CHAPTER 4
MATERIALS AND METHODS

Over the past few years there has been increasing interest to explore the nutritional functionality of prebiotics as nutraceutical. Fructooligosaccharide (FOS) or Oligofructose is currently endorsed as promoting the growth of healthy intestinal bacteria. As a result FOS as a food ingredient has prompted much research on their possible health effects in managing obesity, lowering glycemic index and blood cholesterol, modulating gut flora and lowering endotoxemia. The present study was undertaken to study the **"Sensory evaluation of fructooligosaccharide (FOS) added popular recipes of India and its role in modulating anthropometric indices, gut flora and lipopolysaccharide (LPS) in obese young adults of urban Vadodara".**

This chapter summarizes the experimental plan and discusses the methods and materials used to accomplish the objectives of the study in four phases.

Phase I

Development and Standardisation of fructooligosaccharide (FOS) incorporated popular recipes of India and studying their various organoleptic attributes and overall acceptability.

Section 4.1: Development of FOS incorporated food products

Section 4.1.1: Selection of food products

Section 4.1.2: Procurement of fructooligosaccharide

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Phase II and III

Situational Analysis: mapping the prevalence of obesity and hypertension in banks employees of urban Vadodara (A cross-sectional design) and collection of baseline data of grade-I obese subjects and non-obese subjects in terms of anthropometry profile, medical history, family history of diseases, defecation profile, hunger and satiety, psychological depression status, dependency on habits, dietary intakes, biophysical profile, lipemic profile, plasma LPS levels and gut microbiota (*LAB, bifidobacteria, bacteroides and clostridium*) and understanding the correlations between various parameters.

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Phase IV

Effect of fructooligosaccharide (FOS) supplementation on anthropometry profile, blood pressure, defecation profile, hunger and satiety, psychological depression, dietary intakes, lipemic parameters, plasma LPS levels and gut microbiota (*LAB, bifidobacteria, bacteroides and clostridium*) in obese grade-I adults.

Section 4.4.1	Selection of the subjects for the study
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PHASE I

Development and Standardisation of fructooligosaccharide (FOS) incorporated popular recipes of India and studying their various organoleptic attributes and overall acceptability.

4.1: Development of FOS incorporated food products

This section of the study was carried out to develop FOS incorporated food products *viz. lilva kachori, vegetable parantha, rawa idli* and *chocolate cake* at varying level of FOS addition/substitution. Experimental design for this phase is given in Figure 4.1.

4.1.1: Selection of food products

The potential of incorporating fructooligosaccharide in the popular recipes of India and their sensory qualities were studied by the method of addition and substitution. Total of four products were selected on the basis of their cooking method namely deep frying, shallow frying, steaming and baking. Out of these products that were added with fructooligosaccharide included *lilva kachori, vegetable parantha,* and *rawa idli* at various levels. In *chocolate cake* FOS was partially substituted with fat and sugar. All the products were studied for their sensory characteristics.

4.1.2: Procurement of Fructooligosaccharide (FOS)

Food grade fructooligosaccharide (Make: Meiji Japan; 20 kg bag; Lot no.MMS 182-270) was procured (Powder form) from Mitushi Pharma, Ahmedabad. The fructooligosaccharide was food grade which was apt to alternate fat and sugar. The specifications of the fructooligosaccharide are given in Table 4.1.

Table 4.1: Specification of the fructooligosaccharide used for product development

Items	Results
1. Moisture (w/w %)	2.3
2. Carbohydrate composition (% dry basis)	
Glucose+Fructose+Sucrose	4.1%
Fructooligoscharrides	95.9%
3. Appreance	White powder
4. Granular size	40 mesh pass
5. Ash (w/w %)	Below 0.1
6. total heavy metal (as pb) ppm	Below 1
7. Arsenic (As As_2O_3) ppm	Not detected (detection limit 1 ppm)
8. Foreign taste and odor	Free
9. Foreign substances	Free
10. Microbiological specification	
Mesophilic bacteria	0
Molds and yeast	0
Coliforms	Negative

Meiji Co. Ltd., 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 4.2.

Table 4.2: List of raw ingredients along with their sources used for product preparation

Product	Ingredients	Source/ Brand name
Lilva kachori*	Wheat flour	Wheat variety GW496
	Maida	Uttam brand, Baroda
	Oil	Groundnut oil
	Fresh green peas	Local market, Baroda
	Green chillies	Local market, Baroda
	Garam masala	Local market, Baroda
	Sugar	Local market, Baroda
	Salt	Tata salt
Vegetable parantha*	Wheat flour	Wheat variety GW496
	Potatoes	Local market, Baroda
	French beans, carrots, spring onions and green chilies	Local market, Baroda
	Corn flour	Local market, Baroda
	Spices	Local market, Baroda
	Oil	Groundnut oil
	Salt	Tata salt
Rawa idli*	Semolina	Uttam brand, Baroda
	Curd	Masti dahi (Amul)
	ENO	ENO
	Salt	Tata salt
Chocolate cake*	Maida	Uttam brand, Baroda
	Sugar	Local market, Baroda
	Unsalted butter	Amul
	Egg	Local market, Baroda
	Baking soda and cocoa powder	Wikfeild
	Vanilla essence	
	Milk	Local market, Baroda

