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Original Article

# Growth optimization, antibiogram, and molecular identification of Bacillus species isolated from the human gut

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**Abstract**

The human microbial flora is quite diverse and versatile, playing several beneficial roles in association with the host and deriving nutrition from it. The present study aimed to identify gut microbial flora with potential probiotic activities. Eighteen bacterial isolates were screened from ten male individuals in this study. Seven bacterial isolates, NCCP-2046, NCCP-2031, NCCP-2035, NCCP-2040, NCCP-2041, NCCP-2044, and NCCP-2046, were isolated from the gut samples of volunteer men belonging to various areas of Rawalpindi and Islamabad. These bacterial isolates were cultured on De Man Rogosa and Sharpe Media (MRS), Tryptone Soya Agar (TSA), and Nutrient agar, which showed efficient bacterial growth. The morphological and biochemical characteristics of these bacterial strains were studied under their optimal growth conditions, along with molecular investigations. The antibiotic sensitivity pattern was tested using Kirby-Bauer method, which verified the higher MIC against all eight antibiotics used except for oxacillin. Phylogenetic analysis of only four bacterial isolates was performed based on their 16S rRNA sequences, and their top-hit sequence similarities in NCBI and EzBioCloud.net (95-98% and 94%) verified that these bacterial candidates belong to the *Priestia* and *Staphylococcus* genera. Based on molecular evidence through phylogeny and sequence similarities with previously defined bacterial candidates, the bacterial strains MG-461621 (NCCP-2031), MG-461622 (NCCP-2035), and MG-561934 (NCCP-2046) are presumed to be members of *Priestia* or novel species/genera, while MG-461623 (NCCP-2039) is also found to be a previously identified species of *Staphylococcus*. However, due to decreased similarity with the top-hit sequences, it could also be presumed to represent a member of a novel genus.

**Keywords:** *Priestia*, 16S rRNA, *Staphylococcus*, Human gut, Antibiogram.

## 1. Introduction

Species of the genus *Bacillus* and *Priestia* are rod-shaped and belong to the phylum *Firmicutes.* They include both aerobic and facultative anaerobic species that produce spores during unfavourable conditions. The species in this genus are mostly isolated from soil, but some species are also isolated from food, water, vegetables, animals, and the human gut [1, 2]. It is believed that these species are not natural inhabitants of the human gut, but they colonize the gastrointestinal tract (GIT) when microflora contaminates consumable foodstuff. Additionally, they can colonize the human gut by consuming fermented beans [3].

The mucosal layer of the human gut is composed of the lamina propria, muscularis mucosae, and epithelial cells, which are inhabited by approximately $10^{14}$ microorganisms [4]. These microorganisms play a crucial role as gut microbiota due to their metabolic characteristics, which involve recycling important nutrients and producing essential byproducts [5]. The number of microbes colonizing the human gut is ten times greater than the number of human cells. The colonization of commensal microbiota in the human gut begins shortly after birth and is established

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within the first three years of life, encompassing approximately 1000 species, most of which are anaerobic and unknown species [6, 7].

The initial colonization, composition, and progressive diversity of the gut fluctuate widely in newborns and show significance compared to adults. After ingesting solid foods, usually at around one year of age, intestinal flora begins to localize more effectively than initially [7]. Furthermore, the typical colonization of microorganisms in different parts of the GIT differs due to the physiochemical nature of the stomach and intestine. Major factors such as the pH value of the GIT, food intake, intestinal motility, redox condition, as well as gastric acid, bile, digestive enzymes, and mucus discharge from the host contribute to the composition of the microbial community in the GIT [8].

In several studies, researchers have discovered that *Bacillus* spp. has antimicrobial activity against various pathogenic microorganisms. *B. subtilis* strains, which are used as probiotics, have demonstrated antagonistic abilities against *H. pylori*. This is due to the secretion of the lincomycin antibiotic, which also exhibits inhibitory properties against enteric *E. faecium* and *Shigella flexneri* [9]. Purified bacteriocins, which are secreted by *Bacillus,* have shown a strong inhibitory effect against clinical and foodborne pathogens. Bacteriocins with a small molecular weight, once filtered and purified, exhibit a wide range of antimicrobial activity with low cytotoxicity [10]. Bacteriocin-producing strains of *B. subtilis* have also been isolated [11], showing high levels of antagonistic activity, particularly against bacterial infections that cause foot ulcers. Additionally, *Bacillus* spp. has antimicrobial activity against *Klebsiella* spp. *Bacillus* spp., which can produce bacteriocins, could be used as a biopreservative in food and for treating human and animal infections. In Southeast Asia, food products and medical goods often contain different strains of *Bacillus* used as probiotics. These products may consist of a single strain of *Bacillus* or a combination of other probiotic *Lactobacillus* strains. Such products are being utilized to replace and reduce the traditional use of antibiotics. The strains used in these products are marketed as antibiotic-resistant strains of probiotics. Probiotics help mitigate the risk of transmitting resistant genes to pathogens and commensals in the GIT of both humans and animals, thereby combating antibiotic resistance [12].

Gut bacteria not only provide defensive mechanisms to the host but also help maintain normal gut function. This includes activities such as producing vitamins, promoting gut mobility, absorbing minerals from the intestine, destroying and activating toxins, metabolizing xenobiotic substances, and transforming bile acids [13]. Acetic, propionic, and butyric acids are the short-chain organic acids produced in the colon's proximal region. They serve as a great energy source for the colonic mucosa and peripheral body tissues. Organic acids, formed through the fermentation of complex undigested carbohydrates by colon bacteria, affect bacterial growth in the colon by absorbing colonic water and reducing stool pH. *Burkholderiales* bacteria, such as *Oxalibacter formigenes* and beta-proteobacterium, contribute to health benefits such as regulating oxalic acid homeostasis and preventing kidney stone formation [14]. These studies encourage us to explore the human gut microbiota in order to isolate and identify beneficial strains of *Priestia* and *Staphylococcus* through culturing, biochemical, and molecular investigations. Furthermore, we aim to investigate their probiotic potential and antibiotic susceptibility patterns.

