Probiotic potential of *Bacillus licheniformis* isolated from indigenous traditional fermented beverage

Ranjana Sharma* & Nivedita Sharma

Microbiology Research Laboratory, Department of Basic Sciences, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan-173230, HP, India

E-mails : sharmaranjana0@gmail.com; niveditashaarma@yahoo.co.in

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In this study, a potential probiotic microorganism A3 isolated from *Angoori* was subjected to probiotic characterization. The isolate A3 was identified as *Bacillus licheniformis*. The strain showed broad antagonism against most pathogens, good auto-aggregation capacity, i.e., greater than 40% after 5 hrs, high acid tolerance showing survival of 75.4% at pH 1.0 for 60 min and bile salt tolerance ranging from 80.16% to 84.41%. The isolate was found to be highly sensitive towards all the 16 antibiotics tested. These results indicated that *B. licheniformis* A3 isolated from *Angoori* had a promising potential as good probiotic and could further be exploited for its commercial use.

**Keywords**: *Angoori*, Probiotics, Antagonism, Fermentation, *Bacillus licheniformis*, *Vitis vinifera* L.

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The traditional fermented foods and beverages form most important part of daily diet to people belonging to rural and tribal areas since ancient time. Himachal Pradesh is one of the hilly states of India in northern Himalayas having number of communities, races and cultures intermingled together. There are local variations in the traditional food and beverages from region to region and number of these foods are prepared and consumed by local people. Among them, fruit based beverage, i.e., *Angoori* is a very popular traditional fermented beverage of Kinnaur district of Himachal Pradesh. Kinnaur is situated at 75°21′55″ E longitude and 11°5′20″ N latitude in Himalayan cold temperate zone. This ethnic alcoholic beverage was prepared from local variety of grapes and being used by tribal people of kinnaur since ages during different ceremonies, marriages and above all for their survival in biting cold reeling under -12 °C in peak of winter season. ‘*Phab*’ is a traditional starter culture used as inocula in preparation of *Angoori*. Despite of its high popularity, it has remained confined only to Kinnaur district of HP and has not been commercialized so far perhaps due to difficult geographical conditions of the region and also to preserve the culture which differ from other places in terms of life style of local people. Therefore, this study is very first time being reported here to isolate food grade microorganism from *Angoori* and to explore its probiotic traits for further use.

**Angoori preparation**

A number of grape (Vitis vinifera L.) varieties (purple and green) such as *rangsipay, shonltu white* and *shonltu red* are cultivated in the kinnaur district to make this local alcoholic beverage (wine). In this process grapes are first harvested and washed with clean water followed by crushing using hands. Crushed grapes with the addition of sugar, water and traditional inocula *Phab* are left for fermentation in huge wooden casks for 15 days at 22-25 °C. Must is stirred daily for 5-10 min. After 15 days of fermentation, the fermented juice rises up and is collected in a bottle by using local siphoning apparatus (Fig. 1). This traditional fermented beverage is a rich depository of least explored rare and novel fermenting probiotic strains with immense potential of various health beneficiaries. Therefore, the present study has been carried out to isolate probiotic microorganism from *Angoori* and to study their potential as probiotics so as to widen the use of this potentially active traditional beverage all over India.

*Corresponding author*
Materials and methods

Source of culture

A3 culture was isolated from Angoori, a traditional fermented beverage consumed and prepared by Northern hilly state (Himachal Pradesh) of India. The identity of the bacterial isolate was confirmed as Bacillus licheniformis A3 based on the analysis of 16S rDNA sequence (accession number-KM251712).

Antagonistic spectrum of B. licheniformis A3

Serious food borne pathogens, viz. Staphylococcus aureus IGMC, Enterococcus faecalis MTCC 2729, Listeria monocytogenes MTCC 839, Clostridium perfringens MTCC 1739, Leuconostoc mesenteroides MTCC 107, Bacillus cereus CRI,Ralstonia solani, Aspergillus niger, Rhizopus sp. and Fusarium sp. were used to study antagonistic potential. The test strains were procured from Institute of Microbial Technology (IMTECH, Chandigarh, India), Central Research Institute (CRI, Kasauli, HP, India) and Indira Gandhi Medical College (IGMC, Shimla, HP, India). All these test strains were revived twice for 24 hrs at 37 °C before performing experiments, as all these indicators were preserved in 40% glycerol at -20 °C. Antagonistic activity of B. licheniformis A3 was assessed and the plates were checked for clear zone of inhibition.

Probiotic attributes of B. licheniformis A3

Acid and bile salt tolerance

Tolerance of B. licheniformis A3 to simulated gastric juices was tested and bile salt resistance was performed.

Autoaggregation properties and cell surface hydrophobicity

Autoaggregation capacity of B. licheniformis A3 was determined. Bacterial adhesion to hydrocarbons was tested, was determined and results were expressed according to the method. The final absorbance (A₀) was recorded at 600 nm.

