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Antibiotic resistant microencapsulated probiotics synergistically preserved orange juice

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Abstract

Background: In contemporary medicine, the utilization of various dosage forms of probiotics is increasing both for the treatment of human and animal diseases in Bangladesh. A number of imported pharmaceutical probiotic preparations are available in the local markets at present without justification the scientific information of viability. This study was, therefore, designed to explore the viability of commercial probiotics as well as recommend the consumers for the better products in term of viability. Since probiotics preserved foods have expanded in acceptance, popularity and compliance, the goal of this research was also to investigate the combination effects of lactic acid bacteria (LAB) on development of functional foods like orange juice (OJ).

Methods: Here, we screened five commercially available pharmaceutical probiotic products for rejuvenation and identification of LAB and associated resistance against different classes of antibiotics. Finally, the isolated LAB were microencapsulated and compared with free form of bacteria for biopreservation of OJ.

Results: We observed an inconsistency between the feasible live LAB counts and the declaration of the manufacturing companies. The recovered viable bacteria of pharmaceutical probiotic preparations ranged between $(6.2\text{ -}7.3) \times 10^{10}$ at 37 °C and $(5.33\text{-}7.1) \times 10^{10}$ at 25 °C, the claimed $(9\text{-}10) \times 10^{10}$ colony forming unit (CFU)/g. The encapsulated *Lactobacillus acidophilus* (LAB 1), *L. bulgaricus* (LAB 2), *Lactococcus lactis* (LAB 3) and *Bifidobacterium bifidum* (LAB 4) in OJ was resistant to drop out their viability as quickly as the free-form probiotic bacteria and $>10^6$ CFU/mL were still appeared after 6 wks of storage. Unencapsulated probiotics was found to have a significant reduction in viability in OJ at both 37 °C and 4 °C temperatures. However, the microencapsulation process significantly reduced the loss of viability of four probiotic bacteria as well as the control of acidification of OJ at 4 °C.

Conclusions: The loss of potency and spoiled food associated with pathogenic microbial growth are serious problems in tropical countries including Bangladesh. The biopreserved OJ will become an important functional food due to its expansion of shelf-time, market reputation, profits and innate tastes. This report has an indication that the combination of these four LAB may become good candidate for the development of an OJ with functional characteristics.

Keywords: Microencapsulated lactic acid bacteria, Antibiotic sensitivity, Orange juice, Functional food

Abbreviations: Amx, Amoxicillin; Cef, Ceftazidime; CFS, Cell-free supernatant; Chlor, Chlorampenicol; Cipro, Ciprofloxacin; Dox, Doxycycline; Eryth, Erythromycin; Kan, Kanamycin; LAB 1, *Lactobacillus acidophilus*; LAB 2, *L. bulgaricus*; LAB 3, *Lactococcus lactis*; LAB 4, *Bifidobacterium bifidum*; LAB, Lactic acid bacteria; M:P, ratio 1:5, Methyl paraben and propyl paraben; MB, Methyl-p-hydroxy benzoate; Pen G, Penicillin-G; SB, Sodium benzoate; Strep, Streptomycin; Tet, Tetracycline

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Background

In biopreservation technology, LAB are used to lengthen the shelf life and balance the growth and multiplication of internal pathogens in foods [1, 2]. LAB yield a wide variety of compounds such as lactate, hydrogen peroxide (H_2O_2) as well as antibiotics, bacteriocins that directly inhibit other microorganisms for their depletion of nutrition [3]. LAB are also joined with the formation of several foods and thus have typically recognized as safe (GRAS) status granted by the Food and Drug Administration (FDA) [4–6]. Furthermore, probiotic properties of some LAB provide a number of beneficial effects including regulation of intestinal microbial homeostasis and modulation of local and systemic immune responses [7].

Functional food is generally designated if it gives beneficial effects on body in addition to the classical nourishing value. Functional foods, for example OJ are prepared with bioactive "friendly" LAB [8]. Beyond the functional components, like vitamins and minerals, the inclusion of LAB in OJ can provide an effective means for the generation of healthy food with the increase in life expectancy [8, 9]. Thus, development of foods with probiotic bacteria is one of the supreme research preferences in the food industry as well as nutripharmaceutical companies.

Advances in food science and technology has eliminated but not completely eradicated the food-borne diseases in developed countries. With consumers becoming more concerned about the levels of chemical preservatives present in food, bacteriocins secreted by LAB offer an alternative, more natural approach, while ensuring both food safety and product shelf life [10]. In order to fit the consumer interests with required quality compliance, the classical means of food processing and preservation are being replaced by various advance technologies as like microencapsulation. This trend of techniques has approved the utilization of LAB in biopreserved foods [11–14]. A lot of pharmaceutical probiotic products in various forms spread in the local markets without verification the scientific information such as identity of strains, probiotic properties and also viabilities. This study was, therefore, designed to investigate the viabilities of different commercial sources of probiotic products in Bangladesh as well as compared for the better products to the consumers. Resistance of antibiotic has appeared a serious threat for public health globally and is acquired the interest of health professionals and food scientists in all around the world. Since the consumption of commercial probiotics is increased, it is necessary to give sufficient protection to the consumers from any adverse reactions of the products. The quality and safety considerations of these probiotic strains are the prime prerequisite with resistance to antibiotics as an assessment issue. Since probiotic foods have gained widespread acceptance, the purpose of this study was also to formulate a functional food as prototype of OJ by using different combination of microencapsulated LAB.

