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Screening of *Lactobacillus* spp. from Buffalo Yoghurt for Probiotic and Antibacterial Activity

Abhijit Chowdhury[1], Md. Nur Hossain[1], Nure Jannatul Mostazir[2], Md Fakruddin[1], Md. Morsaline Billah[2] and Monzur Morshed Ahmed[1]*

[1]*Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka-1205, Bangladesh*

[2]*Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh*

Abstract

Since the beneficial effects of viable probiotic bacteria as dietary supplements have gained huge research interest, Lactobacillus spp. with probiotic characteristics are widely used to prepare fermented dairy products such as yoghurts, milk-shakes etc. In this study, eight (08) homemade yoghurt samples were collected from different regions in the country for isolation of probiotic Lactobacillus spp. Among the samples, four (04) isolates were identified as Lactobacillus plantarum based on their growth and biochemical characteristics. The Isolates were resistant to NaCl (1-9%) and bile-salt (0.05-0.3%) and showed good growth in the acidic condition, while maximum growth was observed at pH around 6.0. The isolates were examined for their antibacterial activity against nine (09) different test pathogens and found all pathogens are inhibited their growth to some extent but maximum zone of inhibition was observed against *Bacillus cereus* (53.20 mm) and minimum was against *Staphylococcus aureus* (19 mm) after 72 hour incubation. The results of the present study indicate that, homemade yoghurts in Bangladesh are potential source of probiotic Lactobacillus spp. Further extensive research on isolation and characterization of probiotic organisms from local fermented foods and their growth optimization might be required for development of probiotic enriched food supplements in our country.

Keywords: Probiotic bacteria; *Lactobacillus* spp; Yoghurt; Antibacterial activity; Food supplements

Introduction

Lactic Acid Bacteria (LAB) including *Lactobacillus* spp. are Generally Recognized as Safe (GRAS) bacteria that have been used in the processing of fermented food for centuries [1]. They occur naturally as indigenous microflora in fermented milk products such as yoghurt [2]. There is a growing interest in the use of *Lactobacillus* spp. as probiotics due to the increasing emergence of antibiotic resistance [3].

Increased antibiotic usage is a key factor in the emergence of antibiotic resistant pathogens. Thus there is an urgent need to develop alternatives to antibiotics [4]. Probiotics such as *Lactobacillus* spp. are reported to have inhibitory activity against common human pathogens [5-7]. They are able to produce antimicrobial substances such as bacteriocins which have great potential to be used in therapeutics and as food bio-preservatives [8]. Lactic acid bacteria including *Lactobacillus* spp. are gaining increasing interests worldwide to be used in the prevention, control and treatment of diseases and health maintenance [4]. To confer health benefits, probiotics must overcome physical & chemical barriers such as acid & bile in the small intestine [9].

Considering the above facts in mind, the present study was undertaken to isolate and characterize indigenous *Lactobacillus* spp. from traditional Bangladeshi yoghurt and to assess their anti-bacterial activity against some common human pathogens *in vitro*.

Materials and Methods

Collection and enrichment of samples

A total of eight (08) yogurt samples were collected from local markets of Dhaka, Bogra and Jhenidah districts of Bangladesh and were transported to the laboratory under contained sample box. 10 g of each yogurt sample was separately dissolved in 90 ml of sterile 0.86% normal saline, the samples were then enriched in MRS broth for 24 hours at 37°C.

Isolation & identification of the isolates

The enriched yoghurt samples in MRS broth were taken and streaked on to the MRS agar plates and were incubated in anaerobic jar at 37°C for 72 hours. The suspected *Lactobacillus* spp. were further pure cultured for morphological and biochemical identification. Gram reaction and microscopic study were performed for the isolates of 18 hour culture from MRS agar plates. The biochemical tests performed were Simmon's Citrate Slant test, Indole test, Methyl Red (MR), Voges Proskauer (VP), Oxidase and Catalase tests. Identification of isolates obtained in pure culture was based on Gram staining, morphology, growth characteristics on selective and differential media such as MRS (HI-MEDIA, India) agar and Rogosa SL agar (HI-MEDIA, India) and biochemical test results recommended in the Bergey's Manual of Determinative Bacteriology [10,11].

