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In vitro evaluation of probiotic potential of *Lactobacillus paracasei* BIOTECH 10363 strain isolated from *Tapuy*

[1,*]Malilay, J.K.T., [1]Bautista, J.A.N., [2]Castillo-Israel, K.A.T. and [1]Oliveros, M.C.R.

[1]*Institute of Animal Science (IAS), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB), College, Laguna, Philippines, 4031*
[2]*Institute of Food Science and Technology (IFST), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB), College, Laguna, Philippines, 4031*

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Abstract

Fermented foods can be a source of potentially probiotic microorganisms as they contain different types of lactic acid bacteria. In the Philippines, there are a variety of traditional fermented food products that may harbor probiotics. This study determined the probiotic properties of *Lactobacillus paracasei* BIOTECH 10363 isolated from *Tapuy,* an indigenous alcoholic beverage made from fermented glutinous white rice. The acid and bile resistance, antibacterial, cell surface hydrophobicity, auto-aggregation, NaCl tolerance, hemolytic, and antibiotic sensitivity properties of *L. paracasei* BIOTECH 10363 were evaluated *in vitro*. The isolate displayed resistance to acid (pH 3 to 5) and bile (0.1 to 0.5%). Its cell-free supernatant exhibited strong inhibitory activity against *Escherichia coli*. Higher affinity to xylene and chloroform was observed in *L. paracasei* BIOTECH 10363. This suggests that the cell surface of the isolate has hydrophobic and electron donor (basic) properties. Moreover, the isolate had a strong auto-aggregating phenotype. Cell surface hydrophobicity and strong auto-aggregation activity could enable the isolate to adhere, colonize and survive in the gastrointestinal tract. The isolate could withstand MRS broth with 9% NaCl. It also displayed γ-hemolytic activity in blood agar plates indicating the non-hemolytic nature of the isolate. *L. paracasei* BIOTECH 10363 expressed sensitivity against various cell wall synthesis inhibitors (amoxicillin, ampicillin, augmentin, penicillin, and cefaclor) and protein synthesis inhibitors (streptomycin, chloramphenicol, clindamycin, erythromycin, and tetracycline). However, it displayed intrinsic resistance toward kanamycin. The findings of this study showed the promising potential of *L. paracasei* BIOTECH 10363 as a candidate probiotic strain as it exhibited desirable attributes *in vitro*.

1. Introduction

In recent years, fermented foods are increasingly recognized as a good source of potentially probiotic microorganisms because they normally contain a wide range of lactic acid bacteria (Rezac *et al*., 2018). In the Philippines, there are numerous indigenous fermented food products that have been traditionally part of the local diet. These include cheese and fermented foods made from fish and fishery products, meat and sausages, vegetables and fruits, rice, cassava, sugar cane, coconut and soya (Banaay *et al*., 2013). *Tapuy* is an indigenous alcoholic beverage made from fermented glutinous white rice and produced mainly in the northern part of Luzon Island, Philippines. On its own, *Tapuy* has naturally occurring bioactive compounds such as phenolics, flavonoids, and tannins that act as antioxidants and play a role as free radical scavengers (Hipol and Alma-in, 2018). Furthermore, it contains various microorganisms including many beneficial bacteria (Sanico and Medina, 2020).

Probiotics are cultures of live microorganisms which confer health benefits to the host when administered in adequate amounts (Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO), 2001). They are a diverse group of microbes that consist of beneficial bacteria, molds and yeast. Consumption of probiotics has been linked to improved health and better overall well-being. Numerous scientific works have described the beneficial role of probiotics in the treatment and prevention of acute

*Corresponding author.
Email: *jtmalilay@up.edu.ph*

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infectious diarrhea, antibiotic-associated diarrhea, acute respiratory tract infections, *Clostridium difficile* infection, eczema, inflammatory bowel diseases, infant colic and necrotizing enterocolitis (Liu *et al*., 2018; Sanders *et al*., 2018). Moreover, many potential health benefits are currently being investigated including the effect of probiotics on depression, anxiety, psychological stress, and other brain functions (Sarkar *et al*., 2016). The exact mechanisms of action of probiotics have not yet been fully elucidated. However, some key mechanisms that may be involved include competitive inhibition of pathogens, reduction of gut pH, stimulation of mucosal barrier function, inhibition of bacterial adherence or translocation, increasing mucus production, secretion of antimicrobial peptides (bacteriocins/ defensins) and production of volatile fatty acids (Ng *et al*., 2009; Bermudez-Brito *et al*., 2012; Kechagia *et al*., 2013).

