

Characterization and screening of the potential probiotic lactic acid bacteria and *Bifidobacterium* strains isolated of different biotopes

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Abstract. The use of lactic acid bacteria (LAB) and bifidobacteria species as probiotics may help to reduce antibiotic use for therapeutic, prophylactic and growth promotion in animal husbandry. The choice of the starter cultures has a critical impact on the palatability, processability and nutritional attributes of fermented products. In our research, the aim of this study was to screen and select potent probiotic LAB and *Bifidobacterium* strains isolated from different niches and to evaluate their characteristic features. A total of fourteen LAB and fifty-four *Bifidobacterium* were isolated from four fresh cows and camel's milk, and twenty stool samples of healthy new born infants were identified and characterized by morphology and biochemical tests in order to select most suitable strains according to their technological characteristics including probiotic proprieties, antibiotics resistance and *in vitro* antagonism against food-poisoning bacteria. When the results of tolerance to both gastric and bile juices are taken together it appears that between fourteen LAB and forty-five, the strain *B. longum* BHI 07 has significantly the highest ability to survive during gastrointestinal transit ($P < 0.05$). Therefore, the mixed cultures of *Bf. longum* strains with LAB strains were more active against pathogenic bacteria than the pure one. These results show that bifidobacteria isolated from infants may be useful for improving probiotic formulae with respect to protection against pathogenic bacteria responsible of infection.

Keywords: Human gastrointestinal tract, lactic acid bacteria, probiotic, antibiotic resistance, multidrug resistance, antimicrobial activity

1. Introduction

Lactic acid bacteria as well as *Micrococcaceae* strains, which can convert fermentable carbohydrates into lactic acid [1] used as starter cultures in fermented foods such as cheese, fermented milks, butter and other fermented products [2]. They include species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* [3]. The

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main criteria used to select microbial starters are desirable technological such as growth, acidification rate [4], organoleptic, textural, sensory and nutritional aspects, also synthesis of antimicrobial compounds [5, 6] and antifungal activity [7]. Other selection criteria of probiotic properties were required such as the adhesion to the intestinal mucosa [8, 9], the non-production of biogenic amines, antibiotic susceptibility [8] and the ability to tolerate the presence of pancreatic enzymes [10]. According to previous study probiotics are defined as live microorganisms which when administered in adequate numbers, confer a health benefit to the host by maintaining or improving their intestinal microbial balance [11–13]. Metchnikoff [14] was the first who suggested that the longevity of the LAB-usefulness was due to their high intake. Promotion of human health via functional food is in high demand. Moreover, functional foods are currently produced mainly (but not limited) by the aid of probiotic microorganisms [15]. Consumption of probiotic bacteria via food products is an ideal way to re-establish the intestinal microflora balance for maintaining good health.

There has been much recent interest in the use of various strains of LAB as probiotics. LAB isolated from the gastrointestinal tract (GIT) of animals and humans constitute an important source of new functional bacteria, which can develop biological roles during the gastrointestinal transit (probiotics) or during food processing [16]. The natural adaptation of many LAB to the gut environment and the antimicrobial substances produced by them (organic acids and bacteriocins) has provided these organisms with a competitive advantage over other microorganisms to be used as probiotics [17, 18]. These organisms should possess the ability to cross the barriers from mouth to intestine, such as low pH in the stomach and bile in the duodenum. They should also adhere to the intestinal micelle and exhibit antagonistic activity against harmful bacteria [19]. The health benefits attributed to probiotic bacteria in the literature can be categorized as nutritional and therapeutic [2, 20].

Most probiotic organisms are lactobacilli or bifidobacteria, which are normal inhabitants of the human colon. However, a small number of commensal LAB and bifidobacteria have been reported to be potential probiotics [21]. Borriello et al. [22] recommended that to development of novel ideal probiotics, it should be isolate from the human fecal microflora of healthy volunteers.

In many regions of African and Arabian countries [23–25] and also in Algeria, especially in environments Saharan desert, there are popular butter indigenous dairy products (*lben, raib, shmen...*) made from different milk sources (cow, sheep, goat or camel) which are traditionally produced and consumed. Many studies have focused on the isolation of lactic acid bacteria and *Bifidobacterium* strains from fermented dairy products (raib, smen) [26, 27] and Cheese [28] traditionally fermented olives [26, 27] and fermented sausages [29].

During the last 50 years, microbial infections are a serious public health problem and physical disabilities throughout the world where they are the main cause of the high mortality rates recorded. Infections of the intestinal tract including diarrhea and gastroenteritis are common, affecting people of all ages. The alarming incidence of antibiotic resistance in bacteria becoming a major problem. One of the alternative methods is biological control using antagonistic metabolites produced by microorganisms (probiotics) including lactic acid bacteria [30]. Today, there is a growing need for selection of new strains of LAB and *Bifidobacterium* that not carry transferable antibiotic resistance genes and with favourable health effects on animals or man. Therefore, the main objective of this work was to perform a screening of LAB and *Bifidobacterium* strains previously isolated from raw cows-milk, camel-milk and healthy infant's faeces for potential investigation as starter cultures in the production of fermented feed and foodstuffs such as fermented milk, meat and for development of probiotics.

2. Materials and methods

2.1. Samples

In the present study, the samples were obtained from four fresh cows and camel's milk from various farmhouses in west and south Algeria and twenty stool samples of healthy new born infants' aged 15 months minimum at 10 days postpartum that had no history of antibiotic treatment.

After collection, milk and feces samples were transported to the laboratory in thermoflasks containing ice and they were analyzed on arrival.

2.2. Bacterial strains and culture conditions

Lactobacillus, *Streptococcus*, *Lactococcus*, *Enterococcus* and *Pediococcus* strains were isolated from milk (fourteen strains coded (S1 to S 14). While *Bifidobacterium* strains from infant feces (fifty-four strains) coded (BHI 1 to BHI 54).

One ml of milk-sample was aseptically added into 9 ml of sterile 0.9% NaCl solution. Homogenized and serially diluted. *Lactobacillus* were counted in MRS agar adjusted to pH 5.4 with sodium acetate so that the growth of other organisms could be suppressed [31]. Plates were incubated under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 48 to 72 h until growth was observed. *Lactococcus* were counted in M17 agar (Merck, Darmstadt, Germany) [32] after incubation for 48 h at 30°C. *Pediococcus* were counted in MRS agar. Plates were incubated under aerobic conditions at 37°C for 48 h. *Streptococcus* were counted in M17 agar. Plates were incubated under aerobic conditions at 42°C for 48 h. *Streptococcus* and *Enterococcus* were counted in M17 and MRS agar respectively, Plates were incubated under aerobic conditions at 44°C for 48 h. Isolation of *Bifidobacteria* from infant's feces was carried out following Beeren [33], it was performed on MRS supplemented with 0.05 % cysteine-chloride (Cys-HCl, Sigma) and 2 mg/L of nalidixic acid at pH 6.8. Plates were incubated under anaerobic conditions at 37°C for 48 h to 72 h. Isolation requires strict anaerobic conditions (anaerobic jars with gas-packs). The purification is performed by successive transplanting from Petri plates containing selective medium (MRS medium supplemented with 0.05% cysteine-chloride and 2 mg/L nalidixic acid).

All LAB strains were stored at 4°C for 4 weeks in reconstituted skim milk (10 %) or frozen at - 20°C. *Bifidobacteria* cultures were maintained in skim milk containing 30 % glycerol, 10 % of yeast extract and 0.2 % cysteine-HCl [34], while *Lactobacillus*, *Streptococcus* and *Pediococcus* cultures were maintained in skim milk containing 30 % glycerol, 10 % of yeast extract. Prior to the experiment, the cultures were activated for 72 h at appropriate temperature and subcultured at least three times.

For antimicrobial activity screening, standard bacterial cultures, viz. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Salmonella typhimurium* ATCC 14028, *Bacillus subtilis* (ATCC 6633) and *Bacillus cereus* (ATCC 10876) was obtained from institute pasteur of Alger, Algeria. These pathogenic microorganisms were selected due to their role as pathogens for humans and their presence in the human gut. All the cultures were maintained as per the recommended practices. These pathogens were grown in nutrient broth and incubated at 37°C for 24 h before use.

2.3. Identifications

Strains were identified using an integrated approach that included phenotypic (morphological, biochemical and physiological characterization). The morphology of the colonies and their dimension are studied after that two to three colonies from each medium were re-plated at least five times by streaking on the same medium. Colonies were then examined by Gram staining and those showing morphological characteristics.

Strains were pre-identified according to physiological and biochemical characteristics as described by Mahmoudi et al. [35] & Schillinger and Lücke [36]. Catalase, oxidase and urease test were carried out, also the fermentation of carbohydrate, production of indole, citrate on middle Kempler and Mc Kay are performed. The influence of pH was tested on strains selected at different pH (4, 5, 6.5, 8.5 and 8) and the influence of incubation temperature (25°C, 30°C, 45°C) followed by a growth test in hyper saline environment at 4 %, 6.5 % NaCl. API 50 CH and API 20 A micro-identification systems were used in this step for the identification of species of lactic acid bacteria. The purity of the bacterial isolates was also confirmed by optical microscope observation and Gram staining.

2.4. Assay for functional properties of the Strains

Tolerance of isolated strains to acidic conditions, bile salts and hydrogen peroxide was tested. It's were examined according to Chung et al. [37]; Klingberg et al. [38] with slight modifications. To investigate survival of LAB and *Bifidobacterium* strains under acidic condition. 1 mL of overnight bacterial culture of each culture was inoculated respectively into 5 mL of nutritive broth adjusted to pH values of 2.0, 3.0 and 6 mol.L⁻¹ with HCl. Samples were incubated for 3 h at 37°C. Then, the number of viable LAB and *Bifidobacterium* cells was determined by serial 10-fold dilution in PBS, and 100 µl aliquots were spread evenly on MRS or M17 agar and the residual viable population was determined after 48–72 h of incubation under aerobic or anaerobic conditions. The survival rate was calculated as the percentage of colonies grown on agar compared to the initial bacterial concentration. Each determination was conducted in triplicate.

