How to Cite:
Chauhan, A., & Samant, S. A. (2022). Isolation and identification of kurthia gibsonii from paneer and study its antibacterial activity against intestinal pathogens. *International Journal of Health Sciences*, *6*(S5), 10457–10466. https://doi.org/10.53730/ijhs.v6nS5.11269

Isolation and identification of kurthia gibsonii from paneer and study its antibacterial activity against intestinal pathogens

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Abstract---*Kurthia* is a bacterial genus from the Planococcaceae family and it is more closely related to members of *Lactobacillus, Bacillus*, *Staphylococcus,* and *Streptococcus*. These bacteria are usually found in meat, meat products, milk products, and produce antimicrobial peptides. Antibacterial activity of these peptides has not been reported against intestinal pathogens. This study shows the isolation and identification of *Kurthia gibsonii* from Paneer and study its antibacterial activity against intestinal pathogens. *Kurthia gibsonii* was isolated on De Mann Rogosa sharp agar for identification. Morphologically it is a Gram positive, non-motile, non-spore forming, and Rod-shaped bacteria. Probiotics characteristics of *K. gibsonii* was studied by Acid tolerance, Thermotolerance, Salt tolerance, and Proteolytic activity test. The optimal temperature for growth were 37℃, 45℃, and pH 7.0 respectively. It grew in the presence of 2%, 4%, and 6% NaCl. The organism was identified and confirmed by 16 S RNA sequencing. It was tested for production of Antibacterial peptide by Well Diffusion Assay. The Antibacterial peptide was isolated from *Kurthia gibsonii* by well plate assay and its extraction, purification was done by ammonium precipitation, Dialysis method, and Reverse phase High performance liquid chromatography method. The active antibacterial peptide was eluted out and its inhibitory activity was checked against intestinal pathogenic bacteria ATCC strains namely *E. coli, Shigella, Salmonella.* It was found to show significant antibacterial activity and revealed the possibility of using Bacteriocin as food preservatives detected from *Kurthia gibsonii's* and it can also

Manuscript submitted: 9 March 2022, Manuscript revised: 18 May 2022, Accepted for publication: 27 June 2022

be used in treatment of Gastrointestinal infections along with antibiotic therapy.

Keywords---*Kurthia gibsonii,* paneer, antimicrobial peptide, Enteropathogens.

Introduction

A diverse group of cationic, amphipathic, and aquaphobic antimicrobial peptides synthesised by ribosomal enzymes were termed as Bacteriocin which prevents and kill bacteria that may be closely related to the producer strain. Bacteriocins from gram-positive bacteria such as Staphylococci and Coryneform bacteria have been studied (Nagarajan kayalvizhi et al 2010). Since 1983, FDA from USA has granted a bacteriocin i.e., Nisin A produced by Lactobacillus lactis, Recognized as a Safe food preservative in forty nations. Bacteriocins produced by LAB are being explored extensively in the hopes of finding safest food-grade and biological preservatives (Nagarajan kayalvizhi et al 2010). LAB was the subject of various research, similar bacteriocin are also produced by other classes of bacteria's such as *Enterococci*, *Staphylococci*, and *Corynebacteria* (Nagarajan kayalvizhi et al 2010). Parameters like optimum temperature, salt concentration, and antimicrobial spectrum vary in different bacteriocin like peptides (Nagarajan kayalvizhi et al 2010).

The genus *Kurthia* is classified as 'tentatively' belonging to the *'Coryneform* Group of Bacteria' in the ninth edition of Bergey's Manual of Determinative Bacteriology (Nagarajan kayalvizhi et al 2010). Because members of the genus Kurthia do not have a coryneform morphology. These results of a numerical taxonomic study showed phenotypic similarities among Kurthia and some aerobic *bacillus species*, which supported the taxonomic placement. *Bacillus species* produce a wide range of peptide antibiotics that have a variety of chemical structures. *Bacillus species* are significant in industry and have a long history of being used safely in both food and industry. There have been no peptide antibiotics identified and described from distinct strains of *K. gibsonii* until now. *K gibsonii* has not yet been found to produce bacteriocins or bacteriocin-like compounds. Although some bacteriocins have a narrow spectrum of antibacterial activity, others have wide antibacterial activity against a variety of bacteria which also includes *Listeria monocytogenes* and *Streptococcus pyogenes* (Nagarajan kayalvizhi et al 2010). No gram-positive bacteria, showed the antagonistic activity against *Staphylococcus, Streptococcus, Bacillus Listeriae,* and *Pediococcus* (Shaw. S. Keddie 1983). The present study focuses on isolation of bacterial strain from a paneer sample which produced an antibacterial peptide like bacteriocin which may be used as food preservatives as well used in the treatment of Intestinal Diseases.

