CHAPTER 4

MATERIALS AND METHODS

Research outcomes are only substantial when they are based on unfailing methodologies. Present research "Probiotic profiling and organoleptic evaluation of traditional cereal based fermented drink and its market potential" was conducted in four phases and this chapter is written in hierarchical manner describing the methods and techniques from first to final step of the research.

Phase I - Acceptability of FOS added *ambils* in terms of various organoleptic attributes and overall acceptability using composite score.

Phase II - Probiotic profiling of modified *ambils*

Phase III - Physicochemical properties, nutritional composition of buttermilk and ambils and shelf life of fresh and packaged *ambils*.

Phase IV - Consumer acceptability of packaged ambil and its market potential.

Phase I - Acceptability of FOS added *ambils* in terms of various organoleptic attributes and overall acceptability using composite score.

4.1 Development of FOS incorporated ambils

4.1.1 Procurement of fructooligosaccharide

4.1.2 Procurement of raw ingredients

4.1.3 Preparation of ambils using standard recipe

4.1.4 Modification of ambils and addition of colors, flavors and FOS

4.2 Sensory evaluation of FOS incorporated ambils

4.2.1 Selection and training of panel judges for organoleptic evaluation

4.2.2 Training of the selected panel members

4.2.3 Tools for organoleptic evaluation

4.2.4 Organoleptic evaluation

4.2.5 Statistical analysis

Phase II - Probiotic profile of modified *ambils*

4.3 Preparation of sample for DNA isolation

4.4 Specificity of primers

4.5 Isolation of bacterial DNA

4.6 Polymerase chain reaction and gel electrophoresis

4.7 Sequence analysis of PCR Products

Phase III Physicochemical properties, nutritional composition of buttermilk and ambils and shelf life of fresh and packaged *ambils*.

4.8 Physicochemical analysis

4.8.1 Estimation of Moisture

4.8.2 Estimation of Acidity

4.8.3 Estimation of pH

4.8.4 Estimation of Viscosity

4.9 Nutritional analysis

4.9.1 Estimation of Carbohydrates

4.9.2 Estimation of Protein

4.9.3 Estimation of Total fat

4.9.4 Estimation of Calories

4.9.5 Estimation of Crude fiber

4.9.6 Estimation of Total ash

4.9.7 Estimation of Folic acid

4.9.8 Estimation of Vitamin B1

4.9.9 Estimation of Vitamin B2

4.9.10 Estimation of Vitamin B3

4.9.11 Estimation of Calcium

4.9.12 Estimation of Sodium

4.9.13 Estimation of Potassium

4.9.14 Estimation of Phosphorus

4.9.15 Estimation of Magnesium

4.10 Microbial analysis of buttermilk, fresh ambils and packaged ambils

4.10.1 Total plate count

4.10.2 Yeast and mold

4.10.3 Coliforms

4.11 Shelf life studies for fresh ambils

Phase IV - Packaging of the most acceptable *Ambil*, consumer acceptability and market potential

4.12 Packaging of rice ambil

4.13 Statutory clearances

4.14 Sample size selection

4.15 Selection of subjects for the study

4.16 Inclusion and exclusion criteria for the selection of the subjects

4.17 Study protocol

4.18 Organoleptic evaluation of product by the consumers

4.19 Administration of interviewer based questionnaires

Phase I - Acceptability of FOS added *ambils* in terms of various organoleptic attributes and overall acceptability using composite score.

4.1 Development of FOS incorporated ambils

Standardized recipe of ambil was used (*Ramakrishnan 1977; 1979*) and cereal-millets and flavors based modification were carried out using rice, pearl millet, barley and finger millet. Raw materials were procured from their respective locations and were used to prepare the product.

4.1.1 Procurement of fructooligosaccharide

Food grade 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 was apt alternate for sugar. The specifications of the fructooligosaccharide are given in Table 4.1

Table 4.1 Specification of the Fructooligosaccharide used for ambils

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

4.1.2 Procurement of raw ingredients

Various ingredients and materials that were needed to develop the ambils were collected from the local market of Vadodara. Procurement details of all the raw ingredients and their sources are listed in the Table 4.2

Table 4.2 List of Raw Ingredients along with their Sources used for Product Preparation

Category	Raw material/brand name	Source
Cereals	Rice (RH 204) Pearl millet (RHB 121) Barley (RJ 2035 dwarf) Finger millet (CO-RA 14)	Sri Karan Narendra Agriculture University, Jobner (Rajasthan)
Food flavours	Rose (Flying bird) Khus (Flying bird) Chocolate (Flying bird) Salt (Tata) Jeera powder	Deoomal's speciality shop, Vadodara Deoomal's speciality shop, Vadodara Deoomal's speciality shop, Vadodara Local market, Vadodara Shree Ranchhodray Masala Mill, Vadodara
Food colors	Red (Flying bird) Green (Flying bird) Chocolate (Flying bird)	Deoomal's speciality shop, Vadodara Deoomal's speciality shop, Vadodara Deoomal's speciality shop, Vadodara
Buttermilk		Amul

4.1.3 Preparation of ambils using standard recipe

Rice flour, pearl millet flour, barley flour and finger millet flour were all weighed to 80 g (8%) and then cooked with 520ml (52%) for 10min, 12min, 20 min and 15min respectively. The resulting mixture was then cooled completely at room temperature. Buttermilk was measured to 350 ml (35%) was then added to each of the mixtures and blended in an electric mixer (Panasonic MX AC-220 H Mixer Grinder) to a smooth consistency, without lumps. The total yield was 1 ltr. The smooth consistency beverages were then sieved through 1000 μ sieve and kept for incubation at 35 °C for 4 h.

4.1.4 Modification of ambils and addition of colors, flavours and FOS

The resulting fermented drinks (*ambils*) were prepared above were than modified using four different flavours (0.3%) namely rose, *khus*, chocolate and salt- cumin. Colours (0.3%) were added to according to the respective flavours, red food coloring for rose ambil, green food coloring for khus ambil and brown food coloring for chocolate ambil. Salt - cumin ambil was developed using 0.4% and 0.6% of salt and cumin respectively, no additional food coloring was added to it. The levels of these ingredients were decided on the basis of preliminary trials. FOS (5%) was added to all the *ambil*s and stirred well with all the flavors. The final product was cooled to 5 °C and stored in refrigerator (5–7 °C).

