ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology, 3 (2): 173-180, 2004
ISSN 1682-2978

Comparative Study of Probiotic Cultures to Control the Growth of *Escherichia coli* O157: H7 and *Salmonella typhimurium*

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Abstract: Pure lactobacilli (*Lactobacillus acidophilus*, *L. plantarum* and *L. brevis*) strains isolated from commercial probiotic consortium (Truferm™) and *Bacillus subtilis* ATCC 6633 were investigated for their *in vitro* antagonistic ability against two enteric pathogens (*Escherichia coli* O157:H7 and *Salmonella typhimurium*). Generally, both pathogens were inhibited by the probiotic strains but the inhibition of *S. typhimurium* was greater than that of *E. coli*. Furthermore, *B. subtilis* had a better antagonistic activity compared to that recorded for lactobacilli. Among lactobacilli, *L. acidophilus* exhibited the highest antagonistic activity against both indicator strains. All probiotic strains succeeded to exhibit a positive coaggregation with both faecal pathogens. The maximum coaggregation was reached after 4 h of incubation of indicator strains with lactobacilli and 6 h with *B. subtilis*. *L. acidophilus* recorded the highest coaggregation result. Regarding acid and bile tolerance, *B. subtilis* and *L. acidophilus* could not survive for 1 h at pH 1.0, whereas, *L. plantarum* and *L. brevis* could demonstrate low survival percentages. All strains showed an acid tolerance when incubated at pH 2.0 or 3.0 over a period of 4 h. The highest survival percentage in the acidic conditions occurred with *L. acidophilus*. *B. subtilis* recorded the highest bile tolerance over lactobacilli. The sensitivity response of probiotic as well as indicator strains to antibiotics varied.

Key words: *B. subtilis* ATCC 6633, coaggregation, *E. coli* O157:H7, lactobacilli, probiotic, *S. typhimurium*, tolerance

INTRODUCTION

Probiotic as defined by Fuller[1] is a food (feed) or drug containing live microbe that, when ingested, is expected to confer beneficial physiologic effects to the host through microbial actions. The composition of currently-used probiotics varies from those containing a mixture of many strains to those containing one strain only, but the selection of optimal strains has often been largely empirical. In particular, if the natural microbiota is to serve as a source for strain selection, there are many possibilities to choose from[2].

Probiotics are currently being used for oral therapy and prophylaxis of gastrointestinal disorders[3-6]. These biotherapeutic agents, which are sold commercially for both human and veterinary use, are known to have beneficial effects on the digestive ecosystem and to confer resistance to infections[7-9] .

Many microorganisms have been used or considered for use as probiotics. Because viable and biologically active microorganisms are usually required at the target site in the host, it is essential that the probiotic be able to withstand the host's natural barriers against ingested bacteria[6]. The commonly used probiotics are strains of lactic acid bacteria (e.g., *Lactobacillus* and *Streptococcus*). However, approved probiotics in the European Union are only strains of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Pediococcus* sp. and *Bacillus* spp.[10-11]. Lactic acid bacteria have the longest history as biotherapeutic agent (probiotics) and are still the most common ingredients among those intended for consumption by farm animals[12].

The objective of this study was to investigate the possible antagonistic activity of the lactobacilli and *Bacillus subtilis* ATCC 6633 against *Escherichia coli* O157:H7 and *Salmonella typhimurium* as important pathogens frequently found in animal husbandry and to reduce their pathological consequences for the host. On the other hand, the tolerance of each probiotic strain to bile salts, acidity was investigated to demonstrate its survivability in the small intestine and colon to contribute in the balance of the intestinal microbiota. Moreover, their resistance to antibiotics was also investigated to clarify their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem.

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MATERIALS AND METHODS

Probiotic strains: Truferm™ (Poultry formula) is a highly concentrated, synergistic dry blend (4 x 10^{10} cfu g^{-1}) of lactic acid bacteria (*L. acidophilus, L. plantarum* and *L. brevis*) and digestive enzymes designed for use in poultry rations (Laporte Biochem International, Division of Great Lakes Biochemical Co. Inc. 6120 West Douglas Avenue, Milwaukee, Wisconsin 53218, USA). *Bacillus subtilis* ATCC 6633 strain was also used.

