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Evaluation of Probiotic Bacteria Isolated from Indigenous Honeybee Species of Pakistan

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ABSTRACT: *The honey bee gut is inhabited by many useful bacteria such as Lactic Acid Bacteria (LAB). These LAB possess active probiotic properties that are beneficial to other animals and even humans. The LAB is declared as generally recognized as safe (GRAS). The LAB is non-pathogenic and produces different metabolites having antimicrobial potential. In the current study LAB species isolated from four honey bee species (Apis melifera, Apis dorsata, Apis cerana, and Apis florae) prevalent in Pakistan were identified and investigated for their probiotic potential. The strains were identified using morphological, biochemical, and molecular techniques as genus Lactobacillus and Lactococcus. Current results confirmed that the honey bee gut has a rich source of lactic acid bacteria. Isolated strains were further evaluated for their ability to resist physiological stresses encountered in the human gastrointestinal tract. Safety testing of the isolates such as hemolytic assay revealed that these isolates have γ-hemolytic activity and were adjudged to be safe. Hence the present study concluded that honey bee gut is the source of potential probiotic bacteria and can be used in pharmaceuticals, fermented foods, and nutraceuticals and may be used as a natural food preservative.*

Keyword: Lactic Acid Bacteria, Gastro Intestinal Bacteria, pH and Bile Tolerance, *Apis spp, Lactobacillus*

INTRODUCTION

Honey bee belongs to the family *Apidae* of insects and contains approximately 20,000 species (Zareen et al., 2016). It is present all around the world excluding the extreme Polar Regions. During the last two centuries, honeybees were traditionally kept in Pakistan for honey production (Ansari et al. 2014). Out of four species of honeybee, that are present in Pakistan three of these species *Apis dorsata, Apis florae, Apis cerana* are native to Pakistan whereas *Apis mellifera* was imported from Australia or Russia in 1979 (Anjum et al., 2016). Declines of honeybee colonies are due to colony collapse disorder (CCD), which may occur because of infections, pesticides, contaminated water, antibiotic use, inadequate diet, and inappropriate breeding management have all been suggested as possible reasons for these large-scale losses (Borges and Goodwin, 2021).

Honey bees play a significant role in increasing crop yield production along with their fruit-bearing capacity in forest plants. They primarily consume pollen and nectar as food and produce a sweet liquid called honey (Nicolson and Thornburg, 2007). LAB enters and colonizes the honey stomach through pollen consumption and food exchange with older bees in the colony (Mardan et al., 2011). Probiotic bacteria are those microorganisms that improve the intestinal microbial flora of the host when it is taken in any supplemented food in an adequate amount and gives health benefits to the host. Bermudez-(Brito et al., 2013). Probiotics isolated from gut bacteria are frequently used in many dietary supplements and functional foods (Daisley et al., 2020) Many proteins and other metabolites are produced by these Lactic acid bacteria (LAB) which protect the honey bee from incoming microbial threats. Honeybee-associated Lactic acid bacteria like *Lactobacillus johnsonii, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus apis* are found to have inhibitory effects on honeybee pathogens such as *Paenibaccilus larvae* and *Melissococcus plutonius.*

Both lactic acid and acetic acid bacteria can tolerate very acidic conditions and they can also metabolize sugars to produce organic acid these unique characteristics of LAB aid their growth in the digestive tract of honey bees which is very rich in sugar content and it also inhibits the growth of acid-sensitive bee pathogenic bacteria (Balloi et al., 2011). Lactic acid production, low G + C %, without spore production, gram +ve, and catalase-ve are key characteristics of lactic acid bacteria and

are mostly used as a starting culture in food industries (Gomez, 2016). The presence of LABs in the gut of honey bees has gut-dwelling properties (Pattabhiramaiah et al., 2012). The objectives of the study were to identify the lactic acid bacteria from the honey bee gut of four different species and characterize their probiotic potential.

MATERIALS AND METHODS

Sample collection and culture condition:

Total eight honey bee gut isolates NPL 813 (*Apis florae*), NPL 814 and NPL 815 (*Apis cerana*), NPL 812, NPL 784, and NPL 785 (*Apis dorsata*), NPL 811 and NPL 786 (*Apis mellifera*) were obtained from the National Probiotic Laboratory (NPL) culture collection Faisalabad, Pakistan. These four different honey bee species were collected from hives of Honeybee Research Institute (HBRI), National Agricultural Research Center (NARC), Islamabad in sterilized bottles, and their gut samples were dissected out aseptically. These honey bee isolates were cultivated on MRS (De Man Rogosa Sharpe) agar plate (Neogen, USA) through the streak plate method. The MRS agar plates were incubated at 37 ºC for 24 to 48 hours under aerobic conditions in an incubator as mentioned by Singh et al. (2013).

Bacterial characterization

Morphological characterization

Bacterial plates were visually examined for colony morphologies i.e. size, shape, color, texture, and appearance. For microscopic observation, gram staining was performed according to the protocol of Erkus and Bannigidad (2011). The prepared slides were observed under an Intelligent Inverted Compound Microscope BX63 Olympus at 100X magnification using immersion oil. The microscopic characters (Gram stain reaction, shape, and arrangement) of the cells were 10 recorded.

