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Antimicrobial Potential of *Lactobacillus* and *Bifidobacterium* strains Isolated from Probiotic Antidiarrheal Formulations in Pakistan: An *In Vitro* validation against Diarrheagenic *E. Coli*.

Syed Imran Ali[1], Dr. Baqar Shyum Naqvi [1] and Samiyah Tasleem[2]

[1] Department of Pharmaceutics, University of Karachi, **Pakistan**
[2]Department of Microbiology, University of Karachi, **Pakistan**.

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***Correspondence:** imran4u5@yahoo.com **Accepted:** 11 April. 2019 **Published online: 13** Mayl. 2019

In Pakistan, diarrheal illnesses in children below 5 years of age continued to be an irresistible problem with an increased number every year. Application of probiotics in the prevention and treatment of diarrhea have been clinically validated, comprehensive studies and explored in many products around the world. Aim of the study was to evaluate the antimicrobial activities of twelve *Lactobacillus* and *Bifidobacterium* strains against diarrheagenic *Escherichia coli* isolated from probiotic formulations in Pakistan. MRS, BSM, and Mueller- Hinton media were applied for the growth of probiotic isolates and *E. coli*. Physiological and biochemical methods were utilized for identification of probiotic isolates. Antagonistic activity of isolates were analyzed through Agar spot and well-diffusion methods. Scanning Electron Microscopy supported to scrutinize the change in cell morphology. Statistical tests were adopted with a level of significance $p < 0.05$. All isolates were Gram-positive, non-motile, and catalase negative. The antimicrobial activity of isolates against *E. coli* measured as zone of inhibition (mm). *Lactobacillus reuteri DSM 17938* (26 mm) demonstrated higher effect against diarrheagenic *E. coli*. *Bifidobacterium* strains shown mild antimicrobial activity. Using single or multiple strains of *Lactobacillus* along with *Bifidobacterium spp*. in probiotic pharmaceutical formulations and nutritional supplements is a novel approach for the prevention and management of *E. coli* induced diarrhea. However, more studies are needed to assess the synergistic effects of different probiotics complementarity in pediatric diarrhea.

Keywords: Words Pediatric diarrhea, Probiotic formulations, *In vitro* assessment, *Lactobacillus* and *Bifidobacterium* strains

INTRODUCTION

Globally, diarrheal is the second top listed infectious disease in infants and children under 5 years of age with morbidity of 1.7 billion and mortality of 846,000 cases annually (WHO, 2016). In Pakistan, diarrhea after the respiratory infections is the second primary cause of mortality as infected 87 in every 1000 children below 5 years of age (Mumtaz et al., 2014). Acute diarrhea is developing countries is significantly associated with a wide range of bacteria, viruses, and parasites (Nitiema et al., 2011; Larson et al., 2009). Global enteric multicenter (GEM) study which was conducted in South Asia including Pakistan, observed the involvement of causative pathogens such as *Rotavirus, Cryptosporidium, Shigella,* and *Enterotoxigenic E. coli* in moderate to severe diarrhea in infants and children aged below 5 years of age. (Kotloff et al., 2012). Among bacteria diarrheagenic *E. coli* is more associated with infective diarrhea in infant and children by stimulating the secretion of water and

electrolytes due to toxin induced destruction of absorptive cells of small intestine. (DuPont, 2009; Nataro et al., 2006; Navaneethan et al., 2008; Qadri et al., 2010).

Probiotics are defined as live microorganisms which, when administered in an adequate amount, confer a health benefit to the host. (FAO/WHO, 2001). The US Food and Drug Administration (FDA) classified probiotics in view of "live bio-therapeutics" envisioned for clinical use. (Hoffman, 2008; Sutton, 2008). The intestinal microflora is colonized by different bacterial species around 10 trillion and about 1-2 kg of weight which play a vital role in regulating the physiological and metabolic functions such as fermentation, energy storage, production of amino acids and certain vitamins. In addition, probiotics are important for the maturation and maintenance of body's immune function. (Hara and Shanahan, 2006; Vyas and Ragnanathan, 2012; Kamada et al., 2013). The predominance of probiotic in particular, *Lactobacillus spp.* and *Bifidobacterium spp*. which are the dominant part of gut microflora is a key factor to protect the body against a disease-causing microorganism. (Borchers et al., 2009; Williams et al., 2010; Yan et al., 2007)

