MATERIALS AND METHODS

CHAPTER 4

MATERIALS AND METHODS

Interest in the significant nutritional functions of prebiotics as functional food ingredients has grown over the last decade. Galactooligosaccharide (GOS), is one of the emerging prebiotics naturally present in mammalian milk that might have the capacity to produce beneficial intestinal bacteria conferring potential health benefits, including improved gastrointestinal health. The present study was undertaken to study the "**Presence of Functional Constipation in the Teaching staff of The M.S. University of Baroda and Impact Evaluation of Supplementation of Galactooligosaccharide (GOS) added Gummies on their Gut Health and Constipation Profile – A Randomized Double Blind Placebo Control Trial"**. This chapter outlines the experimental design and discusses the methods and materials used to fulfil the objectives of the study in the following three phases (Table 4.1).

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Screening and identification of functional constipation in University Teaching staff

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Section 4.3.3 Selection of the subjects for the study

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Section 4.3.5 Study protocol

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4.3.7.3 Short chain fatty acid analysis

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Table 4.1: Experimental Design for the study

Phase 1 *Screening for the presence of constipation in the teaching staff of The M. S. University of Baroda*	Phase 2 *Development, Acceptability Trials of GOS addition to standard gummies at varying levels and its shelf life studies*	Phase 3 *Impact evaluation of GOS added gummies on Constipation, Depression, Gut health and Quality of Life of constipated subjects*
Snapshotting the presence of constipation (n=364) - General information - Medical history - Family History of constipation - Personal habits/ Addictions - Physical activity - Perceptions and practices - Constipation Profile ▪ WHO criteria, 1996 ▪ Rome IV criteria, 2016 ▪ Bristol stool scale,1997 - Chrono nutrition profile (Alison 2018) - Frequency of consumption of selected food groups (Krause 2016) - Dietary intake (Krause 2018)	Trained panel (n=8) was recruited for sensory evaluation - Standardisation of gummies ▪ Water (65ml, 75ml, 85ml) ▪ Sugar (50g, 60g, 70g, 80g) ▪ Agar (2g, 2.5g, 3g, 3.5g) ▪ Citric acid (0.48g, 1.9g, 3.8g, 5g) ▪ Sugar substitution with GOS (60%, 80%, 100%) ▪ Addition of sucralose (5g, 5.5g, 6g) - Physico-chemical Properties of GOS gummies ▪ Color intensity ▪ pH/ Acidity ▪ Texture profile ▪ Moisture analysis - Recovery of GOS in 100% GOS gummies - Shelf Life studies of GOS gummies on Day 0, 30, 60, 90 days at 37^{o}C ▪ Sensory properties ▪ Microbial properties (Refai and FAO, 1979). o TPC o Yeast and molds o *E.coli*	Studying the impact evaluation of GOS added gummies (n=48) ▪ Randomised Double blind placebo control technique - Constipation Profile - Depression profile (Becks Depression Inventory 1996) - Quality of life profile (PACQOL, 2004) - Gut Microbiota profile for the following genus & phyla (Krumbeck et al, 2018) ▪ Bifidobacteria ▪ Lactobacillus ▪ Clostridium ▪ Bacteroides ▪ Firmicutes phyla ▪ Bacteroidetes phyla - Short chain fatty acid (SCFA) profile ▪ Acetic acid ▪ Butyric acid ▪ Propionic acid

Phase I
Screening and identification of Functional Constipation in University teaching staff

4.1.1 Statutory clearances

The study protocol was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda and provided the approval number IECHR/ FCSc/PhD/2021/3. Written consent was obtained from the participants who agreed to give baseline information through questionnaires. (Appendix I)

4.1.2 Sample size calculation

The sample size was calculated by a statistics professor in the Department of Statistics, The Maharaja Sayajirao University of Baroda. The sample size calculation is as follows:

$$\frac{Z^2_{\alpha/2} * N * p * q}{e^2 * (N-1) + Z^2_{\alpha/2} * pq}. \quad = \mathbf{241}$$

where,
p = Proportion of constipation = 0.092
q = Proportion of not constipation = 0.908 (Ref: Katusiha et al, 2020)
$Z_{\alpha/2}$ = The value of the standard variate at confidence interval (99%) = 2.576
e = Margin of error = 0.05
Attrition rate=20%

4.1.3 Selection of the subjects for the study

This phase of the study involved screening of teaching staff (n=364) of The M.S. University of Baroda aged between 25-62 years using a cross-sectional study design and purposive sampling technique. Those who met the inclusion criteria of giving their voluntary participation via the informed consent form were requested to fill the validated pretested questionnaires.

4.1.4 Validation and pre-testing of questionnaires

The questionnaire was validated by a gastroenterologist and a general practitioner before pre-testing. It was then subjected to the teaching staff bearing questions on their general information, self and family medical history, personal habits, physical activity, chrono nutrition profile,

perceptions and attitudes related to constipation, dietary practises, food frequency profile and their constipation status.

After obtaining the validation, the questionnaire was sent to 10% of the sample size calculated for this phase for its pre-testing. The suggestions received were incorporated before final data collection.

4.1.5 Administration of validated pretested questionnaires

The pre tested validated questionnaires were administered either via online platform (google forms) or as face to face interviews to collect the baseline information of the subjects. (Appendix I). Online platform was used as the teaching process was on hybrid mode due to the presence of covid pandemic (2020-2021). The questionnaires had the following parameters, explained in detail below:

4.1.5.1 General Information of the subjects

General information of the subjects was collected with regards to their age, sex, type of family, educational level, family income, personal habits, physical activity profile, self-assessed medical history and family history of constipation.

4.1.5.2 Assessment of functional constipation of the subjects

To assess the presence and degree of FC, three tools were used

a) Criteria laid down by World Health Organisation of <3 stools/week (WHO, 1996)
b) Rome IV Criteria (2016) as per The Rome Foundation (Fig 4.1A)
c) Type of stool as per Bristol stool chart (1997) (Fig 4.1B)

The subjects were scored on a scale of 20 to determine the presence of FC. The process is detailed in Fig 4.1C. Presence of <3 stools/ week was scored 4. The categories in Rome IV criteria (with 6 questions) of always, sometimes and never were allotted a score of 2, 1 and 0 respectively. Type of stool was also allotted a score of 4. Hence the total of 8 questions were administered on a scale of 20.

Further, to study the severity of FC among the subjects this score obtained was categorized into 3, mild, moderate severe with ranges of 6-10, 11-15 and 16-20 respectively.

Criteria for Functional Constipation Diagnosis

Onset of constipation symptoms at least 6 months before diagnosis
Below criteria met for the past 3 months

I. Two or more of the following criteria must be present:
   a. Straining with >25% of defecations
   b. Lumpy or hard stools with >25% of defecations
      i. Bristol stool form types 1 and 2
   c. Sensation of incomplete evacuation with >25% of defecations
   d. Sensation of anorectal obstruction/blockage with >25% of defecations
   e. Manual maneuvers required with >25% of defecations
      i. Eg, digital evacuations, support for the pelvic floor
   f. Fewer than 3 spontaneous defecations per week
II. Loose stools are rare without administration of laxatives
III. Insufficient criteria for irritable bowel syndrome

Adapted from Lacy BE, Mearin F, Chang L. *Gastroenterology.* 2016;150(6):1393-1407.

