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Isolation of lactic acid bacteria from chicken gut and its probiotic potential characterization

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Abstract

Probiotics are now best known to be viable microorganisms that plays important role in promoting health by improving the intestinal microbial balance and inhibiting the growth of pathogenic bacterial strains. The aim of the present study was to isolate and select Lactic acid bacteria (LAB) with excellent probiotic potential and antagonism against important pathogenic bacteria**.** Lactic acid bacteria with bacteriocin producing ability were isolated and identified from the gastrointestinal tract (LAB) of chicken. Total of 11 strains were isolated, isolate were characterized morphologically and identification was done through different biochemical tests. For determination of antibacterial activities agar well diffusion method was used. Growth at different percentage concentration of NaCl, bile salt and resistance to various pH were all tested in broth medium. Antibiotic susceptibility was also carried out. All 11 strains showed inhibitory activities against pathogenic bacteria *Klebseilla pneumonia*, *Staphylococcus aurous*, *Escherichia coli* and *Salmonella typhi*. Isolates with code GG,SIYS with inhibition zone of (22mm,23mm)and (21mm,23mm)against *Staphylococci aureus* and *Escherichiacoli* showed best inhibitory activity. All isolated strains were revealed to be tolerant to bile salt at concentration of 3%, showed growth at all percentage concentration of NaCl and survived in both acidic and basic pH but only strain LIC failed to tolerate pH2.Probiotic characterization of the isolated bacterial strains was determined by observing its growth in various pH range (2, 8, 10), bile salt (0.2, 1, 2 and 3.0 %), temperature (25, 37, 45) and NaCl (2, 4, 6 and 8 %). Antibiotic sensitivity pattern showed that the LAB isolates were highly sensitive to Amikacin, Clarithromycin, Amikacin and Oflaxacin but were moderately sensitive to Cefotaxime, Azithromycin, but were resistant to Ampicillin and Tetracycline. The selected LAB were found to exhibits outstanding probiotic potential and can be used as a source of probiotic in human, animal and also as a natural preservative for food.

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Introduction

Lactic acid bacteria (LAB) are most widespread in foods and also make up the intestinal micro biota of humans and animals intestine (Rojo-Bezares *et al.*, 2006). The utilization of probiotics as a supplement on daily basis has become a common practice in commercial poultry feeding, most probably for antibiotic treatment .Lactic acid bacteria or LAB are among the most important bacteria to be utilized as probiotics with large range of metabolites with antimicrobial property which comprise organic acids, diacetyl, hydrogen peroxide, antibiotics and antimicrobial peptides (Akbar *etal.*, 2016:Karimi *etal.*,2008). Bacteriocins are directly produced as bioactive peptides which exhibit bactericidal or bacteriostatic consequence on other microorganisms. Basically have proteins of low molecular weight that after adhering to cell surface receptors get access into target cells. Their antibacterial action mechanism is not same and may consists of formation of pores into the cell wall or cytoplasmic membrane, target cell DNA lyses, interference through specific cleavage of 16srRNA and inhibiting synthesis of peptidoglycan (Todoro *et al.*,2011).

During the last few decades the use of Lactic acid bacteria (LAB) as a antibiotic have been given great importance. They have also been used in a food industry for food preservation and safety either alone or in combination with other conventional treatment. Other applications that are being considered recently is their use as functional food (prebiotics, probiotics and nutraceuticals) and also in human therapy. Lactic acid bacteria can also be used as an alternative to completely eliminate use of artificial ingredients and additives (Stiles M.E., 1996).

Probiotics are known as viable microorganism, which, when present in large numbers, can change or modify the microbiota of a host and results in beneficial health affects (Shida-Nanno, 2008).The role of probiotic in improving intestine health has been suggested for many years. Joint food and Agriculture Organization and World Health Organization has recently redefined probiotics strains as “live microorganisms that, when taken in sufficient amount as part of food, can have health benefits on the host. Probiotics strains are being revealed to be non-pathogenic and safe (Eczema, 2013).