Biochemical Characterization

The biochemical (catalase) test was performed by slide method. A small drop of 3% H_2O_2 was taken on a clean glass slide and one to two bacterial colonies were mixed in a drop with a sterilized inoculating loop. The appearance of gas bubbles indicated a positive catalase reaction (Rahman, 2015).

Molecular Characterization

The molecular identification of bacterial isolates was done by genomic DNA extraction followed by polymerase chain

reaction (PCR) using genus-specific primers.

In DNA Extraction, DNA of overnight growth culture was extracted using Thermo Scientific kit (USA) protocol with minor modification (Mattila et al., 2015).

Gel was prepared. Carefully loaded mixed 1 µl (6x loading dye) with 5 µl of each sample into separate wells and 1 µl DNA ladder into another well. The Gel was visualized under UV light and bands were compared with a DNA ladder this is done by usually documentation Costumbrado et al. (2012). The genomic DNA was amplified with different genus-specific primers by PCR (Bio-Rad, USA). Different ingredients used in a PCR reaction are mentioned below. *Lactobacilli* and *Lactococcus* primers were used in this study Lias et al. (2007) Pepe et al. (2002) Costumbrado et al. (2012). Bacterial species were determined by observing the band size.

Primer Sequence

The following (table 1 and 4) Reverse and Forward Primer sequence was used for NPL No. 811, 812, 813, 784, 785, 786, 788 at 16s rDNA identification of *Lactobacilli*.

Table 1: PCR reaction mixture

Sr. no.	Description	Volume	Final concentration
1	Green PCR reaction mix	12.5µl	1X
2	Forward primer	1.5 µl	0.6µM
3	Reverse primer	1.5 µl	0.6µM
4	Nuclease free water	4.5 µl	-
5	DNA template	5 µl	10pg-1µg

Table 2: Primers used in this study

Sr. #	Target genus	Primer sequence	Annealing temp.	Band size	Ref.
1	*Lactococcus lactis*	NPL-L-lactF GAAGTCGTAACAAGG NPL-L-lactR CAAGGCATCCACCGT	50°C	380	Blaiotta et al. (2002)
2	*Lactobacillus*	NPL-lactoF TGGAAACAGCTGCTAA TACCG NPL-lactoR GTCCATTGTGGAAGATT CCC	62°C	233	(Caufield et al., 2007)

Table 3: PCR Profile *Lactobacilli*

Profile	Temperature	Time	Cycle
Initial Denaturation	95ºC	3 Minute	1
Denaturation	95ºC	30 second	35
Annealing	62ºC	30 second	35
Extension	72ºC	1 Minute	35
Final Extension	72ºC	5Minute	1
Hold	4ºC	∞	-

Table 3: PCR Profile for *Lactococcus Lactis*

Profile	Temperature	Time	Cycle
Initial Denaturation	94ºC	1 Minute	1
Denaturation	94ºC	1 Minute	25
Annealing	55ºC	2 Minutes	25
Extension	72ºC	3 Minutes	25
Final Extension	72ºC	10 Minutes	1
Hold	4ºC	∞	-

Bacterial Growth Curve Experiment under Aerobic Condition

Overnight grown bacterial culture was centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded, and the pellet was washed twice with sterile PBS (pH 7.2). Then bacterial suspension was made by mixing the bacterial pellets in the sterile PBS (pH 7.2). The O. D 600 of bacterial cultures was adjusted 0.5 ± 0.05 through spectra max at 600nm wavelength.

The volume of PBS bacterial suspension was calculated through formula M1V1 = M2V2 for adjusting O.D 0.05±0.005 in MRS broth. Alone MRS broth was used as a negative control (without bacterial culture). The bacterial suspensions were inoculated into sterile fresh MRS broth and incubated at 37ºC. The O.D 600 bacterial cultures were taken in triplicate every 1 hour by transferring the 200µl bacterial culture in 96 well micro-titer plates through spectramax at 600 nm. The bacterial cultures O.D600 were measured and plotted in a line graph (Hu et al. 2017).

pH Assay

The NPL strains were revived from glycerol stocks in 5ml MRS broth at 37ºC for 16-18 hours. Bacterial isolates were sub-cultured twice till O. D600nm lies between 0.2 to 0.8. The bacterial culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded, and the pellet was washed with PBS twice. The pellets were suspended in the PBS and vortex for

making the suspension. The Culture OD was adjusted between 0.2-0.8 through a spectrophotometer at 630 nm. Bacterial culture was calculated by using M1V1 = M2V2 for adjusting OD 0.05±0.005 and transferred into the PBS solution pH 1.5, 3.0, 6.5 and properly mixed. The culture OD was taken at 0 hour and incubated at 37ºC for three hours in PBS for stress treatment. After 3 hours, bacterial culture OD was observed. The bacterial culture was centrifuged at 5000 rpm for 5 minutes and cell pellets were suspended with fresh MRS broth. Initial O. D600nm was taken in triplicates and incubated at 37°C in the incubator. After 5h of incubation, O.D 600 nm of broth (positive control) revived from stress solutions was taken in Spectramax. Results were recorded in triplicates to analyze the pH tolerance of bacteria (Leong et al., 2011).

