CHAPTER 4

METHODS AND MATERIALS

Research outcomes are only substantial when they are based on unfailing methodologies. Present research**"Morbidity Status and Gut Health of Normal and Undernourished School Going Children and its Alteration Upon Feeding them with Fructooligosaccharide Incorporated Ice-Cream''** was conducted in four phases and this chapter is written in hierarchical manner describing the methods and techniques from first to final step of the research.

First phase of the study was the formative research with a Cross-sectional design, wherein the children were assessed for their anthropometric assessments and their parents were interviewed for their socio-economic status, morbidity profile, immunization and past breast feeding practices of their child.

The second phase of the study had the Comparative research design wherein nourished and undernourished children were studied based on the baseline values of their morbidity profile, gut microflora and serum IgA levels.

Randomized placebo control trial was used in the third phase of the research wherein 60 undernourished children were randomly stratified into placebo and experimental group and then their diets were supplement with placebo and FOS ice-cream respectively. The impact was analyzed based on the pre and post values of anthropometric measurements, gut microflora, morbidity

profile, and serum IgA levels.

The fourth or the last phase was undertaken to compile various recipes based on prebiotics viz. FOS and Inulin standardized and developed in the "Department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat", this book was further distributed to counseling centers. Experimental plan of the study is pictured in Fig.4.1.

Material and methods chapter of the present research will precede based on the following stated outlines:

4.1. Location the study

4.2. Selection of the subjects

4.3. Anthropometric Measurements

4.5. Survey

4.6. Analytical Methods

4.7. Dietary intake analysis

4.8. Serum IgA analysis

4.9. Study design for randomized clinical trial

4.10. Statistical Analysis

4.11. Ethical Committee approval and study registration

Fig. 4.1. Study Plan: "Morbidity Status And Gut Health Of Normal And Undernourished School Going Children And It's Alteration Upon Feeding Them With Fructooligosaccharide Incorporated Ice-Cream"

Phase I:

Prospective study: Cross sectional design

One school selected from urban Vadodara

All the children studying in class I-V (n=218) were screened for:

- Anthropometric measures and their Nutritional Status was determined.
- Parents of these children were interviewed for:
- Past 1 month Morbidity profile of: diarrhea, common colds, flatulence, constipation, stomach ache
- Past Breast feeding practices and immunization status of the child

Phase II:

Observational Comparative Study: Case-control design

Nourished (n=30) and Undernourished (n=80) children were compared for:

- Morbidity profile
- Serum IgA levels
- Gut microflora: *Bifidobacteria*, *Lactic acid bacteria* and *E.coli*
- Dietary Intake

Various determinants of Nutritional Status were also ascertained

Phase III:

Experimental Comparative study: Randomized Clinical Placebo trial

- 60 Undernourished children were randomly stratified and were divided into two groups: Placebo and Experimental
- Experimental group was intervened with FOS incorporated ice-cream and Placebo group with Placebo ice-cream for 30 days

Post data was collected on:

- Morbidity profile
- Serum IgA levels

Phase IV:

Compilation and Propagation of a Bi-lingual Booklet entitled "Prebiotic: Our Gut Guardians" based on prebiotic incorporated recipes developed and standardized in the Department of Foods and Nutrition, Faculty of Family and Community Sciences, The M.S. University of Baroda, Vadodara, Gujarat.

4.1. Location of the study

Using convenient sampling procedure, one semi-private school in the vicinity of the M.S. University of Baroda was selected from urban Vadodara, Gujarat (Fig. 4.2). Authorities of the school were priorly counseled about the importance and implication of the present research. Written permission (Appendix 1) was sought from the principal of the school before initiating the screening process.

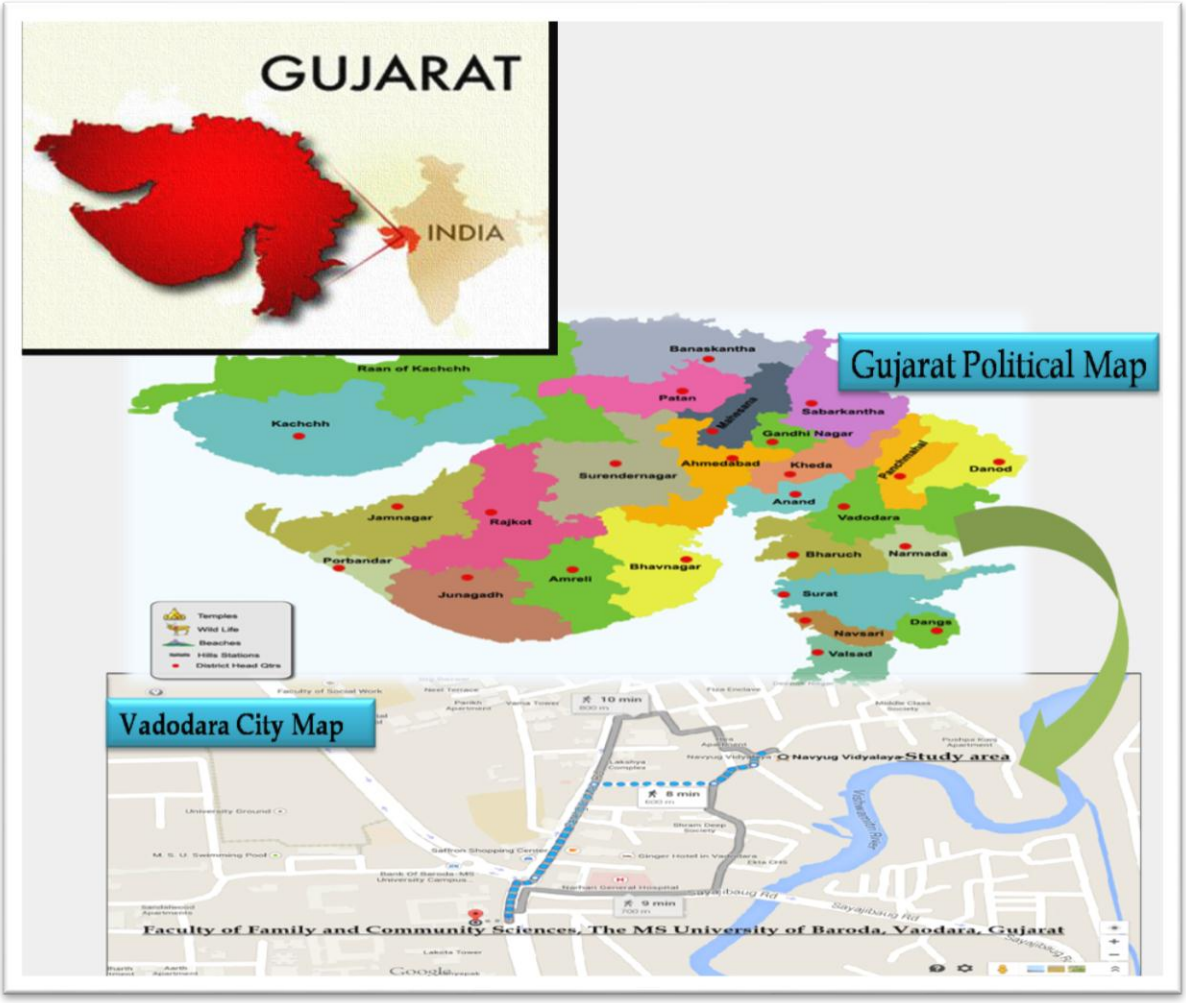


Fig.4.2 Location of the study area

4.2. Selection of the subjects:

All the children studying in class I-V (n=218) were enrolled for screening with respect to anthropometric measurements. Morning hours were allotted to conduct screening process without disturbing the regular schedule of the classes. In-charge teachers of every class were briefed about the objectives and implications of the study and were requested to maintain the decorousness in the children for the appropriate execution of the screening process.

