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 increasing satiety could help to assist obesity control. The present study was undertaken to study the **"Sensory evaluation of Fructooligosaccharide (FOS) added foods and its impact on gut health and biochemical parameters in obese industrial employees of rural Vadodara"**. This chapter outlines the experimental design and discusses the methods and materials used to fulfill the objectives of the study in three phases.

Phases in study

Phase I: Sensory evaluation of FOS added popular Indian food products.

Phase II: Comparative analysis of obese and normal weight subjects of an industry for their anthropometric parameters, nutrient intake, fecal gut microflora, GLP-1, LPS, hunger and satiety.

Phase III: Anthropometric and metabolic responses of obese subjects to supplementation of FOS.

PHASE I

Sensory evaluation of FOS added popular Indian food products

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Comparative analysis of obese and normal weight subjects of an industry for their anthropometric parameters, nutrient intake, fecal gut microflora, GLP-1, LPS, hunger and satiety

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PHASE III

Anthropometric and metabolic responses of obese subjects to supplementation of FOS

4.3.1 Study food and mode of intervention

4.3.2 Statistical Analysis

PHASE I

Sensory evaluation of FOS added popular Indian food products

This section of the study was conducted to assess the acceptability of FOS added food products *viz.* Buttermilk*,* Lemon juice*,* Milk*,* tomato Soup*, Potato curry, Dal, Kadi, Kheer* and *Khichdi* at varying level of FOS addition.

4.1.1 Procurement of Fructooligosaccharide (FOS)

Food grade Fructooligosaccharide (Beneo Raftiline P95 BAG 25 kg, Orafti, Belgium) was procured in a packet of 25 kgs from Brenntag Ingredients India Pvt. Ltd. The

fructooligosaccharide was of food grade which can be used as fat and sugar replacer. The specifications of the fructooligosaccharide are given in Table 4.1.1.

Table 4.1.1: Specification of the Fructooligosaccharide used for addition

Specifications	Analysis	Values	Range
Orafti Physical/chemical. 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

4.1.2 Selection of food products

Total of nine products were selected *viz.* Buttermilk, Lemon juice, Milk, tomato Soup, *Potato curry, Dal, Kadi, Kheer* and *Khichdi* since these food products are the most commonly consumed in Gujarat region, they were considered as a vehicle for FOS addition at five levels 2.5%, 4%, 5%, 6% and 7.5% All the products were studied for organoleptic characteristics experimental design given in Figure 4.1.1.

4.1.3 Procurement of raw ingredients

Ingredients and materials that were needed to develop FOS added products were collected from the local market of Vadodara, Gujarat. 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
Buttermilk*	buttermilk	Sugam (salted jeera chas)
Lemon juice*	Lemon Sugar water	Local market, Vadodara Local market, Vadodara
Milk*	Milk Sugar	Amul (skimmed milk) Local market, Vadodara
Soup*	*Tomato* *Onion* *Potato* *Garlic, Ginger, Green chilly* *Sugar, Salt* *Water*	Local market, Vadodara Local market, Vadodara Local market, Vadodara Local market, Vadodara Local market, Vadodara Local market, Vadodara
Potato curry*	Potato Tomato Ginger, Green chili Coriander leaves Oil Salt, spices Water	Local market, Vadodara Local market, Vadodara Local market, Vadodara Local market, Vadodara Fortune (groundnut oil) Local market, Vadodara
Dal*	Red gram *Dal* Ghee Salt, spices Water	Rajdhani brand, Vadodara Amul (cow) Local market, Vadodara
Kadi*	Bengal gram powder Chas Oil Salt, Spices	Uttam brand, Vadodara Sugam brand, Vadodara Fortune (groundnut) Local market, Vadodara
Kheer*	Milk Rice Sugar Cardamom powder, nuts (almonds, raisins) Water	Amul (gold) India Gate basmati, Vadodara Local market, Vadodara Local market, Vadodara
Khichdi*	Red gram *Dal* Rice Tomato Ghee Salt, Spices Water	Local market, Vadodara India Gate basmati, Vadodara Local market, Vadodara Amul (cow) Local market, Vadodara

4.1.4 Standardization and addition of fructooligosaccharide to the following products:

Standardisation is the process of implementing and developing technical standards. Formulation and implementation of guidelines, rules, and specifications for common and repeated use, aimed at achieving optimum degree of order or uniformity in a given context, discipline, or field.

