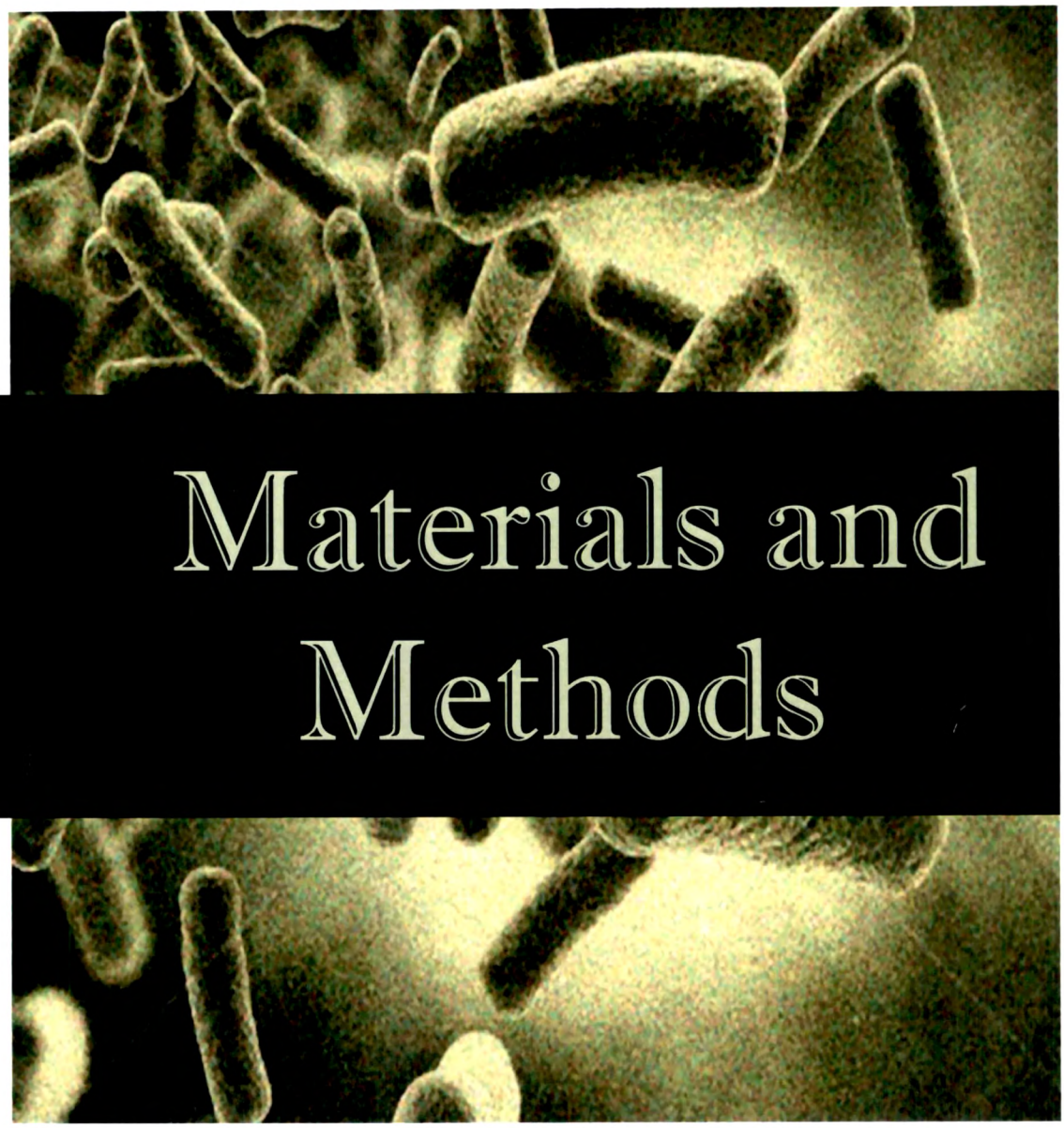


Materials and Methods

CHAPTER 4

MATERIALS AND METHODS

In recent years, there has been increasing interest in the important nutritional roles of prebiotics as functional food ingredient. Among all the natural prebiotics, Fructooligosaccharide (FOS) or Oligofructose is currently marketed as promoting the growth of healthy intestinal bacteria. As a result FOS as a food ingredient has triggered much research on their possible health effects like lowering glycemic index, blood glucose level, blood cholesterol and could help to assist diabetic control. The present study was undertaken to study the **"Acceptability trials of fructooligosaccharide (FOS) substituted food products and impact evaluation of FOS supplementation in type 2 diabetic adults in terms of their glycemia, gut incretin (GLP-1) and gut microbiota"**. This chapter outlines the experimental design and discusses the methods and materials used to fulfill the objectives of the study in three phases.

PHASE I

Development of FOS incorporated food products and studying their various organoleptic attributes, overall acceptability and the recovery of FOS during processing of these products using HPLC technique.

Section 4.1: **Development of FOS incorporated food products**

Section 4.1.1: Procurement of fructooligosaccharide

Section 4.1.2: Selection of food products

Section 4.1.3: Procurement of raw ingredients

Section 4.1.4: Standardization and substitution of FOS to the products

Section 4.1.5: Assessment of Physical and organoleptic properties of the products

a) Principle

b) Standardization of the method

c) Determination of fructooligosaccharide

d) Calculation and Expression of Results

Section 4.1.6: Selection and training of panel judges for organoleptic evaluation

Section 4.1.7: Tools for organoleptic evaluation

Section 4.1.8: Statistical Analysis

Section 4.2: Recovery of FOS during processing

PHASE II and III

Situational analysis of type 2 diabetic subjects in health clinic of The M.S. University of Baroda and Impact of Fructooligosaccharide supplementation on glycemic, lipemic parameters, GLP-1 and gut microbiota in diabetic adults.

Section 4.3.1 Statutory clearances

Section 4.3.2 Power of the study and sample size

Section 4.3.3 Selection of the subjects for the study

Section 4.3.4 Inclusion and exclusion criteria of the subjects

Section 4.3.5 Study Protocol

Section 4.3.6 Administration of Interviewer based Questionnaires

Section 4.3.7 Anthropometric Measurements

Section 4.3.8 Biophysical Investigations

Section 4.3.9 Biochemical Evaluation and Assay methods

Section 4.3.10 Determination of the gut microbiota

Section 4.3.11 Study food and mode of intervention

Section 4.3.12 Statistical Analysis

PHASE I

Section 4.1: Development of Fructooligosaccharide (FOS) incorporated food products and studying their organoleptic attributes and overall acceptability

This section of the study was conducted to develop FOS incorporated food products *viz. chapati, thepla, dhokla* and *patra* at varying level of FOS substitution

4.1.1: Procurement of Fructooligosaccharide (FOS)

Food grade Fructooligosaccharide (Beneo Raftiline P95 BAG 25 kg, Orafti, Belgium) was procured in a pack of 25 kgs from Brenntag Ingredients India Pvt. Ltd. The fructooligosaccharide was food grade which was best suitable as fat and sugar replacer. The specifications of the fructooligosaccharide are given in Table 4.1.1

4.1.2: Selection of food products

The possibilities of incorporating fructooligosaccharide in the popular Indian foods and their organoleptic qualities were studied by the method of substitution. Total of four products were selected on the basis of processing conditions. Out of these products that were substituted with fructooligosaccharide included *Chapati, Thepla, Dhokla* and *Patra,* in which fructooligosaccharide was added at various levels. All the products were studied for physical and organoleptic characteristics.