Methods

Sample collection and processing

Samples of different pharmaceutical probiotics were obtained from local market. The brand names and respective pharmaceutical companies of probiotic products were Probio (Square Pharmaceuticals Ltd.); Prolacto (Drug International Limited); Protik Vitality (Kemiko Pharmaceuticals Ltd.) Bangladesh; Preotik (Meridian Medicare Ltd. India) and TS6 (Tensall Bio-Tech Co. Ltd. Taiwan) and the samples were designated as S1 to S5 (Table 1). For rejuvenation, recovery and enumeration of viable probiotic bacteria, the plate counts and spectroscopic assay (OD_{690nm}) were performed as described previously [15]. Briefly, approximately 1 g of powder of individual pharmaceutical product (capsule or sachet) was mixed homogeneously with sterile phosphate buffered saline (9 mL PBS, pH 7.0) and kept in an incubator for 1 h at 25 °C. Then, the specimens were diluted in 10-times serially with PBS and 100 μL of each diluted sample was then mixed well with deMan Rogosa and Sharpe (MRS) agar (Difco, USA) and incubated under anaerobic conditions at 25 °C and 37 °C temperatures as well as pHs (4.5 and 6.5) under anaerobic and aerobic conditions for 24–48 h. After 48 h of incubation, 3–4 colonies were taken up and shifted to MRS broth and incubated for 24 h. The pure cultures of colonies were streaked on MRS agar plates and incubated for further 24 h at 37 °C. Individual strains were relocated to 2 mL MRS broth and purified by repeatedly plating on MRS agar. Then, the entire strains were examined the morphology with microscope and pure cultures were preferred

Table 1 Common lactic acid bacteria in probiotic products available in Bangladesh

No.	Lactic acid bacteria	Market sample				
		S1	S2	S3	S4	S5
i.	*Lactobacillus acidophilus* (LAB 1)	√	√	√	√	√
ii.	*Lactobacillus bulgaricus* (LAB 2)	√	√	×	×	×
iii.	*Lactococcus lactis* (LAB 3)	×	×	√	×	√
iv.	*Lactobacillus casei*	×	×	×	×	√
v.	*Lactobacillus ramnosus*	×	×	×	√	×
vi.	*Bifidobacterium bifidum* (LAB 4)	√	√	×	√	√
vii.	*Bifidobacterium infantis*	×	×	×	×	√
viii.	*Bifidobacterium longum*	×	×	×	√	√
ix.	*Strepcoccus thermophyllus*	×	×	×	√	×
x.	*Saccharomyces boulardi*	×	×	×	√	×

√ indicated 'present' and × indicated 'absent'

for additional studies. The final concentrations of overnight cultured colonies were made in uniformity values as 1.0×10^9 CFU/mL by spectroscopy (OD_{690nm}) prior to antibiotic sensitivity tests using several antibiotic discs. Four LAB including *Lactobacillus acidophilus* (LAB1), *L. bulgaricus* (LAB2), *Lactococcus lactis* (LAB3) and *Bifidobacterium bifidum* (LAB4) were identified according to the methods described earlier [16]. The purified bacterial strains were kept at -20 °C in MRS broth with an additive, 20 % (v/v) glycerol for future studies.

In vitro antimicrobial activities

For primary screening of antagonistic activities, the individual LAB 1- 4 strains were transferred in MRS broth (50 mL) and mixed well in a shaker for 30 min and then cultured for 48 h at 37 °C. The cultures of LAB 1-4 were centrifuged for 15 min at 6000 rpm and the supernatants were decanted into sterile test tubes marked as cell free supernatants (CFS 1- 4). To examine the mechanisms of antimicrobial activities, the supernatants, CFS 1- 4 were adjusted to pH 7.0 with 5 N sodium hydroxide (NaOH) solution to exclude the effect of pH. The neutralized CFS1- 4 were further reacted with 2 mg/mL catalase solution (pH 7.0) for 0.5 h at 25 °C to minimize the suppressive activities of hydrogen peroxide (H_2O_2). Finally, the mixtures of CFS1- 4 were treated with 0.5 μg/mL proteinase K and incubated at 37 °C for 4 h. The mixtures of the CFS1-4 were decontaminated with membrane filter (pore diameter, 0.22 μm) before loading to the well plates. For preliminary screening of bacteriocin like inhibitory substance (BLIS) activity, the respective cultures of LAB were inoculated in 100 mL of MRS broth medium and incubated for 48 h at 37 °C in culture flasks with optimum physicochemical conditions. Aliquots (10 mL in each) of CFS1-4 extracted with ethyl acetate 1:2 separately by vigorous vortexing for 5 min and repetitively for 2 times. Then, ethyl acetate layers from each isolated cultures were collected, evaporated at room temperature, individually marked as extracts (Ext 1- 4) and tested for BLIS activities against pathogenic bacteria. For synergistic antimicrobial activity, the different combinations (1:1, 1:1:1 and 1:1:1:1) of BLIS extracted from LAB1-4 were mixed together and carried out the experiment. The antimicrobial activities of the LAB were performed by the agar well diffusion method accordingly by Georgieva et al. [17] with minor adjustment against pathogenic bacteria and fungi that were obtained from ICDDR'B, Dhaka. Shortly, the fresh culture (24 h) of pathogenic bacteria was immersed uniformly in nutrient agar. Then different concentrations (25, 33, 50 and 100 μL or μg) of CFS1-4 or Ext1-4 were applied and the agar plates were incubated at 37 °C for 24 h. The diameters of inhibitory zone were measured. The experiments were performed three times and the averages of the clear zones (greater than 6 mm in diameter) were recorded.

Antibiotic resistance test

In this study, the 10 antibiotic discs were used to determine antibiotic sensitivity of pathogenic bacteria and LAB1-4 strains. The antibiotic discs such as amoxicillin (30 μg); ceftazidime (30 μg); ciprofloxacin (5 μg); tetracycline (30 μg); chloramphenicol (30 μg); erythromycin (15 μg); doxycycline (30 μg); kanamycin (5 μg); penicillin-G (10 μg) and streptomycin (10 μg) were purchased from HiMedia, India. For comparison, three food chemical preservatives such as sodium benzoate (0.1 % w/v); methyl-ρ-hydroxy benzoate (0.1 % w/v) and the (1:5) solution of methyl- and propyl paraben (0.1 % w/v) were used in this study. The susceptibility tests for each pathogenic bacterium and LAB were performed using disc diffusion method [15]. The antibiotic susceptibilities were determined by the inhibitory zone formation and compared according to Vandepitte et al. [18].

