FUNCTIONAL, SAFETY AND TECHNOLOGICAL PROPERTIES OF *Lactobacillus acidophilus* BIOTECH 1900

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ABSTRACT

The study aimed to assess the probiotic potential of *Lactobacillus acidophilus* BIOTECH 1900. The isolate was evaluated *in vitro* for a series of functional, safety and technological properties that could enable its use as potential probiotics. In terms of its physiological functionality, the isolate was able to tolerate pH 3.0 and 0.3% bile salts with a resistance rate of 56.43% and 89.00%, respectively. It displayed a strong antagonistic activity against *Escherichia coli* and *Staphylococcus aureus*. The isolate demonstrated a strong auto-aggregating phenotype with 84.40% and 87.97% activity after a 3- and 5-hour incubation, respectively. However, it exhibited a low cell surface hydrophobicity in xylene (3.51%). Considering its safety aspects, the isolate exhibited γ-hemolysis. It was susceptible or moderately susceptible to antibiotics including amoxicillin, ampicillin, augmentin, penicillin G, erythromycin, clindamycin, chloramphenicol and tetracycline. However, it was resistant to cefaclor, kanamycin and streptomycin. Regarding its technological properties, the isolate demonstrated tolerance up to 9% (w/v) NaCl. It also exhibited the same behavior with regard to lactic acid production and viable cell count in different milk systems (cow, buffalo and goat's milk). The results show that *L. acidophilus* BIOTECH 1900 can be labeled as potential probiotics as it displayed desirable functional, safety and technological properties.

Key words: lactic acid bacteria, *Lactobacillus acidophilus*, milk, probiotics

INTRODUCTION

Probiotics are cultures of live microorganisms which confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2001). They are generally recognized as safe (GRAS) microorganisms of a viable single strain or mixed cultures that can be applied to medical and veterinary functions (Holzapfel and Schillinger, 2002). They include a wide range of microbes such as bacteria, yeasts, and molds. Among these microorganisms, lactic acid bacteria (LAB) are regarded as one of the most significant groups of probiotics

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known to have various beneficial effects on the gastrointestinal (GI) tract. They are naturally associated with the mucosal surface of animals, e.g., small intestine and colon. In addition, they are indigenous to food-related habitats including milk.

LAB are gram-positive, catalase-negative, usually non-motile, non-spore forming, non-pathogenic rods and cocci (Khalid, 2011). They are considered as industrially important bacteria as they contribute to the preservation, flavor, and texture of various fermented food and beverages (Walstra *et al*., 2006). In the dairy industry, *Lactobacillus* species have been conventionally used in the manufacture of various kinds of fermented dairy products such as fermented milk, acidophilus milk, cheese and yogurt. They are also considered as one of the most frequently used LAB with an application as probiotics (Holzapfel *et al*., 2001).

The incorporation of probiotics for the enhancement of the therapeutic value of various food products has become a popular trend in the food industry (Williams, 2010). Today, *Lactobacillus acidophilus* is one of the most common and well-known probiotic microorganisms (Ashrafuzzaman *et al*., 2015). However, not all strains of *L. acidophilus* exhibit probiotic characteristics. Only a few strains are able to fulfill the general criteria for the selection of probiotics which include safety, functional, and technological characteristics. Therefore, the objective of this study aimed to: (1) assess the probiotic properties of *L. acidophilus* BIOTECH 1900 based on its acid, bile and sodium chloride tolerance, antibiotic susceptibility characteristics, cell surface hydrophobicity, auto-aggregation, antibacterial and hemolytic activity; and (2) determine its behavior with regard to lactic acid production and viable cell count parameters in different milk systems (cow, buffalo and goat's milk).

