	Statistical Analysis Plan
	Sponsor: SOFAR **Protocol number: PSC-DS BIGI**

PSC-DS BIGI

Randomized Clinical Trial to Evaluate the Efficacy of Bifidobacterium BB-12® in the Treatment of Infantile Colic

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The signatures on this page indicate review and approval of the Statistical Analysis Plan, version 1.0, dated 16/01/2018.

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LIST OF ABBREVIATIONS

AE	Adverse Event
BB	Bifidobacterium animalis subsp. Lactis
CMED	Concomitant medication
CRF	Case Report Form
DNA	Deoxyribonucleic acid
g	grams
GCP	Good Clinical Practice
IC	Infantile Colic
ITT	Intent-To-Treat Population
NOPD	Non-Protocol Deviation
OTH	Other
PD	Protocol Deviation
PP	Per Protocol Population
SAE	Serious Adverse Event
SAF	Safety Population
sIgA	secretory IgA
WITH	Treatment withdrawal

1. INTRODUCTION

1.1. CHANGES FROM THE STUDY PROTOCOL

Reduction of the number of cries from baseline to the end of study (after 28 days), presence of infantile colic at the end of study (after 28 days), reduction of the number of regurgitations and vomiting episodes will not be evaluated since information about these secondary objectives is not collected in the eCRF.

The analysis of Bifidobacterium BB-12® effect on gut microbiota, on the fecal production of peptides from innate immune system (Calprotectina, β-defensin 2, LL37) and on fecal short chain-fatty acids production (butyrate and propionate) will be provided by the center to Sponsor and reported in a separate document.

1.2. DISCLOSURE OF THE RESULTS

Dissemination of results will not be limited. No independent statisticians nor reviewers are foreseen.

2. STUDY OBJECTIVES

For the rationale of this study, please refer to Chapter 1.0 of the Study Protocol #3 dated 16 May 2016.

2.1. PRIMARY OBJECTIVE

The primary objective is to estimate the treatment's success rate, defined as the percentage of infants who have more than a 50% reduction in the average daily duration of cries after 28 treatment days from baseline.

2.2. SECONDARY OBJECTIVES

- Reduction of the number of cries from baseline to the end of study (after 28 days) and presence of infantile colic at the end of study (after 28 days);
- reduction of the number of regurgitations and vomiting episodes;
- number of infections of the respiratory system, gastrointestinal system, urinary system or skin during the study period;
- evacuative frequency;
- consistency of the fecal mass;

- Bifidobacterium BB-12® effect on gut microbiota, on the fecal production of peptides from innate immune system (Calprotectina, β-defensin 2, LL37) and on fecal short-chain fatty acids production (butyrate and propionate).

2.3. EXPLORATORY OBJECTIVES

Not Applicable.

3. BACKGROUND AND RATIONALE

3.1. STUDY DESIGN

This is a prospective randomized double blind placebo controlled clinical trial.

Enrolled patients are treated with a dietary supplement containing Bifidobacterium subsp.lactis animalis BB-12® (Bifidolactis Infant) or Placebo, provided free of charge by SOFAR S.p.A.

The trial is conducted at the Department of Translational Medical Sciences, "Federico II" University of Naples as the sole participating center (Principal Investigator: Prof. Roberto Berni Canani).

3.2. TREATMENT GROUPS AND RANDOMIZATION

Enrolled infants are randomized at T1 to Bifidobacterium BB-12® or Placebo.

Randomization is done according to a randomization list obtained by a specific software and provided by the Coordinator Center.

The treatment regimen can be summarized as follows:

- Group I: patients who take Bifidobacterium BB-12® (Bifidolactis Infant), 6 drops a day (guaranteeing a billion of living cells) for 28 consecutive days;
- Group II: patients who take Bifidolactis Infant Placebo 6 drops a day for 28 consecutive days.

3.3. STUDY POPULATION

Inclusion criteria

Patients are included in the study if they meet all the following criteria:

- Exclusively breastfed healthy infants of both sexes, aged ≤ 7 weeks.
- Diagnosis of IC according to Rome III criteria.
- Written informed consent of the parent/tutor.

