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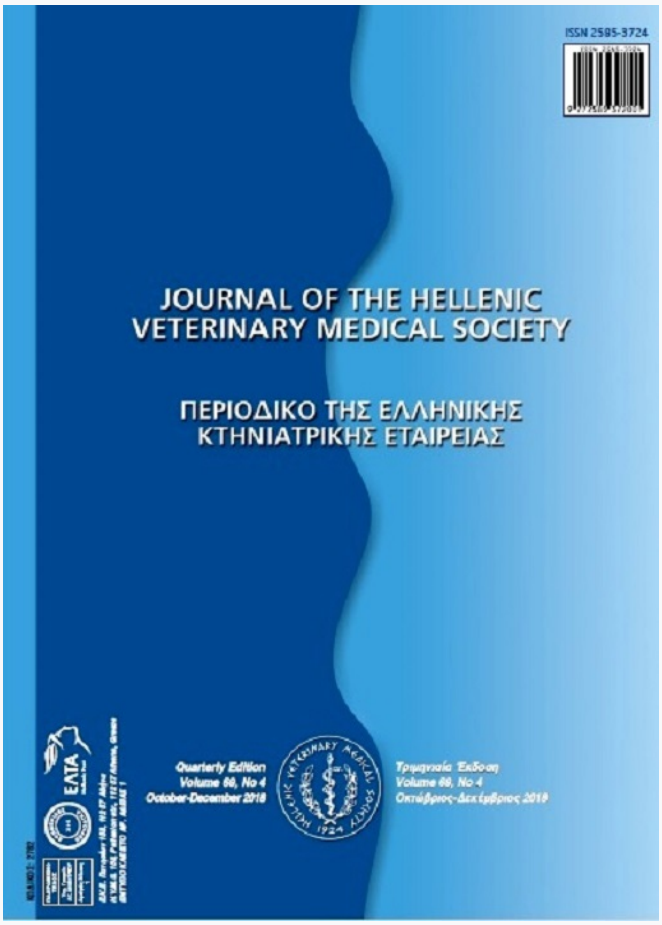

Evaluation of Novel Probiotic Bacillus Strains Based on Enzyme Production and Protective Activity Against Salmonellosis

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Research article
Ερευνητικό άρθρο

Evaluation of *Bacillus* Strains as Probiotic Based on Enzyme Production and In Vitro Protective Activity against Salmonellosis

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ABSTRACT. Probiotic strains of *Bacillus* spp. are used in industrial poultry production because of their ability to produce enzymes enhancing the absorption of nutrients and to reduce the risk of *Salmonella* spp. infection. The aim of this study was to isolate native potential probiotic *Bacillus* spp. with the ability to produce enzymes and adhere to intestinal epithelial cells in order to prevent *Salmonella* Typhimurium infection. First, 25 samples of chicken feces were collected from 7 industrial poultry farms in Golestan province located in Northern Iran. *Bacillus* species from samples were isolated on nutrient agar. These strains were evaluated for the ability of producing amylase and phytase and their probiotic characteristics such as bile salt, acid and antibiotic resistance, the ability to attach to intestinal epithelial cells and inhibit *Salmonella* Typhimurium invasion. Then selected isolates were identified based on *16S rDNA*. Results showed that from 86 isolated, 4 *Bacillus* strains had desirable characteristics such as the ability to produce phytase and amylase and having suitable probiotics features. We identified K03, K02, and K20 isolates as *Bacillus tequilensis* and K20 as *Bacillus subtilis*. *Bacillus tequilensis* K03 showed the highest attachment ability to intestinal epithelium cells and could inhibited *Salmonella* Typhimurium attachment.

Keywords: Attachment, *Bacillus*, Poultry, Probiotic, *Salmonella*

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INTRODUCTION

Chicken intestinal infections result in decreased production, increased mortality and cause decline in food economics and safety (Natsos *et al*., 2016). Using probiotics can improve feed conversion ratio (FCR) by preventing intestinal diseases in chicken (Papatsiros *et al*., 2013; Knarreborg *et al*., 2008). The World Health Organization (WHO) defined probiotics as "live micro-organisms that when administered in adequate amounts, confer a health benefit on the host" (Jorgen, 2005).