Fig 4.1 : (A) Rome IV Criteria

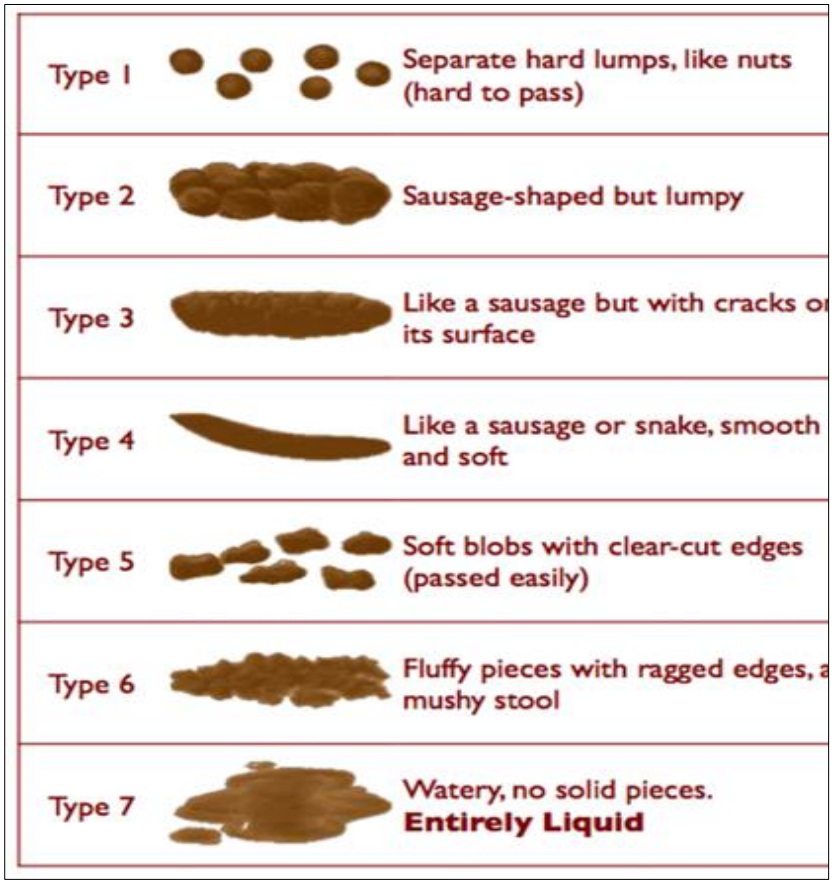


Fig 4.1 (B) Bristol Stool Chart

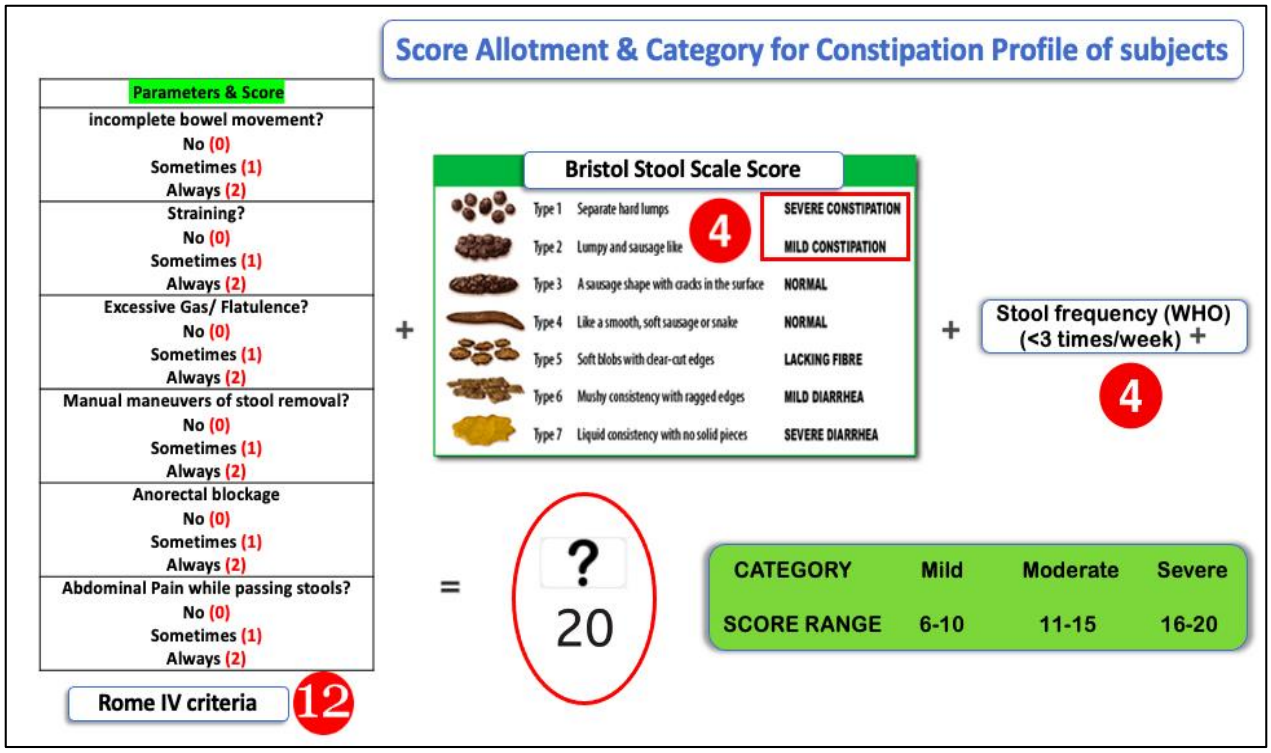


Fig 4.1 (C): Scoring and Categorisation for constipation profile

4.1.5.3 Assessment of chrono nutrition profile of the subjects

Chrono nutrition profile was studied in terms of a free day and weekend. With respect to the chrono nutrition profile, questions on six components (Fig 4.2) were administered which included interval between first and last meal, last meal and sleep onset, frequency of eating at night and skipping breakfast and the largest meal consumed daily. The responses for these parameters were scored as shown for a free day, weekend, and weekday. Appropriate scoring was allotted (Fig 4.2) to obtain the total score in free day and weekend.