The following recipes (Buttermilk, Lemon juice, Milk, tomato Soup, *Potato curry, Dal, Kadi, Kheer* and *Khichdi)* were standardized this was followed by addition of FOS at the level of 2.5%, 4%, 5%, 6%, 7.5% (Pasricha and Rebello 1998).

a) Procurement of Butter Milk

Two hundred grams of standard Sugam salted Jeera butter milk was procured freshly from the dairy and was refrigerated at 7^0 C until use (Plate 4.1.1a).

b) Standardization of Lemon juice

Standard lemon juice was prepared by adding 90 ml of fresh lime juice and 120 g of sugar to 1050 ml of water (Plate 4.1.1b).

lemon juice	
Ingredients	**amount**
Water	180g
Lemon juice	24g
Sugar	10g

c) Procurement of Milk

Two hundred grams of pasteurized Amul (Taza) skimmed milk was procured freshly from the dairy and was refrigerated at 7^{o}C until use, sugar was added at 15% (Plate 4.1.1c).

d) Standardization of tomato Soup

Standard tomato soup was prepared using 600 g of tomatoes, 600 ml water, 125g onion, 100 g potato, 4 cloves of garlic, 10 g ginger, 1 green chili, 5 g sugar and salt to taste. All the ingredients were together pressure cooked for10 min at 95^{o}C and then

cooled, blended and sieved. The soup thus obtained was then boiled up to 3 min (Plate 4.1.1d).

Tomato soup/ serving (200g)	
Ingredients	**amount**
Tomato	120g
Water	110
Garlic	1 clove
Ginger	2g
Green chilly	1/2
Potato	16g
Onion	20g
Sugar	2g
Salt	To taste

e) Standardization of *Potato curry*

Standard *Potato curry* was prepared using 300 g of potatoes, 250 g of tomatoes, 5 g of ginger, 1 green chili, coriander leaves, asafetida, cumin seeds and salt to taste. Cut and fry tomatoes in oil and spices till they form thick gravy now add peeled sliced potatoes. All the ingredients were together pressure cooked for 15 min at 95° C (Plate 4.1.1e).

***Potato curry*/serving (200g)**	
Ingredients	**amount**
Potato	80g
Tomato	70g
Ginger	2g
Green chilly	1/2
Water	100
Oil	5g
Cumin seeds	Half pinch
Asafetida	Half pinch
Salt	To taste

f) Standardization of *Dal*

Standard *Dal* was prepared by pressure cooking 250 g of bengal gram *Dal* that contain 1L water, turmeric powder and salt for 15 minutes, *Dal* was seasoned by the addition of cumin seed, red chili powder and asafetida to 15 ml ghee (Plate 4.1.1f).

Dal/serving (200g)	
Ingredients	**amount**
Toor *Dal*	40g
Water	160g
Oil	5g
Turmeric powder	1/2tsp
Salt	To taste
Cumin seeds	Half pinch
Asafetida	Half pinch

g) Standardization of *kadi*

Standard *Kadi* was prepared by adding 20 g besan to 1000 ml chas; this was added to seasoning prepared from 30 ml of oil, 2g asafetida, 2g turmeric and 4g chili powder. The mixture was continuously stirred till the boiling on slow flame (Plate 4.1.1g).

Kadi/serving (200g)	
Ingredients	**amount**
Buttermilk	250g
Besan	7g
Oil	5g
Salt	to taste
Fenugreek seeds	0.5g
Mustard seeds	0.5g
Cumin seeds	0.5g
Asafetida	1 pinch
Chili powder	1/2tsp

h) Standardization of *Kheer*

To 1100 ml pasteurized full crème (Amul Gold) milk, 55 g of rice and 100 ml water was added and boiled for 30 min an a slow flame. To this 30 g of sugar, cardamom powder 3g, 20 g of almonds and 10g raisins was added thus the standard *Kheer* was prepared (Plate 4.1.1h).

Kheer/ serving (200g)	
Ingredients	**amount**
Milk	340g
Rice	16g
Water	40g
Sugar	25g
Cardamom powder	1-2 pinches
Chopped almonds	10g
Raisins	6g

i) Standardization of *Khichdi*

Standard *Khichdi* was prepared using 50g rice, 50 g of red gram *Dal*, 25g of tomatoes, 10g ghee, 2g red chili powder, 2g turmeric powder, 2g of cumin seeds and salt to taste. Cut and fry tomatoes in ghee and spices till they become soft, to this add washed rice, *Dal* and 400 ml water. All the ingredients were together pressure cooked for 15 min at 95^{o} C (Plate 4.1.1i).

Khichdi/serving (200g)	
Ingredients	**amount**
Toor *Dal*	25g
Rice	25g
Water	150g
Ghee	5 g
Salt	to taste
Turmeric	two pinches
Red chili powder	two pinches
Cumin seeds	two pinches