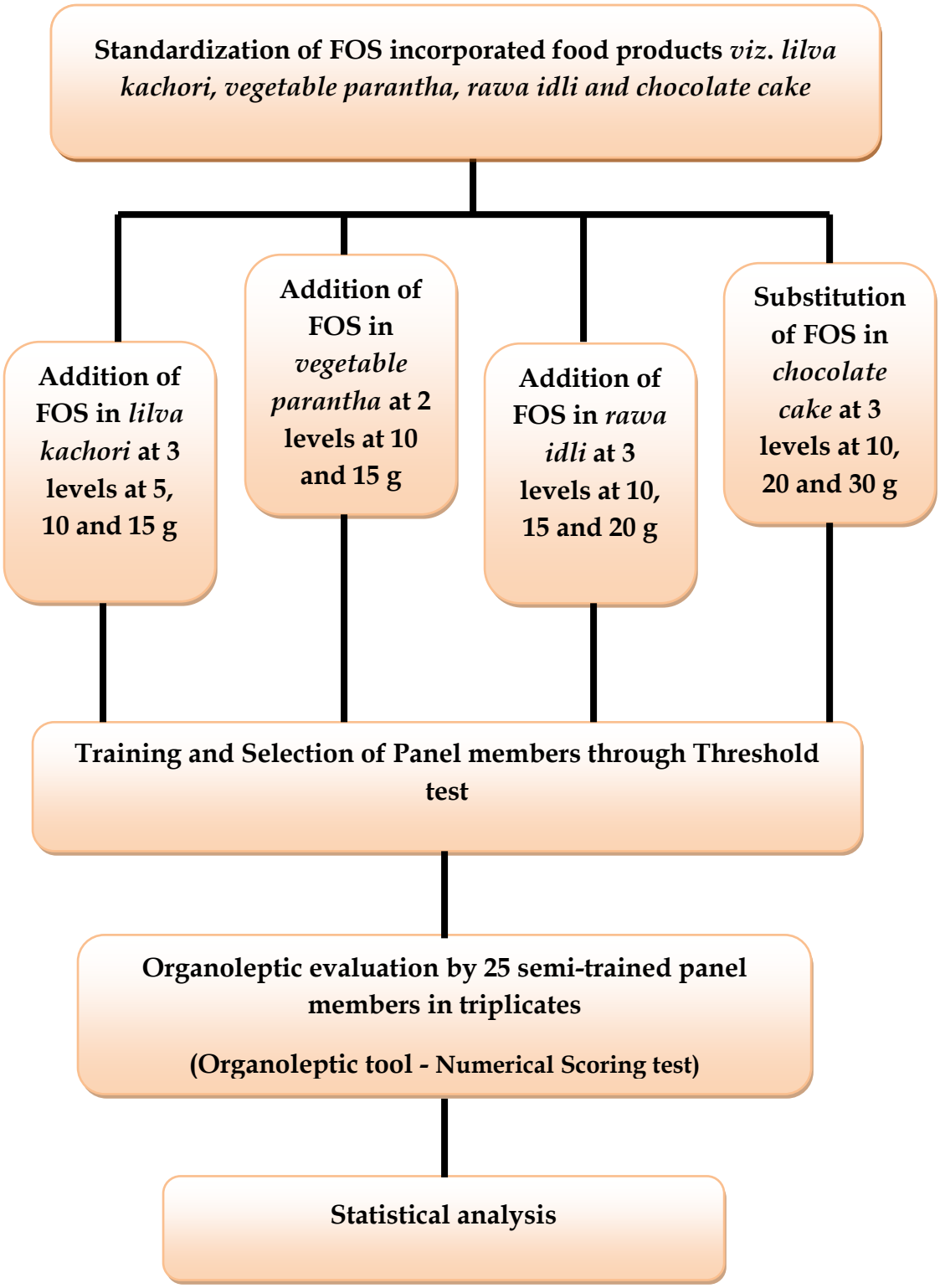

Figure 4.1: Experimental design of Phase I of the study

4.1.4: Standardization and addition and substitution of fructooligosaccharide to the following products

a). Lilva kachori: Standard kachori (one serving) dough was prepared using 80 g maida, 20 g whole wheat flour, 20 ml oil, ¼ tsp. salt and 60 ml water. All the ingredients were kneaded well to make the dough. Further, it was divided into ten equal parts for making balls. Balls were rolled and put into the mold. Fresh green peas (without shell) were taken and crushed once. Til seeds, green chilles, garam masala, 5 g sugar and salt were added and sauté for 2 min. 10 g stuffing was filled in the flattened dough kept in the mold with cover. The mold was closed to give the desirable shape. The stuffed kachori's were deep fried in fresh hot oil (1600°-1700° C) for one minute. Addition of FOS to the stuffing was at three levels, i.e. 5 g, 10 g, 15 g.

b). Vegetable parantha: For preparing standard vegetable parantha (one serving) dough; 120 g wheat flour, 6 g salt, 15 ml oil, and 75 ml water were kneaded together to make the dough. Further, it was divided into three equal parts for making balls which were rolled. Potatoes, french beans, carrots, spring onions, and green chillies were washed properly and chopped in small pieces. 15 ml oil was taken in a pan and all vegetables were cooked for 15 min. 5 g corn flour powder, salt and spices were added to the vegetables. 30 g stuffing was filled in one parantha and rolled again. Parantha's were shallow fried on medium flame for 3 min. each side with ½ tsp. of oil. FOS was added at 2 levels, i.e. 10 g and 15 g in the stuffing.

c). Rawa idli: *Idlis* were prepared (one serving) using 60 g rawa, 3 g of salt and 30 g of curd. 100 ml of water and 1/4 tsp. of ENO were added to this mixture and mixed well. This batter was kept for 25 min. rest. Further it was steamed in a steamer for 15 min. FOS was added at 3 levels, i.e. 10 g, 15 g and 20 g in the batter.

d). Chocolate cake: For preparing standard cake (one serving) one egg added in a bowl and beaten for 5 minutes, ½ tea spoon vanilla essences added to the egg mixture and beaten for 5 min., 40 g powdered sugar added and beaten for 5 min., 40 g unsalted butter added and again beaten for 5 min. in another bowl 50 g of maida, 5 g of cocoa powder, and ½ tsp of baking soda sieved and added to egg mixture and beaten for 15 minutes. Oven was preheated at 150^0 C for 15 min., an aluminum cake mold greased and dusted with some maida on it. The batter of cake poured in the mold and put in oven at 180^0 centigrade for 20 min. FOS was added at 3 levels, i.e. 10 g, 20 g and 30 g in the batter.

4.2: Sensory evaluation of FOS incorporated food products

In this section sensory evaluation of all four (*viz. lilva kachori, vegetable parantha, rawa idli* and *chocolate cake*) FOS incorporated recipes had done at varying level of FOS addition/substitution. Overall acceptability also assessed. Selection and training of judges was done followed by tool preparation.

4.2.1: Selection and training of judges for organoleptic evaluation

Screening of Panelists

In this section, selection of panel members was carried out. Students from the Maharaja Sayajirao University of Baroda, Faculty of Family and Community Sciences, Department of Foods and Nutrition were subjected to threshold testing.

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 I (i), (ii). Each perspective panel member was given three sets of the solution i.e. Set 1, Set 2 and Set 3 having six solutions of different concentrations of salt, sugar and citric acid

respectively and was 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 organoleptic characteristics of the food products.

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.

4.2.2: Development of score cards for the organoleptic evaluation of the products

Score cards were developed for organoleptic evaluation (Appendix II) where in tools for evaluation is listed. For organoleptic evaluation Numerical Scoring Test tool were selected.

4.2.3: Organoleptic evaluation

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 texture, color, taste, mouthfeel 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.

4.2.4: Statistical analysis

Statistical analysis was performed using Microsoft excel 2007. Results were expressed as mean values ± standard deviations of all the four products. ANOVA and student's t test was performed to determine the significant differences in various levels of FOS added and substituted food products. Percent increase and decrease was also determined to compare the FOS added and substituted products from standard products.

a) Lilva kachori

b) Vegetable parantha

c) Rawa idli

d) Chocolate cake

e) Panelist evaluating the products

Plate 4.1: (a-e): FOS incorporated *lilva kachori, veg. parantha, rawa idli* and *chocolate cake* and panelist evaluating the products

Phase II and III

Situational Analysis: mapping the prevalence of obesity and hypertension in banks employees of urban Vadodara (A cross-sectional design) and collection of baseline data of grade-I obese subjects and non-obese subjects in terms of anthropometry profile, medical history, family history of diseases, defecation profile, hunger and satiety, psychological depression status, dependency on habits, dietary intakes, biophysical profile, lipemic profile, plasma LPS levels and gut microbiota (LAB, bifidobacteria, bacteroides and clostridium) and understanding the correlations between various parameters.

This section of the study was design to see the prevalence of obesity in the banks of urban Vadodara and also a comparative analysis was made between non-obese and obese grade-I adults to understand the correlation amongst various parameters with BMI. Experimental design for these phases is given in Figure 4.2.

4.3.1: Statutory clearances

The Institutional Ethics Committee for Human Research (IECHR) of the Foods and Nutrition Department, The M.S. University of Baroda approved the study proposal and provided the medical ethics approval number **(IECHR/2012/13)**. The study also approved and registered under Clinical Trial Registry of India with registration no. **CTRI/2016/02/006611**. The details of the study can also be obtained from www.ctri.nic.in. 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 III).

