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Shakshi Sharma
Microbiology Research Laboratory, Department of Basic Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Nivedita Sharma
Microbiology Research Laboratory, Department of Basic Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Corresponding Author:
Shakshi Sharma
Microbiology Research Laboratory, Department of Basic Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan (India), Himachal Pradesh, India

Preparation of probiotic enriched functional beverage of Kodo millet (*Paspalum scrobiculatum*) a nutritionally enriched absolute new product for commercialization

Shakshi Sharma and Nivedita Sharma

Abstract
In the present study, probiotic enriched nutritional malt beverage has been prepared from kodo millet grains collected from different districts of Himachal Pradesh, India. Malt beverage was prepared in four sets by adding different combinations of in-house probiotic cultures i.e. Set A with *Pediococcus acidilactici* L1, Set B with *Lactobacillus plantarum* L2, Set C with *Lactobacillus fermentum* F3, Set D with a consortium of *Pediococcusacidi lactici* L1, *Lactobacillus plantarum* L2 and *Lactobacillus fermentum* F3 along with a control (without probiotics). These were then further subjected to nutritional, microbiological and sensorial analysis. The nutritional content in set D containing 53.11% antioxidants, 24.2g proteins, 5.5g fats, 14.5g carbohydrates and 8.3g crude fiber was found to be higher as compared to all other sets. The microbiological evaluation indicated that the lactic acid bacteria were found to be predominant during the 5th day of storage. Sensorial analysis of the Set D showed maximum acceptability on 9 point hedonic scale, thus proving it to be the best in terms of taste, texture and color.

Keywords: Millet, beverage, probiotics, lactic acid bacteria, antioxidants, ready to eat

1. Introduction
Millets are one of the important cereals besides wheat, rice and maize and have been used as staple food for thousands of years by people in Asian and African countries before rice became a common commodity to man. Millets are important foods in most underdeveloped countries because of their ability to grow under adverse weather conditions like limited rainfall (Yang *et al* 2012) [28]. The most important cultivated species of millets are: Proso millet (*Panicum miliaceum*), Foxtail millet (*Setaria italica*), Japanese barnyard millet (*Echinochloa frumentacea*), Finger millet (*Eleusine coracana*) and Kodo millet (*Paspalum scrobiculatum*). Among these, kodo millet (*Paspalum scrobiculatum)* is grown primarily in India, and also in the Philippines, Indonesia, Vietnam, Thailand, and in West Africa where it originated. The kodo millet (Paspalum scrobiculatum) is known by different names such as cow grass, rice grass, ditch millet, Native Paspalum, or Indian Crown Grass. It is a very hardy crop that is drought tolerant and can survive on marginal soils where other crops may not survive, and can supply 450– 900 kg of grain per hectare. The grain varies in color from light red to dark grey and is enclosed in a tough husk that is difficult to remove.
Millets are important food crops in developing countries, and contain major and minor nutrients in remarkable amounts (Sarita and Ekta, 2016) [23]. Millets are far ahead of other cereals in terms of their mineral contents and nutritional value and play a very significant role in human nutrition because of their multiple qualities. They are also used as nutraceuticals as their antioxidant contents are much higher than all major cereal crops. Millets are reportedly beneficial in curbing asthma, migraine, blood pressure, diabetic heart disease, atherosclerosis and heart attack while their high fiber contents prevent gall stone formation. Hence, millets are termed as "nutri-cereals". In addition, because of their important contribution to national food security and potential health benefits of combating various diseases, millet grains are now receiving increased attention from food processors, technologists, and nutritionists (Shahidi and Chandrasekara, 2013) [5]. Kodo millet is very easy to digest; it contains a high amount of lecithin and is excellent for strengthening the nervous system. It contains high amounts of polyphenol, an antioxidant compound, is rich in fiber content and low on fat as compared to other different types of millet. It is however an irony that kodo millets have been the most neglected among all millets, hence there is a need to increase awareness about their superior nutritional quality and make them one of the important commodities of our food basket.

