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Antibacterial Activity of Probiotic Bacteria Isolated From Broiler Feces and Commercial Strains

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Background: The extensive use of antibiotics in animal farms to promote the growth rate and prevent the enteric pathogen has led to the development of antibiotic-resistant bacteria and drug residues in the birds body. In the recent years, probiotics have been constantly studied for their inhibitory effects on pathogenic bacteria.
Objectives: The current study aimed to assess the effect of magnesium oxide on controlling serum phosphorus levels and evaluate its side effects.
Materials and Methods: Antibacterial activity of local and commercial probiotic bacteria was investigated using colony overlay assay. Then antibacterial activity of local and commercial probiotics against each pathogen, *Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus* were compared.
Results: Local strain of lactic acid bacteria had significantly higher antibacterial activity compared to those of the commercial probiotics. Local probiotics showed a significantly stronger activity against *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli* compared to all commercial probiotics.
Conclusions: Administration of mono strain of *Lactobacillus salivarius* ES1, or co-administration of ES1 and L. salivarius ES6, is not only more effective than commercial probiotics against Salmonella spp., Staphylococcus spp. and *E.coli*, but also, will have no negative effects on micro flora balance of local birds.

Keywords: Antibacterial Activity; Probiotics; Broiler feces; Commercial; Local

1. Background

Poultry represent nearly 20% of all the meat produced worldwide. It is a source of protein that plays an important role in human diet. It is important to remember that there are several avian infections which can be transmitted to humans. During broiler production, fecal contamination may occur (1). In many countries, as European Food Safety Authority (EFSA) reports show, poultry production and poultry products can be a potential source of human salmonellosis. In the United States, it is estimated that 1.4 million humans contract salmonellosis once a year and the annual cost of this disease, including lost productivity, is $3 billion (2, 3). In the poultry industry, poultry pathogens are controlled by antibiotics. However, the use of dietary antibiotics resulted in common problems such as the progress of drug-resistant bacteria, drug residues in the birds body and imbalance of normal micro flora (4). Drug-resistant bacteria like Staphylococcus spp., *Escherichia coli* and Enterococcus spp. can be transmitted from poultry to humans through the food chain and other sources, leading to potential therapeutic failures in both humans and animals (5). Therefore, public pressure to reduce the use of antimicrobials has influenced development of alternative medicines or methods to reduce pathogens, including probiotics. In broiler nutrition, probiotic species of Lactobacillus, Bacillus, Streptococcus, Bifidobacterium, Candida, Enterococcus, Aspergillus, and Saccharomyces have a favorable effect on broiler performance, modulation of intestinal micro flora and pathogen inhibition (6). In agriculture, probiotics are alternatives of antibiotics that not only control enteric pathogens, but also promote growth (7). Therefore the development of new and more effective probiotic products that can be licensed for animal use received considerable interest.

2. Objectives

The current study aimed to investigate the antimicrobial activity of 16 potential probiotic bacteria isolated from local birds, then, compare the antibacterial activity of putative local probiotic strains that showed moderate to high antibacterial effect, with six commercial probiotic bacteria.

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3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

The probiotic and pathogenic bacterial strains used in the present study, the appropriate media, and culture conditions are listed in Table 1. All commercial probiotic strains were provided by Alborz University Bacterial Collection. Local potential probiotics were isolated from broiler feces of tropical area of Iran, by Agricultural Biotechnology Research Institute of Iran (ABRII). All pathogens were obtained from Pasture Institute Culture Collection, Iran. Strains were maintained at 70°C in 15% (w/w) glycerol onto Cryobank cryogenic beads (Pro-lab Diagnostics, UK). Appropriate plates according to Table 1, were inoculated from the stock culture collection and incubated for 24 or 48 hours at 37°C. All culture media were purchased from Merck (Germany); anaerobic condition was created by Gas Pack (Merck).

