

**BIOACCESSIBILITY OF IRON FROM FINGER MILLET
BASED SUPPLEMENTARY FOOD WITH PROBIOTICS**

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**BIOACCESSIBILITY OF IRON FROM FINGER MILLET
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CERTIFICATE

This is to certify that the thesis entitled “**BIOACCESSIBILITY OF IRON FROM FINGER MILLET BASED SUPPLEMENTARY FOOD WITH PROBIOTICS**” submitted by **Miss. KALYANI UMESH KAMATAGI**, for the degree of **MASTER OF HOME SCIENCE** in **FOOD SCIENCE AND NUTRITION** to the University of Agricultural Sciences, Dharwad is a record of research work carried out by her during the period of her study in this University, under my guidance and supervision, and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

**DHARWAD
JULY, 2019**

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1. INTRODUCTION

Consumers today are highly conscious of nutritionally and functionally rich therapeutic foods. A considerable amount of interest has been focused towards the development of probiotic products. The idea of using probiotics is a method to promote good health and disease prevention through natural and biotic applications to daily food. In recent years, there has been a renewed interest in using microorganisms due to their beneficial aspect in health restoration and disease treatment. Several microorganisms, under the name of “probiotics”, have been proposed and used in a wide range of clinical trials, ranging from diarrheal disease to cancer prevention (Fuller, 1994; Kaur *et al.*, 2001).

The term probiotics are “viable, defined microorganisms in sufficient numbers, which alter the microflora in a compartment of the host and by that exert beneficial health effects in the host” (Scholz-Ahrens *et al.*, 2007). Some types of probiotics are lactobacillus, bifidobacteria, *Saccharomyces boulardii*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Leuconostoc*, etc. The majority of probiotic products available in the marketplace contain species of *Lactobacillus* and *Bifidobacterium*, which are the main genera of Gram-positive bacteria currently characterized as probiotics (Anon., 2001). Among the *Lactobacillus* species *L. acidophilus*, *L. johnsonii*, *L. casei*, *L. rhamnosus*, *L. gasseri*, and *L. reuteri* are known as probiotics. *Bifidobacterium* strains include *B. bifidum*, *B. longum*, and *B. infantis* (Heller *et al.*, 2001). There are some properties that define microorganism to possess probiotic property viz., resistance to acid and bile, attachment to the human gut epithelial cells, colonization in the human intestine, production of antimicrobial substances including bacteriocins, good growth characteristics and beneficial effects on the human health. One of the most important characteristics of a probiotic strain is that it must be nonpathogenic and generally recognized as safe (GRAS). Probiotics must also present certain desirable characteristics, such as maintenance of viability during processing and storage, ease of application in products and resistance to changes during physico-chemical processing of the food (Prado *et al.*, 2008). These bacteria should not be pathogenic, toxic, mutagenic, or carcinogenic in the host organism, must be antagonistic to pathogens and be genetically stable without a plasmid transfer mechanism, especially concerning

antibiotic resistance; they must survive during digestion and possess the ability to adhere and colonize the gut mucosa, promoting immuno-stimulation without inflammatory effects (Saarela *et al.*, 2000). It is important to report that these bacteria should be present in a dairy food to a minimum level of 10^6 CFU g^{-1} or the daily intake should be about 10^8 CFU g^{-1} , with the aim to compensate for the possible reduction in the number of the probiotic microorganisms during the passage through the gut (Shah 2007). Further, these microorganisms must present good technological properties showing good multiplication in the milk, promoting sensorial properties suitable in the product and being stable and viable during storage, so they can be manipulated and incorporated in food products without losing the viability that must be at least of 10^6 – 10^7 CFU g^{-1} (Anon, 2001).

Foods are carriers for the delivery of probiotics to the human body. It helps to regulate their colonization, buffer the probiotic through the gastrointestinal tract and contain other functional ingredients, such as bioactive components, which may interact with probiotics to alter their functionality and efficacy (Ranadheera *et al.*, 2010). Traditionally, probiotics have been added to yogurt and other fermented dairy products (Penna *et al.*, 2007). As fermentation process involves mixed cultures such as yeast, lactic acid bacteria (LAB) and fungi (Blandino *et al.*, 2003), traditional fermented foods viz., *adai*, *agbelim*, *atole*, *ben- saalga*, *boza*, *dosa*, *idli*, *kecap*, *kenkey*, *kimchi*, *kishk*, *kisra*, *koko*, *mahewu*, *mawe*, *ngari*, *ogi*, *sauerkraut*, *som-fug*, *tarhana*, *temphe* and *uji* are ample source of microorganism and some of them show probiotic characteristics (Rivera-Espinoza and Gallardo-Navarro, 2010). Dairy products such as yogurts and fermented dairy beverages (Majamaa and Isolauri 1997; Urbanska *et al.*, 2009), cheeses (Medici *et al.*, 2005), and ice creams (Caglar *et al.*, 2008) are the main vehicle for probiotic supplementation. At present probiotic bacteria are mainly incorporated into dairy products such as cheese, yogurt, ice cream and other dairy desserts. Limitations of dairy products are lactose intolerance, undesirable cholesterol content, requirement for cold storage facilities, low viability of probiotic bacteria due to some factors including titratable acidity, pH value and hydrogen peroxide, oxygen concentration, storage temperature, interactions with other microorganisms in the products, and lactic and acetic acid concentrations (Heenan *et al.*, 2004 & Castro-Cislaghi *et al.*, 2012). Hence, there is a need to explore non-dairy-based probiotic products. There are some marketed

probiotic products such as beverages as well as supplements in the form of tablets, capsules and freeze dried preparations *viz.*, Multibionta, Enterogermina, Reuterina, UltraLevure and Florastor (Schrezenmeir and de Vrese, 2001; Berni-Canani *et al.*, 2007). This has led to development of probiotic products from various food matrices including fruits (Lavermicocca 2006), vegetables (Yoon *et al.*, 2006), legumes (Heenan *et al.*, 2004) and cereal products like juice, beverages, composite mix, supplementary food and weaning food (Helland *et al.*, 2004).

Supplementary food is given mainly to combat micro-nutrient deficiency which is difficult to meet through daily diet. It is composed of multigrain flour which is energy dense, protein rich and contains minerals (Sguassero *et al.*, 2012). Although, it contains the required nutrients, its bioaccessibility is very low. In the view of popularity of probiotic and its therapeutic use, it was proposed to formulate probiotic supplementary food based on finger millet which can be a better source of bioavailable iron. Therefore, in the present investigation, an attempt was made to develop an indigenous food mixture with the following objectives:

Objectives of the study:

1. To assess the nutrient composition, anti-nutritional factors and bioaccessibility of iron in finger millet based supplementary food.
2. To assess the role of probiotic bacteria on the bioaccessibility of iron from the composite mix.
3. To evaluate the shelf life of supplementary food incorporated with probiotics.

2. REVIEW OF LITERATURE

Food, now-a-days, has changed and is perceived not only as a source of nutrition but also as therapeutic agent. Clinical nutrition and pathology has established an interrelationship between food intake, gut microbial ecology and human health. Trillions of bacteria comprising hundreds of beneficial species, which are friendly organisms called probiotics; means “for life”, resides in human intestinal tract. Establishment of the microbial equilibrium towards a beneficial one with the help of specific dietary components like probiotics, have opened the gateway for the development of functional foods. Once a functional food is ready, the next prime importance is the shelf life of these products. So, the technology for extending the shelf life of health promoting foods needs to be expanded. Limited literature is available on probiotic food mixes hence literature pertaining to dairy probiotic foods is presented under the following headings:

- 2.1 Development of Supplementary foods
- 2.2 Nutrient composition, sensory evaluation and shelf life of supplementary foods
- 2.3 Probiotics: definition and historical perspectives
- 2.4 Supplementary foods with probiotics
- 2.5 Health benefits associated with probiotics
- 2.6 Maintenance of normal micro flora and therapeutic dosage
- 2.7 Viability of probiotics
- 2.8 Bioaccessibility of iron and shelf life of supplementary foods with probiotic

2.1 Development of Supplementary foods

Vanalli *et al.* (2003) developed a supplementary sports food prepared with sorghum (M35-1), soybean, sugar and skim milk powder at 40:15:25:20 proportion was organoleptically highly acceptable and met the ideal protein energy ratio of athletes.

Beverage prepared in milk using supplementary sports food was highly acceptable. Supplementary sports food stored in aluminium box and polyethylene cover had shelf-life of 12 weeks with excellent organoleptic qualities."

Sumathi *et al.* (2009) standardized variables for extrusion cooking of pearl millet and physicochemical characteristics and nutritional qualities of the millet and legume-based extruded supplementary foods were determined. The millet was mixed with legumes (30%) and defatted soy (15%) separately and extruded to prepare ready-to-eat supplements to children and mothers. The foods based on the millet and legumes and also the millet and soy contained 14.7% and 16.0% protein with 2.0 and 2.1 protein efficiency ratio values, respectively. The shelf-life of the foods was about 6 months in different flexible pouches at ambient storage conditions.

Kunyanga *et al.* (2012) developed a low-cost food supplement using different traditionally processed local foods, consisting of cereals, legumes, nuts, fish, and vegetables, to meet the nutrient requirements for vulnerable groups in Kenya. Four food supplements were developed and evaluated by taste panel procedures. The product containing amaranth grain, pigeon pea, sweet potato, groundnuts, and brown sugar was found to be the most acceptable supplement.

Khanam *et al.* (2013) developed two types of ready-to-eat supplementary foods by roller drying based on wheat, soy protein concentrate, whey protein concentrate, and green gram flour and were fortified with vitamins and minerals to meet the one third of the Recommended daily allowance (RDA). The supplementary food formulations contained 20– 21% protein, 370–390 kcal of energy and 2,300µg of β-carotene per 100 g serving. The bioaccessibility of iron was 23%. Sensory studies indicated that the products were acceptable with a shelf life of one year under ambient temperature. The product could be served in the form of porridge with water/milk or in the form of small *laddu*.

2.2 Nutrient composition, sensory evaluation and shelf life of supplementary foods

Platel *et al.* (2010) investigated the influence of malting of finger millet, wheat, and barley on the bioaccessibility of iron. Malting increased the bioaccessibility of iron

>3-folds from the two varieties of finger millet and increased by >2-fold from wheat, but no such increase in barley. Thus, malting could be an appropriate food-based strategy to derive iron maximally from food grains.

Salve *et al.* (2011) showed that supplementary foods were formulated from locally available wheat flour, soybean flour and chick pea flour using household technologies like blending and roasting. The proximate composition of product used for preparation of supplementary food fortified with 10% skimmed milk powder contained higher amount of protein and other nutrients. They contained proteins (16.2 to 21.1%), fat (1.9 to 4.5%), fiber (1.28 to 1.78%), ash (0.7 to 1.40%) and carbohydrates (67.66 to 77.2%). The total energy varied from 350.7 to 395.8 Kcal. The various minerals *viz.*, calcium, phosphorus and iron were found to increase on supplementation with 10% skimmed milk powder. Shelf life of the product was good in both polyethylene and laminate packaging materials for the period of three months.

Shilpa and Lakshmi (2012) conducted a study on comparison of enhancement in bioaccessible iron and zinc in native and fortified high-phytate oilseed and cereal composites by activating endogenous phytase. They enhanced the bioaccessibility of minerals in high-phytate oilseed and cereal flour mixes by activating the intrinsic phytase of wheat flour. The flour mixes were fortified with iron and zinc separately for comparison. The flour mixes were incubated at optimum conditions of temperature and pH for phytase activation. Phytase activation enhanced bioaccessible iron by 43–162% in native and 40–168% in fortified wheat–soya, 83–192% in native and 97–240% in fortified wheat–groundnut flour mixes in relation to control flour mixes. Bioaccessibility of zinc was enhanced by 87–183% in native and 30–113% in fortified wheat–soya, 31–65% in native and 61–186% in fortified wheat–groundnut flour mixes. Endogenous phytase activation was effective in enhancing bioaccessibility of iron and zinc in native and fortified flour composites economically.

Kunyanga *et al.* (2012), developed four supplementary foods consisting of cereals, legumes, nuts, fish, and vegetables. The acceptable mix was evaluated for nutritional composition and shelf-life. The acceptable supplement contained 453.2 kcal energy, 12.7 g crude protein, 54.3 g soluble carbohydrates, 20.8 g crude fat, and 10.1 g crude fiber per 110 g. The micronutrient contents were 93.0 mg calcium, 172.4 mg

magnesium, 2.7 mg zinc, 5.7 mg iron, 0.8 mg vitamin B₁, 0.2 mg vitamin B₂, 7.9 mg niacin, 100 µg folic acid, and 140 µg retinol equivalent per 110 g. The supplement also contained 21% total essential amino acid in addition to appreciable levels of palmitic, stearic, oleic, linoleic, and α -linolenic fatty acids. The shelf-life study showed that it could be stored in different packaging materials (polythene bags, gunny bags, and kraft paper) at 26°C without deleterious effects on its chemical composition for up to 4 months.

Malting finger millet reduces tannin (brown millet) and phytic acid content, and improves ionisable iron and soluble zinc significantly (Pradeep and Guha, 2011). Malting of finger millet reduces protein content, but improves protein efficiency ratio (PER), bioavailability of all minerals and has pronounced effect in lowering anti-nutrients (Desai *et al.*, 2010; Krishnan *et al.*, 2012).

2.3 Probiotics: definition and historical perspectives

Probiotics have emerged as the major nutritional factor impacting and influencing gastrointestinal physiology and function. Probiotics in Greek means ‘for life’ and was first used by Lilley and Stillwell (1965) to describe “substances secreted by one micro-organism to stimulate the growth of another-as an antonym to ‘antibiotic’, Parker (1974) further defined probiotics as an organism and substances, which contribute to intestinal microbial balance. The term probiotics was first coined by Parker (1974) to describe the use of microbes in animal health and nutrition.

The scientific basis for the use of live microbes in the prevention and treatment of infections dates back to over 100 years, when the Russian scientist Elie Metchnikoff in 1907 hypothesized that bowel health and prolongation of life could be possible by replacing or reducing the number of ‘putrefactive’ bacteria in the gut with lactic acid bacteria (Anukam and Reid *et al.*, 2007).

Fuller (1989) further refined this description and redefined probiotics as ‘a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance’.

Naidu *et al.* (1999) defined probiotics as “microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract”. Probiotic active substance is a cellular complex of Lactic acid bacteria (LAB) that has a capacity to interact with host mucosa and may beneficially modulate the immune system independent of LAB’s viability.

Probiotics are dietary supplements containing potentially beneficial bacteria or yeasts. According to the currently adopted definition by FAO/WHO (2001) probiotics are: “Live microorganisms which when administered in adequate amount confer health benefit on host”.

2.4 Supplementary foods with probiotics

Khandelwal *et al.* (2015) developed a weaning mix from underutilized crops (finger millet, barnyard millet, black soybean, amaranth grain). Malting and fermentation were adopted as ways of improving cereal protein quality and decreasing anti-nutritional property and probiotic weaning mix was produced using probiotic strain *Lactobacillus plantarum*. The probiotic fermentation resulted in favourable changes in nutritional profile of weaning mix resulting 48.2% increase in protein and 92.3% decrease in tannin content. The optimized set of independent variables (cereals (FM+BM): legume: 70:30 fermented for 25 h with 2.9% inoculums concentration) obtained after statistical analysis, was used to develop final probiotic weaning mix.

