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Screening of Indigenous Strains of Lactic Acid Bacteria for Development of a Probiotic for Poultry

M. A. Karimi Torshizi, Sh. Rahimi*, N. Mojgani[1], S. Esmaeilkhanian[2] and J. L. Grimes[3]
Department of Poultry Science, College of Agriculture, Tarbiat Modares University
Tehran, P.O. Box 14115-336, Iran

ABSTRACT : In an attempt to develop a probiotic formulation for poultry feed, a number of lactic acid bacteria (LAB) were isolated from chicken intestinal specimens and a series of *in vitro* experiments were performed to evaluate their efficacy as a potential probiotic candidate. A total of 650 LAB strains were isolated and screened for their antagonistic potential against each other. Among all the isolates only three isolates (TMU121, 094 and 457) demonstrated a wide spectrum of inhibition and were thus selected for detailed investigations. All three selected isolates were able to inhibit the growth of *E. coli* and *Salmonella* species, although to variable extent. The nature of the inhibitory substance produced by the isolates TMU121 and 094 appeared to be associated with bacteriocin, as their activity was completely lost after treatment with proteolytic enzymes, while pH neutralization and catalase enzyme had no effect on the residual activity. In contrast, isolate TMU457 was able to resist the effect of proteolytic enzymes while pH neutralization completely destroyed its activity. Attempts were made to study the acid, bile tolerance and cell surface hydrophobicity of these isolates. TMU121 showed high bile salt tolerance (0.3%) and high cell surface hydrophobicity compared to the other two strains studied, while TMU094 appeared the most pH resistant strain. Based on these results, the three selected LAB isolates were considered as potential ingredients for a chicken probiotic feed formulation and were identified to species level based on their carbohydrate fermentation pattern by using API 50CH test kits. The three strains were identified as *Lactobacillus fermentum* TMU121, *Lactobacillus rhamnosus* TMU094, and *Pediococcus pentosaceous* TMU457. (**Key Words :** Lactic Acid Bacteria, Probiotics, *In vitro*, Broiler)

INTRODUCTION

The gram positive lactic acid bacteria (LAB) have been well known for thousands of years for their important role in the food industry due to their fermentative capacities (McKay and Boldvin, 1990). In recent years, the role of these bacteria in health and functionality of human and animal intestine has been well emphasized, mainly because of their ability to lower the pH and to produce antimicrobial agents (Delves-Broughton, 1990; Ten Brink et al., 1994; Collins et al., 1998). Controlled introduction of bacterial cultures to chicks was originated by Nurmi and Rantala (1973) for salmonella control and, subsequently, for improvement of performance by numerous authors (Tortuero, 1973; Mulder et al., 1997; Jin et al., 1998a, b). Those bacterial cultures demonstrating antibacterial actions and possessing desirable properties were later termed probiotics. Probiotics are live, nonpathogenic bacteria that contribute to the health and balance of the intestinal tract (Fuller, 1989). They are given orally to poultry to help the birds fight illness and disease. Addition of probiotics to feed is one of the alternatives to be used as a replacement for antibiotics which have created great public concerns due to emergence of antibiotic resistance (Reid and Friendship, 2002; Patterson and Burkholder, 2003).

Most common routes of administering probiotics are feed and drinking water supplementation and, much less frequently, *in ovo* injection, spray and oral dosing (Ghadban, 2002). In all of the above-mentioned applications of probiotics, the microorganisms must pass through the gastrointestinal tract to their targets, such as the lower part of the intestines and ceca. Essentially, these microorganisms must survive low pH and high concentrations of bile salts

* Corresponding Author: Sh. Rahimi. Tel: +98-21-44196522, Fax: +98-21-44196524, E-mail: rahimi_s80@yahoo.com
[1] Department of Biotechnology, Razi Vaccine and Serum Research Institute, Karaj, Iran.
[2] Department of Biotechnology, Animal Science Research Center, Esfehan, Iran.
[3] Department of Poultry Science, North Carolina State University, USA.
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available in upper parts of the gastrointestinal tract to reach the hind gut in an active and functional form and to exert their beneficial properties (Kim et al., 2006). In our research, we aimed to evaluate the probiotic potential of the LAB strains isolated from chicken intestinal specimens under *in vitro* conditions.

