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GENETIC IDENTIFICATION AND ANTAGONISTIC EFFECTS OF *BIFIDOBACTERIUM* ISOLATED FROM AL-JABAL AL-AKHDAR HONEY SAMPLES AGAINST HUMAN PATHOGENS

***Dr. Nagea Abdalsadiq**

Department of Zoology, Faculty of Science, Omar Al-Mukhtar University (OMU), El-Beida-Libya, P.O. BOX 919 El-Beida-Libya.

Corresponding Author: Dr. Nagea Abdalsadiq
Department of Zoology, Faculty of Science, Omar Al-Mukhtar University (OMU), El-Beida-Libya, P.O. BOX 919 El-Beida-Libya.

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ABSTRACT

Probiotics are described as live bacteria that, when given in sufficient concentrations, provide health advantages to the host. Probiotic bacteria are the favored microorganism to a diversity of industries. The main purpose of this work was to screen probiotic bacteria isolated from honey for their antagonistic effects against resistance pathogen and characterization them using 16s rDNA. The results showed that, the probiotic bacteria were able to prevent growth of pathogens and exhibited significant inhibition of the growth of the Gram positive and Gram-negative bacteria growth as assessed using the microtiter plate. The four bacteria strains were identified three Lactiplantibacillus plantarum and one Bifidobacterium hapali tyrs. This study concluded that these secondary metabolite from probiotic bacteria can be used as a natural antimicrobial agent, added to food formulations to prevent of pathogenic microorganism's growth and can be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, reducing the use of synthetic drugs and chemicals.

KEYWORDS: Bifidobacterium, Probiotic bacteria, Antibacterial, Antagonistic effects y, Human pathogens 6s rDNA PCR.

1. INTRODUCTION

LAB has an important function in human and animal digestive systems. In humans, some of these bacteria can be found as commensal in oral cavity, the intestinal tract, and the vagina, and beneficially influence these human ecosystems. As such, they are potential candidates for application as probiotics. In addition, they are of economic significance in the food sector. The natural microflora of many fermented foods such as milk, meats, vegetables, and cereal products is predominated by LAB which serve as preservatives by lowering the pH to 4 (due to lactic acid formation) and hence suppressing the development of the majority of other microorganisms. The lowered pH can also change the food-texture by precipitating some proteins. However, the fermentation and the growth capacity of LAB are self-limited due to their sensitivity to acidic pH environments (Stiles & Holzapfel, 1997, Nikolaev Y.A. & Plakunov V 2007, Mukherjee, P.K. & Chandra et al 2004). The assessment of Antagonistic Activities of probiotic bacteria (lactic acid bacteria) indicated that the *L. animalis ATCC35046*, *L. paracasei A20* and L. agilis CCUG31450 exhibited a greater Antagonistic activity against several resistance human pathogens (Fernandes et al., 2013, Rodrigues et al 2007). The use of honey as traditional medicine to treat infection has been documented in the world's oldest medical literatures. it has been known to possess antimicrobial property as well as wound-healing activity. Its immunomodulatory property is relevant to wound repair too. The antimicrobial activity in most honeys is due to the enzymatic production of hydrogen peroxide. Several studies have reported the presence of probiotic bacteria isolated in honey and honeybee. *Lactobacillus* and *Bifidobacterium* were isolated from stomach of honeybees (Olofsson & Vasquez, 2008; Eva *et al*, 2009, Saharan et al 2014). *Gluconobacter* and *Lactobacillus* were isolated from ripening honey (Ruiz and Rodriguez, 1975), and *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* were isolated from Ethiopian honey wine Recently, these LABs were also isolated in honeybee-gut (Audisio *et al*, 2011). These microorganisms possess interesting properties not only for the food industry but also for the benefit of health. Therefore, this study aims to isolate probiotic bacteria from local honey in Al-Jabal Al-Akhdar, Libya.