In the current era, there is an urgent need to develop a product that can meet the daily requirements of nutrition by supplementation and is simultaneously beneficial for health.
Traditional fermented foods are receiving extensive scientific attention globally and many traditional preparations have been analyzed for their microbiological, enzymologial and biochemical changes (Omemu *et al*, 2007) [19]. Various millet based traditional preparations are available throughout the world and fermentation of millets is a common practice. Kodo millet can be used for traditional as well as novel foods preparation. It is gaining importance as a gluten free food and is an important component of multigrain gluten free food products. Thus, kodo millet has great potential for being utilized in different food systems by virtue of its nutritional quality and economic importance. The functional properties of foods are further enhanced by processing techniques such as sprouting, malting, and fermentation (Hotz and Gibson 2007) [9]. Commonly consumed traditional millet products include porridge, flat bread, steam cooked couscous and fried products which can be made from native as well as parboiled millet flour.
Functional foods with probiotics are gaining popularity worldwide at rapid scale and these have become extremely popular among consumers recently (Saarela *et al*. 2000) [21]. Lactic acid bacteria (LAB) are the most accepted and safe probiotic microorganism to be used as a functional food and have the best probiotic potential. Beverages are drinks that are consumed for their thirst quenching properties or for their stimulating effect. Generally beverages are prepared from fruits and cereal grains, and can be alcoholic as well as non-alcoholic. Beverages derived from kodo millet have enormous health benefits and can be easily consumed by celiac and diabetic patients as they do not contain gluten and has low-GI (Glycemic index). Keeping in view the high potential of converting the underutilized millet grains into value-added food and beverages, the present study attempts to prepare kodo millet malt beverage for the first time that is enriched with in house potential probiotics with an aim to deliver the probiotic health benefits to the mankind.

2. Materials and Methods

2.1 Collection of samples

Kodo millet grains were collected from different districts i.e. Kangra, Mandi and Hamirpur of Himachal Pradesh, a hilly himalyan state of India and brought to the laboratory. All samples were segregated, cleaned and stored in air tight containers till further use.

2.2 Preparation of nutritionally enriched malt beverage

2.2.1 Malting

Kodo Millet seeds (250 g) were soaked in water for germination at 27 ˚C for 12 h, and the water was changed frequently to prevent excessive growth of microorganisms. The millet seeds were then allowed to germinate at room temperature i.e. 27± 2 ˚C for 48 h. After germination, the seeds were dried at a moderate temperature not exceeding 75 ˚C in an oven, and sprouted grains were later dried to final moisture content of nearly 10-12%. These grains were then roasted uniformly at 70-80 ˚C by using conventional toasting pan and grinded. The malt so obtained was then pulverized to convert it into ready to eat (RTE) form. 1500 ml of distilled water was added to the powdered malt thus obtained and the mixture boiled at 100 ˚C for 20 min to reduce microbial load. The obtained liquid was then filtered through sterilized sieve and then again heated at 100 ˚C for 10 min after adding sugar (3% concentration), later it was allowed to cool at room temperature.

2.2.2 Fortification of malt with probiotics

To enhance the nutritional value of malt beverage, it was fortified with in house potential probiotic strains, *Pediococcus acidilactici* L1, *Lactobacillus plantarum* L2and*Lactobacillus fermentum* F3 vide accession number KM251713, KM251714 and KC242235 at an inoculam size of 10^8 cfu/ml. The different combination of probiotics were added to prepare four sets of malt beverage i.e. Set A *Pediococcus acidilactici* L1 (1.5 ml), Set B *Lactobacillus plantarum*L2 (1.5 ml), Set C *Lactobacillus fermentum* (1.5 ml), Set D *Pediococcus acidilactici* L1, *Lactobacillus plantarum* L2 and *Lactobacillus fermentum* F3 (0.5 ml each), so as to check the quality attributes of malt beverage contributed by addition of probiotic cultures (monoculture/ consortium).The schematic representation of beverage preparation has been shown in Figure 1 and 2.

2.2.3 Evaluation of quality attributes of malt beverage

The variable quality attributes of probiotic enriched malt beverage were determined with the purpose of preparing a healthy drink.

2.2.4 Sensorial evaluation

Nine-point hedonic scale method as given by Amerine, (4) was followed for conducting the sensory evaluation of probiotic food products. The panels of 10 judges were selected to evaluate malt beverage.

2.2.5 Microbial evaluation during storage

The colony count was observed during storage period by standard spread plate method. MRS agar was used to enumerate lactic acid bacteria while nutrient agar, yeast extract agar and PDA were used to enumerate total aerobic mesophilic bacteria including yeast and mold, respectively.