MATERIALS AND METHODS

Isolation and Identification of LAB

Samples from the digestive tracts of healthy broilers at market age (42-50 days of age) were cultured in MRS broth (Merck, Germany) and subcultured in MRS agar under micro-aerophilic conditions at 37°C, until pure isolates were obtained. A total of 659 isolates were identified as belonging to lactic acid bacteria based on their gram reaction, morphology and catalase test.

The selected LAB strains were further identified to species level by subjecting them to biochemical reactions and identifying them according to the key in Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1994). Finally, these species were confirmed by using API 50CH test kits (Bio Merieux, France) according to the manufacturer's instruction.

All cultures were maintained as stocks in MRS broth at -20°C, with 15% glycerol.

Inhibitory activity

Antagonistic activity of the isolated LAB strains (659) against a number of pathogenic bacteria was determined by the agar well diffusion method (Schillinger and Lücke, 1989; Jin et al., 1996; Juven et al., 1991, 1992). Only those strains demonstrating a wide spectrum of activity were selected for further studies. The antibacterial activity of these strains against poultry pathogens, including *Salmonella* (serotypes Enteritidis, Pullorum and Typhimurium) and *E. coli* (serotypes O1:K1, O2:K1 and O78:K80), was also determined by the above-mentioned method. To determine the nature of the inhibitory substance produced by the LAB isolates demonstrating antibacterial activity, the cell free supernatant of these strains was subjected to 0.5 mg/ml of enzymes including pepsin, trypsin, lysozyme and catalase (Lewus et al., 1991). The remaining activity was determined after 2 h of incubation at 37°C by the agar well diffusion method. Furthermore, to rule out the possibility of organic acids accounting for the inhibitory action, the cell free supernatants of the LAB isolates were neutralized with 1 M NaOH and the remaining activity determined.

Acid tolerance

The freshly grown lactic acid bacteria in MRS broth were centrifuged at 5,000 g for 10-15 min at 4°C and the pellet collected in a sterile tube. The pellet was washed twice with PBS, pH 7.0 before inoculation in MRS broth adjusted to pH 2.0 (by addition of 1 M HCl). The mixture was enumerated for the presence of viable cells after 2 h by measuring the OD (650 nm) and survival count. The strains exhibiting pH tolerance of 2.0 exceeding 1 h of incubation were considered as selection criteria (Gilliland et al., 1984; Toit et al., 1998).

Bile tolerance

Bile salt tolerance was assayed by the method of Gilliland and Walker (1990). The growth of the LAB strains was monitored in MRS broth supplemented with 0.3 and 1.0% W/V of bile salts (Quelab, Canada). At hourly intervals, the bacterial growth was monitored by measuring absorbance at 650 nm for a period of 12 h. The bile tolerance of each strain was based on the time in minutes required for the absorbance value to increase by 0.3 units (Erkkila and Petaja, 2000).

Cell surface hydrophobicity

The bacterial adhesion to hydrocarbons (BATH) test was adopted to screen the isolated lactic acid bacteria for their cell surface hydrophobicity, using hydrocarbon (hexadecane) by the method of Gusils et al. (1999).

Antibiotic sensitivity

Antibiotic susceptibility of the three selected isolates was determined using commercial antibiotic discs (Padtan Teb, Iran). The diameters of clear zone appearing around the discs were measured and recorded using a digital caliper (Treagan and Pulliam, 1982). All the tests were run in triplicate and the results compared to determine the susceptibility of the strains to given antibiotics.

