



Inhibition of Pathogenic Microbes by the Lactic Acid Bacteria *Limosilactobacillus Fermentum* Strain LAB-1 and *Levilactobacillus Brevis* Strain LAB-5 Isolated from the Dairy Beverage Borhani

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Abstract

Lactic acid bacteria (LAB) with prominent antimicrobial effects against pathogens have been reported in several milk-based and plant-based foods. Borhani is a popular beverage prepared from the ingredients of both dairy and plant origins and is believed to be highly beneficial for health. Herein, we report the pathogen-inhibitory activity of two borhani-associated lactic acid bacteria (LAB), *Limosilactobacillus fermentum* strain LAB-1 and *Levilactobacillus brevis* strain LAB-5. Their antimicrobial activity was primarily assessed using the cell free supernatant (CFS) by agar diffusion technique in which both strains showed strong antimicrobial effects against several pathogenic and spoilage microorganisms including *Acinetobacter baumannii*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella abony*, *Salmonella typhi*, *Shigella flexneri*, and *Staphylococcus aureus*. The *L. fermentum* strain with its ability to inhibit all the target pathogens appeared to be more effective with larger inhibition-zone formation as compared to the *L. brevis* strain which also successfully inhibited all pathogens but had relatively little effects on *A. baumannii*. The extent of their inhibitory effect was further assessed by co-culture inhibition assay in which growth of the test microbes was monitored for 24 hours in presence of the CFS. The CFS of both lactic acid bacteria could effectively inhibit growth of the pathogenic microbes for a significant period of time. While the



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L. fermentum strain could almost completely stop growth of all test organisms, the *L. brevis* strain was particularly effective against *Shigella flexneri* and the *Salmonella* species. Our study, therefore, suggests the presence of beneficial lactic acid bacteria in borhani which can be of important use as antimicrobial agents in functional foods and therapeutics to help acquire protection against drug resistant pathogens.

Introduction

Inhibition of pathogenic bacteria by the microbes that naturally exist in our regular diets can be remarkably beneficial to our health considering the pathogens' biocontrol and disease prevention. Foods that offer such additional benefits than providing mere energy are also highly desirable to the health-conscious consumers.¹ When the pathogen-inhibition is of concern, lactic acid bacteria (LAB) comes before all other microbes for their strong antimicrobial activity reported against many microorganisms.² LAB comprise a large group of heterogeneous bacteria that are generally recognized as safe (GRAS) for our consumption.³ Bacteria in this group are generally Gram-positive, catalase-negative, non-spore forming, and devoid of cytochromes.⁴ They prefer an initial pH of 6 to 7 for their growth and produce a large amount of lactic acid which is the main end product of their an aerobic carbohydrate fermentation.⁵ Today, most bacteria used as probiotics in the functional foods and therapeutics, or as starter cultures in the industrial food-processing are the LAB.⁶ LAB have been isolated from a variety of sources but carbohydrate rich environments such as plants, vegetables, and fruits have been frequently reported as their sources of isolation.^{7,8} Dairy products and fermented foods and beverages also comprise a very good source of LAB.⁹ Among the various species identified in fermented foods, *Lactobacillus* appeared to be the most dominant genus of LAB, followed by *Pediococcus* in most cases.¹⁰ While these bacteria offer a wide range of health benefits,¹¹ their antimicrobial activity is recognized as the most important property in both starter culture and probiotic applications.¹² The antimicrobial activity of lactic acid bacteria is mainly due to the secretion of various organic acids such as lactic acid, formic acid, and acetic acid which reduces the pH, and is thought to be the principal mechanism of pathogen-inhibition.¹³ They also cause accumulation of molecules like hydrogen peroxide,

carbon dioxide, ethanol, acetaldehyde, bacteriocins and bacteriocin-like substances which also possess antimicrobial activities. In addition to providing bioprotection against pathogenic and spoilage microorganisms, the natural LAB of the fermented foods and beverages also help prolonging their shelf life by the reduction of pH and fermentation of sugars into organic acids.¹⁴

