CHAPTER V

SUMMARY AND CONCLUSION

After six months, a baby's requirements for nutritional and energy requirements start to exceed and hence is not fulfilled by mother's milk, necessitating the introduction of complementary foods. At this age, a baby is also developmentally prepared to eat other foods. This shift from breast milk to homemade foods are called as complementary feeding(WHO, 2020). Fermentation occurs mainly by a diverse group of microflora, which grows naturally with lactic acid bacteria generally overpowing the other flora as it is difficult to control, sometimes not safe since they are liable to contamination by pathogen. Therefore the need is to replace the prevailing uncontrolled fermentation with a pure culture probiotic so as to obtain a consistent product quality. The probiotic complementary food mixes was designed in the present study to help restore and maintain the natural flora in the gut and support the immune system of the 6-24 months along with it reduces the diarrheal frequency as it is one of the root causes of childhood disability that is a contributor towards the 3rd major cause of mortalities under the age of five (Semwal *et al*., 2015).Since there is no such data on probiotification of complementary foods, an attempt was made to assess the acceptability of the developed probiotic complementary food mixes based on locally available cereals, legumes and to study its efficacy on the Experimental rats.

Definition of the problem

The 3rd significant cause of malnutritionmortality and morbidity is diarrhoea, among under five children especially in the developing countries like India. World Health Organisation (WHO,2020) stated that death of children (below age five) due to diarrhoeal disease is almost 8 percentage. ‘It is, thus, clear that this disease is a major public health issue in India’ (Omona*et al*., 2020). UNICEF, (2021). According to UNICEF, (2021), malnutrition, pneumonia, diarrhoea, and malaria account for the majority of fatalities among children under five. The five nations comprising India, China, Congo, Nigeria, and Pakistan collectively account for 50% of the 4.249 million child fatalities from diarrhoea. The National Family

Health Survey (NFHS-5) confirms that childhood diarrhoea is prevalentin urban areas as6.2% and rural areas as 7.7% in the year 2019-21.

Objectives of the study: To

A. Develop probiotic complementary food mixes from locally available cereals and legumes
B. Assess the physico-chemical and functional properties of the developed complementary food mixes
C. Study the shelf life properties of the developed complementary food mixes
D. Evaluate the *In-vivo* efficacy of developed probiotic complementary food mixes through animal model.

Methodology:

Following the guidelines given for complementary foods by the Indian Council of Medical Research (ICMR), different food ingredients were procured from local market of Coimbatore, Tamil Nadu, India toformulate complementary food mixes under the present study including rice (*Oryza sativa*), finger millet (*Eleusine coracana*),pearl millet (*Pennisetum glaucum*), malted wheat flour (*Triticum*), mung bean (*Vigna radiata*), soyabean (*Glycine max*), sesame seeds (*Sesamum indicum*) and pumpkin seeds (*Cucurbita sp*.) and were processed using standard protocols. Nutritional attributes of the raw ingredient flours like crude fat, crude protein, fibre, available carbohydrate and moisture with other functional ingredients were determined by using standard procedures by AOAC official methods. Mineral elements that was estimated included calcium (Ca) determined by using flame photometer according to the method A.O.A.C 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).

Based on the guidelines given by Bureau of Indian Standards (2006) for complementary foods, six different complementary food compositions (T_1 to T_6) were formulated under the present study. The formulation with the highest energy density (ED) was subjected to further probiotification. The total energy content of the formulations was determined by ICMR, (2010) method. The energy density value of the formulations between 300 – 550 kcal per 100g of sample were selected for further probiotification.

The probiotic cultures were sought from the National Collection of Dairy Cultures (NCDC), ICAR – National Dairy Research Institute Karnal, Haryana. The probiotics selected were *Lactobacillus fermentum* (NDRI-141), *Lactobacillus casei* (NDRI 297), *Lactobacillus rhamnosus* (NDRI-353) and *Lactobacillus plantarum* (NDRI-375). The mixes were inoculated with the probiotic strains through standard protocols separately and were analysed for microbial viability. The mixes with highest viable cells after Probiotification were designated as CFM I (T_4) and CFM II (T_6).

