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Full Length Research Paper

Potential probiotic of *Lactobacillus johnsonii* LT171 for chicken nutrition

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The objective of this research was to investigate the potential probiotic of *Lactobacillus johnsonii* LT171. It had aggregation (60 min) and antibacterial effects against *Salmonella* Enteritidis*, Salmonella* Typhimurium and *Escherichia coli* O78:K80. It showed amylase and protease activity and high clear zone in culture medium containing calcium phytate; cell surface hydrophobicity, 85.21 ± 7.27%; resistance to acidic condition (pH 3 for 90 min) and bile salts (in culture medium containing 0.075% ox gall). Also it had resistance to nalidixic acid and neomycine. This research showed appropriate probiotic properties of *L. johnsonii* LT171 for chicken nutrition. Hence this strain can complement the characteristics of other strains in multistrain probiotics because of its high clear zone in culture medium containing calcium phytate.

Key words: *Lactobacillus johnsonii* LT171, probiotic, chicken.

INTRODUCTION

The important role of gastrointestinal microflora in the health and nutrition of animals and humans is increasingly recognized. Probiotics are defined as live microbial food supplements that beneficially affect the host by improving intestinal microbial balance (Fuller, 1989). Among the potential probiotics, lactic acid bacteria (LAB) is reported to have important effects on animal performance (Chou and Weimer, 1999).

One of the most reported lactic acid bacteria as a source of chicken probiotic is *Lactobacillus johnsonii*. La Ragione et al. (2004) and Van Coillie et al. (2007) examined the ability of *L. johnsonii* to prevent the colonization of pathogens in chicken. On the other hand, *L. johnsonii* belongs to the group of obligatory homofermentative bacteria that have high total antioxidative activity. This group of Lactobacilli was found to have a relatively much stronger inhibitory activity in an anaerobic environment and has also been shown to have no inhibitory activity toward any species or isolate of the genus *Lactobacillus* (Annuk et al., 2003). Hence this research was conducted to investigate the most important properties of *L. johnsonii* LT171, an indegenus poultry-derived isolate, for potential probiotic. Adhesion ability to the mucus and antibacterial effects against pathogens is the major criteria in the selection of LAB for probiotic candidates (Garriga et al., 1998; Ehrmann et al., 2002). The adherence capability of a bacterial strain to the digestive tract is presumably a prerequisite for its colonization (Bouzaine et al., 2005). These attributes (adhesion ability and antibacterial effects) of LAB provide competitive exclusion against enteric pathogens and reduce their colonization. Competitive exclusion concept was applied first to the domestic fowl by Nurmi and Rantala (1973) when they attempted to control a severe outbreak of *Salmonella* Infantis in Finnish broiler flock. Hence, the examination of LAB ability for this concept has been done in many studies (Edens et al., 2007; La Ragion et al., 2004).

Since LAB can reduce the number of pathogens in the gastrointestinal tract by production of bacteriocins and especially organic acids, the antibacterial effects of *L.*

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johnsonii LT171 were investigated in this research. The tests of aggregation and cell surface hydrophobicity are representative for adhesion ability of LAB to the mucus, indirectly (Garriga et al., 1998; Ehrmann et al., 2002; Taheri et al., 2009) and co-aggregation is thought to be linked to the ability to interact closely with undesirable bacteria (Gusils et al., 1999). These attributes are another characteristics representing competitive exclusion of this strain against enteric pathogens. Therefore, they were examined as well. On the other hand, recent studies have focused to improve the probiotic efficacy and represent more applicable supplements in poultry nutrition. The beneficial effects of expression of α-amylase, phytase, β-glucanase, xylanase and cellulase enzymes in the *Lactobacillus* (Scheirlinck et al., 1990; Liu et al., 2005, 2007; Yu et al., 2008) show the importance of enzymatic activities in bacterial strains of chicken probiotics. A probiotic that has the enzymatic activities can improve digestion especially in newly-hatched chicks. Hence, the enzymatic activities of this strain were also examined. Resistance to pH and bile salts is of great importance in survival and growth of bacteria in the intestinal tract and thus, is a prerequisite for choosing suitable probiotics. Tolerance to acidic condition and bile salts of *L. johnsonii* LT171 were investigated in this study as well as other tests.

MATERIAL AND METHODS

Chemicals

All chemicals used in this study were of analytical grade, mostly purchased from Sigma Chemical Co., St. Louis, MO, USA. MRS (De Man, Rogosa and Sharpe) and nutrient broth media were purchased from Merck, Darmstadt, Germany and Oxoid Ltd., Basingstoke, UK, respectively.