Multiple mode of actions have been proposed concerning the antimicrobial effects of probiotics. *Lactobacillus spp.* inhibits the attachment of pathogenic bacteria to gut epithelial cells by the formation of mucin, lipids, free protein, and immunoglobulins. (González et al., 2012; Collado et al., 2005). Several strains of *Bifidobacterium* and *Lactobacillus*, produce bacteriostatic and bactericidal substances such as reuterin and bacteriocins are biologically active antimicrobial compounds which inhibit the growth of pathogenic bacteria. (Hawrelak et al., 2013). Production of organic acids, in particular, lactic acid and acetic acid are considered as strong antimicrobial substances responsible for the antagonistic activity of probiotic bacteria against enteropathogens (Makarova et al., 2006; Alakomi et al., 2005). Lowering the pH of the intestine by probiotics suppress the colonization of proteolytic bacteria. (Alverdy et al., 2003; Fric, 2002). Many strains of probiotics belong to *Bifidobacterium* and *Lactobacillus* act as "colonization barrier", compete with pathogenic bacteria for the attachment sites and inhibit their growth. (Hawrelak et al., 2013; Kamada et al., 2013)

MATERIALS AND METHODS

Collection of Samples

Culture of enteropathogenic bacteria *E. coli* was obtained from the laboratory of Hamdard hospital then used after undertake identification test. Isolates of probiotics were obtained from the antidiarrheal probiotic formulations intended for infants and children in Pakistan (Table 1).

Table 1: Sources of Probiotic Isolates

Probiotic Strain	Product
Lactobacillus acidophilus LA-5	PPF-1
Lactobacillus reuteri DSM 17938	PPF-2
Lactobacillus paracasei 431	PPF-3
Lactobacillus casei 431	PPF-3
Lactobacillus rhamnosus 343	PPF-4
Lactobacillus GG	PPF-5
Lactobacillus bulgaricus	PPF-6
Lactobacillus plantarum 299v	PPF-6
Bifidobacterium longum BB-12	PPF-7
Bifidobacterium lactis Bb-12	PPF-8
Bifidobacterium bifidum	PPF-8
Bifidobacterium breve	PPF-9

These probiotic samples were collected in air tight leak proof, clean and sterile wide-mouthed container free from any disinfectant residue. Collected samples were immediately transferred to laboratory for microbial analysis and refrigerated at low temperature (-4 ^{0}C) to avoid any possible contamination and deterioration.

Isolation and Enumeration of LABs

Selected strains of *Lactobacilli* were inoculated on MRS broth (deMan, Rogosa and Sharpe, 1960) for 3 days at 37 ^{0}C under anaerobic conditions. Sub-cultured the broth samples on MRS agar for 3 to 6 days at 37 ^{0}C under anaerobic conditions (Murray et al., 1999; Downes et al., 2001). On the other hand, designated strains of *Bifidobacterium* were inoculated on BSM broth for 60 hours under anaerobic conditions at 37 ^{0}C, then sub-cultured on respective BSM agar again under the same conditions and temperature for 48 hours. (Thitaram *et al.,* 2005). MRS agar which contains isolates of *L.acidophillus* and *L.dulbrueckii* also added with 0.5% salicin and 0.05% cysteine to improve the specificity of the medium (Shah, 2000; Hartemink et al., 1997). Adjusted the pH of the media to 5.2 to 6.5 by the addition of 0.1 N solution of sodium hydroxide (NaOH) and

hydrochloric acid (HCl) guided through pH meter. The repeated streaking on MRS and BSM agar was done in order to get the pure colonies of each culture. Appeared colonies were elected for identification and biochemical tests and used for the evaluation of antimicrobial activity against diarrheagenic pathogens.