The ability of the strains to grow in the presence of bile was determined according to the method of Vinderola and Reinheimer [39]. Each strain was inoculated (2 % v/v) into MRS or M 17 broth with 0.3, 0.5 and 1 % (w/v) of bovine bile (Sigma-Aldrich, Saint Louis, Missouri, USA). Cultures were inoculated at appropriate temperature and after 24 h; optical densities (OD) of the cultures were measured at A 620 nm every two hours during the experiment period and compared within the different concentrations of bile salts for each bacterial culture used and with a control culture (without bile salts). This was expressed as the percentage (%) ± standard deviations (sd) of growth at OD 620 in the presence of bile salts compared to the control. All of the experiments were carried out in triplicate. All strains belonging to the LAB or *Bifidobacterium* selected for the functional properties will be the subject for later studies.

2.5. Assay for technological properties of the Strains

2.5.1. Acidifying activity

To determine the acidifying activity, a modified method Accolas et al. [40] was used, with using NaOH (Panreac Quimica, Spain) (N/9) in the presence of phenolphthalein indicator (Sigma-Aldrich, Germany) (1 % in alcohol) and measuring the pH by using a pH meter (*HANNA Instrument*, pH 211, Woonsocket, Romania) of the culture after 6, 24, and 48 h.

2.5.2. Kinetics-Growth

In pure culture, the kinetics growth of LAB and *Bifidobacterium* strains is followed by counting on agar medium appropriate and at different time (0 h, 2 h, 4 h, 6 h...up to 72 h). The colony-forming units (CFU) were estimated. LAB and *Bifidobacterium* strains selected for technological properties will be the subject of assessment of antagonistic activity.

2.5.3. Determination of antibiotic susceptibility testing of the strains

Antibiotic sensitivity or resistance of the selected isolates was determined by the standardized technique of diffusion of Phillips et al. [41] & Zhou et al. [42] toward 11 commercial antibiotics discs: Fusidic acide (10 µg), Erythromycin (15 µg), chloramphenicol (30 µg), Penicillin (10 U), Gentamicin (10 µg), Streptomycin (10 µg), Ampicillin (10 µg), Cefotaxime (30 µg). All discs from Bioanalyse (France) except Amoxycillin (25 µg) (Himedia, India), Oxacillin (1 µg) (bioMérieux, Marcy-l'Etoile, France), Cefalaxin (30 µg) Bio-rad (Marnes-la-Coquette, France).

A bacteria suspension was made by picking one or two colonies from agar plates using a sterile loop and making a suspension in sterile broth medium and left for 18 h. Using a sterile swab, the suspension was applied on the surface of Mueller-Hinton agar in sterile Petri dishes, and the strips were placed on the surface using sterile forceps. Two antibiotic reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were tested with the same technique as the control strains. The results are interpreted according to the recommendations of the French-Society-Committee of sensitivity (2007) and NCCLS (national comity of clinical laboratory standars). The result (average of 3 reading) were expressed as sensitive (S) and resistant (R),

No Sensitive (NS), intermediaries (I) by precisely measuring the diameters of the clear zone appearing around the discs with using a digital caliper [43].

2.6. Assay for antimicrobial activity

The ability of the supernatants from *Bf. bifidum* both single culture and the interaction between *Bf. bifidum* and the five LAB strains selected to inhibit the growth of pathogenic microorganisms was investigated using well-diffusion agar method described by Tagg et al. [44]. The antimicrobial activity was determined by measuring the diameter of the inhibition zones (clear agar) with calipers [45]. Two independent replicates were conducted for each experiment. One or two colonies from agar plates using a sterile loop and making in five mL of sterile broth medium and incubated for a night. Overnight (18 h) cultures of such organism were centrifuged at $3000 \times g$ for 20 min. The pH of the aqueous phase was adjusted to $6.5-7 \pm 0.1$ with 1N NaOH and then filter-sterilized using $0.22 \mu\text{m}$ filters before being subjected to the antibacterial assay. Agar was seeded with 1 % (v/v) of an overnight of pathogenic culture and poured into Petri dishes (90 mm in diameter). Welles of 6 mm were cut out of the agar and loaded with $100 \mu\text{L}$ of each filter-sterilized-supernatant. The plates were left at 5°C for 2 hr, to allow migration and diffusion of the tested supernatant. Control wells filled with sterile nutritive broth and standard antibiotic solution of $100 \mu\text{g/ml}$ Tetracycline (Sigma-aldrich) were performed as negative control and positive control, respectively. Plates were then incubated for 24 h under appropriate conditions for each specific pathogen.

2.7. Statistical analyses

Data were statistically analyzed with IBM SPSS Statistics Base 22.0 software for Windows. One-way ANOVA analysis of variance was carried to identify significant differences between means on quantitative parameters, with the significance level at $\alpha = 0.05$. *T* test and correlation was used to perform multiple comparisons between means. All tests were repeated at least three times to calculate the means and standard error.

3. Results and discussion

3.1. Results of identification of the lactic bacteria

The colonies developed on MRS, M17, Columbia and MRSc agar medium were further studied. From these, sixty-eight strains were isolated. They were appeared small, circular in shape, lenticular, more elongated or round with regular contour, whitish and cream color with varying diameter (Fig. 1A).

The result of microscopic appearance assumes that these selected isolates were Gram-positive cells with cocci or bacillus form which the grouping mode differs from species to another: shells in pairs (*Enterococcus* sp.) (Fig. 2E); in diplococci and tetrad (*Pediococcus* sp.) (Fig. 2B); in clusters (*Lactococcus* sp.) (Fig. 2D) or in chains (*Streptococcus* sp.) (Fig. 2A); long bacilli in pair or chain (*Lactobacillus* sp.) (Fig. 2C), short-curved bacilli (*Bifidobacterium* sp.) (Fig. 2F).

The purified strains were all Gram positive, catalase and oxidase negative, none motile, none sporulating, producing no gas from glucose. These characteristics are typical of LAB and *Bifidobacterium* strains.

The biochemical results of the strains isolated from milk and with reference to the studies reported by Desmazeand et al. [46] and Guirand [47] showed that the fourteen strains belong to the species quoted in the Table 1. While, the strains isolated from newborn feces were gelatinase negative, with no indole production and resisting up to 2 % of bile salt, citrate permease positive, nitrate reductase negative, no production of gas from glucose, growth in anaerobic with the exception of BHI 10, 23–25, 27–30, 36–41 (Table 2). Based on the previous tests, we assume that these selected isolates were likely to be *Bifidobacterium* strain. The results showed that all strains ferment some sugars (glucose, fructose, maltose and lactose).

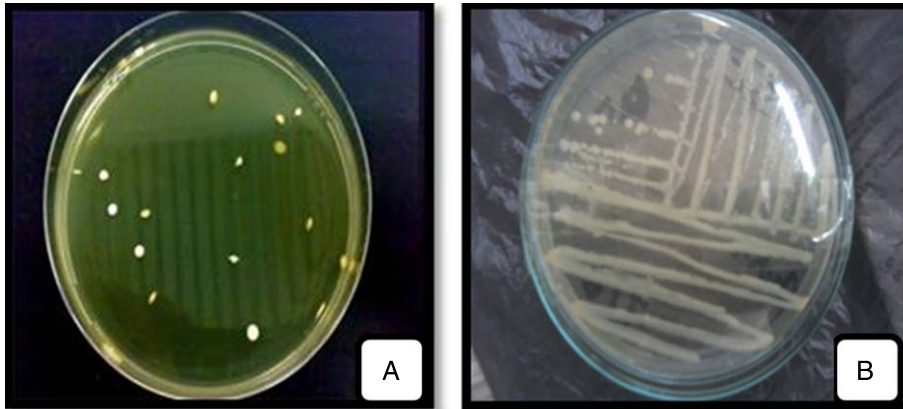


Fig. 1. Macroscopic observation of LAB isolated (A) and purified (B) strains on Agar medium.

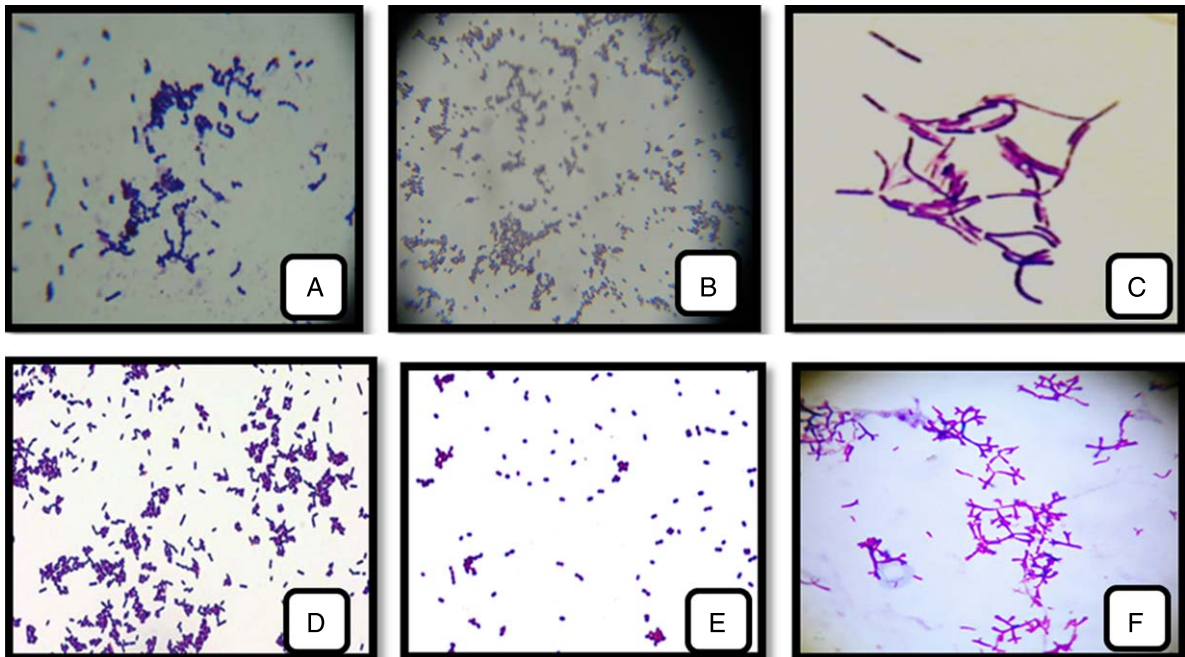


Fig. 2. LAB and *Bifidobacterium* strains isolated from cow's, camel's-milk and infants feces. (A): *Streptococcus thermophilus*; (B) : *Pediococcus cerevisiae*; (C) : *Lactobacillus bulgaricus*; (D) : *Lactococcus lactis*; (E): *Enterococcus faecalis*; (F) : *Bifidobacterium bifidum* (1000 x).