4.3. Anthropometric Measurements

Anthropometry is the measurement of body dimensions to characterize skeletal and tissue development, and effect relationship between nutrient and level of well-being of the body is assessed. In the present study, 218 children were assessed and all anthropometric measurements were made using the guidelines adopted at the NIH sponsored Arlie Conference [Lohman *et al.,* 1988].

4.3.1. Weight

It is the most widely used and simplest reproducible anthropometric measurement. It indicates the body mass and is a composite of all body constituents like water, minerals, fat, protein, bone, etc. [Robinson *et al.,* 1988].

Technique- A platform weighing scale to the nearest 100 g was used to measure weight. Children were weighed in their school uniform but were asked to remove their belt, tie and shoes, and to stand without leaning against or holding

anything. Scale was 'zeroed' before taking any weight, and was calibrated using standard weights after every third subject.

4.3.2. Height

It is a linear measurement made up of the sum of four components i.e. Legs, Pelvis, Spine, and Skull [Jelliffe, 1966].

Technique- A spring- loaded non-stretchable tape was used to measure the height of the children. A convenient flat wall was identified at the clinic site for the measurement of height. The child was made to stand barefoot with the arms hanging freely by the sides. Heels of the feet were placed together with the medial (inner) border of the feet at an angle of 60 degrees. The scapula and the buttock were ensured to be in contact with the measuring wall. The head was held in the Frakfort plane (with the tragus of the ear and the lateral angle of the eye in a horizontal line). Height was recorded to the nearest 0.1 cm after the child inhaled fully and maintained the erect position without altering the load on the heels. In this position, a mark was made on the wall and height was recorded with a measuring tape and two consecutive reading were taken for precision.

4.4. Nutritional Status

Nutritional status was determined by using WHO standards of BMI for age and gender (2007) and were classified under the 3 grades of undernutrition.

Children were classified as Mildly (Grade I undernutrition), Moderately (Grade II undernutrition) and Severely (Grade III undernutrition) underweight when the BMI-for-age and gender was below the median by -1SD, -2SD -3SD respectively [UNICEF, 2009; WHO, 2007].

4.4.1. Body Mass Index (BMI)

Body mass index (BMI) for age, was used to classify the nutritional status of a child. BMI is calculated by using the following stated formula [WHO, 1998], per age. The WHO (2007) developed standards to assess the growth of a child. Appendix 7a (boys) and 7b (girls) reflects the median for measuring the child's nutritional status with the Standard Deviation (SD) value for BMI age and gender. Under-nutrition is associated with deficit in behavior and development of the brain's anatomy, neurochemistry, and metabolism [Black *et al.*, 2005].

$$\textbf{Body Mass Index} = \frac{\textbf{Weight (kg.)}}{\textbf{Height (mt.)} \times \textbf{Height (mt.)}}$$

4.5. Survey

To obtain a better picture and significant determinants of nutritional status of the child, a survey was conducted using interview method to gather information related to the child.

4.5.1. Interview of the parents

After conducting the screening process, a notice was sent to all the parents of the children regarding the study objectives with a request of their participation in seeking various information about their child and family background. Parents of 161 children out of 218 participated and were interviewed for various aspects using pretested structured questionnaire (Appendix 4). Of the 161 parents interviewed, children of 153 were falling in the categories of nourished (37) or undernourished (116) status and therefore they were selected for further assessment. One of the school teacher with fluent command in the local dialect helped the researcher for obtaining precise information from the parents. Diary notices were sent each day to the respective class-room in order to priorly inform the parents for their attendance in the school. During the course of interview, groups of parent were made aware about the study and its implication and their written consent regarding their child's further participation in the study was also obtained (Appendix 2 and 3a, 3b, 3c).

4.5.1. Background Information

The background information from the parent was collected on the following aspects:

- **Date of birth and gender of the child**- parents were asked to specify the date of birth and gender of the child in order to find out the accurate age of the child, required for further assessment of nutritional status.

  To find out various other social determinants of nutritional status of children, their parents were interviewed for the following:

  - **Type of family and Total family members**- parents were asked about their family's size and structure, whether it is nuclear, joint or extended along with total number of people residing with them.
  - **Total family income-** economy inevitably impacts one's health and therefore parents were asked to report not only father's but their total family income in order to further analyze its impact on the nutritional status of children of the present study.
  - **Mother and Father's Education and Occupation-** educational status of parents have a deciding role in shaping their children's health, which is why parents were asked to report their own literacy/educational statuses.
  - **History of Exclusive breast feeding (EBF) Immunization Profile of the child-** plethoric research suggests that EBF influence the health status of children even at later stages [WHO, 2007], similarly

reiteration for immunization is also well-known, therefore this aspect is employed in order to see their association and impact on nutritional status and other parameters of health of the children of present study.

- **Child's Appetite-** mothers and fathers of the children were probed about their child's usual appetite and were graded as normal, low, and very low for further analysis.

4.5.2. Past Medical History- incidence of infection or any other disease or disorder worsens the child's regular regime of health, therefore past one month medical history was obtained from the parents in order to analyze the statistical relevance of morbidity and it's relationship with present nutritional status of the child, also to analyze and justify the impact of FOS on predisposed morbidity of the child. Before interrogating them, parents were made aware of the signs and/or symptoms of the respective disease/disorder and then were asked to recall the previous month's status of their child. Parents were specifically interrogated for the "presence and frequency" of the following:

- **Diarrhea**

  If their child suffered with three or more loose/liquid stool per 24 hours or one to three abnormally loose stools per 24 to 48 hours [Johnston, 2010].

- **Common colds**

  If their child had Cough, Nasal Congestion, Sore throat, and/or Fever and/or headache [University Health Services, Rochester, 2009].

- **Stomach-ache**- if their child complaint any discomfort in the upper/lower region of stomach. Reasons of stomach-ache were not questioned.

- **Constipation-** if the child had small amounts of hard, dry bowel movements, usually fewer than three times a week [University Health Services, Berkley, 2009] or hard and/or painful and/or infrequent and/or irregular stools.

- **Flatulence-** if the child complained about any of the following :

  Passing wind often • smelly flatus • loud flatus • abdominal distension and discomfort • rumblings in the lower abdomen [www.betterhealth.vic.gov.au].

- **History of Atopic Dermatitis:** if the child use to have intensely itchy red, splotchy, raised lesions to form in the bends of the arms or legs, face, and neck which may or may not weep, crack, swell, and crust over.

