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BACILLUS PUMILUS ISOLATED FROM HEALTHY HONEY BEES AND ITS EFFECTS ON SOME BIOLOGICALASPECTS IN MICE

Abdulaziz A.Y. Abbas*[1] and Abdelmalek M. Amran[2]

[1]Department of Biotechnology and Food Technology, Faculty of Agriculture and Veterinary Medicine/Thamar, University of Thamar, Yemen.

[2]Department of Veterinary Medicine, Faculty of Agriculture and Veterinary Medicine/Thamar, University of Thamar, Yemen.

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***Corresponding Author**
Abdulaziz A.Y. Abbas
Department of Biotechnology and Food Technology, Faculty of Agriculture and Veterinary Medicine/Thamar, University of Thamar, Yemen.

ABSTRACT

Several bacterial Strains belonged to *Bacillus pumilus* have been isolated and identified from several natural sources. Some these the strains are widely applied in industrial microbiology such as probiotics. The aim of the present work was to evaluate the biological activity of *B. pumilus* isolated from the digestive tract of healthy honey bees as probiotics. The isolation and purification were done using nutrient agar then the primary and secondary identification were performed by HiBacillus TM Identification Kit and 16S rRNA analysis. Procedures and experiments were performed using 60 adult healthy male mice Mus musculus, which were grouped into three groups, the first group was control while the second and third were treated with sterile normal saline solution and *B. pumilus* suspension of *B. pumilus* respectively. The results showed that the digestive tracts of honey bees that used in this work contained B. pumilus strains. The data obtained from the animal testing reported that the *B. pumilus* strain used in this study have a positive impact on the tested biological parameters. The study concluded that the selected *B. pumilus* strain has a role in a role in improving the health status of mice.

KEYWORDS: *Bacillus pumilus*, Honey bees, probiotic, mice, biological parameters.

1. INTRODUCTION

A *Bacillus pumilus* is a Gram-positive, aerobic, spore-forming bacillus belonging to Bacillus subtilis group. *B. pumilus* is widely used in industrial microbiology, such as the production of

many of fermented foods, based on its efficiency to degrade a lot of the pollutants, it used in the treatment of wastewater and the degradation of environmental pollutants. *B. pumilus* have the ability to produce several biological compounds such as vanillin, keratinase, xylanase, alkaline serine protease and several other bioactive substances.[3,4,10,14,16] A lot of scientific reports confirmed that there are several strains of *B. pumilus* have Symbiotic relationships with some plants leading to promote growth of plant by improving the nitrogen fixation, uptake of nutrients, producing antibacterial and antifungal agents against plant pathogenic microbes and by reducing metal toxicity.[7,12]

Numerous *B. pumilus* strains isolated from several environmental sources were applied as microbial probiotics for animals.[6] However, numerous *B. pumilus* are harmful to human, animals, and plants. Some the strains have been isolated associating with human and plant diseases, and some the trains have the ability to produce toxins that cause some diseases in human.[9] A pathogenic *B. pumilus* strains have been studied by[18], the results confirmed that the strains were lack of phage-ressitant system and suggested that bacteriophages could be a potential biological agent for control of the pathogenic *B. pumilus* strains. The present study was aimed to isolate a novel *B. pumilus* strain to evaluate its ability as bacterial probiotics in animals.

2. MATERIALS AND METHODS

2.1. Isolation of microorganisms

B. pumilus associated with the digestive tract of healthy honey bees was isolated using 100 honey bees. The digestive tracts of honey bees were extracted according to[17], and a sterilization of outer surface of the extracted digestive tracts was done using ethanol solutions (70%) and sodium hypochlorite solution (7%). Washing of the outer surface of the sterilized digestive tracts was performed three times by normal sterile saline solution (0.89% NaCl). The sterile digestive tract was homogenized in phosphate buffer saline (pH, 7.2) then decimal serial dilutions were done using the normal sterile saline solution. 0.1 ml of each dilution were spread on a surface of a nutrient agar plate (Oxoid, UK) and aerobically, incubated at 30 ±2 °C for 48 hours. Each single colonies on the surface of the growth medium were purified on a new nutrient agar plate, the purification was replicated three times to obtain a pure culture. All single (pure) cultures were stored at - 80°C using sterile nutrient broth containing sterile glycerol (30%).