Vidyalaxme *et al.* (2012) developed a probiotic ragi malt (functional food) that has been prepared using ragi and probiotic *Leuconostoc mesenteroides* (Lm) and *Bacillus subtilis natto* (Bs), alone and in combination, for antagonistic activity against *Vibrio cholerae* (Vc). *In vitro* studies using pure cultures showed that each probiotic strain (Lm or Bs) was able to inhibit the planktonic growth of Vc as well as its ability to make biofilms and adhere to extracellular matrix proteins (fibronectin, Fn) that may function *in vivo* as initial ports of entrance of the pathogen. The combination of both probiotic strains (Lm plus Bs) produced the strongest activity against the Vc. When both cultures were used together in the ragi malt the antimicrobial activity against Vc was enhanced due to synergistic effect of both probiotic strains. The inclusion of both

probiotic strains in the functional food produced higher amounts of beneficial fatty acids like linoleic and linolenic acid and increased the mineral content (iron and zinc). The viability and activity of Lm and Bs against Vc was further enhanced with the use of adjuvants like ascorbic acid, tryptone, cysteine hydrochloride and casein hydrolysate in the ragi malt. In sum, the intake of probiotic ragi malt supplemented with Lm and Bs may provide nutrition, energy, compounds of therapeutic importance and antagonistic activity against Vc to a large extent to the consumer.

Wakil and Kazeem (2012) developed an infant weaning food from sorghum - cowpea blends fermented with four combinations of starter organisms (*Lactobacillus plantarum* and *Saccharomyces cerevisiae* (AB1), *Lactobacillus plantarum* and *Pediococcus acidilactici* (AB2), *Pediococcus acidilactici* and *Saccharomyces cerevisiae* (AB3), and *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Saccharomyces cerevisiae* (AB4). The weaning food produced using the Sample AB1 had the highest (26.50%) protein content and the least anti-nutrient content while Sample AB4 showed the highest value of anti-nutrients. Porridges made from the formulated blends were rated above average in terms of over-all acceptability. The samples fermented with the three combinations (AB4) had the highest preference rating. It was concluded that fermentation of sorghum-cowpea formulated weaning blends with combinations of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* (AB1) had the highest nutritional contents and the least anti-nutrients and may be recommended for good quality weaning food.

Palacios *et al.* (2008) conducted a study to select strains with high phytate-degrading activity from human feces, and evaluate their suitability for the bread making process. Twenty-three *bifidobacterial* strains (13 from infants and 10 from adults) were isolated, belonging to the species *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium catenulatum*. The phosphatase and phytase activities of these strains were evaluated as well as their ability to degrade InsP6 during growth. The fermentative ability of the strain showing the highest phytate-degrading activity (*B. longum*. BIF307) was determined in whole wheat bread making. The use of the selected bifidobacterial strain as starter during whole wheat fermentation resulted in bread with similar technological quality than the control (in absence of bifidobacteria) and crumb with lower levels of inositol phosphates. Uses of the selected *Bifidobacterium* strain in whole

wheat bread making process could provide potential nutritional benefits by decreasing the anti-nutrient content of the product.

Garcia *et al.* (2015) conducted a study to develop a fermented soybean drink with improved nutritional quality and source of probiotic bacteria by including as starter for fermentation *Lactobacillus casei* strains modified to produce phytase enzymes from bifidobacteria. The *L. casei* strains showed a good adaptation to develop in the soy drink but they needed the addition of external carbohydrates to give an efficient acidification. The strain expressing the *B. pseudocatenulatum* phytase was able to degrade more than 90% phytate during product fermentation, whereas expression of *B. longum spp. infantis* phytase only led to 65 % hydrolysis. In both cases, accumulation of myo-inositol triphosphates was observed. In addition, the hydrolysis of phytate in soy drink fermented with the *L. casei* strain expressing the *B. pseudocatenulatum* phytase resulted in phytate/mineral ratios for Fe (0.35) and Zn (2.4), which were below the critical values for reduced mineral bioavailability in humans. This investigation showed the ability of modified *L. casei* to produce enzymes with technological relevance in the design of new functional foods.

Hoppe *et al.* (2015), conducted a study by adding probiotics to foods to test the hypothesis that non-haem iron absorption from a fruit drink is improved by adding *Lactobacillus plantarum* 299v (Lp299v). The study revealed that the mean iron absorption from the drink containing 10^9 CFU of the probiotic strain Lp299v (28.6%) was significantly higher than from the control drink (18.5%). The fruit drink with 10^{10} CFU of probiotic strain Lp299v gave a mean iron absorption of 29.1%, whereas the control drink gave an absorption of (20.1%). The difference in iron absorption between the 10^9 CFU Lp299v and the 10^{10} CFU Lp299v drinks was not significant ($P= 0.941$). In conclusion, intake of probiotics can increase iron absorption by approximately 50 % from a fruit drink having an already relatively high iron bioavailability.

2.5 Health benefits associated with probiotics

Present day clinical research has shown that different probiotic strains can prevent and treat diarrhea, irritable bowel syndrome, treat inflammatory bowel disease and prevent colon cancer. There is strong evidence that *Lactobacillus rhamnosus* GG

decreases the duration and severity of rotavirus-associated diarrhea in children. These can also show promise in the treatment of some forms of inflammatory bowel disease and colon cancer. And there is strong evidence for their efficacy in some clinical scenarios as they are now widely used in many countries by consumers and in clinical practice (Boyle *et al.*, 2006; Santosa *et al.*, 2006).

Various health benefits such as colon cancer, anti-atherogenic properties have been attributed to probiotics (Nikhilesh *et al.*, 2007; Vibha and Yadav, 2007).

Probiotics has also been shown to reduce the incidence of allergy at-risk infants through administration of *L. rhamnosus* GG to infants during the first half-year of life (Ouwehand, 2007).

Functional food ingredients such as prebiotics and probiotics could affect a beneficial modification in the composition and activities of gut microflora of infants by increasing positive flora components. The prebiotic approach aims to increase resident bacteria that are considered to be beneficial for human health, e.g. Bifido bacteria and Lactobacilli (Parracho *et al.*, 2007).

Probiotics are able to improve immunological factors that are related to overweight health problems. In addition, probiotics have been shown to be able to prevent the development of diabetes which is highly linked to obesity (Sofia *et al.*, 2010)

Several reports have described their health benefits on gastrointestinal infections, antimicrobial activity, improvement in lactose metabolism, reduction in serum cholesterol, immune system stimulation, anti-mutagenic properties, anti-carcinogenic properties, anti-diarrheal properties, improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection by addition of selected strains to food products (Abatenh *et al.*, 2018, Sharma *et al.*, 2012, Imasse *et al.*, 2007; Shah, 2007).

Probiotics may improve child growth through the prevention of infections and micronutrient deficiencies as they have been shown to improve the absorption of certain nutrients (calcium, zinc and vitamin B12) (He *et al.*, 2005; Scholz-Ahrens *et al.*, 2007) and reduce the risk of anaemia (Sazawal *et al.*, 2010).

Benefits associated with probiotics include reducing symptoms of intestinal diseases such as infection by pathogens (e.g. *Helicobacter pylori*, *Escherichia coli* and *Salmonella* strains), inflammatory bowel disease or irritable bowel syndrome. Probiotics have also been reported to decrease rotavirus shedding and diarrhoea (Muller *et al.*, 2010).

2.6 Maintenance of normal micro flora

Yoghurt with specific strains of live active cultures has been demonstrated to help maintain the normal intestinal micro biota balance and suppress harmful bacteria in intestine. A particular strain used in yoghurt, *Lactobacillus* strain GG, aids in treatment and prevention of antibiotic associated diarrhea, traveller's diarrhea, and acute diarrhea in children. In adults, this particular strain of *Lactobacillus* has been shown to stimulate bowel function by altering the microflora and suppressing fermentation in the intestine. Yoghurt with *Lactobacillus gasseri* may be beneficial for older adults with "atopic gastritis" a condition that predisposes to intestinal infections and constipation (Salminen, 1996).

Regular ingestion of probiotics supplemented foods can, therefore, lead to nutritional and health benefits. Short- term benefits of consuming probiotics include improved lactose digestion and alleviation of GI disturbances or vaginal infections. With the exception of improved lactose digestion (which should occur in less than 2-4 hours after consumption) and other effects will not be seen immediately. Daily consumption of an adequate level of probiotics is recommended, especially for long term benefits. For optimal health benefits a daily dose of 100 million to 10 billion probiotic bacteria is a good target (Sharma and Bikash, 2006).

Probiotic hypothesis precludes that the addition of certain exogenous microorganisms to the intestinal tract will have positive health benefits. It is therefore, imperative and important that probiotics strains are of human origin since some health promoting effects may be species dependent. Probiotic bacteria consumed even in high numbers are not known to become permanent colonizers of human gut and rarely detectable in fecal or intestinal samples beyond a couple of weeks after ingestion (Sanders, 1999).

The challenges associated with the introduction and maintenance of high numbers of viable probiotic cultures into foods include the form of the probiotic inoculant used, process conditions, reconstitution conditions, ability of the probiotic culture to grow and retain viability in the food environment and maintenance of probiotic characteristics in the food product throughout the time of consumption (Muller *et al.*, 2010).

2.7 Viability of probiotics

Kurmann and Rasic (1991) suggested achieving optimal potential therapeutic effects, the number of probiotics organisms in a food should meet a suggested minimum of $> 10^6$ CFU/ml as satisfactory level.

It has been suggested that a product should contain at least 100 g of a probiotics food, with 10^7 cells/g, a level paralleling current Japanese recommendations (Ishibashi and Shimamura, 1993) but considerably higher numbers have been proposed by others (Lee *et al.*, 1995).

This criterion is referred to as the “therapeutic minimum” (Rybka and Kailasapathy, 1995). One should aim to 10^8 live probiotics cells per day. Regular consumption of 400-500 g/week of bio-yoghurt, containing 10^6 viable cells per ml would provide these numbers (Tamine *et al.*, 1995).

The therapeutic minimum needs to be consumed regularly for transfer of probiotics effect to consumers has been proposed by The International Dairy Federation (1997) that in probiotics foods, the specific microorganisms shall be viable, active and abundant at that level of at least 10^7 CFU/g in the product to the date of minimum durability” (Ouwehand and Salminen, 1998).

Currently, there are no legal recommendations for consumption of probiotics in foods. However, it is generally accepted that health benefits from consumption of probiotics strains should be demonstrated through controlled clinical trials and that the manufacturer should provide advice on the minimum dose and duration of use of each individual strain or product (FAO/WHO, 2001).

3. MATERIAL AND METHODS

The present research on “Bioaccessibility of iron from finger millet based supplementary food with probiotics” was carried out in the Department of Food science and Nutrition, College of Community Science, UAS, Dharwad during 2015-17. The details of the materials used and methods employed are categorized here under:

- 3.1 Procurement of raw materials
- 3.2 Processing the grains
- 3.3 Estimation of nutritive value and phytochemical compounds of grains and product.
- 3.4 Role of probiotic bacteria on the bioaccessibility of iron from the composite mix.
- 3.5 Shelf life of supplementary food incorporated with probiotics.
- 3.6 Statistical analysis

3.1 Procurement of raw materials

Finger millet (DHFM 78-3) was procured from AICRP, Millet; Wheat (NIAW 1415) was procured from AICRP, Wheat; Green gram (DGGV 2) was procured from A.I.C.R.P., M.U.L.La.R.P., Probiotic strains LS44, LS14 was procured from A.I.C.R.P.-E.A.A.I. (Bioconservation technique), M.A.R.S., U.A.S, Dharwad and commercial probiotic culture *Lactobacillus acidophilus* form DANISCO; and skimmed milk powder was procured from local market, Dharwad, Karnataka.

3.2 Processing the grains

The raw grains were dried in hot air oven at $105^{\circ}\text{C} \pm 10^{\circ}\text{C}$, finely powdered and subjected for analysis.

Germination: The grains were soaked for 8 hours and germinated for 48 hours (finger millet), 36 hours (wheat) and 24 hours (green gram). The seeds were dried in hot air

oven at $45^{\circ}\text{C} \pm 10^{\circ}\text{C}$, the husk and germs were removed, powdered and subjected for analysis.

Malting: The germinated grains were dried, husk and germs were removed, roasted, powdered and sieved using muslin cloth to obtain the malt and was subjected to analysis.

Supplementary food: For the preparation of supplementary food, the formulation developed in the Department of Food Science and Nutrition, U.A.S., Bengaluru, was used and modified suitably. It was modified by mixing two parts of malted finger millet, one part of malted green gram and one part of wheat malt *i.e.*, 2:1:1 to which 10% of milk powder was added.

3.3 Nutritive value, anti-nutritional factors and bioaccessibility of grains and product

Finger millet, green gram and wheat were analyzed for proximate composition, anti-nutritional factors and minerals of raw flour, germinated flour, malted flour and composite flour. The proximate principles such as protein, fat, and ash were analyzed. The carbohydrate (by difference) and energy values were computed. The nutraceutical such as dietary fiber and minerals (iron, zinc, and calcium) were analyzed on dry weight basis.

3.3.1 Proximate composition

The proximate composition was analyzed in all the samples in triplicates using standard procedures.

Moisture

About 10 g of sample (flour) were transferred into moisture cups and dried in oven at 105°C till the weight of the moisture cups remained constant (Anon., 2005). Moisture was calculated using the formula-

$$\text{Moisture content (\%)} = \frac{\text{Initial weight of the sample} - \text{Final weight of the sample}}{\text{Weight of the sample}} \times 100$$

3.3.1.2 Fat

Moisture free sample was weighed in moisture free thimble. Fat was extracted refluxing thimbles in SOCS PLUS apparatus using petroleum ether (boiling point: 60-80°C) as solvent (Anon., 2005). Per cent fat was calculated by using the formula.

$$\text{Crude fat (\%)} = \frac{[\text{Initial weight (g)} - \text{Final weight (g)}]}{\text{Sample weight (g)}} \times 100$$

3.3.1.3 Protein

The nitrogen content of moisture free samples was estimated by Kjeldhal method (Anon., 1990) using Pelican Kelplus equipment. Crude protein of all the grain samples (in flour form) were calculated by multiplying total nitrogen with the factor 6.25.

$$\text{Protein (\%)} = \frac{(\text{Titrate} - \text{Blank}) \times \text{Normality of HCl} \times 14.007 \times 6.25}{\text{Sample weight (g)}} \times 100$$

3.3.1.4. Total Ash

About 5 g of dried sample was transferred into a crucible, which was heated on oven till the contents were completely charred. The charred sample was ignited in muffle furnace at 600°C for four to five hours, cooled in desiccator and weighed. Complete ashing was ensured, weighed and repeated till two consecutive constant weights were obtained.

The per cent total ash was calculated as

$$\text{Ash content (\%)} = \frac{\text{Weight of the ash}}{\text{Weight of the sample}} \times 100$$

3.2.1.5. Crude fibre

Crude fibre was estimated by the acid alkali digestion method. Fat free sample was hydrolyzed with dilute sulphuric acid (0.255 N) and dilute alkali (0.313 N). The residue obtained after digestion was dried in a crucible and its weight was recorded

(We). The dried residue was then ashed in a muffle furnace at 600 °C for three hours and its weight (Wa) was recorded. The difference between these two digits (We-Wa) was calculated as per the formula below (Anonymous., 2005) and expressed in terms of per cent.

$$\text{Crude fibre (\%)} = \frac{\text{We} - \text{Wa}}{\text{Weight of fat free sample (g)}} \times 100$$

We = Weight of residue with crucible

Wa = weight of ash with crucible

3.3.1.5. Carbohydrate

Carbohydrate content was computed by difference method.

$$\text{Carbohydrate (g/100 g)} = 100 - [\text{Protein (g)} + \text{Fat (g)} + \text{Ash (g)} + \text{Crude fiber (g)} + \text{Moisture (g)}]$$

3.3.2. Dietary fibre

Soluble (SDF), insoluble (IDF) and total component (TDF) of dietary fiber of moisture and fat free samples were estimated by using standard enzymatic gravimetric method (Asp *et al.*, 1983).