- **Any other disease or hospital stay-** to rule out any chronic serious disorder in the child, parents were questioned whether their child has been ever hospitalized and in case of 'yes' the reason was further probed and reported**.**

4.5.3. Frequency of intake of Fermented foods:

Parents were asked about commonly consumed fermented foods like *kadhi; chaach; curd; srikhand* by their child and was recorded using modified food frequency questionnaire (FFQ) (Appendix 4) in order to speculate its association with the prevalent nutritional status of child. Details of the number of times the food items was consumed on a daily, weekly, monthly, or yearly basis was elicited using the Food frequency questionnaire. The FFQ was thus finalized with columns to collect information on frequency of consumption during the past one year (per day/week/month/year).

4.6. Determination of the Gut Microbiota

The gut microbiota was determined with respect to fecal- *Lactic acid bacteria*, *Bifidobacteria* and *E.coli*. The steps involved in the determination of the fecal flora were:

4.6.1. Collection and Storage of the fecal sample
4.6.2. Sterilization of the glass wares
4.6.3. Preparation and Sterilization of dilution blanks
4.6.4. Preparation and Sterilization of Media
4.6.5. Preparation and inoculation of sample
4.6.6. Incubation and enumeration of Lactic acid bacteria
4.6.7. Incubation and enumeration of Bifidobacteria
4.6.8. *Incubation and enumeration of E.coli*
4.6.9. Colony counting

4.6.2. Collection and Storage of the fecal sample

Both parents and children were instructed in their local language about the usage of air tight sterile clinicols and were asked to bring their stool samples just before coming to school at 7 a.m. Stool samples collected from the children were immediately kept in thermocol box with gel packs and stored at -20^{o}C within an hour of collection, until analysis.

4.6.3. Sterilization of the glass wares

Sterile 90mm individually packed petri-plates, supplied by Tarsons and Co ® were used and opened under the laminar only at the time of plating. All the

other glass wares such as beakers and conical flask were sterilized before use. The micro-tips and spatula were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs. pressure. The other instruments which were used like the weighing balance were all sterilized by alcohol flaming using 70 % alcohol.

4.6.4. Preparation and Sterilization of dilution blanks

For the preparation of dilution blanks 1 gram of peptone was dissolved in 1000 ml of distilled water. This solution was dispensed in portion of 100 ml in 10 dilution bottles as depicted in Fig 4.3. These were autoclaved at 121°C for 15 minutes. The bottles were cooled at room temperature before putting them to use [IGNOU, 2005].

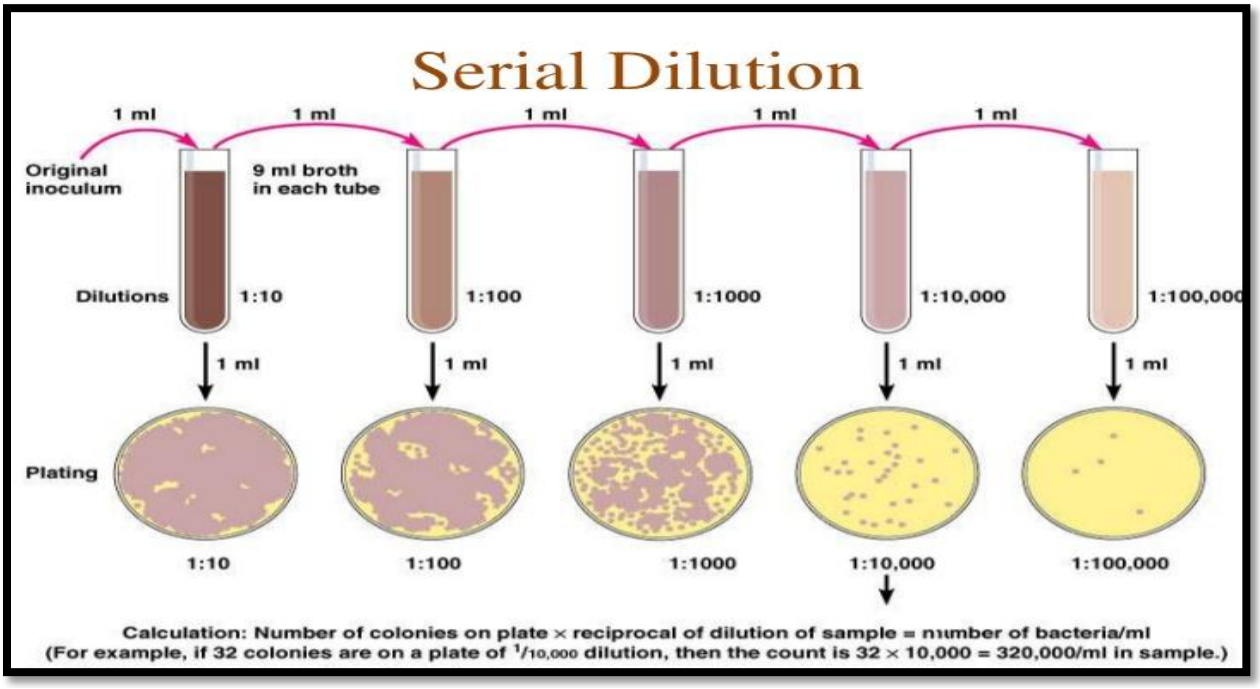


*Fig. 4.3.***Process of serial dilution as applied in the microbial analysis of gut flora of undernourished children**

4.6.5. Preparation and Sterilization of Media

Media used for different gut flora is presented in Table4.1. The prepared media of *Bifidobacteria* and LAB were autoclaved at 121°C for 15 minutes whilst EMB agar was prepared just before pouring. The prepared media were then poured into sterile petri-plates and allowed to set.

Table 4.1 Details of media used in determining the gut flora of school going children

Gut Flora	Incubation Condition	Incubation Duration	Temperature	Media Used (Brand)
Bifidobacteria	Inside anaerobic jar with a anaerobic gas pack	48 hours	37°C	Bifidobacterium Agar (Hi.Media®)
Lactic acid bacteria	Inside dessicator with calcium carbonate	48 hours	37°C	MRS Lactic acid bacillus agar (Hi.Media®)
E.coli	Inside incubator	24 hours	37°C	EMB (Eoisin Methylene Blue) Agar (Hi.Media®)

4.6.6. Preparation and inoculation of sample

One gram of fresh fecal sample was accurately weighed after thawing at room temperature for an hour and homogenized in 99 ml of 0.1% peptone water to provide 1% (w/v) fecal slurry. One ml of slurry was diluted serially in peptone water using stomacher and 0.1 ml of sample was pipetted from each of the dilutions to the petri plates containing respective media. The above procedure was carried out inside laminar flow that ensured a sterile environment thereby preventing contamination from outside (Plate 4.1, Plate 4.2).

