Evaluation Of Antagonistic And Aggregation Property Of Probiotic Lactic Acid Bacteria Isolated From Bovine Milk

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Abstract --Lactic acid bacteria (LAB) are essential ingredients in probiotic foods, intestinal microflora, and dairy products that are capable of coping up with harsh gastrointestinal tract conditions and are available in a variety of environments. The objective of this study is to evaluate the probiotic property of LAB isolated from bovine milk. Milk samples were collected from local dairy farms. Samples were obtained using sterile test tubes and transported to a laboratory in the icebox for further biochemical characterization. Preliminary Physiological and biochemical identification of LAB isolates was conducted by growing on MRS agar after ten-fold serial dilution. Seven of the best isolates were selected for the evaluation of the probiotic property. The LAB isolates were checked for resistance to antibiotics and their antimicrobial activity by disc diffusion assay and agar well diffusion assay respectively. Bile salt hydrolase activity of isolates were studied by growing isolates in BSH medium with bile salt. Cell surface property of isolates was assayed by studying their autoaggregation and coaggregation percentage with S. aerues. All isolates were found BSH positive. In addition, BCM2 and BGM1 were susceptible to all antibiotic disks except BBM1 which was resistant to all antibiotic disks. BCM1 and BGM1 had the highest autoaggregation and coaggregation potential respectively. Since all LAB isolates showed gastro intestinal tolerance and good cell surface property they could be considered as good potential probiotic candidates for treatment and probiotic starter culture preparation.

Keywords: probiotic: aggregation,antibiotic susceptibility, antimicrobial activity, Bacteriocin, Bovine milk, Lactic acid bacteria, Probiotics

1.0 Introduction

Recent reports about the widespread and indiscriminate use of antibiotics for disease management, the promotion of livestock growth and the rise of antibiotic-resistant pathogens have resulted in increased interest in the use of probiotics and their antibacterial metabolites as alternative antimicrobial approaches for infection treatment and prevention[1]. Initially, probiotics were thought to have a positive impact on the host by improving its intestinal microorganisms balance, thereby inhibiting bacteria and viruses. *Lactobacillus* and *Bifidobacteria* are the most common types of probiotic strains, but some yeasts and bacilli have also probiotic features. Probiotic is a modern-day phrase, literally meaning "for life" and is used to name bacterial association with beneficial effects on human and animal health[2]. Despite initial controversies, probiotic studies have progressed significantly over the past two decades and major progress has been made in selecting and characterizing different probiotic species along with important health benefits. As WHO states The term "probiotic"coined to describe "live micro-organisms that confer a health benefit on the host when administered in adequate amounts". An ecological assessment of the gut flora is necessary in order to understand its importance to human health. A reliable probiotic product requires proper identification of the bacterial species and description of species label on the product container. This is important because several recent reports have shown that the identified of recovered microorganisms does not always correlate to the product label[3]. It is one of challenges to select appropriate probiotic strains that would be able to perform efficiently in the gastrointestinal tract. Strain preference generally based on stress-resistant: e.g. low pH, high osmolality, and bile tolerance. The ability of probiotic strains to hydrolyze bile salts also considered one of the top parameters for probiotic strain selection. In addition to these physiological assays, molecular studies are now undergoing to determine the genetic basis for gastric longevity and functionality [4]. Probiotics or microorganisms that promote health can be commonly used to prevent and cure various diseases. The selection of these microorganisms is variable (dairy products, drugs, dietary supplements) and the choice should be primarily based on the fact that the positive effect on the human body and must also be clinically proven . Some probiotic strains have been isolated from healthy human intestinal flora (*Bifidobacteria and Lactobacilli*) and maintain functionality through the gastrointestinal tract, as verified by clinical studies. These are used in pharmaceutical products, food products, and more recently in dairy products[5]. Already in vitro experiments were determined to meet certain demands like acid tolerance, bile tolerance, adhesion to mucosal and epithelial surfaces, antimicrobial activity against certain pathogenic bacteria and bile salt hydrolase activity. The importance of these parameters is still under debate as there are concerns of importance, differences in vivo and in vitro, and a lack of standardization. Since there are no specific parameters necessary for all probiotic applications, the best way to determine the properties of a strain is to target the effect on basic physiological function[6]. Initially, LAB from dairy products were probably the best candidates for enhancing microbiological protection and also as potential probiotic bacteria. Most LABs are derived from fermented foods or human intestinal microbiota, are now deliberately introduced as probiotics to our nutrition. The safety status of any new organism must be investigated thoroughly using studies such as sequencing of 16S rDNA, metabolic and enzyme activities[7]. Probiotic microorganisms in the gastrointestinal tract need to avoid adverse conditions[8]. An important step in choosing possible probiotic candidates is to determine their tolerance to the GI tract's extreme conditions. The first obstacle to be passed is the mouth with a high lysozyme level in the human saliva; the stomach with low pH and digestive enzymes (i.e. pepsin); and the upper intestine with bile[9]. Bile salt is another barrier that must be tackled in the small intestine by probiotic bacteria. It is considered as a requirement for bacteria's colonization and metabolic activity in the host's small intestine. Bile salts from cholesterol catabolism are surface-active chemicals formed in the liver[10]. They are the main components of bile that can destroy the cell membrane structure and therefore harmful to live cells. Bile is a yellow-green aqueous solution that contains bile acids, cholesterol, phospholipids,

