CHAPTER 4

METHODS AND MATERIALS

Significant research findings are established on credible methodologies. Under this chapter all methodology and experimental design [Consort] that were used are mentioned and discussed in details to accomplish the objectives of the study.

STAUTORY CLEARANCE FROM ETHICAL COMMITTEE

Before practically implementing the study, the study protocol was approved by the, 'Institutional Ethical Committee for Human Research', Faculty of Family and Community Sciences, The M.S. University of Baroda in compliance with the guidelines issued by Indian Council of Medical Research. The medical ethics approval number obtained was IECHR/2018/16. Since there was no drug involved in this trail, approval from Drug Controller General of India [DCGI] was not need.

Present research "**Role of Fructooligosaccharide, Buttermilk and Biogenic metabolites released from fermented beverage (Ambil) as a communicator between gut and brain**" was conducted in eight phases.

The first phase of the study quantified biogenic metabolites (β Casomorphin and Casoxin c) in ambil using High-Performance Liquid Chromatography. The second phase was the formative research with a cross-sectional design, wherein the presence of mild to moderate depression among the subjects in the age group of 19-30 in the Faculty of Family and Community Sciences the Maharaja Sayajirao University of Baroda, Vadodara was snapshot. The third, fourth, fifth, and sixth phase of the study was a randomized clinical trial, experimental comparative study where, the impact of supplementation with ambil, Fructooligosaccharide (FOS), tetra packed buttermilk and fresh buttermilk was studied in modulating the gut floras (*Lactobacillus, Bifidobacteria* and *E. coli*), checking depression, blood serum cortisol and defecation profile in mild to moderately depressed subjects respectively. In the seventh phase of the present study, it was evaluated which intervention had the highest impact in reversing depression scores, cortisol levels, and modulating gut microbiota composition. In the last phase, an audio-visual animated aid was developed as a means

of IEC material to make general people aware of the increasing trend of depression and how to cope with it.

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

4.0 Experimental design for the study.

4.1 Quantification of biogenic metabolites Casoxin c and ß Casomorphin in prebiotic enriched fermented beverage by high pressure liquid chromatography (HPLC)

