STUDY OF IN-VITRO PROBIOTIC PROPERTIES AND ANTIBIOTIC RESISTANCE IN LACTOBACILLI ISOLATED FROM COMMERCIAL PROBIOTIC PRODUCTS IN PAKISTAN

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ABSTRACT: The aim of the study was analysis of *in-vitro* probiotic properties and antibiotic resistance in lactobacilli isolated from commercial probiotic products from Pakistan. Lactobacilli were identified by biochemical testing and genus specific polymerase chain reaction. Probiotic properties including tolerance to low pH and bile salts, auto-aggregation, co-aggregation and antimicrobial activity, and antibiotic resistance pattern of all isolates was determined. A total of 14 lactobacilli isolates were recovered from nine products while three products had no lactobacilli. All isolates except AB6 were tolerant to acidic condition (pH: 2). All isolates except AB13 showed growth in presence of bile salts (0.5%). Lactobacilli showed variable auto-aggregation (01-97%) and co-aggregation with *E. coli* and *S. enteritidis* (37.5±7.7-92.4±7.3 and 31.6±6.4-95.8±4.0, respectively). Nine isolates showed activity against *S. enteritidis* while six isolates had activity against *E. coli*. Lactobacilli showed higher level of resistance to meropenem (100%), imipenem (92.85%), polymyxin B (92.85%), kanamycin (92.85%) and aztreonam (78.75%), intermediate level of resistance to vancomycin (64.2%), gentamycin (57.14%) and ciprofloxacin (42.85%), low level of resistance to ampicillin (35.71%), bacitracin (35.71%), penicillin (28.5%) and tetracycline (28.50%), and no resistance to erythromycin and chloramphenicol. It was concluded that transferable antibiotic resistance is present in commercial probiotics which may pose a threat to public safety.

Keywords: Probiotics, *Lactobacillus*, Antibiotic resistance, polymerase chain reaction, Pakistan.

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INTRODUCTION

Probiotics are live microorganisms which when administered in adequate amount confers a health benefit to the host (FAO, 2006). Probiotics provide huge benefits including prevention or control of gastrointestinal problems, lactose intolerance, irritable bowel syndrome, cancer and allergies, strengthen intestinal microbiota (Nagpal *et al.*, 2012), and enhance immunity and overall health status of host (Zhang *et al*., 2016). Probiotics can stop or inhibit the growth of pathogens (Parvez *et al*., 2006) and reduce mycotoxins in gut (Azeem *et al.*, 2019) . Probiotics inhibits pathogens through different mechanisms *i.e.* competition for nutrients, production of toxic conditions and compounds (volatile fatty acids, low pH, and bacteriocins) and competition for space (Nawaz *et al*., 2011). Emergence of antibiotic resistance compelled scientist to develop alternatives i.e. bacteriophages (Siddique *et al*., 2018), nutraceuticals (Doğan *et al.*, 2018) to combat bacterial infections. Probiotics are also used as an alternative growth promoter instead of antibiotics in livestock and poultry production which may help in controlling emergence of antibiotic resistance (Li *et al*., 2019).

Probiotic effects of a product are dependent on different factors *i.e.* properties of strains used, production techniques, delivery system and host. Probiotics or any microbe intentionally added in food chain should be safe *i.e.* it should not produce a disease in host and should have no transferable antibiotic resistance. Although lactobacilli, major organism used as probiotic, have acquired Generally Recognized As Safe (GRAS) status (FAO, 2006), presence of transferable antibiotic resistance has been reported in lactobacilli (Das *et al*., 2012; Saleem *et al*., 2018). Probiotics are regulated as food supplement, or as drug in different countries (Mack, 2005). Probiotics are marketed with different nutritional and health claims. Use of probiotics is on the rise throughout the world including Pakistan. Pakistan import huge quantities of probiotics for human as well as for poultry and livestock. Development of indigenous probiotics is still at nascent stage in Pakistan (Asghar *et al*., 2016; Arif *et al.*, 2018). An organism should fulfill certain pre-requisites before being claimed as probiotic. These pre-requisites include identification to at least specie level and tolerance to physicochemical barriers of host. Probiotic should be of host origin, safe and provide at least one benefit to the host (Asghar *et al*., 2016) . It is important that claims, microbiological quality, and safety