and biliverdin pigment. It is synthesized in the pericentral liver hepatocytes, inter digestively deposited and stored in the gallbladder, and released into the duodenum after intake of food. Bile acts as a biological detergent that emulsifies and solubilizes lipids and thus plays an important role in the digestion of fat. The bile detergent product also provides strong antimicrobial activity, mainly through dissociation of membrane lipids. Bile acids synthesized from cholesterol, cholic and chenodeoxycholic acid, de novo in the liver[11]. Hypercholesterolemia (elevated levels of blood cholesterol) is considered a major contributing factor for the development of the cardiac disease, and although pharmaceuticals (e.g. statins or bile acid sequestrants) are useful in treating this condition, they are often expensive and may have negative side effects[12]. Antimicrobial behavior against pathogenic bacteria is another important feature to be regarded in the quality of probiotic strains to maintain a healthy microbial balance in the GIT. Most of the antagonistic activity was due to the production of probiotic strains of antimicrobial compounds or metabolites such as organic acids, hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetine, carbon dioxide, reuterine, reutericycline, and bacteriocins. This behavior is coupled with the competitor exclusion in which probiotic strains interact with the pathogen[13],[21]. Due to their possible use as non-toxic and healthy additives for food preservation and prevention of food spoilage by food-borne Gram-positive pathogenic bacteria, LAB bacteriocins have gained attention. They have significant potential for extending food's shelf life, as well as for human medicine, as potential therapies or supplements[13].

Antibiotics are substances that can prevent the development or induce death identified certain microorganisms. Antibiotics commonly prescribed as chemotherapeutic agents that inhibit the growth and reproduction of bacteria. The vulnerability to antibiotics is a significant probiotic feature for LAB[14]. They are used as chemotherapeutic agents inhibiting the development and reproduction of bacteria. Antibiotics are incredibly useful and important for medical and veterinary use if prescribed correctly. Use of the antibiotics may be followed by adverse side effects such as allergies and so many customers avoid such antibacterial drugs even under severe conditions. For these reasons, antibiotics may lose their leading position among the most effective drugs[7]. In many different bacterial species, including LAB, antibiotic resistance is present and the responsible genes are detectable in strains with resistant phenotypes. Phenotypic methods define the susceptibility or resistance of LAB to antibiotics[15].. LAB is classified as resistant or susceptible by particular breakpoints into species and genera in the revised guidance of the European Food Safety Authority (EFSA) (European Commission, 2008). While antibiotic resistance is not a medical problem in most LAB species (because LAB does not cause infections), transmissible genes of antibiotic resistance may be passed to human or animal pathogens from LAB. Due to this, it is not advisable to use antibiotic-resistant LAB in food or feed[16]. The ability of aggregation and bind to the intestinal epithelium is one of the most important characteristics of lactobacilli and key criteria for the selection of probiotic strains[17]. In several ecological niches, self-aggregation, i.e. aggregation between microorganisms of the same strain, and co-aggregation, i.e. aggregation of genetically different strains, has significant importance. By obtaining an appropriate mass to form biofilms or bind to the host's mucosal surfaces, the bacterial strain will aggregate. In addition to adhering to host cells, host tissue extracellular matrix, or inorganic surfaces, many bacteria also have the ability to bind themselves. This self-binding is called auto-aggregation or autoagglutination and is one of the first steps in biofilm formation, as well as surface colonization[18]. Auto-aggregation is identified macroscopically as the accumulation of bacterial clumps at the bottom of the tubes of culture. In auto-aggregation, these clumps are formed by the same type bacteria, e.g. through pure culture. This is contrary to coaggregation, the mixture of bacteria with different strains or even different species[48],[49]. Recent studies confirm surface proteins mediate auto-aggregation. In some cases, carbohydrates can also serve as auto agglutinins, particularly exopolysaccharides. The polysaccharide intercellular adhesin (poly-N-acetylglucosamine; PNAG) of staphylococci is an example of exopolysaccharide agglutinin Another example of carbohydrate-mediated auto-aggregation can be found in *Campylobacter jejuni*, where the auto aggregative phenotype depends on flagella glycosylation. Extracellular DNA (eDNA), which is mostly part of the matrix of biofilm, may also act as an agglutinin [19],[50],[53].

Recent studies reveal that physicochemical characteristics of the cell surface, such as hydrophobicity, may have an effect on auto-aggregation and coaggregation.The correlation between hydrophobicity and adhesion ability has been observed in lactobacilli[19]. Upon contact with bile, the aggregation of lactobacilli decreases due to changes in surface hydrophobicity. It is not well understood how these surface properties or other bacterial components are altered in the presence of bile[20]. The objective of this study was to define and characterize the probiotic properties of the LAB with probiotic traits from bovine milk including antibiotic resistance, antibacterial activity against S. aureus, and aggregation properties.

2 Materials and Methods

2.1 Sampling

Dairy samples obtained from local dairy farms (Diary Treatment Farm and Bahubali Milk Farm) in Greater Noida, Delhi, NCR, India. Samples were collected using a sterile tube and carried in an icebox to the laboratory and stored in a refrigerator until analysis. Samples coded as BCM1, BCM2, BCM3, BBM1, BBM2, BBM3, and BGM1. BCM refers to milk from a bovine cow, BBM refers to milk from buffalo's and BGM refers to bovine goat's milk.

2.2 Enumeration and isolation of LAB

10 ml of each milk sample was homogenized in 90 ml of 0.9 percent sterilized saline water. A 10-fold serial dilution (10^{-1} to 10^{-6}) was prepared for each sample using a 1 ml homogenate. 100 µl dilution was spread over low pH MRS agar media and incubated for 48 hours at 32 °C. Colonies with typical LAB characteristics were selected randomly and sub-cultured to pure colonies after primary microscopic and macroscopic examinations. Colonies with LAB characteristics were chosen for further physiological and biochemical studies.

2.3 Identification of LAB

2.3.1 Gram staining

Gram test was conducted by a modified protocol described by Brucker (Brucker ,1986)

2.3.2 Catalase test

A modified methodology by Taylor[46] used for catalase test procedure.

2.3.3 Motility test

Cell motility was studied by observing the formation of turbidity according to [47].