Isolation of the naturally existing beneficial lactic acid bacteria from foods and their specific applications is an important endeavor. Unless being isolated and proved to possess specific beneficial properties, however, no microbe can be considered for probiotic application. This study was, therefore, designed to obtain and characterize microbes having antimicrobial activity from foods that are commonly eaten and are also likely to carry beneficial LAB species. Borhani in this regard appeared to be an ideal candidate to us since it is a beverage believed to be highly beneficial for health and digestion. Moreover, it is prepared from ingredients of dairy and plant origins both of which have been known to be good sources of LAB.¹⁵ Consequently in the present study, we report the isolation of two borhani-associated LAB species *Limosilactobacillus fermentum* strain LAB-1 and *Levilactobacillus brevis* strain LAB-5 and their characterization with regards to their antimicrobial activity. Extent of the antimicrobial effect was assessed against several clinically relevant Gram positive and Gram negative test organisms. Strains of these test microbes are often reported to carry resistance to multiple antibiotics (MDR pathogens). Therefore, their inhibition by the native food-associated LAB species demands special importance. The two LAB species, *L. fermentum* and *L. brevis*, were both described in previous studies to offer a few beneficial effects including the inhibitory activity against some pathogenic and spoilage microorganisms.¹⁶⁻¹⁸ Few strains

of both the LAB groups are already being used as starter cultures, and some are also being considered as potential candidates for probiotic applications in functional foods and medicines.¹⁹⁻²³ However, their beneficial properties were mostly found to be strain specific.²⁴ Hence, in order to provide specific insights into the probiotic potential of the two isolates, we performed out a careful assessment of their antimicrobial activity which is recognized as the most important pre requisite for a microbe to be considered for probiotic applications.^{12,25}

Materials and Methods

Isolation, Purification and Preservation of Bacteria

For bacterial isolation, five different borhani samples collected from local shops were mixed and plated on de Man, Rogosa and Sharpe (MRS) agar with or without cysteine-supplementation.¹⁵ Colonies with unique morphological appearances were selected and streaked on MRS agar to obtain pure cultures. Glycerol stock of each isolate was prepared and preserved at -20°C and -80°C.^{26,27} Before use, the isolates were revived in MRS broth and subsequently transferred and grown in fresh medium.

PCR, Sequencing and Identification

The whole genome sequence of LAB-1 and its annotation and identification have been previously reported.²⁸ For 16S rRNA gene sequencing of LAB-5, its genomic DNA was extracted from a single colony as previously described.²⁹ Fragment of the 16S rRNA gene was amplified by conducting PCR with the universal primers 27F and 1492R, and sequencing was performed as described before.³⁰ The 16S rRNA gene sequence was analyzed using NCBI's BLAST search program against the GenBank database. The identification was based on the sequence similarity, total score, and number of hits obtained against a particular phylotype.²⁷

Accession Numbers

The 16S rRNA gene sequence and WGS can be found in the NCBI database under the accession numbers OM980643 and JAJTII000000000.

Preparation of Cell Free Supernatant (CFS)

CFS of the LAB isolates was prepared by centrifugation of the respective cultures at 10,000×g for 20 min at 4°C. The supernatant so obtained was preserved as 1 mL aliquots at -20°C until used.

Test Strains

The test strains used in antimicrobial assay in the present study are known to have spoilage and/or pathogenic implications and include both Gram-positive and Gram negative microbes listed in Table 1.

Table 1: List of the test strains used in antimicrobial assays.

Gram Negative

Acinetobacter baumannii ATCC 7978
Escherichia coli ATCC 25922
Klebsiella pneumoniae ATCC 13883
Pseudomonas aeruginosa ATCC 9027
Salmonella abony ATCC 14028
Salmonella typhi ATCC 14028
Shigella flexneri ATCC 9199

Gram Positive

Bacillus cereus ATCC14574
Bacillus subtilis ATCC 6633
Staphylococcus aureus ATCC 6538

Agar well Diffusion Assay

Antimicrobial activity was screened by agar diffusion assay following the protocol reported by Coman *et al.*, 2014 with modifications.³¹ 1 mL activated culture of each test strain having a cell density equivalent to 0.5 McFarland standard was added to 20 mL of previously autoclaved warm (42-55°C) Mueller Hinton agar and the suspension was subsequently poured on to petri dishes. Upon solidification of the medium, wells having a diameter of 5 mm was punched using a sterile cork borer and 80 µL of the LAB CFS was placed inside the well. The plates were subjected to a 3 h pre-incubation at 4°C followed by a 24 h incubation at 37° C. Inhibitory activity of the CFS was confirmed by inability of the test strains to grow around the well as indicated by the formation of clear halos. Diameter of the inhibition zones was measured in mm from the edges of the wells.

