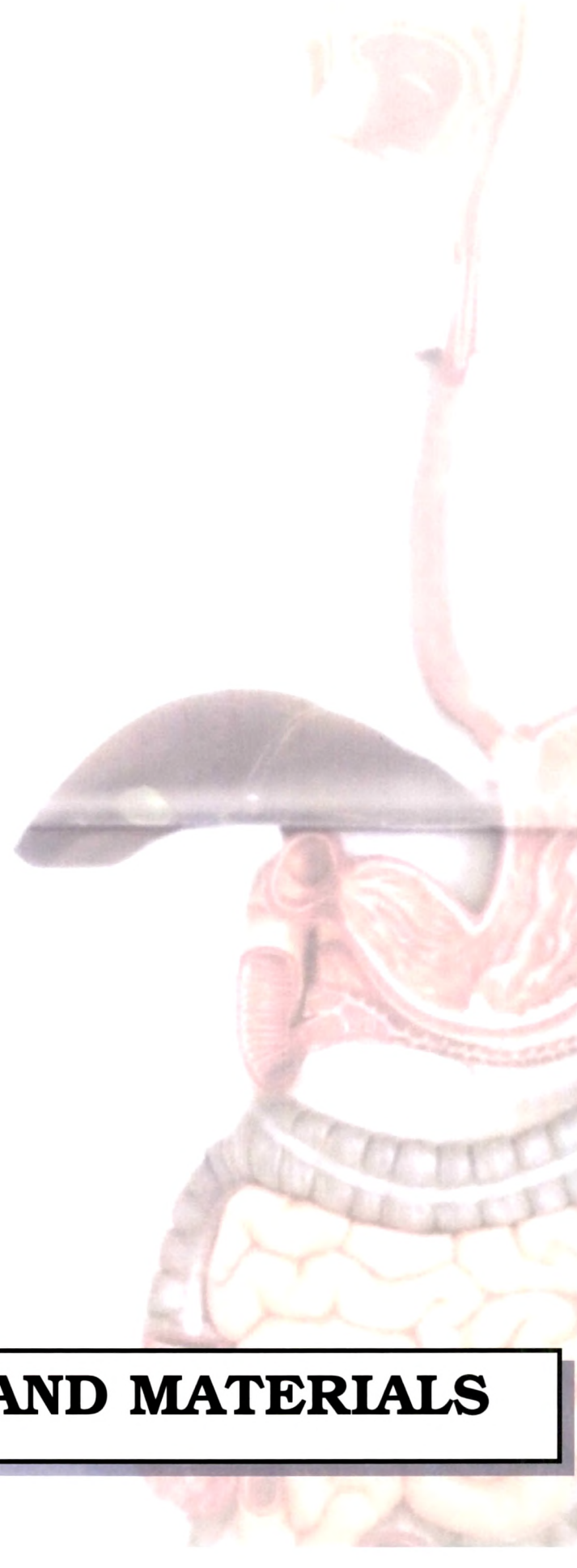

METHODS AND MATERIALS

CHAPTER 4

METHODS AND MATERIALS

Recently a lot of interest has been generated in inulin as prebiotic food which is known to exert beneficial effects in health and disease. However, there is lack of data for inulin content of commonly consumed Indian foods. Also growing physiological knowledge offers new possibilities for the availability of prebiotic rich foods to improve the health of population. This can be achieved by developing inulin rich foods and testing for their acceptability as value added products. Recent evidence has indicated the use of dietary oligosaccharides in improving the conditions of hyperlipidemia, diabetes and hypertension through improvement in gut microflora. Also few studies were undertaken to improve the health parameters in elderlies, therefore an attempt was made to study the effect of inulin supplementation in diets of institutionalized elderlies. This chapter outlines the experimental design and discusses the methods and materials used to fulfill above mentioned objectives of the study in three phases.

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Phase I – Determination of Inulin content of various foods.

This phase of the study was conducted to analyze inulin content of selected popularly consumed raw foods as natural ingredient and to determine its level in the processed wheat based foods.

Section 4.1.1 Purchase and preparation of samples for analysis:

Popular raw food samples included cereals viz wheat (*Triticum aestivum*) -GW496, GW-1, Arnej-8206; rice (*Oryza sativa)*-GR-9; pearlmillet *(Pennisetum typhoideum)*-GHB-526; maize *(Zea mays)* -GM-6 and legumes namely blackgram (*Phaseolus mungo roxb*) -T-9; greengram (*Phaselous aureus roxb*) -K-851; bengalgram (*Cicer arietinum*)-GG-1; redgram (*Cajanus cajan)*–BDN-2; soyabean (G*lycine max Merr*). The grains were procured from the research station whereas fruits (apple-*Malus sylvestris*) and vegetables (fenugreek leaves-*Trigonella foenum graecum*, spinach-*Spinacia oleracea*, cabbage-*Brassica oleracea var. capitata*, brinjal-*Solanum melongena*), roots and tuber (onion-*Allium cepa*, garlic-*Allium sativum*), spices and condiments (fenugreek seeds- *Trigonella foenum graecum*) and processed foods (oat bran, wheat bran, bread, banana chips) were purchased from the local markets of five different zones of Vadodara. Banana chips were selected because of its high consumption in the local region The fruit and vegetable samples were mixed, peeled and the edible portion was then sliced and dried in the oven at 60°C. Three common foods preparations namely '*Chapati'* - Indian flattened unfermented bread cooked for less than a minute by roasting on hot plate, *'puri'*-flattened wheat dough deep fried less than a minute until puffed and *'porridge* were prepared using GW-496 variety of wheat using the standard recipes (Pasrachia and Rebello 1998).