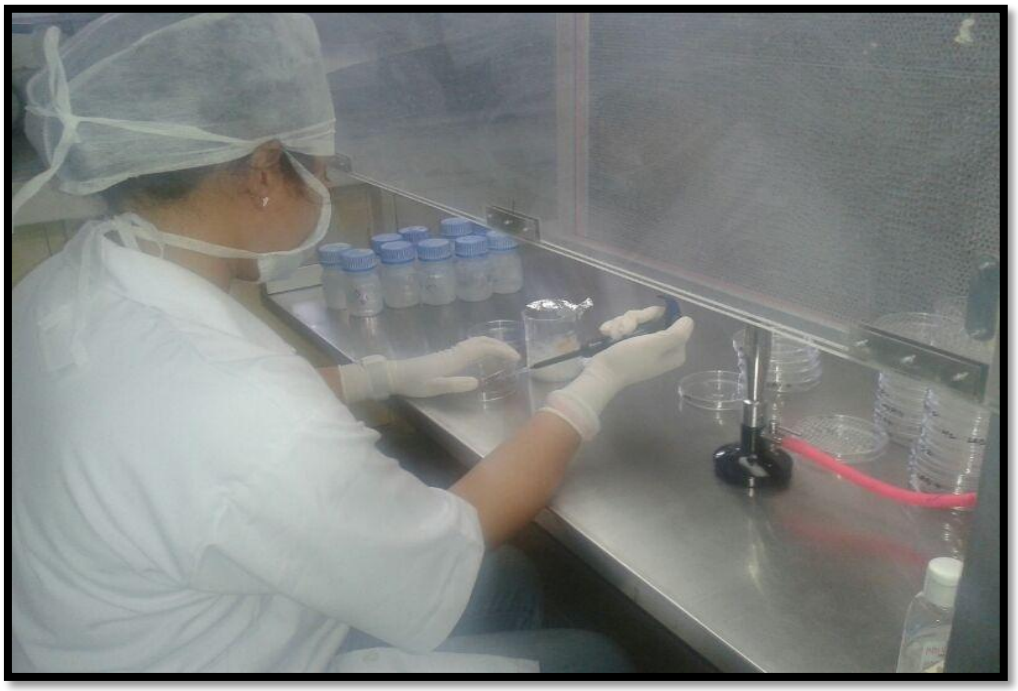


Plate 4.1: Preparation of sample dilutions in Laminar Air Flow Cabinet

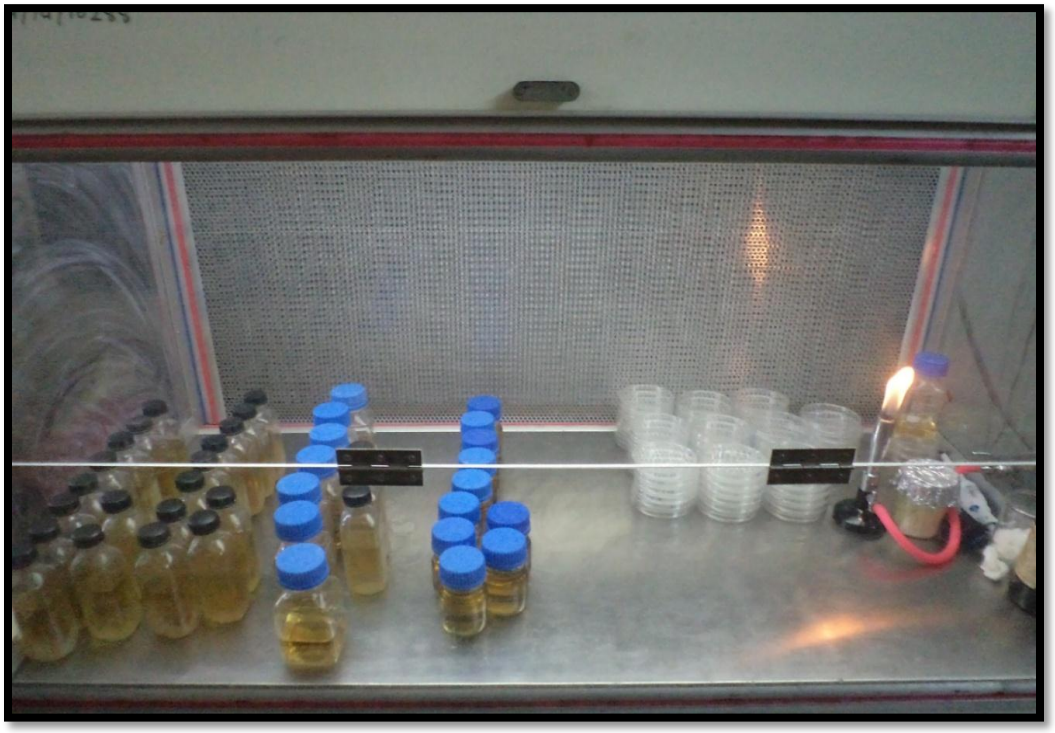


Plate 4.2: Laminar air flow cabinet depicting dilution bottles and petri plates before inoculation

4.6.7. Incubation and enumeration of Lactic acid bacteria

Petri plates of *Lactic acid bacteria* were placed in a dessicator as it is a facultative anaerobe, and the dessicators were then placed in the incubator at 37°C were counted on colony counter after 48 hours of incubation.

4.6.8. Incubation and enumeration of Bifidobacteria

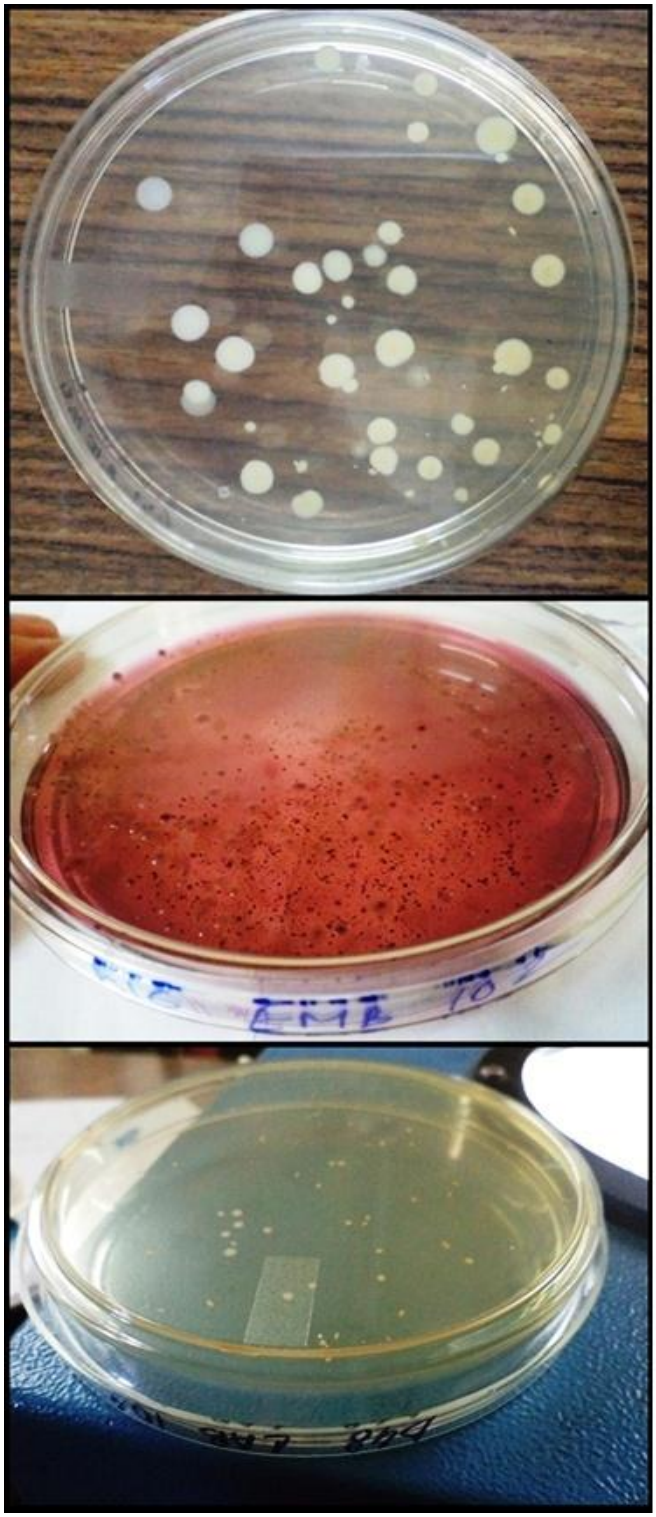
The plates of *Bifidobacteria* were incubated at 37°C placed in the anaerobic jars using anaerobic gas-packs procured from Hi.Media® were counted on colony counter after 48 hours of incubation.