The identification of species has been completed by the fermentation of Carbohydrates with used of the API gallery (Tables 3 and 4) and the results were compared to the literature [48–50].

Therefore, all the various strains isolated and identified as LAB and *Bifidobacterium* have been screened for their acid and bile resistance.

Table 1
Biochemical and physiological characteristics of the isolated strains from fresh milks samples

Biochemical Characteristics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxydase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indol production	+	-	+	-	+	-	-	-	-	-	-	-	-	-
Mobility	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrat permease	-	-	-	+	-	-	+	+	-	-	-	-	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RM	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	-	+	+	+	-	-	-	-	-	+	-	-
LDC	-	-	-	-	-	+	+	+	-	-	-	-	+	+
ODC	-	-	-	-	-	+	+	+	-	-	-	-	+	+
<i>Fermentation of sugars</i>														
Glucose	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Lactose	+	+	+	+	-	+	+	+	+	+	+	±	+	+
Saccharose	±	+	-	+	-	+	+	-	-	±	+	±	+	+
Mannitol	-	+	-	+	-	-	+	+	+	+	-	+	+	+
Production of H ₂ S	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Production of CO ₂ from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Resistance to bile salts (02%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Physical Characteristics</i>														
Test of thermoresistance 65°C	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Growth at pH= 3.2	±	-	+	-	±	±	±	±	-	-	±	±	±	±
Growth at pH= 4	+	±	+	±	-	+	+	+	-	-	-	+	+	+
Growth at pH=6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH=9.6	-	±	-	±	-	-	-	-	-	-	-	-	-	-
Growth in presence of NaCl 4%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in presence of NaCl 6.5%	-	+	-	+	-	+	+	+	+	-	-	+	+	+
Growth at T 30°C	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Growth at T 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at T 45°C	-	+	-	+	-	-	-	-	-	+	+	+	+	-
Species	Lc 01	Ec 01	Lc 02	Ec 02	Pc 01	Pc 02	Lb 01	Lb 02	Lc 03	Sc 01	Sc 02	Sc 03	Lb 03	Lb 04
	<i>Lc. Lactis</i>	<i>Ec. faecium</i>	<i>Lc. cremoris</i>	<i>Ec. Faecalis</i>	<i>Pc. cerevisiae</i>	<i>Pc. acidilactici</i>	<i>Lb. bulgaricus</i>	<i>Lb. Lactis</i>	<i>Lc. Lactis</i>	<i>Sc. boris</i>	<i>Sc. Thermophilus</i>	<i>Sc. agalactiae</i>	<i>Lb. bulgaricus</i>	<i>Lb. lactis</i>

+: positive reaction; -: negative reaction; ±: variable.

Table 2
Biochemical and physiological characteristics of the isolated strains infant's feces

Biochemical Characteristics	BHI 01	BHI 02	BHI 03	BHI 04	BHI 05	BHI 06	BHI 07	BHI 08	BHI 09	BHI 10	BHI 11	BHI 12	BHI 13	BHI 14	BHI 15	BHI 16	BHI 17	BHI 18	BHI 19	BHI 20
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxydase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Indol production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mobility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrat permease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LDC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ODC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of H ₂ S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of CO ₂ from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reductase	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Resistance to bile salts (02%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Physical Characteristics																				
Test of thermoresistance 46,5°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH=4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH=6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH=8.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in presence of NaCl 4%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in presence of NaCl 6.5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at T 30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at T 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at T 45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: positive reaction; -: negative reaction; ±: variable.

Table 2a
Biochemical and physiological characteristics of the isolated strains infant's feces (SUITE)

Biochemical Characteristics	BHI 21	BHI 22	BHI 23	BHI 24	BHI 25	BHI 26	BHI 27	BHI 28	BHI 29	BHI 30	BHI 31	BHI 32	BHI 33	BHI 34	BHI 35	BHI 36	BHI 37	BHI 38	BHI 39	BHI 40
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxydase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
Indol production	-	-	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-
Gelatinase	-	-	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Mobility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrat permease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+	±	±	+	+	+	+	+	+
LDC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ODC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of H ₂ S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of CO ₂ from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reductase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Resistance to bile salts (02 %)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Physical Characteristics																				
Test of thermoresistance 46,5°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH=4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH=6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH=8.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in presence of NaCl 4%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in presence of NaCl 6.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at T 30°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at T 37°C	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at T 45°C	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

+: positive reaction; -: negative reaction; ±: variable.

Table 2b
Biochemical and physiological characteristics of the isolated strains infant's feces (SUITE)

Biochemical Characteristics	BHI 41	BHI 42	BHI 43	BHI 44	BHI 45
Catalase	-	-	-	-	-
Oxydase	-	-	-	-	-
Urease	-	-	-	+	-
Indol production	-	-	-	-	-
Gelatinase	-	-	-	-	-
Mobility	-	-	-	-	-
Citrat permease	+	+	+	+	+
VP	+	+	+	+	+
RM	+	+	+	+	+
ADH	+	+	-	+	+
LDC	-	-	-	-	-
ODC	+	+	+	+	+
Production of H ₂ S	+	+	+	+	+
Production of CO ₂ from glucose	+	+	+	+	+
Nitrate reductase	+	+	+	+	+
Resistance to bile salts (02 %)	+	+	+	+	+
<i>Physical Characteristics</i>					
Test of thermoresistance 46.5°C	+	+	+	+	+
Growth at pH = 4	-	-	-	-	+
Growth at pH = 6.5	+	+	+	+	+
Growth at pH = 8.5	-	-	-	-	-
Growth in presence of NaCl 4 %	+	+	+	+	+
Growth in presence of NaCl 6.5 %	-	-	-	-	-
Growth at T 30°C	+	+	+	+	+
Growth at T 37°C	+	+	+	+	+
Growth at T 45°C	+	+	+	+	+

+: positive reaction; -: negative reaction; ±: variable.

3.2. Tolerance under acidic and bile conditions

According to the suggestion of FAO/WHO [51], it is mandatory to perform a preliminary *in vitro* assessment before assessing the probiotic properties of bacterial strains, such as the capacity for ensure survival during passage through the gastro-intestinal tract [52–54] and have their health promoting effects as metabolically viable active cells when they arrive in the colon [55].

3.2.1. Survival rate at low pH

In this work, the abilities of fourteen (14) LAB strains and forty-five (45) *Bifidobacterium* strains to endure low pH (2.0, 3.0 and 5.0) were examined for acid tolerance, only (11) LAB and 07 strains were selected as resistant to low pH.

Survival rates of the isolates varied during incubation (Figs. 3 and 4). All LAB and *Bifidobacterium* strains survived at pH 3.0 and 5.0 but, BHI 21, 43, 42, 15 and 22 were the most acid sensitive strains after 4 h of incubation of all strains tested. However, at pH 2.0, Ec 01, BHI 34, 43, 32, 42, 22, 44, 16 and 9 strains were losing viability in less than 3 h of incubation, while Pc 01 and Pc 02 strains were losing viability in less than 2 h of incubation whereas, Sc 01, Sc 02, Sc 03 strains than 1 h. Kim et al. [56] reported that *Lactococcus lactis* subsp. *cremoris* and *L.lactis* subsp. *lactis* isolated from milk lost their viability at pH 2.0

Table 3
 Characters fermentation of LAB strains isolated from fresh milks samples

Biochemical Characteristics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxydase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indol production	+	-	+	-	+	-	-	-	-	-	-	-	-	-
Mobility	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Curat permease	-	-	-	+	-	-	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RM	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	-	+	+	+	-	-	-	-	-	+	-	-
LDC	-	-	-	-	-	+	+	+	-	-	-	-	+	+
ODC	-	-	-	-	-	+	+	+	-	-	-	-	+	+
Fermentation of Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of Lactose	+	+	+	+	-	+	+	+	+	+	+	±	+	+
Fermentation of Saccharose	±	+	-	+	-	+	+	+	-	±	+	±	+	+
Fermentation of Mammitol	-	+	-	+	-	-	-	-	+	+	-	+	-	-
Production of H ₂ S	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Production of CO ₂ from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Resistance to bile salts (02 %)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Physical Characteristics</i>														
Test of thermoresistance 65° C	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Growth at pH= 3.2	±	-	+	-	±	±	±	±	-	-	±	±	±	±
Growth at pH= 4	+	±	+	±	-	+	+	+	-	-	-	+	+	+
Growth at pH= 6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH= 9.6	-	±	-	±	-	-	-	-	-	-	-	-	-	-
Growth in presence of NaCl 4 %	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in presence of NaCl 6.5 %	-	+	-	+	-	+	+	+	+	-	-	+	+	+
Growth at T 30° C	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Growth at T 37° C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at T 45° C	-	+	+	+	-	-	-	-	-	+	+	+	-	-
Species	Lc 01	Ec 01	Lc 02	Ec 02	Pc 01	Pc 02	Lb 01	Lb 02	Lc 03	Sc 01	Sc 02	Sc 03	Lb 03	Lb 04
	<i>Lc. Lactis</i>	<i>Ec. Faecium</i>	<i>Lc. cremoris</i>	<i>Ec. Brevalis</i>	<i>Pc. carnisstae</i>	<i>Pc. acidilactici</i>	<i>Lb. bulgaricus</i>	<i>Lb. Lactis</i>	<i>Lc. Lactis</i>	<i>Sc. borris</i>	<i>Sc. Thermophilus</i>	<i>Sc. agalactiae</i>	<i>Lb. bulgaricus</i>	<i>Lb. lactis</i>

Table 4
 Characters fermentation of *Bifidobacterium* strains isolated from infants feces

