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INTRODUCTION

Tribhuvan University Journal of Microbiology (TUJM) is an official, peer reviewed, biomedical journal of the Central Department of Microbiology. It is published annually and publishes articles in the category of original article, review article, case report, letter to the editor.

The aim of the TUJM is to promote the publication of articles related to microbiology. Authors do not have to pay for submission, processing or publication of articles in TUJM.

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THE EDITORIAL PROCESS

The manuscript will be reviewed with the understanding that it has not been submitted to other journal at a time or has not been published or accepted for publication elsewhere. Manuscript is reviewed for originality, scientific and technical ideas, and significant message. The poor articles with insufficient originality, serious scientific and technical mistakes and lack of significant message will be rejected. Manuscript is sent to expert reviewer without revealing the identity of the authors to the reviewers. Each manuscript is then reviewed by the TUJM editor based on the comments of the reviewers and make final decision for publication or rejection of the manuscript.

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Author's guidelines

1. The preparation and presentation of manuscripts

Manuscripts should be drafted as concisely as possible. By submission of a manuscript to the journal, all authors warrant that they have the authority to publish the material and that the paper, or one substantially the same, has neither been published previously, nor is being considered for publication elsewhere.

2. Format of papers

The manuscript must be typed double-spaced on A4 size white paper with Times New Roman font, size of 12 points (In hard printing-Book Antiqua). Individual papers have a limit of approximately 4000 words, including figures and tables. The pages should be numbered consecutively beginning with the title page. The first page should show: (a) the title; (b) name(s) of author(s) and place(s) where the work was done; (c) an abbreviated running headline not exceeding 35 letters and spaces; (d) the name, complete mailing address, email address, telephone and fax numbers of the author to whom all correspondence should be addressed and who will check the proofs. English language used in the manuscript should be of a publishable standard.

3. Submissions

Authors are advised to submit their manuscripts through e-mails (cdm1990@microbiotu.edu.np, manita_aryal11@yahoo.com, upendrats@gmail.com, or adhikarinaba2004@yahoo.com) or electronic copy and three hard copies of the manuscript to the Research Management Cell, Central Department of Microbiology at Kirtipur. A signed cover letter mentioning that the article has not been submitted elsewhere for publication should be submitted with the manuscript.

3. 1 Full-length papers

The paper should have new concepts or the recording of facts. The manuscript should be prepared for a wide readership. As far as possible, the paper should present the results of an original scientific research. The paper will have the following sections:

(a) ABSTRACT: A brief summary of about 150-200 words, should give the major findings of the investigation under the following headings: Objectives; Methods; Results; Conclusion. A list of between four and six keywords should be added.

(b) INTRODUCTION: A balance should be maintained between the pure and applied aspects of the subject.

(c) MATERIALS AND METHODS: Ensure that the work can be repeated according to the details provided. By submission of a manuscript, the authors consent that biological material, including plasmids, viruses and microbial strains, unobtainable from national collections will be made available to members of the scientific community for non-commercial purposes subject to national and international regulations governing the supply of biological material. In the case of a new diagnostic PCR, you should consider the need for an internal amplification control. Ethical approval letter Reg no. form authorised institution should be given if applicable.

(d) RESULTS: Well-prepared tables and figures must be a feature of the 'Results' section because they convey the major observations to readers. Information provided in tables and figures should not be repeated in the text, but attention on the importance of the principal findings of the study should be focused.

(e) DISCUSSION: This must not recapitulate the results and should explain the meaning of results.

(f) CONCLUSION: The conclusion should be based on results.

(g) ACKNOWLEDGEMENTS:

(h) REFERENCES:

APA Style: Detailed Referencing Guide for Authors

Tribhuvan University Journal of Microbiology (TUJM) follows the APA 7th Edition referencing style. Authors must ensure that every in-text citation appears in the reference list and vice versa.