4.3.2: Power of the study and sample size

Sample size calculation: The sample size estimates were based upon two sided confidence level of 95%, confidence interval of 4 and a power of 90%.

Sample size was calculated using formula for finite population, where the population is less than 50,000. This formula was selected as the population of bank employees working in private sector banks in Vadodara city is limited to 1500 employees. Using the formula for infinite population a figure of 600 subjects was arrived. This figure of 600 was put in the formula of finite population and the final sample size was calculated to 428 subjects for screening.

4.3.3: Selection of the banks and subjects for the study

Selection of banks for the study

List of private banks was taken from the website of Indian Banks Association (www.iba.org.in /viewmembanks.asp?id=3). Eighteen out of listed 24 private banks exist in Vadodara city. Six banks (A total of 10 different branches) in different areas of Vadodara city were conveniently selected based on the permission obtained from the administration department of the banks to organize the health screening camp.

Selection of the subjects for the study

A total of five hundred and ninety five (595) bank employees irrespective of age and gender were screened (Phase II) for their anthropometric measurements, body fat percentage and blood pressure. These subjects were classified under the various categories of BMI (non-obese and obese).

Out of 595 subjects screened, 100 bank employees with normal BMI and 100 bank employees with obesity grade-I without any disorders as mentioned in inclusion and exclusion criteria were selected for phase III.

People in the eligible age group were briefed on the objective and benefits of the study, and were motivated to participate by providing an informed consent. Proper record of the eligible members who refused to participate in the study was maintained.

4.3.4: Inclusion and exclusion criteria for selection of the subjects

Inclusion criteria for selection of the subjects

For Phase II:

- All the employees of the banks screened

For Phase III:

- BMI between 18.5 – 22.9 kg/m^2 (For non-obese; n=100)
- BMI between 25-29.9 kg/m^2 (For obese; n=100)
- Age 25 - 35 years old
- Willingness to participate in the study

Exclusion criteria for selection of the bank employees (For Phase III)

- Subjects with following confirmed disorders were excluded from the study.
    - ✓ Hypertension
    - ✓ Diabetes mellitus
    - ✓ Cardiovascular disorder
    - ✓ Thyroid hormone disorder
    - ✓ Renal disorder
    - ✓ Locomotor disorder
    - ✓ Cancer
    - ✓ AIDS
    - ✓ Psychological disorder
- Subjects who are likely to undergo surgery during study period
- Subjects who have participated in any investigational study in last 4 weeks
- Subjects who followed any weight loss regime (Gymming, dieting, or taking any other nutraceuticals)
- Subjects who were taken any kind of anti-biotic drugs.

4.3.5: Study Protocol

A total of five hundred and ninety five (595) bank employees irrespective of age and gender were screened (Phase II) for their anthropometric measurements, body fat percentage and blood pressure. These subjects were classified under the various categories of BMI and hypertension.

Out of 595 subjects screened, 100 bank employees with normal BMI and 100 bank employees with obesity grade-I without any disorders as mentioned in inclusion and exclusion criteria were selected for phase III.

Information regarding socio-economic profile, general habits, dietary intake, and physical activity pattern, personal 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. Physical activity pattern was measured using WHO-Global physical activity questionnaire-2. Dietary analysis was done using 24hr dietary recall and food frequency questionnaire.

Preface assessment of the subjects was carried out with respect to lipid parameters, and plasma LPS levels. Gut microbial counts levels were assessed in terms of *lactobacilli, bifidobacteria, clostridium* and *bacteriodes.*

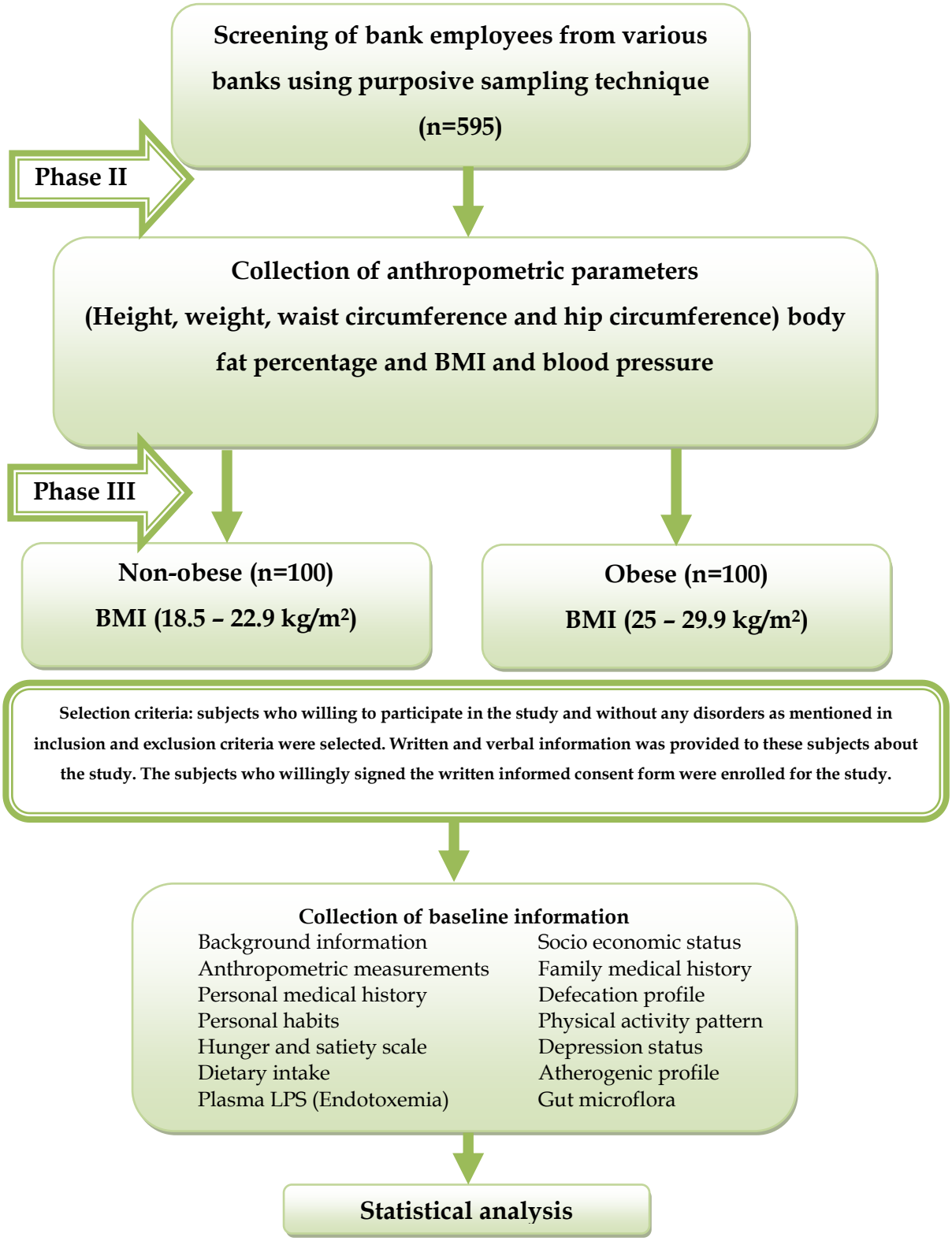

Figure 4.2: Experimental design for Phase II and phase III of the study

4.3.6: Administration of interviewer based questionnaires (Appendix IV)

Background information

General information with regards to name, age, sex, date of birth, contact number, address, email id, religion, and type of family, number of family members, educational level, per capita income, occupation, personal habits, family and medical history of the subjects, and other complications was collected from the subjects.