2.3 Nutritional evaluation of malt beverage:

2.3.1 Proteins

Sample of 0.5 – 1 g weight along with 0.5 g digestion mixture (2.5 g SeO_2 + 20 g $CuSO_4.5H_2O$ + 100 g K_2SO_4) was digested in 25 ml concentrated H_2SO_4 for 5h or till it became colourless. Digestion flasks were allowed to cool overnight at room temperature. The digest was transferred to 100 ml capacity volumetric flask and made up to volume with glass distilled water. The nitrogen was estimated by modified Kjeldhal method.

$$\text{Nitrogen (\%)} = \frac{\text{(Sample titre - blank titre) x Normality of HCL x 14 x 100}}{\text{= Weight of sample x 1000}}$$

Crude protein (%) in the sample was then calculated by multiplying percent nitrogen with the factor 6.25 (Ranganna, 1997).

2.3.2 Carbohydrates

The phenol-sulphuric acid method was used to estimate carbohydrates as described by Sadasivam and Manickam. To the diluted sample, 1ml of phenol solution [5% (v/v)] was added and mixed properly in a test tube. Then, 5 ml of 96% (v/v) sulphuric acid was added and the sample was shaken well. The tubes were kept in a water bath at 25-30°C for 20 min; the absorbance was recorded at 490nm and compared

with the standard curve prepared with glucose. The standard curve was prepared using different concentrations i.e. 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of glucose.

2.3.4 Fat content

Dried sample of 5 g was extracted with petroleum ether in Soxhlet extraction apparatus for 6 hr. The ether extract was filtered in pre-weighed beakers. Petroleum ether was evaporated completely from the beakers and the increase in weight of beaker represented the fat content. The fat (%) obtained was estimated as (g fat/ g dry biomass) × 100 (Folch, 1957) [7].

2.3.5 Antioxidants(Free Radical Scavenging Activity, FRSA)

DPPH (2, 2-diphenyl-1-picrylhydrazyl) was used as a source of free radical. A quantity of 3.9 ml of 6×10^{-5} mol/L DPPH in methanol was put into a cuvette with 0.1 ml of sample extract and decrease in absorbance was measured at 515 nm for 30 min or until the absorbance become steady. The remaining DPPH concentration was calculated using the following equation: Free radical scavenging activity (%) = Ab(b)- Ab(s)

Where
Ab(b) = Absorbance of blank
Ab(s) = Absorbance of sample
Antioxidant activity was determined by DPPH using the method described by Brand *et al*. 1995 [4].

2.3.6 Crude fiber

Distilled water (200 ml) was added to the sample (100 g) and the contents were brought nearly to a boil. After adding 25 ml of 50% (w/v) sodium hydroxide solution, the contents were boiled for five minutes. The material was transferred to the previously weighed screen and washed thoroughly with water until sodium hydroxide was completely removed. The presence of sodium hydroxide was checked by using phenolphthalein indicator. The contents were dried at 100 ºC for 2 h in hot air oven and fiber content was expressed in percentage (AOAC, 2007) [2].

$$\text{Fiber content (\%)} = \frac{\text{loss in weight (g)}}{\text{weight of sample (g)}} \times 100$$

2.4 Statistical analysis

Data pertaining to the physicochemical attributes of probiotic product was analyzed by completely randomized design (CRD) factorial while data on sensorial evaluation was analyzed using randomized block design (RBD) as described by Mahony.

2.5 Results and Discussion

Kodo millet grains were collected from different districts i.e. Kangra, Mandi and Hamirpur of Himachal Pradesh, India for the formulation of nutritionally enriched fortified probiotic drink/ beverage. Table 1 shows the nutritional potential i.e. proteins, carbohydrates, crude fibers, antioxidants and total fat contents of raw kodo millet grains which were recorded as 3.80g protein, 54mg/g carbohydrates, 9.7% crude fibers, 44% antioxidants, 0.12g of crude fat and 1.28µg/ml of flavonoids respectively (Sharma et *al.*, 2017) [25]. The nutritional value of kodo millets was found to be higher than that of popular cereal grains like rice, wheat, barley which makes kodo millet highly desirable for the preparation of nutraceutically enriched food products. Haldimani et al. 1995 reported that millets are rich source of nutrients and contain 60–70% dietary carbohydrates, 6–19% protein, 1.5–5% fat, 12–20% dietary fibre, 2–4% minerals, and several other phytochemicals. Highest phenolic content has been reported for kodo (368 mg/g) followed by finger (brown variety), little, foxtail and barnyard millet. Kodo Millet also offers several health benefits to consumers. These crops lack gluten and hence can be consumed by people suffering from celiac disease. Millet consumption could lower the glycemic response, which is helpful for the treatment of type II diabetes. Inclusion of millet in the human diet can also lower the risk of duodenal ulcers, anemia and constipation.