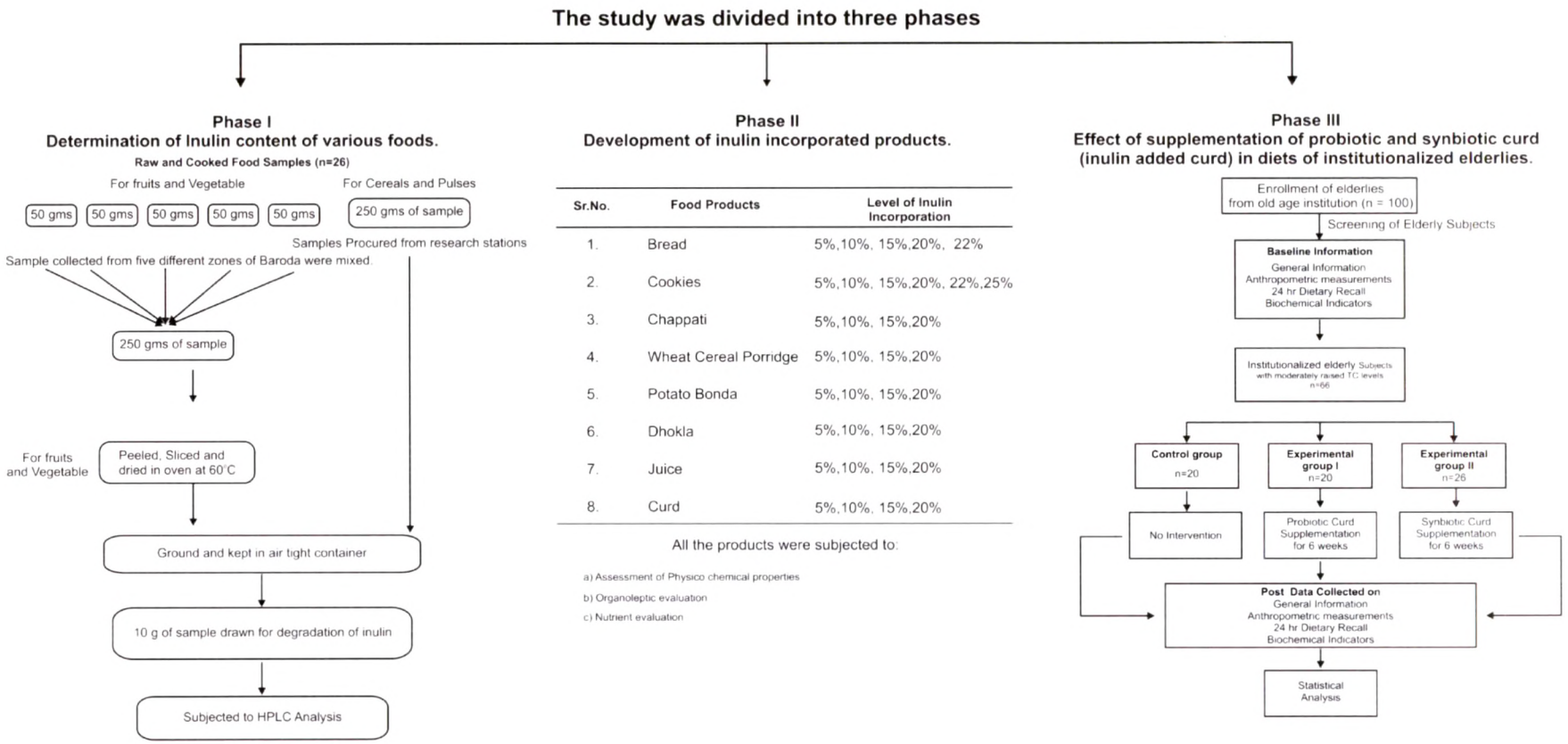

Fig. 4.1 Experimental Plan of the Study

All the samples were powdered by grinding in the milcent mill and transferred into air tight containers until analysis. The results of moisture content are presented in Table 4.1.

Table 4.1: Moisture (g%) Content in Samples Selected for Analysis.

Food items	Moisture g%	Dry matter g%	Food items	Moisture g%	Dry matter g%
Cereals			**Fruits and vegetables**		
Wheat-GW1	7.00	93.00	Apple	84.60	15.40
Wheat-GW496	8.50	91.50	Fenugreek	89.55	10.45
Wheat-Arnej 8206	8.34	91.66	Spinach	90.35	9.65
Pearl millet	8.40	91.60	Brinjal	76.56	23.44
Rice	10.32	89.68	Cabbage	72.55	27.45
Maize	11.56	87.44	Garlic	61.25	38.75
Chapati	34.25	74.75	Onion	87.10	12.90
Porridge	62.92	73.08	**Spices**		
Puri			Fenugreek seeds	8.10	91.90
Pulses and Legumes			**Processed Foods**		
Green gram	8.58	91.42	Bread	39.10	60.90
Bengal gram	7.60	92.40	Banana chips	01.33	98.67
Black gram	9.25	90.75			
Red gram	9.3	90.70			
Soyabean	7.94	92.06			

Section 4.1.2 Reagents and standards

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

Section 4.1.3 Analysis of the samples using HPLC

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

a) **Principle:** The method is based on specific hydrolysis of fructans using an inulinase enzyme (from *Aspergilus Niger* Sigma Aldrich Chemicals Ltd). The standards of fructose (D-fructose extra pure Qualigens Ltd.), glucose (D-glucose pure Qualigens Ltd) and sucrose (Sucorse pure-merck chemicals Ltd) were used as calibration samples. Inulin content in the samples was calculated based on the

comparison of glucose, fructose and sucrose determination before and after hydrolysis.

b) **Standardization of the method:** The high performance liquid chromatography was calibrated for the determination of inulin by injecting water based standard solutions containing different concentrations 1000, 2000, 3000 and 4000 ppm of fructose into HPLC with similar operating conditions. The chromatograms of various concentrations are depicted in appended in Appendix I (i). The standard curve plotted for various concentrations is depicted in Appendix I (ii), which shows that there is no significant variation in the determined concentration, indicating that the detector response is valid at various concentrations of sugars.

c) **Determination of Inulin:** For determining inulin in common foods, 150 ml of 0.1M acetate buffer solution was added to 10 g of the food sample at pH=4.5 and homogenized. The covered beaker was placed in the shaking water bath at 85°C for 20 minutes to dissolve the sample and cooled to 60° C. To this 100µl inulinase enzyme was added and incubated in shaking water bath at 60°C for 30 minutes for total digestion of the inulin. Lead hydroxy acetate up to 3ml was added to precipitate the protein and finally the volume of the mixture was made to 200 ml with water, homogenized and filtered through filter- paper. Filtrate was transferred into a capped vial and subjected to HPLC assay with this solution using 85% acetonitrile and 15% water as mobile phase and the separation was performed at an ambient temperature of 30°C at a flow rate of 0.4ml/min.

The quantity of fructose obtained by HPLC (R1) represented the total amount of fructose in the sample. A duplicate sample of ca 10 g was weighed accurately and proceeded exactly in the same manner as for the test sample, without the addition of inulinase (R2) (Figure 4.2). R3 represents the amount of fructose obtained by HPLC from sucrose. The steps carried out for the determination of inulin content in the food samples are depicted in Plate 4.1(a-f).

d) **Calculation and expression of results**: The amount of fructan dietary fiber (FDF) is given by %β-FDF= 1.1(R1-R2)100 / P (1) for samples containing no sucrose and β-FDF= 1.1(R1-(R2+R3)100/P (2) for samples containing sucrose R1= concentration of the total fructose (g/l); R2= concentration of the free fructose (g/l); R3=concentration of the fructose (g/l) from sucrose; P= mean

mass (g/l) of the duplicate test sample, where P=5 times the dry mass of the test sample taken. Empirical conversion factor is 1.1 for the fructose to fructan, assuming the ratio of 80 to 85% fructose and 15% glucose in the fructan polymer.

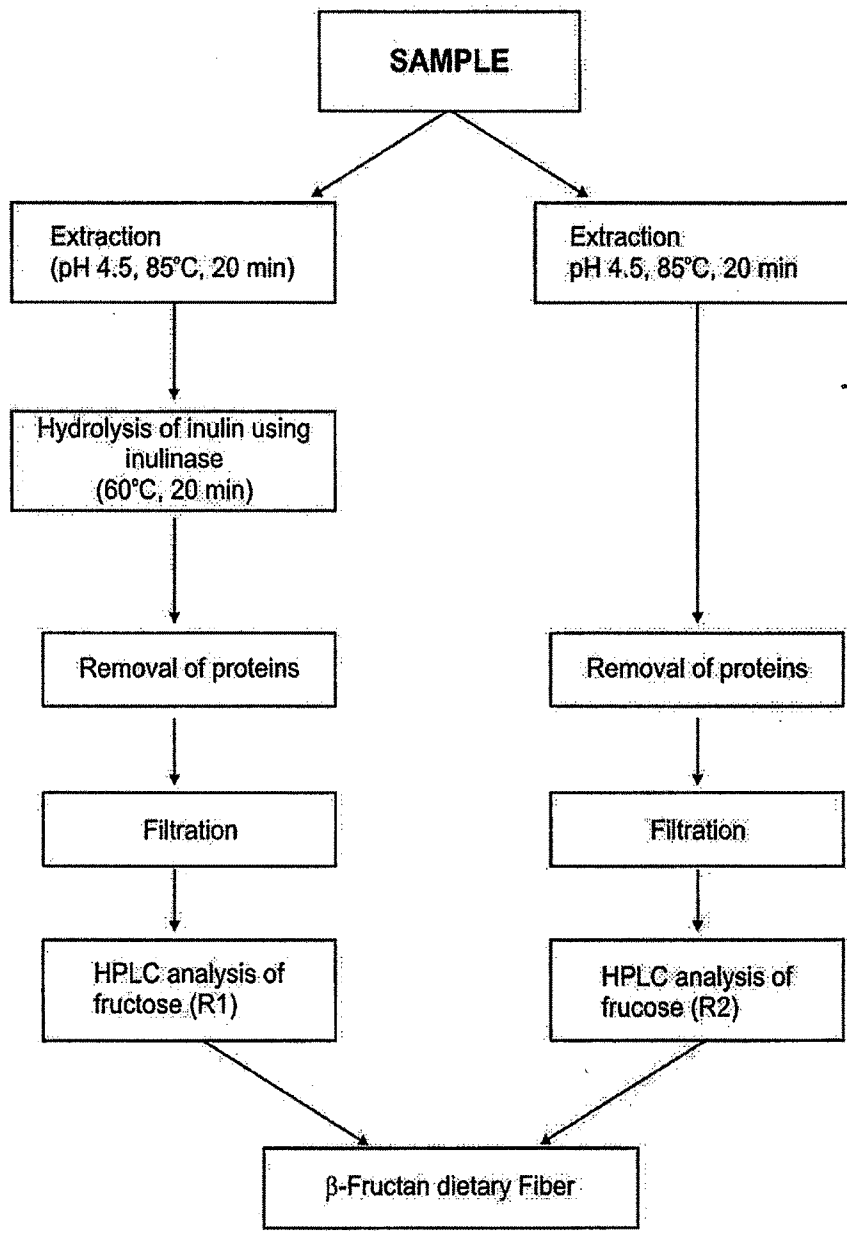

Fig. 4.2 β- Fructan Determination

(a) Dried samples stored in air tight containers

(b) Pre treatment of samples for HPLC assay

(c) Weighing of sample for analysis

(d) Adjusting of pH using pH meter

(e) Injecting of the ready sample

(f) Printing of chromatograms

Plate 4.1(a-f): Steps in Determination of Inulin Content in Food Samples

Phase II – Development of inulin incorporated products.