For evaluating the acceptability of the developed formulations, score card method was selected. In the present study, a score card consisting a table utilizing the Hedonic ratings of nine point scale (Peryam and Pilgrim, 1957) from like extremely to dislike extremely. The IHEC Approval number is AUW/IHEC/FSN-21-22/XPD-33.The product has been patent published under the name “A process of preparation of probiotic complementary food mix and product thereof” with application number 202241007163”.

The physical, nutritional, phytonutrient, free radical scavenging activity of the Probiotic complementary food mixes were studied through the standard protocols as described in Appendix I. The *In vivo* quality efficacy for the developed complementary food mixes was conducted through animal model. The IAEC Approval number is AIW:IAEC.2020:FSN:01.The impact of feeding probioticated CFM I and CFM II on the gut microflora of rats through fecal microbial count were done through the standard protocol described by Barai *et al.,*(2018). Net protein ratio (Bender and Miller, 1953) was used to evaluate the protein quality. It focuses on weight loss in protein free group to the weight gain in the group fed with the protein diet. The food conversion efficiency was calculated as per method of Shingari and Sapra(1991). The blood glucose and total blood protein levels were measured by SIEMENS *in vitro* diagnostic. The shelf life properties of the developed complementary food mixes were assured through peroxide value, free fatty acid content, microbial viability and total fungal count, effect of metallized polyethylene packaging on moisture content of developed in the Probiotic complementary food mixes as per standard methods described in Appendix I. All data was analyzed using SPSS v.17.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at P<0.01and P<0.05.

The salient findings of the study are as follows:

- The moisture content pre and post processing (per 100 g) of rice flour was decreased from 11.20±0.01 g to 3.46±0.06 g, pearl millet flour 11.20±0.05 g to 3.90±0.10 g, finger millet flour 5.58±0.20 g to 3.50±0.10 g, wheat flour 11.01±0.15 g to 4.01±0.10, mung bean flour 10.00±0.05 g to 3.36±0.10, soyabean flour 7.05±0.10 g to 5.02±0.10 g sesame seeds flour 11.20±0.10 g to 3.30± 0.10 g and pumpkin seeds 10.31±0.15 g to 0.31± 0.10g respectively.
- The available carbohydrate content pre and post processing (per 100 g) of rice flour 72.04±0.05 g to 82.94±0.005 g, pearl millet flour 70.00±0.07 g to 75.00±0.010 g finger millet flour 74.03±0.05 g to 88.50±0.015 g, wheat flour 63.86 ± 0.005g to 55.86±0.01 g, mung bean flour 54.09±0.05 g to 73.00±0.005 g, soyabean flour 30.10±0.06 g to 19.08±0.005 g sesame seeds flour, 20.30±0.015 g to 25.40±0.005 g , pumpkin seeds flour 9.90± 0.06 to 11.90± 0.001 g respectively.
- The crude protein content pre and post processing (per 100 g) of rice flour was 5.85±0.01 g to 6.00±0.005 g, pearl millet flour 10.40±0.50 g to 11.40±0.005 g, finger millet flour 7.42±0.05g to 7.80±0.001g, wheat flour 10.50±0.03 g to 30.00±0.005 g, mung bean flour, 20.02±0.05 g to 23.32±0.001 g/100g, soyabean seeds flour 34.06±0.10 g to 39.28±0.005 g, sesame seeds flour, 18.12±0.10 g/100g to 19.20±0.005 g, and pumpkin seeds flour 25.10±0.15 to 30.10±0.001 g respectively.
- The fat content pre and post processing (per 100 g) of rice flour was 1.13±0.02 g to 3.14±0.38 g, pearl millet flour 3.80± 0.14 g to 4.08± 0.14 g,

finger millet flour, 6.20± 0.06 g to 1.20± 0.05 g/100g, wheat flour, 11.05±0.05 g to 9.80± 0.002 g, mung bean flour, 1.05± 0.001 g to 1.48± 0.25 gsoyabean seeds flour 19.08± 0.01 g to 10.48± 0.13 g/100g, sesame seeds flour, 36.50± 0.50 g to 45.90± 0.10 g, and pumpkin seeds flour 49.50± 0.15 g to 51.90± 0.15 g respectively.

