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RESEARCH ARTICLE

Identification and *In Vitro* Evaluation of Species Specific Probiotic for Feeding Broiler Chicken Using Probiotic Scores

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ABSTRACT

The study was undertaken to isolate and identify suitable species specific probiotic for feeding broiler chicken. Probiotic organisms were obtained from the gastrointestinal tracts of eight broiler chicken. Out of 24 bacterial isolates from gastro intestinal tract, four were identified as *Lactobacillus* based on morphological, physiological and biochemical tests which are specific for *Lactobacillus* genus. These four *Lactobacillus* isolates were further identified at species level as *L.acidophilus, L.crispatus, L.salivarius* and *L.fermentum* based on sugar fermentation tests. The identified *Lactobacilli* species were screened for probiotic properties by *in vitro* tests like acid tolerance, bile tolerance and antimicrobial activity against *E.Coli* in agar well diffusion assay. 'Probiotics score' was considered as yardstick to identify the best species specific among the probiotic organisms for feeding broiler chicken. 'Probiotic score' for chicken, among the isolated *Lactobacilli* species, was formed by considering the better *Lactobacillus* for acid tolerance at pH 2, bile tolerance at 0.3 per cent bile acid in the MRS medium and antimicrobial activity against *E.coli* in agar well diffusion assay. The best *L*actobacillus species for acid tolerance, bile tolerance and antimicrobial activity were *L.acidophilus* (optical density 2.000±0.001), *L.fermentum* (optical density of 0.218±0.010) and *L.salivarius* (Inhibition zone 26 ±0.30 mm) *respectively.* Hence, from the results obtained from Probiotic score it is concluded that *L. salivarius* which has the maximum score (93.4/300) was chosen as best species specific probiotic and can be used for feeding broiler chicks.

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INTRODUCTION

Probiotics are live microbial feed supplements, which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). Crawford (1979) defined probiotics as a culture of specific living microorganisms, primarily *Lactobacillus* spp. that are implanted in the organism and ensure the rapid and effective establishment of a beneficial intestinal population. Modes of action of probiotics in poultry include 1) maintaining a beneficial microbial population by "competitive exclusion" and "antagonism" (Fuller, 1989); 2) improving feed intake and digestion Nahanshon *et al.* (1992 and 1993); 3) altering bacterial metabolism Cole *et al.* (1984). The pre-condition for probiotic microbe to favour animal's performance is colonization in the gut which is best attained if the organism being administered originates from the gut of same species (Gibson and Fuller, 2000). It is inevitable to identify the predominant probiotic bacteria to evolve the species specific probiotic organisms. Hence, to assess the suitable species specific probiotics with maximum probiotic properties for feeding broiler chicken, a new scoring system for selecting probiotics was exclusively used in this study.

MATERIALS AND METHODS

Identification of Probiotics

The study was conducted based on standard procedures to identify the probiotic organisms (Reque, 2000). Morphological, physiological and biochemical tests were performed on the 24 isolates which were collected from different parts like oesophagus, duodenum, ileum, ceacum and colon of the gastrointestinal tracts. The samples were collected from different slaughter shops across the zones of Chennai city. Decimal Dilution of the collected samples suspended in sterile MRS Broth for enrichment and incubated at 37°C for 48 h under anaerobiosis. Pure cultures were maintained in MRS agar at 4°C for short-term use and lyophilised for preservation. Selection of strains was made by identification and antimicrobial activity.

Gram staining method

Cover the sample in the glass slide with crystal violet and allow it to set for 60 seconds. Rinse with slow running water. Cover sample with Gram's Iodine and let rest for 60seconds. Rinse with water. Decolorize with decolorizing reagent 95% ethyl alcohol for 30 seconds. Cover sample with Safranin for 60 seconds. Rinse with water and allow it to dry for viewing under microscope.

To identify the *Lactobacillus* species, sugar fermentation tests were performed Lan *et al*. (2003). 1% peptone water was performed to identify the species of the isolated pure culture of the lactic acid bacteria. Sugar discs were added in each test tube and then autoclaved for sterility. Ten microlitre of the pure culture were inoculated into the sterile peptone water containing sugar discs. After incubation for 24 hours, three drops of Andrade's reagent were added to study the sugar fermentation pattern. The experiment was conducted in triplicate. Development of pink colour was indicated as positive sign and absence was indicated as negative sign. Marginal colour development was indicated by positive sign followed "$+_W$". The results of sugar fermentation test were compared with Bergey's table (Kandler and Weiss, 1986) to identify the *Lactobacilli* species.