MATERIALS AND METHODS

Lactobacillus acidophilus BIOTECH 1900 was acquired from the Philippine National Collection of Microorganisms, Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños. The strain was maintained on de Man, Rogosa, Sharpe (MRS) agar (HiMedia®, Mumbai, India) stab at 4°C after a 24-hour growth at 37°C. For routine analysis, the stock culture was activated twice in MRS broth (HiMedia®, Mumbai, India). Indicator strains, *Escherichia coli* (laboratory isolate) and *Staphylococcus aureus* BIOTECH 1350, were maintained on Brain Heart Infusion (BHI) agar (HiMedia®, Mumbai, India) slants. For working stocks, indicator strains were propagated in BHI broth (HiMedia®, Mumbai, India). The inoculum density of *L. acidophilus* BIOTECH 1900 was standardized prior to the conduct of *in vitro* studies on its probiotic properties. The cells were harvested through centrifugation (5,000 x g, 10 minutes, 4°C) and washed twice with sterile quarter-strength Ringer's solution. The culture was adjusted to give a turbidity equivalent to a 0.5 McFarland standard.

The acid and bile resistance rates of the isolate were determined using the method of Kumar and Kumar (2015) with slight modifications. For acid tolerance assay, 1 mL of the activated culture was inoculated into 10 mL MRS broth acidified to pH 1.0, 2.0, 3.0, 4.0, 5.0 and 7.0. The optical density (OD) was recorded at 560 nm after incubation (37°C for 24 hours). The standard for acid tolerant strain was set at pH 3.0 with more than 50% resistance rate. The acid resistance (%) was expressed as the percentage of growth at OD_{560nm} in different pH levels compared with the control (pH 7.0). For bile tolerance assay, 0.2 mL of the activated culture was inoculated into 10 mL MRS broth containing different concentrations (0.1 to 0.9 % w/v) of bile salt (Sigma-Aldrich, St. Louis, Missouri, USA). MRS broth

without bile salt served as the control. The OD was also measured at 560 nm after a 24-hour incubation at 37°C. The standard for bile tolerant strains was set at 0.3% with more than 50% resistance rate. The bile resistance (%) was expressed as the percentage of growth at OD_{560nm} in the presence of bile salts (0.1 to 0.9%) compared with the control.

Prior to the conduct of antibacterial activity assay, the cell-free supernatant (CFS) of the isolate was prepared using the method described by Saadatzadeh *et al*. (2013) with slight modifications. An overnight grown culture was centrifuged at 4,000 x g for 15 minutes at 4°C. The supernatant was then divided into two parts: one part of the CFS was left with its initial acid pH and the rest was neutralized to pH 6.5. Both supernatants were filter-sterilized through a 0.22 µm Millex®-GP syringe filter unit (Sigma-Aldrich, St. Louis, Missouri, USA). The antibacterial activity of the isolate against selected indicator strains was determined by standard well-diffusion assay using Mueller Hinton (MH) agar (HiMedia®, Mumbai, India) plates. The wells (≈6 mm) were filled with 100 µl CFS of the isolate. All MHA plates were incubated at 37°C for 18 to 24 hours. The diameter of the clear zones around each well was measured and scored according to Akabanda *et al*. (2014). The diameter of the zone was interpreted as follows: no inhibition (<1 mm), weak (1 to 4 mm), moderate (4 to 8 mm) and strong inhibition (8 to 12 mm).

The cell surface hydrophobicity (CSH) assay was carried out as described by Ji *et al*. (2015) with some modifications. An overnight grown culture was harvested by centrifugation (5,000 g, 10 min, 4°C) and washed twice with phosphate buffered saline (PBS). It was re-suspended with PBS and its absorbance was measured at 580 nm (reading 1). Xylene (Univar®, Ajax Finechem Pty Ltd, Australia) was added at a ratio of 1:1. The suspension was mixed for 2 minutes and allowed to stand for 30 minutes at 37°C for the separation of the two phases. The water-soluble layer (reading 2) was measured at 580 nm for the calculation of hydrophobicity.

$$Hydrophobicity\ (\%) = \frac{(OD_{580nm}\ reading\ 1 - OD_{580nm}\ reading\ 2)}{OD_{580nm}\ reading\ 1}\ x\ 100$$