4.2 Location of the study

4.3 Screening of the subjects for depression

4.3.1 Interpreting the Beck's Depression Inventory

4.4 Survey

4.5 Dietary intake analysis

4.6 Intervention trials for mild to moderate depressed subjects

4.6.1 Sample size calculation

4.6.2 Study Design for clinical trials

4.6.3 Inclusion and Exclusion criteria to be part of the research

4.6.4 Supplements monitoring plan

4.6.5 Compliance monitoring

4.7 Post data collection

4.8 Procurement of the intervention supplements

4.9 Determination of the Gut Microbiota

4.9.1. Collection and Storage of the fecal sample

4.9.2. Sterilization of the glass wares

4.9.3 Preparation and Sterilization of dilution blanks

4.9.4 Preparation and Sterilization of Media

4.9.5 Preparation and inoculation of sample

4.9.6 Incubation and enumeration of *Lactic acid bacteria*

4.9.7 Incubation and enumeration of *Bifidobacteria*

4.9.8 Incubation and enumeration of *E. coli*

4.9.10 Colony counting

4.10 Assessment of cortisol levels

4.11 Statistical Analysis

4.11.1 Chi Squared [$\chi 2$] Test

4.11.2 Student's 't' test

4.11.3 Analysis of Variance (ANOVA) or 'F' Test

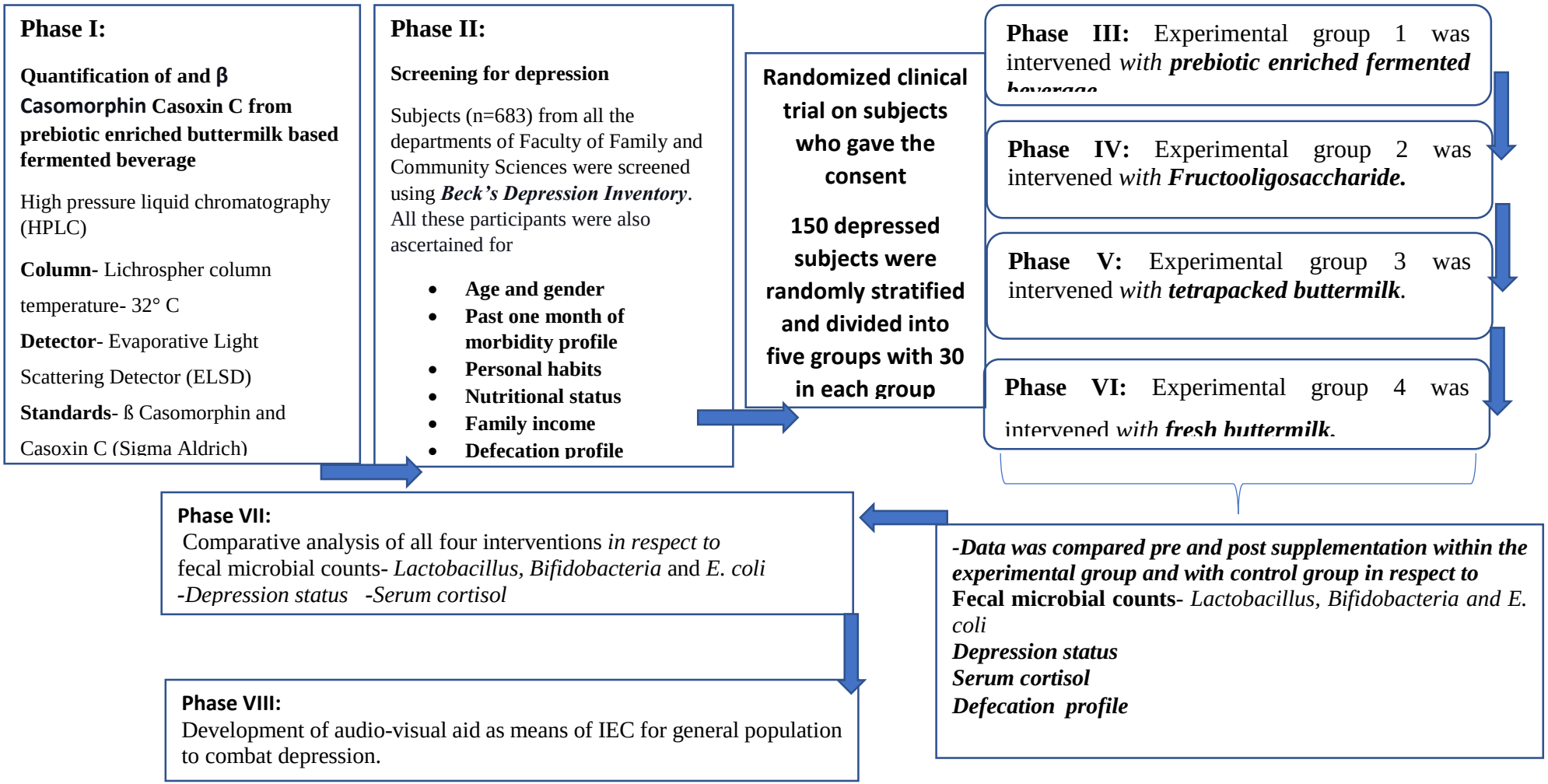
4.11.4 Post Hoc LSD test or Fisher's Least Significant Difference (LSD)

4.11.5 Paired ‘t’ test

4.11.6 Pearson’s correlation

4.12 Development of IEC material

Figure 4.1: Experimental design for the study: 'Role of Fructooligosaccharide, Buttermilk and Biogenic metabolites released from fermented beverage (Ambil) as a communicator between gut and brain'



4.1 Quantification of biogenic metabolites from Fructooligosaccharide enriched buttermilk based fermented beverage

Biogenic metabolites viz. Casoxin c and ß Casomorphin were quantified from ambil (Fructooligosaccharide enriched buttermilk-based fermented beverage) using high-pressure liquid chromatography (HPLC) through Evaporative Light Scattering Detector. The column used was Lichrospher, and the temperature was maintained at 32° C. Standards for ß Casomorphin and Casoxin C were purchased from Sigma Aldrich (Chen and Innis, 2004).

1 **Preparation of mobile phase:** buffer solution was prepared by mixing HPLC grade acetonitrile and water in the ratio 82:18.

2 **Standard solution**- standards of ß Casomorphin and Casoxin C 102.25mg each were dissolved in two separate volumetric flask of 50 ml containing mobile phase maintaining the temperature 20°C.

3 **Sample solution** - Ambil 200ml was poured in rotary flask and allowed to stay in vacuum condition (105°C for 2 hours). Dried sample (100mg) was then dissolved in 50 ml volumetric flask containing mobile phase (buffer solution: HPLC grade acetonitrile and water 82:18 at 20°C)

Calculating the percentage of ß Casomorphin and Casoxin C: Flow rate was adjusted to separate the standards. The instrument was equilibrated by pumping mobile phase through it until a stable baseline was obtained. Peak areas of the sample solution were measured. Chromatograms of the standard and sample solution were recorded. **Percentage of Casoxin C and ß Casomorphin was calculated from the formula:**

Weight of standard/ weight of sample x peak area of sample/ peak area of standard x factor

4.2 Location of the study

Department of Clothing and Textiles, Department of Extension and Communication, Department of Family and Community Resource Management, Department of Food and Nutrition, Department of Human Development and Family Studies in the vicinity of The Faculty of Family and Community Sciences, The MS University of Baroda, Gujarat were purposively selected for carrying out the current research**.** Written permission was sought from the head of respective departments within the faculty. Permission was also granted from the psychologist, MARG Health centre, The MS University of Baroda before initiating the screening process [Appendix I].