Chrononutrition behavior descriptions and scoring cutoffs for the Chrononutrition Profile

Chrononutrition Cutoff	Description	Format	Scoring Cutoffs (Poor; Fair; Good)
Eating Window	Duration between first eating event and last eating event	HH:MM	> 14:00 12:01 to 14:00 ≤12:00
Breakfast Skipping	Frequency of breakfast skipping	Days/Week	≥ 4 days/week 2-3 days/week 1 day/week or less
Evening Latency	Duration between last eating event and sleep onset	HH:MM	≤2:00 2:01 to 6:00 >6:00
Evening Eating	Risk of eating late in the waking day	HH:MM	≥23:00 20:00 to 22:59 < 20:00
Night Eating	Frequency of night eating	Days/Week	≥ 4 days/week 2-3 days/week 1 day/week or less
Largest Meal	Meal in which largest amount of food is eaten	Meal Name	Dinner/Supper Lunch Breakfast

Note. Diary data should be averaged across data collection period. Questionnaire data should be weighted for weekdays and weekends.
Note. Poor values assigned a score of 2; fair values assigned a score of 1; good values assigned a score of 0.
Note. When calculating risk for breakfast skipping and night eating frequency as reported in the diary, we recommend calculating the proportion of days missed. ≥57% should be coded as 2, 28.5% to 42.8% should be coded as 1, and ≤14.2% should be coded as 0.

Fig 4.2: Parameters for assessing Chrono nutrition profile (Allison 2018)

4.1.5.4 Assessment of Dietary profile of the subjects

The dietary profile included questions on dietary practices including the dietary habits, frequency of chewing, their food frequency profile with respect to fibrous and non-fibrous foods, processed foods, water, salt and sugar intake.

A subset of the population who were suffering from functional constipation (n=40) as per the above mentioned profile were subjected to a 24-hour diet recall for 3 consecutive days at baseline to assess their dietary intake with respect to estimated average allowance (EAR) and recommended dietary intake (RDA) guidelines as per NIN ICMR.

4.1.6 Statistical analysis

The data obtained was entered in google spreadsheet. The data was cleaned and subjected to appropriate statistical analysis. Data analysis was performed JASP software (MAC version) 2022. Results were expressed in terms of mean values, number percent and standard deviation for the variables. Pearson correlation (r) was used to assess the associations between constipation status, medical profile, personal habits, physical activity status, dietary practises, type of diet consumed, food frequency profile and their nutritional profile with respect to the macronutrients and fiber intake. Chi square test ($\chi 2$) was used to assess the association between the constipation status and chrono nutrition profiles on a free day, working day and holiday and food their frequency profile. The levels of significance (p) were assessed at values of 0.05, 0.01 and 0.001.

Phase II Development of Galactooligosaccharide (GOS) added gummies and study their acceptability Trials and shelf life studies

4.2.1: Procurement of Galactooligosaccharide (GOS) and other raw materials

Galactooligosaccharide (95%) with the necessary certifications and toxicological testing was purchased from Tata Chemicals, Pune. All additional ingredients used for the preparation of gummies (agar, sugar, citric acid, sucralose, natural colours and flavours) were FSSAI certified and purchased from the local markets and e-commerce websites.

4.2.2. Procurement of equipments and reagents

Chemical analysis of the most acceptable GOS gummy post acceptability trials was carried out in Food and Drug Laboratory, Vadodara, India. Lovibond Tintometer Colorimeter (Model F) was used for color estimation. Electronic pH meter (Cole-Parmer P100) was used for pH analysis. Karl Fischer Moisture Titrator (Model: MKV-710M) was used for moisture analysis. For texture profile analysis, texture analyser (Brookfield CT3 version 3.0 Texture Analyser) was used and Recovery analysis was carried out using HPLC (Brand: Agilent Technologies, Model: LC Agilent 1260 Infinity). For conducting recovery analysis of GOS, High Performance Liquid Chromatography (HPLC) grade chemicals were used.

4.2.3 Development of standardised and GOS added gummies

4.2.3.1 Standardisation of recipe of gummies

The basic ingredients required for the preparation of gummies include agar, sugar, and water. The ingredients (sugar and water) were cooked to a soft ball stage, followed by the addition of agar, citric acid, and natural colours and flavours. The gummies were poured into the mould and allowed to set in the refrigerator for 1 hour (Fig 4.3). The gummies were then dried for 48 hours under the fan (Plate 4.1; Plate 4.2). The standardised process was conducted with varying levels of each ingredient such as water content (55ml, 65ml, 75ml, 85ml), sugar content (50g, 60g, 70g, 80g), agar content (2g, 2.5g, 3g, 3.5g) and citric acid content (0.48g, 0.95g, 1.9g, 3.8g). All the products were subjected to organoleptic testing in triplicates to select the standardised gummy.

Plate 4.1: Gummies prepared in bulk

Plate 4.2: Drying of gummies prepared in bulk

4.2.3.2 Substitution with GOS and Sucralose

Sugar content in the standardised gummies was substituted with varying levels of galactooligosaccharide (60%, 80%, 100%) and subjected to organoleptic testing. In an attempt to make the 100% substituted galactooligosaccharide gummies more acceptable, the gummies were prepared with varying levels of artificial sweetener (sucralose) (5g, 5.5g, 6g) and subjected to organoleptic testing by the panel.