Samples were gelatinized. The protein and starch contents were removed by enzymatic digestion. Total dietary fiber was precipitated by treating with 4 volumes of ethanol and contents were filtered off with celite. Insoluble dietary fiber was filtered similarly and soluble dietary fiber was precipitated from the filtrate of insoluble dietary fiber. The residue was dried (D₁), incinerated in muffle furnace and its weight (I₁) was recorded. The difference in the two weights (D₁ - I₁) was taken as insoluble dietary fiber.

Soluble (SDF), insoluble (IDF) and total dietary fiber (TDF) contents were calculated by the following formula

$$\text{IDF (\%)} = \frac{(D_1 - I_1) - B_1}{W} \times 100$$

$B_1 = (\text{crucible weight with celite} - \text{weight of dry crucible})$

$$\text{SDF (\%)} = \frac{(\text{D}_2 - \text{I}_2) - \text{B}_2}{W} \times 100$$

$B_2 = (\text{crucible weight with celite} - \text{weight of dry crucible})$

Where, W - Sample weight (g)

D - Weight after drying (g)

I - Weight after incineration (g)

B – Weight of ash free blank (g)

$\text{TDF (\%)} = \text{SDF (\%)} + \text{IDF (\%)}$

3.3.3 Minerals

3.3.3.1. Preparation of mineral solution

The mineral solution of samples was prepared by using standard wet ashing method (Anon., 2005). The sample was treated with a mixture of mineral acid (triacids mixture- HNO_3 , H_2SO_4 and HClO_4) and heated for more rapid decomposition. The volatile constituents disappear and non- volatile mineral elements enter into the solution. Heating was continued until content was reduced to few ml of clear white residue. The residue was dissolved in HCL (6N), filtered and made to a known volume (100ml) with distilled water for various elemental analyses.

3.3.3.2. Estimation of minerals

Iron, zinc, copper was estimated using standard AOAC procedure using Atomic Absorption Spectrophotometer and calcium was estimated by EDTA titrimetric method (Derderian *et al.*, 1961).

3.3.4 Phytochemical composition

3.3.4.1. Phytic acid

Phytic acid in grains was determined as phytin-phosphorus (Appendix XI) as outlined by Thompson and Erdman (1982).

3.3.4.2. Total polyphenols

Total polyphenols were estimated using spectrophotometric method- FCR assay (Singleton and Rossi., 1965)

3.3.4.3. Tannins

Tannins were estimated by the modified Vanillin assay (Price *et al.*, 1978).

3.4 Role of probiotic bacteria on the bioaccessibility of iron from the supplementary food

3.4.1 Incorporation of probiotics in supplementary food

Probiotics was added to the supplementary food in the form of encapsulated beads. Encapsulation was done by sodium alginate. The probiotic culture and sodium alginate was mixed to form a solution. This solution was dropped in calcium carbonate solution to form beads and left in it for an hour. These beads are then dried for further use. Potential probiotic strains LS44, LS14 and commercial probiotic culture *Lactobacillus acidophilus* were added after encapsulation in the food ensuring 10^9 CFU/g. The probiotic strain's influence on iron bioaccessibility was tested individually as well as in combination.

3.4.2 Bioaccessibility of iron from supplementary mix and with probiotics

The influence of probiotic bacteria on iron bioaccessibility was assessed by *in-vitro* gastro-intestinal simulation method as described by Luten *et al.* (1996).

3.5 Shelf life of supplementary food incorporated with probiotics

Supplementary food incorporated with probiotics was packed in HDPE packaging material and was kept for storage at two different temperatures 30° C and 40° C and at a relative humidity of 80 per cent for 15 days and 30 days respectively. The samples were drawn at five days' intervals and moisture and free fatty acids were estimated.

Moisture was estimated as 3.3.1.1 and free fatty acid was estimated by titrating the sample against potassium hydroxide in the presence of phenolphthalein indicator (Jitendra *et al.*, 1983).

Viability of probiotic cells:

The composite supplementary mix with encapsulated probiotic cultures were stored at 30°C and 40°C and samples were drawn at frequent intervals to test the thermal death rate noted as per the method by King *et al.* (1998) and Toledo (1991).

Prediction of shelf life:

Shelf life of the product was determined by Q_{10} values. A tool used in accelerated storage is “Rule of ten,” or Q_{10} , which is the factor by which the rate of spoilage increases when the temperature is raised by 10°C. Q_{10} allows for the prediction of storage life of a product under real life conditions based on the results of testing conducted at high temperatures. It is unit less and can be calculated with the equation below:

The rule of Ten (Q_{10})

$$Q_{10} = \frac{R_1^{(10/T_1 - T_2)}}{R_2}$$

Where,

T = Temperature ($^{\circ}\text{C}$), R_1 = Rate of reaction at T_1 and R_2 = Rate of reaction at T_2

$AAR = Q_{10}((AAT - AT)/10)$

$AATD = DRTA/AAR$

Where,

AAR = Accelerated aging rate, $AATD$ = Accelerated aging time duration,

$DRTA$ = Derived real time aging, AAT = Accelerated aging temperature and
 AT = Ambient temperature (22°C)

Q_{10} = Accelerated aging factor, ($Q_{10} = 2$ = Industrial standard)

For most products Q_{10} value is 2.0, which means that for every increase in 10°C , the rate of chemical reaction will double. When calculating the shelf life of a product using Q_{10} value of 2.0, the predicted shelf life is 32 weeks (8 months).

3.6. Statistical analysis

Following suitable statistical tests were applied for interpretation of data using the SPSS software version 23.0. Mean and standard deviation was used to interpret the data. One way *i.e.* two factor analysis of variation (ANOVA) techniques were used to compare the values. Multivariate analysis of variance (MANOVA) techniques were used to interpret the data of the shelf life study.

4. EXPERIMENTAL RESULTS

The experimental results of the study entitled “Bioaccessibility of iron from finger millet based supplementary food with probiotics” conducted with the objectives to assess nutritional composition, phytochemical compounds, physical parameters, bioaccessibility and shelf life study of soybean genotypes are presented under the following sub-headings.

- 4.1 Physical parameters of raw and processed products.
- 4.2 Nutrient composition of raw and processed grains.
- 4.3 Phytochemical content of raw and processed grains.
- 4.4 Mineral content of raw and processed grains.
- 4.5 Bioaccessibility of composite mix with probiotics.
- 4.6 Accelerated storage study of composite mix with probiotics.

4.1 Physical parameters of raw and processed products

4.1.1 Water activity

Water activity was estimated and the results for which are presented in Table 1. The water activity of raw finger millet was 0.12 which increased significantly in germinated finger millet flour to 0.63. It reduced to 0.28 in malted finger millet flour. In raw green gram, aW was 0.58 which rose to 0.83 in germinated product and decreased to 0.43 in malted green gram. Similar trend was seen in wheat as well. Raw wheat's aW was 0.22 which increased in germinated wheat flour to 0.71 and malting decreased the aW to 0.13. The aW of composite mix was 0.23.

4.1.2 Color of raw and processed grains

The results pertaining to color has been presented in Table 2. The L-value for raw finger millet was found to be 52.10 which significantly increased in germinated finger millet flour (65.94). Malted finger millet (70.56) was more towards lighter side of L values. The 'a' values were all positive 'a' values. The 'a' value for raw, germinated

Table 1. Water activity of raw and processed grains

Sample		aW
Finger millet	Raw	0.12 ^f ± 0.01
	Germinated	0.63 ^c ± 0.04
	Malted	0.28 ^e ± 0.04
Green gram	Raw	0.58 ^c ± 0.04
	Germinated	0.83 ^a ± 0.04
	Malted	0.43 ^d ± 0.01
Wheat	Raw	0.22 ^e ± 0.01
	Germinated	0.71 ^b ± 0.01
	Malted	0.13 ^f ± 0.02
Composite Mix		0.23 ^e ± 0.03
F-value		185.344 ***
SEm		0.02
CD at 5%		0.07

Values in a column followed by different letters are significantly different according to DMRT at the 0.05 level

Table 2. Color of raw and processed grains

Sample		L	a*	b*
Finger millet	Raw	52.10 ^h ± 0.42	7.95 ^b ± 0.07	3.33 ^g ± 0.14
	Germinated	65.94 ^c ± 0.25	5.40 ^d ± 0.08	8.75 ^e ± 0.07
	Malted	70.56 ^d ± 0.16	3.83 ^e ± 0.07	7.22 ^f ± 0.13
Green gram	Raw	33.49 ⁱ ± 0.33	-9.18 ^h ± 0.05	26.67 ^b ± 0.16
	Germinated	54.43 ^g ± 0.08	-8.85 ^g ± 0.10	26.15 ^b ± 0.02
	Malted	56.53 ^f ± 0.13	3.88 ^e ± 0.10	29.72 ^a ± 0.30
Wheat	Raw	91.14 ^c ± 0.04	8.77 ^a ± 0.06	29.04 ^a ± 0.63
	Germinated	94.29 ^b ± 0.35	7.13 ^c ± 0.02	25.15 ^c ± 0.06
	Malted	96.06 ^a ± 0.11	5.52 ^d ± 0.01	24.68 ^c ± 0.12
Composite Mix		70.07 ^d ± 0.05	1.81 ^f ± 0.07	9.35 ^d ± 0.07
Total mean		73.96 ± 28.65	6.33 ± 2.59	19.00 ± 10.15
F-value		32525.167***	2971.735***	3748.985***
SEm		0.16	0.05	0.17
CD @ 5 %		0.51	0.16	0.54

Values in a column followed by different letters are significantly different according to DMRT at the 0.05 level

and malted finger millet were recorded to be 7.95, 5.40 and 3.83, respectively. The 'b' values increased significantly ($p \geq 0.001$) representing the increasing yellowness of the product. The 'b' values were recorded as 3.33, 8.75 and 7.22 for raw, germinated and malted finger millet, respectively. The lightness (L-values) of green gram increased significantly ($p \geq 0.001$) in germinated (54.43) and malted (56.53) forms compared to raw green gram which was 33.49. The 'a' values for green gram were negative representing the greenness of the raw (-9.18) and germinated green gram (-8.85) flour. It changed significantly in malted form which was in positive side of 'a' value recorded as 3.88.

4.2 Nutrient composition of raw and processed grains

The proximate composition of raw, germinated and malted forms of finger millet, green gram and wheat were estimated and results are depicted in Table 3. In finger millet the raw flour had a moisture content of 14.42 per cent and it increased in case of germinated flour and reduced after malting. There was no significant change in fat, protein and ash of both germinated and malted finger millet as compared to its raw form. But a highly significant difference in moisture, total carbohydrate and energy were observed. The moisture content of germinated finger millet increased (37.32%) and it decreased in malted (4.37%) form which was highly significant. Crude fiber varied significantly in all three (raw, germinated and malted) forms. It increased after germination and decreased after malting. Carbohydrate and energy decreased in germinated form and increased significantly in malted form of finger millet.

In raw wheat flour, moisture was found to be 10.81 per cent, protein 13.73 per cent, fat 1.62 per cent, ash 1.77 per cent, crude fiber 3.75 per cent, total carbohydrate 72.09 per cent and energy 358Kcal. Germination brought about significant changes in moisture (36.68%), protein (18.68%), crude fiber (5.63%), total carbohydrate (41.49%) and energy (254Kcal) at the level of $P \leq 0.05$. No such changes were seen in fat (1.53%) and ash (1.63%) content compared to its raw form. Malting decreased the moisture content to 4.72 per cent and showed no significant changes in malted wheat protein (18.37%) compared to germinated wheat (18.68%). A significant difference was observed in crude fiber (1.65%), total carbohydrate (74.01%) and energy (383Kcal). Moisture increased significantly in germinated wheat and reduced after malting. Protein

Table 3. Proximate composition of raw and processed grains (g/100 g)

Sample		Moisture	Protein	Fat	Ash	Crude fibre	Total CHO	Energy
Finger millet	Raw	14.42 ^d ± 0.13	7.48 ^g ± 0.19	1.48 ± 0.02	2.40 ^b ± 0.11	3.57 ± 0.06	74.22 ^c ± 0.45	341 ^e ± 0.71
	Germinated	37.32 ^b ± 0.27	7.28 ^g ± 0.34	1.49 ± 0.34	2.33 ^b ± 0.11	5.97 ± 0.01	51.58 ^g ± 0.52	249 ^g ± 2.12
	Malted	4.37 ⁱ ± 0.78	6.83 ^g ± 0.13	1.39 ± 0.32	2.07 ^{bc} ± 0.08	2.63 ± 0.10	85.35 ^a ± 0.28	382 ^{ab} ± 2.12
Green gram	Raw	12.40 ^e ± 0.02	24.34 ^b ± 0.18	1.35 ± 0.13	3.54 ^a ± 0.07	3.09 ± 0.03	58.38 ^f ± 0.27	343 ^e ± 1.41
	Germinated	55.16 ^a ± 0.12	23.20 ^c ± 0.20	1.28 ± 0.14	3.39 ^a ± 0.39	5.10 ± 0.02	16.98 ⁱ ± 0.21	172 ^h ± 0.00
	Malted	6.51 ^g ± 0.03	27.09 ^a ± 0.52	1.53 ± 0.31	3.47 ^a ± 0.05	1.65 ± 0.02	61.41 ^e ± 0.13	368 ^c ± 1.41
Wheat	Raw	10.81 ^f ± 0.03	13.73 ^f ± 0.22	1.62 ± 0.07	1.77 ^{cd} ± 0.16	3.75 ± 0.69	72.09 ^d ± 0.49	358 ^d ± 0.00
	Germinated	36.68 ^c ± 0.23	18.68 ^d ± 0.18	1.53 ± 0.11	1.63 ^d ± 0.16	5.63 ± 0.02	41.49 ^h ± 0.09	254 ^f ± 2.12
	Malted	4.72 ^h ± 0.28	18.37 ^d ± 0.49	1.42 ± 0.05	1.49 ^d ± 0.00	1.97 ± 0.02	74.01 ^c ± 0.83	383 ^a ± 0.71
Composite Mix		5.01 ^h ± 0.06	14.63 ^e ± 0.16	1.29 ± 0.05	1.79 ^{cd} ± 0.03	3.20 ± 0.02	77.29 ^b ± 0.02	379 ^b ± 0.00
F-value		25.809.05***	1283.88***	0.66 ^{NS}	51.84***	90.435 ***	5117.51***	5657***
Sem		0.11	0.21	-	0.11	0.16	0.28	0.96
CD at 5%		0.35	0.65	-	0.35	0.49	0.9	3.03

Values in a column followed by different letters are significantly different according to DMRT at 5 % level

increased significantly after germination with not much increase in malted form. Crude fiber increased after germination and decreased after malting. The difference was significant at the level of $P \leq 0.05$. Both energy and carbohydrate decreased after germination and increased after malting.