of imported or indigenously developed probiotics are strictly monitored and regulated as these may not fulfill their claims. Probiotics are produced as capsule, powder, tablet, or added in different food products *i.e* yogurt and cheese. As probiotics are live microbes, it is imperative that these reach to their target site in sufficient quantities and remain viable to exert their effect. Viability depend on manufacturing process, delivery system and strain as well (Das *et al*., 2012). A probiotic product developed from same strain in different environments and production facilities may have different capabilities (de Simone and Hepatology, 2018). Commonly available probiotic products in Pakistan include Protexin Soluble, Max-Grow, CBT XL, Max Econo Vital, SiloSolve F.C, Bovamine Daily, BioStabil, Ecotec, Gitpro, Uflora, Ovipro, Hi FLORA, Ultra Probiotics, Nestle Lactogrow, Amybact, etc.

Keeping in mind the overall growth of probiotics worldwide and an increasing trend of using probiotics in Pakistan, current study was designed to analyse *invitro* probiotic properties and antibiotic resistance in lactobacilli from commercial probiotic products intended for use in human and poultry.

MATERIALS AND METHODS

Isolation and identification: Commercial probiotic products (n=12) sold for both human beings (n=09) and poultry (n=03) were used in this study. The research was conducted in the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. Firstly, the isolation of *Lactobacillus* was done by spread plate method. For this, 10-fold dilution of products in Phosphate Buffer Saline (PBS) was prepared and 100 µL from each dilution was spreaded over MRS (deMan Rogosa and Sharp) agar. The plates were incubated at 37^{o}C for 48 hours in anaerobic conditions. Plates were checked after incubation and enumeration was done (Sutton, 2011). Colony characteristics were observed *i.e.* color, size and margins.

Molecular identification DNAs were extracted by a commercially available kit (GeneAll Biotechnology, South Korea) following manufacturer's instructions. The isolates were confirmed by PCR using genus specific primers *i.e.* XB5-F (5'-GCCTTGTACACACCGCCCGT-3') and LbLMA1-R (5'-CTCAAAACTAAACAAAGT-3') as described previously (Nawaz *et al.*, 2011). The PCR products (~250 bp) were then analyzed by gel electrophoresis which confirmed the presence of *Lactobacillus* in samples.

Tolerance to Low PH: Tolerance to low pH and bile salts was determined as described previously (Nawaz *et al*., 2011). The exponentially growing isolates (AB1-AB14) in MRS broth were centrifuged; after centrifugation supernatant was removed and pellet was washed three times with sterile distil water and adjusted to 1.0 McFarland unit by taking O.D at 600 nm. Isolates (100 µL) were added in phosphate buffer saline having pH 2, 3, 4, 7 and incubated for 90 min at 37^{o}C. Tolerance to pH was determined by re-culturing 100 µL of isolates, treated with different pH, in 10 mL MRS broth for 48 hours at 37°C. After incubation, 200 µL from each tube was shifted to 96 well plate and optical density (O.D.) was measured at 600 nm.

Bile salt tolerance: Tolerance to bile salts was determined as described previously (Nawaz *et al*., 2011). Exponentially growing isolates (~1.0 McFarland) were added in MRS tubes having different bile salt concentrations (0.5, 1 and 2%) and incubated at 37˚C for 24 hours followed by recording the optical density at 600 nm.

Co-aggregation and auto-aggregation: The ability of bacteria to auto-aggregate and co-aggregate was determined as described previously by Wagner *et al*., (2008). Overnight grown cultures of tested isolates were centrifuged at 5000 ×*g* for 20 minutes. The pellet was washed three times with sterile distilled water and then suspended in PBS (pH 7.0). The isolates were added (200 µL) in 96 well plate and $O.D_{600}$ (optical density at 600 nm) was determined at different time interval *i.e.* 0min, 15min, 30min, 45min, 60min, 2 hours, 3hours, 4 hours, 5 hours and 24 hours. Similarly, co-aggregation test for lactobacilli was performed by mixing equal volume (100 µL) of tested isolate and indicator microbes in 96 well plates. Absorbance was monitored at different time interval at 600 nm.