In vitro assays to select *Lactobacillus* possessing maximum probiotics property

The *Lactobacillus* species that possess the maximum probiotics property among the identified species was determined through acid tolerance test, bile tolerance test and antimicrobial activity. For evaluating the identified probiotics, *in vitro* tests like acid tolerance, bile tolerance and anti microbial tests were performed. The method of Khalil *et al*. (2007) was followed for acid tolerance and bile tolerance tests.

Acid tolerance test

Overnight cultures of the test isolates were inoculated (1% v/v) in MRS broth (Oxoid) previously adjusted to pH values 2, 3, and 4 with 1 N NaOH or HCl. The cultures were incubated aerobically at 37oC for 6 h. Culture turbidity was monitored at 650 for growth at hourly intervals. The control comprised MRS broth adjusted to pH 6. The experiment was conducted in triplicate.

Bile salt tolerance test

Bile-resistance was determined using the broth assay. Overnight cultures (1% v/v) were inoculated in MRS broth (control cultures) and MRS broth containing 0.2, 0.3 and 0.4 (w/v) oxbile and incubated aerobically at 37 o C for 6 h. The pH of control and test cultures was adjusted to 6 with 1 N HCl or NaOH. Cultures turbidity was hourly monitored spectroscopically for growth at 650 nm. The control comprised MRS broth without bile. The experiment was conducted in triplicate.

Agar well diffusion test

The inhibitory activities of culture supernatant of identified *Lactobacilli* were tested against *E.coli* by the agar well diffusion assay following the procedure of Schillinger and Lucke (1989). *Lactobacillus* species, showed beneficial effects on resistance to infectious agents like *Escherichia coli* by Jin *et al*. (1998). Identified *Lactobacillus* species were grown in MRS broth for 24 hours at 37°C under anaerobic conditions. Cells were removed by centrifugation (4000 g for 30 min at 4°C). The pH of the supernatant was adjusted to 6.0 with 10 M NaOH and supernatant was filtered through 0.45 µm – pore-size membrane (Millipore). The culture supernatants were concentrated five times using rotary evaporator according to the method of Strompfova *et al.* (2003). Portions of 35 ml of Muller-Hington agar were autoclaved and cooled to about 48°C and then 100 µl of overnight cultures of *E.Coli* containing approximately 2 x 10^7 cells per ml were added. The inoculated medium was then poured into plates and wells of 6 mm in diameter were cut. Aliquots of supernatants from different *Lactobacillus* isolates were dispensed into wells and plates were incubated overnight at 37°C with appropriate atmosphere. The diameter of clear zones of growth inhibition around each well was measured and reported (Swida and Binek, 2005). The experiment was conducted in triplicate for each of identified *Lactobacillus* species.

Probiotics scoring system

The idea to develop a probiotics score was conceived and used exclusively for this study. 'Probiotic score' for chicken, among the isolated *Lactobacilli* species, was formed by considering the acid tolerance at pH 2, bile tolerance at 0.3 per cent bile acid in the MRS medium and antimicrobial activity against *E.coli* in agar well diffusion assay.

Formulas involved in the probiotic scoring system		
Per cent value for *r species* in acid tolerance (AT) / bile tolerance (BT)/Antimicrobial activity (AMA)	=	$\frac{\text{O.D in AT/ BT/ AMA for r species}}{\text{O.D. in AT/ BT/ AMA for } r \text{ species} + x \text{ species} + y \text{ species} + z \text{ species}} \times 100$
Cumulative point for *r species*	=	Percent value for *r species* in acid tolerance + Percent value for *r species* in bile tolerance + Percent value for *r species* in anti microbial activity

Table 1: Results of morphological, physiological and biochemical tests conducted to isolate *Lactobacilli* from chicken gut

Tests for *Lactobacillus* identification	Isolates from the gastrointestinal tract of broiler chicken																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Morphological tests:																								
Grams staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape	R	C	C	R	R	R	R	R	R	C	C	C	R	R	R	R	R	R	R	R	R	R	R	R
Physiological Tests:																								
Growth at 15ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biochemical tests:																								
Catalase	-	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	+	+
Production of gas glucose	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of NH_3 from arginine	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The probiotic score was arrived by assessing the cumulative points generated by each species for acid tolerance, bile tolerance at third hour of incubation and antimicrobial activity on overnight incubation. The cumulative points were calculated by summing up the per cent value of each test results for individual species versus total of all four species for that test. The per cent value of each test result for individual species versus total of all four species for a particular test was calculated by dividing the result of the individual species by total of results obtained for all the four species for that particular test and then multiplied by 100. Whichever organism resulted in highest cumulative points was considered to have highest probiotics score and hence the best species specific probiotics (SSP).