Section 4.2.1 Procurement of Inulin

Food grade inulin (Beneo Raftiline ST BAG 25 kg, Orafti, Belgium) was procured in a pack of 20 kgs from S.A Pharmachem Pvt. Ltd. The inulin was food grade which was best suitable as fat and sugar replacer. The specifications of the inulin are given in Table 4.2.

Table 4.2: Specification of the Inulin used for Substitution and Addition

Specifications	Analysis	Values	Range
Orafti phys/chem. Parameters packaged	Dry matter	98%	95.5-98.5
	pH	6.7	5.0-7.0
	Conductivity(uS)	18	0-249
Orafti Spectrometry packaged	Sucrose(%DM)	3.7%	0.0-8.0
	Inulin	95.9%	90.1-100.0
	Glucose + fructose	0.4%	0.0-4.0
Orafti microbiology	Total counts (/gd.m.)	0	0-1000
	Yeasts	0	0-20
	Moulds	0	0-20

Section 4.2.2 Selection of products

The possibilities of incorporating inulin in the popular Indian foods and their organoleptic qualities were studied by the method of substitution and addition. Total of eight products were selected on the basis of processing conditions. Out of these products that were substituted with inulin included bread, cookies, *Chapati and dhokla* and products in which inulin was added included cereal pulse porridge, *potato bonda*, orange juice and fermented milk. All the products were studied for physicochemical, organoleptic and nutritional characteristics.

Section 4.2.3 Procurement of raw ingredients

Various ingredients and materials that are needed to develop inulin enriched products were collected from local market of Vadodara. The list of raw ingredients and their sources according to the type of recipe is listed in the Table 4.3.

Table 4.3: List of Raw Ingredients along with their Sources used for Product Preparation.

Product	Ingredients	Source/ Brand name
Bread and cookies	Refined wheat flour	Local market
	Yeast	Baker's yeast
	Sugar	Tata
	Salt	Tata
	Water	Potable
	Fat	Nutrela soyabean oil
	Eggs	Local market
	Baking powder	Butterfly Baking powder
Chapati	Wheat flour	Wheat variety GW496
Wheat porridge	Broken wheat	Uttam brand, Baroda
	Green gram	local market
	Oil	Sundrop heart
Potato bonda and dhokla	Potatoes	Local market
	Bengal gram flour	Uttam brand, Baroda
	Rice flour	Uttam brand, Baroda
Juice	Oranges	Local market
Curd	Curd	Amul masti dahi

* The detailed recipes of the standard products are appended in appendices II (i-vi).

Section 4.2.4 Standardization and Substitution of inulin to the following products:

a) **Bread**: Bread was developed by slight modification in straight dough method (Bennion 1939) in the second step of its preparation where the kneading of the dough was carried out in commercial food preparing machine (Hobart Corporation Troy, OHIO made in U.S.A) instead of manual kneading. Rest of the method of bread preparations remained the same. Five types of bread were prepared wherein the refined wheat flour in the standard bread was replaced with 5%, 10%, 15%, 20% and 22% of inulin.

b) **Cookies**: Cookies were standardized after repeated trials (Standard recipe is appended in II (ii). Six cookies samples were developed wherein fat was replaced with 5%, 10%, 15%, 20%, 22% and 25% level of inulin.

c) ***Chapati***: *Chapati*es are unleavened flat breads normally prepared from whole wheat flour. The whole wheat flour was replaced in the standard *Chapati* recipe

(Pasrachia and Rebello, 1998) with 5%, 10%, 15%, 20% and 22% level of inulin substitution.

d) **Dhokla**: *Dhokla's* were prepared by a standard recipe (Pasrachia and Rebello, 1998) with slight modification wherein rice pulse mix flour was substituted with inulin at 5%, 10%, 15%, 20% level of substitution.

Section 4.2.4 Addition of inulin to the products

a) **Cereal pulse porridge:** Cereal pulse porridge was prepared by the standard recipe (Pasrachia and Rebello, 1998). Inulin was added to 100g of the raw ingredients of the product at 5%, 10%, 15% and 20% level.

b) **Potato bonda:** Potato bonda was prepared by the standard recipe (Pasrachia and Rebello, 1998). Inulin was added to the standard recipe at 5%, 10%, 15%, 20% level of addition.

c) **Orange juice:** Fresh oranges were procured from local markets of five different zones of Baroda. The oranges were peeled and juice was extracted. Inulin was added at same levels as for porridge and bonda.

d) **Fermented milk:** Standard "masti dahi" was procured from Amul dairy outlet. Four samples with addition of inulin at different levels (5%, 10%, 15% and 20%) were developed.

Section 4.2.5 Assessment of Physico-chemical properties of standard and inulin enriched recipes:

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

a) **Physicochemical evaluation of Bread:**

i. **Water retention (ml):** Water retention was measured as the weight of the bread minus the weight of the bread ingredients.

ii. **Loaf volume(ml):** Loaf volume was calculated by multiplying length, breadth and height of the loaf of the bread.

iii. **Loaf weight**: The loaf weight of the bread was recorded using a digital weighing balance.

iv. **Total Baking loss**: This was calculated using a formula (Korus et al 2006)

v. **Total baking loss %** = {(250 g of dough –weight of the cold bread)/250 g}*100

b) Physicochemical evaluation of Cookies:

i. **Width of the cookies (W):** Width of the cookies was measured by laying six cookies edge to edge with a help of a scale rotating them to 90° and re-measuring the width of the cookies in cm and then taking average value.

ii. **Thickness of the cookies (T):** Thickness or height of the cookies was measured by stacking six cookies on top of one another and taking the average thickness (T) of the cookies in cm.

iii. **Spread Ratio**: Spread ratio was calculated by dividing the average value of width (W) by average value of thickness (T) of cookies.

iv. **Percent Spread Ratio:** Percent spread ratio was calculated by dividing the spread ratio of supplemented cookies with the spread ratio of control cookies and multiplying by 100.

c) Physicochemical evaluation of *Chapati*:

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

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

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

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

Calculation: WAP (in ml) = W1-W2

W2= amount of water left

W1= amount of water measured initially

d) Physicochemical evaluation of Dhokla

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

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

e) Physicochemical evaluation of Cereal Pulse Porridge

i. **Cooked weight:** The weight of the cooked cereal pulse porridge was recorded using electronic balance.

ii. **Time of steaming:** This refers to the time required for pressure cooking the cereal pulse porridge.

iii. **Water retention (ml):** Water retention was measured as the weight of the cereal pulse porridge minus the weight of the cereal pulse porridge ingredients.

f) Physicochemical evaluation of Potato Bonda

i. **Oil absorption:** Oil absorbed was calculated by subtracting the amount of oil left in frying pan from the total amount of oil taken for frying.

ii. **Frying time**: time for frying the potato bondas was recorded in minutes

iii. **Frying temperature**: Frying temperature was recorded in Celsius. The temperature of oil was monitored with a digital thermometer (spectrochem Pvt. Ltd. Mumbai).

g) Physicochemical evaluation of sweet orange juice

i. **Juice recovery:** For determination of juice weight was divided by the fruit weight and multiplied by 100.

ii. **Density of juice:** Density of the juice was determined by taking the ratio of mass/volume of the juice.

iii. **pH of the juice:** The pH of the juice was determined by using pH strips (Indikrom papers, product no 38211) and were compared with the standard

h) **Physicochemical evaluation of fermented milk**

pH of the fermented milk: The strength of the acidic nature of the fermented milk was determined using pH analysis through pH strips (Indikrom papers, product no 38211) and were compared with the standards.