Auto-aggregation assay was performed according to Melgar-Lalanne *et al*. (2015) with slight modifications. An overnight grown culture was harvested by centrifugation (5,000 x g, 10 minutes, 4°C) and washed twice with phosphate buffered saline (PBS). It was then re-suspended with PBS and its optical density was standardized to 0.5 ± 0.01 at 600 nm. Cell suspension (4 mL) was mixed by vortexing for 10 seconds and the auto-aggregation activity was determined after 3 and 5 hours of incubation at room temperature. A portion of upper suspension (0.1 mL) was transferred to another tube with 3.9 mL of PBS and the absorbance was measured at 600 nm. The auto-aggregation percentage is expressed as follows:

$$\text{Autoaggregation (\%)} = 1 - \frac{\text{Absorbance at time} = \text{ 3 or 5 hours}}{\text{Absorbance at time} = \text{ 0 hour}} \times 100$$

Hemolysis was determined by the method described by Halder *et al*. (2017) with slight modifications. Briefly, an overnight grown culture was streaked on sterile blood agar (HiMedia®, Mumbai, India) plates (BAP) supplemented with 5% defibrinated sheep blood. The plates were incubated at 37°C for 72 hours. The BAP were examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green-hued zones around colonies)

or γ-hemolysis (no zones around colonies).

The antibiotic resistance of *L. acidophilus* BIOTECH 1900 was assessed using the standard disc diffusion assay (Tambaker and Bhutada, 2010). An overnight grown culture was streaked over the entire surface of MRS agar plates. Antibiotic discs (MASTDISCS™, Mast Group Ltd, Merseyside, UK) were then placed on the surface of the agar (4 discs per plate). The plates were incubated at 37°C for 24 hours. The diameter of the halo was measured after incubation and the results were expressed in terms of the inhibition zone diameter (mm).

For sodium chloride tolerance assay, an overnight grown MRS broth culture of the isolate (1% v/v) was inoculated into sterilized MRS broth adjusted with different concentrations (1 to 10%, w/v) of NaCl (Univar®, Ajax Finechem Pty Ltd, Australia). The plates were incubated at 37°C for 24 hours and the growths were determined using turbidity method previously described by Hoque *et al*. (2010).

For the determination of the isolate's behavior in different milk system, 12% reconstituted skim milk (RSM) was prepared and sterilized at 10 psi for 10 minutes. The isolate was then inoculated (1% v/v) in sterilized RSM to generate starter culture. Cow, buffalo and goat's milk samples were subjected to compositional analysis to determine their fat and protein content using the Ekomilk-Ultra Milk Analyzer (Eon Trading, Bulgaria) and by performing the AOAC (2005) Kjeldahl method, respectively. Titratable acidity (expressed as % lactic acid) of the milk samples was determined using AOAC (2005) titration method and the pH was measured using the digital pH meter model 3505 (Jenway, Staffordshire, UK). The sterilization of the milk was carried out by autoclaving at 10 psi for 10 minutes. The sterile milk medium was inoculated with 2% (v/v) starter culture. Single strain fermentation was carried out at 37°C for 24 hours. The viable count, titratable acidity and pH were also determined after fermentation. The viable counts were obtained using the pour plating method.

All determinations were performed in triplicate. The resistance rates (acid and bile tolerance), antimicrobial activity, cell surface hydrophobicity, auto-aggregation activity and antibiotic susceptibility data were subjected to descriptive analysis. A one-way analysis of variance (ANOVA) in Completely Randomized Design (CRD) was used to analyze the data on fat, protein, titratable acidity, pH and viable count of the different milk media inoculated with *L. acidophilus* BIOTECH 1900. All statistical analyses were carried out using the Statistical Analysis System (SAS) release 9.4 (SAS Institute, Inc., Cary, NC) software. Comparison of treatment means was done using the Scheffe's test at and considered significant at 5% significance level.