2.3.4 Temperature tolerance test

The ability of LAB isolates to grow at different temperature ranges was studied by culturing LAB isolates at different temperature ranges by adjusting the incubator to 10 °C, 15 °C and 40 °C. Sample growth patterns were recorded by measuring their OD using a spectrophotometer and subculture to agar medium whether colony form or not.

2.3.5 Carbohydrate fermentation test

Isolated lactic acid bacteria were tested for fermentation of sucrose, lactose, mannitol, dextrose, and maltose as per standard procedure

2.4 Evaluation of probiotic property of LAB

2.4.1 Preparation of 0.5 MacFarland standard

MacFarland standard was prepared by mixing 9.95 ml of 1%(v/v) sulphuric acid with 50 µl of 1%(w/v) barium chloride dehydrate ($BaCl_2.2H_2O$). McFarland standard tube was then sealed with paraffin to prevent evaporation and stored in dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked using a spectrophotometer. For the 0.5 McFarland standards, the absorbance was adjusted at a wavelength of 600nm and distilled water was used as a blank solution. The 0.5 McFarland standards were vigorously agitated to turbidity using a vortex mixer before use. As per microbiological standard, a 0.5 McFarland standard is comparable to a bacterial suspension of $1.5x10^8$ colony-forming units (CFU/ml) (NCCLS, 2000).

2.4.2 Test organism and inoculum preparation

The broth culture of test organism Methicillin-Resistant Staphylococcus Aureus (MRSA)was obtained from Sharda University, department of medical microbiology. 50% (v/v) glycerol solution was prepared by mixing 20 ml of distilled water and 20 ml of 100% glycerol solution for long term preservation purpose, then 900 µl BHI broth containing staphylococcus aureus was mixed with 200 µl. 50%(v/v) glycerol solution in a 2 ml Eppendorf tube and preserved in -20°C deep freeze for further usage. Fresh *S.aureus* culture inoculated to BHI broth and incubated for 24 hours at 37°C. standard test pathogenic *S.aureus* inoculum was prepared by sub culturing fresh cells from BHI broth to BHI agar medium and incubated for 24 hours at 37°C. 2-3 colonies were picked up by wire loop aseptically into the sterile normal saline solution and the turbidity was adjusted to 0.5 McFarland's standard solution (a concentration of 1.5 X 10^8 CFU/ml).

2.4.3 Antibiotic resistance assay

The LAB isolates were checked for resistance to antibiotics by the disc diffusion method. Standard antibiotics discs used to determine the resistance of isolates to a variety of antibiotics. The disc placed on the surface of the Muller-Hinton agar plate seeded with LAB isolates and incubated at 37°C for 24 hrs, after the incubation the diameter hallo zone around the disc measured using a ruler. Ampicillin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), streptomycin(10µg) and vancomycin(30µg) from himedia company were used for antibiotic susceptibility test. Isolates classified as sensitive, resistance and intermediate according to the inhibition zone diameter in agreement with clinical and Laboratory Standards Institute tables (CLSI, 2014). All analysis were done in triplicate.

2.4.4 Antimicrobial activity

The agar-well diffusion method employed for the antimicrobial activity assay. *Staphylococcus aerues* (MRSA) made in sterile normal saline solution and adjusted to 0.5 McFarland's standard. Mueller Hinton (MH) agar plate uniformly seeded with 100 µl of *S. aureus* and spread with a sterilized cotton swab. The plates left on the bench for excess fluid to be absorbed. Wells of 6 mm in diameter, 5 mm deep and about 2 cm apart punched in the MH agar with a sterile cork-borer. Then 100 µl of isolate filled into each well till its fullness. Inoculated plates incubated at 37 ºC overnight. After a 24-hour incubation period, zones of inhibition measured in mm. All analyses were done in triplicate.

2.4.5 Auto-aggregation assay

Autoaggregation assays performed according to[19] with some modifications.All Freezed seven LAB isolates were thawed using a water bath and sub-cultured to MRS broth in a 10 ml test tube. Isolates were incubated at 37°C for 48 hours. The broth was centrifuged at 10,000 RPM at 4°C for 10 minutes and the supernatant discarded to the beaker and the pellet washed three times using PBS buffer pH 7.0. After washing the pellet three times, the pellet was resuspended in a 10 ml PBS solution. The pellet and PBS vortex until the pellet completely dissolve in the PBS solution. The optical density of each isolate was studied using a spectrophotometer at time 0 h, 2hrs, 16 hrs., and 24 hrs. The experiment was done in triplicate.

$$\boldsymbol{Auto-aggregation\ percentage} = \frac{[\boldsymbol{At-A0}]}{\boldsymbol{A0}} \times \mathbf{100}$$

2.4.6 Co-aggregation assay

The preparation of cell suspensions for coaggregation was the same as for the auto-aggregation analysis. Equal volumes (5 mL) of the cell suspensions of a LAB strain and the pathogen strain staphylococcus aerues mixed and optical density at OD 600 immediately measured (designated A0). OD600 measured at time t, 2hrs.16hrs and 24 hrs. of incubation at 37 °C. Percent of coaggregation calculated using the following equation.

$$\boldsymbol{Co-aggregation\ percentage} = \frac{[\boldsymbol{At-A0}]}{\boldsymbol{A0}} \times \mathbf{100}$$

2.4.7 Statistical analysis

All statistical analyses were conducted using SPSS software version 20.0. All the experiments were conducted in triplicate and data was analyzed and compared statistically

using ANOVA at a 95% level of significance. A probability value at $p \leq 0.05$ was considered statistically significant. Data are presented as mean values ± standard deviation calculated from triplicate determinations.

3.0 Result

Table 1: Morphological, physiological and biochemical characterization of lactic acid bacteria isolates.
+=growth - =no growth

Carbohydrate fermentation result was positive for all isolates.The physiological and biochemical charactesrics of LAB isolates is tabulated on table 1.