Table 4.1.1: Specification of the Fructooligosaccharide used for Substitution

Specifications	Analysis	Values	Range
Orafti Phys/chem. Parameters packaged	Dry matter	96.2%	95.5-98.5
	pH	6.6	5.0-7.0
	Conductivity(uS/cm)	6	0-249
Orafti Spectrometry packaged	Sucrose(%DM)	3.7%	0.0-8.0
	Oligofructose	96.3%	92.3-100.0
	Glucose + fructose	0.4%	0.0-4.0
Orafti Microbiology	Total counts (per gd.m.)	0	0-1000
	Yeasts	0	0-20
	Molds	0	0-20

Orafti P95, 2011

4.1.3: Procurement of raw ingredients

Various ingredients and materials that were needed to develop FOS enriched products were collected from the local market of Vadodara. The list of raw ingredients and their sources according to the type of recipe is listed in the Table 4.1.2

Table 4.1.2: List of Raw Ingredients along with their Sources used for Product Preparation

Product	Ingredients	Source/ Brand name
Chapati*	Wheat flour	Wheat variety GW496
Thepla*	Wheat flour	Wheat variety GW496
	Bengal gram flour	Uttam brand, Baroda
	Fenugreek leaves	Local Market, Baroda
	Green chilies, Garlic, ginger	Local Market, Baroda
	Oil	Groundnut oil
Dhokla*	Bengal gram flour	Uttam brand, Baroda
	Semolina	Uttam brand, Baroda
	Curd	Masti Dahi (Amul)
	Baking powder	
Patra*	Bengal gram flour	Uttam brand, Baroda
	Patra leaves, Mint leaves and Tamarind	Local Market, Baroda

* The detailed recipes of the standard products are appended in appendices I

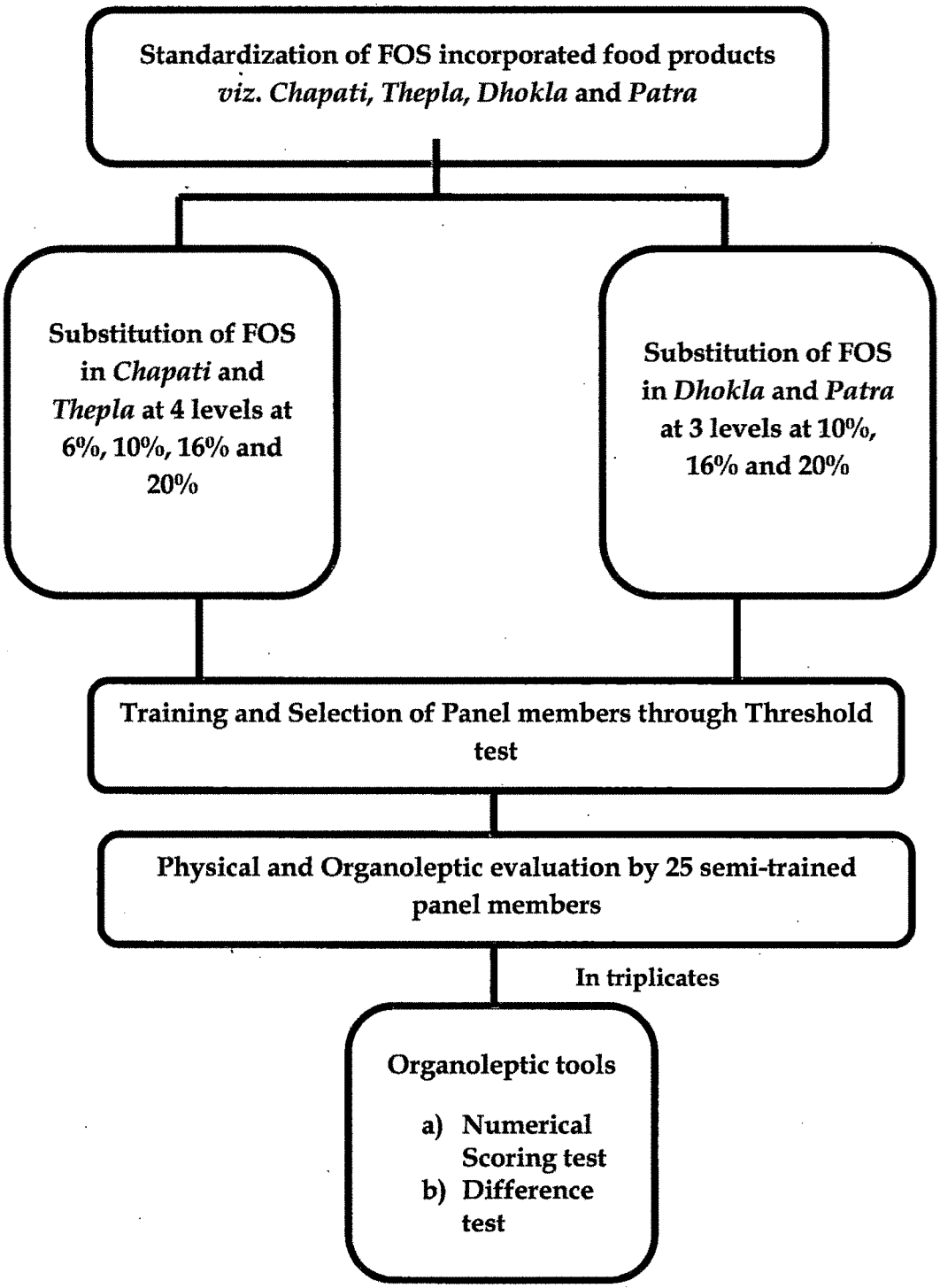


Figure 4.1.1: Experimental design of Phase I of the study

Section 4.1.4 Standardization and Substitution of fructooligosaccharide to the following products:

a) ***Chapati***: *Chapati*es are unleavened flat breads which were prepared using 100 g whole wheat flour and kneaded with water to get a desired consistency of dough. After that it was flattened and roasted (Appendix I). The whole wheat flour was replaced in the standard *Chapati* recipe (Pasrachia and Rebello 1998) with 6%, 10%, 16%, 20% level of fructooligosaccharide substitution. For preparing standard *chapati,* 100 ml water was added to 100 g wheat flour and was kneaded to make the dough. Dough was divided into five equal parts for making balls which were flattened, roasted for a few seconds and then puffed. Modification in the quantity of water was done in the FOS added flour in order to prepare the dough of desirable elasticity.

b) ***Thepla:*** Standard *Theplas* were prepared using 100 g wheat flour and bengal gram flour mix as base material, 50 g fenugreek leaves, 80 ml water, 10 ml oil, 10 g garlic-ginger and chilli paste and 2 g spices. All the ingredients were kneaded together to make the desirable dough which was divided into five equal parts for making balls, further it was flattened and shallow fried at medium flame (Appendix I). FOS was substituted in the base material at 4 levels at 6%, 10%, 16% and 20%. Modification in the quantity of water was done in the FOS added flour in order to prepare the dough of desirable elasticity.

c) ***Dhokla***: *Dhoklas* were prepared by a standard recipe (Pasrachia and Rebello 1998) with slight modification with cereal pulse mix flour. Standard *dhoklas* were prepared using 100 g bengal gram flour and semolina mix, 70 g curd, 2 g spices, 10ml oil, 20 ml water and baking soda and this batter was mixed and stirred for 10 min and thereafter leavened for 20 mins (Appendix I). FOS was substituted in the base material at 3 different levels *viz.* 10%, 16% and 20%. Added water was

gradually reduced in the FOS added batter to prevent thinning to obtain desirable consistency of the batter.

d) ***Patra:*** For preparing standard *patra,* batter was made using 100 g Bengal gram flour, 50 ml water, 2 g spices and 5 g tamarind. This batter was layered on 60 g Colocasia leaves which was rolled and kept for steaming for 20 mins (Appendix I). It was further cut into small pieces and sautéed using 10ml oil, 2 g gingelly seeds and 5 g coriander leaves. Base flour of Bengal gram flour was substituted with FOS at 10%, 16% and 20%. Added water was gradually reduced in the FOS added batter to prevent thinning to obtain desirable consistency of the batter.

4.1.5: Assessment of Physical properties of standard fructooligosaccharide enriched recipes

All the recipes were assessed for the Physical properties for standard and FOS incorporated products. The tests used are described as follows:

a) **Physical evaluation of *Chapati*:**

i. **Dough weight (g):** The dough weight of the *chapati* was recorded using a digital weighing balance.

ii. **Cooked weight (g):** The cooked weight of the *chapatis* was recorded using digital weighing balance.

iii. **Water absorption power:** This property mainly refers to the amount of water that is required to be added to the flour to make the dough of desired consistency.