Isolation of bacterial strain

L. johnsonii LT171 was isolated from digestive tract (ileum) of broiler chicken and identified by sequencing the 16S rRNA. Three strains of avian origin, *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Escherichia coli* O78:K80 were obtained from the Veterinary Department at the University of Tehran, Iran. They were used examining antagonistic activity and co-aggregation behavior of *L. johnsonii* LT171.

Detection of enzymatic activities

Enzyme (that is, amylase, protease, lipase and phytase) activities were conducted according to Taheri et al. (2009). In order to detect the amylase, lipase and phytase activities, the strain was sub-cultured and then spot-inoculated onto relevant medium, which was incubated anaerobically (in anaerobic jars; Sharif Azmayeshgahi Co., Tehran, Iran) for 48 h at 37°C. After incubation, the halo zone surrounding colony was measured with a caliper. For amylase activity, the strain was sub-cultured on mMRS (modified MRS: MRS prepared in the lab, with 0.25% starch instead of whole glucose) broth. Activity was examined using a medium that consisted of meat peptone (0.5%), yeast extract (0.7%), NaCl (0.2%), starch (2%) and agar (1.5%). For detecting the clear zones of amylase activity, dense Lugol's solution was poured over the plates. In the case of lipase activity, the MRS broth containing olive oil (1%) and Arabic gum (1%) was used to sub-culture the strain. Activity was detected by using a medium that consisted of tryptone (0.1%), yeast extract (0.5%), NaCl (0.05%), olive oil (0.1, 0.5 or 1%), Arabic gum (1%) and agar (1.5%).

The MRS broth which contained 0.25% calcium phytate (Sigma-Aldrich Co., St. Louis, MO, USA) was used to sub-culture the strain and the medium consisted of glucose (1.5%), calcium phytate (0.5%), NH_4NO_3 (0.5%), KCl (0.05%), $MgSO_4.7H_2O$ (0.05%), $MnSO_4.7H_2O$ (0.02%), $FeSO_4.7H_2O$ (0.001%) and agar (1.5%) (adjusted to pH 7.0 by $Ca(OH)_2$) was used for detection of clear zone.

For detection of protease activity, the strain was cultured on MRS broth and after anaerobic incubation for 24 h at 37°C, 30 µl of culture supernatant was transfered onto a disc placed over a medium consisting of skim milk (1%) and agar (1.5%). Clear zones surrounding disc was measured with a caliper.

Detection of antagonistic activity

For detection of inhibitory activity, the well diffusion assay described by Schillinger and Lucke (1989) was used. Plates containing solidified nutrient agar (20 ml) overlaid with 10 ml of soft nutrient agar (0.7% agar in nutrient broth) were inoculated with 5 µl of an overnight culture of *S.* Enteritidis*, S.* Typhimurium or *E. coli* O78: K80. Wells were made in the agar and 30 µl of the culture supernatant of *L. johnsonii* LT171 was transferred into the well. The plates were incubated aerobically for 24 h at 37°C, after which they were examined for clear inhibition zones around the wells. The test was carried out two times with duplicates each time. This test was evaluated by the disc diffusion assay as well. The procedure was the same as described before except that the paper discs (Tadbir Fan Azma Co., Tehran, Iran) were used instead of the wells.

Aggregation test

The test was performed by the method of Reniero et al. (1992). Aggregation was scored positive when bacteria gravitated to the bottom of the tubes, leaving a clear supernatant fluid. The test was examined every 15 min for 2 h.

Cell surface hydrophobicity test

Cell surface hydrophobicity was determined by the method of Rosenberg et al. (1980). The strain was harvested after 18 h of growth, washed twice and resuspended in physiological saline solution to an optical density (OD) of 0.5 at 600 nm. To test tubes containing 3 ml of washed cells, 1 ml of toluene was added. The mixtures were blended on a vortex for 90 s. The tube was left to stand for 15 min for separation of the two phases and the OD_{600} of the aqueous phase was then measured. Hydrophobicity was calculated as the percentage decrease in the OD_{600} of the bacterial suspension due to partitioning of cells into the hydrocarbon layer. Percentage of hydrophobicity = [(OD_{600} before mixing - OD_{600} after mixing) / OD_{600} before mixing] x100.

