Study Protocol

"Efficacy of Bifidobacterium bifidum on Symptoms of Infantile Colic and Atopic Dermatitis: A Randomized Controlled Trial"

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1. Introduction and Scientific Rationale

Bifidobacterium bifidum is one of the principal commensal microorganisms of the human intestinal microbiota and exhibits a marked tropism for early life. It is a bacterial species highly adapted to the neonatal gut ecosystem owing to its ability to efficiently utilize both host-derived glycans, particularly human milk oligosaccharides (HMOs), and intestinal mucin. This metabolic versatility is supported by a broad repertoire of extracellular glycosidases and by the presence of sortase-dependent pili, which promote adhesion to the intestinal epithelium, mucosal colonization, and interaction with the host immune system.

The degradation of HMOs by B. bifidum plays a crucial role in shaping the intestinal microbiota. The released degradation products—including fucose, sialic acid, and lacto-N-biose (LNB)—serve as substrates for other beneficial bacterial species, thereby promoting cross-feeding mechanisms and supporting the establishment of a healthy microbial community during early life.

Whereas the growth of B. bifidum is naturally sustained by HMOs in breastfed infants, formula-fed infants exhibit a marked depletion of this key microbial species. Nevertheless, the ability of B. bifidum to stably adhere to the intestinal mucosa through sortase-dependent pili and to metabolize endogenous mucin

suggests that it may successfully colonize the infant gut even in the absence of breastfeeding. In this context, B. bifidum may act as a functional substitute by restoring cross-feeding interactions and promoting the immune tolerance typically observed in healthy neonates. Consequently, B. bifidum is considered one of the primary early intestinal colonizers capable of significantly influencing neonatal immune development. This effect appears particularly evident in breastfed infants, as consistently reported in the available literature.

Reduced abundance of bifidobacteria has been associated with both infantile colic, as defined according to the Rome IV criteria, and atopic manifestations, including atopic dermatitis. Both conditions share features of immune and intestinal immaturity and are characterized by an increased prevalence of gut dysbiosis, involving depletion of Bifidobacterium species and expansion of gas-producing microorganisms.

Taken together, current evidence suggests that Bifidobacterium bifidum represents a key bacterial species whose depletion is associated with adverse outcomes along a clinical continuum that includes infantile colic and atopic dermatitis. Considering its specialization in HMO metabolism, its strong capacity to adhere to the neonatal intestinal mucosa, and its distinctive role in promoting immune tolerance, it is of particular interest to systematically investigate its use not only for the management of infantile colic in formula-fed infants—who lack exposure to the HMOs naturally present in human milk—but also for the early modulation of symptoms associated with atopic disease.

Despite the strong biological rationale and encouraging evidence reported for other Bifidobacterium and Lactobacillus species, no randomized controlled trials have specifically evaluated the efficacy of Bifidobacterium bifidum in formula-fed infants with infantile colic. Furthermore, no structured clinical data are currently available regarding its potential effect on symptoms associated with atopic dermatitis.

The aim of the present study is therefore to evaluate the efficacy of Bifidobacterium bifidum supplementation in formula-fed infants diagnosed with infantile colic according to the Rome IV criteria, while also investigating its potential impact on the control of symptoms associated with concomitant atopic dermatitis.

2. Study Objectives

2.1 Primary Objective

To determine whether the daily administration of a probiotic formulation containing Bifidobacterium bifidum BFP19® and Bifidobacterium bifidum BFP29® (Bifipral® oral drops) reduces crying duration in infants diagnosed with infantile colic according to the Rome IV criteria, compared with placebo.

2.2 Secondary Objectives

To evaluate changes in the frequency of crying episodes, sleep quality, bowel movement frequency, and stool consistency.
To assess changes in the fecal microbiota, as determined by shotgun metagenomic sequencing, and in fecal short-chain fatty acid (SCFA) concentrations between baseline and the end of treatment.
In participants with concomitant atopic dermatitis, to evaluate changes in the SCORAD (Scoring Atopic Dermatitis) index before and after treatment.

2.3 Primary Outcome

Treatment response, defined as a ≥50% reduction in the mean daily crying duration compared with baseline.

Baseline crying duration will be calculated as the average of the values recorded during the three consecutive days preceding the baseline visit, in accordance with the methodological standards adopted in probiotic clinical trials and the recommendations of the Rome IV criteria.