## 2. Materials and Methods

### 2.1 Moral authorization

The selected specimens were collected from the stool samples of male participants and processed in the laboratory. Since the specimens were collected indirectly, and no individuals were involved, there is no need to obtain ethical approval from the ethical committee.

### 2.2 Study area and specimen collection

The current study was conducted at the Institute of Microbial Culture Collection of Pakistan, at NARC and Quaid-i-Azam University in Islamabad. A total of 10 participants, aged between 24 and 35 years, were selected. Stool specimens were collected in sterile containers, carefully handled, and labelled for the screening and examination of bacterial strains. Data/records regarding health status, including weight, diet, age, and medical history, were documented byfrom the participants.

### 2.3 Specimen processing

The collected samples were directly inoculated onto de Man Rogosa and Sharpe agar (MRS) culture medium. Serial dilutions in phosphate-buffered saline with a pH ranging from 5.5-6.5 were made before inoculating on culture plates using the spread plate technique. After initial growth, the colonies were further cultured and confirmed on nutrient agar and trypticase soy agar (TSA) and incubated aerobically at 37°C for 24-48 hours. Later, the bacterial isolates were enriched multiple times on MRS agar for bacterial strain recovery.

### 2.4. Identification of bacterial isolates

The selective and desired bacterial strains were identified morphologically and biochemically after obtaining pure colonies. Colonial morphology was observed using a phase contrast microscope and further examined using a scanning electron microscope. Molecular testing was conducted solely on the selected bacterial strains. The DNA of the bacterial strains was extracted using the specified method [15].

A total of 50 µl of solution was prepared, consisting of 1 µl DNA extract, 25 µl of TAKARA pre-mix *Ex-Taq*, 2 µl of universal forward 9F primer (5'-GAGTTTGATCCTGGCTCAG-3'), and 2 µl of 1510R universal reverse primer (5'-GGCTACCTTGTTACGA-3'). Additionally, 20 µl of PCR water was used for the PCR amplification of 16S rRNA. The final amplified PCR product was sent for sequencing (http://dna.macrogen.com). The species-level identification of bacterial strains was done using EzBioCloud-Taxon server. Nucleotide sequences were aligned using the Clustal X software and assembled by BioEdit. These nucleotide sequences of bacterial strains were blasted at NCBI in order to obtain closely related sequences of 16S rRNA species for the construction of a phylogenetic tree and molecular evolutionary analysis using MEGA software version 7 [16]. After processing, all the nucleotide sequences obtained from sequencing were submitted to the GenBank Database at NCBI with the accession numbers MG461621-MG461622, MG461623 and MG561934.

### 2.5 Optimization of bacterial isolates

#### *2.5.1 pH tolerance test*

The acid tolerance properties of bacterial isolates were observed via a method described by the previous study [17] because bacterial growth is quite sensitive to the pH environment. To estimate the acid tolerance of bacterial culture, a 200 µl cell suspension with a concentration of $10^8$ CFU/ml from the pure culture of each bacterial isolate's growth was inoculated onto MRS broth with different pH levels (pH adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5). The optimized culture of each sample was observed by measuring the absorbance at 600 nm in each tube after 48 hours of incubation. The pH resulting in the best bacterial growth was chosen for further experiments. The trial was conducted in triplicate, and the mean of each sample was calculated. The same trial was conducted for temperature, but with different temperature levels (28°C, 30°C, 34°C, 37°C, 40°C, and 45°C). After incubation, the OD at 600nm wavelength of each sample was observed. The temperature that resulted in the best bacterial growth was used for further study.

#### *2.5.2 Bile tolerance test*

The survival of bacterial isolates was also evaluated with different bile salt concentrations in MRS broth (Oxgall, Merck). Fresh culture was used after growth in MRS broth at 37°C in an incubator, with a final bile salt concentration of 0.3%. The culture was centrifuged and resuspended in MRS culture broth. One ml of fresh culture from each sample was mixed with different bile salt concentrations (0.1%, 0.2%, 0.3%) and allowed to incubate for 4-5 hours. Afterwards, 1 ml of each sample was incubated on MRS culture plates, and the sample was allowed to incubate for 18-24 hours at 37°C. After incubation, the growth of each bacterial isolate was examined carefully, and CFU/ml was calculated for each concentration. The experiment for all samples was conducted in triplicate.

The susceptibility pattern of all bacterial isolates was observed using the Kirby-Bauer disc diffusion method. Commercially available antibiotic discs (OXOID) were used, including ceftriaxone (CRO 30µg), levofloxacin (LEV 5µg), tobramycin (TOB 10µg), oxacillin (OX 1µg), erythromycin (E 15µg), norfloxacin (NOR 30µg), cefotaxime (CTX 30µg), and tetracycline (TE 30µg). Mueller Hinton Agar (MHA) medium was used to determine the sensitivity pattern of the isolates. The culture plates were incubated at 37°C for 18-24 hours. After incubation, the zone of inhibition of each antibiotic was measured in mm following CLSI standards.