Fermentation of sugars	BHI 01	BHI 02	BHI 03	BHI 04	BHI 05	BHI 06	BHI 07	BHI 08	BHI 09	BHI 11	BHI 12	BHI 13	BHI 14	BHI 15	BHI 16	BHI 17	BHI 18
L-Arabinose	-	+	-	+	-	-	+	-	-	-	+	-	-	+	-	+	-
D-Ribose	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+
Xylose	-	±	±	+	±	±	-	-	±	-	±	-	±	+	±	±	±
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	±	±	±	±	±	-	-	±	+	±	-	±	±	±	±	±
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	±	-	-	±	-	-	-	-	-	±	-	-	-	±	-	-	-
D-Sorbitol	±	-	-	±	-	-	-	-	-	±	-	-	-	±	-	-	-
Salicine	+	-	-	+	-	-	-	±	-	+	-	-	-	+	-	-	-
Cellobiose	±	-	-	+	-	-	-	±	-	±	-	-	-	+	-	-	-
Maltose	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	±	+	+	+	+	±	+	+	+	+	+
D-Saccharose	+	+	+	+	+	+	±	+	+	+	+	±	+	+	+	+	+
D-Trehalose	±	-	-	±	-	-	-	±	-	±	-	-	-	±	-	-	-
Inuline	±	-	±	±	-	±	-	±	±	±	-	-	±	±	±	-	±
D-Mélezitose	±	+	-	+	+	-	-	±	-	±	+	-	-	+	-	+	-
D-Raffinose	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Amidon	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-
Gluconate	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-
Species	<i>breve longum infantis</i> <i>Adolescentis longum infantis</i> <i>bifidum thermophilum infantis</i> <i>bifidum longum bifidum infantis</i> <i>Adolescentis Infantis longum infantis</i>																

Table 4a

Fermentation of sugars	BHI 19	BHI 20	BHI 21	BHI 22	BHI 26	BHI 31	BHI 32	BHI 33	BHI 34	BHI 35	BHI 42	BHI 43	BHI 44	BHI 45
L-Arabinose	-	-	+	-	+	-	-	+	-	+	-	-	-	+
D-Ribose	+	-	+	+	+	+	-	-	+	+	-	+	+	+
Xylose	-	-	±	±	+	±	-	+	-	±	-	-	±	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	±	+	+	+	+	+	+
D-Mannose	+	-	±	±	±	±	-	±	+	±	-	+	±	±
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	±	-	-	-	±	-	-	-	±	-	-	±	-	±
D-Sorbitol	±	-	-	-	±	-	-	-	±	-	-	±	-	±
Salicine	+	-	-	-	+	-	-	-	+	-	-	+	-	+
Cellobiose	±	-	-	-	+	-	-	-	±	-	-	±	-	+
Maltose	+	-	+	+	+	+	-	+	+	+	-	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	±	+	+	+	+	±	+	+	+	±	+	+	+
D-Saccharose	+	±	+	+	+	+	±	+	+	+	±	+	+	+
D-Trehalose	±	-	-	-	±	-	-	-	±	-	-	±	-	±
Inuline	±	-	-	±	±	±	-	-	±	-	-	±	±	±
D-Mélezitose	±	-	+	-	+	-	-	-	±	+	-	±	-	+
D-Raffinose	+	-	+	+	+	+	-	+	+	+	-	+	+	+
Amidon	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Glucosate	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Species	<i>breve</i>	<i>bifidum</i>	<i>longum</i>	<i>infantis</i>	<i>Adolescentis</i>	<i>infantis</i>	<i>bifidum</i>	<i>sis</i>	<i>breve</i>	<i>longum</i>	<i>bifidum*</i>	<i>breve</i>	<i>infantis</i>	<i>Adolescentis</i>

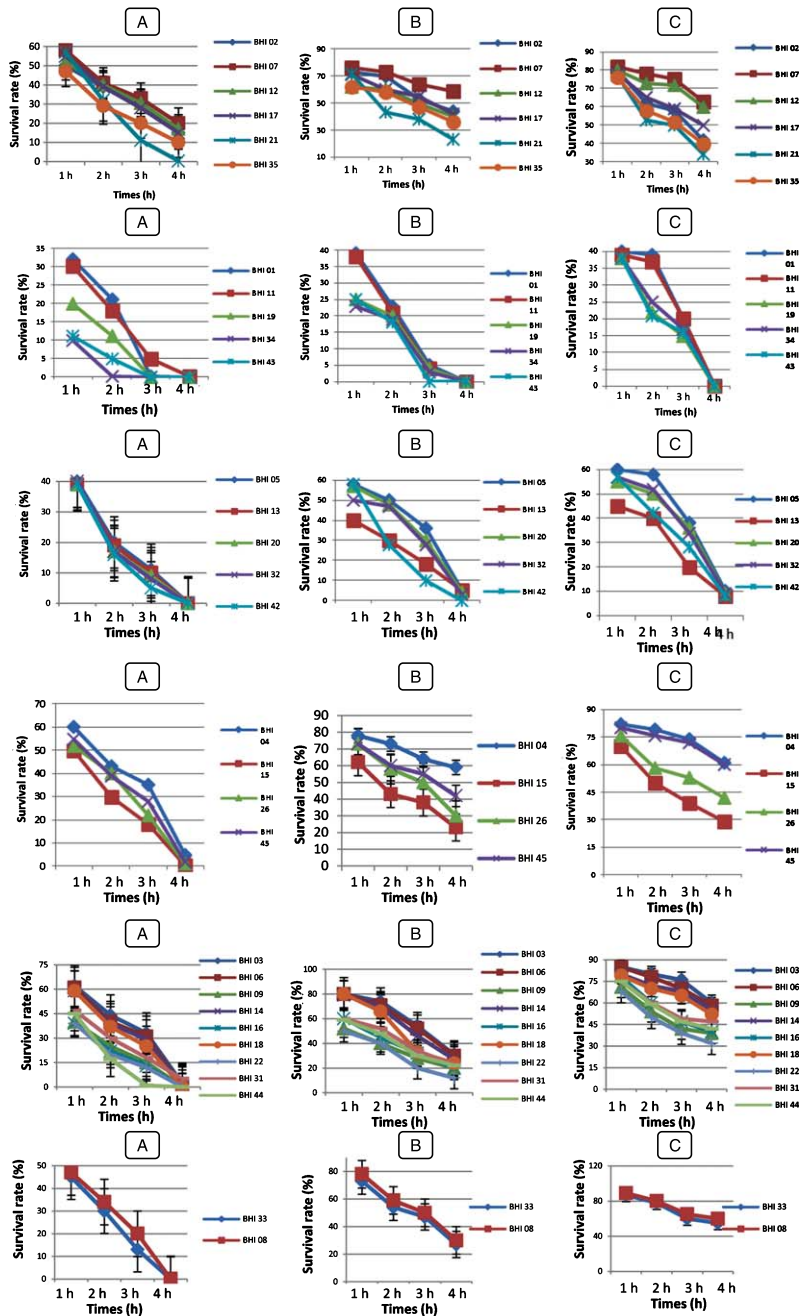


Fig. 3. Survival rate of *Bf. longum* (BHI 02, BHI 07, BHI 12, BHI 17, BHI 21, BHI 35), *Bf. breve* (BHI 01, BHI 11, BHI 19, BHI 34, BHI 43), *Bf. bifidum* (BHI 05, BHI 13, BHI 20, BHI 32, BHI 42), *Bf. adolescentis* (BHI 04, BHI 15, BHI 26, BHI 45); *Bf. infantis* (BHI 03, BHI 06, BHI 09, BHI 14, BHI 16, BHI 18, BHI 22, BHI 31, BHI 44); *Bf. suis* (BHI 33); *Bf. thermophilum* (BHI 08), at pH 2 (A); pH 3 (B); pH 5 (C) after 4 h at 37°C. Vertical bars represent standard deviations of the mean.

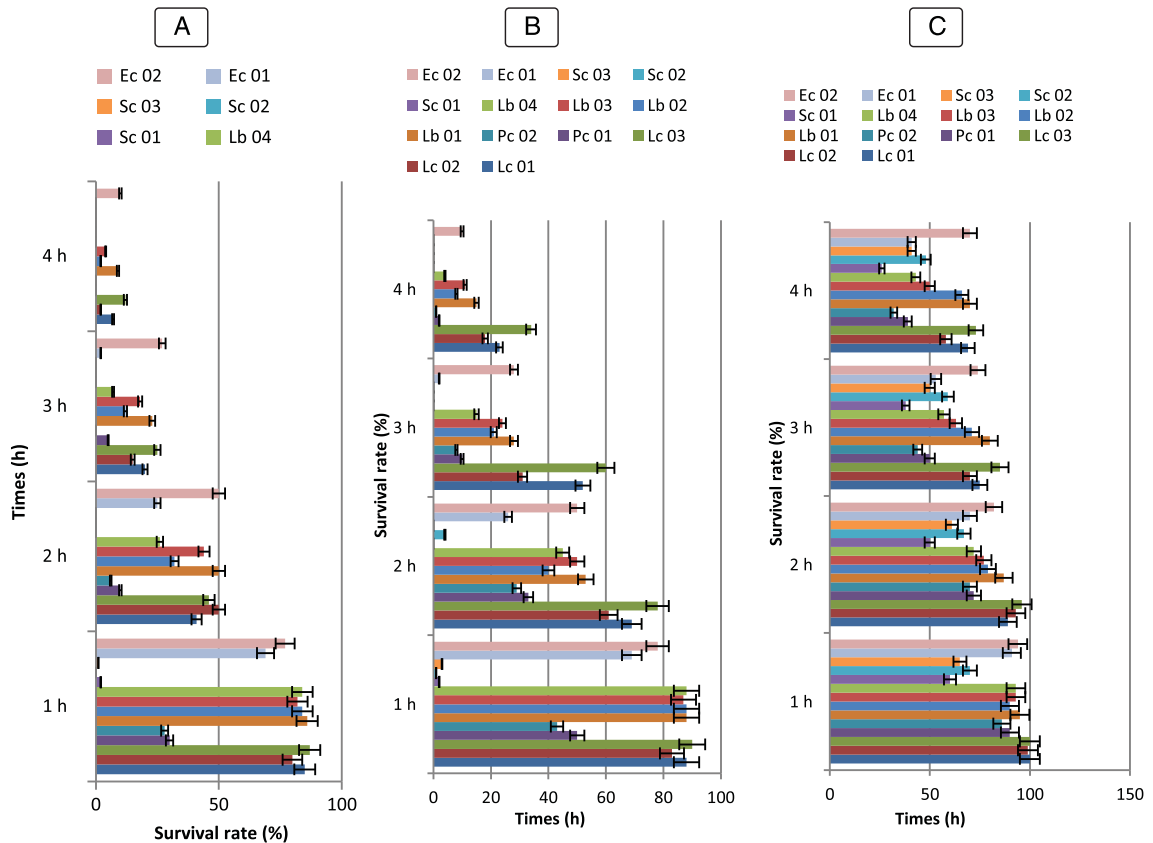


Fig. 4. Survival rate of LAB strains under acidic condition at pH 2 (A); pH 3 (B); pH 5 (C) after 4 h at 37°C. Vertical bars represent standard deviations of the mean. Survival rate at bile salt

Bf. bifidum (BHI 07) showed significantly higher survival rate (SR = 63 %) after 4 h of incubation ($P < 0.05$) than any other isolates and followed by *Bf. adolescentis* (BHI 04), *Bf. infantis* (BHI 03), *Bf. thermophilum* (BHI 08), *Bf. suis* (BHI 33). However, LAB strains were showed higher survival rate were *Lc. cremoris* (Lc 03) with 73 % and followed by Pc 01, Lb 01, Sc 02 and Ec 02. When we compare our results with these reported by Xanthopoulos [57] *Lactobacillus* spp. isolates (*L. acidophilus*, *L. gasseri*, *L. rhamnosus* and *L. reuteri*) from infant feces, their survival ability to tolerance low pH (pH 3.0) ranged between 0.01 – 68.3 %. Our strains have shown a very good performance.