The selection criteria to be considered for selection of LAB as probiotics: need to be safe ,have to retain viability/activity during delivery, must be resistant to acid environment and must also be resistant to bile salt ,must have good adherence capacity so that can easily colonize the gastrointestinal tract ,ability for production of substances with antibacterial property, capable to stimulates immune response and must also harbor capacity to improve metabolic activity such as vitamins production, assimilation of cholesterol and lactose digestion ability to help lactose intolerant people(Savadago *et al* .,2006).

The main objectives of the study were to isolate lactic acid bacteria LAB from gastrointestinal tract of non-broiler chicken. To determine antagonistic activity against pathogenic bacterial strains. Physiological and biochemical characterization of isolated LAB strains in order to assess their potential applications as a probiotic supplement based on resistance in conditions similar to that of intestinal tract.

Material and methods

Isolation of lactic acid bacteria from gastrointestinal tract of chicken

The chicken gastrointestinal tract was used as source of LAB.Almost22 to 25g of each part of the intestine tract was integrated into 250ml of normal saline (0.9 % NaCl) for 5 min.Selected serial dilutions were poured over sterilized MRS agar (Himedia, India) supplemented with 0.02% bromobically incubated at 37 ^{o}C for almost 24-48h.Isolated colonies were selected only from the highest dilutions of each MRS agar plate based on it morphological differences with other colonies. Purification of colonies was made by sub-culturing each colony 2-4 times on MRS agar. Characterization of isolated bacterial cultures wasthen made by Gram stain,cell morphology and also by catalase tests. Only catalase negative and gram-positivecolonies were stored at -20 ^{o}C in MRS

broth provided with (25%) glycerol. But in case of further analysis, preserved strains were sub _cultured in MRS broth at 37°C for 24h.

Catalase test

An isolated bacterial colony was streaked off on a glass slide and than almost one to two drops of 3 % hydrogen peroxide (Merck, Germany) was added on to it. The absence of bubbles presents the negative response while presence of bubbles implies positive response of bacteria to catalase test (Nelson and George, 1995).

Carbohydrate fermentation

The isolated LABs were grown in MRS broth, incubated at 37 °C for about 24h. Phenol red was used as a indicator for this test. Different sugars namely, arabinose, sucrose, maltose, lactose (BDH, UK), sorbitol (GPR, UK) and glucose (R & M Chemicals, UK) were used. To each 100 ml of medium 0.1 g (0.1 % w/v) of each sugar was added. Each test tube was supplied with 5 ml of each mixture, were than sterilized for 15 min at 121°C.A single colony of the bacteria was inoculated into test tubes under study. Test tube that changes from purple to yellow indicated Positive test and negative test was represented by no colour change (Thoesen, 1994).

Antimicrobial assay for screening antimicrobial activity

Isolated bacterial strains cultures were inoculated to MRS broth incubated at 37 ˚C for almost 48hrs.After incubation period 2ml of supernatant was used for the antimicrobial activity by agar well diffusion method against the four pathogenic test organisms. An overnight culture of test bacteria that were grown in their respective media at 37 °C were diluted in accordance to 0.5 Mcfarland standard (Khunajakr *et al.*, 2008). An amount of 20ml of sterile BHI molten media were poured into Petri dishes and allowed to solidify. Then 100µl of each tested bacterial strains were spread over agar plates. Again the plates were allowed to dry and wells (each of 7mm in diameter) were made by sterile borer, almost 100µl of isolated bacterial culture were loaded into wells, were than incubated at 37 °C for 24h for test bacterial isolates.

Probiotic characterization of isolated bacterial strains

pH tolerance

The isolated bacterial cultures were inoculated into sterile MRS of various pHi.e. 4, 6 and were incubated at 37°C aerobically for 24 to 48hrs.After incubation period MRS broth cultures were observed for turbidity.

Temperature tolerance

For the determination of growth at various temperatures, MRS broth was inoculated with colony of fresh overnight culture of LAB and incubated at 25, 37, and 45 °C for 24 h. The growth was evaluated by spreading on MRS agar and monitors their growth. The test was performed in triplicates.

Bile salt tolerance

Bacterial strains were inoculated into MRS broth having different concentrations of bile salts (0.2, 1, 2 and 3.0%), incubated at 37°C for 48h.Then 0.1ml inoculums was transferred to MRS agar by pour plate method and incubated at 37 °C for 24 to48h.The development of LAB culture on MRS agar plates were used to consign isolates as bile salt tolerant.