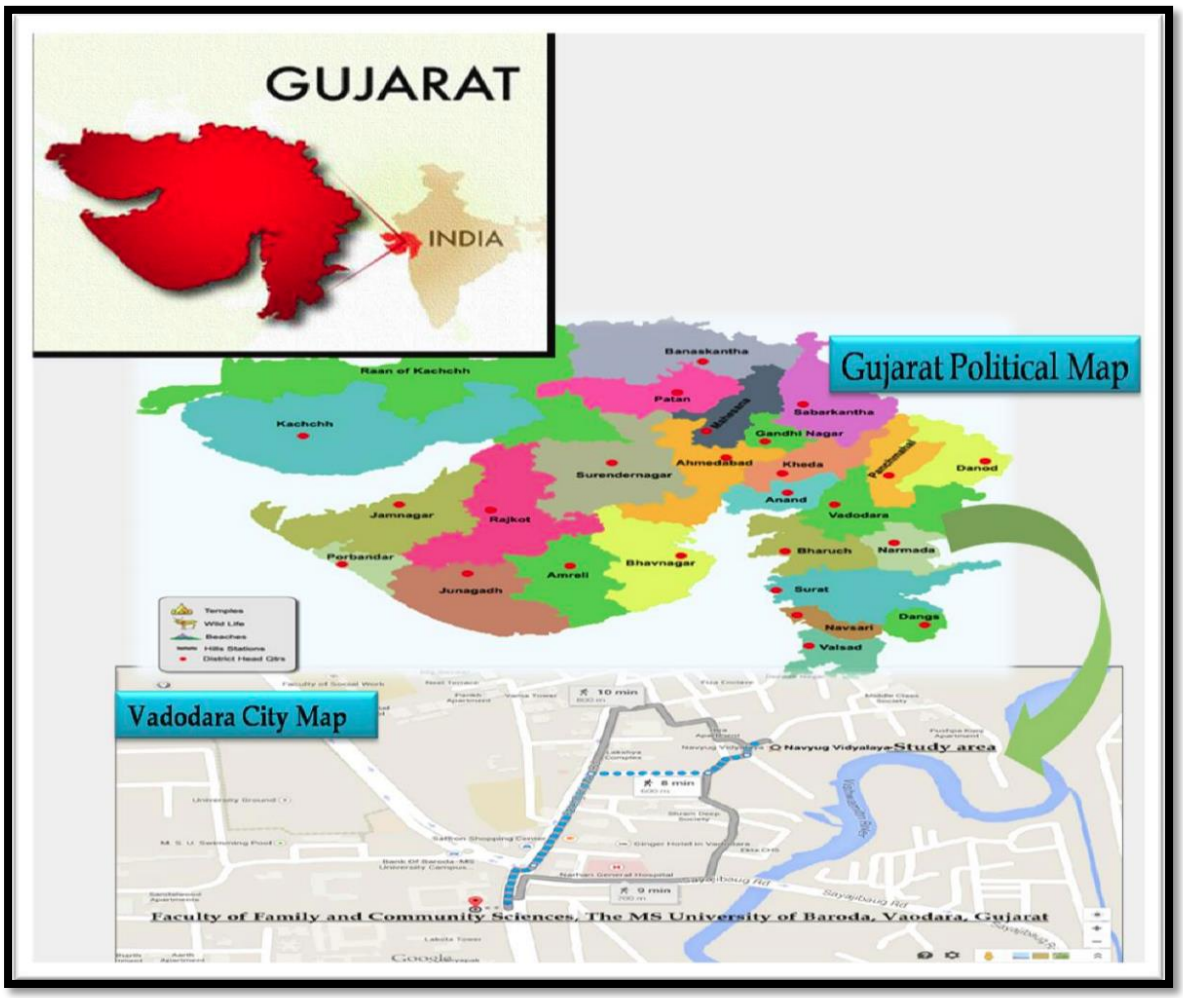


Figure 4.2: Location of the study area

4.3 Screening of the subjects for depression

To understand depression, we need to look into two primary components that is cognitive component [mood] and somatic component [sleep and appetite].

One of the most respected and commonly used instruments for measuring depression is **Beck's Depression Inventory** [BDI] [Appendix II]. BDI consists of 8 cognitive and 13 somatic sub scales for assessment of depression.

Cognitive sub-scale consisted attributes such as pessimism, past failures, guilty feelings, punishment feelings, self-dislike, suicidal thoughts or wishes, self-criticalness, and worthlessness

Somatic sub-scale consisted attributes such as sadness, loss of pleasure, crying, agitation, loss of interest, indecisiveness, loss of energy, change in sleep patterns, irritability, change in appetite, concentration difficulties, tiredness and/or fatigue, and loss of interest in sex (Engstrom, 2018).

Subjects aged 19-30 years who gave the duly filled and signed informed consent [Appendix III] to fill the questionnaire and Beck's Depression Inventory (BDI) were screened using a purposive sampling procedure from The Faculty of Family and Community Sciences, The MS University of Baroda, Gujarat. BDI was administered to 683 subjects to determine the presence of depression. Based on the scores subjects were classified into the normal, mild, borderline clinical, moderate, and severe categories. Participants were further oriented regarding the objective and the implications of the study before assessment.

4.3.1 Interpreting the Beck's Depression Inventory: To evaluate BDI we need to add up the all scores marked by employee for each of the twenty-one questions marked on the right-side of each question. The range of score would be from zero to highest total of sixty-three for the whole test. This would mean that employee either circled zero for all question or rated highest score of three for all twenty-one questions. Score cut-offs for evaluating depression is mentioned in the Table 4.1 below.

Table 4.1: Score Cut-offs for Becks Depression Inventory

Scores	Levels of depression
01-10	These ups and downs are considered normal
11-16	Mild mood disturbance
17-20	**Borderline clinical depression**
21-30	Moderate depression
31-40	Severe depression over
40	Extreme depression

4.4 Survey

A questionnaire-based survey was conducted to get a better picture of the subjects enrolled. The questionnaire collected background information on the following aspects: date of birth, gender, type of family, total family members, total family income, education, past medical history, medication, and hospitalisation in the previous six months.