Bacteria and growth conditions: *E. coli* and *Salmonella* were isolated from broiler chicken from poultry farm in Behera Governorate, Egypt. The two isolates were then purified and identified as *E. coli* O157: H7 and *Salmonella typhimurium* using Gram staining and standard biochemical tests (Triple sugar and lysine iron agar, urease test and serological tests-Difco-O, Vi polyvalan antiserum for Salmonellae identification). For the maintenance of *E. coli* O157: H7 serotype, semisolid agar (4%) medium was used, whereas *S. typhimurium* was maintained on brilliant green agar (Oxoid CM329) and the cultures were kept at 5°C until use.

The microbial components of the commercial product (Truferm™) were isolated using de Man, Rogosa and Sharp (MRS) agar (Oxoid) supplemented with 25 μg ml^{-1} tetracycline and 3 μg ml^{-1} nystatin for lactobacilli. Cultivation of lactobacilli strains were performed in MRS broth (Oxoid) anaerobically at 37°C. On the other hand, *Bacillus subtilis* ATCC 6633 strain was originally cultivated in tryptone yeast extract (TBY, Difco) broth (0.8% tryptone, 0.5% yeast extract and 0.5% NaCl) at 37°C. The solid medium for cultivation of *B. subtilis* was TBY broth solidified with 1.5% agar. Lactobacilli were identified and confirmed by morphology examination, Gram staining and use of API 50 CH strips (BioMérieux, Marcy- l' Étiole, France) and other complementary tests according to the criteria of Bergey's Manual of Determinative Bacteriology[13]. All Stock cultures were stored at -70°C in 40% glycerol.

Determination of antagonistic activity by an *in vitro* assay: Antagonistic activity was determined by the double layer method[5]. The inoculum of the probiotic (Truferm™ and *B. subtilis* ATCC 6633) was prepared in brain heart infusion (BHI) broth medium (Oxoid) incubated at 37°C for 18 h. The inoculum was spotted (10^4 cfu $spot^{-1}$) onto the surface of BHI agar medium (in a 9 cm Petri-dish) with a toothpick, in triplicate to ascertain the ability of each strain to inhibit the pathogen. The cultures were incubated for 48 h at 37°C. After incubation, the cells were killed by exposure of each dish to 4 ml chloroform for 20 min. The chloroform residue was allowed to evaporate and the agar in each dish was overlaid with 5 ml of BHI soft agar (0.75%) which had been inoculated with 0.1 ml (2 x 10^9 cfu ml^{-1}) of an 18 h culture at 37°C of *S. typhimurium* or *E. coli*. After 48 h of incubation, the plates were evaluated for the presence of zones of growth inhibition. The final inhibition zone diameter corresponded to the difference between the total inhibition zone and the diameter of the colony. The experiment was repeated twice and each reading represents the mean of six observations.

Aggregation test: The aggregation test was performed as described by Reniero *et al.*[14]. Aggregation was scored positive when clearly visible sand-like particles, formed by aggregated cells, sedimented to the bottom of the tubes, leaving a clear supernatant fluid within 2 h.

Spectrophotometric coaggregation assay: Bacterial strains were grown at 37°C for 18 h in MRS broth. Suspensions of *Lactobacillus* strain, *B. subtilis*, *E.coli* or *S. typhimurium* were adjusted to an optical density (OD) of 0.5 measured at 660 nm. Equal volumes (0.5 ml) of each bacterial suspension were mixed, blended on a vortex mixer including controls and the OD of the suspensions was measured after 4 h. Control tubes only contained 1.0 ml of either bacterial suspension. Percentage coaggregation was calculated using the equation of Handley *et al.*[15]:

$$\frac{(A+B)/2-C}{(A+B)/2}\times 100$$

Where A represents the OD (660 nm) of control tube of probiotic strain measured at time t. B represents the OD (660 nm) measured at same time t of the control tube of the indicator strain. C represents the OD (660 nm) measured for the suspension mixture of both indicator and probiotic strains after the same period. To quantify the time-course of coaggregation, readings were taken at 2, 4, 6, 12 and 24 h. The experiment was repeated twice and each reading represents the mean of six observations.

Tolerance to acidic pH: Probiotic strains were grown in MRS broth (Oxoid) at 37°C overnight, then subcultured into fresh MRS broth and incubated for another 24 h[2]. The cultures were centrifuged at 5000 rpm for 10 min at 4°C (Chilspin MSE Fisons centrifuge), the pellets washed in sterile phosphate-buffered saline (PBS), pH 7 and resuspended in PBS. Each strain was diluted 1/100 in PBS at pH 0.5, 1, 2 and 3. Incubation times were 1, 2 and 4 h. The bacteria were then transferred to MRS media and broth and incubated anaerobically for lactobacilli and aerobically for *B. subtilis* at 37°C overnight. Counts of

surviving cells were determined by plating on MRS agar. The experiment was repeated twice and each reading represents the mean of three observations.