Isolates	Shape	Catalase test	Growth at different temperature (°C)			Gram test result	Motility test	Surface	Margin	Elevation	Color
			15	30	45						
BCM1	Cocci	-	+	+	+	+	Non motile	Smooth	Entire	Convex	white
BCM2	Rod	-	+	+	+	+	Non motile	Rough	Entire	Convex	white
BCM3	Cocci	-	+	+	+	+	Non motile	Rough	Entire	Convex	white
BBM1	Tetrad	-	+	+	+	+	Non motile	Smooth	Entire	Convex	white
BBM2	Rod	-	+	+	+	+	Non motile	Smooth	Entire	Convex	white
BBM3	Cocci	-	+	+	+	+	Non motile	Smooth	Entire	Convex	white
BGM1	Cocci	-	+	+	+	+	Non motile	Smooth	Entire	Convex	white

All isolates were susceptible to Ampicillin, chloramphenicol, and vancomycin (except BBM1), BBM1 was exceptionally resistant against all antibiotics including Vancomycin.
Table 2 antibiotic susceptibility of LAB isolates against standard antibiotics. Diameters of inhibition zones are expressed in millimeters; the diameter of the disc was 6 mm. Data are arithmetical means of three measurements, R=resistant, S=susceptible, I=intermediate

Isolates	Ampicillin	Chloramphenicol	Streptomycin	Tetracycline	Vancomycin
BCM1	17 (S)	14 (I)	11 (R)	17(S)	13 (I)
BCM2	32 (S)	27 (S)	8 (R)	32(S)	22 (S)
BCM3	26 (S)	24 (S)	12 (I)	26(S)	21 (S)
BBM1	8 (R)	9 (R)	9 (R)	8(R)	14 (R)
BBM2	30 (S)	28 (S)	7 (R)	30(S)	23 (S)
BBM3	32 (S)	25 (S)	8 (R)	32(S)	19 (S)
BGM1	20 (S)	20 (S)	14 (I)	20(S)	19 (S)

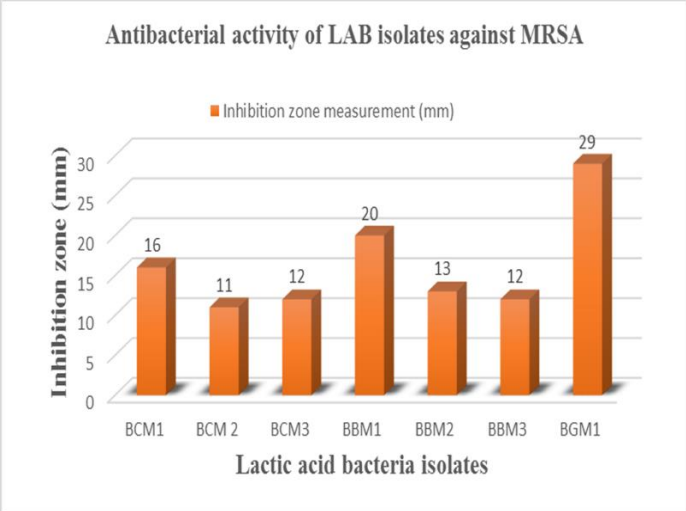


Fig 1 Antimicrobial activities of neutral pH CFS of LAB isolate against S. aureus, Data is expressed as mean ± SD (n = 3).
All the seven Lactobacillus isolates showed antagonistic effects against MRSA, but the degrees of antagonism differed among the Lactobacillus isolates. BGM1 record the highest antagonistic activity with the zone of inhibition 29 mm whereas BCM2 showed the least antibacterial activity (11 mm) against methicillin-resistant staphylococcus aerues. BBM1 records the second-highest record on its antagonistic activity with a 20 mm inhibition zone.

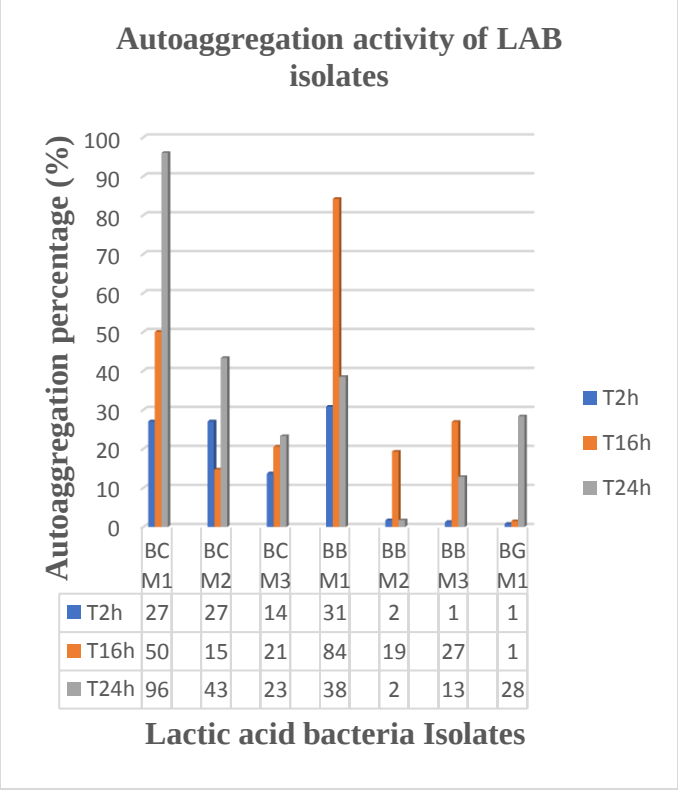
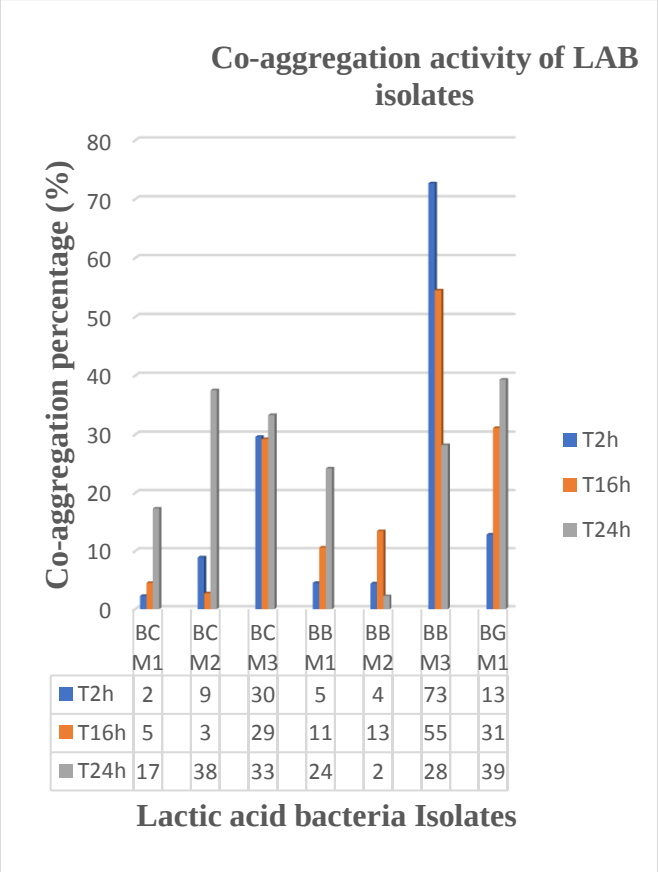