4.5 Dietary intake analysis

Diet determines different aspects of one's health. 24 hours diet recall (Srilakshmi, 2007) for three days was taken from all the enrolled subjects through self-administered questionnaire [Appendix IV]. Details of all the meals consumed throughout the day was taken into account. Total energy intake, nutrient components such as carbohydrates, protein, fat, iron, calcium, zinc, vitamin C and non-nutrient component such as total dietary fiber were calculated. The cooked value of the food taken in each meal was converted into raw values and then entered in Diet Soft Software (Kaur, 2007) to obtain the nutrient intake of each individual. Frequency of consumption of prebiotic, probiotic and fermented beverages like *buttermilk*, *kadhi*, *chaach*, *curd*, *shrikhand* was also noted and recorded. Details of the number of times the food items were consumed on a daily, weekly, monthly, or yearly basis was elicited using the Food frequency questionnaire (Srilakshmi, 2007).

4.6 Intervention trials for mild to moderate depressed subjects

Shortlisted subjects were intervened with prebiotic and fermented beverages.

4.6.1 Sample size calculation

Assuming that 50% of the population falling in the category of mild to moderate depression have one or other complication and expecting a reduction of 20% in mean depression levels, the required sample size is based on following calculations (Ye and Yi, 2017).

$$\text{Sample size} = (Za)^2 * pq/d^2$$

where, p= 50

q= 100-p, thus q= 50

Confidence interval at 95%

d= 100-B error

= 100-80 =20

therefore, sample size= $(1.96)^2 50*50/(20)^2$

Thus, the required sample size in each arm is 24, considering the dropouts we took 30 subjects in each group.

4.6.2 Study Design for clinical trials

Systemized Random Sampling was used to segregate mild to moderately depressed subjects into five experimental groups with 30 individuals in each supplementation group.

4.6.3 Inclusion and Exclusion criteria to be part of the research

To be the part of any supplementation group subjects had to fulfil certain criteria. Inclusion criteria for the research participants were BDI score between 11 to 30 and the written consent to participate in the study.

Subjects reporting history of severe medical conditions mentioned in figure 4.2 and frequent consumption of prebiotics, probiotics and fermented beverage which was taken into account through health and lifestyle questionnaire were excluded from the

study. Those who fell in the category of severe depression (scored above 30 on BDI) were asked to seek expert help and given reference of the psychologist, MARG Health centre, The MS University of Baroda. Subjects were encouraged to report if they feel uneasy or start any medication during the course of the study. Table 4.2 portrays Inclusion – Exclusion criteria for selection of research subjects.

Table 4.2: Inclusion – Exclusion criteria for selection of research subjects

Inclusion criteria	Exclusion criteria
•Age group of 19-30 •Scored between 11-30 on BDI •Written consent for participation in the research	•Scored more than 30 on BDI •Hypertension • Diabetes mellitus • Cardiovascular Disorder • Thyroid Hormone Disorder • Valve Replacement Surgery • Gastric surgery or Perforation • Renal Disorder • Cancer / AIDS • Heavy physical activity • Weight loss regime • Subjects on any mental therapy •Hospitalization in last six months •Reported frequent consumption of pre/probiotic •Reported consumption of fermented beverage on daily basis

4.6.4 Supplements monitoring plan

Ambil 200 ml, liquid FOS 10 ml, tetrapacked buttermilk 200 ml and fresh buttermilk 200 ml were intervened to different experimental groups for 45 days respectively. No intervention was given to control group.

Subjects who were supplemented with ambil and tetrapacked buttermilk were given seven packets every week consecutively for 6 weeks in a row. Subjects in the experimental group on fresh buttermilk intervention were asked to collect buttermilk daily from the mess, where the record book was maintained. FOS was supplied in 500 ml food grade plastic container.

4.6.5 Compliance monitoring

Compliance was monitored with appropriate follow ups using daily SMS reminders and phone calls fortnightly. A compliance calendar [Appendix V] was especially designed and distributed to all the participants to document daily consumption of supplements and any unusual symptoms or side effects if observed. Calendars were collected back after the completion of intervention period. All the participants were required to send daily picture of themselves consuming the product and return the empty cartoon post intake.

4.7 Post data collection

Post data was collected on the parameters similar to the baseline i.e., Beck's Depression Inventory, serum cortisol, defecation profile in terms of stool frequency and gut microflora (*Lactobacillus, Bifidobacteria* and *E. coli).* Figure 4.4 represents experimental design for randomized control trial.

4.8 Procurement of the intervention supplements

Ambil

Ambil used for supplementation was made up using rice powder and buttermilk enriched with fructooligosaccharide following standardized recipe (Ramakrishnan; 1979).

Fructooligosaccharide

Liquid fructooligosaccharide (25 litres bottle; Type L95; Batch No. FL915071-T) was procured from Tata Chemicals Limited -Innovation Centre, Mumbai.

The fructooligosaccharide was food grade which can be used as fat and sugar replacer.

Specifications of the fructooligosaccharide are given in Table 4.3

Buttermilk

Buttermilk 200ml tetrapacked were procured from Amul, Gandhinagar. Fresh buttermilk was obtained from Paras, Vadodara Dairy Baroda.