Bile Assay

The NPL strains were revived from glycerol stocks in 5ml MRS broth at 37ºC for 16-18 hours. Bacterial isolates were sub-cultured twice till O. D600nm lies between 0.2 to 0.8. The bacterial culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded and the pellet was washed with PBS twice time. The OD was adjusted in PBS between 0.2-0.8 through spectramax at 600 nm. The bacterial culture was calculated by using the formula M1V1 = M2V2 to adjust the OD to 0.07. The culture was transferred into MRS broth containing 0%, 0.15 %, and 0.3% and mixed through overtaxing followed by incubation at 37ºC. The OD 600 nm was taken at 0 hour and log phase in triplicate in 96 well microplates. Results were recorded in triplicates to analyze the bile tolerance level of bacteria (Ehsani et al., 2012).

Hemolysis Assay

Overnight incubated strains were streaked on blood sheep Agar (Merck, Germany), and incubated at 37 º C for 48 hours. Zones were observed around the streaked culture Mandal et al., (2017). Hemolysis was categorized as no clear halos as non-hemolytic, clear hemolysis zone as β-hemolytic or completely hemolytic, and a greenish halo as α-hemolytic or partially hemolytic.

Glycerol Stock Preparation

The glycerol stocks of each purified isolate were made by subculturing on MRS broth. The broth cultures were preserved by adding glycerol (20% v/v). The mixture was transferred to labeled sterile eppendorf tubes. The label includes their respective NPL number, date of preservation, and initials of the related personnel. Each isolate was

preserved in triplicate cryovials one as mother stock and the other two as working. The stocks were kept in labeled cryo-boxes and stored at –40°C in a Biomedical freezer for further use (Sieo et al., 2014).

RESULTS

Morphological Characterization

In the present study, a total of eight bacterial isolates were taken from the National Probiotic Laboratory (NPL) culture collection Faisalabad, Pakistan. They were as follows: NPL 813 (*Apis florae*), NPL 814 and NPL 815 (*Apis cerana*), NPL 812, NPL 784, and NPL 785 (*Apis dorsata*), NPL 811 and NPL 786 (*Apis mellifera*) shown in figure 1. The identification was performed according to morphological and microscopic characteristics. The bacterial isolates were revived from glycerol stocks on MRS agar plates after 24 to 48 hrs, of incubation at 37ºC under aerobic conditions. All the isolates were gram-positive. Out of eight strains, morphologically six isolates NPL 813 (*Apis florae*), NPL 812, NPL 784, NPL 785 (*Apis dorsata*), NPL 811, NPL 786 (*Apis mellifera*) were bacilli and two isolates of NPL 814, NPL 815 (*Apis cerana*), were Coccus. All were found to be non-motile or non-spore former. The macroscopic characteristics of all the isolates were shown in Fig. 1.

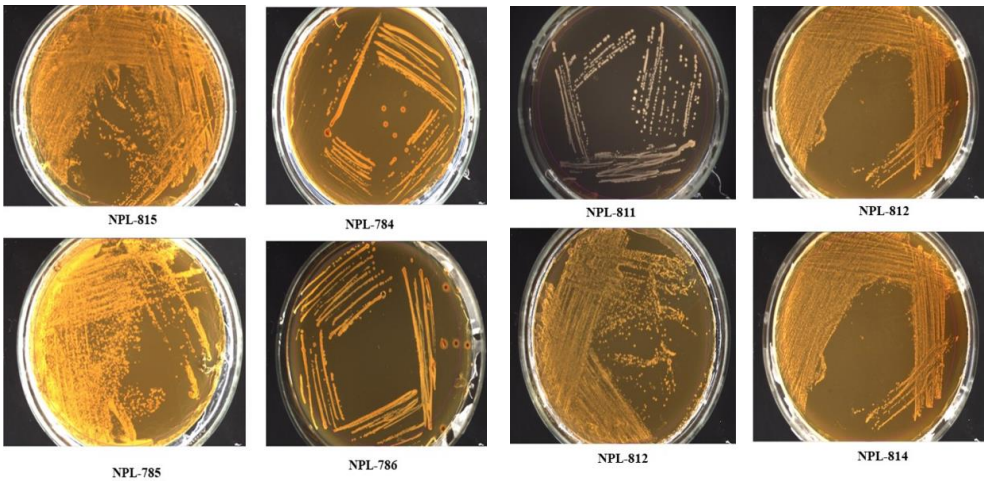

Fig. 1. Growth of honey bee isolates on MRS agar plates after 24-48 hours incubation at 37ºC

Biochemical characterization

All eight isolates of NPL was shown catalase negative. They are not formed bubble on glass slide when drop the H_2O_2 and converted it into H_2O.

Molecular Identification

The genus specific primers of *Lactobacilli* were used to amplify the genomic DNA of gram-positive rods whereas specie specific primers were used for gram positive cocci. The bands represent result after gel electrophoresis of PCR product. The DNA fragment of 380bp and 250 bp was separated on 2% agarose gel along DNA ladder. The observations on gel was taken by putting it in gel documentation system shown in Fig. 2.

