CHAPTER III

METHODOLOGY

The methodology followed to pursue the research pertaining to this present study on "**Assessment of nutritional and functional properties of probiotic complementary food mixes from locally available cereals and legumes"**are detailed in this chapter under the following heads:

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PHASE II

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3.8. Shelf life studies of the developed probiotic complementary food mixes.

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3.8.2. Free fatty acid of developed probiotic complementary food mixes.

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3.8.4. Total fungal count in the probiotic complementary food mixes

3.8.5. Effect of metallised polyethylene packaging on moisture content of developed probiotic complementary food mixes

3.9. Data collection and statistical analysis

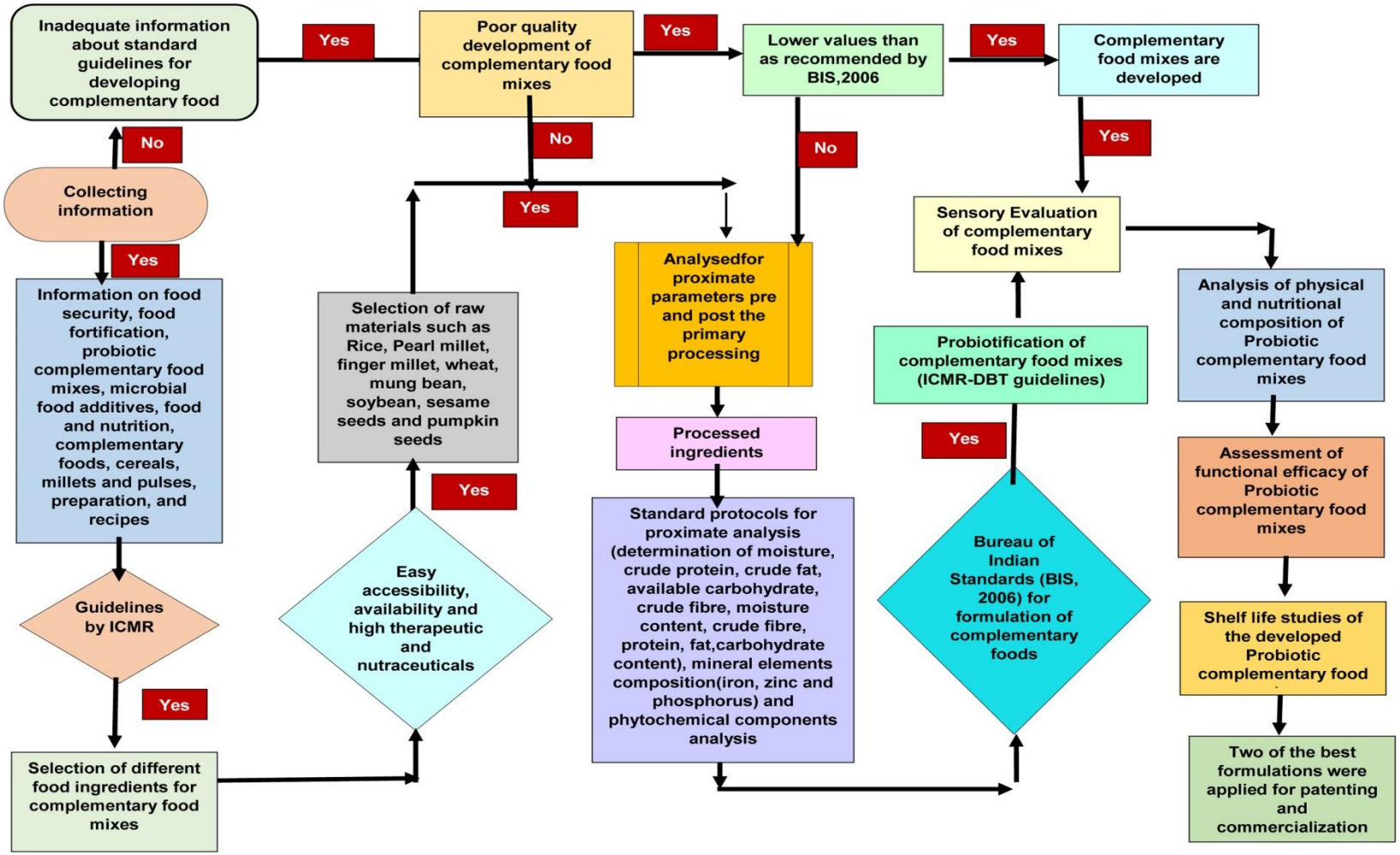


Fig.3.1. Conceptual framework of the present study

3.1. Phase I

Sample collection

3.1.1. Procurement of raw materials

Using the recommendations for complementary foods provided by the Indian Council of Medical Research (ICMR, 2000), different raw ingredients for formulating complementary food mixes were selected for the present investigation. Rice, pearl millet, finger millet, wheat, mung bean, soyabean, sesame seeds and pumpkin seeds were procured from Coimbatore, Tamil Nadu, India and selected for their easy accessibility, availability and high therapeutic and nutraceuticals properties. The samples were stored in High Density Poly Ethylene (HDPE) virgin containers for future use.

3.1.2. Processing of raw materials

The raw ingredients used for the study were ground to fine flour for incorporating into the formulations for the development of complementary food mixes. The primary processing of cereals and legumes is an important part of their preparation before ultimate usage. The raw ingredients were analysed pre and post the primary processing to study about the changes in the nutritional profile of the raw ingredients. Processing techniques changes the nutritional quality of the food, resulting in either increase or decrease of nutrients, phytochemicals or digestibility and availability of nutrients. The final product after processing is presented in Plate 1.

3.1.2.1. Processing of rice into flour

Rice samples were properly cleaned and washed for the purpose of removing debris and other foreign particles. Cleaned rice samples were oven dried for 6 hours at 60-65 °C. Dried samples were milled in electric grinder and sieved by 100 mesh sieve to obtain fine flour. The flour obtained were roasted for fifteen minutes (80-110˚C) and stored in High Density Poly Ethylene (HDPE) virgin containers before use in a refrigerator. The study methodology was detailed by Southan (2006) with modifications. The different phases of processing rice into flour are presented in Fig. 3.2.

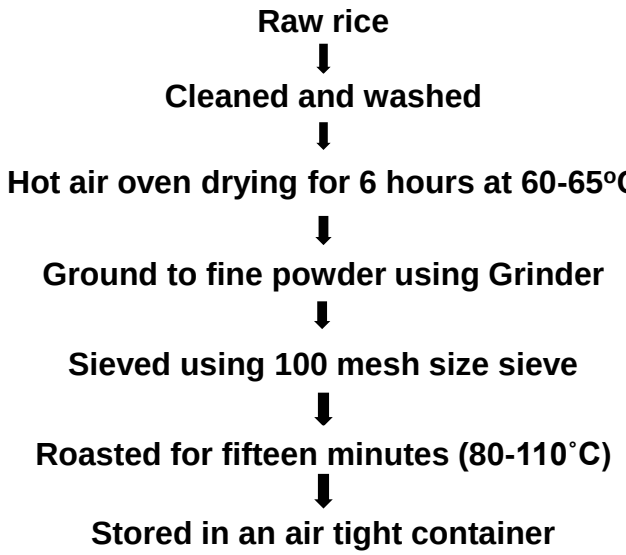


Fig. 3.2. Flow diagram of processing of rice into flour

3.1.2.2. Processing of pearl millet, finger millet and mung bean into flour

Pearl millet flour was processed by the approach given by Florence *et al*., (2014) with modifications, finger millet was processed by the approach given by Taynath *et al.,* (2018) with modifications, and mung bean was processed by the approach given by Patil *et al*., (2011) with modifications respectively. Raw ingredients were properly cleaned and washed for the purpose of removing debris and other foreign particles. The millets samples were then dried using hot air oven drying for 4 hours (60-65˚C) and mung bean was dried using hot air oven drying for five hours (60-65˚C) respectively. The millet samples were then roasted for 10 minutes at 120˚C and mung bean was roasted for 10 minutes at 125˚C respectively. All the raw ingredients were milled in mechanized grinder separately.Millets flour was sieved by 100 mesh sieve and mung bean flour was sieved by 80 mesh sieve to obtain fine flour. Flours were then separately stored in High Density Poly Ethylene (HDPE) virgin containersbefore use in a cool temperature at 4^{o}C. The detailed steps of processing flour are shown in Fig. 3.3.