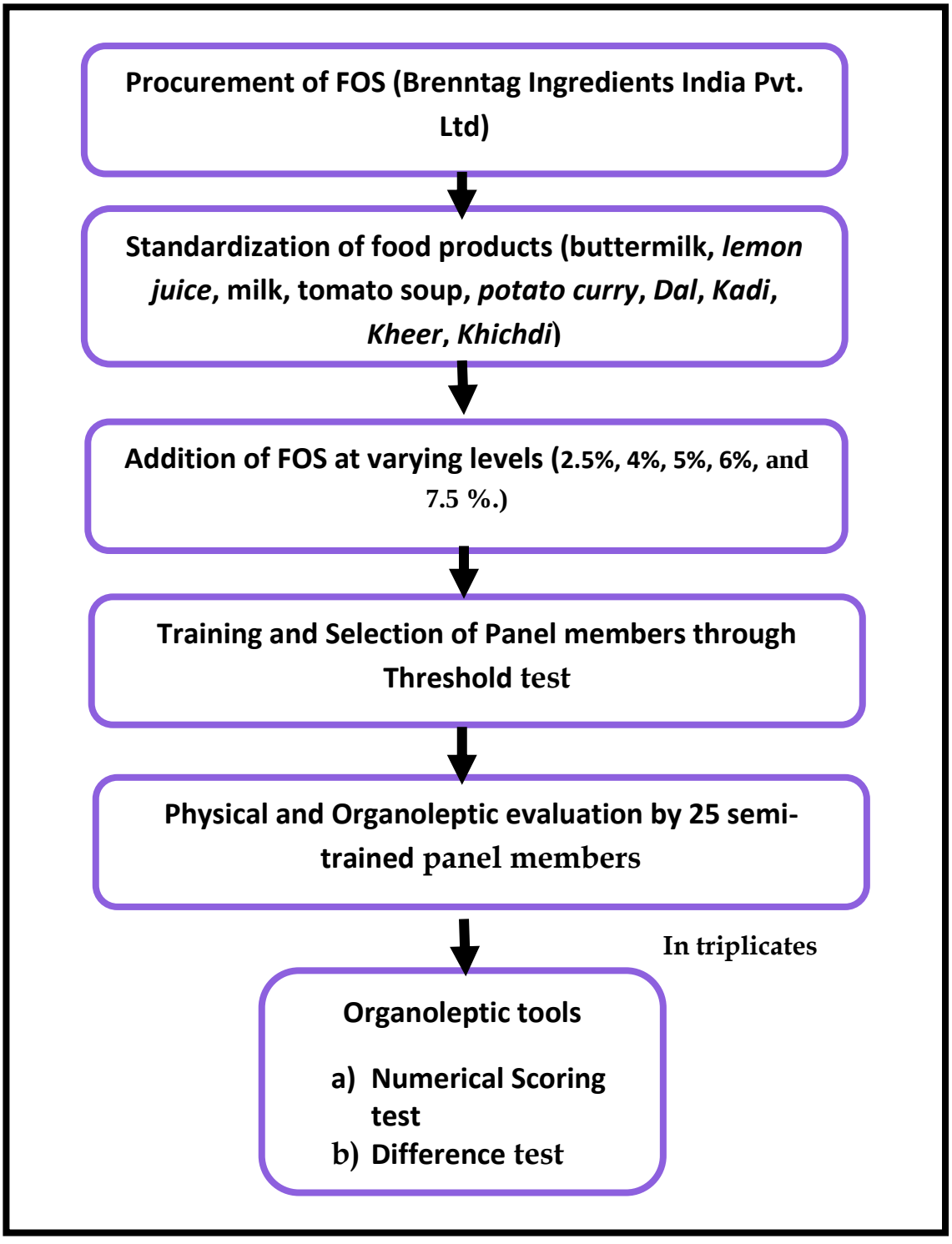

Figure 4.1.1: Flowchart indicating experimental design of Phase I

4.1.5 Selection and training of judges for organoleptic evaluation

a) Screening of Panelists: In this section, 25 panel members were selected from the Department of Foods and Nutrition, Faculty of Family and Community Science, The Maharaja Sayajirao University of Vadodara, Gujarat 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. Score card for the same was formulated and pre tested (Appendix I (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 which were arranged in random order. The participants were required 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 panellists. Subjects who qualify the threshold test were included further in evaluating the physical and organoleptic characteristics of the food products.

c) Training of the selected panel members: For imparting the basic knowledge and understanding of visual and organoleptic characteristics of the products a training tool was developed. 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 II).

4.1.6 Tools for organoleptic evaluation

Organoleptic evaluation tools selected were

a) Hedonic rating scale and
b) Difference test (ISI 1972)

a) Hedonic rating scale: Application of this test was done for organoleptic and visual evaluation. Panelists were asked to evaluate and score essential attributes that were needed to be scored like color and appearance, taste, after taste, consistency and overall acceptability. The maximum score of each

attribute was 10. These tests were done to assess each attribute of all the nine products for all the samples.

b) **Difference Test**: This test was performed to measure the effect of process (addition of FOS) change on quality of the product. 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. Score cards were given where the panelist were asked to rate FOS added products as compared with the standard products for color and appearance, taste, after taste, consistency and overall acceptability in term of equal, superior, inferior.

Plate 4.1.1 (a-i): FOS added buttermilk, lemon juice, milk, tomato soup, *Potato curry*, *Dal*, *Kadi*, *Kheer*, *and Khichdi*

4.1.7 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel (2007) software. Results were expressed as mean values ± standard deviations of all the nine Indian recipes. ANOVA was performed to determine the significant differences in various levels of FOS added food products. Chi square was used to determine the extent of differences that existed amongst the varying level of FOS added products in terms of being equal, superior or inferior to the standard products.