RESULTS AND DISCUSSION

L. acidophilus BIOTECH 1900 displayed decreasing optical density and percentage resistance with decreasing pH level as shown in Table 1. At pH 5, it displayed the highest resistance rate of 98.28%. The standard for probiotics was set at pH 3 with a resistance rate of more than 50% (Kumar and Kumar, 2015). The isolate could be considered as an acid tolerant strain as it displayed resistance of 56.43% at pH 3. On the other hand, it was not resistant at pH 1 and 2 as indicated by the percentage resistance lower than 50%, suggesting that it was mostly killed by the acid stress. Generally, *Lactobacillus* strains are able to retain their viability when exposed to pH ranging from 2.5 to 4 but displayed a loss of viability at

lower pH values (Jacobsen *et al*., 1999; Dunne *et al*., 2001; Maragkoudakis *et al*., 2006). According to Matsumoto *et al*. (2004), acid tolerance of bacteria was related to the induction of H^+-ATPase activity in which glucose and other simple sugars provide the ATP pool required, allowing optimal H^+ extrusion by F_0F_1ATPase. The F_0F_1ATPase is a known mechanism that gram-positive organisms use for protection against acidic conditions (Cotter and Hill, 2003).

Table 2 shows that *L. acidophilus* BIOTECH 1900 displayed decreasing optical density and percentage resistance with increasing bile salt concentration. The standard for probiotics was set at 0.3% bile concentration with a resistance rate of more than 50% (Kumar and Kumar, 2015). The isolate was able to tolerate 0.1 and 0.3% bile concentrations with percentage resistance of 93.91% and 89.00%, respectively. On the other hand, it was not resistant at 0.5% to 0.9% bile concentrations as indicated by the percentage resistance lower than 50%. Bile salts can influence the intestinal microflora by acting as antimicrobial molecules (Fontana *et al*., 2013). Their strong lipophilic steroid ring targets the cell membrane of the microorganisms, in which they disturb the lipid packaging and disrupt the proton motive force, thereby causing cell death (Kurdi *et al*., 2006). The bile resistance of microorganism has been attributed to the various mechanisms including active efflux of bile acids or salts, bile salt hydrolysis, and changes in the architecture and composition of

Table 1. Optical density and resistance rate of *L. acidophilus* BIOTECH 1900 in MRS broth adjusted with different pH levels.

pH Level	**Optical Density (absorbance at 560 nm)**	**Resistance Rate (%)**
1	0.7817 ± 0.0070	28.95 ± 0.26
2	0.8460 ± 0.0013	31.33 ± 0.05
3	1.5235 ±. 01860	56.43 ± 0.69
4	2.2320 ± 0.0135	82.94 ± 0.50
5	2.6447 ± 0.0127	98.28 ± 0.47

Data are presented as mean ± standard deviation.

Table 2. Optical density and resistance rate of *L. acidophilus* BIOTECH 1900 in MRS broth adjusted with different concentrations of bile salts.

Bile Salt Concentration (%)	**Optical Density (absorbance at 560 nm)**	**Resistance Rate (%)**
0.1	2.4850 ± 0.0048	93.91 ± 0.18
0.3	2.3550 ± 0.0070	89.00 ± 0.26
0.5	1.2165 ± 0.0304	45.98 ± 1.15
0.7	0.3898 ± 0.0081	14.73 ± 0.31
0.9	0.2787 ± 0.0031	10.53 ± 0.12

Data are presented as mean ± standard deviation.

the cell membrane and cell wall (Ruiz *et al*., 2013).

L. acidophilus BIOTECH 1900 displayed a strong inhibitory activity against the indicator organisms when the cell-free supernatant (CFS) was used. Its inhibitory capacity against *E. coli* and *S. aureus* BIOTECH 1350 were 15 mm and 13 mm, respectively. The antibacterial activity of microorganisms is mostly attributed to the production of acetaldehyde, acetoin, carbon dioxide, diacetyl, ethanol, hydrogen peroxide, organic acids (acetic, benzoic, formic and lactic acid), bacteriocin, and antimicrobial peptide (Corr *et al*., 2007; Yuksekdag and Aslim, 2010; Todorov *et al*., 2011; Messaoudi *et al*., 2013; Shokryazdan *et al*., 2014). In this study, no activity was reported when the neutralized CFS was used. This would suggest that no bacteriocin-like activity exists in the CFS of the isolate. The observed inhibition may be the result of the action of organic acids since the inhibitory compound was completely lost when the CFS was neutralized to pH 6.5.