Exclusion criteria

Patients are excluded from this study if they meet any of the following criteria:

- Birth weight < 2500 g.
- Gestational age < 37 weeks.
- APGAR 5 minutes < 7.
- Formula feeding.
- Stunting/loss of weight (< 100 g/weeks from birth to the last reported weight).
- Neurological diseases.
- Known or suspected food allergy.
- Gastroesophageal reflux disease.
- Use of substances that alter gut microbiota (probiotics, prebiotics, antibiotics, gastric acidity inhibitors) in the last 2 weeks prior the enrollment.
- History of fever and/or infectious diseases in the last 2 weeks prior to enrollment.
- Ongoing systemic infections.
- History of congenital infections.
- Chronic intestinal diseases (cystic fibrosis or other forms of primitive pancreatic insufficiency)
- Primitive or secondary malformations of the gastrointestinal tract (such as esophageal atresia, intestinal atresia, short bowel syndrome, malrotation).
- Metabolic diseases.
- Genetic diseases and chromosomal abnormalities.
- Primary or secondary immunodeficiencies.
- Not sufficient reliability or presence of conditions that may result in non-compliance/adherence of the patient to the Protocol.
- Previous participation in this study.

3.4. STUDY DRUG AND DOSING

Parents/tutors receive two vials of dietary supplement Bifidobacterium BB-12® (Bifidolactis Infant) or Bifidolactis Infant Placebo for the entire treatment period (28 days); each vial is provided together with a dispenser and contains drops for 21 days.

Bifidolactis Infant and Placebo are packaged in indistinguishable boxes.

One vial of the investigational drug contains 6 ml and one billion of living cells.

The vial contains sunflower oil (Heliantus annus L.) and the cap contains Bifidobacterium BB-12® (Bifidobacterium animalis subsp. Lactis, strain deposited DSM 15954) and maltodextrin.

An adequate supply of product is provided free of charge by SOFAR S.p.A.

Reference samples of Bifidolactis Infant and Placebo are deposited at the SOFAR S.p.A. quality control department.

Infants receive 6 drops a day; drops should be dosed on a spoon and mixed with mother's milk.

3.5. PROHIBITED MEDICATIONS

Probiotics/prebiotics/synbiotics and other anti-colic drugs should be avoided during the study.

3.6. SAMPLE SIZE

According to the results of a previous trial that looked at a probiotic's effect on infants with CI, it is estimated that when the sample size in each group is 33, the study will have 80% power to detect an absolute difference of 35% in the treatment success rate (15% in the Placebo group and 50% in the treatment group) with a 0,05 alpha level.

The number of infants that will be included in the study is 80, with a dropout rate expected for a maximum of 20%.

3.7. SCHEDULE OF TIME AND EVENTS

The total duration of the study is established in 5 weeks for each infant.

All infants should enter a 1 week run-in period during which probiotic fermented milk, probiotic dietary supplements and prebiotic dietary supplements should be avoided. After this period, if the diagnosis of CI is confirmed, the infant is eligible for the study and is randomized to investigational treatment or Placebo.

During the first visit (T0), the Investigator assesses inclusion/exclusion criteria and tells parents of the study objective and design.

After having parents/tutor written informed consent, the Investigator evaluates family medical history and the infant's clinical condition, and instructs parents on how to record data in the diary about: number and duration of daily cries, fecal consistency and evacuative frequency for the subsequent 7 days.

After 7 days (T1) a new visit is planned; if the diagnosis of CI is confirmed, a fecal sample is collected and the infant is assigned to Bifidobacterium BB-12® or to Placebo, according to the randomization list. Infants should take treatment for 4 weeks.

The Investigator tells parents the product daily dose and instructs them on how to administer the product accurately.

All infants are examined weekly by the Investigator for 28 days from randomization.

At each visit (T2, T3, T4, T5) the infant's clinical conditions are recorded, diary completeness is checked and parents/tutor receive a new dairy for data collection.

All anthropometric data are collected at T0, T1 and T5.

At T1 and T5, a fecal sample (3 g) is collected for each infant to evaluate: β-defensin 2, LL37, Calprotectina (ELISA method), sIgA, intestinal microbiota (through 16SrRNA amplification and quantification and DNA microarrays) and short-chain fatty acids (butyrate and propionate) through gas-chromatography.

Six visits are planned during the study according to the flow chart shown in Table 2.6-1:

Table 2.6-1: Flow Chart

Visit	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**
Days	**1**	**7**	**14**	**21**	**28**	**35**
Inclusion / Exclusion criteria	X	X				
Written Informed Consent	X					
Anthropometric data	X	X				X
Randomization		X				
Investigational treatment		X	X	X	X	
Medical history	X					
Clinical data	X	X	X	X	X	X
Physical examination	X	X	X	X	X	X
Diary completeness check		X	X	X	X	X
Fecal samples		X				X

4. ANALYSIS POPULATIONS

The following analysis sets are defined:

- Per Protocol Set (PP): all randomized patients who complete the study without any significant protocol violation.
- Intention to Treat Set (ITT): all randomized patients who receive at least one dose of study treatment. Following the intent-to-treat (ITT) principle patients will be analyzed according to the treatment they have been assigned to at the randomization visit.
- Safety Set: all randomized patients who receive at least one dose of study treatment. Patients will be analyzed according to the actual treatment received.