Lactic acid bacteria (LAB) are often considered one of the most important groups of probiotic microorganisms (Sadiq, 2022). The most widely used LAB with an application as probiotics belong to the two main genera of *Lactobacillus* and *Bidifobacterium* (FAO/ WHO, 2006). Although, microorganisms from other genera are also being used such as *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Sporolactobacillus* and *Streptococcus* (Kailasapathy, 2013). Today, *L. paracasei* is one of the most frequently utilized probiotics in food, dietary supplements and pharmaceutical products (Fijan, 2014; Stefanovic *et al*., 2017). *Lactobacillus paracasei* is a gram-positive, catalase-negative, non-spore-forming, rod-shaped bacterium. It is usually found in milk and dairy products, fermented sausages, vegetables and wine (Gobbetti and Minervini, 2014).

Many previous studies have concentrated on the isolation, identification and characterization of microorganisms in *Tapuy*. However, there is very limited information available on the probiotic potential of lactic acid bacteria isolated from this local rice wine. Therefore, the aim of this study was to assess the acid and bile tolerance, antibacterial against *Escherichia coli*, cell surface hydrophobicity, auto-aggregation, NaCl tolerance, hemolytic and antibiotic susceptibility properties of *L. paracasei* BIOTECH 10363 isolated from *Tapuy*.

2. Materials and methods

2.1 Bacterial strains and culture conditions

An active culture of *L. paracasei* BIOTECH 10363 was acquired from the Philippine National Collection of Microorganisms (PNCM), National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB), College, Laguna, Philippines. The pure culture of the isolate was obtained using the four-quadrant streak plate method (Sanders, 2012). Isolated colonies from the fourth quadrant were picked and subcultured into *Lactobacillus* de Man, Rogosa, Sharpe (MRS) agar stabs. Inoculated tubes were then incubated at 37°C for 18 to 24 hrs. After incubation, the prepared agar stabs were stored and served as stock cultures. For working stocks, the purified stock culture was activated by two sequential transfers in MRS broth before each use.

For the propagation of the indicator strain, *Escherichia coli* (laboratory isolate) was streaked in Brain Heart Infusion (BHI) agar slants using a sterilized inoculating loop. Inoculated tubes were then incubated at 37°C for 18 to 24 hrs. After incubation, the prepared agar slants were stored and served as stock cultures. For working stocks, *E. coli* was propagated (1% v/v) in BHI broth for 18 to 24 hrs at 37°C. All working stocks were kept in the refrigerator at 4°C for storage.

2.2 Standardization of inoculum density

Bacterial cells were collected from the working stock of *L. paracasei* BIOTECH 10363 using a laboratory bench-top refrigerated centrifuge (5,000×*g*, 10 mins, 4° C). Cells were washed twice in 10 mL sterile ¼-strength Ringer's solution and then resuspended in the same solution. The resulting suspension was photometrically adjusted to that of a 0.5 McFarland standard (Kumar and Kumar, 2015).