2. MATERIALS AND METHODS

2.1 Isolation of probiotic bacteria from honey sample

The samples used in this study were local honey from Al-Jabal Al-Akhdar, Libya (Hannon, Zaater and Sidr). Approximately 10 g of honey samples were suspended in 90 ml peptone water (0.1 % w/v) in stomacher bags and the bags were manually agitated. Then, the addition of 1 mL into 9 mL of de Man, Rogosa & Sharpe (MRS) broth (Oxoid CM359) and the incubation was at 30o C for 24 to 48 hr followed by diluting serially with peptone water (0.1% w/v). Subsequently, 0.1 mL was spread plated on several adapted media specifically, MRS agar (Oxoid) MRS agar with 0.8% CaCO3 MRS agar with 1% glucose, tomato juice agar with 0.8% CaCO3, and tomato juice agar with 1% glucose. The incubation of plates was under anaerobic situation in anaerobic jar at 37o C for 48 hr or until the bacterial colonies grown sufficiently in size. The testing of colonies for catalase activity with 4% H2O2 and the streaking of catalase negative colonies on MRS agar that contained 0.8% CaCO3 was kept warm at 37o C for 48 hr to attain pure colonies. The isolates' validation for Gram staining and culture purity was inspected using morphology and microscopic. All negative catalase and gram-positive LAB isolates were preserved in MRS broth with 15% of glycerol and set aside at −20o C for more inspection (Kheadr, 2006, Velraeds).

2.2 Preparation of cell free supernatant (CFS)

Seventy bacterial isolates were pre-cultured and grown in MRS broth medium containing 5% crude incubated in shaker at 37°C, 120rpm for 72 hrs. CFSs from LAB strains were obtained to screen biosurfactant producing LAB and to evaluate their inhibiting activity against the indicator pathogen strains using the agar well-diffusion test. CFS were sourced from MRS broth cultures by centrifugation (Jouan Br4i, France) at 10,000 g for 10 min at 4ºC. To prevent inhibition because of pH reduction due to organic acids, the pH of the CFSs was determined at 6.2 with the use of 1 N NaOH. Any inhibition by hydrogen peroxide was also removed by adding catalase. The CFSs were subjected to filter-sterilisation through 0.22 µm pore-size filters (Schleicher & Schüll, Dassel, Germany) (Rodrigues *et al.*, 2006).

2.3 Determination of antagonistic activities of probiotic bacteria supernatant

Antagonistic activities of probiotic bacteria supernatant using microtiter plates was performed as described by Gudina et al. (2010). Briefly, an aliquot of 150µl of a washed bacterial suspension in Nutrient broth adjusted to 0.5 McFarland standard turbidity (a final density of 108 CFU ml-1) was added to each well of a sterile 96-well microtiter plate together with 200 µl of probiotic bacteria supernatant. Control wells contained Nutrient broth and bacterial suspension without supernatant. All microtiter plates were incubated at 30 ºC for 24, 48, and 72 h. The optical density of each well was recorded at 560 nm in micro-ELISA auto reader (Model 680, BioRad). The percentage growth of pathogenic bacteria was determined based on the following formula: Percentage growth of pathogenic bacteria (%) = [1 – (ODc)/ OD0] × 100

Where: ODc is the optical density of the well with a supernatant c and pathogen.

OD0 is the optical density of the pathogen suspension with no supernatant (control).

Triplicate assays were conducted, and the mean of optical density was taken.

2.4 Genotypic Identification of Selected probiotic bacteria Isolates using 16s rDNA

Out of eighteen probiotic bacteria, best antagonistic activities (four isolates) were gnomically identified according to the method described by Jarvis & Hoffman (2004). The extraction of total genomic DNA from an overnight culture in 20 ml MRS broth at 30 ºC was done employing the Master Pure™ Gram positive DNA Purification Kit (USA). One ml of overnight culture was subjected to centrifugation 11500 rpm for 10 min at 25ºC (Eppendorf centrifuge 5804 R) and the pellet was then retrieved. To the pellet 150 µl of TE buffer was added and subjected to incubation at 37 ºC overnight. 1 µl of proteinase K (50 µg/µl, Sigma) was mixed with 150 µl of gram-positive lysis solution and then added to TE buffered mixture and subjected to thorough mixing. What followed was the incubation of the sample at 65-70 ºC for 15 min and then vertexing at 5 min intervals. The next step was to place the sample on ice for 5 min. Then 175 µl of MPC protein precipitation reagent was added to every sample, followed by vertexing and centrifugation at 13,000 rpm for 10 min at 4 ºC (Eppendorf centrifuge 5804 R). The CFSs were then moved to new tubes and the pellets discarded. 1 µl of RNase II (5 µg/µl) was added to each sample followed by thorough mixing. The samples were subjected to incubation at 37 ºC for 30 min; 500 µl of isopropanol was added to the CFS, followed by centrifuging at 4ºC for 10 min at 13,000 rpm (Eppendorf centrifuge 5804 R). Isopropanol was eliminated with an Eppendorf pipette, but the DNA pellet was kept in place. The pellets were washed with 200 µl ethanol 70% and subjected to centrifugation at 5, 000 rpm for 2 min at room temperature. The removal of the ethanol was done with care and the DNA was suspended again with 35 µl of deionized water and stored at -20 ºC for future study.