Section 4.2.6 Selection and training of judges for organoleptic evaluation

a) **Screening of panelists:** In this section, selection of panel members was carried out (Fig. 4.3). Students and staff of department of Foods and Nutrition were subjected to threshold testing.

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

Threshold is defined as a stimulus scale at which a transition in a series or judgment occurs. For conducting this test, score card for the same was formulated and pre tested (Appendix III). Each perspective panel member was given two sets of the solution i.e. Set 1 and Set 2 having six solutions of different concentrations of salt and sugar respectively and 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.

c) **Training of the selected panel members:** A training tool was developed for imparting the basic knowledge and understanding of visual and organoleptic characteristics of the products. Training was given using the same. The prerequisites which were looked into before and at time of organoleptic evaluation have been listed in Appendix IV.

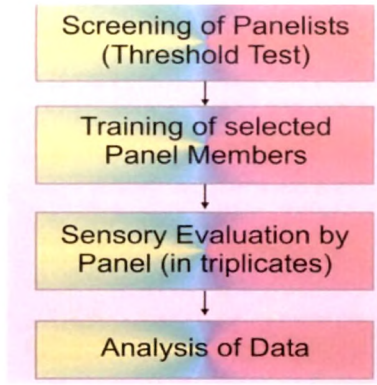

Fig. 4.3 Steps for Organoleptic Evaluation

d) Development of score cards for the organoleptic evaluation of the products: Score cards were developed for organoleptic evaluation (AppendixV) where in tools for evaluation are listed.

Section 4.2.7: Tools for organoleptic evaluation

Organoleptic evaluation tools selected were

a) Numerical Scoring test and

b) Multiple difference test (ISI 1972)

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

b) Multiple difference test: This test was used to measure the effect of process change on quality of the product or by formulation for product improvement. It measures more than one test variable per session. Each panelist was served 4-6 samples depending upon the number of test variables. One sample was known standard. The panelists compared each coded sample with the known standard. Whatever score the panelists assigned to the blind standard was subtracted from the score assigned to the test variable. Direction and degree of freedom was also judged.The development and evaluation of inulin incorporated recipes is depicted in plates 4.2 (a-f)

(a) Weighing of raw ingredients for product development.

(b) Frying of inulin incorporated potato bonda

(c) Breads taken out from oven after processing

(d) Inulin incorporated cookies

(e) Different type of inulin enriched products

(f) Sensory evaluation carried out by panel members

Plate 4.2 (a-f): Development and organoleptic evaluation of inulin incorporated products

Section 4.2.8: Nutrient analysis of recipes

The nutrient analysis of recipes was carried out in the following manner.

a) **Preparation of the sample for analysis**: For the recipes raw ingredients were purchased from five different markets of Vadodara city. Equal weights of each were pooled together to obtain a primary sample, out of which a sub- sample was drawn which was then used for preparations of the standard recipe.

b) **Parameters analyzed:** The inulin incorporated recipes was subjected to analysis for various parameters including moisture, total protein, fat, crude fiber and total ash content (ISI 1982; ISI 1984). The carbohydrate content was calculated by difference.

i) **Moisture**: Ten gram of the sample was weighed into a weighed moisture box and dried in the oven at 100 $^{\circ}$ C and cooled in a dessicator. The process of heating and cooling was repeated till a constant weighed was achieved.

$$\text{Moisture \%} = \frac{\text{Initial weight-Final weight}}{\text{Weight of the sample}} \times 100$$

ii) **Total proteins:**

Principle: The estimation of nitrogen was done by Kjeldahl method, which is based on the principle that organic nitrogen when digested with sulphuric acid in the presence of a catalyst (selenium oxide, mercury or copper sulphate) is converted into ammonium sulphate. Ammonia liberated by making the solution alkaline is distilled into a known volume of a Standard acid, which is then back-titrated. The protein is obtained by multiplying the nitrogen value with 6.25.

Reagents:

a) Digestion moisture : 98 parts K_2SO_4 + 2 parts $CuSO_4$
b) 40% NaOH
c) N/10 NaOH
d) N/10 H_2SO_4
e) Methyl red indicator: 0.1 g of the indicator dissolved in 60 ml of alcohol and water added to make to 100 ml.

Procedure:

The sample (1g) was weighed into dry Kjeldahl flask. About 5 g digestion mixture and 20 ml of pure conc. H_2So_4 were added to the sample and the mixture was digested by heating for 4 to 5 h. Glass beads were added to prevent bumping. After the content of the flask became clear, the digestion was continued for 1 h. The contents of Kjeldahl flask were cooled, diluted with distilled water and the mixture made alkaline by adding excess of 40% NaOH (75 ml). A Small Quantity of pumice powder was added to prevent bumping during distillation. The ammonia liberated was distilled into a receiver containing 25 ml of N /10 H_2So_4. The excess of acid in the receiver was back against N/10 NaOH using 3 drops of methyl red indicator .A reagent blank was similarly digested and distilled. The titre value was subtracted from the value obtained for the sample to get the true titre value 'b'.

Calculation:

If 'a' g of the sample is taken and if 'b' and 'c' ml of alkali of normality 'd' were required for back titration and to neutralize 25 ml of N/10 H_2So_4 respectively then,

The protein content in g/100 g sample is $\frac{(c-b) \times 14d \times 6.25}{a \times 1000} \times 100$

iii) Fat: Fat was estimated as crude ether extract of the dry material. The dry sample (10 g) was weighed accurately into a thimble and plugged with cotton. The thimble is then placed in the Soxhlet apparatus and extracted with anhydrous ether for about 16 h. the ether extract is filtered into a weighed conical flask. The flask containing the ether extract was washed 4 to 5 times with small quantities of ether and the washings were also transferred. The ether was then removed by evaporation and the flask with the residue dried in an oven at 100 ° C, cooled in a dessicator and weighed.

iv) Total Ash: The sample was weighed accurately into tarred platinum crucible (which has been previously been heated to about 600° C and cooled). The crucible was placed on a clay pipe triangle and heated first over a low flame till all the material was completely charred, followed by heating in muffle furnace for 5h at 600° C. It was then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible was again heated in a muffle furnace for 1 hr, cooled and weighed. This was repeated till two consecutives weights are the same and the ash was almost white or grayish white in colour.

Fat content (g/100 g sample) = Weight of the ether extract X100

Wt of the sample (Equivalent to fresh sample taken)

v) Carbohydrates: The Carbohydrate content was calculated by subtracting the nutrients and moisture from 100. The energy value was computed by multiplying the identified values for carbohydrates, fat and protein with 4, 9 and 4 respectively (at-water constants) (ISI 1984).

Phase III – Effect of supplementation of probiotic and synbiotic fermented milk (inulin added fermented milk) in diets of institutionalized elderlies.

This Phase of the study was undertaken to examine the effects of the daily intake of probiotic fermented milk and synbiotic fermented milk (probiotic fermented milk with the addition of 15 g of inulin) on the gut microflora, fasting blood lipids, plasma glucose, hemoglobin levels and general health of institutionalized elderly men and women with mildly raised TC and TG levels. The sections for this phase of the study are being discussed under the following heads:-

Section 4.3.1 Ethics Committee Approval: Approval to undertake the study was obtained from the Institutional ethics committee of The M.S. University of Baroda. Written informed consent was obtained from each one of the subjects who participated in this study (Appendix VI).