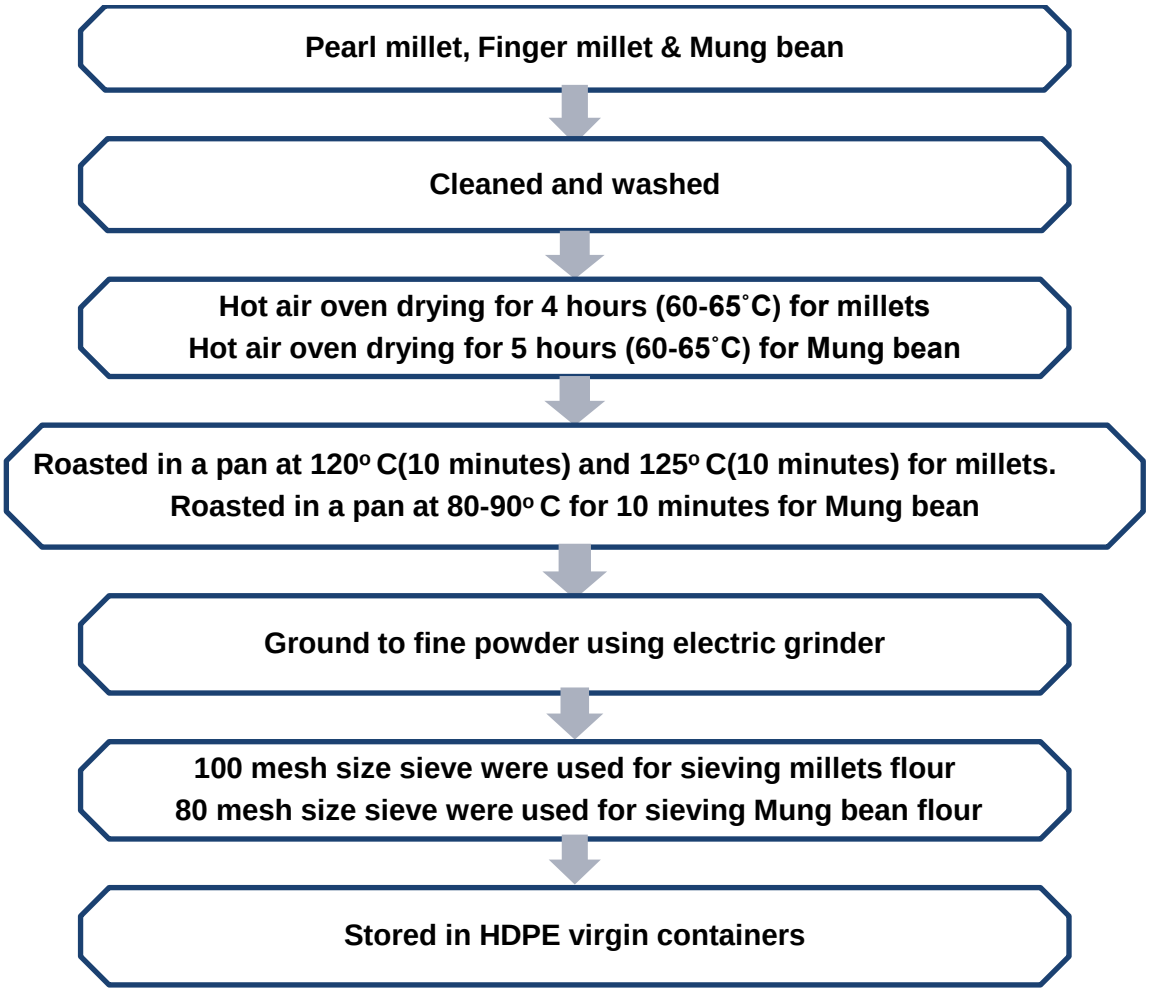


FIG. 3.3.Flow diagram of processing of pearl millet, finger millet and mung bean into flour

3.1.2.3 Processing of soyabean into flour

The process used in the processing was detailed by Adelekan (2013) with modifications. Soyabean seeds were soaked overnight for 12 hours in double the concentration of water. The soaked water was decanted and boiled for 30 minutes. The removal of seeds was done manually through hands. After the removal of skin the soyabean seeds were washed till the water becomes clear. The samples were then dried using hot air oven drying for 5 hours (60-65˚C) and were milled in mechanized grinder to make flour and sieved by 100 mesh size sieve and stored in High Density Poly Ethylene (HDPE) virgin container for further use.The detailed steps of processing soyabean flour are shown in Fig. 3.4.

