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Article - Human and Animal Health

Multienzyme and Antibacterial Potential of Bacteria Isolated from gut of Asian Honey Bee (*Apis cerana Indica*), Lahore Using Culture Dependent Method

Iram Liaqat[1*]
https://orcid.org/0000-0002-4638-8253.

Muhammad Mubin[2]
https://orcid.org/0000-0001-8319-1962

Maleeha Anwar Chaudhry[1]
https://orcid.org/0000-0003-3052-1520

Najma Arshad[3]
https://orcid.org/0000-0002-0244-6757

Muhammad Afzaal[4]
https:// orcid.org/0000-0002-2369-2658

Chaman Ara[5]
https://orcid.org/0000-0003-4567-0078

Nazish Mazhar Ali[1]
https://orcid.org/0000-0001-8740-4515

Andleeb Anwar Sardar[6]
https://orcid.org/0000-0003-0708-8925

Umer Farooq Awan[6]
https://orcid.org/0000-0003-3955-2905

Sumera Sajjad[7]
https://orcid.org/0000-0001-6982-5965

Farzana Rashid[7]
https://orcid.org/0000-0002-5519-3175

Shaukat Ali[1]
https://orcid.org/0000-0003-2481-1978

[1]GC University, Microbiology Laboratory, Department of Zoology, Lahore, Pakistan; [2]University of Agriculture, Centre of Agricultural Biochemistry and Biotechnology, Faisalabad, Pakistan; [3]University of Lahore, Department of Zoology, Lahore, Pakistan; [4]GC University, Sustainable Development Study Centre, Lahore, Pakistan; [5]University of the Punjab, Department of Zoology, Lahore, Pakistan; [6]GC University, Department of Botany, Lahore, Pakistan; [7]Lahore College for Women University, Department of Zoology, Lahore, Pakistan.

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*Correspondence: iramliaq@hotmail.com; Tel.: +92-300-6477459 (I.L.).

HIGHLIGHTS

- The current study focuses on antibacterial potential of bacterial isolates from gut of Asian HB, *Apis cerana Indica* F. (Hymenoptera: Apidae), against human pathogens.
- PCR study confirmed the presence of bacteriocins by successful amplification of important antimicrobial peptide biosynthesis genes *spaS* and *spoA.*
- Results suggested HB gut is a home to bacteria that possess antimicrobial activity and important enzymes with antimicrobial potential
- This is the first report demonstrating the antimicrobial potential of bacteria isolated from gut of HB (*A. cerana*) against human pathogens.

Abstract: The bacteria residing in the gut of honey bees (HB) has demonstrated a significant role in protecting bees against various pathogens, production of honey and wax. However, no information exists about the antibacterial potential of bacterial isolates from gut of Asian HB, *Apis cerana Indica* F. (Hymenoptera: Apidae), against human pathogens. This study aims to investigate the antibacterial and multienzyme potential of aerobic bacteria from *A. cerana* gut using culture dependent approach. A total of 12 HB gut bacteria were characterized morphologically and biochemically. These strains were further screened for their antimicrobial activity against pathogenic human microorganisms *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Bacillus licheniformis* and *Bacillus subtilis* using cross streak (primary screening) and agar well diffusion methods (secondary screening). Preliminary characterization of cell-free supernatant (CFS) of two promising isolates was performed by measuring lactic acid concentrations, enzymatic digestion of antimicrobial compounds, stability over a range of temperature, pH and amplification of *spaS* (subtilin) and *spoA* (subtilosin) genes. In primary screening, among 12 HB isolates, eight strains showed statistically significant highest zones of inhibition ($p \leq 0.05$) against *E. coli*, *K. pneumoniae* and *P. aeruginosa*. 16S rRNA sequencing revealed that *these isolates belong to Bacillus genus*, identified as *B. tequilensis*, *B. pumilus*, *B. xiamenensis*, *B. subtilis*, *B. amyloliquefaciens*, *B. safensis*, *B. licheniformis*, *B. altitudinis* (Accession numbers: MT186230-MT186237). Secondary screening revealed that among eight isolates, *B. subtilis* and *B. amyloliquefaciens* showed statistically significantly strong inhibition ($p \leq 0.05$) against all tested pathogens. Antibiotic susceptibility testing revealed that both isolates were resistant to antibiotics and possesses proteolytic, lipolytic and cellulolytic activities. The nature of the compound causing inhibitory activity was found to be proteinaceous and showed stability over a wide range of temperature as well as pH. PCR study confirmed the presence of bacteriocins by successful amplification of important antimicrobial peptide biosynthesis genes *spaS* and *spoA.* These results suggest that the HB gut is a home to bacteria that possess antimicrobial activity and important enzymes with antimicrobial potential. To our knowledge, this is the first report demonstrating the antimicrobial potential of bacteria isolated from gut of HB (*A. cerana*) against human pathogens.

Keywords: honey bees; gut microbes; antimicrobial activity; *B. amyloliquefaciens*; *B. subtilis; A. cerana.*

INTRODUCTION

Increasing antibiotic resistance against bacteria causing serious multiple infections in humans has left scientists with fewer options of successful treatment. There is a need to find new agents having antimicrobial properties [1]. Microorganisms that live and colonize the digestive tracts of animals including insects are known as gut microbiota [2-3]. In insects, the role of gut microbes is directly related to improved ecology, agriculture and medicine. The herbivorous insects, in particular those living in large colonies, for example, honey bees (HB) inhabit diverse ecosystems and microorganisms having symbiotic association with insects also show great diversity. These microbes produce a variety of bioactive secondary metabolites, which prevent attack of pathogenic bacteria and /predatory species by empowering host immune system and contributing to defensive strategies such as behavioral or mechanical responses [4].

Honey bees play a significant role in human wellbeing both by pollination of important food crops and provision of honey. Globally, more than fifty percent of 115 common food commodities rely on honey bee pollination to produce seeds and fruits. Further, the cost of agricultural goods produced as a result of HB pollination is estimated to be approximately $15 billion/year in the U.S. alone. This indicates that colony health of HB hives is very important and management of its deteriorations due to several bee diseases is a serious concern worldwide [5]. For decades, various antibiotics and chemicals have been used to control HB diseases. For example, tetracycline was used to prevent Paenibacillus larvae and Melissococcus plutonius growth to limit American foulbrood (AFB) and European foulbrood, respectively. However, later research proved that both HB gut microflora and their products pay significant role to prevent and combat bee diseases caused by P. larvae [6].

The gut of HB is a complex environment which supports the growth of great diversity of microbial flora (Streptococcus sp., L. rigidus, Enterobacter sp., Proteus sp., Clostridium sp., Flavobacterium sp., Achromobacter sp., Citrobacter sp., Klebsiella sp., Pseudomonas sp., Bacillus sp., and Gram-negative bacteria). Microbial diversity of HB gut fluctuates depending upon various factors, for example, during the flowering season of rape Wang and coauthors [7] reported the Bacillus group dominant bacteria in HB stomachs. Bacillus group was observed to be the dominant bacteria in HB stomachs [7]. Similarly, gut microbiota of summer and winter HB has been observed to diversify. Studies have shown that the HB gut supports growth of both lactic acid bacteria (LAB) and Fructophilic Lactic Acid Bacteria (FLAB) [8], particularly

L. kunkeei [9]. LAB prefers glucose and FLAB prefers fructose as a growth substrate. The gut of Slovakian HB was found to be mainly colonized by anaerobic, rather than aerobic bacteria belonging to Coliforms, Enterococci, Staphylococci, Bacillus sp., Pseudomonas sp., microscopic fungi and yeast [9]. Additionally, a great variety of different microorganisms can be transferred from HB gut to honey [10]. There are reports where honey showed better antibacterial activity compared to antibiotics. Likewise, pure form of honey has proven the best against many serious pathogenic bacteria E. coli, Shigella sp., Salmonella sp., V. cholerae and other Gram –ive as well as Gram +ive microoorganisms. The observed high antibacterial potential of honey is the result of acidity, hydrogen peroxide, osmotic effect and phytochemical factors [11].