NaCl tolerance

For Nacl tolerance determination of isolated LAB strains, MRSbroth adjusted with varying concentration of Nacl (2%, 8%, and 10%). After sterilization, each test tube was inoculated with fresh overnight culture of bacterial isolates and was incubated at 37°C for almost 24h.

After incubation period growth in all test tubes were determined by observing their turbidity. Double positive sign (++) was used to indicate maximum growth, while normal growth was represented by single positive sign (+) and (-) sign was used for no growth. Our experimental results have the similarities with the investigations of Elizete and Carlos, lactobacilli from gastrointestinal tract of swine were bearable to 4-8% NaCl (Elizete *et al.*, 2005).

Antibiogram determination

For this agar diffusion method was used to know the antibiotic susceptibility patterns of isolated LAB strains on MRS agar plates, Muller Hinton base medium was used. Eight antibiotics were used including Oflaxacin, Azithromycin, Kenamycin, Tetracycline, Amikacin, Ceftriaxone, Clindamycin and Ampicillin in the current study.

The antibiotic discs were placed on the agar plates, were than incubated at 37 ºC for 48h.The inhibition zones diameter were measured and the results were presented as sensitive and resistance according to CLSI standard.

Results and discussion

Bacteria were gram-positive, cocci round shaped and occurred as tetrad and in pairs. The catalase test is the most important test being useful for the identification of bacteria because this test is quite simple. In catalase test results, no bubble were observed thus indicated that all the isolated bacterial strains were catalase negative and were unable to result in the decomposition of H_2O_2 to produce O_2.

Table 1. Antimicrobial activity of LAB isolated from gut of chicken.

S.No	Cods	Zone of inhibition			
		Staphylococci aureus	*Escherichiacoli*	*Salmonella typhi*	*Klebsiella pneumonia*
1	CC	16mm	18mm	15mm	14mm
2	SIW	19mm	21mm	15mm	16mm
3	LIB	13mm	14mm	11mm	14mm
4	GW	16mm	16mm	12mm	15mm
5	SIG	15mm	18mm	13mm	15mm
6	CYS	18mm	18mm	14mm	17mm
7	LIGW	17mm	17mm	11mm	16mm
8	GG	22mm	23mm	16mm	15mm
9	SIYS	21mm	23mm	16mm	14mm
10	GO	14mm	14mm	12mm	14mm
11	LIC	12mm	17mm	11mm	15mm

Isolated bacteria fermented sorbitol, arabinose, lactose, glucose but all failed to ferment maltose and 5 did not ferment sucrose as shown in Table 4. On the basis of carbohydrates fermentation and morphological characteristic organisms were identified to be *Pediococcus acidophilus, Pediococcus parvulus*. The main reason for carbohydrate fermentation test was that it's used to investigate the ability of bacteria if they can ferment carbohydrates. Phenol red broth base medium was used as an indicator to distinguish the bacteria in accordance to carbohydrate fermentation. Lactose fermentation by LAB was investigated by Ahmed and Kanwal (2004).Lactose intolerant are unable to utilize lactose because of absence of β-galactosidase enzyme. This problem could be resolved if probiotic LAB are added to milk, thus enable the lactose intolerant touse products without elevating breath hydrogen or other symptoms(Fooks *etal*, 1999.

Screening antibacterial activity of isolated bacterial

In present study, the antimicrobial spectra of isolated LAB strains were carried out against *Staphylococcusaureus, Escherichia. coli, Salmonella typhi* and *Klebsiella pneumonia* results contained in Table 1 and images shown in Fig1.CC showed maximum inhibition zone against E.coli of 18mm followed by *Staphylococcus aureus* of 16mm,*Salmonella typhi* of 15mm and minimum zone of inhibition against *Klebsiella pneumoniae* of 14mm.SIW showed maximum inhibition zone against Escherichia coli of 21mm followed by *Staphylococcus aureus* of 19mm,Salmonella typhi of 15mm and *Klebsiella pneumoniae* of 16mm. LIB showed