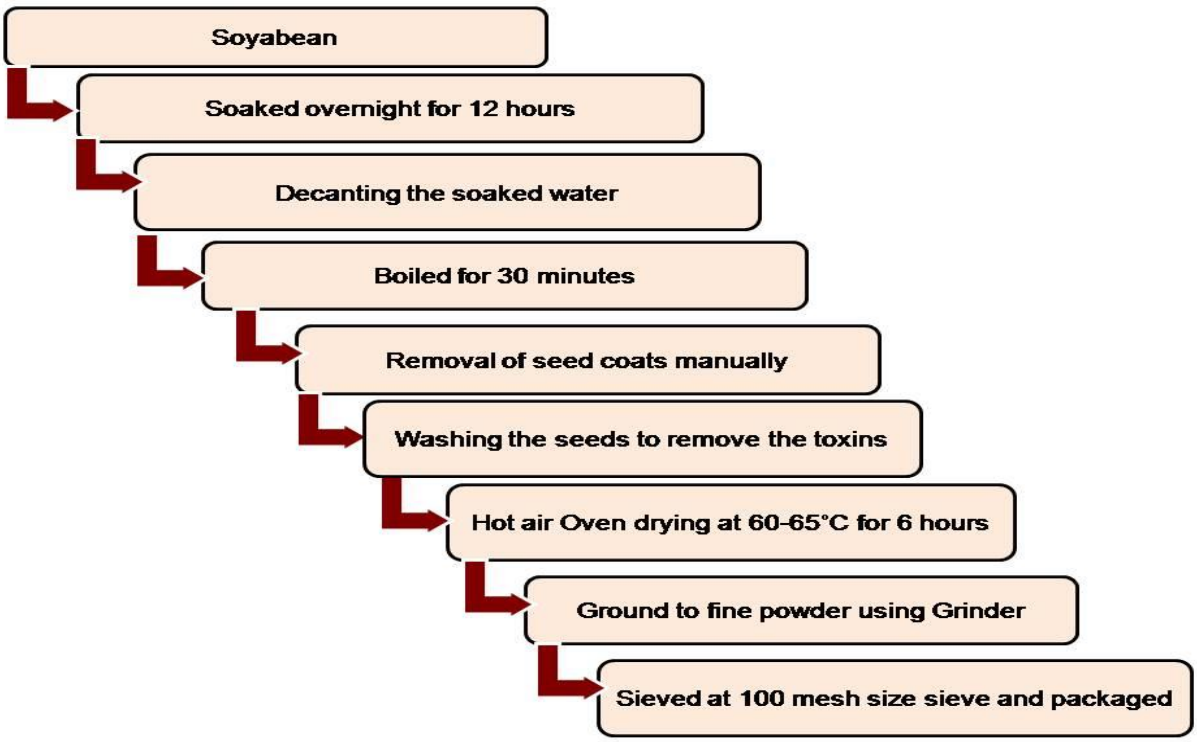


FIG. 3.4.Flow diagram of processing of soyabean into flour

3.1.2.4. Processing of oil seeds into flour

The method used for processing sesame seeds was described by Animashaun *et al*., (2011)with modifications and pumpkin seeds were processed with modification of method detailed by Zema *et al*., (2015). Whitesesame seeds were cleaned and roasted at 60°C in a pan for 5 minutes and pumpkin seeds were sundried for 20 hours and roasted for 5 minutes at 60°C. The roasted seeds were then grinded using grinder and 36 mesh size sieves were used for sieving both the flour. Polyethylene Terephthalate (PET) containers were used to store the flours in refrigerators at 4^{o}C. The detailed steps of making sesame seed and pumpkin seed flour are shown in Fig. 3.5. Plate 1 and Plate 2 depicts the raw and processed cereals, millets, pulses and oil seeds.

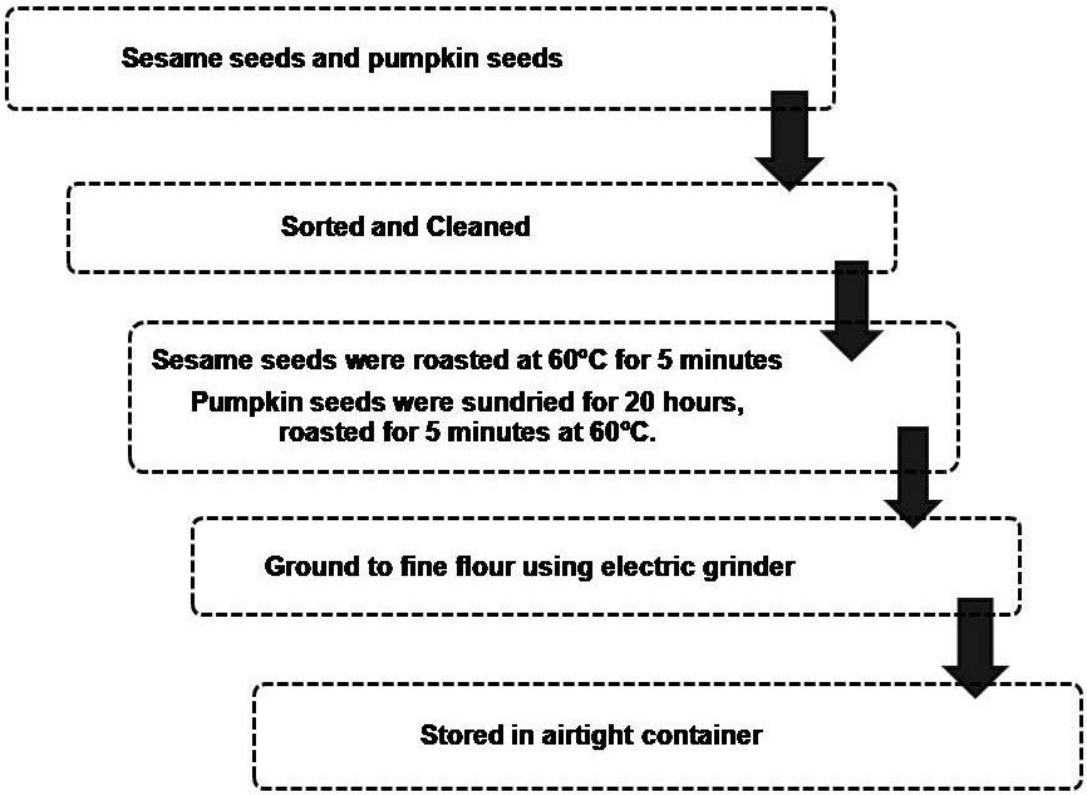


FIG. 3.5.Flow diagram of processing of sesame seeds and pumpkin seeds into flour

Rice

Rice flour

Pearl millet

Pearl millet flour

Finger millet

Finger millet flour

Plate 1. Raw and processed cereals and millets used for the study

Mung bean

Mung bean flour

Soyabean

Soyabean flour

Sesame seeds

Sesame seeds flour

Pumpkin seeds

Pumpkin seeds flour

Plate 2. Raw and processed pulses and oil seeds used for the study

3.1.3. Development of the Amylase rich flour

The study's method was detailed by Bala *et al*., (2014) with modifications. The wheat grains were steeped in water double the concentration for 12 hours and water was changed after every six hours. The soaked seeds were put in muslin cloth bags for 72 hours in air rest and allowed to germinate. Water was sprinkled from time to time because of the summer season. The samples that had been germinated were oven dried for 28 hours at 60 to 65°C, and rootlets were manually removed in a winnowing tray. The samples were ground into fine flour using an electric grinder, and then sieved using an 80 mesh sieve. Polyethylene Terephthalate (PET) containers were used to store the flours in refrigerator at 4^{o}C. The detailed phases of Amylase rich flour is depicted in Fig. 3.5. Plate 3 shows germinated wheat and its flour.