2.2. Primary identification

The isolated and purified bacteria were stained using Gram method and microscopic characteristics were observed using a digital light microscopy (Motik, China). To perform the primary identification, a HiBacillus™ Identification Kit (Himedia, india), which includes 12 tests (malonate, voges Proskauer's, citrate, o-nitrophenyl-β-d-galactopyranoside (ONPG), nitrate reduction, catalase, arginine, sucrose, mannitol, glucose, arabinose, trehalose) for identifications of *Bacillus* spp. was used following to manufacturer's instructions.

2.2.1. Identification by 16S rRNA analysis

To obtain a bacterial template DNA, a single and pure cultivated bacteria are used. A pure colony of the cultivated bacterium was selected and suspended in 0.5 ml of sterile normal saline solution (0.89% NaCl) using centrifuge tube (1.5 ml). The bacterial suspension was centrifuged at 10,000 rpm for 10 min then the supernatant was disposed in ethanol solution (70%), after that the pellet was resuspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). The suspension of pellet was incubated at 56°C or 30 min then heated at 100°C for 10 min using a water bath. To perform a polymerase chain reaction, 1 µl of template DNA was treated with 20 µl of a PCR reaction solution using AGAGTTTGATCMTGGCTCAG (27F) and TACGGYTAC CTTGTTACGACT T (1492R) according to.[5] The amplification program performed 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragments (~1400 bp) were amplified from the bacteria. A positive control (Escherichia coli genomic DNA) and a negative control were included in the PCR. To purify the PCR products, unincorporated PCR primers and dNTPs from the PCR products were removed using a Montage PCR Clean up kit (Millipore). The purified PCR products of approximately 1,400 bp were sequenced using CCAGCAGCCGCGGTAATACG (518F) and TACCAGGGTATCTAATCC (800R) primers according to.[11] Sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

2.3. Procedures and experiments

60 adult healthy male mice Mus musculus domesticus were selected to obtain bodyweight 25±1 gm then used in the present biological experiment. All mice were grouped into three groups (20 mice for each group), the first group (control group) was dieted a standard mice diet, the second group was dieted with standard mice diet and treated with 0.20 ml of sterile

normal saline solution (Orally, the solutions was given daily), the third group was treated as in the second with the replacement of the sterile normal saline solution with the bacterial suspension (The bacterial suspension was prepared from pure culture of *Bacillus pumilus* in a sterile normal saline solution (106 cell/ml)). Body weight and mortality were recorded daily whereas blood samples were collected at the fourth weeks from an orbital sinus[13], in a sterile plastic tube containing ethylene diamine tetraacetic acid (EDTA) as an anticoagulant agent. A vet-animal blood counter (ABC, ABX-France) was used to determine blood constituents (WBCs, RBCs, HGB, HCT, PLT, MCV, MCH and MCHC) while blood compounds (total protein, total cholesterol, insulin, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH)) were determined according to.[1]

2.4. Statistical analysis

A completed random design (CRD) was applied in this study and statistical analyses of data were done using IBM SPSS Statistics 23.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification

The aim of this study was to evaluate the biological effects of *B. pumilus* strain isolated from healthy honey bees on several biological parameters in mice. The microscopic features results showed that some the bacteria isolated from the digestive tract of healthy honey bees were rod-shaped, Gram-positive, aerobic, spore-forming bacteria. All the isolates that have previous microscopic features were selected as *B. pumilus* then the biochemical tests using HiBacillusTM Identification Kit (Himedia, india) were done to distinguish between *Bacillus* species. The data in table 1 reported that there are some bacterial isolates may be *B. pumilus* strains where each Voges Proskauer's, citrate, ONPG, catalase, sucrose, mannitol, glucose, arabinose and trehalose positive; and malonate, nitrate reduction and arginine negative bacterial isolates were considered *B. pumilus* strains. The primary base-biochemical tests identification was confirmed by 16S rRNA analysis using BLAST pairwise alignment in GenBank (Figure 1). Depending on the BLAST tree, the bacterial isolate is likely that it will be *B. pumilus*.