The characteristics of a bacteria that could be identified as probiotic are: non-pathogenic, resistant to gastric acidity and bile salts while passing through the digestive system, having the ability to produce digestive enzymes, facilitative for the digestion and absorption of nutrients, competitive with important pathogenic bacteria such as *Salmonella* spp. in attachment to intestinal epithelium cells, and being able to neutralize internal toxins (Gaggìa *et al*., 2010).

Among the probiotics used in chicken diets, the *Bacillus* spp. can withstand environmental conditions and survive for a long time by producing spores (Nicholson, 2002). It has been shown that species of the genus *Bacillus*, in addition to naturally occurring in soil, are found at high levels in chicken feces (Nicholson, 2002). The bacteria can remain stable in the gastrointestinal tract of the chicken and have probiotic beneficial effects (Nicholson, 2002).

Some of the *Bacillus* spp. is capable of producing biofilm in animal intestines helping the bacteria to resist changes and stresses and also to protect the intestine against attachment of the pathogenic bacteria such as *Salmonella* spp. to the intestinal epithelial cells (Thirabunyanon and Thongwittaya, 2012).

The *Bacillus* spp. have many applications by producing enzymes such as α-amylase and phytase (Lee *et al*., 2012). These enzymes can decompose anti-nutrient agents in food, increase nutrient bioavailability, break down some chemical bonds, strengthen the indigenous enzymes and finally increase production efficiency (Lee *et al*., 2012).

The major source of phosphorus in plant-derived foods, especially in cereal grains, is phytate phosphate (Askelson *et al*., 2014). It accounts for 50 to 80 percent of total phosphorus in grain and legumes, respectively (Askelson *et al*., 2014). Phytase can help releasing the phosphorus in chicken's intestinal track and making it available for absorption (Askelson *et al*., 2014).

Starch is the most abundant combination of carbohydrates in cereals, such as wheat and corn (Latorre *et al*., 2016). The presence of amylase producing bacteria in broiler diets helps the digestion of insoluble starch (Latorre *et al*., 2016).

The *Salmonella* spp. is one of the major foodborne pathogen in poultry industry which also causes infection in humans (Mouttotou *et al.,* 2017).

Using probiotics capable to attach to intestinal epithelial cells and compete with *Salmonella* spp. is a safe alternative method to antibiotic therapy (Thirabunyanon and Thongwittaya, 2012).

The purpose of this study was to isolate and identify potential native and suitable probiotic *Bacillus* spp. bacteria in Golestan province of Iran with the ability to produce enzymes and high affinity to intestinal epithelial cells. These selected native probiotic *Bacillus* spp. bacteria could be used in poultry diets to improve the production quality, reduce antibiotic usage, lower the incidence of various related diseases and to prevent *Salmonella* Typhimurium infection in human and poultry.

MATERIALS AND METHODS

Sampling and Isolation of *Bacillus* strains

Twenty five samples of chicken feces were collected from 7 farms located in Golestan province in Northern Iran, with the capacity of rearing 20,000 chicken each farm. These farms were under windowless management with full automatic feeding system and were not using any kind of antibiotics in their diet. The specimens were collected in peptone water and were transferred to the laboratory within 2h under sterile condition. In order to omit vegetative and non-spore forming bacteria, samples were put in 90˚C bain-marie for 30 minutes. *Bacillus* isolates were insulated on nutrient agar (QueLab-393506, Canada) plates, after 48 hours of incubation at 37˚C. Isolates were evaluated by Gram staining, sporulation staining, catalase and hemolysis tests (Barbosa *et al*., 2005).

Determination of α-amylase enzyme activity

All *Bacillus* strains were grown in tryptic soy broth (TSB, Merck-105459-0500, Germany) at 37˚C for 24 hours. Then 10 µl with 10^8 cfu/mL of each strain were

placed at the center of starch agar (Merck-1.01252.1000, Germany). After incubation, the α-amylase producer strain was selected by starch solubilisation zone around the colony (Latorre *et al*., 2016). Amylase activity was measured by DNS method. The reaction mixture containing 1% soluble starch, 0.1 M Tris/HCl buffer (pH 8.5), 0.5 ml enzyme solution was incubated at 37˚C for 30 minutes. The reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) then heated for 10 minutes in boiling water and cooled in 4˚C. For estimating the enzyme activity, glucose standard curve was drown (Nwokoro and Anthonia, 2015).