Co-aggregation test

Reid et al. (1988) suggested that the dominance of inhibitor-producing lactobacilli on the urogenital epithelium of humans and the ability of these organisms to interact closely with uropathogens such as *E. coli* would constitute an important host defense mecha-

Table 1. Different attributes of *L. johnsonii* LT171 (this research) in compare to *L. crispatus* LT116 (a candidate strain as a source of probiotic; Taheri et al. 2009).

Parameter	Strains	
	***L. johnsonii* LT171**	***L. crispatus* LT116**
Antibacterial activity[1]	1.2	0.91
Amylase activity	+[2]	+++
Clear zone in culture medium containing calcium phytate	+++	+
Protease activity (mm)	15.05 ± 2.75	14.2 ± 2.54
Lipase activity	-	-
Aggregation time[3] (min)	60	15
Cell surface hydrophobicity (%)	85.21 ± 7.27	92.14 ± 3.7

[1]The mean of growth inhibition radius (mm) against all pathogens (*S.* Enteritidis*, S.* Typhimurium and *E. coli* O78:K80).
[2]+++, Halo zone was more than 3 mm; +, halo zone was just detected as less than one mm
[3]Time needed to give a clear supernatant fluid, lower aggregation time indicates more aggregation of each strain.

nism against infection. Hence co-aggregation was investigated according to Jin et al. (1998). Suspension of the strain, *E. coli* O78: K80, *S.* Enteritidis or *S.* Typhimurium was adjusted in phosphate buffer (pH 7) to an OD_{600} of 0.5. A suspension (0.5 ml) of each pathogen and a similar suspension (0.5 ml) of the strain was placed together in a test tube and mixed thoroughly using a vortex. The OD_{600} of the bacterial mixture was measured after incubation for 4 h at 37°C. Control tubes contained 1 ml of a suspension of each bacterial species. The percentage of co-aggregation was calculated using the equation of Handly et al. (1987):

$$\text{Percentage of co-aggregation} = \{[(PC+LC)/2-(P+L)] / (PC+LC)/2\} \times 100$$

where PC and LC represent the optical densities in control tubes containing only pathogen or *Lactobacillus* after 4 h of incubation, respectively; P+L represent the optical density of the mixed culture after the same period of incubation.

Bile salts tolerance test

Overnight culture of the strain was centrifuged (Beckman, J-6M; Beckman Coulter, Fullerton, CA, USA) at 7500 g for 5 min at 4°C. After re-suspending the pellets in the phosphate buffer (pH 6), it was diluted 1×10^{-5}. Subsequently, the counts of viable cells were determined by growing the suspensions on MRS containing 0.075, 0.15, 0.3 and 1% (w/v) ox-bile (Fluka Biochemika, Sigma-Aldrich, Buchs, Switzerland) anaerobically at 37°C for 48 h (Garriga et al., 1998).

Acidic pH tolerance test

Cell suspension was prepared as above and then diluted 1×10^{-5} in phosphate buffer at pH 3 and 6. The suspensions were then incubated for 90 min at 37°C. The viable cells were then counted by growing the suspensions on MRS agar anaerobically at 37°C for 48 h (Garriga et al., 1998).

Antibiogram test

Antibiotic sensitivity of the strain was determined as follow: the density of bacterial suspension was adjusted until the visible turbidity was equal to 0.5 McFarland standard. The inoculum was spread evenly over the entire surface of the plates contained MRS agar. Subsequently, paper discs containing the antibiotics (Tadbir Fan Azma Co, Tehran, Iran) were laid on the plates and incubated anaerobically at 37°C and growth inhibition was examined after 24 h.

RESULTS AND DISCUSSION

We have earlier screened 332 LAB based on aggregation time, antibacterial effects, enzymatic activity, cell surface hydrophobicity, co-aggregation, tolerance to bile salts and acidic condition and finally selected *Lactobacillus crispatus* LT116 as a source of chicken probiotic because of its predominant characteristics in comparison to the other isolated strains from the gastrointestinal tract of chickens (Taheri et al., 2009). We reported the enzymatic results of LAB as “nd”, not detected (without enzymatic activity); “+”, halo zone was just detected as less than one mm (low enzymatic activity) and “+++”, halo zone was more than 3 mm that was noticeable compared to other strains (high enzymatic activity). Since all methods used in this study was similar to their research, the results of *L. johnsonii* LT171 are represented in comparison with their findings (Table 1). The results showed that *L. johnsonii* LT171 had no extra lipase production; however, it showed low amylase activity and high clear zone in culture medium containing calcium phytate. Although Sreeramulu et al. (1996) and Zamudio et al. (2001) showed the high phytase activity of *Lactobacillus plantarum* and *L. amylovorus* among *Lactobacillus*, respectively, they did not examine the phytase activity of *L. johnsonii* in their studies. Halo zone for protease activity was 15.05±2.75 mm. *L. johnsonii* LT171 had antibacterial effect against all pathogens (*S.* Enteritidis*, S.* Typhimurium and *E. coli* O78:K80). Also it had aggregation, 60 min and cell surface hydrophobicity, 85.21 ± 7.27%.