2.4 Secondary Outcomes

Change in the frequency of crying episodes.
Change in sleep quality, assessed by the following parameters:
Total sleep duration over a 24-hour period (average of the three recorded days);
Number of nocturnal awakenings;
Longest uninterrupted nighttime sleep episode.
Change in bowel movement frequency and stool consistency, assessed using the modified Bristol Stool Scale for infants.
Changes in the fecal microbiota composition, evaluated by shotgun metagenomic profiling.
Changes in fecal short-chain fatty acid (SCFA) concentrations from baseline to post-treatment.
In participants with atopic dermatitis, change in the SCORAD score between baseline and the end of treatment.

3. Study Procedures
3.1 Study Design

This is a randomized, double-blind, placebo-controlled, parallel-group (1:1) clinical trial. Randomization will be performed using a block randomization scheme and stratified by age (<3 months vs. 3–5 months) to minimize variability associated with the physiological maturation of the gut microbiota and age-related crying patterns.

3.2 Study Duration
Total study duration: 2 years.
Duration for each participant: 9 weeks, consisting of a 1-week run-in period followed by 8 weeks of treatment, with scheduled study visits.
The 7-day observational run-in period is intended to:
confirm the diagnosis of infantile colic according to the Rome IV criteria;
familiarize parents or legal guardians with completion of the daily diary;
collect the baseline data required for efficacy analyses.
A safety follow-up visit will be conducted 2 to 4 weeks after completion of treatment.

3.3 Treatment

Participants will receive either the investigational probiotic product or placebo, administered once daily at a dose of five oral drops, preferably in the morning before feeding. The investigational product and placebo will be indistinguishable with regard to packaging, appearance, color, odor, consistency, and labeling to preserve blinding.

To ensure precise characterization of the investigational product, the probiotic concentration will be expressed as colony-forming units (CFU) per daily dose. Bifipral® oral drops contain a combination of Bifidobacterium bifidum BFP19® and Bifidobacterium bifidum BFP29®. The planned daily dose (five drops) provides a total of 4×10^9 CFU, corresponding to 2×10^9 CFU of each strain. Expressing the dose in CFU per daily administration ensures accurate dose standardization and facilitates comparison with other clinical trials investigating probiotic interventions.

Treatment adherence (compliance) will be assessed by counting the remaining product in returned bottles. Compliance below 80% will be considered inadequate and will result in exclusion from the per-protocol analysis.

Throughout the study, only supportive measures considered compatible with the protocol and not expected to interfere with the study endpoints will be permitted. Parents or legal guardians will receive standardized verbal and written instructions regarding approved behavioral interventions for symptom management (e.g., comforting techniques, soothing strategies, and non-pharmacological modifications of the infant's routine), prohibited treatments during the study, and procedures for contacting the study team.

The independent use of other probiotics, simethicone, systemic antibiotics, or any medications or products potentially affecting the intestinal microbiota will not be permitted. However, the investigator may authorize their use when deemed clinically necessary. Such decisions will be based on an individual clinical assessment and may include the following circumstances:

persistence or significant worsening of inconsolable crying;
clinically relevant disturbances in sleep or feeding;
clinically significant infant distress;
substantial family stress related to the infant's symptoms;
lack of response to approved supportive behavioral measures;
any additional condition judged by the treating pediatrician to be in the infant's best interest.

Parents or legal guardians will be instructed not to introduce any concomitant medication without first contacting the study team. They will receive a dedicated telephone number and email address to report clinical concerns, ask questions regarding product administration, or discuss the need for additional medications during the study.

The safety and well-being of the infant will always take precedence over methodological considerations. The introduction of rescue therapy, when clinically indicated, will be evaluated on a case-by-case basis by the investigator according to the infant's clinical condition.

All concomitant treatments will be documented in both the Case Report Form (CRF) and the daily diary. If a concomitant therapy is considered incompatible with the study objectives, the participant may be excluded from the per-protocol analysis while remaining eligible for the scheduled safety follow-up.