Broth Inhibition Assay

Inhibitory effect of the CFS was also assessed by a modified co-incubation broth assay.³² The test strains were activated and grown at 37°C until cell

density reached to an OD₆₀₀ of 1.0. 50 µL of this activated culture was inoculated in 2 mL of nutrient broth containing 400 µL of the CFS and the mixture was incubated at 37°C at 180 rpm in an orbital shaker for 24 h. In the control reaction, sterile MRS broth was used instead of the CFS. Growth of the test strains was monitored by measuring the OD₆₀₀ at 0 h, 2 h, 4 h, 8 h and 24 h of incubation. Inhibition of growth was determined using the following formula.

Growth inhibition (%) = $(A_C - A_S)/A_C \times 100$, where AC is the absorbance of control and A_S is that of sample at 600 nm.

Statistical Analysis

All experiments were performed in triplicate and the data are expressed as mean ± standard deviation (SD). The results were statistically evaluated using t-test and P value less than 0.05 was considered to be statistically significant.

Results

Molecular identification and taxonomic assignment
Molecular identification of the LAB-1 strain has been previously reported based on the nucleotide identity of its whole genome sequence and the 16S rRNA gene sequence extracted from the genome sequence,²⁸ whereas identification of the LAB-5 strain was carried out by the amplification and sequencing of a fragment of its 16S rRNA gene followed by sequence identity analysis. Nucleotide BLAST of the 16S rRNA gene sequences of LAB-1 and LAB-5 against the NCBI standard database revealed a high homology, with identity scores more than 99% to the respective genes of several *L. fermentum* and *L. brevis* strains respectively. The BLAST results with the taxonomic lineage, score, and the number hits obtained for a particular phylotype have been presented in Table 2.

Table 2: Molecular identification of the LAB-1 and LAB-5 strains. Results of nucleotide BLAST of their 16S rRNA gene sequences. Uncultured or unidentified organisms have been removed.

Isolates	Lineage	Score	No. of Hits
LAB-1	root		2007
	.Bacteria		2004
	..Bacilli		1997
	...Lactobacillaceae		1995
 <i>Limosilactobacillus</i>		1985
 <i>Limosilactobacillus fermentum</i>	2368	1973
 <i>Limosilactobacillus oris</i>	2368	1
 <i>Limosilactobacillus fermentum</i> 3872	2368	1
 <i>Limosilactobacillus fermentum</i> F-6	2368	1
 <i>Limosilactobacillus fermentum</i> CECT 5716	2368	1
 <i>Limosilactobacillus fermentum</i> IFO 3956	2368	1
 <i>Limosilactobacillus</i> sp.	2303	7
 <i>Lactobacillus</i> sp.	2290	1
 <i>Lactocaseibacillus paracasei</i>	2287	1
 <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	2285	1
 <i>Lactobacillus</i> sp. T17/4F	2283	1
 <i>Lactobacillus</i> sp. JCM 7751	2276	1
..... <i>Lactobacillus acidophilus</i>	2276	1	
..... <i>Lactobacillus</i> sp. CR-6AS	2268	1	
LAB-5	root		540
	.Bacteria		539
	..Bacilli		534
	...Lactobacillaceae		529

.... <i>Levilactobacillus</i>		509
.... <i>Levilactobacillus brevis</i>	2567	502
.... <i>Levilacto bacillus brevis</i> KB290	2562	1
.... <i>Levilactobacillus brevis</i> ATCC 367	2562	1
.... <i>Levilactobacillus brevis</i> BSO 464	2556	1
.... <i>Levilactobacillus</i> sp.	2556	2
.... <i>Levilactobacillus brevis</i> ATCC 14869 = DSM 20054	2556	2
.... <i>Lactobacillus</i> sp. L01	2567	1
.... <i>Lactobacillus</i> sp. NBRC 106029	2564	1
.... <i>Lactobacillus</i> sp. NBRC 106045	2564	1
.... <i>Lactobacillus</i> sp.	2562	8
.... <i>Lactobacillus</i> sp. SMG65	2562	1
.... <i>Lactobacillus</i> sp. NBRC 106022	2560	1
.... <i>Lactobacillus</i> sp. NBRC 106046	2560	1
.... <i>Lactobacillus</i> sp. ABR11NW.F58	2560	1
.... <i>Lactobacillus</i> sp. NBRC 107227	2558	1
.... <i>Lactobacillus</i> sp. safa1	2556	1
.... <i>Lactobacillus</i> sp. SMG100	2556	1
.... <i>Lactobacillus</i> sp. NBRC 106044	2556	1
... <i>Bacillus amyloliquefaciens</i>	2562	1
... <i>Weizmannia coagulans</i>	2556	1

Table 3: Inhibition zone (mm) produced against the test strains in agar diffusion assay. The asterisk denotes a statistically significant difference between the two isolates at the 0.05 level.