4.6.9. *Incubation and enumeration of E.coli*

Petri plates of *E.coli* were directly placed in the incubator at 37ºC for 24 hours.

4.6.10. Colony counting

After completion of incubation period (Plate 4.3) of the respective bacteria, petri plates were then placed on colony counter. The colonies that appeared in the range of 30 – 300 were converted in to log counts after multiplying with their dilution factors [Ramona *et al.,* 2000] and further used for statistical analysis.



a. Plate of Bifidobacteria

b. Plate of E.coli

c. Plate of Lactic acid bacteria

Plate 4.3. Plates showing typical growth of *Bifidobacteria, E.coli,* and *Lactic acid bacteria*

4.7. Dietary intake analysis

Dietary intake is one of the essential aspect of one's health, it provides a greater outlook for individual's choices of food which ultimately reflects his/her health. Though it was difficult to obtain 24 hour dietary recall from the children, therefore at the time of interview, a self-administered diet recall card (Appendix 5) was handed over to parents and they were also briefed about the method of writing and interpreting the diet recall of the child. In case, of illiterate parents, the elder child of the family was called and briefed about the same. In this manner, a three day 24 hour dietary recall of 80 undernourished and 30 nourished children was collected for the study. Parents were asked to provide details of all the major meals consumed by the child throughout the day, along with additional beverages, snacks, sweets, pickles, etc. along with added sugar and salt.

Total energy intake, nutrient components such as carbohydrates, protein, fat, iron, calcium, zinc, vitamin C and non-nutrient component such as total dietary fiber were calculated. The cooked value of the food intake of each meal was priorly converted into raw values and then entered in Diet Soft Software [Kaur, 2007] to obtain the nutrient intake of each child.

4.8. Serum IgA analysis [Dia Sys,2007]

The human immunoglobulin classes (IgG, IgA, IgM, IgE and IgD) are a group of functionally and structurally closely related glycoproteins. Human IgA has a molecular weight of about 160000 dalton and consists of two identical heavy chains and two identical light chains which are bound together by disulfide bonds in a characteristic Y-shaped form. Serum IgA is produced by plasma cells (B-cells) and represents about 15% of all soluble immmunoglobulin classes. About 90% of the serum IgA is monomeric the rest is dimeric and polymeric.

The main function of serum-IgA is to bind to antigensand trigger further catabolism of the antigen. Decreased serum-IgA concentrations occur in primary as well as in secondary immunodeficiency syndromes. A high increase of one immunoglobulin class due to multiple myeloma may result in a decrease in other immmunoglobulin classes like IgA. Increased loss of IgA due to severe enteritis may result in a decreased concentration. Increased IgA concentrations can be observed in severe infections and autoimmune diseases. Especially inflammatory processes of the liver may result in increased serum IgA levels assay.

Sera of 80 undernourished and 32 nourished children were collected at the school by trained and certified technician and stored at -20°C until analysis and serum IgA was determined using following stated immunoturbidimetric method:

Principle

Endpoint determination of the concentration IgA through photometric measurement of antigen-antibody-reaction between antibodies to human IgA and IgA present in the sample.

The ready to use **Reagents (R_1 and R_2)** had following Components and Concentrations

R1: TRIS pH 7.5 100 mmol/L

NaCl 180 mmol/L

Polyethylenglycol (PEG) detergents, stabilizers

R2: TRIS pH 8.0 100 mmol/L

NaCl 180 mmol/L

Anti-human IgA antibody (goat) with stabilizers

Storage Instructions and Reagent Stability

The reagents were stable up to the end of the indicated month of expiry, if stored at 2 – 8 °C and contamination should be avoided. Reagent should not be freezed.

- **Materials required:**
  - NaCl solution 9 g/L
  - General laboratory equipment

Specimen

- Serum, heparin plasma or EDTA plasma

  Stability: 7 days at 15 – 25 °C

3 months at 2 – 8 °C

6 months at - 20 °C

It does not apply in case of repeated freezing.

Contaminated specimens should be discarded.

- **Assay Procedure for Analyzers**

Wavelength	570 nm
Optical path	1 cm
Temperature	37 °C
Measurement	Against reagent blank

Steps Involved in IgA determination:

	Blank	**Sample**
Sample	-	2 µL
Dist. water	2 µL	-
Reagent 1	250 µL	250 µL
	Mix, incubate for 3 – 5 min., read absorbance (A1), then add:	
Reagent 2	50 µL	50 µL
	Mix, incubate for 3 min., read absorbance (A2)	

Calculation: **A = [(A2 – A1) sample] - [(A2 – A1) blank]**

The concentration of IgA in unknown samples is derived from a calibration curve using an appropriate mathematical model such as logit/log. The calibration curve is obtained with 5 calibrators at different levels and NaCl solution (9 g/L) for determination of the zero value.

Stability of calibration: 4 weeks

- **Specifications for Calibrators and Controls**

For the calibration of automated photometric systems the DiaSysTruCal Protein calibrator set or the TruCal Protein high calibrator is recommended. The assigned values of the calibrators have been made traceable to the IFCC/BCR/CAP Reference Material for 15 Plasma Proteins CRM 470.

Reference Value:

Age	Serum IgA Levels
4-<7 years:	29-256 mg/dL
7-<10 years:	34-274 mg/dL
10-<13 years:	42-295 mg/dL

Serum IgA values obtained from the laboratory were matched with above stated reference levels and then subjected to further statistical evaluations.

