Research Article

Development of a composite food using dephytinized grains through probiotic fermentation

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ABSTRACT

Introduction and Aim: Over three billion people are malnourished globally, due to inconsistent supply of foods with essential nutrients due to presence of antinutrients in most plant foods. Phytic acid, an antinutrient in plant products binds to proteins and minerals, making them unavailable for absorption and leading to malnourishment. The present investigation aimed at screening of phytase producing probiotic strains that can reduce phytic acid content from commonly used cereals and pulses through fermentation and to develop a composite food.

Methods and Materials: Probiotic strains were isolated from probiotic capsule, curd, fruits like apple, orange, tomato, grape, pomegranate and chikoo. The isolates were then screened for production of phytase on MRS medium containing calcium phytate along with a positive strain *Lactobacillus plantarum*.

Results: Out of 13 strains only 2 strains were found positive for phytase (isolate 6 and 7). Preliminary biochemical studies indicated that the isolates are *Lactobacillus* spp. There was a considerable reduction in phytic acid content in rice, wheat, ragi, barley, green gram, horse gram, chickpea and soybean after fermentation. *Lactobacillus plantarum* reduced phytic acid (16.1- 90.02%) in all cereals and pulses used. However, isolate 6 could reduce 91.3% phytic acid in wheat which was significantly more than the positive control strain. A functional food was formulated using fermented cereals and pulses in the ratio 3:1 respectively.

Conclusion: Both *Lactobacillus plantarum* and isolate 6 showed significant results in reducing phytic acid. However, their efficacy needs to be confirmed through *in-vivo* studies and also sensory evaluation for superior nutritional quality and safety of the formulation.

Keywords: Phytic acid; cereals and pulses; phytase; *Lactobacillus*; composite food

INTRODUCTION

The use of plants to meet the world's food needs is vital to human survival. The grains contain 15-40% protein, sufficient amount of carbohydrates, high amounts of phosphorous, iron, calcium and are also rich in vitamins (A, B, C, D). Since pulses contain high amounts of amino acids, they are consumed in developing countries together with low-protein and high calorie foods that are staple diets of those areas instead of animal proteins, which are both expensive and rare (1). Seeds of legumes and other plant sources in their raw state contain wide varieties of antinutrients which are potentially toxic. They include, toxic amino acids, saponins, cyanogenic glycosides, tannins, phytic acid, gossypol, oxalates, goitrogens, lectins (phytohemagglutinins), chlorogenic acid and inhibitors of protease and amylase (2).

Phytic acid (myo-inositol hexakis phosphate) or phytate as a salt occurs naturally throughout the plant kingdom and is the storage form of phosphorus in all grains and oil seeds (3). It accounts for 50-80% of the total phosphorus in different cereals. About 62-73% and 46-73% of the total phosphorus within cereal grains and legume seeds are in the form of organically bound phytin phosphorus, respectively (4). Phytic acid accumulates in storage sites of seeds

and apparently chelates other minerals forming complex phytate salt (5). The phosphorus bound within phytic acid is mostly unavailable to monogastric animals due to lack of phytase, an enzyme that hydrolyses phytic acid (2). Phytic acid acts as a strong chelator, forming protein and mineral-phytic acid complex, the net result of which is reduced protein and mineral bioavailability (6). Phytic acid is reported to chelate metal ions such as calcium, magnesium, zinc, copper, iron and molybdenum to form insoluble complexes that are not readily absorbed from gastrointestinal tract. Phytic acid also inhibits the action of gastrointestinal tyrosinase, trypsin, pepsin, lipase and amylase (6).

Phytic acid in cereal foods can be degraded completely by adding exogenous phytase, an enzyme that successively removes phosphate groups from phytic acid until it no longer binds iron (7) or by activating the native phytases by a combination of processes such as soaking, germinating, and fermenting (8). Phytases from fungi have been extensively used as animal feed to improve the bioavailability of minerals that are otherwise chelated by phytic acid (9). Fermentation is also widely used to improve the nutritional and functional qualities of food products (10) by way of reducing phytic acid from cereals (11).

Probiotics are reported to render potential health benefits in humans and animals due to their interaction with gastrointestinal tract. Hence, they are widely used to prepare fermented foods (12).