Pure isolates were grown in an MRS broth medium and allowed to incubate overnight. After incubation, a 20% glycerol stock solution (300µl) was added to each culture (700µl) in an Eppendorf tube and preserved at -80°C following the method followed previously [18]. The viability of cultures preserved in frozen glycerol stock was assessed after four months of storage. 100µl of each sample was inoculated into nutrient broth and allowed to incubate for 48 hours at 37°C. After growth, one ml of culture was inoculated on an MRS culture medium, and the viability of each sample was determined as CFU/ml after incubating for 48 hours at 37°C. The data were analyzed using SPSS version 20.0 and Excel.

All the data were analyzed using SPSS version 20.0 and GraphPad version 8.0. A p-value lied less than 0.05 was considered as significant.

## 3. Results

The human gut microbiota from ten participants was used in this study to investigate their probiotic potential and test antibiotic susceptibility profiling. Out of these, 18 species were isolated from the samples, and seven gram-positive bacterial isolates were collected on an MRS medium at 37°C under aerobic conditions. Bacterial isolates were initially selected based on selective media as well as gram-staining and were named NCCP-2031, NCCP-2035, NCCP-2039, NCCP-2040, NCCP-2041, NCCP-2044, and NCCP-2046. The colonial morphology of the growth is shown in Fig. 1. Based on molecular data, the bacterial strains MG-461621 (NCCP-2031), MG-461622 (NCCP-2035), and MG-561934 (NCCP-2046) exhibited 97.73%, 96.83%, and 94.77% similarity with *Priestia endophytica*, respectively. MG-461623 (NCCL-2039) exhibited 95.10% resemblance to *Staphylococcus petrasii* (Fig. 2 & 3). Presumably, if the nucleotide sequences have a similarity of less than 97% with the top-hit sequence in the databases, it verifies the novelty of the bacterial isolates, considering

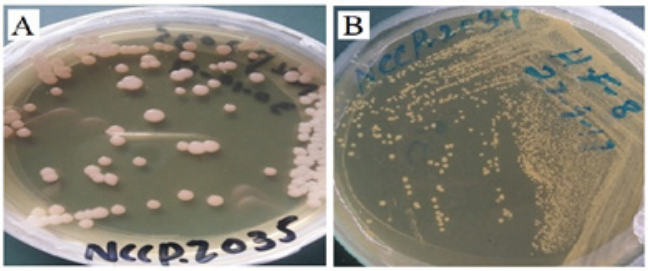


**Fig. 1.** A) Illustrate colonial morphology of gram-positive rod and B) illustrate gram-positive cocci on MRS culture plate.

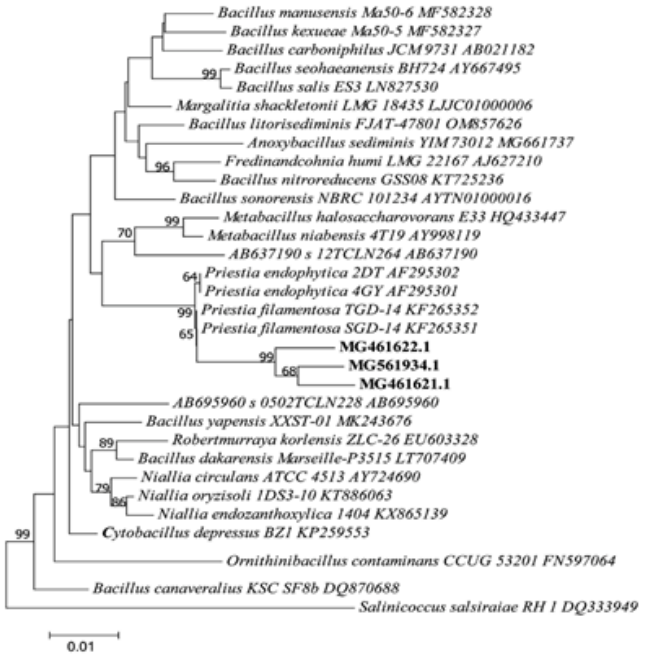


**Fig. 2.** The phylogenetic tree was constructed using the Neighbor-Joining method [1] utilizing 16S rRNA nucleotide sequences MG-461622, MG-461621, and MG-561934 from closely related species of *Bacillus* and *Priestia*. To ensure a better tree topology, *Salinicoccus salsiraiae* with accession number DQ-333949 was used as an outgroup. Only tree nodes with a bootstrap value above 60 percent are represented.

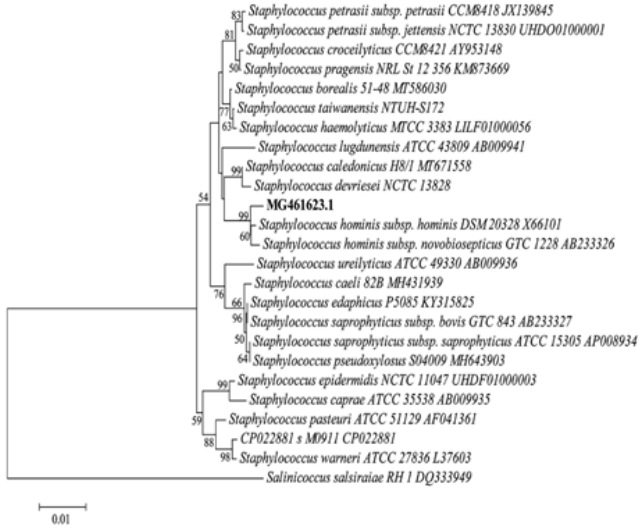


**Fig. 3.** The phylogenetic tree was constructed based on 16S rRNA nucleotide sequences with accession number MG-461623, along with closely related sequences from the genera *Staphylococcus*. MEGA 7 was used to construct the tree using the Neighbor-Joining model (1), with bootstrap values above 50 percent represented in the tree. The out-group bacterial member *Salinicoccus salsiraiae* was included to enhance the topology of the phylogenetic tree.

subject to the total size of the amplified DNA sequence.