Biochemical characterization of the isolates

Carbohydrate utilization: Carbohydrate utilization capability of the isolates was tested according to Forouhandeh et al. [3]. Glucose, xylose, sucrose, fructose, galactose, lactose, maltose, trehalose, ribose, rhamnose, mannitol and dextrose were used in this study as carbohydrate.

Sodium chloride (NaCl) tolerance: For determination of NaCl tolerance, all the isolates were grown in MRS broth supplemented with different concentrations of NaCl (1-9%). The broths were inoculated with 100 µl overnight culture of the isolates and incubated anaerobically

***Corresponding author:** Monzur Morshed Ahmed, Industrial Microbiology Laboratory, Institute of Food Science & Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka- 1205, Bangladesh, E-mail: monzur_29@yahoo.com

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at 37°C for 24 hour. After 24 hour incubation, growth was determined using a spectrophotometer, reading the optical density at 600 nm [12].

Bile salt tolerance: Bile salt tolerance of the isolates was investigated by determining their growth in MRS broth containing different levels (0.05, 0.1, 0.15, 0.3 and 0.5) of bile salts (Ox-gall). Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 h under anaerobic condition. Optical densities were measured using a spectrophotometer at 560 nm after 24 h incubation [12].

Optimization of culture pH: For the determination of optimum culture pH growth of the isolates, 100 µl overnight culture of the isolates were inoculated into MRS broth with varying pH ranging from 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The inoculated broths were then incubated in anaerobic condition for 24 h at 37°C. Growth of the bacterial isolates was measured using a spectrophotometer at 560 nm.

Organic acid production: Quantification of organic acids produced by the isolates was performed according to Hoque et al. [13]. MRS broth supplemented with 10% skim milk inoculated with 1% (v/v) or 100 µl overnight culture of the isolates and incubated in anaerobic condition at 37°C for 72 hour. Fermented samples were collected in every 24 h, 48 h and 72 h and liquids of coagulated milk were separated by filtration. After filtration, the pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was done through titration with 0.1 N NaOH using phenolphthelin as pH indicator.

Antibacterial activity of the isolates: Agar overlay method was used to determine the antimicrobial activities of the isolated *Lactobacillus* spp. [14]. Nine different human pathogens belonging to both gram-positive and gram negative groups such as *Bacillus subtilis* (laboratory strain), *Bacillus megaterium* (laboratory strain), *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 65154, *Salmonella paratyphi* (laboratory strain), *Vibrio parahaemolyticus* ATCC 17802 were used in this study as test pathogen. Antibacterial activity was further characterized by determining whether bacteriostatic or bactericidal. The test was performed by swabbing of the growth inhibition zone. The swab was streaked onto nutrient agar plate and incubated aerobically at 37°C for 24 hours. The presence of growth in nutrient agar plate was interpreted as an inhibitory activity i.e. bacteriostatic, while no growth was interpreted as bactericidal.

Results

Growth and morphology on selective media

A total of four (04) isolates among others were identified as *Lactobacillus plantarum*. The isolates were subjected to grow on selective MRS agar media and produced round shape, off-white to cream colour, shiny colonies those were quite similar to the reference *Lactobacillus* spp. grown on MRS agar media (Figure 1A). Isolates when Gram stained, found rod shaped, short-medium chain and positive in Gram reaction those all are typical characteristics of *Lactobacillus* spp. (Figure 1B). The isolates were able to grow at pH between 4.0 and 8.0, but the optimum growth was observed at pH between 5.5 and 6.5 when grown in MRS broth at 37°C.

