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Original Research

A Study of Trans-Himalayan Fermented Flour 'Tattakhar': A Disappearing Immunity-boosting Superfood

Abstract

Aesculus indica tree (Hippocastanaceae), known as Himalayan chestnut – and locally as 'Khanor' – grows abundantly in the Trans-Himalayan region of India. The fruits of this tree contain high medicinal compounds like aescin and flavonoids, which possess many health benefits including anti-inflammatory and anti-cancer properties. This study investigated the nutritional value of fermented flour made from the seeds of *Aesculus indica* tree – tattakhar. Results showed that not only is it rich in nutrients, especially minerals, but it also has a high therapeutic potential. This is due to the presence of fermenting activities and probiotic bacteria – which have been globally proven as immunity enhancers – thereby giving tattakhar status as a functional food. The objective of the present study was to highlight the health potential and nutritional quality of tattakhar with the aim of repopularizing it among consumers and giving it superfood status. The flour was prepared using the seeds of *Aesculus indica*. A nutritional analysis, evaluating total carbohydrates, total proteins, reducing sugars, moisture content, crude fibres, total soluble sugars and pH was performed. Lactic acid bacteria were isolated from the fermented flour and safety was evaluated by observing their DNase production, gelatinase production and haemolytic activity. The potential of strain *Bacillus sp.* TK3 was assessed for antimicrobial activity, antioxidant analysis and bacteriocin production. The bacteriocin produced by isolated lactic acid bacteria proved to be a good source to inhibit food borne pathogenicity. A scaling up and adaption of fermentation methodology, coupled with commercial promotion, would afford tattakhar the potential to become one of the most sought-after health promoting and immunity-boosting foods commercially available.

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Introduction

The Himalayan horse chestnut tree (*Aesculus indica*) – locally known as 'Khanor' – is an indigenous plant with many nutritional and medicinal properties. It grows toa height of about 30 meters and a spread of about 12 meters and is widespread in the Himalayan lowlands, particularly between the areas of Kashmir, Uttarakhand and Himachal Pradesh of North-western Himalayan region [1]. In Himachal Pradesh, it is present in abundance in the Chuwar valley of Mandi district, situated between 31°13'50" and 32°04'30" North latitude and between 76°37'20" and 77°23'15" East longitude at a height of 3,300 meters above sea level [2].

Overall, this tree bears a very high medicinal value. Parts of the tree have long been used in traditional Indian medicine for the treatment of some skin diseases, rheumatism, relieving headaches and as an astringent [3]. The seeds and fruits of the tree have been reported in literature for their activity against P-388 lymphocytic leukaemia, nasopharyngeal carcinoma and stomach ailments [4]. The fruits of the plant are rich in minerals and the oil content of the seeds has been reported to be 2.02% [5].

In Himalayan hillocks of Himachal Pradesh, a unique practice is observed with the fruits of horse chestnut – they are washed, dried, crushed and then fermented traditionally to make a nutritional flour known as 'tattakhar', which has high medicinal value [6]. Fruits are crushed into smaller pieces and washed repeatedly in a bamboo basket, which facilitates the removal of bitter saponins. The crushed pieces are then left to ferment naturally for 5–7 days. The crushed and fermented seeds are dried, cleaned and stored in gunny bags before being ground in at the local water flour mill (gharatt) to prepare fermented flour. This fermented flour is rich in nutrients and is used for making many rich delicacies [7]. Fermented products are considered beneficial for health due to the presence of fermenting microorganisms, which are mostly probiotics. Probiotics have become extremely popular worldwide due to their immense health benefits including improved gut health and enhanced immunity.