The bacterial isolates MG461621 and MG561934, with similarity levels of 96.83% and 94.77% respectively. The *Priestia endophytica* isolated from the midgut field-caught adult *Aedes aegypti* isolated by Godrigo *et al*., in 2020 (unpublished). Additionally, *Priestia filamentosa* was isolated from the same source by Eida *et al*. in 2018 (unpublished). The bacterial isolate with accession number MG461621 showed 97.73% similarity to *Priestia filamentosa*, which was isolated by Burgos and Arevala in 2020 (unpublished).

Molecular investigation also verifies that the bacterial isolate with code MG-461623 showed sequence similarities of 95.10% with the *Staphylococcus* strain isolated by Yadav and Krishnamurthi in 2021 (unpublished data). The phylogenetic analysis further confirms its taxonomy by clustering with described bacterial species with a high bootstrap value (Fig. 3).

### 3.1 Bacterial isolates optimization

The growth of bacterial isolates was checked across different temperature ranges, as mentioned in the methodology. The maximum growth pattern was observed in the range of 34-37°C, as determined by OD (550-600nm). A gradual decrease in the growth rate was observed as the temperature increased from 40°C onwards. A control was also conducted, which showed no absorption on the spectrophotometer. The optimum temperature for the studied bacterial isolates was 37°C and was used for further growth (Fig. 4).

The maximum growth of bacterial isolates was observed at pH 6.0. As the pH increased from 6 to 7 and above, a continuous decrease in the OD was observed for the growth of all bacterial isolates. The absorbance levels noted for each bacterial isolate varied. At pH 8, most of the bacterial isolates exhibited no growth. Therefore, the optimum pH (pH 6.0) was used for further study of bacterial growth. The growth of isolates on different pH levels is given in Fig. 5.

### 3.2 Tolerance against bile salt

The concentration of bile salts in the intestine fluctuates, making it very difficult to predict the bile concentration at any given position. This is because the secretion of bile salts depends entirely on the type of food consumed. The results presented in both Fig. 6 and Fig. 7 describe the optimal growth of bacterial isolates on MRS medium after zero hours of incubation at both 0.2% and 0.3% bile concentrations. However, the growth of bacterial strains decreased after one hour of incubation at the same concentration. As the incubation period extended, the growth of bacterial isolates continued to decrease at both 0.2% and 0.3% bile concentrations. After four hours of incubation, the minimum growth of all isolates was observed, with varying values for each bacterial isolate. Furthermore, the reduced growth observed at 0.3% bile was greater than