Method: Take measured amount of water in a beaker and add to the flour gradually to make the dough of appropriate consistency

Calculation: WAP (in ml) = W1-W2

W2= amount of water left

W1= amount of water measured initially

b) Physical evaluation of *Thepla*:

i. **Dough weight (g):** The dough weight of the *thepla* was recorded using a digital weighing balance.

ii. **Cooked weight (g):** The cooked weight of the *thepla* was recorded using digital weighing balance.

iii. **Water absorption power:** (As per section 4.1.5 a-iii)

c) Physical evaluation of *Dhokla*

i. **Dough weight (g):** The dough weight of the *dhokla* was recorded using a digital weighing balance.

ii. **Cooked weight (g):** The cooked weight of the *dhokla* was recorded using digital weighing balance.

iii. **Water absorption power:** (As per section 4.1.5 a-iii)

iv. **Time of steaming**: This refers to the time recorded in minutes required for steaming the batter for *dhokla* preparation

v. **Bulk Density**: The bulk density of the dhokla was determined by measuring the weight of the known volume of the prepared *dhokla*. The product was cut into a cube form and was measured for its length, breadth and thickness and weighed on an electronic balance. The bulk density was calculated by dividing the weight of the sample by volume.

d. Physical evaluation of *Patra*

i. **Dough weight (g):** The dough weight of the *patra* was recorded using a digital weighing balance.

ii. **Cooked weight (g):** The cooked weight of the *patra* was recorded using digital weighing balance.

iii. **Water absorption power:** (As per section 4.1.5 a-iii)

iv. **Time of steaming:** Noted on a digital watch

4.1.6: Selection and training of judges for organoleptic evaluation

a) **Screening of Panellists:** In this section, selection of panel members was carried out. Students of the department of Foods and Nutrition were subjected to threshold testing.

b) **Threshold test (Ranganna 1995)**

Threshold is defined as a stimulus scale at which a transition in a series or judgment occurs. For conducting this test, score card for the same was formulated and pre tested (Appendix II (i), (ii)). Each perspective panel member was given two sets of the solution i.e. Set 1 and Set 2 having six solutions of different concentrations of salt and sugar respectively and were arranged in random order. The participants were asked to identify and rank the samples in increasing order of concentration of taste from the test solutions offered. Three successive trials were conducted for screening of the panelists. Subjects who succeed to pass the threshold test were included further in evaluating the physical and organoleptic characteristics of the food products.

c) **Training of the selected panel members:** A training tool was developed for imparting the basic knowledge and understanding of visual and organoleptic characteristics of the products. Training was given using the same.

d) **Development of score cards for the organoleptic evaluation of the products:** Score cards were developed for organoleptic evaluation (Appendix III) where in tools for evaluation is listed.

4.1.7: Tools for organoleptic evaluation

Organoleptic evaluation tools selected were

a) Numerical Scoring test and
b) Difference test (ISI 1972)

a) **Numerical Scoring Test:** Application of this test was done for visual and organoleptic evaluation. Panelists were asked to evaluate and score essential quality attributes that were needed to be scored like volume, color, taste, aroma etc. The maximum score of each attribute was 10. These tests were done to assess each attribute of all the four products for all the samples.

b) **Difference Test**: This test was used to measure the effect of process change on quality of the product or by formulation for product improvement. It measures more than one test variable per session. Each panelist was served 4-6 samples depending upon the number of test variables. One sample was known standard. The panelists compared each coded sample with the known standard. Whatever score the panelists assigned to the blind standard was subtracted from the score assigned to the test variable. Direction and degree of freedom was also judged. The development and evaluation of FOS incorporated recipes is depicted in plates 4.1(a-d)

a) Chapati

b) Thepla

b) Dhokla

c) Patra

Plate 4.1.1 (a-d): FOS incorporated *chapati, thepla, dhokla* and *patra*

4.1.7 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS 17.0v) software. Results were expressed as mean values $\pm$ standard deviations of all the four products. ANOVA was performed to determine the significant differences in various levels of FOS substituted food products. Percent increase and decrease was also determined to compare the substituted products from standard products.

Section 4.2: Recovery of FOS during processing

This phase of the study was conducted to determine the recovery of FOS in chapati, thepla, dhokla and patra at minimum (6%) and maximum (20%) level of FOS substitution during processing.

a) **Preparation of sample for analysis:** Four food products *chapati, thepla, dhokla* and *patra* were prepared without adding FOS (n=4), with FOS at 6% level (n=4) and at 20% level (n=4). After preparation of the products

they were kept in oven at 100^{0}C for 2 hours for drying. Thereafter when products were dried moisture was measured, grinded and kept in air tight containers for determination of FOS by HPLC analysis.

Table 4.2.1: Moisture (g%) Content in Samples Selected for Analysis

Products	Moisture (g%)	Dry matter (g%)
Chapati	25	75
Thepla	27	73
Dhokla	35	65
Patra	30	70

b) **Reagents and Standards:** HPLC-gradient grade acetonitrile was purchased from Merck (Darmstadt, Germany). Standards of d-(+)-glucose anhydrous, d (−)-fructose and sucrose came from Fluka (Buchs, Switzerland). Stock solutions (1 g/L) were prepared in ultrapure water (Millipore, Bedford, MA, USA) and stored in darkness at 5 °C. Deionized water was purified with a Milli-Q water system (Millipore Corporation, MA, USA). The inulinase was provided by Sigma (Sigma–Aldrich, Madrid, Spain).

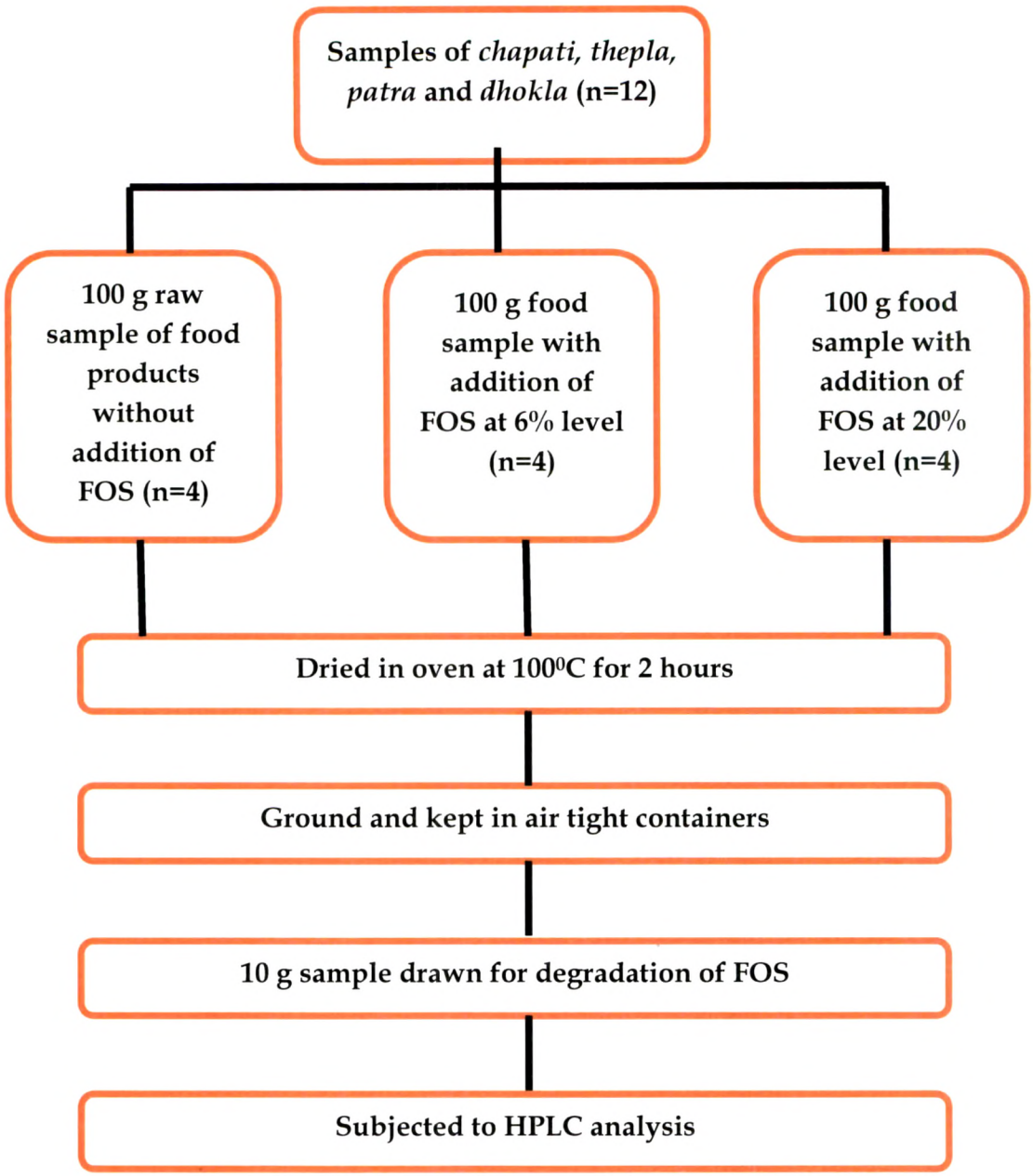


Figure 4.2.1: Experimental Plan for recovery of FOS in food products

c) Analysis of the samples using HPLC

The samples were analyzed using a method of 'Ceria -Belgium' which is based on the procedure outlined by Dysseler et al 1999.

Principle: The method is based on specific hydrolysis of fructans using an inulinase enzyme (from Aspergilus Niger Sigma Aldrich Chemicals Ltd.). The standards of fructose (D-fructose extra pure Qualigens Ltd.), glucose (D-glucose pure Qualigens Ltd.) and sucrose (Sucorse pure-merck chemicals Ltd) were used as calibration samples. FOS content in the samples was calculated based on the comparison of standard FOS and other peak of sugars are calculated as glucose, fructose and sucrose determination before and after hydrolysis.