PHASE II

Comparative analysis of obese and normal weight subjects of an industry for their anthropometric parameters, nutrient intake, fecal gut microflora, GLP-1, LPS, hunger and satiety

In phase II, 273 total working population, 110 subjects (38 normal weight adults with BMI 18.5-22.9 kg/$m^2$and 72 were obese adults with BMI 25-30 kg/m^2 aged between 25-50 years) were screened and selected from Larsen and Toubro plant, Ranoli, Vadodara based on inclusive and exclusive criteria. Purposive sampling method was done to enroll subjects. Of these 110, 30 normal weight and 60 obese gave consent for their participation and intervention trial. Relevant data was obtained through patient medical records, face to face interview and direct measurements like anthropometric measurements, physical activity levels, dietary profile, biochemical analysis like glucagon like peptide-1 (GLP-1), lipopolysaccharide (LPS) and fecal gut microflora in terms of *Lactic acid bacteria, Bifidobacteria* and Enteric pathogen.

4.2.1 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/55). Duly filled informed consent (Appendix VII) was obtained from the participants prior to the enrollment, who agreed to give baseline information through questionnaire and give sample of blood and stool for biochemical and microbiological analysis respectively.

4.2.2 Selection of the subjects for the study

A total of 95 adults aged between 25-50 years were selected, out of which 30 were having normal weight (BMI between 18.5 – 22.9 kg/m^2) and 65were obese (BMI ≥ 25-30 kg/m2) from Larsen and Toubro plant, Ranoli, Vadodara. 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 during the study period should remain unaltered.

Drop outs:

There were total 5 drop outs during the study period all of them were from experimental group. Two drop outs were due to non-compliance of study protocol, 2 went out of station and 1 started doing exercise.

4.2.3 Inclusion and Exclusion criteria of the subjects Normal weight subjects

Inclusion criteria

- BMI between 18.5 – 22.9 kg/m2
- Age 25 - 50 years old
- Working at L&T Ranoli (Vadodara)

Exclusion criteria

- Hypertension
- Diabetes mellitus
- Cardiovascular Disorder
- Thyroid Hormone Disorder
- Valve Replacement Surgery
- Gastric surgery or Perforation
- Renal Disorder
- Locomotor Disorder
- Cancer / AIDS
- Psychological disorder

- Heavy Physical Activity

Obese subjects

Inclusion criteria

- BMI between≥ 25-30 kg/m2
- Age 25 - 50 years old
- Working at L&T Ranoli (Vadodara)

Exclusion criteria

- Hypertension
- Diabetes mellitus
- Cardiovascular Disorder
- Thyroid Hormone Disorder
- Valve Replacement Surgery
- Gastric surgery or Perforation
- Renal Disorder
- Locomotor Disorder
- Cancer / AIDS
- Psychological disorder
- Heavy Physical Activity

4.2.4 Study Protocol

In the present study 95 subjects were screened and selected based on inclusion and exclusion criteria from Larsen and Toubro plant, Ranoli, Vadodara, out of which 30 were normal weight adults (BMI 18.5-22.9 kg/m^2) and 65 were obese adults aged between 25-50 years gave their consent. Purposive sampling method was done to enroll subjects, Information regarding socio demographic profile, general habits, dietary intake, and physical activity pattern, medical and family history, Gut incretin (GLP-1), LPS and microbial parameters with respect to *lactobacilli, Bifidobacteria* and Enteric pathogen was collected using pretested semi structured questionnaire (Appendix III). Physical activity pattern was measured using WHO-Global physical

activity questionnaire (Appendix VI). Dietary analysis was done using 24hr dietary recall and food frequency questionnaire (Appendix V, IV) experimental design given in Figure 4.2.1.