1. Journal Articles

A. One Author

Format:

Author, A. A. (Year). Title of the article. *Title of Journal*, *Volume*(Issue), page range. https://doi.org/xxxxx

Example:

Sharma, P. R. (2021). Antimicrobial resistance patterns in clinical isolates. *Journal of Medical Microbiology*, *70*(4), 001–010. https://doi.org/10.xxxx/

jmm.2021.123

In-text citation:

- Parenthetical: (Sharma, 2021)
- Narrative: Sharma (2021)

B. Two Authors

Format:

Author, A. A., & Author, B. B. (Year). Title of the article. *Title of Journal*, *Volume*(Issue), page range. DOI

Example:

Thapa, S., & Karki, R. (2020). Biofilm formation by hospital pathogens. *Microbiology Research*, *18*(2), 45–53.

In-text citation:

- (Thapa & Karki, 2020)
- Thapa and Karki (2020)

C. Three or More Authors

Format:

Author, A. A., Author, B. B., & Author, C. C. (Year). Title of article. *Journal Name*, *Volume*(Issue), pages. DOI

Example:

Joshi, D., Singh, A., Gupta, N., & Mehta, K. (2022). Molecular detection of resistant bacteria. *International Journal of Microbiology*, *25*(1), 33–41.

In-text citation (always):

- (Joshi et al., 2022)

2. Books

A. One Author Book

Format:

Author, A. A. (Year). *Title of the book*. Publisher.

Example:

Prescott, L. M. (2018). *Microbiology*. McGraw-Hill Education.

B. Two Authors Book

Format:

Author, A. A., & Author, B. B. (Year). *Title of book*. Publisher.

Example:

Tortora, G. J., & Funke, B. R. (2019). *Microbiology: An introduction*. **Pearson.**

C. Three or More Authors Book

Format:

Author, A. A., Author, B. B., & Author, C. C. (Year). *Title of book*. Publisher.

Example:

Madigan, M. T., Bender, K. S., Buckley, D. H., & Stahl, D. A. (2021). *Brock biology of microorganisms*. Pearson.

3. Websites and Online Documents

A. Individual Author

Format:

Author, A. A. (Year, Month Day). Title of page. Website Name. URL

Example:

Singh, R. (2023, May 12). Antimicrobial resistance trends. Microbiology Online. https://www.example.com

B. Organization as Author (No Individual Author)

Format:

Organization Name. (Year). *Title of the webpage*. URL

Example:

World Health Organization. (2023). *Antimicrobial resistance*. https://www.who.int

In-text citation:

- (World Health Organization [WHO], 2023) → first citation
- (WHO, 2023) → subsequent citations

4. WHO Reports

Format:

World Health Organization. (Year). *Title of the report*. WHO. URL

Example:

World Health Organization. (2021). *Global antimicrobial resistance and use surveillance system (GLASS) report*. WHO. https://www.who.int

5. CDC Reports

Format:

Centers for Disease Control and Prevention. (Year). *Title of the report*. CDC. URL

Example:

Centers for Disease Control and Prevention. (2022). *Antibiotic resistance threats in the United States*. CDC. https://www.cdc.gov

In-text citation:

- (Centers for Disease Control and Prevention [CDC], 2022)
- (CDC, 2022)

6. CLSI Guidelines

CLSI documents are treated as organizational reports.

Format:

Clinical and Laboratory Standards Institute. (Year). *Title of guideline* (Standard number). CLSI. URL

Example:

Clinical and Laboratory Standards Institute. (2023). *Performance standards for antimicrobial susceptibility testing* (M100). CLSI.

In-text citation:

- (Clinical and Laboratory Standards Institute [CLSI], 2023)
- (CLSI, 2023)

7. Important APA Rules for TUJM Authors

- Use sentence case for article and webpage titles.
- Use title case for journal titles.
- Italicize journal name, volume number, and book titles.
- Include DOI whenever available.
- URLs should not end with a period.
- Use et al. for three or more authors in all in-text citations.

Abbreviations and units

The Journal uses SI units: g/l; d, h, min, s (time units) but week and year in full; probability is p; centrifugation conditions relative to gravity (g or rpm). Please refer to the Biochemical Journal 'Instructions to Authors'.