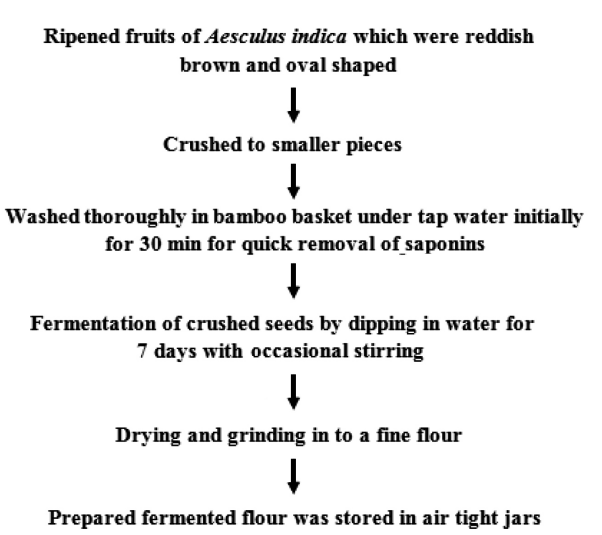
The horse chestnut fruit itself is shown to have high medicinal and nutritional value, therefore tattakhar flour is also rich in these properties. Until now, the role of fermentation in the nutritional and microbiological profile of this rare traditional food, tattakhar, has not been studied. The present study was therefore undertaken with the objective to determine the nutritional value and microbiological profile of tattakhar flour and to repopularize this immunity boosting superfood among consumers before it disappears.

Materials and methods

Collection of *Aesculus indica* fruits

Himalayan horse chestnut (*Aesculus indica*) ripened fruits were collected from different districts of Himachal Pradesh and brought to the laboratory. All samples were segregated, cleaned and stored in airtight containers for use in the preparation of fermented tattakhar flour.

Figure 1 Methodology for the preparation of fermented flour of tattakhar



Nutritional analysis

Total carbohydrates

Carbohydrate content was determined using the phenol-sulphuric acid method. One mL of phenol was added to the sample extract followed by the addition of 5 mL of 96% sulphuric acid. After 10 minutes the contents were shaken well and the tubes were placed in a water bath at 25–30°C for 20 minutes. Total carbohydrates were estimated at 490 nm [8].

Total proteins

Total proteins were estimated using Lowry's method after extracting proteins in 5 mL of extraction buffer. After extraction, each sample was centrifuged and the supernatant was used for protein estimation [9].

Reducing sugars

Reducing sugar estimation was done using the Dinitrosalicylic acid (DNS) method. One mL of flour extract was added to the test tube and equalized the volume to 3 mL with water. To this solution, 3 mL of DNS reagent was added, and the solution was heated for five minutes after which 1 mL of 40% Rochelle salt solution was added. Reducing sugars were estimated at 540 nm [10].

Moisture content

The moisture content of tattakhar was determined by the standard AOAC method. Briefly, 5 g of fermented flour was dried in an air circulating oven at 105°C for 72 hours to constant weight [11].

Crude fibres

Fibres were determined according to the Maynard (1970) method where the dried sample was made fat free, followed by successive digestion with acid and base and the crude fibre was obtained as a residue after digestion [12].

Total soluble sugars

Total Soluble Sugars (TSS) were determined by the standard AOAC method using a hand refractometer (Erma model). Five grams of tattakhar were mixed in 5 mL of distilled water to make a suspension and were observed using a hand refractometer [11].

pH

The pH of the flour was measured by the standard AOAC method using a pH meter (Cyberscan, 510). Briefly, 10 g of tattakhar was suspended in 50 mL of distilled water and allowed to stand for 30 min followed by measurement of pH of the suspension [11].

Isolation of lactic acid bacteria from tattakhar

Predominant aerobic and anaerobic microflora in tattakhar were isolated using the two-fold serial dilution technique, followed by spread plate method. After preparing two-fold serial dilution, the appropriate dilution was plated on nutrient agar and MRS agar medium and were incubated for 24 hours under anaerobic conditions using anaerobic gas packs. After incubation at 37°C for 24 hours, several colonies that had developed on the surface of plates were enumerated. The colonies were subjected to gram staining and catalase reaction. The isolates were then subjected to biochemical fingerprinting, i.e. carbohydrate fermentation, methyl red test, Voges-Proskauer test and citrate utilization test and were tentatively identified.

Microbial profile

The colony count of isolated lactic acid bacteria (LAB), i.e. T1, T2, T3, T4 and TK3 was observed by the standard spread plate count method. Stock was made by adding 1 mL of sample in 9 mL of distilled water and serially diluted by serial dilution range of 10-1 to 10-9. A 1mL sample from each dilution was mounted by spread plate method on sterilized petri plates containing solidified MRS agar medium to enumerate LAB [13].

Evaluation of safety assessments

DNase production

Isolates *Lactobacillus* T1 sp., *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactococcus sp.* T4 and *Bacillus sp.* TK3 were spot inoculated on the DNase agar medium plates and incubated at 37oC for 24 hours [14].