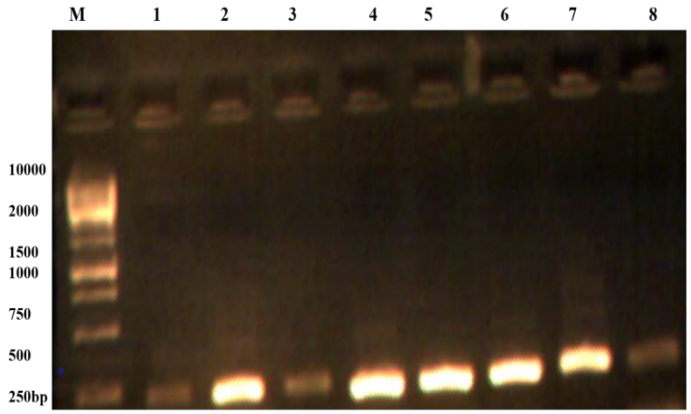

Fig. 2. PCR amplification of honey bee isolates of *Apis dorsata* (NPL-785, 784, 785), *Apis melifera* (NPL-811, 786), *Apis florae* (NPL-813) and *Apis cerana* (NPL-814, 815)

Bacterial Growth Curve Experiment

All the isolates were grown in MRS broth. The growth curve of *Apis dorsata*, isolates NPL784, 785, 812 showed that log phase from 2nd to 10th hours under aerobic condition Figure 3. The growth curve of *Apis melifera* isolates NPL 811 and 786 showed that the log Phase from 3rd to 7th hour hours, 2nd from 12th respectively under aerobic conditions are shown in Figure 4. The growth curve of *Apis florae* isolates NPL 813 showed that the log phase from the 2nd to 7th hour is presented in Figure 5. The growth curve of *Apis cerana* isolates NPL 813 showed

that the log phase from the 3rd to 7th hours is given in Fig. 6.

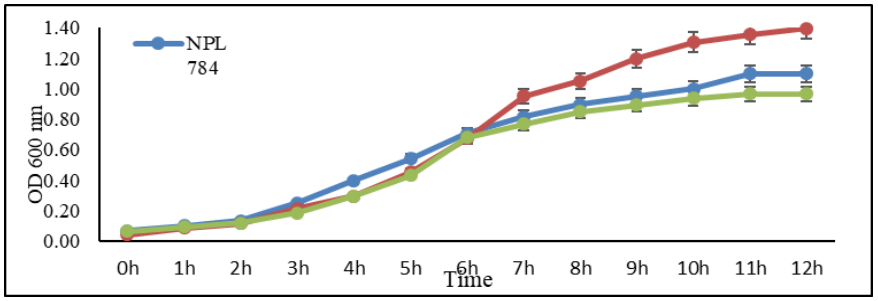

Fig. 3. Growth curve assays of honey be e isolate *(Apis dorsata)* under aerobic condition

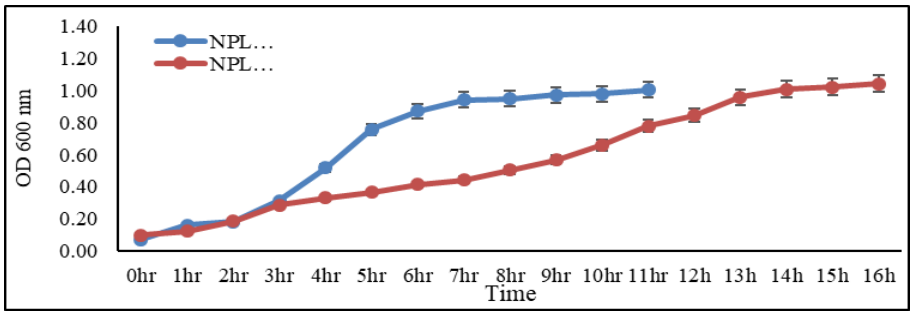

Fig. 4. Growth curve assays of honey bee isolate (*Apis melifera*) under aerobic condition

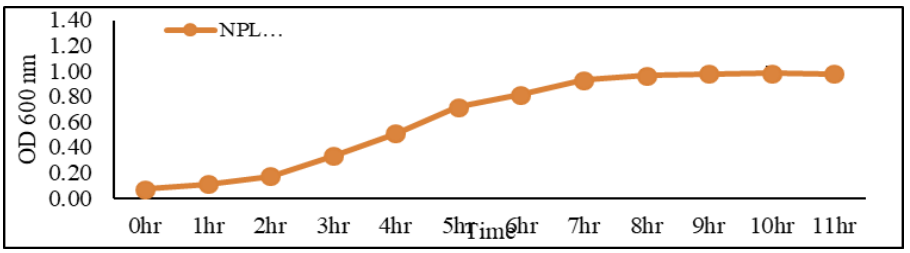

Fig. 5. Growth curve assays of honey bee isolate (*Apis florae*) under aerobic condition