2.6 Preparation of nutritionally enriched fermented malt beverage

The nutritional content of kodo millet grains showed an increase after malting. The antioxidant content of malted grains was found to be 48% while that of raw grains was 45%. The malting process is responsible for changes in the composition of barley and malt grains as it causes modifications and degradation of endogenous phenolic compounds which has a great impact on the overall antioxidant capacity of malt. The antioxidant activity of barley grains was found to be significantly higher after roasting (16.8% to 108.2%) (Sharma and Gujral, 2011) [24]. The protein content of malted grains also increased (9.5 g) leading to high protein levels in the endosperm, starch/protein compacting, limiting endosperm hydration and enzyme modification during the malting process (Yuhong *et al*. 2008) [10]. Similarly, the total fat content of kodo millet was found to be 5.3g in malted as compared to 1.20g in raw millet. However, the carbohydrate content (12.11g) showed decrease after malting, while the crude fiber content (7.7 g) increased. An evaluation and comparision of nutritive values of the prepared sets of malt beverages per 100gm with control (no added probiotic culture) has been presented in Table 1 and Figure 3. Chemical analysis of the malt beverages showed that set D (24.20 g) had the highest protein content followed by set A (23.5 g), set C (23 g) and set B (20) while the control showed the lowest amount of protein (19.50 g). Table 1 shows that the antioxidant in the set D i.e. 53.11% was significantly higher than the other sets of beverages prepared i.e. set C with 52.34% of antioxidant and control with 48.00%. Similarly set D contains significantly higher amount of crude fibers (8.3 g) than set B (8.20) followed by lower crude fiber contents in set A, set C and control. Among all the sets of beverages prepared, set D showed the maximum levels of nutritional components i.e. antioxidants, protein, total fat, carbohydrate content and crude fiber as compared to the other sets of beverages prepared (Fig. 4). The result of the experiment indicates that the set D contains consortium of potential probiotic strains contributing to the nutritional quality enhancement of malt beverage as compared to the other prepared sets A, B and C having monoculture of probiotic strain

Malting is an important process which is used to enhance the nutritional/ organoleptic status of kodo millet. Malting induces positive effect on the grains as the main objective of malting is to promote the development of hydrolytic enzymes, which are not present in non-germinated grain. Fermentation and germination reduces anti-nutrients in millet, improves nutrient bio-availability, and can synthesize certain amino acids and increase the availability of vitamins. The protein content in whole grains of minor millets varied from 4.76% in

Finger millet to 13.10% in Foxtail millet, which were followed by Little millet, Kodo millet and Finger millet (Scientific correspondence, 2014). Kodo millet has the highest free radical (DPPH) quenching activity followed by great millet (sorghum) and finger millet (Hegde and Chandra, 2005). Kodo millet and little millet are also reported to have 37% to 38% of dietary fiber, which is the highest among the cereals (Malleshi and Hadimani 1993; Antony *et al*. 1996).

Table 1: Nutritional chart of malt beverage

Nutritional evaluation of kodo millet	Raw kodo millet	Control Malted kodo millet (without adding probiotics)	Malted beverage with adding probiotics				CD
			Set A*	Set B**	SetC ***	Set D****	
Antioxidants (%)	45.0	48.0	51.28	50.54	52.34	53.11	0.81
Proteins (g)	3.80	9.5	23.5	20	23	24.2	1.16
Total Fat (g)	1.20	5.3	5.3	4.8	4.6	5.5	0.16
Carbohydrate (g)	55.0	13.9	12.11	12.15	14.12	14.15	0.08
Crude fibers (g)	6.80	7.5	7.7	8.2	7.9	8.3	0.18
CD0.05	1.35	0.94	0.43	0.89	0.87	0.24	