Asian HB, Apis cerana Indica F. (Hymenoptera: Apidae), is a social insect and native to Pakistan. Disayathanoowat and coauthors [12] studied the bacterial diversity of midgut of A. cerana. Authors isolated aerobic bacteria by culturing on five different media and characterized fifty eight isolates using biochemical tests and 16S rRNA gene sequencing. Two dominant phyla Gamma-proteobacteria and Firmicutes with abundant bacterial species Enterobacter cloacae, Klebsiella oxytoca and K. pneumonia were found. Bacterial isolates were observed to possess protease and lipase activities and some members of Klebsiella showed effective inhibition of AFB disease [12]. Likewise, Sudhagar and coauthors [13] studied the gut colonizing bacteria of A. cerana from India. Authors reported diversified gut microbial communities inhabiting different elevations and observed Bacillus sp. as predominant genus at higher elevation, while Serratia sp., Klebsiella sp. and Enterobacter sp. was dominant in coastal and plain areas. There is established data from other countries on the antibacterial potential of A. cerana gut bacteria against various bee diseases [14-15]. Furthermore, various authors reported LAB [16-17] and Bacillus sp. [16] residing in the HB gut producing bioactive substances with potential to be used as alternatives to antibiotics against human and animal infections [11].

Further, symbiotic relationship of HB with different insects involves the great diversity of microbes, most of which provide nutritional benefit to host. In some bees, for example stingless bee, nest is made up of plant-based complex biomolecules for example, lipids, proteins and cellulose. In order to make bee nest, these complex biomolecules are degraded by hydrolytic enzymes produced by bacteria residing in HB gut. It was reported that most of these isolates have cellulase, protease and lipase activities [18-19], hence also aid in food digestion by these bees [20]. Antimicrobial activities of HB have been reported to be linked with the presence of antimicrobial peptides such as spaS (subtilin) and spoA (subtilosin) [21]. spoA is a circular anionic antimicrobial peptide produced by B. subtilis [22]. spaS is a linear lantibiotic, resembles to nisin, and is commonly used in food preservation [23].

To date, no information exists about the antibacterial potential of bacteria isolated from A. cerana gut against human pathogens around the globe. Most of research has focused on HB (preferably A. mellifera) gut bacterial potential to control bee diseases in countries other than Pakistan. In this study, we used a culture dependent method to isolate, identify and characterize aerobic bacteria from the gut of A. cerana. Antibacterial activities of the HB gut isolates against both Gram positive and Gram negative human pathogens were studied. Following that, bacterial proteolytic, lipolytic and cellulolytic activities were analyzed using modification of existing enzyme assays. Preliminary characterization of CFS inhibitory activity was performed by measuring lactic acid concentrations, enzymatic digestion, and stability over a range of temperature, pH and amplification of spaS (subtilin) and spoA (subtilosin) genes. This study will provide the primary information about cultureable aerobic gut bacteria of A. cerana from Lahore, Pakistan as well as their antibacterial and multienzyme potential.

MATERIAL AND METHODS

Sample collection and dissection

Honey bees (HB) were collected from three local beekeepers in Lahore, Pakistan and washed with 95% alcohol before dissection to prevent surface contamination. Whole alimentary canals of bees were dissected aseptically with the help of sterile forceps under a laminar flow cabinet [24]. Dissected parts were kept in sterile 0.85% (w/v) saline solution, homogenized and plated within half hour of collection.

Isolation of bacteria and morphological characterization

Known aliquots of homogenate were spread on brain heart infusion (BHI) agar plates, tryptic soy agar, Lactobacilli MRS agar and incubated for 37-48 hours at 37 ºC and relative humidity (80%). Incubation period was extended until visible colonies appeared on plates. Colonies were purified by streaking and re-streaking. Different colonies were selected on the basis of colony morphology, Gram's staining and motility test [25].

Biochemical tests and preparation of cell free supernatant (CFS)

Following morphological characterization and Gram staining, 12 Gram positive HB gut isolates were found to be morphologically different and selected for biochemical characterization. Different biochemical tests such as catalase test, carbohydrate test, voges proskauer test, methyl red test, starch hydrolysis test (glucose, lactose and sucrose) were performed. Cell free supernatants (CFS) were prepared by culturing the HB gut isolates in nutrient broth for 24-48 hours at 37 ºC and then centrifuged at 6000rpm for 25 minutes [20]. Supernatants were filter sterilized through 0.22 µm filter papers and stored at -20 ºC till further analysis.

Determination of antibacterial activity

The antibacterial activity of HB gut isolates was determined using two protocols i.e. primary screening and secondary screening.

Primary screening (Cross streak method)

In primary screening method, all 12 HB gut isolates were tested against five human pathogens *Escherichia coli* (MN900682)*, P. aeruginosa* (MN900691)*, K. pneumonia* (MN900695)*, B. licheniformis* (MN900686) and *B. subtilis* (MN900684) using cross streak method]. Briefly, plates were inoculated with single streak of HB gut isolates and incubated for 24-48 hours at 37°C. Bacterial pathogens were streaked at 90° angle and again incubated. Zone of inhibition (in mm) was measured after 24 hours. Experiment was performed in triplicates.

Genomic DNA extraction, PCR and sequencing

Genomic DNA of HB gut isolates (HB1, HB2, HB5, HB6, HB8, HB9, HB10 and HB12) with significant antibacterial activity was extracted using GeneJET Genomic DNA purification kit [26]. Five µL of genomic DNA was used for gel electrophoresis and sharp bands of DNA were observed under UV trans-illuminator. Polymerase chain reaction (PCR) was performed to amplify the 16S rRNA gene using a Techne thermo cycler (PROGENE) under standard conditions. It was performed in a PCR tube containing 29.5µL of DNA grade water, 0.5µL of Taq polymerase, 4µL of MgCl2, 5µL of 10× PCR buffer, 5µL of dNTP mixture, 1µL of Forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′), 1µL of reverse primer (5′-AAGGAGGTGATGATCCAGCCGCA-3′) and 4µl of template DNA using standard conditions. Amplified products were analyzed by gel electrophoresis (75V, 30 min) on 1.0% agarose gel and visualized under UV trans-illuminator. Amplified gene fragments were purified and cleaned using GeneJET Genomic DNA extraction kit. Amplified 16S rRNA genes were sent for sequencing to Axile scientific Singapore. The representative sequences obtained were analysed using Chromas Lite software. Corrected sequences were searched using NCBI BLAST to compare with the nearest matched species. These sequences were deposited in the GenBank nucleotide sequence databases (accession numbers MT186230-MT1862307). A phylogenetic tree from 16S rRNA sequence was built with the neighbor joining method using MEGA 10 software and the genetic distance of each strain was determined [26].

Secondary screening (Agar well diffusion method)

Secondary screening was performed using agar well diffusion method. Nutrient agar plates were prepared and wells of 6mm diameter were made using sterilized cork borer. Test cultures were spread on plates uniformly with the help of sterilized cotton swabs. Nutrient broth and rifampicin (Rif-50 µgmL-1) were used as positive and negative controls respectively. Plates were incubated for 24 hours at 37ºC and diameter of zones of inhibition (mm) was assessed to measure the antibacterial activity. Inhibition zones greater than ≥10 mm were considered as positive.