Section 4.3.2 Selection of subjects:

A total of hundred elderly men and women were enrolled from old age institutions of urban Vadodara for this phase of the study according to the inclusion and exclusion criteria for the selection of the subjects. Out of these, subjects above 60 years with hypercholesterolemia were assigned to each of the three groups as mentioned below:

a) Group I (control group) (n=20)
b) Group II (Probiotic fermented milk supplementation group) (n=20)
c) Group III (Synbiotic Fermented milk supplementation group) (n=26)

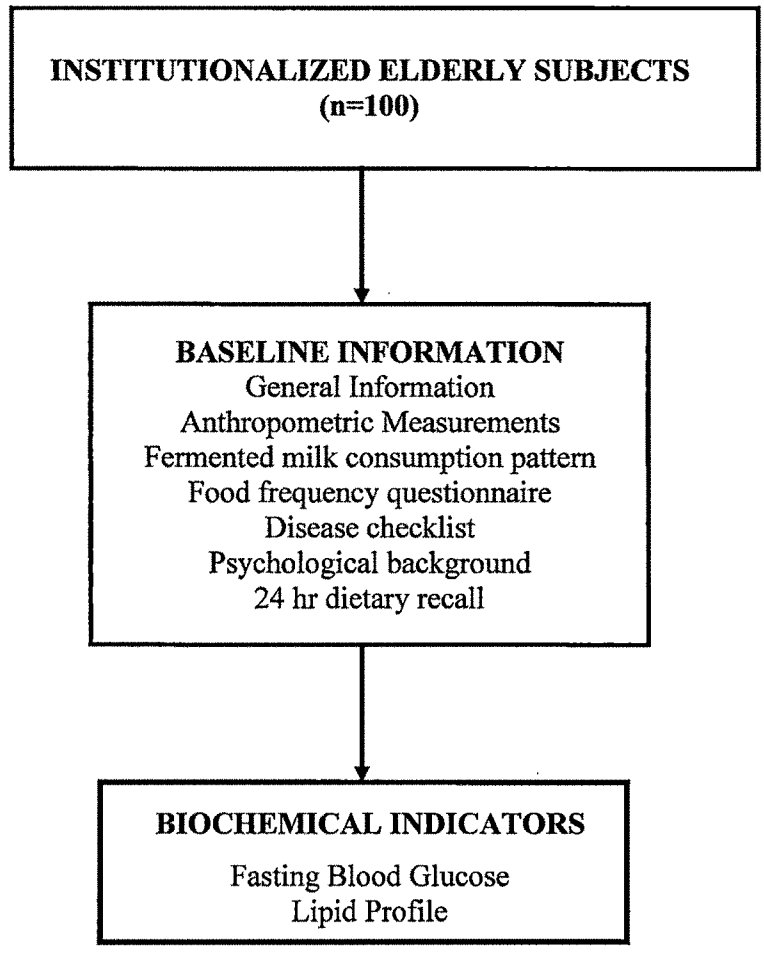

Fig. 4.4.a Selection of Participants at Baseline

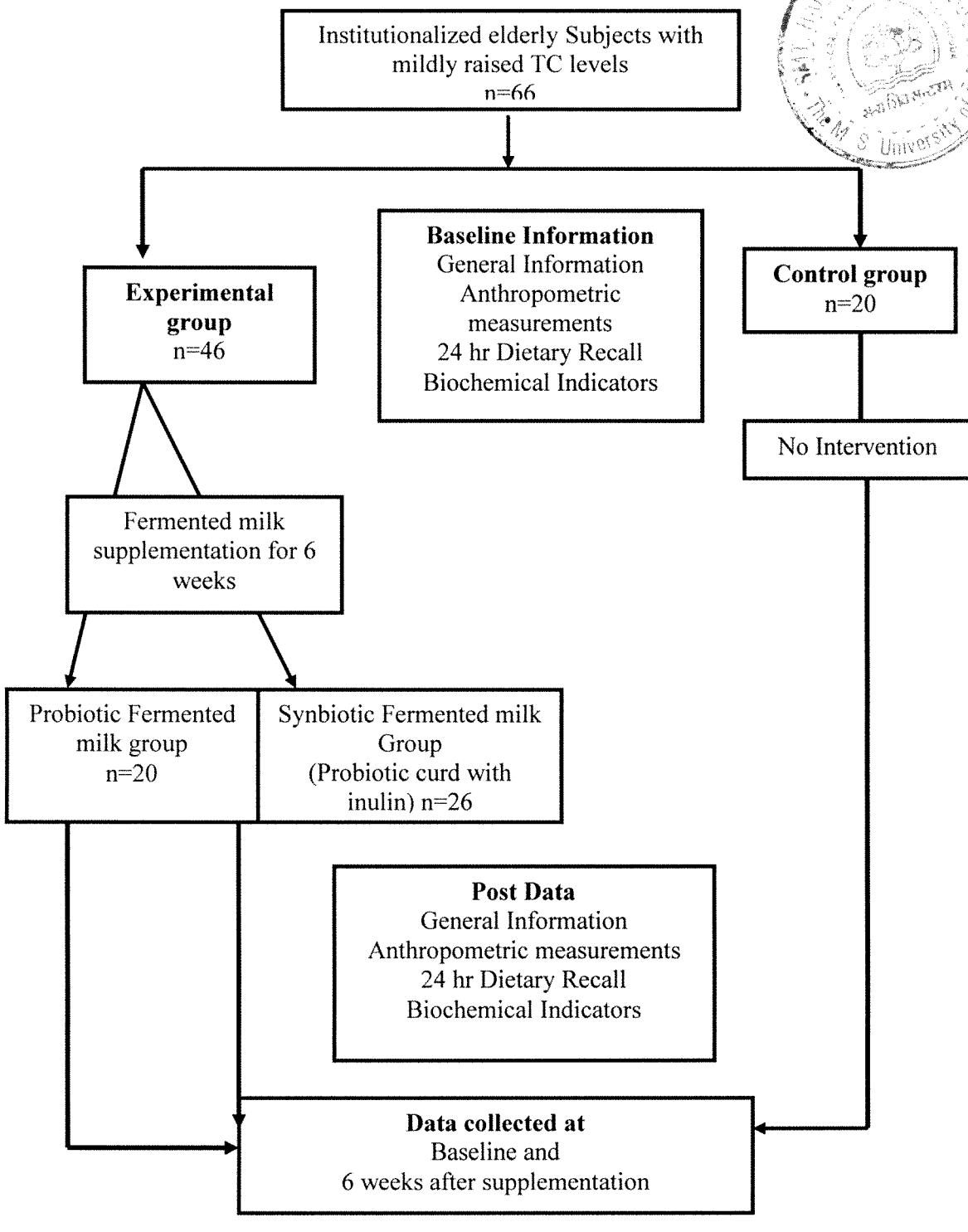

Fig. 4.4.b Experimental Plan for Supplementation of Fermented Milk

a. Inclusion criteria for selection of the subjects

i. Subjects were above 60 years of age

ii. Subjects were not taking any medication for the treatment of hyperlipidemia or any other drug which would affect their plasma lipid levels.

iii. Willingness of the subjects to participate in the study.

iv. Willingness to consume fermented milk daily for a period of six weeks

b. Exclusion criteria for selection of the subjects:

i. Subjects not having any history of heart disease.

ii. Subjects who are not suffering from any other major health complication.

Section 4.3.3 Administration of Interviewer based Questionnaires

Subjects 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. Set of 9 questiones were administered on the subjects and baseline information was gathered and willing members were informed about the venue, and reminded of coming to the camp in a fasting state. During the camp, blood samples were taken, blood pressure measured and anthropometric were taken.