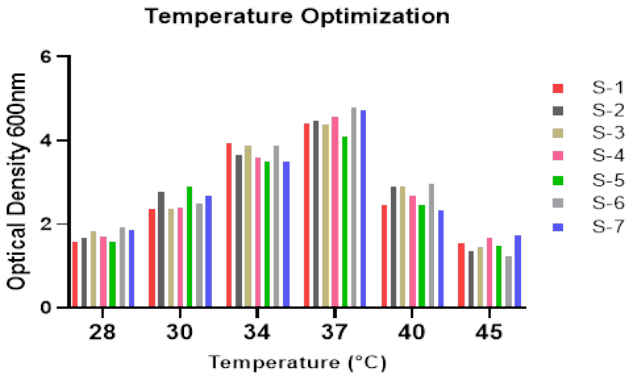


**Fig. 4.** Bacterial growth at different temperatures.

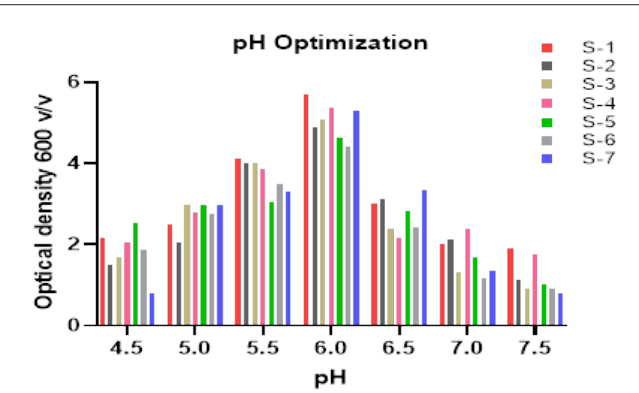


**Fig. 5.** The growth of bacterial isolates observed at different pH values.

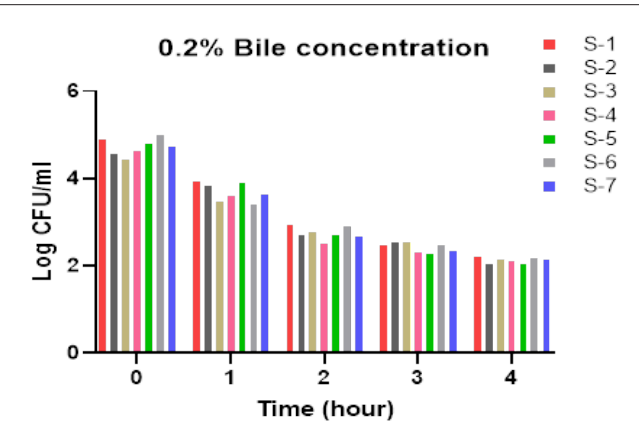


**Fig. 6.** Tolerance at 0.2% bile concentration at 0 to 4 hours.

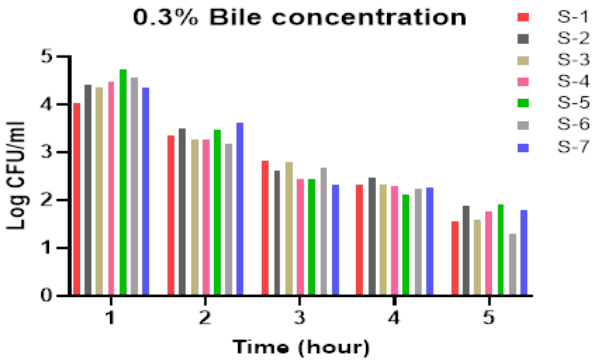


**Fig. 7.** Tolerance is at a bile concentration of 0.3% within 0 to 4 hours.

the bacterial growth at 0.2%. The growth of isolates at both 0.2% and 0.3% bile concentrations was compared after four hours of incubation. The results revealed that the CFU/ml fluctuated more significantly, ranging from 2.01 to 2.21, at 0.2% bile concentration, while the CFU/ml remained relatively stable, ranging from 1.31 to 1.91, at 0.3% bile concentration (Fig. 8).

### 3.3 Susceptibility pattern of bacterial isolates

The antibacterial susceptibility pattern was determined using the Kirby-Bauer disc diffusion method. All isolates showed distinct zones of inhibition for each antibiotic on the MHA medium after overnight incubation at 37°C (Fig. 9). With the exception of NCCP-2031, all bacterial isolates exhibited resistance to oxacillin. The bacterial isolates demonstrated overall good sensitivity, with varying zones of inhibition for each isolate to levofloxacin compared to other antibiotics, followed by norfloxacin, tobramycin and tetracycline. Each isolate exhibited different zones of inhibition for various commercially available antibiotic discs. The p-value was found to be significant (p=0.0128) when the Chi-square test was applied, and the data were cross-tabulated.

### 3.4 Viability test

The viability test of all isolates after 4 months showed that most of the isolates grew well on the culture medium (MRS) after 48 hours of incubation at 37°C. The growth of each isolate was counted as CFU/ml. The NCCP-2044 bacterial strain exhibited the highest growth on the MRS medium, followed by NCCP-2031, while NCCP-2044 showed the lowest growth compared to the other bacterial isolates (Fig. 10).

## 4. Discussion

This study aimed to isolate beneficial bacterial strains from human faecal samples that potentially produce antibiotics, and vitamins. These strains could be utilized as probiotics or in commercial food processing. To achieve this goal, bacterial strains were isolated and characterized from the gastrointestinal tract of normal male volunteers. To screen these strains, specific criteria were established, including tolerance to acidic conditions, bile concentration, and temperature. Finally, all the isolates were tested with various antibiotics, and the MIC was recorded.

In the current study, bacterial isolates were exposed to different pH levels to determine their tolerance and optimum pH for growth. A total of 18 isolates were screened in the preliminary tests. Based on the results of the preliminary tests, 7 isolates were selected for further testing.