Determination of phytase enzyme activity

The ability to produce phytase enzyme was screened by using phytase specific medium (PSM). The pure cultures were placed at the center of PSM agar and incubated at 37˚C for 62 h. After incubation, the strains with the ability to produce phytase enzyme were selected by a clear zone around the colony (Kumar *et al*., 2013). For phytase activity measurement, 0.1 ml of enzyme solution, 0.9 ml of 2 mM sodium phytate and 0.1M Tris-HCl buffer (pH 7) were used. Incubation was done at 37˚C for 10 minutes. The reaction was stopped by adding 0.75 ml of 5% tricholoroaccetic acid. The released phosphate was measured by adding 1.5 ml of color reagent containing 2.5% ammonium molybdate solution, 5.5% sulfuric acid and 2.5% ferrous sulfate solution (Demirkan *et al*., 2014).

Bile salts and acid resistance

Resistance of selected bacteria to bile salts and acid were measured according to Razmgah *et al.* (2016) and Cenci *et al.* (2006) respectively.

Antibiotic resistance of *Bacillus* strains (MIC method)

The antibiotic resistance pattern of selected *Bacillus* strains was determined according to Sorokulova *et al.* (2008).

Attachment and invasion assay of *Bacillus* spp. and *Salmonella* Typhimurium to the intestinal epithelial cells

Cells of the intestinal epithelial cell line (Caco2) were cultured in dulbecco´s modified Eagle´s minimal medium (DMEM; Sigma-Aldrich, USA) adding 10% fetal calf serum inactivated by heating at 56ºC for 30 min, 1% (v/v) L-glutamine and 1% Streptomycin (10 mg/ml and 10-103 IU/ml). Cells were incubated in 5% CO_2 at 37ºC and attachment and colonization tests were done according to Thirabunyanon and Thongwittaya (2012).

The ability of *Bacillus* spp. to inhibit *Salmonella* Typhimurium attachment to Caco-2 cells was assayed by two methods of exclusion and competition, after Caco-2 monolayers at 90% confluence in a 12-well plate were washed twice with PBS (phosphate buffered saline, pH 7.4) (Zhang *et al*., 2010).

In the exclusion assay, Caco-2 monolayers were inoculated with 300 µl of *Bacillus* spp. suspension (10^7 CFU/well) in DMEM medium and incubated in 5% CO_2 at 37ºC for 1 h then 100 µl of *Salmonella* Typhimurium suspension (10^7 CFU/well) in DMEM medium was added. Finally incubation was done in 5% CO_2 at 37ºC for 1 h.

In the competition assay, Caco-2 monolayers were inoculated with 400 µl of *Bacillus* spp. suspension (10^7 CFU/well) and *Salmonella* Typhimurium suspension (10^7 CFU/well) in DMEM medium and incubated in 5% CO_2 at 37ºC for 1 h.

Molecular identification based on *16S rDNA*

The DNA of selected *Bacillus* strains was extracted using the lysozyme enzyme digestion according to Araújo *et al.* (2004). For amplification of a 1500 bp fragment from *16S rDNA* region was performed with universal primers, 27F-5/ -AGAGTTTGATCCTGGCTCAG-3/ and 1492R-5/ - ACGGCTACCTTGTTACGACTT -3/.The 25 µl PCR reaction mixture consisted of 200 ng template DNA, 2.5 µl of PCR 10x Buffer, 200 µM dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primers and 1 unit of Taq DNA polymerase enzyme and distilled water. Amplification cycling included primary denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing of primer at 58 °C for 1 minute, extension at 72 °C for 30 seconds, and final extension at 94 °C for 5 minutes. Then, the PCR products were electrophoresed on 1.5% agarose gel. The 100 bp molecular weight marker (Sina-Clon, Tehran, Iran) was used as a molecular marker. After purification of the PCR product from the agarose gel, the samples were sent for sequencing to Bioneer Company (Daejeon, Republic of South Korea) (Araújo *et al*., 2004).

Statistical and phylogenetic analysis

Data obtained from sequencing were edited by bioinformatic software of Chromas Pro. and saved in FASTA format. Then GenBank of EzTaxon database was used to identify the bacteria (Chun *et al*., 2007). The phylogenetic tree was drawn by MEGA 5 software and neighbor-joining method (Saitou *et al*., 1987., Tamura *et al*., 2011). The statistical data analysis was done using GraphPad Prism v7.03 statistical software for selected *Bacillus* strains.