Biochemical characterization

For biochemical characterization, catalase, oxidase, indole, MR, VP and citrate tests were conducted and found negative for all the isolates. Carbohydrate utilization test was performed to investigate whether the isolates can ferment lactose, sucrose, glucose, maltose, galactose, fructose, mannitol, ribose, dextrose, xylose, rhamnose and threlose. The results described in table 1 show that all the isolates were able to ferment given carbohydrates.

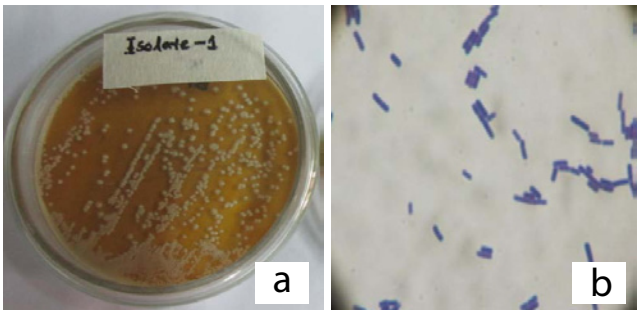

Figure 1: (a) typical colony characteristics of the isolates grown on MRS agar medium; (b) Microscopic view of the isolates when Gram stained.

Identification of isolates

On the basis of biochemical results, all the isolates belonged to *Lactobacillus plantarum* according to Bergey's Manual of Determinative Bacteriology [10].

Probiotic and antibacterial activity

Bile-salt and sodium chloride (NaCl) tolerance of the isolates were conducted to determine whether the isolates posses probiotic activity within themselves. The isolated *Lactobacillus* strains from yogurts were able to tolerate 1-9% w/v concentration of NaCl in the MRS broth. Figure 2A shows that all the isolates maintained good growth up to 3% conc. of NaCl and growth declined sharply with the increase of salt concentration in the broth. On the other hand, *Lactobacillus* isolates were able to maintain good growth and multiplication up to 0.1% w/v supplementation of bile-salt in MRS broth. Figure 2B shows that growth of *Lactobacillus* declined with increased bile-salt supplementation. The results indicate that the isolated *Lactobacillus* spp. might have potential to be used as probiotic bacteria provided toxicity and other researches are carried out.

Quantification of Organic Acid and Determination of pH Value

The present experiment indicates that organic acid production was increased with the incubation time. On the other hand, pH of the media decreased with the increasing acid production. From the results showed in figure 3 Highest acidity (1.8%) was observed after 72 h incubation at 37°C for *Lactobacillus* spp. isolated from Bogra (Y_1). On the other hand, other probiotic bacteria isolated from yoghurt of Dhaka showed the acid (2.12%), Jhenidah region also showed the acid (2.07%) and acid (1.98%) value after 72 h incubation.

Determination of antimicrobial activity

The selected isolates were examined to investigate their antimicrobial activity by modified agar overlay method. For this purpose, strains were subjected against the indicator microorganisms such as *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi*, *Salmonella paratyphi* and *Vibrio parahaemolyticus*

The diameter of inhibition zones (Figure 4 and 5) show that all of the isolates have antibacterial effect on the indicator microorganisms. Whether the isolates are bacteriostatic or bacteriocidal, confirmation

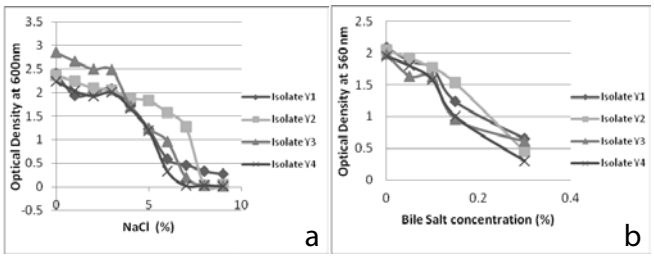

Figure 2: Sodium chloride (NaCl) and bile-salt tolerance of isolated *Lactobacillus* spp.