Table 1: The biochemical tests used to identify *Bacillus pumilus* using HiBacillus™ Identification Kit (Himedia, india).

Biochemical test	Malonate	Voges Proskauer's	Citrate	ONPG	Nitrate reduction	Catalase	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose
Results	-	+	+	+	-	+	-	+	+	+	+	+

ONPG = o-Nitrophenyl-β-D-Galactopyranoside, + = positive and - = negative.

3.2. Animal experimentation

A figure 2 shows the biological effects of the *B. pumilus* on body weight (gm) and mortality (%) of the mice. The data confirmed that *B. pumilus* has a positive impact on the body weight (gm) and mortality (%) of the mice. The *B. pumilus* led to a significant ($P<0.05$) increase in the body weight and a significant ($P<0.05$) decrease in the mortality. The biological effects of the *B. pumilus* on the blood constituents and compounds were arranged in table 2.

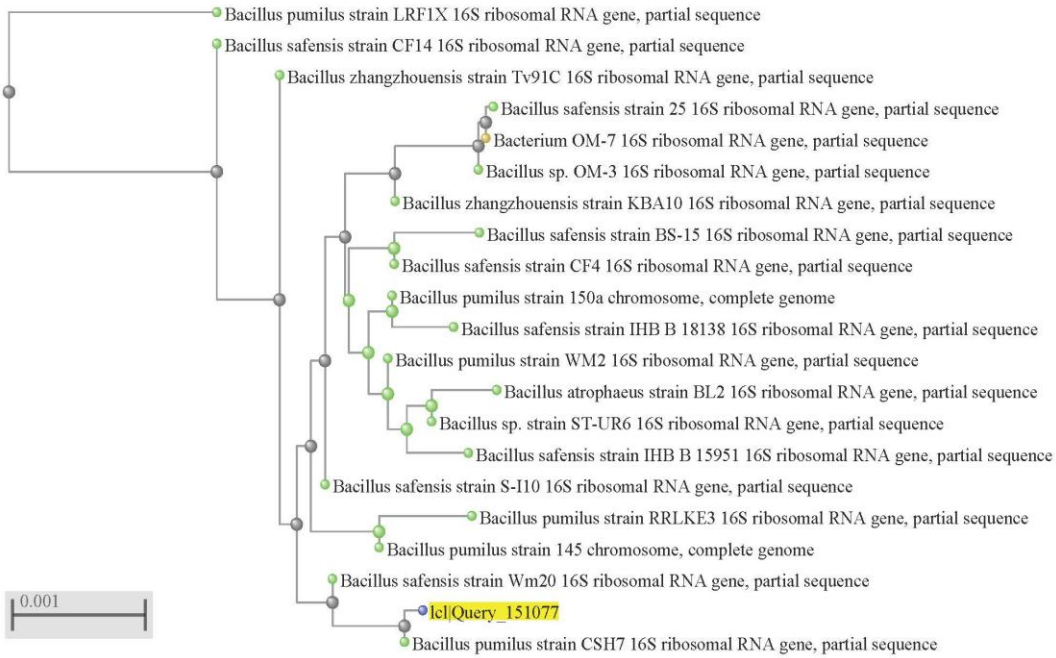

Figure 1: Identification by BLAST tree suggests that the isolate is *Bacillus pumilus* ((lcl]Query_151077). A BLAST tree produced by a BLAST pairwise alignment in GenBank (http://www.ncbi.nlm.nih.gov/blast/treeview).

Differences in the mean concentration of the biological parameters were not significant in all groups. The level of the blood constituents and compounds of mice, which were investigated in this work were within normal levels comparing to the levels mentioned by Mitruka and Rawnsley.[8] The data obtained from this study reported that *B. pumilus* has no unfavorable influence on total protein, glucose, insulin, total cholesterol, WBCs, RBCs, HGB, HCT, PLT, MCV, MCH, and MCHC.