Control: without inoculam
Set A*: P. acidilacticiL1
Set B**: L. plantarum L2
Set C***: L. fermentumF3
Set D****: P. acidilacticiL1 + L. plantarum L2+ L. fermentumF3

2.7 Microbiological evaluation

The different sets of fermented malt beverages were stored for a period of upto 5 days to check the storage stability of the prepared drink. The microbial count in the prepared malt beverage was determined on the 1th, 3rd and 5th day of fermentation by potential probiotic bacterial strains. The data regarding viable colonies of different microbes i.e. bacteria, LAB, yeast and mold were expressed in terms of log cfu/ ml. An increase in number of bacterial cells during storage conditions was observed in each treatment. The data obtained from analysis of the samples were evaluated by variance of analysis. On the 1st day, the LAB count in the varied sets of prepared malt beverages was found as: Set A (10.20), Set B (10.05), Set C (10.18) and Set D (10.20) in terms of log cfu/ml as compared to control where no growth was observed. On the 3rd day of storage, growth was observed for LAB, yeast and other bacteria except mold in the control, the LAB count for Set D (13.80) was maximum as compared to control and other sets (A, B and C). Viable count was found to be maximum for set A, B, C and D on the 5th day with log cfu/ml 14.80 for set A, 16.50 for set B, 12.85 for set C and 16.50 in Set D as compared to control (4.50). The mesophilic bacteria, yeast and mold were observed to be below the detection limit as shown in Fig. 5, whereas lactic acid bacteria were found to be the predominant microflora of the malt beverages. The latter inhibit food borne and pathogenic bacteria that causes several diseases. Statistically, it had been confirmed that no significant change occurred in viability of microbial cells during storage and set D contained the highest number of beneficial LAB as compared to control and other prepared sets i.e. A, B and C. Fermented beverages were also observed to have increased shelf life without the need of adding any type of preservatives due to the production of much acid and alcohol, which can act as natural preservatives. Ingestion of LAB has been suggested to confer a range of health benefits including immune system modulation (Bielecka *et al.* 2002; Tannock, 2001) [3, 27], increased resistance to malignancy and infectious illness (Krasaekoopt *et al.* 2003) [12], besides leading to enhancement in the nutritional content of the prepared product. Lango and Antony (2014) studied the microbial count of "koozh", a fermented beverage made from millet flour and rice and found that the prepared beverage was dominated by lactic acid bacteria which was instrumental in repressing the broad spectrum of pathogens. Besides the high quality of polyphenols also contributed strongly to antimicrobial activity and imparted shelf stability to its products.

2.8 Sensorial evaluation

Since kodo millet malt beverage is gluten free, it can be safely consumed by celiac patients. The non starchy polysaccharides of the millet form bulk of its dietary fiber constituents and offer several health benefits including delayed nutrients absorption, increased faecal bulk and lowering blood lipids (Sharma *et al.*, 2017) [25]. Malting and fermentation of cereals not only improve the sensory quality but also the nutritional quality of the end products (Kazanas and Fields 1981; Chavan *et al.* 1988) [11, 6]. Freshly prepared malt beverage samples were assessed by 10 panelist using a 9 point sensory hedonic scale for some sensory parameters (*viz*. appearance/colour, flavour, texture, taste and overall acceptability), as described by Amerine. In a sensory evaluation, malt beverage set A was least accepted whereas malt beverage set D had a maximum acceptability as it scored 7.97 out of 10, which showed that this prepared set had a better taste, flavor and texture
(Fig.6). Statistically sensorial evaluation was carried out by Randomized Block Design (RBD). The result showed significantly higher acceptability effect of set D based on different treatments on sensory attributes of malt beverage. The results of above experiment also proved that the probiotic bacterial strain had a significant influence on the overall acceptability of the product.