Microbial nomenclature

The Latin binomial name of micro-organisms, plants and animals (other than farm animals) must be givenat first mention in the text; thereafter the generic name will be abbreviated in such a way that confusion isavoided when dealing with several genera all beginning with the same letter, viz. *Pseudomonas*, *Proteus*, *Pediococcus*, etc. (see list of abbreviations below). Subspecies are italicized (*Corynebacterium diphtheria* subsp. *mitis*; groups and types are printed in Roman and designated by capital letters or Arabic figures (e.g. *Staphylococcus aureus* group A).

Common names will not have an initial capital letter nor will they be underlined in the manuscript, viz. pseudomonad, salmonellas. The specific name will be given in full in the captions to tables and figures. Major ranks are written in Roman with an initial capital (e.g. Enterobacteriaceae).

At the first citation of a serotype the genus name is given followed by the word 'serotype' and then the serotype name. Names of serotypes should be in Roman type with the first letter capitalized (for example *Salmonella* serotype Typhimurium). Subsequently the name should by written with the genus (abbreviated) followed directly by the serotype name (for example S. Typhimurium).

Nucleotide sequences

1. Nucleotide sequence data should be deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries and the accession number referenced in the manuscript.

2. Sequence data should only be included if they are new (unpublished), complete (no unidentified nucleotides included) and if the sequence information itself provides important new biological in sights of direct relevance to the question addressed in the manuscript. Generally, sequences should not be submitted if the same gene has been reported in another species unless a comparison with related sequences contributes important new information.

3. Presentation of nucleotide sequences should include clear indications of nucleotide numbers and points of interest, e.g. promoter sequences, ribosome binding sites, mutations, insertions, probe sequences, etc. In the case of comparisons, nucleotides which differ between the sequences should be readily visible to the reader, e.g. by the use of bold face, shading, boxing or by the use of a dash to represent identical nucleotides. The font size used in the manuscript should facilitate appropriate reduction of the figure.

Statistics

Tests must be presented clearly to allow a reader with access to the data to repeat them. It is not necessary to describe every statistical test fully, as long as it is clear from the context what was done. In particular, null hypotheses should be clearly stated. Authors are urged to give consideration to the assumptions underlying any statistical tests used and to assure the reader that the assumptions are at least plausible. Authors should be prepared to use nonparametric tests if the assumptions do not seem to hold.

Tables

Tables must be prepared using the same word processing package as the manuscript text. They should not be embedded but be placed immediately following the main text. Do not submit tables separately. Tables must not include ruled vertical or horizontal lines with the exception of headers and a footer. The use of explanatory footnotes is permissible and they should be

marked by the following (shown in order of preference): *, †, ‡, §, ,**, †† etc.

Figures

Figures may be line drawings or photographs. They may be uploaded to the online submission site as separate files or included within the manuscript following the text and any tables. Do not embed figures in the text. All graphs, charts and diagrams must be submitted in a finished form and at their intended publication size. Authors are advised that poor quality figures may delay the publication of their paper. Symbols or keys representing data series in graphs and charts must not be shown on the figure itself but be included in the legend typed on a separate sheet.

Photographs

These must be of good quality and high contrast. The magnification must be indicated by adding a bar representing a stated length. Composite photographs can reduce the numbers that require publication. The Journal will not accept figures illustrating SDS-PAGE and agarose gels, with multiple lanes, where lane order has been rearranged using digital imaging software. The figure should also show sufficient of the gel to reveal reference markers (e.g. the sample origin and a tracker dye, or a lane of molecular mass markers). Captions should be set out in the same manner as that used for figures.

Supporting data

Data that is integral to the paper must be made available in such a way as to enable readers to replicate, verify and build upon the conclusions published in the paper. Any restriction on the availability of this data must be disclosed at the time of submission.

Data may be included as part of the main article where practical. We recommend that data for which public repositories are widely used, and are accessible to all, should be deposited in such a repository prior to publication. The appropriate linking details and identifier(s) should then be included in the publication and where possible the repository, to facilitate linking between the journal article and the data. If such a repository does not exist, data should be included as supporting information to the published paper or authors should agree to make their data available upon reasonable request.

Footnotes

Not permitted other than on the first page of a manuscript where they are used to show the author's change of address and the address for correspondence.

Experimental hazards

Chemical or microbiological hazards that may be involved in the experiments must be explained. Authors should provide a description of the relevant safety precautions adopted or cite an accepted 'Code of Practice'.