Table 1. Bacterial Strains, Media and Culture Condition

Bacterial Species	Strain	Origin	Media/Atmosphere/Temperature
Commercial probiotic strains			
Bifidobacterium bifidum	*B94*	DSM	TOS/anaerobic/37^{o}C
Lactobacillus casei	*L26*	DSM	MRS/anaerobic/37^{o}C
Lactobacillus acidophilus	*L10*	DSM	MRS/anaerobic/37^{o}C
Lactobacillus plantarum	*299 v*	Biogaia	MRS/anaerobic/37^{o}C
Lactobacillus reuteri	*protectis*	Biogaia	MRS/anaerobic/37^{o}C
Local probiotic strains			
Lactobacillus salivarius	ES 01	ABR II	MRS/anaerobic/37^{o}C
L. salivarius	ES 02	ABR II	MRS/anaerobic/37^{o}C
Lactobacillus reuteri	ES 03	ABR II	MRS/anaerobic/37 ^{o}C
L. reuteri	ES 04	ABR II	MRS/anaerobic/37 ^{o}C
L. salivarius	ES 06	ABR II	MRS/anaerobic/37^{o}C
L. salivarius	ES 07	ABR II	MRS/anaerobic/37^{o}C
L. salivarius	ES 08	ABR II	MRS/anaerobic/37 ^{o}C
L. salivarius	ES 09	ABR II	MRS/anaerobic/37 ^{o}C
L. salivarius	ES 10	ABR II	MRS/anaerobic/37^{o}C
L. salivarius	ES 11	ABR II	MRS/anaerobic/37 ^{o}C
L. salivarius	ES 12	ABR II	MRS/anaerobic/37^{o}C
L. salivarius	ES 13	ABR II	MRS/anaerobic/37^{o}C
L. reuteri	ES 14	ABR II	MRS/anaerobic/37 ^{o}C
L. reuteri	ES 15	ABR II	MRS/anaerobic/37^{o}C
L. reuteri	ES 16	ABR II	MRS/anaerobic/37^{o}C
L. reuteri	ES 17	ABR II	MRS/anaerobic/37^{o}C
Pathogens			
Pseudomona aeroginosa	PTCC 1707		Luria–Bertani agar/aerobic/37^{o}C
Enterohemorrhagic E. coli	PTCC 1399		Luria–Bertani agar /aerobic/37^{o}C
Clostridium difficile	PTCC 1765		Clostridial (RC) agar /anaerobic/37^{o}C
Enterococcus hirae	PTCC 1239		Luria–Bertani agar /aerobic/37^{o}C
Salmonella enterica	PTCC 1709		Luria–Bertani agar/aerobic/37^{o}C
Staphylococcus aureus	PTCC 1431		Luria–Bertani agar /aerobic/37^{o}C

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Table 2. Mean Inhibition Zone of Six Local and Commercial Probiotic Bacteria Isolates Against Reference Pathogens

-	*E.coli Enterohemorrhagic*	*Salmonella enterica*	*Staphylococcus aureus*	*Mean of antibacterial activity*
Local strains				
Lactobacillus salivarius ES 1	16	18	3	7.5
L. salivarius ES 6	12	15	9	11
L. salivarius ES 7	7	8	5	7.8
L. salivarius ES 11	10	12	6	7.5
L. salivarius ES 13	6	10	4	7
L. salivarius ES 15	7	18	2	7
Commercial strains				
Bifidobacterium bifidum B94	3	0	4	4.6
L. acidophilus L 10	8	21	3	7.5
L. casei L 26	10	10	4	6.5
L. plantarum 299v	7	10	3	7.3
L. reuteri Protectis	12	16	2	8.1

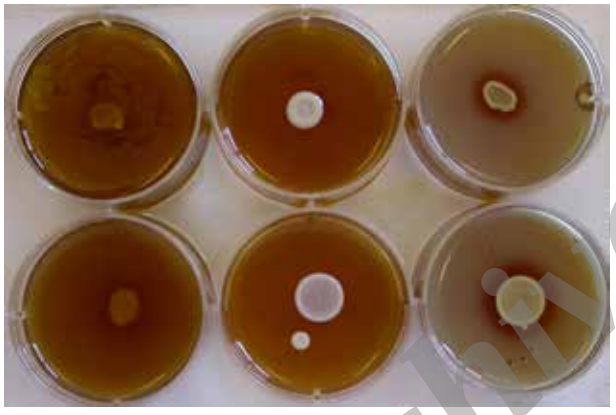

Figure 1. Antibacterial activity of probiotic strains using colony overlay assay, a: Antibacterial activity assay of *Bifidobacteriumbifidum* against Sallmonella spp., no zone of inhibition, b: overlay without any bacteria, C: Antibacterial activity of *Bifidobacteriumbifidum* against *E. coli*.

3.2. Antibacterial Activity test

Antibacterial activity was investigated using colony overlay assay according to the modified method of Jacobsen et al. (8). Briefly, overnight culture (10 7 CFU ml-1) of putative probiotic strains were inoculated at 5 μL spot on MRS agar plates (3 spots/plate) and incubated at 37°C for 24 hours under appropriate conditions, to allow colonies to develop. The plates were aerated for 20 minutes before overlaying with 10 mL of 0.7% (w/v) appropriate agar at 45 °C, previously inoculated with 10 μL (107 CFU mL −1) of an overnight culture of the indicator pathogen strain. MRS agar plates without any putative probiotic spots were also poured with 0.7% (w/v) containing 10 μL of the indicator pathogenic strain as a control. The plates were incubated aerobically at 37 °C, except the plates of *Clostridium difficile* which were incubated anaerobically at 30 °C. Inhibition zones around the spots at any of the incubation time points (8, 24 and 48 hours) were examined and scored. The experiment was carried out three times in duplicate (Figure 1).Among the tested strains, putative local probiotic strains that showed moderate- to high- anti-pathogenic effect were then selected for further study.