Identification and Biochemical Testing

Gram staining and different biochemical tests such as catalase and sub-cultured motility were performed for identification of isolated strains of probiotics by using the formed and suspected colonies of enumerated probiotic strains.

Gram Staining

Colonies of *Lactobacilli* and *Bifidobacterium* were picked from the MRS agar and BSM agar culture, mixed with one drop of sterile distil water and smeared on a sterilized glass slide followed by the heat fixed. The slides were stained with crystal violet and incubated at ambient temperature for one minute then washed with sterile distil water and applied Gram's iodine solution and incubate for one minute. Finally stain the slides with Safranin for 60 seconds then rinsed and dried the slides and observe under the microscope. The similar process was repeated for each isolated strains from the samples. Observations were recorded in terms of morphology.

Catalase Test

Many microorganisms other than lactis acid bacteria, have an ability to break hydrogen peroxide ($2H_2O_2$) into water and oxygen. Thus, the formation of gas bubbles in the incubated culture depict the existence of catalase enzyme. Since, the lactic acid bacteria are catalase negative. Observer previously prepared MRS and BSM culture and pour the 3% solution of hydrogen peroxide and incubate overnight. Colonies with no gas bubble formation were selected for further studies. Results were recoded for each isolated strains collected from different samples. (Nelson et al., 1995)

Sub-cultured Motility Test

The motility of isolated strains were examined through the microscopic observation of slides prepared according to Hanging-drop wet method. The observation for each strain was recorded. (McFadden, 2000)

Preparation of Enteropathogens Test Plates

Mueller Hinton agar susceptibility plates were prepared by pouring it into sterilized 100 ml of Petri dish with 100 mm diameter to get the even depth of 4 mm from the bottom surface which is obtained by adding 30-32 ml of Mueller Hinton agar into Petri dish of 100mm. Finally cooled the susceptibility plates to room temperature

Preparation of Inoculum

The process of inoculum was performed near the flame to avoid the risk of contamination. The inoculum was prepared with the help of sterile wire loop and isolated cultures *E. coli* suspended by touching the topmost of colonies of cultures present in a test tube containing 2-3 ml of Mueller Hinton Broth. The same method was adopted for the inoculum of each selected diarrheagenic microorganisms. The test tubes were incubated at 37 0C for few hours until the standard of turbidity achieved to McFarland 0.5 M standard. After 3-4 hours, similar procedure was repeated for each isolates.

Culture Inoculation

Broth suspension of selected pathogens was sub-cultured initially by dipping the sterile cotton swab into the prepared suspension through rotation and pressing the swab towards the wall of test tube facing to fluid in order to get the excess of inoculum present in swab. The cotton swab was streaked evenly on the prepared Muller Hinton agar surface. The same procedure was repeated three times subsequent with rotating the plates in order to ensure an even spreading of inoculum then allow the inoculum to dry.

Antimicrobial Activity of Probiotic Isolates against Pathogens.

Agar Spot test

Agar spot test was used to determine the antibacterial activity of isolated lactis acid bacteria against selected pathogens (Tejero-Sarinena et al., 2012). Overnight serially diluted cultures of probiotics bacteria of 10^7-10^9 CFU/ml was spotted on the surface of MRS agar and incubated further at 37 ^{0}C for 24 hours under anaerobic condition in order to allow further colonies then incubated plates were overlaid with previously inoculated plate with pathogenic bacteria (10^7-10^9 cells per ml). All plates were incubated at 37 ^{0}C for 24 hours under anaerobic condition. Repeated the procedure for each strain of isolated strains of probiotics. Diameter of clear zone of inhibition

around the spotted LAB was measured by Vernier caliper and recorded.