Method

1. Collection of Sample

Collected from nearby local dairy

2. Isolation and Identification of bacterial strain *K. gibsonii*

Bacterial strain *K. gibsonii* was isolated from the paneer in local dairy of Navi Mumbai, Maharashtra, India. This strain was grown in De Mann Rogosa sharp medium (Hi Media, Mumbai, India) followed by serial dilution and incubate at 37℃ with constant agitation (Prawan. K et al 2017). The bacterial strain *K. gibsonii* was identified by physiological and biochemical features. Further, Using the specific primers PSL forward (5'-AGGATTAGATACCCTGGTAGTCCA-3') and primer XB4 Reverse (5'- GTGTGTACAAGGCCCGGGAAC -3') the 16S rRNA gene sequence was amplified. Initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; last elongation at 72°C for 7 minutes in a ProFlexTM (Applied Biosystem, Metropolis India). The 3500 DX genetic Analyzer was used to purify and sequence the amplified PCR result. For taxonomic resolution, Analyse the sequence by using the NCBI Blast site (Nagarajan kayalvizhi et al 2010 and Fiucci. G et al 2004).

3. Study the probiotic characteristics of *K. gibsonii*

Probiotics characteristics of *K. gibsonii* was studied by Acid tolerance, Thermotolerance, Salt tolerance, and Proteolytic activity test. MRS broth was used to examine the inhibiting substances described above. Growth was observed at 620 nm after a 24-hour incubation period at varied NaCl concentrations of 2%, 4%, and 6% and temperatures of 15°C, 24°C, and 45°C (4). Proteolytic activity was checked by increasing the temperature at 70°C for 30 min to inhibit the protease enzyme activity. Incubate the samples with the chemicals for 1 h at 37℃. At the working concentration of 50 percent (v/v) organic solvents were used with detergents Tween 80 (10% v/v), Acetone (50% v/v), Chloroform (50% v/v) of concentrations. After centrifugation at 10000 g for 20 min, supernatant was tested for antimicrobial activity against indicator bacteria (Nagarajan kayalvizhi et al 2010 and S. Oh et al 2000).

4. Detection and Screening of Bacteriocin (Antibacterial peptide)

Kurthia gibsonii was cultivated in 10 ml MRS broth at pH 6.5 for 24 h at 37 °C. After incubation, the cell culture was heated at 70 °C for 30 min to assure inhibition of protease activity, then cooled at room temperature and centrifuged (5000 rpm for 20 min at 4 °C). In order to eliminate the antimicrobial effect of organic acids, the pH of the supernatants was adjusted to 6.5 with 10 M NaOH solution. The inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase from bovine liver followed by filtration through a 0.2 µm pore-size cellulose acetate. The agar-well diffusion method was used to test bacteriocin activity. 200 µl of Bacillus cereus (indicator microorganism) was inoculated on de man rogosa sharp media. 100 µl of cell free supernatants (Kurthia gibsonii) was filled inside 4 mm diameter wells. Phosphate-buffered saline (PBS) will be used as negative control After incubating the plates at 37°C for 24 hours, the inhibitory zone was observed. Antimicrobial activity was measured in the sample that resulted in an inhibitory zone (Gaspar .C et al 2018; Kaur. R and Tiwari S. K 2016, and S. Oh et al 2000)

5. Purification of bacteriocin (antimicrobial peptide)

K. gibsonii was grown for 48 hours in sterile flasks containing 100 mL MRS broth at 37°C. After incubation, centrifuged cells were collected (5000 revolutions per minute, 25 minute). The pellet was discarded and the supernatant was resuspended [12]. 50% Ammonium sulphate was added gradually with constant stirring. After 24 hours at 4°C, the broth was centrifuged (5000 rpm, 25 minute). The resuspend the pellet in 5 mL of Distilled water as a buffer and discard the supernatant. Purification of the protein was done using a dialysis bag (Dialysis membrane -60, Himedia, Mumbai) after which the protein was confirmed using the Bradford Assay. An Agilent Technologies 1260 infinite HPLC unit equipped with an analytical reversed phase C18 column was used for retention time measurement when dialysed sample were loaded. Bacteriocin was detected as a peak with a retention time ranging from 35.0 to 45.0 minutes. Two solvents were used for the gradient separation that is 95% Mili Q water (Millipore, USA) and 5 % acetonitrile, 100% Acetonitrile. 1ml min^{-1} flow rate was set for the mobile phase. This gradient was used for further process (0% solvent B for 3 min, 0-40% solvent B for 45 min followed by 40-100% solvent B for 5 min and then back to 100% solution A). At the time of different HPLC run, Active fractions were collected at the 35 min to 45 min retention time and then pooled lyophilized [4,12]. The fractions which show inhibitory activity against indicator organisms were pooled together and subjected to Disc diffusion assay for bacteriocin activity (Goh H F et al 2015).