Moisture in untreated green gram flour was found to be 12.40 per cent, protein 24.34 per cent, fat 1.35 per cent, ash 3.54 per cent, crude fiber 3.09 per cent, total carbohydrate 58.38 per cent and energy 343Kcal. On germinating green gram, the moisture increased significantly to 55.16 per cent. Protein decreased after germination (23.20%) compared to untreated green gram. It showed no significant changes in ash (3.34%) and fat (1.28%) content. Carbohydrate and energy reduced after germination (16.98% and 172Kcal) which again increased significantly after malting (61.41% and 368kCal) respectively. Moisture reduced in green gram after malting (6.51%) and showed significant increase in protein (27.09%) compared to both untreated (24.34%) and germinated (23.20%) green gram flour. Significant changes were observed in crude fiber (1.97%). It increased after germination and decreased after malting with the significance at the level of $P \leq 0.001$.

The raw, germinated and malted forms of finger millet, green gram and wheat along with the composite mixture were analyzed for dietary fiber content which is shown in Table 4. The total dietary fiber and soluble dietary fiber in raw finger millet was found to be 11.34g/100 g and 1.64g/100 g which showed no significant changes after germination (11.87 g/100 g and 1.05g/100 g) and malting (10.99 g/100 g and 1.15g/100 g), respectively. Whereas, insoluble dietary fiber increased from 9.71g/100 g to 10.82 g/100 g after germination. It was found to decrease after malting process to 9.84g/100 g.

Total dietary fiber (22.16g/100 g) and soluble fiber (3.69g/100 g) content of raw green gram were on par with that of total and soluble dietary fiber in germinated (21.75g/100 g and 1.38g/100 g) and malted (21.28g/100 g and 2.46g/100 g) form. There was a significant increase in insoluble dietary fiber after germination from 18.48g/100 g in raw green gram to 19.29g/100 g, respectively. Malting had no significant effect on insoluble dietary fiber content.

Table 4. Dietary fibre content of raw and processed grains (g/100 g)

Sample		Insoluble Dietary fibre	Soluble Dietary fibre	Total Dietary fibre
Finger millet	Raw	9.71 ^e ± 0.28	1.64 ^{abc} ± 0.05	11.34 ^{de} ± 0.23
	Germinated	10.82 ^d ± 0.13	1.05 ^c ± 0.02	11.87 ^{de} ± 0.15
	Malted	9.84 ^e ± 0.46	1.15 ^c ± 0.03	10.99 ^e ± 0.42
Green gram	Raw	18.48 ^b ± 0.60	3.69 ^{ab} ± 2.71	22.16 ^a ± 3.31
	Germinated	19.90 ^a ± 0.04	1.38 ^{bc} ± 0.23	21.75 ^{ab} ± 0.19
	Malted	19.29 ^a ± 0.38	2.46 ^{abc} ± 0.91	21.28 ^a ± 0.19
Wheat	Raw	9.81 ^e ± 0.25	2.05 ^{abc} ± 0.64	11.86 ^{de} ± 0.88
	Germinated	11.42 ^d ± 0.14	3.10 ^{abc} ± 0.11	15.24 ^c ± 0.25
	Malted	10.90 ^d ± 0.06	3.82 ^a ± 0.45	14.00 ^{cd} ± 0.39
Composite Mix		15.80 ^c ± 0.18	2.89 ^{ab} ± 0.31	18.69 ^b ± 0.49
F-value		391.502***	2.360 NS	30.127***
SEm		0.22	-	0.84
CD @ 5 %		0.68	-	2.65

Values in a column followed by different letters are significantly different according to DMRT at the 0.05 level

In raw wheat flour, total dietary fiber was found to be 11.86g/100 g which showed significant rise after germination (15.24g/100 g) and malting (14.00 g/100 g) although germinated and malted values were on par with each other. Soluble dietary fiber content in raw wheat (2.05g/100 g) was on par with both germinated (3.10 g/100 g) and malted (3.82 g/100 g) form. Insoluble dietary fiber in raw wheat flour was 9.81g/100 g which increased after germination (11.42 g/100 g) and decreased after malting (10.90 g/100 g).

4.3 Phytochemicals in raw and processed grains

The effect of germination and malting on anti- nutrients such as phytic acid, total phenols and tannins was studied. The results related to phytochemical content of raw, germinated and malted finger millet, green gram and wheat is presented in Table 5. It reveals that both germination and malting helps to reduce anti- nutritional factors. In finger millet, phytic acid content reduced from 680.93 mg/100 g in raw form to 471.74 mg/100 g in germinated form. Malting further reduced phytate to 260.25 mg/100 g. Similar trends were seen for both green gram and wheat as well. Green gram showed 262.48 mg/100 g of phytic acid content in its raw form which decreased in germinated (199.35 mg/100 g) and malted (79.22 mg/100 g) form. In raw wheat flour, phytic acid content was 437.67 mg/100 g which decreased to 406.51 mg/100 g and 208.58 mg/100 g after germination and malting respectively. Polyphenol and tannins also showed same trend as phytate in finger millet, green gram and wheat. Polyphenols decreased significantly from 305.10 mg CE/100 g in raw finger millet to 204.21 mg CE/100 g and 178.04 mg CE/100 g in germinated and malted form. Tannin content reduced as well from 18.32 mg TAE/100 g in raw finger millet to 7.20 mg TAE/100 g and 5.89 mg TAE/100 g in germinated and malted forms, respectively.

In raw green gram, polyphenol content was found to be 231.28 mg CE/100 g which reduced to 199.35 mg CE/100 g after germination and further decreased to 79.22 mg CE/100 g after malting. Polyphenols in wheat also decreased after germination (88.01 mg CE/100 g) and malting (60.61 mg CE/100 g) compared to their raw form (370.24 mg CE/100 g). In raw green gram, tannins content was found to be 416.49 mg TAE/100 g which reduced to 409.68 mg TAE/100 g after germination and further decreased to 242.70 mg TAE/100 g after malting. Tannins in wheat also decreased after

Table 5. Phytochemical content of raw and processed grains

Sample		Phytic acid (mg/100 g)	Polyphenols (mg CE/100 g)	Tannins (mg TAE/100 g)
Finger millet	Raw	680.93 ^a ± 5.78	305.10 ^b ± 9.28	18.32 ^d ±0.62
	Germinated	471.74 ^b ± 8.38	204.21 ^d ± 7.42	7.20 ^e ±0.06
	Malted	260.25 ^c ± 6.53	178.04 ^e ± 10.78	5.89 ^e ±0.23
Green gram	Raw	262.48 ^c ± 1.21	231.28 ^c ± 6.57	416.49 ^a ±6.37
	Germinated	199.35 ^c ± 1.45	237.13 ^c ± 2.79	409.68 ^a ±5.22
	Malted	79.22 ^d ± 91.44	151.17 ^f ± 3.33	242.70 ^c ±1.12
Wheat	Raw	437.67 ^b ± 3.80	370.24 ^a ± 12.68	3.88 ^e ±0.32
	Germinated	406.51 ^b ± 5.76	88.01 ^g ± 9.61	3.03 ^e ±0.12
	Malted	208.58 ^c ± 6.18	60.61 ^h ± 10.83	1.23 ^e ±0.08
Composite Mix		216.98 ^c ± 4.62	145.59 ^f ± 3.25	370.54 ^b ±5.28
F-value		71.43***	254.20***	7273.48***
SEm		20.76	5.92	2.21
CD @ 5 %		65.41	18.65	6.96

Values in a column followed by different letters are significantly different according to DMRT at the 0.05 level

germination (3.03 mg/100 g) and malting (1.23 mg TAE/100 g) compared to their raw form (3.88 mg TAE/100 g).

4.4 Mineral composition of raw and processed grains

Table no. 6 shows the results for mineral content in raw and processed grains. The iron content of finger millet decreased significantly after germination (3.56mg/100 g) and increased again after malting (7.49 mg/100 g) compared to its raw form (4.28 mg/100 g). Whereas, calcium content increased after both germination (182.38 mg/100 g) and malting (210.51 mg/100 g). Zinc increased significantly from 0.24 mg/100 g in raw finger millet to 0.64 mg/100 g after germination which was decreased after malting (0.40 mg/100 g). A significant decrease was observed in copper content after germination (0.21 mg/100 g) which increased after malting (0.88 mg/100 g) compared to raw finger millet (0.79 mg/100 g).

The process of germination (5.52 mg/100 g) and malting (6.45 mg/100 g) increased the iron content significantly in green gram which initially had 5.03 mg/100 g of iron in its raw form. Calcium and zinc were found to have no significant changes after germination (82.24 mg/100 g and 0.75 mg/100 g) compared to raw green gram (80.24 mg/100 g and 0.76mg/100 g) but increased significantly after malting to 96.91 mg/100 g and 0.86mg/100 g, respectively. The copper content in raw green gram was 1.07mg/100 g which decreased after germination (0.28 mg/100 g) and increased significantly after malting (0.54 mg/100 g) at the level of $P \leq 0.001$. germination and malting brought about significant increase in iron (4.24 and 4.92 mg/100 g), calcium (79.52 and 152.20 mg/100 g) and zinc (0.60 and 0.96mg/100 g) content of wheat compare to iron (3.36 mg/100 g), calcium (43.12 mg/100 g) and zinc (0.15 mg/100 g) content in raw wheat, respectively. Copper was found to increase after germination (0.90 mg/100 g) and reduced significantly after malting (0.39 mg/100 g) compared to its raw form (0.83 mg/100 g).

Table 7 shows the results for biochemical profile of composite supplementary flour mix. The supplementary mix constituted 2:1:1 ratio of malted finger millet, green gram and wheat and 10 per cent milk powder. The composition of the mix used for further studies is depicted in table 5. In supplementary mix, total dietary fiber was

Table 6. Mineral content of raw and processed grains (mg/100 g)

Sample		Iron	Calcium	Zinc	Copper
Finger millet	Raw	4.28 ^e ±0.18	136.90 ^d ± 0.88	0.24 ^g ±0.01	0.79 ^d ±0.00
	Germinated	3.56 ^f ±0.19	182.38 ^b ±3.35	0.64 ^e ±0.02	0.21 ^g ±0.02
	Malted	7.49 ^a ±0.33	210.51 ^a ±1.23	0.40 ^f ±0.00	0.88 ^c ±0.02
Green gram	Raw	5.03 ^d ±0.03	80.24 ^f ± 0.10	0.76 ^d ±0.01	1.07 ^b ±0.01
	Germinated	5.52 ^c ± 0.28	82.62 ^f ±1.87	0.75 ^d ±0.00	0.28 ^g ±0.05
	Malted	6.45 ^b ± 0.13	96.91 ^c ± 0.59	0.86 ^c ±0.00	0.54 ^e ±0.02
Wheat	Raw	3.36 ^f ± 0.20	43.12 ^g ± 0.13	0.15 ^h ±0.01	0.83 ^{cd} ±0.02
	Germinated	4.24 ^e ± 0.042	79.52 ^f ± 0.99	0.60 ^e ±0.05	0.90 ^c ±0.03
	Malted	4.92 ^d ± 0.06	152.20 ^c ± 0.16	0.96 ^b ±0.01	0.39 ^f ±0.04
Composite Mix		6.05 ^b ± 0.10	160.80 ^b ± 0.83	1.28 ^a ±0.06	1.19 ^a ±0.08
F-value		104.74***	3037.925***	367.14***	178.42***
SEm		0.13	0.98	0.02	0.02
CD at 5%		0.4	3.08	0.07	0.07

Values in a column followed by different letters are significantly different according to DMRT at the 0.05 level

Table 7. Physico-chemical profile of supplementary food per 100 g

Sl. No.	Component	Amount
1.	Moisture %	5.01 ± 0.06
2.	Protein (g/100 g)	14.63 ± 0.16
3.	Fat (g/100 g)	1.29 ± 0.05
4.	Ash (g/100 g)	1.79 ± 0.03
5.	Crude fibre (g/100 g)	3.20 ± 0.02
6.	Total CHO (g/100 g)	77.29 ± 0.02
7.	Energy (kCal)	379 ± 0.00
8.	Insoluble dietary fibre (g/100 g)	15.80 ± 0.18
9.	Soluble dietary fibre (g/100 g)	2.89 ± 0.31
10.	Total dietary fibre (g/100 g)	18.69 ± 0.49
11.	Iron (mg/100 g)	6.05 ± 0.10
12.	Calcium (mg/100 g)	160.80 ± 0.83
13.	Zinc (mg/100 g)	1.28 ± 0.06
14.	Copper (mg/100 g)	1.19 ± 0.08
15.	Phytic acid (mg/100 g)	216.98 ± 4.62
16.	Polyphenols (mg CE/100 g)	145.59 ± 3.25
17.	Tannin (mg TAE/100 g)	370.54 ± 5.28
18.	L*	70.07 ± 0.05
19.	a*	1.81 ± 0.07
20.	b*	9.35 ± 0.07
21.	aW	0.23 ± 0.03

Values indicate mean ± SD of three replications

18.69 g/100 g which had 15.80 g/100 g of insoluble dietary fiber and 2.89g/100 g of soluble dietary fiber. Phytate content of composite mix was found to be 216.98g/100 g, polyphenol content was 145.59 mg CE/100 g and tannin content was 370.54 mg TAE/100 g. In supplementary mix, iron content was 6.05 mg/100 g, calcium (160.80 mg/100 g), zinc (1.28 mg/100 g) and copper content was 1.19 mg/100 g.

4.5 Bioaccessibility of iron from supplementary food with probiotic cultures

The supplementary mix with added encapsulated probiotic beads of LS44, LS14 and commercial strain individually and in combination (LS44+LS14) was tested for bioaccessible iron. The result for which is shown in Fig 1. The bioaccessible mineral of supplementary mix (control) was found to be around 50 per cent of total iron. It increased to 82 per cent in supplementary mix with probiotic LS-44. The combination of probiotics (LS44 + LS14) showed the highest bioavailable iron which was nearly 89 per cent. Supplementary mix with LS-14 had 74 per cent bioaccessible iron which was on par with commercial strain (72%).