Standardization of the method: The high performance liquid chromatography was calibrated for the determination of fructooligosaccharide by injecting water based standard solutions containing different concentrations 1000, 2000, 3000 4000 and 5000 ppm of FOS into HPLC with standard operating conditions. The chromatograms of various concentrations are depicted in Figure 5.1.2.1 of Chapter 5 (Phase I, section 5.1). The standard curve plotted for various concentrations is depicted in Figure 5.1.2.2 of Chapter 5 (Phase I, section 5.1), which shows that there is no significant variation in the determined concentration, indicating that the detector response is valid at various concentrations of sugars.

Determination of Fructooligosaccharide: For determining FOS in food products, 150 ml of 0.1M acetate buffer solution was added to 10 g of the food sample at pH=4.5 and homogenized. The covered beaker was placed in the shaking water bath at 85°C for 20 minutes to dissolve the sample and cooled to 60° C. To this 100µl inulinase enzyme was added and incubated in shaking water bath at 60°C for 30 minutes for total digestion of the FOS. Lead hydroxy acetate up to 3ml was added to precipitate the protein and

finally the volume of the mixture was made to 200 ml with water, homogenized and filtered through filter- paper. Filtrate was transferred into a capped vial and subjected to HPLC assay with this solution using 100% water as mobile phase and the separation was performed at an ambient temperature of 30°C at a flow rate of 0.4ml/min.

Calculation and expression of results: The amount of total FOS is given by (R1-R2) which is the difference of inserted Sample area divided by Standard area (variation of 1-2 RT is taken into account due to the errors). This whole equation is then multiplied by Standard weight per dilution (10) multiplied by total volume (200 ml) after addition of water per sample weight and this equation is multiplied by 100 as potency of FOS. The formula for calculation is given below.

$$FOS = \frac{R1 - R2}{Std.Area} * \frac{\text{Std. wt.}}{Dilution} * \frac{\text{Total volume}}{Sample\ wt.} * 100$$

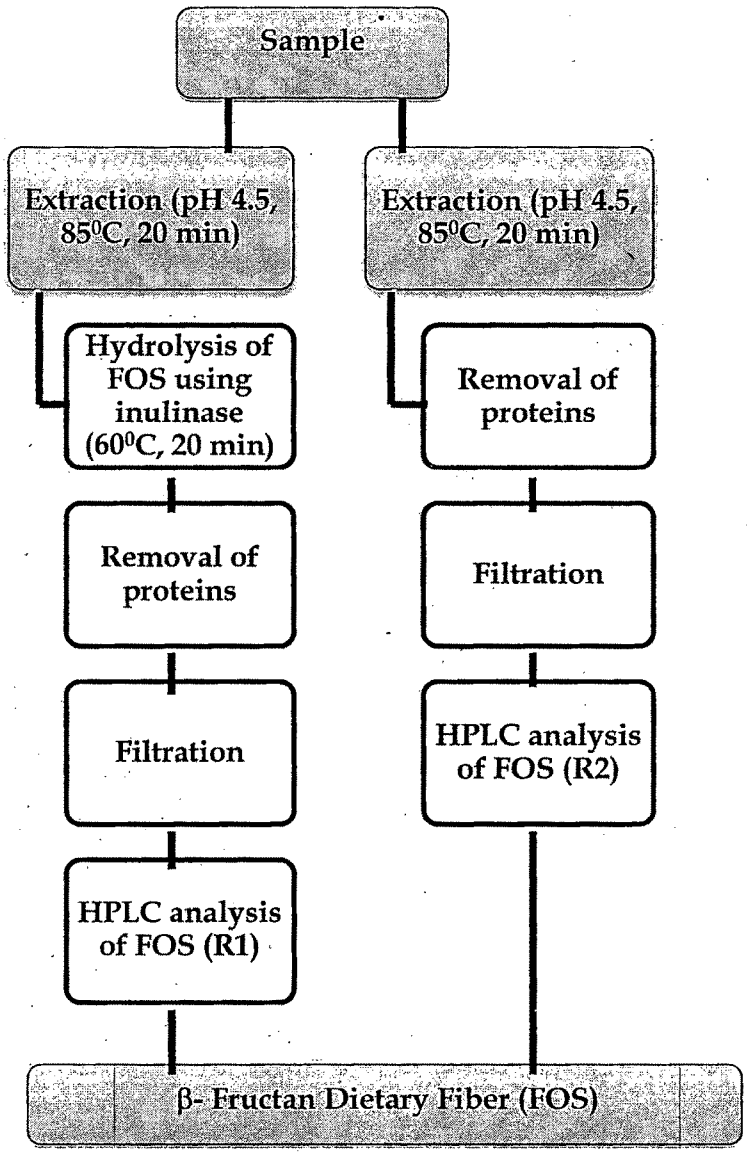
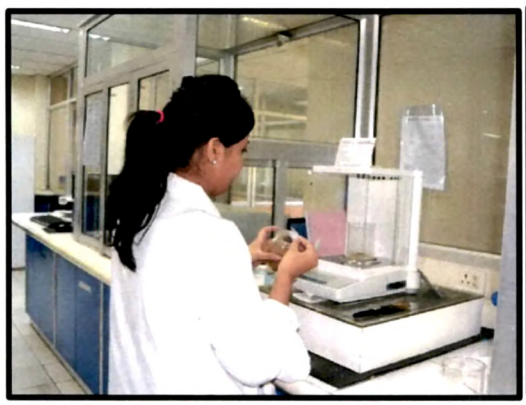


Figure 4.2.2: Determination of β- Fructan Dietary Fiber (FOS) using HPLC
(Dysseller et al 1999)

(a) Dried samples stored in air tight containers

(b) Pre-treatment of samples



(c) Weighing the sample for analysis

(d) Adjusting of pH using pH meter

(e) Injecting the sample into HPLC system

(f) Printing of chromatograms

Plate 4.2 (a-f): Steps in Determination of FOS Content in Food Samples

PHASE II and III

Situational analysis of type 2 diabetic subjects in health clinic of The M.S. University of Baroda and impact of fructooligosaccharide (FOS) supplementation on glycemic, lipemic parameters, gut incretin (GLP-1) and gut microbiota in diabetic adults.

The study involved 120 known diabetic male and female adults aged between 40-70 years, who attended the University health clinic of The Maharaja Sayajirao University of Baroda at Vadodara. The subjects were university staff members who voluntarily agreed to participate in the study. Purposive sampling method was done to enroll subjects who were on oral drugs; metformin and sulfonylurea. Relevant data was obtained through patient medical records, face to face interview and direct measurements like anthropometric measurements, biophysical determination, biochemical analysis like fasting Blood Sugar (FBS), post prandial blood sugar (PP2BS), glycated hemoglobin (HbA_{1c}), glucagon like peptide-1 (GLP-1), lipemic parameters and gut microflora in terms of *Lactic acid bacteria, Bifidobacteria* and Enteric pathogen. The patients who satisfied the inclusive criteria of the study were randomly divided in to control (n = 20) and experimental group (n = 45) with the help of randomized tables and were given 10 g FOS for 8 weeks and examined the effect of daily intake of FOS for the same parameters as mentioned above. Experimental design of the study is depicted in Figure 4.3.1

4.3.1 Statutory clearances

The Medical Ethics committee of the Foods and Nutrition Department, The M.S. University of Baroda approved the study proposal and provided the Medical ethics approval number (F.C.Sc/FND/ME/56). Written consent was obtained from the participants who agreed to give baseline information through questionnaire and give sample of blood and stool for biochemical and microbiological analysis respectively (Appendix IX).

4.3.2 Power of the study and sample size

The power of the study and sample size was calculated from Medical statistics online calculators developed by General Clinical Research Center Program, Massachusetts General Hospital and National Institutes of Health. The probability is 90 percent that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is 1.500 units. This is based on the assumption that the within-patient standard deviation of the response variable is 2.