Test strains	Inhibition zone (\pm SD)	
	LAB-1	LAB-5
Gram negative		
<i>A. baumannii</i>	9 (\pm 1)	Not measured
<i>E. coli</i>	8.67 (\pm 1.16)	3.34 (\pm 0.58)
<i>K. pneumoniae</i>	9.34 (\pm 1.53)	3 (\pm 2.65)
<i>P. aeruginosa</i>	10.67 (\pm 1.16)	5.67 (\pm 0.58)
<i>S. abony</i> *	7.67 (\pm 2.09)	3.34 (\pm 0.58)
<i>S. typhi</i>	6.34 (\pm 0.58)	3.67 (\pm 0.58)
<i>S. flexneri</i>	9.5 (\pm 0.5)	1.34 (\pm 1.16)
Gram positive		
<i>B. cereus</i>	10 (\pm 1.74)	1 (\pm 1.74)
<i>B. subtilis</i>	9.67 (\pm 1.53)	3 (\pm 2.65)
<i>S. aureus</i> *	5 (\pm 1.74)	3.5 (\pm 1.33)

Pathogen Inhibition in Agar Diffusion Assay

Antimicrobial activity of the two lactic acid bacteria was primarily tested by agar diffusion assay using the cell free supernatant (CFS) against seven Gram-negative (*A. baumannii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. abony*, *S. typhi*, *S. flexneri*) and

three Gram-positive bacteria (*B. cereus*, *B. subtilis*, *S. aureus*). Presence of the inhibitory activity was found in the CFS of both isolates with a broad antimicrobial spectrum (Table 3). The *L. fermentum* strain was able to inhibit all the test strains with a minimum inhibition zone

of 9 mm against most of the pathogens. The *L. brevis* strain showed similar antimicrobial spectrum but with relatively smaller inhibition

zones (<6 mm) and a very minor zone against *A. baumannii*. Both isolates were most effective against *P. aeruginosa* in the diffusion assay.

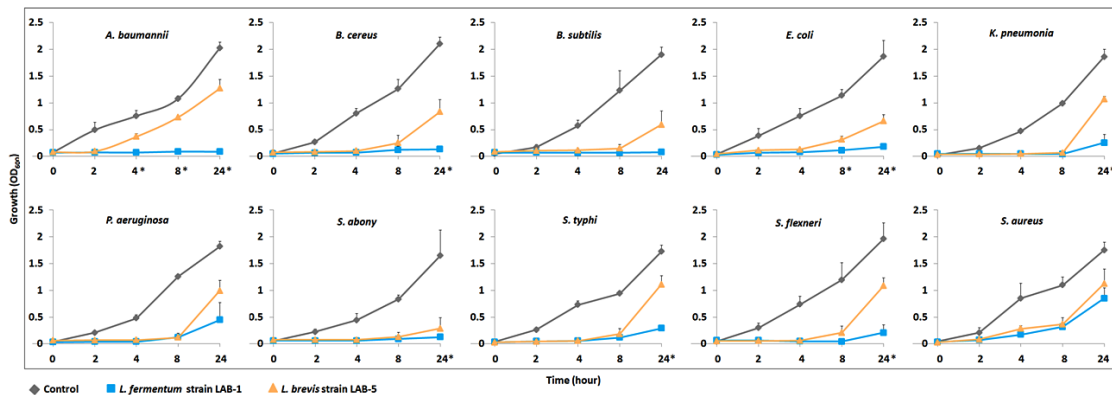


Fig. 1: Co-incubation growth curves. Interference of the LAB isolates on the growth of the test organisms evaluated by incubating each of the test strains alone and with the CFS prepared from the *L. fermentum* or *L. brevis* strain. The asterisk denotes a statistically significant difference between the two isolates at the 0.05 level.