Gelatinase production

Gelatinase enzyme production was determined by inoculating 20 µl of 12-hour-old cultures on plates containing MRS agar supplemented with 3% gelatin. The plates were incubated at 37°C for 24 hours and then flooded with saturated ammonium sulphate solution [15].

Haemolytic activity

Haemolytic activity was determined by spot inoculating fresh overnight bacterial cultures on sheep blood agar plates supplemented with 5% human blood and incubated at 37°C for 24 hours [16].

Antimicrobial activity

The antimicrobial activity of five LAB isolates, i.e., *Lactobacillus* T1 sp., *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactococcus sp.* T4 and *Bacillus sp.* TK3 was analysed using the spot-on-lawn method [17]. Serious food-borne illness/spoilage causing bacteria, i.e., *Listeria monocytogenes*, *Clostridium perfringens*, *Staphylococcus aureus*, *Bacillus cereus*, *Leuconostoc mesenteroides*, *Escherichia coli*, *Enterococcus faecalis* and *Salmonella typhimurium* were used as test strains to assess antagonistic potential of isolates.

Antioxidant analysis

Flavonoid content was determined by the aluminium chloride colorimetric method [18]. Total flavonoids in the test sample were expressed as mg quercetin equivalent/g of sample. The antioxidant potential of the sample was estimated by DPPH free radical scavenging assay [19]. Extract (0.1 mL) was mixed with 1 mL of 0.1 mM DPPH. After 30 minutes of incubation at room temperature, absorbance was measured at 517 nm. Ascorbic acid was used as standard. Total phenolic content of extracts was determined by Folin-Ciocalteu reagent method as described by Bray and Thorpe [20].

Bacteriocin production

Isolated LAB *Lactobacillus* T1 sp., *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactococcus sp.* T4 and *Bacillus sp.* TK3 were further tested for their bacteriocin production potential against test indicators, i.e., *Listeria monocytogenes*, *Clostridium perfringens*, *Staphylococcus aureus*, *Bacillus cereus*, *Leuconostoc mesenteroides*, *Escherichia coli*, *Enterococcus faecalis* and *Salmonella typhimurium*. One mL inoculum of each indicator bacteria (1.0 OD) was swabbed onto pre-poured sterilized nutrient agar plates using sterilized cotton buds. Using the well-diffusion method, a well of 7 mm diameter and 5 mm depth was cut on the lawn laid in the nutrient agar plates with the help of sharp borer. One OD culture of bacterial isolates was centrifuged at 12,000 rpm at 4°C for 15 minutes. The culture supernatant was poured into the wells and the well-diffusion method was repeated with crude preparations of isolates against their respective indicator. The plates were then incubated at 37°C for 24 hours and the zones of inhibition formed around the wells were measured [21].

Results and discussion

Tattakhar prepared from Himalayan horse chestnut fruits is a nutritionally rich traditional fermented food of Himachal Pradesh (**Fig. 2**).

Figure 2 Tattakhar (*Aesculus indica*) fermented flour

Compared with other nuts and seeds, these fruits are relatively low in calories and fat and rich in minerals, vitamins and phytonutrients that benefit health. In addition to the nutritional properties of horse chestnut fruits, tattakhar is known for its medicinal properties, induced as a result of therapeutic probiotics produced from fermenting LAB.

Nutritional analysis of tattakhar flour

The nutritional value – proteins, reducing sugars, total carbohydrates, crude fibres and minerals (ash content) – of tattakhar was analyzed. The results obtained are presented in **Table 1** and show that the nutrition content of this fermented flour is high. Total proteins in tattakhar flour were found to be 28.22% – which compares closely with the protein-rich soybean powder (36.49%). Total carbohydrate content of the flour was found to be 71.0 mg/g – which is equivalent to the carbohydrate content of wheat and could therefore be considered as a supplement for wheat flour. Amount of ascorbic acid was 6.6% and crude fibre content was 0.44 mg/g. The presence of 9.45% ash content indicates a very high probability of a large amount of minerals in tattakhar flour, thereby highlighting its nutritionally-enriched characters. In a similar study, tattakhar flour was analyzed for its proximate composition and the following results were observed: moisture content 5.45%; protein (6.13 %); ash content (3.42%); total carbohydrate 89.09% [22].