3.4 Study Population

The study will enroll formula-fed infants aged ≤5 months attending the outpatient clinics of the Division of Gastroenterology and Digestive Endoscopy at the Santobono-Pausilipon National Referral Hospital, Naples, and the Division of Pediatrics at Sant'Andrea University Hospital, Rome, who have been diagnosed with infantile colic according to the Rome IV criteria, with or without concomitant atopic dermatitis

3.5 Inclusion Criteria

Participants must meet all of the following criteria to be eligible for enrollment:

Formula-fed infants aged ≤5 months.
Diagnosis of infantile colic according to the Rome IV criteria, with or without concomitant atopic dermatitis.
Written informed consent obtained from the parents or legal guardians prior to enrollment.

3.6 Exclusion Criteria

Participants meeting any of the following criteria will be excluded from the study:

Age >5 months.
Exclusive breastfeeding.
Mixed feeding (breast milk and formula).
Absence of a diagnosis of infantile colic according to the Rome IV criteria.
Presence of any of the following conditions:
chronic diseases;
malignancies;
primary or secondary immunodeficiency;
chronic infections;
autoimmune diseases;
inflammatory bowel disease (IBD);
celiac disease;
inherited metabolic disorders;
cystic fibrosis or other chronic pulmonary diseases;
congenital cardiovascular, respiratory, or gastrointestinal malformations;
neurological or neuropsychiatric disorders;
vegetarian or vegan diet.

3.7 Study Procedures

The study includes a 7-day observational run-in period, designed to confirm the diagnosis of infantile colic according to the Rome IV criteria and to familiarize parents with completion of the daily diary. At the end of the run-in period (Visit 1 – Baseline), eligible participants will be randomized in a 1:1 ratio to receive either Bifipral® oral drops or placebo. Randomization will be generated using a computerized permuted block randomization system. Allocation concealment will be ensured through a randomization list maintained by an independent data manager and inaccessible to the clinical investigators.

The treatment period will last 8 weeks. Parents or legal guardians will administer five drops of the assigned study product once daily, preferably in the morning before feeding, in accordance with the storage instructions provided. The active product contains 2×10^9 CFU of Bifidobacterium bifidum BFP19® and 2×10^9 CFU of Bifidobacterium bifidum BFP29® per daily dose. The investigational product and placebo will be indistinguishable with respect to packaging, color, weight, odor, taste, and labeling, thereby maintaining blinding throughout the study. Investigators, parents, and all study personnel will remain blinded to treatment allocation for the entire duration of the trial.

At Visit 1, parents will receive the daily diary (Appendix 1) together with standardized instructions for recording:

duration and frequency of crying episodes;
sleep parameters;
bowel movement frequency;
stool consistency.

Parents will also record any deviations from the prescribed study treatment. Treatment adherence will be evaluated through review of the diary and verification of returned study bottles. Compliance below 80% may constitute grounds for exclusion from the per-protocol analysis.

During the treatment period, standard supportive therapies that are not expected to affect the intestinal microbiota (e.g., emollients for atopic dermatitis) will be permitted. The use of other probiotics, systemic antibiotics, simethicone, or medications potentially affecting the study outcomes will be prohibited unless considered clinically necessary. Any concomitant treatment or therapeutic modification will be documented in the Case Report Form (CRF).

The study includes three scheduled clinical visits:

Visit 1 (Baseline; after the 7-day run-in period)
Clinical assessment.
Dispensing of the investigational product.
Collection of the first fecal sample.
Distribution of the daily diary.
Visit 2 (End of Treatment; after 8 weeks)
Collection of completed daily diaries.
Clinical assessment.
Collection of the second fecal sample for:
gut microbiota analysis by shotgun metagenomic sequencing;
fecal short-chain fatty acid (SCFA) analysis.
Safety Follow-up Visit (2–4 weeks after treatment completion)
Final clinical assessment.
Recording of essential post-treatment clinical data.
Documentation of any adverse events (AEs) or serious adverse events (SAEs).

Fecal samples will be collected by parents using standardized collection kits. Following collection, samples will be stored in the home freezer (−20°C) for no longer than 24 hours before being transported to the clinical center, where they will be stored at −80°C until analysis.

Microbiota profiling and SCFA analyses will be performed at the La Fontana Medical Analysis Laboratory (Piacenza, Italy).

Adverse events (AEs) and serious adverse events (SAEs) will be recorded during all study visits and, when necessary, through telephone contact. If an adverse reaction to the investigational product is suspected, the treating pediatrician may discontinue treatment, and the event will be managed in accordance with applicable regulatory requirements.