4.3.3 Selection of the subjects for the study

A total of 120 known type 2 diabetic male and female adults aged between 40-70 years, were selected who attended Health clinic of The Maharaja Sayajirao University of Baroda. Those who met the inclusive criteria of the study were further requested to participate in the intervention program of the study. The willingness of the subjects was considered for participation through the informed consent letter. They were counselled about the benefits of FOS and requisites of the study that the diet, physical activity and drug usage during the study period should remain unaltered

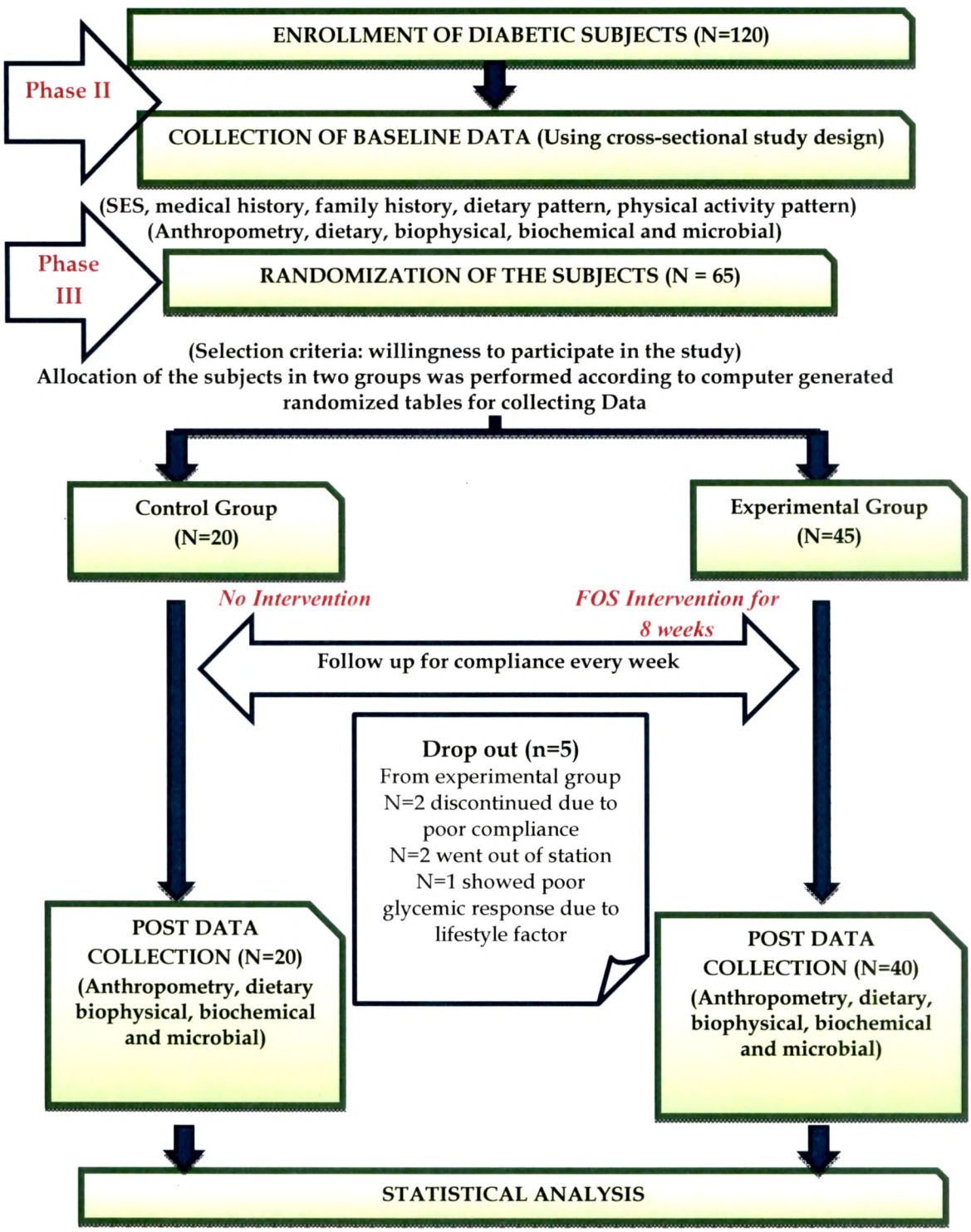


Figure 4.3.1: Experimental Design of Phase II and III

4.3.4 Inclusion and Exclusion criteria of the subjects

Inclusion Criteria:

- Patients with either sex between age group 40 - 70 yrs
- Patients diagnosed with Type 2 Diabetes Mellitus who are on oral diabetic drugs (Metformin and sulfonylurea)
- Patients with fasting blood glucose <200 mg/dl and/or postprandial blood glucose <350 mg/dl
- Patients with HbA_{1c} >7%
- Patients with BMI <35
- Patients willingness for FOS supplementation
- Non smokers
- Non alcoholic

Exclusion Criteria:

- Patients suffering from any of the following conditions were excluded from the study.
  - Type 1 diabetes mellitus
  - Patients with severe hyperglycaemia (FBS > 200 mg/dl; PP2 >350 mg/dl)
  - Patients with total cholesterol >260 mg/dl and serum triglycerides >300 mg/dl
  - Patients with HbA_{1c} <7%
  - Severe renal, hepatic or respiratory disorder
  - Patients with haematological disorder
  - Patients with BMI >35
  - Severely Anaemic
- Show allergic symptoms to study food
- Patients who are likely to undergo surgery during study period
- Patients who have participated in any investigational study in last 4 weeks
- Smokers, alcoholic

4.3.5 Study Protocol

In the present study 120 known type 2 diabetic adults (both male and female) of the age group 40–70 y were included who were on oral hypoglycemic drugs (Metformin and Sulfonylurea). Information regarding socio demographic profile, general habits, dietary intake, and physical activity pattern, clinical, medical and family history was collected using pretested semi structured questionnaire. Subjects were screened for various parameters in terms of anthropometric, dietary, biophysical, biochemical and microbial parameters. General information and information on socio economic status was collected using a pre-tested semi-structured questionnaire. Physical activity pattern was measured using WHO-Global physical activity questionnaire. Dietary analysis was done using 24hr dietary recall and food frequency questionnaire.

Preliminary examination of the subjects was conducted with respect to body weight, height, waist circumference, hip circumference, blood pressure, lipid parameters, FBS, PP_2BS, HbA_{1c} and GLP-1 values. Gut microbial counts levels were assessed in terms of *lactobacilli, bifidobacteria* and enteric pathogen. Based on the results of the preliminary examination and willingness to further participate in the study, 65 diabetic subjects were enrolled in the study trial. The subjects were randomly assigned using computer generated random tables to either control or experimental group. Informed consent from the subjects was obtained prior to supplementation. The experimental group was asked to consume 10 grams of FOS per day for 60 days along with their meals. Subjects were required to provide fecal samples and blood samples before and at the end of each intervention. Fecal samples were collected for determining microbial counts in terms of *lactobacilli, bifidobacteria* and enteric pathogen and blood samples were collected in the fasted state for FBS and lipid analysis, just after the meal for GLP-1 analysis and after 2 h of meal for PP2BS estimation. Anthropometric measurements and systolic and diastolic blood pressure (BP) were measured by the investigator before and after each

intervention period. Subjects were advised not to alter their usual calorie intakes and physical activity pattern and were asked to document any unusual symptoms or side effects and to keep a diary of illness and medications. All subjects filled up a 3-day diet dairy of their habitual dietary intakes before and after each intervention arm. Subjects were followed up every week for compliance and felt side effects if any (Appendix X).

Primary variable:

Glycated hemoglobin

Secondary variables:

BMI, Age, FBS, PP2BS, GLP-1, Lipid profile, Fecal microbial counts, Dietary intake, Physical activity level.

4.3.6 Administration of Interviewer based Questionnaires

Subjects who met the inclusive criteria of the study were briefed on the objective and benefits of the study, and were motivated to participate by providing an informed consent. Set of questions were administered on the subjects and baseline information was gathered (Appendix IV).

a. Background information

General information with regards to age sex, religion, type of family, educational level, per capita income, personal habits, family and medical history of the disease, duration of disease, other complications and medications was collected from the subjects.

b. Activity pattern

In order to understand epidemiology of chronic diseases like Diabetes, and plan effective intervention, it is necessary to assess physical activity pattern. Physical activity pattern of the subjects was determined using was assessed using Global Physical Activity Questionnaire (GPAQ) given by the WHO

2008 (Appendix V). It collects information on physical activity participation on three domains i.e. activity at work, travel to and from places; recreational activities and sedentary behavior. Assessment of GPAQ was done using following analytical procedure:

i) **Physical Activity Level (PAL)**: this was used as a composite index of physical activity patterns and was calculated as:

PAL=24 hour energy expenditure*/ Basal Metabolic Rate**

*Sum of all reported activities computed for a single day

**Basal Metabolic Rate (BMR)

Males

30-60 yrs= [(47.2*Wt(kgs)+(66.9*Ht(m))+3769]/1440
>60= [(36.8*Wt(kgs)+(4719.5*Ht(m))+4481]/1440

Females

30-60 yrs= [(36.4*Wt(kgs)+(104.6*Ht(m))+3619]/1440
>60= [(38.5*Wt(kgs)+(2665.2*Ht(m))+1264]/1440

Table 4.3.6: Cut Offs for Physical activity levels (PAL)

Criteria	PAL values
Sedentary	<1.4
Moderately active	1.55-1.60
Heavily active	>1.6

ii) Expenditure for each activity:

In order to calculate energy expenditure for each of the components of activity, BMR per minute was computed. For each reported activity, a metabolic equivalent (MET) was applied. Thus higher MET values indicate higher levels

of physical activity. Total energy expenditure for a particular activity was thus computed as:

BMR/Min*MET*Duration of activity in minutes

c. Food Frequency Questionnaire (FFQ):

Lists of foods were categorized according to the food groups and their frequency of consumption was obtained as per daily, weekly, monthly or yearly basis (Appendix VI). For obtaining the information of prebiotic and probiotic rich foods a list of 26 commonly consumed probiotic and prebiotic foods were prepared (Appendix VII). 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).

d. 24 h Dietary recall:

A 24-h recall gathered from a group of individuals can be used to characterize the 'usual' (foods that the respondent consumer on a typical day) diet pattern of the population from which they are sampled, since intra-individual variation in diet is unimportant when examining group level dietary patterns. A three day 24-dietary recall was collected before and after the intervention. This questionnaire (Appendix VIII) was interviewer administered, since it is seen that this method produce better results as the interviewer can probe for forgotten items or common diet patterns.

