EXPLORING THE PROBIOTIC PROFICIENCY OF DAIRY-DERIVED LACTIC ACID BACTERIA AND THEIR ANTIMICROBIAL EFFICACY AGAINST MULTI-DRUG RESISTANT DIARRHEAL AND URO-PATHOGENS

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ABSTRACT

Multidrug resistance (MDR) poses a global health threat, necessitating the exploration of alternative solutions. Probiotics, especially lactic acid bacteria (LAB), offer promising options against the impending crisis due to their recognized safety and potential health benefits. Probiotic potential characterization and selection of candidate LAB strains are highly crucial in probiotic product formulation. This study aimed to identify LAB from dairy product yogurt and evaluate their potential probiotic properties, i.e. aggregation capacity; tolerance to gastric and intestinal conditions; as well as antimicrobial potency. Ten LAB isolates were characterized based on colony characteristics, cellular morphology, and biochemical tests. The LAB isolates, both single and in mixed consortia, displayed a time-dependent increase in auto-aggregation, ranging from 21% to 71% after 5 hours of incubation. Isolate SW2 exhibited the highest auto-aggregative capability (65%). Co-aggregation studies revealed varying degrees of co-aggregation between probiotic LAB and pathogens, with some isolates showing stronger interactions (YD3, SW1, and SW2). Mixed consortia from sample TT demonstrated the highest co-aggregative ability with all tested pathogens. These findings highlight the potential of these isolates to form protective clusters, aiding in their survival and colonization within the gastrointestinal (GI) tract, besides the competitive exclusion of pathogens. The isolates demonstrated good tolerance to simulated gastric and intestinal conditions, as indicated by their non-significant reduction (only 1-2 log) in the bacterial count after 180 minutes of treatment. These findings indicate that LAB isolates can withstand harsh GI conditions, highlighting their suitability as probiotics. Antimicrobial profiles of the LAB isolates were evaluated using radial streak method and turbidimetric microtiter plate assay against eight MDR diarrheal and Uro-pathogens (n=4 for each). LAB isolates SKY1, SW1, SW3 and TT1 exhibited the highest antimicrobial activities; while pathogens DP2, UP41 and UP42 showed the most sensitivity. Exhibited antimicrobial activity of the LAB isolates points to their potential as formidable weapons against MDR infections. Overall, the results indicate that dairy-derived LAB isolates used in this study exhibit potential probiotic traits. Further research is warranted for their mechanisms, safety, efficacy, and use in probiotic supplement development.

KEYWORDS: Multidrug resistance (MDR), Probiotics, Lactic acid bacteria (LAB), Antimicrobial activity.

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Introduction

The word "probiotic" comes from the Latin prefix "pro" and the Greek word "bios", which jointly imply "for life" or "in support of life". The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) jointly define probiotics as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (Bajagai, et al., 2016). They confer many health benefits, i. e. boosting the immune system, improving digestion, balancing gut microflora, improving mental health, keeping the heart healthy, lowering cholesterols and managing weight and so on (Bermudez-Brito, et al., 2012, Khalighi, et al., 2016). Microbial strains to be included in probiotic supplementation should have precise criteria as these are concerned with the safety of use, and functional and technological characteristics. There are three main requirements; (a) the microorganisms must be alive during administration, (b) they must be supplied in a dose that is sufficiently high to have a health-promoting impact; and (c) the host must experience a benefit from the microorganisms (Zielińska and Kołożyn-Krajewska 2018). Some additional features include tolerance to gastric and intestinal conditions (gastric enzymes, low pH, high concentration of bile salts)(Sanders 2014). The consumption of foods containing probiotics has been in practice since ancient times (Bull, et al., 2013). Due to their claimed health benefits, commercial production of probiotic-containing foods or dietary supplements are increasing day by day (Fenster, et al., 2019).

Lactic acid bacteria (LAB) are a kind of probiotic microorganisms that have drawn a lot of attention due to their probiotic potential. LAB are a varied collection of bacteria that produce lactic acid as the main end product during carbohydrate fermentation. They are often present in myriad of fermented foods such as yogurt, sauerkraut, kimchi, kefir, kombucha, pickles etc. (Marco, et al., 2006). The LAB includes different genera of bacteria such as *Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, Enterococcus and Bifidobacterium* (Holzapfel, et al., 2001, Harzallah, et al., 2013). LAB have a long history of safe consumption and are considered beneficial to human health. They play a crucial role in maintaining a balanced gut microbiota; modulating immune responses; enhancing nutrient absorption; inhibiting pathogenic bacterial invasion through competitive exclusion and the production of antimicrobial substances (like bacteriocins), maintaining the intestinal barrier, and many other processes (Wilson, et al., 1988, Parvez, et al., 2006, Sanders, et al., 2016).

Antimicrobial resistance (AMR) is a pressing challenge in global health, recognized as one of the top 10 threats facing by the World Health Organization (WHO). Based on the varied resistance patterns of bacteria, AMR may be divided into three categories: multidrug resistance (MDR), extensively drug resistance (XDR), and pan drug resistance (PDR). Non-susceptibility to three or more antibiotic classes is characterized as MDR (Magiorakos, et al., 2012). Pathogens causing diarrhea and urinary tract infections (UTIs) are of utmost priority due to their raising MDR profiles. A high prevalence of plasmids and antimicrobial resistance markers in diarrheagenic *E. coli* has been reported in Bangladesh (Mahmud, et al., 2021), that warrants its immediate management. Changing patterns in uro-pathogens' antimicrobial resistance have also been noticed (Manjunath, et al., 2011), rendering the previously effective antibiotics futile. It is a potent threat to humankind as near future no antibiotic will be effective against these pathogens (Vivas, et al., 2019). Many alternative approaches have been proposed and researches are in progress (Ołdak and Zielińska 2017, Mishu, et al., 2022, Suchi, et al., 2023). There is mounting evidence to support the use of probiotics as an alternative to antibiotics for the treatment and prevention of bacterial infection (Wan, et al., 2019).