In the event of withdrawal of consent, loss to follow-up, or premature discontinuation of treatment, every reasonable effort will be made to collect essential final study data (e.g., by telephone follow-up or return of the completed diary). The management of missing data will be described in the Statistical Analysis Plan.

3.8 Pharmacovigilance and Adverse Event Management

All adverse events (AEs) occurring during the study will be documented in the Case Report Form (CRF), irrespective of their suspected causal relationship with the investigational product.

Adverse events (AEs) and serious adverse events (SAEs) will be collected and classified according to the International Council for Harmonisation – Good Clinical Practice (ICH-GCP) guidelines. Serious adverse events (SAEs) and suspected unexpected serious adverse reactions (SUSARs) will be reported by the

Principal Investigator to the Sponsor and the Ethics Committee within the timelines required by applicable regulations and ICH-GCP.

Parents or legal guardians will be instructed to promptly notify the investigator or the study team of any unexpected clinical event or worsening of the infant's condition.

For each adverse event, the following information will be recorded:

date of onset;
duration;
severity;
outcome;
any treatment administered;
assessment of the causal relationship with the investigational product.

If an adverse event is suspected to be related to the investigational product, the investigator may decide to temporarily or permanently discontinue treatment in the best interest of the participant. All adverse events will be followed until complete clinical resolution, stabilization, or determination of the final outcome.

4. Sample Size Calculation and Statistical Analysis Plan
4.1 Primary Endpoint

The primary endpoint of the study is the proportion of infants achieving a clinical response after 8 weeks of treatment, defined as a ≥50% reduction in the mean daily crying duration compared with baseline.

Baseline crying duration will be calculated as the average of the values recorded during the three consecutive days preceding the study visit using the structured parental daily diary.

4.2 Sample Size Calculation

Based on published randomized controlled trials investigating probiotic treatment for infantile colic, the expected responder rate in the placebo group is estimated to be approximately 25–30%.

An absolute difference of at least 25% between the treatment groups was considered clinically meaningful, corresponding to an expected responder rate of approximately 50–55% in the probiotic group.

The sample size calculation was performed assuming:

a two-sided significance level (α) of 0.05;
a statistical power of 80–85% (β = 0.15–0.20);
comparison of two independent proportions.

Under these assumptions, the estimated minimum sample size required is 48 evaluable participants.

To increase the statistical robustness of the study, improve the precision of treatment effect estimates, and account for anticipated dropouts, protocol deviations, and incomplete diary data (estimated at approximately 15–20%), the planned sample size was increased to 64 infants, corresponding to 32 participants per treatment arm.

This sample size is expected to preserve adequate statistical power despite losses to follow-up and to improve the interpretability of secondary and exploratory outcomes.

4.3 Randomization

Participants will be randomized in a 1:1 ratio using permuted block randomization, stratified by age (<3 months vs. 3–5 months) to minimize variability associated with the physiological maturation of both crying behavior and the intestinal microbiota.

No individual matching between participants is planned. However, age stratification is expected to ensure an adequate balance of the principal prognostic factors between treatment groups.

4.4 Analysis Populations

The primary analysis will be performed according to the Intention-to-Treat (ITT) principle and will include all randomized participants who received at least one dose of the assigned study treatment.

A Per-Protocol (PP) analysis will also be conducted as a sensitivity analysis, excluding participants with major protocol deviations or treatment adherence below 80%.

4.5 Statistical Analysis

The proportion of treatment responders (≥50% reduction in crying duration) will be compared between treatment groups using the Chi-square test or Fisher's exact test, as appropriate.

Treatment effects will be expressed as:

absolute risk difference (ARD);
relative risk (RR);
95% confidence intervals (95% CI).

Continuous secondary outcomes—including crying duration, sleep parameters, and SCORAD score—will be analyzed using parametric or non-parametric statistical tests, depending on the distribution of the data.

Within-group changes over time will be evaluated using paired statistical tests, whereas between-group comparisons will be performed using tests for independent samples.

4.6 Subgroup Analysis

Among participants with concomitant atopic dermatitis, changes in the SCORAD score from baseline to Week 8 will be evaluated.