Supporting information

Authors wishing to submit supporting information material (such as multimedia adjuncts, large data sets, extra colour illustrations, bibliographies or any other material for which there is in sufficient space in the print edition of the Journal) must do so at the time of first submission. This supporting information is an integral part of the article and will be reviewed accordingly. The availability of supporting information should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting information' and providing titles of figures and tables.

Letter of Conflict of Interest (If applicable)

3.2 Review Articles

Preparation of manuscript

The review manuscript should not be simply a review of past work or be concentrated largely on unpublished results from the laboratory. There should be a distillation of early and present work within the field to show progress and explain the present interest and relevance. It is essential at the planning stage to realize that there is a limit to the number of pages available. The final manuscript must not exceed 4000 words with double-spaced typing, including references. The Tables and Figures must be considered as part of the text and the pages available for text reduced accordingly. References can make a heavy demand on the pages available to you, and it is suggested that you select key references only.

Manuscript presentation

The headings in these review articles are of the author's choice. The first page of the manuscript must give only (a) the title; (b) name(s) of author(s) and address; (c) an abbreviated title to be used for the running title not exceeding 35 letters and spaces; (d) the name, postal and e-mail address of the author to whom all correspondence should be addressed and who will check the proofs. A short SUMMARY of 150-200 words

must be included, as well as an INTRODUCTION, DISCUSSION, CONCLUSION (possibly referring to future prospects) sections. References must be chosen carefully as their number is limited by the size limitation of the review article.

3.3 Letters to the editor

The Chief Editor will consider letters which will provide further debate on a particular topic arising from the publication of a paper. Author(s) of the paper will be sent an edited copy of the letter and they will have the right of reply. Both letters will be published in the Journal.

3.4 Notes to the editor

The Chief Editor will consider notes which will provide further confirmatory information on a particular topic, or a novel aspect of a methodology (e.g. detection) or a microorganism (e.g. virulence factor) for which results are preliminary but the impact for Microbiology deemed to be important and requires rapid publishing. Notes should be concise (2000 words; including references), with no headings and present results in 1 table or 1 figure only. The abstract should be a brief summary of the work under the following four headings: Objectives; Methods and Results; Conclusion; Significance and Impact of the Study.

3.5 Short Communication

1. Manuscript type & Purpose

- The short communication should present **concise, novel, or preliminary findings** that are important but may not require a full-length article. (E.g., small epidemiological surveys, brief case-series, methodological notes, initial findings, etc.)
- Should not duplicate content already published elsewhere.

2. File format & General Formatting

- Use A4-size paper.
- Double-spaced text throughout.
- Use **Times New Roman**, 12-point font.
- Pages numbered consecutively from the title page.
- Running header (abbreviated title) not exceeding 35 characters/spaces on first page (if applicable).
- Manuscript submitted electronically (Word/RTF) — optionally also hard copies per TUJM's submission policy.

3. Manuscript Components and Structure

Since TUJM currently lists categories like "full-length papers", "review articles", "letters to editor" or "notes" — but not a formally defined "short communication" — the template below adapts from their "Notes to the editor" guideline with modifications for clarity.

Suggested structure:

1. Title Page

- Title of manuscript (concise, informative).
- Author name(s) and affiliations / institutional addresses.
- Corresponding author's full mailing address, email, telephone/fax.
- Running headline (abbreviated title), ≤ 35 letters/spaces (if used).

2. Abstract

- Short summary of key points. Because this is a short communication / "note-style", you may adapt a brief structured abstract under headings such as: Objectives / Aim; Methods; Key Findings (or Results); Conclusion / Significance.
- **Word limit:** ~150–200 words. (Same as full-length abstracts.)
- Provide **4–6 keywords** following the abstract.

3. Main Text

- **Introduction / Background:** Brief rationale — why this study or observation matters; what gap is being addressed. Since space is limited, keep concise.
- **Materials and Methods** (or Methods / Approach): Enough detail so that someone else could repeat the work, if relevant. Include ethical approval, strain/sample sources, etc., if applicable.
- **Results (and/or Observations):** Present key findings clearly. Use tables or figures if needed, but **limit total display items** (consider 1 table +/– 1 figure, but ensure conciseness). In "notes" TUJM allows only one table or one figure.
- **Discussion/Interpretation:** Briefly discuss significance, context, implications. Do not repeat all results. Focus on interpretation, limitations, and potential relevance.
- **Conclusion:** Summarize the main message or recommendation arising from the results/observations.