- The crude fibre content pre and post processing (per 100 g) of rice flour was 2.05±0.01 g to 3.05±0.001 g, pearl millet flour 1.88±0.015 g to 3.49±0.001 g, finger millet flour 2.50±0.06 g to 3.65±0.005 g, wheat flour, 3.50±0.05 g to 3.12±0.001 g, mung bean flour, 2.05±0.06 g to 3.91±0.005 g, soyabean seeds flour, 4.00±0.50 g to 4.91±0.001 g, sesame seeds flour, 2.50±0.010 g to 3.01±0.005 g and and pumpkin seeds flour 4.50±0.15 g to 5.84±0.001 g/100g respectively.
- The impact of malting on the nutrient composition of wheat (*Triticum aestivum)* flour was compared with the raw wheat flour by independent t -test. It was observed that germination and malting has an impact on the nutrient composition of wheat. It was revealed that the nutritional content of malted wheat flour significantly (P<0.01) improved than the raw wheat flour.
- The highest content of calcium among cereals was found in finger millet i.e 330.01±0.01 mg. Among pulses the highest calcium content was found in soyabean seeds 220.01±0.010 mg per 100 g. Among oil seeds sesame seeds had the highest calcium content i.e 1330.01±0.010 mg in 100 g. The study reported that finger millet contains 6.71±0.01 mg of iron. Among pulses, soyabean had the highest iron content i.e11.01±0.005 mg per 100 g. Among oil seeds sesame seeds had the highest content of iron content i.e 3.30±0.011 mg in 100 g. Finger millet had highest zinc content of 2.79±0.05 mg in 100 g. Among pulses, soyabean had the highest zinc content of 3.31±0.010 mg in 100g. Among the oil seeds, zinc was found to be highest in sesame seeds 12.11±0.010 mg. Among the cereals, the phosphorus content was found to be highest in malted wheat flour i.e 811.00±0.005 mg. Among the pulses it was found to be highest in soyabean 675.01±0.015 mg in 100 g. Among the oilseeds, phosphorus was found to be highest in sesame seeds 551.69±0.010 mg.

- The results showed highest phytate content in finger millet, among cereals and millets i.e 699±0.50 mg/100g. Pearl millet had the highest amount of oxalates among all the cereals and millets i.e. 39 mg/100g. In the present study tannin content was reported to be highest in pearl millet i.e 280±0.10 mg/100g.
- Out of the six test samples, T_6 developed using Pearl millet flour + Mung bean flour + malted wheat flour +Sesame Seeds flour + Pumpkin Seeds flour had the energy density value of 443.49 Kcal/100g sample or 1855 kJ. T_4 developed using *Rice* flour + Mung bean flour + malted wheat flour +Sesame Seeds flour + Pumpkin Seeds flour had the energy density value of 437.50 Kcal or 1830 kJ after $T_{6.}$
- Among all four test samples, T_4A4 inoculated with *Lactobacillus plantarum* showed highest cell viability (6.0×10^8 cfu/ml) after freeze drying. Thus, T_4A4 having highest microbial cell count was designated as CFM I. T_6B2 inoculated with *Lactobacillus casei*showed highest cell viability (7.2×10^8 cfu/ml) after freeze drying. Thus, T_6B2 having highest microbial cell count was designated as CFM II.
- Resistance to gastric acidity studies reported that at neutral pH 7 the total number of colonies in CFM I was found to be 5.0×10^8cfu at 3 hours and 4.6×10^8 cfu at 6 hours and CFM II was found to be 6.5×10^8cfu at 3 hours and 6.1×10^8cfu at 6 hours.
- Bile acid resistance studies reported thatat bile acid concentrations of 0.0 and 0.3 percent, the colonies were too numerous to count, at which it was revealed that the lactic acid bacillus was viable at a bile acid concentration 0.5 and 1.0 percent, which would enable the Lactobacilli to overcome the effect of bile in the gastro intestinal tract.
- Activity of Antibiotics on common enteropathogens revealed that Ampicillin antibiotic had a significant inhibitory effect on both pathotypes EHEC (15±0.03 mm) and O157:H7 (18±0.01 mm)
- Activity of Probiotic Complementary food mixes on common enteropathogens concluded that that CFM II had a significant inhibitory