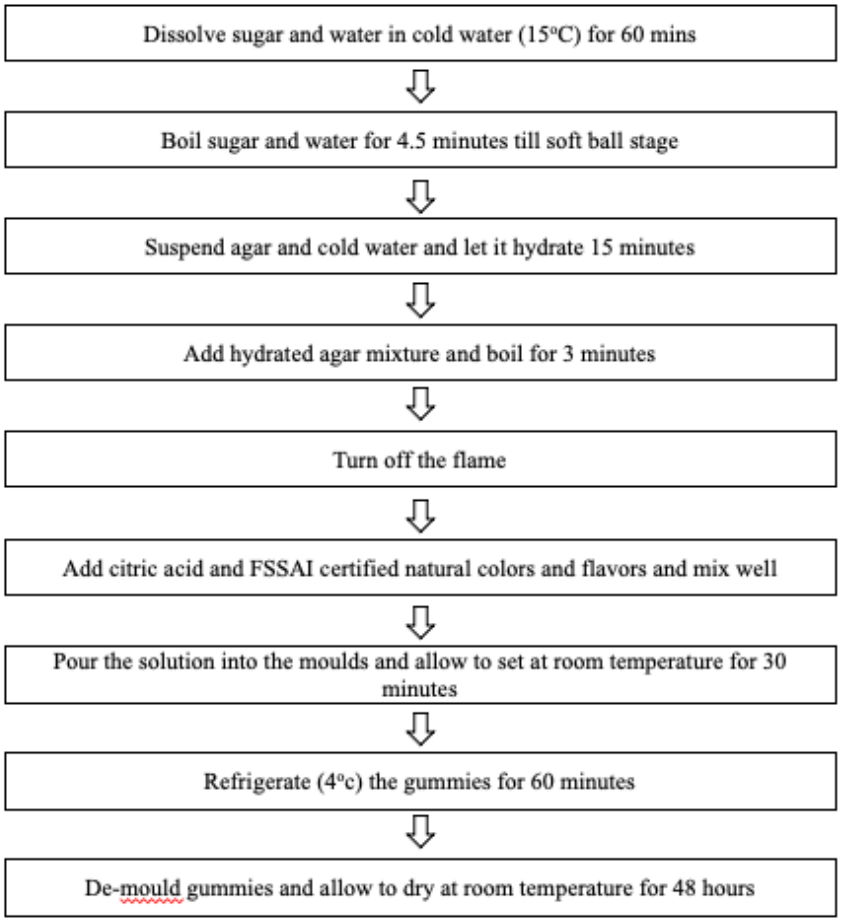


Fig 4.3: Process flow chart and Preparation of standard gummies

Plate 4.3 Staff performing threshold test

Plate 4.4 Panel members performing sensory evaluation

4.2.4: Selection and training of panel judges for sensory evaluation

4.2.4.1 Selection of panel members using threshold test

A threshold test (Fig 4.3) was conducted to select the panelists to perform sensory evaluation for the varying standardised recipes (Appendix II). Different solutions of sweet, salt, and sour with varying concentrations were given for tasting. Panelists who were able to identify the solutions correctly and gave consent were selected to be trained for sensory evaluation (Rangana, 1986) (Appendix III).

4.2.4.2 Training of the Panel members

The selected panel members were trained for a period of 7 consecutive days to evaluate the standardised gummy for its sensory attributes like colour and appearance, mouthfeel, texture, taste, and aftertaste (Silva et al, 2014).

The objective of training was to increase panellists' sensory accuracy while giving them a fundamental understanding of how sensory evaluation functions. Through training, panel members also improve their capacity to define, recognise, and explain sensory experiences regarding gummies. The general step-by-step procedure for training in dairy products is summarised in the paragraphs that follow (Diako et al, 2019).

a) The sensory panellists (assessors or evaluators) were informed of the fundamental requirements of sensory evaluation, including what they should and shouldn't do.

b) Assessors were trained about the following: - the product's appealing and undesirable qualities - the proper scorecard terminology to use, the scoring technique, and the sequence of observations.

c) Samples used for training and testing were representative of the range normally found in the market in terms of origin, style, and quality.

4.2.5: Tools for organoleptic evaluation

Score cards were developed for sensory analysis were:

a) Numerical scoring test for standardisation (Appendix V)
b) Difference test (IS, 1971) for obtaining the most acceptable GOS gummy (Appendix VI)

4.2.5.1 Numerical scoring testing for standardisation

Informed consent was obtained from the selected trained panel (Appendix IV). Sensory evaluation of the gummies was performed using the numerical scoring test where the panellists were asked to evaluate the sensory attributes of the gummies such as colour and appearance, flavour, texture, mouth feel, taste, after taste, and overall acceptability on a scale of 10 for each attribute for all the samples with varying concentrations (IS, 1971). The test was conducted in triplicates (Plate 4.4).

4.2.5.2 Difference test (IS, 1971) for obtaining the most acceptable GOS gummy

The difference test was used to measure the superiority, inferiority, or equal in terms of taste for the coded test samples against the standard gummy. The 100 percent galactooligosaccharide substituted gummy with varied quantities of artificial sweetness (sucralose) was evaluated using the difference test (IS, 1971).The panellists were asked to compare three coded samples to the standard gummy during the test, which was conducted in triplicate. Sample A, B, and C contained 5g, 5.5g, and 6g of artificial sweetener, respectively.

4.2.6: Assessment of physicochemical characteristics of the most acceptable GOS gummies

4.2.6.1 Evaluation of physicochemical properties of the 100% GOS substituted gummies:

The 100% GOS substituted gummies were crushed using a mortar and pestle and were utilised for measuring colour intensity, moisture, and pH in triplicates. The analyses were carried out at the Food and Drug Laboratory, Vadodara, India.

Colour intensity

The crushed sample (2.5g) was dissolved in 10 ml of methanol, placed in a glass cell inside the lighting cabinet, and its colour intensity was assessed using a Lovibond Tintometer and compared with Lovibond colour racks of known colour characteristics. The racks are varied until a visual colour match is found for the light from the sample and its colour can then be expressed in Lovibond® units. The colour was matched by adjusting the yellow and red racks of the instrument in triplicates (Gupta and Sheth, 2015).

Moisture content

The moisture content was determined by the Karl Fischer Titrator method to obtain a direct analysis of the sample. The crushed sample of gummies (5g) was solubilised in methanol and other reagents (Karl Fischer reagent) with a rotation speed of 5 RPM. The resulting moisture content was displayed digitally after 10 minutes (Robert A Martin, 1977).

pH content

The sample (2.5g) was crushed and dissolved in 50ml of distilled water by incubation for 10 minutes at 80 °C in a water bath. The dissolved samples were cooled, filtered and checked for its pH using an electronic pH meter at ambient temperature. Results were obtained after calibration with a buffer solution of pH 4.0 and 9.2 (Yadav et al, 2021).

Texture profile

Texture profile analysis was carried out using a texture analyser to provide insight into how the samples behave when chewed. Using circular probes (TA-TX2 50mm) moving at a speed of 1 mm/second, the gummy was placed on the base plate and compressed twice with a pause of 3 seconds (Cano-Lamadrid et al, 2020). The parameters assessed were hardness, adhesiveness, springiness, cohesiveness, gumminess, and chewiness in triplicates.

4.2.6.2 Recovery of GOS from the 100% Galactooligosaccharide substituted gummies

High performance liquid chromatography analysis for determining the GOS content of the gummies was carried out as explained by shodex.com with some modifications using an Inertsil NH2 (amino) column (15 mm x 4.6 mm, 32^{o}C) and a refractive index (RI) detector (32^{o}C). 1g GOS gummy was weighed and dissolved in 50 ml distilled water by heating to 100 ^{o}C and subjected to vacuum filtration. Limit of quantification of GOS standards was 4µg/µl. 20µl of the sample was injected into the column, where GOS was eluted using a mobile phase of Acetonitrile and water (ACN: H2O, 70:30 v/v) at a flow rate of 1.0 mL/min for 30 min. The analysis was carried out in triplicates.

The following formula was used to calculate the amount of GOS present in a gummy:

Concentration of GOS in one piece of gummy (3.5g) =

$$\frac{\text{Average area of the sample concerned}}{\text{Average area of the standard GOS}} \quad \text{X } 100$$

4.2.7: Shelf life analysis of the 100% GOS gummy

Shelf quality tests were performed on the 100 percent GOS substituted gummies, which were packaged in High density polyethylene (HDPE) bottles and kept in an incubator at an accelerated temperature of 37°C. They were subjected to:

a) Sensory evaluation
b) Microbial analysis

4.2.7.1 *Sensory evaluation* with similar parameters for standardisation using a numerical scoring card on days 0, 30, 60, 90, and 180

4.2.7.2 *Microbial testing* was carried out on days 0, 30, 60, 90, and 180 with respect to *E. coli*, total plate count (TPC), and yeast and mold count (Refai and FAO, 1979). The results were expressed as Log_{10}CFU/g of sample. The media used for the microbial evaluation were violet red bile agar, potato dextrose agar and nutrient agar for the colonisation of *E. coli*, yeast and mold count and total plate count (TPC) respectively and incubated for 24, 48 and 72 hours respectively.