Fig 2 Auto-aggregation activity of LAB isolates as measured at 2,16 and 24hrs of incubation at 37 °C Data are expressed as mean ± SD (n = 3) In the present study, BBM1 show the highest auto-aggregation percentage after two hours of incubation with percentage value 30.76 % and BGM1 has the lowest auto-aggregation percentage after

two hours of incubation with auto-aggregation percentage 0.78% whereas BBM1, BCM1, and BCM3 shows the highest auto-aggregation potential after 16 hours of incubation with 84.16 %,50%, and 20.54% respectively. BCM1shows the highest auto-aggregation percentage at 96.3% followed by BCM2 with 43.3% auto-aggregation percentage, whereas BBM2 has the lowest auto-aggregation percentage with 1.75% after 24 hours of incubation at 37° C. BBM3 and BCM3 showed the highest record of coaggregation percentage with 72.72% and 29.68% after 2 hours of incubation on the contrary BCM1 had the lowest coaggregation percentage with 2.25%. BBM3(54.15%) and BGM1(31.1%) record the highest coaggregation percentage after 16 hours of incubation whereas BCM2 is the lowest in 16 hours incubation time. BGM1 and BCM2 record the highest co-aggregation percentage against S. aureus with 39.3% and 37.5% after 24 hours of incubation respectively. BBM2 has the lowest co-aggregation value of 2.24%. unlike the other isolates BBM3 co-aggregation value decrease in a row with incubation time.



	BCM1	BCM2	BCM3	BBM1	BBM2	BBM3	BGM1
T2h	2	9	30	5	4	73	13
T16h	5	3	29	11	13	55	31
T24h	17	38	33	24	2	28	39

Fig 3 Co-aggregation activity of LAB isolates, OD measured at 2,16 and 24hrs of incubation at 37 °C Data are expressed as mean ± SD (n = 3)

4.0 DISCUSSION

LAB is a group of bacteria with the shape of rod or coccus, gram-positive, catalase-negative, non-motile, homofermentative or heterofermentative, and able to grow in low acid condition.All isolates showed the biochemical ,physiological properties of lactic acid bacteria(Table 1). A key feature of probiotic bacteria is that they should not bear any transmittable genes of antibiotic resistance[21]. Because of the possibility of passing genes of antibiotic resistance horizontally from LAB to pathogens, the current European Union regulations require that the LAB used in the food and feed production need to be sensitive to clinically or veterinary relevant antibiotics[5]. It's also recommended for all bacterial products intended for use as feed additives, it is necessary to establish the susceptibility of the competent strain to a specific range of human or veterinary antimicrobials[22]. The evaluation of the antibiotic resistance profile of probiotic isolates was carried out to confirm the absence of transferable antibiotic resistance genes **(Table 2**). As per CLSI breakpoint rates, this study confirmed that all isolates were susceptible to Ampicillin, chloramphenicol, and vancomycin (except BBM1), supporting their safety as putative probiotic strains**.** The result of this study is in line with [23]. It is well documented that vancomycin (belonging to the antibiotics of the glycopeptide blocks the synthesis of peptidoglycan, which is an essential structural element of the cell wall [8]. Since the genes resistant to vancomycin are chromosomally located available in lactobacilli, Lactobacillus strain resistance to vancomycin is assumed not to be transferable horizontally [24]. With respect to antibiotic resistance, it is better to use probiotics with antibiotic sensitivity properties. Probiotics sensitivity to antibiotics may not tend to the horizontal transfer of antibiotics resistance gene to pathogens[25]. Several reports have shown that LAB is generally resistant to various antibiotics[43][51],[52]. One of the functional properties used to describe probiotics is the synthesis of antimicrobial compounds such as organic acids, short-chain fatty acids, and bacteriocins. LAB's mechanism of antagonistic activity against pathogenic strains is well studied and reviewed by (Su˘skovi´c et al.,2010). The ability to produce various antimicrobial compounds may be one of the essential features which enable the probiotic strain effectively compete and exclude pathogen survival in the intestine and the expression of a probiotic effect on the host[26]. LAB and Bacillus sp. anti-microbial activity against pathogenic bacteria is attributed to different types of inhibitory metabolites especially bacteriocin[27],[28]. H_2O_2 is one of the primary metabolites that LAB can produce and can contribute to its antagonistic activity. H_2O_2 production is also considered beneficial for food preservation and serves as a barrier for pathogenic microorganisms development[29]. In this study, LAB isolates showed high inhibitory activity against the *S. aureus* **(Fig 1)**. This finding is in line with[23],[30] who reported that Lactobacillus-produced antimicrobial substances have great potential to inhibit pathogenic microorganism. [31]states the antagonistic activity of lactobacilli in co-culture with pathogenic bacteria is primarily due to the production of organic acids that lead to a reduction in pH, although also generate some other substances. The acidic conditions in the stomach can even increase this antimicrobial activity[32]. In addition, these probiotic properties may be partly based on the generation of specific lactic acid concentrations in the gastrointestinal tract, which inhibits the growth of Gram-negative pathogenic bacteria in collaboration with a detergent such as bile salts[33]. The antimicrobial activity of organic acids is due to the reduction of pH as well as the undissociated type of molecules. It has been reported that the low external pH induces acidic cell cytoplasm, while the non-dissociated lipophilic acid will passively diffuse across the membrane. The undissociated acid operates by collapsing the gradient of the electrochemical