Proper record of the eligible members who refused to participate in the study was maintained.

a. Background information: General information with regards to age sex, religion, educational level, marital status, income etc was collected from the subjects (VII-a).

b. Food Frequency Questionnaire (FFQ): Details of the number of times the food items was consumed on a daily, weekly, monthly or yearly basis was elicited using the FFQ. Food list contained 110 food items. Certain food items were found to be uncommon and almost similar in terms of their nutrient content and method of preparation. These foods were therefore clubbed together in respective categories and the food items were divided in subcategories, for instance, 'gulabjamun' & 'jalebi' were kept in the same category of 'Deep fried sweets': similar snacks 'Samosa', 'Pakora', and 'Kachori' were kept in one category. The FFQ was thus finalized with columns to collect information on frequency of consumption during the past one year (per day/week/month/year). Seasonality of fruits and vegetables were ascertained by

contacting the main fruit and vegetable vendors in the city and mentioned in the FFQ the duration for which fruits and vegetables were available (Annexure VII-b).

c. 24 h dietary recall: Many studies have found that approximately six 24-hr dietary recalls scattered throughout a year can be used to estimate the intake of most nutrients. However, a single 24-hr 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 of 100 institutional elderly people was therefore collected for the study. The subject was asked to recall all food items and beverages consumed on the previous day. This questionnaire (appended in appendix VII-c) 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-hr dietary is used to estimate the food intake of an individual over the period of 24 hours, referring to previous day/night. The 24 hr dietary recall was done for all the subjects to collect information on the intakes of calories, proteins fat, iron, calcium and vitamin C. 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. The portion size was estimated using volume measures, circular measures and numbers. A set of five vessels (Ladle, 'Katori', Table spoon, Tea spoon and Glass) was used as an aid to estimate volumes. Items like *Chapati*es, puri or parathas were estimated using a set of 4 circular models-8 cms, 12 cms, 16 cms and 18 cms.

The details of the preparation of various recipes were obtained from the person who cooked in the institution first in terms of household measures and then in terms of standard cups and spoons and their nutritive value was obtained. An account of raw ingredients used in each of the reported preparations was obtained and weighed on

Braun Scale with 10 g sensitivity. Also total cooked amount of each preparation was thus measured and volume to weight conversion of the ingredients made using the formula give below

$$\text{Individual intake in terms of raw equivalents (g)} = \frac{\text{Total raw amount of each ingredient}}{\text{Total cooked amount (g)}} \times \text{Individual intake of cooked amount (g)}$$

Nutrient content of diet was calculated using ICMR Food composition tables given in the nutritive value of Indian foods (Gopalan et al 2004).

d. Activity pattern

A checklist containing various types of activities along with the time spent to perform each activity was used to assess the activity pattern of elderly subjects. Besides sleep the various activities were grouped under categories (Appendix VII-d). The subjects were classified as having active and sedentary lifestyles according to WHO classification. Those individuals indulging in moderate physical activity for at least 30 minutes for 3-4 times in a week were categorized as active (WHO 2004).

e. Fermented milk consumption pattern

Information on fermented milk consumption pattern of the subjects was collected using questionnaire and reasons for avoiding it were identified (Appendix VII-e.

f. Disease profile

The subjects were asked to report the various illnesses experienced in the past two months and the recurring problems of the gastrointestinal tract using the checklist as appendicized in appendix VII [vii (a-f)].

g. Psychological background

Data on their psychological background was obtained using Geriatric Depression Inventory which was included in the questionnaire. The subjects were classified as mild, moderate and severely depressed based on the GDI scores (Yesvage et al 1984) (Appendix VII-g).

Geriatric Depression Inventory (GDI) Scale

Categories	Score
Normal	1-10
Mild	11-15
Moderate	16 – 20
Severe	≥21

Source: Yesavage et al 1983

Section 4.3.4 Anthropometric Measurements

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

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

Technique- A platform weighing scale to the nearest 100gm was used to measure weight. The subject was weighed in standard indoor clothing, bare feet and without leaning against or holding anything. Scale was 'zeroed' before taking any weight, and was calibrated using standard weights after every third subject. Calibration values were maintained (Appendix VII- h).

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

Technique- A spring- loaded non-stretchable tape was used to measure the height of the subjects. A convenient flat wall was identified at the clinic site for the measurement of height. The subject was made to stand barefoot with the arms hanging freely by the side. Heels of the feet were placed together with the medial (inner) border of the feet at an angle of 60 degrees. The scapula and the buttock were ensured to be in contact with the measuring wall. The head was held in the Frakfort

plane (with the tragus of the ear and the lateral angle of the eye in a horizontal line). Height was recorded to the nearest 0.1 cm after the subject inhaled fully and maintained the erect position without altering the load on the heels. In this position, a mark was made on the wall and height was recorded with a measuring tape. Two consecutive reading were taken.

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

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

d) Hip Circumference:

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

Computed Anthropometric Indices

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

$$\text{BMI (kg/m}^2\text{)} = \frac{\text{Wt (kg)}}{\text{Ht. (m}^2\text{)}}$$

BMI Cut offs: (2004)

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 (WHO 2004; North American Association for the study of Obesity 2001).

Table 4.4 Revised BMI cut offs:

Classification	BMI (kg/m^2)
Underweight	**< 18.5**
Normal	**18.5 – 22.9**
At risk	**23 – 24.9**
Obese I	**25 – 29.9**
Obese II	**> 30**

f) 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)

It is computed as-

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

Table 4.5 Cut offs for Waist –Hip Ratio (WHR)

	Males	Females
Waist (cms)*	>90	**>80**
WHR > 0.9 **	Obese	**-NA-**
WHR > 0.8 **	**-NA-**	**Obese**

The WHO Asia Pacific criterion for abdominal obesity.

(*National Cholesterol Education Program – Adult Treatment Panel – III Guidelines)

(**Enas, 2005)

Section 4.3.5 Biophysiological Investigations

a) 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 in it (Chatterjee 1976). Sitting blood pressure of subjects was measured using the AND Digital Blood Pressure Monitor UA-767PC (Saitama, Japan) 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).

Table 4.6 Classification of Blood Pressure for Adults

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

(Joint National Committee – VII (JNC VII), 2003)

Section 4.3.6 Biochemical parameters

After the overnight fast, venous blood sample was collected in clean, sterilized vaccutainers and allowed to stand at room temperature for 15 minutes. Serum was immediately separated and stored at -70^0 C temperatures. The blood was then analyzed for Glucose and Total Lipid Profile using standardized kits.

a. Estimation of blood sugar:

It was estimated by the GOD/POD method using an enzymatic kit procured from Glaxo, India. Glucose is oxidised by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of peroxidase (POD) oxidises the chromogen, 4-aminoantipyrine/phenolic compound to a red coloured compound. The intensity of the red colored compound is proportional to the glucose concentration and is measured at 505nm (490-530nm).

b. Estimation of Total Cholesterol

Cholesterol was estimated using enzymatic kits [Chema] by the enzymatic end-point method. Cholesterol esters are hydrolysed to free cholesterol and fatty acid by cholesterol esterase (CHE). The free cholesterol is then oxidised by cholesterol oxidase (CHOD) to cholest-4-en-one and hydrogen peroxide. Liberated hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce quinonimine, a red coloured complex. The intensity of colour produced is directly proportional to the total cholesterol in the sample, which is measured at 500nm (Hg 546nm). The final colour is stable for 60 minutes.

(a) Cholesterol esters $\xrightarrow{CHE}$ Cholesterol + Fatty acids

(b) Cholesterol + O_2 $\xrightarrow{CO}$ Cholesterol 4en-3 one +H_2O_2

(c) $2H_2O_2$ + 4 Aminoantipyrine + Phenol $\xrightarrow{\text{Peroxidase}}$ 5 Quinoneimine + $4H_2O$

c. Estimation of High Density Lipoprotein (HDL)- Cholesterol

Enzymatic kits procured from Glaxo India was used for Direct HDL-cholesterol estimations. The assay consists of two distinct reaction steps. Firstly, cholesterol esterase, cholesterol oxidase and subsequently catalase eliminate the VLDL-cholesterol, LDL-cholesterol and chylomicrons. Secondly, cholesterol ester is hydrolysed by cholesterolesterase to cholesterol and fatty acid. The cholesterol is then oxidised to cholestenone and hydrogen peroxide by cholesterol oxidase. Hydrogen peroxide in the presence of peroxidases reacts with 4-aminoantipyrine and HDAOS to produce a quinone pigment. The intensity of the quinonimine dye produced is directly proportional to the cholesterol concentration when measured at 600nm.

d. Determination of Low Density Lipoproteins (LDL) Cholesterol

The LDL-C values were calculated using the formula.