Given the anticipated limited sample size of this subgroup, these analyses will be considered exploratory and will not be used to draw definitive conclusions regarding treatment efficacy.

No adjustment for multiple comparisons will be performed for secondary outcomes or subgroup analyses.

4.7 Missing Data

Missing daily diary data will be handled using the Last Observation Carried Forward (LOCF) approach for the ITT analysis, acknowledging its potential to attenuate treatment effects.

Participants with less than 70% of diary data available during the final two weeks of treatment will be excluded from the analysis.

4.8 Microbiota and SCFA Analyses

Changes in the fecal microbiota between baseline and Week 8 will be analyzed using the Wilcoxon signed-rank test for paired comparisons and PERMANOVA (Permutational Multivariate Analysis of Variance) to compare the overall microbial community composition between treatment groups.

Fecal short-chain fatty acid (SCFA) concentrations will be compared using either the Student's t-test or the Mann–Whitney U test, according to data distribution.

4.9 Blinding and Emergency Unblinding

The randomization sequence will be generated by an independent statistician.

The treatment allocation list will be maintained by the independent data manager and will remain inaccessible to the clinical investigators throughout the study.

Emergency unblinding will be permitted only in the event of a Serious Adverse Event (SAE) when knowledge of the assigned treatment is considered essential for the participant's clinical management, in accordance with a predefined and documented emergency unblinding procedure.

Editorial suggestion: This version follows the style commonly adopted in protocols published in journals such as Trials, BMJ Open, and BMC Pediatrics, using dedicated subsections (Primary Endpoint, Sample Size, Analysis Populations, Statistical Analysis, Missing Data, etc.) rather than a single continuous paragraph. This organization improves readability while preserving the original scientific content.

5. Dissemination of Study Results

In accordance with the International Council for Harmonisation – Good Clinical Practice (ICH-GCP) guidelines, the Principal Investigator will prepare a final study report and will be responsible for the dissemination and publication of the study findings.

Participants and/or their parents or legal guardians will be informed of the study results considered relevant within three months following completion of the study.

6. Ethical Considerations and Informed Consent

This study involves the prospective collection of new clinical data and biological samples within the framework of a randomized, controlled interventional clinical trial.

All study data will be pseudonymized using unique participant identification codes and processed in accordance with applicable national and international data protection regulations.

Written informed consent will be obtained from the parents or legal guardians of each participant by the investigators or appropriately delegated study personnel before any study-specific procedures are performed.

7. Study Funding and Conflict of Interest

As this is an interventional clinical study, appropriate insurance coverage will be provided for all study participants in accordance with applicable clinical trial regulations.

The study is financially supported by Pharmaextracta S.p.A. (Via G. Natta 28, 29010 Pontenure, Piacenza, Italy; Tax Identification Number 01419710338), represented by its Chief Executive Officer, Dr. Maurizio Speroni.

The Sponsor will provide the investigational product (Bifipral®) and will cover the costs associated with the microbiological analyses specified in this protocol.

The funding organization will have no role in:

the operational conduct of the study;
data collection;
data management;
statistical analysis;
interpretation of study results;
decisions regarding publication of the findings.

The Principal Investigator and all members of the research team declare that they will conduct the study with full scientific independence and in accordance with the principles of Good Clinical Practice (ICH-GCP) and all applicable regulatory requirements.

Any actual or potential conflicts of interest will be disclosed according to institutional procedures and communicated to the responsible Ethics Committee.

8. Ethics Committee and Competent Authorities

The Sponsor will submit the study protocol together with all required documentation to the competent Ethics Committee for review and approval.

The study will commence only after formal approval has been granted and communicated to the Principal Investigator in accordance with applicable regulatory requirements.

9. Risk–Benefit Assessment

All study procedures are non-invasive and consistent with standard clinical practice; therefore, the anticipated risks to participants are considered minimal.

The expected benefit is considered significant, as the study will provide a rigorous evaluation of the efficacy of Bifidobacterium bifidum supplementation in the management of common pediatric conditions, including infantile colic and associated atopic manifestations.

10. Benefits for Participants and Society

No guaranteed direct clinical benefit is anticipated for individual participants.

However, the knowledge generated by this study may contribute to the development of more effective preventive and therapeutic strategies for infantile colic and atopic dermatitis, thereby benefiting future pediatric patients and the wider community.

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