Antibiotic susceptibility test

Using Kerby-Bauer disc diffusion method [27], different antibiotics (ampicillin; Am-50 μgmL^{-1}), erythromycin; Ery-20 μgmL^{-1}) and rifampicin; Rif-50 μgmL^{-1}) were used to check the susceptibility pattern of the selected HB gut isolates. The discs were prepared by placing 20µL of antibiotic on disc and drying aseptically in laminar flow. These discs were placed aseptically on media plates inoculated with HB gut isolates and incubated overnight at 37°C. On the basis of presence or absence of zones of growth inhibition, HB gut isolates were considered sensitive/resistant.

Physiological characterization

Physiological characterization was performed by studying growth curve and investigating the effect of pH and temperature on growth of selected HB gut isolates. For temperature, test tubes were inoculated with 100µL of fresh bacterial culture except for control and incubated overnight at 30, 37, 40 and 50ºC. Likewise, for optimum pH determination, pH of nutrient broth was adjusted to 4, 6, 7, 8 and 10 using 1 M HCl or 1 M NaOH nutrient broth followed by inoculation as described above and incubation for 24 hours at 37ºC. Growth curve was determined by inoculating autoclaved 250 mL nutrient broth (pH=7) with overnight pre-inoculum of selected HB gut isolates in 500 mL pre autoclaved Erlenmeyer flask at 250 rpm except for control under sterilized conditions. First optical density was taken at zero-time interval spectrophotometrically. Afterwards, flasks were incubated at 37ºC in shaking incubator and OD600 was monitored at various time intervals to capture lag, log, stationary and death phase of HB isolates over a 24 hours' time span.

Measurement of proteolytic, lipolytic and cellulolytic activities

Following slight modifications of method by Rahman and coauthors [28], proteolytic activity was determined using skimmed milk agar specifically. Briefly, selected HB gut isolates were cultured at 37˚C with shaking in the production medium comprising of trypticase soy broth (Oxoid Ltd., Basingstoke, UK) having 1% tryptone. Overnight broth culture was centrifuged and 0.1 mL supernatant was mixed with 1 mL azocasein in Tris–HCl at 37ºC. 10% trichloroacetic acid (Sigma, USA) was added to terminate the reaction. It was followed by incubation and centrifugation. The supernatant was mixed with equal volume of 1M NaOH. OD was then measured at 450 nm using a microplate reader.

Lipolytic activity was determined using specific tributyrin agar medium following protocol by Kanwar and coauthors [29] with minor modifications. Bacterial isolates were grown in the production medium comprising trypticase soy broth (TSB) supplemented with 1% olive oil, 1% yeast extract, and 0.5% $CaCl_2$. Overnight culture was centrifuged and supernatant was mixed with 0.1M phosphate buffer. Afterwards, freshly prepared 0.02M p-nitrophenyl palmitate in isopropanol was added to supernatant at 37˚C and 175×*g* shaking. Ethanol (0.1 mL of 90%) was added to stop the reaction and absorbance was measured at 410 nm. p-nitrophenol was used as standard to prepare the calibration curve [30].

Following modifications of method by Liang and coauthors [31], cellulolytic activity was determined using carboxymethyl cellulose (CMC) agar specifically for this purpose. Briefly, both bacterial isolates were grown in the production medium (pH 7.0) made of TSB, 2% CMC, and 1% yeast extract. Overnight culture was centrifuged and supernatant was mixed with 1% CMC in 0.1M phosphate buffer for 30 min. The reaction was stopped by adding DNS reagent (1% (w/v) 3,5-dinitrosallicylic acid, 2M NaOH and sodium potassium tartrate followed by incubation for 15 min at 100˚C. OD was measured at 540 nm. Glucose was used as standard to prepare the calibration curve. Experiments were performed in triplicates.

Preliminary characterization of antibacterial compounds

Determination of lactic acid concentrations

Following manufacturer's protocol, concentrations of D-lactic acid/L-lactic acid were measured in the CFS of both isolates using D/L-lactic acid test kit (Roche Boehringer, Germany). Obtained data were processed using equations provided in protocol to correctly measure the concentration of each acid (D/L-lactic acid) in sample.

Enzymatic digestion of antibacterial compound

In order to assess the nature of the antibacterial compound that causes growth inhibition of tested pathogens, CFS of both isolates was treated with seven enzymes catalase, proteinase K, pepsin, trypsin, chymotrypsin, lipase and protease (Sigma) overnight. Briefly, 200 *µ*l aliquots of the CFS were mixed with equal quantity of enzymes for 24 hours at incubation temperature (50, 70 and 90ºC) and pH (4, 6, 8 and 10) appropriate for optimal activity of respective enzyme. Two controls *i.e*., (i) enzyme with sterile MRS media, and (ii) enzyme diluent with CFS were used in parallel. After incubation period of 24 hours, pH of all samples was neutralized to 6.0 +/- 0.2 and antibacterial activity was measured compared to indicator strain *P. aeruginosa using* agar well diffusion assays.

Amplification of subtilin and subtilosin genes

To determine whether *B. subtilis* and *B. amyloquefaciens* possess bacteriocins responsible for antibacterial activity, two genes *i.e.,* subtilin (*spaS*) and subtilosin (*sboA*) were amplified using previously reported specific primers [spaSFwd -AAGTTCGATGATTTCGATTTGGATG, spaSRev – AGTTACAAGTTAGTGTTTGAAGGAA [32]*;* sboAFwd-CGCAAGTAGTCGATTTCTAACA, sboARev CGCAAGTAGTCGATTTCTAACA [23]. Master mix having genomic DNA of *both isolates*, primers, nucleotides, buffer and Taq polymerase was prepared. PCR was performed following denaturation for 30 s at 95˚C, annealing for 30 s at 54˚C (*spaS*) or 53˚C (*sboA*), and elongation for 1 min at 65˚C for a total of 35 cycles. Obtained sequences were analysed using chromas lite and deposited at GenBank (MT490214) for subtilis and (MT490213, MT495613) for subtilosin of *B. amyloliquefaciens* and *B. subtilis* respectively.

Statistical analysis

All experiments were performed in triplicates. The mean and standard deviation of zones of inhibition were calculated. One way analysis of variance (one-way ANOVA) followed by post- hoc Turkey's test was performed using SPSS software (Statistical Package for Social Sciences) version 21 Chicago, IL, USA to determine significant differences at $P \leq 0.05$ among different groups.

RESULTS

Isolation and morphological characterization of HB gut bacteria

A total of twelve colonies (HB1-HB12) were found to be morphologically different from other colonies on the surface of BHI agar. Five colonies (HB3, HB4, HB7, HB9 and HB12) were irregular in shape and seven (HB1, HB2, HB5, HB6, HB8, HB10, HB11) were circular. All colonies were opaque and white in color. Gram's staining revealed that all isolates (HB1-HB12) were Gram-positive, rod shape and motile (Table S1). Biochemical tests revealed that only two isolates (HB2 and HB7) were catalase and voges proskauer negative. All isolates fermented three sugars (glucose, lactose and sucrose) except for one isolate (HB11), which was unable to ferment lactose. All isolates were able to hydrolyze starch and only two HB isolates (HB2 and HB7) were negative for methyl red (Table S2).