Socio-economic status

General information with regards to educational level, income and occupation was collected from the subjects using the Kuppuswamy's Socioeconomic Status Scale (Sharma, 2012).

Defecation profile

Information was collected using the defecation score given by Tokunaga regarding the various aspects like frequency, quantity, odor, color, hardness, and feeling after defecation (Tokunaga, T., Nakada, Y., Tashiro, Y., Hirayama, M., and Hidaka, H., 1993).

General Physical Activity Questionnaire (GPAQ2–WHO 2007)

A checklist containing various types of activities along with the time spent to perform each activity was used to assess the activity pattern of the subjects (WHO, 2012).

Hunger and satiety scale

A score card was used to rate the degree of hunger and satiety on different meal timings before and after meals developed by Lisa Burgoon MS, RD, LD, Sports Nutritionist, Sportwell Center, McKinley Health Center, University of Illinois at Urbana – Champain, 1998. (Table 4.3)

Table 4.3: Hunger and satiety score card

Scale	Score
Famished, starving	**1**
Headache, weak, cranky, low energy	**2**
Want to eat now, stomach growls and feels empty	**3**
Hungry - but could wait to eat, starting to feel empty but not there yet	**4**
Not hungry, not full	**5**
Feeling satisfied, stomach feels full and comfortable	**6**
Feeling full, definitely don't need more food	**7**
Uncomfortably full	**8**
Stuffed, very uncomfortable	**9**
Bursting, painfully full	**10**

Psychological depression status

Data on their psychological background was obtained using Becks Depression Inventory which was included in the questionnaire. The subjects were classified as mild, moderate and severely depressed based on the BDI scores (Beck, Ward, Mendelson, Mock, & Erbaugh, 1961).

Food Frequency Questionnaire (FFQ)

A brief detail was taken on the number of times the food item was consumed on a daily, weekly, fortnightly, monthly, yearly or rarely basis. Food list contained 46 food items. Food items were clubbed together in respective food group's categories. The FFQ was thus finalized with columns to collect information on frequency of consumption during the past one year (per day/week/month/year). Fruits were divided into 3 sub-categories according the fiber content present.

24 hour 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. This

questionnaire 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 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 (Longvah, Ananthan, Bhaskarachary, and Venkaiah, 2017) using Diet Soft Software (Dt. Gurdeep Kaur AIIMS, 2007).

4.3.7: 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, Roche, and Martorell, 1988).

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 digital platform weighing scale to the nearest 100gm was used to measure weight. The subject was weighed in standard office 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. Calibration values were maintained.

Height

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

Technique- Stedometer was used to measure the height of the subjects. A convenient flat wall was identified at the bank 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 Frankfort 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.

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). (Table 4.4)

Technique- The subject was made to stand erect with the abdomen relaxed and the arms at the sides. The circumference was recorded using the Flexi 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.

Table 4.4: Cut offs for waist circumference

Gender	**Waist** circumference **(cm)**	Abdominal o**besity**
Female	≤80	Absent
	≥80	Present
Male	≤90	Absent
	≥90	Present

Source: (World Health Organization, 2008)

Hip Circumference

Technique- Hip circumference was measured at the point yielding the maximum circumference over the buttocks. The Flexi 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.

4.3.7.1: Computed Anthropometric Indices

Body Mass Index (BMI)

The BMI is a convenient and valid measure of adiposity and found to be positively correlated with blood pressure and plasma lipid concentrations and therefore with the risk of cardiovascular disease (Brown et al., 2000).

It is computed as- $$\text{BMI (kg/m}^2\text{)} = \frac{\text{Weight (kg)}}{\text{Height (m}^2\text{)}}$$

BMI Cut offs

A relatively new classification of BMI has been recommended by WHO for the Asians. Under this, a BMI of more than 25 kg/m^2 is considered obese for Asian Indians in contrast to 30 for other population (Misra et al., 2009, World Health Organization, 2000). (Table 4.5)

Table 4.5: Revised BMI Cut Offs for Asian adults:

BMI (kg/m^2)	**Classification**	**Risk of Comorbidities**
≤18.5	Underweight	Low (but risk of other clinical complications)
18.5–22.9	Normal	Increased but acceptable risk
23.0–24.9	Overweight	At Risk-increased
25.0–29.9	Obesity grade I	Moderate
≥ 30.0	Obesity grade II	Severe

Source: Adapted from (World Health Organization, 1995, 2000, 2004; Misra et al., 2009)

Waist- Hip Ratio (WHR)

This ratio gives an idea of central adiposity. High WHR often indicates an atherogenic lipid profile that tremendously enhances the cardiovascular risk (Suk et al., 2003). (Table 4.6) It is computed as-

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

Table 4.6: Cut offs for waist–hip ratio (WHR)

Gender	**WHR**	At risk
Female	≤0.85	No
	≥0.85	Yes
Male	≤0.90	No
	≥0.90	Yes

Source: (World Health Organization, 2008)

4.3.8: Biophysical Investigations

Blood Pressure

The diastolic and systolic blood pressures were assessed, as Hypertension is the major risk factor of CVD. Blood pressure is the lateral pressure exerted by blood on vessel walls while flowing (Forrester, Diamond, Chatterjee, and Swan, 1976). Sitting blood pressure of subjects was measured using the AND Digital Blood Pressure Monitor UA-767PC (Saitama, Japan) on the right arm. (Table 4.7)

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 position 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 >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., 2003).

Table 4.7: Classification of blood pressure for adults

Category	Systolic, MmHg	Diastolic, MmHg
Hypotension	<90	<60
Desired	90–119	60–79
Pre-Hypertension	120–139	80–89
Stage 1 Hypertension	140–159	90–99
Stage 2 Hypertension	160–179	100–109
Hypertensive Emergency	≥180	≥110

Source: "Understanding blood pressure readings", American Heart Association, January 2011

Body composition analysis

Digital body fat monitor (Omron healthcare co. ltd Japan; model no. HBF–306-C1; SN: 2010100047IUF) was used to measure Body fat percentage, Basal metabolic rate and Body mass index. It also displayed graphical interpretation of body types like lean, lean normal, muscular, latent obesity and obese.

Technique: Place hands around grips with middle finger resting in the depression. Correctly position thumbs on top of grip (thumbs up). Adhere your palms firmly to both upper and lower electrodes. Switch the start button on and hold till the results are displayed. (Table 4.8)

Table 4.8: Classification of percent body fat

Discription	% **Body Fat (Male)**	% **Body Fat (Female)**
Essential fat	2-5 %	10–13%
Athletes	6–13%	14–20%
Fitness	14-17%	21-24%
Acceptable	18-24%	25-31%
Obese	≥25%	≥32%

Source: (Muth, 2009)

4.3.9: Biochemical analysis and assay method

Estimation of Lipemic parameters (For Phase III)

Subjects were asked to fast overnight, than venous blood sample was collected in clean, sterilized yellow cap vacutainers and allowed to stand at room temperature for 15 minutes. Serum was separated from total blood and immediately stored in dry ice until analysis. The blood was then analyzed for Total Lipid Profile using standardized kits at City Lab, Vadodara.