This study aims to isolate and characterize LAB strains from yogurt and evaluate their probiotic potential. The probiotic attributes under investigation include auto-aggregation, co-aggregation, resistance to gastrointestinal conditions, and antimicrobial activity against multidrug-resistant pathogens. The findings of this study will contribute to the understanding of the probiotic potential of LAB and provide valuable insights for the development of LAB-based functional probiotic products.

Materials and Methods

Collection of Probiotic Samples

To isolate lactic acid bacteria, four yogurt samples were collected from different areas of old Dhaka, Bangladesh. The samples were- ULTRA Probiotic yogurt drink (YD); Shakti+ Misti doi (SKY); Gazi sweets misti doi (SW) and Tasty treat sweet yogurt (TT). Initial pH of the samples ranged between 3-5. The samples were homogenized with 0.85% normal saline and diluted by 10-fold serial dilution. 100µL of each homogenized samples were then spread on the MRS (de Man, Rogosa, and Sharpe) agar plates and plates were incubated at 37°C for 48 hours. Total ten isolates were obtained from the samples and used for further characterization. Moreover, mixed cultures of the yogurt sample were also assessed in terms of some of the probiotic characterization assays.

Collection of Test Pathogens

Eight multidrug-resistant test pathogens, four from diarrheal patients (DP1, DP2, DP3, DP6) and four from UTI patients (UP41, UP42, UP45, UP46) were received from the Department of Microbiology, University of Dhaka. All the isolates were previously characterized as *Escherichia coli*.

Presumptive Identification of LAB isolates

The isolates were characterized by observation of colony characteristics (size, shape, colour, texture and opacity), microscopic morphology observation by gram staining and some routine biochemical tests like Kliger's Iron Agar (KIA) test, Motility Indole Urease (MIU) test, citrate acid utilization, oxidase, catalase, methyl red, Voges-Proskauer, salt tolerance at 2%, 4% and 8% NaCl (Ismail, et al., 2018).

Aggregation assay

To determine the aggregative capability of the LAB isolates, auto-aggregation and co-aggregation assay was performed by a slight modification to Zawistowska et al. (Zawistowska-Rojek, et al., 2022).

Auto-aggregation assay

Overnight grown probiotics cultures were centrifuged at 10,000 rpm for 10 minutes at 4°C. After discarding the supernatants, pellets were washed twice with PBS. The pellets were re-suspended in PBS so that the absorbance reaches 0.25 (± 0.05) at 600nm and mixed thoroughly by vortex. The mixture was separated in 5 aliquots (each aliquot carrying 1) and incubated at 37°C without agitation. The absorbance of each aliquot was taken at the time interval of 1 hour, 3 hours and 5 hours and calculated percent auto-aggregation using the following formula:

$$\% \text{ Auto-aggregation} = [1 - A_t/A_0] \times 100\%;$$

Here A_0 and A_t stand for the $\text{absorbance}_{600nm}$ values at 0 h and $\text{absorbance}_{600nm}$ values at the specified time points.

Co-aggregation assay

Centrifugation was performed on overnight cultures of pathogens and probiotic isolates at 4°C for 10 minutes at 10,000 rpm. Discarding supernatants, pellets were given two PBS washes. The pellets were re-suspended in PBS so that the absorbance reaches 0.25 (± 0.05) at 600nm and mixed thoroughly by vortex. Equal volumes of probiotics and pathogens (1ml for each) were mixed in 5 aliquots. 2 sets of 5 control aliquots were prepared for probiotics only and pathogens only (1ml in each). After thorough mixing, the aliquots were incubated at 37°C without agitation. The absorbance of each aliquot was taken at 5 hours' time intervals. Percentages of co-aggregation were calculated using the following formula:

$$\% \text{ Co-aggregation assay} = [(A_{pat} + A_{probio})/2 - A_{mix}]/ [(A_{pat} + A_{probio})/2] \times 100;$$

Here, the absorbance of the individual pathogen and probiotic bacterial suspensions in control aliquots is represented by the letters A_{pat} and A_{probio}, whereas the absorbance of the

combined bacterial solution at the prescribed time is represented by the letter A_{mix}.

Simulation of tolerance to gastric and intestinal conditions

Tolerance to simulated gastrointestinal conditions was performed with slight modification from the previous study (Choi, et al., 2018). To determine the tolerance to gastric conditions, simulated gastric juice was prepared by suspending 3g/L of pepsin (Sigma-Aldrich) in a sterile saline solution (0.5% NaCl, w/v). The pH of the solution was finally adjusted to 3.5 by adding 1M HCl.

The overnight grown probiotic cultures were re-suspended in PBS after a double wash. The bacterial suspension (200 µL) was then added onto the mixture of simulated gastric juice (1 mL) and sterile saline solution (300 µL). After that, the mixture was incubated at 37°C for 60 and 180 minutes. Total viable bacterial counts were measured by repeated dilution plating on MRS agar plates using spread plate and drop plate techniques at the mentioned time intervals. On the other hand,1g/L of pancreatin together with 0.3% bile salts was mixed in a sterile saline solution to prepare simulated intestinal juice. The pH of the solution was adjusted to 8 by adding 1M NaOH. Similar methods as tolerance to gastric simulation were used to determine the tolerance to simulated intestinal conditions.