2.5 Gel Electrophoresis

2.5.1 Amplifying the PCR products from universal bacterial primer was subjected to analysis for expected size.

Two µl of each amplification mixture was put through electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TEA buffer for 45 min and 110 V. DNA molecular mass marker (250 to 10,000 bp) molecular ladders from 1st Base, was the standard. Following electrophoresis staining of the gels in ethidium bromide was carried out and after rinsing the gels were observed and photographs taken with UV transilluminator (Bio-Rad Laboratories, Segrate, Italy). The partial 16S rDNA, Lbp11 and LMM

primers sequences were established by 1st Base, Malaysia and comparison was made of the sequences and the databases (Gen-Bank).

2.6 Statistical Analysis

Results were shown as the mean ± standard deviation and all measurements were done in triplicate. A one-way ANOVA ($P < 0.05$) applying the Tukey multiple-comparisons using SPSS software was used for the evaluation of the. statistically significant differences of the conditions tested in the different assays. There was a significant difference if $P < 0.05$.

3. RESULTS

3.1 Isolation of probiotic bacteria using different media

Isolation of probiotic bacteria form honey was successfully achieved in MRS broth followed by plating in selective media. Eighteen of probiotic bacteria that isolated from honey were identified as probiotic bacteria because they produced clear zone on MRS agar supplied with CaCO3, Gram positive and catalase negative as in table (1) and figure (1, 2, 3). Lactic acid bacteria present in Hannon honey from Al-Jabal Al-Akhdar, Libya were isolated from MRS with 1 % glucose and from MRS with 0.8 % $CaCO_3$, while lactic acid bacteria from Zaater and Sidr honey was isolated only from MRS with 0.8 % $CaCO_3$. The highest number of Lactic acid bacteria ($>10^{5/}$ml) was isolated from Hannon honey (pH 5.2), followed by Sidr honey ($> 10^4$/ml, pH 4.4) then Zaater honey ($> 10^3$/ml, pH 4.13). The microorganisms in honey have been reviewed by Snowdon and Cliver (1996), however, the microbiota associated with honey is still not fully understood. Several studies have reported the presence of lactic acid bacteria isolated in honey and honeybee. *Lactobacillus* and *Bifidobacterium* were isolated from stomach of honeybees (Olofsson & Vasquez, 2008; Eva *et al*, 2009). *Gluconobacter* and *Lactobacillus* were isolated from ripening honey (Ruiz and Rodriguez, 1975), and *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* were isolated from Ethiopian honey wine. Recently, these lactic acid bacteria were also isolated in honeybee-gut (Audisio *et al*, 2011). These microorganisms possess interesting properties not only for the food industry but also for the benefit of health. Results of biochemical and morphological tests showed bacterial diversity of dairy products. Study by Afridi et al., (2020). bacterial isolates were identified as *Bacillus* and *Clostridium spp*, according to biochemical and morphological characteristics.

Table 1: Phenotypic characterization of probiotic bacteria isolated from honey samples using different media incubated at 37 ºC for 24 h[a].

Sample code	Source	pH of honey	Media	Catalase reaction	Gram reaction	Morphology of cell	Number of LAB
				-	+	Rod	Positive
HH1	Hannon	5.2	MRS + Caco3	-	+	Cocci	10^{-5}
HH2	Hannon	5.2	MRS + Glucose	-	+	Rod	10^{-4}
HZ	Zaater	4.4	MRS + Caco3	-	+	Rod	10^{-5}
HS	Sidr	4.1	MRS + Caco3	-	+	Rod	10^{-3}