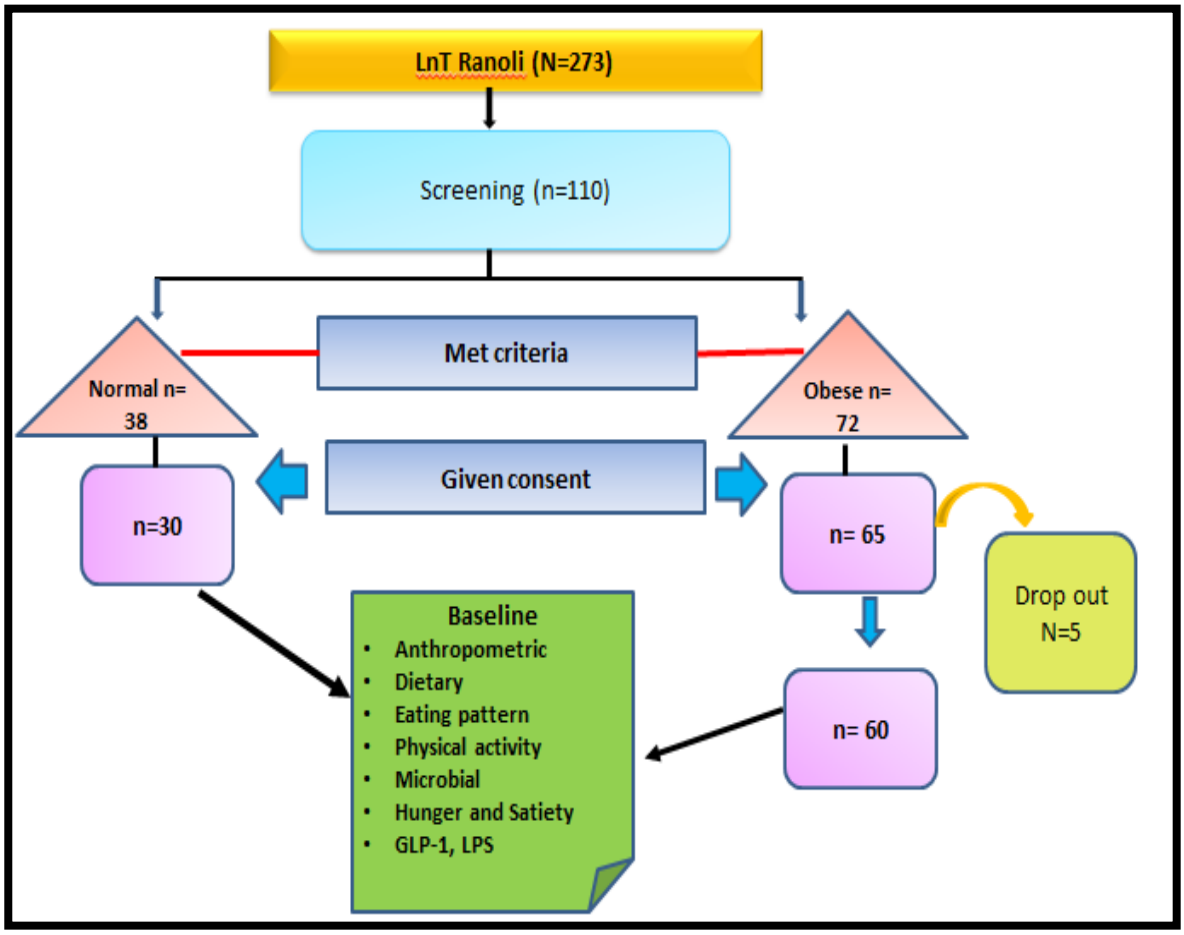

Figure. 4.2.1: Flowchart indicating experimental design of Phase II

Primary variable:

Weight

Secondary variables:

BMI, WC, WHR, GLP-1, LPS, Fecal microbial counts, hunger, Satiety and Dietary intake.

4.2.5 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, personal habits, family and medical history of the disease, duration of disease and other complications was collected from the subjects.

b) Activity pattern

Physical activity pattern of the subjects was assessed using Global Physical Activity Questionnaire (GPAQ) given by the WHO 2008 (Appendix VI). It collects information on physical activity pattern 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 (cut offs Table 4.2.1):

PAL=24 hour energy expenditure*/ Basal Metabolic Rate

*Sum of all reported activities computed for a single day

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

Table 4.2.1: 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 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):

A semi-structured food frequency questionnaire was prepared for various food groups; their frequency of consumption was obtained as per daily, weekly, monthly or occasionally basis (Annexure V).

d) 24 h Dietary recall:

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. Twenty four hour dietary recall for 3 consecutive days (Annexure IV) was taken for all the subjects to collect information on the intakes of various nutrients. Subjects were asked to provide details of all the major meals consumed throughout the previous day including beverages, snacks, sweets, pickles, papad etc. 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. The subjects were encouraged to respond in volume, number and size of the food item consumed using standardized cups and spoons. The reported volumes were later on converted into raw amounts using standardized recipes for population of Baroda. These values were entered into Diet Cal Software (Dt. Gurdeep Kaur AIIMS, 2008) to calculate the amount of various nutrients consumed by the person in a day.

4.2.6 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) Height and Weight: *Technique*- Height was measured by a non-elastic fiber-glass tape. Body weight was taken by research-grade, portable Salter scales

standardized using 5 kg standard weights and readings were recorded to the nearest 0.1kg.

b) 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) (Cut offs-Table 4.2.3).

Technique- For measuring WC, participants were asked to stand with their feet together and arms placed on the sides. The tape was placed through the midpoint between the inferior margin of the last rib and the crest of the ilium in the mid auxiliary plane and the measurement was taken to the nearest 0.1cm, without the tape compressing the skin.

c) 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 compressing the skin. The measurement was noted to the nearest 0.1 cm.

d) Computed Anthropometric Indices

i. Body Mass Index (BMI): The BMI is a convenient and valid measure of adiposity (cut offs Table 4.2.2).

It is computed as-

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

Table 4.2.2: 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**

Source: *Asia Pacific Classification WHO 2004*

Ii. Waist- Hip Ratio (WHR): This ratio gives an idea of central adiposity (cut offs Table 4.2.3).