effect on both pathotypes O157:H7 (28±0.01 mm) and EHEC (26±0.05 mm). Similarly CFM I also showed inhibitory effect on both pathotypes O157:H7 (26±0.05 mm) and EHEC (25±0.001 mm).

- The bulk density of CFM I ($p<0.05$) (0.52±0.01 g/ml) was not significantly different when compared to CFM II (0.54±0.005 g/ml). The sedimentation volume of both CFM I (5.80±0.01 ml) and CFM II (5.11±0.01 ml) significantly decreased at ($p<0.01$) level after probiotification
- The results of the present study revealed that the swelling capacity of probiotic complementary food mixes CFM I 17.43±0.01% and CFM II 19.00±0.10% significantly ($p\leq0.01$) increased after inoculation of probiotic strain.
- The water holding capacity of Probiotic complementary food mixes CFM I and CFM II were 200±1.00% and 191±1.00% respectively and was significantly lower less than that of untreated ones.
- The fat holding capacity of CFM I and CFM II were 200.66±0.57 % and 211.00±1.00 % respectively and was significantly lower than the untreated ones.
- Viscosities of both the mixes CFM I and CFM II at a rotational speed of 60 rpm were lower, when compared to that of the commercial complementary food formula (4591.3^{a} ± 1.52 cP to 1517.1^{c} ± 0.7 cP) at the same rotational speed.
- Adhesiveness of the CFM I and CFM II Probiotic complementary food mixes ranged from 0.21±0.03 J to 1.16 ±0.03 J. Stickiness varied from -4.35 ±0.57 to -5.63 ± .06 g. Stringiness varied between 0.11±0.03 to 2.30±0.04 mm, and resilience ranged from 0.14±0.05 to 0.10±0.04.
- The moisture content of CFM I and CFM II developed were 3.24±0.01g and 3.85±0.01 g per 100g of sample and were not significantly different at ($p\leq0.05$) from each other, but were significantly lower from the untreated ones. The available carbohydrates content of CFM I and CFM II were 80.24± 0.66 g and 72.83± 0.15 g per 100g of sample respectively. The Crude protein content of Probiotic complementary food mixes of CFM I and CFM II were 12.88±0.02 g and 14.90±0.09 g. The fat content of Probiotic

Complementary food mixes CFM I and CFM II were 5.84±0.02 g and 6.60±0.05 g respectively. The crude fibre content of CFM I and CFM II were 5.62 ± 0.02 g and 6.12 ± 0.07 g respectively. The total mineral content of CFM I and CFM II were 1.23 ± 0.01 g and 2.00 ± 0.05 g respectively.