4. Acknowledgments (if needed)

- Funding, institutional support, contributions, conflict-of-interest disclosure, etc.

5. References

- Use the **APA style**, consistent with TUJM's instructions.
- For more than three authors, use "et al." in text citations.
- List references in ascending order by date (or alphabetical when same date authors).

6. Tables / Figures (if any)

- **Preferably no more than one table or one figure** (1 Figure + 1 Table).
- **Tables:** created in same word-processing file, included near relevant text (not at the very end). Use footnotes instead of vertical/horizontal border lines (except header/footer).
- **Figures/Photos:** high quality, good contrast; if microscopy or gel images, include scale bar / marker; avoid digital rearrangement of lanes/gels.
- Figure caption / legend should be placed on a

separate sheet or just below figure. Ensure clarity, include magnification or scale, labeling, etc.

4. Length and Scope Considerations

- Since TUJM does not formally define "short communication", authors should aim for **conciseness** — e.g., ~ **1,500–2,000** words (excluding large background introduction), but must still provide sufficient methodological and interpretative clarity.
- Use minimal references — only those necessary to support context, methodology, or comparison.

5. Submission & Ethical/Administrative Requirements

- Manuscripts must be **original**, not submitted elsewhere, and not previously published.
- Submit via email to the TUJM submission contacts (as per journal's "Submissions" page), along with a signed cover letter with a statement that the work is not under consideration elsewhere.
- If biological material (microbial strains, plasmids, viruses, etc.) is not publicly available, author must commit to make them available for non-commercial research upon request.
- Ethical approval letter / registration number must be included if human/animal samples are used (as per TUJM full-length papers guideline).

6. What to Emphasize / Ideal Uses of Short Communications in TUJM

- Brief epidemiological surveys or incidence reports (e.g. distribution of a microbial species in a defined population).
- Preliminary data of ongoing research, especially if of potential public health or clinical significance.
- Novel methodological notes — e.g. a new, efficient diagnostic method, or validation of a simpler test.
- Single-case or small-series reports of unusual or rare microbial isolates, antibiotic resistance patterns, etc.
- Observational data that may prompt larger investigations — e.g. emergence of a new pathogen/ resistance pattern, outbreak notifications

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ORIGINAL RESEARCH

SHORT COMMUNICATION

EDITORIAL

The One Health Approach: A Critical Pathway for Public Health and Sustainable Development in the Developing World

One Health, which views interdependencies between human, animal, and environmental health, is receiving increasing attention across the globe as an effective way to handle the complexities associated with health issues. In developing countries, like Nepal, where the economic survival of human populations is ecologically interwoven with animals, wild animals, and the natural environment, One Health is no longer a theoretical or academic model, but the need of the day.

Nepal is an exemplary situation for applying the concepts of One Health. There is a large number of population that depends on agriculture and animal resources as a means of living. Direct contact between human and animal populations and minimal bio-security measures make them vulnerable to incidents of transmitting and acquiring diseases from each other. These include rabies, bird flu, brucellosis, Leptospirosis, Q-Fever, Tick Borne Viral diseases, and Japanese Encephalitis, which continue to threaten the community at large. Furthermore, these diseases continue to have an increased impact on the rural sectors and the commonly deprived sections of the community, thereby worsening the already prevailing disparity between those who are able to access better healthcare and those who are deprived.

In terms of public health, the significance of the One Health approach lies in the fact that it enhances disease prevention and control by overcoming the limitations of the old-fashioned sectorial approaches. Indeed, the old-fashioned approaches may create problems related to delayed disease outbreak identification and consequently poor disease control and prevention. The aforementioned problems can lead to weaknesses in disease prevention and control. Morever, the COVID-19 pandemic has shown that the human-animal-environment interface has international implications and that the one health approach can contribute to the prevention of future pandemics.