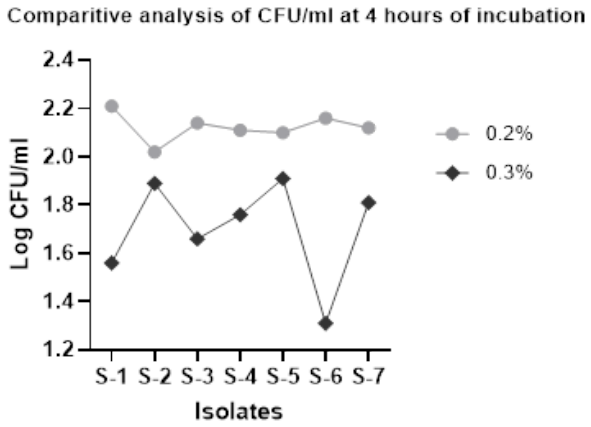


**Fig. 8.** Comparison after 4 hours of incubation with a bile concentration of 0.2 to 0.3%.

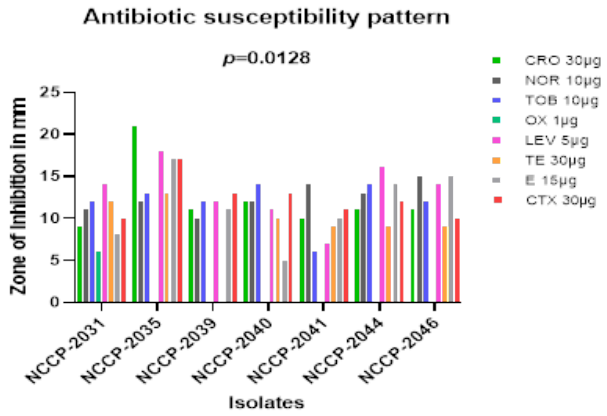


**Fig. 9.** Graphical representation of bacterial susceptibility patterns.

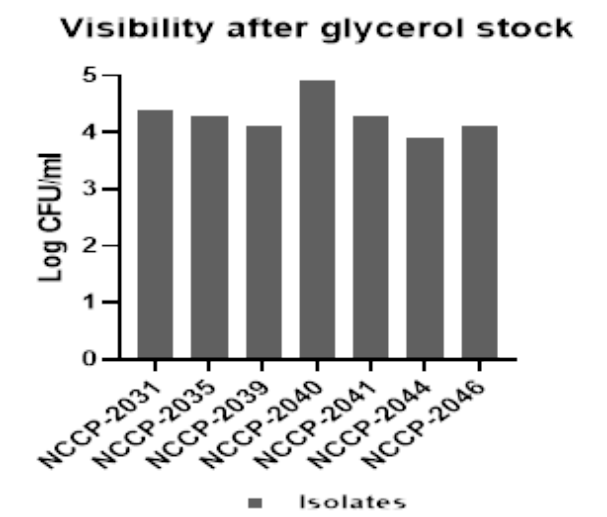


**Fig. 10.** Showed the colony-forming units for each isolate after reactivation from the glycerol stock.

In this study, all the isolates showed efficient growth patterns at different pH levels. The ability to achieve optimal growth at the desired temperature is a characteristic of beneficial bacteria. Additionally, increasing the temperature of fermentation limits the growth of undesirable microorganisms [19, 20].

The most important characteristic of beneficial bacteria is the ability to survive and proliferate in environments with varying pH levels (acidic or alkaline) [21]. Microorganisms' potential activities and growth are highly affected by low pH levels. The food we consume typically stays

in our stomach for approximately four hours. In order to digest the food, the stomach secretes approximately 2.5 liters of gastric juice daily, which is highly acidic in nature. The acidity levels in the constituents of the gastrointestinal tract vary in different parts. The stomach has the highest acidity level, ranging from 1.5 to 3.0 pH. Therefore, it is crucial for microorganisms to bypass this highly acidic condition. However, spores of *Bacillus* spp. can resist this condition and remain viable in the small intestine, where they convert into their vegetative form. *Bacillus* spp. tolerate this harsh condition and colonize the intestine, providing beneficial approaches to the host [22, 23].

Bile is secreted into the upper small intestine (upper duodenum) through a bile duct produced by the liver. Bile is a yellow-green solution. The concentration of bile salt mainly depends on the intake of food and may vary from individual to individual, ranging from 0.2-2% (wt/vol) [24]. The main function of bile salts is to dissolve and emulsify ingested fat as well as disperse lipids present in bacterial cell membranes. Bile salts enter bacterial cells, destroying DNA strands and causing oxidative stress [25, 26]. Furthermore, it is assumed that bile salts also cause the misfolding of proteins and may induce the expression of molecular chaperones in many other different bacteria [27, 28]. All bacterial isolates were found to tolerate 0.2 and 0.3% bile salt concentrations for up to four hours of incubation in bile salt.

Despite the availability of modern-day molecular identification techniques, biochemical tests are still used for the preliminary identification of microbes. Both genotypic and phenotypic identification tests have their importance. All the isolates were found to be catalase-positive, gram-positive, and oxidase-negative. On the other hand, molecular identification techniques can be used to find similarities to already identified specific species whose data is available in online libraries, but these techniques are not very helpful in identifying the minute fermentation differences found in them. Compared to previous techniques, molecular identification is now considered more effective [29-31].

Overall analysis observed during experimental work for characterization is depicted. The cells appeared Gram-stain positive and were observed to be long rods. They were arranged in long chains resembling filaments and were non-motile. This result aligns with the findings of the previous study [32]. Colonies grown on MRS media exhibited aerobic characteristics, with circular shapes, smooth edges, and a shiny surface. Growth was observed at 37$^{o}$C over a 24-hour incubation period. *Staphylococcus petrasii* was initially identified in clinical samples by [3]. The bacteria showed growth within a temperature range of 28$^{o}$C to 45$^{o}$C, and pH levels of 4.5 to 7.5, with a bile salt concentration of 0.2 to 0.3%. Optimal growth occurred at temperatures of 34$^{o}$C and 37$^{o}$C, with pH levels ranging from 5.5 to 6.0. After 4 hours of incubation, the CFU/ml values ranged from 1.5 to 2.5. No growth was observed below 20$^{o}$C, at pH 4.0, or bile salt concentrations above 0.3%. Viability was assessed using glycerol stock, and showed CFU/ml values of 4 to 5 log for all the isolates. Antibiotic susceptibility patterns were determined for all seven bacterial strains except for Oxacillin, which demonstrated the minimum MIC. NCCP 2031, on the other hand, showed resistance to Oxacillin.

Molecular studies confirmed that three of the isolated bacterial strains, namely MG-461622, MG-461621, and MG-561934, showed a close resemblance to the members of the *Priestia* genus, specifically *Priestia endopytica*. The results were further validated by the phylogenetic analysis shown in Fig. 2. It is also presumed that another bacterial isolate, MG-462123, exhibits similarity with a member of the *Staphylococcus* genus due to a lower similarity index (less than 98%) with the top-hit candidates in both the NCBI and EzBioCloud.net databases. However, due to its lower similarity (94%), it can be assumed that the isolated bacterial candidate could be a member of a novel genus. Detailed investigation must be carried out to confirm this. All strains showed novelty except NCCP-2039, which showed 100% similarity and clustered with *Staphylococcus petrasii* in the phylogenetic tree. The isolates were screened from stool samples and were never reported from the human gut before this study, so re-sampling was needed from those volunteer subjects as well. To demonstrate and guarantee strain novelty, it is not only based on high or identical similarity on the 16S rRNA gene sequence. Blast similarity of the 16S rRNA sequence <97% with the respective genus is required to authenticate the strain through DNA-DNA hybridization along with DNA fingerprinting, to obtain information at the subspecies and strain level. To claim novelty, it is essential to perform high-performance liquid chromatography (HPLC), which is recommended for determining the G+C content of the new strains and genus [34]. Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that NCCP-2031 (nucleotides NO.1365), NCCP-2035 (nucleotides NO.1550), NCCP-2040 (nucleotides NO.1398), NCCP-2041 (nucleotides NO.1266), NCCP-2044 (nucleotides NO.1484), and NCCP-2046 (nucleotides NO.1483) closely formed a cluster with *Bacillus filamentosus.* However there is great fluctuation present in all closely related clusters with *B. filamentosus*, NCCP-2039 (nucleotides NO.1514) clusters exactly with *Staphylococcus petrasii*.