(a) (b) (c)

Plate 4.1 Preparation of rice ambil

(a) (b) (c)

Plate 4.2 Preparation of pearl millet ambil

(a) (b) (c)

Plate 4.3 Preparation of barley ambil

(a) (b) (c)

Plate 4.4 Preparation of finger millet ambil

Plate 4.5 Colour and flavor added rice ambil

4.2 Sensory evaluation of FOS incorporated ambils

4.2.1 Selection of panelists

In this section, selection of panel members were carried out. Teachers and Post Graduate students from the Maharaja Sayajirao University of Baroda, Faculty of Family and Community Sciences, Department of Foods and Nutrition were subjected to threshold testing.

Threshold test (Rangana 2010)

Threshold test was defined as a stimulus scale at which a transition in a series or judgment occurs. For conducting this test, score card for the same was formulated and pre tested (Appendix I). Each perspective panel member was given three sets of the solution i.e. Set 1, Set 2 and Set 3 having six solutions of different concentrations of salt, sugar and citric acid respectively and was arranged in random order. The participants were asked to identify and rank the samples in increasing order of concentration of taste from the test solutions offered. Three successive trials were conducted for screening of the panelists. Subjects who succeed to pass the threshold test were included further in evaluating the organoleptic characteristics of the food products.

4.2.2 Training of the selected panel members

A training tool was developed for imparting the basic knowledge and understanding of visual and organoleptic characteristics of the products. Training was given using the same.

4.2.3 Tools for organoleptic evaluation

Composite Scoring Test tool was selected as a tool for organoleptic evaluation (Appendix II). In the score cards, codes for the samples were mentioned when given to the panel members for evaluation which were then decoded before entry into excel sheet and analysis.

Composite scoring test

Organoleptic evaluation was carried out on the *ambil* samples containing different flavors using . Twenty five semi trained panellists were selected using the sensitivity threshold test. The panel members were asked to rate the samples for colour (10), consistency (10), taste and flavor (30), mouthfeel (10), after taste (10), absence of defects (10), and overall acceptability (20) using a composite score analysis in triplicates (Rangana, 2010).

4.2.4 Organoleptic evaluation

Application of this test was done for visual and organoleptic evaluation. Panelists were asked to evaluate and score essential quality attributes that were needed to be scored like color, consistency, taste, mouthfeel etc. These tests were done to assess each attribute of all the four flavours for all the cereal-millet based ambils.

Plate 4.6 Sensory evaluation of ambils by panel members

4.2.5 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS 17.0v) software. Results were expressed as mean values + standard deviations of all the four products. ANOVA was performed to determine the significant differences in various levels of FOS substituted food products, followed by Fishers Least Significant Difference (LSD) test to identify statistical differences among the means. Triplicate measurements were taken for each analysis. Results from all the tests were expressed as means ± standard deviations (SD).

Phase II - Probiotic profile of modified *ambils*

Probiotic profiling of ambils were done in Genelon Institute of Life Sciences, Bangalore. All the ambils were prepared using the same process under standard conditions. The samples were diluted and subjecting to DNA isolation, PCR and gel electrophoresis. Sequence analysis of the PCR products was done to match and identify the Lactobacillus and Bifidobacterium strains.

4.3 Preparation of sample for DNA isolation

MRS (de Man, Rogosa, and Sharpe)-agar plates were used for the isolation of species. The fermented drink that was developed was subjected to serial dilutions (10 folds). Then, 1 mL of the samples was added to 10 mL of MRS (de Man, Rogosa and Sharpe) broth (Difco Laboratories, Detroit, Mich., USA) as the specific growth medium for the strains. After 24 h of anaerobic growth (37°C), 0.02 mL of the diluted solutions was spread for 48 h on MRS agar media. The Petri plates were incubated for 72hrs at 37°C. Once the bacterial colonies developed, they were transferred to 200µl of PCR water and mixed thoroughly**.**

Plate 4.7 Preparation of dilutions of ambil samples for DNA isolation

4.4 Specificity of Primers

Novel species-specific primers for identification of the target species of Lactobacillus and Bifidobacterium were designed from 16S rRNA, 16S-23S rRNA intergenic spacer region, and 23S rRNA sequences. The specificity of the species-specific primer pairs were evaluated by PCR with genomic DNAs purified from the reference strains, which are listed in Table 4.3.

Table 4.3 Species specific primers used in the study
(Kwon et al, 2004; Yeon et al, 2007; Matsuki et al, 2003)

Target bacteria	Name of primers	Primer sequence 5'-3'	Length (bp)
L. rhamnosus	Rham-F	GTCGAACGAGTTCTGATTATTG	22
	Rham-R	GAACCATGCGGTTCTTGGAT	20
L. acidophilus	Lacido-F	CACTTCGGTGATGACGTTGG	20
	Lacido-R	CGATGCAGTTCCTCGGTTAAGC	22
L. reuteri	L.reuteri-F	GGCGGAACGTTGAATATTGT	20
	L.reuteri-R	ATTTTGGGGGAATCATAGCC	20
L. casei	hyb15-F	GGCAGTTGTAGGGCCAGTAA	20
	hyb15-R	CTCGAGCTGCACTGCTCTT	19
B. adolescentis	BiADO-F	CTCCAGTTGGATGCATGTC	19
	BiADO-R	CGAAGGCTTGCTCCCAGT	18
B. bifidum	BiBIF-F	CCACATGATCGCATGTGATTG	21
	BiBIF-R	CCGAAGGCTTGCTCCCAAA	19
B. longum	BiLON-F	TTCCAGTTGATCGCATGGTC	20
	BiLON-R	GGGAAGCCGTATCTCTACGA	20
B. lactis	Bflac-F	GTGGAGACACGGTTTCCC	18
	Bflac-R	CACACCACACAATCCAATAC	20

The bacteria were isolated from the food samples. Isolation was performed by the routine microbiological procedure and inoculation on a solid medium. Selective media were used for LAB and BIF was MRS plates. In most of the cases more than one colony was observed on the surface of the plates. The cultural and morphological characteristics were examined within the help of microscope. Different types of microorganisms were observed, majority of them belonged to Gram positive rods and cocci shaped bacteria. Subculturing of the isolate was done until pure isolates were obtained. Once pure colonies were obtained they were cultured in MRS and M17 and stored at 4°C in refrigerator until it was used.