2.3 Acid resistance assay

The acid tolerance assay was adapted from the method previously described by Kumar and Kumar (2015) with slight modifications. A volume of the working stock (1 mL) was inoculated into tubes containing modified MRS broth (10 mL) with different pH levels (1, 2, 3, 4, 5 and 7). MRS broth adjusted to pH 7 served as the control. The inoculated tubes were then incubated for 18 to 24 hrs at 37°C. After incubation, the optical density at 560 nm (OD_{560nm}) was measured and the acid resistance (%) was calculated using the formula below:

$$\% \text{ Resistance} = \frac{\text{Increment of } OD_{560nm} \text{ in MRS broth with pH 1,2,3,4, 5}}{\text{Increment of } OD_{560nm} \text{ in MRS broth with pH 7 (control)}} \times 100$$

2.4 Bile salt resistance assay

The bile salt tolerance of *L. paracasei* BIOTECH 10363 was assessed using the method previously described by Kumar and Kumar (2015) with slight modifications. A volume of the working stock (0.2 mL) was inoculated into tubes containing modified MRS

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broth (10 mL) with different concentrations (0.1, 0.3, 0.5, 0.7 and 0.9 % w/v) of ox gall bile salts. MRS broth without ox gall bile salts served as the control. The inoculated tubes were incubated at 37°C for 18 to 24 hrs. After incubation, the optical density at 560 nm was measured and 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.

2.5 Antibacterial activity assay (well-diffusion assay)

The antagonistic effect of *L. paracasei* BIOTECH 10363 against *E. coli* was evaluated using a standard agar well diffusion assay. Around 100 µl of the working stock of *E. coli* was streaked on the Mueller Hinton agar (MHA) plate surface using sterile cotton swabs. Agar wells (6 mm) were then made using sterilized pipette tips.

Cell-free supernatant (CFS) of *L. paracasei* BIOTECH 10363 was obtained following the method of Saadatzadeh *et al*. (2013) with slight modification. CFS was separated from the working stock of *L. paracasei* BIOTECH 10363 using a laboratory bench-top refrigerated centrifuge (4,000×*g*, 15 mins, 4°C). The supernatants were then filter-sterilized using a Millex®-GP syringe filter unit with a pore size of 0.22 µm. A portion of the CFS was used to assay bacteriocin production by adjusting the pH to 6.5 while the rest was left with its initial pH.

Around 100 µl of CFS of *L. paracasei* BIOTECH 10363 were then loaded in each agar well. The plates were kept for 1 hr at ambient temperature for diffusion and incubated for 18 to 24 hrs at 37°C. The diameter of the zone of inhibition (ZOI) was measured and recorded in mm after incubation. The obtained ZOI was interpreted according to Akabanda *et al*. (2014).

2.6 Microbial adhesion to hydrocarbons assay

The microbial adhesion to hydrocarbons (MATH) assay was adopted from the modified method of Ji *et al*. (2015). Briefly, bacterial cells were collected from the working stock of *L. paracasei* BIOTECH 10363 using a laboratory bench-top refrigerated centrifuge (5,00×*g*, 10 mins, 4°C). Cells were washed twice in sterile PBS (phosphate-buffered saline) and then resuspended in the same buffer solution. The optical density at 580 nm (OD_{580nm}) of the resulting suspension was determined and referred to as reading 1. Equal volumes of the suspension and solvents (xylene, chloroform, or ethyl acetate) were mixed for 2 mins using a vortex mixer (Uzusio VTX-3000L model, LMS Co., Ltd., Tokyo, Japan). The two phases were left to separate for 30 mins at 37°C. A portion of the aqueous phase was collected, and its optical density was measured at 580 nm (reading 2). The cell surface hydrophobicity was computed using the formula below:

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

2.7 Auto-aggregation assay

Auto-aggregation activity was measured following the method previously described by Melgar-Lalanne *et al*. (2015) with some modifications. Bacterial cells were collected from the working stock of *L. paracasei* BIOTECH 10363 using a laboratory bench-top refrigerated centrifuge (5,000×*g*, 10 mins, 4°C). Cells were washed twice in sterile PBS and then resuspended in the same buffer solution. The optical density at 600 nm (OD_{600nm}) of the resulting suspension was adjusted to 0.5±0.01. Around 4 ml of the cell suspension was thoroughly mixed for 2 mins using a vortex mixer. Auto-aggregation activity was assessed at 3- and 5-hr incubation at ambient temperature. An aliquot (0.1 ml) was taken from the upper suspension and pipetted onto a 3.9 mL sterile PBS solution. The absorbance of the resulting mixture was determined at 600 nm and the auto -aggregation activity of the tested strain was computed as follows:

$$\text{Autoaggregation (\%)} = 1 - \frac{A_t}{A_0} \times 100$$

Where A_t = Absorbance at time (t) = 3 or 5 hrs and A_0 = Absorbance at t = 0 hr

2.8 Halotolerance assay

A volume (1% v/v) of the working stock of *L. paracasei* BIOTECH 10363 was inoculated into tubes containing modified MRS broth (10 mL) with different concentrations (1 to 10%, w/v) of sodium chloride (NaCl). MRS broth without NaCl served as the control. The inoculated tubes were then incubated at 37°C for 18 to 24 hrs. After incubation, bacterial growth was examined using the turbidimetric method of Hoque *et al*. (2010). The growth was interpreted as follows: (-) = no growth; (+) = normal growth; and (++) = maximum growth.

2.9 Hemolytic activity assay

Hemolytic activity assay was performed following the modified method of Halder *et al*. (2017). Sterile and defibrinated sheep blood was procured from the Animal Disease Diagnostic Laboratory, Veterinary Teaching Hospital, College of Veterinary Medicine (CVM), UPLB. The blood was subjected to a sterility test. Briefly, sterile blood agar plates (BAP) were aseptically prepared by mixing blood agar base (infusion agar) and 5% (v/v) sheep blood. The prepared BAPs were then incubated for 48 hrs at 37°C. After incubation, BAPs

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were investigated for the presence or absence of microbial growth. All test plates remained clear indicating the sterility of the sheep blood used in the study.

One loopful of the working stock of *L. paracasei* BIOTECH 10363 was streaked on the sterile BAP surface. Inoculated BAPs were incubated for 72 hrs at 37°C. After incubation, the plates were then examined for the presence of clear zones (β-hemolysis) or green-hued zones (α-hemolysis) around individual colonies. The absence of such zones in the BAPs indicates γ-hemolysis.

2.10 Antibiotic susceptibility test

The antibiotic susceptibility pattern of *Lactobacillus paracasei* BIOTECH 10363 was determined semi-quantitatively using a standard disc diffusion assay. Briefly, one loopful of the working stock of *L. paracasei* BIOTECH 10363 was streaked on the MRS agar plate surface using sterile cotton swabs. Using an aseptic technique, antibiotic discs were laid on the surface of agar plates using sterile forceps. The plates were then maintained at 40°C for 1 hr for diffusion and then incubated for 24 hrs at 37°C. After incubation, the diameter of the zone of inhibition (ZOI) around the antibiotic disc was measured and recorded in mm. The results were categorized as resistant (R), moderate susceptible (MS), or susceptible (S) according to the CLSI interpretative standards described previously by Charteris *et al*. (1998).

2.12 Ethical statement

The study did not include any human and/or animal experiments.

2.13 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Data on antibacterial activity, MATH assay, auto-aggregation activity, NaCl tolerance assay, hemolytic activity, and antibiotic susceptibility were subjected to descriptive analysis. Data on acid and bile tolerance assays were analyzed using a one-way analysis of variance (ANOVA) in a completely randomized design (CRD) with pH levels and bile concentrations as treatments, respectively. SAS® (Statistical Analysis System) OnDemand for Academics (SAS Institute Inc., Cary, NC) software was used to conduct all statistical analyses. Scheffe's post hoc test was employed for pairwise comparison of treatment means at a 5% level of significance.

3. Results and discussion

3.1 Acid resistance of Lactobacillus paracasei BIOTECH 10363

Table 1 displays the optical density and resistance rate of *L. paracasei* BIOTECH 10363 in modified MRS broth with various pH levels. The isolate exhibited decreasing optical density and resistance rate with decreasing pH levels. The highest resistance rate of *L. paracasei* BIOTECH 10363 was observed in MRS broth adjusted to pH5. It differed significantly (P<0.05) from the resistance rate observed in MRS broth adjusted to pH 1, 2, 3 and 4. According to Kumar and Kumar (2015), *Lactobacillus* species are considered acid resistant when the resistance rate at pH3 is 50% or higher. In this study, *L. paracasei* BIOTECH 10363 can be labeled as an acid-resistant strain since it had a resistance rate of 57.63% at pH 3. However, it was not resistant at pH 1 and 2, implying that the survival of the isolate was greatly reduced at pH values lower than 3. Several acid-resistant strains have also been identified in previous studies including *L. paracasei* KW3110 (Nishida *et al*., 2008), *L. paracasei* CT12 (Romero-Luna *et al*., 2020) and *L. paracasei* PCR140 (Akmal *et al*., 2022).