The 24-h dietary recall is used to estimate the food intake of an individual over the period of 24 h, referring to previous day/night. The 24 h dietary recall was done for all the subjects to collect information on the intakes of calories, proteins fat, carbohydrates and fibers. Subjects were asked to provide

details of all the major meals consumed throughout the previous day, along with additional beverages, snacks, sweets, pickles, etc. along with added sugar and salt. The subject was probed to remember any forgotten item, and it was made sure that no expression of opinion, feelings or suggestions was made could lead to affect respondent's answer. Subjects were shown the set of standardized set of utensils and encouraged to respond to the quantity, number and size of the food item consumed on the basis of the same. An account of raw ingredients used in each of the reported preparations was obtained and weighed on Braun Scale with 10 g sensitivity.

Nutrient content of diet was calculated using ICMR Food composition tables given in the nutritive value of Indian foods (Gopalan et al 2004) using Diet Soft Software (Dt. Gurdeep Kaur AIIMS, 2007).

4.2.7 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, all anthropometric measurements were made using the guidelines adopted at the NIH sponsored Arlie Conference (Lohman et al 1988)

a) 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. The subject was weighed in standard indoor clothing, bare feet and without leaning against or holding anything. Scale was 'zeroed' before taking any weight, and was calibrated using standard weights after every third subject.

b) Height: It is a linear measurement made up of the sum of four components i.e. Legs, Pelvis, Spine and Skull (Jellife 1966).

Technique- A spring- loaded non-stretchable tape was used to measure the height of the subjects. A convenient flat wall was identified at the clinic site for the measurement of height. The subject was made to stand barefoot with the arms hanging freely by the side. 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 subject 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. Two consecutive reading were taken.

c) Waist Circumference: Circumference of the waist is an important indicator of the risk of CVD when calculated with Hip circumference to give Waist-Hip Ratio (WHR) (Walker et al 1996).

Technique- The subject was made to stand erect with the abdomen relaxed and the arms at the sides. The circumference was recorded using the constant tension, spring loaded tape at the narrowest part of the abdomen between the ribs and iliac crest. This was done with measurer facing the subject and identifying the natural waist (i.e. the point of narrowing). The measurement was taken to the nearest 0.1 cm at the end of a normal expiration, without the tape compressing the skin.

d) Hip Circumference:

Technique- Hip circumference (HC) was measured at the point yielding the maximum circumference over the buttocks. The constant-tension spring loaded measuring tape was placed around the buttocks in a horizontal plane at this level without compression the skin. The measurement was noted to the nearest 0.1 cm.

Computed Anthropometric Indices

e) Body Mass Index (BMI): The BMI is a convenient and valid measure of adiposity.

It is computed as-

$$\text{BMI (kg/m}^2\text{)} = \frac{\text{Weight (kg)}}{\text{Height (m}^2\text{)}}$$

Table 4.3.7.1: BMI Cut-offs

Category	BMI
Underweight	**<18.5**
Normal	**18.5-22.9**
Overweight	**23-24.9**
Obese grade I	**25-29.9**
Obese grade II	**30-34.9**
Obese grade III	**≥ 35**

Asia Pacific Classification 2004

f) **Waist- Hip Ratio (WHR):** This ratio gives an idea of central adiposity.

It is computed as-

$$WHR = \frac{\text{Waist Circumference (cm)}}{\text{Hip Circumference (cm)}}$$

Table 4.3.7.2: Cut offs for Waist –Hip Ratio (WHR)

	Males	Females
Waist (cm)	>90	**>80**
WHR > 0.9 (Male)	Obese	**-NA-**
WHR > 0.8 (Female)	**-NA-**	**Obese**

The WHO Asia Pacific criterion for abdominal obesity (WHO 2004)

4.3.8 Bio-physiological Investigations

Blood Pressure: Blood pressure is the lateral pressure exerted by blood on vessel walls while flowing in it. Sitting blood pressure of subjects was measured using the standard electric sphygmomanometer on the right arm.

Technique- Blood pressure measurements were taken after the subject was made to sit down quietly for at least 5 minutes. The bare arm of the subject was supported and positioned at heart level. A cuff of suitable size was evenly applied to the exposed upper arm, with the bladder of the cuff positioned over the brachial artery. The bladder length was at least 80% and width at least 40% of the circumference of the arm.

The cuff was snugly wrapped around the upper arm and inflated to 30 mmHg above the pressure at which the radial pulse disappears. The cuff was deflated at rate greater than 2 mmHg/beat. If initial readings were high, several further readings were taken after 5 min. of rest. On each occasion two or more readings were averaged. For diastolic reading the disappearance of

sound was used; Muffing of sound was used if sound continued towards zero (Adams et al 2002; Thomas G et al 2005).

Table 4.3.8 Classification of Blood Pressure for Adults

Blood Pressure Classification	SBP	DBP
Normal	< 120	< 80
Pre-hypertension	120 - 139	80 - 89
Stage I Hypertension	140 - 159	90 - 99
Stage II Hypertension	> 160	> 100

Joint National Committee (JNC VII), 2003

4.3.9 Biochemical Evaluation and Assay methods

After the overnight fast, venous blood sample was collected in clean, sterilized vacuum containers and allowed to stand at room temperature for 15 minutes. For determination of postprandial blood glucose, blood was collected after 2 hours of a meal. Serum was immediately separated and stored at -80^0 C until analysis. The blood was then analyzed for Glucose and Total Lipid Profile using standardized kits.

a. Fasting blood glucose (FBS) and Postprandial blood glucose (PP2BS)

FBS was estimated using enzymatic reference method with hexokinase (Reinauer et al 2002). Hexokinase (HK) catalyzes the phosphorylation of glucose by ATP to form glucose 6 phosphate and ADP. To follow the reaction, a second enzyme, glucose 6 phosphate dehydrogenase (G6PDH) is used to catalyze oxidation of glucose 6 phosphate by NAD^+ to form NADH. The concentration of the NADH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340nm.

D-Glucose + ATP $\xrightarrow{HK}$ D-glucose-6-phosphate+ADPD

Glucose-6-phosphate + NADH $\xrightarrow{G6DH}$ D-6-phsphogluconate +NADH

b. Glycated Hemoglobin (HbA_{1c})

HbA_{1c} was quantified assayed using IFFC and FDA approved automated dedicated high performance liquid chromatography (HPLC) method (IFFC 2002). The principle involved ion exchange of HPLC. The samples are automatically diluted on the d-10, injected into the analytical flow path, and applied to the analytical cartridge. The d-10 delivered a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobin is separated based on their ionic interactions, then pass through the flow cell of the filter photometer, where change in the absorbance at 415 nm are measured. The d- 10 software performs reduction of raw data collected from each analysis; two level calibrations are used for quantization of HbA_{1c} values. A sample report and a chromatogram are generated for each sample.

The A_{1c} area is calculated using an Exponentially Modified Gaussian (EMG) algorithm that excludes the labile A_{1c} and carbamylated peak areas from the A_{1c} peak area. HbA_{1c} covers all fractions; this includes labile HbA_{1a}, HbA_{1b} and HbA_{1c}. The former two fractions are labile and hence do not represent the stable or long term change. HbA_{1c} represents the true long term glycemic control.