The present work is aimed at screening of phytase producing probiotic strains for the reduction of phytic acid extracted from commonly used cereals and pulses and to develop a composite food through probiotic fermentation to combat malnourishment and iron- deficiency disorders.

MATERIALS AND METHODS

All the chemicals and reagents used in this investigation were of analytical grade and all the standards were obtained from Sigma USA. *Lactobacillus plantarum* strain was obtained from Microbiology Department of CFTRI, Mysuru and the culture was maintained at 4°C in the refrigerator.

Cereals and pulses

Rice (*Oryza sativa*), wheat (*Triticum vulgare*), ragi (*Eleusine coracana*), barley (*Hordeum vulgare*), green gram (*Vigna radiata*), horse gram (*Macrotyloma uniflorum*), chick pea (*Cicer arietinum*) and soybean (*Glycine max*) were collected from a local grocery shop at Kushalnagar, Kodagu District, Karnataka, India.

Probiotic capsule (Sporlac DS) available in the pharmacy, curd (dairy), fruits like apple (*Malus spp*), orange (*Citrus reticulata*), tomato (*Solanum lycopersicum*), grape (*Vitis vinifera*), pomegranate (*Punica granatum*), and chikoo (*Manilkara zapota*) were used for isolation of probiotic strains through standard serial dilution method. After serially diluting all the raw materials, appropriate dilutions were plated on Mans Rogosa and Sharpe (MRS) medium and the plates were incubated at 37°C for 24-36hrs. The developed colonies were isolated and characterized further.

Qualitative screening of probiotics for phytase production

About 13 probiotic bacterial isolates along with known *Lactobacillus plantarum* (13) were screened for phytase production by inoculating on to modified MRS medium supplemented with 0.5% calcium phytate and the plates were incubated at 37°C for 24-

48 hrs. The strains that produced zone of hydrolysis around the colonies were selected as positive cultures for phytase production.

Characterization of phytase producing cultures

The positive strains were microbiologically characterized through simple staining, Gram's staining and colony morphological studies. Biochemical characterization was also carried out for the positive strains. The tests include indole test, methyl red test, voges- Proskauer test, Citrate utilization test, Catalase test and Sugar fermentation test.

Submerged fermentation for phytase production by probiotics

The phytase positive strains were cultivated in MRS broth in two sets that is one set with and the other without 0.5% calcium phytate to check for constitutive or inductive nature of the enzyme. About 25 ml aliquots of MRS broth were inoculated with overnight cultures of *Lactobacillus plantarum* and other phytase producing isolates. The flasks were incubated for 72 hrs at 37°C and 150 rpm in a shaker incubator. The culture broth was filtered through Whatman No 1. filter paper and the filtrate were used as crude phytase preparation for further quantitative assay. Phytase enzyme activity was determined by the method of Heinonen and Lahti (14). Soluble protein in the phytase enzyme sample was estimated by the method of Lowry *et al.*, (15). One Unit is the activity of the enzyme to release 1 µM of inorganic phosphorus from the substrate in 1 min at 40°C.

Extraction and estimation of phytic acid from cereals and pulses

The cereals and pulses such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), ragi (*Eleusine coracana*), barley (*Hordeum vulgare*), horse gram (*Macrotyloma uniflorum*), cowpea (*Vigna unguiculata*), green gram (*Vigna radiata*) and soybean (*Glycine max*) were selected and their phytic acid was extracted. The extraction and spectrophotometric estimation of phytic acid was carried out by the method of Gao *et al.*, (2007) (16).

Calculation:

$$\text{Phytic acid (mg/100g)} = \frac{\text{Sample conc.} \times \text{Initial dilution (10 ml)} \times \text{Final dilution (25ml)}}{\text{Weight of the sample (0.5g)} \times \text{Aliquot taken for further analysis (3ml)}}$$

Fermentation of cereals and pulses

15 g of coarsely ground cereals and pulses were fermented in 100 ml of MRS broth inoculated with 1% inoculum of 18 hrs old hyper phytase producers. The flasks were incubated at 37°C for 72 hrs in an incubator shaker at 200 rpm. Then the samples were centrifuged at 8000 rpm at 4°C for 10 minutes to

collect the pellets. The pellets were then washed with distilled water and centrifuged again with water at 8000 rpm and 4°C for 10 minutes. The pellets were dried in an oven at 50°C overnight and were finely ground using pestle and motor and estimated for phytic acid content.