Total cholesterol (TC) was estimated using end point enzymatic colorimetric technique (Richmond, 1973). Enzymatic colorimetric method (GPO/PAP) with glycerol phosphate oxidase and 4-aminophenazone was used to assess triglycerides (Fossati and Prencipe, 1982). HDL, LDL and VLDL fraction of

cholesterol was determined using enzymatic, colorimetric method (CHOD/PAP) without sample pretreatment (Sugiuchi et al., 1995).

Total Cholesterol (TC)

Total cholesterol was estimated using end point enzymatic colorimetric technique. Cholesterol esters are cleared 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, 1973b). (Table 4.9)

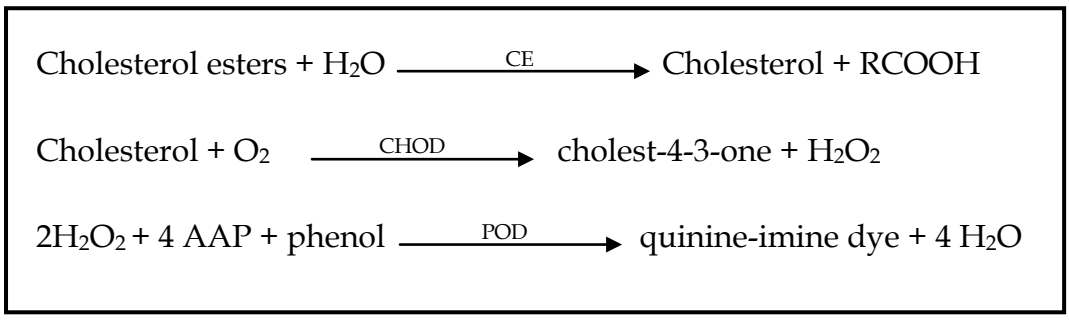

Table 4.9: Cut offs for serum cholesterol

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

Source: (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001)

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). (Table 4.10)

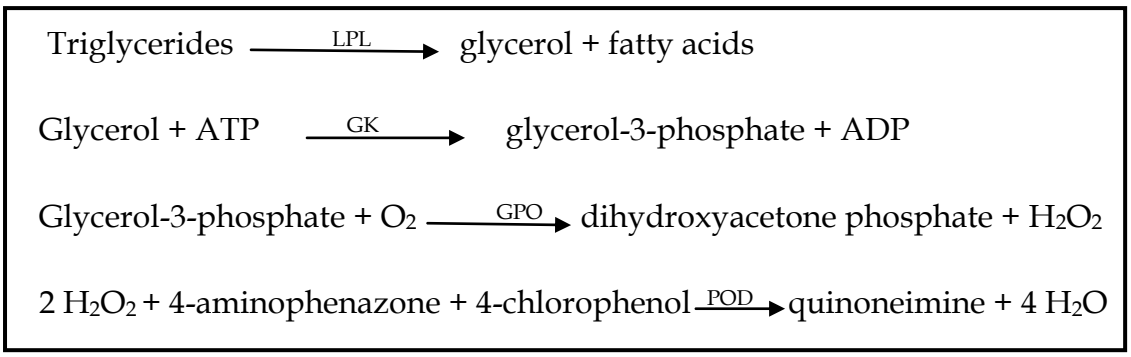

Table 4.10: Cut offs for Triglycerides

Classification	Triglycerides Value (mg/dl)
Optimal	≤100
Normal	101-150
Borderline	150-199
High	200-499
Very High	≥500

Source: (Miller et al., 2011)

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 et al., 1995). (Table 4.11)

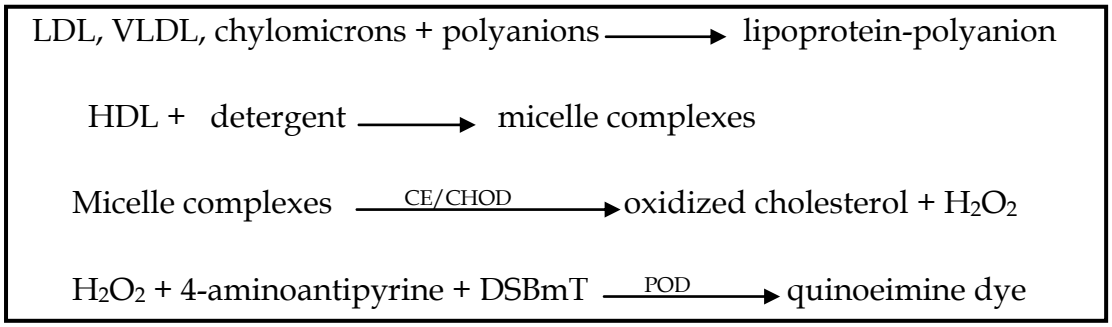

Table 4.11.: Cut offs for HDL-Cholesterol

Classification	HDL Cholesterol Value (mg/dl)
Low	**≤40**
Optimal	**41-59**
High	**≥60**

Source: (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001)

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 et al., 1995). (Table 4.12)

Step 1: LDL, VLDL, chylomicrons + detergents $\longrightarrow$ 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 $\longrightarrow$ released cholesterol

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

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

Table 4.12.: 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**

Source: (Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2001)

Determination of plasma lipopolysaccharide (LPS)

Lipopolysaccharides (LPS), also known as lipoglycans, are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals.

Subjects were asked to fast overnight; than venous blood sample was collected in clean, sterilized EDTA-treated lavender cap vacutainers and immediately centrifuged at 10000 rpm for 2 min. Plasma was separated from total blood and immediately kept in dry ice until stored at -20°C, the sample was centrifuged again after thawing before the assay. Grossly hemolyzed samples are not suitable for use in this assay. (Table 4.13)

Principle of the assay

The microtiter plate provided in the kit **(Cusabio, Catalog No. CSB-E09945h**) has been pre-coated with an antibody specific to LPS. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for LPS and Avidin conjugated to

Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain LPS, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of LPS in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Detection range

6.25 pg/ml-400 pg/ml. The standard curve concentrations used for the ELISA's were 400 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml, 25 pg/ml, 12.5 pg/ml, 6.25 pg/ml.

Specificity

This assay recognizes recombinant and natural human LPS. No significant cross-reactivity or interference was observed.

Sensitivity

The minimum detectable dose of human LPS is typically less than 1.56 pg/ml. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.

Storage

1. Unopened test kit was stored at 2-8°C upon receipt and the microtiter plate was kept in a sealed bag.
2. Opened test plate was stored at 2-8°C in the aluminum foil bag with desiccants to minimize exposure to damp air.
3. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 OD or greater at 450nm wavelength is acceptable for use in absorbance measurement.

Table 4.13.: Materials provided in the kit

Reagent	Quantity
Assay plate	1
Standard	2
Sample diluent	1 x 20 ml
Biotin-antibody diluent	1 x 10 ml
HRP-avidin diluent	1 x 10 ml
Biotin-antibody	1 x 120µl
HRP-avidin	1 x 120µl
Wash buffer	1 x 20 ml(25×concentrate)
TMB substrate	1 x 10 ml
Stop solution	1 x 10 ml

Reagent preparation

Bring all reagents to room temperature before use.