Assessment of antimicrobial activity of LAB isolates against test pathogens

Radial streak method

Initial characterization of the antimicrobial activities of probiotic isolates against MDR clinical pathogens was performed using the radial streak method according to Coman et al. (Coman, et al., 2014). Probiotic isolates were grown overnight and OD_{600} was adjusted to 0.1. Each probiotic isolate was inoculated on MRS agar plate by covering a circular area in the center of the Petri dish. The plates were incubated at 37°C for 48 hours. Then, the pathogens (0.5 McFarland standard) were streaked by radial lines of inoculum from the border to the centre of the plate and incubated at 37°C. After 24 hours' incubation, zones of inhibition were observed around probiotic isolates and measured in mm from the periphery of the isolates.

Turbidimetric assay using microtiter plate

Inhibitory action of LAB CFS (cell free supernatant) against the MDR pathogens was observed by turbidimetric microtiter plate assay with some modifications of the method described previously (Scillato, et al., 2021). To prepare the cell free supernatant, probiotic isolates were grown in MRS broth at 37°C for 48 hrs. The cultures were centrifuged at 10000 rpm for 10 mins at 4°C. The supernatants were collected in a tube and passed through a sterile 0.45 µm pore sized syringe filter to get the cell free supernatants. The assay was carried out in 96-well microtiter plates. The plates were designed in the following fashion; wells containing only 100 µL media considered as media blank, wells containing 50µL media along with 50 µL pathogen as growth control, wells containing 100 µL only CFS as CFS blank, wells containing 50 µL CFS and 50 µL pathogen. Initial absorbance of the plate was taken at 600 nm immediately after inoculation. The plate was then incubated at 37°C for 24 hours and final absorbance was taken. Considering the growth control as 100%, the reduction in percentage of growth in CFS containing wells was observed. Antimicrobial activity of the CFS of the mixed cultures was also assessed.

Results

Presumptive identification of lactic acid bacteria

All 10 isolates have shown almost similar colony characteristics and cellular morphology (Table 1). Most of the colonies were small, round, white in color, smooth and opaque. Two exceptions in colony morphology were observed for YD3 and YD4 that showed large and irregular shaped colonies. All the isolates were gram positive cocci. Biochemical characterization revealed that these isolates exhibited negative results in catalase, oxidase, gas and H_2S production, motility, indole, urease, VP and citrate test. In case of methyl red test, all the isolates (except one ambiguous result for YD1) showed a positive reaction. On the basis of the results exhibited by the isolates we can presumably identify them as lactic acid bacteria (LAB)(Ismail, et al., 2018) (Table 2).

Table 1. Colony characteristics and microscopic observation of the LAB isolates collected from yogurt samples

Isolates	Colony characteristics	Microscopic observations	
		Cellular morphology	Gram reaction
YD1	Small, round, white, smooth, opaque	Cocci, cluster, small	+
YD2	Small, round, white, smooth, opaque	Cocci, small	+
YD3	Large, irregular, milky white, smooth, opaque	Coccoid, large	+
YD4	Large, irregular, milky white, smooth, opaque	Coccoid, irregular	+
SKY1	Small, round, white, smooth, opaque	Cocci, irregular	+
SKY2	Small, round, white, smooth, opaque	Cocci, cluster, small	+
SW1	Small, round, white, smooth, opaque	Cocci, small	+
SW2	Small, round, white, smooth, opaque	Cocci, small	+
SW3	Small, round, white, smooth, opaque	Cocci, small, chain	+
TT1	Small, round, white, smooth, opaque	Cocci, small	+

Table 2. Biochemical profiles of the LAB isolates collected from yogurt samples

Biochemical tests		Isolates no									
		YD1	YD2	YD3	YD4	SKY1	SKY2	SW1	SW2	SW3	TT1
Catalase		-	-	-	-	-	-	-	-	-	-
Oxidase		-	-	-	-	-	-	-	-	-	-
KIA	B/S	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y
	G/L	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	Gas	-	-	-	-	-	-	-	-	-	-
	H_2S	-	-	-	-	-	-	-	-	-	-
Salinity endurance	2%	+	++	++	++	++	++	++	++	++	++
	4%	-	+	+	+	++	+	+	+	+	+
	8%	-	-	-	-	+	+	-	-	++	+
MIU	Motility	-	-	-	-	-	-	-	-	-	-
	Indole	-	-	-	-	-	-	-	-	-	-
	Urease	-	-	-	-	-	-	-	-	-	-
MR		+/-	+	+	+	+	+	+	+	+	+
VP		-	-	-	-	-	-	-	-	-	-
Citrate		-	-	-	-	-	-	-	-	-	-
Presumptive identification		Lactic Acid Bacteria (LAB)									

Here, '+' indicating positive reaction, '-' indicating negative reaction; B= butt, S= slant; G= glucose fermenter, L=lactose fermenter; Y=Yellow; for salt endurance '++' indicating dense growth,'+' indicating slight growth, '-' indicating no growth; +/- indicating ambiguous reaction or undecided.

Auto-aggregative capability of the isolated LAB

All the isolates as well as mixed cultures of the individual samples showed a time-dependent increase in auto-aggregative ability. The highest auto-aggregation for all isolates was exerted after 5 hrs of incubation. The percentages of auto-aggregation after 5 hours' time span was found in between 21% to 71%. The highest percentage was observed in mixed cultures of sample TT, whereas lowest was observed in YD2. In case of the single isolates, SW2 showed the highest percentage of auto-aggregation that is around 65%. There was no significant difference between single culture and mixed cultures. Graphical representations of the percentages of auto-aggregation are shown in Figure 1.