Antibiotic susceptibility testing: Antibiotic susceptibility pattern of isolates was done using Kirby Bauer method on MRS agar plates (Boyle e*t al*., 1973) against 14 antibiotics which include: Penicillin, Erythromycin, Tetracycline, Gentamycin, Polymyxin B, Meropenem, Kanamycin, Aztreonam, Chloramphenicol, Imipenem, Vancomycin, Bacitracin, Ampicillin, Methicillin and Ciprofloxacin. Zones of inhibition were measured in).

Antimicrobial activity of lactobacilli: The antimicrobial activity of the isolates was determined by well diffusion assay against *S. enterica* ATCC13076 and *E. coli* E1 as described previously (Asghar *et al.*, 2016) . Indicator organisms were obtained from Department of Microbiology. Cell free supernatants of the isolates exponentially growing in MRS broth were prepared by filtration and collected in sterile tubes. Inoculum of test isolates *i.e. Salmonella enterica* and *Escherichia coli* (~0.5 McFarland) were spread over nutrient agar plates, wells were made sealed and 100 µL of supernatant was suspended in respective wells. Plates were incubated for 24 hours at 37°C. Zones of inhibition were measured in mm.

RESULTS

Recovery and enumeration of total lactobacilli from different probiotic products is given in table-1. Out of 12 different commercial probiotic products, lactobacilli were successfully recovered from nine products while lactobacilli were not detected from three products. Out of nine products from which lactobacilli were recovered, number of lactobacilli strains in 08 products were as per claims while one product (No.10) only had one type of lactobacilli which was against the claims (strains). Enumeration results revealed that different products had different counts of total viable lactobacilli (Not detected to 9.1±0.07 log10 CFU/g). Out of 12 products a total of 14 lactobacilli (AB1-AB14) were recovered and identified by cultural, biochemical and molecular characteristics. Probiotic properties *i.e.* resistance to low pH, tolerance to bile salts, auto-aggregation, co-aggregation, and activity against *E. coli* and *S. enteritidis* are shown in table-2. None of the isolates showed growth in MRS at pH 2 while all isolates except AB6 showed growth at pH 3 and pH 4. All isolates except AB13 could grow in MRS supplemented with 0.5% bile salts. All isolates except AB10 and AB13 showed growth in MRS containing 1% bile salts. Fifty percent of isolates (7/14) also showed growth at 2% bile salts. AB3, AB4, AB5 and AB6 showed good auto-aggregation (>50%) while other isolates showed low to moderate level of auto-aggregation (1-42%). Co-aggregatation of isolates with *E. coli & S. enteritidis* was 37.5±7.7-92.4±7.3 and 31.6±6.4-95.8±4.0, respectively. Isolate AB4 showed maximum co-aggregation with *Escherichia coli* after 24 hours. Antimicrobial activity against *E. coli* and *S. enteritidis* revealed 06 isolates (AB1, AB7, AB8, AB9, AB10, and AB14) had activity against *E. coli* while 09 isolates (AB4-AB10, AB12, AB14) had activity against *S. enteritidis*. It was also evident that 05 isolates (AB7-AB10 and AB14) had activity against both of the indicator organisms. Antibiotic resistance profile of all isolates against is shown in table 3. Isolates showed higher level of resistance of resistance to meropenem (14/14, 100%), imipenem (13/14, 92.85%), polymyxin B (13/14, 92.85%), kanamycin (13/14, 92.85%), and aztreonam (11/14, 78.75%), intermediate level of resistance to vancomycin (9/14, 64.2%), gentamycin (8, 57.14%) and ciprofloxacin (6/14, 42.85%), and low level of resistance to ampicillin (5/14, 35.71%), bacitracin (5/14, 35.71%), penicillin (4/14, 28.5%) and tetracycline (4/14, 28.50%). All isolates were sensitive to erythromycin and chloramphenicol. AB4, AB8, AB 9 and AB14 were resistant to penicillin while AB1, AB8, AB9, and AB14 were resistant to tetracycline.

Table-1: Isolation and enumeration of lactobacilli from commercial probiotic products.