The cell surface hydrophobicity of *L. acidophilus* BIOTECH 1900 in xylene was 3.51%. According to Sharma and Sharma (2017), *Lactobacillus* strains with more than 40% affinity to non-polar solvents are generally more hydrophobic. Therefore, in this study, the isolate was considered hydrophilic. The hydrophilicity of bacteria results from the presence of polysaccharides in their cell surface (Pelletier *et al*., 1997). LAB intended for probiotic applications are preferred to have hydrophobic surface characteristics since hydrophobicity plays an important role in adhesion of microorganisms to intestinal epithelial cells (Xu *et al*., 2009).

L. acidophilus BIOTECH 1900 demonstrated an auto-aggregation activity of 84.40% and 87.97% after a 3- and 5-hour of incubation, respectively. The results indicate that the isolate exhibited a strong auto-aggregating phenotype as it had an activity of more than 40% (Wang *et al*., 2010). According to Janković *et al*. (2012), auto-aggregation is a property characterized by clumping of cells which belong to the same strain of bacteria. It plays an important role for probiotics in their adhesion, colonization, and persistence to oral cavity, gastrointestinal and urogenital tract (Nikolic *et al*., 2010). It was reported that the presence of proteins, glycoproteins, teichoic and lipoteichichoic acids on the cell wall surface may be involved in aggregation ability of microorganisms (Ramiah *et al*., 2008; Li *et al*., 2015).

L. acidophilus BIOTECH 1900 exhibited γ-hemolytic activity (no hemolytic activity) when grown in sheep blood agar as indicated by the absence of clear or green-hued zones around its colony. The result is in agreement with many reports which revealed the non-hemolytic activity of LAB (Carasi *et al*., 2014; Yadav *et al*., 2016; Ida Muryany *et al*., 2017). This finding may indicate the non-pathogenic nature of the isolate. Other virulence markers, however, should be thoroughly studied to further confirm its safety properties.

L. acidophilus BIOTECH 1900 expressed variable sensitivity to the inhibitors of the cell wall synthesis (Table 3). It showed susceptibility to all penicillins and β-lactamase studied, i.e. amoxicillin, ampicillin, augmentin, and penicillin G. However, it showed resistance to a second-generation cephalosporin antibiotic, cefaclor. When inhibitors of the protein synthesis were used, it exhibited susceptibility or moderate susceptibility to chloramphenicol, erythromycin, clindamycin, and tetracycline. However, it displayed resistance to kanamycin and streptomycin. According to Gueimonde *et al*. (2013), lactobacilli are normally susceptible to the cell wall-targeting penicillin, β-lactamase and low concentrations of many protein synthesis inhibitors including chloramphenicol, macrolides (e.g. erythromycin), lincosamides (e.g. clindamycin), and tetracycline but are

Table 3. Antibiotic susceptibility pattern of *L. acidophilus* BIOTECH 1900 using disc diffusion array on MRS agar.

Antibiotics	Concentration (μg)	Zone of Inhibition (mm)[1]	Interpretation[2]
Cell wall synthesis inhibitors			
Amoxicillin	10	36	Susceptible
Ampicillin	10	35	Susceptible
Augmentin	30	39	Susceptible
Penicillin G	10	44	Susceptible
Cefaclor	30	12	Resistant
Protein synthesis inhibitors			
Kanamycin	30	0	Resistant
Streptomycin	10	11	Resistant
Chloramphenicol	30	17	Moderately Susceptible
Erythromycin	15	32	Susceptible
Clindamycin	2	21	Susceptible
Tetracycline	30	29	Susceptible

[1]Inhibition zone diameters are means from triplicate determination. Diameters of the discs (6 mm) are inclusive.
[2]Susceptibility was expressed as susceptible, moderately susceptible and resistant (Charteris *et al*., 1998).

more resistant to cephalosporin (e.g. cefaclor) and aminoglycosides (e.g. kanamycin and streptomycin).