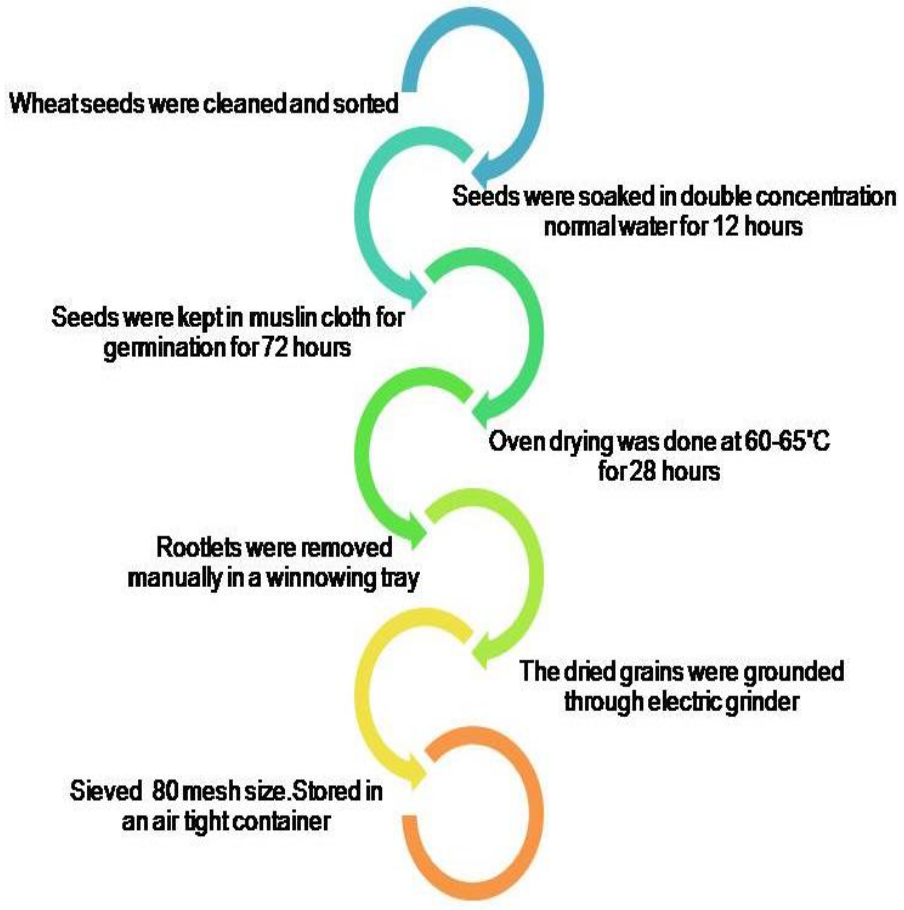


Fig. 3.6. Processing of amylase rich flour

Plate 3. Germinated wheat and its flour

3.2 Phase II

Determination of nutritional characteristics of raw ingredients

3.2.1. Proximate analysis of raw ingredients

Consumer's curiosity in certain foods with health promoting qualities or physiologically active food ingredients, also known as "functional foods" has exploded recently. A food containing a dietary component that may or may not be nutrient which targets one or more functions in human body, to produce favourable results to support particular health claims causing positive health effects. To put it another way, meals that include these functional foods have health-promoting characteristics in addition to their nutritional worth.

Nutritional attributes of the raw ingredients flour like moisture, crude protein, fat, available carbohydrate and crude fibre with other functional ingredients were determined by using standard procedures. Moisture content and crude fibre of the samples were determined following the AOAC (2010) Official Methods (Raghuramulu *et al.,* 2003), protein was analysed estimating organic nitrogen by the AOAC (2000) (Kjeldahl method). Fat was analysed by the Soxhlet method using AOAC (2000) method. The available carbohydrate content was determined by Anthrone method (Hedge and Hofreiter, 1962). The standard protocols are presented in Appendix I.

3.2.2 Mineral composition of raw ingredients

Mineral elements that were estimated included calcium (Ca) determined by using flame photometer according to the method A.O.A.C (1984) using atomic absorption spectroscopy (AAS), iron(Fe) was determined according by Ranganna (1997) by using spectrophotometer, zinc (Zn) content was estimated in the sample by using Atomic Absorption Spectrophotometer, phosphorus (P) was determined by Photometric method by Wheeler and Ferrel (1971). The standard protocols are presented in Appendix I.

3.2.3. Estimation of phytochemical components of raw ingredients

The term "phytochemical" refers to metabolites that are protective and vary in structure from high molecular weight proteins to simple amino acids and oligosaccharides with distinct biological effects.The tannins were determined by Folin - Ciocalteu method given by Tambe and Bambar (2014). The saponin content estimation method was used from Fenwick and Oakenfull (1981). Phytates were determined by Latta and Eskin (1980). The standard protocols are presented in Appendix I.

3.3 Phase III

Formulation and standardization of complementary food mixes

3.3.1 Formulations of complementary food mixes

The formulation of complementary foods mixes for the study had been done in accordance with Bureau of Indian Standards (BIS, 2006) standards, which state that the product is intended to be mixed with milk or water before consumption and that complementary foods should not contain cereals and legumes combined less than 75 percent. The processed flours of cereals/millets, legumes and oil seeds were used in formulating six different formulations namely T_1, T_2, T_3, T_4, T_5 and T_6.

Table 3.1. Formulation of complementary food mixes for estimating the energy density values in 100 gm

TREATMENTS	INGREDIENTS							
	Cereals/millets				Pulses/ legumes		Oilseeds	
	R (%)	PM (%)	FM (%)	MWF (%)	MB (%)	SB (%)	SS (%)	PS (%)
T_1	50	-	-	5	-	30	10	10
T_2	-	50	-	5	-	30	10	10
T_3	-	-	50	5	-	30	10	10
T_4	50	-	-	5	30	-	10	10
T_5	-	50	-	5	30	-	10	10
T_6	-	-	50	5	30	-	10	10

(R=*Rice*, PM=*Pearl millet*, FM=*Finger millet*, MB=Mung bean, SB=Soyabean, SS=Sesame seed, PS= Pumpkin seed).

3.3.2 Calculation of Energy Density (ED) value of the formulations

The estimated amount of carbohydrate, protein, and fat from all the raw ingredients for each test sample was added, and the total energy density of the formulations was calculated by multiplying by a constant factor of 4 for carbohydrate and protein, and 9 for fat (ICMR, 2000). Six test samples namely T_1, T_2, T_3, T_4, T_5 and T_6were formulated using different level of incorporation. The energy density value of the complementary food mixes between 300 – 550 kcal per 100g of sample as recommended by World health organization (WHO, 2001)was selected and was further subjected to Probiotification. The samples selected for the study are presented in Plate 4 and Plate 5.

Plate 4. Complementary food mixes T_4[ED value (437.50 kcal)]

Plate 5. Complementary food mixes T_6 [ED value (443.49 kcal)]

3.4 Phase IV

Probiotification of complementary food mixes

3.4.1. Location of study

The experiments on Probiotification were conducted in the Microbiology lab, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, Tamilnadu, India. The scholar was trained in handling the equipments and instruments for a period of one month under the supervision of a Biotechnologist and the research supervisor guidance. The Probiotification was done taking in consideration the ICMR-DBT guidelines for probiotics.

3.4.2. Procurement of bacterial cultures

The probiotic cultures namely *Lactobacillus plantarum, lactobacillus fermentum, Lactobacillus rhamnosus, Lactobacillus casei*were obtained from National collection of dairy cultures (NCDC), Indian council of Agricultural Research – National Dairy Research Institute, Karnal, Haryana, India. The probiotic cultures are shown in Plate 6.

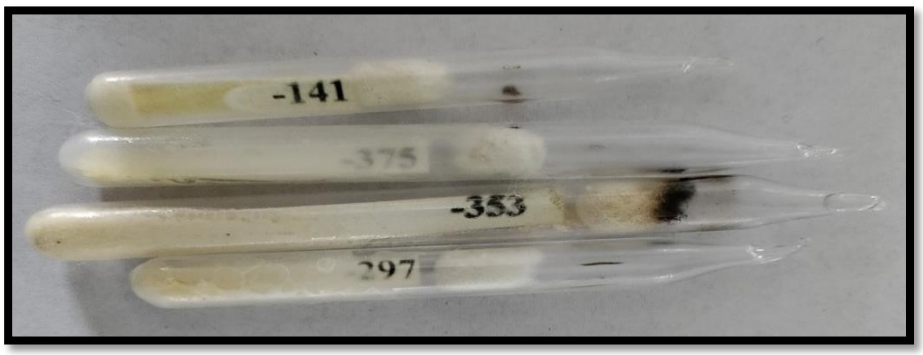


Plate 6. Probiotic cultures from NDRI, Karnal, Haryana

3.4.3. Storage of bacterial cultures

The bacterial strains that were purchased were in freeze dried state, in an ampoule amount of sterile hydrating fluid and with a ready to use cotton swab also called as Kwik- Stik. Strains were kept at -20˚C to -80˚C in a deep freezer.