Microbiological profile of tattakhar

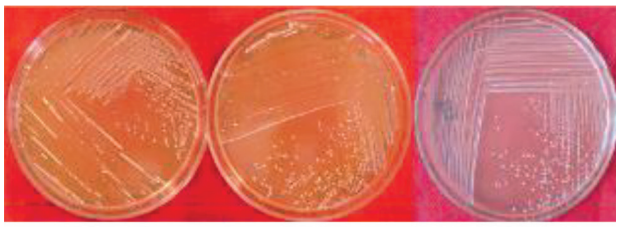
The microbiological profile of tattakhar flour was studied for the very first time to explore rare and novel fermenting microorganisms. To assess its microbiological profile, tattakhar flour was collected from different parts of Himachal Pradesh. In total, ten bacteria were initially isolated, from which the five most predominant isolates showing high microbial count, i.e., ranging from 6.7 to 7.9 log CFU/mL were studied further. Out of these five isolates, three were found to be gram-positive rods (on MRS agar medium), one was gram-positive cocci (on MRS agar medium), while the fifth was gram-positive rod obtained on nutrient agar medium with white, punctiform and raised colonies on media plates (**Fig. 3**).

All isolates were catalase positive, able to ferment carbohydrates as they produced acid and gas, utilized citrate, MR +ve and VP-ve and were not able to produce hydrogen sulphide (H2S).

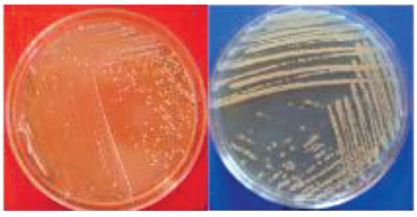
Table 1 Nutritional value of tattakhar flour

Sr No.	Nutrient	Content
1.	Total protein (%)	28.22
2.	Reducing sugar (mg/g)	0.018
3.	Total carbohydrates (mg/g)	71.0
4.	Crude fibre (mg/g)	0.44
5.	Phosphorus (%)	18.30
6.	Iron (%)	11.50
7.	Calcium (%)	15.0
8.	Antioxidants (%)	45.0
9.	Ascorbic acid (%)	6.6
10.	TSS (°B)	11.2
11.	Moisture (%)	0.49
12.	Ash (%)	9.45
13.	pH	4.74

Figure 3 Morphological characteristics of bacteria isolated from tattakhar flour



Lactobacillus sp. T1 *Lactobacillus sp.* T2 *Lactobacillus sp.* T3



Lactobacillus sp. T4 *Bacillus sp.* TK3

Based on morphological and biochemical examination, these isolates were tentatively identified as *Lactobacillus sp.* T1, *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactobacillus sp.* T4 and *Bacillus sp.* TK3 respectively. These five isolates had a microbial load >106 CFU/g of sample, thus fulfilling the WHO criteria for products to qualify as probiotics (106 CFU/g of load). This measurement is deemed adequate for the probiotic to establish, colonize and flourish in a healthy gut and to exert beneficial health effects like improved digestion, strengthening immunity, lowering cholesterol, anti-obesity and anticarcinogenic in nature. Moreover, these fermenting bacteria show high antioxidant potential, bacteriocin production and a broad antagonistic spectrum against deadly human pathogens and can therefore be categorized as effective probiotics with high therapeutic effect [23].

Antimicrobial spectrum

To detect the antimicrobial activity of the LAB, they were grown in nutrient/MRS broth and tested against challenging foodborne pathogens, i.e., *Listeria monocytogenes*, *Clostridium perfringens*, *Staphylococcus aureus*, *Bacillus cereus*, *Leuconostocmesenteroides*, *Escherichia coli*, *Enterococcus faecalis* and *Salmonella typhimurium*. All five isolates, i.e., *Lactobacillus sp.* T1, *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactobacillus sp.* T4 and *Bacillus sp.* TK3 were found to substantially inhibit the challenging foodborne pathogens. (**Fig. 4**). A maximum zone size of 29 mm and 25 mm was depicted during the growth cycle of the isolate TK3 and T2 against *S. aureus* and *B. aureus* respectively, after the 44th hour of incubation.