## 5. Conclusion

In the current study, all the isolates screened from human gut samples were found to be susceptible to eight commonly used antibiotics, except for NCCP-2031, which showed susceptibility to Oxacillin (OX) 1µg. No MIC was measured in other bacterial strains against it. Better efficacy was observed under various growth conditions, with the isolates being mesophilic and neutrophilic and showing good efficacy at 0.3% bile concentration. Out of the four bacterial isolates, one showed high diversity in the gut samples. NCCP-2039 was identified as a strain with 100% similarity to *Staphylococcus petrasii*. The phylogenetic tree revealed that the other bacterial isolates were positioned differently in the tree and had a lower similarity index with close candidates. However, for complete taxonomic characterization, a detailed investigation must be carried out to meet the classification criteria set by Bergey's manual.

## Conflicts of interest

The authors declare no conflict of interest.

## Data availability statement

All the data has been included in this manuscript.

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**Authors' contributions**
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**References**

1. Alou MT, Rathored J, Khelaifia S, Michelle C, Brah S, Diallo BA, Raoult D, Lagier J-C (2015) Bacillus rubiinfantis sp. nov. strain mt2T, a new bacterial species isolated from human gut. New Microbes New Infect 8: 51-60. doi: 10.1016/j.nmni.2015.08.007
2. Kotb E (2015) Purification and partial characterization of serine fibrinolytic enzyme from Bacillus megaterium KSK-07 isolated from kishk, a traditional Egyptian fermented food. Appl Biochem Microbiol 51 (1): 34-43. doi: 10.1134/S0003683815010079
3. Muhammad I, Muhammad M, Asher R, Shuaibu A (2024) In vitro assessment of the Larvicidal activity of Bacillus thuringiensis israelensis (Vectobac 12AS formulation) on Anopheles mosquito larvae. Cell Mol Biomed Rep 4(1): 9-16. doi: 10.55705/cmbr.2023.391224.1115
4. Clark JA, Coopersmith CM (2007) Intestinal crosstalk–a new paradigm for understanding the gut as the "motor" of critical illness. Shock 28 (4): 384. doi: 10.1097/shk.0b013e31805569df
5. Daniel R (2005) The metagenomics of soil. Nat Rev Microbiol 3 (6): 470. doi: 10.1038/nrmicro1160
6. Honda K, Takeda K (2009) Regulatory mechanisms of immune responses to intestinal bacteria. Mucosal Immunol 2 (3): 187. doi: 10.1038/mi.2009.12
7. Bischoff SC, Krämer S (2007) Human mast cells, bacteria, and intestinal immunity. Immunol Rev 217 (1): 329-337. doi: 10.1111/j.1600-065X.2007.00520.x
8. Gerritsen J, Smidt H, Rijkers GT, Vos WM (2011) Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr 6 (3): 209. doi: 10.1007/s12263-011-0229-7
9. Pinchuk IV, Bressollier P, Verneuil B, Fenet B, Sorokulova IB, Mégraud F, Urdaci MC (2001) In Vitro Anti-Helicobacter pylori Activity of the Probiotic Strain Bacillus subtilis 3 Is Due to Secretion of Antibiotics. Antimicrob Agents Chemother 45 (11): 3156-3161. doi: 10.1128/AAC.45.11.3156-3161.2001
10. Abdhul K, Ganesh M, Shanmughapriya S, Vanithamani S, Kanagavel M, Anbarasu K, Natarajaseenivasan K (2015) Bacteriocinogenic potential of a probiotic strain Bacillus coagulans [BDU3] from Ngari. Int J Biol Macromol 79: 800-806. doi: 10.1016/j.ijbiomac.2015.06.011
11. Joseph B, Dhas B, Hena V, Raj J (2013) Bacteriocin from Bacillus subtilis as a novel drug against diabetic foot ulcer bacterial pathogens. Asian Pac J Trop Biomed 3 (12): 942-946. doi: 10.1016/S2221-1691(13)60186-5
12. Chesson A, Franklin A, Aumaître A, Sköld O, Leclercq R, von Wright A, Guillot J (2002) Opinion of the scientific committee on animal nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human and veterinary importance. Directorate C—Scientific Opinions, European Commission Health and Consumer Protection Directorate-General, Brussels, Belgium. doi: 10.2903/j.efsa.2005.183
13. Macfarlane S, Steed H, Macfarlane GT (2009) Intestinal bacteria and inflammatory bowel disease. Crit Rev Clin Lab Sci 46 (1): 25-54. doi: 10.1080/10408360802485893
14. Jalanka-Tuovinen J, Salonen A, Nikkilä J, Immonen O, Kekkonen R, Lahti L, Palva A, de Vos WM (2011) Intestinal microbiota in healthy adults: temporal analysis reveals individual and common core and relation to intestinal symptoms. PLoS ONE 6 (7): e23035. doi: 10.1371/journal.pone.0023035
15. Ahmed I, Yokota A, Fujiwara T (2007) A novel highly boron tolerant bacterium, Bacillus boroniphilus sp. nov., isolated from soil, that requires boron for its growth. Extremophiles 11 (2): 217-224. doi: 10.1007/s00792-006-0027-3
16. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4 (4): 406-425. doi: 10.1093/oxfordjournals.molbev.a040454
17. Bassyouni RH, Abdel-all WS, Abdel-all MGFS, Kamel Z (2012) Characterization of Lactic Acid Bacteria Isolated from Dairy Products in Egypt as a Probiotic. World J Agric Sci 8 (4): 486-494. doi: 10.5829/idosi.wjas.2012.8.4.64178
18. Fonseca F, Marin M, Morris G (2006) Stabilization of frozen Lactobacillus delbrueckii subsp. bulgaricus in glycerol suspensions: freezing kinetics and storage temperature effects. Appl Environ Microbiol 72 (10): 6474-6482. doi: 10.1128/AEM.00998-06
19. Menconi A, Kallapura G, Latorre JD, Morgan MJ, Pumford NR, Hargis BM, Tellez G (2014) Identification and characterization of lactic acid bacteria in a commercial probiotic culture. Biosci Microbiota Food Health 33 (1): 25-30. doi: 10.12938/bmfh.33.25
20. Ibourahema C, Dauphin RD, Jacqueline D, Thonart P (2008) Characterization of lactic acid bacteria isolated from poultry farms in Senegal. Afr J Biotechnol 7 (12). doi: 10.5897/AJB08.159
21. Hawaz E (2014) Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activities against pathogenic bacteria. Afr J Microbiol Res 8 (13): 1419-1425. doi: 10.5897/AJMR2013.6616
22. Vinderola C, Reinheimer J (2003) Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. Food Res Int 36 (9-10): 895-904. doi: 10.1016/j.foodres.2003.01.001
23. Huang Y, Adams MC (2004) In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Int J Food Microbiol 91 (3): 253-260. doi: 10.1016/j.ijfoodmicro.2003.07.001
24. Dawson P (2002) Bile secretion and the enterohepatic circulation of bile acids. Gastrointest Liver Dis: 1051-1064. doi: 10.1016/B978-1-4160-0245-1.50089-4
25. Kandell RL, Bernstein C (1991) Bile salt/acid induction of DNA damage in bacterial and mammalian cells: implications for colon cancer. Nutr Cancer 16 (3-4): 227-238. doi: 10.1080/01635589109514161
26. Sokol RJ, Winklhofer-Roob BM, Devereaux MW, McKim Jr JM (1995) Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids. Gastroenterology 109 (4): 1249-1256. doi: 10.1016/0016-5085(95)90589-9
27. Flahaut S, Hartke A, Giard J-C, Benachour A, Boutibonnes P, Auffray Y (1996) Relationship between stress response towards bile salts, acid and heat treatment in Enterococcus faecalis. FEMS Microbiol Lett 138 (1): 49-54. doi: 10.1111/j.1574-6968.1996.tb08135.x