All LAB strains with the exception of *Pediococcus* and *Streptococcus* strains, *Bf. bifidum* BHI 07, *Bf. breve* BHI 01, *Bf. longum* BHI 05, *Bf. adolescentis* BHI 04, *Bf. infantis* BHI 03 were the most acid-tolerant strains, retaining around 20 – 50 % viability for up to 2 h at pH 2.0. Similar studies have been done by Al-Saleh [58] reported that *Streptococcus thermophilus* DSM 20617 and *Bifidobacterium infantis* DSM 20088 were the most acid sensitive of all bacterial strains tested. These strains completely lost their viability after 1.5 h at pH 2, but at pH 3, the strains improved the acid tolerance with the exception of *Streptococcus thermophilus*.

3.2.2. Survival rate to tolerate bile salts

Another critical factor influencing the viability of microorganisms in the stomach is the presence of bile salt. The abilities of bile salt resistance of all LAB and *Bifidobacterium* strains tested for their ability to survive in

gastric juice were examined. The results showed that all isolates grew at concentrations of at least 0.3 % (w/v) bile extract and the maximum value of bile resistance varied from 9 to 32 (Fig. 5 and 6). In comparison to the control culture, there was no significant difference ($P > 0.05$). BHI 34, 43, 20, 42 and 15 *Bifidobacterium* strains could not grow in 1 % after 3 h of incubation. *B. bifidum* strain (BHI 07) showed the maximum resistance at 1.0 % concentrations while the *B. longum* (BHI 02, 12, 17, 35) and *B. infantis* (BHI 03, 06, 14, 18) strains indicates relatively strong bile salt tolerance.

In order to survive and establish within the human GIT, some of the desirable properties of probiotics include their ability to resist the acidity (pH 2.5 – pH 3.5) of the stomach [59] and the exposure to bile in the upper part of the intestine. Gilliland et al. [60] and Goldin et al. [61] reported that 0.3 % bile as a suitable concentration for selection of probiotics. In the present study, the exposure to pH 2 showed to be a very discriminating factor with three strains Lc 03, Lb 01 and Ec 02 out 14 LAB strains, also five *B. longum* (BHI 07, 12, 17, 02 and 35) out 45 strains surviving after 4 h of exposure. These strains also performed well in the acid and bile assay, suggesting their survival through the stomach and their ability to compete in the human intestine.

These results are in agreement with those of Pyoung Il Kim et al. [62] reported that large variation in the survival of the isolates in gastric juice and growth in the medium containing 0.3 % (w/v) bile and strain dependent [63]. Xanthopoulos et al. [57] reported that *Lactobacillus* spp. isolates (*L. acidophilus*, *L. gasseri*, *L. rhamnosus* and *L. reuteri*) from infant feces were tested for their ability to tolerance low pH (pH 3.0) and bile salt. Survival of the test strains ranged between 0.01 – 68.3 % at pH 3.0 and 10.3 – 57.4 % at 0.15 % bile salts. Another research has shown that all cells of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* lost their viability at pH 2.0 and 0.2 % bile salts [64]. When we compare our results with these results, our strains have shown a very good performance at low pH and bile salts.

Three LAB strains and 38 *Bifidobacterium* strains were excluded based on these criteria in the current study and only 11 LAB strains and 7 *Bifidobacterium* strains were selected for further examinations. When the results of tolerance to both gastric and bile juices are taken together it appears that the strain *B. longum* BHI 07 has significantly the highest ability to survive during gastrointestinal transit ($P < 0.05$). Therefore, it can be potentially used as probiotic strains.

3.3. Technological properties

The ability to acidify the milk was investigated for the 17 strains selected as described above. Among 11 LAB and 7 *Bifidobacterium* strains examined, 5 LAB and one *Bifidobacterium* strains were fast acidifying isolates and produced lactic acid.

The measurement of initial and final pH shows a decrease in pH in the medium containing the lactic strains isolated. This decrease is shown in Fig. 7.

The values of acidity found show variations more or less important between the different species ($p < 0.05$). They vary between 1.5 and 2.8 g of lactic acid/ 100 mL to T_0 , after 6 h the acidity starts to gradually increase ($p < 0.05$) and varied between 1.9 and 3.8 g of lactic acid/ 100 mL. After 48 h of incubation, the pH of the culture medium decreased to values lower than 3.94 and the acidity reached values to 10 g of lactic acid/ 100 mL for the strain Lb 02. While, the strain Pc 02 was presented significantly the highest acidifying capacity with Δ pH = 2.58 ($p < 0.05$) following by Lb 02 (Δ pH = 2.36) (data not shown). While *Sc. thermophilus* (Sc 02) and *Sc. agalactiae* (SC 03) strains were the most weakly acidifying with Δ pH = 1.05 and 1.08, respectively developing an acidity of 3.4 and 3.1 g of lactic acid/ 100 mL. For the seven (7) *Bifidobacterium* strains, during fermentation and after 48 h of incubation, the pH values changed from 6.41 to 4.86 with Δ pH = 0.38 and 1.84, respectively (data not shown). The strains *Bf. longum* (BHI 05) and *Bf. bifidum* (BHI 07) performed the fastest acidification with Δ pH = 1.90 and 1.84, respectively and reached the highest level of viable counts with $\mu = 6.58$ Log (UFC/mL) while *Bf. suis* (BHI 33) showed the lowest growth with $\mu = 0.53$ h⁻¹ (data not shown). A slower acidification was observed for *Bf. adolescentis* and *Bf. suis* (BHI 04, 33) reaching pH 6.35 and 6.41 after 48 h

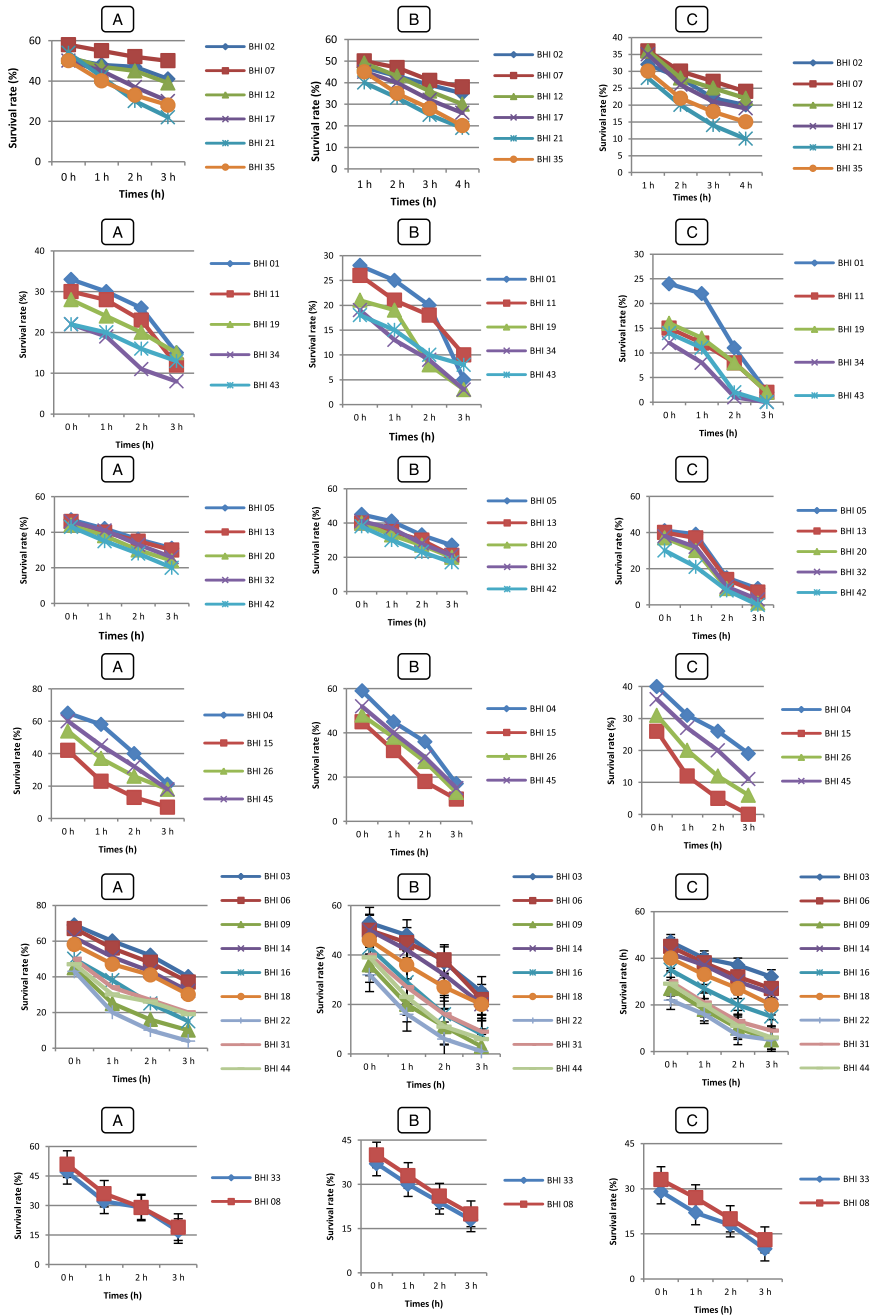


Fig. 5. Survival rate of *Bf. longum* (BHI 02, BHI 07, BHI 12, BHI 17, BHI 21, BHI 35), *Bf. breve* (BHI 01, BHI 11, BHI 19, BHI 34, BHI 43), *Bf. bifidum* (BHI 05, BHI 13, BHI 20, BHI 32, BHI 42), *Bf. adolescentis* (BHI 04, BHI 15, BHI 26, BHI 45); *Bf. infantis* (BHI 03, BHI 06, BHI 09, BHI 14, BHI 16, BHI 18, BHI 22, BHI 31, BHI 44); *Bf. suis* (BHI 33); *Bf. thermophilum* (BHI 33), at 0,3% (A), 0,5% (B), 01% (C) of bile salts ($n=3$). Vertical bars represent standard deviations of the mean.