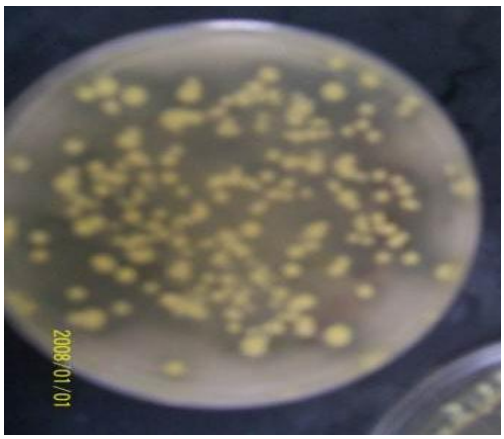

Figure 1: LAB Isolates Producing Clear zone on Modified MRS-$CaCO_3$ Agar

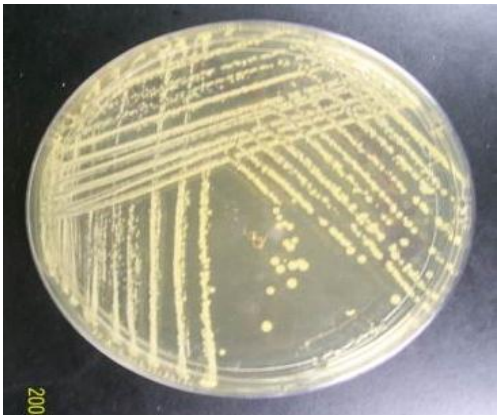

Figure 2: Lactic Acid Bacteria Pure Colony.

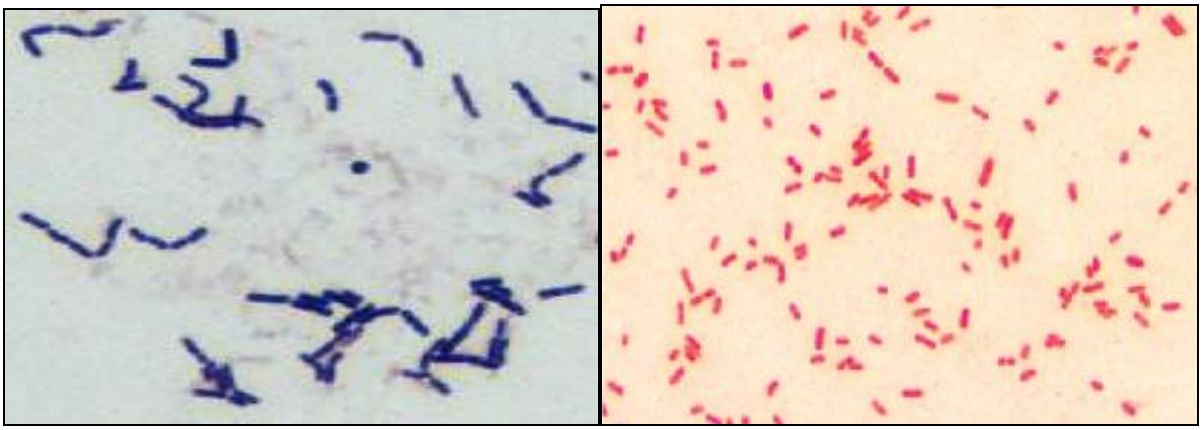

Figure 3: Gram Positive of probiotic bacteria: Rod on the left and cocci on the right.

3.2 Determination of antagonistic activities of probiotic bacteria supernatant in microtiter plate

Honey has the ability to inhibit microorganisms such as pathogenic bacteria, spoilage fungi and yeast and viruses. The antibacterial effect of honey especially against Gram positive bacteria is well documented (Molan, 1997; Bogdanov, 1997). Several strains of bacteria were isolated from honey and demonstrated antimicrobial activity against both gram negative and positive pathogenic and spoilage bacteria (Ibarguren *et al*., 2010; Lee *et al*., 2008). Antagonistic activities of probiotic bacteria supernatant five pathogen namely *Proteus mirabilis*, S. *aureus*, *Bacillus. subtilis*, K. *Pneumoniae* and *Candida. albicans* was evaluated using microtiter plate. All supernatant of probiotic bacteria isolated from three honey type caused more than 90% growth inhibition of bacterial pathogens after 24 h incubation (Table2 and figure 4).