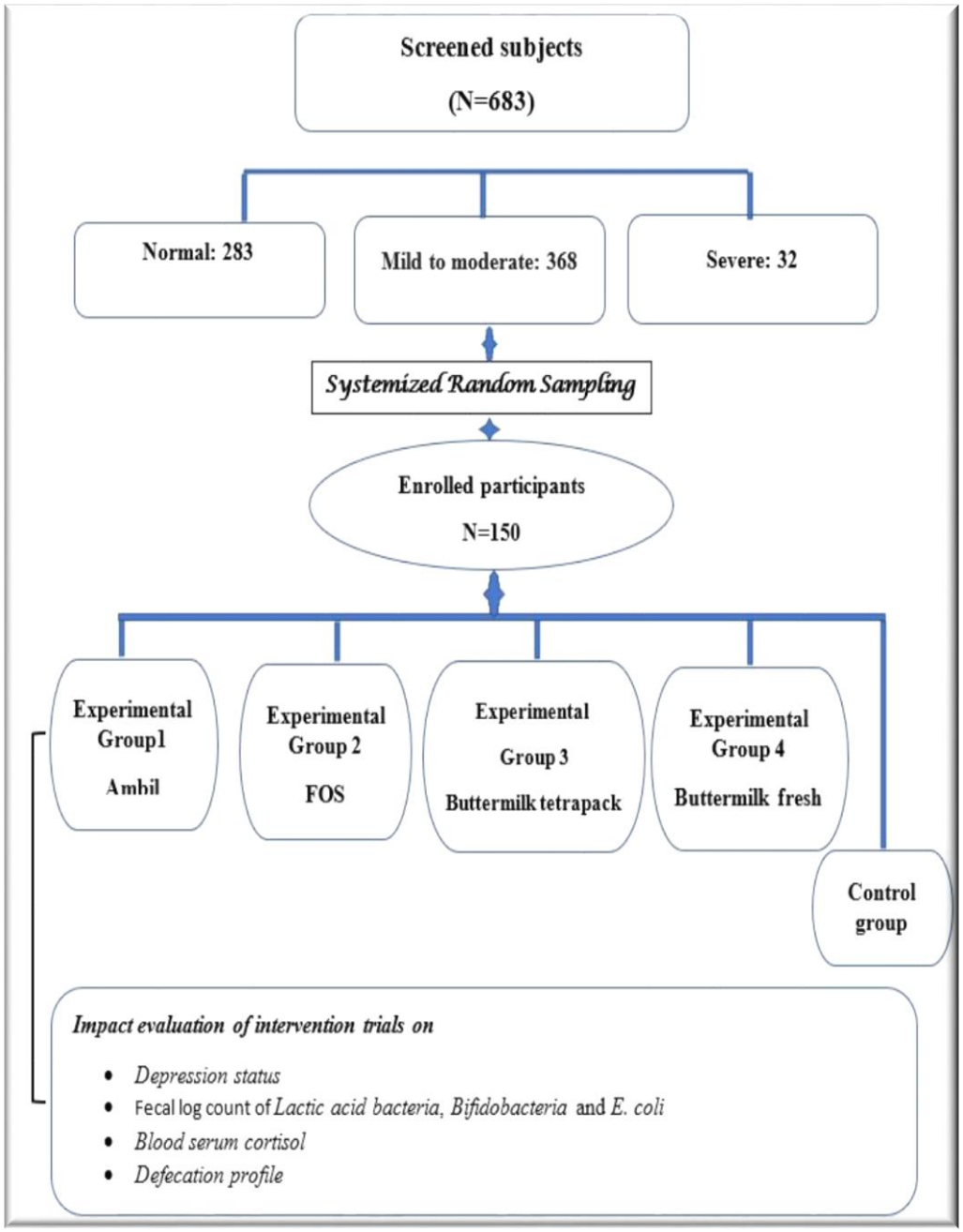


Figure 4.3: Experimental design for randomized control trial

Table 4.3 Specifications of Fructooligosaccharide used for supplementation

Items		Product Code - Fossence(L-95)
		Specifications
Sensory	Appearance	Clear to light yellow translucent and sticky liquid
	Insoluble	No visible insoluble
	Smell	Special FOS smell
	Taste	Good sweet taste and no odor
Physicochemcial Index	Dry matter (Solid material) % ≥	75
	pH value	5.5~7.5
	Total FOS (on dry matter basis) (%)≥	55
	Ash(%)≤	0.4
	Transmittance(%)≥	85
Hygienic Index	Arsenic(mg/kg) ≤	1.0
	Lead(mg/kg) ≤	5
	Total bacteria number(cfu/g) ≤	1000
	Coliforms （MPN/100g） ≤	30
	Pathogenetic germs (Salmonella, Shigella, Staphylococcus)	None

Plate 4.1: Ambil supplemented to experimental group

Plate 4.2: Packing of Fructooligosaccharide for distribution

4.9 Determination of the Gut Microbiota

The gut microbiota was determined with respect to fecal- ***Lactic acid bacteria, Bifidobacteria*** and ***E. coli*** (Ramona et al., 2001).

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

4.9.1. Collection and Storage of the fecal sample

4.9.2. Sterilization of the glass wares

4.9.3 Preparation and Sterilization of dilution blanks

4.9.4 Preparation and Sterilization of Media

4.9.5 Preparation and inoculation of sample

4.9.6 Incubation and enumeration of *Lactic acid bacteria*

4.9.7 Incubation and enumeration of *Bifidobacteria*

4.9.8 Incubation and enumeration of *E. coli*

4.9.10 Colony counting

4.9.1 Collection and Storage of the fecal sample

The subjects were given air tight sterile clinicols which were labelled properly to avoid inconvenience, for collection of their stool samples. They were asked to bring their stool samples just before coming to department. Samples collected were immediately stored at appropriate temperature (-20°C) in deep freezer and were analyzed within 2 days after collection.