The bacterial DNA was isolated from each of these colonies and was subjected to PCR amplification, electrophoresis and sequencing (Abed, 2013; Abdulamir et al., 2010). Results of the sequence were compared with existing sequences in the NCBI database using the Blast N programme.

4.5 Isolation of bacterial DNA

Resuspension buffer/ TE buffer (200 µl) was added to 200 µl of the PCR mix. To this 200 µl of lysis buffer was added followed by 50 µl of proteinase K and mixed thoroughly. This mix was then incubated for 10mins at 60°C followed by addition of 200 µl of chilled ethanol, and was kept at room temperature for 1 min. Silica gel (100 µl) was added to this mix, centrifuged at 6000rpm for 2mins and the supernatant was discarded. Wash buffer was then added 500 µl and centrifuged at 6000rpm for 2mins, supernatant was discarded, this process was repeated twice and the final collected sample was dried at 60°C for 2-3 mins in dry bath. 200 µl of elution buffer (10ml of 1M TIS + 200 µl of 0.5 M EDTA) was added and centrifuged at 6000 rom for 2mins. The final product was collected and subjected to agarose gel electrophoresis.

4.6 Polymerase chain reaction and gel electrophoresis

The sequence of the primer set 16S forward - was 5'…..GGA ATC TTC CAC AAT GGA CG…..3' and the primer set 16S reverse - was 5'…..CGC TTT ACG CCC AAT AAA TCC GG …..3' (Abdulamir et al., 2010); amplifications were carried out in 50 µl volumes containing (2 pmol/µl) of each primer, ammonium sulphate and KCl buffer 2.5 µl each, Magnesium chloride buffer 5 µl, dNTPs 1 µl, Taq. DNA polymerase 0.5 µl and DNA template 2 µl.

Amplification was achieved in 35 cycles (Plate 4.8). Prior to the first cycle, DNA was denatured at 95°C for 5 min subsequently, and each cycle consisted of denaturation at 95°C for 30 s, followed by annealing at 55°C for 1min. Elongation was carried out at 72.5°C and the extension time at 2 min subsequently. A final elongation was performed at 72°C for 10 min, and the holding temperature was for 10 s. PCR product was separated by electrophoresis. 1.5% (w/v) agarose gel (0.3gms) was added to 30ml of TA-EDTA (1 x TAE buffer) in an erlenmeyer flask and heated until dissolved properly. Propedium iodide (4 µl) was added and the gel was kept in a tray to solidify. 2 µl of loading dye and 10 µl of the PCR mix was loaded to 7 µl of ladder. Then, DNA bands were visualized by ultraviolet (UV) illumination at 300 nm wave-lengths on UV trans illuminator system (Herolab/Germany), and then photographic pictures were taken for each gel (Sambrook and Russell, 2001).

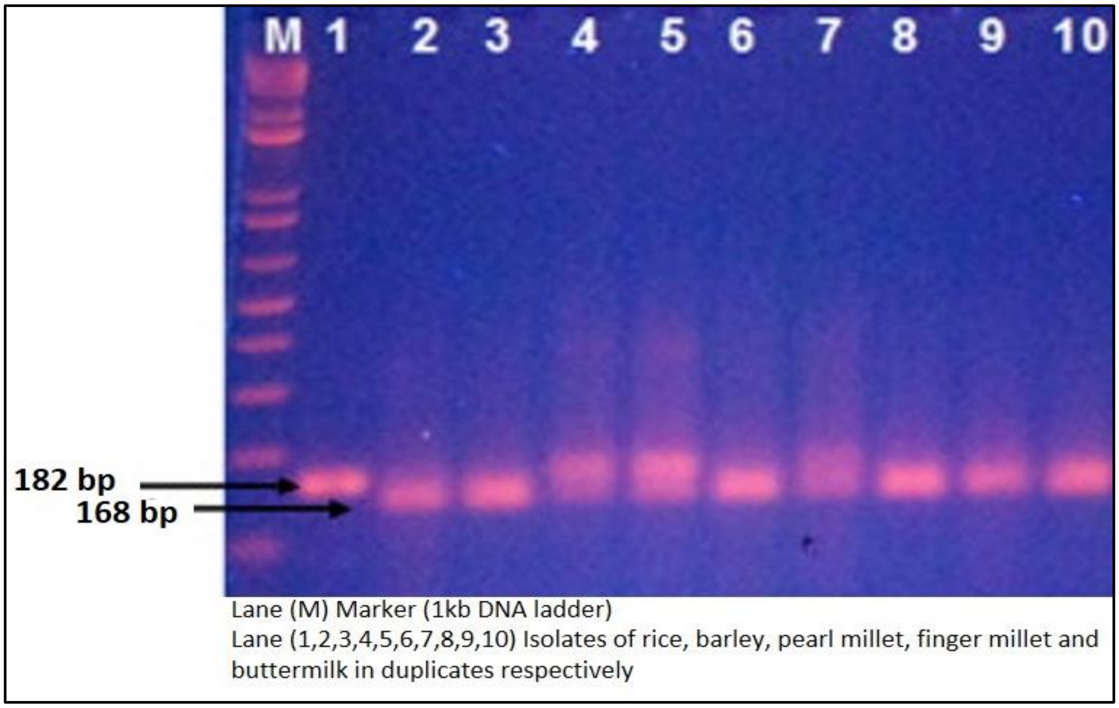

Plate 4.8 Agarose gel electrophoresis (1%) showing the amplification of the PCR products for 55 minutes at 100volt

4.7 Sequence Analysis of PCR Products

PCR products of probiotic samples were extracted from agarose gel using a QIA quick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The sequencing of the amplified DNA fragment was carried out with an ABI PRISM 3700 DNA analyser. The DNA sequences were analysed using the Vector NTI software Version 7. The isolates were then identified and discriminated by blasting their sequences with BLAST software (http://blast.ncbi.nlm. nih.gov/Blast.cgi). The strains were then compared with the sequences deposited in NCBI and GenBank.