Preparation of orange juice (OJ)

The high quality oranges (scientific name *Citrus sinensis*) were selected, segmented and the seeds were removed manually from the slices. The extraction of OJ was done by using blender and sterile filter. The OJ was stored in sterilized clean containers at 4 °C for sequential works. It is to be mentioned that no chemical preservatives were added to the OJ and a long shelf life were determined accordingly [19, 20]. In order to select for suitable growth condition of LAB, freshly prepared OJ (unadjusted pH 3.95-4.2) was modified for pH range (2.0-7.0) with citric acid-sodium citrate buffer solutions (Sigma Chemical Co.) and the viability of LAB in OJ was examined. For turbidity test, the absorbance of OJ before and after inoculation of LAB was determined at 540 nm with the help of UV spectrophotometer. The buffered OJ was inoculated with individual LAB1-4 and several blending of LAB as indicated previously and their viabilities for 4-6 wks duration were measured in MRS broth medium using 0.1-1.0 % inoculum, incubating at 37 °C. The MRS broth was removed completely by repeated washing in sterile PBS before inoculating the bacterial cells into OJ. Different combinations of LAB suspensions were added in aliquots of OJ (50 mL in each) and incubated them for 48 h at 37 °C. Then, free-form LAB in OJ was preserved for 5-6 wks at 4 °C in order to measure the viability of LAB.

Individual LAB1- 4 were cultured statically in 100 mL MRS broth for 18 h at 37 °C, centrifuged for 15 min at 4 °C with speed 4000 × g and collected the pellets after rinsed with sterile PBS (pH 7.4). Finally, individual 25 mL of LAB 1-4 solutions were prepared to obtain as

approximately 10^8 CFU/mL for both free-form and microencapsulated LAB. For microencapsulation, LAB 1-4 integrated into sodium alginate following a slight modification of method of Shah and Ravula [21]. Summarily, 100 mL of sterilized sodium alginate solution (3 % w/v) was mixed well with PBS washed LAB 1-4 solution (25 mL) at 21 °C. The suspension of alginate and LAB 1-4 was dispensed gently into a beaker containing a solution of 1.0 gm Tween 80 in 600 mL vegetable oil in order to form an emulsion. This emulsion was mixed well at constant speed (200 rpm) and mixed gradually with 0.1 M calcium chloride solution into beaker until phase separation occurred in the emulsion. The beads of calcium alginate were preserved by decanting the oil phase after 0.5 h, and stored for 10 h at 4 °C in order to allow the beads for complete hardness before being handled the experiments. The enumeration of free form of LAB and microencapsulated organisms were performed using methods illustrated by Zhang et al. [22] and Sohail et al. [13]. Enumeration of the LAB 1-4 in OJ was accomplished by consecutive 6 wks by culturing these organisms in MRS medium for 48 h in an anaerobic jar at 37 °C.

Statistical analysis

All the experiments performed here were carried out in triplicate and the results were expressed as mean ± S.D (standard deviation). Analysis of statistical data was evaluated by using Student's *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, n.s.: not significant, throughout the manuscript).

Results

Probiotic bacteria, most of the *Lactobacilli* strains, collected from different pharmaceutical industries were rejuvenated and rescued successfully on MRS media and their statistics was given in Table 1 and Fig. 1. The obtained bacteria have a wide variety of colony morphologies and finally four distinguishing bacteria were selected for identification and characterization [16]. The outcomes indicate that several probiotic strains were present in the corresponding brands and this was expected with the specifications of the respective companies. However, the bacteria counts indicated that all of the samples had lower numbers of probiotics than that declared by their producers (Fig. 1) and the results were consistent with other reports [23, 24]. Most importantly, the viable bacteria counts in S4 and S5 were between 100 and 1000 times lower than claimed in their respective datasheets.

Antimicrobial activities

The antagonistic activities of cell-free supernatants (CFSs) of LAB against pathogenic Gram- positive and negative bacteria, and fungi were detailed in Tables 2, 3 and 4. The results suggested that CFS1 and 3 had significant antimicrobial activities against all tested pathogenic bacteria and fungi and the CFS2 and 4 had moderate suppressive properties (Table 3). The antimicrobial activities of LAB were originated by the secretion of lactic acid, H_2O_2 or bacteriocin-like metabolites. To evaluate the inhibitory mechanism, at first the CFS1- 4 were treated with 5 N NaOH to eliminate the effects of secreting acids indicating that the CFS1- 4 contained H_2O_2 or bacteriocin-like metabolites. The results of neutralization of acid in CFS1- 4 indicated a mild decrease of antibacterial and antifungal properties. In order to determine if a combination of LAB would have a synergistic inhibitory effect on the growth of certain pathogens, we used different fractions of CFSs and extracts