4.6 Shelf life study of the finger millet based supplementary food with probiotics

4.6.1 Effect of storage on moisture content of probiotic supplementary food

The effect of accelerated storage on moisture content has been shown in Table 8. The samples were treated at two different temperatures 30 °C and 40 °C at a constant humidity (Rh 80). The samples were withdrawn on five days' interval three times *i.e.*, on 05th, 10th and 15th day. The initial moisture content of supplementary food at 30 °C was 4.60 per cent which increased to 6.39 per cent, 7.41 per cent and 10.59 per cent in supplementary food withdrawn on 05th, 10th and 15th day, respectively. At 40 °C, the moisture content initially was 3.34 per cent which increased to 5.92 percent, 6.37per cent and 8.74 per cent as seen on 05th, 10th and 15th day, respectively. In supplementary food added with LS-44 the moisture content at 30 °C was initially 4.74 per cent which increased to 7.39 per cent in sample withdrawn on 05th day. There was no significant change in moisture content as seen in sample withdrawn on 10th day (7.80%) and increased

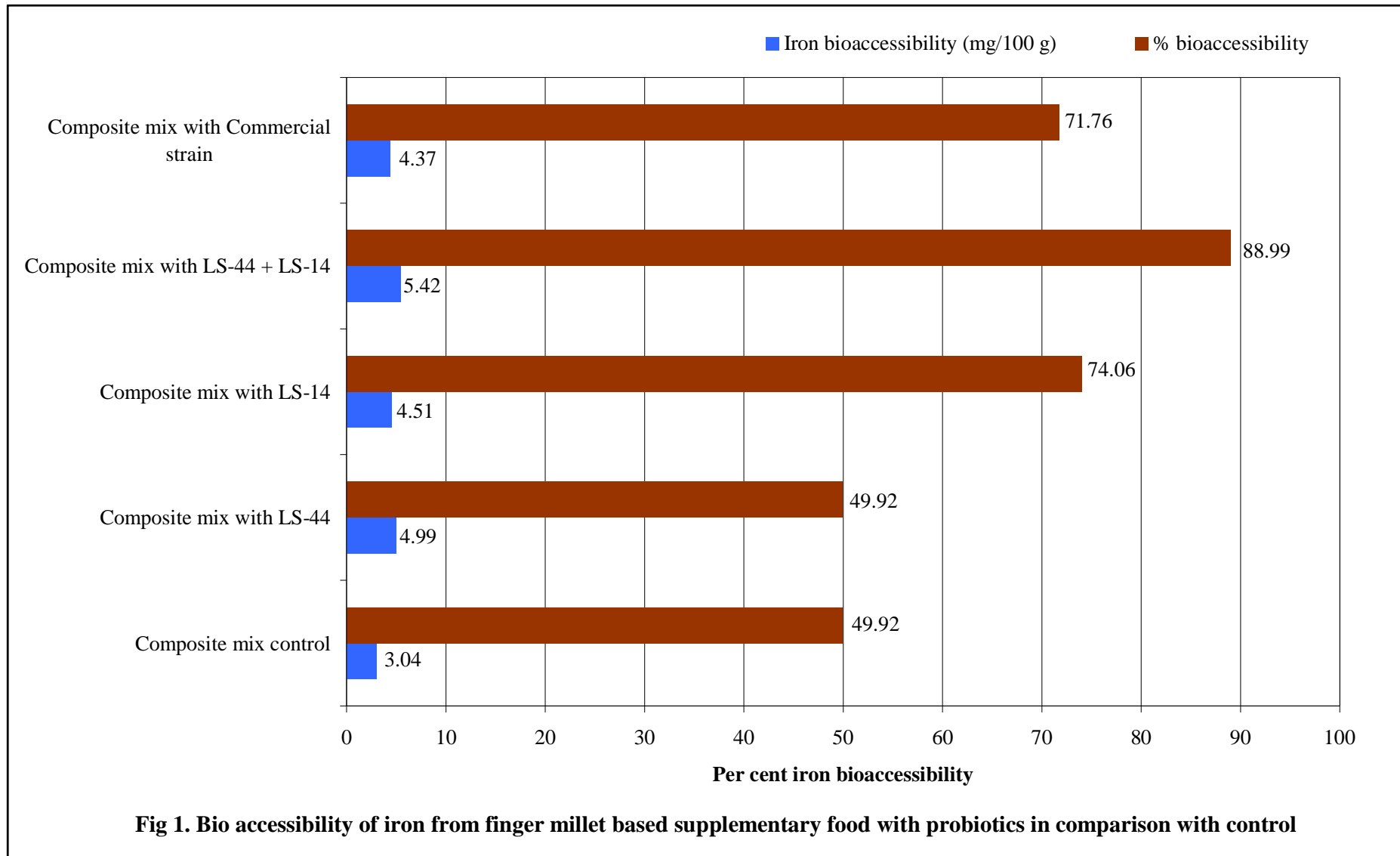
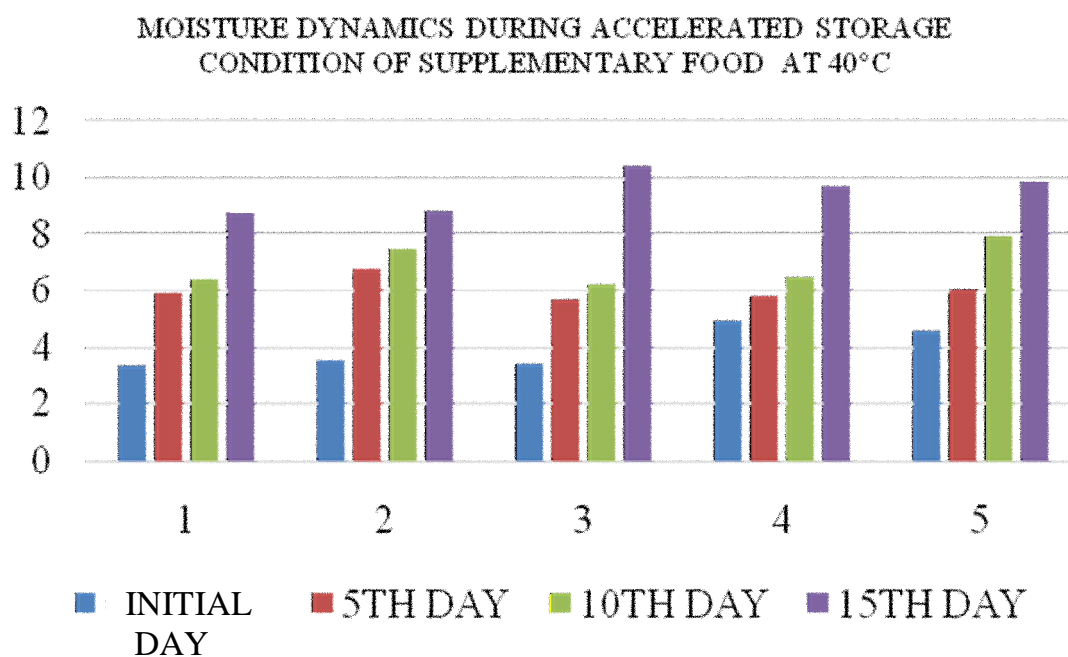
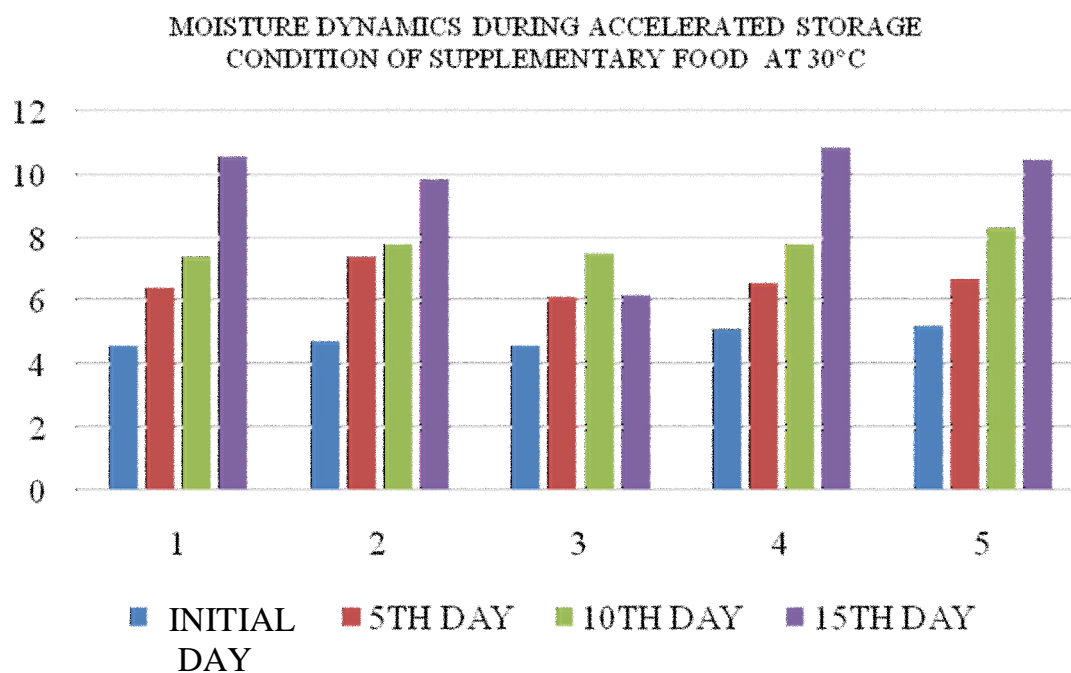


Table 8. Effect of accelerated storage on moisture content of probiotic supplementary food

Sample	Initial		05 th day		10 th day		15 th day		Total Mean	
	30 ^o C	40 ^o C	30 ^o C	40 ^o C	30 ^o C	40 ^o C	30 ^o C	40 ^o C	30 ^o C	40 ^o C
Supplementary Mix (SM)	4.60±0.51	3.34± 0.13	6.39±0.07	5.92± 0.74	7.41±0.16	6.37± 0.18	10.58±0.01	8.74 ± 0.03	7.25±2.33	6.09 ±2.07
SM + LS44	4.74±0.02	3.55 ±0.13	7.39±0.07	6.78± 0.01	7.80±0.08	7.50 ± 0.06	9.86±0.11	8.83± 0.21	7.45±1.95	6.66 ±2.08
SM + LS14	4.57±0.06	3.41 ± 0.22	6.14±0.07	5.66 ± 0.33	7.50±0.18	6.18 ± 0.23	11.27±0.04	10.45±0.03	7.37±2.65	6.42 ±2.73
SM + (LS44+ LS14)	5.10±0.01	4.92± 0.08	6.53±0.11	5.79 ± 0.08	7.80±0.24	6.50 ± 0.13	10.86±0.11	9.71 ± 0.10	7.57±2.27	6.73 ±1.94
DANISCO: LAB cultures (commercial strain)	5.21±0.11	4.60± 0.59	6.70±0.13	6.04 ± 0.03	8.31±0.01	7.97 ± 0.04	10.47±0.01	9.83 ± 0.04	7.67±2.08	7.11 ±2.12
	F-value				SEM		CD			
Temperature	341.568***				0.06		0.121			
Sample	2575.382***				0.104		0.21			
Storage time	27.1728***				0.12		0.242			
Temperature × storage time × sample	3.963 ***				0.207		0.419			



1-Supplementary food (SF- control), 2- SF with LS44, 3- SF with LS14, 4- SF with LS44+14 and 5- SF with Commercial strain.
T1- 30°C, T2- 40°C, Rh- 80

Fig. 2. Effect of accelerated storage on keeping quality of supplementary food with and without probiotics (Moisture)

significantly in sample withdrawn on 15th day to 9.86 per cent. At 40 °C, the moisture content initially was 3.55% and it increased to 6.78%, 7.50% and 8.83 per cent in 05th, 10th and 15th day.

Similar trend was seen in moisture content of supplementary food added with LS-14, LS-44+LS14 and commercial strain. In supplementary food with LS-14 at 30 °C showed initial moisture of 4.57 which increased to 6.14 per cent and 7.50 per cent in samples drawn on 5th and 10th day. The moisture content increased significantly on 15th day to 11.27 per cent. At 40 °C, the initial moisture recorded was 3.41 per cent which increased to 5.66 per cent, 6.18 per cent and 10.45 per cent as seen on 05th, 10th and 15th day, respectively. The combination of LS-44 and LS-14 in composite mix showed initial moisture of 5.10 per cent at 30 °C which increased to 6.53 per cent, 7.80 per cent and 10.86 per cent in 05th, 10th and 15th day samples, respectively. The changes in moisture content at 40 °C initially were 4.92 per cent and it increased to 5.79 per cent, 6.50 per cent and 9.71 per cent on 5th, 10th and 15th day, respectively.

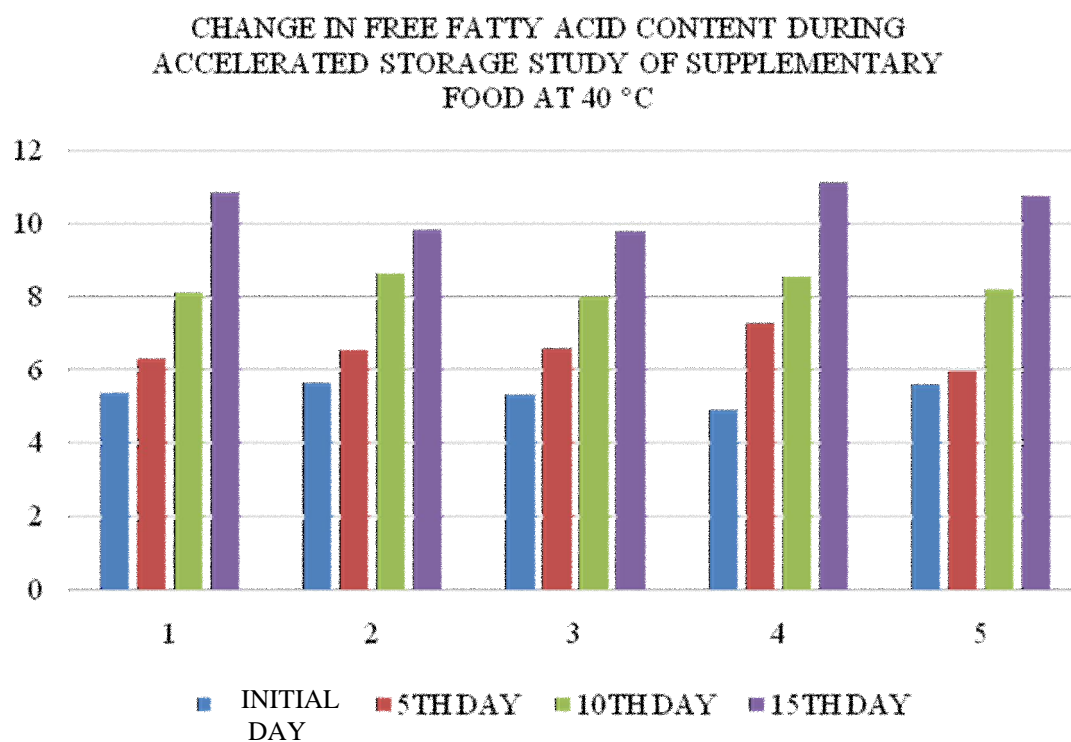
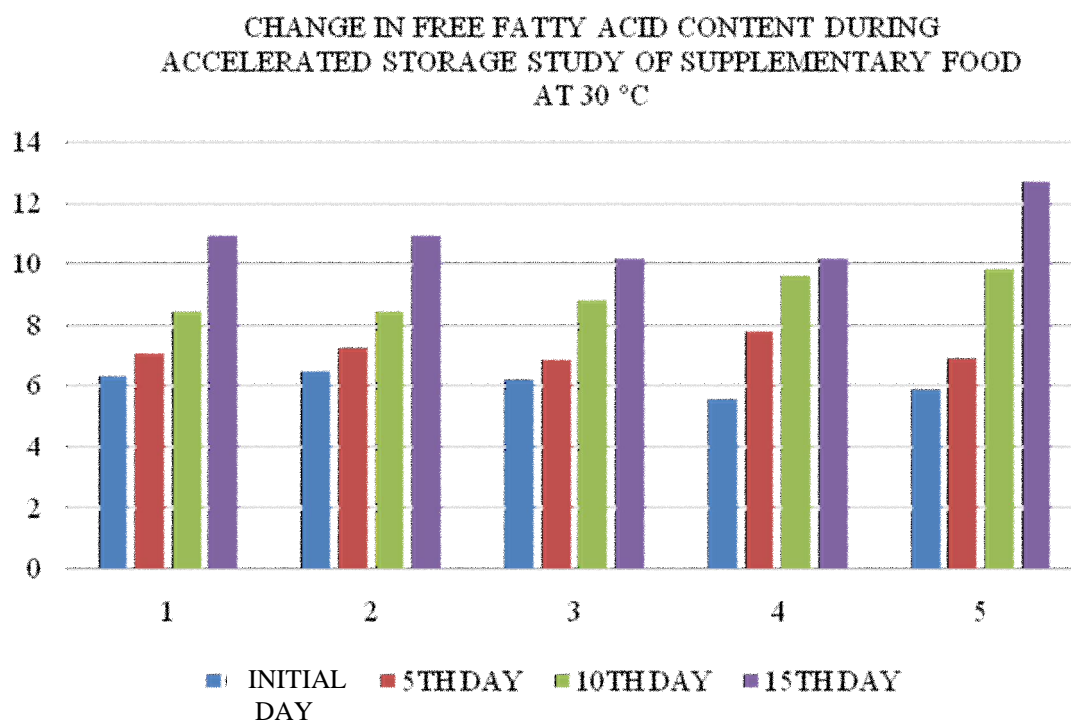
Supplementary food added with commercial strain also had same trend of changes in moisture levels. At 30 °C, initial moisture noted was 5.21 per cent which increased with storage time to 6.70 per cent, 8.31 per cent and 10.47 per cent on 05th, 10th and 15th day, respectively. At 40 °C, initial moisture content was 4.60 per cent, 6.04 per cent on 05th day, 7.97 per cent on 10th day and 9.83 per cent on 15th day. The highest moisture content at 30 °C and 40 °C on 15th day was seen in LS-14 with 11.27 per cent and 10.45 per cent, respectively. The moisture content at 30 °C was slightly higher when compared to moisture content at 40 °C at relative humidity 80.

