The Tolerance of Organic Formula Milk and Its Fecal Microbiome Characteristic in Infants

Study Protocol

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Summary:

An experimental study aims to investigate the tolerance of organic formula milk on infants supplemented with organic formula milk. This study also observes gut microbiota, short chain fatty acids, nutritional status, and atopic manifestation on infants supplemented with organic formula milk. This study will be done on 50 subjects, with an age of 6-7 months old, 38-42 weeks of gestation, had a birth weight ranging from 2700 grams to 4200 grams, not suffering from any major congenital anomaly, not severely stunted at birth, has a normal thyroid function, not suffering any prominent gastrointestinal disease, not having a severe disease at the beginning of study, and has an approval from their parents. Subjects' diet will be added an organic formula for infant for 3 months, and they will be monitored regularly, since this study starts, at each month, and at the end of this study. The subjects' gut microbiomes will be calculated at every session of monitoring by collecting their fecal samples, and brought to laboratory. Anthropological data (weight, height, body mass index), atopic manifestation, IL-6 and IL-10 will also be collected.

Introduction:

An enormous number of microbiotas inhabit the human intestinal tract. The microbiota forms a unique and complex gut ecosystem by interacting with the host, and this affects the health and diseases of the host in various ways, including the modification of the immune system. Butyrate produced by the gut microbiota promotes differentiation of peripherally derived regulatory T cell (pTreg) in colonic lamina propria through epigenetic modification. Besides, it induces B cell Lymphocyte to secrete IgA (sIgA) which will be transferred to intestinal lumen. sIgA plays an important role in antigen binding and clearance. Additionally, gut microbiota can inhibit histone deacetylases (HDACs) as well. All these functions may have the inflammation control not only in the gut but also in other parts of the body. [1].

The microbiota hypothesis states that the disruption of mucosal regulatory immune responses caused by the perturbation of microbiota due to Western lifestyle factors leads to the development of both Th1- and Th2- mediated disease. It is necessary to have a complex

microbiota in order to prevent some diseases such as allergy, diarrhea, autoimmune disorder, etc. The depletion or deletion of bacterial communities, as associated with an elevated serum IgE level, increases the circulating basophil population, thereby exaggerating the Th2 cell responses and allergic inflammation. It has been clarified that gut microbiota plays a critical role in oral tolerance in mouse model. Various studies have revealed that the microbiota plays an important role in pathogenesis of allergic diseases. It remains unclear how the differences in age, region, dietary intake or other environmental factors such as allergen exposure or infection, affect this role [2,3]. Candidate bacterial taxa have been reported it still remain unclear which bacteria (or other microbes), in which numbers and combinations, and when during the gut colonization process may prevent allergic diseases and asthma [4]. Some different microbiota may produce the same metabolome. For example, one of SCFA (short chain fatty acid), pyruvate acid is produced by Bacteroides and Clostridium as well.

Imbalance of gut microbiota or dysbiosis may start in early life. The increasing in cesarean section, formula feeding and the use of antibiotics during pregnancy or infancy have the significant impacts on microbial homeostasis.

Objectives of Trial:

1. **Primary Objective**
    a. To know the acceptability of organic formula milk on infants
2. **Secondary Objective:**
    a. To determine gut microbiota characteristics on infants supplemented with organic formula milk
    b. To determine gut short chain fatty acids on infants supplemented with organic formula milk
    c. Nutritional status including body mass index.
    d. Atopic manifestation

Hypothesis:

H0 : Organic formula milk is not well accepted by infants
H1 : Organic formula milk is well accepted by infants

Trial Design:

Observational study

Study Population:

Number of samples:
50 subjects

Inclusion criteria:

- Healthy infants 6-7 months of age.
- Gestational age 38-42 weeks
- Birth weight >2700 and <4200 gram
- Not suffering from a major congenital anomaly, severely stunted at birth, not having a thyroid problem, not suffered from prominent gastrointestinal diseases, severe diseases at the time of inclusion (severe pneumonia, severe dehydration, etc)
- Parents want to follow the study by signing the informed consent

Exclusion Criteria:

- Subjects are in the severe disease condition at the time of recruitment
- Severe acute malnutrition

- Have conditions that will influence the nutritional status such as moderate to severe dehydration, organomegaly, edema.

Product Description:

Organic formula milk + solid food/breast feeding

Arla Baby&Me Organic Infant Formula 6-12 months, contains:

Skimmed milk, Lactose, Vegetable oils (Palm oil, Rapeseed oil, Soy bean oil, Coconut oil), Galactooligosaccharide (GOS), Concentrated whey protein powder, Fructooligosaccharide (FOS), Arachidonic acid from M. alpina, Docosahexaenoic acid from C. cohnii, Minerals (Tricalcium disphosphate, Calcium carbonate, Calcium hydroxide, Potassium chloride, Tripotassium citrate, Trisodium citrate, Sodium chloride, Magnesium chloride, Ferrous sulphate, Zinc sulphate, Copper sulphate, Potassium iodide, Manganese sulphate, Sodium selenite), Vitamins (Retinyl acetate, Cholecalciferol, D-alpha tocopherol, Phytomenadione, L-ascorbic acid, Ascorbyl palmitate, Thiamine hydrochloride, Nicotinamide, Calcium-D-pantothenate, Pyridoxine hydrochloride, D-biotin, Folic acid), Emulsifier (Lecithin E322).