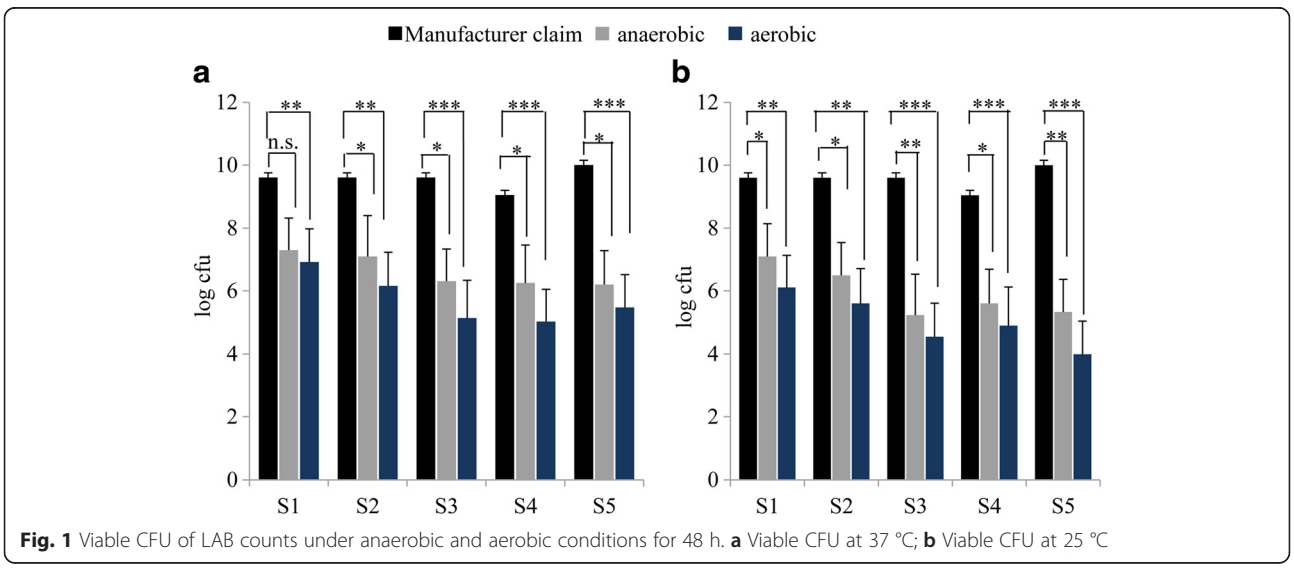

Fig. 1 Viable CFU of LAB counts under anaerobic and aerobic conditions for 48 h. **a** Viable CFU at 37 °C; **b** Viable CFU at 25 °C

Table 2 Antagonistic activities of LAB with different physicochemical treatments. Screening of antimicrobial activities of LAB after different treatment of cell free supernatants (CFS)

Treatment	*B. cereus*	*B. subtilis*	*Staphyl. aureus*	*E. coli*	*Shi. dysenteriae*	*Shi. sonnei*	*Saccharomyces cerevisiae*	*Aspergillus niger*	*C. albicans*
LAB1 CSF (100 μl)	++++	++++	++++	++++	++++	++++	+++	+++	+++
Neutralized by NaOH	+++	+++	++	+++	+++	++	+++	++	++
Catalase	++	++	++	+++	++	++	++	++	+
Protease K	+	-	+	+	+	++	-	+	+
Heat 121 °C for 15 min	+++	+++	+++	+++	+++	+++	++	++	++
LAB2 CSF (100 μl)	++	+++	++	++	+	++	+	+	+
Neutralized by NaOH	++	+++	++	++	+	++	+	+	+
Catalase	++	++	++	++	+	++	+	+	+
Protease K	+	+	+	+	-	-	-	-	+
Heat 121 °C for 15 min	+	+	+	+	-	-	-	-	+
LAB3 CSF (100 μl)	++++	+++	+++	+++	+++	++++	++	++	++
Neutralized by NaOH	+++	+++	+++	+++	++	+++	+/-	+	+
Catalase	++	++	++	++	++	++	+	+	++
Protease K	+	-	+	+	+	++	++	+/-	+
Heat 121 °C for 15 min	+++	++	++	+++	++	++	++	+	++
LAB4 CSF (100 μl)	++	+	+++	+++	++	++	++	++	+
Neutralized by NaOH	+	+	++	++	++	++	++	++	+
Catalase	++	+	+++	++	++	++	++	++	+
Protease K	+	+	-	-	-	+	+	-	+
Heat 121 °C for 15 min	++	+	++	++	++	++	++	++	+

-, No effect; +, 10.1-15 in mm; ++, 15.1-20 in mm; +++, 20.1-25 in mm; ++++, >25.1 in mm

Table 3 Antimicrobial activities of cell free supernatant (CFS) of LAB

Pathogenic organisms	Lactic acid bacteria cell free supernatant combination (100 μl)											Standard
	100 μl				50 μl + 50 μl			33 μl + 33 μl + 33 μl			25 μl in each	
	CFS 1	CFS 2	CFS 3	CFS 4	CFS (1 + 2)	CFS (1 + 3)	CFS (1 + 4)	CFS (1 + 2 + 3)	CFS (1 + 3 + 4)	CFS (2 + 3 + 4)	CFS (1 + 2 + 3 + 4)	Strep (10 μg)/ Nys (30 μg)
Gram positive bacteria						Zone of inhibition (mm)						
B. cereus	25	20	27	17	15	28	23	18	30	22	32	23
B. subtilis	24	22	31	12	17	29	22	22	31	25	33	28
Staphyl. aureus	28	18	31	22	16	30	28	21	32	24	38	27
Gram negative bacteria						Zone of inhibition (mm)						
E. coli	25	17	28	22	16	27	21	17	34	25	36	25
Shi. dysenteriae	25	15	30	21	14	27	22	15	31	21	37	30
Shi. sonnei	26	19	27	18	14	28	21	18	35	24	39	21
Pathogenic fungi						Zone of inhibition (mm)						
S. cerevisiae	22	13	18	16	13	22	23	16	26	17	28	21
A. niger	19	15	20	15	13	23	24	16	27	15	27	20
C. albicans	26	12	18	12	15	26	25	20	29	17	31	23

Strep streptomycin and *Nys* nystatin

Table 4 Antimicrobial activities of LAB extracts

Pathogenic organisms	Lactic acid bacteria extract combination (100 μg)											Standard
	100 μg				50 μg + 50 μg			33 μg + 33 μl + 33 μg			25 μg in each	
	Ext 1	Ext 2	Ext 3	Ext 4	Ext (1 + 2)	Ext (1 + 3)	Ext (1 + 4)	Ext (1 + 2 + 3)	Ext (1 + 3 + 4)	Ext (2 + 3 + 4)	Ext (1 + 2 + 3 + 4)	Strep (30 μg)/ Nys (30 μg)
Gram positive bacteria	Zone of inhibition (mm)											
B. cereus	19	15	21	16	11	23	22	17	25	21	27	22
B. subtilis	18	17	19	13	12	24	24	21	24	22	29	29
Staphyl. aureus	22	15	20	18	12	25	23	18	23	20	32	22
Gram negative bacteria	Zone of inhibition (mm)											
E. coli	19	17	20	19	12	24	20	14	25	25	26	24
Shi. dysenteriae	21	15	21	18	13	23	22	16	24	21	27	22
Shi. sonnei	20	12	20	19	13	24	19	17	25	24	29	25
Pathogenic fungi	Zone of inhibition (mm)											
S. cerevisiae	15	11	17	14	12	21	21	15	21	15	25	21
A. niger	15	12	16	12	12	22	22	14	22	16	24	20
C. albicans	17	10	13	10	11	23	23	18	24	14	23	23