proton or by affecting the permeability of the cell membrane, resulting in disturbance of the transport of substrates. Organic acids have a significant inhibitory effect on gram-negative bacteria. [34]. The recovery of a balanced oral microbiota is typically based on the ability of probiotics to inhibit the development of oral cavity-related pathogens[35]. The result of this study is align line with previous studies by[36]. It has been studied that the ability to bind to epithelial cells and mucosal surfaces is an important property for probiotics. Cell adhesion is a cascade of stages involving attachment between the bacterial cell membrane and interacting surfaces. Several studies examined the composition, structure, and interactions associated with bacterial adhesion to intestinal epithelial cells [19],[36],[37]. The bacterial strain can form aggregation by achieving a sufficient mass to form biofilms or attach to the host's mucosal surfaces[38]. According to [39], good auto-aggregation ability must be greater than to 40%, and any LAB strains with less than 10% are considered to have weak auto-aggregation potential. This result indicates except BBM2, all LAB isolates are a potentially high capability of adhering to epithelial cells and mucosal surfaces (Fig 2). The auto-aggregation mechanism of LAB is reported to accompanied by proteins in the supernatant culture and proteins or lipoproteins on the cell surfaces[40]. It is a desirable probiotic trait of bacterial trapping in an aggregated form that enhances the stability of microbial strains in the gastrointestinal tract (GIT) where microbes exposed to uninhabitable gastrointestinal environments[27]. A recent report revealed that extracellular polysaccharides on the cell surface could be involved in the aggregation[29]. Probiotic bacteria's ability to co-aggregate can allow them to form a barrier that prevents pathogenic bacteria from colonizing[41]. The potential to bind closely with other bacteria is correlated with co-aggregation[42]. This aggregation ability of LAB species in the presence of pathogenic microbes may create a protective mechanism to stop pathogens from colonizing in the human gut [43]. Pathogens cause serious illness to humans, particularly when they can colonize and control the human gut [44].Co-aggregation is an important approach for evaluating the close association between LAB and pathogenic bacteria, as LAB has surface binding proteins specific to surfaces and bacteria[45]. In this study, All the tested LAB isolates were found excellent in co-aggregating with pathogenic strain *S. aureus(Fig 3)*. The co-aggregation with pathogenic strain enhances the LAB's potential probiotic property which makes them efficient in preventing colonization by pathogenic bacteria. Studies indicated that the LAB's ability to co-aggregate in the presence of gut pathogens would improve the LAB's probiotic properties. This finding confirms the potential probiotic properties of LAB isolates. This study is in line with previously documented results by[38], [52], [54], [55], [56].

5.0 CONCLUSION

The findings of this in vitro study showed that all the seven isolates (except BCM2) of LAB were able to exhbit probiotic features , and while most of them were susceptible to antibiotics. From all the isolates, BBM1 had the best ability to survive in 0.3% bile, whereas BGM1 had the highest antimicrobial property and coaggregation potential. BCM1 and BGM1 had the highest autoaggregation and coaggregation potential respectively. Since all seven isolates of LAB showed strong antagonistic activity against *S. aerues* they could be considered as good potential probiotic candidates for treatment and infection prevention. LAB display numerous antimicrobial activities due to the production of organic acids, but also of other compounds, such as ethanol, hydrogen peroxide, diacetyl, reuterin, and bacteriocins. This study underlines the important role of LAB that may play a key role in the food industry as a probiotic starter culture.

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6.0 REFERENCES

[1] Shokryazdan, P. *et al.*, "Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains," Biomed Research International *(2014).*

[2] George Kerry, R., J. K. Patra, S. Gouda, Y. Park, H. S. Shin, and G. Das."Benefaction of probiotics for human health: A review." Journal of Food and Drug Analysis. 26.3(2018):927–939

[3] Gueimonde,M. and S. Salminen, "New methods for selecting and evaluating probiotics." Digestive and Liver Disease 38.2(2006): 242–247

[4] Dunne,C. *et al.*, "In vitro selection criteria for probiotic bacteria of human origin.," *American* Journal of Clinical Nutrtion 73(2011):386–392

[5] Temirova, G. "Antibiotic Resistance And Probiotic Properties Of Lactic Acid Bacteria Isolated From Camel Milk And Shubat" University Of Eastern Finland, Faculty of Science and Forestry 2016**.**

[6] Kechagia.M., *et al.*, "health benefits of probiotics: A review."ISRN nutrition 2013:7