Phase III Physicochemical properties, nutritional composition of buttermilk and ambils and shelf life of fresh and packaged *ambils*.

4.8 Physicochemical analysis

4.8.1 Estimation of Moisture

5 g of the sample was weighted and taken in a tared porcelin dish (W). The sample was crushed at the bottom of the dish with the glass rod. Dish was placed in hot air oven maintained at 105°C and dried for at least 3 hours. Dish was cooled in a dessicator and weighted in g (W2) (Ranganna, 2010)

Where,

Tare weight of dish—(W) g

Weight of dish with samples—(W1) g

Weight of dish + sample after keeping in oven (W2) g

Calculation:

$$\text{Percent moisture content} = \frac{\text{loss in weight} \times 100}{\text{Initial weight of sample}}$$

$$= \frac{(W1\text{-}W2) \times 100}{(W1—W)}$$

4.8.2 Estimation of Acidity

The acidity of ambil was determined against 0.1 N NaOH solution using phenolphthalein as an indicator. As ambil was an opaque liquid the end point of the titration using phenolphthalein indicator was difficult. To overcome this difficulty an auxiliary indicator like rosaniline acetate was used 10 ml of the thoroughly mixed sample was pipette out in a pair of porcelain dishes. It was diluted with equal volumes of freshly boiled and cooled water. To one of the samples 1ml of phenolphthalein indicator was added and to the other 1ml rosaniline acetate bench solution. It was then titrated against 0.1 N NaOH solution delivered from a burette protected with soda-lime guard tube. Near the end point alkali was added drop-wise until the color of the sample change to a pink shade, which matches to the tint of the sample in the other basin containing rosaniline acetate solution. The titrable acidity was expressed as % lactic acid (Ranganna, 2010).

Calculation:

Titratable acidity = 0.9 × V1 × N1

Where, V1 = Quantity of NaOH solution require for titration

N1 = Normality of NaOH solution

4.8.3 Estimation of pH

The pH was determined by using the digital (Lab-India) electronic pH meter.

4.8.4 Estimation of Viscosity

Viscometer was determined using multi-range ACM 52301 digital viscometer (Weiber).

4.9 Nutritional analysis

4.9.1 *Calculation of Carbohydrates*

Total carbohydrate content of foods was calculated by difference test. Under this approach, the other constituents in the food (protein, fat, water, alcohol, ash) were determined individually, summed and subtracted from the total weight of the food. This was referred to as total carbohydrate by difference and was calculated by the following formula:

Carbohydrate (gm) = 100 - (Wt. in gms [protein + fat + water + ash + alcohol] in 100 g of food)

4.9.2 *Estimation of Protein*

100mg powder was weighed in 30ml digestion flask and 1.9±0.1g of K_2SO_4 and 80±10mg of HgO, 2ml of H_2SO_4 were added and mixed well. Boiling chips/ glass beads were added to digest the sample until colourless over digestion rack (40 minutes at 370°C). It was cooled and water was added along the side of the flask to dissolve solid and transfer quantitatively to distillation apparatus with successive rinsing with water. 100ml conical flask containing 5ml of boric acid solution was placed with a few drops of mixed indicators in such a way that the tip of the condenser dipping inside the solution. 10 ml sodium hydroxide-sodium thiosulphate solution was added to the digest in the apparatus through the funnel and rinse with water. Ammonia was distilled and collected in boric acid. The colour changed from violet to green was an indication of ammonia absorbed (collected at least 15-20 ml of distilled amount). The tip of condenser was rinsed with water and titrated the distilled sample against standard HCl / H2So4 (0.02N) until the appearance of original violet colour as the end point. It was run blank and digested similarly with equal volume of water after washing the distillation apparatus by the back suction with excess of water (Ranganna, 2010).

$$N\% = \frac{(\text{ml HCl in sample}) \text{ X } (\text{ml HCl in blank}) \text{ X normality of acid X } 14.01 \text{ x } 100}{\text{Weight of the sample (mg)}}$$

1 N of acid= Gram equivalent weight dissolved in 1.0 liters of solution

4.9.3 Estimation of Total fat

Fat was estimated as crude ether extract of the dry material. The dry sample (5 - 10 g) was weighed accurately into a thimble and plugged with cotton. The thimble was placed in a Soxhlet apparatus and extracted with petroleum ether for about 16 h. The ether extract was filtered into a weighed conical flask. The flask containing the ether extract was washed 4 - 5 times with small quantities of ether and the washings were also transferred. The ether was then removed by evaporation, the flask with the residue dried in an oven at 80 - 100°C, cooled in a dessicator and weighed (Raghuramulu et al., 2003).

$$\text{Fat content (g/lOOg)} = \frac{\text{Wt. of ether extract x } 100}{\text{Wt. of sample}}$$

4.9.4 Calculation of total calories

The Atwater system was used to determine the energy values. This system uses factor to estimate available energy from protein, fat, carbohydrates and alcohol component of food item. Energy was calculated by using the general Atwater factor of 4 kilocalorie (kcal) per 'g' protein, 9 kcal per 'g' fat and 4 kcal per 'g' carbohydrate.

Energy (kcal) = (4 kcal/g x g protein) + (4 kcal/g x g carbohydrate) + (9 kcal/g x g fat).

4.9.5 Estimation of Crude fiber

2.5 g of the sample was transferred into the beaker, 200 ml of boiling sulphuric acid was added and connected with the digestion apparatus. It was boiled for exactly 30 minutes, filtered through filtering cloth and wash with hot water until it was free from acid. The residue was transferred on the cloth into the flask with 200 ml of boiling sodium hydroxide solution. The flask was connected immediately with the digestion apparatus and boiled further for exactly 30 minutes. The flask was removed and immediately filtered through Gooch crucible or alundum crucible. Washed with hot water until it was free from alkali and then with 10 ml of alcohol. It was dried at 105-110 °C in an air oven for about 2 hours and cooled to room temperature in desiccator and weighed. The lowest weight was noted which was considered as the weight of crucible and contents after drying. The contents were incinerated in the electric muffle furnace at 600+20 °C for about 30 minutes, cooled to room temperature in desiccator and weighed. The process of 30-minute drying, cooling and weighing was repeated until the difference between two successive weighing was less than 1 mg. The lowest weight was noted was considered as the weight of crucible and ash after incinerating. The difference between the two weighing was the weight of crude fibre (Ranganna, 2010).