Statistical analysis

All quantitative data were subjected to analysis of variance using SAS (SAS, 1990). A test of least significant differences was used to separate means; differences between means were considered statistically significant at $p<0.05$ (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

A total of 659 isolates appearing as gram positive cocci or rods, catalase negative and non-spore forming were identified as lactic acid bacteria (LAB) and were further screened for their probiotic properties *in vitro*. If a probiotic preparation is used mainly for excluding potential pathogenic microorganisms, antagonistic properties are of utmost importance (Huis in't Veld and Shortt, 1996). In our studies, only three isolates demonstrated a wide spectrum of inhibitory action when tested against each other and were

Table 1. Some important morphological and biochemical data used in isolate identification

	P. pentosaceus TMU457	*L. fermentum* TMU121	*L. rhamnosus* TMU094
Morphology	Cocci*	Rod	Rod
L-arabinose	+	-	-
D-xylose	+	-	-
L-sorbose	-	-	+
L-rhamnose	+	-	+
Inositol	-	-	+
D-mannitol	-	-	+
D-sorbitol	-	-	+
Methyl-αD-Glucopyranoside	-	-	+
N-acetyl glucosamine	+	-	+
Amygdalin	+	-	+
Arbutin	+	-	+
D-maltose	-	+	+
D-sucrose	-	+	-
D-trehalose	+	-	+
D-melezitose	-	-	+
Gentibiose	+	-	+
D-turanose	-	-	+
D-xylose	-	-	+
D-tagatose	+	-	+
Potassium 2-Keto gluconate	+	-	-

* Diplococcus.
+ : Able to ferment the substrate. - : Not able to ferment the substrate.

thus selected for detailed investigations (Table 2). These isolates were further identified according to their morphological and cultural properties and an API 50 CHL kit analyzed by API LAB Plus software version 4.0 database (Bio Merieux, France). A brief list of important morphological and biochemical results used in identification procedures is illustrated (Table 1). The selected LAB isolates were able to inhibit the growth of some poultry pathogens against which they were tested, although to a variable extent. All three strains appeared more effective in inhibiting the growth of *Salmonella* than *E. coli* serotypes. *S.* Pullorum was found to be the most susceptible of all the *Salmonella* serotypes tested in this study. Similar results have been observed previously by Jin et al. (1996).

As reported earlier, the antagonistic activity of LAB towards pathogens can be attributed to the production of bactericidal substances like bacteriocins, organic acids and hydrogen peroxide (Upreti and Hinsdill, 1975; Joerger and Klaenhammer, 1986). During our investigations we evaluated the nature of the inhibitory substance produced by the three selected LAB strains (Table 3). The inhibitory effects of the culture supernatants of LAB isolates were retained after treatment with catalase and lysozyme, which eliminates the involvement of hydrogen peroxide and carbohydrate moiety in inhibitory actions. In contrast to *P. pentosaceus* TMU457, treatment of the culture supernatant fluids of *L. fermentum* TMU121 and *L. rhamnosus* TMU094 with proteolytic enzymes completely destroyed the inhibitory effects in these two strains, indicating the protein nature of the antagonistic substances. Consequently, the antagonistic action in these two species may be attributed to the presence of bacteriocin or bacteriocin-like substance. However, the culture supernatant fluid of *P. pentosaceus* TMU457 lost its inhibitory action after pH neutralization, showing the possibility of organic acids in antagonistic effects in this isolate. Similarly to our results, Gusils et al. (2002) reported that the inhibitory action of 100 LAB strains isolated from the gastrointestinal tract of pigs was not affected by catalase treatment, while pH neutralization resulted in complete loss of activity.