RESULTS

Isolation of *Bacillus* spp.

Spore-forming bacteria were selected by heat treatment of chicken feces. Thirty-four pure colonies were selected for further analysis. These strains were named K1_K34. All of the isolates were catalase-positive, oxidase-positive and non-hemolytic.

α-amylase enzyme assay

K03 strain was the only isolate with a significant zone of clearance on starch agar and the ability to produce α-amylase enzyme (Figure 1). Using the equation, the percentage of starch solution by selected *Bacillus* strain was calculated. Also, by drawing the standard curve (Figure 2), the linear regression equation (Equation 2) was used to calculate the production of α-amylase by the strain (Equation 3) (Table 1).

% soluble starch = (OD control-OD sample) / OD control * 100 (Equation 1).

Y = 0.0001234 * X + 0.05034 (Equation 2).

Where Y = OD supernatant, X = starch concentration remain.

X-total starch: starch concentration consumption

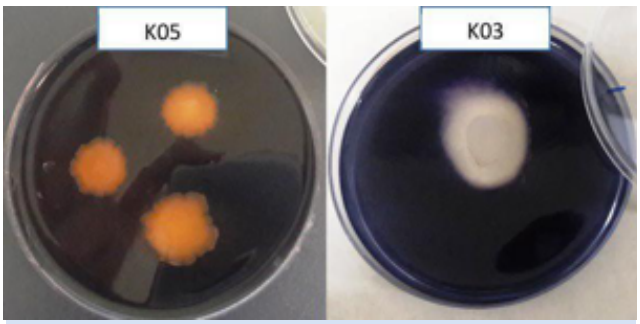


Fig 1: *Bacillus* spp. with the ability to produce the α-amylase enzyme: K03 (+), K05 (-)

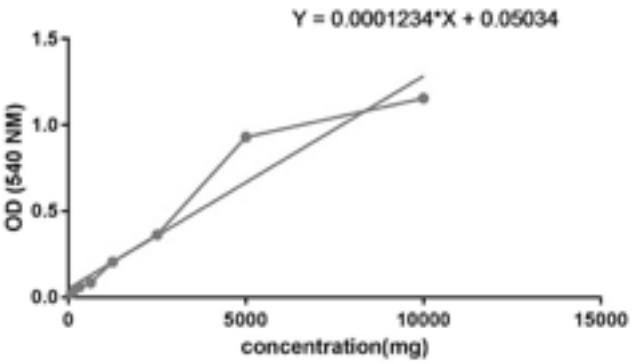


Fig 2: α-amylase enzyme standard curve

Table 1. Amount of α-amylase enzyme production

Starch solution (%)	Produced α-amylase enzyme (u/ml)	Strain
60.62	36.7±1.3	K03

Amount of amylase (u/ml) = Starch concentration consumption / 10min (Equation 3).

Phytase enzyme assay

Four *Bacillus* strains of K03, K02, K20 and K10 showed a clear zone in plate assay.

Percent of dissolution capacity of phosphorus was investigated in these strains (Equation 4). The standard curve was drown by standard phytase (Figure 3) and the linear regression equation (Equation 5) was prepared and the rate of phytase production by strains (Equation 6) was calculated (Table 2).

Percent of dissolution capacity of phosphorus = (OD control-OD sample)/OD control*100 (Equation 4)

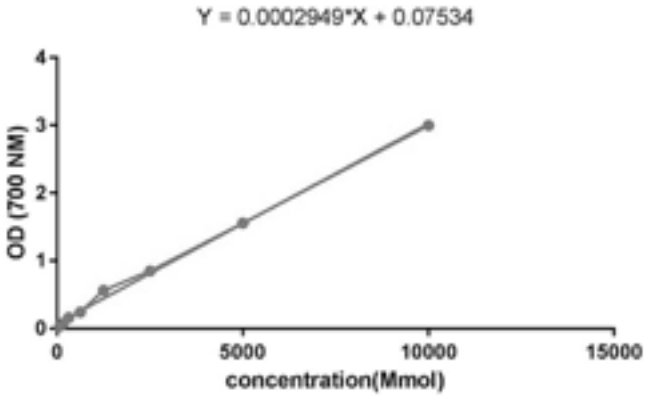


Fig 3: Phytase enzyme standard curve

Table 2. Amount of phytase enzyme production

Phosphorus solution (%)	Phytase production (u/ml)	Strain
45.97	22.33±1.2	K10
33.15	15.95±1.8	K02
30.28	14.53±2.3	K20
10.25	4.56±1.1	K03

Standard linear regression formula: Y = 0.0002949 * X + 0.07534 (Equation 5)

Y = OD supernatant

X = Calcium phytate concentration remains

Total Calcium Phytat-X = Calcium phytate concentration consumption

Amount of phytase (u/ml) = Calcium phytate concentration consumption / 30min (equation 6).