[7] Temirova, G. "Antibiotic Resistance And Probiotic Properties Of Lactic Acid Bacteria Isolated From Camel Milk And Shubat" University Of Eastern Finland, Faculty of Science and Forestry 2016**.**

[8] Rushdy, A.A., and E. Z. Gomaa, "Antimicrobial compounds produced by probiotic Lactobacillus brevis isolated from dairy products," Ann. Microbiol 63.1(2013):81–90

[9] Bartolomé, B., T. Requena, A. García-Ruiz, A. Esteban-Fernández, D. González de Llano, and M. V. Moreno-Arribas, "Assessment of probiotic properties in lactic acid bacteria isolated from wine." Food Microbiol 44(2014): 220–225

[10] Havenaar,R. and J. H. J. Huis In't Veld, "Probiotics: A General View," Lactic Acid Bacteria *1*(1992):151–170,

[11] Begley, M., C. Hill, and C. G. M. Gahan, "Bile Salt Hydrolase Activity in Probiotics Bile Salt Hydrolase Activity in Probiotics." Applied and Enviromental Microbiology 72.3(2006):

[12] [Schuster,H. "Improving lipid management - To titrate, combine or switch," International Journal of Clinical Practice.58.7(2004):689–694

[13] Fricourt, B.V., S. F. Barefoot, R. F. Testin, And S. S.

Hayasaka, “Detection and Activity of Plantaricin F an Antibacterial Substance from Lactobacillus plantarum BF001 Isolated from Processed Channel Catfish.” Journal of Food Protection 57.8(1994):698–702
[14] Sahoo,T.K., P. K. Jena, N. Nagar, A. K. Patel, and S. Seshadri, “In Vitro Evaluation of Probiotic Properties of Lactic Acid Bacteria from the Gut of Labeo rohita and Catla catla,” Probiotics Antimicrobial Proteins 7.2(2015): 126–136
[15] Patel, A.R., N. P. Shah, and J. B. Prajapati. “Antibiotic Resistance Profile of Lactic Acid Bacteria and Their Implications in Food Chain.” World Journal of Dairy & Food Sciences 7.2(2012):202-211
[16] Egerva, M., H. Lindmark, S. Roos, G. Huys, and S. Lindgren, “Effects of Inoculum Size and Incubation Time on Broth Microdilution Susceptibility Testing of Lactic Acid Bacteria.” Antimicrobial Agents And Chemotherapy 51.1(2007):394–396
[17] Candela, M. *et al.*, “Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production.” Internatonal Journal of Food Microbiology 125.3(2008):286–292
[18] Trunk, T., H. S. Khalil, and J. C. Leo, “Bacterial autoaggregation.” AIMS Microbiology 4.1(2018): 140–164.
[19] Del Re, B., B. Sgorbati, M. Miglioli, and D. Palenzona, “Adhesion , autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum.” Letters in Applied Microbiology 2000: 438–442
[20] Kos, B., J. Šušković, S. Vuković, M. Sĭmpraga, J. Frece, and S. Matošić, “Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92,” Journal of Applied Microbiology *94.*6(2003):981–987 Brucker M C (1986) ‘Gram Staining A Useful Laboratory Technique. Journal of Nurse-Midwifery 31(3), pp. 0–2.
[21] Borah, T., B. Gogoi, A. Khataniar, M. Gogoi, A. Das, and D. Borah, “Probiotic characterization of indigenous Bacillus velezensis strain DU14 isolated from Apong, a traditionally fermented rice beer of Assam.” Biocatalysis and Agricultural Biotechnology 18(2019).
[22] Vankerckhoven,V. *et al.*, “Biosafety assessment of probiotics used for human consumption : recommendations from the EU-PROSAFE project.” Trends in Food Science & Technology 19 (2008):102–114
[23] Sahoo, T.K., P. K. Jena, N. Nagar, A. K. Patel, and S. Seshadri, “In Vitro Evaluation of Probiotic Properties of Lactic Acid Bacteria from the Gut of Labeo rohita and Catla catla,” Probiotics and Antimicroial. Proteins 7.2(2015):126–136.
[24] Morrow, L.E., V. Gogineni, and M. A. Malesker, “Probiotic , prebiotic , and synbiotic use in critically ill patients.” Lippincott Williams & Wilkins vol. 18.2(2012):186–191
[25] Ayyash, M., A. Abushelaibi, S. Al-Mahadin, M. Enan, K. El-Tarabily, and N. Shah, “In-vitro investigation into probiotic characterisation of Streptococcus and Enterococcus isolated from camel milk.” *LWT* - Food Science and Technol 87(2018):478–487
[26] Argyri, A.A. *et al.*, “Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests,” Food Microbiology 33.2(2013): 282–291
[27] Sakandar, H.A., S. Kubow, and F. A. Sadiq, “Isolation and in-vitro probiotic characterization of fructophilic lactic acid bacteria from Chinese fruits and flowers,” LWT Food science and technology 104(2019):70–75
[28] Caggia, C., M. De Angelis, I. Pitino, A. Pino, and C. L. Randazzo, “Probiotic features of Lactobacillus strains isolated from Ragusano and Pecorino Siciliano cheeses,” Food Microbiology 50(2015) :109–117
[29] Sabir, F., Y. Beyatli, C. Cokmus, and D. Onal-darilmaz, “Assessment of Potential Probiotic Properties of Lactobacillus spp ., Lactococcus spp ., and Pediococcus spp . Strains Isolated from Kefir.” Journal of food science 75.9(2010):568–573
[30] Çadirci, B.H. and S. Çitak, “A Comparison of Two Methods Used for Measuring Antagonistic Activity of Lactic Acid Bacteria,” Pakistan Journal of NutrtiTION 4.4(2005):237–241
[31] Millette, M., F. M. Luquet, and M. Lacroix, “In vitro growth control of selected pathogens by Lactobacillus acidophilus- and Lactobacillus casei-fermented milk,” Letters in Applied Microbiology 44.3(2007): 314–319
[32] Ganzle, M.G., S. Weber, and W. P. Hammes, “Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins.” International Journal of Food Microbiology 46 (1999) 207–217
[33] Gahan,C.G.M., and C. Hill, “The interaction between bacteria and bile.” FEMS Microbiology Reviews 29 (2005) 625–651
[34] Musikasang, H., A. Tani, A. H-kittikun, and S. Maneerat, “Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract,” World Journal of Microbiol. Biotechnology 25.8(2009):1337–1345
[35] Reid, G., J. A. Younes, H. C. Van Der Mei, and G. B. Gloor, “Microbiota restoration : natural and supplemented recovery of human microbial communities,” Nature Publishing Group.9.1(2010): 27–38
[36] Pelletier, C., C. Bouley, C. Cayuela, and S. Bouttier, “Cell Surface Characteristics of Lactobacillus casei subsp . casei , Lactobacillus paracasei subsp . paracasei , and Lactobacillus rhamnosus Strains,” Applied And Environmental Microbiology 63.5(1997):1725–1731
[37] Parvez, S., K. A. Malik, S. A. Kang, and H. Kim, “Probiotics and their fermented food products are beneficial for health.” Journal of Applied Microbiology 100(2006):1171–1185
[38] Grze, L., M. Carmen, and S. Salminen, “Evaluation of aggregation abilities between commensal fi sh bacteria and pathogens”Aquaculture 357(2012):412–414
[39] Wang, C., P. Lin, C. Ng, and Y. Shyu, “Anaerobe Probiotic properties of Lactobacillus strains isolated from the feces of breast-fed infants and Taiwanese pickled cabbage,” Anaerobe 16.6(2010):578–585
[40] Jena, P.K., D. Trivedi, K. Thakore, H. Chaudhary, S. S. Giri, and S. Seshadri, “Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota,” Microbiology and Immunology 57.6(2013):407–416
[41] Collado, M.C., J. Meriluoto, and S. Salminen, “Author ’