Table 4.3.9.1: Cut offs for HbA_{1c}

Classification	HbA_{1c} Value
Good	≤6
Borderline	7-8
Poor	>8

American Diabetes Association 2007 standards

c. Total Cholesterol (TC)

Total cholesterol was estimated using end point enzymatic colorimetric technique. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide formed effects the oxidative coupling of phenol and 4-aminoantipyrine to form a red quinine-imine dye. The colour intensity of the dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance at 512 nm (Richmond W 1973)

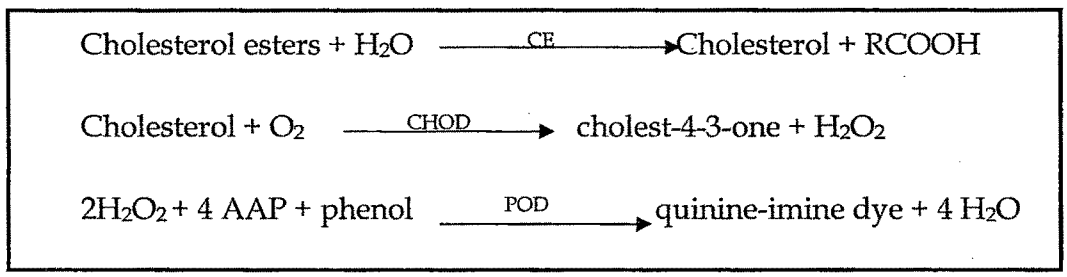


Table 4.3.9.2: Cut offs for Serum Cholesterol

Classification	Cholesterol Value (mg/dl)
Desirable	< 200
Borderline High	200-239
High	>240

NCEP-Adult Treatment Panel (NCEP-ATP3) guidelines, 2002

d. Triglycerides (TG)

Enzymatic colorimetric method (GPO/PAP) with glycerol phosphate oxidase and 4-aminophenazone was used to assess triglycerides. Triglycerides are hydrolyzed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed

by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). In the presence of peroxidase (POD), hydrogen peroxide affects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form red coloured quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample (Fossati P and Prencipe L 1982).

Triglycerides $\xrightarrow{LPL}$ glycerol + fatty acids

Glycerol + ATP $\xrightarrow{GK}$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{GPO}$ dihydroxyacetone phosphate + H_2O_2

2 H_2O_2 + 4-aminophenazone + 4-chlorophenol $\xrightarrow{POD}$ quinoneimine + 4 H_2O

Table 4.3.9.3: Cut offs for Triglycerides

Classification	Triglycerides Value (mg/dl)
Desirable	< 150
Borderline High	150-199
High	200-499
Very High	≥500

NCEP-Adult Treatment Panel (NCEP-ATP3) guidelines 2002

e. HDL Cholesterol (HDL-C)

HDL fraction of cholesterol was determined using enzymatic, colorimetric method (CHOD/PAP) without sample pretreatment. The principle of HDL-Cholesterol direct is based on the absorption of synthetic polyanions to the surface of lipoproteins. LDL, VLDL and chylomicrons are thereby transformed into a detergent resistant form, where as HDL is not. Combined

action of polyanions and detergent solubilises cholesterol from HDL, but not from LDL, VLDL and chylomicrons. Solubilized cholesterol is oxidized by the sequential enzymatic action of cholesterol esterase (CE) and cholesterol oxidase (CHOD). The hydrogen peroxide formed reacts with N, N-bis (4-sulfonyl)-m-toluidine (DSBmT) and 4-aminoantipyrine (4-AAP) in the presence of peroxidase (POD) and forms a red quinoneimine dye. The color intensity of the red quinoneimine dye formed is directly proportional to the HDL-Cholesterol concentration. It is determined by measuring the increase in absorbance at 552 nm (Sugiuchi J et al 1995).

LDL, VLDL, chylomicrons + polyanions ⟶ lipoprotein-polyanion

HDL + detergent ⟶ micelle complexes

Micelle complexes $\xrightarrow{CE/CHOD}$ oxidized cholesterol + H_2O_2

H_2O_2 + 4-aminoantipyrine + DSBmT $\xrightarrow{POD}$ quinoeimine dye

Table 4.3.9.4: Cut offs for HDL-Cholesterol

Classification	HDL Cholesterol Value (mg/dl) Female	HDL Cholesterol Value (mg/dl)Female
Low	<45	< 35
Optimal	45-55	45-55
High	>55	>45

NCEP-Adult Treatment Panel (NCEP-ATP3) guidelines, 2002

f. LDL Cholesterol (LDL-C)

Enzymatic colorimetric method (CHOD/PAD) was used for the direct estimation of LDL, HDL, VLDL and chylomicrons are specifically hydrolyzed by a detergent. The released cholesterol content in these lipoproteins reacts immediately in the enzymatic action of cholesterol esterase (CE) and cholesterol oxidase (CHOD) generating hydrogen peroxide. The latter is consumed by a peroxidase (POD) in the presence of 4-aminoantipyrine to

generate a colorless product. During this first step, LDL particles remain intact. The reaction of LDL cholesterol is initiated by the addition of another detergent together with a coupler, N,N-bis(4-sulfonyl)-m-toluidine (DSBmT). The second detergent releases cholesterol in the LDL particles which are subjected to the enzymztic reaction in the presence of coupler to produce a coloured product. The colour intensity of the red quinoneimine dye formed is directly proportional to the LDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 520nm (Sugiuchi J et al 1995).

Step 1: LDL, VLDL, chylomicrons + detergents ⟶ release cholesterol

Cholesterol $\xrightarrow{CE/CHOD}$ oxidized cholesterol + H_2O_2

H_2O_2 + 4-aminoantipyrine + DSBmT $\xrightarrow{POD}$ colorless product

Step 2: LDL + detergent ⟶ released cholesterol

Cholesterol $\xrightarrow{CE/CHOD}$ oxidized cholesterol + H_2O_2

H_2O_2 + 4-aminoantipyrine + DSBmT $\xrightarrow{POD}$ quinoeimine dye

Table 4.3.9.5: Cut offs for LDL-Cholesterol

Classification	LDL Cholesterol Value (mg/dl)
Optimal	< 100
Near optimal/Above optimal	100-129
Borderline High	130-159
High	160-189
Very high	>190

NCEP-Adult Treatment Panel (NCEP-ATP3) guidelines 2002

g. Determination of Glucagon like Peptide-1 (GLP-1) (7-36amide):

Assay Principle: This ELISA is designed, developed and produced for the quantitative measurement of bioactive GLP-1 (7-36) in plasma sample. The assay utilizes the two-site "sandwich" technique with two selected GLP-1 (7-36) specific antibodies. Assay standards, and test samples are directly added to wells of a microplate that is coated with streptavidin. Subsequently, a mixture of biotinylated GLP-1 (7-36) specific antibody and a horseradish peroxidate (HRP) conjugated GLP-1 (7-36) specific antibody is added to each well. After the first incubation period, a "sandwich" immunocomplex of "Streptavidin - Biotin-Antibody -GLP-1(7-36) - HRP conjugated antibody" is formed and attached to the wall of the plate. The unbound HRP conjugated antibody is removed in a subsequent washing step. For the detection of this immunocomplex, each well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to GLP-1 (7-36) on the wall of the microtiterwell is directly proportional to the amount of GLP-1 (7-36) in the sample.

Technique: (1) Place a sufficient number of streptavidin coated microwell strips/wells in a holder to run GLP-1 (7-36) standards, controls and unknown samples in duplicate.

(2) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	SAMPLE 2
B	STD 1	STD 5	SAMPLE 2
C	STD 2	C1	SAMPLE 3
D	STD 2	C1	SAMPLE 3
E	STD 3	C2	SAMPLE 4
F	STD 3	C2	SAMPLE 4
G	STD 4	SAMPLE 1	
H	STD 4	SAMPLE1	

(3) Prepare GLP-1 (7-36) Antibody Mixture: mixing GLP-1 Tracer Antibody and Capture Antibody by 1:21 fold dilution of the Tracer Antibody (30229) and by 1:21 fold dilution of the biotinylated Capture Antibody (30230) with the Tracer antibody Diluent. For each strip, it is required to mix 1 Ml of the Tracer Antibody Diluent (30017) with 50 FL the Capture Antibody and 50 FL of the Tracer Antibody in a clean test tube.

(4) Add 100 CL of standards, controls and test samples into the designated microwell.

(5) Add 100 CL of GLP-1 (7-36) Antibody Mixture to each well

(6) Cover the plate with one plate sealer and incubate plate at 2-8°C, static for 20 - 24 hours.

(7) Remove plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 FL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.

(8) Add 200 CL of ELISA HRP Substrate into each of the wells.

(9) Cover the plate with one plate sealer and also with aluminum foil to avoid exposure to light.

(10) Incubate plate at room temperature, static for 20 min.

(11) Remove the aluminum foil and plate sealer. Add 50 CL of ELISA Stop Solution into each of the wells. Mix gently.

(12) Read the absorbance at 450nm/620 nm within 10 minutes in a microplate reader.