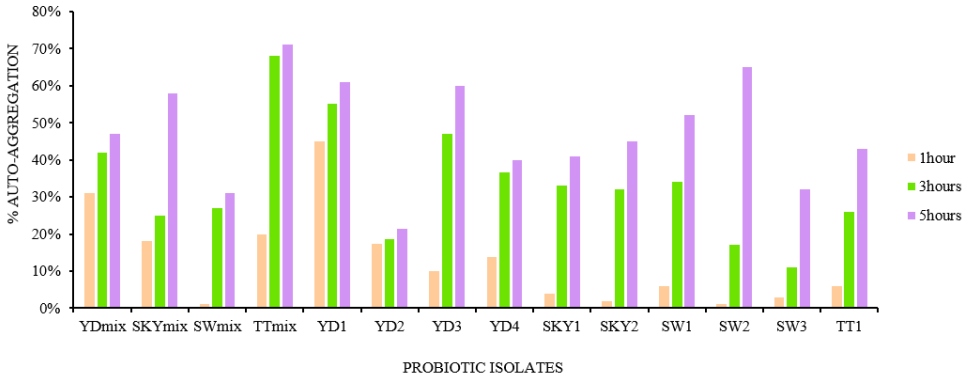

Figure 1. Auto-aggregative capability of LAB isolates evaluated after the time intervals of 1 hour, 3 hours and 5 hours' incubation in PBS at 37°C. An increase in the auto-aggregative ability of each isolate was observed throughout 5hours time incubation. The percentage range for auto aggregation was in between 21% to 71% where the highest was found for TTmix and the lowest was for YD2.

Co-aggregative capability of probiotic LAB with pathogens

A diversified result in percentages of co-aggregation of the probiotic mixed cultures and single isolates with pathogens was observed after 5 hours of incubation time. The lowest 2% and the highest 55% co-aggregative ability was calculated among all the isolates and mixed cultures. Single isolates, YD3, SW1, and SW2 were found more active and exhibited higher co-aggregation with pathogens than others. SW3 and TT1 showed the poorest results with most of the pathogens among the isolates. Mixed consortia of sample TT exhibited the highest co-aggregative ability with all pathogens, while mixed consortia of SW showed the least ability among the mixed cultures of the sample. The overall least co-aggregation was found with the pathogens of UTI samples; contrastingly the highest with the pathogens from the diarrheal patients. The graphical representations of the percentages of co-aggregation with pathogens are shown in Figure 2.

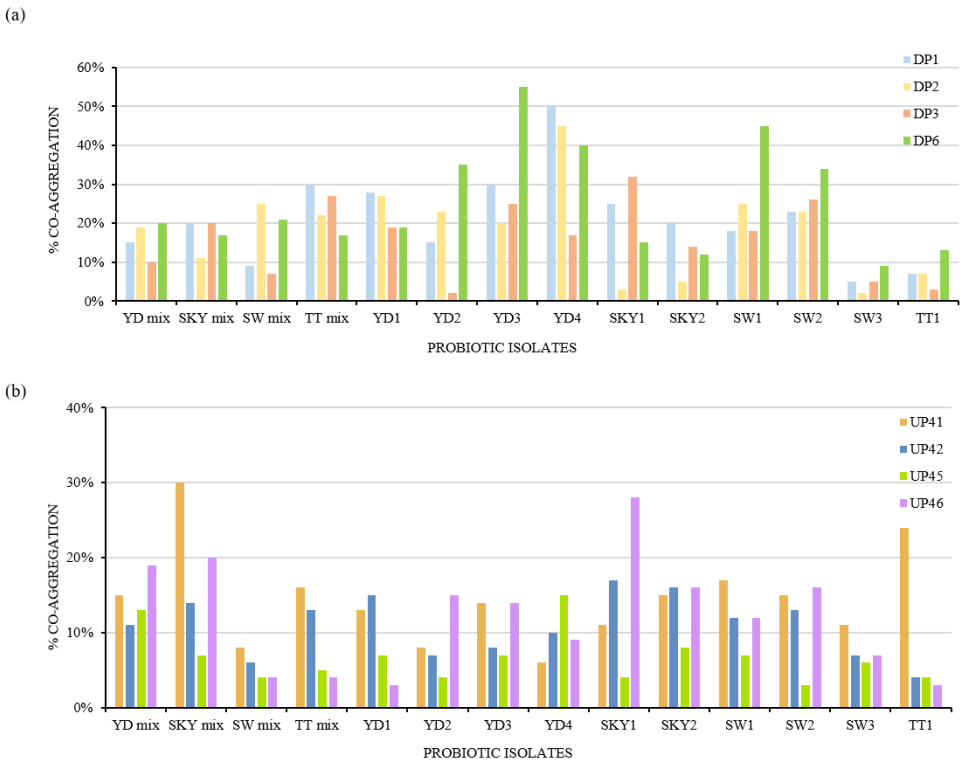

Figure 2. Co-aggregative capability of LAB isolates with (a) diarrheal pathogens (DP) and (b) Uro-pathogens (UP) after 5h incubation at 37 °C in PBS. The highest 55% (YD3 with DP6) and the lowest 2% (YD2 with DP3) co-aggregation was observed among the isolates.