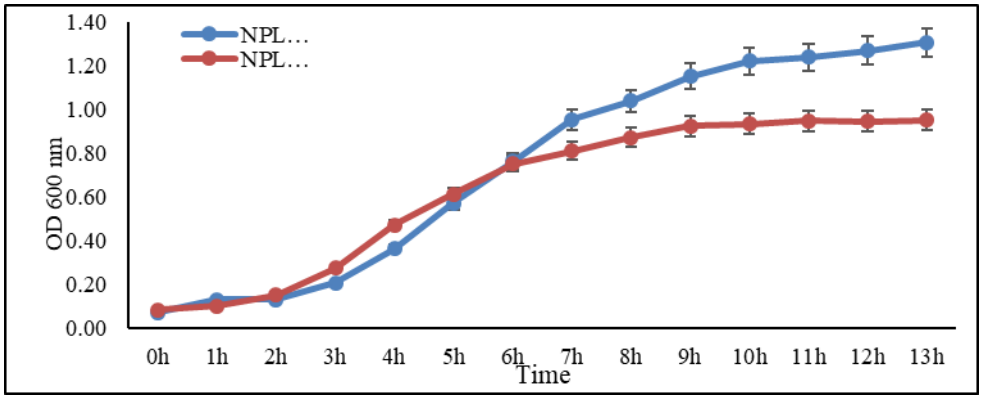

Fig. 6. Growth curve assays of honey bee isolate (*Apis cerana*) under aerobic condition

pH Assay

The results of the present study are shown in figure 7. The sustainability of *lactobacilli* decreased after incubation at pH 1.5 and pH 3.0. The comparison of all strains showed similar pH tolerance by showing significant differences in the cell viability after 3 hours of treatment. The strains were not stressed at pH 6.5 which was thought to reduce the viability of the test bacteria. As a result, all strains continued to exhibit normal growth.

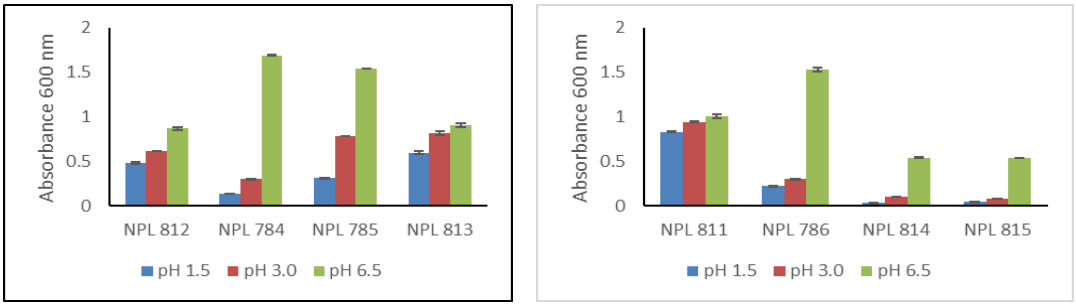

Fig. 7. pH assay for (A) *NPL 812, 784, 785 (Apis dorsata) and NPL 813 (Apis florae)* (B) NPL 811, 786 (*Apis melifera*) and NPL 814, 815 (*Apis cerana*) under aerobic condition

Bile Assay

All NPL strains were resistant to bile concentration with a minor reduction of growth. As all the strains tolerated bile salts so were surviving well in the host intestine which showed their capacity to be resistant to bile salts that may be considered as an antimicrobial molecule. This consequently showed the potential use of LAB as a probiotic, because if LAB can resist the concentration of bile salt then they could

manage to colonize in host intestine as its normal flora. The bile tolerance results of all the NPL strains. Nine NPL were able to grow when cultured at bile salt concentrations of 0.3% and 0.15% (average concentration of bile salt depending upon individual's gastric condition and food ingested) at 1, 2, and 7 hours of incubation Fig. 8 and 9.

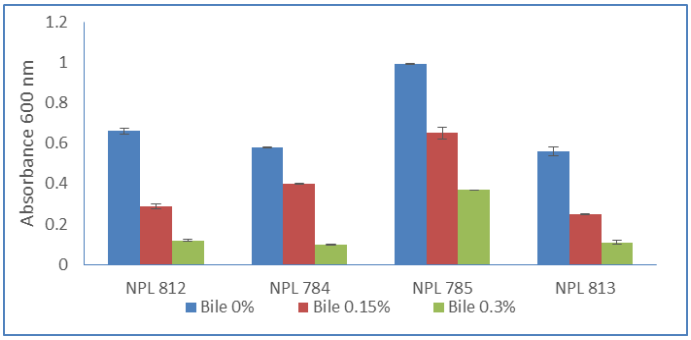

Fig. 8. Bile assay for NPL 812, 784, 785 (*Apis dorsata*) and NPL 813 (*Apis florae*) under aerobic condition

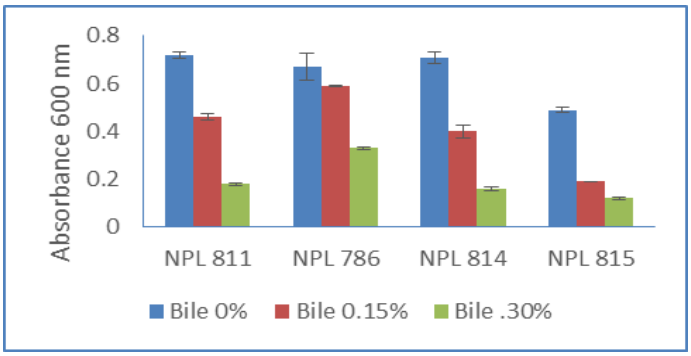

Fig. 9. Bile assay for NPL 811, 786 (*Apis melifera*) and NPL 814, 815 (*Apis cerana*) under aerobic condition

Hemolysis Assay

None of the honey bee isolates formed any zones around the colonies streaked on the blood agar.