1. **Wash Buffer: (**If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved) 20 ml of Wash Buffer Concentrate was diluted into deionized or distilled water to prepare 500 ml of Wash Buffer.

2. **Standard**: The standard vial was centrifuged at 6000-10000rpm for 30s. Reconstitute the Standard with 1.0 ml of Sample Diluent. This reconstitution produces a stock solution of 400 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (400 pg/ml). The Sample Diluent serves as the zero standard (0 pg/ml). Fresh standard was prepared for each assay. All the standards were used within 4 hours and were discarded after use.

3. **Biotin-antibody:** The vial was centrifuged before opening and the diluted to the working concentration using **Biotin-antibody Diluent**(1:100), respectively.

4. **HRP-avidin**: The vial was centrifuged before opening and diluted to the working concentration using **HRP-avidin Diluent**(1:100), respectively.

Other supplies required

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm. Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.

Assay procedure

All reagents and samples were brought to room temperature before use. All samples, standards, and controls were assayed in duplicate. All the reagents were added directly to the liquid level in the well. It was made sure that the pipette should avoid contacting the inner wall of the well.

1. Hundred µl of Standard, Blank, or Sample was added per well. It was covered with the adhesive strip and Incubated for 2 hours at 37°C.
2. The liquid of each well was removed (Note: don't wash).
3. Hundred µl of Biotin-antibody working solution was added to each well and Incubated for 1 hour at 37°C (Biotin-antibody working solution may appear cloudy). It was the warm up to room temperature and mixed gently until solution appears uniform.
4. Each well was aspirated and washed, the process was repeated three times for a total of three washes. Wash: Each well was filled with Wash Buffer (200µl) and was allowed to stand for 2 minutes, and then liquid was removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel (Note: Complete removal of liquid at each step is essential to good performance).

5. Hundred µl of HRP-avidin working solution was added to each well. Microtiter plate was covered with a new adhesive strip and Incubated for 1 hour at 37°C.

6. Ninety µl of TMB Substrate was added to each well and Incubated for 10-30 minutes at 37°C. The plate was kept away from drafts and other temperature fluctuations in the dark.

7. Fifty µl of Stop Solution was added to each well when the first four wells containing the highest concentration of standards develop obvious blue color (Note: If color change does not appear uniform, gently tap the plate to ensure thorough mixing).

8. Optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

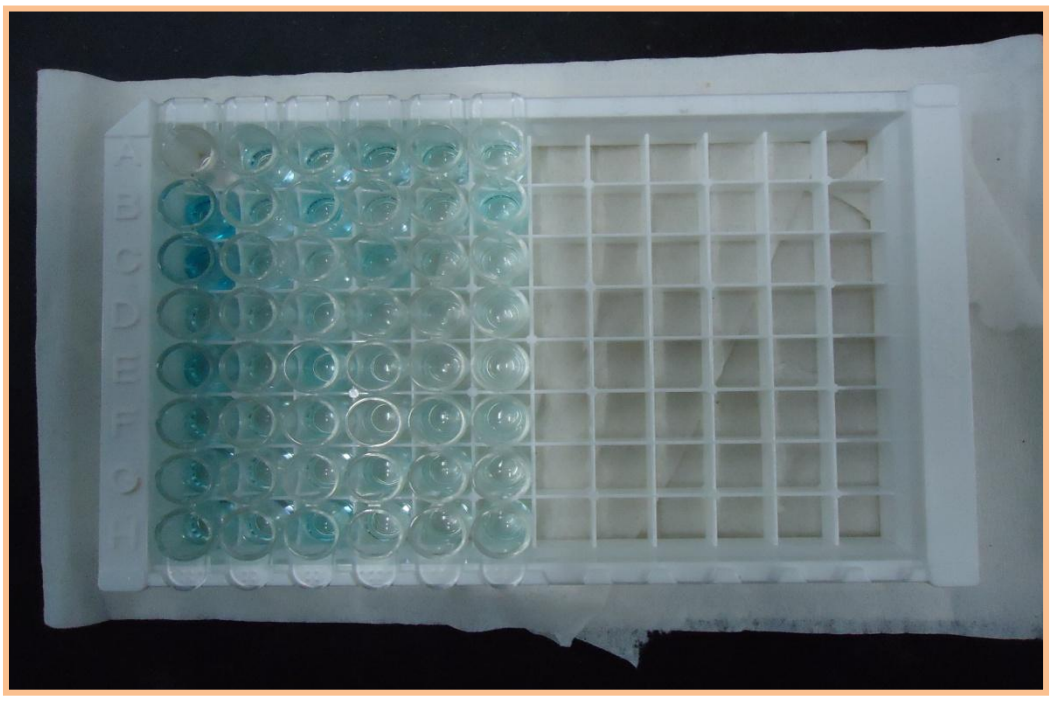

Plate 4.2: 96 well assay plate with samples

Calculation of results

The professional soft "Curve Exert 1.3" to make a standard curve was used. Average of duplicate readings for each standard, control, and sample was calculated and the subtracted from the average zero standard optical density.

A standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, a standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the LPS concentrations versus the log of the O.D. and the best fit line was determined by regression analysis (Note: If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor).

Limitations of the procedure

- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Standard Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Standard Diluent and repeat the assay.
- Any variation in Standard Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

Technical hints

- Centrifuge vials before opening to collect contents.
- When mixing or reconstituting protein solutions, always avoid foaming.

- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless or light blue until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless or light blue to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

4.3.10: Determination of gut microflora

Parameters analysed: Gut microflora was determined with respect to species of *Bifidobacteria, Lactobacillus, Bacteriodes,* and *Clostridium* from the stool samples of the subjects.

a) Collection of Sample: Stool samples were collected in sterile container directly placed in dry ice. All samples were collected fresh and thereafter carried to the department's microbiology laboratory. The samples were processed immediately within 4 hours of collection.

b) Sterilization of 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.

c) **Preparation of the sample:** One gram of fecal sample was weighed in a vacuum weighing balance and was used for the estimation.

d) **Homogenization and Dilution:** For the preparation of dilution blanks 1 g of peptone was dissolved in 1000 ml of distilled water (0.1% w/v peptone and 0.005% w/v of NaCl). 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. One gram of stool sample was added in Stomacher bag. To this 99ml of peptone water was added. Homogenization was done using a Stomacher Blender (Bag Mixer 400 VW) at 200 rpm for 1 minute.

e) **Serial Dilution :** Ten-fold Serial dilution was performed from 10^{-2} to 10^{-12} (WHO/FAO 1979)

f) **Preparation and sterilization of media:**

Lactobacillus **-** Hi Media MRS Agar M 641 (67.15 g in 1000 ml distilled water).

Bifidobacterium – Hi Media Bifidobacterium Agar M 1396 (49.3 g in 1000 ml distilled water).

Clostridium – Hi Media Anaerobic Agar M 228 (58 g in 1000 ml distilled water).

Bacteriodes – Hi Media Anaerobic Basal Agar M 1635 (45.9 g in 1000 ml distilled water).

The prepared media was autoclaved at 121^0C for 15 minutes and then poured into sterile petri plates and was allowed to set.

g) **Plating:** 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.