3.4.4. Selection of Media

Nutrient Agar and de Man Rogosa Sharpe (MRS) broth were selected as the suitable medium for the growth and culturing of probiotic bacteria.

3.4.5. Retrieval of NDRI - NCDC Cultures

The glass was cracked by putting red hot wire or glass rod to a file cut that had been made on the ampoule at the centre of the cotton wool stopper. The pointed part was then carefully removed after allowing the air to enter slowly. The cotton stopper and upper portion of the ampoule were dumped into a disinfecting solution. The samples were added to a 5 ml test tube of MRS broth using a Pasteur pipette after the open end of the ampoule had been flamed aseptically. A subculture was cultured for 24 hours in the proper gaseous environment at 37°C. Before employing the revived cultures for the research in the best possible way, they were subcultured twice (NDRI, NCDC Catalogue, 2016), retrieved from http://www.ndri.res.in/ncdc/Members/NCDC_Catalogue_2016.pdf.

3.4.6. Culturing the bacteria (Agar & broth)

Nutrient Agar and de Man Rogosa Sharpe (MRS) broth at temperature 37°C for 12-24 hours were selected as the suitable medium for the growth and culturing of probiotic bacteria.

3.4.7. The morphological and biochemical characterization of bacterial cultures were done by the methods described by Giuliano *et al.,* (2019).

3.4.8. ***In vitro*** **probiotics assays:** Resistance to gastric acidity and bile acid resistance of food mixes at different pH values at different exposure time were done by standard protocol given by Awan and Rahman (2005). Antimicrobial activity was carried out by disc diffusion method (Daoud *et al.,* 2015).

3.4.9. **Standard stock solution to estimate the accurate bacterial count was estimated using** the standard protocol given by Zapata and Ramirez (2015) with modifications. The 0.5 McFarland turbidity standards are presented in Plate 7.

Plate 7: Standard stock solution to estimate the accurate bacterial count

3.4.10 Preparation of pure inoculum of probiotic strains prior to inoculation

MRS broth (100 ml) was prepared and autoclaved for sterility. 4 conical flasks of 50 ml were used for the process. Pure culture from agar plates were mixed in 50 ml conical flask with 25 ml of MRS broth and kept in a BOD incubator for 24 hours at 37^{o} C. The bacterial cultures grown in broth were then centrifuged for 5000 rpm for 15 minutes once to receive pure culture. Supernatant were taken as pure culture and added with 10 ml of normal saline. These cultures can be stored in refrigerator (4 ^{o} C) till use. The method used was given by Okhonlaye and Michael (2015) with modifications and shown in Plate 8.

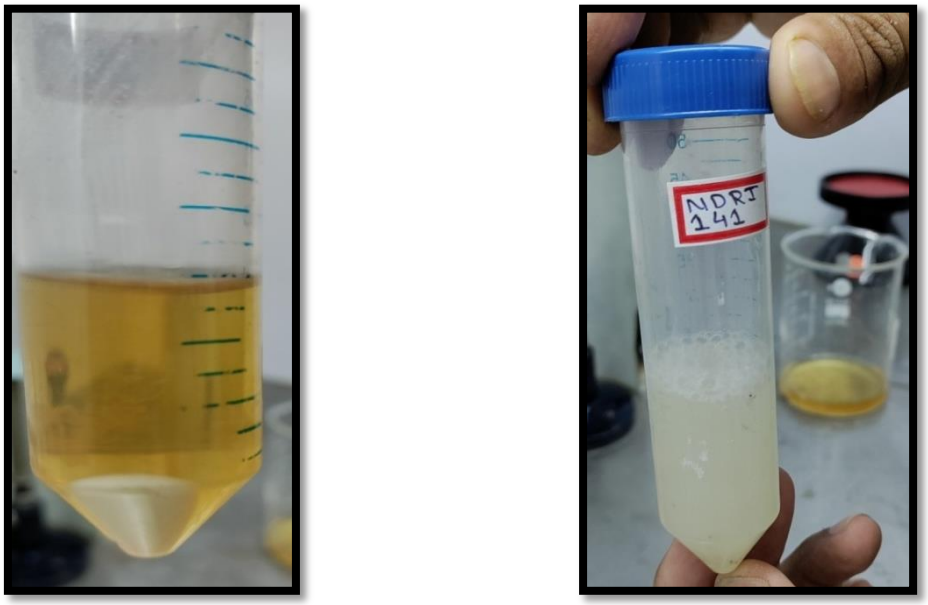


Plate 8. Preparation of pure suspension of probiotic strains prior to inoculation

3.4.11 Preparation of Inoculums

The density of bacterial cell suspension/inoculum was compared to the freshly prepared 0.5 McFarland turbidity standard scale by holding them both in front of a light against a contrast background. The density of inoculum was adjusted by diluting it with more sterile normal saline if needed. The standard method was derived from Ntsamo (2020) with modifications and shown in Plate 9.

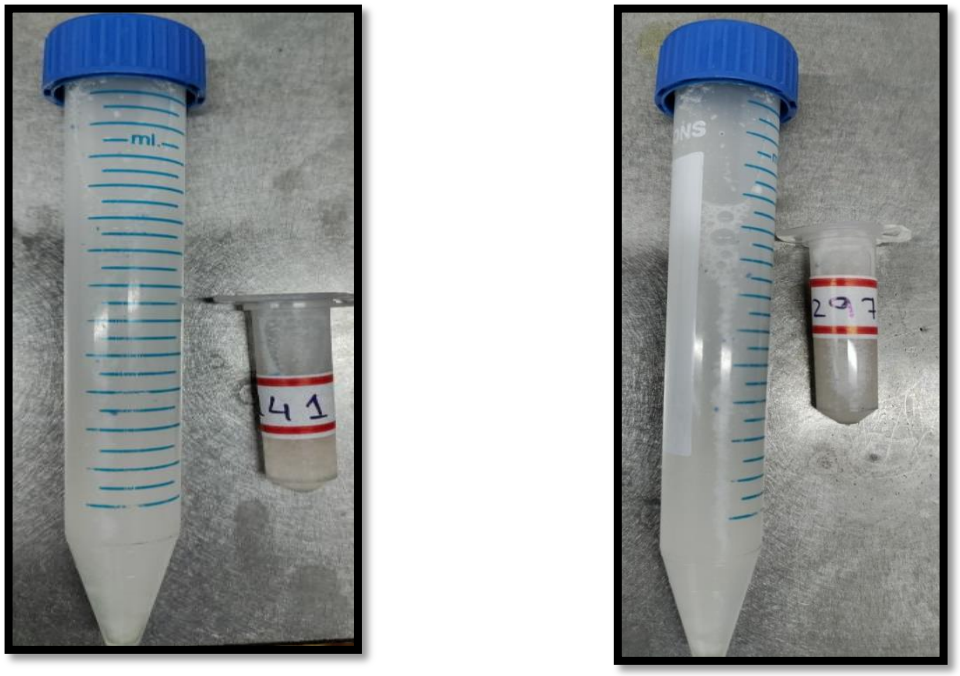


Plate 9. Preparation of inoculums with required colony forming units

3.4.12. Fermentation/Probiotification of Complementary food mixes by inoculation of bacterial cultures

Submerged fermentation (1:3) was carried out by adding 297 ml of sterile distill water aseptically in 100 ml of test samples and mixed thoroughly in a homogenizer and sterilized at a temperature of 121^{o}C for 20 minutes. The fermentation jars were cooled and inoculated at 10^{8} CFU m/l, tightly covered with aluminium foil to keep it away from contamination and kept undisturbed in a BOD incubator for 24 hours at 37^{o} C .The standard method was derived from Ntsamo (2020) with modifications and shown in Plate 10.