4.6.2 Effect of storage on free fatty acid content of probiotic supplementary food

The effect of accelerated storage on free fatty acid content has been shown in Table 9. The sample was treated at two different temperatures 30 °C and 40 °C at a constant humidity (Rh 80). The samples were withdrawn on five days' interval three times *i.e.*, on 05th, 10th and 15th day. The initial free fatty acid content of supplementary food at 30 °C was 5.39 per cent which increased to 6.32 per cent, 8.12 per cent and 10.84 per cent in supplementary food withdrawn on 05th, 10th and 15th day, respectively. At 40 °C, the free fatty acid content initially was 6.34 per cent which increased to 7.08 per cent, 8.45 per cent and 10.97 per cent as seen on 05th, 10th and 15th day, respectively.

Table 9. Effect of accelerated storage on free fatty acid content of probiotic supplementary food

Sample	Initial		1 st month		2 nd Month		3 rd Month		Total Mean	
	30 ^o C	40 ^o C	30 ^o C	40 ^o C	30 ^o C	40 ^o C	30 ^o C	40 ^o C	30 ^o C	40 ^o C
Supplementary Mix (SM)	5.39±0.04	6.34 ± 0.03	6.32±0.19	7.08± 0.06	8.12±0.05	8.45 ± 0.31	10.84±0.11	10.97 ±0.11	7.66±2.22	8.21 ±1.89
SM + LS44	5.64±0.02	6.47 ± 0.15	6.55±0.01	7.3050 ±	8.67±0.18	8.93 ± 0.08	9.84±0.11	10.24 ±0.18	7.67±1.78	8.23±1.56
SM + LS14	5.34±0.11	6.25 ± 0.16	6.62±0.09	6.87± 0.45	8.05±0.10	8.85 ±0.18	9.83±0.08	10.20 ± 0.09	7.46±1.79	8.04 ± 1.69
SM + (LS44+ LS14)	4.95±0.08	5.60 ± 0.13	7.32±0.19	7.82 ± 0.05	8.58±0.04	9.62± 0.05	11.14±0.04	10.24 ±0.18	8.00±2.39	8.32 ± 1.93
DANISCO: LAB cultures (commercial strain)	5.60±0.20	5.90 ± 0 .06	6.01±0.06	6.91± 0.08	8.21±0.01	9.87 ±0.04	10.78±0.13	12.74 ±0.18	7.65±2.21	8.85±2.86
Total	5.38±0.27	6.11± 0.35	6.56±0.47	7.19 ± 0.40	8.33±0.28	9.14± 056	10.48±0.58	10.87 ± 1.03	7.69±1.89	8.33 ± 1.95
	F-value		SEM		CD					
Temperature	341.568***		0.06		0.121					
Sample	2575.382***		0.104		0.21					
Storage time	27.1728***		0.12		0.242					
Temperature × storage time × sample	3.963 ***		0.207		0.419					



1-Supplementary food (SF- control), 2- SF with LS44, 3- SF with LS14, 4- SF with LS44+14 and 5- SF with Commercial strain.

T1- 30°C, T2- 40°C, Rh- 80

Fig. 3. Effect of accelerated storage on keeping quality of supplementary food with and without probiotics (Free fatty acids)

In supplementary food added with LS-44 the free fatty acid content at 30 °C was initially 5.64 per cent which increased to 6.55 per cent, 8.67 per cent and 9.84 per cent in sample withdrawn on 05th, 10th and 15th day, respectively. At 40 °C, the free fatty acid content initially was 6.47 per cent and it increased to 7.31 per cent, 8.93 per cent and 10.24 per cents on 05th, 10th and 15th day, respectively.

Similar trend was seen in free fatty acid content of supplementary food added with LS-14, LS-44+LS14 and commercial strain. In supplementary food with LS-14 at 30 °C showed initial free fatty acid was 5.34 per cent which increased to 6.62 per cent, 8.05 per cent and 11.14 per cent in samples drawn on 5th, 10th and 15th day, respectively. At 40 °C, the initial free fatty acid recorded was 6.25 per cent which increased to 6.87 per cent, 8.85 per cent and 10.34 per cent as seen on 05th, 10th and 15th day, respectively.

The combination of LS-44 and LS-14 in supplementary food showed initial free fatty acid of 4.95 per cent at 30 °C which increased to 7.32 per cent, 8.58 per cent and 9.83 per cent in 05th, 10th and 15th day samples, respectively. The changes in free fatty acid content at 40 °C initially were 5.60 per cent and it increased to 7.82 per cent, 9.62 per cent and 10.20 per cent on 5th, 10th and 15th day, respectively.

Supplementary food added with commercial strain also had same trend of changes in free fatty acid levels. At 30 °C, initial free fatty acid noted was 5.60 per cent which increased with storage time to 6.10 per cent, 8.21 per cent and 10.78 per cent on 05th, 10th and 15th day, respectively. At 40 °C, initial moisture content was 5.90 per cent, 6.91 per cent on 05th day, 9.87 per cent on 10th day and 9.98 per cent on 15th day. The highest free fatty acid content at 30 °C was seen in LS14 and at 40 °C, commercial strain showed highest free fatty acid content as seen on 15th day withdrawn samples.

4.6.3 Prediction of shelf life

4.6.3.1 Computation of shelf life

T_1 - 30 °C; R_1 - 15 days

T_2 - 40 °C; R_2 - 30 days

Where,

T_1 is the temperature maintained at 30 °C for 15 days (R_1) and T_2 is the temperature maintained at 40 °C for 30 days (R_2).

$$Q_{10} = (40/30)^{10/(30-15)} = 2$$

Based on Q_{10} value, AAR was computed

$$1. AAR_1 = 2^{((30-22)/10)} = 1.74$$

$$AATD_2 = 15/1.74 = 51.69 \text{ days} = 7.4 \text{ Weeks}$$

$$2. AAR_2 = 2^{((40-22)/10)} = 3.48$$

$$AATD_2 = 90/3.48 = 25.85 \text{ days} = 3.69 \text{ Weeks} = 3.7 \text{ Weeks}$$

Where,

AAR= Accelerated aging rate

AATD= Accelerated aging time duration

DRTA= Derived real time aging

AAT= Accelerated aging temperature

AT= Ambient temperature (22 °C)

Q_{10} = Accelerated aging factor ($Q_{10}= 2$ = Industrial standard)

Ambient Temperature = 22 °C

Table 10 indicates the shelf life equivalency based on AATD, considering $Q_{10}= 2$, at 30 °C was 7.4 weeks and at 40 °C it was 3.7 weeks.

Table 11 indicates the shelf life prediction based on changes in moisture and free fatty acid. All the products can be stored at 30 °C up to 29.6 weeks or 7.4 months and at 40 °C was 16 weeks or 4 months.

Accelerated Aging Time Duration is 52 days for every 90 days of desired shelf life. The study ran the sample at 30 °C and 40 °C and found that the product tested at 40 °C expired after approximately 4 weeks.

Based upon $Q_{10} = 2$, the shelf life would be

Table 10. Shelf life equivalency (in weeks) of probiotic supplementary food and control

Temperature	Shelf life equivalency (Weeks)
30 °C	7.4
40 °C	3.7

Table 11. Estimation of shelf life based on changes in moisture and free fatty acid content of composite mix

Sample	Based on changes in moisture and free fatty acids	
	Shelf life (Weeks) at 30 °C	Shelf life (Weeks) at 40 °C
Supplementary Mix (SM)	29.6	16
SM + LS44	29.6	16
SM + LS14	29.6	16
SM + (LS44+ LS14)	29.6	16
DANISCO: LAB cultures (commercial strain)	29.6	16

$$4 * (2 * 2) = 16 \text{ Weeks} = 4 \text{ months at } 40^\circ\text{C}$$

$$7.4 * (2 * 2) = 29.6 \text{ Weeks} = 7.4 \text{ months at } 30^\circ\text{C}$$

4.6.4 Effect of accelerated storage on thermal death rate (%) in supplementary food with probiotic cultures

Table 12 gives the thermal death rate of the encapsulated probiotic cultures when stored at 30°C and 40°C. The samples were exposed to two different temperatures 30 °C and 40 °C for 24 hours (drawn at six-hour interval) and for four hours (drawn at one-hour interval) respectively, and were calculated for thermal death rate to indicate the cell viability. The thermal death rate increased with exposure time. The initial death rate was zero for all samples. For supplementary food with LS44 the death rate per cent at 30 °C increased from 1.03 per cent at six-hour interval to 1.16 per cent, 2.25 per cent and 3.71 per cent at 12, 18 and 24 hours' interval, respectively. At 40°C, the death rate per cent increased from 0.91% at one-hour to 0.32 per cent, 0.72 per cent and 0.96 per cent at two, three and four-hour interval, respectively. In supplementary food with LS14 the death rate per cent at 30°C was 0, 0.98 per cent, 1.02 per cent, 3.04 per cent and 4.01 per cent at initial, six, 12, 18 and 24 hours' exposure. At 40°C, the per cent death rate was zero initially which increased to 0.23 per cent, 1.76 per cent, 3.25 per cent and 5.59 per cent at one, two, three and four-hour interval.

Supplementary food with combination of probiotics *viz.*, LS44+LS14 showed similar per cent death rate as in mix with LS14. The per cent death rate increased from 0.98 per cent at six-hour interval to 1.2 per cent, 3.04 per cent and 4.01 per cent at 12, 18 and 24 hours of exposure. The death rate at 40°C was 0, 0.24 per cent, 1.70 per cent, 4.09 per cent and 4.74 per cent as seen at 1hour, 2hours, 3 and 4 hours' interval. Supplementary food with commercial LAB culture beads also showed similar trend. The per cent death rate was 0, 1.06 per cent, 1.20 per cent, 3.14 per cent and 4.15 per cent as seen at 0, 6, 12, 18 and 24 hours' interval. At 40°C, it was zero initially which increased to 0.20 per cent on first hour interval, 1.74 per cent, 4.30 per cent and 4.89 per cent was observed on second, third and fourth hours' interval.

Table 12. Effect of accelerated storage on thermal death rate (%) in supplementary food with probiotic cultures

Composite mix with LAB cultures	Temperature									
	30°C					40°C				
	Period interval (in hrs)					Period interval (in hrs)				
	0	6	12	18	24	0	1	2	3	4
SM + LS44	0	1.03±0.02	1.16 ±0.05	2.25±0.10	3.71±0.18	0	0.91 ± 0.00	0.32± 0.01	0.72 ± 0.01	0.96 ± 0.08
SM + LS14	0	0.98 ± 0.014	1.02 ±0.05	3.04±0.18	4.01±0.20	0	0.23±0.02	1.76 ±0.04	3.25 ±0.05	5.59 ±0.09
SM + (LS44+ LS14)	0	1.23± 0.01	1.1 ±0.16	2.65± 0.10	3.86±0.18	0	0.24 ±0.00	1.70 ±0.01	4.09 ±0.04	4.74 ±0.04
SM+ DNISCO. (Commercial strain)	0	1.06 ±0.01	1.20 ± 0.23	3.14±0.13	4.15±0.19	0	0.20 ±0.01	1.74±0.04	4.30 ±0.05	4.89 ±0.05
	F-value		SEM			CD				
Temperature	0.668 ^{NS}		0.024			-				
Day	6476.21***		0.05			0.10				
Sample	633.907***		0.04			0.09				
Temperature × day × sample	146.487***		0.08			0.17				

5. DISCUSSION

Supplementary foods are the foods which provide nutrients which are missing in the regular diet. Iron deficiency anemia is one of the major nutritional problems worldwide and WHO reports 50.10 per cent prevalence of anemia among pregnant women in India as of 2016 (Anon, 2016). Malted finger millet supplementary food is a traditional food given to all age groups after six months of age. Finger millet is the only rich source of calcium (Devi *et al.*, 2014) among cereals. Although the millet has fair amount of iron, the bioavailability is very less (Hemalatha *et al.*, 2007). Supplementary mixes have been developed by combining different grains to avail maximum nutritional benefits by various researchers (Minhas *et al.*, 2010). The present combination chosen developed by UAS, Bengaluru was modified by addition of 10 per cent milk powder. The selected supplementary food has a balance of amino acids, rich in calcium and has prebiotic ingredients. Probiotic organisms change the gut environment of the host and provide beneficial health effects. The nutritive and therapeutic value of staple based food mixes can be enhanced by the addition of probiotics. The present study was conducted to know the iron bioaccessibility of finger millet based supplementary food with addition of different probiotic cultures. The results obtained in the study are discussed and presented under following headings:

- 5.1 Physico-chemical and nutritional composition of supplementary food, individual and processed grains
- 5.2 Effect of probiotics on iron bioaccessibility
- 5.3 Shelf life study of the supplementary food

5.1 Physico-chemical and nutritional composition of supplementary food, individual and processed grains

The water activity of pure water is 1. The upper limit of a_w is 0.6 above which there is risk of microbial growth in the product. Water activity is defined as the ratio of the partial pressure of water in the atmosphere in equilibrium with the substrate (e.g., a food) to that of the atmosphere in equilibrium with pure water at the same temperature and is expressed on a scale of 0 to 1 where one is for pure water. Most bacteria, for

example, do not grow at water activities below 0.91, and most molds cease to grow at water activities below 0.70. No microbial proliferation is seen in aW below 0.60 (Fontana A. J. Jr., 2007). The aW increased significantly in germinated forms of all samples which was seen to decrease in malted samples which might be due to thermal processing *viz.*, roasting (Table 1).

The surface color was measured to record 'L', 'a' and 'b' values for raw, germinated and malted forms of finger millet, green gram and wheat. The L* is the lightness coefficient, ranging from 0 (black) to 100 (white) on a vertical axis. The a* is purple-red (positive a* value) and blue-green (negative a* value) on a horizontal axis. A second horizontal axis is b*, that represents yellow (positive b* value) or blue (negative b* value) color. Digital colorimeter shows L-values, which denote the degree of whiteness, was chosen to represent the color of samples. The L* is the lightness coefficient, ranging from 0 (black) to 100 (white) on a vertical axis. The a* is purple-red (positive a* value) and blue-green (negative a* value) on a horizontal axis. A second horizontal axis is b*, that represents yellow (positive b* value) or blue (negative b* value) color. In finger millet and green gram, the a* and b* values have changed significantly. This change might be due to loss of color while germination. Germination and malting both increases the lightness of the product and b* value lean more towards yellowness and a* value show lesser redness (Table 2).