6. Effect of Bacteriocin (Antibacterial peptides) on ATCC strains

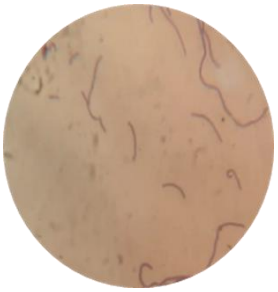
The antibacterial activity of the antimicrobial peptide was tested against 3 common gastrointestinal pathogens namely *E. coli* ATCC 24922, *Shigella flexneri* ATCC 12022, and *Salmonella typhimurium* ATCC 14028 using the Disc diffusion method. Agar plate was inoculated with loop full indicator bacteria, and a sterial disc was dipped in extracted protein for 3 hours before being placed on it. Incubate the plates at 37°C for 24-48 hours, the inhibitory zone was observed. Antimicrobial activity was measured in the sample that resulted in an inhibitory zone (Deshmukh P. V et al 2013 and Saif. F. A et al 2016).

Result

1. Isolation and Identification of bacterial strain

The bacteria isolated from paneer sample was gram-positive, nonspore forming, straight rod which shows aerobic growth (figure 1, 2). Biochemically, isolate belongs to the genus *Kurthia*. The strain indicates 99.6% identity with the sequence of *K. gibsonii* while analysing with 16S rRNA gene sequence.

Figure 1. Isolation of Kurthia gibsonii



2. Microscopic structure of Kurthia gibsonii

2. Study the probiotic characteristics of *K. gibsonii*

Salt tolerance and different temperature were tested on *K. gibsonii* isolates using MRS broth which shows growth against temperature 37℃, 45°C, and 65°C with pH 7 and growth on NaCl concentrations of 2%, 4%, and 6% respectively. Chemicals such as acetone, chloroform and detergent Tween 80 has not shown any effect on antibacterial peptide while testing (Table 1).

Treatment	Growth
Temperature Treatment	
37°C	Growth
45°C	Growth
60°C	Growth
pH	
5	Growth
6	Growth
7	Growth
Salt Tolerance	
2% Nacl	Growth
4% Nacl	Growth
6% Nacl	Growth

3. Detection and Screening of Bacteriocin (Antibacterial peptide)

Partially purified bacteriocin was found to be resistant to protease enzyme, but a bacteriocin solution treated with Catalase did not affect the activity. The fact that catalase had no effect on the bacteriocin inhibitory activity suggested that the inhibition is not due to hydrogen peroxide.

These data indicate that the inhibitory substance is proteinaceous in nature.
The bacteriocin production of Kurthia gibsonii was tested against B. cereus using the agar well diffusion method. The zone of inhibition is shown in Kurthia gibsonii (figure 3).

Figure 3. Showing zone of inhibition due to bacteriocin production

4. Purification of Bacteriocin (antibacterial peptide)

Antibacterial peptide extracted from *K. gibsonii* showed a production of antibacterial peptide that is bacteriocin which was detected in the screening test and showed good antibacterial activity against Indicator organisms that is Bacillus cereus. The protein was loaded to RP-HPLC and run for 1 hour. The active bacteriocin was eluted out from 35 to 45 min (figure 4) and inhibited *E. coli, Shigella,* and *Salmonella* ATCC strains.