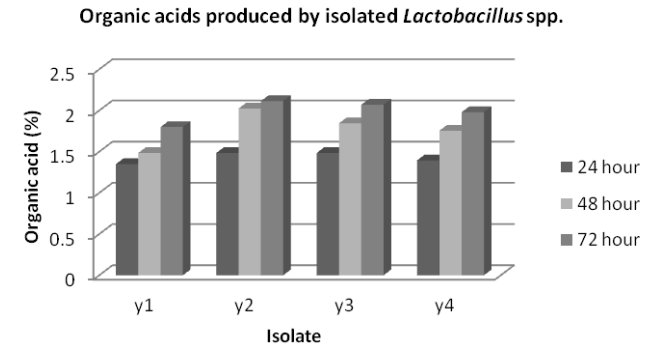

Figure 3: Organic acids produced by isolated *Lactobacillus spp*. at 37°C.

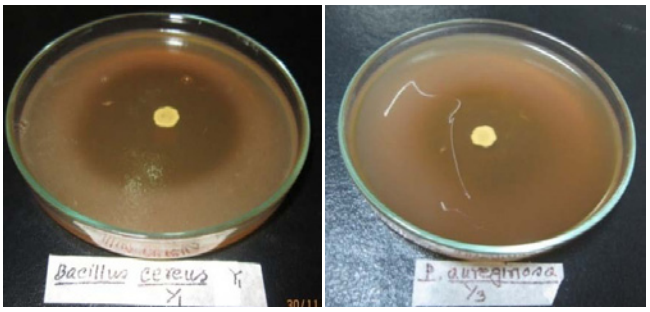

Figure 4: Antimicrobial Activity of isolated *Lactobacillus spp*. Clear zone indicates inhibition of bacterial growth.

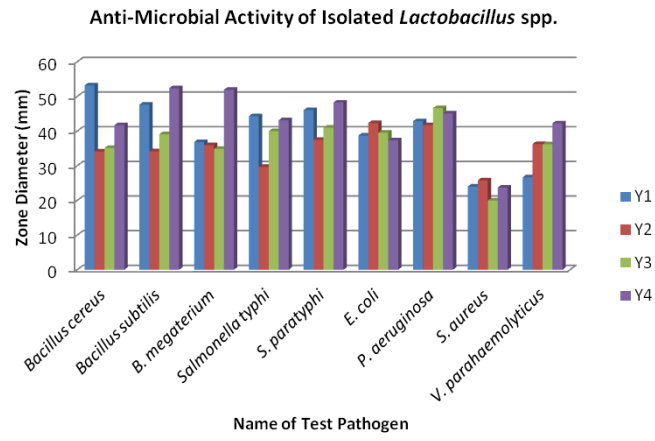

Figure 5: Antimicrobial activity of isolated Lactobacillus spp. against test pathogens.

Test parameters	Results			
	Isolate- Y_1	Isolate -Y_2	Isolate- Y_3	Isolate- Y_4
Gram Stain	+	+	+	+
Simmon's Citrate Slant test	-	-	-	-
Indole tes	-	-	-	-
Methyl Red (MR) test	-	-	-	-
Voges Proskauer (VP) test	-	-	-	-
Oxidase test	-	-	-	-
Catalase tests	-	-	-	-
Glucose fermentation	+	+	+	+
Xylose fermentation	+	+	+	+
Sucrose fermentation	+	+	+	+
Fructose fermentation	+	+	+	+
Galactose fermentation	+	+	+	+
Lactose fermentation	+	+	+	+
Maltose fermentation	+	+	+	+
Trehalose fermentation	+	+	+	+
Ribose fermentation	+	+	+	+
Rhamnose fermentation	+	+	+	+
Mannitol fermentation	+	+	+	+
Dextrose fermentation	+	+	+	+

(+= positive result (Gram positive in case of Gram staining and Capability to ferment in case of sugar fermentation); - = negative result (Gram negtive in case of Gram staining and unability to ferment in case of sugar fermentation))

Table 1: Biochemical reactions and carbohydrate fermentation by the isolates.