Bile resistance: MRS-broth (Difco) supplemented with 0.2, 0.3, 0.4 and 2.0% (w/v) oxgall (dehydrated fresh bile, Difco) was prepared. The latter was inoculated with 1% (v/v) 0.5 McFarland overnight lactobacilli and *B. subtilis* suspensions. The cultures were incubated at 37°C. Control cultures were without oxgall. Absorbance was measured at 560 nm every 30 min[16] against the corresponding non-inoculated blanks. The experiment was repeated twice and each reading represents the mean of three observations.

Resistance to antibiotics: The antibiotic resistance was evaluated using the agar plate method. Muller-Hinton agar (Difco) was used as the basal medium for bacterial growth. Culture suspensions were obtained after incubation at 37°C in MRS broth and spread on agar plates at 0.5 McFarland. Multipositional disc dispenser applied standard discs (Unipath Limited, Basingstoke, Hampshire, UK). The plates were incubated 24 h at 37°C. The Inhibition zones were measured and then compared with the values of susceptibility interpretative breakpoints issued by the National Committee for Clinical Laboratory Standards[17].

Statistical analyses: The data were analyzed using Statistica program version 4.5 (StatSoft.IN, USA).

RESULTS

Antagonistic activity: The antagonistic activity of the four-probiotic strains against the two faecal indicator strains (*E.coli* and *S. typhimurium*) was tested (Table 1). In the agar, spot test showed the inhibition of the growth of *S. typhimurium* around the colonies of each of *B. subtilis*, *L. acidophilus*, *L. brevis* and *L. plantarum* in a decreasing order. On the other hand, the four-probiotic strains demonstrated a relative decrease in the inhibition record (37% for *B. subtilis*, 40% for *L. acidophilus*, 58% for *L. plantarum* and 48% for *L. brevis*) against *E. coli* compared to that obtained for *S. typhimurium* with the same inhibition order.

Autoaggregation and coaggregation tests: All microorganisms used in this study including the indicator strains as well as probiotic candidates were tested for their autoaggregation potentials (Table 2). The time required for significant ($p<0.05$) autoaggregation was between 10 and 100 min. The indicator strains showed a weak autoaggregation property (≥180 min for *E. coli* and ≥220 for *S. typhimurium*). On the other hand, the aggregation among lactobacilli was much clearer (10-65 min). The coaggregation properties with the two indicator strains *E. coli* and *S. typhimurium* were investigated (Table 3). The best coaggregation properties were achieved with *L. acidophilus* and *L. plantarum*. Although *B. subtilis* showed lowest autoaggregation results, its coaggregation profile is comparable to lactobacilli. Coaggregation was not affected by different pH values (data not shown).

Table 1: The inhibitory effect of the probiotic strains against *E. coli* O157: H7 and *S. typhimurium*

	Inhibition zone diameter (mm) against the indicator strain	
Probiotic strain	*E. coli* O157: H7	*S. typhimurium*
B. subtilis	36±1.64	57±0.58
L. acidophilus	33±1.79	55±1.02
L. plantarum	22±4.01	52±3.04
L. brevis	28±2.01	54±2.45

± represents standard deviation

Table 2: Microorganisms used in this study and their autoaggregation profile

Microorganism	Origin	Aggregation time (min)*
E. coli	Broiler	≥180
S. typhimurium	Broiler	≥220
B. subtilis	ATCC	100
L. acidophilus	Truferm™	60
L. plantarum	Truferm™	10
L. brevis	Truferm™	65

*The time required to produce a clear supernatant fluid

The effect of physiological saline pH: The results of acid tolerance (survival at various pH values) showed that all tested probiotic strains survived an incubation period of 4 h at pH 2.0 (except *L. plantarum*) and pH 3.0 (Table 4). The only surviving strains (with low survival percentages) after incubation at pH 1.0 for 1 h were *L. acidophilus* and *L. brevis*. Generally, *L. acidophillus* survived acidic conditions better than the rest of the probiotic strains. A significant ($p<0.05$) decrease in the survival percentage was noted when the exposure time progresses for *L. brevis*, *L.acidophilus* and *B. subtilis*, whereas, *L. plantarum* recorded a constant low survival percentage (at 1 and 2 h incubation). No growth occurred after incubation at pH 0.5 for 1 h (data not shown).