4.2.8 Statistical analysis

Statistical analysis was performed using Microsoft Excel and the statistical package for IBM (SPSS version 34) software. Results were expressed in terms of mean values and standard deviation for the prepared gummies. ANOVA- one way variance was performed to determine any significant difference among the samples with varying concentrations. Chi square test was used to access the extent of differences among the scores obtained for the difference test, which was used to study the superiority or inferiority between the standard gummy and the 100% galactooligosaccharide and sucralose added gummy at a p value of 0.05. ANOVA- one way variance was used to determine any significant change in the shelf life of the gummies over the period of 6 months at a p value of 0.05.

Phase III

Impact evaluation of supplementing GOS gummies to subjects suffering from FC on their constipation profile, gut microflora, SCFA profile, depression status and quality of life

This phase of the study was conducted to assess the impact evaluation of supplementation of GOS gummies to the teaching staff (n=35) from The Maharaja Sayajirao University of Baroda at Vadodara suffering from functional constipation. The subjects who satisfied the inclusion and exclusion criteria of the study were randomly divided in to control (n=18) and experimental group (n = 17) with the help of double randomization process and were given 10 g GOS or placebo for 4 weeks. The effect of daily intake of GOS was examined with respect to subjective parameters including their constipation profile, depression status and quality of life and objective parameters of gut health with respect to gut microflora and short chain fatty acid profile quantification. Experimental design of the study is depicted in Fig 4.6.

4.3.1 Statutory clearances

The study protocol was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda and provided the approval number IECHR/ FCSc/PhD/2021/3. The intervention trial has also been registered in CTRI, ICMR bearing approval number **CTRI/2021/10/037474** (Appendix XIII)**.** Written consent was obtained from the participants who agreed to give baseline information through questionnaires (Appendix VII)

4.3.2 Sample size calculation

The sample size was calculated by a statistics professor in the Department of Statistics, The Maharaja Sayajirao University of Baroda. The sample size calculation is as follows:

$$n = [(Z\,\alpha/2 + Z\,\beta)\,2 \times \{(p\,1\,(1-p\,1) + (p\,2\,(1-p\,2))\}]/(p\,1 - p\,2)\,2$$

where,
p 1 = proportion of subject effectiveness by treatment A = 0.7,
p 2 = proportion of subject effectiveness by Placebo = 0.2,
p 1 -p 2 = clinically significant difference = 0.5

Z α/2 : This depends on level of significance, for 5% this is 1.96
Z β : This depends on power, for 80% this is 0.84
Level of significance = 5%, Power = 80%, Type of test = two-sided

Based on above formula the sample size required per group was 38. Keeping an attrition rate of 20%, the total sample size required was 48.

Since no Indian study on adult population to detect functional constipation using Rome IV criteria was available, a similar study conducted in Japan was used as the reference (Souza et al, 2018).

4.3.3 Selection of the subjects for the study

Male and female teaching staff (n=38) of The M.S. University of Baroda aged between 25-62 years suffering from functional constipation as per the constipation profile which used to screen during the cross-sectional phase, was recruited for this phase of the study. Those who satisfied the inclusion and exclusion criteria of the study were further requested to participate for the double blind intervention program. The willingness of the subjects was considered for participation through the informed consent letter (Appendix VII). They were counseled about the benefits of GOS and requisites of the study that the diet, physical activity and their lifestyle pattern during the study period should remain unaltered.

4.3.4 Inclusion and exclusion criteria of the subjects

Inclusion criteria:

- Subjects who were a teaching faculty in The M.S. University of Baroda
- Subjects having symptoms related to functional constipation (as per the profile) for >6 months
- Subjects in the age group of 25-42 years
- Subjects willing to participate in the double blind intervention trial

Exclusion Criteria:

- Subjects with a history of Inflammatory Bowel Syndrome (IBS)/ Inflammatory Bowel Disease (IBD)

- Subjects who underwent intestinal resection or had a history of GI diseases like hiatal hernia, fissures, haemorrhoids and others
- Subjects with chronic metabolic diseases such as symptomatic cardiovascular disease, diabetes or hyperglycaemia, hypertension, renal, hepatic, respiratory disorder, cancer or current active treatment of cancer
- Subjects on antibiotics/ regular use of NSAIDs (E.g., aspirin)/any medication for any chronic illness use within the last 12 weeks prior to enrolment
- Subjects on excessive alcohol intake (more than two drinks for men and one drink for women daily) or smoking
- Subjects who were consuming probiotics, prebiotics, or synbiotic on a regular basis
- Female subjects who were pregnant or lactating
- Subjects intolerant to lactose and/or allergic to any of the ingredients used for production of gummies.
- Subjects with BMI <18kg/m^2 and >24kg/m^2

4.3.5 Study protocol

The selected subjects were subjected to a double blind randomised placebo controlled clinical trial. The subjects were requested not to take the gummies in empty stomach and not to share the gummies with others as they were in experimentation phase. The subjects were asked to limit intake of prebiotics, probiotics or synbiotics, if taking any. The process is described in detail below.

4.3.5.1 Randomisation, allocation and blinding

Constipated adults (n=48) were randomly assigned to one of the groups— control group (n=24) receiving placebo, experimental group (n=24) receiving 100% GOS gummies—through a randomization list prepared using computer generated random numbers. The allocation ratio followed was 1:1. The list was prepared by a senior professional in the Department of Foods and Nutrition who was not otherwise involved with the study. Appropriate coding was generated and allotted to the subjects to maintain blinding between the groups (Smith PG et al, 2015).

4.3.5.2 Study Intervention details

The 2 intervention groups received kits with the gummy bottle, compliance sheet, uricols (stool collection container), a black polythene bag and a thank you card for participating in the trial (Plate 4.5). The kits for control subjects contained standard sugar gummies and kits for experimental group contained the GOS gummies (Plate 4.6). Subjects were provided with gummies for 4-week intervention period and were instructed to consume 4 gummies daily containing 10g GOS. At the end of the 4-week intervention period, subjects provided stool samples along with the post data on their constipation profile, depression profile and quality of life with the help of validated questionnaires.