Table 1. Optical density and resistance rate of *L. paracasei* BIOTECH 10363 in MRS broth adjusted with different pH levels.

pH level	Optical density (absorbance at 560 nm)	Resistance rate (%)
1	0.7637±0.0113	26.60±0.39^{e}
2	0.8843±0.0148	30.81±0.52^{d}
3	1.6542±.0047	57.63±0.16^{c}
4	2.2168±0.0101	78.14±0.35^{b}
5	2.7417±0.0150	96.64±0.53^{a}
	P-value:	<0.001

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different (P≤0.05).

Low pH can cause changes in the structural and functional integrity of cellular components such as proteins. These changes can disrupt nutrient transport and energy generation, impairing microbial growth (Ray, 2004). Microorganisms employ a myriad of resistance mechanisms when exposed to acidic environments including F_0F_1ATPase proton pumps, macromolecule protection and repair, cell membrane modification, amino acid decarboxylation and deamination, metabolic regulation and protection from organelle (Guan and Liu, 2020). In lactobacilli, the acid resistance is mostly attributed to their ability to regulate and maintain a steady gradient between the intracellular (cytoplasmic) and extracellular pH through the extrusion of a proton by F_0F_1ATPase proton pumps (Corcoran *et al*., 2005). This

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mechanism would allow microorganisms to survive and remain viable in the acidic environment of the stomach during the digestion process.

3.2 Bile resistance of Lactobacillus paracasei BIOTECH 10363

Table 2 shows the optical density and resistance rate of *L. paracasei* BIOTECH 10363 in modified MRS broth with different bile salt concentrations. The isolate displayed decreasing optical density and resistance rate with increasing bile concentration. The highest resistance rate of *L. paracasei* BIOTECH 10363 was observed in MRS broth with 0.1% (w/v) bile salts. It differed significantly (P<0.05) from the resistance rate observed in MRS broth supplemented with 0.5%, 0.7% and 0.9% (w/v) bile salts. However, it was not significantly different from the resistance rate obtained in MRS broth with 0.3% (w/v) bile salts. According to Kumar and Kumar (2015), *Lactobacillus* species are considered bile resistant when the resistance rate at 0.3% bile salt concentration is 50% or higher. In this study, *L. paracasei* BIOTECH 10363 can be labeled as a bile-resistant strain since it displayed a resistance rate of 88.56% at 0.3% (w/v) bile salts. However, the isolate was not resistant at bile salt concentrations higher than 0.5%.

Table 2. Optical density and resistance rate of *L. paracasei* BIOTECH 10363 in MRS broth adjusted with different concentrations of bile salts.

Bile salt concentration (%)	Optical density (absorbance at 560 nm)	Resistance rate (%)
0.1	2.4600±0.0079	90.18±0.29[a]
0.3	2.4160±0.0060	88.56±0.22[a]
0.5	2.1345±0.0252	78.25±0.92[b]
0.7	0.9677±0.0568	35.47±2.08[c]
0.9	0.3883±0.0144	14.24±0.53[d]
	P-value:	<0.001

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different (P≤0.05).