Development of composite food

All the tested cereals and pulses were finely pulverized using pestle and mortar followed by mixing of equal amounts of all the cereals and equal amounts of all the pulses. A composite food was then formulated using fermented cereals and pulses in the ratio of 3:1 of cereals and pulses respectively. Jaggery and cardamom were also added to the final product as sweetener and flavouring agents respectively.

Statistical analysis

Data are presented as the standard error means ($\pm$ SEM;17). Values were considered significant at $P < 0.05$ and are indicated in the tables as English alphabets.

RESULTS AND DISCUSSION

Isolation of probiotics

Probiotics were isolated from probiotic capsule, curd and also from fruits such as apple (*Malus domestica*), grape (*Vitis vinifera*), pomegranate (*Punica granatum*), orange (*Citrus sinensis*), tomato (*Solanum lycopersicum*) and chikoo (*Manilkara zapota*). Thirteen isolates were obtained on MRS agar medium and their colony morphology is given in table 1.

Table 1: Isolation of probiotics from various sources

Isolate	Source	Colony Morphology
1	Capsule	White, small, raised, moist colony
2	Capsule	White, small, flat, dry colony
3	Capsule	White, large, raised, moist colony
4	Capsule	White, small, flat colony
5	Capsule	White, medium sized, raised, moist colony
6	Curd	White, small, raised, moist colonies
7	Curd	White, small, flat, moist colony
8	Curd	Cream coloured, small, raised, moist colonies
9	Grape	Cream coloured, medium sized, raised colony
10	Pomegranate	Cream coloured, flat colony
11	Pomegranate	White coloured, small colony
12	Orange	White coloured, raised, moist colony
13	Tomato	White coloured, small colony

Qualitative screening of probiotics for phytase production

All the isolates were qualitatively screened for phytase enzyme production by point inoculation method on modified MRS medium containing 0.5% calcium phytate along with positive control

Lactobacillus plantarum. Isolates 6 and 7 were found positive for phytase production [Fig. 1]. Both the isolates were found in curd sample. Isolate 6 had white, small, raised, moist colonies, while isolate 7 had white, small, flat, moist colonies. The diameter of the zone of inhibition is given in table 2.

Fig. 1: Qualitative screening of probiotics on MRS agar medium containing calcium phytate. **A.** *Lactobacillus plantarum*, **B.** Isolate 6 and **C.** Isolate 7

Table 2: Zone of hydrolysis produced by phytase producers on MRS agar with calcium phytate

Organism/Isolate	Zone of hydrolysis (mm)
<i>Lactobacillus plantarum</i>	4.0
Isolate 6	2.5

Isolate7

2.0

Characterization of probiotics

Microbiological characterization

All 3 phytase positive bacterial isolates such as *Lactobacillus plantarum*, isolate 6 and Isolate 7 were subjected to simple and Gram's staining procedures in order to determine their shape morphology and

Gram's nature. The result showed that all three strains were coccobacilli. Preliminary identification, morphological characterization such as positive growth on MRS agar medium, colony colour and characteristics, positive gram tests suggest that the strains were lactic acid bacteria (18).

Fig. 2: Formulation prepared using fermented cereals and pulses. **A.** *Lactobacillus plantarum*. **B.** Isolate 6

Biochemical characterization

The results obtained through biochemical characterization indicated that the isolates were catalase negative and IMViC test negative (table 3), there by these isolates were confirmed as

Lactobacillus spp (19). All three strains were able to ferment glucose, sucrose and lactose, producing acids. The results obtained in the present investigation were found similar to the findings of Chowdhury *et al.*, (20).

Table 3: Biochemical characterization of the isolates

Biochemical Tests	<i>Lactobacillus plantarum</i>	Isolate 6	Isolate 7
Indole test	-	-	-
Methyl red test	-	-	-
VP test	-	-	-
Citrate Utilization test	-	-	-
Catalase test	-	-	-
Sugar fermentation test	+	+	+

Phytase Activity

Phytase activity of the two strains was not significantly different with and without calcium phytate. Quantitative estimation showed lower phytase activity in isolate 7, however in qualitative test it showed larger zone of inhibition that may be due to other nonspecific enzymes such as acid phosphatases. Hence for all further studies only isolate 6 was used along with positive strain *Lactobacillus plantarum*.