It is computed as-

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

Table 4.2.3: Cut offs for Waist Circumference (WC) and Waist –Hip Ratio (WHR)

Cut offs (WHO 2000)	category
Waist Circumference for males (≥90cm)	**Abdominal Obesity**
WHR for males (≥0.95)	**Abdominal Obesity**

Source: *The WHO Asia Pacific criterion for abdominal obesity (WHO 2000); Zimmet PZ, Alberti 2006*

4.2.7 Body composition analysis

Digital Body Fat Monitor (Omron Healthcare co. Ltd Japan; Model no. HBF – 306 –C1; SN: 2010100047 IUF) was used to measure Body fat percentage. 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.

4.2.8 Blood Pressure

Blood pressure is the lateral pressure exerted by blood on vessel walls while flowing in it. 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) (cut offs Table 4.2.4).

Table 4.2.4 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**

Source: *Joint National Committee (JNC VII), 2003*

4.2.9 Hunger and satiety scale

A score card was used to rate the degree of hunger and satiety, 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.

SCORE CARD FOR APPETITE – SATIETY SCALE

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

4.2.10 Biochemical Evaluation and Assay methods

Venous blood sample was collected in clean, sterilized vacuum containers and allowed to stand at room temperature for 15 minutes. Plasma was immediately separated and stored at -80^0 C until analysis. It was then analyzed for GLP-1 (glucagon like peptide) and LPS (lipopolysaccharide).

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

Assay Principle: A high sensitive ELISA (enzyme-linked immunosorbent assay) kit is produced for the exclusively quantitative determination of glucagon-like peptide-1 (7-36) level in plasma and EDTA-plasma sample among human, rat, mouse, goat, etc. 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 peroxidase (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 microtiter well 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 respective corrected 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.

b) Determination of 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.

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.

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

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.

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.

Sample collection and storage

Plasma: Plasma was collected using citrate, EDTA as an anticoagulant. It was centrifuged for 15 minutes at 1000 g within 30 minutes of collection and then immediately assayed (samples were stored at -20°C, the sample was Centrifuged again after thawing before the assay).

Note: Grossly hemolyzed samples are not suitable for use in this assay.

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 were 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.

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.

4.2.11 Determination of the gut microflora

The gut microflora was determined with respect to the microorganisms- *Lactic acid bacteria*, *Bifidobacteria* and Enteric pathogen in fecal sample.

The steps involved in the determination of the fecal microflora 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 subjects were given air tight sterile containers for collection of stool then the sample was transferred in ice boxes and carried to the laboratory where it was stored at appropriate temperature (-20°C) in deep freezer The sample was used within 2 days for enumeration after collection.

b) Preparation of the sample:

One gram of fecal sample was weighed on a sterilized 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 glasswares were kept in a hot air oven at 180^0 C for 2 hours for sterilization. The micro-tips were sterilized by autoclaving them 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 swabs and 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 transferred in portion of 100 ml in 10 dilution bottles. These bottles were autoclaved at 121^0C for 15 minutes and cooled at room temperature before putting them to use.

e) Preparation and Sterilization of media

The media used for the enumeration of *Bifidobacteria* was *Bifidobacterium agar* supplied by HiMedia. The prepared media was autoclaved at 121^0C 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 Hi-Media. It is grid scored on the base and is irradiated to ensure perfect sterility. These plates are specially developed for microbial testing (HiTouch Flexi plate series manual 2007). The Flexi plates were kept inside the 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

Frozen fecal sample was first thawed and brought to room temperature; one gram of fecal sample was accurately weighed and mixed homogeneously 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 4.2.2. 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 4.2.1) to ensures a sterile environment thereby preventing contamination from outside. The plates of *Bifidobacterium* were then incubated at 37^{o}C placed in the anaerobic jar in the incubator with the gas packs and read after 48 hours. Flexi plates of *Lactic acid bacteria* were placed in a desiccator (plate 4.2.2) as it is a facultative anaerobe and Enteric pathogen were directly placed in the incubator. After 48 hours of incubation the colonies were counted on colony counter (Cintex colony counter, Dadar Mumbai) 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).