Bile is extremely toxic to microorganisms, especially to those that are not adapted to intestinal conditions. It causes structural changes in the cell membrane by disturbing the lipid packaging and disrupting the proton motive force, ultimately leading to cell death (Kurdi *et al*., 2006). Microorganisms use a multitude of bile resistance mechanisms such as the bile-efflux systems, cell membrane and cell wall modification, bile salt hydrolysis, general stress response, protection against oxidative damage and global glycolytic reorganizations (Ruiz *et al*., 2013). Generally, the bile resistance of lactic acid bacteria is attributed to the active extrusion of the bile acids or salts that accumulate in the cytoplasm using bile-efflux systems. A number of these efflux pumps or transporters have been identified and characterized in lactobacilli (Whitehead *et al*., 2005; Pfeiler and Klaenhammer, 2009). This system would permit microorganisms to survive and persist in the intestinal tract where they can exert their health benefits to the host.

3.3 Antibacterial activity of Lactobacillus paracasei BIOTECH 10363

The inhibitory capacity of *L. paracasei* BIOTECH 10363 against an indicator microorganism is shown in Table 3. When the cell-free supernatant (CFS) was used, the isolate demonstrated strong inhibitory activity against *Escherichia coli*. However, this activity was not detected when the CFS was neutralized to pH 6.5. This finding implies that the CFS of the isolate lacks bacteriocin and bacteriocin-like activities and the observed inhibition could be due to the action of organic acids and other antimicrobial compounds.

The antimicrobial activity of *L. paracasei* strains against *E. coli* has also been described in previous studies (Islam *et al*., 2012; Coman *et al*., 2014; Smetankova *et al*., 2014). However, the degree of inhibition differs between strains. The antimicrobial activity of lactic acid bacteria has been mostly attributed to the production of acids (e.g., acetic acid, benzoic acid and lactic acid) and other primary metabolites such as acetaldehyde, acetoin, carbon dioxide, diacetyl, ethanol, fatty acids, hydrogen peroxide, reuterin and reutericyclin (Šušković *et al*., 2010; Shokryazdan *et al*., 2014). Biologically active peptides with antimicrobial properties may also be produced by other strains (Todorov *et al*., 2011; Messaoudi *et al*., 2013). These antimicrobial compounds may beneficially modulate the intestinal ecology by preventing the pathogenic colonization of the gastrointestinal tract.

Table 3. Antibacterial activity of *L. paracasei* BIOTECH 10363 against *E. coli*.

Supernatant	pH	Inhibition diameter (mm)[1]	Interpretation[2]
Cell-free Supernatant	5.12	12.33±0.47	Strong inhibition
Neutralized Cell-free Supernatant	6.50	0.00±0.00	No inhibition

[1] Inhibition zone diameters are means from triplicate determination. Diameters of the wells (6 mm) are inclusive.

[2] The diameter of the zone was interpreted according to Akabanda *et al*. (2014): <1 mm = no inhibition (-); 1 to 4 mm = weak (+); 4 to 8 mm = moderate inhibition (++); and 8 to 12 mm = strong inhibition (+++).

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3.4 Cell surface hydrophobicity of Lactobacillus paracasei BIOTECH 10363

The cell surface properties of *L. paracasei* BIOTECH 10363 were determined photometrically using the MATH assay (Table 4). The adhesion of microorganisms to xylene, a non-polar solvent, reflects cell surface hydrophobicity. According to Sharma and Sharma (2017), *Lactobacillus* strains with more than 40% affinity to non-polar solvents are generally more hydrophobic. In this study, the affinity of *L. paracasei* BIOTECH 10363 in xylene was 82.79%. Furthermore, the isolate had a higher affinity to chloroform (acidic solvent and electron acceptor) than ethyl acetate (basic solvent and electron donor). The results suggest that the cell surface of the isolate has hydrophobic and electron donor (basic) properties. Several highly hydrophobic strains have also been described in previous studies including *L. paracasei* subsp. *paracasei* (Dewan and Tamang, 2007) and *L. paracasei* subsp. *paracasei* NIRD H831 (Harty and Knox, 1991). The hydrophobicity of bacteria is associated with the presence of proteinaceous and hydrophobic compounds on their cell surfaces (Pelletier *et al*., 1997). In lactobacilli, a number of these compounds have been characterized including the surface layer (S-layer) proteins and lipoteichoic acid (Krasowska and Sigler, 2014). Cell surface hydrophobicity is an essential property of probiotics as it aids in the adhesion and colonization of these microorganisms to the intestinal mucosa (Xu *et al*., 2009).

