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Survey of Probiotic *Bacillus* in Poultry Farm of Arak, Iran

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Probiotic bacteria with an ability to change micro flora of intestine play an important role in body's health. Probiotics are also suitable alternatives for antibiotics. The objective of this study is to obtain the indigenous *Bacillus* strains isolated from poultry farms of Arak city of Iran and to evaluate their probiotic properties. A total number of 41 samples of fresh fecal matters were collected from 8 poultry farms of Arak city. Probiotic properties of the strains were then determined (as for their resistance against acid, bile, pepsin, gizzard extract and production of antimicrobial compounds). In this study, a total number of 140 *Bacillus* isolates were screened of which 14 isolates were utilized for the purpose of preventing hemolysis in the forthcoming studies. Study of probiotic properties showed that strain 8 was resistant at 100% against Hydrochloric acid and strain 5 was resistant at 100% against bile salts. More than 80% of the selected strains were resistant to gizzard extract and pepsin. All strains possessed an antimicrobial activity against the common poultry pathogens. This study exhibited that the selected bacillary isolates possessed probiotic properties and they could be used as a poultry probiotic after thorough examinations and field tests.

Key words: Probiotics, Screening, *Bacillus*, Antimicrobial activity.

Today, one of the problems poultry industries is excessive use antibiotics for two purposes: 1. Growth induces, 2. Prevention and treatment infectious diseases. The growth stimulus is low dosage of antibiotics that suppression of harmful natural microbial flora and causes more nutrients absorption and more growth in animal.

For instance bacteria *Enterococcus hirae* is a part of intestine's natural microbial flora in poultry and it is nonpathogenic. However, this type of bacteria causes the thickening of lining in the walls of an animal's intestine through its activity, resulting in low absorption of nutrients and poor growth by thepoultry[1,2]. Growthinducing antibiotics restrain these bacteria. Also, for the purpose of preventing infectious diseases, a great number of poultry farmers introduce doses of various antibiotics to newlyhatched chicks' food rations from early days onward so they may reduce animals' chances of contracting bacterial diseases. This uncontrollable method of utilizing antibiotics has created numerous difficulties of which the most significant ones include: enhancement of resistance for poultry's pathogenic bacteria due to consistent and long-term contact with antibiotics thus resulting in higher mortality rate in animals, transference of drugs' resistance to human' or

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other animals' pathogenic bacteria via genetically exchanges and existence of great quantities of antibiotics in produced meat and eggs.In order to solve these sorts of problems and reduce the consumption of antibiotics in poultry farming industry, today in most regions of the world and particularly in advanced countries,probiotic products are consumed. The consumption of these products in Iran also, which are entirely of import nature, is on an increase[3,4].

The word "probiotic"which has Latin roots, means "for life" and it was first used around 100 years ago by Metchnikoff, a microbiology scientist. Today, this word defines a live microorganism entering an animal's digestive tract (such as human, cattle, poultry anaquatic) and it is capable of surviving and reproducing there having beneficent effects upon its host's health and survival[5]. The probiotic microorganisms are subdivided into three categories of bacteria, yeasts, and molds. The examples of each category are as the followings: *Lactobacillus acidophilus* for bacteria, *Saccharomyces boulardii* for yeasts, and *Aspergillus oryzae* for molds. Each species of animal possesses an array of specific probiotics for itself which originate from the microbial flora of the animal's digestive tract having perfect adaptability with the animal's physical and chemical metabolic conditions and being completely nonpathogenic[6].

Altogether, it is possible to conclude the effects of probiotics in the following phrases: controlling beneficial microbial population within digestive tract, increasing rate of receiving food and improving digestion and therefore promoting growth, causing change in microbial metabolism in the digestive tract, preventing the pathogenic and harmful microbes from inhabiting inside of the digestive tract, stimulating the immune system and neutralizing intestinal toxins[7]. Among all various types of bacterial probiotics used for poultries, the bacteria of genus *Bacillus* are able to withstand harsh environmental conditions and stay alive for long periods because of spore production. Experiments have shown that these bacteria are found at great frequencies in poultry feed and they are able to constant remain inside an animal's digestive tract and applying their beneficial probiotic effects[8].