Bile tolerance: Bile tolerance of lactobacilli and *B. subtilis* was investigated (Fig. 1). *L. acidophilus* and *L. plantarum* (Fig. 1a and b) demonstrated significant changes of absorbance ($p<0.05$) at 0.2 and 0.3% bile concentrations. *L. plantarum* was the least tolerant among the lactobacilli, whereas, *L. brevis* (Fig. 1c) showed the highest tolerance. On the other hand, *B. subtilis* recorded the highest

Table 3: The ability of lactobacillus strains and *B. subtilis* to coaggregate with *E.coli* O157: H7 and *S. typhimurium*

	Percentage reduction at O.D. 660 nm									
	Escherichia coli					*Salmonella typhimurium*				
	After (h)									
Probiotic strain	2	4	6	12	24	2	4	6	12	24
B. subtilis	46±2.1	50±2.1	60±2.5	46±3.4	45±3.4	51±1.8	61±2.4	67±1.2	22±1.9	18±0.9
L. acidophilus	48±4.2	98±4.2	80±3.7	70±2.5	62±2.3	87±2.1	90±2.1	60±1.6	55±0.7	47±0.4
L. plantarum	36±1.8	67±1.6	55±2.0	49±1.2	49±1.3	67±1.4	69±1.6	58±2.0	50±1.2	47±1.3
L. brevis	28±1.4	52±1.1	48±1.3	46±1.2	43±1.0	55±0.9	59±1.0	51±0.7	46±1.6	40±1.0

Results are expressed as the percentage reduction after 2, 4, 6, 12 and 24 h in the optical density of a mixed suspension compared to the summed O.D. (at 660 nm) values for the individual suspensions and are the mean of six observations. ± represents standard deviation

Table 4: Survival percentages of *B. subtilis* and lactobacillus strains after their incubation in phosphate buffered saline at various pH values

	Survival (%) after incubation at							
	pH 1.0		pH 2.0			pH 3.0		
Probiotic strain	1h	2h	1h	2h	4h	1h	2h	4h
B. subtilis	0±0.0	0±0.0	41±1.7	22±1.3	2±0.9	55±2.0	45±2.2	35±2.1
L. acidophilus	3±0.0	0±0.0	64±2.9	43±2.2	5±1.2	76±2.3	76±3.4	46±1.9
L. brevis	2±0.1	0±0.0	50±3.4	40±3.2	3±0.6	65±2.1	57±3.2	22±1.0
L. plantarum	0±0.0	0±0.0	7±1.0	7±1.1	0±0.0	12±1.3	12±1.5	6±0.9

± represents standard deviation

Table 5: *Antibiotic sensitivity profile of indicator and probiotic strains*

		Inhibition zone diameter and their interpretative breakpoints					
Antibiotic	Disc Content (µg)	*E.coli* O157: H7	*S. typhimurium*	*B. subtilis*	*L. acidophilus.*	*L. plantarum*	*L. brevis*
Amikacin	30	S (19±0)	S (20±1)	R (10±0)	R (9±0)	R (6±0)	R (11±0)
Amoxicillin	10	R (6±1)	S (30±1)	R (7±1)	R (7±1)	R (7±1)	R (7±1)
Ciprofloxacin	30	S (20±1)	S (22±0)	S (24±1)	S (20±1)	S (17±1)	S (19±1)
Erythromycin	15	S (25±1)	I (17±1)	R (6±1)	R (6±1)	R (6±1)	R (6±1)
Gentamicin	10	S (18±0)	S (22±1)	S (28±0)	R (13±1)	S (28±0)	R (13±1)
Kanamycin	30	S (30±1)	S (33±0)	S (16±1)	R (7±0)	R (7±0)	S (16±1)
Lincomycin	2	S (30±1)	R (10±0)	R (6±0)	R (6±0)	R (6±0)	R (6±0)
Nalidixic acid	5	R (6±1)	R (6±0)	S (28±0)	S (28±0)	S (36±0)	S (30±0)
Ofloxacin	10	S (18±0)	S (19±1)	R (6±1)	R (6±1)	R (6±1)	R (6±1)
Oxytetracycline	30	S (23±1)	S (25±0)	S (20±0)	R (6±1)	R (6±1)	R (6±1)
Penicillin G	10	R (12±0)	R (12±0)	S (24±0)	S (20±0)	S (20±0)	S (20±0)
Streptomycin	10	I (13±0)	S (19±1)	R (12±0)	R (12±0)	R (12±0)	R (12±0)
Sulbactam	20	S (19±1)	S (22±1)	S (20±1)	S (18±1)	S (17±1)	S (21±0)
Tetracycline	30	R (12±0)	I (15±1)	R (25±0)	R (20±0)	R (21±0)	R (23±0)
Vancomycin	30	S (13±1)	S (13±0)	S (22±0)	R (6±0)	R (6±0)	R (6±0)