A patient who receives at least one dose of study treatment will be defined as a patient who comes back at Visit T2.

The primary efficacy analysis will be performed on the ITT population and on the PP population as supportive. The secondary efficacy analysis will be performed only on the ITT population. The safety analysis will be performed on the Safety population.

Patients violating the criteria defined above will be excluded from any analyses.

The assignment of patients to analysis populations will be summarized before the database lock in the Patient Validation Document. Final assignment of patients to analysis populations will be approved by the Sponsor study team.

The list of protocol deviations leading to the exclusion from the analysis populations is reported in the Protocol Deviation Handling Document (PDHD).

Non-protocol deviations (NOPDs) detected through SAS® programming are not protocol violations but they lead to exclusion from analysis populations; they are listed in the following Table 3-2:

Table 3-2: List of non-protocol deviations

Deviation code	Description	Exclusion from analysis population
NOPD1	Patient who was not randomized	PP/ITT/Safety
NOPD2	Randomized patient who did not have receive at least one dose of study treatment	PP/ITT/Safety

* NOPDs = Non-protocol deviations

5. VARIABLES

5.1. EFFICACY VARIABLES

PRIMARY EFFICACY PARAMETERS

- Treatment's success rate: percentage of infants who have more than a 50% reduction in the average daily duration of cries after 28 treatment days.

SECONDARY EFFICACY PARAMETERS

- Number of cries from baseline to the end of study (after 28 days) and presence of infantile colic at the end of study (after 28 days).
- Number of regurgitation and vomit.
- Number of infections of the respiratory system, gastrointestinal system, urinary system or skin during the study period.
- Daily evacuative frequency.
- Daily consistency of the fecal mass, evaluated through Bristol stool scale.

- Bifidobacterium BB-12® effect on gut microbiota, on the fecal production of peptides from innate immune system (Calprotectina, β-defensin 2, LL37) and on fecal short chain-fatty acids production (butyrate and propionate).

5.2. SAFETY VARIABLES

Adverse Events

Adverse events are defined as the appearance of undesirable sign(s) (included laboratory abnormal values), symptom(s) or medical condition(s) temporarily associated to a drug.

An Adverse Event will be defined as a Serious Adverse Event if the event:

- results in death

- is life-threatening

- requires in-patient hospitalization or prolongation of existing hospitalization

- results in persistent or significant disability/incapacity

or

- is a congenital anomaly/birth defect

An event that compromises patient safety will be considered as a Serious Adverse Event.

Physical examination

Physical examination is evaluated at each visit. The following locations will be analyzed, considering if the result was normal, abnormal on not evaluated: hairs/skin, lymph nodes, eyes, ears/nose/throat, breast, respiratory, cardiovascular, abdomen, urogenital, pelvic, rectal, musculoskeletal, neurologic, mental status.

Anthropometric data

Anthropometric data are collected at Visits T0, T1 and T5: weight (g), length (cm), cranium measure (cm), weight (g) / length (cm).

6. DEFINITION AND GENERAL METHODOLOGY

6.1. GENERAL METHODOLOGY

All statistical tables, listings and figures will be produced using SAS® for Windows release 9.4 (64-bit) or later (SAS Institute Inc., Cary, NC, USA). Continuous data will be summarized by mean,

standard deviation (SD), median, 1st and 3rd quartile, minimum and maximum. Categorical data will be presented by absolute and relative frequencies (n and %) or contingency tables.

Percentages will be computed considering patients with non-missing information, if not differently specified.

DEFINITION OF BASELINE

The baseline period for the present study is defined as visit T0.

CODING OF THERAPIES AND MEDICAL TERMS

Clinical/surgical history and adverse events will be coded using MedDRA version 19.1 ITA; prior / concomitant medications will be coded using WHO Dictionary B2 Q4 2015.

MISSING OR PARTIAL DATES

Not Applicable.

HANDLING OF MISSING DATA/IMPUTATION RULES/CENSORING RULES

Not Applicable.

HANDLING OF DROP-OUT PATIENTS

Not Applicable.

MULITPLICITY ISSUES

Not Applicable.