Certain *Bacillus* strains are capable of producing biofilms and formation of these materials in the gastrointestinal tract of an animal can cause resistance against physical and chemical changes for these bacteria. Moreover, the existence of these biofilms in the gastrointestinal tract protects the animal against pathogenic agents' inclusion. *Bacillus subtilis* cells, for instance, are able to create a complex biofilm and sporulate under such condition. Spores from the bacteria genus *Bacillus* could also be utilized for processing probiotic products by whose advantages, with respect to the growth cells, it is possible to imply the followings: low production cost, facile production, resistance to production processes, and shelf life longevity in a wide temperature range due to production of spores[9,10].

In two separate studies, Nisbet and Silvia and their associates demonstrated that the spores of certain probiotic *Bacillus* isolates are resistant in conditions similar to the digestive tract and the bilious salts. One of the most notable properties of certain *Bacillus* isolates was their repelling capability against the pathogenic bacteria. The commercial products which underwent scrutiny did not have the strength to hinder the growth of the common poultry's pathogenic bacteria at all and this was while the isolated indigenous bacteria by producing the antimicrobial compounds conveniently prevented the pathogenic bacteria from growing[11,12].

The objective of this study is to isolate the indigenous *Bacillus* strains, which possess tremendous probiotic properties, from the poultry farms of Arak city in Iran, so that through their usage one is able to produce indigenous probiotic products which are of proper to be consumed in poultries and also decrease risks of epidemics of different diseases.

MATERIALS AND METHODS

Sampling

For this purpose after selecting 8 poultry farms (the poultry farms were randomly selected out of the total 72 farms in Arak city), a total number 41 samples were collected into tubes containing peptone water from chicks' fresh fecal matters using sterile swaps and the samples then were transported to the laboratory immediately.

Samples' enrichment

In order to eradicate bacterial growth and activate the spores, samples' enrichment was performed in the laboratory. Heat treatment was implemented for our purpose in such manner that the test tubes containing fecal samples were placed in water bath and they were subjected to the temperature of 80°C for 15 minutes[9,13].

Isolating and identifying bacteria

First, nutrient agar medium(Merk of Germany) was prepared and then the heat treated fecal samples were culture on the nutrient agar plates by streak method to purify the *Bacillus* isolates. After the growth process, the *Bacillus* isolates were diagnosed using gram stain, spore stain, catalase test, hemolysis test, blood agar, motility test, Potassium hydroxide3% and growth in 10% saline solution. Considering the aforementioned tests, we attempted to isolate gram positive, sporulating, catalase positive, and hemolysis negative *Bacillus* bacteria.

Preserving the bacteria

For the purpose of preserving the bacteria, different levels of concentrations of glycerol, (40% to 50%) in medium of BHI broth (Brain Heart Infusion broth, Merk of Germany) were used and they maintained freeze at -20°C. To prevent the cultures from contamination and to keep the bacteria in an active state, slant agars and repeated passages (one pass in every two weeks) were used and the bacteria were kept at refrigerator temperature.

Studying resistance against salt

A quantity of sodium chloride 10%(100gr/ Lit) was added to the nutrient agar and then the *Bacillus* isolates were cultured on plates containing salt and were incubated at 37°C for 48 h[11].

Studying resistance against Hydrochloric acid

To perform this test, BHI broth medium was created and then each one of probiotic *Bacillus* isolates was inoculated on them. The broth cultures were placed on shaker and incubated at 37°C for 24 h (preculture). In the next stage, 2 flasks for each single isolate were designated to be poured with 45 ml of BHI broth each. One of the flasks reached an acidic state at pH of 2 by adding Hydrochloric acid (Merk of Germany). In the following step after preculture, a volume of 5 ml of the obtained *Bacillus* isolates were inoculated into each of the flasks. Later, both flasks underwent Spectrophotometer testing at 600nm wavelength at 0 h, 2 h, 4 h, 6 h, and 24 h time intervals and their OD were measured and recorded (underlying basis for this task was turbidity testing)[14].

Studying resistance against bilious salts

This test was similar to the previous test with only this difference that instead of hydrochloric acid, bilious salts (sodium cholate, sodiumd eoxycholate) (Merk of Germany) having the quantity of 2gr/Lit at 1:1 ratio was utilized.