Antibacterial activity via primary screening (Cross streak method)

Cross streak method was used to check the antibacterial activity of all 12 Gram-positive, motile HB gut isolates against *P. aeruginosa, E. coli, B. licheniformis, K. pneumoniae* and *B. subtilis.* Among twelve HB isolates, gut isolates HB1 and HB12 showed significantly higher zones of inhibition (ZI; 7-11mm; $p \leq 0.05$) against *E. coli*. Isolates HB5 and HB6 showed highest ZI (7-8 mm; $p \leq 0.05$) against *K. pneumoniae* and both HB9, HB10 showed highest ZI against *P. aeruginosa* (8 ± 0.57; $p \leq 0.05$). Eight HB gut isolates showed significant inhibitory activity against both Gram-positive and Gram-negative pathogens (Figure. 1a, b), hence subjected to 16S rRNA sequencing and secondary screening.

a)

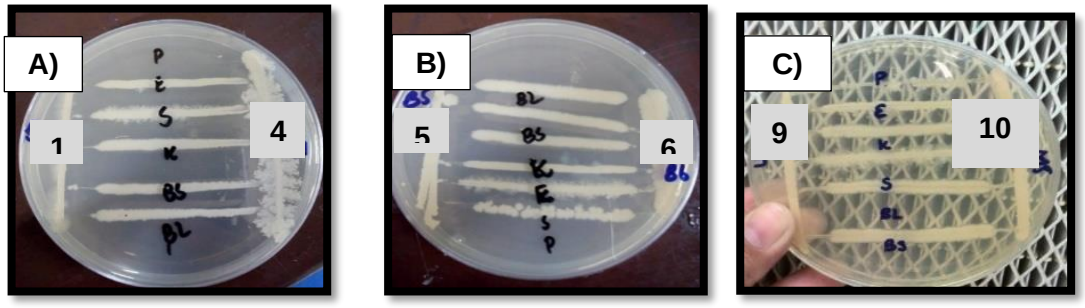

Figure 1a. Antimicrobial activity of some honey bee (HB) gut isolates against human pathogens using cross streak method (A) HB gut isolates HB1 and HB4 against pathogens (B) Inhibition by isolate HB5 and HB6 (C) Inhibition of pathogens by isolates HB5 and HB6. P, *P. aeruginosa;* E, *E. coli;* B.L, *B. licheniformis;* S, *B. subtilis;* K, *K. pneumonia.*

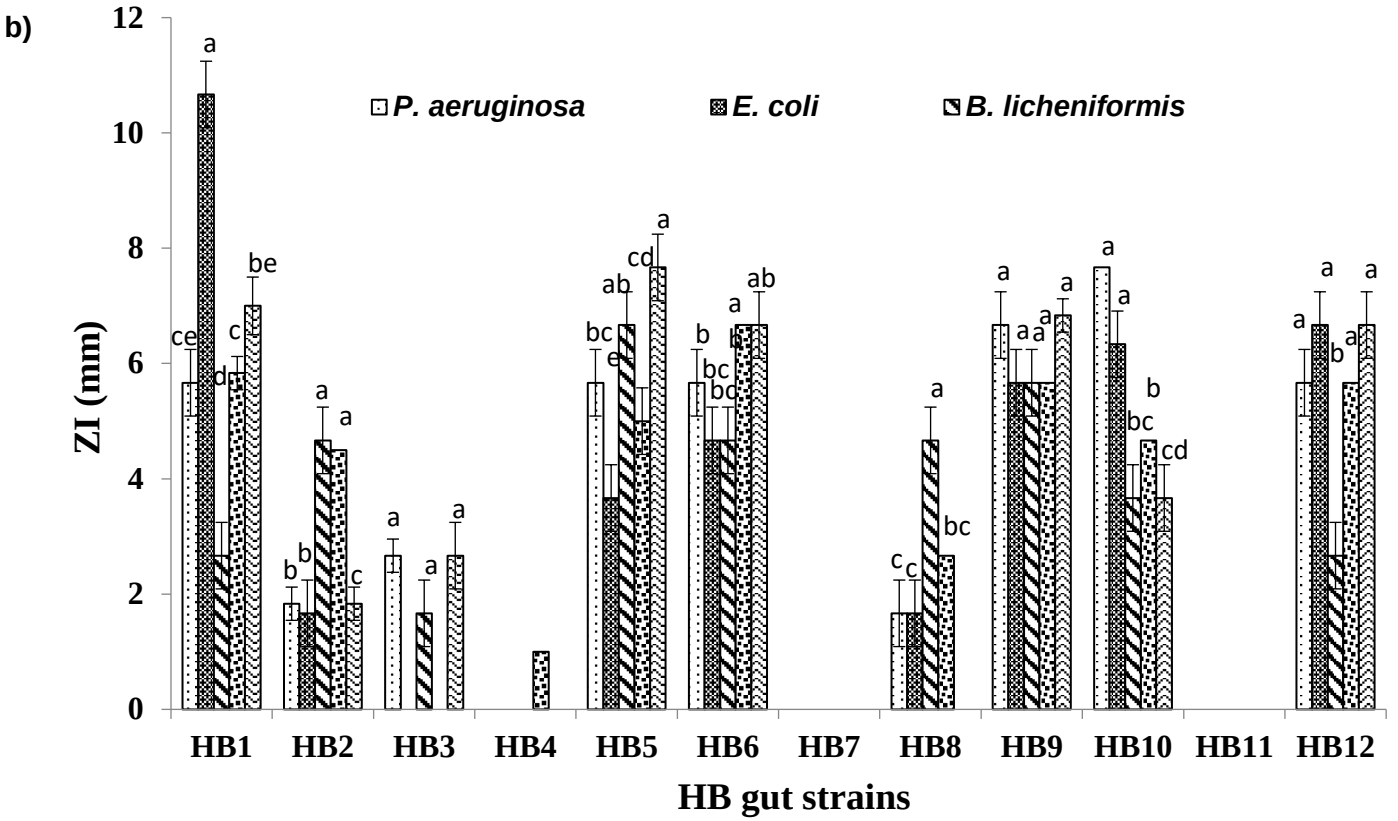

Figure 1b. Antimicrobial activity of twelve honey bee (HB) gut isolates (HB1-HB12) against five human pathogens (*P. aeruginosa, E. coli, B. licheniformis, Salmonella* sp., *K. pneumoniae*) using cross streak method. Among twelve isolates, eight HB gut isolates (HB1, HB2, HB5, HB6, HB8, HB9, HB10, HB12) showed significant inhibitory activity against both Gram-positive and Gram-negative pathogens. One-way analysis of variance (ANOVA) with post hoc turkey test was used to determine the level of significance. Bars with different superscripts are significantly different at $P \leq 0.05$.

16S rRNA gene sequencing

Eight HB gut isolates (HB1, HB2, HB5, HB6, HB8, HB9, HB10 and HB12) were identified upto species level using partial 16S rRNA gene sequence analysis. Comparison of HB gut isolates with sequences from BLAST programme at National Centre for Biotechnology Information (NCBI) database revealed that eight gut isolates belong to the evolutionary clade of genus *Bacillus.* These isolates were *B. subtilis* (HB1, MT186233), *B. xiamenensis* (HB2, MT186232), *B. altitudinis* (HB5, MT186237)*. B. safensis* (HB6, MT186235), *B. licheniformis* (HB8, MT186236), *B. amyloliquefaciens* (HB9, MT186234), *B. tequilensis* (HB10, MT186230) and *B. pumilus* (HB12, MT186231) (Figure 2).