Agriculture is another area of equal importance where One Health can bring changes. Animal health is directly associated with food security, nutritional status, as well as earning capacity. Poor health of the animal affects it indirectly through low productivity, financial losses, and overuse of antibiotics. The latter causes antimicrobial resistance (AMR). AMR is an emerging problem in Nepal as a result of unregulated use of antibiotics both at the human medicine counter and animal health services.

Environmental health is the base of the Three-One Health components and has relevance in the context of the ecologically vulnerable environment of Nepal. Environmental degradation, such as unplanned forestation and climate change, affects the ecology of diseases and thereby increases the susceptibility of people to new pathogens. Environmental Health promotes responsible environmental policies and climate-resilient policies in relation to health.

Apart from the benefits to human health outcomes, the One Health approach also meets the overall goals of development. One Health will contribute to the control of poverty and the advancement of economic growth. For a country such as Nepal, implementing One Health will lead to improved governance. This will result from cooperation among different ministers and the use of data to make decisions. The use of One Health will prove to be cost-effective because the expenditure needed to provide health care in human beings will not be necessary. In essence, the One Health approach provides a holistic and pragmatic framework through which developing nations can address problems that intertwine food security, human health, and natural resource management. To Nepal, adoption of One Health is no longer an issue of choice but one of need: for improving public health, protecting agriculture, safeguarding the environment, and promoting sustainable development.

Upendra Thapa Shrestha, Associate Editor
Dr. Dev Raj Joshi, Editor in Chief
Tribhuvan University Journal of Microbiology (TUJM)

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Tribhuvan University Journal of Microbiology

ORIGINAL RESEARCH

Antibacterial Activity of Bacteriocin Like Compound Extracted from Lactic Acid Bacteria Isolated from Farm Soil, Curd, and *Gundruk*

Anshuya Sigdel[1], Anita Thapa Magar[1], Rama Khadka[1*]

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ABSTRACT

Objectives: This study was focused on examining the antimicrobial properties of bacteriocin like compound extracted from Lactic acid bacteria (LAB) isolates from curd, farm soil, and *Gundruk* samples.

Methods: A total of 30 samples of farm soil (10), curd (10) and *Gundruk* (10) were collected from a Kathmandu district and identified strains of LAB. Dot plate technique was used for screening of bacteriocin, then bacteriocin was extracted from precipitation method. Antimicrobial activity was done from cell-free suspension by Agar well diffusion method.

Results: In this study, 86% of LAB were isolated and identified as *Streptococcus* species (46.67%), *Lactobacillus* species (23.33%) and *Pediococcus* species (16.67%). Out of the 26 isolates, 7 isolates (23.33%) produced bacteriocin. The antibacterial activity demonstrated inhibition zones ranging from 7–16 mm for farm soil isolates, 10–20 mm for curd isolates, and 7–18 mm for *Gundruk* isolates. From mixed extraction of bacteriocin (1:1 of *Pediococcus* spp and *Streptococcus* spp), antibacterial activity was shown to all test bacteria except *S. aureus* ATCC 43300.

Conclusion: This study concluded that LAB isolates from *Gundruk* exhibited the highest antibacterial activity (18–21 mm), compared to farm soil and curd isolates, highlighting their potential as a more effective natural antimicrobial source.

Keywords: Farm soil, curd, *Gundruk*, Lactic acid bacteria and Bacteriocin

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive bacteria that produce lactic acid from various sugars through the fermentation process, which covers a wide range of health benefits (Leska, 2023). LAB is facultative anaerobic, which means that they can grow in both oxygen-rich and oxygen-poor environments, allowing them to adapt to different niches (Ganzle, 2015). LAB has a significant and extensive impact on the field of food technology. They are naturally found in fermented food and have been identified in soil, water, manure, and sewage. So, LAB are considered an important group of probiotic bacteria (Ekundayo, 2014).