Studying resistance against pepsin

This test was similar to resistance to acid test, and the acid-treated flask with hydrochloric acid possessing the pH of 2 received a quantity of 1 mg/ml of pepsin (a protolyticenzyme present in chicken's stomach). 1 ml of the previously prepared preculture was added to both flasks and the contents were incubated at 37°C for 2 h and then bacterial plate count was done to the dilution of 10^{-10} using pourplate method[15].

Studying the resistance against gizzard extract

Contents of 5 chicken gizzards were collected and weighed and they were mixed with distilled water which weighed up to twice as much of the quantity their total weight. The mixture was centrifuged at 1000 rpm for 30 min and then separated supernatant and pH of its was measured. In the next phase, the supernatant was filtered using a sterilized 0.2 micron filter and then the obtained gizzard extract was freeze at -18°Cuntil the time of the usage. Next, a volume of 5 ml from the one night old *Bacillus* cultures were poured into each one of 2 sterilized tubes and they were centrifuged at 7000rpm for 10 min. The supernatants of both tubes were discarded and the *Bacillus* cells remaining deposit of one of the tubes was mixed with 1 ml of previously prepared gizzard extract and other tube was mixed with 1 ml of physiological saline serum and both tubes were incubatedat 37°C. Then their bacteria were counted by using pourplate method (up to the dilution of 10^{-10})[16].

Studying the production of antimicrobial compounds using Top Agar technique

The probiotic *Bacillus* isolates were blotted into nutrient agar plates using a bottom loop. After the passage of 24 h incubation when cultures had adequately grown, under

microbiological Laminar flow the plates' lids were removed and into eachplate'slid add a volume of 1 ml of formalin to kill the bacteria and release their antimicrobial compounds. The plates were maintained in the same condition for 15 min. Next, a 1% suspension of pathogenic bacteria (*E.coli* and *Salmonella* spp) in a melted nutrient agar was prepared and it was placed as layers on the blotted plates at a specified volume. The plates were incubatedat 37°C so that their antimicrobial effects of the *Bacillus* extract could be determined[17,18,19,20].

RESULTS

Sampling and purifying

Altogether, 41 samples from fresh chicken fecal matters were transported to the laboratory and after purification a total of 140 uncontaminated plates of *Bacillus* isolates were obtained.

Identifying the isolates

To identify the bacteria gram stain test, spore stain test, catalase test and antibiotic Vancomycin sensitivity tests were used. All of the isolates behaved as gram positive and catalase positive, contained spores and resistant to Vancomycin.To screen the pathogenic isolates, hemolysis test was conducted in blood agar medium(Merk of Germany) for which half of the isolates were positive hemolysis and some others that had a weak hemolysis at first and later completed full hemolysis after a period of 48 h. All the isolates possessing positive hemolysis were set aside and ultimately a total of 14 isolates with negative hemolysis remained upon which the forthcoming studies were conducted[21,22].

Studying the isolates' probiotic properties

The *Bacillus* isolates 1 through 14 that were previously separated from the whole were tagged arbitrarily and by this manner we studied the probiotic properties which are mentioned as the followings:

Studying the resistance of the separated isolates against salt

The isolates were cultured on a BHI medium containing 10% sodium hydrochloride and they were incubated at 37°C for 48 h. The results were as the followings (Table 1).

Studying the *Bacillus* capability of producing antimicrobial compounds against chicken's pathogens, *Salmonella* spp and *E.coli*,by using Top Agar technique

All the isolates exhibited antimicrobial activities against *E.coli* and *Salmonella* spp (the strains of pathogenic *E.coli*, and *Salmonella gallinarum* the cause of poultry typhoid and *Salmonella pullorum* the cause of poultry white bacillary diarrhea, which were separated from the carcasses of the poultry farms casualties). This activity was observed as the pathogens' zones of inhibition (the dilution for using the pathogens was at 0.5 McFarland by the *Bacillus* probiotics on the plates (Table 2)[17,18,19,20].

Studying the separated isolates' resistance against Hydrochloric acid and Bile

Isolates 5 and 8 in ranking order displayed the highest resistance against the acid and the bile. Isolates 4 and 13 had the lowest resistance against the acid and the bile. Findings from the tests are included in table 3[23].