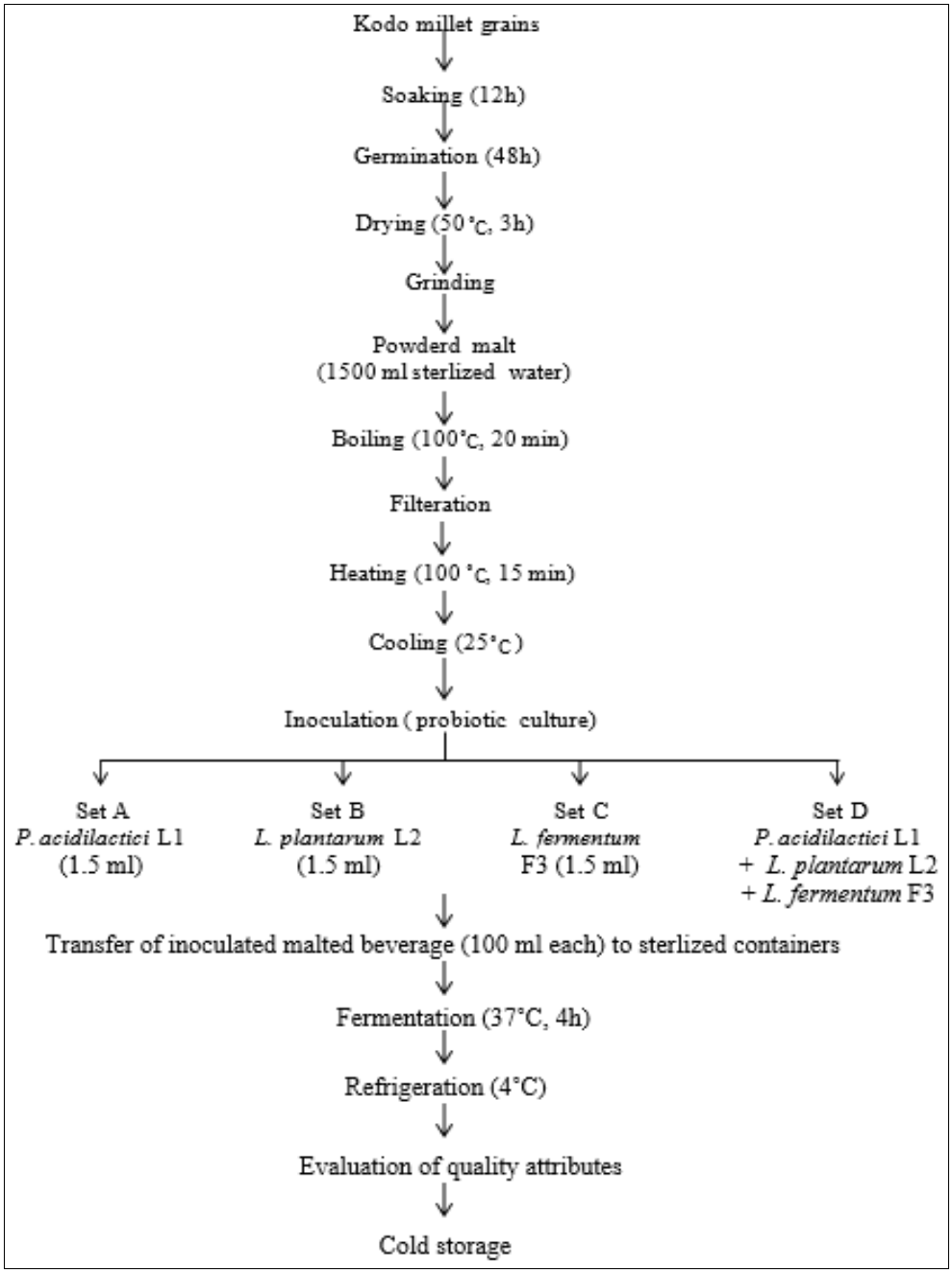


Fig 1: Preparation of malt beverage

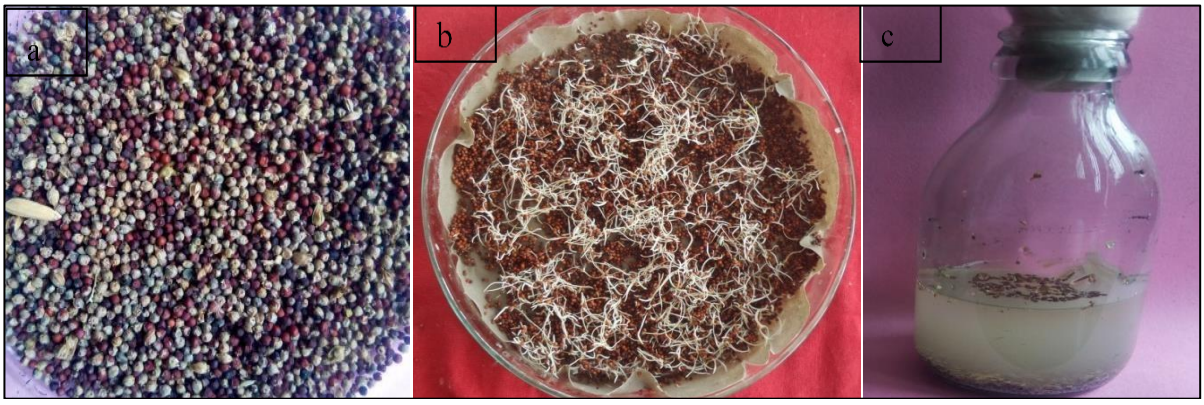


Fig 2: Preparation of kodo millet malt beverage (a) Kodo millet grains. (b) Germination of millet grains. (c) Filterate of malt beverage