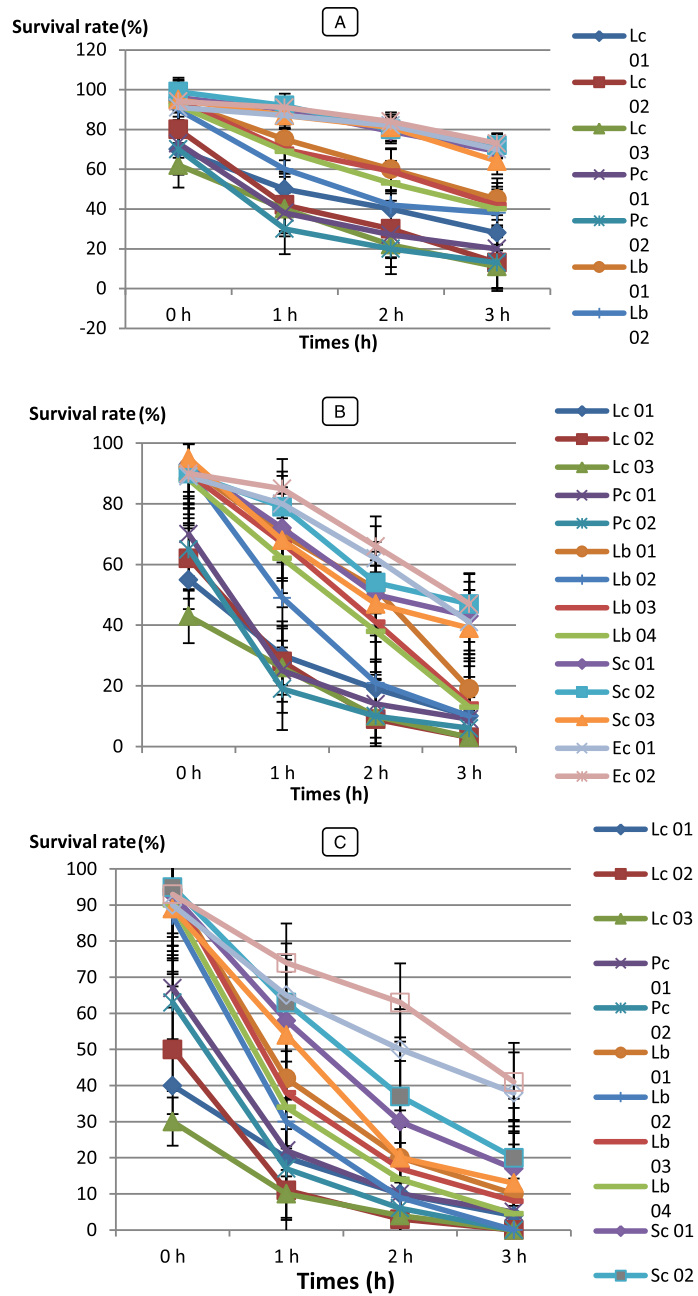


Fig. 6. Survival rate tolerance at 0,3 % (A), 0,5 % (B), 01 % (C) of bile Salts of LAB strains (◆) *Lc. lactis* (Lc 01), (■) Lc 02, (▲) Lc 03, (◇), (×) Pc 01, (*) Pc 02, (●) Lb 01, (○) Lb 02, (+) Lb 03, (-) Lb 04, (◊) Sc 01, (◊) Sc 02, (◊) Ec 01, (□) Ec 02. Error bars show the SD of the mean of triplicate analyses.

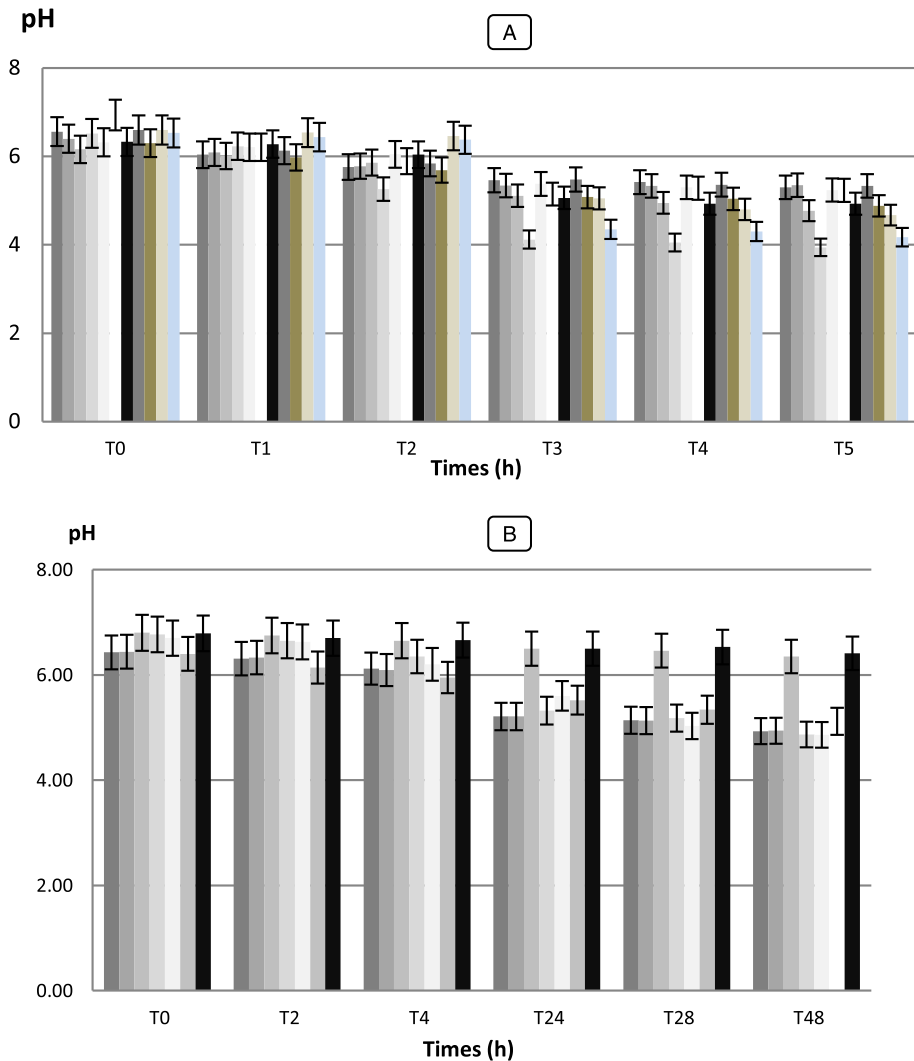


Fig. 7. Acid production of the medium measured by recording the pH by LAB strains (A) and *Bifidobacterium* strains (B) selected for their capacity to survive during passage through the gastro-intestinal tract. Data points are shown with 02-h intervals. Determinations were carried out twice with a maximum standard deviation of ± 0.05 . The LAB and *Bifidobacterium* strains were added at a level of 10^6 CFU/g. Error bars show the SD of the mean of triplicate analyses. (A) LAB strains: *Lc. cremoris* (Lc 02), *Lc. lactis* (Lc 03), *Pc. cerevisiae* (Pc 01), *Pc. acidilactici* (Pc 02), *Sc. boris* (Sc 01), *Sc. thermophilus* (Sc 02), *Sc. agalactiae* (Sc 03), *Ec. Faecium* (Ec 01), *Ec. Faecalis* (Ec 02), *Lb. bulgaricus* (Lb 01), *Lb. lactis* (Lb 02), *Bf. breve* (BHI 01), *Bf. infantis* (BHI 03), *Bf. Adolescentis* (BHI 04), *Bf. bifidum* (BHI 05), *Bf. Longum* (BHI 07), *Bf. thermophilum* (BHI 08), *Bf. suis* (BHI 33).

of fermentation. Our results showed that there is a positive correlation ($r=0.98$ for *Bf. bifidum* strain, $r=0.970$ for *Bf. adolescentis* strains) between the Acidifying capacity of the strains correlation is significant at the 0.05 level.

The results obtained in this study with regard to the technological properties of LAB and *Bifidobacterium* strains were shown a very important with those obtained by Herreros et al [28] observed that *L. lactis* subsp.

Table 5
Antibiotic sensitivity test of selected LAB and *Bifidobacterium* isolates

Symbol μg/disc	Inhibition zone										
	AM 10 μg	FA 10 μg	E 15 μg	OX 1 μg	C 30 μg	P 10 U	FOX 30 μg	AX 25 μg	CN 10 μg	S 10 μg	CE 30 μg
<i>Lc. lactis</i>	R	R	R	R	R	R	R	R	S	S	S
<i>Pc. acidilactici</i>	R	R	R	R	I	R	R	R	S	S	I
<i>Sc. boris</i>	R	R	R	R	R	R	R	R	S	S	I
<i>Ec. faecalis</i>	S	I	I	R	I	R	R	R	S	S	S
<i>Lb. Lactis</i>	S	S	S	S	I	I	I	S	S	S	S
<i>Bf. breve</i>	S	S	S	R	S	S	R	S	S	S	I
<i>Bf. infantis</i>	S	S	S	S	S	S	S	S	S	S	S
<i>Bf. adolescentis</i>	S	S	S	S	S	S	R	S	S	S	I
Bf. bifidum	S	S	S	R	S	R	R	I	I	S	I
<i>Bf. longum</i>	S	S	S	S	S	S	S	S	S	S	S
<i>Bf. thermophilus</i>	S	S	S	R	S	R	S	S	S	S	I
<i>Bf. suis</i>	S	S	S	S	S	R	S	S	S	S	S
<i>Escherichia coli</i> ATCC 25922	S		–	–	S	–	I	S	S	S	S
<i>Staphylococcus aureus</i> ATCC 25923	S		S	S	I	I	I	S	S	S	S

R: resistant, I: intermediate, and S: sensitive; AM, Ampicillin; FA, Fusidic Acid; E, Erythromycin; OX, Oxacillin; C, Chloramphenicol; P, Penicillin; FOX, Cefoxitin; AX, Amoxicillin, CN, Gentamicin, S, Streptomycin; CE, Cefotaxime.

lactis strains were those which showed the highest acidifying capacity, developing an acidity of 0.40 g.100 mL⁻¹ lactic acid after 6 h and 0.65 – 0.70 g.100 mL⁻¹ after 12 h.