Table 4. Cell surface hydrophobicity of *L. paracasei* BIOTECH 10363.

Solvents used	Solvent properties	Hydrophobicity (%)
Xylene	Non-polar solvent	82.79±0.28
Chloroform	Acidic solvent and electron acceptor	53.64±0.39
Ethyl acetate	Basic solvent and electron donor	1.45±0.13

Values are presented as mean±SD of triplicates.

3.5 Auto-aggregation activity of Lactobacillus paracasei BIOTECH 10363

The sedimentation rate of *L. paracasei* BIOTECH 10363 is shown in Table 5. The highest auto-aggregation activity of the isolate was observed after 5 hrs of incubation. According to Wang *et al*. (2010), strains with activity higher than 40% are strongly auto-aggregating. In this study, the tested strain exhibited a strong auto-aggregating phenotype as it had an activity of 89.53%. This finding is in agreement with previous studies which reported the strong auto-aggregation activity of several strains of *L. paracasei* (Bhagat *et al*., 2019; Fonseca *et al*., 2021; Sadeghi *et al*., 2022). However, the observed activity varies from strain to strain, indicating that auto-aggregation is a strain-specific property.

Auto-aggregation is the characteristics of the bacteria belonging to the same strain to clump or self-aggregate together to achieve high cell density (Janković *et al*., 2012). This property enables probiotics to reach viable counts necessary for their adhesion, colonization, and survival in the oral cavity, gastrointestinal tract, and urogenital tract (Collado *et al*., 2008; Nikolic *et al*., 2010). Moreover, it also impedes the adherence of pathogens to the host's intestinal mucosa by forming a barrier via biofilm formation (Schellenberg *et al*., 2006). Microorganisms intended for probiotic applications should have high cell surface hydrophobicity and strong auto-aggregation activity.

Table 5. Auto-aggregation activity of *L. paracasei* BIOTECH 10363.

Incubation time	Auto-aggregation activity (%)
3 hrs	86.93±0.32
5 hrs	89.53±0.68

Values are presented as mean±SD of triplicates.

3.6 Salt (NaCl) tolerance of Lactobacillus paracasei BIOTECH 10363

Lactobacillus paracasei BIOTECH 10363 was inoculated on MRS agar at 37°C with different concentrations of salt (Table 6). The tested strain had maximum growth in modified MRS broth with 5% NaCl and could withstand up to 9% NaCl. The result agrees with previous studies which reported that many probiotic potential lactobacilli were able to tolerate up to 9% NaCl (Chowdhury *et al*.,2012; Rahman *et al*., 2016; Ahmadnejad and Dolatabadi, 2021). Most microorganisms use a variety of solutes such as

Table 6. Salt tolerance of *L. paracasei* BIOTECH 10363.

NaCl concentration	Results	Interpretation[1]
1%	++	Maximum growth
2%	++	Maximum growth
3%	++	Maximum growth
4%	++	Maximum growth
5%	++	Maximum growth
6%	+	Normal growth
7%	+	Normal growth
8%	+	Normal growth
9%	+	Normal growth
10%	-	No growth

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

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inorganic ions (often K^+) and osmolytes (compatible solutes) to overcome osmotic stress caused by salt (Wood, 2015). These solutes accumulate in the cell cytoplasm and restore the balance between the intracellular and extracellular osmotic pressures (Welsh, 2000). The most common solutes identified in lactic acid bacteria are acetylcholine, carnitine, choline, glycine betaine, choline, carnitine, dimethylsulfonioacetate, and proline (Kets, 1997; Marrec, 2011). The accumulation of these solutes is a prerequisite for the survival of probiotics during osmotic stress.