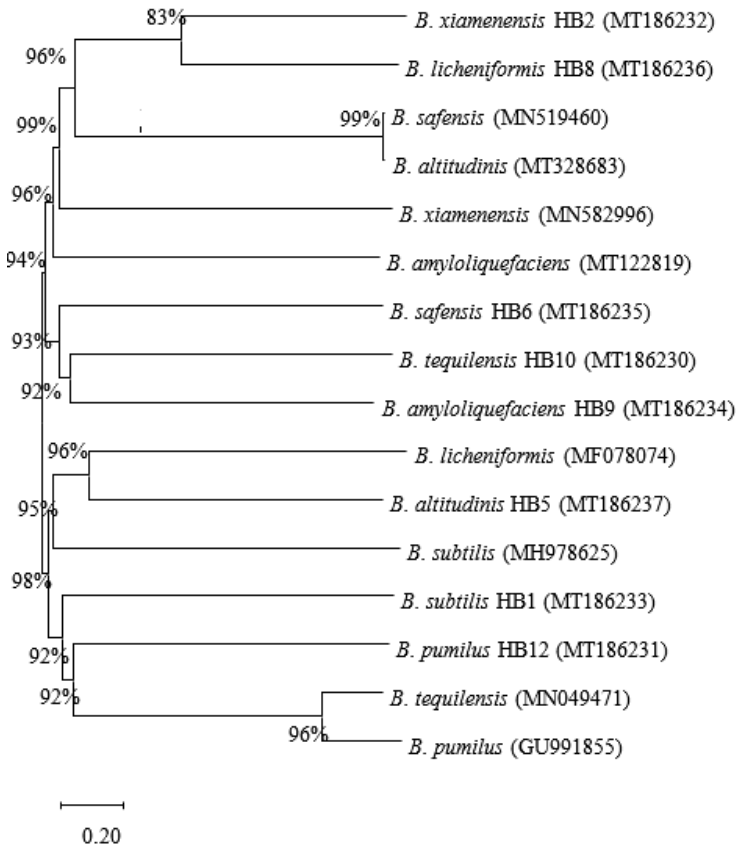

Figure 2. Phylogenetic study of 16S rRNA partial genome sequences of promising honey bee (HB) bacteria (HB1, HB2, HB5, HB6, HB8, HB9, HB10, HB12). Bacteria isolated from gut of HB were identified using 16S rRNA partial gene sequenicng and compared with the sequences from National Centre for Biotechnology Information (NCBI). The analysis was conducted with MEGA10 using neighbor-joining method and the genetic distance of each strain was determined.

Antibacterial activity (agar well diffusion method)

Secondary screening of eight identified HB gut isolates (HB1, HB2, HB5, HB6, HB8, HB9, HB10 and HB12) was performed using agar well diffusion method. *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) showed highest inhibition against all pathogens and their greatest inhibitory activity was against *E. coli* (ZI; 13 ± 0.577 mm; 14 ± 0.330, $p \leq 0.05$). *B. altitudinis* (HB5) showed significant inhibitory activity against *E. coli* and *B. licheniformis* (6-8mm; $p=0.0161$) only while *B. safensis* (HB6) showed highly significant inhibition against *K. pneumoniae* (12mm; $p \leq 0.01$) (Figure 3). Additionally, *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) were the only isolates able to inhibit growth of *P. aeruginosa* (8-11 mm; $p \leq 0.05$). This led us to select *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) for further antibiotic susceptibility testing, physiological characterization, extracellular enzyme assays and gene study.

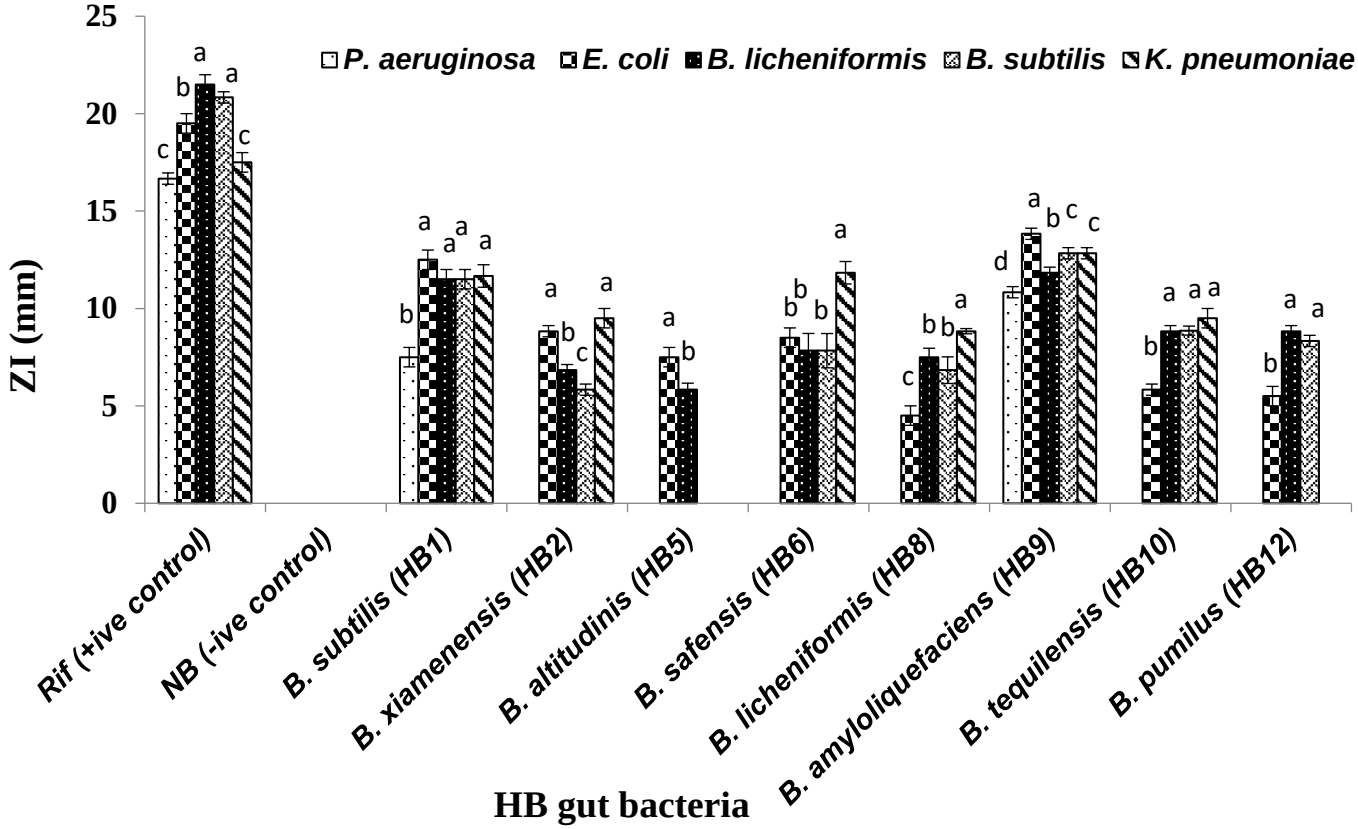

Figure 3. Antimicrobial activity of eight honey bee (HB) gut isolates (HB1, HB2, HB5, HB6, HB8, HB9, HB10, HB12) against five human pathogens (*P. aeruginosa, E. coli, B. licheniformis, Salmonella* sp., *K. pneumoniae*) using agar well diffusion method. One-way analysis of variance (ANOVA) with post hoc Tukey test was used to determine the level of significance. Bars with different superscripts are significantly different at $P \leq 0.05$.

Antibiotic susceptibility profile

Both isolates [*B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9)] showed sensitivity against the three antibiotics (ampicillin, erythromycin and rifampicin) used in this study. Both isolates were more susceptible to rifampicin, (ZI= 5-6 mm) and less susceptible to erythromycin and ampicillin (ZI=3-4 mm) (Figure 4).