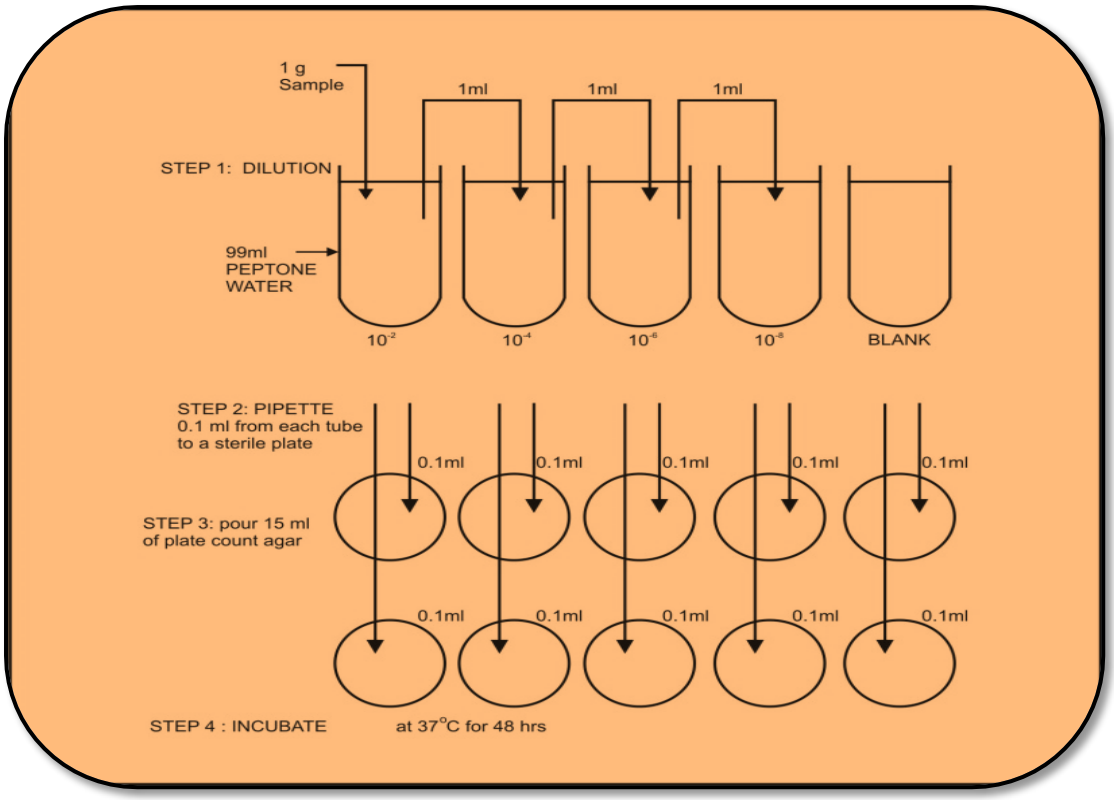

Figure. 4.2.2: Serial dilution technique along with Steps of Inoculation and Incubation

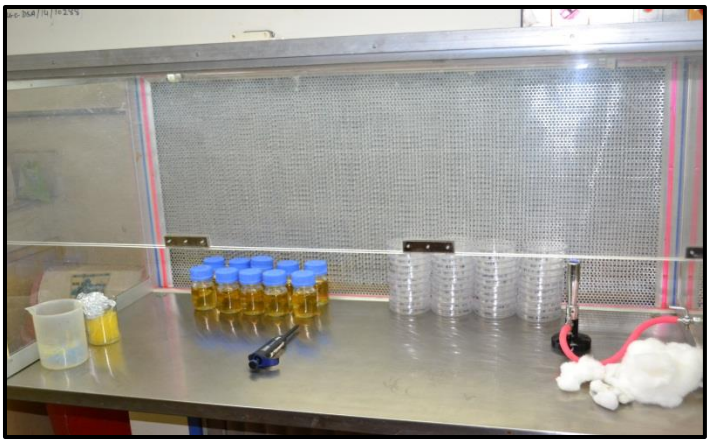

Plate 4.2.1: Laminar flow depicting plates inoculated with samples and media

Plate 4.2.2: Anaerobic Jar and Desiccator Placed inside Incubator with plates inoculated with fecal samples for determination of *Bifidobacteria, Lactobacilli and* Enteric pathogen

4.2.12: Statistical Analysis

The data was entered in Microsoft Office Excel (2007). 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. The significance levels were set at 5% by two sided tests. Multivariate linear regression was carried out with the fecal gut microbial count as dependent variables to observe its relation with various biochemical and anthropometric indices. Pearson correlation was observed between anthropometric, GLP-1, LPS and fecal gut microflora count.

PHASE III

Anthropometric and metabolic responses of obese subjects to supplementation of FOS

Obese subjects who satisfied the inclusive criteria of the study were randomly divided in to placebo control (n = 30) and experimental group (n = 30) with the help of randomized tables and were given 12 g FOS/dextrose for 60 days and examined for the effect of FOS intake daily for the same parameters as mentioned above. Experimental design of the study is given in Figure 4.3.1.

Study protocol

Based on the results of the preliminary examination and willingness to further participate in the study (Appendix VII), 60 obese subjects were enrolled in the study trial. The subjects were randomly assigned using computer generated random tables to either placebo control or experimental group. Informed consent from the subjects was obtained prior to supplementation. The experimental and placebo control group was asked to consume 12 grams of FOS/dextrose per day for 60 days along with breakfast/lunch. 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 for GLP-1 and 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 and to keep a diary of illness and medications. All subjects filled up a 3-day diet diary 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 VIII).

4.3.1 Study food and placebo

The FOS used as study food for the intervention was food grade FOS derived from chicory roots was procured in a pack of 25 kgs from Brenntag Ingredients India Pvt. Ltd, Mumbai whereas and dextrose (anhydrous) used for the placebo was procured from Hexon laboratory Pvt. Ltd .