Calculations,

Crude fibre, by weight = 100(W1-W2)/W
Where,
W was weight of sample, g
W1 was weight of crucible and contents after drying, g.
W2 was weight of crucible and ash after incinerating, g

4.9.6 Estimation of Total ash

5 g of the sample was weighted and taken in a tared crucible dish. The sample was crushed in the dish with glass rod. The sample was ignited on a blue flame of a burner till the smoke was given off. Then the crucible was cooled in a dessicator and weigh of the dish was noted in g (Ranganna, 2010).

Observation: Weight of dish + sample after drying = W2(g)
Weight of dish + sample after ignition = W3(g)

Calculation: Weight of ash = weight of dish after ignition – tare weight of dish = (W3 – W)

$$\text{Ash\%} = \frac{\text{Weight of ash} \times 100}{\text{Weight of sample}}$$

$$= \frac{w3 - w \times 100}{w1 - w}$$

4.9.7 Estimation of Folic acid

A known amount of sample containing about 100mg of folic acid was placed in a 100ml flask. 50ml of potassium hydrophosphate solution was added, and the mixture was heated to above 60°C with swirling until the sample is properly dispersed, cooled to room temperature and volume was made upto 100ml with potassium hydrophosphate solution. The centrifuge solution was filtered. An aliquot of the clear solution containing about 1mg folic acid was transferred to a 100ml volumetric flask and diluteed to volume with potassium hydrophosphate solution. This solution was used for color development and estimation. 1ml sodium nitrite solution and 1ml of 5N hydrochloride were added to all tubes. It was mixed and allowed to stand for 2min, 1ml of ammonium sulphate solution was added and mixed with swirling. 1ml of N- (1-naphdryl) ethylene diamine dihydrochloride solution was added, mixed and allowed to stand for 10min. 1gm of sodium chloride and 10ml of isobutyl alcohol were added and shakeen vigorously for 2-3min. The iso-butyl alcohol layer was separated by centrifugation and about 9ml of the clear supernatant layer was removed. Color of the iso butyl alcohol was read at 550mm within 25min using iso butyl alcohol as blank (Ranganna, 2010).

Calculation, Folic acid (mg/ml)

$$0.4\,C = \frac{A1 - A2}{A2 + A3 - (A1+A4)}$$

Where, C = concentration of the working standard of folic acid in mg/ml

A1, A2, A3, A4 are absorbance of tubes 1,2,3,4 respectively.

4.9.8 Estimation of Vitamin B1

The test was based on the oxidation of thiamine to thiochrome, which fluoresces in UV light. Under standard conditions and in the absence of other fluorescing substances, the fluorescence was proportional to the thiochrome present, and hence, to the thiamine originally in solution (Raghuramulu et al, 2003).

Preparation of vitamin extract: About 10 - 20 g of the homogenized vegetable samples were treated with 10 ml of acetate buffer and the mixture blended in a blender, (if necessary) for thorough mixing. To the slurry, 5 ml of enzyme suspension containing 150 mg of taka-diastase and 75 mg of papain in 5 ml of acetate buffer was added. An enzyme blank containing the buffer and enzymes but without the sample was also run simultaneously. A few drops of toluene were added and the mixture was incubated overnight at 37°C. The enzymes were inactivated by heating in a water bath at 80°C for 5 - 10 min. The solution was cooled, the mixture filtered and the clear filtrate collected. Aliquots of this solution were used for estimating thiamine.

Procedure: 20 ml of the vitamin extract was treated with 10 ml of basic lead acetate solution, mixed well and centrifijged. A suitable aliquot of the supernatant (20 or 25 ml) was treated in another centrifiige with 3 ml of 30% sulphuric acid and 12 ml of water (or enough water to make the total volume to 40 ml), mixed well and centrifiiged. 2 ml of 40% NaOH was added to three 100 ml separating funnels (one of them served as a blank, the other as the experimental and third as recovery). 0.8 ml of 2% potassium ferricyanide solution was added to the experimental and recovery, shaken well and 10 ml of thiamine extract was added to the separating funnels. To the recovery 1 ^ig of pure thiamine solution was added and the contents allowed to stand for 2 min after shaking. Then 15 ml of isobutyl alcohol (water saturated) was added and shaken for 2 min. The aqueous layer was rejected. If the alcohol layer was turbid, 1 ml of ethyl alcohol was added to clear the solution. The fluorescence of the isobutyl alcohol was measured in a flourimeter using the excitation wavelength of 370 nm and emission wavelength of 445 nm. The calculation was done after making due allowances for the dilution and the blank.

Calculation:

$$\mu\text{g thiamine/100 g sample} = \frac{\text{Experimental - Blank}}{\text{Recovery - Experimental}} \times \frac{100}{\text{Wt. of the sample}} \times \text{Dilution}$$

4.9.9 Estimation of Vitamin B2

The native fluorescence of riboflavin in neutral pH was used for the chemical estimation (Raghuramulu et al, 2003). Procedure: To about 25 ml of vitamin extract (prepared as in analysis of thiamine), one or two drops of caprylic alcohol was added, followed by 3 ml of fi-eshly prepared 4% potassium permanganate solution. The mixture was stirred well and within 2 min, 3 ml of 1:1

H202:water solution was added to discharge the permanganate color and the pH adjusted to 7.0 with NaOH. The volume was made up to 35 ml, the solution was filtered and the fluorescence of the filtrate was measured in a flourimeter using the appropriate filters.

Calculation:

The fluorescence of a known aliquot of the filtrate was - A 1

µg of riboflavin was added and the reading noted - B

A small pinch of sodium hydrosulphite was added to destroy the riboflavin only and recorded –C

Then the quantity of riboflavin in the solution taken for fluorimetry = $\frac{A - C}{B - A} \times 1\mu g$

From this, the riboflavin content of the sample was calculated making due allowances for dilution and weight of the sample.