An Agar-well Diffusion Method

Isolated probiotics stains belong to *Lactobacillus* and *Bifidobacterium* were grown on MRS agar and BSM gar respectively and incubated at 37 ^{0}C for 24 hours under anaerobic condition then diluted to 0.5 McFarland standard and filled into the wells of 6 mm media which was cut into three wells of 6 mm in diameter contained with inoculated Mueller-Hinton agar pathogenic culture containing 10^7-10^9 cells per ml. The plates were incubated at 37 ^{0}C for 24 hours. The diameter of zone of inhibition around each wall was measured with Vernier caliper and recorded for further analysis. (Ayeni et al*.,* 2009)

Statistical Analysis

Results were analyzed with SPSS 24 version. Statistical analysis was done by comparing mean and Pearson correlation coefficient with the level of significance $p < 0.05$. In addition, liner regression and ANOVA test were also performed to reveal the difference between the antibacterial activities of the LAB and *E.coli.*

Scanning Electron Microscopy of Antagonistic Activity of Probiotics

In-depth observation concerning the antimicrobial activity of probiotics against tested pathogens was done through scanning electron microscopy (SEM) at Centralized Science Laboratory, University of Karachi. Cultured colonies were selected under fume hood then culture was centrifuged at 5000 rpm for 15 minutes. The bacterial supernatant was discarded and bacterial cells were fixed in McDowell-Trump reagent at pH 7.2 for 2 hours then washed them with 0.1 M of phosphate buffer and centrifuged again for 10 minutes at 5000 rpm. The pallet was re-suspended in phosphate buffer solution and again centrifuged. Osmium tetroxide (1%) was used to fix the pallet for an hour. Washed the sample twice a time with distilled water for 10 minutes. Dehydrated the sample with ethanol at different concentrations of 50%, 75%, 95%, and 99% subsequently added 1 ml of hexamethyldisilazane in sample tube for 10 minutes then allow the sample to dry at room temperature. Finally, sample was coated with up to 300°A gold and observe the images under scanning electron microscopy.

RESULTS

Gram Staining, Catalase and Motility Test

Gram staining was done for the identification of isolated strains and observed under the microscope as Gram-positive, cocci and rod-shaped bacteria. Catalase test due to their uncomplicatedness is considered as most suitable method for the recognition of LABs. In carrying out the catalase test, no gas bubbles (O_2) were observed due to the decomposition of H_2O_2 demonstrating the catalase negative characteristics of isolated strains associated with *Bifidobacterium* and *Lactobacilli* genera. (Schillinger, 1999). In terms of motility, Hanging-drop wet method was performed and observed that tested healthy bacteria were non-motile confirming their origin to *Bifidobacterium* and *Lactobacilli*. (Tamang et al., 1996).

Table 2: Diameter of Zone of Inhibition (mm*) by Isolated Probiotics Strains on Diarrheagenic Pathogens Incubated under Anaerobic Conditions at 37^{0}C for 24 h.

Code	Probiotic Strain	E. Coli
Z1	*Lactobacillus acidophilus LA-5*	24
Z2	*Lactobacillus reuteri DSM 17938*	26
Z3	*Lactobacillus paracasei 431*	22
Z4	*Lactobacillus casei 431*	22
Z5	*Lactobacillus rhamnosus 343*	24
Z6	*Lactobacillus GG*	22
Z7	*Lactobacillus bulgaricus*	22
Z8	*Lactobacillus plantarum 299v*	18
Z9	*Bifidobacterium longum BB-12*	18
Z10	*Bifidobacterium lactis Bb-12*	19
Z11	*Bifidobacterium bifidum*	24
Z12	*Bifidobacterium breve*	20

*Millimeter

Table 3: Pearson Correlation Coefficient

		Probiotics	***E.coli***
Probiotics	Pearson Correlation	1	
	p-value		
E.coli	Pearson Correlation	0.432*	1
	p-value	.0161	

Diarrheagenic *E. coli* inhibited by the isolated probiotics as their significance value is less than 0.05

Table 4: ANOVA Analysis: Effectiveness of Isolated Probiotics to Tested Pathogens

Model		**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
1	Regression	2.575	5	.515	33.680	.000[b]
	Residual	.092	6	.015		
	Total	2.667	11			
b. Predictors: (Constant), *E.coli.*						