- Sodium content for CFM I and CFM II was 2.32 ± 0.26 and 5.72 ± 0.24 ppm. Potassium content for CFM I and CFM II was 235.18±1.02 and 432.38±0.33 ppm. Calcium content for CFM I and CFM II was 87.53±0.40 and 259.58±0.38 ppm. Magnesium content for CFM I and CFM II was 6.02±0.02 and 6.85±0.30 ppm. Manganese content for CFM I and CFM II was 9.85±0.30 and 15.48±0.42 ppm. Iron content for CFM I and CFM II was 10.26±0.21 and 13.65±0.52 ppm. Zinc content for CFM I and CFM II was 4.31±0.35 and 4.84±0.14 ppm. Copper content for CFM I and CFM II was 11.49±0.45 and 14.15±0.21 ppm. Phosphorous content for CFM I and CFM II was 40.91±0.38 and 55.11±0.99 ppm.
- The free radical scavenging activity of developed CFM I and CFM II were 72.2% and 74.3% respectively. Highest free radical scavenging activity was observed in CFM II.
- The phytochemical parameters of CFM I and CFM II were flavonoids content were 107.96±0.83 and 130 ± 0.95 mg/100g. Alkaloids content were 23.60 ± 0.36 and 41.06± 0.51 mg/100g. Cyanogenic Glycosides content were 16.18 ± 0.18 and 26.53 ± 0.45 mg/100g. Tannins content were 4.74 ± 0.01 and 6.29 ± 0.06 mg/100g. Oxalates content were 0.47 ± 0.01 and 0.55 ± 0.01 mg/100g. Trypsin inhibitor content was 2.59 ± 0.02 and 4.20 ± 0.25 mg/100g. Saponins content was 9.01 ± 0.01 and 13.41 ± 0.07 mg/100g and Phytate content was 0.24 ± 0.02 and 1.68 ± 0.03 mg/100g.
- The protein digestibility of the Probiotic complementary food mixes of CFM I was 73.46±0.50 % and CFM II was 70.26±0.37 %.
- Assessment of the relationship between viscosity and protein digestibility as done by Pearson product-moment correlation coefficient showed a significant positive correlation between the viscosity and protein digestibility.

- The total starch of CFM I was 78.72±0.50% and 61.03±0.50% resistance starch of CFM I was 2.06±0.03% and CFM II was 2.17±0.02%, digestible starch of CFM I was 70.23±0.23% and CFM II was 65.05±0.05%, rapidly digestible starch of CFM I was 20.88±0.68% and CFM II was 19.11±0.10% and starch digestion index for CFM I was 20.72±0.63% and CFM II was 20.84±0.29%.
- Out of the four formulations prepared, the best scored in overall acceptability was CFM II which was selected for *in vivo* study for further assessment of quality parameters of the developed complementary food.
- The Antidiarrheal efficacy of probiotic complementary food mixes in castor oil induced diarrhoeal Wistar strain albino rats revealed that the percentage of diarrheal inhibitions were 13.02% for negative control group, 70.61% for positive control group and 64.86% for experimental Group A and 68.91% for experimental Group B respectively
- The initial total microbial load of Experimental Group A was 10.2×10^{8} cfu/g. A significant decrease ($P\leq0.01$) in total microbial load was observed after supplementation with CFM I from a total count of 9.6×10^{8}cfu/g on the 1st day of supplementation to 4.2×10^{8}cfu/g at the end of supplementation period.
- The Experimental Groups supplemented with test diets CFM I and CFM II, the highest net protein intake was observed in Group D supplemented with test diet CFM II. The mean protein intake of Group D was 63.25±0.56 g protein followed by Group C fed with test diet CFM I. The mean protein intake of Group C was 26.11±0.67 g.
- The maximum food intake and highest weight gain was observed in group III fed with CFM II, followed by group II fed with CFM I and group I fed with rat ration.
- The blood glucose levels of the Wistar strain albino rats fed with the formulated test diets (Group II and III) did not differ significantly ($p\leq0.05$) until 7thday of the experiment. It can be concluded that different formulated test diets had no effect on the fasting blood glucose levels of the experimental animals on day 0 and 7. However, on day 14th and 21st , the

blood glucose levels of the experimental groups were statistically different at ($p \leq 0.05$) from each other. The decrease in the blood glucose levels was more in group II followed by group III. Group III fed with CFM II, followed by group II fed with CFM I and group I fed with rat ration.