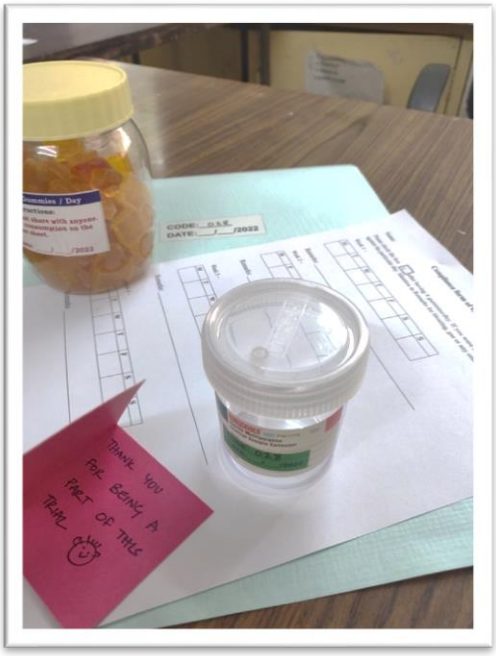


Plate 4.5: Contents of intervention kit

Plate 4.6: Gummies packed in bottle after drying

4.3.5.3 Monitoring and Compliance

Compliance was monitored with appropriate follow ups and side effects experienced if any, using daily SMS reminders and phone calls weekly. A calendar was especially designed and distributed to all the subjects to document daily consumption of gummies and any unusual symptoms or side effects if observed. Compliance sheet (Appendix IX) were collected back after the completion of intervention period. They were asked to inform the researcher

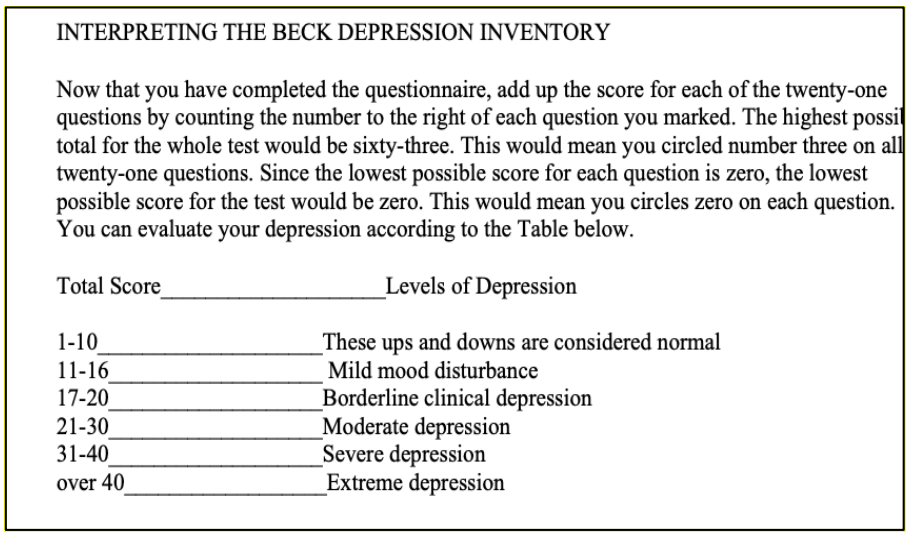
immediately as well as record for any pain, straining, difficulty passing stools and adverse effects (excessive pain, regurgitation or vomiting) in the compliance sheet during the intervention period.

4.3.6 Administration of validated pretested 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 (Appendix VII). The subjective assessment in this phase involved questions with respect to their dietary profile in terms of 24 hour dietary recall (Appendix VIII); constipation profile (Fig 4.1; Appendix X), depression status in terms of Beck's Depression Inventory (BDI II,1996) (Appendix XI) and quality of life in terms of patient assessment of constipation on quality of life (PAC-QOL) (Appendix XII) on baseline and post intervention.

Depression status

The subjects were asked 21 situational questions and their responses were scored on 63 which were and they were further categorised into different levels of depression (Fig 4.4)



INTERPRETING THE BECK DEPRESSION INVENTORY

Now that you have completed the questionnaire, add up the score for each of the twenty-one questions by counting the number to the right of each question you marked. The highest possi total for the whole test would be sixty-three. This would mean you circled number three on all twenty-one questions. Since the lowest possible score for each question is zero, the lowest possible score for the test would be zero. This would mean you circles zero on each question. You can evaluate your depression according to the Table below.

Total Score	Levels of Depression
1-10	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

Fig 4.4: Categories of Depression as per BDI II (1996)

Quality of life (PAC-QOL) assessment

PACQOL measures the physical, mental, social and functional aspects of an individual's life (Besley et al, 2010). The questions asked were related to the extent or intensity of symptoms faced, how constipation affects their daily life, their feelings related to constipation, life with constipation and degree of satisfaction with the treatment provided. For each of the 28 questions the subjects were asked to rate for each of the parameters on a scale of 1-5.

4.3.7 Evaluation of gut microflora and fecal SCFA of the selected respondents and Assay methods

Gut heath was determined with respect to Fecal microflora quantification using Real time PCR technique in terms of *Bifidobacteri*a sp., *Lactobacillus sp., Clostridium sp., Bacteroides sp.* and major phyla Firmicutes and Bacteroidetes which constitutes for 90% of the human gut microbiome, along with short chain fatty acid (SCFA) analysis using Gas Chromatography technique with respect to acetic acid, propionic acid and butyric acid. The steps involved are detailed below:

4.3.7.1 Collection fecal sample, storage and analysis

Fecal samples were collected in air tight sterile uricols and stored right away at -80°C until DNA extraction was completed for RTPCR analysis. Fecal samples were also subjected to SCFA analysis.

4.3.7.2 *DNA extraction*

DNA from the stool samples was extracted using the QIAamp DNA stool mini kit (Cat. No. 51504, Qiagen, Hilden, Germany).

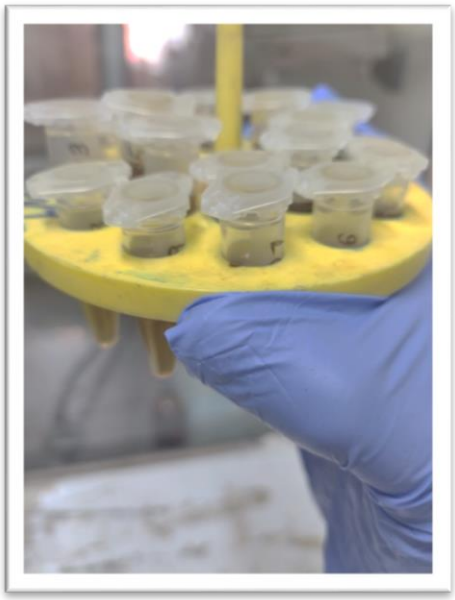


Plate 4.7: Coded Eppendorf tubes

180-220 mg of stool was weighed in 2 ml eppendorf tubes for further processing. Thereafter, 1.4 µl of ASL buffer was added to the stool sample and vortexed for 1 minute until thoroughly homogenized. The suspension was heated for 5 min at 70℃ (95℃ for gram positive bacteria) and vortexed for 15 second and centrifuged the sample at 14,000rpm for 1 minute to pellet stool sample. 1.2 ml of the supernatant was pipetted into a new 2 ml micro centrifuge tube discard pellet. 15 µl proteinase K was taken into a new tube and added 400 µl of supernatant to it. 400 µl of AL buffer was added and vortexed 15 sec. 400 µl of ethanol was added to the lysate and mix by vortexing (Plate 4.7). Complete lysate was added from previously step to the QIAamp column and centrifuged at 14000rpm for 1 minute place the QTAamp spin column in a new 2 ml collection tube and the filtrate was discarded. 500 µl of AW1 buffer was added to the spin column and centrifuged at 14000rpm for 1 minute. The QIAamp column was placed in a new 2 ml collection tubes and the filtrate was discarded. 500 µl AW1 buffer were added to the QIAamp spin column and centrifuged 3 minute and the filtrate was discarded. The QIAamp spin column was transferred into a new tube and 100 µl AE buffer was added on to the QIAamp membrane. Incubation was done at room temperature for 1 minute centrifuged at 14000rpm for 1 minute and the DNA was eluted (Plate 4.8).