3.3. Comparison of Antibacterial Activity of Local and Commercial Probiotics

Local putative probiotics that had a mean antibacterial activity higher than 6 mm were selected and their activity were compared with those of the commercial probiotics. Among pathogens, probiotics are mainly used to prevent and combat Salmonella spp., *E. coli*, and Clostridium spp. infections (9). *Staphylococcus aureus* infection is also one of the most common infections among small flocks. Therefore, antibacterial effects of local and commercial probiotics on each pathogen were compared.

3.4. The Antibacterial Activity of Local and Commercial Probiotics against Important Birds Pathogens

3.4.1. Anti-Salmonella Activity

Anti-Salmonella effects of local and commercial probiotics were compared.

3.4.2. Anti-E. coli Activity

E. coli infection may cause a large variety of diseases, including yolk-sac infection of chicks, reproductive disorders and peritonitis in layers, and septicemia in growers. Anti-E.coli effects of local and commercial probiotics were compared.

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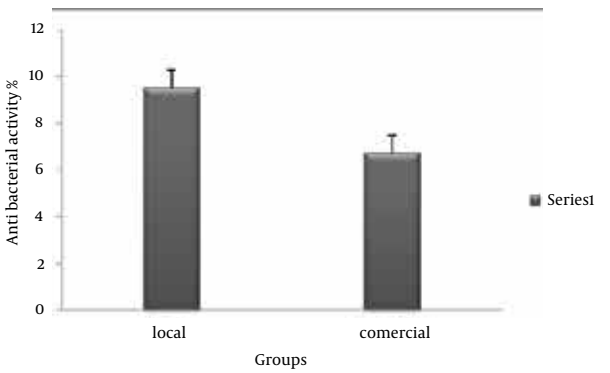

Figure 2. Antibacterial activity of local and commercial probiotic strains against reference pathogens using colony overlay assay. Inhibition zones were measured and statistically evaluated using student's t-test with *P < 0.05. Data are expressed as Mean ± SD.

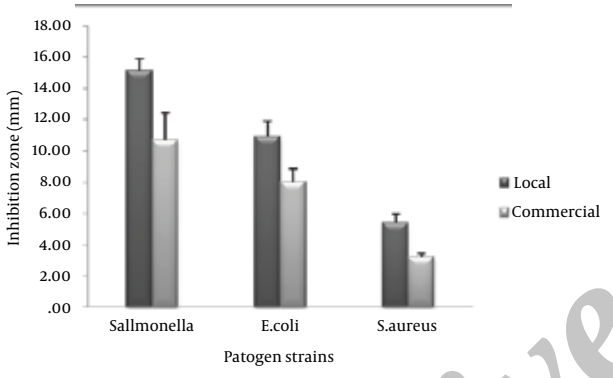

Figure 3. Antibacterial activity of local and commercial probiotic strains against three reference pathogens using colony overlay assay. Inhibition zones were measured and statistically evaluated using student's t-test with * P < 0.05, ** P < 0.005. Data are expressed as Mean ± SD.

3.4.3. Anti Staphylococcus aureus Activity

Staphylococcus aureus is widespread in the environment and causes a variety of opportunistic infections in poultry, especially in the tropical regions. Anti-Staphylococcus aureus effects of local and commercial probiotics were compared.

3.5. Statistical Analysis

The results are mean of experiments carried out in duplicate in three different occasions. Data are presented as mean ± standard deviation (Mean ± SD). The results were analyzed by Student's t-test to verify significant differences at the level of 5% (P < 0.05).

4. Results

The inhibition zones around the local potential probiotics were measured. Among them six strains that had mean antibacterial activity higher than 6 mm were selected to compare with commercial probiotics (Table 2).

4.1. Comparison of Antibacterial Activity of Local and Commercial Probiotics

Table 2 indicates the inhibition zone against pathogens. Taken as a whole, local strains of lactic acid bacteria had significantly higher antibacterial activity compared to commercial probiotics (Figure 2).

4.2. The Antibacterial Activity of Local and Commercial Probiotics against Important birds Pathogens

4.2.1. Anti *Sallmonella Activity*

In order to determine the anti-Sallmonella activity of local and commercial probiotics, colony overlay assay was used; the zone of inhibition against *Sallmonella spp*. is shown in Table 2. The anti-Salmonella activity of local probiotics was significantly higher than those of all commercial probiotics (Figure 3). *Lactobacillus acidophilus* had the highest activity against *Salmonella* species.