Tolerance to simulated gastric and intestinal condition

All the isolates showed a non-significant reduction in the bacterial count after the treatment with freshly prepared gastric juice and intestinal juice for 180 minutes. They showed mostly a 1-2 log reduction in the bacterial count with respect to the initial bacterial count proving the isolates to have good tolerance against adverse gastric and intestinal conditions. Only exception was found for YD3 which survived 60 minutes treatment but could not survive 180 minutes treatment in both cases. The tolerance profiles of the probiotic isolates to simulated gastric and intestinal conditions are presented in Tables 3 and 4, respectively.

Table 3. Tolerance profile of the LAB isolates to simulated gastric juice. The results are represented as CFU/mL (colony forming unit/mL)

Isolates	Bacterial Count (CFU/mL)		
	Time interval (in minutes)		
	0	**60**	**180**
YD1	TNTC	4.7×10^{7}	4×10^{7}
YD2	TNTC	3.1×10^{7}	3.5×10^{7}
YD3	5×10^{6}	10×10^{6}	-
YD4	1.125×10^{8}	1.5×10^{7}	1×10^{7}
SKY1	7×10^{8}	3.3×10^{6}	3.3×10^{6}
SKY2	1.2×10^{7}	1.5×10^{6}	2.36×10^{6}
SW1	3.56×10^{8}	3.7×10^{7}	7.8×10^{7}
SW2	3.08×10^{8}	7.1×10^{7}	2.06×10^{7}
SW3	TNTC	4.7×10^{8}	4.7×10^{7}
TT1	1.13×10^{8}	3.3×10^{7}	7×10^{7}

Table 4. Tolerance profile of the LAB isolates to simulated intestinal juice. The results are represented as CFU/mL (colony forming unit/mL)

Isolates	Bacterial Count (CFU/mL)		
	Time interval (in minutes)		
	0	60	180
YD1	TNTC	4.6×10^{7}	3.9×10^{7}
YD2	TNTC	3.7×10^{7}	3.1×10^{7}
YD3	5×10^{6}	1×10^{4}	-
YD4	1.125×10^{8}	2×10^{7}	1.13×10^{7}
SKY1	7×10^{8}	3.5×10^{7}	1.8×10^{8}
SKY2	1.2×10^{7}	7.4×10^{7}	1.7×10^{7}
SW1	3.56×10^{8}	7.6×10^{7}	8.5×10^{7}
SW2	3.08×10^{8}	7.4×10^{7}	1.46×10^{8}
SW3	TNTC	1.22×10^{8}	1.22×10^{8}
TT1	1.13×10^{8}	2.5×10^{7}	1.1×10^{8}

Antimicrobial profiles of the probiotic samples against multidrug-resistant pathogen

Growth inhibition pattern observed by radial streak method

Clear zones of growth inhibition of the pathogens were found around the radial streak lines of the probiotic isolates. A wide range of inhibition zone, lowest 2mm to highest 18mm was measured against the MDR pathogens. The Isolates from the sample YD exhibited the least activities, whereas isolates from the SKY, SW, and TT exhibited the highest activities against both types of pathogens. (Figure 3)

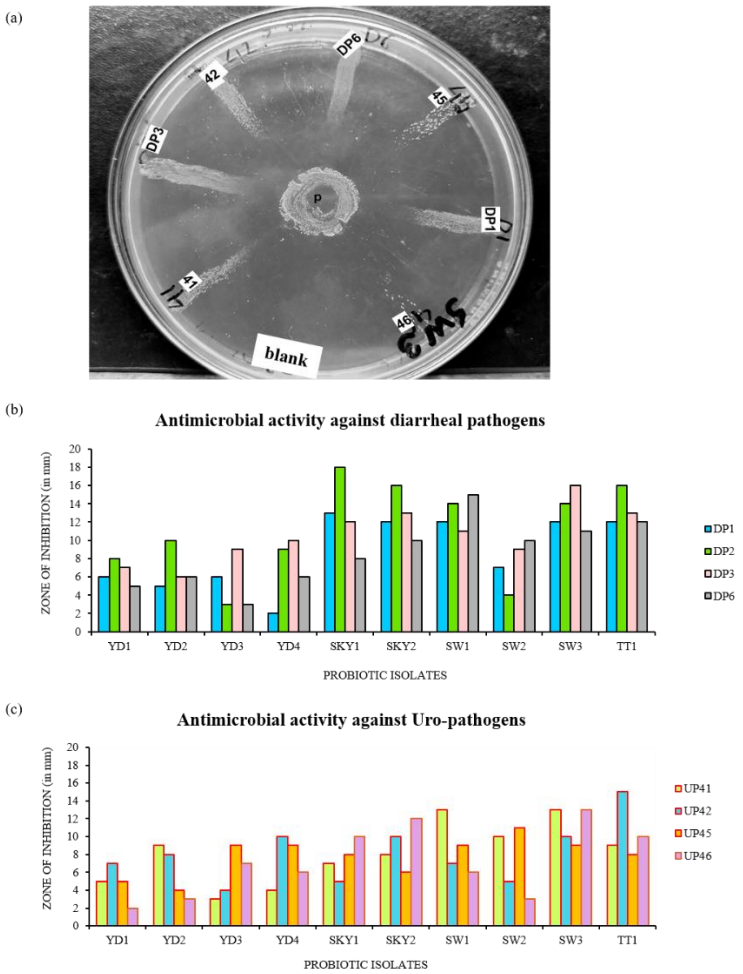

Figure 3. Antimicrobial activity of LAB isolates against pathogens by radial streak method. (a) Representative figure of a radial streak plate. Culture at the center of the plate denoting as P represents the growth of LAB isolates. DP1, DP3, DP6 diarrheal pathogens and 41,42, 45, 46 Uro-pathogens. **Zone of growth inhibition of (b) diarrheal pathogens (DP) and (c) Uro-pathogens (UP)** was measured in millimeters. SKY1 exhibited the highest zone of inhibition (18mm) against DP2, while YD1 and YD4 both exhibited the lowest zone of inhibition (2mm) against UP46 and DP1 respectively.