Name of the test organism	Isolate Y_1	Isolate Y_2	Isolate Y_3	Isolate Y_4
Bacillus cereus	+	-	+	+
Bacillus subtilis	+	+	-	-
Bacillus megatorium	-	-	-	_
Salmonella typhi	-	-	+	-
Salmonella paratyphi	+	+	-	-
E. coli	+	+	+	+
Pseudomonas aureginosa	+	+	+	+
S. aureus	+	-	-	-
Vibrio parahaemolyticus	-	+	+	+

(- = Bacteriocidal; += Bacteriostatic)

Table 2: Bacteriocidal and Bacteriostatic activity of isolate Y_1, Y_2, Y_3 and Y_4

tests were conducted, in this case swabs were taken from each clear zone of the test organism and were streaked on to the nutrient agar plates for growth. Depending on the growth, the bacteriostatic and bacteriocidal activities are classified in the table 2. Presence of growth of the indicator organism was interpreted as an inhibitory activity, called bacteriostatic, while no growth was interpreted as bacteriocidal.

Discussion

The goal of this research work was to isolate and characterize potential probiotic bacteria from yoghurt samples of Bangladesh and to assess their anti-bacterial activity against some common pathogenic bacteria. Based on the morphological characteristics four (4) isolates were identified as *Lactobacillus* spp. from yoghurt samples. After gram staining the isolated bacteria were rod shaped, convex, rough, smooth, shiny, irregular, circular, gram positive, facultative anaerobic, non-spore forming which indicate them to be the member of *Lactobacillus* spp [15]. The significant growth of the isolates at pH 6.5 on MRS – agar plates in anaerobic conditions further confirmed their identification as *Lactobacillus* spp. [16]. Oxidase, catalase and IMViC test of selected isolates gave same results as *Lactobacillus* spp. All of the isolates were Indole, MR, VP, Citrate, Oxidase and Catalase negative, the results are similar with the findings of Elizete and Carlos [17].

Among the carbohydrates used in this study, all the four isolates were able to ferment glucose, sucrose, fructose, lactose, xylose, ribose, galactose, maltose, mannitol, trehalose, rhamnose and dextrose. It indicates that they are able to grow in variety of habitats utilizing different type of carbohydrates.

pH is an important factor which can dramatically affect bacterial growth. To be used as probiotic, organisms have to tolerate low pH of human gut. The isolated *Lactobacillus* spp. can tolerate a wide range of pH (1-9) and grow well at acidic pH (1-5). NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria and probiotic organisms have to withstand high salt concentration in human gut [18]. The current results showed that *Lactobacillus* spp. isolated from yoghurts was able to tolerate 1-9% of NaCl and good growth was observed at 1-5% NaCl (Figure 2A). In this present study, 0.05-0.3% bile salt were supplemented in the growth media, as it corresponded to that found in the human intestinal tract and 0.3% is the maximum concentration that is present in healthy men [12]. Therefore, before selection of probiotic bacteria for human consumption it must be endurable to 0.3% bile concentration [19]. *Lactobacillus* spp. isolated in this study was resistant to 0.3% bile salt. All of the isolates are able to survive and grow in 0.3% bile salt concentration.

The present experiment indicates that organic acid including lactic acid production was increased with the incubation time and the pH of the media decreased with the increasing acid production. From the results table, highest acidity (1.8%) and lowest pH (3.63) was observed after 72 h incubation at 37°C for *Lactobacillus* sp. isolated from Bogra yoghurt. On the other hand, other probiotic bacteria isolated from yoghurt of Dhaka region of Bangladesh showed the acid (2.12%) and lowest pH (3.62), Jhenidah region also showed the acid (2.07%), lowest pH (3.64) and acid (1.98%), lowest pH (3.70) value after 72 h incubation. This investigation indicates that, there is a minor variation in organic acid production by Lactobacilli due to their regional variation.