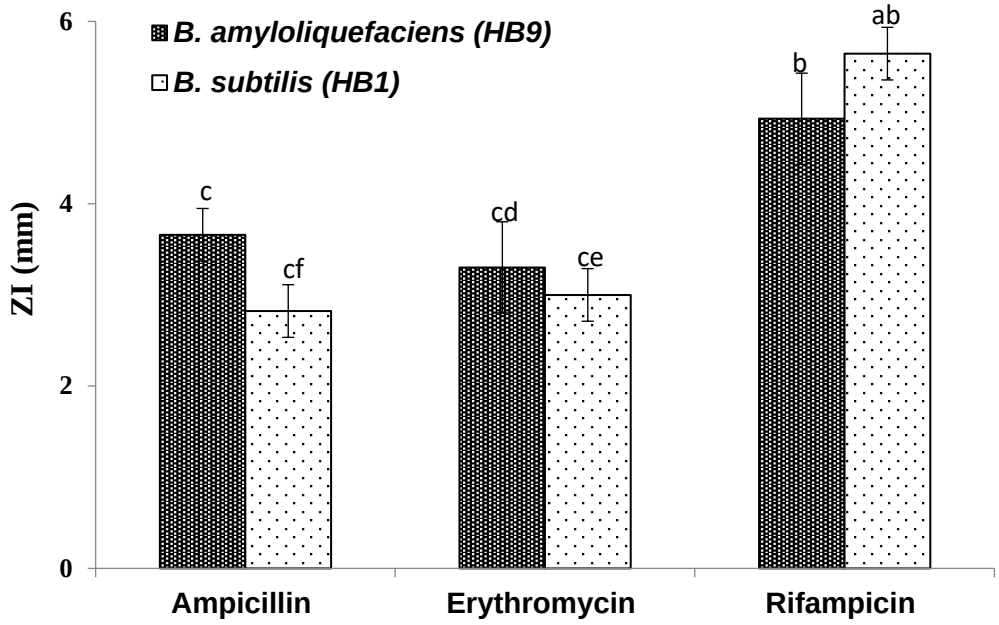

Figure 4. Antibiotic susceptibility profile of honey bee (HB) gut isolates *B. amyloliquefaciens* (HB9) and *B. subtilis* (HB1). Three antibiotics rifampicin (50 μgmL^{-1}), ampicillin (50 μgmL^{-1}) and erythromycin (20 μgmL^{-1}) were used. One-way analysis of variance (ANOVA) with post hoc Tukey test was used to determine the level of significance. Bars with different superscripts are significantly different at $P \leq 0.05$.

Growth curve study, pH and temperature effect

Growth curve of isolates indicated that both *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) showed lag phase of 1 hour and log phase of 3 hours. Afterwards, stationary phase lasted for 3-4 hours in both isolates. Following stationary phase, death phase was observed (Figure 5). *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) were characterized for their optimum temperature and pH. It was observed that both isolates showed best growth at temperature 37ºC and pH 7 (Figure 6).

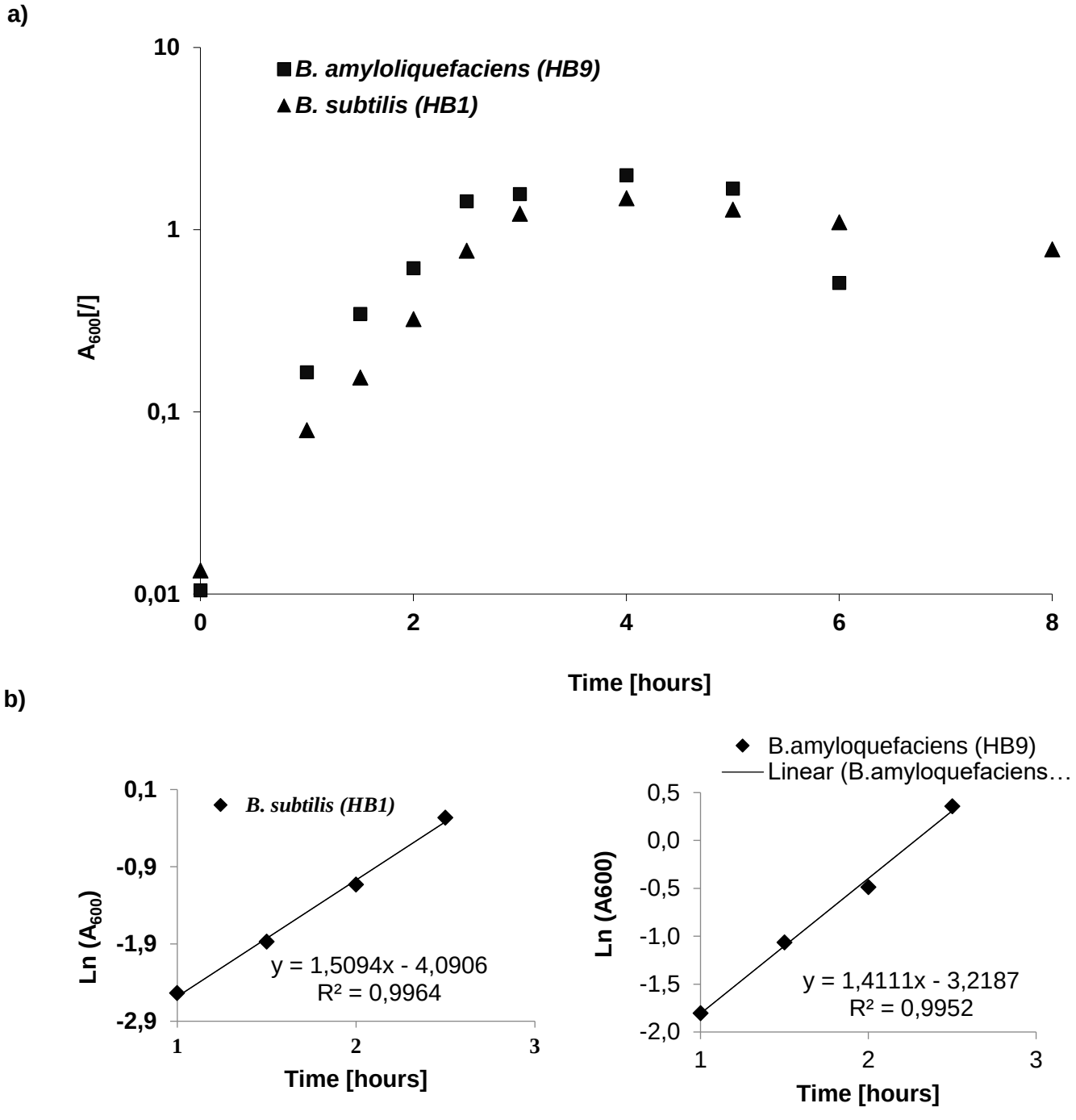

Figure 5. Growth curve of two promising honey bee (HB) gut isolates *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9). **a).** Bacterial growth was determined by OD_{600}. Data were obtained from the average of three independent experiments **b)**. Specific growth rate of two isolates *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9). It was measured by taking OD measurements at fixed intervals and plotting the growth with Ln (OD) at the y-axis (exponential phase) and time at x-axis to find linear curve from 3-4 points.