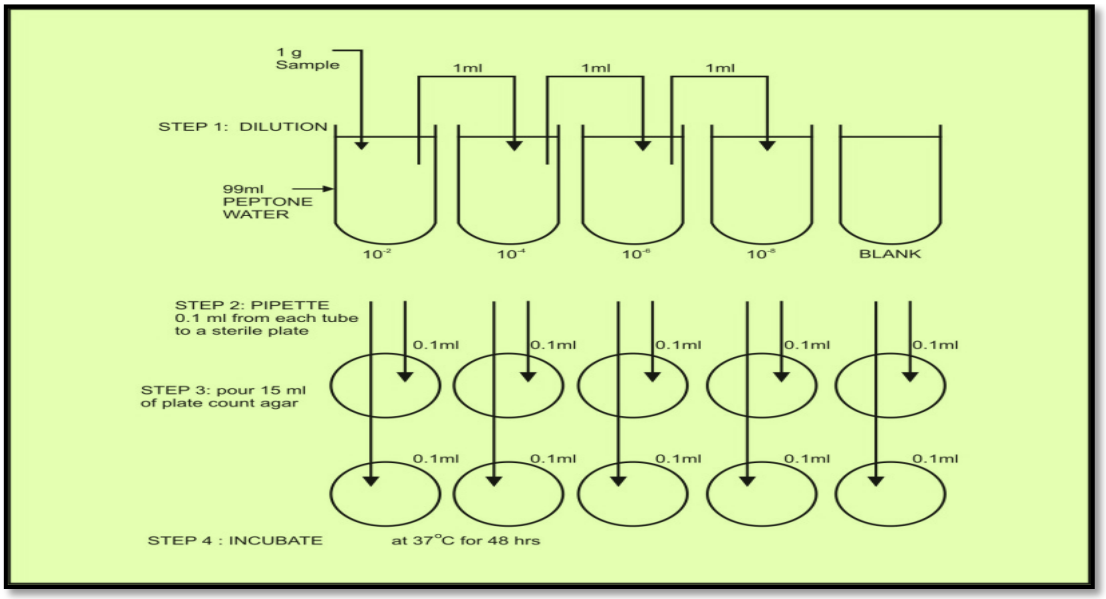

Fig. 4.3: Serial Dilution technique along with Steps of Inoculation and Incubation

h) Incubation: Anaerobic Jars were used for the petriplates of obligate anaerobes like *Bifidobacterium, Clostridium, and Bacteriode.* Gas pack was opened up and kept in the anaerobic jars. The Plates of *Lactobacillus were* kept in the Dessicator. All plates were kept for Incubation at 37^0 C for 48 – 72 hours.

i) Counting and Recording of data: After 48 hrs. of incubation colonies were counted using a digital colony counter and data was recorded. 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).

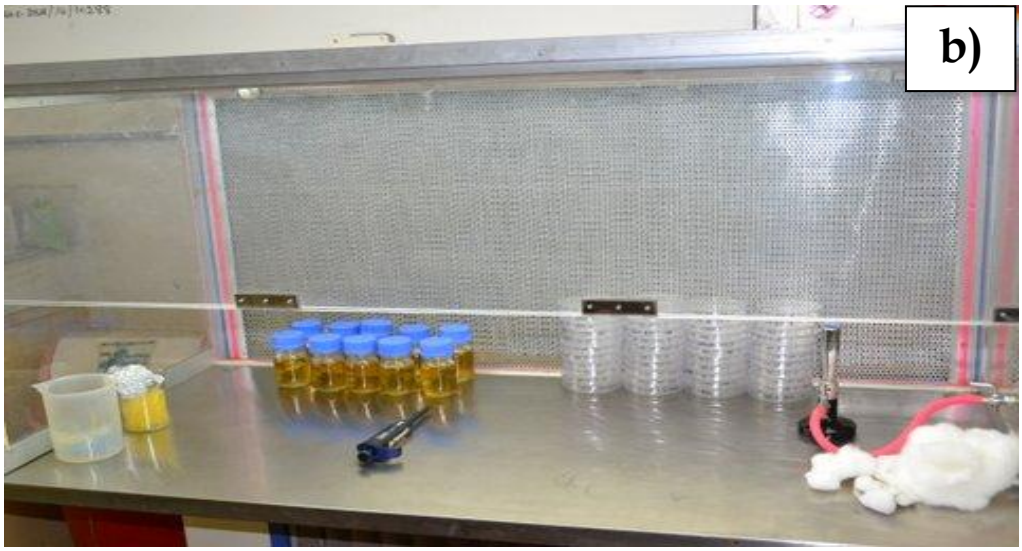

Plate 4.3: a) sampling in the laminar flow; b) Serial dilution in laminar flow

4.3.11: Statistical Analysis

The data was entered in an excel spreadsheet. The data was cleaned and verified and subjected to appropriate statistical analysis. Statistical analysis was performed using Microsoft excel 2007. Results were expressed as mean values ± standard deviations. Student *t* test and X^2 test was performed for the comparison between non-obese and obese for the various biochemical,

microbial and anthropometric parameters. The significance levels were set at 5% by two sided tests.

Pearson correlation and multivariate linear regression was calculated using the Statistical Package for the Social Sciences (SPSS 17.0 version, SPSS Inc.,Chicago, IL,USA) and to understand relations amongst various parameters with BMI.

Phase IV

Effect of Fructooligosaccharide (FOS) supplementation on anthropometry profile, blood pressure, defecation profile, hunger and satiety, psychological depression, dietary intakes, lipemic parameters, plasma LPS levels and gut microbiota (*LAB, bifidobacteria, bacteroides and clostridium*) in obese grade-I adults.

This section of the study was designed to evaluate the impact of FOS supplementation on obese (grade-I) young adults of urban Vadodara. For achieving the desired objectives, a total of 116 obese subjects were enrolled and randomly divided in two groups i.e. experimental and placebo groups which received FOS (20 g) and dextrose (20 g) respectively for 90 days and examined the effect of daily intake of FOS on anthropometry profile, blood pressure, defecation profile, hunger and satiety, psychological depression, dietary intakes, lipemic parameters, plasma LPS levels and gut microbiota (*LAB, bifidobacteria, bacteroides and clostridium*). Post intervention the sample size remained as 51 in experimental group and 32 in placebo group, after considering the dropouts due to various reasons. Experimental design of the study is depicted in Figure 4.4.1

4.4.1 Selection of the subjects for the study

A total of 116 obese subjects were enrolled and randomly divided with the help of random tables in two groups i.e. experimental and placebo groups which received FOS (20 g) and dextrose (20 g) respectively for 90 days.

Subjects were briefed on the objective and benefits of the study, and were motivated to participate by providing an informed consent. Proper record of the eligible members who refused to participate in the study was maintained.

4.4.2 Inclusion and exclusion criteria for selection of the subjects

Inclusion criteria for selection of the subjects

- BMI between 25-29.9 kg/m^2 (For obese; n=100)
- Age 25 - 35 years old
- Willingness to participate in the study

Exclusion criteria for selection of the bank employees

- Subjects with following confirmed disorders were excluded from the study.
  - ✓ Hypertension
  - ✓ Diabetes mellitus
  - ✓ Cardiovascular disorder
  - ✓ Thyroid hormone disorder
  - ✓ Renal disorder
  - ✓ Locomotor disorder
  - ✓ Cancer
  - ✓ AIDS
  - ✓ Psychological disorder
- Subjects who are likely to undergo surgery during study period
- Show allergic symptoms or discomfort to study food
- Subjects who have participated in any investigational study in last 4 weeks
- Subjects who followed any weight loss regime (Gymming, dieting, or taking any other nutraceuticals)
- Subjects who were taken any kind of anti-biotic drugs.