One of the most important properties for a probiotic to provide health benefits is that it must be able to overcome physical and chemical barriers such as acid and bile in the

Table 2. Antagonistic activity demonstrated by the three selected LAB strains assayed by measuring the zone diameter around the wells (Mean±SD)

LAB	*Escherichia coli*			*Salmonella*		
	O78:K80	O2 :K1	O1:K1	Enteritidis	Pullorum	Typhimurium
P. pentosaceus TMU457	$16.4^{a,**}\pm 0.9$	$14.1^{a,*}\pm1.0$	$16.7^{a,*}\pm1.4$	$17.3^{a,*}\pm0.6$	$20.3^{a,**}\pm1.5$	$17.7^{a,**}\pm1.5$
L. fermentum TMU121	$11.7^{b}\pm2.1$	$10.2^{b}\pm1.3$	$11.9^{b}\pm2.1$	$13.7^{b}\pm1.5$	$16.0^{b}\pm1.0$	$13.0^{b}\pm1.0$
L. rhamnosus TMU094	$10.5^{b}\pm1.3$	$10.4^{b}\pm2.0$	$12.4^{b}\pm2.0$	$14.7^{b}\pm1.5$	$17.0^{b}\pm1.0$	$14.0^{b}\pm1.5$

[a, b] Means with different superscripts in columns are significantly different (* p<0.05, ** p<0.01).

Table 3. Nature of antagonistic properties exerted by culture supernatant of three selected LAB

Supernatant treatment	*P. pentosaceus* TMU457	*L. fermentum* TMU121	*L. rhamnosus* TMU094
Control	+	+	+
pH neutralization	-	+	+
Catalase	+	+	+
Pepsin	-	-	-
Trypsin	+	-	-
Lysozyme	+	+	+

+ Inhibitory effect observed/- Inhibitory effect not observed.

Table 4. Acid and bile tolerance and degree of hydrophobicity of three selected LAB strains (Mean±SD)

Selected LAB	% Survivability at pH 2.0 after 2 h	OD (650 nm) in the presence of 0.3% bile salt after 12 h	Hydrophobicity (%)
P. pentosaceus TMU457	55.89^{b}±1.86	35.00^{b}±4.58	3.64^{b}±0.19
L. fermentum TMU121	51.58^{b}±1.48	296.67^{a}±15.28	79.94^{a}±9.70
L. rhamnosus TMU094	67.76^{a}±2.66	43.67^{b}±3.51	93.53^{a}±3.10

[a, b] Means with different superscripts in columns are significantly different ($p<0.01$).

Table 5. Antibiotic susceptibility profile of three selected LAB

Antibiotics	*P. pentosaceus* TMU457	*L. fermentum* TMU121	*L. rhamnosus* TMU094
Amikasin	S	I	R
Enrofloxacin	R	R	R
Cephalexin	R	R	R
Gentamicin	S	S	S
Kanamycin	S	S	I
Chloramphenicol	S	S	I
Tetracycline	R	S	R
Trimethoprim +Sulfamethoxazole	R	I	R
Lincospectin	R	S	S
Flumequin	I	S	I
Novobiocin	R	R	R
Nalidixic acid	R	S	R
Synotrime	S	S	S
Penicilin	R	R	R
Vancomicin	R	R	R
Bacitracin	I	S	I
Neomycin	R	R	R

R = Resistant; I = Intermediate; S = Susceptible.

gastrointestinal tract (Gibson et al., 2000). Stresses to organisms begin in the stomach, with pH between 1.5 and 3.0, and in the upper intestine which contains bile (Lankaputhra and Shah, 1995; Corzo and Gilliland, 1999). Although stomach pH can be as low as 1.0, in most *in vitro* assays pH 3.0 has been preferred (Garriga et al., 1998; Suskovic et al., 2001). Thus, survival at pH 3.0 for 2 h and at a bile concentration of 1,000 mg/L is considered an optimal acid and bile tolerance for probiotic strains (Usman, 1999). The acid and bile tolerance of the three selected LAB isolates is presented in Table 4. In this study, one of the strains designated TMU094, identified as *L. rhamnosus*, was able to resist low pH values of 2.0 and showed maximum growth at this pH within an hour of incubation. However, *L. fermentum* TMU121 showed moderate resistance to this pH value and lost about half of its viable counts within an hour of incubation, while *P. pentosaceus* TMU457 showed a sharp decline in its growth rate immediately after exposure to this pH (2.0). This phenomenon has previously been observed in a number of probiotic bacteria where a substantial decrease in the viability of strains was often observed at pH 2.0 or below (Hood and Zottola, 1988; Gupta et al., 1996). However, as reported by Prasad and his co-workers (1999), the probiotic strains are likely to be buffered by food or other carrier matrix molecules following consumption and are thus not likely to be exposed to the extremes of pH in the stomach.