Product No.	Intended Use/Host	Number of lactobacilli strains as per label	Lactobacilli Count (Mean Log_{10} ± S.D/g	Number of lactobacilli strains recovered
1	Human	02	4.6±0.09	(AB1, AB2)
2	Human	02	ND	N.D
3	Human	02	9.1±0.07	2 (AB3, AB4)
4	Human	02	4.5±0.3	2 (AB5, AB6)
5	Human	02	7.7±0.2	2 (AB7, AB8)
6	Poultry	N.S	5.5±0.3	2 (AB9, AB10)
7	Poultry	01	1±0.06	1 (AB11)
8	Human	01	ND	N.D
9	Human	01	8.5±0.4	1 (AB12)
10	Human	02	6.3±0.2	1 (AB13)
11	Poultry	03	ND	N.D
12	Human	01	5.7±0.2	1 (AB14)

N.D: Not detected, SD: standard deviation, NA: Not Applicable

Table-2: *In-vitro* probiotic properties of lactobacilli isolated from commercial probiotics.

Isolates*	*In-vitro* probiotic properties of lactobacilli isolated from commercial Probiotics										
	Tolerance to low pH			Tolerance to Bile Salts			Percent Auto-aggregation at 24 hrs	Percent Co-aggregation with pathogenic bacteria at 24 hrs		In vitro activity of CFSs against pathogenic bacteria (ZOI, mm)	
	2	3	4	0.5%	1%	2%		*E.coli*	*S. enterica*	*E.coli*	*S. enteric*
AB1	-	+	+	+	+	+	1.02±0.8	66.8±3.0	45.6±3.3	8	N.D
AB2	-	+	+	+	+	+	15±1.5	64.2±4.1	34.0±4.2	N.D	N.D
AB3	-	+	+	+	+	-	84±5.6	79.3±5.0	95.3±6.0	N.D	N.D
AB4	-	+	+	+	+	-	96±15.0	92.4±7.3	95.8±4.0	N.D	8
AB5	-	+	+	+	+	-	97.4±11.0	74.5±5.6	94.3±3.7	N.D	10
AB6	-	-	-	+	+	-	96.3±11.0	60.8±3.8	94.1±6.2	N.D	8
AB7	-	+	+	+	+	-	2.9±0.1	37.5±7.7	31.6±6.4	9	10
AB8	-	+	+	+	+	+	36±2.5	46.9±4.1	51.7±4.0	8	10
AB9	-	+	+	+	+	+	42.7±5.2	46.2±3.6	43.4±3.9	8	9
AB10	-	+	+	+	-	-	20±2.3	53.5±3.4	45.3±3.0	8	9
AB11	-	+	+	+	+	+	23.9±4.0	64.5±4.2	74.5±3.8	N.D	N.D
AB12	-	+	+	+	+	+	18.5±2.0	59.3±7.0	59.3±7.6	N.D	8
AB13	-	+	+	-	-	-	18±3.0	57.4±5.9	53.1±7.3	N.D	N.D
AB14	-	+	+	+	+	+	26±5.5	46.3±3.4	47.7±5.0	14	12

N.D: Not detected, SD: standard deviation, ZOI: Zone of Inhibition

Table-3: Antibiotic susceptibility pattern of lactobacilli (n-14) isolated from commercial probiotics as determined by disc diffusion method.

Antibiotics	Disc (μg)	AB1	AB 2	AB3	AB4	AB5	AB6	AB7	AB8	AB9	AB10	AB11	AB12	AB13	AB14	Total Resistant N (%)
Penicillin	?	S	S	S	R	S	S	S	R	R	S	S	S	S	R	4(28.5)
Ampicillin	?	S	S	R	R	S	S	S	R	R	S	S	S	S	R	5(35.71)
Meropenem	?	R	R	R	R	R	R	R	R	R	R	R	R	R	R	14(100)
Imipenem	?	R	R	R	R	R	R	R	R	R	R	R	R	R	S	13(92.85)
Aztreonam	?	R	R	S	S	R	R	R	R	R	R	S	R	R	R	11(78.75)
Bacitracin	?	S	S	R	R	S	S	S	S	S	S	S	R	R	R	5(35.71)
Polymyxin B	?	R	R	R	R	R	R	R	R	R	R	S	R	R	R	13(92.85)
Vancomycin	?	S	S	R	S	R	S	R	R	R	R	S	R	R	R	9(64.2)
Erythromycin	?	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0(0)
Gentamycin	?	R	R	S	S	R	S	R	S	S	R	S	R	R	R	8(57.14)
Kanamycin	?	R	R	R	R	R	R	R	R	R	R	S	R	R	R	13(92.85)
Tetracycline	?	R	S	S	S	S	S	S	R	R	S	S	S	S	R	4(28.5)
Chloramphenicol	?	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0(0)
Ciprofloxacin	?	R	R	S	S	S	R	S	R	R	S	S	S	S	R	6(42.85)