Phytic acid in cereals and pulses

Phytic acid content in cereals and pulses were estimated before and after fermentation. There was a significant reduction in phytic acid content after fermentation using the two strains. The results are given in table 4. In the present study, phytate content

in rice was reduced to 55.67%, which is found to be significant than other traditional processing like soaking and germination. It was found that soaking and germination reduced phytic acid upto 42- 59% and 4- 40% respectively (21). In the present investigation phytic acid content in wheat got reduced upto 91.3% after probiotic fermentation. It was reported earlier that in roasted wheat 51.8% reduction and in germinated seed 31% reduction was observed (11). During fermentation phytic acid in ragi was reduced upto 76.84%. However, a reduction in phytic acid upto 61.5 and 49.2% in roasted and germinated ragi respectively was also reported (11). In barley 62.48% of phytic acid was reduced whereas, in soaked barley it was found to be 58% (21).

Table 4: Phytic acid content in cereals and pulses before and after fermentation

Cereals/ Pulses	Phytic acid in raw sample (mg/100g)	Phytic acid after fermentation with <i>L. plantarum</i>	% reduced after fermentation with <i>L. plantarum</i>	Phytic acid after fermentation with Isolate 6	% reduced after fermentation with Isolate 6
Rice	6.0 ± 0.02 ^a	2.66 ± 0.03 ^b	55.67	4.33 ± 0.02 ^a	27.84
Wheat	23.33 ± 0.05 ^a	2.03 ± 0.02 ^b	91.30	1.0 ± 0.004 ^b	95.72
Ragi	27.68 ± 0.02 ^a	6.33 ± 0.02 ^b	77.14	9.0 ± 0.02 ^b	67.50
Barley	5.33 ± 0.03 ^a	2.0 ± 0.02 ^b	62.48	2.33 ± 0.01 ^b	56.30
Green gram	31.67 ± 0.03 ^a	3.67 ± 0.03 ^b	88.42	3.67 ± 0.008 ^b	88.42
Horse gram	39.33 ± 0.06 ^c	33.0 ± 0.04 ^a	16.10	31.33 ± 0.05 ^a	20.35
Cow pea	16.33 ± 0.03 ^a	1.53 ± 0.003 ^b	90.64	4.0 ± 0.02 ^b	75.51
Soybean	42.0 ± 0.02 ^a	16.67 ± 0.02 ^b	60.31	22.43 ± 0.03 ^b	46.60

The letters 'a' and 'b' indicates significant reduction in phytic acid content in each of the cereals and pulses after fermentation using *Lactobacillus plantarum* and Isolate 6

During fermentation phytic acid in green gram was reduced upto 88.42%. However, a reduction of 35.2 and 33.4% in pressure cooked and germinated green gram respectively has been reported (22). In the present study, fermentation reduced phytic acid content in horse gram upto 20.35%. But, in sprouted horse gram only 18% phytate was reduced (23). A significant reduction in phytic acid content was observed in soybean (60.31%). However, phytic acid content in soybean could be reduced by soaking and cooking only upto 39 and 28% respectively (24). A formulation was made using fermented cereals and pulses with lowered phytic acid content however efficacy needs further validation through *in-vivo* studies.

CONCLUSION

Phytic acid is one of the predominant antinutrients found in almost all cereals and pulses. It is very clear from earlier studies that phytic acid could be eliminated through various processing techniques and by using GRAS microorganisms producing phytase enzyme. The results obtained in the present investigation also strengthens the earlier findings with *Lactobacillus plantarum* reducing phytic acid content considerably in all the tested cereals and pulses. However, isolate 6 could reduce 91.3% phytic acid in wheat which was more than that of *Lactobacillus plantarum*. If this isolate could even reduce gluten content in wheat it could be a wonder strain in food industry. This needs to be confirmed. Phytate- degrading activity in *Lactobacillus* species might improve the nutritional quality of fermented grains that enhance the functionality of food products. Thus, fermented cereals and pulses could be employed in the formulation of a nutritionally superior composite food to combat malnutrition.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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