4.9.10 Estimation of Vitamin B3

Niacin reacts with cyanogen bromide to give a pyridinium compound which undergoes rearrangement yielding derivatives. These derivatives couple with aromatic amines to give yellow colored pigment. Under proper conditions, the intensity of the yellow color produced is proportional to the amount of niacin present.

Pipette out 0.1mL to 0.5mL of the standard niacin solution into a series of test tubes. Two or three different volumes of samples solution in other test tubes (0.1-0.5mL) were taken. Volume was made up to 6mL with distilled water in all the tubes. 3mL of cyanogen bromide was added using a burette and shaken well. After 10 min1mL of 4% aniline was added to each tube. Yellow color developed after 5 min at 420nm against a reagent blank.

Calculation,

Standard graph was drawn with the readings of the standard niacin. Niacin content was found in the sample volume taken and g weight of the sample was calculated.

***Estimation of* Minerals**

Preparation of dry ash solution:

The ash (as analyzed under proximate constituents) was moistened with a small amount of glass distilled water (0.5 - 1.0 ml) and 5 ml of distilled hydrochloric acid was added to it. The mixture was evaporated to dryness on a boiling water bath. Another 5 ml of hydrochloric acid was added again and the solution evaporated to dryness as before. 4 ml of hydrochloric acid and a few ml of water was then added, the solution warmed over a boiling water bath and filtered into a 100 ml volumetric flask using Whatman No. 40 filter paper. After cooling the volume was made up to 100 ml and suitable amounts were used for estimation of phosphorus, iron and calcium (Raghuramulu et al, 2003).

4.9.11 Estimation of Calcium

Calcium was precipitated as calcium oxalate. The precipitate was dissolved in hot dilute H2SO4 and titrated against standard potassium permanganate (Ranganna, 2010).

Procedure: An aliquot (25 ml) of the ash solution obtained by dry ashing to a 250 ml conical flask was pipetted and diluted to 150 ml with water. A few drops of methyl red indicator was added and the mixture was neutralized with ammonia till the pale pink color changes to yellow. The solution was heated to boiling point, 10 ml of ammonium oxalate was added and boiled again for a few min. Glacial acetic acid was added till color of the mixture was distinctly pink. It was allowed to stand at room temperature for at least 4 h or preferably overnight. Filtered through Whatman No. 42 paper and washed with warm water, till the filtrate was oxalate free. (Since HCl had been used for preparing the ash solution, it was convenient to test for the absence of chloride using AgNOs). 5 - 10 ml of dilute H2SO4 was addedon the filter paper, the point of the filter paper was broken with a pointed glass rod and transferred to the conical flask. The solution was heated to 70°C and titrated against 0.01 N KMn04 to a permanent pale pink color.

Calculation: 1 ml of 0.0 IN KMn04 = 0.2004 mg of calcium If the normality of standard KMn04 solution was not exactly O.OIN, the following formula can be used.

$$\text{Calcium mg/100g} = \frac{\text{Titre X Normality of KMn04X 0.2004 X Total volume of ash solution X 100}}{\text{ml of ash solution taken for estimation X Wt. of sample taken for ashing}}$$

4.9.12 Estimation of Sodium

Flame photometric method was used to estimate sodium using standard curve between concentration and percent luminosity of sodium.

An aliquot was diluted so that it contains less than 10ppm of sodium. Sufficient HCl was added so that the concentration of acid is the same as that in the standard solution. The diluted extract was atomized in a calibrated flame photometer with the wavelength set at 589nm and the transmittance at 100% for the top standard solution of sodium. The instrument was monitored periodically with the top standard solution (Ranganna, 2010).

Calculation,

$$\text{Sodium (mg/100gm)} = \frac{\text{ppm found from the standard curve x volume made up x dilution x100}}{\text{Wt of sample x 1000}}$$

4.9.13 Estimation of Potassium

An aliquot of ash solution containing less than 150 ppm potassium was diluted, sufficient amount of HCl was added so that the concentration of acid was same as that in the standard solution. The diluted extract was atomized in a calibrated flame photometer with the wavelength dial set at 768 nm and the transmittance set at 100% for the top standard solution of potassium. The instrument was checked periodically with the top standard solution. The concentration was noted from the standard curve (Ranganna, 2010).

Calculation,

$$\text{Potassium mg/100g} = \frac{\text{ppm found from standard curve} \times \text{volume made up} \times \text{dilutions (if any)} \times 100}{\text{Weight of sample} \times 1000}$$

4.9.14 Estimation of Phosphorus

Phosphorus contents were determined by the colorimetric method. To an aliquot (0.1 ml) of the mineral solution of ammonium molybdate, 1 ml of hydroquinone and 1 ml of sodium carbonate solutions were 38 added in this order. The volume was then made to 15 ml with distilled water and the solution was mixed thoroughly. After 30 min the optical density of this solution was measured in a photoelectric colorimeter, against a reagent blank (prepared in the same way as the test except that the test solution was omitted) using a red filter (660 nm). The phosphorus content of the sample was read from a standard curve prepared with standard phosphate solution (range 0.01 - 0.1 mg P) following the same procedure as described above (Ranganna, 2010).

Calculation,

$$\text{Phosphorus mg/100g} = \frac{\text{ppm found from standard curve} \times \text{volume made up} \times \text{dilutions (if any)} \times 100}{\text{Weight of sample} \times 1000}$$

4.9.15 Estimation of Magnesium

HNO3 was added to the calcium free filtrate (obtained from the filtrate after precipitation of calcium as oxalate) the 30ml of conc. and the solution was evaporated completely on a boiling water bath. Five ml of conc. HCl and 100 ml of water were added in solution and stirred well with a glass rod. It was followed by the addition of 10 ml of 10% ammonium phosphate solution and 5 ml of 10% sodium citrate solution and the mixture was stirred well. After adding 2- 3 drops of methyl red indicator to the mixture and the solution was neutralized with the addition of 1: 4 dilute ammonia. Strong ammonia (25 ml) was then added and stirred vigorously and the mixture was left to stand overnight and then filtered through Whatman No.40 or 44 filter paper followed by washing to make free from chlorides using 1:10 dilute ammonia(tested with HNO3+silver nitrate solution). The funnel with the precipitate on the filter paper was dried in an oven. The filter paper was then transferred to a weighed crucible (the crucible was previously heated, cooled and weighed) and ashed slowly over a burner. It was then kept in a muffle furnace at 900 ºC for 2hrs. The crucible and the contents were recooled in a desiccators and weighed to get mangnesium as its pyrophosphate (Ranganna, 2010).