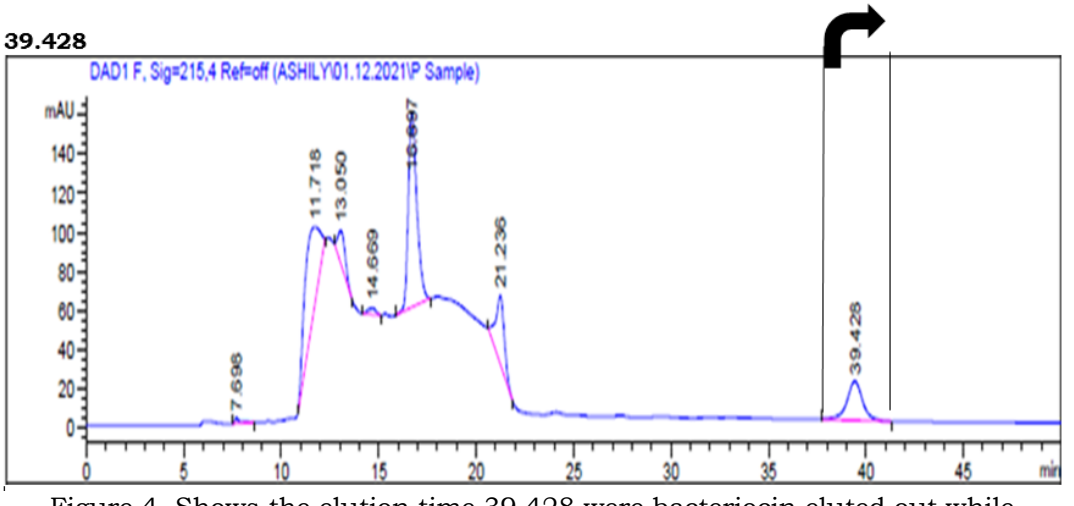


Figure 4. Shows the elution time 39.428 were bacteriocin eluted out while running RP-HPLC.

5. Effect of bacteriocin (Antibacterial peptides) on ATCC strains

In our study, we selected 3 pathogenic organisms i.e., *E. coli* ATCC 24922, *Shigella flexneri* ATCC 12022 *and Salmonella typhimurium* ATCC 14028 as they are the common cause of GI infections. Hence, we report a bacteria K. gibsonii

which has antibacterial activity against gastric pathogens. The activity of antibacterial peptides against *E. coli* ATCC 24922, *Shigella flexneri* ATCC 12022 and *Salmonella typhimurium* ATCC 14028 was tested using the Disc diffusion method and the inhibitory zone was observed around 08 mm, 08 mm and 05 mm (figure 5).

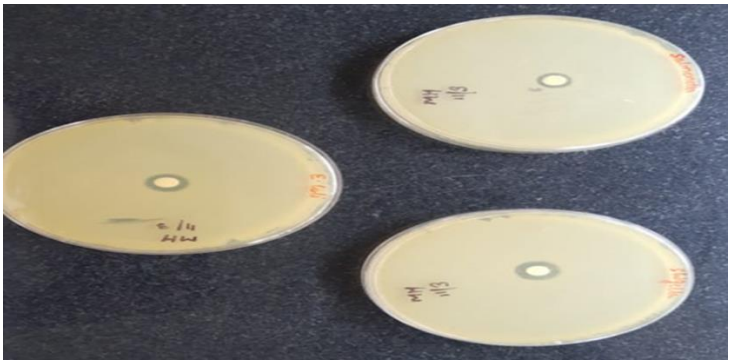


Figure 5. ATCC strains of E. coli, Shigella and Salmonella shows zone of inhibition which shows antibacterial activity of K. gibsonii.

Discussion

1. Isolation and Identification of bacterial strain

India is known as an agro country with a large vegetarian population. As a source of animal proteins, milk is an important part of such people's diets. Dairy products have a long history of contributing to society's social, economic, and nutritional well-being. Milk and milk products have been valued as a complete diet since Vedic times. Basically, milk is consumed as a liquid in our country but half of the milk is used as Ghee, curd, butter, khoa, paneer, cheese, chhach, ice cream, and milk powders (Rupp. M et al 2016). Paneer is an essential indigenous product made by heating milk and then acid coagulating it with a suitable acid such as citric acid, lactic acid, tartaric acid, alum, or sour whey (Rupp. M et al 2016). With the exception of soluble whey proteins, lactose, and minerals, paneer includes all of the milk ingredients. Paneer has a high fat and protein content while having a low lactose content explained by Sunil Kumar (Kumar. S et al 2014).

Kurthia is a bacterial genus from the Planococcaceae family and more closely related to members of Lactobacillus, Bacillus, Staphylococcus and Streptococcus. Kurthia species are known to produce chitinous enzymes (Paul M. K. et al 2016). However, antibacterial peptide i.e., Bacteriocin production by this organism has not been reported yet. These bacteria usually found in meat, meat products, milk products.

In the present study, bacteria isolated from paneer sample was gram-positive, nonspore forming, straight rod which shows aerobic growth and the strain indicates 99.6% identity with the sequence of K. gibsonii while analysing with 16S rRNA gene sequence. The partial 16S rRNA gene sequence of this strain showed

99.6% identity with those of K. gibsonii SSOG3, K. gibsonii LB, K. gibsonii TY-06, K. gibsonii JM107, and K. gibsonii SegA3 (Nagarajan kayalvizhi et al 2010). Previous study by Shaw S and Keddie also explain the same things regarding Kurthia physiological identification as well as the Taxonomical placements (Shaw. S. Keddie 1983).