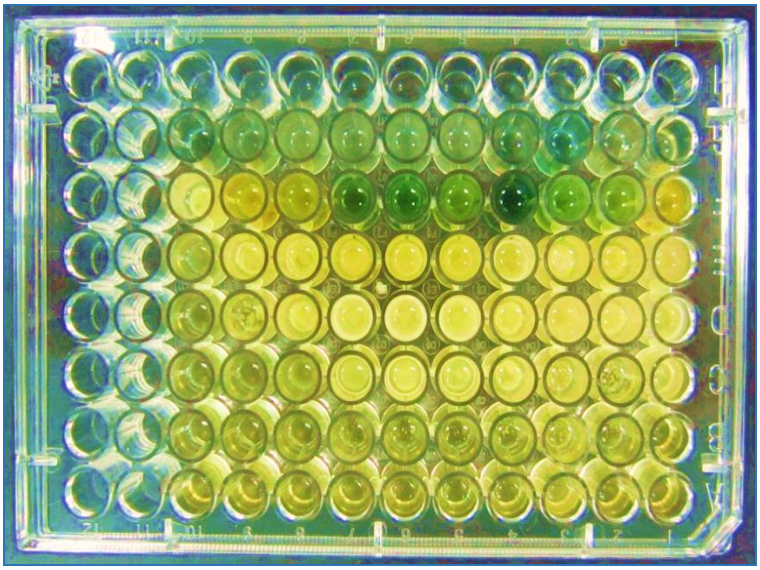

Figure 4: Antagonistic activities of probiotic bacteria supernatant against pathogens in microtiter plate.

Similarly study by Olofsson & Vasquez, (2008) which isolated *Lactobacillus* and *Bifidobacterium* with antimicrobial activity. All probiotic bacteria isolate (HH1, HH2, HZ and HS) completely inhibited *S. epidermis* and *P.mirabilis* after 24h of incubation. However, some probiotic bacteria supernatant allowed growth of the target fungus and bacteria as shown by SH against *C. albicans* after and HH1 and HH2 against *P.mirabilis* and *B. subtilis* after 24h incubation. In agreement with this study, c strain LB from human that showed antimicrobial activity against *K. pneumoniae*, *Enterobacter* spp., *S. Typhimurium*, *E. coli*, *L. monocytogenes*, *S. flexneri* and *P. aeruginosa*. These results provide evidence that supernatant of probiotic bacteria isolated from honey efficiently inhibits growth a wide range of pathogenic microorganisms.

Table 2: Antagonistic activities of probiotic bacteria supernatant against pathogens.

Pathogens	Probiotic bacteria			
	HH!	HH2	HZ	HS
S. aureus	NG	NG	NG	NG
P. mirabilis	NG	NG	NG	NG
B. subtilis	4.7±0.1[a]	6.0±0.4[a]	NG	8.1±0.1[a]
K. Pneumoniae	7.1±0.01[a]	7.3±0.1[a]	NG	NG
C. albicans	NG	NG	2.5.5±1.3[a]	20.2±0.1[b]

Several strains of probiotic bacteria were isolated from honey and demonstrated antimicrobial activity against both gram negative and positive pathogenic and spoilage bacteria (Ibarguren *et al*.,010; Lee *et al*., 2008). Olofsson & Vasquez, (2008) isolated novel probiotic bacteria in the genera *Lactobacillus* and *Bifidobacterium* from honeybee stomach, and the same isolates were also detected in honey. Lactic acid bacteria are known for their antimicrobial activity specially lactobacilli (Klaenhammer, 2001). Coconnier *et al*., (1997) isolated *L. acidophilus* strain LB from human that showed antimicrobial activity against *K. pneumoniae*, *Enterobacter* spp., *S.* Typhimurium, *E. coli*, *L. monocytogenes*, *S. flexneri* and *P. aeruginosa*. Recently, Fathabad & Eslamifar *et al*., (2011) reported that strain of *Lactobacillus paraplantarum* isolated from tea leaves has antimicrobial activity against *E. coli* and *S. Typhimurium*.