The salt tolerance of *L. acidophilus* BIOTECH 1900 was determined in MRS broth with different concentration of NaCl (1 to 10%) after a 24-hour incubation (Table 4). The isolate was able to tolerate up to 9% NaCl with a maximum growth up to 4% NaCl. This test gave an indication of the osmotolerance level of a LAB strain. According to Wood (2015), some microorganisms may overcome salt stress by accumulating or releasing solutes, thus, attenuating water fluxes. Those solutes include inorganic ions (often K^+) and organic molecules known as osmolytes or compatible solutes.

Table 5 shows the microbial and physicochemical properties of three milk media (cow, buffalo and goat's milk). Buffalo's milk had significantly ($P<0.05$) higher fat (7.91%) and protein content (4.02%) compared to goat (4.13% fat, 3.63% protein) and cow's milk (3.31% fat, 3.27% protein). However, no significant difference ($P>0.05$) was observed in terms of their initial pH and acidity (% lactic acid). After a 24-hour fermentation, the developed acidity (% lactic acid), pH level and viable cell count (log cfu/ml) of the sterile buffalo's milk inoculated with *L. acidophilus* BIOTECH 1900 had no significant differences ($P>0.05$) compared to cow and goat's milk. The results indicated that *L. acidophilus* BIOTECH 1900 demonstrated the same behavior with regard to lactic acid production and viable cell count in different milk systems such as in cow, buffalo and goat's milk. The developed acidity and pH of the milk media ranged from 0.37% to 0.40% lactic acid and 4.80 to 4.88, respectively. Moreover, their viable cell counts ranged from 9.07 to 9.16 log cfu/ml. The International Dairy Foundation (IDF) suggested a minimum number of 7 log cfu/ml of the product consumed to confer the health benefits associated with probiotic consumption

Table 4. Halotolerance activity of *L. acidophilus* BIOTECH 1900 to different concentrations of NaCl in MRS broth.

NaCl Concentration (%)	*L. acidophilus* BIOTECH 1900[1]
0	++
1	++
2	++
3	++
4	+
5	+
6	+
7	+
8	+
9	+
10	-

[1]Maximum growths were indicated as double positive sign (++), normal growths as single positive sign (+) and no growth as negative sign (-) for NaCl.

Table 5. Viable count and physico-chemical properties of different media (cow, buffalo and goat's milk) inoculated with *L. acidophilus* BIOTECH 1900.

Parameters	Milk Media			*P*-value
	Cow	Buffalo	Goat	
Fat	3.31 ± 0.16[c]	7.91 ± 0.11[a]	4.13 ± 0.06[b]	<0.0001
Protein	3.27 ± 0.07[c]	4.02 ± 0.13[a]	3.63 ± 0.08[b]	0.0002
pH				
0 hr[ns]	6.71 ± 0.04	6.81 ± 0.06	6.73 ± 0.04	0.0683
24 hr[1ns]	4.88 ± 0.03	4.83 ± 0.04	4.80 ± 0.06	0.1482
Acidity (% lactic acid)				
0 hr[ns]	0.14 ± 0.01	0.15 ± 0.01	0.16 ± 0.02	0.2556
24 hr[1ns]	0.37 ± 0.01	0.38 ± 0.02	0.40 ± 0.03	0.2589
Viable count (log cfu/mL)[1ns]	9.07 ± 0.01	9.13 ± 0.06	9.16 ± 0.05	0.0883

Data are presented as mean ± standard deviation.
[1]Incubation was carried out for 24 hours at 37^{0}C.
[a-c]Means within row with different superscript are significantly different ($P \leq 0.05$).
[ns]Not significant at $P > 0.05$.

(Kaur *et al*., 2014). In this study, *L. acidophilus* BIOTECH 1900 was able to surpass the recommended level set by the IDF.

It can be concluded that *L. acidophilus* BIOTECH 1900 displayed desirable probiotic properties based on acid, bile and sodium chloride tolerance, antibiotic susceptibility

characteristics, auto-aggregation, antibacterial and hemolytic activity. The isolate also exhibited the same behavior with regard to lactic acid production and viable cell count in different milk system (cow, buffalo or goat's milk). Further studies on the technological, safety and health-promoting properties are recommended before it can be utilized for commercial applications.

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