S=susceptible; I=intermediate; R=resistant

Results (in parentheses) represent the means of six independent measurements of the inhibition zone diameter with standard deviation (±). S, R and I were interpreted for each individual test-antibiotic following the interpretative breakpoints issued by NCCLS

tolerance pattern over lactobacilli and demonstrated significant changes of absorbance ($p<0.05$) at 0.2, 0.3, 0.4 and 2% bile concentrations (Fig. 1d).

Antibiotic sensitivity pattern: All probiotic strains were resistant to amikacin, amoxicillin and tetracycline. *Lactobacilli* only demonstrated resistance to ofloxacin and vancomycin, whereas *B. subtilis* was sensitive to vancomycin and resistant to ofloxacin (Table 5). The antibiotic sensitivity profile of the indicator strains was also investigated. *E.coli* O157: H7 showed resistance to amoxicillin, nalidixic acid, penicillin G and tetracycline, whereas *S. typhimurium* showed resistance to lincomycin, nalidixic acid and penicillin G.

DISCUSSION

In the past twenty-five years, a great concern was directed towards finding methods to control enteric infection in general and *Salmonella* in particular. Enteric organisms are of great importance to poultary industry as they render the nutrients non-available[18], in addition to being pathogenic to poultry and man. In this study, the potential of some probiotic strains to control the enteropathogens *E. coli* and *S. typhimurium* was investigated. The results obtained demonstrated variable antibacterial activity among lactobacilli (and *B. subtilus*). The potential of the lactobacilli and *B. subtilis* to control

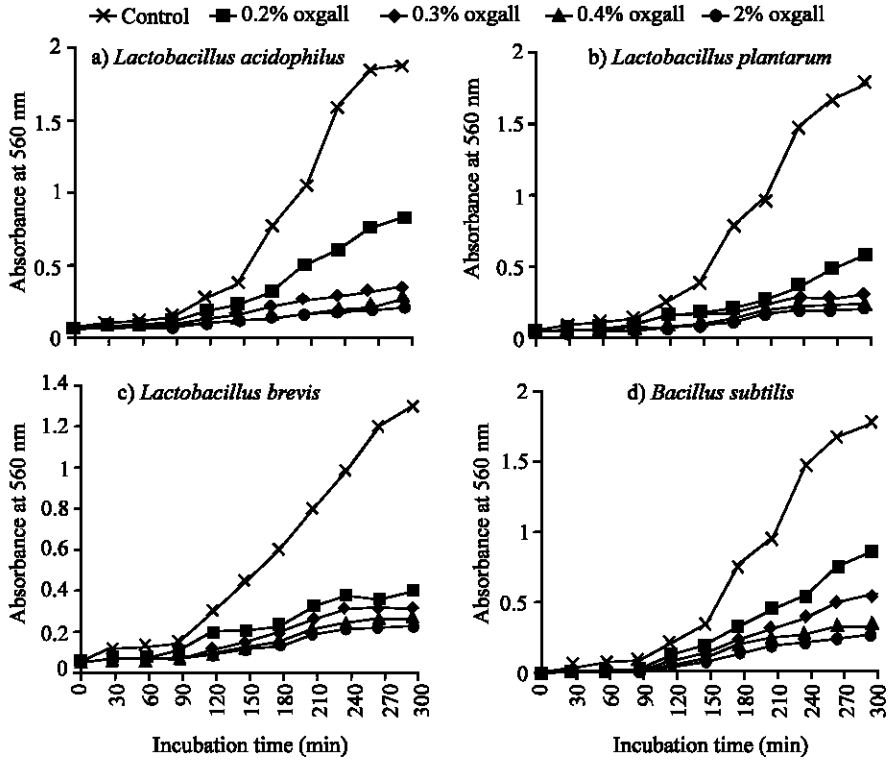

Fig. 1: The growth profiles of lactobacillus strains (1a-1c) and *B. subtilis* (1d) as measured by the absorbance at 560 nm in the presence of 0.2-2% oxgall. Control cultures lack the presence oxgall

these intestinal pathogens with probiotic properties is a valuable feature for considering their application in food development.