Glycerol Stock

The easy way to store bacterial strains for a longer period is to preserve them in glycerol stock. All eight NPL isolates

glycerol stock was placed at -40˚C for further use.

DISCUSSION

The current study was designed to evaluate the probiotic properties of some lactic acid bacteria (LAB). These LAB were isolated from honey bees collected from NARC Islamabad, Pakistan. MRS agar medium was used for the isolation of lactic acid bacteria (LAB). As the cultures were grown on MRS medium, all the isolates were subjected to morphological and biochemical characterization. Selected isolates were found to be gram-positive and catalase negative. *Lactobacilli* were gram-positive rods that occur in chains, pairs, or singly. They were facultative anaerobes, non-endospore forming, and non-motile bacteria. *Lactococcus lactis* isolated from honeybees found was coccoid, gram-positive, facultative anaerobes, non-endospore forming, non-motile, catalase-negative that occurs in chains, pairs, or singly.

The NPL strain was isolated from the gut of *Apis Mellifera* honey bee species of Pakistan obtained from an apiary maintained by NARC Islamabad. The physiology and biochemical characteristics of selected LAB isolated are similar to those described in Bergey's Manual Determinative of Bacteriology for genera *lactobacillus* and *Lactococcus* Grimont and Grimont (2005).

Probiotic properties of the isolated bacteria were evaluated and their pH and bile assay. Probiotics help to improve digestion and boost non-specific immunity Elzeini et al. (2021; Safonov (2020). Out of these isolated bacteria, all were able to grow at as low as pH 1.5 and at pH 3.0. All isolates retained their viability at this low pH, but their growth was insignificant. Our study was corroborated by a study, where isolated strain showed insignificant growth withstanding low pH of 1.5 and 3.0 El Sohaimy et al. (2016). When the pH of the medium was increased from pH 3 to pH 6.5, there was a gradual increase in the growth of bacteria. This shows that our isolated strains can survive in a human gastric environment which has a pH of 1.5 to 3.0. All eight NPL strains were grown at low pHs of 1.5 and 3.0 in PBS. After exposure to pH, all eight strain retain their viability. These results agree with other studies, where *lactobacillus* strains can maintain their viability when exposed to low pH.

Bile concentration in the small intestine varies and ranges up to 0.3% w/v in humans which is necessary for the metabolic activity of bacteria and in

contributing balance of intestinal microflora Shehata et al. (2016). LAB enduring the bile concentration is considered as bacteria of choice for probiotic activity. All eight strains were able to tolerate bile concentrations of 0.15% and 0.3 %. The NPL strain 785 was more tolerant to this bile concentration concerning other strains. The strain was more tolerant of 0.15% bile than 0.3% concentration. Similar results were found in two different studies observing reduced viability and diminished growth at similar concentrations Arcand et al. (2005) Bianchi et al. (2014). Thus strain 785 proved to be a more beneficial probiotic bacteria based on its characteristics. All other strains maintained their viability and downscale culture at the given conditions with lesser probiotic activity but fulfilled the basic criteria.

Hemolysis on blood sheep agar is a safety consideration that should not be observed in the potential probiotic strains for their selection. Along with hemolysis, antibiotic resistance is another phenomenon that if present excludes the potentially probiotic strain from being applied for human or animal consumption (Food, 2002). In the current study, all strains were screened for hemolysis, and all the strains with γ-hemolysis (i.e. no hemolysis). The findings were supported by previous studies on hemolysis with similar results Zoumpopoulou et al. (2013) Viale et al. (2014).

Molecular analysis is used for the definitive identification of the strains. Out of eight isolated strains, six isolates belonged to the *lactobacillus* genus (75%) and two belonged to the *Lactococcus* genus (25%). The ratio of *Lactobacilli* species was higher as compared to *Lactococcus* showing that they are more prevalent probiotic bacteria. The *Lactococcus* strains were identified as *Lactococcus lactis* species. The *lactobacilli* were predominant in a similar conducted study with (10%) and *Lactococcus lactis* were found to be (10%) in the study Edward et al. (2018).

CONCLUSION

It was concluded in the study that the bacteria isolated from the honey bee gut have probiotic potential. The bacteria were gram-positive and molecular technique identified them as *Lactobacillus* and *Lactococcus*. *Lactobacillus* were found in abundant and comprised 78% of the isolates. The probiotic potential activity can be used in food, pharmaceutical, and nutraceutical industries for the well-being of living organisms.