4.9.2 Preparation of the sample

One gram of faecal sample was weighed on a sterilized balance and was used for bacterial enumeration.

4.9.3 Sterilization of the glass wares

All the petri dishes and the other glass wares such as beakers and conical flasks were sterilized before use. The petri dishes were kept in the petri dish box and the other glassware's were kept in a hot air oven at 180^0C for 2 hours for sterilization. The micro-tips were decontaminated by autoclaving at 121^0C for 15 minutes at 15 lbs. pressure. The other instruments which were used like the weighing balance and spatula were all sterilized by alcohol swabs and alcohol flaming using 70 % alcohol. Sterile petri-plates were used and opened under the laminar only at the time of plating.

Plate 4.3: Sterilization of Petri plates in hot air oven

Plate 4.4: Sterilization of dilution bottles in autoclave

4.9.4 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 [IGNOU, 2005].

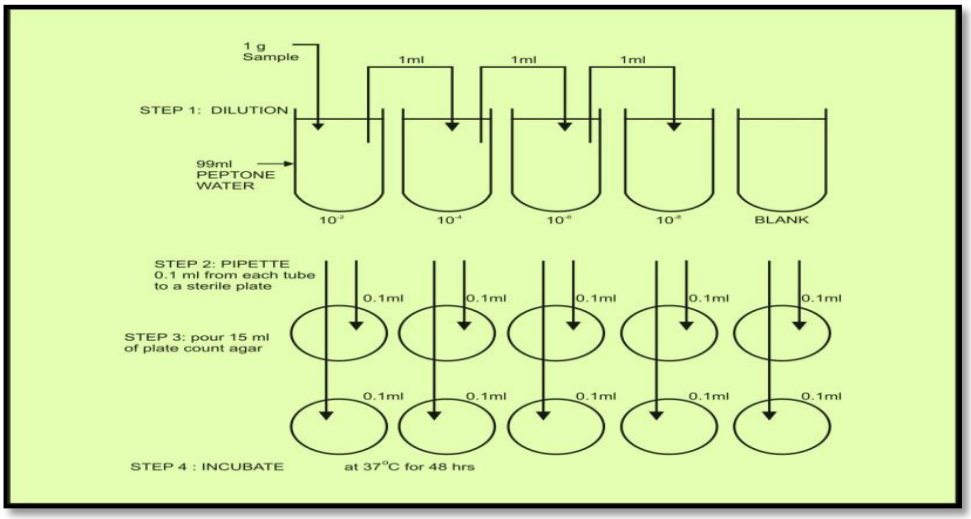


Fig. 4.4: Serial Dilution technique along with steps of Inoculation and Incubation

Plate 4.5: Sterile bottles filled with peptone water used for serial dilution

4.9.5 Preparation and Sterilization of media

The media used for the enumeration of *Bifidobacteria* was Bifidobacterium agar, MRS Lactic acid bacillus agar and Violet Red Bile Agar was used for *Lactic acid bacteria* and *E. coli* respectively (Table 4.1). Media were supplied by Hi. Media. The prepared media of Bifidobacteria and LAB were autoclaved at 121ºC for 15 minutes whilst EMB agar was prepared just before pouring. The prepared media were then poured into sterile petri-plates and allowed to set inside the laminar flow under UV light. Media used for different gut flora is presented in Table 4.4

Table 4.4: Details of media used in determining the gut flora of the subjects

Gut Flora	Incubation Condition	Incubation Duration	Temperature	Media Used (Brand)
Bifidobacteria	Inside anaerobic jar with an anaerobic gas pack	48 hours	37ºC	Bifidobacterium Agar (Hi. Media®)
Lacticacid bacteria	Inside dessicator with calcium carbonate	48 hours	37ºC	MRS Lactic acid bacillus agar (Hi. Media®)
	Inside incubator	24 hours	37ºC	Violet Red Bile Agar (Hi. Media®)

a) Violet Red Bile Agar

b) MRS Lactic acid bacillus agar

c) Bifidobacterium agar

Plate 4.6: Preparation of media

Plate 4.7: Prepared media ready to get in water bath

4.9.6 Preparation and inoculation of sample

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 using stomacher and 0.1 ml of sample was pipetted from each of the dilutions to the petri plates containing respective media. The above procedure was carried out in duplicates for each sample inside laminar flow that ensured a sterile environment thereby preventing contamination from outside.

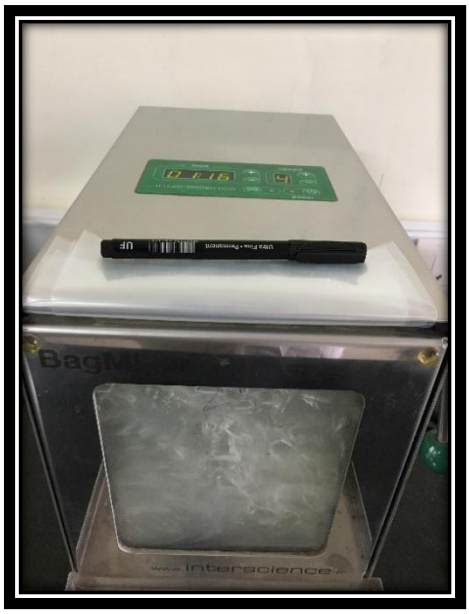


Plate 4.8: Mixing of stool sample with peptone water in stomacher to form slurry

Plate 4.9: Inoculation of bacterial culture in laminar flow

4.9.7 Incubation of Lactic acid bacteria

Petri plates of Lactic acid bacteria were placed in a desiccator as it is a facultative anaerobe, and the desiccator were then placed in the incubator at 37ºC for 48 hours of incubation.