3.4. Resistance of LAB to antibiotic

The cultures were demonstrated sensitive (S) and resistant (R) by observing the inhibitory zone against tested antibiotics taking into consideration the clinical break points presented by the FEEDAP panel [65]. The results of antibiotic susceptibility testing are shown in Table 5. The isolates were analyzed by the diffusion method in Muller-Hinton agar for eleven antibiotics.

In the present study, it was found that the *Bifidobacterium* isolates were sensitive to ampicillin, fusidic acid, erythromycin, oxacillin, chloramphenicol, penicillin, amoxicillin, gentamicin, streptomycin and cefotaxime, with the exception of *Bf. breve*, *Bf. adolescentis* strains were resistant to Cefoxitin whereas *Bf. longum*, *Bf. thermophilus*, *Bf. suis* were resistant to Oxacillin. While *Bf. bifidum* strain was resistant to three antibiotics (oxacillin, penicillin, cefoxitin) and also it has been intermediate to three antibiotics (amoxicillin, gentamicin, cefotaxime). Recent studies have reported that, among the different *Bifidobacterium* species, *B. breve* possesses resistant levels to several antibiotics that are higher than those of other *Bifidobacterium* species [66].

Previous studies document that *Bifidobacterium* were highly resistant to kanamycin, gentamicin, fusidic acid, streptomycin, polymyxin B, nalidixic acid, paromomycin, neomycin and susceptible to penicillin G [67–71], metronidazole, tetracycline, cephalotin, aztreonam, bacitracin, rifampicin and bacitracin [72, 73].

Although, *Lc. Lactis*, *Pc. Acidilactici*, *Sc. boris* strains were resistant to eight antibiotics tested (ampicillin, fusidic acid, erythromycin, oxacillin, chloramphenicol, penicillin, amoxicillin) but it's have been sensitive to the three antibiotics (gentamicin, streptomycin and cefotaxime). Delgado et al. [74] reported that *Lactobacillus*, *Lactococcus* and *Leuconostoc* species show resistance to high levels of cefoxitin (MIC ≥ 30 μg.ml⁻¹) whereas, *Bifidobacterium* species are very sensitive. However, resistant strains to these agents have also been identified

[74, 75] and several genes providing such resistance have been studied; e.g., a chloramphenicol-resistance *cat* gene has been found in *Lactobacillus reuteri* [76] and *Lactobacillus plantarum* [77]; different erythromycin-resistance genes (*erm*) which have been found in many species [78, 79] and a number of tetracycline resistance genes –tet (K, M, O, Q, S, W, 36) [80]. While, more studies reported that *Lc. lactis* have been shown resistant to chloramphenicol, clindamycin, streptomycin, erythromycin and tetracycline [81–83]. It was found to contain at least three different plasmid-encoded antibiotic resistance determinants (for tetracycline [tet (S)], chloramphenicol and streptomycin) [84] and tet (M) gene [85].

The knowledge of genetic mobility of antibiotic resistance genes constitutes an important task that should be explored. In fact, one of the essential definitions of a safe micro-organism that can be added to food preparation (e.g., *B. lactis*, *B. breve*, and *B. bifidum* probiotic strains) should be the absence of mobile antibiotic-resistance genes. Although it is clear that simultaneous application of susceptible probiotics with oral antibiotics is generally unreasonable, in the case of microbial infection it might be possible to use these resistant probiotics in combination with appropriate antibiotics [86].

Bifidobacteria are increasingly employed in the production of functional foods, only a few studies [69, 87] have been conducted in order to define the resistance/sensitivity to antibiotics of bifidobacteria. Antibiotic resistance is not a common trait of dairy and intestinal LAB and *Bf.* species. However, several strains were considered to be resistant to some antibiotics, and were thought to harbour acquired resistance genes [88]. European Commission, it can be concluded that the isolates do not carry any resistant genes and can be safely be used as starter cultures. The resistance gene reservoir hypothesis suggests that beneficial and commensal bacterial populations may play a role in the transfer of antibiotic resistance to pathogenic and opportunistic bacteria [89, 90].

The large numbers of LAB and *Bifidobacteria* in fermented products and in the GIT help in the appearance of different resistant mechanisms via mutation. In addition, LAB can also acquire resistances from other bacteria in the environment. Once a LAB becomes resistant, the determinant is amplified and may be transmitted to another host. Therefore, checking for signs of transferable antibiotic resistance in starter strains and bacteria used as feed and food additives is essential; quite apart from the need for their complete physiological and technological characterization [8]. Thought susceptibility levels are species dependent. LAB and bifidobacteria have probably acquired most antibiotic resistances by conjugation. Therefore, strains intended to be used in feed and food systems should be systematically monitored for resistances in order to avoid their inclusion in starters and probiotics. The widespread use of LAB and bifidobacteria in fermented foods and dairy products has a long history of safety, and it is generally assumed that the risk of infection from ingested bacteria is very low [91, 92]. At the same time, the use of LAB and bifidobacteria species as probiotics may help to reduce antibiotic use for therapeutic, prophylactic and growth promotion in animal husbandry [93].

3.5. *In vitro* inhibition of pathogen growth

Antimicrobial activity is a very important criterion for selection of the potential-antagonists-starter and probiotic-culture against harmful bacteria. The inhibition ability of supernatants from selected strain BHI 07 *Bf. bifidum* and five LAB selected strains (*Lc. Lactis*, *Pc. Acidilactici*, *Sc. boris*, *Ec. Faecalis*, *Lb. Lactis*) for their acid and bile resistance, performed the fastest acidification and reached the highest level of viable counts was determined against 4 pathogens, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10876, *Salmonella typhimurium* ATCC 14028 by the agar diffusion test to evaluate their antagonistic activity against potentially bacterial pathogens.

The inhibition of pathogenic bacteria, causing diarrhea or other diseases in the human intestine, is a desirable property for probiotic bacteria [74]. The positive control used (tetracycline) produced inhibition zones for all pathogens with diameters ranging from 14 to 29 mm (data not shown).

All the LAB and *Bifidobacterium* isolates exhibited wide spectrum of antibacterial activity against the pathogenic tested strains (*E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10876) but toward the pathogen strain *Salmonella typhimurium* ATCC 14012, *Bf. bifidum*, *Lc. Lactis*, *Sc. boris*, *Ec.*

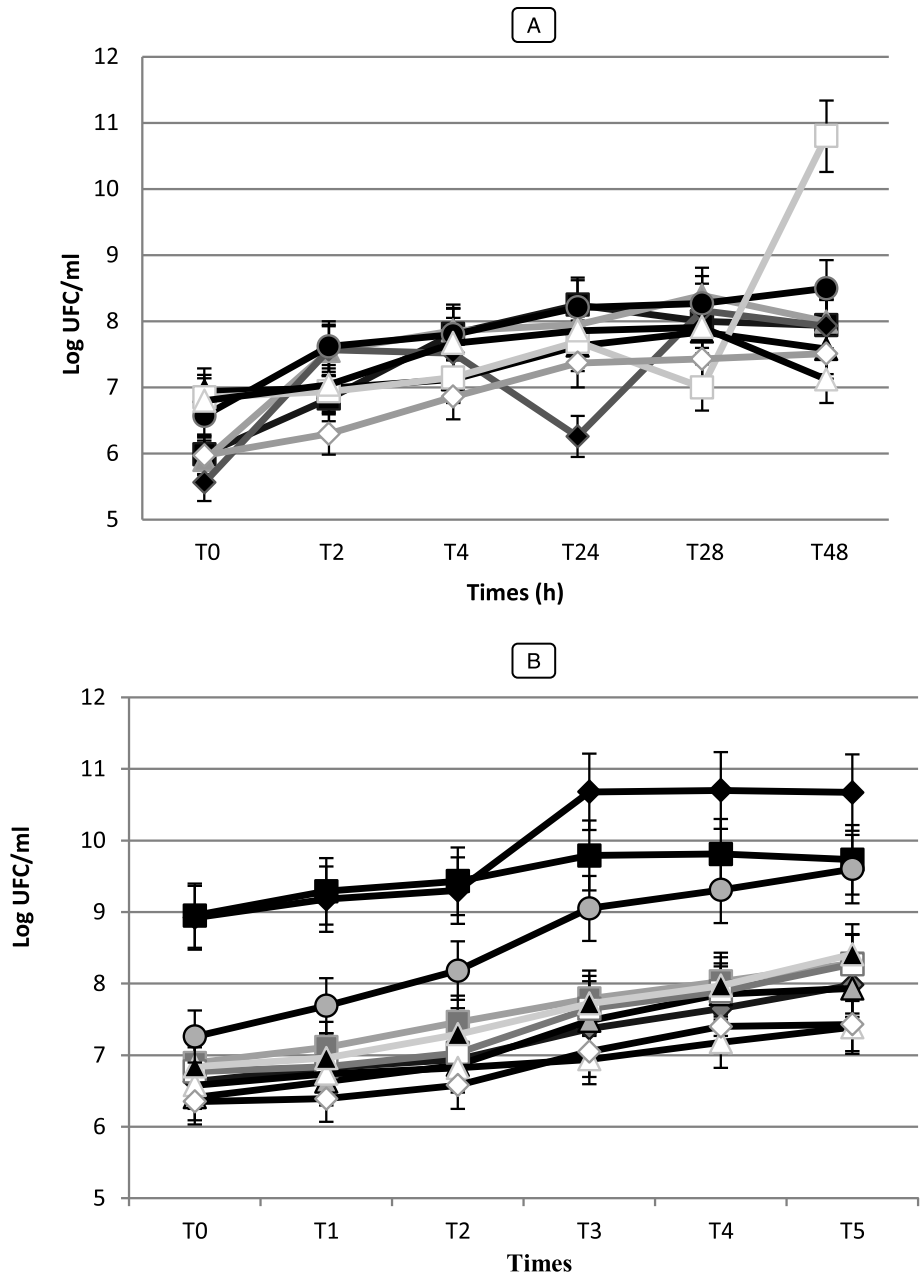


Fig. 8. Growth (Log UFC/ ml) of *Bifidobacterium* strain (A) : (■) *Bf. breve* (BHI 01), (◆) *Bf. infantis* (BHI 03), (▲) *Bf. Adolescentis* (BHI 04), (●) *Bf. bifidum* (BHI 05), (□) *Bf. Longum* (BHI 07), (◇) *Bf. thermophilum* (BHI 08), (Δ) *Bf. suis* (BHI 33). LAB strains (B) : (○) *Lc. cremoris* (Lc 02), (◐) *Lc. lactis* (Lc 03), (◑) *Pc. cerevisiae* (Pc 01), (◒) *Pc. acidilactici* (Pc 02), (◓) *Sc. boris* (Sc 01), (◔) *Sc. thermophilus* (Sc 02), (◕) *Sc. agalactiae* (Sc 03), (◖) *Ec. Faecium* (Ec 01), (◗) *Ec. Faecalis* (Ec 02), (◘) *Lb. bulgaricus* (Lb 01), (◙) *Lb. lactis* (Lb 02), in skim milk at 37°C. Vertical bars represent standard deviations of the mean.