Another important characteristic for a probiotic that enables it to survive and then grow and exert its action in the small intestine is its bile tolerance. Selection of bile tolerant strains at bile concentrations between 0.1 and 4.0% w/v in growth media is typical (Gilliland and Speck, 1977; Mayara-Makinen et al., 1983). Gilliland et al. (1984) reported that bile tolerant lactobacilli occur in high numbers in the upper section of the intestine. Although the bile concentrations in the gastro-intestinal tract vary, the mean intestinal bile concentration is believed to be 0.3% w/v (Sjovall, 1959; Gilliland et al., 1985). In our studies, the most bile resistant strain was *L. fermentum* TMU121 which was able to survive in the presence of high bile salt concentrations (0.3%), while *P. pentosaceus* TMU457 showed minimum growth in the presence of this salt. Owing to the high tolerance of bile salt by *L. fermentum* TMU121, we expect the strain to be effective in bile salt deconjugation and consequently effective in lowering serum cholesterol in broilers. This phenomenon was also observed by Begley and his colleagues (2005), who reported that hydrolysis of bile salts by bile tolerant bacteria results in increased usage of cholesterol to synthesize new bile salts and this resulted in lowering of serum cholesterol levels. However, it is worth mentioning that too high bile salt hydrolase activity in the intestinal lumen can result in low availability of conjugated bile salts needed for lipid digestion (Kim and Lee, 2005).

The beneficial effect of probiotic bacteria has been attributed to their ability to colonize human and animal gastrointestinal tracts (Dharmawan et al., 2006). Several factors are involved in the adhesion of probiotics; one is the hydrophobicity of the bacterial cell surface. It has been reported by Wadström and his co-workers (1987) that, compared to hydrophilic strains, hydrophobic lactobacilli adhered better to intestinal epithelial cells. As bacterial cells alter their membrane fluidity under various environmental conditions, growth conditions may have a profound effect on the fatty acid composition of their lipids and subsequently on the hydrophobicity and adhesion ability of bacterial strains. As Table 4 depicts, *L. rhamnosus* TMU094 had the highest cell surface hydrophobicity compared to *L. fermentum* TMU121 and *P. pentosaceus* TMU457. As suggested by others, the high cell surface hydrophobicity of the mentioned strain could indicate its potential to attach to

the epithelial cell lining of the intestine and resist the movement of digesta (Yu et al., 2007).

Antibiotic susceptibility profiles of selected LAB are shown in Table 5. All three LAB had some degree of antibiotic resistance against some of the tested antibiotics. In a previous report, Nemcova et al. (1997) investigated the antimicrobial susceptibility of 13 lactobacilli strains isolated from suckling piglets and found they were susceptible to many common feed additive antibiotics. Although it is clear that simultaneous application of susceptible probiotics with oral antibiotics is generally unreasonable, in the case of microbial infection it might be possible to use these resistant probiotics in combination with appropriate antibiotics (Marounec and Rada, 1995).

To conclude, we might suggest the three selected LAB strains, namely *Lactobacillus rhamnosus* TMU094, *Lactobacillus fermentum* TMU121 and *Pediococcus pentosaceus* TMU457, to be potential candidates in developing a probiotic formulation for poultry feed. Although all three strains varied in some of their properties, when used in combination they could provide beneficial and desirable properties as a probiotic.

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