Randomization of subjects and mode of intervention of study food and placebo:

All the subjects were randomly divided into two groups and codes were allocated to each group (1and2). Color codes were given to test food and placebo (green, orange) and then codes were given to these colors same as codes of the study group i.e. 1 and 2 (green-1, orange-2) by the third party, then test food and the placebo were distributed among subjects so that color code 1 will go to group1 and 2 will go to group 2. Twelve grams of FOS (study food) and dextrose (placebo) was given in powder form each packed in auto sealed

sachets. The subjects were asked to incorporate FOS/dextrose in to water along with the breakfast/lunch for period of 60 days

4.3.2 Statistical Analysis

The data was entered in Microsoft Office Excel (2007). 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 anthropometric measurements, LPS, GLP-1, satiety and fecal gut microbial counts. The significance levels were set at 5% by two sided tests. Student *t* test and chi square was performed for the comparison between placebo control and experimental group for the various biochemical, microbial and anthropometric parameters.

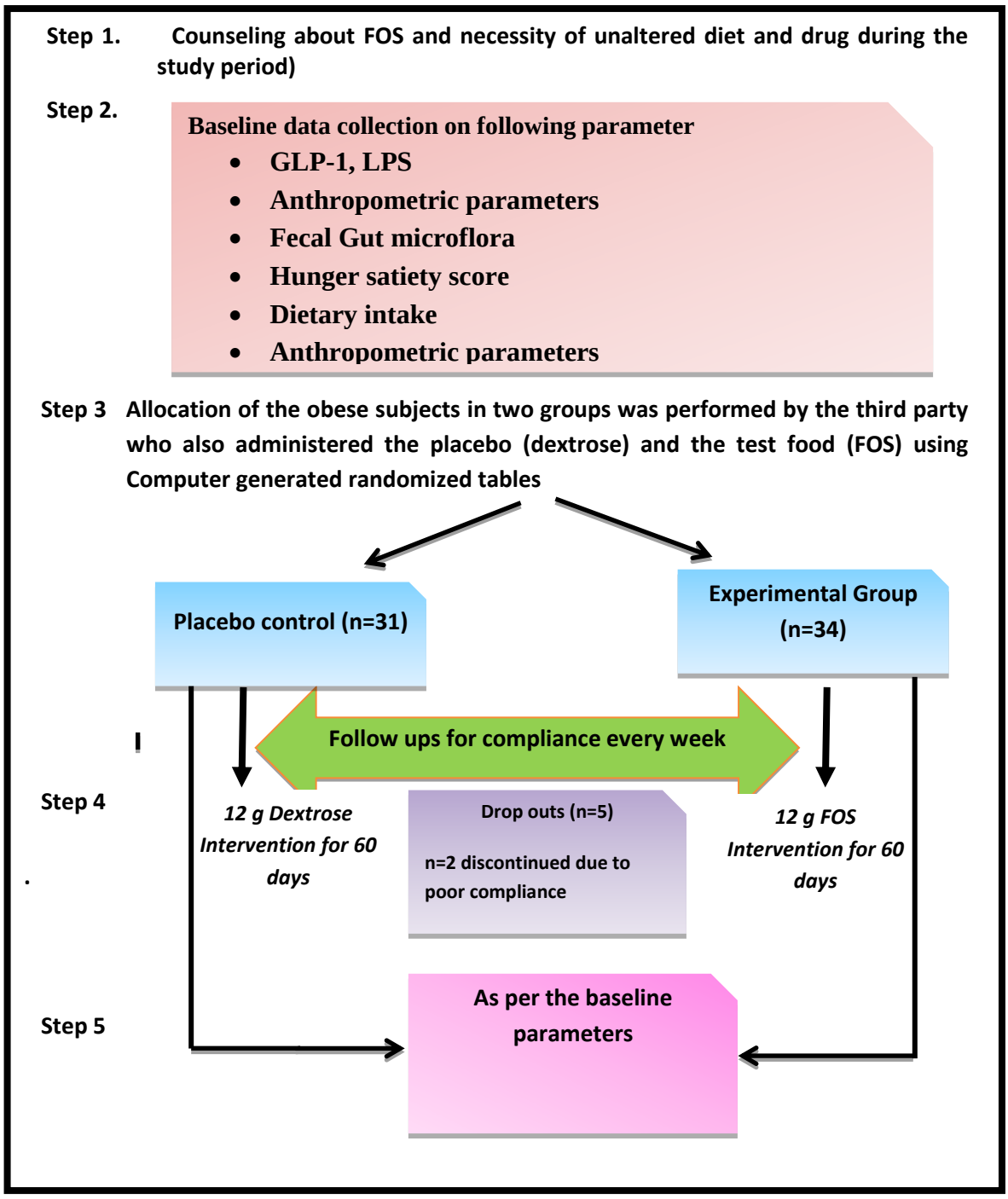

Figure 4.3.1: Flow chart indicating experimental design of Phase III