4.4.3 Study Protocol

A total of 116 obese subjects were enrolled and randomly divided with the help of random tables in two groups i.e. experimental and placebo groups which received FOS (20 g) and dextrose (20 g) respectively for 90 days. Experimental design for this phase is given in Figure 4.4.

Information regarding dietary intake, defecation profile depression profile, hunger and satiety score were collected using pretested semi structured questionnaire before and after intervention. Anthropometric profile, biophysical, biochemical and microbial parameters were also assessed before and after intervention. Dietary analysis was done using 24hr dietary recall. Assessment of the subjects was carried out with respect to lipid parameters, and plasma LPS levels and gut microbial counts levels in terms of *lactobacilli, bifidobacteria, clostridium* and *bacteriodes* before and after intervention.

Informed consent from the subjects was obtained prior to supplementation. The subjects were asked to consume 20 g of supplementation for 90 days along with their meals. Subjects were asked 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, clostridium* and *bacteriodes* before and after intervention and blood samples were collected in the fasted state lipid analysis and plasma LPS analysis. 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. 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.

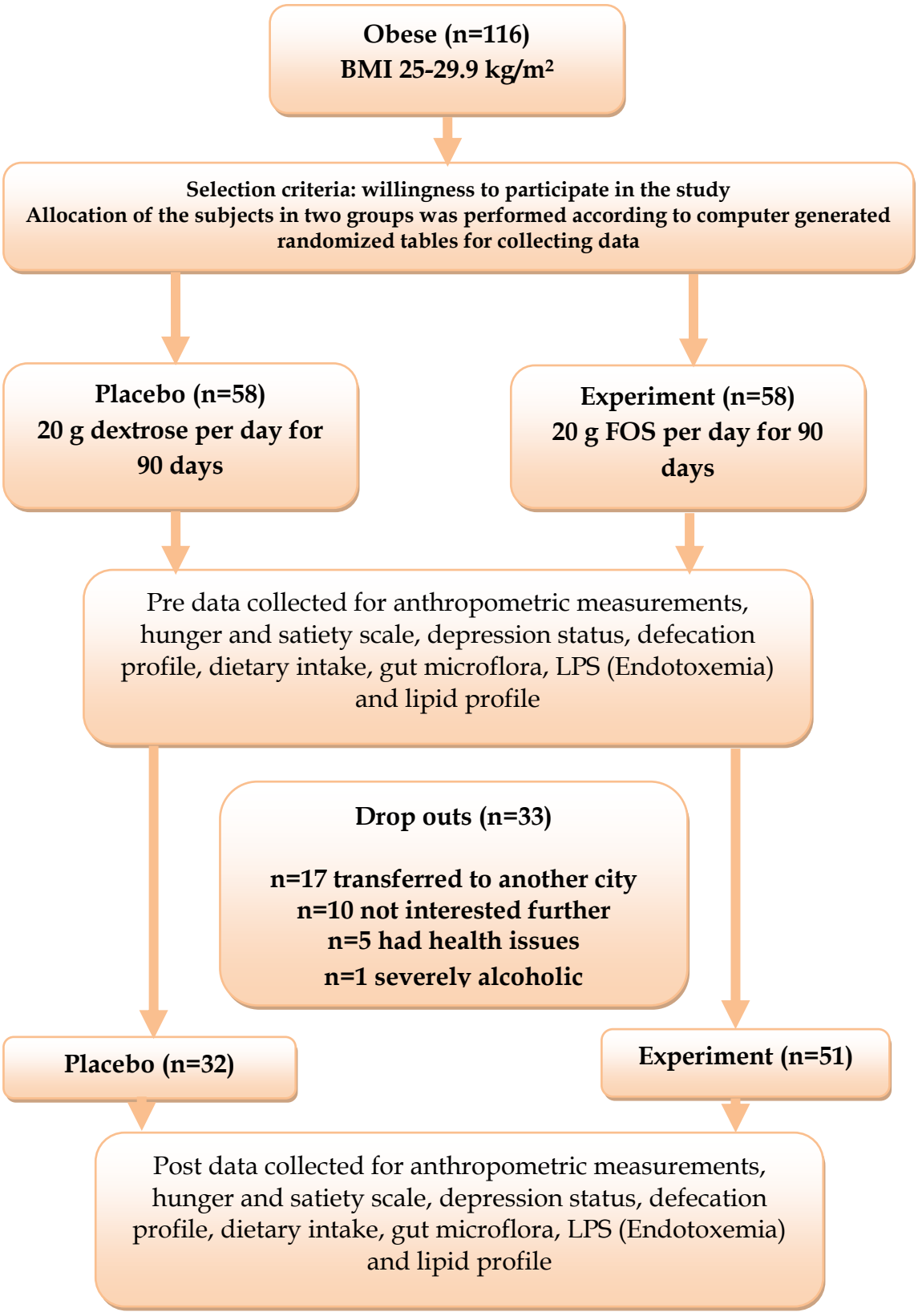

Figure 4.4: Experimental design of Phase IV of the study

4.4.4 Study food and mode of intervention

a) Intervention agent: Fructooligosaccharide - Dose: 20 gms for 3 months.
b) Comparator agent: Dextrose – Dose: 20 gms for 3 months

Adverse effects of supplementing FOS

FOS is "Generally Recognized As Safe". FOS, ingested at up to 20g/day in adults, appears to be safe and well tolerated (WHO, 2000).

The FOS and dextrose was given in powder form packed in two sachets, 10 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), dal* or water and consume along with the meals.

4.4.5 Methods opted for randomized trials

Method of generating randomization sequence:

Computer generated randomization

Method of allocation concealment:

Centralized

Who generated the allocation sequence:

Staff from department, not involved in the study

Who enrolled participants:

Investigators

Who assigned participants to their groups:

Random allocation using computer generated randomization numbers

Blinding/Masking:

Participant, investigator and outcome assessor blinded

4.4.6 Trial monitoring plan

The FOS and placebo (Dextrose) were supplemented to the experimental and control group respectively in powder form in sachet of 10 g (2sachets/day). Sachets were given on weekly basis, total 14 sachets were provided to one subject in one week. A compliance card was prepared for each subject and each subject had asked to tick (√) in the card on daily basis after consuming 2 sachets and the investigator visited once in a week for checking the compliance and collection of empty sachets. After 3 months all ticks (√) were counted and compliance was calculated.

Compliance Card

Name: _____

Subject Code: _____

Period: _____

1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

- Please make a circle on each day on which you consumed the given sachets.
- Please return empty and unconsumed sachets.

Figure 4.5: compliance card for the subjects

4.4.7 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 Microsoft excel 2007 and 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. The significance levels were set at 5% by two sided tests. Student *t* test was performed for the comparison between control and experimental group.

4.4.8 Details of drop outs

There were total 33 drop outs during the study period. Out of 33, 7 were from experimental group and 26 were from placebo group in which 17 subjects were transferred to another city, 10 subjects lost their interest further, 5 subjects had some health issues and one was dropped out due to severely alcoholic.