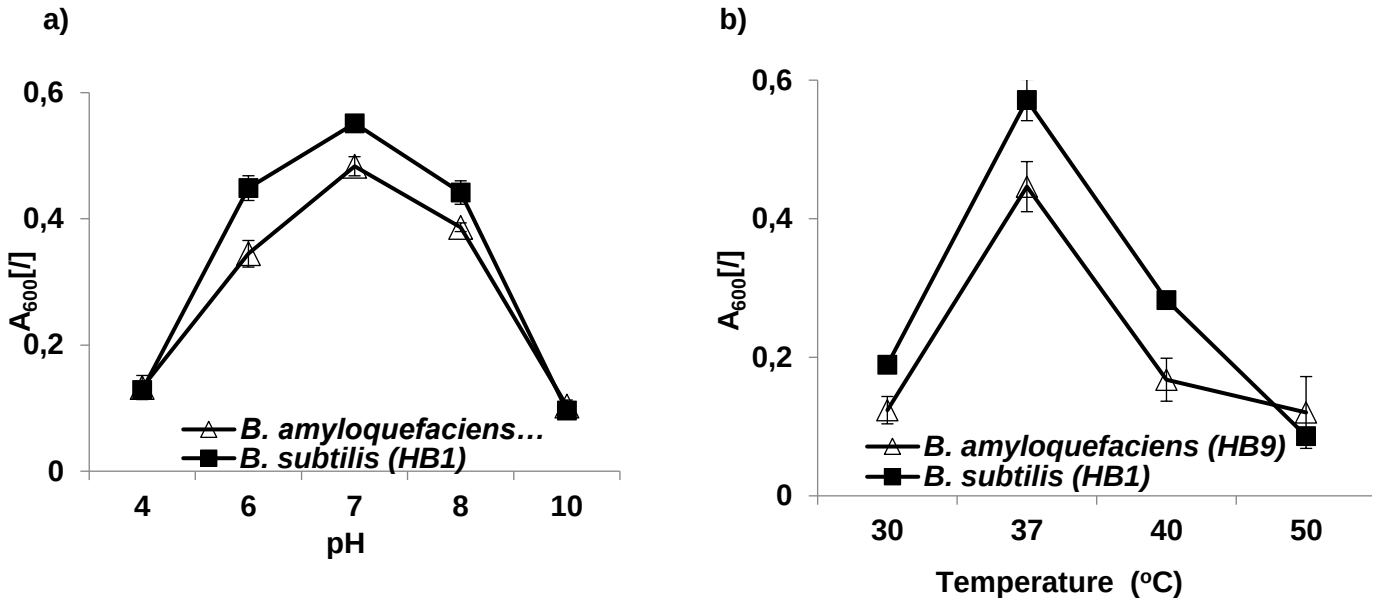

Figure 6. Effect of pH and temperature on two promising honey bee (HB) gut isolates *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9). Bacteria were grown in LB medium at **a)** various pH (4, 6, 7, 8 and 10) and **b)** temperatures (30, 37, 40 and 50ºC). OD_{600} indicated maximum growth at pH 7 and temperature 37ºC. Results were expressed as Mean ± SD. Experiments were performed in triplicates (n=3).

Extracellular enzyme activities of bacterial isolates

Cellulase, protease and lipase activities of both isolates *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) revealed that they possess all the three enzyme activities (Table 1). Briefly, cellulolytic activities were found to be high for both *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) (ranging from 0.70 to 0.75 U/mL) (Table 1). *B. amyloliquefaciens (*HB9) showed the highest proteolytic activity (2.48 U/mL), while the lowest was found from *B. subtilis* (1.20 U/mL). Lastly, *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) showed almost equal lipolytic activity (0.80-0.82 U/mL) (Table 1).

Table 1. Extracellular enzyme activity of two promising HB gut bacteria

Bacterial isolates	Extracellular enzyme activity (U/ml)		
	Proteolytic	**Lipolytic**	**Cellulolytic**
B. subtilis (HB1)	1.20 ± 0.05	0.82 ± 0.04	0.70± 0.01
B. amyloliquefaciens (HB9)	2.48 ± 0.06	0.80 ± 0.02	0.75 ± 0.02

Measurement of lactic acid concentrations

Both isolates *i.e*., *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) produced very small amount of D- and L-lactic acid. Results showed total production of 0.17 g l^{-1} D-lactic acid per sample, which was equal to blank. Concentrations of L-lactic acid was averaged to 2.23-2.25 g l^{-1} in both isolates respectively, which was higher from 0.16 g l^{-1} concentration of blank (Table 2).

Table 2. D- and L-lactic acid concentration in cell-free supernatant

Sample	ΔA_D	[D-Lactic acid] (g l^{-1})	ΔA_L	[L-Lactic acid](g l^{-1})
Blank	0.05 +/- 0.00	0.19 +/- 0.01	0.05 +/- 0.00	0.16 +/- 0.01
B. subtilis (HB1)	0.05 +/- 0.00	0.17 +/- 0.09	0.68 +/- 0.01	2.25 +/- 0.02
B. amyloliquefaciens (HB9)	0.05 +/-0.00	0.17 +/- 0.02	0.68 +/- 0.00	2.23 +/- 0.03

Enzyme digestion and effect of temperature and pH on antibacterial activity

Table 3 summarizes the results of CFS treatment with seven enzymes. Almost complete loss of activity in the presence of proteinase K and pepsin, and significantly decreased activity upon treatment with trypsin and chymotrypsin was observed, thus indicating proteniacious nature of antibacterial activity. Temperature and pH also had no effect on antibacterial activity of the studied CFS (Table 4).

Table 3. Effect of enzymatic digestion on antimicrobial activity

Enzyme	Enzyme concentration (mg mL^{-1})	Enzyme diluent	Zone of inhibition (mm)	
			B. subtilis **(HB1)**	***B. amyloliquefaciens*** **(HB9)**
Trypsin	20	1 mmol l^{-1} HCl	3.0 +/- 0.26	3.0 +/- 0.26
Pepsin	10	10 mmol l^{-1} HCl	3.0 +/- 0.50	3.0 +/- 0.50
Proteinase K	10	ddH_2O	2.0 +/- 0.29	3.0 +/- 0.29
Chymotrypsin	10	1 mmol l^{-1} HCl	5.0 +/- 0.58	5.0 +/- 0.58
Catalase	10	50 mmol l^{-1} Kpi (pH 7)	12.0 +/- 0.58	11.0 +/- 0.58
Lipase	20	ddH_2O	11.0 +/- 0.76	11.0 +/- 0.76
Protease	10	ddH_2O	6.0 +/- 0.29	6.0 +/- 0.29

Table 4. Effect of temperature and pH on antimicrobial activity

Temperature (˚C)	pH	Exposure time (min)	Antimicrobial activity	
			B. subtilis **(HB1)**	***B. amyloliquefaciens*** **(HB9)**
40	4	10	+	+
		60	+	+
60	6	10	+	+
		60	+	+
80	8	10	+	+
		60	+	+
100	10	10	+	+
		60	+	+

Genetic study of bacteriocins

PCR study showed that both *B. subtilis* (HB1) and *B. amyloliquefaciens* (HB9) were positive for the functional gene-encoding subtilosin (*spoA*). BLAST study of DNA sequence of amplified PCR product from both isolates showed 98-100% similarity with subtilosin A of *B. subtilis* (WP_087992738.1). However, *spaS* gene encoding subtilin protein was only successfully amplified in *B. subtilis* (HB1) having 83% homology with subtilin of *B. subtilis* (QIR30939.1).

DISCUSSION

Resistance of bacteria to antibiotics is emerging rapidly worldwide which is endangering the efficiency of antibiotics. Antibiotic resistance has become a global burden to health sector [33]. This study was carried out to study promising bacteria from gut of honeybees which can produce novel bioactive compounds as alternative to antibiotics. Using culture based methods, white, rod shape bacteria were selected and purified on nutrient agar. The purified bacteria were further characterized morphologically and biochemically. Morphological identification showed variations in colony appearance, opacity, margin and elevation. Biochemical characterization revealed that almost all bacteria had ability to ferment carbohydrates and hydrolyze the starch. These biochemical tests were comparable to the findings by Audisio and coauthors [34], who reported Gram positive rod shaped bacteria from HB gut with ability to ferment carbohydrates and

hydrolyze starch. Similarly findings were also reported by Audisio [35], who isolated Gram positive, motile and catalase positive HB gut.

Antibacterial activity of HB gut bacteria using cross streak method revealed that these bacteria have the tendency to inhibit the pathogens. HB gut bacteria exhibited broad spectrum antibacterial activity with variable inhibition zones against *E. coli, B. licheniformis*, *P. aeruginosa, K. pneumoniae* and *B. subtilis*. The antibacterial activity of HB gut isolates against both Gram positive and Gram negative bacteria observed in this study might be due to the bactericidal effect of protease sensitive bacteriocin like substances, production of hydrogen peroxide and organic acids [36]. However, mechanism of antibacterial action may have other attributions. For example, Janashia and coauthors [37] reported that antibacterial activity of HB LAB is due to synergistic action of several other secondary metabolites in addition to bacteriocins. Likewise, Yoshiyama and coauthors [38] analysed the transcription level patterns of antibacterial peptide genes from LAB of HB gut and verified these as immune activators.