3.6 Hemolytic activity of Lactobacillus paracasei BIOTECH 10363

There were no notable zones around the colonies of *L. paracasei* BIOTECH 10363 when grown in sheep blood agar. This indicates that the isolate displayed gamma (γ)-hemolytic activity (i.e., no hemolytic activity). Gamma (γ)-hemolytic microorganisms do not usually produce hemolysin, a compound that destroys red blood cells (RBCs) in the blood agar plates (Sohail *et al*., 2021). The absence of hemolysin guarantees that opportunistic virulence will not occur in the tested strain. The finding of this study corroborates with previous reports which described the gamma (γ)-hemolytic nature of *L. paracasei* (Le *et al*., 2019; Huang *et al*., 2021; Sornsenee *et al*., 2021). This suggests the non-hemolytic nature of the tested strain. However, other virulence factors such as DNAse, gelatinase, and coagulase activity must be fully examined before the isolate can be used as probiotics in foods and pharmaceuticals.

3.7 Antibiotic susceptibility of Lactobacillus paracasei BIOTECH 10363

The antibiotic susceptibility of *L. paracasei* BIOTECH 10363 against 11 antibiotics was evaluated using the disc diffusion method. The average diameters (mm) of the zone of inhibition are shown in Table 7. The tested strain was susceptible or moderately susceptible against various cell wall synthesis inhibitors such as aminopenicillins (amoxicillin and ampicillin), penicillin (augmentin and penicillin G) and second-generation cephalosporin antibiotic (cefaclor). Furthermore, the isolate displayed the same sensitivity against several protein synthesis inhibitors including streptomycin, chloramphenicol, lincosamides (clindamycin), macrolides (erythromycin) and tetracycline. Generally, *Lactobacillus* species are generally susceptible or sensitive to cell wall synthesis inhibitors and low concentrations of chloramphenicol, lincosamides, macrolides and tetracycline (Darsanaki *et al*., 2013; Gueimonde *et al*., 2013). On the other hand, the tested strain was found to be resistant to kanamycin. This finding is in agreement with previous studies which reported the intrinsic resistance of many *Lactobacillus* species toward kanamycin and other aminoglycosides (Benavides *et al*., 2016; Štšepetova *et al*., 2017; Zhang *et al*., 2022). The observed resistance is caused by a lack of cytochrome-mediated electron transport, a system that is normally involved in the uptake of antibiotics in microorganisms (Narayanan and Narayanan, 2019).

4. Conclusion

Lactobacillus paracasei occurs naturally in milk and dairy products, fermented sausages, vegetables, and wine. It is one of the most common and well-known probiotic microorganisms in food, dietary supplements, and pharmaceutical preparations. However, not all strains of *L. paracasei* demonstrate probiotic properties.

Table 7. Antibiotic susceptibility pattern of *L. paracasei* BIOTECH 10363 using disc diffusion assay on MRS agar.

Antibiotics	Concentration (μg)	Zone of inhibition (mm)[1]	Interpretation[2]
Cell wall synthesis inhibitors			
Amoxicillin	10	27	Susceptible
Ampicillin	10	28	Susceptible
Augmentin	30	28	Susceptible
Penicillin G	10	35	Susceptible
Cefaclor	30	16	Moderately Susceptible
Protein synthesis inhibitors			
Kanamycin	30	8	Resistant
Streptomycin	10	13	Moderately Susceptible
Chloramphenicol	30	23	Susceptible
Clindamycin	2	20	Susceptible
Erythromycin	15	29	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).

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Only a few strains are able to fulfill the general criteria for the selection of probiotics. In the present study, *L. paracasei* BIOTECH 10363, a gram-positive, catalase-negative, non-spore-forming, rod-shaped lactic acid bacterium isolated from *Tapuy* (Philippine rice wine) was assessed for its probiotic potential. The isolate showed promising *in vitro* performance in terms of functional, technological, and safety properties. It displayed resistance to acid and bile, an antagonistic effect against *E. coli*, cell surface hydrophobicity, strong auto-aggregation activity, tolerance to NaCl, γ-hemolytic activity, and antibiotic susceptibility to various cell wall and protein synthesis inhibitors. This study provides baseline information regarding the probiotic potential of *L. paracasei* BIOTECH 10363.

Conflict of interest

The authors declare no conflict of interest.

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