Strep streptomycin and *Nys* nystatin

isolated from LAB1- 4 and tested against pathogenic bacteria and fungi (Tables 3 and 4). A sequential synergistic suppressive effect was observed for the combinations of CFS1,3; CFS1,3,4 and CFS1,2,3,4 and significantly reduced multiplication of different pathogens such as *Staphylococcus aureus*, *Shigella sonnei* or *Candida albicans*. These data suggested that combination of LAB 1, 2, 3 and 4 strains showed a maximal antagonistic activity against pathogenic organisms. From the results, it is suggested that the co-cultures of these strains would be beneficial in controlling the growth of pathogens in acidic media like fruit juices. To investigate the bacteriocin like inhibitory substance (BLIS) activity, the Ext1, 2, 3 or 4 were mixed in different proportions and tested against *Staphyl. aureus*, *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Shi. dysenteriae* and *Shi. sonnei* (Table 4). The BLIS of Ext1 showed the highest inhibition against *Staphyl. aureus*, *Shi. dysenteriae, Shi. sonnei, B. cereus, E. coli* and *B. subtilis* where the zones of inhibition were 2.2, 2.1, 2.0, 1.9, 1.9 and 1.8 cm respectively. However, the antimicrobial activities were increased when the extracts were given in different combinations. Ext1 and 3 showed a better activity, Ext 2 and 4 showed mild to moderate activities against all the test organisms. Interestingly, the combined form, Ext 1- 4 exhibited the greater synergistic antagonism against *Staphyl. aureus, B. subtilis* and *Shi. sonnei*.

Susceptibility of LAB to antibiotics

The LAB1-4 and pathogens like *B. cereus, B. subtilis, Staphyl. aureus, E. coli*, *Shi. dysenteriae* and *Shi. sonnei* varied in their sensitivity to different antibiotics. The results suggested that the LAB 1 and 2 were resistant to different antibiotics whereas LAB 3 and 4 were susceptible only for amoxicillin, ciprofloxacin and penicillin G. It was noted that all of the LAB were susceptible to the food preservatives and the zone of inhibition was less than 10 mm which assured resistance to antibiotics.

Survival rates of free-form and microencapsulated LAB in OJ

The survival rates of both unencapsulated and encapsulated *Lactobacilli* included into OJ was given in Fig. 2a and b. Unencapsulated LAB in co-cultures degenerated quickly in the OJ within 4 wks and there were no viable LAB left by the fifth wk (Fig. 2a). In free-form, most of the LAB in monocultures and co-cultures appeared in comparable depletion of viability. However, a synergistic action occurred when LAB 1- 4 was given in combination and the viable bacteria were found to be maximal than that of individual cultures and other co-culture system. Free-form LAB disappeared gradually when compared to monocultured bacteria in the OJ within 4 wks. At the beginning of fifth wk, the viable bacteria were approximately 10^4 CFU/mL. Since, OJ was acidic and the survival rate of multiple co-cultured probiotic bacteria was enhanced than any monocultured bacteria. The deprivation of encapsulated bacteria in OJ was apparently stable in respect to their viability as compared to the free-form bacteria and the bacteria were present about 10^6 CFU/mL at the end of 6 wks (Fig. 2b).

Biopreservation and stability of OJ

The results of preservative activity of LAB were shown in Fig. 3. The variation of pH in OJ inoculated unencapsulated and microencapsulated LAB and their preservative