Plate 10. Probiotification of complementary food mixes by inoculation of bacterial cultures

3.4.13. Freeze Drying

The fermented samples were than freeze dried at - 53˚C for continuously 72 hours till the product reach to a free flowing state according to the process outlined by Martin and Christ, (2014) and Ciurzynska and Lenart, (2011). The freeze drying is shown in Plate 11.

Plate 11. Freeze drying of samples

3.4.15. Revival studies (Periodically)

To estimate the microbial load in the fermented complementary food mixes total viable bacterial counts were calculated as described by da Silva in 2016. Peptone water was mashed and mixed into the samples. For the counting of aerobic viable bacteria, subsamples were decimally diluted, and 0.1 ml aliquots were spread out on nutrient agar (NA), MacConkey agar (MCA), and potato dextrose agar (PDA). PDA plates were incubated at room temperature of 25 °C for 3-5 days whereas NA and MCA plates were incubated at 37°C for 24-48 hours. After that, the colonies were calculated through colony counter in colony forming units per gram (cfu/g) of the samples. The Stuart scientific colony counter was used to do duplicate counts of each item.The standard protocol by da Silva (2016) was used with modifications.

Phase V

3.5 Sensory evaluation of probiotic complementary food mixes

The evaluation of food in sensory evaluation is done through all the senses as the individual interact with the food being examined. Usual examination involves a judgment on both texture and colour. The smell sense is used to assess the aroma of the meal and also influences how flavour is perceived in general. The ability to distinguish between sour, sweet, salty, and bitter flavour components depends heavily on one's sense of taste (Srilakshmi, 2010). In the sensory evaluation laboratory of the Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher education for Women, Coimbatore, the acceptability of the formulated mixes was assessed using a nine-point Hedonic scale.

3.5.1 Formulation of score card

For evaluating the acceptability of the developed formulations, score card method was selected. According to Piggott and Hunter (1999) scoring is a form of rating using a numerical score where the numbers form an interval or the ratio scale. In the present study, a score card was made consisting a table utilizing the Hedonic ratings of nine point scale (Peryem and Pilgrim, 1957) from like extremely

to dislike extremely. The qualities taken into consideration were colour, flavour, consistency, appearance, taste and overall acceptability. The nine-point Hedonic scale method was used to measure the consumer acceptability of the formulated products where the food samples were served to the panelists and asked to rate the product. The score card used for evaluating the formulations is given in the Appendix II.

3.5.2 Conduct of acceptability trials

A trained and semi-trained panel of 30 individuals from the Department of Food Science and Nutrition at the Avinashilingam Institute for Home Science and Higher Education for Women in Coimbatore conducted the sensory evaluation. On a nine-point hedonic scale, scores were given. The sensory qualities of the developed complementary food mixes were evaluated for acceptance.

Forthe acceptability of the complementary food mixes, all of the formulations were prepared into porridge with milk and jaggery powder. A simultaneous presentation of the score cards and porridges, both served at room temperature, were made carefully to ensure that they had the right consistency and flavour. In a quiet laboratory that was well-ventilated and lit, the sensory evaluation session took place. At the time of evaluation, the judges were in good health status. Judges were seated comfortably in chairs at a suitable height at counters.Before the start of the sensory session, the panelists received the proforma for the sensory evaluation and were briefed on it. Water was also given to the panelists to help them get over their taste bud overload. The panelists assessed the samples based on their colour, appearance, flavour, consistency, and overall acceptability. Each quality's scores were added together and averaged. The formulations with the highest overall acceptability scores were chosen from the prepared formulations to be used in the *in vivo* studies and for a subsequent evaluation of the physico-chemical parameters of the developed complementary food mixes.The IHEC approval number is AUW/IHEC/FSN-21-22/XPD-33. The conduction of acceptability trials are shown in Plate 12.

Plate 12. Conduction of acceptability trials

3.6 Analysis of physical and nutritional composition of probiotic complementary food mixes

3.6.1. Physical evaluation of probiotic complementary food mixes

The physical characteristics of any material are essential for developing food products with additional value. As consumer's food choices increases, food industry increasingly relies on ingredients that give foods good functional characteristics in addition to nutritional value. The physical characteristics of food are particularly important for storage quality, processing of ingredients and quality. Understanding the physical properties of food products are helpful in handling, preparing, processing, preserving, packaging, storing, and distributing food, understanding physical qualities is crucial.

Bulk density was measured by the method suggested by Lewis, (1987).Swelling capacity was estimated by Appiah *et al.,* 2011.Viscosity was measured by the modified method of Hallic and Kelly, (1959) by using Visco basic plus viscometer. Water absorption capacity of the flours was determined by the method of Sosulski *et al*., (1976). The same method was used for estimating oil absorption instead of water oil was used. Dispersibility was calculated by Kulkarni *et al*., (1991). Texture was analysed by the modified method given by Mantihal *et al.,* (2019) by using a TA.XT-Plus Texture analyser.

3.6.2. Nutritional evaluation of probiotic complementary food mixes for moisture, crude protein,fat, crude fiber, available carbohydrate and total minerals were calculated by standard protocol mentioned in Appendix I. The standard protocol for Iron, zinc, copper, manganese, potassium, magnesium, sodium, calcium,

phosphorus were determined by Atomic Absorption Spectrophotometry (AAS) and are mentioned in Appendix I. *In vitro* protein digestibility and *In vitro* starch digestibility was estimated using modified method detailed by Walter *et al.,* (1983) and Som *et al.,* (1992).

3.6.3. Estimation of free radical scavenging activity (RSA) of probiotic complementary food mixes was done using DPPH method according to modified method given by Vani*et al.,* (1997) presented in Annexure-I

3.6.4. Estimation of phytochemical components of probiotic complementary food mixes.

Phytochemicals such as flavonoids, alkaloids, terpenoids, glycosides, tannins, phenolic, and saponins were analyzed by both qualitative and quantitative methods. The tests were done in sterilized environments in triplicates using standard protocols mentioned in Appendix I.

Phase VI

3.7 Assessment of functional efficacy of probiotic complementary food mixes through *in- vivo* animal studies

50-80 g of body weight, weanling healthy wistar albino rats of both sexes were procured from Animal house of Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu. The IAEC Approval number is AIW: IAEC.2020:FSN: 01(Appendix III).