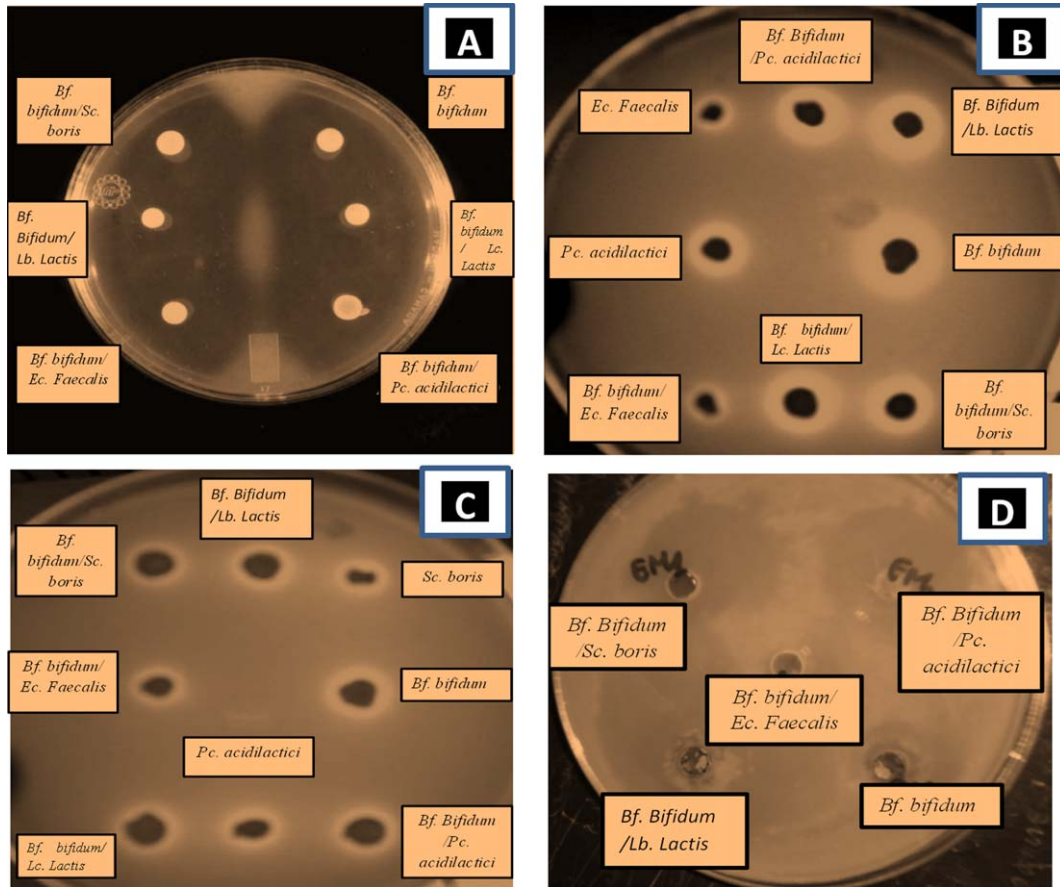


Fig. 9. Agar-well diffusion showing inhibition zones from antimicrobial activity of supernatants- LAB and *Bf. bifidum* BHI 07 grown alone or in combination with lactic acid bacteria grown in skim milk either alone or in combination with LAB: *Lc. Lactis*, *Pc. Acidilactici*, *Sc. Boris*, *Ec. Faecalis*, *Lb. Lactis*, on A) *E. coli*, B) *S. aureus*, C) *Bacillus cereus*, D) *Salmonella typhimurium*. Analyses were carried out using Muller-Hinton agar. Clear area is zone of inhibition.

faecalis in single trials and *Bf. bifidum* in mixed culture with *Sc. boris*. Strains showing a clear zone (Fig. 9) of lateral extension greater than 2 mm are considered as producers of antibacterial substances [94]. This inhibition could be due to the production of inhibitory substances, such as organic acids, bacteriocins or peroxide hydroxide [95].

E. coli ATCC 25922 strain was the most sensitive to the presence of *Bf. bifidum* in mixed culture with *Sc. boris* showing an inhibition of 35 ± 0.83 mm following by *Bf. bifidum* in mixed trial with *Lc. lactis* (31 ± 1.12 mm) but *Bf. bifidum* strain on single trials showing somewhat low inhibition effect (25 ± 1.83 mm). *Sc. boris* in single trials (9 ± 0.8 mm) (Table 6).

Staphylococcus aureus ATCC 6538 was the most sensitive to the presence of *Bf. bifidum* in mixed culture with *Lc. lactis* (37 ± 1.54 mm) following by *E. coli* ATCC 25922 and *Bacillus cereus* ATCC 10876 to the presence of *Bf. bifidum* in mixed trial with *Ec. Faecalis* (35 ± 0.83 mm; 34.5 ± 0.45 , respectively). But the inhibitory effect of *Bf. bifidum* strain in single trial on pathogenic bacteria was somewhat low. However, a loss of inhibitory activity was observed in *Lb. Lactis*; *Pc. acidilactici* in single trial and *Bf. bifidum* in mixed trial with the same

Table 6

In vitro inhibition of the growth of pathogens by *Bf. bifidum* and LAB strains tested. Results display the diameter of the inhibition zones (mm)

Strains	Diameter (mm) of inhibition zones			
	<i>E. coli</i> ATCC 25922	<i>Staphylococcus aureus</i> ATCC 6538	<i>Bacillus cereus</i> ATCC 10876	<i>Salmonella typhimurium</i> ATCC 14028
Single cultures				
<i>Bf. bifidum</i>	25 ± 1.83	24 ± 1.23	21 ± 1.49	1.2 ± 0.45
<i>Lc. Lactis</i>	17.5 ± 0.9	19 ± 0.09	13 ± 1.36	0.8 ± 0.5
<i>Pc. acidilactici</i>	21 ± 1.2	23 ± 0.05	19 ± 0.02	3.78 ± 0.14
<i>Sc. boris</i>	09 ± 0.8	14 ± 1.03	10 ± 0.09	0.3 ± 0.91
<i>Ec. Faecalis</i>	18 ± 1.05	22 ± 0.17	20 ± 1.06	0.9 ± 0.67
<i>Lb. Lactis</i>	20 ± 1.53	18 ± 1.4	23 ± 0.38	3.5 ± 0.65
Mixed cultures				
<i>Bf. bifidum</i> / <i>Lc. Lactis</i>	31 ± 1.12	37 ± 1.54	27 ± 0.16	2.5 ± 0.34
<i>Bf. bifidum</i> / <i>Pc. acidilactici</i>	28 ± 0.71	28 ± 0.48	25 ± 0.69	4 ± 0.12
<i>Bf. bifidum</i> / <i>Sc. boris</i>	29 ± 1.41	29.5 ± 0.15	22 ± 1.50	1.7 ± 0.39
<i>Bf. bifidum</i> / <i>Ec. Faecalis</i>	35 ± 0.83	32 ± 1.67	34.5 ± 0.45	2.5 ± 0.78
<i>Bf. bifidum</i> / <i>Lb. Lactis</i>	27 ± 0.21	33 ± 0.31	32 ± 1.18	3.7 ± 1.3

The strains examined include strains that survived exposure to pH 2 and to 0.3% oxgall for at least 1 h as well as.

Values are means ± S.E. of triplicate analyses.

strains (Table 6). This finding confirms the results of previous studies [96–98]. Therefore, the mixed cultures of *Bf. bifidum* strains with LAB strains were more active than the pure one. This result was also been found by Kheadr [99] who noted a remarkable antibacterial activity of *B. longum* DSM 20097 against both *Staphylococcus aureus* and *Bacillus subtilis*. When grown as co-cultures with *Lb. paracasei*, *Lb. acidophilus* or *Lb. plantarum*, *B. longum* appeared to be more active at inhibiting such indicator organism.

Antimicrobial activity of *bifidobacteria* was first noticed by Tissier [100], who described various types of antagonistic effect of *B. bifidum* against *E. coli*. Other reports described numerous antagonistic activity or specific antimicrobial activity in *bifidobacteria* linked to the production of lactic and acetic acids, or to bacteriocins [101–103]. Yildirim and Johnson [102] described a bacteriocin (bifidocin B) produced by *B. bifidum* NCFB 1454, which was found to be active against certain species of *Listeria*, *Bacillus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* and is believed to be encoded by a 8.4 kb plasmid.

4. Conclusion

In conclusion, the result of screening procedure of LAB and *Bifidobacterium* strains isolated from different niches as probiotic bacteria showed that BHI 07 *Bf. bifidum* strain was considered candidates to be used as putative probiotic starter cultures in fermented foods. The efficiency of the antimicrobial activity of the isolated strains needs to be confirmed *in vivo* and in the food matrix. Further study is needed to characterize the antimicrobial activity of the isolates reported in the present study and evaluate their effectiveness at inhibiting the pathogenic bacteria and their use as an alternative inexpensive and natural remedy to restore and maintain health.

In this context, the result is very important for the selection of probiotic bacteria and may be could have a broad application as probiotic strains. They may play an important role in the food industry as starter cultures, co-cultures or bioprotective cultures, to improve food quality and safety or as probiotic therapeutics appropriate for clinical practice. In addition, sensitivity or intrinsic resistance of the majority of the strains to a recommended

set of antibiotics make them safe for use in different products for animal consumption or human for treatment of infection diseases of microbial origin.

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