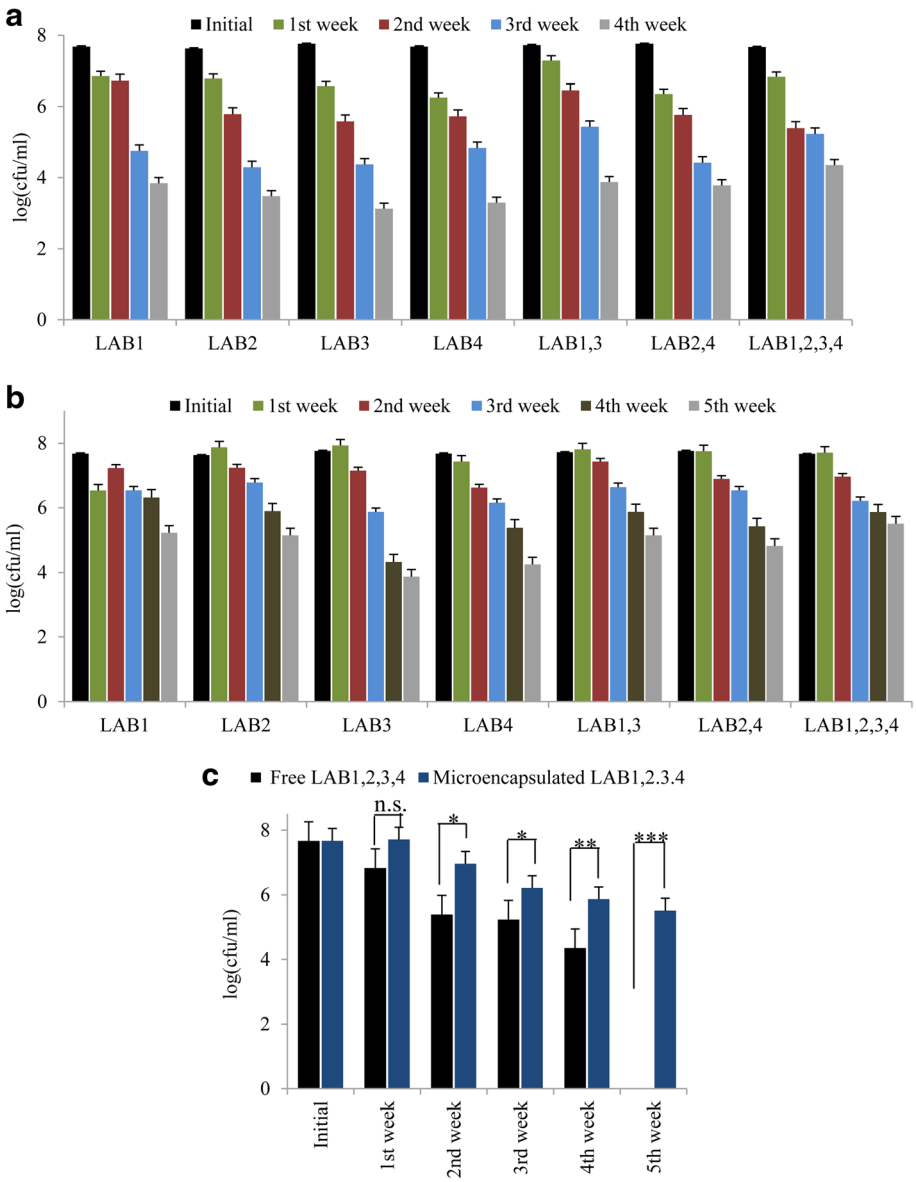

Fig. 2 LAB preserved OJ stored at 4 °C over a period of 5 wks. **a** Free form LAB; **b** Microencapsulated LAB; **c** Comparison of viable CFU of free form and microencapsulated LAB

effects during 4-6 wks storage condition were delineated in Fig. 3. An identical fashion in the fall of pH was observed in both free-form and encapsulated LAB 1-4 in OJ. However, the terminal pH of OJ immersed with encapsulated LAB during 6 wks storage condition was larger than that inoculated with free form bacteria. The pH of OJ entrapped free-form bacteria reduced from 3.94 to 2.71 at the end of 4 wks (Fig. 3a). On the other hand, the pH of the juice comprised of encapsulated LAB dropped to only 3.3 during that period of storage (Fig. 3b). This data confirms that microencapsulated LAB state had a more endurable and feasible for survival condition and was consistent with Ding group [20].

The preservative action of LAB had been attributed due to the decreasing pH (≥4) by secreting organic acids and this property was effective to inhibit the growth of pathogenic microorganisms, the causative agents of food spoilage and diseases [25]. By maintaining the lower pH, LAB can prolong the shelf life of fermented food. In order to examine the preservative action of LAB in OJ, we evaluated the variation of pH and light transmission in OJ only; OJ contaminated with pathogenic bacteria (PB); OJ inoculated with LAB1-4 and chemically preserved OJ (preservative PR; propyl- and methyl paraben, ratio 5:1). The LAB1-4 inoculated OJ lowered the pH from 3.94 to 3.3 and light transmission was approximately

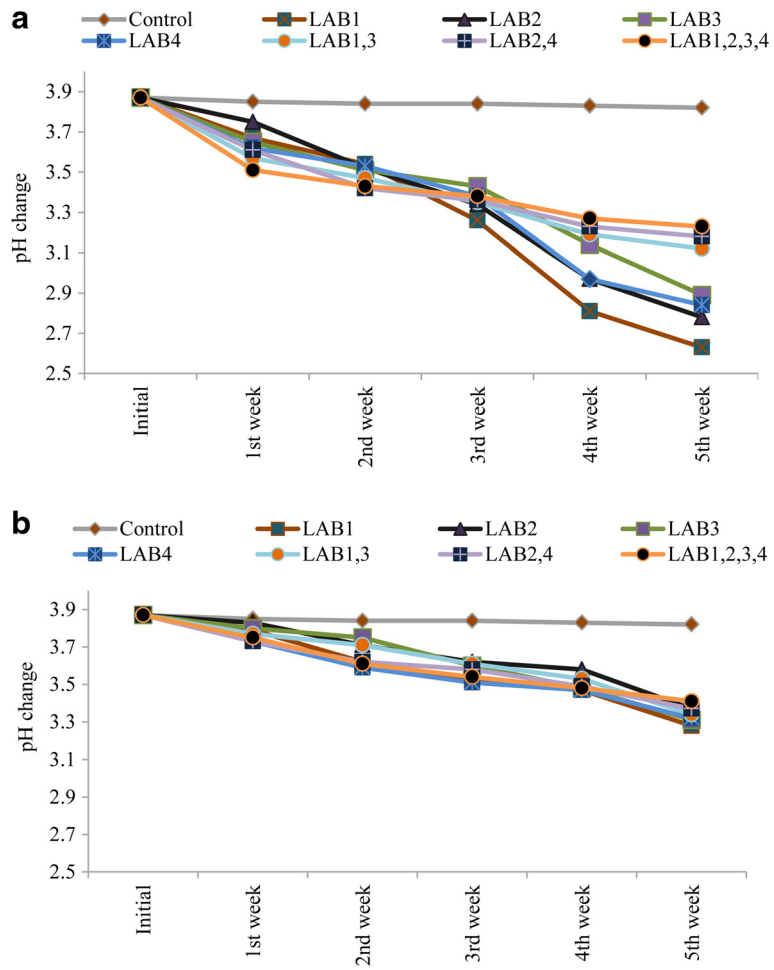

Fig. 3 pH change in OJ containing free and microencapsulated LAB. **a** Free LAB containing OJ; **b** Microencapsulated LAB containing OJ

50 % (Fig. 4a and b). At the end of 48 h, pH increased in free-form OJ due to somehow microorganisms contaminated the juice, and in PB infected OJ. Comparing to LAB1-4 in OJ, the OJ had almost stable pH when chemical preservative was used (Fig. 4a). After 48 h, turbidity also increased in only OJ and OJ containing PB. However, the light transmission were approximately similar in LAB inoculated OJ and PR inserted OJ (Fig. 4b).