4.9.1. Experimental Trials:

Present study consisted of two trials. The first trial was a case control trial where a comparative analysis was exercised for nourished and undernourished children in terms of various subjective and objective parameters as depicted in Fig 4.4

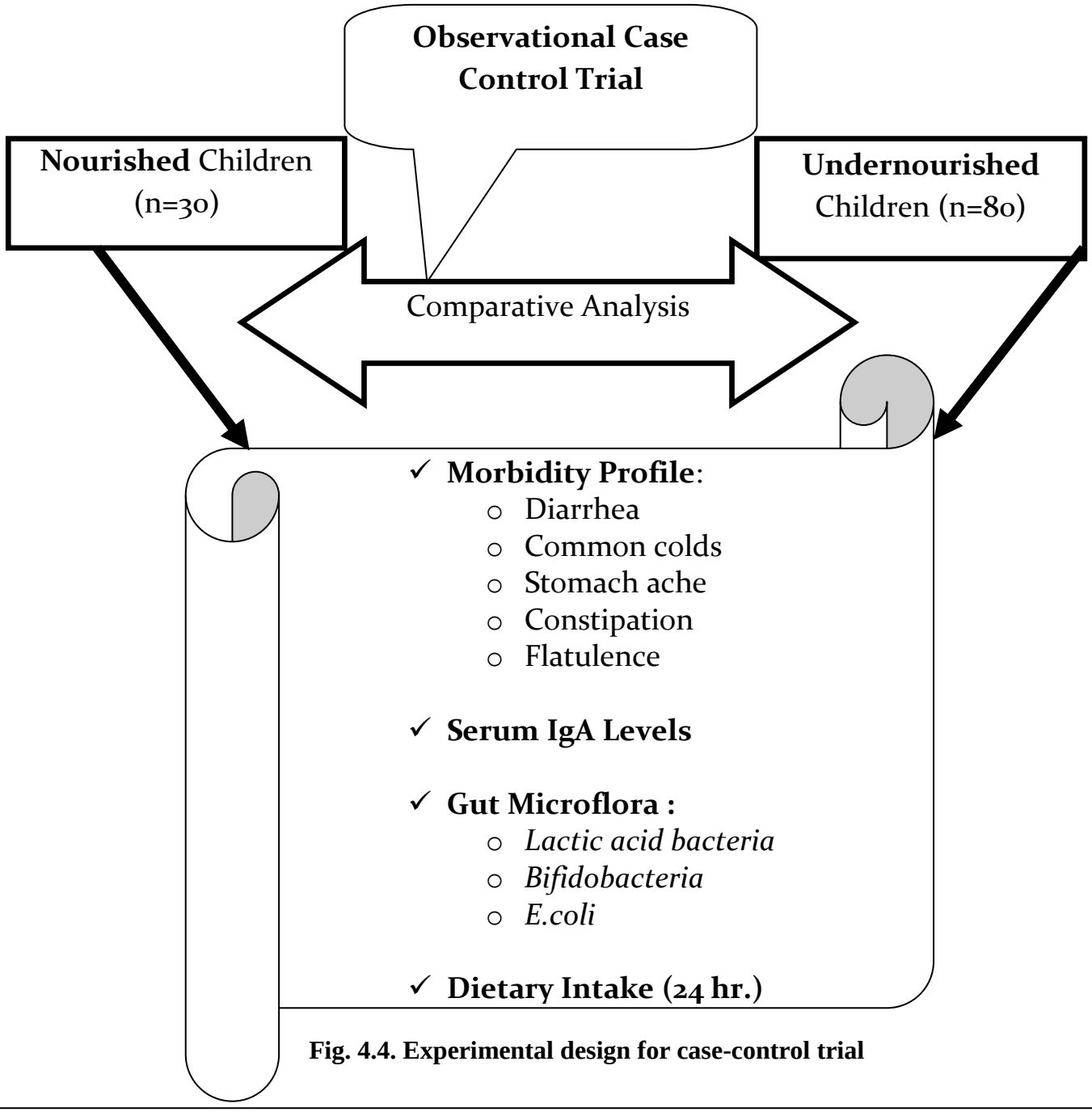


Fig. 4.4. Experimental design for case-control trial

4.9. Randomized Clinical Placebo Trial

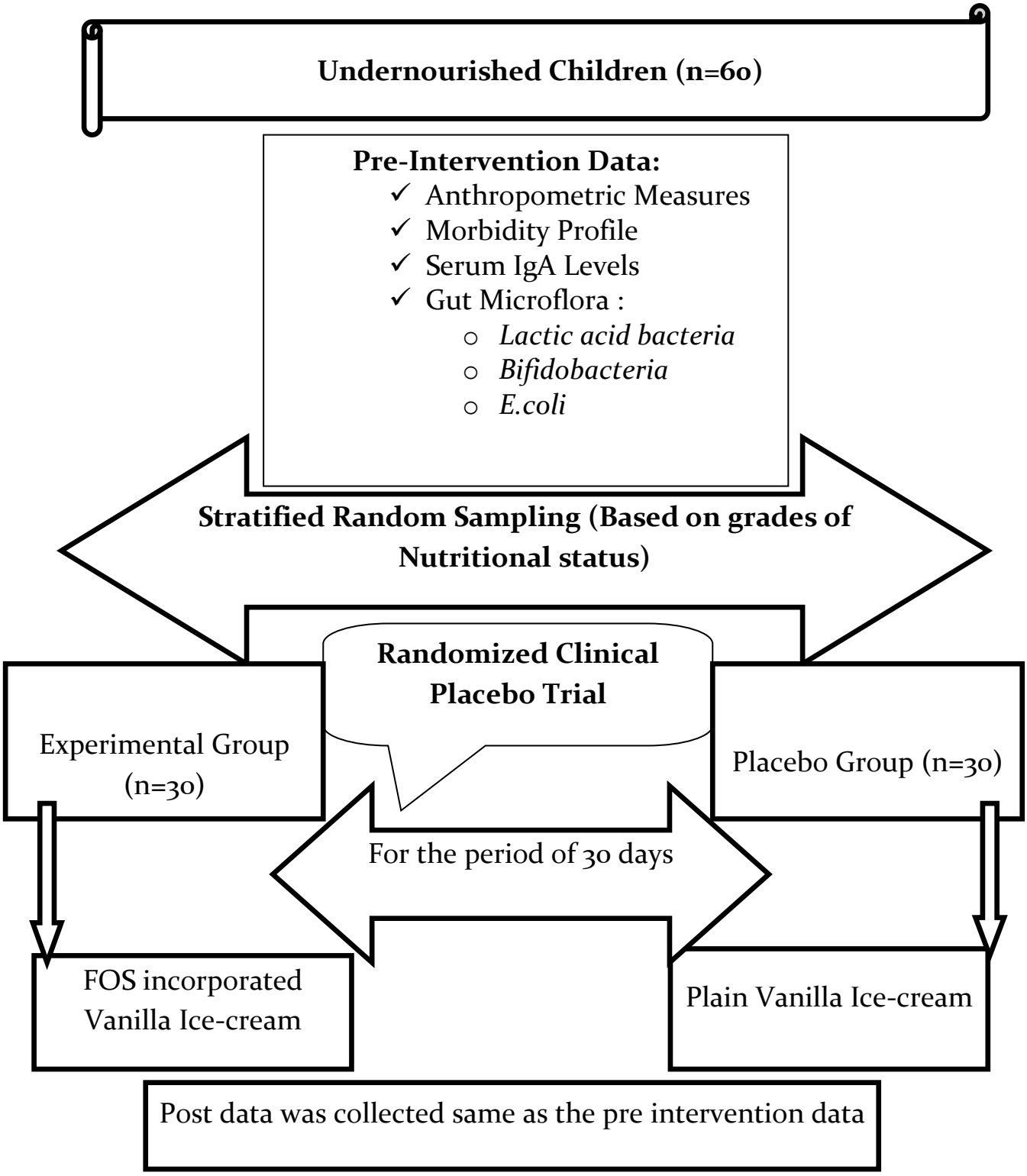


Fig. 4.5. CONSORT for the intervention trial

To evaluate the impact of FOS on morbidity profile, serum IgA levels, gut microflora with respect to *Lactic acid bacteria, Bifidobacteria*, and *E.coli*, and nutritional status of undernourished children, a randomized clinical placebo trial was undertaken based on the following stated details*:*

4.9.1. Selection of subject

Sixty undernourished subjects belonging to three grades of undernutrition were randomly stratified into two groups- experimental and placebo after prior consent for participation in the intervention program (refer CONSORT in Fig.4.5).