Pathogen Inhibition in Broth Assay

To obtain a more precise and more direct evaluation of inhibition, the antimicrobial effect was further estimated by broth inhibition assay in which growth of the above pathogenic microbes was tracked from 0 to 24 h in presence of the CFS. The growth rate and the inhibition kinetics are presented in Figure 1 and 2. The test strains were almost completely suppressed by the *L. fermentum* CFS after 2, 4, and 8 h of incubation. In a few of the test strains, e.g., *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, *S. flexneri*, and *S. aureus*, small growths were observed after 24 h which probably was due to the decreasing amount of the antimicrobial substances being used up over time (Figure 1). The relatively less affected test strain was found to be *S. aureus* followed by *S. typhi* (Figure 2). Similar to the findings in the agar diffusion assay, antimicrobial activity of *L. brevis* was found to be less effective than *L. fermentum* in all cases. The isolate could largely inhibit the pathogens' growth until 4h or 8h, and a reduced growth was observed afterwards (Figure 1). *A. baumannii* and *S. aureus* exhibited relatively better resistance towards the inhibitory activity of *L. fermentum* (Figure 2).

Discussion

In the present study, we have assessed antimicrobial activity of two lactic acid bacterial strains,

L. fermentum LAB-1 and *L. brevis* LAB-5 indigenous to the popular South Asian beverage borhani. The two lactic acid bacteria showed a broad spectrum of antimicrobial activity with different extents of inhibitory efficiencies. Both isolates could significantly impede the growth of all the test organisms in both primary and secondary assessments. Although the inhibitory effects varied against specific pathogens in the two methods, the degree of inhibition was generally found same, i.e., the *L. fermentum* strain appeared to be more efficient than the *L. brevis* strain in both assay methods. The inhibition area in the agar diffusion assay was at least two to three fold higher with the use of *L. fermentum* CFS than that obtained with *L. brevis* (Table 3). The diffusion assay is an easy, rapid and one of the most widely used methods for the assessment of antimicrobial activity in microbial and plant extracts.³³ However, this technique relies, to some extent, on smooth diffusion of the sample across the agar and movement of certain antimicrobial compounds is hampered by their interaction with the extrinsic substances or contaminants present in the agar.³² Hence, inhibitory activity of the lactic acid bacteria was further examined by broth inhibition assay in which cell-free culture filtrate of each isolate was co-cultured with the pathogenic microbes and changes in the pathogens' growth were recorded.

The growth stopped almost completely for a significant period of time which confirmed

presence of strong antimicrobial activity in the lactic acid bacteria.

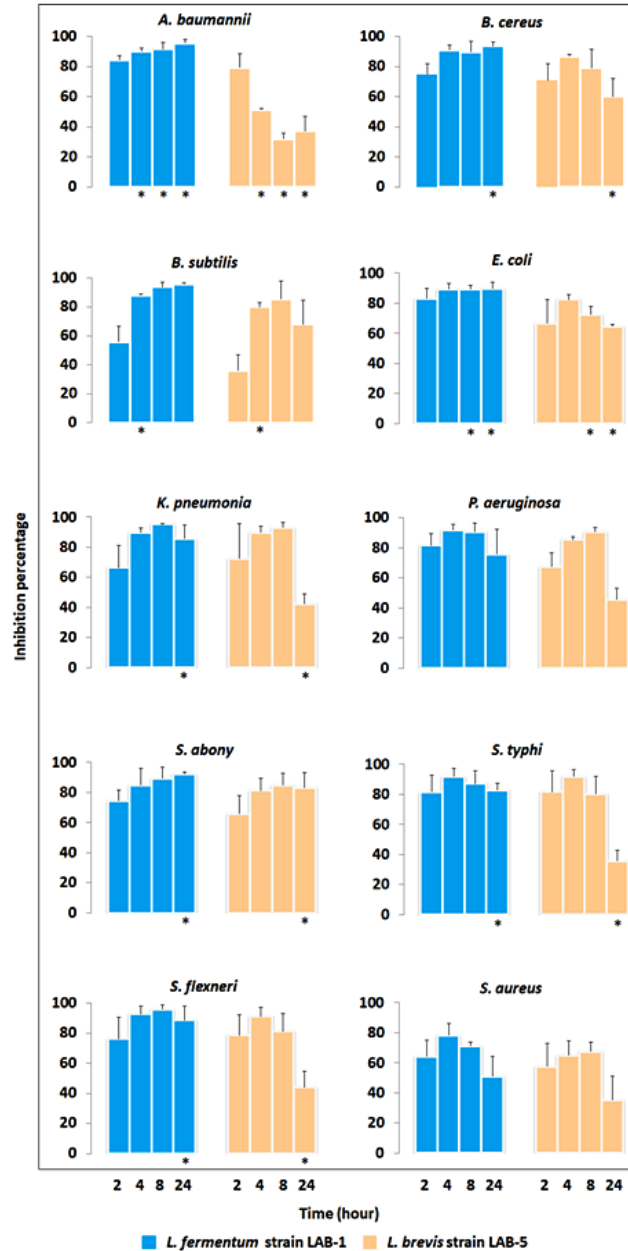


Fig. 2: Antagonistic effects of the lactic acid bacteria. Inhibition (%) of the pathogens' growth by the CFS of the lactic acid bacteria. The asterisk denotes a statistically significant difference between the two isolates at the 0.05 level.