4.9.8 Incubation of Bifidobacteria

The plates of Bifidobacteria were incubated at 37ºC placed in the anaerobic jars using anaerobic gas-packs procured from Hi. Media® for 48 hours.

4.9.9 Incubation of E. coli

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

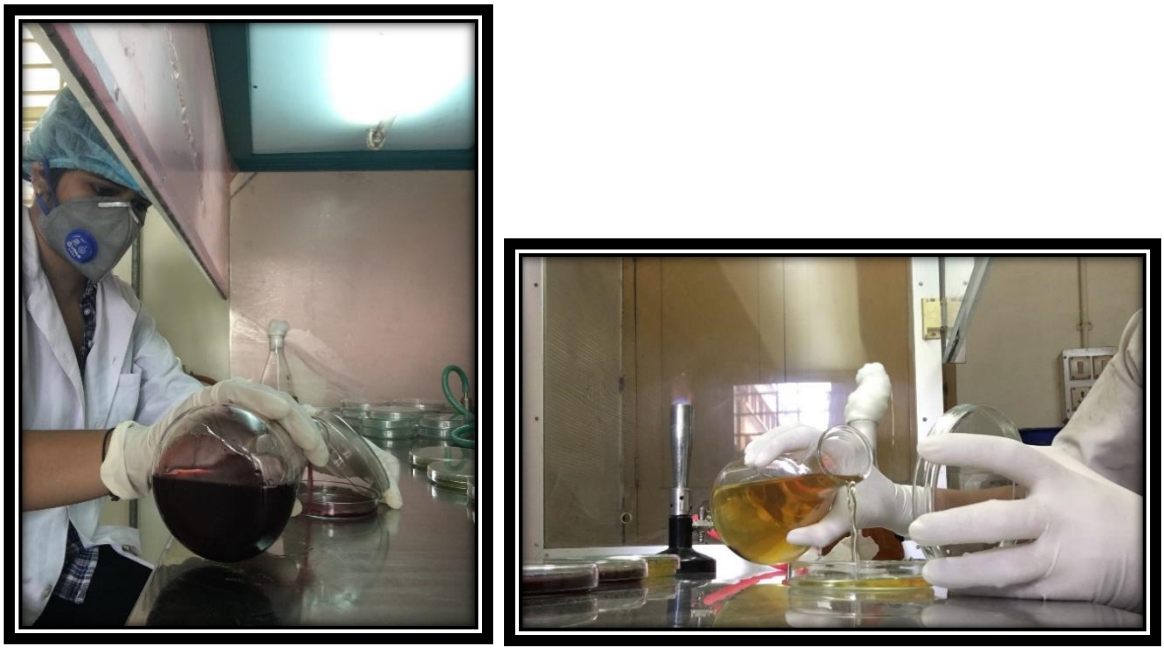


Plate 4.10: Setting of media using Pour Plate Technique

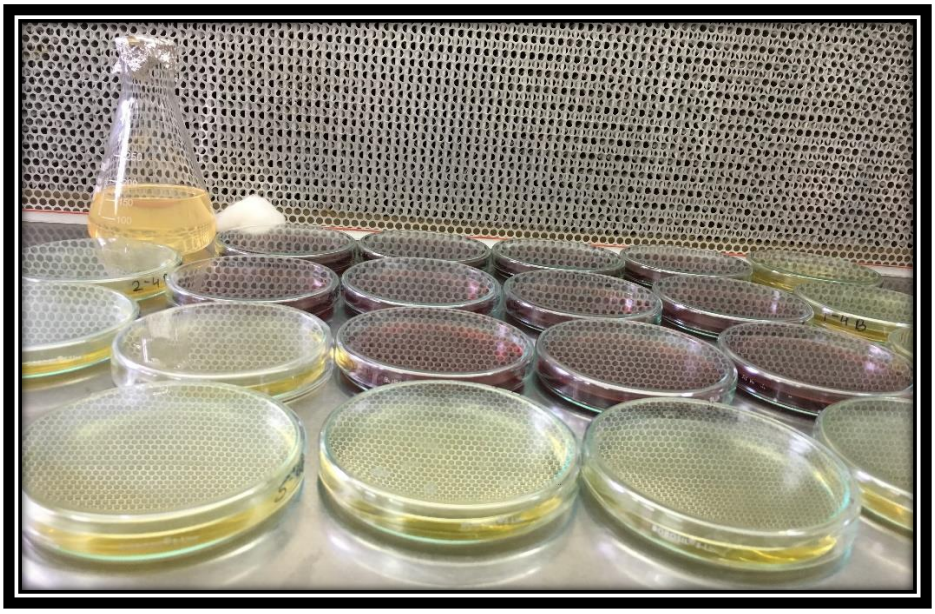


Plate 4.11: Petri plates containing media and inoculated bacteria

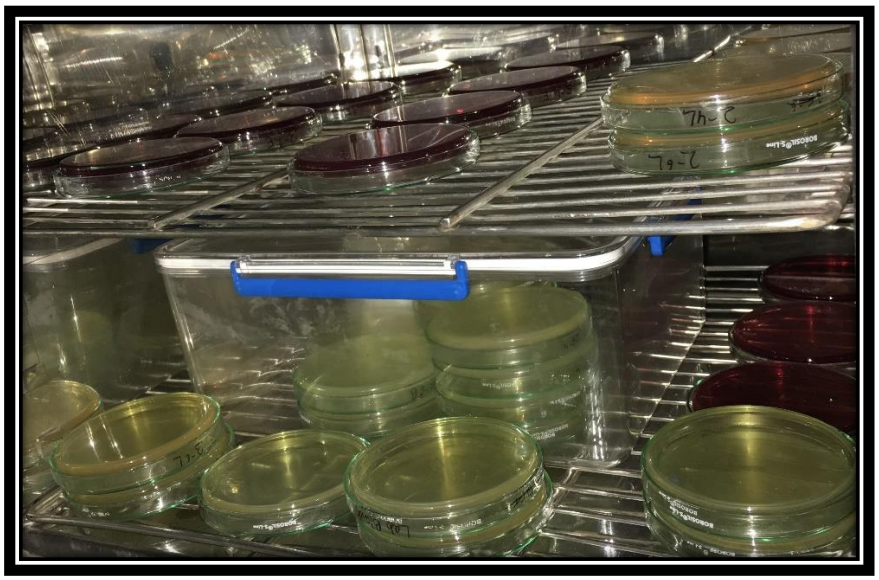


Plate 4.12: Anaerobic Jar and Desiccator Placed inside Incubator with plates inoculated with fecal samples for determination of *Bifidobacteria, Lactobacilli and Enteric pathogen*

4.9.10 Colony counting

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

4.10 Assessment of cortisol levels

Cortisol levels were determined in blood serum, by an expert lab technician.

- The flow of blood in the arm was stopped by wrapping an elastic band around the upper arm, which made the veins in arm to become more visible and easier to insert the needle.
- Alcohol was used to clean the site on the skin where the needle was inserted. The needle was inserted into the vein, and blood was collected in a tube that's attached to the needle.
- After the needle was removed from the skin, cotton or gauze was placed on the site of the needle insertion. A bandage was used to secure the cotton or gauze.
- Blood was drawn into plain tubes, preserved freezed at −4°C and then separated for subsequent measurement of cortisol in serum with the IMMULITE. The volume of serum required was 10 µl. Cortisol was determined at pharmacological lab from blood serum by Chemi Luminescent Immuno assay in ROCHE machine.

Plate 4.13: Collection of blood sample by an expert lab technician for cortisol analysis

To provide evidence of significance of the data observed during the course of research, statistical analysis is an inevitable tool. Present research was validated using analytical software. The data was entered in an excel spreadsheet (MS, 2007). The data was cleaned, verified, and subjected to appropriate statistical analysis using Statistical program for social sciences (Trial version 20.0).

4.11.1 Chi Squared [χ2] Test

Chi-square is a versatile statistical test used to examine the significance of relationships between two (or more) nominal-level variables. It is a non-parametric test of significance and therefore used for all the qualitative data in the present research. The significance levels were set at 95% by two sided tests. Chi square, in the present research was utilized to determine the significance of difference based on gender, age, religion, education, family type and income and to determine the significance of difference in the incidence of stool frequency in different categories of depression.