Calculations,

$$\text{Magnesium (mg/100gm)} = \text{Wt. of ash} \times \frac{48.64}{222.6} \times \frac{100}{\text{ash solution taken for estimation}} \times \frac{100}{\text{Wt. of sample}} \times 1000$$

4.10 Microbial analysis of buttermilk, fresh ambils and packaged ambils

4.10.1 Total plate count

The method was used for determining the total number of viable bacteria in product and consists of mixing appropriately diluted sample with a suitable nutrient agar medium in a Petri dish and counting bacterial colonies developed after incubated at a 37°C temperature for 24 hrs time. The samples were thoroughly mixed so it formed the uniform consistency. The eleven gram of sample transferred in 99ml dilution blank to get 1:10 dilution of the product. 1ml of suspension from this solution transferred to 9ml dilution blank to have 1:100 dilutions. 1 ml portion from three selected dilutions poured into sterile Petri dishes in duplicates. Then SPC a previously melted 10-15ml added to each plate and cooled to 45°C. These plates inverted and incubated at 37°C for 24 hours. After the completion of incubation period the plates were removed and counted the colonies and the results expressed as total viable count per gram of the product (Manuals of food quality control, 1967; Practical Manual Environmental Microbiology, 2009).

4.10.2 Yeast and mold

The samples were thoroughly mixed so to form uniform consistency of ice-cream Sample. The eleven gram of sample transferred in 99ml dilution blank to get 1:10 dilution of the product. 1ml of suspension from this solution transferred to 9ml dilution blank to have 1:100 dilutions. 1ml portions from the above dilutions poured into sterile Petri dishes adjusted aseptically, the pH of potato dextrose agar to 3.5 by adding calculated amount of sterile tartaric acid solution at the time of pouring plates. The agar poured in the plates and mixed the content well. The agar allowed to cooled and set. The plates were inverted and incubated at 21-25°C for 3 days. The number of colonies in plate counted and expressed results as number of yeasts and number of molds per gram of the product (Manuals of food quality control, 1967; Practical Manual Environmental Microbiology, 2009).

4.10.3 Coliform count

Coliform Test with Solid Media–Transferred appropriate volume (0.1 to 1.0 ml) of suitable dilution into sterile plates. Added with 10- 15 ml of violet red bile agar or desoxycholate agar with a view to increasing the sensitivity of tests, larger test portions (up to 4.0 ml) may be used with 15-20 ml of the medium. Quite often 10 ml of 1:10 dilution was distributed in 2-4 plates using 15-20 ml of the medium per plate. Mixed the contents thoroughly and allowed to solidify (within 5-10 minutes); added 3-4 ml of the same melted medium in each plate as an overlay to completely cover the surface to inhibit formation of surface colonies. Inverted and incubated for 18-24 hours at 37± 0.5oC. Dark red colonies measuring 0.5 mm or more on uncrowded plates were considered to be coliform bacteria. Count such colonies and report as 'coliform colonies per g' using two significant figures and substituting decimals for whole number where necessary (Manuals of food quality control, 1967; Practical Manual Environmental Microbiology, 2009).

4.11 Shelf life studies

Shelf life studies were conducted on all fresh ambils and packaged rice ambil (Appendix III). All the freshly prepared *ambils* were stored in refrigerator at 4°C ±1 in glass bottles with a tight fitting lid. The storage stability was studied for a period of 5 days, or till the product became unacceptable in terms of its physical appearance and aroma. Organoleptic characteristics and physical and microbial parameters were studied including pH, acidity, viscosity, peroxide value, whey separation, and microbial parameters including TPC, Coliform, yeast and mold. Packaged rice ambil was studied for a period of 6 months for organoleptic characteristics and physicochemical parameters.

Phase IV - Packaging of the most acceptable *Ambil*, consumer acceptability and market potential

4.12 Packaging of rice ambil

Rice ambil was prepared using 40kg rice flour and 260 litre water, and was cooked at 65°C for 12minutes. The mixture was then cooled up to 45-50°C and 175 litres of buttermilk was added. The buttermilk and rice mixture was incubated at 35°C for 4 hours and pH was maintained at 4.6. Salt and cumin were added and ambil was filled in large containers and kept in cold storage overnight. The prepared ambil was subjected to Ultra-high temperature processing followed by packaging in 200ml Tetra packs. The fresh rice ambil was heated to 132°C for 3 seconds and was packaged in 200ml tetra packs (Plate 4.9 - 4.11).

Plate 4.9 Bulk production of rice ambil at Amul **Plate 4.10 UHT processing machine**

Plate 4.11 Tetra packing machine

4.13 Statutory clearances

The Medical Ethics committee of the Foods and Nutrition Department, The M.S. University of Baroda approved the study proposal and provided the Medical ethics approval number (F.C.Sc/FND/ME/55). Permissions were obtained from the (i) Head and Dean of Dept. of Food and Nutrition, Faculty of Family and Community Sciences, The MS University of Baroda to enroll the participants of Food Fest held in the campus, (ii) Principles of Experimental School of The MS University of Baroda, Vadodara and (iii) Head officer of Jalaram Vrudhashram, Nizampura and Premdas Jalaram ashram, Warasia. Duly filled informed consent (Appendix II and IV) was obtained from the participants prior to the enrollment, who agreed to taste and rate the product and fill the questionnaire.

4.14 Sample size selection

Sample size was calculated based on the population census of Gujarat *(Census of India, 2011; Directorate of Economics and Statistics Government of Gujarat, 2011)* for all the different age groups. All the subjects selected were a representative of the total percentage population of that age group *(Census of India, 2011; Directorate of Economics and Statistics Government of Gujarat, 2011).*

4.15 Selection of subjects for the study

4.15.1 Consumer acceptability

Five age groups were defined which included elderlies, adults, adolescents, school going children and pre-schoolers. The consumers were selected by convenient sampling technique from a Food-fest organized at Faculty of Family and Community Sciences, Vadodara, Experimental School of The MS University, Vadodara, Old age homes and a local supermarket of Vadodara, depending on the subject's willingness to taste the product. A total of 518 subjects were selected using the percentage census of population. The consumer acceptability trials were continued until the target number of consumers from each group was reached (Appendix .