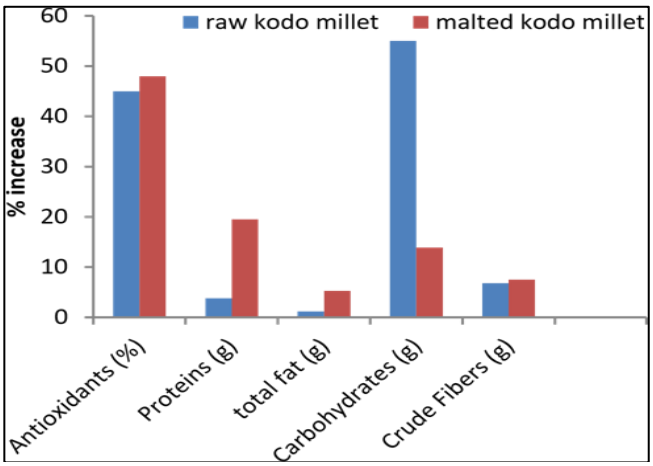


Fig 3: % change in nutritional content of malted kodo millet as compared to the raw millet

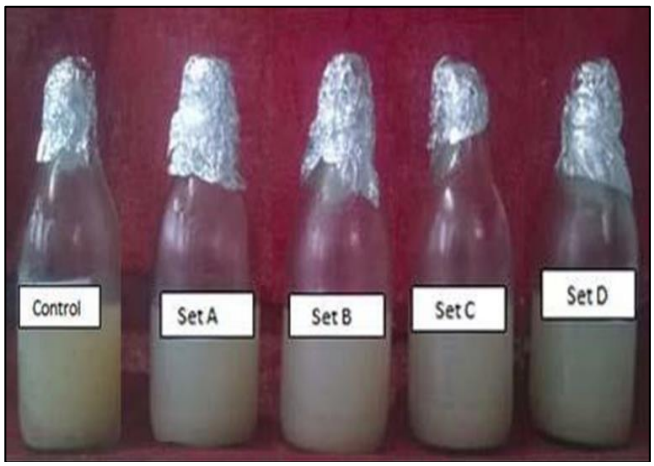
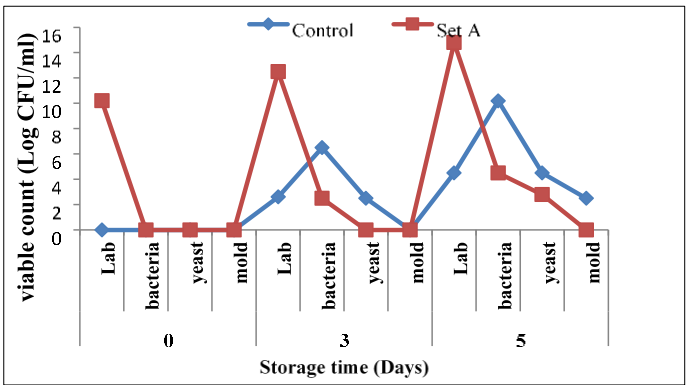
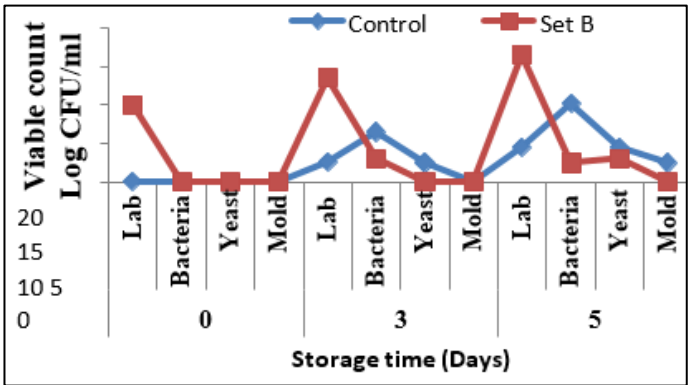