maximum inhibition zone against *Escherichia coli* and *Klebsiella pneumoniae* of 14mm,followed by *Staphlococcus aureus* of 13mm and *Salmonella typhi* of 11mm.Maximum inhibition zones were produced against all test microorganisms by strains SIYS with inhibition zone against *Staphlococcus aureus* of 21mm, *Escherichia coli* of 23mm,*Salmonella typhi* of 16,*Klebsiella pneumoniae* of 14mm and GG produced inhibition zone against *Staphylococcus aureus* of 22mm,*Escherichia coli* of 23mm,*Salmonella typhi* 16mm,and *Klebsiella pneumoniae* of 15mm). GW showed maximum inhibition zone against *Staphylococcus aureus*, *Escherichia coli* of 16mm followed by *Klebsiella pneumoniae* of 15mm and *Salmonella typhi* of 12mm.SIG showed maximum inhibition against *Escherichia coli* of 18mm followed by *Staphylococcus aureus* , *Klebsiella pneumonia*e of 15mm and *Salmonella typhi* of 13mm.CYS showed maximum inhibition zone against *Staphylococcus aureus, Escherichia coli* of 18mm followed by *Klebsiellapneumoniae* of 17mm and *Salmonella typhi* 14mm.LIGW showed maximum inhibition against *Staphylococcus aureus*, *Escherichia coli* of 17mm followed by *Klebsiella pneumoniae* of 16mm and *Salmonella typhi* of 11mm.GO showed maximum inhibition against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* of 14mm and *Salmonella typhi* of 12mm.LIC maximum zone was showed against *Escherichia coli* of 17mm,followed by *Klebsiella pneumoniae* of 15mm, *Staphylococcus aureus* of 12mm and *Salmonella typhi* of 11mm.LAB strains showing effective inhibition spectra can be used as probiotics to replace chemical antibiotics in animal and in fish feed industry. Considering probiotic properties, all the isolated strains can be used as potential probiotics with further detailed studies.

Table 2. Growth at different temperature and NaCl salt tolerance of selected LAB isolates.

S.No	Codes	Temperature (°C)			NaCl Concentration (%)			
		25	37	45	2	4	6	8
1	CC	++	++	+	++	++	++	+
2	SIW	++	++	+	++	++	++	+
3	LIB	++	++	++	++	++	++	++
4	GWG	++	++	+	++	++	++	++
5	SIG	++	++	++	++	+	+	+
6	CYS	++	++	+	++	++	+	+
7	LIGW	++	++	+	++	++	++	++
8	GG	++	++	+	++	++	++	++
9	SIYS	++	++	+	++	++	++	++
10	GO	++	++	+	++	++	+	+
11	LIC	++	++	++	++	++	+	+

Legend: ++=Good growth, +=Growth.

Temperature

In recent study being carried out, all 11 isolated strains of LAB were able to grow at 25 ˚C,37 ˚C and 45°C results shown in Table 2but one strain was found thermo tolerant as it showed growth at 50˚C. This temperature range was used during the study to investigate if bacterial strains can grow within range of around body temperature or not. If isolated strains failed to survive within the present temperature range then they would been unable to continue to exist in gut of human, animal and its most important criteria for selection of probiotic bacteria, the results of this study were found positive in case of all 11 isolates.

NaCl

In this study all 11 isolated bacterial strains were able to tolerate 2-10% NaCl concentration results shown in Table 2.NaCl having an inhibitory action can prevent the growth of various types of bacteria. If the lactic acid bacteria are not tolerant to NaCl than they would

be unable to show their action in the availability of NaCl, so it was far important to test the isolated strains resistance to NaCl.

Bile salt

Isolated LAB strains survived in 0.2, 1, 2 and 3.0% of bile salt results contained in Table 3.The isolates not only survived in above mentioned concentration of bile salts but also multiplied well. In this study design, 0.2,1,2 and 3.0% of bile concentration were used, as it is equalent to that found in the human intestine tract and in healthy men almost 0.3% of bile is present (Graciela *et al.*, 2001).Being tolerant to high bile salt concentration these isolates are expected to be effective in deconjugation of bile salts and thus in turn can lower cholesterol level in serum of boilers.