LAB are present in the environment (soil), contributing to microbial diversity and the overall health of the soil ecosystem. Soils are dynamic environments with fluctuating moisture, temperature, and nutrient availability. Although LAB in soil may not be as extensively studied as fermented foods or the human microbiome, their importance in maintaining soil health and their contribution to ecological balance are increasingly recognized (Wu et al., 2021). It is found in yogurt (*Lactobacillus bulgaricus, Streptococcus thermophilus*), sauerkraut, kimchi, pickle (*Lactobacillus brevis*), cheese, raw vegetables, soil, plant materials, and honey. They are also found in milk and milk Products. They are also gut flora, which is present in the human intestine and maintains gut health (Zhong et al., 2022).

Certain strains of LAB are classified as probiotic bacteria having beneficial effects on the human digestive system. Probiotics contribute to a healthy balance of microorganisms in the gut, aid digestion, and have potential immunomodulatory and anti-inflammatory effects (Ganzle, 2015). LAB produces

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different compounds like organic acids, hydrogen peroxide, diacetyl, carbon dioxide, etc. to kill other microorganisms. Bacteriocins are also produced by LAB, which have specialized peptides having antimicrobial activity and able to specifically target similar bacterial strains or harmful bacteria (Zotta, 2017). The growing global threat of antibiotic resistance has demanded a renewed focus on research into novel antibacterial agents. Isolation and characterization of LAB for the production of antibiotics has proven to be an important attempt in the research.

Many of LAB are still poorly understood, and their potential to produce antibiotics is unknown. These LABs can produce antibiotics that help fight harmful bacteria. (Doo et al., 2024). The study of comprehensive and systematic analysis of soil bacteria with the potential to produce antibiotics was done advanced microbiological and molecular techniques (Shabana et al., 2013). There are several studies in LAB which were isolated from different samples like milk, milk products, fermentation products, soil etc. However, LAB produced secondary metabolites known as bacteriocin having antibacterial activity against pathogenic bacteria were less studied. So, the main aim of study was to extract bacteriocin like compound from Lactic Acid Bacteria (LAB) isolates from curd, farm soil and *Gundruk* samples and perform its antimicrobial activity against highly resistance bacteria. Furthermore, this study has done comparative study of LABs producing bacteriocin from different sources.

METHODS

Study design, area, site, and duration

A descriptive cross-sectional study was conducted and a total of 30 samples were collected from the Kathmandu district, including 10 samples each from farm soil, curd, and *Gundruk*. This study was done from December 2023 to April 2024.

Sample collection and processing

The Convenience sampling method was used for the sample collection. The samples were collected in sterile zip-lock bags to prevent moisture loss during transportation, and then the samples were transported to the Microbiology laboratory of Padmakanya Multiple Campus.

Isolation of Lactic Acid Bacteria

LAB were isolated on MRS (de Man Rogosa Sharpe) agar containing 1% (w/v) $CaCO_3$. One gram of each sample was suspended in 9 ml of sterile phosphate buffer and serially diluted (10^{-1} to 10^{-6}), and 1 ml of each dilution was spread on the agar plates. The plates were then incubated at 37°C for 48 hrs (*Kazemipor* et al., 2012).

Characterization of Lactic Acid Bacteria

From the pure culture of obtained LAB, the morphology of the colony was studied. They were characterized by using various techniques such as Gram staining, oxidase test, catalase test, motility test, spore staining, and fermentation test, as stated in Bergey's manual of determinative bacteriology.

Sub-culture of test bacteria

Test bacteria, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus* spp, as well as *E. coli* ATCC 25922 and *S. aureus* ATCC 43300, were obtained from the Central Department of Microbiology, Kritipur, Kathmandu and sub-cultured on Nutrient agar. The bacteria were confirmed by Gram staining and biochemical tests and pure cultures were used to evaluate the antibacterial activity of LAB.