Discussion

The overestimation of probiotic amounts in all pharmaceutical products tested could be due to many factors. It might be happened that firstly, sub-optimal methodological procedures were used with respect to rehydration conditions and sample homogenization [15, 26]. However, this factor alone was not sufficient to explain the large differences in CFUs reached and those claimed. Therefore, other potential problems have occurred: 1) a large number of bacteria were destroyed during different stages of processing and sometimes, the manufacturing companies intentionally exaggerated the contents in their probiotic products [27, 28], 2) the product was not dried enough and a high water activity in the powder was detrimental to viability during storage, 3) an efficient antioxidant was absent in the product, 4) packaging did not prevent the entrance of moisture or oxygen in the product during storage, 5) storage temperature was not cold enough or there was indiscriminate handling and storage conditions (cold chains) [23]. It was reported that probiotic products should be consumed regularly, at a daily intake of approximately 10^9 viable cells [29]. However, in our current study, among five commercial probiotic product none of them were shown to have the minimum level. Thus, it is impractical and questionable that the health recovery from the low quality products can be acquired by the insufficient number of viable probiotics [30]. Thus, this study provides a meaningful and useful fundamental data on exactness and reliability of information regarding probiotic bacteria described on the leaflets of pharmaceutical products where legislations

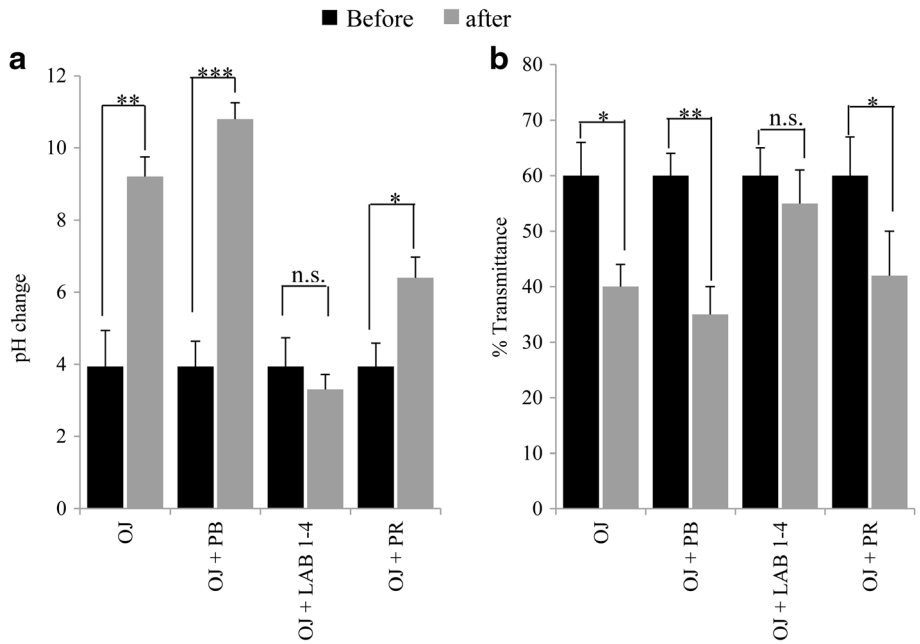

Fig. 4 Effects of pH and transmittance of light on inoculation of LAB1- 4 in OJ. **a** pH change; **b** transmittance of light. OJ, orange juice; PB, pathogenic bacteria; LAB, lactic acid bacteria; PR, food preservative

and regulations about the labeling of products are recently obscures [31]. The ideal situation should be as is done in Canada, where the stated counts must be obtained at the terminal of storage time, and higher populations than those claimed are therefore packaged in order to allow for viability losses during storage. At a minimum, as done by some manufacturers in the USA, a disclaimer is made on the package which states that the CFUs are only those when the product left the factory; this warns consumers that CFUs could be lower should the product suffer abuse during storage.

Because LAB produce some known organic acids like lactic acid or acetic acid, which are important for enabling the LAB to suppress pathogenic Gram-negative and -positive bacteria [32]. Thus, the inhibitory activities of LAB might be associated with the production of organic acids or acidic molecules. After exclusion of H_2O_2 by catalase, the resulting mixtures of CFSs also moderately lowered the inhibition activities of all LAB. Whenever bacteriocin-like metabolites were digested by proteinase K, the antagonistic activities of all LAB dramatically abolished indicating that bacteriocin-like metabolites had a greater role in inhibition of pathogenic bacteria and fungi. Many of bacteriocin-like metabolites are either heat stable or heat-labile. So, we checked the effects of heat after treating at 121 °C for 15 min and found that CFS1 and 3 were stable and active against pathogenic bacteria and fungi, and the other CFSs lost their antimicrobial activities.

Antibiotics have been necessary to combat against infectious agents and have been associated with the increase of life span. However, antimicrobial resistance has become gradually a global threat of public health where antibiotics are no longer effective against pathogenic bacteria. The boundless antibiotic resistance is attributed to excessive, inappropriate or indiscriminate application of antibiotics in many area of the world as a result of chromosomal resistance [33, 34]. Some ecological communities (e.g., the human gut) are occupied by a special group of microorganisms, called commensal bacteria. The majority of these bacteria inhabited the niches of human gut and most of the cases, these bacteria were supposed to harmless for health. However, sometimes in close proximity, LAB could transfer genetic material carrying antibiotic resistance (AR) genes to highly infectious pathogens [35, 36]. For these purposes, we investigated the antibiotic resistant screening against the LAB (Table 5). Our results indicate some variations of antibiotic resistances with other reports [15, 37]. These resistances of antibiotics and other pathogenic bacteria were not only conferred by intrinsic genes but also achieved motile genetic elements. This is an important mechanism for transmission of mobile elements (such as plasmids and transposons) from one bacterium to another by conjugation. Indeed, this phenomena has been observed in vitro where antibiotic resistant gene is shifted from one *Lactobacillus* to another or from *Lactobacilli* to another species including pathogens like *Staphylococcus* [37, 38].