s personal copy Measurement of aggregation properties between probiotics and pathogens : In vitro evaluation of different methods." Journal of Microbiological Methods 71 (2007) 71–74

[42] Malik, A. *et al.*, "Coaggregation among Nonflocculating Bacteria Isolated from Activated Sludge." Applied And Environmental Microbiology 69.10(2003):6056–6063

[43] Vidhyasagar, V., and K. Jeevaratnam, "Evaluation of Pediococcus pentosaceus strains isolated from Idly batter for probiotic properties in vitro," Journal of Functional Foods (2012):1–9

[44] Karmakar, S., R. S. Khare, S. Ojha, K. Kundu, and S. Kundu, "Development of Probiotic Candidate in Combination with Essential Oils from Medicinal Plant and Their Effect on Enteric Pathogens : A Review," Gastroenterology Research and Practice (2012)

[45] Soleimani, N.A., R. K. Kermanshahi, B. Yakhchali, and T. Nejad, "Antagonistic activity of probiotic lactobacilli against Staphylococcus aureus isolated from bovine mastitis.," African Journal of Microbiology Research 4.20(2010):2169–2173 Brucker, M.C.''Gram Staining A Useful Laboratory Technique.'' Journal of Nurse-Midwifery 31.3(1986):0–2.

[46] Taylor, W.I, D, Achanzar ''Catalase Test as an Aid to the Identification of Enterobacteriaceae.'' Applied Microbiology, 24.1(1972): 58–61.

[47] Abiola, C., , V. O, Oyetayo''Isolation and Biochemical Characterization of Microorganisms Associated with the Fermentation of Kersting ' s Groundnut (Macrotyloma geocarpum.'' Research Journal of Microbiology. Science Alert, 11.2(2016): 47–55.

[48] Ochiai, K., Y. Kamino, and T. Ikeda, "Effect of co-aggregation on the pathogenicity of oral bacteria,"Journal of medical microbiology 39(1993):183–190

[49] Guerry, P., "Campylobacter flagella : not just for motility," TRENDS in Microbiology 15.10(2007)

[50] Arenas, J., Cano, S., Nijland, R., van Dongen, V., 1Rutten, van der Ende, A., Tommassen,J "The meningococcal autotransporter AutA is implicated in autoaggregation and biofilm formation."17(2015):1321–1337

[51] Zommiti, M., E. Bouffartigues, O. Maillot, and M. Barreau, "In vitro Assessment of the Probiotic Properties and Bacteriocinogenic Potential of Pediococcus pentosaceus MZF16 Isolated From Artisanal Tunisian Meat ' Dried Ossban .'" 9(2018):1–15

[52] Hummel, A.S., et al., C,Hertel, W, Holzapfel, C, Franz "Antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria Antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria." vol. 73.3(2007): 730–739

[53] Das, t., P. K. Sharma, H. J. Busscher, H. C. Van Der Mei, and B. P. Krom, "Role of Extracellular DNA in Initial Bacterial Adhesion and surface aggregation." Applied And Environmental Microbiology 76(10):3405–3408

[54] Bao, Y,, Y, Zhang,Y, Zhang, Y, Liu, S., Wang, X, D, Y, Wang H, Zhang "Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products." Food Control21.5(2010): 695–701,

[55] Sanz,Y, M. C. Collado, M. Gueimonde, and M. Herna, "Adhesion of Selected Bifidobacterium Strains to Human Intestinal Mucus and the Role of Adhesion in Enteropathogen Exclusion." Journal of Food Protection,68.12(2005):Pages 2672–2678

[56] Cardell, E. and V. Za, "Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese : initial characterization of plantaricin TF711 , a bacteriocin-like substance produced by Lactobacillus plantarum TF711." Journal of Applied Microbiology 99(2005):, 77–84