Antimicrobial effect of LAB CFS observed by turbidimetric assay

A diversified result was observed in the CFS activities of the probiotic isolates by a turbidimetric assay using microtiter plates. Figures 4 and 5 depict the antimicrobial activity of LAB observed by co-culturing LAB CFS with diarrheal and Uro-pathogens, respectively. Two types of results were observed in board categories; one reducing the growth of pathogens, another enhancing the growth of pathogens. Among all the pathogens, the growth of DP1, DP2, UP41, UP42, and UP46 was more or less inhibited by CFS of single isolates and mixed consortia. In terms of DP1, CFS of mixed consortia exhibited comparatively better inhibition than single isolates, whereas CFS of single isolates showed better antagonism against DP2, UP41, UP42, UP45, and UP46. However, besides inhibitory activities, CFS of some single isolates enhanced the growth of pathogens. For example, DP6 showed 2% and 10% enhanced growth than control with the CFS of SKY2 and SW3 respectively; DP3 manifested 24%, 1%, and 23% more growth with the CFS of YD4, SKY1 and TT1 respectively; 43% of enhanced growth was observed in uro-pathogen UP45 due to the presence of CFS from SKY mixed consortia. Overall, only considering the reduction in growth of the pathogens, the highest 98% reduction by CFS of YD1 was found against UP42 and the lowest 17% reduction by CFS of SKY2 against DP1.

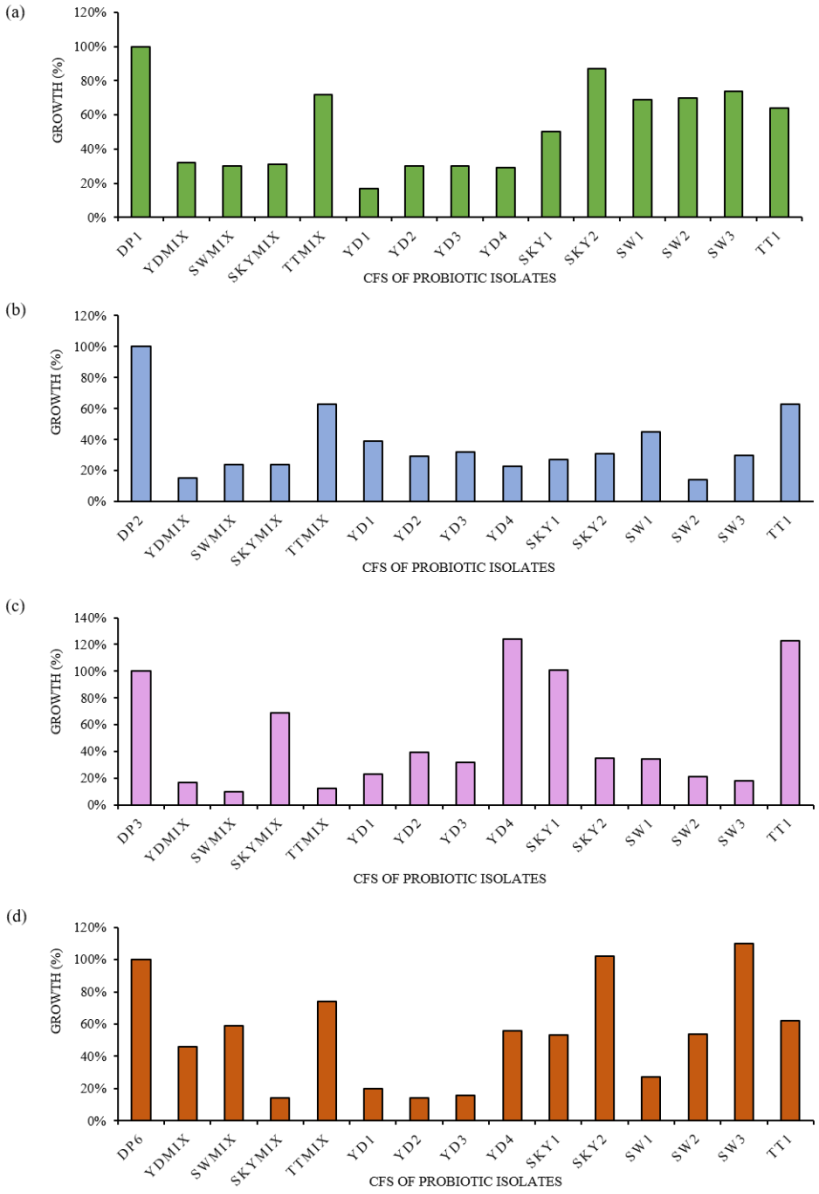

Figure 4. Antimicrobial activity of LAB isolates against diarrheal pathogens by microtiter plate turbidimetric method. Growth rate of diarrheal pathogens (DP) **(a) DP1, (b)DP2, (c) DP3 and (d) DP6** in the presence of LAB CFS after 24 hours' incubation at 37°C. The growth rate of pathogens without CFS was assigned to 100%. The bacterial growth was determined at OD_{600nm}. Reduction in growth percentages of diarrheal pathogens was observed in most of the cases, however, enhanced growth of pathogens was observed in a few cases. The CFS of SWmix exerted the highest 90% growth reduction of DP3, while SKY2 exerted the lowest 13% of DP1.