Findings related to the resistance against pepsin

Isolates 7, 8, 10 and 12 showed promising results in the test for production of the antimicrobial compounds in relation to the acid and the bile and, therefore, only these 4 isolates were used in this test. Isolate 10 showed the lowest resistance and its growth in the presence of pepsin was limited. In contrast, isolates 7, 8 and 12 exhibited resistance against pepsin and their growth was not interrupted or limited. The results of the isolates' resistance against pepsin are displayed in table 4 where the percentage of isolates' resistance against pepsin

Table 1. Studying the resistance of the separated of isolates against sodium hydrochloride

Isolate	Resistance
1	+
2	-
3	-
4	-
5	-
6	-
7	-
8	-
9	+
10	+
11	+
12	-
13	+
14	+

is derived from dividing the number of grown colonies in normal medium over the number of the colonies grown medium containing pepsin. *B.licheniformis* was also used as the control strand in this test (Table 4)[15].

Findings related to the resistance against gizzard extract

4 isolates of 7, 8, 10 and 12 exhibited the highest degree of resistance in the production of antimicrobial compounds and also against the acid

Table 2. Comparison of the zones of inhibition for *E.coli* and *Salmonella* produced by the *Bacillus* probiotic isolates

Bacillary isolates	Diameter of zone of inhibition 1 *E.coli* (cm)	Diameter of zone of inhibition 2 *E.coli* (cm)	Diameter of zone of inhibition 3 *Salmonella*(cm)	Diameter of zone of inhibition 4 *Salmonella* (cm)	Average diameter of zones of inhibition 1 & 2 *E.coli* (cm)	Average diameter of zones of inhibition 1 & 2 *Salmonella* (cm)
1	5.1	5	4.8	4.6	5.05	4.7
2	4.6	5	4.9	4.6	4.8	4.75
3	4.9	4.7	4.6	4.8	4.8	4.7
4	4.8	4.6	4.2	4.4	4.7	4.3
5	4.7	4.5	4.4	4.4	4.6	4.4
6	4.7	4.8	5	4.9	4.75	4.95
7	3.5	4.7	4.7	4.5	4.1	4.6
8	4.3	4.9	4.5	4.3	4.6	4.4
9	4.3	4.5	4.9	4.8	4.4	4.85
10	4.7	4.8	4.5	4.7	4.75	4.6
11	4.6	4.7	3.9	4.1	4.65	4
12	3.7	4.1	4.8	4.4	3.9	4.6
13	4.1	5.1	4.3	4.7	4.6	4.5
14	4	4.1	4	4.1	4.05	4.05

Table 3. The separated *Bacillus* isolates resistance against Hydrochloric acid and Bilious salts (the resistance of the strains is described as the numerators)

Bacillary isolates	Treatment with Acid	Treatment with Bile	Bacillary isolates	Treatment with Acid	Treatment with Bile
1	41.6%	27%	9	28.42%	67%
2	72.2%	70%	10	33.3%	60%
3	28%	50%	11	45.6%	12.8%
4	21.2%	69%	12	81%	35.2%
5	66.6%	100%	13	72%	0
6	32.2%	20.6%	14	27%	43%
7	91.7%	74.5%	*B.subtilis*(control)	73%	69%
8	100%	70%	*B.licheniformis*(control)	76%	73%

Table 4. Studying the isolates' resistance against pepsin

Bacillary isolates	Number of colonies In environmentcontaining Pepsin	Number of colonies in normal environment	Percentage of resistance against Pepsin
7	44.25×10^6	65×10^6	68.07%
8	70×10^5	105×10^5	66.6%
10	32×10^4	32.6×10^6	0.98%
12	56×10^6	85×10^6	65.08%
B.licheniformis(control)	66×10^6	87×10^6	75.88%

and the bile and therefore, only these isolates were used in this test. Isolate 10 at 0.2% revealed the lowest degree of resistance against the gizzard extract whereas isolates 7, 8 and 12 displayed the highest degree of resistance compared to the control bacteria. Such great resistance is considered to be as a suitable property of a type of bacteria and therefore, such specific bacteria could be utilized as a probiotic. The produced results from the study of the isolates' resistance against the gizzard extract are presented in table 5 in which the percentage of resistance for the isolates was computed through dividing the number of grown colonies in the normal medium over the number of colonies grown in the medium containing gizzard extract. Needful to mention that *B.licheniformis* was also used as the control strain in this test (Table 5)[16].