6.2. STUDY PATIENTS

Patient Disposition

A complete description of patient disposition will be provided on the enrolled patients overall and by treatment group. The number and percentage of patients entered at each visit, as well as the number of patients who completed and discontinued the study will be provided with the reasons for discontinuation. A complete description of the number of protocol violators and the number of patients per deviation will be summarized in the Patient Validation Document.

The numerousness of the analysis population will be described overall and by treatment group and the reason excluding the patient from any analysis population will be provided.

Patient Baseline Characteristics

Demographic and baseline characteristics will be summarized overall and by treatment group on the Intention To Treat population.

Demographic/diagnosis information includes:

- Sex

- Age (days) at baseline will be computed in terms of days elapsed from birth date to informed consent date. When dates of parents' informed consent signatures are different, the latest date will be considered for the computation.
- Diagnosis of IC, according to Rome III criteria, for infants <= 4 months
- Type of childbirth (natural or caesarean)
- Gestational age (weeks) will be described by means of descriptive statistics for continuous data, while days will be only listed.
- Birth weight (g) will be described by means of descriptive statistics for continuous data, while its percentile will be only listed.
- APGAR 5 minutes score
- Positive family history of allergic diseases
- Exposure to second-hand tobacco smoke within the home environment
- Positive family history of functional gastrointestinal diseases
- Clinical/surgical history will be evaluated checking if the infant had any clinical/surgical relevant event in the past.

  If the infant had a relevant event that could exclude him or her from the study, a category was required: ears/nose/throat, ophthalmic system, respiratory system, cardiovascular system, gastrointestinal system, hepatobiliary/pancreas, renal system, urogenital system, neurologic system, blood/lymphatic, endocrine/metabolic, musculoskeletal system, skin, psychiatric, other (allergies included). The frequency and proportion of patients who reported each category at least once will be summarized and presented by category, ATC Code Level 2 and Preferred Term. Patients could have more than one specification.

Other background characteristics will be evaluated at visit T0 and will include information about infant's parents:

- Number of terminated pregnancies of the mother
- Parents' smoking habit (if the father/the mother were smokers).

6.3. PRIOR AND CONCOMITANT MEDICATIONS

Prior / concomitant medications will be evaluated checking if the infant took any therapy in the past / during the study and will be presented by System Organ Class, Preferred Term and ongoing status on the Intention To Treat population and on the Safety population respectively

6.4. DRUG EXPOSURE AND TREATMENT COMPLIANCE

Exposure will be computed as the time in days elapsed from the date of the first study drug administration (Visit T1) to the date of the last study drug administration (if applicable) or to the date of the day before Visit T5.

6.5. EFFICACY EVALUATION

Efficacy data will be summarized by treatment. Primary variables will be analyzed on the ITT and PP populations while secondary variables will be analyzed on the ITT population only.

6.5.1. Primary efficacy analysis

Treatment's success rate.

Duration of crying will be collected daily in the diary during the entire study period. The average daily duration will be defined as the mean duration of all cries recorded during the day in the "Evaluation of crying" section (i.e. sum of episodes' duration/number of cries during the day).

Weekly mean will be defined as the mean of the calculated average daily durations during the selected week and will be described by means of descriptive statistics for continuous data. Mean changes from baseline (i.e. mean of the first Week) to the mean of the selected week will be computed as well.

Treatment success rate will be evaluated in terms of reduction of duration of cries, comparing mean weekly duration of the last Week (from T4 to T5) and mean weekly duration of Week 1 (from T0 to T1). A success will be defined as a reduction >=50%.

The proportion of patients with a success in the two groups will be summarized by means of descriptive statistics for categorical data and analyzed by means of a Chi-Square test.

6.5.2. Sensitivity analysis on primary endpoint

Not Applicable.

6.5.3. Secondary efficacy analysis

- Number of cries

  Number of cries are collected daily in the diary during the entire study period.

  Weekly mean of cries will be defined as the mean number of cries reported in the "Evaluation of behavior" section during the week (i.e. number of episodes/number of days with episodes) and will be described by means of descriptive statistics for continuous data.

  Mean changes from baseline (i.e. mean of the first Week) to the mean of the selected week will be analyzed too.

Moreover, in order to compare the two groups of patients, a t-test on the change of weekly mean of cries at last Week will be performed. In case of data not normally distributed, a Wilcoxon/Mann-Whitney test will be used.

- Number of infections of the respiratory system, gastrointestinal system, urinary system or skin during the study period.

  Number of infections will be described on the ITT population, even if infections are defined using safety parameter. In fact, an infection will be defined as an Adverse Event with SOC equal to "Infections and Infestations".