Aggregation shows clumping of strains together and also adhesion ability to the epithelial cells indirectly but in a strong way (Garriga et al., 1998; Ehrmann et al., 2002; Taheri et al., 2009). It has been reported that bacteria

Table 2. Antibiogram profile of *L. johnsonii* LT171.

Antibiotic	Halo zone (mm)
Ampicillin (10 µg)	22
Bacitracin (10 units)	30
Chloramphenicol (30 µg)	30
Doxycycline (30 µg)	6
Erythromycin (15 µg)	25
Furazolidon (100 µg)	7
Florfenikol (30 µg)	28
Gentamicin (10 µg)	14
Kanamycin (30 µg)	24
Lincomycin (2 µg)	3
Nalidixic acid (30 µg)	0
Neomycine (30 µg)	0
Oxytetracyclin (30 µg)	6
Penicillin (10 units)	28
Streptomycin (10 µg)	12
Tetracycline (30 µg)	3
Vancomycin (30 µg)	20

which show high aggregation (or in other words, low aggregation time), also have high cell surface hydrophobicity and adhesion ability to the mucus (Taheri et al., 2009).

Aggregation and cell surface hydrophobicity of the bacteria could be used instead of the examination of adhesion ability to the mucus (Taheri et al., 2009), because there is a strong relationship among these characteristics especially between aggregation time and adhesion ability to the epithelium of the digestive tract (Garriga et al., 1998). The results of this study showed no co-aggregation between *L. johnsonii* LT171 and enteric strains. Jin et al. (1998) showed that there was poor co-aggregation between selected LAB and *E. coli* strains. These authors indicated that most isolated bacteria had co-aggregation of between 1 to 4.6%. With regard to the pH of feed in the gastrointestinal tract of the chicken (Church and Pond, 1974), it was thus preferred to examine the survivability of *L. johnsonii* LT171 at pH 3 for 90 min. This strain was completely resistance to this pH. Garriga et al. (1998) screened LAB with regard to their tolerance to pH 3 and they showed that pH 3 did not decrease the number of LAB. The effects of bile salts on the survival of lactobacilli have been investigated by several authors and are thought to be linked to the ability to de-conjugate bile acids (Tannock et al., 1997). *L. johnsonii* LT171 showed resistance in culture medium containing 0.075% bile salts. Antibiogram profile is also important, since many antibacterial chemicals are used as feed additives in poultry farms. There is no information about minimum inhibitory concentration break points for *Lactobacillus* in the NCCLS (data is only for pathogenic bacteria), hence only the size of halo zone of *L. johnsonii* LT171 is represented in Table 2. There was not any growth inhibition against this strain in nalidixic acid and neomycine antibiotics. In general, *L. johnsonii* LT171 had appropriate probiotic properties. This strain can complement the characteristics of other strains in multistrain probiotics and be more effective in such supplements. Timmerman et al. (2004) showed that multistrain or multispecies probiotics were more effective than monostrain probiotics for resistance to pathogens and performance in animals. Since our previous results (Taheri et al., 2009) showed that *L. crispatus* LT116 (a candidate strain) had low clear zone in culture medium containing calcium phytate, *L. johnsonii* LT171 with high activity of this attribute can be useful in probiotic that contained *L. crispatus* LT116 plus *L. johnsonii* LT171. Noticeable characteristics of *L. crispatus* LT116 are high amylase activity and aggregation (Taheri et. al., 2009) and of *L. johnsonii* LT171 is high clear zone in culture medium containing calcium phytate. Hence these strains can complement the characteristics of each other in a doublestrain probiotic.

A separate study will be required for the evaluation of *L. johnsonii* LT171 and *L. crispatus* LT116, alone or in combination, as a probiotic supplement on chicken performance.

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