Tolerance to bile salts and acid

All strains which produced enzymes, were resistant to bile salts. Among these strains, the 2 strains of K03 and K20 showed the highest resistance to bile (Figure 4).

Table 3. Influence of pH on growth parameters of *Bacillus* strains

pH			Strain
7	4	2	
7.246±0.2457	6.554±0.04846	6.322±0.2803	K03
7.540±0.06247	6.253±0.2526	6.429±0.1276	K02
8.517±0.4375	7.751±0.02694	6.301±0.3010	K20
8.246±0.2457	7.440±0.1617	6.246±0.2457	K10

All strains of *Bacillus*-producing enzymes grew in pH 2, pH 4 and pH7 (Table 3).

Antibiotic resistance analysis

Bacillus strains were sensitive to all antibiotics listed by EFSA in 2012 (Table 4).

Adhesion capability of *Bacillus* spp. to intestinal epithelial cells

The adhesion ability of *Bacillus* spp. to intestinal epithelial cells is shown in Figure 5. Affinity ranged between 1.3-1.9 log CFU/well among the isolates. K03 strain showed the highest adherence ability to intestinal epithelial cells (Figure 6).

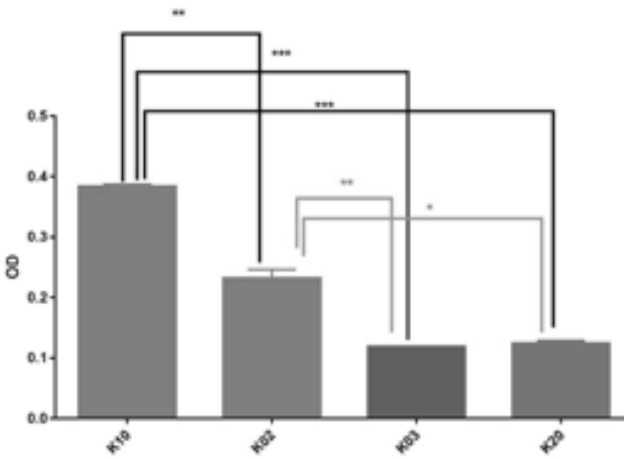
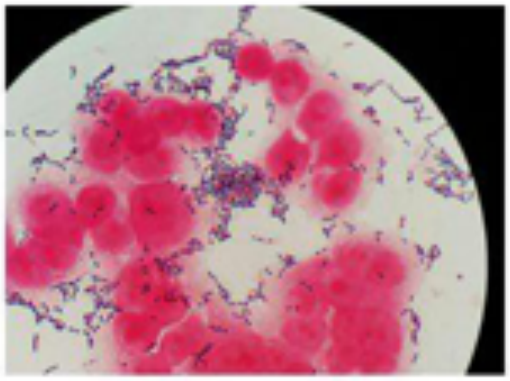


Fig 4: Bile tolerance of selected *Bacillus* spp.
*: P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001

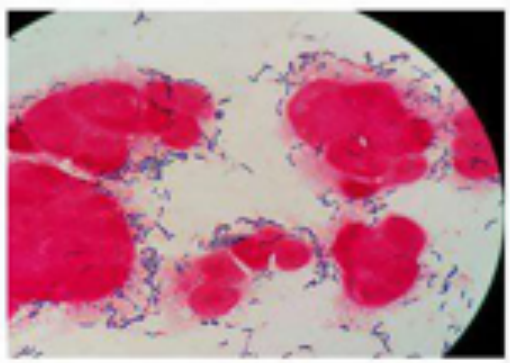
Table 4. Antibiotic resistance of *Bacillus* strains (MIC method)