4.9.2. Inclusion and Exclusion criteria

The inclusion criteria included selection of children from both the gender, studying in class I-V (age 5-12 years), and belonging to a lower income group.

Overweight and Obese Children and children suffering from any severe or chronic diseases such as celiac disease, juvenile diabetes, HIV, cancer, renal disorder and liver disorder were excluded from the trial. Presence of these diseases was confirmed from either the medical records or as reported by the parents.

4.9.3. Intervention

Selected undernourished subjects were supplemented with FOS (10g) incorporated ice cream and sucrose incorporated ice-cream daily for 30 days over a period of 45 days before lunch in the month of March 2013. For ethical reasons, all the children who had given the consent for participation in the study were also offered ice-cream. Cups were color coded for placebo and FOS added ice-creams (Plate 4.4 and 4.5) and the identity cards worn by the children around their necks in experimental group and control were tagged with red and blue colors respectively.

In order to prevent the children from exchanging the ice-cream cups during the course of intervention, they were made to sit in two different groups (Plate 4.4)

Plate 4.4. Children consuming ice-cream during intervention period

4.9.4. Procurement and logistics of ice-cream

Recipe of FOS incorporated ice-cream was developed (Fig. 4.6) and standardized at Amul Dairy® Vadodara, Gujarat, India, a part of Gujarat Co-operative Milk Marketing Federation. Thermo-stably packed boxes of ice-cream cups were delivered at the school every morning by the diary authorities, based on the weekly schedule as given by the researcher.

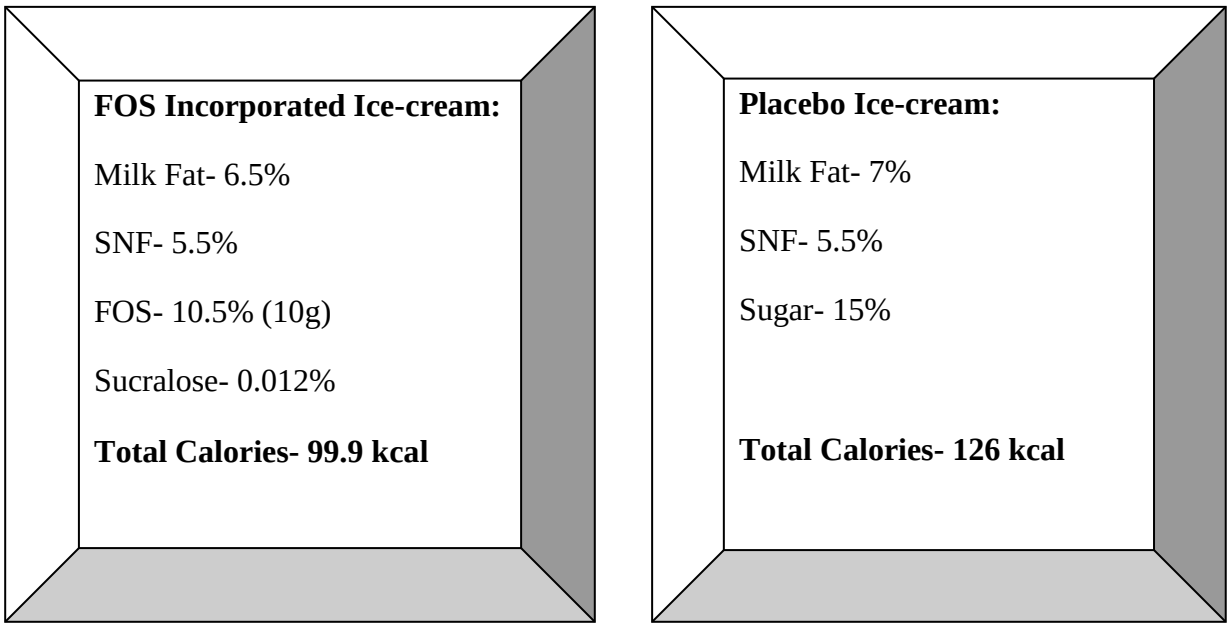


Fig.4.6.Composition of Ice-cream (per cup) as reported by Amul co-operative limited.

4.9.5. Collection of Post Data

Post data was collected on the parameters similar to the baseline such as serum IgA, gut microflora, anthropometric measurements, and morbidity status (Fig.4.5).

4.9.5.1. Compliance data on records of the diarrhea and common cold occurrence

A calendar (Appendix 6) was especially designed and distributed to the parents/guardians of the children to mark the incidence of diarrhea and common colds during the course of intervention. Calendars were collected back after the completion of intervention period.

Plate 4.5. Red labeled cup of FOS incorporated ice-cream

Plate 4.6. Blue labeled cup of placebo ice-cream

4.10. Development of a bilingual booklet based on prebiotic added recipes entitled "Prebiotic: Our Gut Guardians"

A compilation of recipes incorporated with prebiotic *viz.* inulin and FOS, in a form of bilingual booklet (Appendix 8) was undertaken, in order to bring out a ready reckoner of prebiotic rich foods. These recipes were standardized and developed by the various researchers working in the field of prebiotics at the department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda. The recipes were computed for an appropriate portion size considering the maximum allowance of prebiotic that would result in most acceptable products and then segregated based on their suitability for meal timings (Fig 4.6).

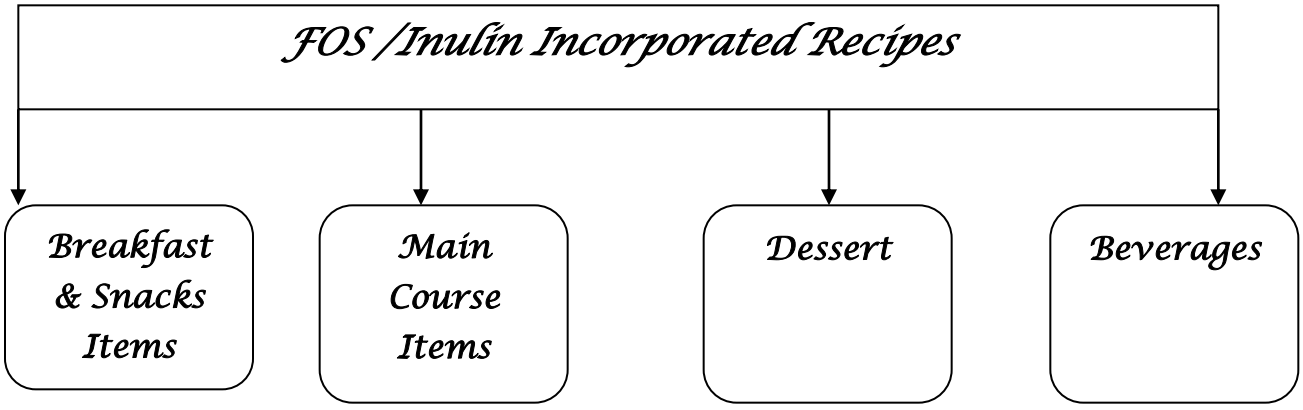


Fig.4.7 Segregation of contents of bilingual recipe booklet

4.11. Statistical Analysis

To provide an evidence of significance of the data observed during the course of research, statistical analysis is an inevitable tool. Present research was validated using below stated analytical software (Table4.2). The data was entered in an excel spreadsheet (MS, 2007). The data was cleaned, verified, and subjected to appropriate statistical analysis.