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CONFLICT OF INTEREST

Author's declare there is no conflict of interest.

REFERENCES

1. Al-Ghamdi A, Khan KA, Ansari MJ, Almasaudi SB, Al-Kahtani S (2018). Effect of gut bacterial isolates from Apis mellifera jemenitica on Paenibacillus larvae infected bee larvae. Saudi J. Biol. Sci. 25(2): 383-387.
2. Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou EZ, Tassou CC (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiol. 33(2): 282-291.
3. Audisio MC, Torres MJ, Sabaté DC, Ibarguren C, Apella MC (2011). Properties of different lactic acid bacteria isolated from Apis mellifera L. bee-gut. Microbiol. Res. 166(1): 1-13.
4. Bansal S, Singh A, Mangal M, Sharma, SK (2013). Isolation and characterization of lactic acid bacteria from fermented foods. Vegetos. 26: 325-330.
5. Bibi S, Husain SZ, Malik RN (2008). Pollen analysis and heavy metals detection in honey samples from seven selected countries. Pak. J. Bot. 40(2): 507-516.
6. Blaiotta G, Pepe O, Mauriello G, Villani F, Andolfi R, Moschetti G (2002). 16S–23S rDNA intergenic spacer region polymorphism of Lactococcus garvieae, Lactococcus raffinolactis and Lactococcus lactis as revealed by PCR and nucleotide sequence analysis. Syst. Appl. Microbiol. 25(4): 520-527.
7. BorgesD GN, Goodwin HP (2021) Effects of prebiotics and probiotics on honey bees (*Apis mellifera*) infected with the microsporidian parasite *Nosema ceranae*. Microorg. 9(39): 481.
8. Caufield P, Li Y, Dasanayake A, Saxena D (2007). Diversity of lactobacilli in the oral cavities of young women with dental caries. Cari. Res. 41(1): 2-8.
9. Endo A, Salminen S (2013). Honeybees and beehives are rich sources for fructophilic lactic acid bacteria. Syst. Appl. Microbiol. 36(6): 444-448.
10. Elzeini HM, Ali AA, Nasr NF, Elenany YE, Hassan AA (2021) Isolation and identification of lactic acid bacteria from intestinal tract of honey bee *Apis mellifera* L. in Egypt. J. Apicult. Res. 60(2):349–357

11. Engel P, Martinson VG, Moran NA (2012). Functional diversity within the simple gut microbiota of the honey bee. Proc. Nat. Acad. Sci. 109(27): 11002-11007.
12. Fontana L, Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S, Gil A (2013). Sources, isolation, characterisation and evaluation of probiotics. *Br. J. Nutr*. 109(S2): S35-S50.
13. Food J (2002). Agriculture Organization of the United Nations and World Health Organization Working Group: Report on Drafting Guidelines for the Evaluation of Probiotics in Food, London.
14. Grimont P, Grimont F (2005). Bergey's Manual of Systematic Bacteriology (2): The Proteobacteria, Part B-The Gammaproteobacteria.New York: Springer.
15. Gómez NC (2016) Use of Potential Probiotic Lactic Acid Bacteria (LAB) Biofilms for the Control of Listeria monocytogenes , Salmonella Typhimurium , and Escherichia coli O157 : H7 Biofilms Formation. Front. Microbiol. (7): 1–15.
16. Halder D, Mandal M, Chatterjee SS, Pal NK, Mandal S (2017). Indigenous probiotic Lactobacillus isolates presenting antibiotic like activity against human pathogenic bacteria. J. Biomed. 5(2): 31.
17. Hamdi C, Balloi A, Essanaa J, Crotti E, Gonella E, Raddadi N, Manino A. (2011). Gut microbiome dysbiosis and honeybee health. J. Appl. Entomol. 135(7): 524-533.
18. Hassanzadazar H, Ehsani A, Mardani K, Hesari J (2012). Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Paper presented at the Veterinary Research Forum.
19. Hiremath P, Bannigidad P (2011). Automated Gram-staining characterisation of bacterial cells using colour and cell wall properties. Int. J. Biomed. Eng. Technol. 7(3): 257-265.
20. Jeyaprakash A, Hoy MA, Allsopp MH (2003). Bacterial diversity in worker adults of Apis mellifera capensis and Apis mellifera scutellata (Insecta: Hymenoptera) assessed using 16S rRNA sequences. J. Invertebr. Pathol. 84(2): 96-103.
21. Jones JC, Myerscough MR, Graham S, Oldroyd BP (2004). Honey bee nest thermoregulation: diversity promotes stability. Sci. 305(5682): 402-404.
22. Khan HU, Anjum SI, Sultana N, Khattak B (2016). Honey production potential of the honey bee (*Apis mellifera*) in Karak and Kohat, Pakistan.