Antibiotic (μg)	K02	K03	K10	K20	EFSA 2012 *Bacillus* spp. mg/L
Ampicillin (10)	16	15	16	14	n.r.*
Vancomycin (30)	1	2	1	1	4
Gentamycin (10)	1	1	1	1	4
Kanamycin (30)	3	2	4	2	8
Streptomycin (10)	4	4	3	4	8
Erithromycin (15)	2	1	1	2	4
Clindamycin (2)	1	1	3	2	4
Tetracyclin (2)	2	2	1	1	8
Chloramphenicol (30)	4	3	4	1	8

* not required



A



B

Fig 5: Attachment ability of *Bacillus* spp. to intestinal epithelial cells (A:K03/B:K02)

Inhibitory capability of *Bacillus* spp. against *Salmonella typhimurium* adherence to intestinal epithelial cells

The results showed that all the selected *Bacillus* spp. have inhibitory effects on the attachment of *Salmonella* Typhimurium to Caco-2 cells. Among them K03 strain showed more inhibitory strength than the others in both methods of exclusion and competition assay (Table 5).

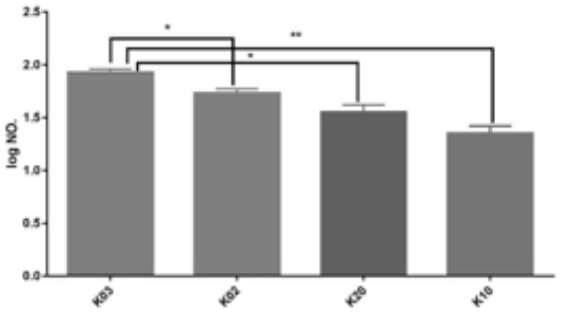


Fig 6: The adherence activity of Bacillus spp. to intestinal epithelial cells *: $P \leq 0.05$; **: $P \leq 0.01$

Phylogenetic identification of *Bacillus starins*

The analysis of 16S rDNA gene similarity showed K10 strain had the highest similarity to *Bacillus subtilis* and K03, K02 and K20 strains closely belonged to *Bacillus tequilensis* (Figure 7). Both strains are probiotic bacteria (Thirabunyanon and Thongwittaya, 2012; Parveen *et al*., 2016).

DISCUSSION

In vivo models of probiotic applications of *Bacillus* spp. have shown that these bacteria can enhance the absorption of food and have protective effects against infections (Ouwehand *et al*., 2002). In this study *Bacillus* strains were isolated from chicken feces with the specific ability of enzyme production and inhibition of invasive *Salmonella* Typhimurium.

Table 5. Evaluation of inhibition of *Salmonella* Typhimurium attachment to Caco-2 cells by exclusion and competition methods

Strain	Exclusion experiment *S.*Typhimurium (CFU/well *10^5)	Competition experiment *S.*Typhimurium prevented from adherence to Caco-2 (%)	*S.typhimurium* (CFU/well *10^5)	*S.*Typhimurium prevented from adherence to Caco-2 (%)
*S.*Typhimurium (control)	2.46±0.18		2.53±0.37	
K03	1.16±0.23*	53	2.18±0.18*	14
K02	1.64±0.31*	34	2.29±0.29	10
K20	1.93±0.12*	22	2.28±0.19	10
K10	1.95±0.23*	21	2.30±0.21	10

Data are mean standard deviation of three independent experiments, *S.* Typhimurium alone served as a control. Asterisk (*) indicates means which were significantly different from the control value ($p \leq 0.05$). % prevented from adherence to Caco2 cells = (control- test)/control