Table4.2. Record of software used for statistical analysis in the research

Name of the Program	**Version**	**Tests applied**
Microsoft excel	2007	➢ Number and Percent calculation ➢ Calculating age and BMI of the children ➢ Student's 't' test ➢ Paired 't' test ➢ Quartiles for serum IgA ➢ Graphical representation of data
Statistical program for social sciences	Trial version 20.0	➢ Spearman's correlation ➢ Pearson's correlation ➢ Linear regression ➢ Post hoc LSD test
Epi info.	7.0	➢ Chi square ➢ Odds ratio

4.11.1. Odds Ratio [OR]/Risk Ratio

An **odds ratio** (OR) is a measure of association between an exposure and an outcome. The OR represents the **odds** that an outcome will occur given a particular exposure, compared to the **odds** of the outcome occurring in the absence of that exposure [Szumilas, 2010].

Odds ratio (OR) was calculated to find out significant determinants of undernutrition and morbidity in the cross-sectional phase of the study (Phase I). Data was filtered using excel filters, for the required parameter and then entered in Epi info (7.0, CDC), odds >1 was considered significant at 95% of confidence interval.

Present research consists of OR analysis for:

- ✓ Determining the odds of occurrence of undernutrition, having diarrhea and common colds against the exposures like total family income, literacy and occupational status of parents, gender of the child, immunization status and past of breast feeding practices of the children, etc.

4.11.2. Chi Squared [χ^2] Test

Chi-square is a versatile statistical test used to examine the significance of relationships between two (or more) nominal-level variables. It is anon parametric test of significance and therefore used for all the qualitative data in the present research. The significance levels were set at 95% by two sided tests.

Chi square, in the present research was utilized to:

- ✓ To determine the significance of difference based on gender in different classes of primary school going children.
- ✓ To determine the significance of difference in the incidence of morbidity in nourished and undernourished children and also in different grades of undernutrition.

4.11.3. Spearman's Correlation

Spearman's rank correlation coefficient or Spearman's rho, named after Charles Spearman and often denoted by the Greek letter "**σ** "(rho)is a nonparametric measure of statistical dependence between two variables. It assesses how well the relationship between two variables can be described using a monotonic function. If there are no repeated data values, a perfect Spearman correlation of +1 or −1 occurs when each of the variables is a perfect monotone function of the other [Wikipedia, 2015].

Data imported to SPSS [trial version 20.0], the first phase of the study consisted of various qualitative variables which were ranked (scored) appropriately for carrying out Spearman's correlation in order to:

- ✓ Determine if there exist an association between grades of nutritional status and total income of the family, child appetite, presence/absence of morbidity, fulfillment of exclusive breast feeding, occupational and literacy status of parents.

4.11.4. Student's't' test

Student's t-test is applied for testing difference between means of two independent samples. In order to find out the significance of difference in parameters of two different groups Student's't' test was exercised, the significance levels were set at 95% by two sided tests.

Independent 't' test used in the present research for:

- ✓ Determining the significance of difference in the mean counts of gut microflora, dietary intake, and serum IgA levels of nourished and undernourished children.
- ✓ Determining the significance of difference in the initial and final values of the mean counts of gut microflora, serum IgA, anthropometric measurements, incidence of diarrhea and common colds in placebo and experimental groups.

4.11.5. Analysis of Variance (ANOVA) or 'F' Test

Analysis of variance (ANOVA) is a collection of statistical models used in order to analyze the differences between group means and their associated procedures (such as "variation" among and between groups), developed by R. A. Fisher**.** In its simplest form, ANOVA provides a statistical test of whether or not the means of several groups are equal, and therefore generalizes the t-test to more than two groups. As doing multiple two-sample t-tests would result in an increased chance of committing a statistical type I error ,ANOVAs are useful in

comparing (testing) three or more means (groups or variables) for statistical significance (wikipedia.com). Significance level was set at 95% by two sided test, ANOVA in the present research used for:

- ✓ Determining the significance of difference in gut microflora, serum IgA levels of different grades of undernutrition.
- ✓ Determining the significance of difference of gut flora, BMI, incidence of diarrhea and common colds in different quartiles of serum IgA levels.

4.11.6. Post Hoc LSD test or Fisher's Least Significant Difference (LSD) Test

Post hoc tests are designed for situations in which the researcher has already obtained a significant omnibus F-test (ANOVA) with a factor that consists of three or more means and additional exploration of the differences among means is needed to provide specific information on which means are significantly different from each other.

Post hoc LSD was applied in the present research to

- ✓ Determine the significance of multiple comparison of gut microflora in different grades of undernutrition.
- ✓ Determine the significance of multiple comparisons in different quartiles of serum IgA levels for gut flora, BMI, incidence of diarrhea and common colds.

4.11.7. Linear Regression

In a cause and effect relationship, the **independent variable** is the cause, and the **dependent variable** is the effect. **Least squares linear regression** is a method for predicting the value of a dependent variable *Y*, based on the value of an independent variable *X*.

Stepwise linear regression model was used in SPSS and Body mass index (BMI) of the children was controlled as dependent variable, for:

- ✓ Predicting BMI against the independent variables viz. serum IgA levels, gut microflora, incidence of diarrhea, common colds, stomach ache, flatulence and constipation.
- ✓ Predicting BMI against the independent variable viz. serum IgA levels, gut microflora, incidence of diarrhea and common colds in different quartiles of serum IgA.

4.11.8. Paired't' test

A paired t-test measures whether means from a within-subjects test group vary over two test conditions. The paired t-test is commonly used to compare a sample group's scores before and after an intervention. The third phase of the study had the objective of analyzing the impact of FOS or placebo supplementation to undernourished children for which this test was applied and the significance levels were set at 5% by two sided tests in order to:

- ✓ Determine the significance of difference obtained before and after the supplementation of FOS or placebo in the gut microflora, serum IgA levels, anthropometric measurements and morbidity profile in both the groups *viz.* placebo and experimental.

4.12. Ethical Committee Approval and Study Registration

The study protocol was approved by the Medical Ethics committee of the Foods and Nutrition Department, The M.S. University of Baroda- –"Institutional Ethical Committee for Human Trials" in compliance with the guidelines issued by Indian Council of Medical Research with the medical ethics approval number (No.F. C. Sc FN IECHR/2012/14).

The study is also registered under clinical trial registration of India (CTRI/2015/03/005615). The details of the study can also be obtained from www.ctri.nic.in.

All the parents were priorly informed and their written consent was obtained for their children to be undertaken for the trial.