The animals were monitored from the time of gestation to the time of delivery. The pups were then monitored for 21 days until weanling period and were confined in polypropylene cages with 6 rats per cage and allowed to acclimatize to experimental condition for 7 days. All the animals in the cages were numbered from 1 to 6. Animals were kept in typical laboratory settings with accessibility to a regular balanced diet and clean drinking water*ad-libitum*with 12:12 hour light/dark cycle at ambient temperature, 12-25°C (Plate 13 and Plate 14). The Control groups fed with rat ration and Experimental Groups fed with probiotic complementary food mixes with highest viable cell count. For growing or weaning rats, a daily dietary dose of 15 g/rat can be given (National research council, 1995). The prepared complementary food mixes is shown in Plate 14. The

standard protocol detailed in this study was adopted by modifying method of Barai *et al*., (2018).

Plate 13. Grouping of Wistar strain albino rats

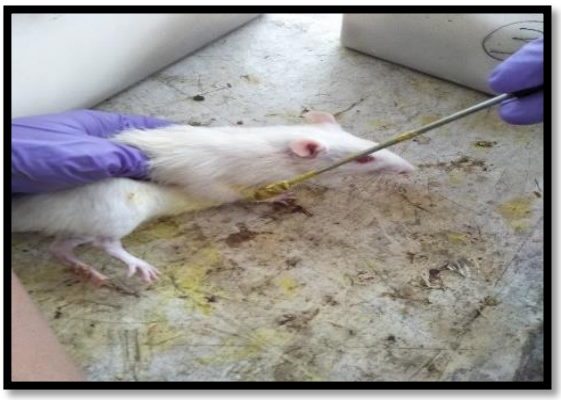


Plate 14. Marking of Wistar strain albino rats

3.7.1. Efficacy of probiotic complementary food mixes on the castor oil induced diarrhoeal Wistar strain albino rats

Rats were housed into cages made of propylene and kept starved for 24 hours while having access to water. Loperamide (3 mg/kg) was given to experimental rats in the positive control group one hour before administrating castor oil. Except for the control group, all rats received 0.5 ml of castor oil orally, and each cage was coated with white blotting paper. Stool or any other fluid materials that stained the blotting paper placed under each cage with a floor were considered signs of diarrhoea. Stools when became loose, muddy or watery was considered to as diarrhea (Barai *et al.,* 2018) (Appendix I). After the infection was developed, all the groups were fed with probiotic complementary food mixes for 7days twice a day from 24hr of induction of diarrhoea except group B which will be fed by rat ration along with a standard drug Loperamide at 5 mg/kg. Faecal were collected from 0-9 days at an interval of 24 hrs during the time of inoculation of infection as well as complementary food mix feeding. The fecal matter of each rat was collected with Sterile, Hiculture Collection device with a sterile transport cotton swab fixed on a polypropylene stick .Plate 15, Plate 16 and Plate 17 depicts the process of efficacy of probiotic complementary food mixes on the castor oil induced diarrhoeal Wistar strain albino rats. Table 3.2 shows the different grouping of animals.

Table 3.2. Proportion and composition of diets given to the Wistar albino rats

Group	Number of rats	Diet
Standard	6	100% RR
Negative control	6	100% RR
Positive control	6	100 % RR
A	6	100 % CFM I
B	6	100% CFM II

RR = Rat ration, negative control group- Diarrhoeal induced but without medicine, positive control group- diarrhoeal induced with standard drug Loperamide 3mg/kg. Experimental group A- Diarrhoeal induced and fed with CFM I, Experimental group B-Diarrhoeal induced and fed with CFM II.

3.7.2. Gastrointestinal viability of probiotic complementary food mix through fecal microbial count

The study was done on 36 healthy weanling Wistar albino rats each of either sex. Three groups were made namely Control, Experimental Group A and Experimental Group B. Each group consist of 12 rats and was divided into two sub groups. Control group were fed on rat ration, Group A was fed on CFM I and Group B were fed on CFM II. Each group consists of 12 rats. All rats were fed once with 15 g of experimental foods each (CFM I and CFM II) on the first day of study for 7 days. The fecal matter of each rat was collected with Sterile, Hiculture Collection device with a sterile transport cotton swab fixed on a polypropylene stick. The collection was done every day in the morning and stored in a refrigerator (2-8 ^{o}C) till further analysis. After 72 hours of anaerobic incubation at 37°C, the number of Lactobacillus spp. was counted on Rogosa agar (Merck, Darmstadt, Germany). The feeding trials were adopted from Adebiyi *et al.,* (2008).

The study was performed to compare the influence of probiotic on other microbial load in the gastrointestinal microflora till 7days of experimental period.

3.7.3. *In Vivo* protein quality assessment of complementary food mixes by using net protein ratio bioassay

Net protein ratio (Miller and Bender, 1955) is the simplest procedure to evaluate the protein quality.It focuses on weight gain on test protein and weight

loss of protein free group to the weight of test protein diet consumed. The NPR assay is similar to the Protein Efficiency Ratio (PER). Different diets was prepared, according to AIN-93G (Reeves *et al*. 1993).Group A was kept as reference diet (casein), group B was fed with protein-free diet (maple syrup). Group C and D were kept at experimental diets.The Net Protein Ratio (NPR) was determined on the 14th day of the experiment, taking the weight gain of the test group plus the weight loss of protein free diet group in relation to test protein consumption.

Net protein ratio of experimental diets were analysed with the following formula:

Net protein ratio (NPR) = [weight gain on test protein (g) + weight loss of non protein group (g)]/ weight of test protein consumed (g)

Relative protein ratio values (RNPR) were calculated as follows:

RNPR= (NPR of test diet/ NPR of control diet) × 100

Plate 15. Feeding of castor oil

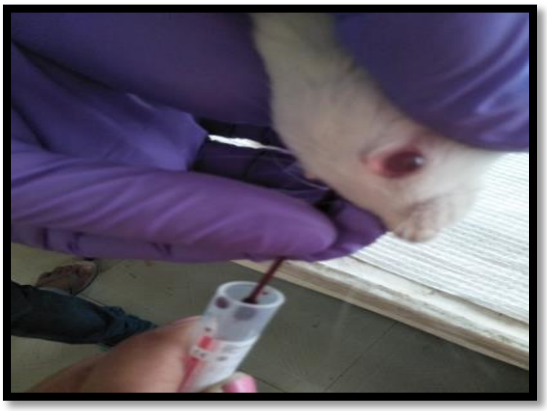
Plate 16. Inoculation of diarrhoea

Plate 17. Collection of anal fecal matter

Plate 18. Weighing of Wistar strain albino rats

Plate 19. Preparation of feed

Puncture from the inner retro-orbital eye for blood collection



Blood collection from retro orbital plexus of albino rats

Plate 20. Collection of blood samples from Wistar albino rats

Table 3.3. Proportion of diets given to the Wistar albino rats

Group	Number of rats	Diet
A	6	100% casein
B	6	100% protein free diet
C	6	100 % CFM I
D	6	100 % CFM II

Experimental group C (CFM I): T_4 + *Lactobacillus plantarum*
Experimental group D (CFM II): T_6 + *Lactobacillus casei*

3.7.4. Food conversion efficiency in albino rats

The following parameters were observed in 30 weanling healthy albino rats of either sex. The feed was given according to the Table 3.4 and depicted in Plate 18 and Plate 19.