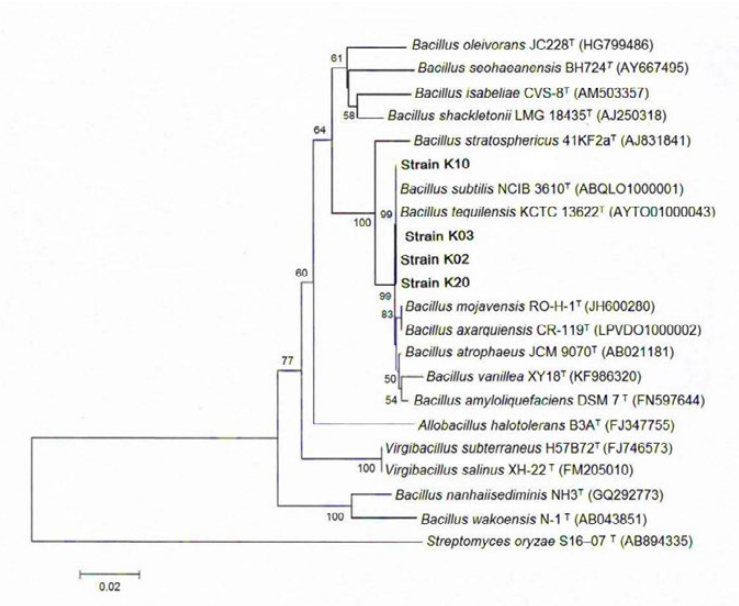


Fig 7: The phylogenic tree of *Bacillus* spp. Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences, showing the position of Bacillus strains and other related genera. GenBank accession numbers are given in parentheses. The 16S rDNA gene sequence of the Streptomyces oryzae S16-07T was used as outgroup. Bootstrap values (%) are based on 1000 replicates. Bar, 0.02 substitutions per nucleotide position.

Khusro et al. (2017) isolated α-amylase producing *Bacillus* strains from chicken, and increased the amount of amylase enzyme production to 136.71 IU/ml by optimizing the culture conditions.

Latorre et al. (2016) isolated 31 strains of *Bacillus* from chicken, which could produce amylase, phytase, protease and lipase enzyme. In the current study, 4 *Bacillus* isolates from chicken feces with the ability to produce phytase enzyme were isolated. From these strains K03 was also able to produce amylase enzyme. Among them the K10 strain had the highest ability to produce phytase enzyme at 22.33 ± 1.2 IU/ml and K03 strain was the superior bacterium in production of 4.56 ± 1.1 U/ml phytase and 36.7 ± 1.3 U/ml α-amylase enzymes.

Seeber et al. (2015) reported that among 69 *Bacillus* isolated from broiler chickens, only three isolates were able to tolerate bile salts with a concentration of 0.037% and acidic conditions (pH 2.0).

Mingmongkolchai and Panbangred (2017) isolated 187 *Bacillus* strains from fresh milk of cattles, pigs and calves, and reported that 7 strains had the ability to produce phytase, cellulase, xylanase enzymes and had the most compatibility with intestinal conditions. The results of the present study showed that all strains of *Bacillus*-producing enzymes were able to withsand bile salts with a concentration of 0.03% and acidic conditions (pH 2.0, 4.0), which were consistent with mentioned studies.

Khusro and Aarti (2015) isolated the strains of amylase producing *Bacillus* from chicken feces. The 16S rDNA identification showed that 5 strains that had the ability to produce amylase enzyme were closer to *Bacillus tequilensis*, *Bacillus subtilis* and *Bacillus licheniformis*, which were further selected to optimize the production of amylase enzyme. In this investigation, phylogenetic studies showed isolated *Bacillus* strain had the highest relationship with *Bacillus subtilis* and *Bacillus tequilensis.*

Thirabunyanon and Thongwittaya (2012) reported that among 117 bacilli isolated from chicken intestines, 10 isolates had inhibitatory abilities against 7 pathogenic bacteria, including *Salmonella* spp. and also had the ability to attach to Caco-2 cells in a variety of conditions ranging from 2.8-4.9 logCFU/well. In the present study a novel probiotic *Bacillus tequilensis* K03 had the highest attachment ability to Caco-2 cells and showed the highest inhibition of *Salmonella* Typhimurium, up to 53% compared to control. According to Kizerwetter-Swida and Binek (2006) the bacteria with a high attachment ability to the Caco-2 cell has a higher ability to inhibit pathogenic bacterial attachments. These results are consistent with a study done by Jankowska and Laubitz (2008) that found a new probiotic bacteria belonged to *Bacillus tequilensis* FR9 acquired from chicken digestive tract, which was capable of inhibiting *Listeria monocytogenes*.

CONCLUSION

In the present study, we isolated a novel starin of *Bacillus tequilensis* K03 from chicken feces that was a potent producer of amylase and phytase with efficient protection activity against *Salmonella* Typhimurium infection *in vitro*. It was resistant to bile and acidic environment of intestinal track. Further investigation is needed, to evaluate its in vivo use as a native probiotic in chicken diets in order to improve the production

quality, reduce antibiotic consumption, lower the incidence of salmonellosis in broiler chickens and human and its native to the region.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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