**Contains milk and soy ingredients.*

Outcomes:

The tolerance will be the primary outcome measured by clinical examination and questionnaire. We will measure the outcome every month. The secondary outcomes, gut microbiomes which will be obtained through fecal samples collection. Fecal DNA extraction, polymerase chain reaction amplification and library construction for bar-coded 16S rRNA gene amplicon sequencing, using the Illumina MiSeq platform will be examined. The 16SrRNA gene amplicon sequences will be analyzed using QIIME1.9.0 and operational taxonomic units (OTUs). Fecal SCFAs will be measured by LC-MS/MS. Data will then be calculated to see if there is a significant difference before and after organic formula feeding. Additionally, we also will measure nutritional status, including body mass index, atopic manifestation, IL-6, and IL-10. Measurements of weight, length, and head circumference will be monitored at every visits. Anthropometric data will be collected at the beginning of study, first visit (4 weeks after) and at second visit (end of study) to evaluate the subjects growth.

Conduct of Trial:

All the guardians or parents of the subjects who participate in this study will be interviewed in order to record the dietary and sign for inform consent. Parents will be given a journal to record the subject's diet, any sign of colic on subjects, their quality of sleep, and wellbeing. All the subjects will be fed organic formula milk as a part of their dietary for 12 weeks. Before feeding the formula, the nutritional status will be measured and the subject's fecal will be collected for microbiome and short chain fatty acid (SCFA) examination. Every month the parent will be called in order to assess for child's sickness, allergy, body weight, length, head circumference, and to review their journal.

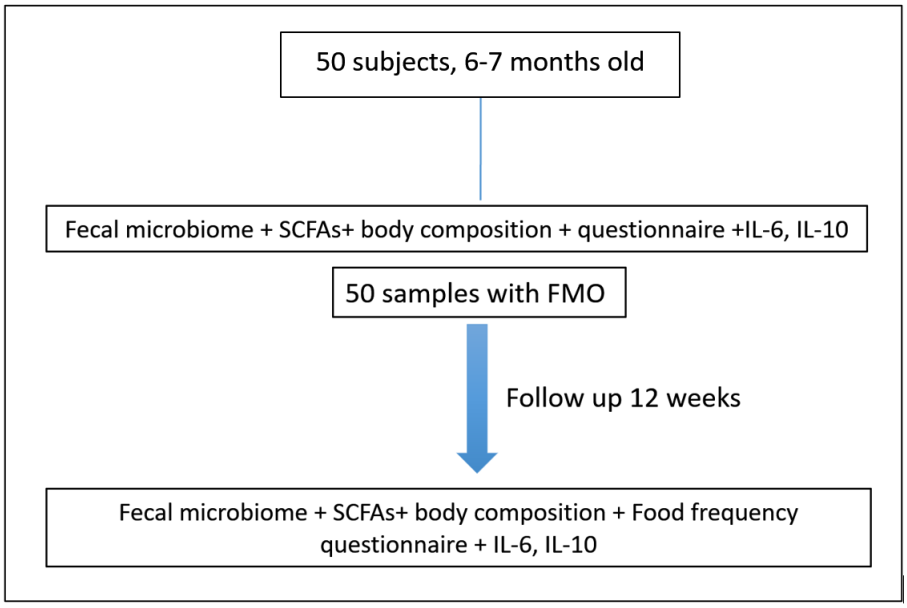

Figure 1. Study Procedure

Description of Visits:

A visit follow up will be done before and after the study, the tolerance, nutritional status (including body mass index) and fecal collection for all subjects will be performed. We will also collect the allergy related data. Each fecal examination will follow a strict cold chain procedure, each sample will be collected directly, bottled, and frozen to minimize error. The frozen samples will be transported in insulated bags with frozen ice blocks before being transferred to -80°C for storage.

Statistical Aspects and Considerations:

A statistical analysis will be performed using r program and STATA 13.0. The Normality of data distribution will be tested using the Shapiro–Wilk test. To evaluate potential associations between factors in relation to fecal microbiome or SCFAs, Spearman's rank correlations will be calculated. A p-value <0.05 will be considered significant.

The model will be adjusted for potential confounders. Estimated associations will be described as odds ratios (ORs) with 95% confidence intervals (CIs). The non-parametric Wilcoxon test for paired samples will be used to compare different laboratory parameters at different times of collecting samples. The Mann–Whitney U test for unpaired samples will be applied to compare different categorical parameter between infants before and after treatment. Multivariable linear regressions will be done to find out the association of different parameters change before and after treatment.

Ethical Aspects: The institutional ethics committee of each participating Hasanuddin University Hospital and Wahidin Sudirohusodo Hospital must be approved the study protocol

References:

1. Ohno H: Gut microbial short-chain fatty acids in host defense and immune regulation. Inflamatian and regeneration.2015;35:114-121.
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4. Sjödin KS, Vidman L, Rydén P, et al. Emerging evidence of the role of gut microbiota in the development of allergic diseases. Cur Opin Allergy Clin Immunol 2016;16:390-395.