LDL-C = TC – [HDL-C + (TG/5)]

e. Estimation of Very Low Density Lipoproteins (VLDL) Cholesterol

VLDL-C was calculated by dividing triglyceride values by five (TG/5).

f. Estimation of Triglycerides

Enzymatic kits using GPO-PAP method [Chema] were used for triglyceride estimations. Triglycerides are hydrolysed by lipase (LPL) to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to glycerol-3-phosphate which is oxidised by the enzyme, glycerol-3-phosphate oxidase (GPO) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-aminoantipyrine and p- chlorophenol in the presence of peroxidase (POD) to produce quinonimine, a red coloured complex, which is measured at 500nm (Hg 546nm). The final colour is stable for 60 minutes.

(a) Triglyceride + H_2O $\xrightarrow{\text{Lipoprotein Lipase}}$ Glycerol + Fatty acids

(b) Glycerol + ATP $\xrightarrow{GK}$ Glycerol – 3 Phosphate + ADP

(c) Glycerol-3-PO_4 + O_2 $\xrightarrow{G\text{-}3\text{-}PO_4}$ H_2O_2 + Dihydroxyacetone Phosphate

(d) H_2O_2 + Aminoantipyrine + DHBS $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + H_2O

g. Hematology: Assessment of hematological indices is based on the principle of cell counting and volumetric analysis. White Blood Cell, Red Blood Cell and Platelet are counted and sized by the Electrical Impedance Method. This method is based on the measurement of changes in electrical resistance produced by a particle passing through a high-resolution cylindrical aperture. Electrodes are submerged in the liquid on each side of the aperture to create an electrical pathway. As blood cells are non-conductive, when they pass through the aperture, they cause an increase in impedance, electric impedance directly proportioned to the cell size. Model no. cellenium 19 for was used for Hematocrit analysis. Cellenium 19 samples the

appropriate dilutions prepared from whole blood to count and size WBC, RBC, PLT and to measure HB.

The dilution specimens are aspirated into the RBC and WBC high resolution apertures under negative pressure. The volumetric assembly contains a precision glass tube (volumetric tube) fitted with two optical sensors. This tube ensures that a precise amount of diluted specimens the volume of diluted specimen.

The instrument counts individual cells and sorts them by size. The number of cells counted by the instrument is much greater than the number counted under a microscope, overcoming the unfavorable influence on the counting by human factors, thereby substantially improving the accuracy and reducing the statistical error. The complete blood count test was carried out by a lab technician.

Section 4.3.7 Determination of the gut micro flora

The gut micro flora were determined with respect to the microorganisms- *Lactic acid bacteria, Bifidobacterium* and *E.coli.*

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

a) Collection of the sample
b) Preparation of the sample
c) Sterilization of the glass wares
d) Preparation and Sterilization of dilution blanks
e) Preparation and sterilization of media
f) Preparation of sample for inoculation and incubation of *Lactic acid bacteria, Bifidobacteria* and *E. coli*
g) Enumeration of micro organisms.
h) Calculation for reporting colony counts.
i) Identification of bacterial species

a. Collection of the fecal sample

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

b. Preparation of the sample:

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

c. Sterilization of the glass wares:

All the petridishes and the other glass wares such as beakers and conical flask were sterilized before use. The petridishes were kept in the petridish box and the other glasswares 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 mins at 15 lbs pressure. The other instruments which were used like the weighing balance and spatula were all sterilized by alcohol flaming using 70 % alcohol.

d. Preparation and Sterilization of dilution blanks:

For the preparation of dilution blanks 1 gm of peptone was dissolved in 1000 ml of distilled water. This solution was dispensed in portion of 100 ml in 10 dilution bottles. These were autoclaved at 121^0C for 15 mins. The bottles were cooled at room temperature before putting them to use (Fig 4.5).

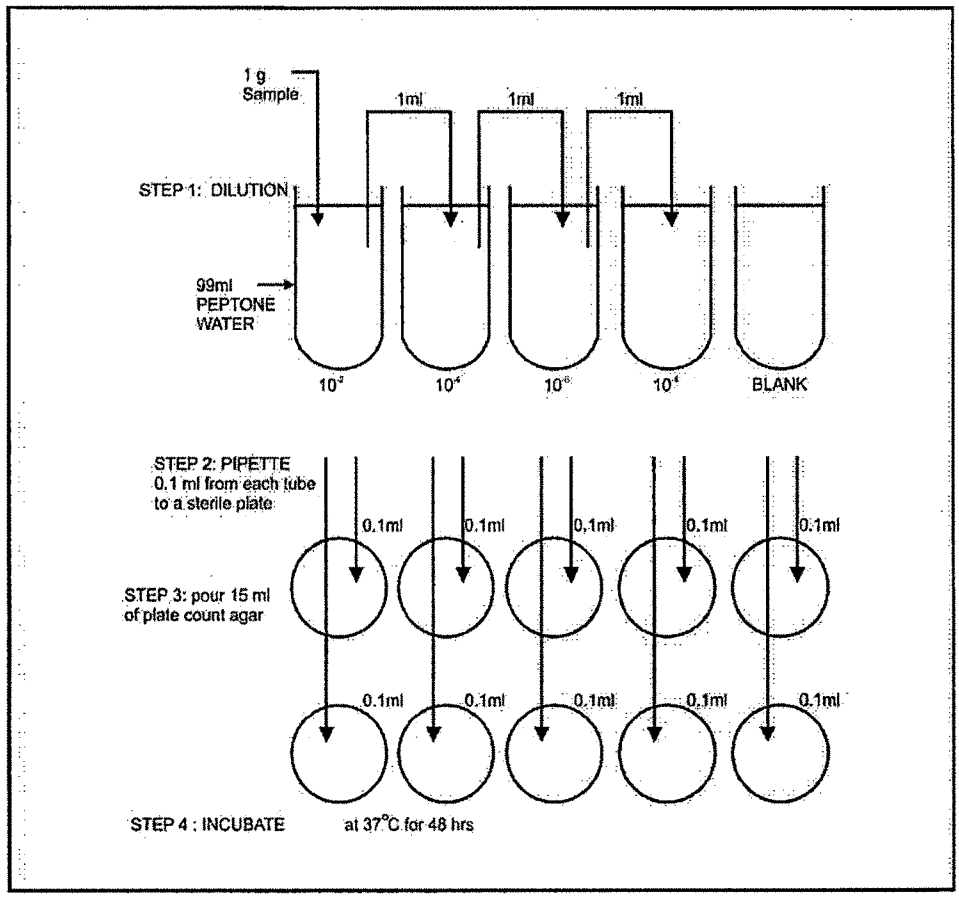

Fig. 4.5: Serial Dilution technique along with Steps of Inoculation and Incubation

e. Preparation and Sterilization of Media

The media used for the enumeration of *Bifidobacterium* was *Bifidobacterium agar* supplied by HiMedia. The prepared media was autoclaved at 121^0C for 15 minutes and then poured into sterile petriplates and was allowed to set.

The enumeration of *Lactic acid bacteria* and *E.coli* was done using ready-*made HiTouch Flexiplates* supplied by HiMedia. HiTouch Flexiplates have ready to use sterile media supplied in flexible disposable plates, 55mm in diameter. It is grid scored on the base and is irradiated to ensure perfect sterility. These plates are specially developed for microbial testing, where not only counts are obtained but it is also possible to select and differentiate between groups of microorganisms (HiTouch Flexiplate series mannual 2007). The Flexiplates were kept inside laminar flow under UV light before using them for inoculation and enumeration of bacteria.

f. Preparation of sample, inoculation and incubation of *Lactic acid bacteria, Bifidobacteria* and *E. coli*

One gram of fresh fecal sample was accurately weighed and homogenized in 99 ml of 0.1% peptone water to provide 1% (wt/vol) fecal slurry. One ml of slurry was diluted serially in peptone water as shown in figure 4.5. Then 0.1 ml of dilution was pipetted from each of the dilutions to the petri plates containing respective medias. The above procedure was carried out inside laminar flow (as shown in plate 4.3 a) that ensures a sterile environment thereby preventing contamination from outside. The plates of *Bifidobacterium* were then incubated at 37°C placed in the anaerobic jar in the incubator (plate 4.4 a) and read after 48 hours. Flexi plates of *Lactic acid bacteria* were placed in a dessicator (plate 4.4 a) as it is a facultative anaerobe and those of *E. coli* were directly placed in the incubator. After 48 hours of incubation the colonies were counted (Appendix VII) and reported (Ramona et al 2001).

f. Enumeration of microorganisms:

The organisms were enumerated using a colony counter and the number of colonies was reported as log values of these colonies per gram of sample (log CFU/gm).

g. Identification of bacterial species:

Species of *Bifidobacterium* and *Lactic acid bacteria* were identified using carbohydrate fermentation kits supplied by HIMEDIA (plate 4.5 a). 2-3 colonies of bacteria were picked by a sterile loop and were diluted in 3ml saline water (1%) to make a homogenous solution. This solution was then pipetted in wells of readymade carbohydrate fermentation kit. Different *lactobacilli* and *bifidobacterium* species have their own identical characteristics to react with specific carbohydrates and produce acid. Using this principle the bacterial species were treated with various carbohydrates and the results obtained were matched with the species identification chart. The species identification is confirmed if the results matched atleast 80% of that mentioned in Bergy's Manual (Buchmann et al 1974).