4.2.2. Anti E. Coli Activity

The anti-*E. coli* effects of local and commercial probiotic bacteria were also compared. The inhibition zones around the local potential probiotics against *E. coli* are shown in Table 2. Local probiotics had a significantly stronger effects on *E. coli* compared to all commercial probiotics (Figure 3). *Lactobacillus salivarius* Es1 had the highest activity against *E. coli*.

4.2.3. Anti Staphylococcus aureus

The anti-Staphylococcus aureus effects of local and commercial probiotic bacteria were also compared. Table 2 shows inhibition zone of local and commercial probiotics against *Staphylococcus aureus*. Local probiotics showed a significantly stronger activity against *Staphylococcus aureus* compared to all commercial probiotics (Figure 3). Lactobacillus *salivarius* Es11 had the highest activity against *Staphylococcus*.

5. Discussion

Antibiotics have been widely used in poultry industry at sub-therapeutic dose, usually in water or feed, for growth promotion, protecting animals against diseases, or therapeutically for treatment of diseases (10). Although such methods are responsible for improving productivity and healthier poultry breeds, the efficacy of antibiotics has been questioned as a result of the increased antibiotics resistant bacterial strains (10, 11). In the recent years, the probiotics used in poultry nutrition are being accepted as potential alternatives to antibiotics (11). After inclusion of probiotics in the diet, improvements in growth performance, favorable microbial balance in gut, and feed efficiency have been reported in broiler chickens (12). There are several

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proposed mechanisms that explain the modes of probiotics actions in poultry: 1. maintaining a beneficial bacterial population by "competitive exclusion" and "bacterial antagonism" (13), 2. improving feed intake and digestion (14), and 3. altering metabolism by increasing digestive enzyme activity, decreasing bacterial enzyme activity, and production of ammonia (12, 15). Chicken is a great example of a young animal which is deprived of getting in touch with its mother or other adults and therefore, it is likely to benefit from supplementation with probiotic preparations designed to restore the protective intestine micro flora (6). Probiotics are mainly used in birds, aiming to prevent and combat potentially pathogenic bacteria specially Salmonella spp., *Escherichia coli* and *Clostridium perfringens*, via competition for nutrients and adhesion sites on the intestinal epithelium, production of antimicrobial components such as bacteriocins, bacteriocin-like substance, hydrogen peroxide and volatile short chain fatty acids, decreasing the pH of the environment, and stimulation of immune system (9).To date, comparison of commercial and local probiotic against pathogens has not been reported. In the first step of this study, the antibacterial activity of local probiotics was compared with those of the commercial lactobacillus probiotics. Since Salmonella spp. are often transmitted to human from poultry and poultry products (3, 16), the poultry industry and public health agencies are focused on eradicating Salmonella species in live birds (17). In the second step, anti-Salmonella, anti-E. coli and anti-Staphylococcus effects of local and commercial probiotic were compared, respectively. The results showed that anti-Salmonella, anti-Staphylococcus and anti-E. coli effects of local probiotics were significantly higher than those of the commercial probiotics. Nouri et al. showed that *Lactobacillus salivarius* and *Lactobacillus crispatus* isolated from chicken gastrointestinal tract can suppress growth of *Salmonella enteritidis* (18). There are several studies which have shown a high antibacterial activity of bacteriocins produced by poultry probiotics against *Escherichia coli*, Salmonella spp., *Staphylococcus aureus*, *Clostridium perfringens*, and Campylobacter spp. (19, 20). Watkins and Miller further suggested that *L. acidophilus* increases competitive gut exclusion against harmful organisms (S. *typhimurium*, *S. aureus* and *E. coli*) in the intestinal tract of the chicken, as suggested earlier by Fuller (21-23). Edens et al. have shown that *L. reuteri* administration in ovo decreases the colonization of Salmonella species and *E. coli*, and also increases rate of intestinal colonization in both chicks and poultries. Furthermore, mortality because of in-hatcher exposure to *E. coli* or Salmonella spp. is reduced by *L. reuteri* (24). Recent studies have shown that *Lactobacillus salivarius* strains isolated from chicken gut could produce bacteriocins with antagonistic activity against Gram-positive bacteria (25). Morishita et al. demonstrated that lactic acid bacteria are more effective for poultry when they have chicken origin (26). Therefore, administration of locally isolated strains will improve digestive gut micro flora as well as combat pathogen infection among birds. In conclusion, administration of mono strain of *L. salivarius* ES1, or co-administration of ES1 and *L. salivarius* ES6, is not only more effective than commercial probiotics against Salmonella, Clostridium, Staphylococcus species and *E.coli*, but also, will have no negative effects on micro flora balance of local birds. Further in vivo studies are necessary to confirm the results.

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