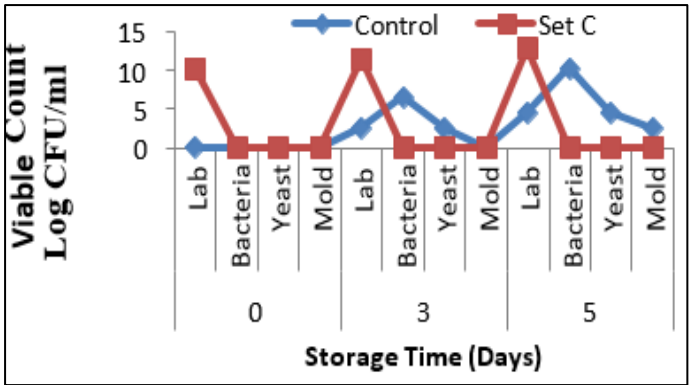
Fig 4: Probiotic enriched malt beverage



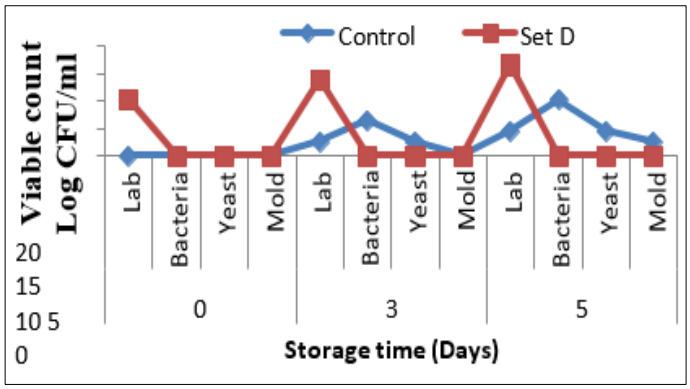
(a)



(b)

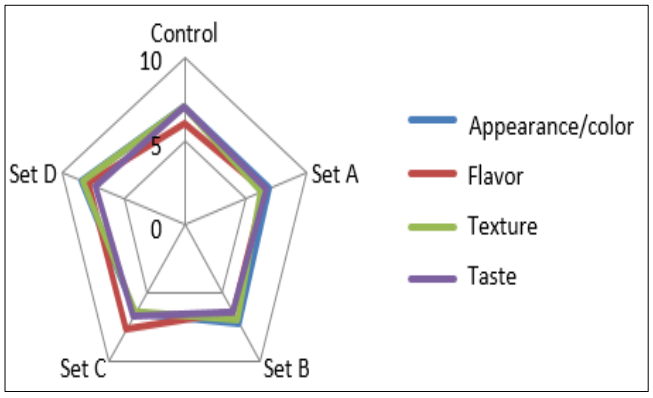


(c)



(d)

Fig 5: Total viable count of malt beverage:



(a) Set A (b) Set B (c) Set C (d) Set D along with Control (without probiotics)

Fig 6: Sensorial evaluation of malt beverage

Conclusions

In the present study, a new probiotic enriched malt beverage has been prepared using kodo millet grains with high nutritional content of antioxidants 53.11%, proteins 24.2g, total fat 5.5 g, carbohydrates 14.15 g and crude fiber 8.3. The procedure involved fermentation of consortium of potential probiotics i.e. *Pediococcus acidilactici* L1, *Lactobacillus plantarum* L2 and *Lactobacillus fermentum* F3. The addition of probiotics to the malt beverage provided longer shelf life to the malt beverage. The sensorial analysis indicated that the malt beverage was good in taste and texture and thus is strongly recommended for human consumption due to the health benefits it offers. The novel functional malt beverage has been assessed with high nutritional value along with other exceptional health benefits and probiotic viability which fulfill the main objectives of the present study.

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