human International Ethics Committee of A.O.U. “Ospedale Maggiore della Carità” of Novara, Italy, approved the study, and written informed consent was obtained from all enrolled patients.

## Conflict of Interest Statement

The authors disclose no conflict of interests.

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## Author Contributions

S.F. and D.S. made substantial contributions to conception and design of the study. E.T. and M.H. carried out the immunological and microbiological experiments. Supervision of experimental procedures, analysis, and interpretation of data was performed by D.S., S.F., E.B., and F.M. The manuscript was written through contributions of all authors. All authors read and approved the final manuscript.

## Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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