2. Study the probiotic characteristics of *K. gibsonii*

In present study *K gibsonii* shows positive result when probiotic characteristics was studied by Acid tolerance, Thermotolerance, Salt tolerance, and Proteolytic activity test. The antibacterial protein was shown to retain the activity within the pH range of 5, 6, and 7 retaining about 100% of its initial activity. This protein was not affected by treatment with chemicals and organic solvents such as acetone, n-butanol, and Tween-20. Nagarajan kayalvizhi has also shown this results of Bacteriocin on *Bacillus lechiniformis* that the bacteriocin protein was not affected by treatment with chemicals and organic solvents such as acetone, n-butanol, and Tween-20 as well as S. Oh had also shown that the L. acidophilus 30SC bacteriocin was active over wide range of pH, and was stable to various heat treatment (Nagarajan kayalvizhi et al 2010 and S. Oh et al 2000).

3. Detection and Screening of Bacteriocin (Antibacterial peptide)

The purified antibacterial protein was resistant to protease enzyme treatment. The similar activity of L. acidophilus 30SC reported by S. Oh shows that the purified bacteriocin was resistant to protease enzyme while tested (S. Oh et al 2000). The compound was stable at different temperatures 37℃, 45°C, and 65°C. Bacteriocin are ribosomal synthesized peptide which show antibacterial activity. Srionnual S have reported that Lactic acid bacteria produces bacteriocin which is safe and used as a food preservative as well as for production of fermented food (Srionnual. S et al 2007). Other genera of bacteria's also known to produces bacteriocin such as Enterococcus, Leuconostoc and streptococcus which shows antibacterial activity against pathogenic bacteria. In our study K. gibsonii showed antibacterial activity against Indicator organisms that is B. cereus. A similar activity has been reported by Hweh fen Goh for another organisms that is Weissella confusa (Goh H F et al 2015). However, there are no reports yet published which shows antibacterial activity of K. gibsonii.

4. Purification of Bacteriocin (antibacterial peptide)

Antibacterial peptide extracted from *K. gibsonii* showed a production of antibacterial peptide that is bacteriocin which was detected in the screening test and showed good antibacterial activity against Indicator organisms that is *Bacillus cereus*. Hweh fen Goh also performed this purification method for *W. confusa* and shows positive results against *B. cereus* (Goh H F et al 2015).

5. Effect of bacteriocin (Antibacterial peptides) on ATCC strains

In present study, three pathogenic organisms were selected i.e., *E. coli* ATCC 24922, *Shigella flexneri* ATCC 12022 *and Salmonella typhimurium* ATCC 14028 as they are the common cause of GI infections and an inhibitory zone was observed.

Feriala A.A. have been reported that Antibacterial activity of Lactic acid bacteria isolated from fruits and vegetables that is *Lactobacillus* and *Lactococcus* were measured against *S. aureus* ATCC6538, *E. coli* ATCC8739, *P. aeruginosa* ATCC9027 showed an inhibitory zone (Saif. F et al 2016). Deshmukh P. V also checked the antibacterial activity of Bacteriocin against *S. aureus, E. coli, P. aeruginosa, Bacillus coagulans, klebsiella pneumoniae* and *Proteus vulgaris* showed an inhibitory zone against RM 1 to RM 10 except RM 5 (Deshmukh P. V and Thorat P. R 2013). The antibacterial peptides isolated from K. gibsonii in this study can be explored further for its physio chemical properties, so that it can be a useful for pharmaceutical industry.

Conclusion

Kurthia gibsonii was isolated from Paneer and Bacteriocin production by this organism was characterised by RP-HPLC. To our knowledge, this is the first report of antibacterial peptides (bacteriocin) from *Kurthia gibsonii* which may be used as food preservatives as well used in the treatment of Intestinal infections.

Acknowledgement

I would like to express my gratitude to Dr Niranjan Patil Metropolis Laboratory, India for providing the research facilities and special thanks to my Colleagues Ashily Rajendran. The isolate of *K. gibsonii* was not submitted in NCBI because this organism is known organism.

Conflict Of Interest:
There is no conflict of interest.

Author's Contributions:
I have made substantial contributions to conception, design, acquisition of data, analysis and interpretation of data. I have been involved in drafting the manuscript. Sharvari Samant revising it and give final approval of the version to be published.

Funding:
Self-funded.

Data Availability:
None.

Ethics Statement:
This has been approved by Institutional Ethical Committee by MGM IHS, Navi Mumbai, Maharashtra.

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