Traditional processes such as germination and malting affect the nutritional composition of the grains. Moisture increased in germinated forms as seeds take up water in order to start the metabolic processes (Shah *et al.*, 2011). It decreased in all the malted forms as it involves thermal processing *viz.*, roasting which reduces the moisture content in the malted products. Protein content was found to increase in both green gram and wheat upon germination and malting (Table 3). The increase in protein in green gram and wheat can be attributed to increase in hydrolytic enzyme activity. In finger millet it decreased which can be attributed to loss of low molecular weight nitrogenous compounds during soaking and rinsing of the millet grains and hydrolysis of lipid and oxidation of fatty acids during germination (Choudhury *et al.*, 2011).

There was gradual decrease in fat and ash content in all the three forms (raw, germinated and malting) in all the three grains which might be due to the utilization of

lipids and other nutrients during germination. The other changes after germination can be attributed to total solid loss during soaking prior to germination (Ehirim *et al.*, 2018) and due to the utilization of nutrients by growing sprouts (El-Adawy, 2002). Crude fiber shows similar trend in raw and processed grain forms. Crude fiber content increases significantly in germinated forms and the reduction is also significant in the malted forms. Increase in crude fiber in germinated forms may be attributed to synthesis of structural carbohydrates like cellulose and hemicellulose which are major contributors of cell wall content (Shah *et al.*, 2011). Carbohydrate content decreased in germinated grains due to absorption of water by the grain. The carbohydrate content increases in malted grains because of loss of moisture after kilning and roasting.

Dietary fiber content (Table 4) showed no significant difference in total dietary fiber content of raw and processed forms of finger millet and green gram. Total dietary fiber reduced significantly in germinated and malted form of wheat compared to raw. Martin-Cabrejas *et al.* (2003) also showed significant increase in total dietary fiber as well as IDF and SDF increased after germination. Similar results have been found in the present study in wheat and finger millet. Even though green gram showed no significant differences in processed forms the results are comparable with Martin-Cabrejas *et al.* (2003).

Raw finger millet had highest phytic acid of 680.93 ± 5.78 mg/100 g which reduced in processed samples. Significant reduction was seen in malted sample compared to raw forms. Phytic acid decreased in both raw green gram and wheat but was not significant in comparison to raw form. It decreased significantly in malted green gram and wheat as well. Fiber rich foods, including cereals and legumes, contain high levels of phytates or phytic acids. Phytic acid is present as phosphorous, which is the storage form, in cereals and is released when germinated (Oghbaei *et al.*, 2017). Soaking, cooking and germinating of raw seeds are known to reduce phytate content (Gunashree *et al.*, 2014 and Khatoon and Prakash 2006). These changes can be attributed to an increase of phytase enzyme. The process of roasting also contributes to the decrease in phytate reduction (Singh and Raghuvanshi 2012). Total polyphenols were also found to decrease significantly in processed forms of all three grains and also tannins. Tannins were found to decrease significantly in all malted forms compared to raw forms of the samples. All these changes in phytochemical composition might be

due to activation of enzymes during soaking, germination and malting process. These processes help synthesize new enzymes. The decrease in polyphenols may be due to the enzyme polyphenol oxidase, decrease in phytates might be attributed to activities of endogenous phytase which help to reduce them. The catabolic endogenous enzymes also contribute to these changes (Table 5).

The mineral composition of raw and processed flours showed significant increase in iron, copper and calcium contents after malting, while zinc decreased in case of finger millet (Table 6). In green gram, malting increased the content of all the minerals significantly, while in wheat iron, calcium and zinc increased significantly whereas, copper decreased significantly. The changes could be due to the metabolic processes and liberation of minerals from the matrix (Sadawarte *et al.*, 2018).

The supplementary food developed constituted 2:1:1 ratio of malted finger millet, green gram and wheat and 10 per cent milk powder. The mix provides protein of 15 per cent, 1.3 per cent fat, 379 Cal. energy, total dietary fiber of 18.69g/100 g which had 15.80 g/100 g of insoluble dietary fiber and 2.89g/100 g of soluble dietary fiber. It has phytate content of 216.98g/100 g, polyphenol content 145.59 mg CE/100 g and tannin content was 370.54 mg TAE/100 g. The mineral content includes iron 6.05 mg/100 g, calcium (160.80 mg/100 g), zinc (1.28 mg/100 g) and copper content of 1.19 mg/100 g (Table 7).

5.2 Effect of probiotics on iron bioaccessibility

The supplementary food was enriched with encapsulated probiotic cultures LS44, LS14, LS44+LS14, commercial culture, which contained 10^9 cfu/g of sample. These mixes with probiotic culture were subjected *in vitro* simulation of gastrointestinal digestion and iron bioaccessibility was measured by atomic absorption spectrophotometer (Fig 1). It was observed that 50 per cent of the iron in the supplementary food was bioaccessible. While the mix with LS44 showed 82 per cent bioaccessibility, in case of mix with LS14, the bioaccessibility was 74 per cent. The combination showed the highest bioaccessibility 89 per cent. The mix with commercial strain had 72 per cent bioaccessible iron. The high bioaccessibility of iron with probiotic cultures indicate a positive influence. The bioavailability of iron from food is

influenced by the various factors like processing, dietary factors, promoters, and inhibitors. In the present study, enhanced bioaccessibility of iron could be attributed to the release of acids during fermentation, which helps in the release of metal ions from the food matrix (Krishnan *et al.*, 2012). Malting is a process where several changes take place facilitating iron bioavailability. It is reported that malting increased the bioaccessibility of iron by >3-fold in finger millet and two fold in wheat. The beneficial effect of malting on the bioaccessibility of iron was due to the combined effects of soaking, germination and heat treatment (Platel *et al.*, 2010). During malting phytases a type of phosphatase which cleaves the phosphate group of phytate (myoinositol hexaphosphates), thereby freeing bound divalent metal ions such as iron, calcium, magnesium and zinc.

5.3 Shelf life study of the supplementary food

The supplementary food with encapsulated probiotic cultures was tested for shelf life at two different temperatures 30° and 40° C at an RH of 80 per cent in HDPE packages. Moisture and free fatty acid were analyzed at regular intervals. The moisture content increased significantly at both the temperatures in all the samples. It could be due to packaging material and the relative humidity in which it was stored. Moisture contributes to the liberation of free fatty acids due to hydrolytic rancidity (Robards, Kerr and Patsalides, 1988), the increase in free fatty acids at both the temperatures could be attributed to this. The shelf life of the samples based on the Q10 values computed indicate that when the samples are kept at 30° C at a RH of 80 per cent, the accelerated aging time duration is 7.4 weeks and at 40° C and at RH of 80 per cent it is 3.7 weeks. With regard to free fatty acid, the shelf life at 30° C at a RH of 80 per cent is 29.6 weeks and at 40° C at a RH of 80 per cent is 16 weeks. Thus indicating the product could be stored up to 7.4 months at 30° C at a RH of 80 per cent. The per cent death rate of the cells increased with exposure time and temperature. 40°C showed the fastest thermal reduction. The highest death rate at 30°C was observed in commercial strains as seen after 24-hours exposure. The highest per cent death rate was observed in supplementary food with LS14 where death rate recorded was 5.59% after four hours of exposure to 40°C temperature. The lowest thermal death rate per cent was seen in composite mix with LS44.

6. SUMMARY AND CONCLUSIONS

- The proximate composition of raw, germinated and malted forms of finger millet, green gram, wheat were estimated.
- The moisture content increased significantly in germinated flour and decreased in malted form of finger millet. Similar trends were observed in wheat and green gram as well.
- Malting significantly increased the protein content of green gram and wheat whereas, in finger millet the protein content decreased non significantly.
- Fat and ash content showed no significant changes in any of the processed grains compared to raw grains.
- Crude fiber varied significantly in all three (raw, germinated and malted) forms. It increased after germination and decreased after malting.
- Carbohydrate and energy decreased in germinated form and increased significantly in malted forms.
- The changes in phytochemicals were significant in processed grains compared to raw forms. The phytic acid content reduced by 38% in malted finger millet, 30% in green gram malt and by 50% in malted wheat.
- Polyphenols also decreased significantly in all the processed grains. In finger millet, malting decreased polyphenol content by 57%, 65% decrease in malted green gram and malted wheat showed 48% decrease compared to raw form.
- Tannins also showed similar trend as showed by phytates and polyphenols. A significant decrease by 32%, 58% and 40% was seen in tannin content of malted finger millet, green gram and wheat when compared to tannin content in raw grain flour.
- Processing the grains helped increase soluble dietary fiber and decreased the insoluble dietary fiber.

- Iron content varied from 4.28 mg/100 g in raw finger millet to 7.49 mg/100 gram in malted form. Iron increased significantly in all the three grains after processing. Raw green gram had 5.03 mg/100 g which increased to 6.45 mg/100 g in malt. Wheat also showed significant increase in iron content.
- Calcium increased by 15%, 12% and 35% in malted finger millet, green gram and wheat, respectively when compared to unprocessed grain flour.
- Zinc increased significantly in raw finger millet in germinated form which was found to decrease in malted form. There were not much changes in zinc content of germinated green gram but it increased significantly in malted form.
- A significant decrease was observed in copper content after germination which increased in malted form significantly compared to raw finger millet. Green gram and wheat also showed the same trend in changes.
- In supplementary mix, total dietary fiber was 18.69g/100 g which had 15.80 g/100 g of insoluble dietary fiber and 2.89g/100 g of soluble dietary fiber and 14.63g/100 g of protein. Phytate content of supplementary mix was found to be 216.98g/100 g, polyphenol content was 145.59 mg CE/100 g and tannin content was 370.54 mg TAE/100 g. In supplementary mix, iron content was 6.05 mg/100 g, calcium (160.80 mg/100 g), zinc (1.28 mg/100 g) and copper content was 1.19 mg/100 g.
- Water activity in all the products was within the safe range.
- The raw and processed samples were tested for color. Germination and malting enhances the lightness of the product and a* values show reduced redness in germinated and malted flour when compared to raw wheat flour and b* values show more of yellowness in both germinated and malted products.
- The bioaccessible mineral of supplementary mix was found to be around 50% of total iron. It increased to 82% bioaccessible iron in supplementary mix with probiotic LS-44. The combination of probiotics (LS44 + LS14) showed the highest bioavailable iron which was nearly 89%. LS-14 showed 74% bioaccessible iron which was on par with commercial strain (72%).

- Based on the changes in moisture and free fatty acid content the shelf life of the products was 29.6 weeks at 30 °C and for 16 weeks at 40 °C.
- The probiotic cell's viability was found to decrease when subjected to higher temperatures. The highest death rate at 30°C was observed in commercial strains as seen after 24-hours exposure. The highest per cent death rate was observed in supplementary mix with LS14 where death rate recorded was 5.59% after four hours of exposure to 40°C temperature. The lowest thermal death rate per cent was seen in supplementary mix with LS44.

Thus, it can be concluded that addition of probiotic cultures to the composite finger millet based supplementary food can increase iron bioaccessibility significantly. The product can be stored for nearly 8 months at 30 °C.

Future line of work

1. Toxicology safety of the probiotic cultures used.
2. In vivo studies on the efficacy of the products for iron absorption.

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APPENDIX I

Estimation of protein

Principle

Organic Nitrogen digested with sulphuric acid in the presence of catalyst is converted to ammonium sulphate. Ammonium liberated by making the solution alkaline is distilled into a known volume of standard acid, which is then back titrated. Protein per cent was calculated by multiplying the nitrogen present by the factor 6.25.

Reagents

- 40 % NaOH (400 g NaOH dissolved in distilled water and volume made up to 1 litre with distilled water)
- 4 % Boric acid (40 g of boric acid dissolved in distilled water and volume made up to 1 litre with distilled water)
- Mixed indicator [0.2 % Bromocresol green and 0.2 % methyl red in 1:2 proportions 0.1 N HCL standardized (for titration)]
- Concentrated H₂SO₄
- Catalytic mixture (K₂SO₄ and CuSO₄) Ratio 5:1

Procedure for digestion

Preheat the digestion system to 250 °C. Take 0.20 to 0.25 g of food sample in the digestion tube. Now add 3-4 g of catalytic mixture and finally add 10 ml of conc. Sulphuric acid to the sample and place the tubes in the digestion block along with manifolds. Ensure the manifolds are fitted properly. Turn 'ON' the tap water inlet to the KEL-FLOW Assembly immediately. Ensure any frothing of samples is there: if frothing is not there then increase the temp to 420 °C. Leave the tubes in the block for 1 hr and after 1 hr ensure the color of the samples are turned into bluish green: if not replace the tubes in the block for some time once the bluish green color appears remove the tubes and place them in the cooling stand.

Distillation

Switch on the POWER switch on Wait for READY signal to glow. Open the tap for condensation. Ensure the overhead tank is filled fully with distilled water and the tap is in ON condition. Ensure 40 % NaOH (in the tank) is filled in the alkali tube up to the loading tube (press the alkali button manually, till alkali gets collected in the sample end tube)

Note: This is initial step only and the same procedure is followed for boric acid also.

Titration

The green colored boric acid was titrated against the 0.1 N HCL until its color turned to pink. A blank was run simultaneously. The titre values obtained were incorporated in the equation below to obtain the per cent nitrogen present in the sample which, in turn, was multiplied by the factor 6.25 to obtain the per cent protein.

$$\text{Percent nitrogen (\% N)} = \frac{(V_a - V_b) \times 0.0014 \times V_1}{V_2 \times W} \times 100$$

Where

V_a – Titre value of sample

V_b – Titre value of blank

V_1 – Volume to which digested sample was made up (100 ml)

V_2 – Weight of sample taken for digestion (0.5 g)

APPENDIX II

Estimation of fat

Principle

The extraction of fat from substances is often tedious and requires through contact and heating with the solvent. This is done in the Socs plus apparatus in which fresh solvent continuously comes into contact with the material to be extracted over a relatively long period of time.

Procedure

Five gram of sample was weighed into a thimble and plugged with fat free cotton wool. The thimble was placed in the Socs plus apparatus attached to a pre-weighed flask and extracted for about 14 - 26 hr. Therefore, the flask was retrieved from the apparatus with as little solvent in it as possible. It was then transferred into an even to evaporate the remaining solvent, leaving behind only the residue or extract. The flask was then cooled in desiccators after which it was weighed to estimate the fat.

$$\text{Fat content (g/100 g of sample)} = \frac{\text{Weight of ether extraction (g)}}{\text{Weight of sample taken (g)}} \times 100$$

APPENDIX III

Estimation of crude fiber

Principle:

During the acid and subsequent alkali treatment, oxidative hydrolytic degradation of native cellulose and considerable degradation of lignin occurs. The residues after final filtration is weighed, incinerated, cooled and weighed again. The loss in weight gives the crude fiber content.

Calculation:

W = Sample weight

W₁ = Weight of the crucibles after oven drying

W₂ = Weight of the crucibles after drying muffle furnace

W₃ = W₁ - W₂

$$\% \text{ of crude fiber} = \frac{W_3}{W_1} \times 100$$

APPENDIX IV

Estimation of dietary fiber

Estimation of dietary fiber was carried out by Asp *et al.* (1983) method.