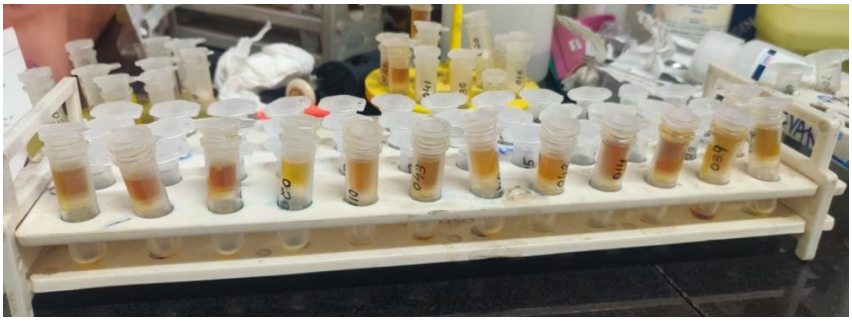


Plate 4.8: Coded Eppendorf tubes with the buffer before DNA elution

DNA was quantified using Thermo Scientific Multiskan sky Spectrophotomter (MA, USA) with high accuracy and reproducibility (Plate 4.9A; Plate 4.9B). Samples were loaded onto the Thermo Scientific µDrop plate and measured the absorbance at 260 nm (Plate 4.9 C). A sample volume of 2 ul samples were measured. A 2 ul sample was pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap was controlled to both 1 mm and 0.2 mm paths.

The instrument was controlled by PC based software, and the data was logged in an archive file on the PC. The sampling arm was opened and the sample was pipetted onto the lower measurement pedestal. The sampling arm was closed and spectral measurement using the operating software on the PC was initiated. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made. When the measurement was completed, sampling arm was opened and the sample was wiped from both the upper and lower pedestals using a soft laboratory tissue wipe.

Plates 4.9 (A) Thermo Multiskan Sky Instrument; (B) Touchscreen control of Multiskan Sky spectrophotometer; (C) µDrop plate for loading upto 16 samples/time

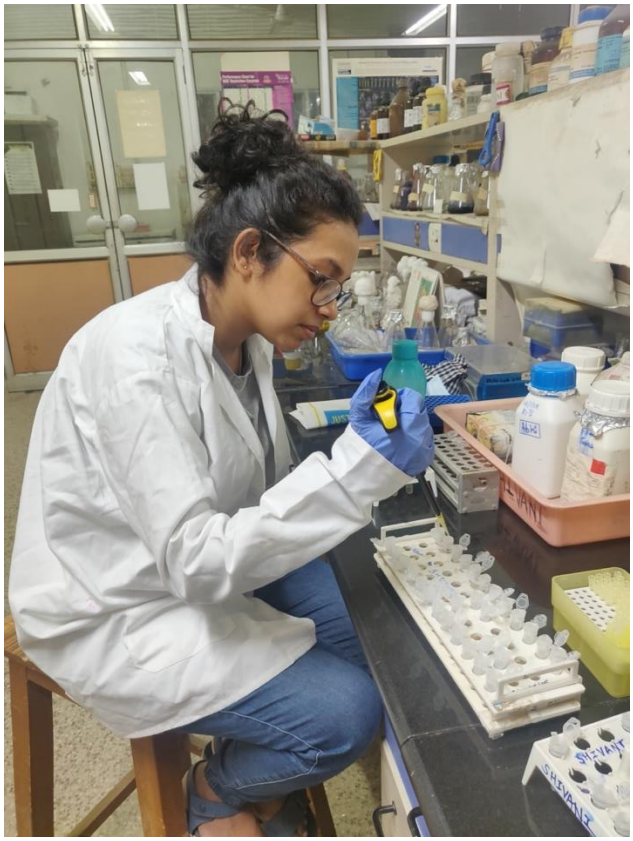


Plate 4.10: Ph.D. student working at Microbiology Department, MSU, Baroda

4.3.7.3 Real-Time Polymerase Chain Reaction (RT-PCR) analysis

Metagenomic DNA (µDrop plate, Multiskan sky spectrophotometer) was used to determine the relative abundance of bacterial genomes, qPCR was used. DNA concentration for the PCR was established by serially diluting the DNA from 5 ng to 0.25 ng, and it was established that 1 ng/reaction was appropriate for all amplifications. PCR amplification of V6-V8 region of bacterial 16S rRNA was carried out using the specific primers for the genus Bifidobacterium, Lactobacillus, Clostridium, Bacteroides and phyla Bacteroidetes and Firmicutes (Fig 4.5). All of the Real-Time qPCR experiments were performed using iTaq Universal SYBR Green Super mix kit (Bio-Rad, Cat. No. 1725124, Hercules, CA). Each PCR reaction constituted a final concentration of 1x standard Taq buffer, 1.75 mM of $MgCl_2$, 200µM of dNTPs, 0.2µM of each primer, 0.5U of Taq DNA polymerase (Sigma Aldrich, USA) and 25ng of template DNA. QuantStudio3 (Life Technologies, Carlsbad, CA) was used for the Real-time PCR amplification, and the PCR conditions followed was initial denaturation at 95 ºC for 10 minutes, initial denaturation at 95 ºC for 10 minutes, denaturation at 95 ºC for 15 seconds,

annealing for 1 minute at a temperature as calculated for 40 cycles, and melt curve analysis was performed at the end of the reaction. The test was conducted in triplicates.