S: Sensitive, R: Resistant

DISCUSSION

This study was done to check that the lactobacilli present in commercially available probiotic products in Pakistan were safe and either had potential for performing their functions. Furthermore it was confirmed whether probiotic products had lactobacilli as per claim or not. Probiotic should be administered in sufficient quantities ($\geq 10^6$ CFU/g) to extract their maximum benefit (Ashraf and Shah, 2011). In this study, three products had no viable lactobacilli while another 05 products had lactobacilli less than 10^5 CFU/gm which indicate that these product may not exert their benefits on consumer and a strict regulation of probiotics claims is needed (de Simone and Hepatology, 2018).

Majority of the lactobacilli isolated in this study showed good compliance to probiotic pre-requisites. In present study, none of the isolate showed tolerance to pH 2 while majority of isolates (13/14) were resistant to pH 3. Sensitivity of potential probiotics to pH 2 and resistance to pH 3 has been reported previously as well (Nawaz *et al*., 2011). Similarly, all lactobacilli except AB13 isolated in this study were tolerant to 0.5% while resistance to 1 and 2% bile salts was lesser. Similar results showing good pH and bile salt tolerance has been reported previously as well. Auto-aggregation and co-aggregation capacity of the majority of isolates also was indicative of their probiotic potential. Auto-aggregation and co-aggregation capacity of probiotics is an indirect measure of their capacity to adhere to intestinal epithelium and activity against pathogens, respectively (Collado *et al*., 2008). Similar aggregation capacities have been reported in many previous studies (Reid *et al*., 1988; Drago *et al*., 1997; Aslim *et al*., 2007; Collado *et al*., 2008; Asghar *et al*., 2016). Activity of probiotics against gut pathogens is an excellent property. In current study many isolates had activity against *Salmonella* (9/14) and *E. coli* (6/14) which indicate their use of probiotics in fighting against gut problems (Kim *et al*., 2012; Asghar *et al*., 2016; Park *et a*l., 2016).

Acquired antibiotic resistance in probiotics is an important safety concern as acquired resistance is transferable to other bacteria. An organisms having acquired antibiotic resistance will serve as reservoir of antibiotic resistance and aggravate the already recalcitrant issue of antibiotic resistance worldwide (Saleem *et al*., 2018). Resistance to penicillin, ampicillin, erythromycin and tetracycline is considered as acquired resistance in lactobacilli while resistance to other antibiotics is specie dependent. Transfer of antibiotic resistance from lactobacilli or probiotic lactobacilli has been reported frequently in recent years (Gueimonde *et al*., 2013; Saleem *et al*., 2018). Presence of tetracycline resistance (AB1, AB8, AB9) and erythromycin resistance (AB1, AB8, AB9 and AB1) is contradictory to previous studies which reported no acquired resistance in the lactobacilli isolated from commercial products (Teuber *et al*., 1999). Acquired antibiotic resistance from probiotics isolated from marketed products has also been reported previously (Kastner *et al*., 2006; Liu e*t al*., 2009). Tetracycline and erythromycin resistant in lactobacilli of different sources have been reported from Pakistan as well (Nawaz *et al*., 2011; Asghar *et al*., 2016*;* Arif *et al*., 2018*;* Saleem *et al*., 2018*).* To best of our knowledge, it is first report of presence of acquired antibiotic resistance in lactobacilli from marketed products in Pakistan. Before approval from the Qualified Presumption of safety, antibiotic resistance of any kind in probiotics and starter culture microorganism should be determine (Gueimonde *et al*., 2013).

Conclusion: It was concluded that substandard commercial probiotic products which do not fulfill their labels were available in market. Furthermore, probiotics may also harbor transferable antibiotic resistance which insinuates for strict monitoring and regulation of probiotic market in Pakistan.

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