Calculations: The GLP-1 (7-36) concentrations for the controls and test samples are read directly from the standard curve using their respectivecorrected absorbance. If log-log graphic paper or computer assisted

data reduction program utilizing logarithmic transformation are used, sample having corrected absorbance between the 2nd standard and the next highest standard should be calculated by the formula:

> Value of unknown= Corrected absorbance (unknown) / Corrected absorbance (2^{nd} Std.)* Value of the 2^{nd} Std.

4.3.10 Determination of the gut microbiota

The gut microbiota was determined with respect to the microorganisms- *Lactic acid bacteria, Bifidobacterium* and Enteric pathogen.

The steps involved in the determination of the fecal flora were:

a) Collection of the sample

b) Preparation of the sample

c) Sterilization of the glass wares

d) Preparation and sterilization of dilution blanks

e) Preparation and sterilization of media

f) Preparation of sample for inoculation and incubation of *Lactic acid bacteria, Bifidobacteria* and Enteric pathogen

g) Enumeration of micro-organisms.

h) Calculation for reporting colony counts.

a. Collection of the fecal sample

The stool samples from the subjects were brought in an air tight sterile container kept with cold packs. The sample was used for the enumeration within 3 hours after the collection of sample.

b. Preparation of the sample:

One gram of fecal sample was weighed in a balance and was used for the estimation.

c. Sterilization of the glass wares:

All the petri dishes and the other glass wares such as beakers and conical flask were sterilized before use. The petri dishes were kept in the petri dish box and the other glassware were wrapped in a paper and kept in a hot air oven at 180^0 C for 2 hours for sterilization. The micro-tips were sterilized by autoclaving at 121^0C for 15 minutes at 15 lbs pressure. The other instruments which were used like the weighing balance and spatula were all sterilized by alcohol flaming using 70 % alcohol.

d. Preparation and Sterilization of dilution blanks:

For the preparation of dilution blanks 1 g of peptone was dissolved in 1000 ml of distilled water. This solution was dispensed in portion of 100 ml in 10 dilution bottles. These were autoclaved at 121^0C for 15 mins. The bottles were cooled at room temperature before putting them to use (Figure 4.4).

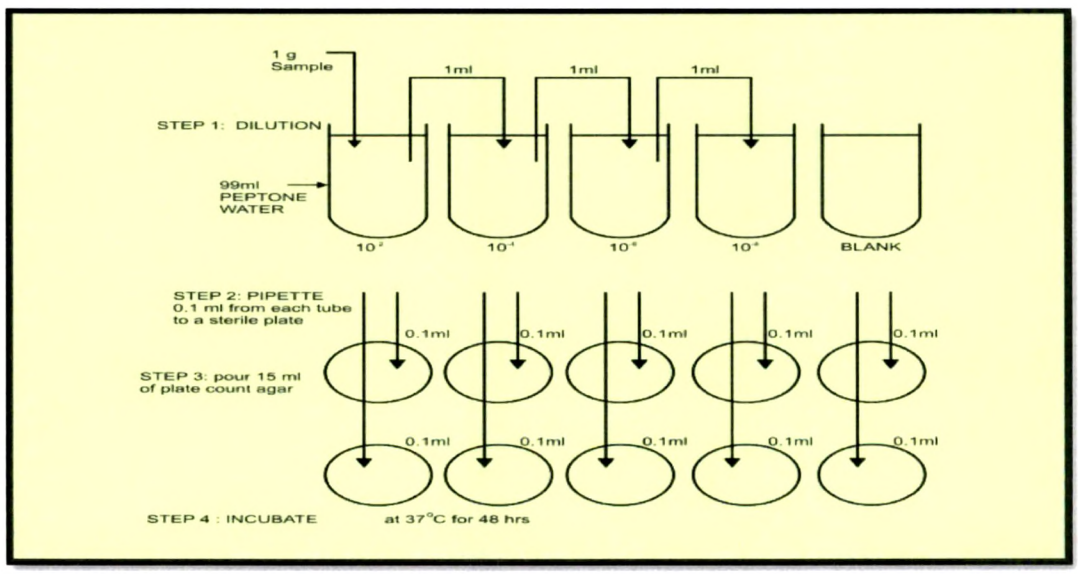


Fig. 4.3.9.1: Serial Dilution technique along with Steps of Inoculation and Incubation

The media used for the enumeration of *Bifidobacterium* was *Bifidobacterium agar* supplied by HiMedia. The prepared media was autoclaved at 121^{0}C for 15 minutes and then poured into sterile petri plates and was allowed to set. The enumeration of *Lactic acid bacteria* and Enteric pathogen was done using ready-*made HiTouch Flexi plates* supplied by HiMedia. HiTouch Flexi disposable petri plates are 55mm in diameter. It is grid scored on the base and is irradiated to ensure perfect sterility. These plates are specially developed for microbial testing, where not only counts are obtained but it is also possible to select and differentiate between groups of microorganisms (HiTouch Flexi plate series manual 2007). The Flexi plates were kept inside laminar flow under UV light before using them for inoculation and enumeration of bacteria.

f. Preparation of sample, inoculation, incubation and enumeration of *Lactic acid bacteria, Bifidobacteria* and Enteric pathogen

One gram of fresh fecal sample was accurately weighed 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 as shown in Figure 4.3.9.1. Then 0.1 ml of dilution was pipetted from each of the dilutions to the petri plates containing respective media. The above procedure was carried out inside laminar flow (as shown in plate 4.3.9.2, 4.3.9.4) that ensures a sterile environment thereby preventing contamination from outside. The plates of *Bifidobacterium* were then incubated at 37°C placed in the anaerobic jar in the incubator with the gas packs (plate 4.3.9.3) and read after 48 hours. Flexi plates of *Lactic acid bacteria* were placed in a desiccator (plate 4.3.9.3) as it is a facultative anaerobe and those of Enteric pathogen were directly placed in the incubator. After 48 hours of incubation the colonies were counted on colony counter and colonies that appeared in the range of 30 - 300 were converted in to log counts after multiplying with their dilution factors (Ramona et al 2001).

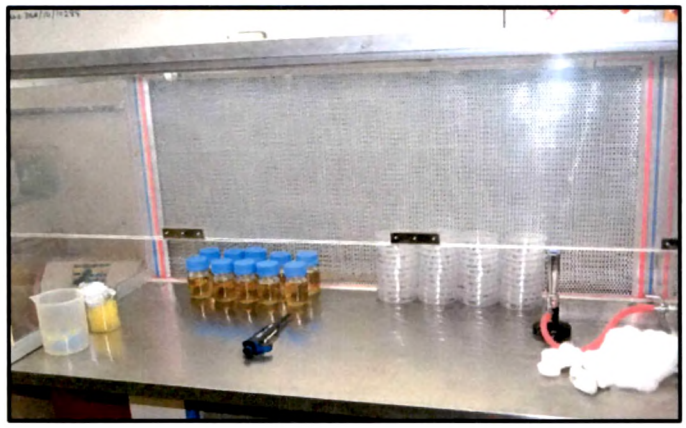


Plate 4.3.9.2: Laminar flow depicting plates inoculated with samples and media

Plate 4.3.9.3: Anaerobic Jar and Desiccator Placed inside Incubator with plates inoculated with fecal samples for determination of *Bifidobacteria, Lactobacilli and Enteric pathogen*

Plate 4.3.9.4: Serial dilution and sampling in the laminar flow

4.3.11: Study food and mode of Intervention

The Fructooligosaccharide (FOS) used for the intervention was derived from chicory roots, procured from Brenntag Ingredients India Pvt. Ltd, Mumbai. The FOS was given in powder form packed in two sachets, 5 g each. The subjects were asked to incorporate FOS in to commonly consumed traditional food items like milk, buttermilk, fermented milk *(Dahi)*, sweetened fermented milk *(lassi)*, or water and consume along with the meals preferably 5g in lunch and 5g in dinner. A pilot study on 10 subjects was carried out for acceptability of quantity of FOS.

4.3.12: Statistical Analysis

The data was entered in an excel spreadsheet. The data was cleaned and verified and subjected to appropriate statistical analysis. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS 17.0 version, SPSS Inc.,Chicago, IL,USA). Mean and standard deviation were calculated. Paired t test was performed to observe the effect of FOS supplementation on blood glucose, lipid profile, anthropometric measurements and gut microbial counts. The significance levels were set at 5% by two sided tests. Student *t* test was performed for the comparison between control and experimental group for the various biochemical, microbial and anthropometric parameters. Multivariate linear regression was carried out with the gut microbial counts as dependent variables to observe its relation with various biochemical and anthropometric indices. Pearson correlation was observed between blood glucose parameters and gut microflora counts.