4.15.2 Market potential

Market potential was studied on the same subjects excluding the age group of less than 10 years (n=429), using a feedback form (Appendix IV). Questions were asked regarding their willingness to purchase the same beverage if available in the market, preference in any different flavor, package size preferred, packaging material and cost expected if introduced commercially in supermarkets of Vadodara.

4.16 Inclusion and exclusion criteria for the selection of the subjects

Inclusion criteria:

- Elderlies (>60 years)
- Adults (40-59 years)
- Young adults (20-39 years)
- Adolescents (10-19 years)
- Preschool and School children (<10 years)

Exclusion criteria: Children below 3 years of age.

4.17 Study protocol

Five hundred and eighteen subjects were selected from the age groups of <10 years, 10-19 years, 20-39 years, 40-59 years and >60 years to study the consumer acceptability of the packaged rice ambil. Forty two subjects were enrolled from old age homes in the age group of >60 years. Similarly 106 subjects from the age group of 40-59 years and 186 subjects from the age group of 20-39 years were selected using convenient sampling technique from a Food-fest organized at Faculty of Family and Community Sciences, Vadodara. Ninety five subjects and 89 subjects were selected from the age group of 10-19 years and <10 years respectively from the college students in the Faculty of Family and Community Sciences and The Experimental school, MS University of Baroda, Vadodara.

4.18 Organoleptic evaluation of product by the consumers

Five hundred and eighteen subjects were selected for the study for all the age groups and were briefed about the objectives and implications of the study. The subjects were given the packaged drink to taste and give hedonic rating according to their liking of the product.

Plate 4.12 Consumer acceptability in Experimental school of MS University of Baroda

Plate 4.13 Consumer acceptability in Food fest held at Dept. of Food and Nutrition, Faculty of Family and Community Sciences MS University of Baroda

4.19 Administration of questionnaires for market potential

To understand the market potential and consumer acceptability of the new concept for packaged rice ambil, market research was conducted in the state of Gujrat with 429 subjects (excluding the age group of less than 10 years). A total set of 12 questions were administered to understand the frequency of consumption of current health drinks, likability towards packaged rice ambil as a health drink option and further commercial acceptability of packaged rice ambil if introduced in the market. Following questions were asked.

4.19.1 Frequency of consumption of curd/ buttermilk

The subjects were asked about the consumption frequency of curd or buttermilk to understand the current pattern of consumption of homemade health drinks. Details of the number of times curd or buttermilk was consumed on a daily, monthly, twice a month or never basis was elicited using the questionnaire.

4.19.2 Frequency of consumption of fruit juices

The subjects were asked about the consumption frequency of fruit juices to understand the consumption pattern. Details of the number of times fruit juices were consumed on a daily, monthly, twice a month and never basis was elicited using the questionnaire.

4.19.3 Frequency of consumption of probiotic beverages

The subjects were asked about the consumption frequency of commercially available probiotic beverages to understand the acceptability of commercial probiotic beverages in the market. Details of the number of times probiotic beverages were consumed on a daily, monthly, twice a month and never basis was elicited using the questionnaire.

4.19.4 Consumer views on the idea of the packaged rice ambil

Consumer views were studied about the idea of the new health beverage ambil. The respondents were given options including “great idea”, “sounds interesting” and “neutral” to choose from.

4.19.5 Consumer views on need of the packaged rice ambil commercially

Consumer views were assessed on commercial need of the packaged rice ambil, and the responses were recorded in terms of “yes”, “no” and “may be”.

4.19.6 Consumer views on whether they will purchase packaged rice ambil if available commercially

Consumer views were assessed on whether they are likely to purchase if packaged rice ambil is made available commercially. This was needed to understand consumer acceptability of packaged rice ambil as a commercial product in the market. The responses were recorded in terms of “yes”, “no” and “may be”.

4.19.7 Consumer preference on price of packaged rice ambil if made available commercially

Consumer views were assessed on what is the range of price that would be acceptable for the consumers for a packaged rice ambil. This was needed to understand the commercial viability of such product in the market in future. Respondents were given following price range options to choose from Rs <10, Rs 10 – 15, Rs 15 – 20, Rs 20 – 25 and Rs 25 – 30

4.19.8 Consumer preference on recommending packaged rice ambil if made available today

Consumer views were assessed on whether they would recommend packaged rice ambil if made available today. This was to further understand their likability toward the product and acceptability of the benefits received from consuming such product. Assumption was made that if consumer is recommending the product to others, they are also likely to use it on their own. The responses were recorded in terms of "yes", "no" and "may be".

4.19.9 Consumer preference on flavors of packaged rice ambil in future

Consumer views were asked on possible flavor that they would like to consume packaged rice ambil in with options given as Cardamom, Chocolate, Mango, Pineapple, Rose, Saffron, and Salt & Cumin. This was asked to help understand the possible flavor options that should be provided to the consumers when the product is launched in the market.

4.19.10 Consumer preference for package size of packaged rice ambil

Consumer views were asked on the possible package sizes that they would like to consume packaged rice ambil in with options provided as 100 ml, 150 ml, 200 ml, 250 ml, > 250 ml. This was asked to understand the operational viability of packaging the rice ambil vs consumer acceptability.

4.19.11 Consumer likability for packaged rice ambil

Consumers were asked in the likert scale of 1 – 9 whether they liked the product or not. This was the most important criteria to understand the acceptability and further potential of such health drink in the market.

4.19.12.Consumer view on the factors affecting the purchase of health drinks in the market

Consumers were asked about the factors that affect their purchase of current health drinks in the market. This was to understand the product aspects that should be considered while deciding the commercial viability of launching packaged rice ambil in the market. Following options were given to the respondents - Appearance, Taste, Texture, Nutrition, Convenience, Price, Odour, Innovation and Brand.