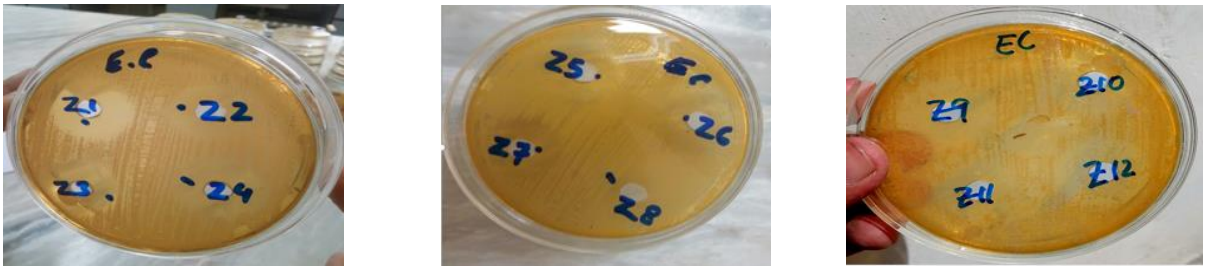
The F value from the above table shows the reliability of dependent variable (effectiveness of probiotics on diarrhea causing *E. coli)* as F = 33.680 is significant at p < 0.05

Table 5: Antagonized Potential of Lactobacillus Compared to Bifidobacterium Isolates

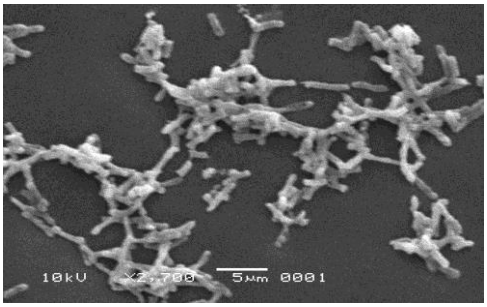
Probiotics		***E. coli***
Lactobacillus	Mean	22.50
	Std. Deviation	2.330
Bifidobacterium	Mean	20.25
	Std. Deviation	2.630

The above table shows the mean value of *E.coli* with *Lactobacillus* 22.50 and *Bifidobacterium* 20.25. Hence, it can be inferred that *Lactobacillus* strains are more effective in the treatment of *E. coli* induced diarrhea.

Figure1. Antimicrobial Activity of *Lactobacillus* and *Bifidobacterium* Isolates against Diarrheagenic *E. coli*



Z1: *L. acidophilus LA-5*, **Z2:** *L. reuteri DSM 17938*, **Z3:** *L. paracasei 431*, **Z4:** *L. casei 431* **Z5:** *L. rhamnosus 343*, **Z6:** *L, GG*, **Z7:** *L. bulgaricus*, **Z8:** *L. plantarum 299v*, **Z9:** *B. longum BB-12*, **Z10:** *B. lactis Bb-12*, **Z11:** *B. bifidum*, **Z12:** *B. breve*



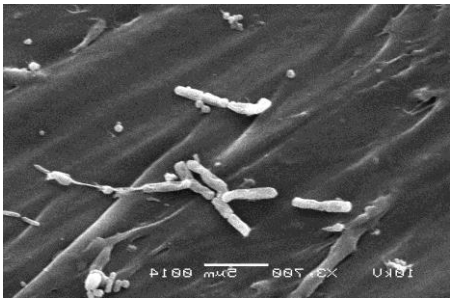


Figure 2 .Images: Scanning Electron Microscopy. (1) Growth of Isolates (2) Treated E. coli with Probiotic Isolates