- The total blood protein levels of the animals fed with normal rat ration (Group I) and the total blood protein levels of the Wistar strain albino rats fed with the developed Probiotic Complementary food mixes (CFM I and CFM II) did not differ significantly ($p \leq 0.05$) until 7^{th} day of the experiment. On day 14^{th}, the total blood protein levels of the experimental groups were statistically different at ($p \leq 0.05$) from each other.
- The free fatty acid values at 0 days for CFM I was 1.06±0.03 % for CFM II was 1.71±0.06 %, at 30 days for CFM I was 3.17±0.28 %for CFM II was 2.78±0.07 %, at 60 days for CFM I was 3.98±0.04 % for CFM II was 3.20±0.05 %, at 90 days for CFM I 4.29±0.05 % was for CFM II was 4.05±0.05%.
- The peroxide values at 0 days for CFM I was 3.05±0.05 m moles/kg fat for CFM II was 4.10±0.09 m moles/kg fat, at 30 days for CFM I was 4.10±0.09 m moles/kg fatfor CFM II was 5.03±0.05 m moles/kg fat, at 60 days for CFM I was 5.18±0.07 m moles/kg fat for CFM II was 6.18±0.03 m moles/kg fat, at 90 days for CFM I 6.95±0.05 m moles/kg fat was for CFM II was 7.05±0.0 m moles/kg fat.
- Both the formulations CFM I and CFM II contained 10^8cfu of viable probiotic cells per ml/g of sample on storage for 60 days at ambient temperature of 20-25^o C.
- The total fungal count of were found to be below detectable level. However on the 75^{th} day of storage, 0.3 x10^1 cfu/g was observed in CFM I and 0.4 x10^1 cfu/g was observed in CFM II respectively.
- With the progression of storage period there was significant increase in moisture content. It was observed that there was no significant increase in the moisture content up to 30 days storage but after 30 days there was significant increase in moisture content.

Conclusion

As per the salient findings, stated above it can be concluded that complementary feeding becomes a necessity for the optimum development of an infant after completion of six months of age. It is evident from the study that probiotic complementary food mixes can be formulated from locally available cereals and legumes and can be processed through simple household technologies. Such probiotic complementary food mixes can serve as potential sources of the much required protein which can treat protein-energy malnutrition along with treating the episodes of infant diarrhea which is observed especially among the rural communityof developing countries.

The present work focused on nutritional enhancement of complementary food mixes from locally available cereals and legumes. Out of the two formulations CFM I and CFM II, CFM II was found to be highly acceptable and possessed high nutritional and functional contents, free radical scavenging activity and nutraceutical potentials.The *in-vivo* studies stated that both the products were effective in reducing the episodes of diarrhea along with it increasing the beneficial bacteria in the gut microflora, increasing the total protein and total weight and maintaining the blood glucose. Dissemination of this technology to the rural areas on large scale will aid the rural farm women to feed a nutritionally balanced feed to their children.The probiotic fermentation resulted in favourable changes in nutritional profile of complementary food mixes resulting in increase in nutritional quality. Since there is no peer reviewed studies on Probiotification of non dairy foods this study creates an awareness to focus on identifying proper fermentation technology hence, it can be recommended that the probiotic fermentation technology possess a good potential to develop nutritionally enriched complementary mixes to improve the quality and quantity of all the nutrients for infants.

Limitations

- The research work has used only *in vitro* models to assess the protein digestibility of the developed complementary food mixes due to Covid-19 restrictions human models were not included for the *in-vivo* trials as consent could not be obtained.

- Further in depth research can be undertaken to develop novel value added products using functional ingredients and their therapeutic role can be studied through human model for management of diseases.
- Due to strict covid-19 lockdown the probiotic strains were present in very limited numbers. Strains like *Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve*. Hence, narrowing down the study to few of available probiotic cultures.

Recommendations for future:

- Commercialisation of the probiotic complementary food mixes by transfer of technology to industries.
- New studies can be done at molecular and cellular level to understand the digestibility of probiotic complementary food mixes which can helps to improve infant health**.**
- Further studies may also be done to assess if the complementary food mixes can also be given to other vulnerable group of populations like pregnant /lactating and elderly people.
- To create awareness on homemade complementary food mixes prepared using locally available cereals and legumes and popularization can be done through social media aids among the masses.
- The concept of Probiotic complementary food mixes can be forwarded to Ministry of Consumer affairs to include in Public Distribution System (PDS) and Targeted Public Distribution System (TPDS) to meet the nutrition security among vulnerable section of a community.
- Recommending to policy makers in Government of India and Government of Tamil Nadu to include this probiotic complementary food mixes in the noon-meal schemes to enhance the nutritional status of beneficiaries and to reduce diarrheal incidences.