16S rRNA sequencing of eight promising HB gut strains revealed that all strains displayed closest similarity with phylum Firmicutes, genus *Bacillus*. Previously, Yoshiyama and Kimura [15] assessed the bacterial diversity in the digestive tract of the *A. cerana* japonica, Japanese honeybee, using 16S rRNA gene sequencing and histological studies. Authors reported that seven bacteria with strong inhibitory activity against *P. larvae* belonged to genus Bacillus. In another study, Pomastowski and coauthors [14] also made similar observations. They isolated the bacterial strains from honey and 16S rRNA sequencing revealed the isolate as *B. tequilensis* (NR 104919). Likewise, Agbagwa and coauthors [39] reported antibacterial potential of *Bacillus* species alongwith antibiotics residues from branded and unbranded honey samples, Nigeria.

Agar well diffusion assay of eight promising HB gut strains was studied using CFS against *E. coli, K. pneumoniae, P. aeruginosa, B. licheniformis* and *B. subtilis*. Only two isolates *B. amyloliquefaciens* and *B. subtilis* inhibited the growth of all tested pathogens. Similar findings were reported by Hasali and coauthors [40], who studied antibacterial activity of HB microbiota using agar well diffusion method and found excellent inhibitory activity of HB microbiota against different pathogens; *S. epidermidis, B. subtilis, P. aeruginosa, E. coli, S. typhimurium, S. aureus* and *L. monocytogenes*. Likewise, Kacaniova and coauthors [6] investigated the antibacterial activity of HB gut bacteria against *P. larvae*. Authors observed that *L. gasseri* and *L. amylovorus* showed strongest antibacterial activity against *P. larvae* compared to *L. fructivorans* which had least antibacterial activity. In contrast, Janashia and coauthors [37] reported that none of his studied HB gut isolates showed any antibacterial activity against tested pathogens.

Following antibacterial activity and 16S rRNA sequencing antibiotic susceptibility profile of *B. amyloliquefaciens* and *B. subtilis* was performed. It was observed that *both isolates* showed less susceptibility to rifampicin compared to ampicillin and erythromycin. Between two isolates, *B. subtilis* was more sensitive towards tested antibiotics compared to the *B. amyloliquefaciens*. Similar results were also reported by Kacaniova and coauthors [6] in which antibiotic resistance of HB gut bacteria was checked against ciprofloxacin, levofloxacin and gentamicin. Their findings revealed that isolated Gram positive bacteria were sensitive to all aforementioned antibiotics. In another study by Gharehyakheh and coauthors [41], susceptibility of HB gut bacteria was checked against commercially available antibiotics (chloramphenicol, kanamycin, erythromycin, penicillin, streptomycin and amoxicillin). Results showed that all isolates showed susceptibility for tested antibiotics with maximum inhibition against chloramphenicol.

When we looked at the optimum growth conditions of the two isolates *i.e.*, *B. amyloliquefaciens* and *B. subtilis*, we found that growth was optimum at 37°C temperature and pH 7. This is similar to the results shown by Albaridi [42]. They observed that HB gut bacteria showed best growth at pH range of 6.5-7.5. Raghavan and coauthors [43] observed the growth of HB gut bacteria at acidic and basic environment, and different temperature range (30-40 °C). Their findings revealed that most stains were resistant to acidic pH, with optimum growth at pH 7 and temperature 37°C.

Enzyme assays were performed to screen hydrolytic enzymes. We observed that both isolates possess cellulose, protease and lipase. Enzymes produced by the bacterial isolates might be involved in the food digestion and breakdown of complex biomolecules for example, carbohydrates and proteins involved in bee nest formation [19]. Insects perform symbiotic relationships with various microorganisms. Symbiotic bacteria of insects not only benefit the host nutritionally [44], but also play important in degradation of nest products and other pathogens by producing various enzymes [20].

To confirm the nature of antibacterial activity, a series of preliminary tests were conducted. Absence of D/L lactic acid and sensitivity of CFS towards protein degrading enzymes suggested its proteniacious nature. Likewise, stability and antibacterial activity at high temperature and pH stresses is in agreement with the

findings observed by Sharma and coauthors [45], who attributed the stability of Bacillus bacteriocins to the presence of unusual amino acids present in CFS. These observations suggest that CFS of both Bacilli isolates possess antibacterial substances belonging to class of bacteriocins.

Presence of bacteriocins in CFS was confirmed by successful amplification of *sboA* and *spaS* genes. Data revealed that CFS of both bacteria possesses subtilosin (encoded by *sboA* gene), similar to bacteriocin. Presence of subtilosin protein was reported both in *B. subtilis* [46] and *B. amyloliquefaciens* [47], previously. In fact, presence of subtilosin protein in *B. amyloliquefaciens* might correspond to the fact that both isolates show close similarity with respect to their 16S rRNA regions and 16S–23S internal transcribed spacer regions [48]. *B. amyloliquefaciens* is diverge species compared to *B. subtilis* and its closely related *B. atrophaeus*. Albano and coauthors [49] did transcriptional profiling of *B. subtilis* and confirmed Rok protein orthologue (encoded by *roc* gene) in *B. amyloliquefaciens that might have transferred* from *B. subtilis* via horizontal gene transfer. Subtilin protein amplification in *B. subtilis* only in this study is in agreement with previously published findings by Abriouel and coauthors [46]. There are several other studies where authors reported that *Bacillus* species produce a diverse range of bacteriocins such as ericin A, subtilin, ericin S, cerein 7A, coagulin and cerein 7B *etc*. These bacteriocins have been observed to show antibacterial activity against food-borne pathogens, and potential in biomedical industry [50]. Similarly, Shelburne and coauthors [51] studied the antibacterial activity of subtilosin in detail and observed broad spectrum activity of bacteriocins against majority of microbes except for encapsulated ones. However, these findings were contradicted by Abriouel and coauthors [46], who suggested subtilosin as an ideal bacteriocin to treat bacterial vaginosis without any harm to healthy microbes.

This study provides evidence that bacteriocins might be important candidates for food preservation. In a study by Burkard and coauthors [52], similar to nisin, subtilin and subtilosin A have been found quite effective against *L. monocytogenes*. Suwanjinda and coauthors [53] used PCR to determine the presence of bacteriocins such as nisin, pediocin and enterocin A in lactic acid bacteria isolated from traditional Thai fermented foods. Although, commercially available nisin is mainly used in food applications, there is continuous need to develop new lantibiotics and unmodified bacteriocins. Problems such as bacteriocins instability in certain foods/environments and low production levels need to be properly addressed. However this needs further investigation by the food industry because range of activity by bacteriocins is often specific and limited.

CONCLUSION

It can be concluded from the present study that microbiota present in the gut of honeybees possess antibacterial activity. They are susceptible to antibiotic and express extracellular enzymes. Laboratory based findings allowed us to select two active isolates *i.e*., *B. amyloliquefaciens* and *B. subtilis* from HB gut with significant antibacterial potential against both Gram-positive and Gram-negative bacteria. Our further observations proved the proteniacious nature and stability of inhibitory activity under stress (high pH and temperature). PCR amplification of *spoA* and *spaS* genes confirmed that antibacterial activity was due to the presence of subtilin and subtilosin proteins, which are closely related to family bacteriocins. Further purification and *in vivo* study will help us to understand safety and toxicity of bacteriocins by determining its potential as probiotics, in food packaging and pharmaceutical applications. Additionally, this study provided preliminary proof that detailed molecular, pharmacological and chemical evaluation of compounds from HB gut bacteria may help us to find novel antibacterial compounds that could help us in solving the problem of antibiotic resistance with possibility to find effective alternatives to antibiotics.

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