4.11.2 Student's 't' test

Student's t-test was applied in order to find out the significance of difference in parameters of two different groups. The significance levels were set at 95% by two sided tests. Independent t' test is used in the present research for:

Determining the significance of difference in the mean counts of depression scores, serum cortisol, gut microflora, and defecation profile of subjects in experimental groups and control group.

4.11.3 Analysis of Variance (ANOVA) or 'F' Test

Analysis of variance (ANOVA) is a collection of statistical models used in order to analyse the differences between group means and their associated procedures (such as "variation" among and between groups), developed by R. A. Fisher. In its simplest form, ANOVA provides a statistical test of whether or not the means of several groups are equal, and therefore generalizes the t-test to more than two groups.

4.11.4 Post Hoc LSD test or Fisher's Least Significant Difference (LSD)

Post hoc LSD was applied in the present research to determine the significance of multiple intervention on depression scores, serum cortisol, gut microflora, and

defecation profile and to rule out which intervention was most effective.

4.11.5 Paired 't' test

A paired t-test measures whether means from a within-subjects test group vary over two test conditions. The paired t-test is commonly used to compare a sample group's scores before and after an intervention. The phases of the study involve analysing the impact of Ambil, FOS and buttermilk to mild to moderately depressed subjects for which this test was applied and the significance levels were set at 5% by two sided tests in order to:

Determine the significance of difference obtained before and after the supplementation in the depression scores, serum cortisol, gut microflora, and defecation profile.

4.11.6 Pearson's correlation

Test was carried out in order to determine if there exist an association between grades of depression, cortisol levels, nutritional status, gut microbiota and defecation profile of subjects enrolled in the research.

4.12 Development of IEC material

Animated movie was developed using the software *Animaker*. Movie script was written keeping in mind the need to create awareness among general population about depression and how to overcome it. Different characters were allotted and dialogues were recorded and synchronized with particular scenes. The movie is named 'RESTART' which aim to ring the wake-up call to restart with new hope, new strength and new thought to overcome depression.