The *in vitro* antagonistic activities obtained in this work are supported by the findings published by Jacobsen *et al.*[19]. This antagonistic activity is possibly due to the antibacterial substances produced by the probiotic strains. Several lactobacillus strains have been found to produce various types of antibiotics for example; *L. acidophilus* produces lactacin, while *L. plantarum* produces plantaricin[20-22]. Lactobacilli also produce hydrogen peroxide and organic acids such as lactic and acetic acids, which inhibit growth of many pathogenic Gram-negative organisms[23]. Production of organic acids lowers the pH of the digestive tract, which creates an unfavourable environment for these pathogens to grow. Beside the low pH maintained within the intestine, the antimicrobial activity may be due to low oxidation-reduction potentials i.e. lowering the oxygen available to pathogens[24,25]. On the other hand, *Bacillus* spp. also produce many kinds of bioactive compounds as secondary metabolites, among them, surfactine, tyrocidine, gramicidine, mersacidin and bacitracin[26-30]. Green *et al.*[31] reported that probiotic preparations of *B. subtilis* were sold commercially in most European Countries, although little is understood about how these bacteria exert their therapeutic benefit.

Both aggregation and coaggregation assays, are simple and reliable methods applicable to a large number of test strains. These properties are thought to be linked to the ability to interact closely with undesirable bacteria[32]. In this work, *B. subtilis* and lactobacilli gave good coaggregation scores, although the former demonstrated lower autoaggregation record. The pronounced disposition for aggregation of lactobacilli has previously been reported by various authors[33-35]. In this work, strains with high coaggregation activity also showed high autoaggregation. A strong inclination to autoaggregation is not always combined with a strong coaggregation property. For example, *L. agilis* and two strains of *L. reuteri* showed no significant coaggregation with pathogenic bacteria but, in contrast, recorded a strong autoaggregation[2]. Bacterial coaggregation was first recognized as highly specific partnerships between streptococci and actinomycetes in the oral cavity[36]. Unlike coaggregation in the oral cavity, which leads to the formation of dental plaque, it has been suggested that inhibitor-producing lactobacilli, which coaggregate with

pathogens of the urinary tract, may constitute an important host defense mechanism against infection[37]. A protective mechanism involving coaggregation between lactic acid bacteria and enteropathogens could operate similarly in the digestive tract[38].

The indigenous gastrointestinal microbiota is regulated by many factors, including non-host factors as *in situ* substrate availability and bacterial interactions[39] as well as host factors, e.g. diet, gastric and pancreatic enzymes, mucus, pH, peristalsis and bile salts. The pH of the gastric juice in chickens and ducks can be as low as 0.5-2.0[2] and after passage through the acidic conditions, it is important that, for probiotic strains, to survive the bile salt in the intestine, the normal level of which is around 0.3%, but may range up to the extreme 2.0% during the 1st h of digestion[16]. Bile resistance of some strains is related to specific enzyme activity-bile salt hydrolase (BSH) which helps hydrolyze conjugated bile, thus reducing its toxic effect[40]. Failure to do so may soundly explain the inefficiency of some commercial preparations of probiotics. BSH activity has most often been found in organisms isolated from the intestines or faeces of animals[41,42]. The two lactobacilli (*L. acidophilus*, *L. gasseri*) tested by Fernandez *et al.*[43] showed bile resistance in the presence of 0.15% bile salts. It was also reported by Usman and Hosono[44] that there was no significant correlation between BSH (deconjugate activity) and bile tolerance.

Resistance of the four-probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections. Unfortunately, both indictor strains used in this work and were resistant to nalidixic acid, this eliminate the use of such nucleic acid inhibitor from their therapeutic regime. On the other hand, the resistance of lactobacilli strains to vancomycin confirms the finding of Salminen *et al.*[45] who reported that vancomycin resistance is an intrinsic property of many lactobacilli.

Collectively, the results obtained in the present study showed the survivability of the probiotic strains in the conditions of high oxgall concentration and low pH values. This will help probiotic strains to reach the small intestine and colon and contributing the balance of the intestinal microbiota. The inhibitory effect of the probiotic strains against enteropathogenic bacteria prevents poultry farms from being a reservoir for food borne disease. Although *B. subtilis* produced satisfactory results as a probiotic candidate, however it is difficult to see how this is active in the gut. It is certainly not an intestinal organism and, since it is a strict aerobe, would not be able to grow in the gut. So further *in vivo* investigations are required before the role of this probiotic strain in the intestinal health can be delineated clearly.

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