Antibiotic sensitivity test

Antibiotic sensitivity of B. licheniformis A3 was determined on solid NA medium by the use of 16 different antibiotic discs. The interaction to antibiotics of isolated strain was tested through HiMedia® Antimicrobial susceptibility test discs. The result was expressed as sensitivity (S) or resistant (R).

Cumulative probiotic potential

The cumulative probiotic potential of B. licheniformis A3 was calculated using standard score card.

Statistical analysis

The experimental data was analyzed by completely randomized design (CRD).

Results and discussion

The data on inhibitory spectrum of B. licheniformis A3 by bit/disc method is shown in Table 1. The inhibition of the growth of pathogens can be through the production of antimicrobial compounds like organic acid, hydrogen peroxide, bacteriocins, etc. Probiotic microorganisms can co-aggregate with pathogens or attach to enterocytes (competitive exclusion) and thus inhibit the binding of enteric pathogens to the intestinal mucosa. Antagonistic activity was mainly seen in case of gram positive bacteria as compared to gram negative one because of presence of lipo-polysaccharide (LPS) in the cell wall of later. The wide spectrum inhibitory activity against challenging and deadly food borne pathogens turn this isolate desirable for exploring its potential for health in food industry. The studies pertaining to explore the antimicrobial activity against food borne pathogens by bit/disc method have been well documented in literature. Thirty Bacillus strains were isolated and screened them for antagonistic activity against gram positive and gram negative bacteria using agar well diffusion method. Geotrichum klebahnii was isolated from a cheese sample which showed an inhibitory activity against food-borne pathogens such as Salmonella sp., Vibrio sp. and Staphylococcus aureus.

Probiotic attributes of B. licheniformis A3 was studied, viz. acid tolerance, bile tolerance, autoaggregation, hydrophobicity and antibiotic sensitivity. Before
reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2.0. *B. licheniformis* A3 strain showed survival of 66% at pH 2.0 after 60 min of incubation period as shown in Table 2. Similar observations were reported, their isolates showed 56-100% survival rate at pH 2 after 3 hrs. Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host. The *B. licheniformis* A3 strain showed 80.16% survival in 2% bile concentration as shown in Fig. 2. Similar observations were obtained by one other author. All isolates demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24hrs at 37 °C.

The sedimentation rate of *B. licheniformis* A3 was measured over a period of 5 hrs. Initially, the percentage of autoaggregation was 7.5 % that increased continuously and finally registering a high percentage of 75 %, respectively. The observed autoaggregation could related to cell surface component, because it was not lost after washing and suspending of the cells in PBS (Fig. 3). Similarly, 23 yeast isolates from traditional fermented foods of Western Himalayas are isolated and found that the autoaggregation ability was in the range of 18.47 to 67.65% for all the tested indigenous yeast isolates.

The probiotic ability to adhere to the intestinal epithelium is regarded as a prerequisite to colonize the human GIT for exerting beneficial effects, such as the exclusion of enteropathogenic bacteria. *B. licheniformis* A3 showed 84 % adhesion towards xylene, 80% towards chloroform and 28% towards ethyl acetate, respectively. The results indicated that the strain was found to be highly hydrophobic (Table 3). Similar observations were reported in which they found 15.24% and 32.2% hydrophobicity towards xylene.

The antibiotic susceptibility of strain makes it crucial for the safety point of view to their use as potential probiotics because probiotic bacteria may act as potential reservoir of antimicrobial resistance
B. licheniformis A3 strain was found to be sensitive to all of the tested antibiotics, viz. ampicillin, gentamycin, chloramphenicol, ofloxacin, tetracycline, co-trimozol, ceftazidime, vancomycin, cefotaxime, cefuroxime, clindamycin, erythromycin, amikacin, ticarcillin, amphotericin-B and penicillin-G, which is desirable for potential probiotic strains. Similar observations were reported. He tested susceptibility and resistance of 15 isolates against 10 available antibiotics. Almost all strains were sensitive to 50% of the 10 antibiotics used in the test but maximum sensitivity was observed for oxacillin and kanamycin.

The pro-biotic potential of the bacterial strains is based upon assigned cumulative pro-biotic score. The pro-biotic potential of B. licheniformis A3 was adjudged 91.7% as shown in Table 4. Since, pro-biotic preparations for commercial launching have to touch the pro-biotic score in the range of 75 to 85%, thus the present study revealed that B. licheniformis A3 have completely fulfilled the criteria of FAO/WHO for earning the status of good pro-biotics.

Conclusion
The observations recorded in this investigation that the strain B. licheniformis A3 isolated from traditional fermented beverage, Angoori of Himachal Pradesh, a Himalayan state of India bearing notably high pro-biotic potential is capable of tolerating high bile salt, able to survive in simulated low gastric pH and showed broadest antagonism against pathogens. In addition, the strain is sensitive to most of the antibiotics used, showed strong autoaggregation of cells and hydrophobicity. Thus B. licheniformis A3 is useful in making the functional food such as Angoori.

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References