Principle: Defatted samples were gelatinized and protein and starch were removed enzymatic digestion.

Reagents

1. 0.1 M sodium phosphate buffer, pH 6.0
2. 4 M HCl
3. 4 M NaOH
4. 95 % ethanol, technical grade
5. 78 % ethanol
6. Acetone, puriss
7. Pepsin NF
8. Pancreatin 4 x NF
9. Termamyl 120 L (Sigma)

Procedure

1. Weigh 1 g of sample with 0.1 mg accurately (W) and transfer to an Erlenmeyer flask. Add 25 ml of 0.1 M phosphate buffer, pH 6.0, and suspend the sample thoroughly.
2. Add 0.1 ml termamyl. Cover the top of flask with aluminum film and incubate in a boiling water bath for 15 min (with occasional shaking).
3. Allow to cool. Add 20 ml of distilled water and adjust the pH to 1.5 with HCl. Rinse the electrode with a few millilitres of water.
4. Add 100 mg of pepsin. Cover the flasks and incubate in a 40 °C water bath with agitation for 60 min.

5. Add 20 ml of distilled water and adjust the pH to 6.8 with NaOH. Rinse the electrode with 5 ml of water.
6. Add 100 mg of pancreatin. Cover the flasks and incubate in a 40 °C water bath with agitation for 60 min.
7. Adjust the pH to 4.5 with HCl.

(A) Residue (Insoluble fiber)

8. Filter through a dry weighed crucible (porosity 2) containing 0.5 g of dry celite as the filter aid. Wash with 2 × 10 ml of distilled water.
9. Wash with 2 × 10 ml of 95 % ethanol and 2 × 10 ml of acetone.
10. Dry at 105 °C to constant weight or overnight. Weigh, after cooling in a desiccator (D1)
11. Incinerate at 550 °C for at least 5 hr. Weigh, after cooling in a desiccator (I1).

(B) Filtrate (Soluble fiber).

12. Adjust the volume of the combined filtrate and washing waters to 100 ml.
13. Add 400 ml of warm (60 °C) 95 % ethanol. Allow to precipitate for 1 hr.
14. Filter through a dry and weighed crucible (porosity 2) containing 0.5 g of celite.
15. Wash with 2 × 10 ml of 78 % ethanol, 2 × 10 ml of 95 % ethanol and 2 × 10 ml of acetone
16. Dry at 105 °C to constant weight or overnight. Weigh, after cooling in a desiccator (D2).
17. Incinerate at 550 °C for at least 5 hr. Weigh, after cooling in a desiccator (I2).

Blank: Insoluble and soluble blank values are obtained by running the procedure without sample (B1 and B2). The blank values should be checked occasionally and when new batches of enzymes are used.

Calculation:

W = Sample weight (g), D = Weight after drying (g), B = Weight of ash free blank (g).

$$\% \text{ Insoluble dietary fiber} = \frac{D1 - I1 - B1}{W} \times 100$$

$$\% \text{ Soluble dietary fiber} = \frac{D2 - I2 - B2}{W} \times 100$$

APPENDIX V

Estimation of micronutrients

Estimation of micronutrients was done using atomic absorption spectrophotometry (Anon, 2000).

Principle: The sample is treated with a mixture of mineral acids (tri or diacid) and heated for more rapid decomposition. The volatile constituents disappear and non-volatile mineral elements enter into solution. Heating is continued until digest is reduced to a few ml of clear white residue. The residue is dissolved in HCl (6 N), filtered and made to known volume with distilled water for various elemental analysis.

Reagents: Triacids mixture: Mix 100 ml of conc. HNO₃ 10 ml of conc. H₂SO₄ and 40 ml of 60% HClO₄ or 30 ml of 72% HClO₄. (HNO₃: H₂SO₄: HClO₄ in the ratio of 10:1:4 respectively).

Procedure: Predigestion of sample with HNO₃: Around 1 g of the sample was transferred into 100 ml conical flask and the samples, wetted with 10 ml of conc. HNO₃ and allowed to stand overnight. The content was then gently heated on a sand bath till the volume of the contents was reduced to 4 ml or less and then cooled.

Digestion with triacids mixture: 5 ml of triacids mixture was added and kept on hot sand bath until evolution of dense white fumes subside leaving about 3 ml of colourless solution in the conical flasks which on cooling gives white residue.

Preparation of test solution: 5 ml of 6 N HCl is added to the residue; the contents swirled and transferred to 50 ml volumetric flasks by filtering through Whatman No.42 filter paper. The procedure was repeated 3 - 4 times with additional quantity of 6 N HCl until all the residue is filtered. The conical flask is rinsed with distilled water and the contents transferred to volumetric flasks. The volume is made up to 50 ml with distilled water washing of the residue on the filter paper. The flask is stoppered and preserved for elemental analysis. The instrument is set to zero with blank. The working standards of element to be determined to AAS is fed to calibrate the instrument. The plant digest is fed and absorbance/concentration of the element recorded.

$$\text{Total (minerals, ppm)} = \frac{\text{Volume made after digestion}}{\text{Weight of sample}} \times \text{ppm from the instrument}$$

$$\% \text{ Fe, Cu, Zn or Mn in sample} = \text{ppm}/10,000$$

APPENDIX VI

Estimation of polyphenols

Polyphenols were estimated using Folin- ciocalteau reagent (Anon., 2000).

Principle: Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produced blue coloured complex (molybdenum blue).

Reagents: 80 % ethanol, Folin- Ciocalteau reagent, Na_2CO_3 , 20 %, Standard stock: 100 mg Gallic acid in 100 ml water. Ten times diluted for a working standard.

Procedure

1. The defatted sample, 0.5 to 1 g was weighed and ground with a pestle and mortar in 10-time volume of 80 % ethanol.
2. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was saved. The residue was re-extracted with five times the volume of 80 % ethanol, centrifuged and the supernatants pooled.
3. The supernatant was evaporated to dryness. The residue was dissolved in a known volume of distilled water (5 ml).
4. The different aliquots (0.2 -2 ml) were pipette out into test tubes.
5. The volume was made up to 3 ml with water, and 0.5 ml of Folin- Ciocalteau reagent is added.
6. After 3 min, 2 ml of 20 % Na_2CO_3 solution was added to each tube and mixed thoroughly.
7. The tubes were placed in boiling water for exactly 1 min, cooled and the absorbance measured at 650 nm against a reagent blank.
8. A standard curve was prepared using different concentrations of gallic acid.

Working Standard:

Take different aliquots of gallic acid (without sample) like 0.2 to 2 ml



Make up the volume up to 3 ml with distilled water



Add 0.5 ml of FCR reagent



Keep it for 3 minutes at room temperature (incubation)



Add 2 ml of Na_2CO_3



Keep for boiling for 1 min in water bath



Cool it and read the absorbance at 650 nm against the reagent blank

Calculation

From the standard curve the concentration of phenols in the test samples is expressed as mg GAE/g material.

APPENDIX VII

Estimation of tannins

Tannins were estimated using Folin-Denis reagent (Schander, 1970).

Principle: Tannins react with phosphotungstomolybdic acid in Folin-Denis reagent in alkaline medium and produce blue colour.

Reagents: Folin- Denis reagent, Na_2CO_3 , Standard stock: 100 mg Tannic acid in 100 ml water, Working standard: 10 ml stock in 1,000 ml water

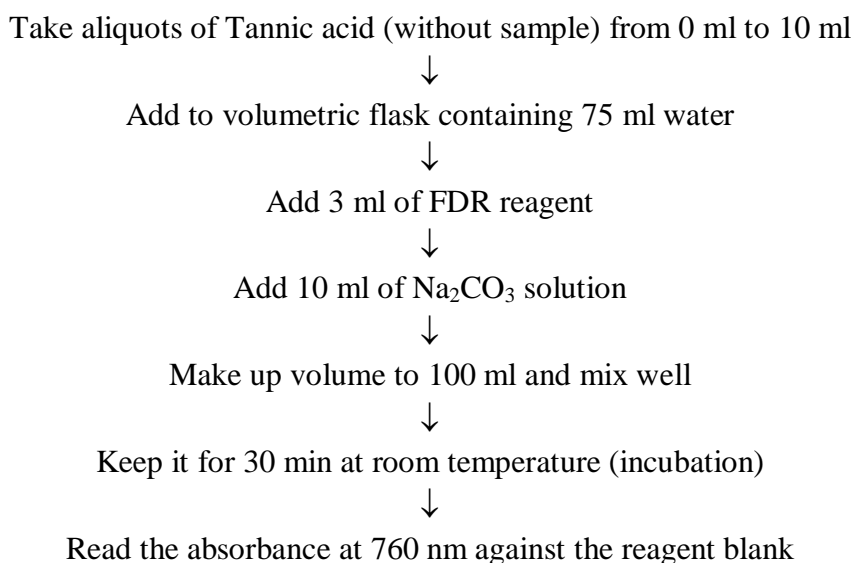
Procedure

Preparation of Sample: The defatted sample, 5 g was weighed and extracted with 85 ml methanol containing one per cent HCL for 30 min with occasional shaking. The content was filtered using Whatman No. 1 filter paper. The filtrate was used for tannin estimation.

Procedure for tannin estimation: To 1 ml of extract 5 ml of Folin- Denis reagent and 10 ml of Na_2CO_3 was added and mixed. The contents were diluted to 1,000 ml. Allowed to stand for 30 min and absorbance was measured at 760 nm.

A standard curve was prepared using different concentrations of gallic acid.

Working Standard:



Calculation: From the standard curve tannin content in the samples is calculated and expressed as tannic acid equivalents

Shelf life study

1. Determination of the test method and duration
Indirect method (Accelerated) = Approximately ¼ of expected
2. Determination of the storage conditions
Elevated storage conditions at 10 degrees C difference.
3. Determine Test Intervals
5 days' interval
4. Determine product samples and protocol
Moisture, FFA and cell viability

This can be done by varying the temperature and relative humidity.

The rule of ten (Q_{10})

$$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$$

$$AAR = Q_{10}^{((AAT-AT)/10)}$$

$$AATD = DRTA/AAR$$

where,

AAR= Accelerated aging rate, AATD= Accelerated aging time duration, DRTA= Derived real time aging, AAT= Accelerated aging temperature, AT= Ambient temperature, Q_{10} = Accelerated aging factor, ($Q_{10}= 2$ = Industrial standard, $Q_{10}= 1.8$ = More conservative option).

Results:

T1	T2
30 °C	40 °C
R1	R2
15	30

$$Q_{10} = (40/30)^{(10/(30-15))} = 2$$

$$1. \text{ AAR1} = 2^{((30-22)/10)} = 1.74$$

$$\text{AATD2} = 15/1.74 = 51.69 \text{ days} = 7.4 \text{ Weeks}$$

$$2. \text{ AAR2} = 2^{((40-22)/10)} = 3.48$$

$$\text{AATD2} = 90/3.48 = 25.85 \text{ days} = 3.69 \text{ Weeks} = 3.7 \text{ Weeks}$$

Accelerated Aging Table based on $Q_{10}=2$, Ambient Temperature = 22 °C

Degrees C	90 days shelf life equivalency (Weeks)
30 °C	7.4
40 °C	3.7

Accelerated Aging Time Duration is 52 days for every 90 days of desired shelf life.

The study ran the sample at 30 °C and 40 °C and found the product tested at 40 °C expired after approximately 4 weeks.

Based upon $Q_{10} = 2$, the shelf life would be

$$4 * (2 * 2) = 16 \text{ Weeks} = 4 \text{ months at } 40 \text{ °C}$$

$$7.4 * (2 * 2) = 29.6 \text{ Weeks} = 7.4 \text{ months at } 30 \text{ °C}$$

Sample	FFA	
	Shelf life (Weeks) at 30 °C	Shelf life (Weeks) at 40 °C
Composite Mix	29.6	16
LS44	29.6	16
LS14	29.6	16
LS44+14	29.6	16
Commercial	29.6	16

APPENDIX VIII

In vitro bioaccessibility of Calcium

Bioaccessibility of calcium was determined using *in vitro* dialyzability according to Luten *et al.* (1996) with simulated peptic and pancreatic digestion. Digestive enzymes and bile salts were purchased from Sigma-Aldrich, Malaysia. A pepsin solution was prepared by dissolving 16 g of pepsin (P-7000, from porcine stomach mucosa) in 100 ml of 0.1M HCl. The pancreatin solution contained 4 g of pancreatin (P-1750, from porcine pancreas) and 25 g of bile extract (B-8631, porcine) with 1000 ml of 0.1M NaHCO₃.

Peptic digestion

Firstly, 10 g of homogenized bread sample was adjusted to pH 2.0 with 6 M HCl and then 3 ml pepsin solution was added. The mixture was brought up to 100ml with distilled water and incubated at 37°C for 2 hours.

Titrateable acidity

A titration was performed in which 20 ml of gastric digest was mixed with 5 ml of pancreatin-bile mixture and the amount of 0.5 M NaOH needed for this mixture to achieve a pH of 7.5 was determined.

Pancreatic digestion

Segment of dialysis tubing (Ø = 20.4 mm; MMCO of 10k Da; Sigma-Aldrich, Malaysia) containing an amount of NaHCO₃, being equivalent to the moles of NaOH needed for the pancreatic digestion (titrateable acidity), was made up to 25 ml with distilled water. The dialysis tube was then placed into the conical flask containing 20 ml of gastric digest and incubated for 30 minutes. After attaining a pH of 7.0-7.5, 5 ml of pancreatin-bile mixture was added, and the incubation continued for another 2 hours.

The content in the dialysis tube was analysed by ICPMS for the calcium contents. Lastly, the bioaccessibility of the calcium was expressed as the following formula:

$$\text{Bioaccessibility (\%)} = \frac{Y}{Z} \times 100\%$$

Where,

Y= dialysate portion of calcium (mg mineral 100 g of sample)

Z= total calcium content (mg mineral/100 g of sample)

BIOACCESSIBILITY OF IRON FROM FINGER MILLET BASED SUPPLEMENTARY FOOD WITH PROBIOTICS

KALYANI UMESH KAMATAGI 2019

**Dr. HEMALATHA S.
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ABSTRACT

Consumers today are highly conscious of nutritionally and functionally rich therapeutic foods. A considerable amount of interest has been focused towards the development of probiotic products. The idea of using probiotics is a method to promote good health and disease prevention through natural and biotic applications to daily food. Keeping this in mind, a supplementary food with probiotics was developed. Different probiotic strains were encapsulated in sodium alginate solution and were formed into beads which were added individually (LS-44 and LS-14) and in combination (LS-44+ LS-14), to study its effect on iron bioaccessibility and were compared with commercially available probiotic strain (DANISCO). The results showed that bioaccessible mineral of supplementary mix was found to be around 50% of total iron. The bioaccessibility of the iron increased to 82% in supplementary mix with probiotic strain LS-44. The combination of probiotics (LS44 + LS14) showed the highest bioavailable iron which was nearly 89%. LS-14 showed 74% bioaccessible iron which was on par with commercial strain (72%). The mix was subjected to accelerated storage study to determine its shelf life. Based on the changes in moisture and free fatty acid content the shelf life of the products was 29.6 weeks at 30 °C and for 16 weeks at 40 °C. Thus, it can be concluded that addition of probiotic cultures to the composite finger millet based supplementary food can increase iron bioaccessibility significantly. The product can be stored for nearly 8 months at 30 °C.