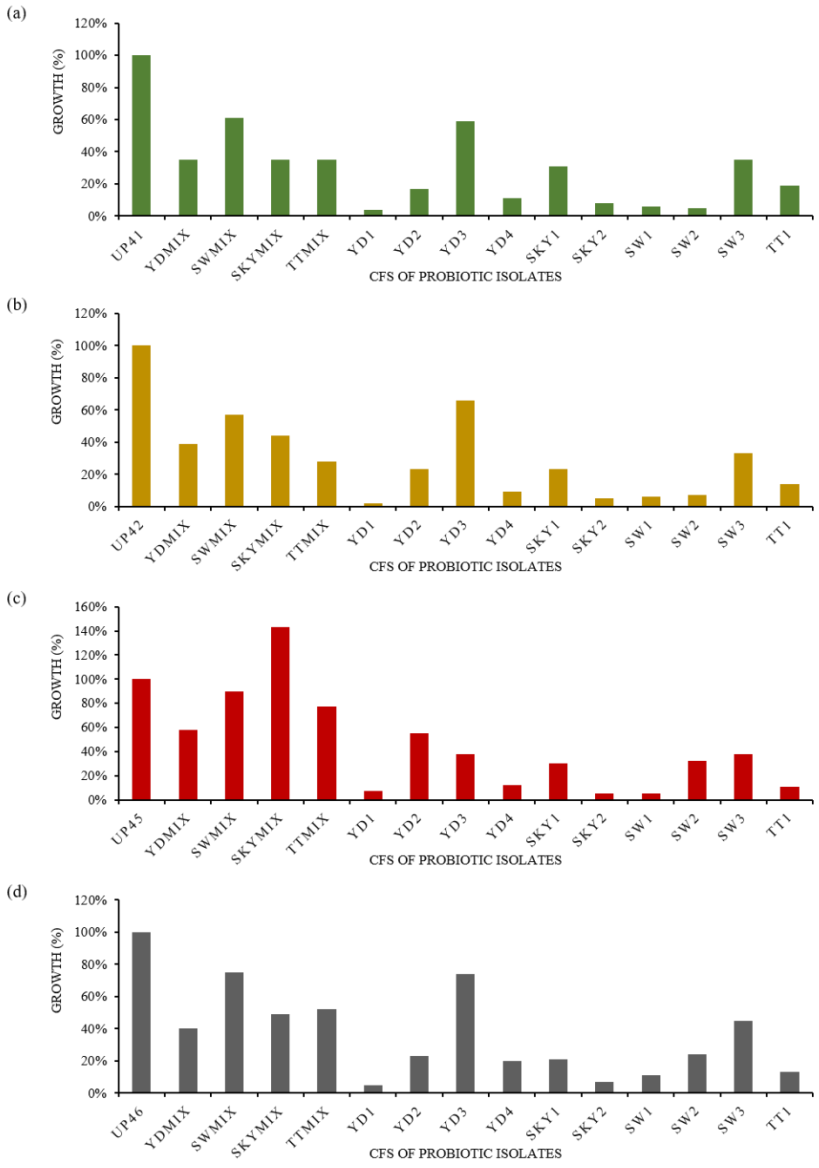

Figure 5. Antimicrobial activity of LAB isolates against Uro-pathogens by microtiter plate turbidimetric method. Growth rate of Uro-pathogens (UP) **(a) UP41, (b) UP42, (c) UP45 and (d) UP46** in the presence of LAB CFS after 24 hours' incubation at 37°C. The growth rate of pathogens without CFS was assigned to 100%. The bacterial growth was determined at OD_{600nm}. Reduction in growth percentages of uro-pathogens was observed in most of the cases, however, in one case, enhanced growth of pathogens was observed. The CFS of YD1 exerted the highest 98% reduction in growth of UP42, while SWmix exerted the lowest 10% reduction in growth of UP45.

Discussion

In recent years, lactic acid bacteria (LAB) have emerged as noteworthy candidates for probiotics, capturing considerable research interest. LAB are commonly found in various fermented foods and are known for their beneficial effects on the gut microbiota and overall human health. The study was conducted to assess the probiotic potential of ten LAB isolated from yogurt.

One of the key probiotic attributes is the auto- and co-aggregative capability of the LAB isolates. Previous studies have reported higher auto-aggregative abilities (60-88%) of LAB isolates (Sadrani, et al., 2014, Dlamini, et al., 2019). Conversely, Li et al. observed lower auto-aggregative abilities ranging from 5.92% to 23.32% (Li, et al., 2015). In line with these findings, our study demonstrated diverse auto-aggregative abilities (ranging from 21% to 71%) among the LAB isolates after 5 hours of incubation. This inconsistency in auto-aggregation capacities across LAB isolates might be attributable to their genetic and physiological variations. The auto-aggregative ability is often associated with cell adherence properties by forming protective clusters, aiding in their survival and colonization within the gastrointestinal tract (Vinderola, et al., 2004). It also plays a crucial role in preventing pathogen colonization (Dlamini, et al., 2019).