DISCUSSION

The purpose of the study to investigate the effectiveness of lactic acid bacteria belong to *Lactobacilli* and *Bifidobacterium* against diarrheagenic *E. coli.* During the study, eight strains of *Lactobacilli* and four species of *Bifidobacterium* were isolated from the commercial antidiarrheal probiotic products available in Pakistan. Isolates were inoculated under anaerobic conditions at 37ºC MRS and BSM broth and agar (Ashwani *et al.,* 2014; Yateem *et al.,* 2008). The grow cultures of isolated probiotics on MRS and BSM-agar were proof of their association with *Lactobacilli* and *Bifidobacterium* through identification and biochemical tests (Elizete *et al.,* 2005). The isolated LABs are Gram-positive, cocci and rod-shaped. Catalase negative and motility tests were also performed in order to get the pure colonies for further procedure. Cultured probiotic bacteria matched with the required characteristics of *Lactobacilli* and *Bifidobacterium spp,* as they are negative catalase and non-motile. The motility of isolated probiotics was examined through Hanging-drop wet method (Tamang et al., 1996). This is in agreement with previous studies suggested that LABs are Gram-positive, catalase negative, and non-motile (Osuntoki *et al.,* 2008; Elizete et al., 2005). Electron microscopic scan was also performed which showed disruption of *E. coli* cells by isolates (Hassan & Peh, 2014; Ray, 2001).

The antimicrobial activities of each isolate were performed through agar spot method by inoculation of probiotic isolates under anaerobic conditions. Similar techniques were used by the researchers (Farhana *et al.,* 2013). Zone of inhibition was measured in millimeter (mm) which indicated the effectiveness of isolates against tested pathogens. The antagonistic activities of LABs are mostly due to the formation of antimicrobial substances such as reuterin, bacteriocins, hydrogen peroxide, acetoin, benzoic and formic acids. In addition, LAB after the fermentation produced certain metabolic end products such as organic acids, carbon dioxide, and ethanol. Antimicrobial activities of LABs varied due to the level of antimicrobial and metabolites production subjected to the type and strain of LAB (Khay et al., 2011; Savadogo, 2004). *Lactobacillus reuteri* showed the significant inhibitory actions against *E. coli* (26 mm) followed *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* (24 mm). Farhana et al., in 2013 isolated the *L.acidophillus* and *L.bulgaricus* from the breast milk and reported their effectivity due to having 10-20 mm and 7 to 22 mm zone of inhibition respectively against *E. coli.* (Fahana et al., 2013). Another study revealed their strong effects against diarrheagenic E. coli. (Abolfazl et al., 2015; Tsai et al., 2007). Yingchun et al., suggested the strong antimicrobial activities of *L. GG, L. rhamnosus, L. paracasei,* and *L.casei* against *E. coli*. due to increased aggregation and adherence properties (Yingchun et al., in 2011).

The antibacterial effects of isolated strains of *Bifidobacterium* including *B. bifidum, B. breve, B. lactis,* and *B. longum* have shown the zone of inhibition ranging from 18 to 24 mm. Another study conducted on 46 species of *Bifidobacterium* suggested that 3 strains of *Bifidobacterium bifidum,* 10 of *Bifidobacterium breve*, and 6 strains of *Bifidobacterium longum* shown strong antimicrobial activity against *E. coil* (Irene *et al.,* in 2012). Another study carried out by Tejero and colleagues who evaluated the 15 strains of Bifidobacterium belong to *B.bifidum, B.breve*, B.lactis, and *B.longum* had effective against *E. coli.* (Tejero et al., in 2012)

During the study it has been seen that *Lactobacilli* strains significantly inhibit the growth

of *E. coli* compared to *Bifidobacterium* due to the formations of organic acids and bacteriocins. This mechanism is agreement with previous findings revealed that *Lactobacilli* species due to additional formation of hydrogen peroxide exhibit more inhibitory effects. (Abolfazl et al., 2015; Ito et al., 2003).

CONCLUSION

The present study counseled that among isolated *Lactobacillus* strains, *Lactobacillus reuteri DSM 17938* has strong inhibitory potential compared to mild inhibitory activity of *Bifidobacterium* species against diarrheagenic *E. coli.* Using single or multiple strains of *Lactobacillus* along with *Bifidobacterium spp.* in probiotic pharmaceutical formulations and nutritional supplements is a novel approach for the treatment and prevention of diarrheal infections. However, more studies are needed to evaluate the synergistic effects of different probiotics complementarity in pediatric diarrhea.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Syed Imran Ali designed and performed the experiment and also wrote the manuscript. Samiyah Tasleem reviewed the manuscript and Baqar Shyum Naqvi has supervised the study. All authors read and approved the final version..

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