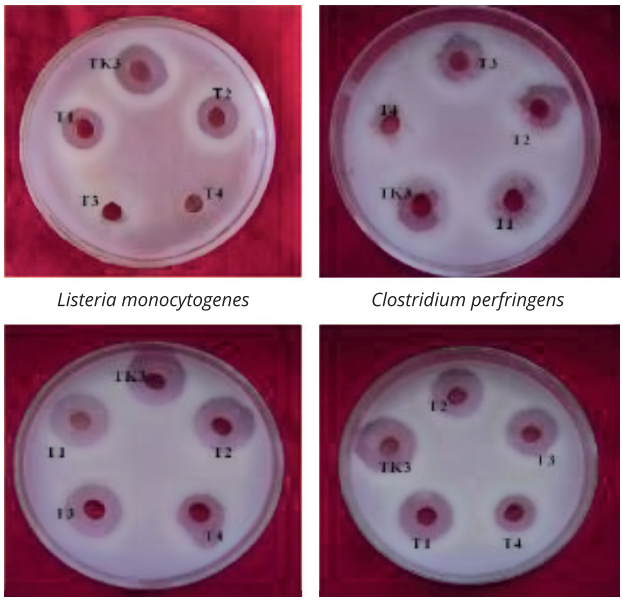
Antimicrobial peptides (AMPs) are produced by every living organism and are considered an important line of defence against invading pathogens. The strong antagonistic effect of these LAB against deadly pathogens turn tattakhar into a strong natural agent with the potential to protect against serious foodborne infections.

Evaluation of safety assessment

DNase activity

The LAB, i.e. *Lactobacillus sp.* T1, *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactobacillus sp.* T4 and *Bacillus sp.* TK3 isolated in the present investigation were found to be negative for the production of DNase enzyme, assigning a completely safe status (**Fig. 5**). Deoxyribonucleases, enzymes that hydrolyze nucleic acids to yield oligonucleotides, are involved in bacterial virulence. Microorganism producing DNase enzyme cannot be used as a probiotic in the food and animal feed industry [24]. Extracellular DNase provides a growth advantage to the pathogens by enlarging the pool of available nucleotides by DNA hydrolysis and helps in dissemination and spread of the pathogens. DNase also aids the evasion of the innate immune response by degrading neutrophil extracellular traps (NETs) [25].

Figure 4 Antagonistic spectrum of lactic acid bacterial isolates against serious foodborne pathogens



Listeria monocytogenes | *Clostridium perfringens*

Bacillus cereus | *Staphylococcus aureus*

Figure 5 Negative DNase activity shown by all strains

Gelatinase activity

All five strains, i.e., *Lactobacillus sp.* T1, *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactobacillus sp.* T4 and *Bacillus sp.* TK3 were assayed for gelatinase activity and showed zero gelatinase activity as there was no clear zone surrounding the colony on gelatin agar plates (**Fig. 6**), thus confirming its safe nature. Gelatinases – the matrix metalloproteinases (MMPs) capable of degrading almost all extracellular matrix (ECM) – and basement membrane components are enzymes mostly produced by pathogenic bacteria. Gelatinase is a zinc metalloprotease, encoded by gelE that is capable of hydrolyzing gelatine, collagen, casein, haemoglobin, and other peptides. As it is encoded by a plasmid gene, gelatinase could mediate binding to the host epithelium and it appears that it also plays an important role in promoting bacterial aggregation during conjugation, facilitating plasmid exchange [25]. Syal and Vohra (2014) studied the production of gelatinase by Geotricum klebahnii to reveal its safety as probiotic microorganisms. LAB are designed to meet food safety, shelf life, technological effectiveness and economic feasibility criteria, of which safety criteria are of prime importance [26].

Haemolytic activity

In the present investigation, *Lactobacillus sp.* T1, *Lactobacillus sp.* T2, *Lactobacillus sp.* T3, *Lactobacillus sp.* T4 and *Bacillus sp.* TK3 were negative for haemolysis on blood agar plates. Because neither clear zones (α-haemolysis) nor green-hued zones (β-haemolysis) were observed around colonies (**Fig. 7)** the safety and non-virulent nature of the product was proven. Probiotics must be non-pathogenic and non-invasive, hence exclusion of pathogenic strains is essential for the selection of safe probiotics [27]. Production of haemolysin by *Lactobacillus* was determined by streaking the actively growing cells on (BHI) agar supplemented with 1% (w/v) glucose, 0.03% L. Arginine and 5% (v/v) of human blood. Plates were incubated at 37°C for 24 hours in anaerobic conditions [24].