3.3 Genotypic Identification of Selected probiotic bacteria Isolates using 16s rDNA

Recently, the molecular biology has developed very fast and that had high impact on the microbiology world. Using the gene sequencing is the most reliable method of identifying the bacteria. 16S rDNA is one of these methods and it has been used for many cases for the identification of the bacteria especially LAB. Molecular characterization of bacterial DNA using universal primer showed clear strain bands with molecular weight 1500 bp (Figure 5). The similarity between each strain in this study and other bacteria in data base was estimated as in table 3. The similarity between probiotic isolates (HH1, HH2, HZ and HS) and bacteria from gene bank was estimated by (97.3%, 93.95%, 99%, 96%). The 16S rRNA gene sequence has been widely used as a molecular method to identify the bacteria especially LAB bacteria. Lim *et al*., (2009)

Table 3: Molecular Identification of probiotic bacterial Isolates.

Bacterial Codes	Identification	Percentage of similarity %	Accession No.
HH1	*Lactiplantibacillus plantarum*	97.3%	SU2765421
HH2	*Bifidobacterium hapali tyrs*	93.9%	LU6784230
HZ	*Lactiplantibacillus plantarum*	99. 3%	MT610988
HS	*Lactiplantibacillus plantarum*	96.93%	MT981291

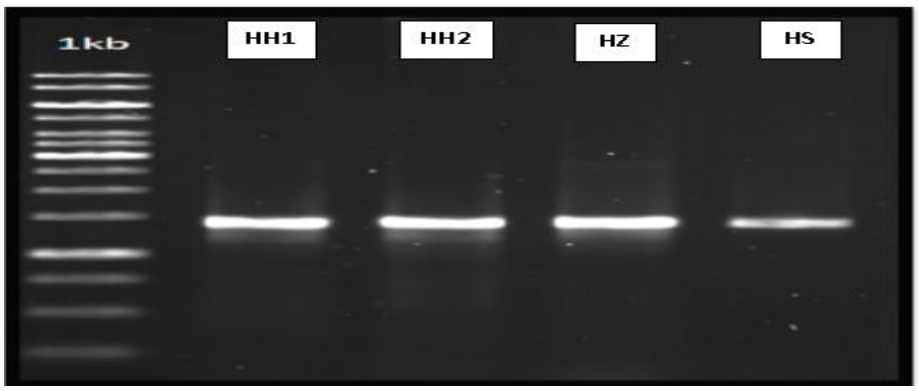

Figure 5: Probiotic bacterial DNA Bands on the 1.5 % Agarose Gel using universal primer.

Reported that *Lactobacillus brevis*, *Enterococcus faecium* and *Pediococcus acidilactici* were isolated from children faces and identified using two universal primers. Another study used for PCR amplification to identify LAB isolates from vagina and the isolates identified as; *L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii* and *Streptococcus* reported by Forney *et al*., (2010). Different accession number were used to keep each bacterial sequences in the Gene Bank database. The accession numbers of probiotic isolates were (SU2765421, LU6784230, MT610988 and MT981291 respectively (Table 3). This study reported the probiotic bacterial isolated from honey had a molecular weight of1500bp. In previous study, three isolates S17, S5 and S13 were identified by 16S rDNA sequencing of gene and similarity with *Bacillus subtilis*, *Bacillus tequilensis* and *benzoelyticu*m was estimated by 97%as indicated by analysis of phylogenetic (Afridi et al., 2020). The phylogenetic tree was constructed by the Neighbour-joining method (MEGA X 10.0.5). Numbers in parentheses are accession number of published sequences. The numbers at the nodes are bootstrap confidence levels (percentage) from 1000 replicates (Jawan et al 2020). Phylogenetic analysis revealed that probiotic strains have at least 97% similarity with *lactobacillus* strains and 93.3% similarity with *Bifidobacterium hapali tyr* (Figure 6, 7).