The co-aggregative properties of lactic acid bacteria (LAB) isolates have shown a diverse range of results in this study. Some isolates exhibited lower percentages of co-aggregation, as low as 2%, while the highest co-aggregation percentage reached 55% after 5 hours of incubation. These findings indicate that co-aggregation abilities are strain-specific and vary among different pathogens, as consistent with previous statements (Collado, et al., 2007, Xu, et al., 2009, Li, et al.,

2015, Sirichokchatchawan, et al., 2018, Dlamini, et al., 2019). Lower co-aggregative ability (less than 10%) of LAB with *E. coli* was observed by Collado et al., while Sirichokchatchawan et al. reported LAB strains exhibiting more than 30% co-aggregation with *E. coli.* Anandharaj et al. observed co-aggregation percentages ranging from 19% to 68% between LAB and *E. coli* (Anandharaj, et al., 2015).

The co-aggregation of LAB with pathogens is closely related to the auto-aggregation of probiotics, as reported previously, strains with higher auto-aggregative capabilities tend to exhibit higher co-aggregation abilities with pathogens (Collado, et al., 2007). Our study does not establish any direct correlation between the auto- and co-aggregation capability, yet we have observed moderate to high co-aggregative abilities of the isolates having the higher auto-aggregative capability, e.g. YD3, SW1 and TT_{mix}. Probiotics' co-aggregative properties can hinder pathogens' capacity to infect hosts and stop the invasion of foodborne pathogens (García-Cayuela, et al., 2014), thus possessing the potential to reduce diarrheal infections. LAB with strong co-aggregative activity can effectively inhibit foodborne pathogenic bacteria from adhering to HT-29 cells, as stated by former research (Choi, et al., 2018). Additionally, during co-aggregation, the concentration of inhibitory substances excreted by LAB may be increased, further contributing to pathogen inhibition (Kaewnopparat, et al., 2013).

Tolerance of LAB isolates to the strident GI condition is another crucial probiotic trait ensuring their survival and colonization within the human digestive system. It allows them to reach the intestines, where they can exert their potential health benefits, such as modulating the gut microbiota, enhancing nutrient absorption, and inhibiting the growth of pathogens (Marco, et al., 2006). Choi and the team conducted a study demonstrating the strong resistance of certain LAB isolates to gastrointestinal conditions (Choi, et al., 2018). In line with these findings, our study also revealed that all the LAB isolates exhibited significant resistance to simulated gastric and intestinal conditions.

The antimicrobial activity of the LAB isolates against multi-drug resistant (MDR) pathogens was examined by radial streak method and micro-titer plate turbidimetric assay incorporating cell free supernatant (CFS) of LAB isolates. Eight MDR isolates obtained from patients having diarrhea and urinary tract infection (UTI) were used as test pathogens.

The radial streak method is a valuable approach for assessing the antimicrobial activities of LAB using active whole cells. This antimicrobial activity is attributed to the production of metabolites such as lactic acid, acetic acid, diacetyl, and bacteriocins by LAB during the experimental period. These bioactive compounds are known to diffuse through the agar medium, effectively inhibiting the growth of pathogens (Coman, et al., 2014). In our study, variety of inhibitory actions, ranging from 2mm to 18mm inhibition zone, were observed against the tested pathogens. Coman and colleagues observed zone of inhibition values between 12mm and 24mm for different LAB strains against *Bacillus cereus* and *Enterococcus faecium* (Coman, et al., 2014). This discrepancy in inhibitory actions can be explained by the MDR profile of our selected pathogens.

Furthermore, the CFS of LAB demonstrated significant inhibitory action against the tested pathogens. However, this antimicrobial activity was specific to LAB strain selected and the pathogen concerned, with highest 98% growth inhibition. Previous studies by Dejene et al. and Dissasa et al. also reported satisfactory inhibitory action of LAB-derived CFS, corroborating to our findings (Dejene, et al., 2021, Dissasa, et al., 2022). Worth Noting, CFS of some isolates unexpectedly enhanced the growth of pathogens by a significant percentage, for reasons that remain unknown. Thus, strain selection with proper characterization is highly recommended in the formulation of probiotic supplements. Studies have suggested that mixed cultures of probiotics may exhibit higher antimicrobial activity compared to monocultures (Kozhakhmetov, et al., 2009). Surprisingly, in this study, the antagonistic profile of single isolates was found to be more active than the mixed consortia in most of the experiments. Further investigations into the complex interactions among consortia may shed light on the underlying causes of these inconsistent results.

Conclusion

The increasing prevalence of multidrug-resistant bacteria poses a significant global threat. Probiotic supplements and foods containing probiotic bacteria are emerging as potential alternatives to antibiotics. LAB are an attractive choice for probiotic strains due to their presence in the normal gut microbiota. In this in vitro study, we successfully isolated and characterized several LAB strains with promising probiotic and antagonistic properties against MDR pathogens. The LAB isolates exhibited diverse auto-aggregative and co-aggregative abilities, indicating strain-specific characteristics. They also demonstrated resistance to simulated gastrointestinal conditions, suggesting their ability to survive and colonize the human digestive system. Moreover, most of the LAB isolates displayed satisfactory antimicrobial activity against MDR pathogens, implausibly, some isolates enhanced pathogen growth. These findings highlight the importance of strain selection and characterization in probiotic formulation. The study provides valuable insights into the probiotic potential of LAB isolates, contributing to the development of LAB-based probiotic products with potential health benefits. Future research directions should include safety assessments, such as antibiogram profiling; identification of specific metabolites (e.g., lactic acid, acetic acid, diacetyl, bacteriocins) responsible for the inhibitory effects of LAB. Additionally, a trial on animal model remains an essential step in establishing a potential strain as a probiotic supplement.

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