  The frequency and proportion of patients who reported at least one infection will be summarized and presented by category, System Organ Class and Preferred Term.

- Evacuative frequency

  Frequency and consistency of stools are collected daily in the diary.

  Stool frequency will be evaluated as the mean of total daily stools reported per week. Mean changes from baseline (i.e. mean of the first Week) to the mean of the selected week will be analyzed.

  Summary statistics of mean stool frequency per week will be provided by means of descriptive statistics for continuous data.

- Consistency of the fecal mass

  Stool consistency will be evaluated as the number and the proportion of patients who reported at least one stool sample of each type per week, according to Bristol scale. Patients could report more than one stool consistency per day (according to the number of daily stools): all the information collected will be analyzed.

Other data from patients' diary will be analyzed:

- Infant's mood

  Infant's mood (calm, asleep, agitated, irritable) is collected daily in the diary and will be evaluated as the number and the proportion of infants who reported at least one mood of each type per week.

- Infant's sleep

  Duration of sleep (in minutes) will be collected daily in the diary during the entire study period. The average daily duration will be defined as the mean duration of all sleeps during the day (i.e. sum of episodes' duration/number of sleeps during the day).

  Weekly mean will be defined as the mean of the calculated average daily durations during the selected week and will be described by means of descriptive statistics for

continuous data. Mean changes from baseline (i.e. mean of the first Week) to the mean of the selected week will be analyzed too.

- Infant's temper

  Duration of temper episodes (in minutes) will be collected daily in the diary during the entire study period. The average daily duration will be defined as the mean duration of all temper episodes during the day (i.e. sum of episodes' duration/number of temper episodes during the day).

  Weekly mean will be defined as the mean of the calculated average daily durations during the selected week and will be described by means of descriptive statistics for continuous data. Mean changes from baseline (i.e. mean of the first Week) to the mean of the selected week will be analyzed as well.

- Infant's feeding

  Feeding time (in minutes) will be collected daily in the diary during the entire study period. The average daily duration will be defined as the mean feeding time during the day.

  Weekly mean will be defined as the mean of the calculated average daily durations during the selected week and will be described by means of descriptive statistics for continuous data. Mean changes from baseline (i.e. mean of the first Week) to the mean of the selected week will be analyzed also.

  Information about way of feeding will be only listed.

6.5.4. Exploratory efficacy analysis

Not Applicable.

6.6. SAFETY EVALUATION

Safety data will be summarized by treatment on the Safety population.

6.6.1. Adverse Events

The incidence of Adverse Events (AEs) and Serious Adverse Events (SAEs) recorded during all the study will be presented. Treatment-emergent events will be considered those AEs with an onset date after the initiation of treatment.

Non-treatment emergent adverse events (i.e. events occurring between T0 and T1) will be listed separately.

Tables reporting a general summary of AEs will be produced by treatment group specifying the number of total events and the absolute and relative frequency of patients with AEs. As

a patient may have more than one AE, the total number of AEs could be greater than the total number of patients.

Absolute and relative frequency of patients with drug related AEs, severe AEs, SAEs, AEs with an outcome of death, AEs leading to permanent discontinuation of treatment, AEs leading to temporarily interruption, AEs leading to hospitalization/prolonged hospitalization will be also reported.

Drug-related AEs will be the AEs with causality equal to ”Probably”, “Possibly” and “NA”; if the relationship to study drug is unknown or missing, the AE will be drug related as well.

AEs will be tabulated by System Organ Class, Preferred Term and severity. The absolute and relative frequency of patients who have experienced each type of event will be presented; if the same patient reported more than one occurrence of the same AE with different intensity, no selection of the one with the worst intensity will be adopted.

SAEs will be summarized similarly.

All serious AEs, study drug related AEs, AEs with an outcome of death, AEs leading to permanent discontinuation of treatment will be listed with all their details.

Relative days, defined as days elapsed from the treatment start to the onset date of the event, will be computed.

6.6.2. Laboratory parameters

Not Applicable.

6.6.3. Vital signs/Physical examination

Physical examination will be summarized by treatment group by means of absolute and relative frequency at each visit.

6.6.4. Other safety parameters

Anthropometric data will be summarized by treatment group by means of descriptive statistics for continuous variables at T1 and T5. Weight (g) / length (cm) will be calculated at each visit. The percentile of each variable will be only listed.

Changes vs. T0 at T1 and T5 will be provided.

6.7. SUBGROUP ANALYSES

Not Applicable.

6.8. INTERIM ANALYSIS/DMC

Not Applicable.

7. REFERENCES

Not Applicable.

8. APPENDIX

Not Applicable.