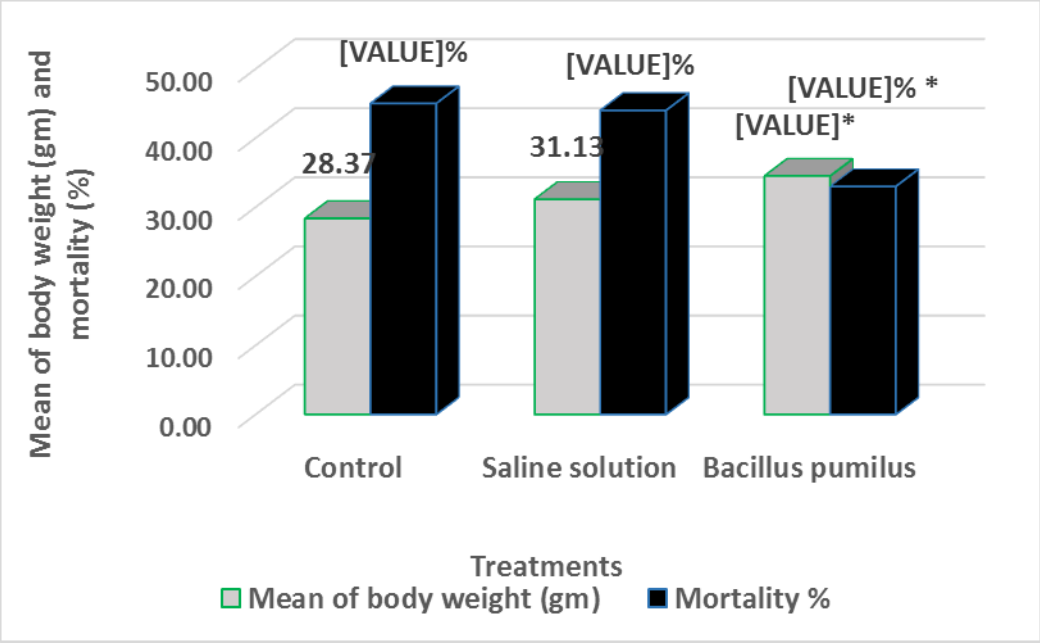

Figure 2: The biological effects of Bacillus pumilus on body weight (gm) and mortality of mice.

There are several *Bacillus* species such as *B. cereus*, *B. clausii*, *B. pumilus* have been carried in numerous commercial probiotic products, and have been characterized for potential attributes including colonization, immunostimulation, and antimicrobial activity that accounted for their probiotic features.[2]

Table 2: Means ±S.E (*n* = 6) of measured blood constituents.

Blood constituents and compounds	Co	Ts	Tb
White blood Cells (WBCs) (^{103}/mm^3)	13.5±0.2	14.2±0.5	14.1±0.2*
Red blood Cells(RBCs) (10^6/mm^3)	10.1±0.5	9.8±0.6	9.7±0.45
Haemoglobin (HGB) (g/dl)	11.3±1	10.9±0.8	11.6±0.7
Hematocrit (HCT)	45.1±	47.1±	46.5±
Mean Corpuscular Haemoglobin (MCH) (pg)	12.3±0.3	13.1±0.6	12.6±0.8
Mean cell volume (MCV) (µm^3)	50.3±2	51.2±2.5	49.7±3.4
Mean Corpuscular Haemoglobin Concentration (MCHC) (g/dl)	25.3±2	24.8±3	24.1±2.5
Platelets(PLT) (10^3/mm^3)	700±11	710±13	699±5

Total protein level (g/dl)	5.7±0.2	4.9±0.4	5.1±0.11
Glucose level (mg/dl)	129±3	135±4	136±2
Insulin level (U/I)	6.2±0.1	6.5±0.3	6.6±
Total cholesterol level (mg/dl)	82.1±	78.3±	79.3±

*All groups are not significantly different ($P<0.05$). Co= control group, Ts= Treated with sterile normal saline solution and Tb= Treated with *Bacillus pumilus*.

A colonization, immunostimulation, and antimicrobial activity of the bacteria are major key to consider these strains as probiotics

CONCLUSIONS

In conclusion, these results suggest that the digestive tract of healthy honey bees could be a natural source of some beneficial microorganisms. In additions, these results presented there a primary evidence to consider the *B. pumilus* strain as novel probiotic through its role in a role in improving the health status of mice.

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