The food intake was determined as the differential between the quantity of food served and the quantity of food left over and the change in weight was determined by weighing the rats daily. The food conversion efficiency was calculated as per method of Shingari and Sapra (1991).

$$\text{Food Conversion Efficiency (FCE)} = \frac{\text{Total quantity of food consumed (g)}}{\text{Body weight gain (g)}}$$

3.7.5. Biochemical parameters

The following parameters were observed in 30 weanling healthy albino rats of either sex. The feed was given according to the Table 3.4.

i) Blood glucose
ii) Total blood protein

Blood samples (5 ml) from the inner retro-orbital plexus of the Wistar albino rat eye was punctured and placed individually in heparinized, sterile centrifuge tubes to measure biochemical markers. The plasma was generated by vortexing the blood samples at 3000 rotation per minute (rpm) for 15 minutes.The samples will be then subjected to further biochemical investigation.The serum was separated as per standard protocols which were later subjected to the above test. The blood glucose level will be measured using SIEMENS Glucose *in*

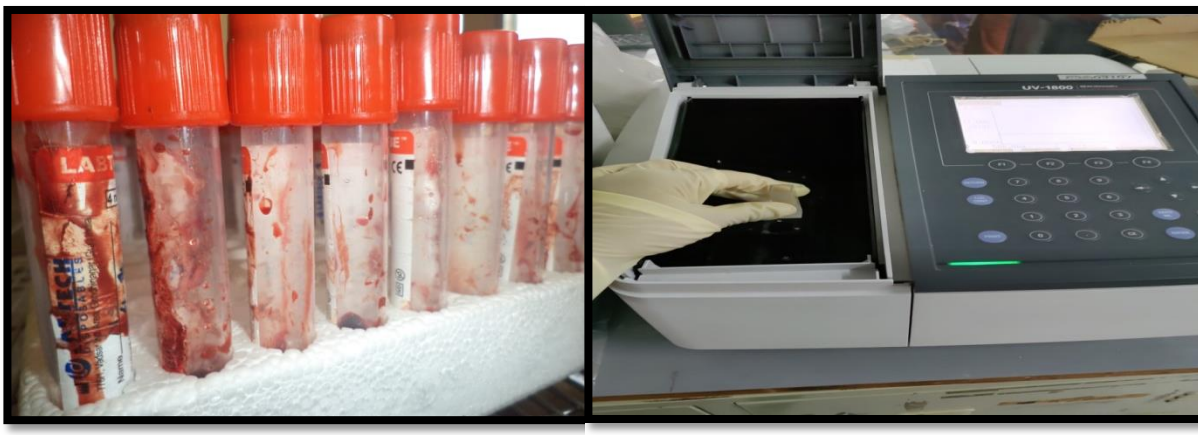
*vitro*diagnostic kit. The total blood protein levels will be measured by using SIEMENS Total Protein *in vitro* diagnostic kit. The feeding trials were adopted from Dutta *et al*., 2019. Plate 20 depicts the process of collection and analysis of blood samples of Wistar strain albino rats.

Table 3.4. Proportion of feeding of Wistar strain albino rats for analysis of biochemical parameters

Group	Number of rats	Diet
I	6	Rat ration
II	6	100 % CFM I
III	6	100 % CFM II

CFM I: T_4 + *Lactobacillus plantarum*

CFM II: T_6 + *Lactobacillus casei*



Blood samples in heparinized centrifuge tubes **Blood samples in heparinized centrifuge tubes**

Plate 21. Analysing of blood samples from Wistar albino rats

3.8. Shelf life studies of the developed probiotic complementary food mixes.

Shelf life study was conducted by storing the mixes CFM I and CFM II at ambienttemperature of 20-25^0 C.

3.8.1. Peroxide value and free fatty acid content of developed probiotic complementary food mixeswas determined using AOAC (2010) method and AOAC (1970)(Appendix I).

3.8.2. Microbial viability and pHin probiotic complementary food mixes across storage.

To verify the viability of microbial food cultures in the formulations, cfu count of viable cells present in the complementary food mixes (cfu/ml) were determined by plate count methodology using Miles and Misra (1938) method for 60 days (Appendix I)

The pH value of probioticated complementary food mixes was accessed with digital pH meter (Eutech Instruments, Germany).

3.8.3. Total fungal count in the probiotic complementary food mixes

The total fungal count was analyzed at an interval of 7 days of the Probiotic complementary food mixes stored from 0^{th} day till 75^{th} day. The shelf life was analyzed by the Standard AOAC (2010) method. TFC was analyzed by the Standard operating procedure, by Colony Count Technique at 35°C for estimating the number of microorganisims (TBC)IS 5402:2012/ISO 4833:2003, BAM, DGHS Manual (Appendix I).

3.8.4. Effect of metallised polyethylene packaging on moisture content of developed probiotic complementary food mixes

The moisture content of the stored samples was estimated or a period of 90 days with a gap of 15 days using AOAC (2010) (Raghuramulu *et al.,* 2003) (Appendix I)

Phase IX

3.9 Data collection and Statistical analysis

Statistics were used to analyze the relationship between different formulations and other parameters. The data generated from the analysis were presented in tables, graphs etc. The statistical methods used in the present investigation are given below. All the data were scrutinized by SPSS v.26.0 for Windows (SPSS Inc., Chicago, IL). The significance level was set to the level of $P< 0.05$ and $P<0.001$. The following were the statistical tests applied in the different phases (Appendix I).

1. The descriptive statistics i.e, mean and standard deviation (SD) were used to evaluate the proximate analysis of raw ingredients, mineral composition of the raw ingredients used for formulations, phytonutrients of raw ingredients used for formulation of mixes, activity of antibiotics on common enteropathogens, activity of probiotic complementary food mixes on common enteropathogens, mineral composition of the probiotic complementary food mixes, qualitative and quantitative phytochemical parameters in probiotic complementary food mixes, *In-vitro* protein and starch digestibility of the probiotic complementary food mixes, impact of feeding probiotic complementary food mixes on the net protein ratio of wistar strain albino rats, Food intake, weight gain and food conversion efficiency in Wistar strain albino rats.
2. Percentage was calculated for qualitative parameters including Free Radical Scavenging Activity (RSA) of the probiotic complementary food mixes,
3. Paired 't' test was applied to compare the impact of a specific treatment on the sample individual (selected sample) which included Impact of malting on the nutrient composition of Wheat (*Triticum aestivum)* flour.
4. Post – Hoc Duncan's test - analysing the multiple comparisons of the mean and standard deviation of physicochemical analysis for probiotic complementary food mixes, acceptability trials of the probiotic complementary food mixes,efficacy of probiotic complementary food mixes on the castor oil induced diarrhoeal Wistar strain albino rats, blood glucose levels of Wistar strain albino rats after feeding with probiotic complementary food mix, shelf life studies of the developed probiotic complementary food mixes
5. ANOVA was used in comparingbetween group variability to within group variability (F-test) in order to test the equality of different treatments on the time of interval on the Impact of feeding probiotic complementary food mixes (CFM I and CFM II) on the gut microflora of Wistar strain albino rats(Analysis of variance).
6. Analysis of simple linear regression was employed to predict the influencing power of the independent variables on dependent variable. The

effect of a factor on dependent variable, simple linear regression was conductedfor analyzing the influence of net body weight on gain/loss of Wistar strain albino rats.

7. Critical differences were calculated to find out the treatment differences.
   Level of significance selected were:
   (P≤0.05) Significant ; (P≤0.01) Significant
   Formula used were from Snedecor Cochran (1967).
8. Karl Pearson's chi-square correlation analysis was applied to assess the association between viscosity and protein digestibility