Table 5. Studying the isolates' resistance against the gizzard extract

Bacillary isolates	Number of colonies In environmentcontaining Pepsin	Number of colonies in normal environment	Percentage of resistance against Pepsin
7	116.6×10^{10}	139.7×10^{10}	79.8%
8	70.5×10^{10}	85.7×10^{10}	82.2%
10	0.57×10^{8}	28.5×10^{8}	0.2%
12	133.1×10^{10}	170×10^{10}	78.3%
B.licheniformis(control)	70×10^{10}	88×10^{10}	80%

DISCUSSION

Probiotics are those live microorganisms such bacteria, yeast and mold that are capable of improving their hosts' life quality. These microorganisms are able to survive through body's immune barriers like those of the stomach and the gastrointestinal tract and finally settle in the small intestine and even on the host's body surface. By exploiting these microorganisms, a host's immune system is invigorated and its resistance against a great many diseases is boosted. Today, the probiotic industry in the world is making a speedy progress and various commercial products are made and marketed using different microorganisms. Recently in Iran, the use of probiotics has also been taken into consideration and commercial products of this nature are available for sale. The use of these products can be efficacious in converting the food ration factor into meat products. A matter which ought to be regarded as extremely significant is that, in addition to prevention of the hard currency flowing out of the country, production of probiotic products can also be, from the scientific aspect, of great value and from another aspect, the indigenous bacteria of Iran have gained an adaptability over time with the country's indigenous and domesticated animals' digestive tracts and they are most definitely capable of improving animals' quality of life[19,24-28].

In this study, 140 strains of indigenous *Bacillus* was isolated from the poultry farms located in Arak city in Iran and their growth and probiotic properties were compared to those of the isolated strands from the commercial products. Significant findings were produced regarding this field of research in such fashion that at the end of the tests, 3 *Bacillus* strains whose probiotic properties were greater than those belonging to the commercial ones and which had a greater growth rate.

A proper probiotic strain must be able to endure a host animal's body conditions and reach its target alive. The conducted tests indicated that strains 7, 8 and 12 respectively had the resistance rates of over 74.5%, 70% and 35.2% against the chicken bile and for the 2 Biochem strains (Feed product Company in Germany), *Bacillus subtillis* and *Bacillus lincheniformis*, these values were equivalent to 69.3% and 73.26% respectively. This fact reveals that the indigenous strands possess a proper resistance capability against the bile and they are able to make passage through it and reach the animal's intestine in adequate numbers.

Of other conditions against which a probiotic bacterium must be resistant is the acidic environment along with pepsin inside a chicken's stomach. The tests indicated that isolate 7, 8 and 12 were resistant to pepsin at approximately 68.07%, 66.6% and 65.08% respectively, while these values

for Biochem strains, *Bacillus subtilis* and *Bacillus lincheniformis* (the samples that were used as controls) were equivalent to 72.96% and 75.88%, respectively. This fact is quite transparent that the indigenous strands possess a proper percentage rate of resistance in surviving the chicken's stomach environment. In another experiment, it was determined that each single one of the 5 strains had the capability of growth in Hydrochloric acid with a pH equaling 1. One of the most important properties of the mentioned strains was their ability to inhibit the growth of the chicken's pathogenic bacteria. The conducted experiments in the manner of in vitro exhibited that these strains are able to prevent *E.coli* and *Salmonella* spp bacteria from growing, seeming that this kind of activity occurs with the production of bacteriocin. The 2 Biochem strains did not display an antibacterial activity against the challenged strains.

By considering the obtained findings from this study, the essential probiotic properties enabling a bacterium to be used as a probiotic and the similar studies performed by the others, it can be tremendously likely to suggest isolates 7, 8 and 12 as the proper probiotic bacterial strains for poultry so that with some supplemental tests such as invivo, safety evaluation and etc. we will be able to exploit them as the indigenous probiotic products.

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