Table 3. pH, Bile salt tolerance and lactic acid production from lactose of selected LAB isolates Legend: ++ = Growth, - = No growth.

S.No	Samples	pH range			Bile Salt Concentration (%)			
		2	8	10	0.2	1	2	3.0
1	CC	++	++	++	++	++	++	++
2	SIW	-	++	++	++	++	++	++
3	LIB	+	++	++	++	++	++	++
4	GWG	++	++	++	++	++	++	++
5	SIG	++	++	++	++	++	++	++
6	CYs	+	++	++	++	++	++	++
7	LIGW	++	++	++	++	++	++	++
8	GG	+	++	++	++	++	++	++
9	SIYs	++	++	++	++	++	++	++
10	GO	+	++	++	++	++	++	++
11	LIC	+-	++	++	++	++	++	++

In the gastrointestinal tract the concentration of bile varies, but almost the mean bile concentration is considered not to be more than 0.03%w/v (Gilliland *et al.*, 1985).

Begley and his colleagues (2005) revealed that the hydrolysis of bile salts by bile resistant bacteria enhanced the utilization of cholesterol and thus results in decreasing serum cholesterol level. Kim and Lee (2005) reported that very high hydrolase activity can decrease the availability of conjugated bile salts required for digestion of lipid. Therefore selection of LAB as probiotics bacteria should be made only if endurable to 0.3 or more %of bile concentration (Gilliland *et al.,* 1984).

pH

pH is the most important factor that can influence bacterial growth. In this study design the growth of isolated LAB strains were observed in various pH value ranges from 2-8.

The results of pH tolerance shown in table 3 ,indicated that all the isolated LAB strains tolerated and survived in both acidic as well as alkaline Ph but only strain LIC failed to tolerate pH 2.

The probiotic bacteria for human use have to survive during the passage through the stomach where the pH is 1.5-3.0, before they arrive at intestinal tract and must remain viable for almost 4 hr or even more (Ouwehand *et al.*, 1999). The time taken for feed to pass through the entire alimentary canal is 2.5 hours (Duke, 1977). Therefore, for bacterial strains in chicken, acid resistance is not that much important as for those in other animals where the feed passage is much slower.

Table 4. Morphological and biochemical characteristics of isolated lactic acid bacteria.

S.No	Isolates code	Cultural characteristics	Morphology	Gram reaction	Catalase test	*Acid and Gas formation*			Gelatinase	pH (3.5-7.0)	Temperature tolerance	NaCl tolerance	Lactic acid production
						Glu	Lac	Suc					
1	CC	Colorless colony	Cocci in tetrad	+	_	+	+	_	_	+	+	+	+
2	SIW	Colony whitish	Cocci inpairs	+	_	+	±	_	_	+	+	+	+
3	LIB	Whitegreencolonies	Small chain cocci	+	_	+	+	+	_	+	+	+	+
4	GW	Colonies were whitish	Cocci with short chain	+	_	+	+	+	_	+	+	+	+
5	SIG	Green colonies	Cocci with short chains	+	_	+	+	+	_	+	+	+	+
6	CYS	Small yellow colonies	Cocci with tetrad	+	_	+	+	_	_	+	+	+	+
7	LIG	Colonies with green	Cocci in pairs	+	_	+	+	+	_	+	+	+	+
8	GG	Green colonies	Cocci in short chain	+	_	+	±	_	_	+	+	+	+
9	SIYS	Yellow diffused colony	Cocci with short chains	+	_	+	+	+	_	+	+	+	+
10	GO	Off-white colonies	Cocci in pairs	+	_	+	+	_	_	+	+	+	+
11	LIC	Colorless colonies	Cocci in pairs	+	_	+	+	+	_	+	+	+	+

Legend: - = Negative, += Positive, Glu = Glucose, Lac = Lactose, Suc =Sucrose.