23. Kwong WK, Moran NA (2016). Gut microbial communities of social bees. Nat. Rev. Microbiol. 14(6): 374.
24. UĞRAŞ S (2017). Isolation, identification and characterization of probiotic properties of bacterium from the honey stomachs of Yigilca honeybees in Turkey.
25. Lamei S, Hu YO, Olofsson TC, Andersson AF, Forsgren E, Vásquez A (2017). Improvement of identification methods for honeybee specific Lactic Acid Bacteria; future approaches. PloS one. 12(3): 0174614.
26. Lee PY, Costumbrado J, Hsu CY, Kim YH (2012). Agarose gel electrophoresis for the separation of DNA fragments. J. Visual. Exp. 62(1): e3923.
27. Mainville I, Arcand Y, Farnworth E (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. Int. J. Food Microbiol. 99(3): 287-296.
28. Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA (2011). A simple and distinctive microbiota associated with honey bees and bumble bees. Mol. Ecol. 20(3): 619-628.
29. Martinson VG, Moy J, Moran NA (2012). Establishment of characteristic gut bacteria during development of the honeybee worker. Appl. Environ. Microbiol. 78(8): 2830-2840.
30. Mathialagan M, Edward YJT, David P, Senthilkumar M, Srinivasan M, Mohankumar S. (2018). Isolation, Characterization and Identification of Probiotic Lactic Acid Bacteria (LAB) from Honey Bees. Int. J. Curr. Microbiol. App. Sci. 7(4): 894-906.
31. Mitsuoka T (1992). The human gastrointestinal tract. InThe Lactic Acid Bacteria Volume 1: The Lactic Acid Bacteria in Health and Disease (pp. 69-114). Boston, MA: Springer US.
32. Nicolson SW, Thornburg RW (2007). Nectar chemistry Nectaries and nectar 215-264: Springer.
33. Olofsson TC, Alsterfjord M, Nilson B, Butler È, Vásquez A (2014).

Lactobacillus apinorum sp. nov., Lactobacillus mellifer sp. nov., Lactobacillus mellis sp. nov., Lactobacillus melliventris sp. nov., Lactobacillus kimbladii sp. nov., Lactobacillus helsingborgensis sp. nov. and Lactobacillus kullabergensis sp. nov., isolated from the honey stomach of the honeybee *Apis mellifera.* Int. J. Syst. Eol. Microbiol. 64(9): 3109-3119.

34. Olofsson TC, Vásquez A (2008). Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Curr. Microbiol. 57(4): 356-363.
35. Pattabhiramaiah M, Brueckner D, Witzel K, Reddy M (2011). Wolbachia Endosymbiont in the workers of European honeybee, *Apis mellifera* carnica. Elect. J. Bio. (7) 81-85.
36. Pisano M B, Viale S, Conti S, Fadda ME, Deplano M, Melis MP, Cosentino S (2014). Preliminary evaluation of probiotic properties of Lactobacillus strains isolated from Sardinian dairy products. Biomed. Res. Int.
37. Rahman S (2015). Probiotic properties analysis of isolated lactic acid bacteria from buffalo milk. Arch. Clin. Microbiol. 7(1).
38. Rauf S, Zareen S, Rehman HU, Hussain, R, Ateeq M, Usman K, Saeed K (2016). An analysis of fidelity among honey bee reared in Gujranwala, Pakistan.
39. Safonov V (2020) Assessment of heavy metals in milk produced by black-and-white holstein cows from Moscow. Curr. Res. Nut. Food Sci. J. 8(2):410–415
40. Sahadeva R, Leong S, Chua K, Tan C, Chan H, Tong E, Chan H (2011). Survival of commercial probiotic strains to pH and bile. Int. Food Res. J. 18(4).
41. Shehata M, El Sohaimy S, El-Sahn MA, Youssef M (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. Ann. Agric. Sci. 61(1): 65-75.
42. Shokryazdan P, Sieo CC, Kalavathy R, Liang JB, Alitheen NB, Faseleh Jahromi M, Ho YW (2014). Probiotic potential of Lactobacillus strains with antimicrobial activity against some human pathogenic strains. Biomed. Res. Int.
43. Solieri L, Bianchi A, Mottolese G, Lemmetti F, Giudici P (2014). Tailoring the probiotic potential of non-starter Lactobacillus strains from ripened Parmigiano Reggiano cheese by in vitro screening and

principal component analysis. Food Microbiol. (38): 240-249.

44. Tajabadi N, Mardan M, Manap MYA, Mustafa S (2013). Molecular identification of Lactobacillus spp. isolated from the honey comb of the honey bee (*Apis dorsata*) by 16S rRNA gene sequencing. J. Api. Res. 52(5): 235-241.
45. Tajabadi N, Mardan M, Manap MYA, Shuhaimi M, Meimandipour A, Nateghi L (2011). Detection and identification of Lactobacillus bacteria found in the honey stomach of the giant honeybee Apis dorsata. Apidol. 42(5): 642.
46. Tajabadi N, Mardan M, Saari N, Mustafa S, Bahreini R, Manap MY (2013). Identification of Lactobacillus plantarum, Lactobacillus pentosus and Lactobacillus fermentum from honey stomach of honeybee. Braz. J. Microbiol. 44: 717-722.
47. Tarpy DR, Mattila HR, Newton IL (2015). Characterization of the honey bee microbiome throughout the queen-rearing process. Appl. Environ. Microbiol. 81(1): 3182-3191.