RESULTS AND DISCUSSION

Isolation and identification

Four isolates were confirmed as *Lactobacillus* genus from the twenty four isolates of gastro intestinal tracts, which has shown Gram positive rods on Gram staining, and negative for catalase and hydrogen peroxide tests reported in Table 1. These four isolates of *Lactobacillus* genus in Table 2 were subjected to sugar fermentation tests as per the procedure followed by Lan (2003) and identified as *L.acidophilus* (isolate 1), *L.crispatus* (isolate 6), *L.salivarius* (isolate 8), and *L.fermentum* (isolate 14) based on sugar fermentation results of Bergey's table (Kandler and Weiss, 1986). Khalil *et al.* (2007) identified *Lactobacillus* genus based on morphological and biochemical tests. The isolates identified in this study are in agreement with (Mitsuoka, 1969) that isolated *L. salivarius, L. acidophilus* and *L. fermentum* from chicken gastro intestinal tract.

Acid tolerance

L. acidophilus, a caecal isolate tolerated lower pH and exhibited maximum growth rate (2.000±0.001) compared to other *Lactobacilli* species (Table 3). Acid tolerance of *L. acidophilus* reported in the study confirm to the finding of Khalil *et al.* (2007). The pH of gastric juice in chicken is as low as 0.5-2 and the time required for feed to pass through the entire alimentary canal is as short as 2.5 hours (Duke, 1977). Taking both these points into consideration, acid tolerance test is a crucial factor to assess the survivability of probiotic in chicken gut. It can be inferred that *L. acidophilus* and possibly *L. salivarius* could possibly pass the crop and gizzard and survive in the small intestine. But, *L. crispatus* and *L. fermentum* may not be able to survive passage through the crop and gizzard and reach the intestine because of their weak tolerance to low pH.

Bile tolerance

In this test, among the four *Lactobacillus* species, *L. fermentum* had the highest bile tolerance and *L. acidophilus* exhibited the least bile tolerance as incubation progressed to three hours in Table 4. Similar results were also reported by Jin *et al.* (1998) and they revealed that growth of *L. fermentum* was not affected by 0.3% chicken bile up to a period of 6 hour incubation. Since the gastro intestinal tract transit time is three hours, third hour of incubation is crucial for assessing the bile tolerance activity.

Table 2: Sugar fermented by *Lactobacillus* isolates from chicken gut

Sugars	Isolate 1	Isolate 6	Isolate 8	Isolate 14
Lactose	+	+	+	+
Sucrose	+	$+_W$	+	+
Fructose	+	+	$+_W$	+
Salicin	$+_W$	+	$+_W$	+
Arabinose	$+_W$	$+_W$	+	-
Sorbitol	-	$+_W$	-	-
Xylose	-	-	$+_W$	-
Maltose	+	+	+	+
Melibiose	-	-	+	+
Mannose	$+_W$	$+_W$	+	+
Rhamnose	-	-	-	+
Raffinose	+	$+_W$	-	-
Inulin	+	$+_W$	-	$+_W$
Galactose	-	+	$+_W$	+
Trehalose	$+_W$	-	$+_W$	+
Mannitol	$+_W$	-	+	+
Amygdalin	+	+	-	-
Esculin	+	+	-	$+_W$
Gluconate	-	-	+	-
Melezitose	-	-	-	-

$+_W$ Mild to weak reaction+Positive reaction-Negative reaction

Table 3: Optical density of MRS medium (pH 2) containing four *Lactobacillus* species (Mean* ± SE)

OD values / Probiotics	Optical Density values at 650 nm (pH 2)			
	1 hour	2 hour	3 hour	4 hour
L. acidophilus	0.1910±0.002[c]	0.1920±0.008[c]	2.000±0.001[b]	0.2030±0.001[b]
L.crispatus	0.1197±0.005[a]	0.1226±0.006[a]	0.1212±0.005[a]	0.1273±0.005[a]
L.fermentum	0.1220±0.001[a]	0.1190±0.005[a]	0.1190±0. 004[a]	0.1270±0.003[a]
L.salivarius	0.1560±0.014[b]	0.1570±0.011[b]	0.1870±0. 009[b]	0.1930±0.002[b]

abc - Means bearing different superscripts in a column differ significantly (P< 0.05)