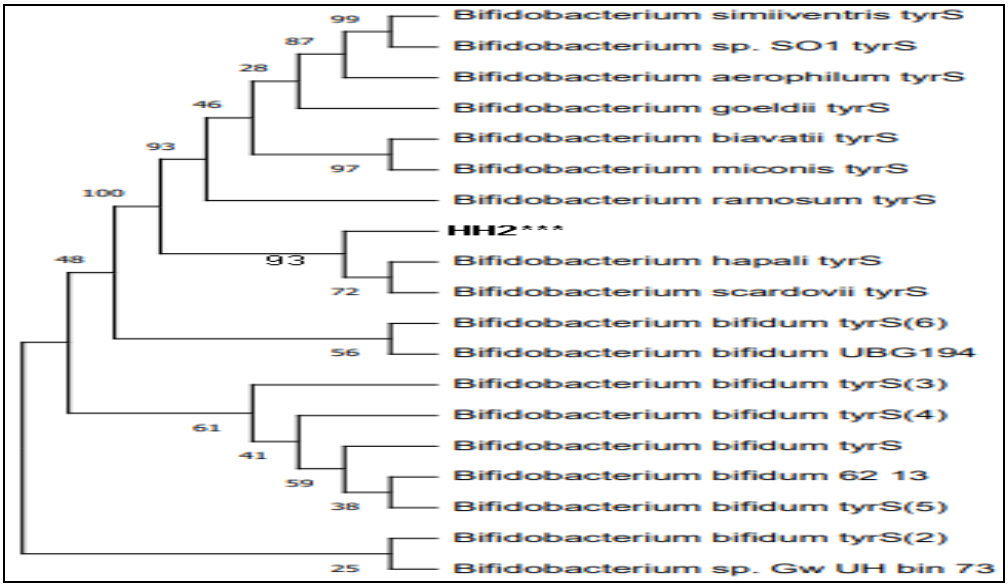

Figure 6: Phylogenetic tree of isolates (HH2) showing the close relatives inferred from 16 S rRNA gene sequence.

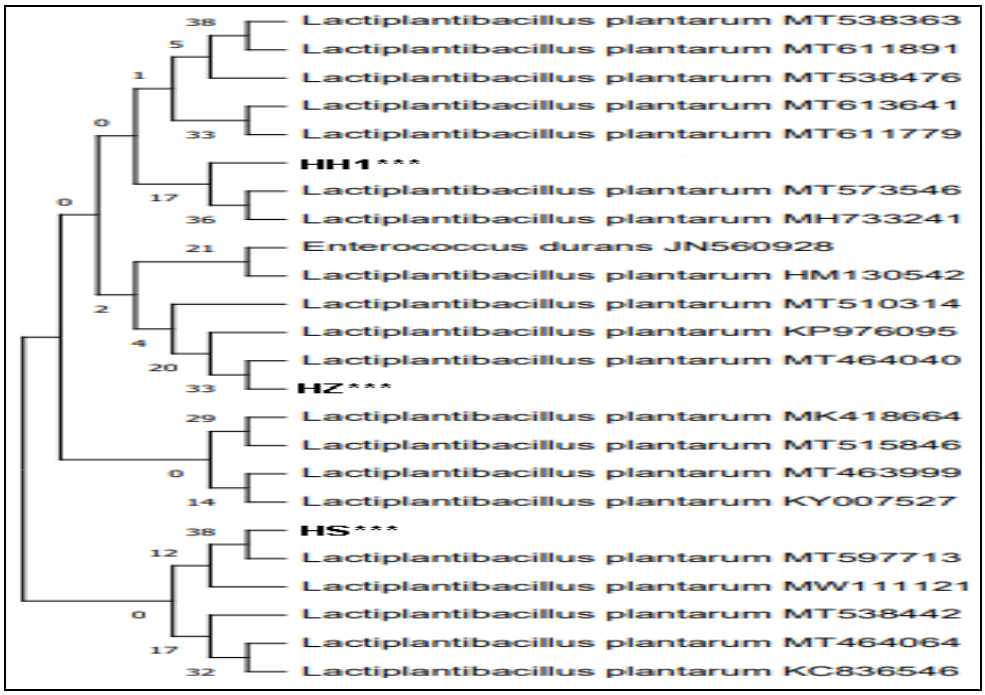

Figure 7: Phylogenetic tree of isolates (HH1, HZ and HS) showing the close relatives inferred from 16 S rRNA gene sequence.

The sequences of these isolates were established and kept in the Gene Bank database under accession number SU2765421, LU6784230, MT610988 and MT981291 respectively.

4. CONCLUSION

A total of eighteen probiotic bacteria were isolated from honey and screened for antagonistic activities. Out of eighteen, four isolates could produce secondary metabolite with antagonistic activities against several resistance human pathogens. Therefore, these secondary metabolite from supernatant of probiotic bacteria can be used as a natural antimicrobial, antiadhesion and anti-biofilms and can be added to food formulations to prevent of pathogenic microorganism's growth.

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