Table 5 Susceptibility of lactic acid bacteria (LAB) to antibiotics and food-preservative

	Amx (30 μg)	Cef (30 μg)	Chlor (30 μg)	Cip (5 μg)	Dox (30 μg)	Ery (15 μg)	Kan (5 μg)	Pen (10 μg)	Strep (10 μg)	Tet (30 μg)	SB (0.1 %)	(M : P 0.1 %)	MB (0.1 %)
LAB 1	8(R)	6 (R)	8 (R)	12	8 (R)	6 (R)	6 (R)	8 (R)	6 (R)	9 (R)	15	14	19
LAB 2	6 (R)	12	8 (R)	12	8 (R)	6 (R)	6 (R)	10 (R)	6 (R)	10 (R)	14	19	14
LAB 3	19	6 (R)	13	18	17	18	6 (R)	10 (R)	19	11	16	21	17
LAB 4	17	6 (R)	6 (R)	13	8 (R)	6 (R)	6 (R)	6 (R)	21	6 (R)	18	22	13
Gram positive bacteria													
B. cereus	6 (R)	28	27	32	19	22	18	17	21	22	11	12	14
B. subtilis	17	23	23	26	6 (R)	12 (R)	22	22	17	19	6 (R)	6 (R)	6 (R
Staphyl. aureus	23	31	24	40	21	6 (R)	18	34	6 (R)	30	6 (R)	6 (R)	6 (R
Gram negative bacteria													
E. coli	34	22	34	33	0 (R)	25	27	0 (R)	23	32	12	16	15
Shi. dysenteriae	6 (R)	31	33	6 (R)	8 (R)	22	10 (R)	16	23	25	10 (R)	6 (R)	6 (R)
Shi. sonnei	28	34	9 (R)	21	23	9 (R)	6 (R)	23	15	18	6 (R	6 (R)	9 (R)
Pathogenic fungi													
S. cerevisiae	6 (NR)	6 (NR)	6 (NR)	0 (NR)	0 (NR)	0 (NR)	8 (NR)	0 (NR)	0 (NR)	0 (NR)	17	18	16
A. niger	0 (NR)	0 (NR)	6 (NR)	0 (NR)	6 (NR)	0 (NR)	0 (NR)	6 (NR)	9 (NR)	6 (NR)	17	20	18
C. albicans	0 (NR)	0 (NR)	6 (NR)	6 (NR)	0 (NR)	6 (NR)	6 (NR)	0 (NR)	0 (NR)	0 (NR)	18	18	15

R resistance, *S* susceptible, *NR* non-response and zone of inhibition in mm

Microcapsules support a suitable anaerobic condition for the growth of susceptible probiotics and give them a physical blockade from the bitter acidic environment in OJ [13, 39]. The interesting results suggested that the higher survival rate of microencapsulated multiple bacteria were found when inoculated into OJ than the free form (Fig. 2c). For significant health benefits, it is evident to stay a large number of viable LAB in the probiotic products [40]. However, several factors can affect the survival rates of LAB in these products [41, 42]. Free-form of bacteria might fermented carbohydrates and released several acids, which in turn lowered the pH during the storage condition. However, a lot of free bacteria were died at the subsequent stages of storage; although the dead probiotics could secrete enzymes for breakdown sugars in the juice and reduced the pH [42, 43]. These data concluded that bacteria in microencapsulation would survive over a longer storage period and could formulate more stable functional foods [43].

In general, probiotic bacteria are utilized in dairy industries for fermentation of sugars in order to provide organic acids such as lactic acid which are associated with the decrease in pH [44]. In this study, LAB 1- 4 survived in the high acidic OJ. A quick fall in pH in the starting of fermentation improved the quality of final products as well as minimized the influence of spoilage bacteria. The suppressive activity of probiotic bacteria against pathogens is due to the accumulation of metabolites such as lactic acid as well as antimicrobial compounds such as H_2O_2 and antibiotics, bacteriocins. However, the grade of quality and amounts among these secreting compounds vary on strains of bacteria, culture conditions and other physical properties.

The food spoilage and associated diseases are the major problems in developing countries [45]. In most cases, low level temperature is applied to enhance the shelf life of food products. The ambient temperature is high in maximum days of a year in many countries like Bangladesh. There is also disrupted and intermittent supply of electricity for maintaining low storage temperature inconvenient for most of the people. As a result, pathogenic bacteria and fungi easily attack the food products due to high storage temperature. Although a wide variety of chemical preservatives are available, the food-grade preservation and marketing must be taken into considerations for extended shelf life [46, 47]. Thus, the concept of preservative action of LAB in OJ will become popular in manufacturing of several products by lowering the pH which prevents the food poisoning and diseases.

Conclusion

From this study, it was indicted the viability of LAB available probiotic products in Bangladesh were less than that of manufacturers claimed. The entire identified LAB showed antimicrobial activities synergistically against common food spoilage pathogenic microbes. The higher number of viable LAB were present in microencapsulated OJ than that of free form and the result indicated that the capability of immobilized LAB preserved OJ for longer time against spoilage owing to restrict the

growth of pathogenic microorganisms in the OJ. Thus, he microencapsulated probiotics would be used in preservation of food and beverage industry in place of chemical preservatives in the food processing and final products. In the near future, the microencapsulated probiotics may become an alternative vehicle for the production of biopreserved food and as a functional food, biopreserved OJ will give the prospective beneficial and protective role in human health.

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Availability of data and materials
All data generated or analyzed during this study were included in this manuscript and the datasets analyzed of the study will be available from the corresponding author on reasonable request.

Authors' contributions
MSH performed all the experiments on the role of LAB in biopreservation. MAAB designed of the study, choice of assay methods, writing and revised manuscript. MIIW assisted in data analysis and interpretation, and ZHM critically reviewed the manuscript. All the authors revised carefully and approved the final manuscript for submission.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
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