Table 4: Optical density of MRS medium containing four *Lactobacillus* species at 0.3 % bile (Mean *± SE)

OD values / Probiotics	Optical Density values at 650 nm (pH 2)			
	1 hour	2 hour	3 hour	4 hour
L.*acidophilus*	0.227±0.009[b]	0.106±0.011[a]	0.101±0.009[a]	0.098±0.006[a]
L.crispatus	0.168±0.010[a]	0.143±0.014[a]	0.087±0.004[a]	0.133±0.004[b]
L.fermentum	0.343±0.006[c]	0.226±0.004[b]	0.218±0.010[b]	0.204±0.001[d]
L.salivarius	0.222±0.022[b]	0.235±0.019[b]	0.191±0.021[b]	0.155±0.028[c]

abc - Means bearing different superscripts in a column differ significantly (P< 0.05)

Table 5: Antimicrobial activity of *Lactobacillus sp* against *E. coli* (Mean* ± SE)

Lactobacillus species	Zone of inhibition (mm)
L.acidophilus	24.00[c] ±0.30
L.crispatus	17.00[b] ±0.36
L.fermentum	15.00[a] ±0.28
L.salivarius	26.00 [d] ±0.30

abcd - Means bearing different superscripts in a column differ significantly (P< 0.05)

Table 6: Probiotic scoring for four *Lactobacillus sp* and isolates

In vitro assays	*L.acidophilus*	*L. salivarius*	*L. crispatus*	*L. fermentum*
Acid tolerance (%)	31.8	29.8	19.3	18.9
Bile tolerance (%)	16.9	31.9	14.5	36.5
Antimicrobial activity (%)	29.2	31.7	20.7	18.2
Probiotic score	77.9	93.4	54.5	73.6

The effect of bile salts on the survival of *Lactobacilli* has been investigated by several researchers and is thought to be linked to the ability to de-conjugate bile acids (Gilliland and Speck, 1977; Tannock *et al*. 1989). The de-conjugation of cholic acid by bile salt hydrolase (BSH) is detrimental to *Lactobacilli* cells and provokes growth inhibition at moderately acidic pH. Therefore, the presence of BSH can also be considered as a reason for different bile tolerance rates for the species specific probiotic strains used in the study although BSH activity was not assessed here.

Agar well diffusion

Antimicrobial activity of *Lactobacillus* species against *E.coli* was measured as the zone of inhibition in agar well diffusion assay and the values are presented in the Table 5. IMViC tests results are positive for indole and methyl red and negative for Voges Proskaeur and citrate, revealed that the isolate was *E.coli* as per Quinn *et.al.* (1992).Each species exhibited significant (P<0.05) difference in their antimicrobial activity. The maximum zone of inhibition was obtained for *L. salivarius* (26.00 mm).

Probiotic scoring system

Probiotic score was maximum for *L. salivarius* (93.4/300) and minimum for *L. fermentum* (54.50/300) *L. acidophilus and L. fermentum* had the score of 77.9/300 and 73.6/300 respectively (Table 6). Similar observation was made by Garriga *et al.* (1998) and Lan *et al.* (2003). They also noted that *L. salivarius sub sp salicinus* inhibited the growth of *E.coli* with an inhibition zone of 7-8 mm which was lower from the zone of inhibition observed in the present study. Thus *L. salivarius* had better antimicrobial activity when compared to other species. The antagonistic action of *Lactobacilli* towards other bacteria was attributed to the production of hydrogen peroxide (Price and Lee, 1970).

In vitro assay for acid tolerance *L acidophilus* showed higher tolerance (O.D value 2.0 ± 0.001) to pH 2 at 3rd hour of incubation. *In vitro* assay for bile tolerance revealed that *L.fermentum* had higher tolerance (OD Value 0.218 ± 0.1) to 0.3% bile at 3rd hour of incubation. In an agar well diffusion assay to measure antimicrobial activity, *L. salivarius* showed higher zone of inhibition (26 ± 0.30 mm) against *Escherichia coli*, a chicken cloacal isolate as indicator organism. Based on these *in vitro* tests maximum probiotics score was secured by *L.salivarius* (93.4).

Conclusion

The results generated from the *in vitro* studies serves as the base for predicting species specific probiotic for chicks using Probiotic score. It is concluded that *L.salivarius* has the maximum probiotic score can be used for feeding broiler chicks.

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