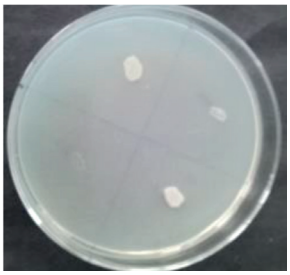


Figure 6 Negative gelatinase activity shown all strains

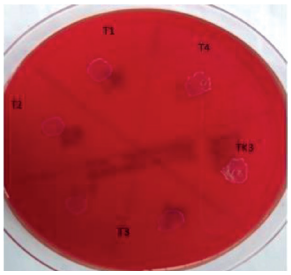


Figure 7 Negative results shown for haemolytic activity

Antioxidant analysis

Tattakhar is rich in almost all important nutrient groups and can be therefore be categorized as a superfood. A scaling up and adaption of its fermentation methodology, coupled with commercial promotion, would afford it the potential to become one of the most sought-after health promoting and immunity boosting foods commercially available.

DPPH is a free radical generating compound and has been used to estimate free-radical scavenging ability of different antioxidant compounds. The methanolic extract of tattakhar flour showed 45.0% scavenging ability. The antioxidant activity of fermented tattakhar flour can be attributed to its potential to act as reducing agents, hydrogen donor, free-radical scavengers and metal chelators [28]. Probiotics from *Lactobacillus* plantarum have been reported to improve growth performance, nutrient utilization, immune status and gut health in livestock. In a study by Izuddin WI *et al* (2020), the antioxidant activity of *L. plantarum* RG14, RG11 and TL1, dietary effects, antioxidant activity, hepatic antioxidant enzymes and ruminal barrier function were investigated [29]. The very high antioxidant potential of 45.0% proves its therapeutic value as an immunity enhancer, which is of high significance among consumers.

Bacteriocin production

In this study, five LAB i.e., T1, T2, T3, T4 and TK3 were tested further to check their bacteriocin production potential. Only one LAB –TK3 – was found positive for bacteriocin production, whereas T1, T2, T3 and T4 were found to be negative. Halos of 28 mm and 26 mm were seen against *S. aureus* and *L. monocytogenes* respectively, after 44 hours of incubation. The bacteriocin produced by the LAB isolated in this study has potential to inhibit foodborne pathogenicity [32]. *Lactobacillus* can upregulate the transcription levels of antimicrobial resistance genes in *Gardernella vaginalis* due to the production of bacteriocins by *Lactobacillus* and induce altered gene transcription in *Gardernella vaginalis* [33].

A study by Chavan et al. (2016) successfully isolated LAB from idli, a fermented food made from of rice batter, urad dal and fenugreek seeds. Ten isolates were able to produce bacteriocin whose antibacterial activity was analyzed by agar well-diffusion assay against indicator organisms. Regarding bacteriocin production, cell growth was started from late log phase itself and maximum was obtained in early stationary growth phase at the 30th hour of the culture. Bacteriocin production is a desirable trait of probiotics because of its antimicrobial nature and thus it is capable of inhibiting or suppressing the growth of many challenging pathogens in the system [34].

Conclusion

In the present study, tattakhar – a traditional fermented flour of Himachal Pradesh made from the fruits of the Himalayan horse chestnut – was assessed for its nutritional profile and presence of probiotics. A nutritional assessment of the flour revealed that it is considerably rich in nutrients, thereby highly recommending its use as a nutritional supplement. In addition, due to the presence of bacteriocin produced by probiotic bacteria, tattakhar has potential therapeutic effects, showing inhibition against many fatal disease-causing pathogens. It can therefore be recommended as a functional food to promote health. The present study also revealed that the nutritional value of tattakhar flour is high when compared with other staple foods used in our daily life. Above-all, the rare health beneficial microflora present in tattakhar impart a strong probiotic characteristic as well as enhancing its nutritional value. Probiotics are live microbial food supplements that benefit the host by improving the intestinal microbial balance. During the last few decades, development of the functional food concept and, more specifically, the application of certain LAB strains as life vaccines, pro- and prebiotics and nutraceuticals have created new perspectives for LAB research and human consumption, attracting the attention of microbiologists, food scientists and health professionals globally.

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