28. Leverrier P, Dimova D, Pichereau V, Auffray Y, Boyaval P, Jan G (2003) Susceptibility and adaptive response to bile salts in Propionibacterium freudenreichii: physiological and proteomic analysis. Appl Environ Microbiol 69 (7): 3809-3818. doi: 10.1128/AEM.69.7.3809-3818.2003
29. Aziz T, Hussain N, Hameed Z, Lin L. Elucidating the role of diet in maintaining gut health to reduce the risk of obesity, cardiovascular and other age-related inflammatory diseases: recent challenges and future recommendations. Gut Microbes. 2024;16(1):2297864. doi: 10.1080/19490976.2023.2297864
30. Aziz T, Naveed M, Makhdoom SI, Ali U, Mughal MS, Sarwar A, Khan AA, Zhennai Y, Sameeh MY, Dablool AS, Alharbi AA, Shahzad M, Alamri AS, Alhomrani M. Genome Investigation and Functional Annotation of Lactiplantibacillus plantarum YW11 Revealing Streptin and Ruminococcin-A as Potent Nutritive Bacteriocins against Gut Symbiotic Pathogens. Molecules. 2023;28(2):491. doi: 10.3390/molecules28020491
31. Aziz T, Khan AA, Tzora A, Voidarou CC, Skoufos I. Dietary Implications of the Bidirectional Relationship between the Gut Microflora and Inflammatory Diseases with Special Emphasis on Irritable Bowel Disease: Current and Future Perspective. Nutrients. 2023;15(13):2956. doi: 10.3390/nu15132956
32. Sonalkar VV, Mawlankar R, Ramana VV, Joseph N, Shouche YS, Dastager SG (2015) Bacillus filamentosus sp. nov., isolated from sediment sample. Antonie Van Leeuwenhoek 107 (2): 433-441. doi: 10.1007/s10482-014-0348-4
33. Pantůček R, Švec P, Dajcs JJ, Machová I, Černohlávková J, Šedo O, Gelbíčová T, Mašlaňová I, Doškař J, Zdráhal Z (2013) Staphylococcus petrasii sp. nov. including S. petrasii subsp. petrasii subsp. nov. and S. petrasii subsp. croceilyticus subsp. nov., isolated from human clinical specimens and human ear infections. Syst Appl Microbiol 36 (2): 90-95. doi: 10.1016/j.syapm.2012.11.004
34. Logan NA, Berge O, Bishop AH, Busse H-J, De Vos P, Fritze D, Heyndrickx M, Kämpfer P, Rabinovitch L, Salkinoja-Salonen MS (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. Int J Syst Evol Microbiol 59 (8): 2114-2121. doi: 10.1099/ijs.0.013649-0