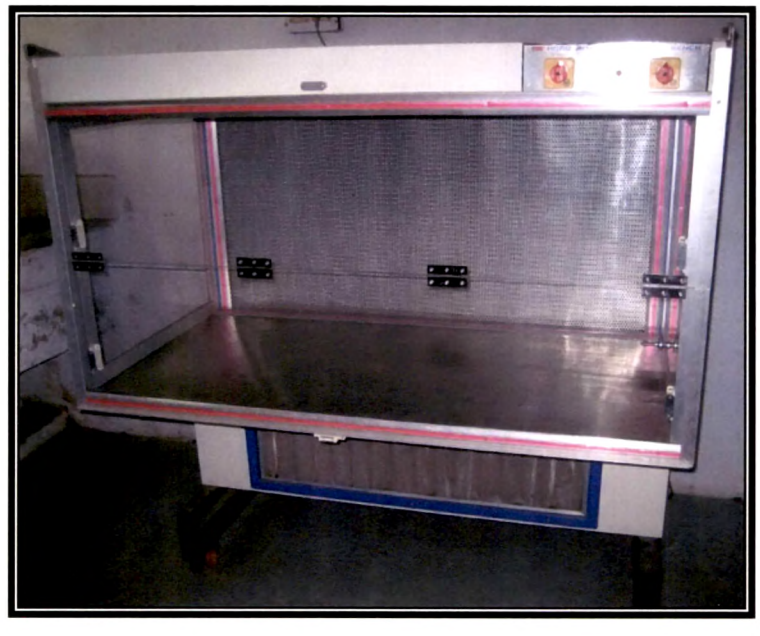

Plate 4.3: Laminar Flow

Plate 4.4: Anaerobic Jar and Dessicator Placed Inside Incubator

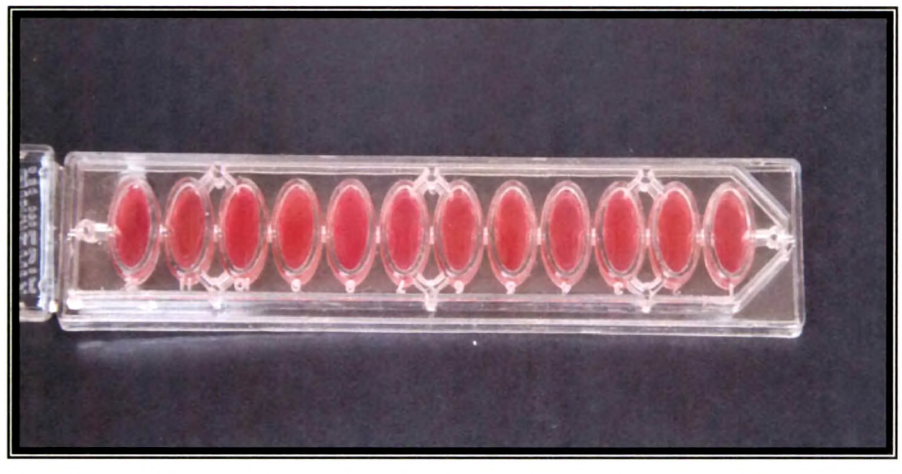

Plate 4.5: Carbohydrate Fermentation Test Kit Used for Identification of Bacterial Species

Section 4.3.8: Supplementation with probiotic and synbiotic fermented milk

Based on the screening of biochemical parameters sixty six subjects were selected and were divided into three groups.

- Group I- Control group:

  The elderly not willing to consume fermented milk formed the control group. This group received no intervention.

- Group II- Probiotic fermented milk supplementation group

  The diets of the elderly subjects were supplemented with 100 g of probiotic fermented milk daily for a period of six weeks. The probiotic fermented milk contained the cultures of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium12* and *Streptococcus thermophilus.*

- Group III- Synbiotic fermented milk supplementation group

  This group was supplemented with 100g of Synbiotic fermented milk (probiotic fermented milk with 15 g of inulin addition).

A fermented milk compliance diary (Appendix VIII) was given to the subjects to keep a record whether they are consuming the fermented milk on a regular basis or not. The compliance diary also consisted of nutrition and health education giving them healthy messages about good dietary habits and physical activity. The subjects were asked to record their fermented milk consumption pattern in terms of:

- Fermented milk taken or not taken
- The amount consumed from 100gms
- Reason for not consuming fermented milk on a particular day

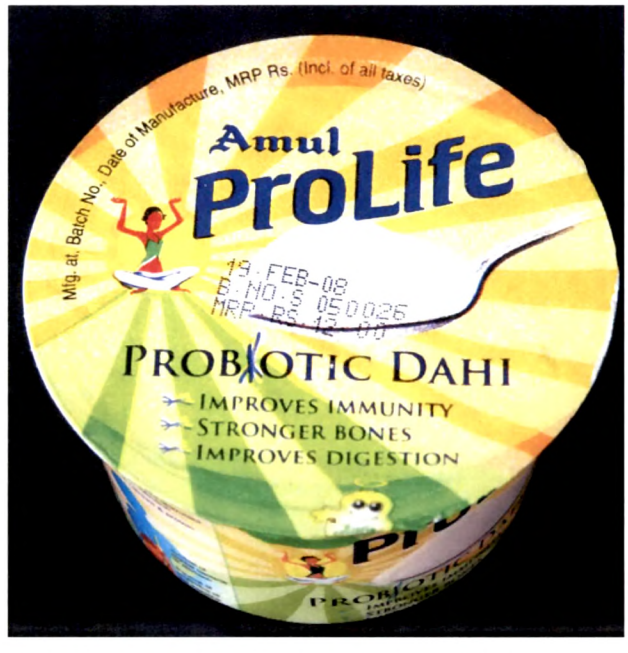

Plate 4.6: Probiotic fermented milk supplemented in diets of elderly subjects

Plate 4.7 a: Method of Distribution of 200 g Probiotic Fermented Milk and Addition of Inulin into 100 g Packaging

Plate 4.7 b: Inoculation of plates with the Sample in Laminar Flow

Plate 4.7 c: Probiotic Bacteria Counting in using Colony Counter

Table 4.7. Nutritional Composition of Probiotic Fermented Milk/100 g

Energy	57 kcal
Total fat	2.5 gm
Saturated fat	1.6 gm
Cholesterol	7 mg
Total carbohydrate	4.5 gm
Protein	4.2 gm
Calcium	183 mg
Phosphorus	158 mg
Sodium	61 mg

Section 4.3.8 Collection of post- intervention data:

The blood and stool samples were collected after the supplementation period of six weeks and analyzed for the various parameters as done at baseline. Data was again taken on their anthropometric measurements, nutrient intakes, fermented milk consumption pattern, disease profile, psychological background, blood pressure and hemoglobin levels of the subjects.

Section 4.4 Statistical Analysis

The data was entered in a computer using an excel spreadsheet. The data was cleaned and verified and subjected to appropriate statistical analysis. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS16.0 version, SPSS Inc.,Chicago, IL,USA.

Mean and standard deviation were calculated. Frequency distribution and percentages were calculated wherever applicable. Paired't' test and 'F' test were done to find out statistical significance between the groups. All tests were considered significant at least at $p<0.05$. Correlation were calculated to find the associations between the various parameters.