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial effects of lactic acid bacteria are incurred by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins [20]. Probiotics including *Lactobacillus*, Bifidobacterium and *Streptococcus* spp. are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favorable effects against disease caused by an imbalance of the gut microflora, several experimental observations have showed a potential protective effect of probiotic bacteria against the development of colon tumors [21].

All the *Lactobacillus* isolates showed to inhibit the test organisms included in this study though they vary in zone of inhibition diameter. Our experimental result showed that, the highest inhibitory activity of isolate Y_1 was demonstrated against *Bacillus cereus* (52 mm) and lowest zone of inhibition was (24.05 mm) against *Staphylococcus aureus* after 72 hour incubation. The highest diameter of inhibition zone of isolate Y_2 was showed against *E. coli* ATCC 8739 (42.35 mm) and lowest zone (25.81 mm) against *Staphylococcus aureus* after 72 hour incubation. Highest diameter of inhibition zone of isolate Y_3 was showed against *Salmonella paratyphi* (44.0 mm) and lowest zone (20.0 mm) against *Staphylococcus aureus* after 72 hour incubation. In case of isolate Y_4 highest zone was found against *Salmonella paratyphi* (48.26 mm) and lowest zone (27.73 mm) against *Staphylococcus aureus* after 72 hour incubation.

In the study by Gharaei-Fathabad and Eslamifar [1] a strain of *Lactobacillus* paraplantarum isolated from tea leaves showed strong inhibitory activity against *Salmonella typhi* (65 mm), *E. coli* (30 mm), Staphylocouus aureus (56 mm), *Enterococcus faecalis* (55 mm) and Citrobacter sp (60 mm). Isolates of the present study have almost similar antimicrobial capability. In the study of Osuntoki et al. [4], *Lactobacillus* spp. isolated from fermented dairy products showed antibacterial activity against some clinically important pathogens such as Enterotoxigenic *E. coli* (4.2 mm), *Salmonella typhi*murium (4.3 mm) and *Listeria monocytogenes* (5.0 mm). Isolates of the present study have better antimicrobial capability than these *Lactobacillus* spp. isolates. Our isolates showed almost similar antagonistic activity against *E. coli* and *Salmonella typhi*murium as compared to *Lactobacillus plantarum* and *Lactobacillus* salivarius isolated by Murray et al. [5] from a botanical probiotic. Isolates of the present study also showed much better antimicrobial activity than that of bacteriocin extracted from lactic acid bacteria strains isolated from fermented milk by Savadogo et al. [21], in which study, bacteriocin produced 8 mm to 12 mm zone of inhibition against *Bacillus cereus*, *Staphylococcus aureus* and *E. coli*.

Inhibition of test organisms was both bactericidal & bacteriostatic type (Table 1). Isolate Y_1 was bactericidal to *Bacillus megaterium*, *Salmonella typhi* and *Vibrio parahaemolyticus* and bacteriostatic to *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa*, *Salmonella paratyphi*. Isolate Y_2 was bactericidal to *Bacillus cereus*, *Salmonella typhi* and Bacillus sutilis and bacteriostatic to *Staphylococcus aureus*, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Vibrio parahaemolyticus* and *Bacillus megaterium*. Isolate Y_3 was bactericidal to Bacillus sutilis, Bacillus megatorium, *Salmonella paratyphi* and *Staphylococcus aureus*, and bacteriostatic to *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio parahaemolyticus*, Bacilluscereus and *Vibrio parahaemolyticus*. Isolate Y_4 was bactericidal to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, Bacillussutilis and bacteriostatic to *Bacillus cereus*; and *Salmonella typhi*, Bacillus megatorium, *Salmonella paratyphi* and *Escherichia coli* ATCC 8739.

Conclusion

The isolated *Lactobacillus* spp. fulfills the required criteria for a probiotic such as tolerance to harsh conditions such as high salt, low pH and high bile salt concentration and can produce bacteriocin extracellularly which inhibits a no of pathogenic organisms. These isolates may be considered potential to be used as probiotic.

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