S. No.	Oligo	Sequence	Annealing temperature
1	Universal	For- 5'-CCTACGGGAGGCAGCAG-3'	64 °C
		Rev- 5'-ATTACCGCGGCTGCTGG-3'	
2	Bacteroides Sp	For- 5'-AAGGGAGCGTAGATGGATGTTTA-3'	63 °C
		Rev- 5'-CGAGCCTCAATGTCAGTTGC-3'	
3	Clostridium Sp.	For- 5'-CGGTACCTGACTAAGAAGC-3'	59 °C
		Rev- 5'-AGTTTGATTCTTGCGAACG-3'	
4	Lactobacillus Sp.	For- 5'-AGCAGTAGGGAATCTTCCA-3'	58 °C
		Rev- 5'-CACCGCTACACATGGAG-3'	
5	Firmicutes Sp.	For- 5'-GGAGCATGTGGTTTAATTCGAAGCA-3'	65 °C
		Rev- 5'-AGCTGACGACAACCATGCAC-3'	
6	Bifidobacterium Sp.	For- 5'-GGGTGGTAATGCCGGATG-3'	62 °C
		Rev- 5'-TAAGCGATGGACTTTCACACC-3'	
7	Bacteroidetes Sp.	For- 5'-GGARCATGTGGTTTAATTCGATGAT -3'	64 °C
		Rev- 5'-AGCTGACGACAACCATGCAG-3'	

Fig 4.5: Primers used in the study with specific temperatures

4.3.7.4 Short chain fatty acid (SCFA) Analysis

SCFA analysis was performed on a sub sample for the control group (n=7) and experimental group (n=8) at baseline and post intervention. SCFA profile was studied with respect to acetic acid, propionic acid, butyric acid, in triplicates. The details of the procedure are mentioned below. The results were expressed as mg/ml.

Preparation of standard stock solution

100ml of each of the standards (acetic acid, propionic acid and butyric acid) was accurately weighed and transferred into a 20ml volumetric flask containing 20 ml volumetric flask, containing 20 ml of the diluent (methanol).

Preparation of standard solution

1ml of the standard stock solution was diluted to 100 ml with the diluent (methanol).

Preparation of test solution

100mg of test sample was accurately weighed and transferred in to a 20ml screw cap bottle. 2ml diluent (methanol) was added sonicated for 4 minutes. The solution was filtered with 0.45µ syringe filter.

Gas Chromatography Analysis

GC was performed on the Shimadzu GC 2010 plus, with RTX1301 column (length-60 m, thickness-0.25 mm, ID-1.4 µm). Temperature programme was started at 60 °C, held for 2 mins and then raised finally to 250 °C at 4 °C/min at which it was held for 15 mins. 1µl sample was injected at 250 °C, at 37psi at a linear velocity of 35cm/sec having a column flow rate of 2.0 ml/min, with He as carrier gas. The purge flow was 3.0ml/min with 5.0 split. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST) library.

4.1.8 Statistical analysis

The data was entered in an excel spreadsheet. The data was cleaned and verified and subjected to appropriate statistical analysis. Data analysis was performed using JASP software (Mac version). Mean and standard deviation were calculated. Paired t test was performed to observe the effect of GOS supplementation on constipation profile, gut health with respect to gut microbial quantification, short chain fatty acid profile, depression status and quality of life on pre and post intervention of standard and GOS gummies to the constipated participants. The significance levels were set at 5% by two sided tests. Paired *t* test was performed for the comparison between control and experimental group for the various subjective and objective parameters. Various associations were studied such as gut flora, SCFA, depression status and QOL with presence of functional constipation at baseline.

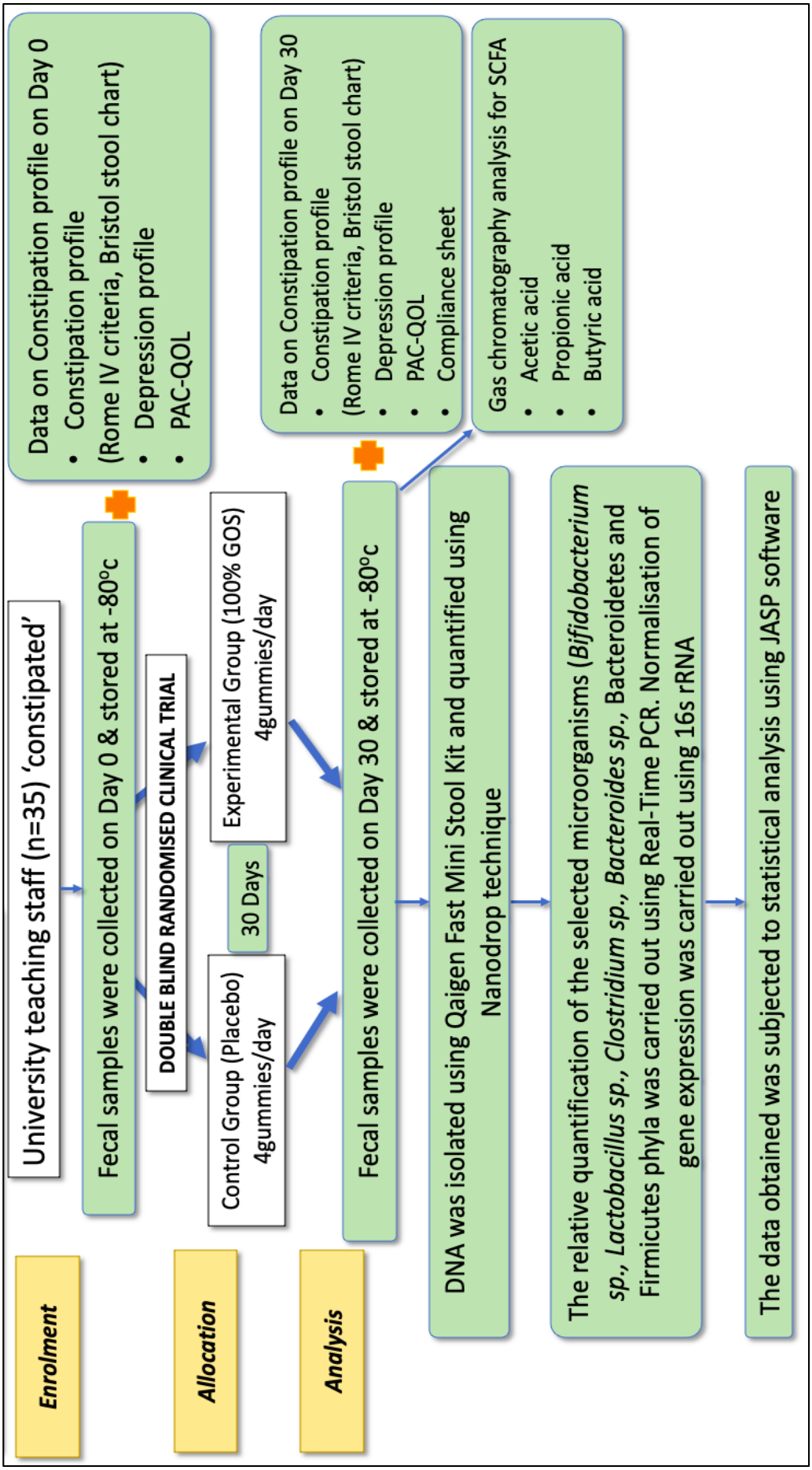


Fig 4.6 Experimental plan for Phase III