Isolation of *L. fermentum* and *L. brevis* from dairy products and other sources were previously described. Their antagonistic activity against a few

bacterial pathogens has also been demonstrated. One of the *L. fermentum* strains isolated from the oral mucosa of healthy children inhibited six *S. aureus*

strains including three methicillin-resistant strains.³⁴ Another orally isolated *L. fermentum* strain, L. f1, was also able to inhibit *S. aureus*.³⁵ The *L. fermentum* strain ING8 isolated from Dahi, an Indian traditional fermented milk, could inhibit *Listeria monocytogenes*, *B. cereus*, and *E. coli*.³⁶ Another strain which was obtained from cattle feces, *L. fermentum* F1, showed antimicrobial activity against *S. aureus* and *E. coli*.³⁰ Few antibacterial strains of *L. brevis* have been reported as well. *Streptococcus mutans*, a pathogen responsible for dental caries was found to be inhibited by a *L. brevis* strain that was isolated from the Korean sauerkraut kimchi.³⁸ Inhibitory effect of another *L. brevis* strain obtained from an Egyptian dairy food was documented against *E. coli*, *S. aureus* and *S. typhi*.¹⁷

In the present study, antimicrobial activity of the LAB isolates was examined against a relatively large number of clinically relevant pathogens that are known to cause a range of serious infections in humans. *A. baumannii*, for example, has been frequently reported in recent years as a causative agent of pneumonia, meningitis, bacteremia, urinary tract infections (UTIs), wound infections, and infections of the skin and soft tissue.^{39,40} *K. pneumoniae*, another major pathogen used in the antimicrobial assay, also causes several nosocomial infections and is responsible for pneumonia, UTIs, blood stream infections and sepsis.⁴¹ Moreover, these infections have the ability to metastatically spread and have high rates of morbidity and mortality. Another opportunistic pathogen, *P. aeruginosa*, is also known to cause serious infections such as malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia.⁴² One of the two *Salmonella* serovars used in the antimicrobial assays, *S. typhi*, causes the life-threatening disease typhoid fever.^{43,44} The other serovar, *S. abony*, is relatively innocuous, but recently it has been implicated with severe invasive disease and lung abscess.⁴⁵ Infections with *S. flexneri* may also develop into life-threatening condition if not carefully treated.⁴⁶ The three Gram positive pathogens used in this work, *B. cereus*, *B. subtilis*, *S. aureus*, are commonly recognized as food poisoning agents and responsible mainly for bacteremia, gastrointestinal and pulmonary illnesses, and skin infections as well.⁴⁷⁻⁵⁰ An additional concern regarding the above infections is the recent advent

of multi drug resistance (MDR) in the pathogenic microbes.^{44,51} All the test pathogens used in the present study for antimicrobial assays are known to carry resistance towards multiple antibiotics.⁵²⁻⁶¹ As a result, treatment of their associated diseases has become very challenging with a few therapeutic options left. In this perspective, natural presence of the antimicrobial lactic acid bacteria in foods and beverages, or their exogenous application in starter culture, functional foods and therapeutics would be of particular benefit to help build protection from the pathogens.

Conclusion

Findings of the present study suggested beneficial lactic acid bacteria to be present in the traditional South Asian beverage borhani. The two species of lactic acid bacteria, *Limosilactobacillus fermentum* LAB-1 and *Levilactobacillus brevis* LAB-5 which were isolated from borhani, showed strong antimicrobial activity in agar-diffusion and co-incubation assays. Both isolates could inhibit a wide spectrum of pathogens with a prolonged antimicrobial activity which highlights their important role in enhancing food safety. Their inhibitory effects against spoilage microbes suggest their potential application as food preservatives as well. Upon further in-vivo assessment of their various functional properties, the isolates could be considered as starter culture, adjunct culture or probiotic culture in the fermented foods, functional foods and medications. It is also worth noting that presence of such beneficial lactic acid bacteria as the resident microflora in borhani indicate the potential of getting additional health benefits from this popular beverage.

Ethical statement

This article does not contain any experiments done with human participants or animals performed by any of the authors.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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