Antibiogram

In the present study sensitivity of isolates was tested against eight different antibiotics. It was found that isolate CC was highly sensitive to Chloramphenical (19mm),Oflaxacin (20mm), Amikacin (19mm), Azithromycin (17mm), was moderately sensitive to Cefotaxime(13mm), Clarithromycin (16mm) but was resistant to Tetracycline and Amipicillin.Strain SIW was highly sensitive to Clarithromycin (20mm), Oflaxacin (21mm), Chloramphenical (18mm), Amikacin (18mm), but was moderately sensitive to Azithromycin (16mm), Cefotaxime (8mm) but was resistant to Tetracycline and Azithromycin. Isolate LIB was highly sensitive to Chloramphenical(19mm), Oflaxacin (19mm), Clarithromycin (17mm), Amikacin (18mm), Azithromycin (15mm), was less sensitive to Cefotaxime(9mm) but was resistant to Tetracycline and Amipicillin. Strain GWG was highly sensitive to Chloramphenical (21mm), Oflaxacin(20mm), Amikacin (18mm), Azithromycin (17mm), moderately sensitive to Chlarithromycin (16mm), Cefotaxime (10mm) and was resistant to Tetracycline and Ampicillin. Isolate SIG was highly sensitive to Chloramphenical (19mm), Oflaxacin (16mm), Amikacin (19mm) Clarithromycin(19mm),moderately sensitive to Cefotaxime(8mm), Azithromycin(15mm) and was resistant to Tetracycline and Amipicillin. Strain CYS was sensitive to Chloramphenical (23mm), Amikacin(19mm), Clarithromycin (19mm) ,and Oflaxacin (21mm), moderately sensitive to Cefotaxime (11mm), Azithromycin (16mm), and were resistant to tetracycline, Ampicillin.Isolate LIGW was highly sensitive to Chloramphenical (22mm), Oflaxacin (21mm), Amikacin (19mm), Clarithromycin (22mm),moderately sensitive to Cefotaxime (11), Azithromycin (16mm) and was resistant to Tetracycline and Ampicillin. Isolate GG was highly sensitive to Chloramphenical (22mm), Oflaxacin (20mm), Amikacin (19mm), Clarithromycin (18mm),was moderately sensitive to Cefotaxime (11mm), Azithromycin (15mm) and was resistant to Tetracycline, Amipicillin.Strain SIYS showed sensitivity to Oflaxacin (21mm), Amikacin (20mm), Clarithromycin (19mm), Azithromycin (19mm),was moderately sensitive to Cefotaxime (12mm) and was resistant to Tetracycline, Chloramphenical, Ampicillin. Isolate GO showed sensitivity to Chloramphenical (20mm), Oflaxacin (22mm), Amikacin (18mm), Azithromycin (17mm), Clarithromycin (18mm) but showed less sensitivity to Cefotaxime (11mm) and was resistant to Tetracycline and Ampicillin, LIC showed sensitivity to Chloramphenical (22mm), Amikacin (18mm), Clarithromycin (15mm) but was less sensitive to Oflaxacin (8mm), Cefotaxime (11mm), Azithromycin (13mm) and was resistant to both Tetracycline, Ampicillin. Present investigation thus demonstrated that antibiotics such as Clarithromycin, Oflaxacin, Amikacin, Azithromycin, Chloramphenical can dramatically decrease the probiotic bacteria from

intestinal microflora, but Tetracycline, Ampicillin will have no influence on the growth of LAB population. From our experiments we revealed that Pediococcus spp. isolated from gut of non-broiler chicken have shown broad range of sensitivity to most of the antibiotics including Chloramphenical. This was possibility due to the fact that no antibiotic were which if used could have contributed to the dissemination of antibiotic in chicken.

Fig. 1. Antibacterial activity of isolates against pathogenic strains of *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Klebsiella pneumonia*.

Conclusion

In this study, All the isolated bacterial strains harbor most common probiotic properties. All 11 isolates were found to have the ability to utilize lactose as part of their metabolism process; all were found negative for catalase test and were active against pathogenic bacteria *viz. S. typhi, E. coli, S.aureus* and *Klebsiella pneumonia*. All 11 isolates were Gram-positive cocci. From this study all the 11 isolated bacterial strains could be used as probiotic in feeding formulation in poultry. Further analysis is necessary on *in vivo* probiotic properties on poultry production. Additional experiment providing the wellbeing of the strain and bacteriocin production and purification need to be considered.

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