Screening of potential Lactic Acid Bacteria for bacteriocin like compound

After characterization, isolated LAB were screened for antibacterial activity using the dot plate technique on Mueller-Hinton agar. Test bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus* species were lawn cultured on the MHA agar, and isolates of *Lactobacillus* species, *Streptococcus* species and *Pediococcus* species were point inoculated. Plates was incubated at 37°C for 24 hrs, after which the zone of inhibition were measured *(*Ma et al.*, 2019).*

Extraction of Bacteriocin like compound

Pure LAB cultures with inhibitory activity were grown in MRS broth (pH 7) at 37°C for 48 hrs. Cultures were centrifuged at 10,000 rpm for 20 min at 4°C to obtain a cell-free supernatant, then neutralized to pH 7 using 1 M NaOH. Bacteriocin was then eluted from Whatman filter paper in potassium phosphate buffer and collected in sterile test tubes *(*Yang et al.*, 1992).*

Determination of antibacterial activity of Bacteriocin like compound by agar well diffusion method

To determine antimicrobial activity, test bacteria were grown in nutrient broth for 4 hrs at 37°C and adjusted to 0.5 McFarland standard. Bacterial suspensions were

swabbed onto Mueller-Hinton agar, and 6 mm wells were made. A 70 µl solution of bacteriocin from soil, curd, and *Gundruk*, along with sterile water (negative control) and ciprofloxacin (positive control) were added to the wells and allowed to diffuse for 15 min. Plates were incubated at 37°C for 24–48 hrs, after which the zones of inhibition were measured *(Zhennai, 2000)*.

Quality control in the laboratory

Quality was also monitored for each laboratory equipment's throughout the study period. Standard culture of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 43300) were used for the interpretation of result in antimicrobial activity.

Data Analysis

All the data was obtained from this study was entered into Microsoft Excel 2016 and analyzed by percentage calculation.

RESULTS

In this study, among 30 samples (farm soil, curd and *Gundruk*), 26 (86.67%) isolates were identified as LAB.

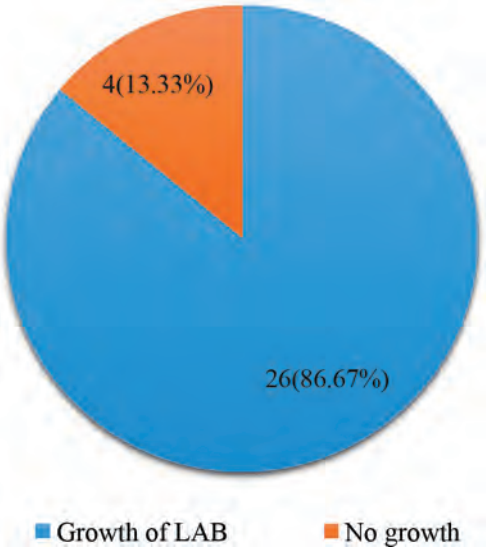


Figure 1: Growth of LAB among total samples

Types of Lactic Acid Bacteria isolated among total samples

Among 26 (86.67%) LAB isolates, 14 (46.67%) *Streptococcus* spp were identified followed by 7(23.33%) *Lactobacillus* spp and 5 (16.67%) *Pediococcus* spp from farm soil, curd, and *Gundruk* samples.

Table 1: Types of Lactic Acid Bacteria isolated among the total samples

Samples	Number of samples	*Lactobacillus* spp N (%)	*Streptococcus* spp N (%)	*Pediococcus* spp N (%)	Total N (%)
Farm Soil	10	4 (13.33)	4 (13.33)	2 (6.67)	10(33.33)
Curd	10	3 (10.0)	5 (16.67)	0	8(26.67)
Gundruk	10	0	5 (16.67)	3 (10.00)	8(26.67)
Total	**30**	**7(23.33)**	**14(46.67)**	**5(16.67)**	**26(86.67)**

N = Number, % = Percentage

Carbohydrate Fermentation test of Lactic Acid Bacteria

In carbohydrates fermentation test, LAB isolates from farm soil, curd and *Gundruk* showed fermentation to different sugars including glucose, lactose, sucrose and fructose.

Table 2: Fermentation test of different carbohydrates by Lactic Acid Bacteria isolates of farm soil samples

Carbohydrates	*Lactobacillus* species (4)	*Streptococcus* species (4)	*Pediococcus* species (2)
Glucose	+	+	-
Lactose	+	+	+
Sucrose	+	-	+
Fructose	+	+	+

+ = Fermenter, - =Non-fermenter

Table 3: Fermentation test of different carbohydrates by Lactic Acid Bacteria isolates of curd samples

Carbohydrates	*Lactobacillus* species (3)	*Streptococcus* species (5)
Glucose	+	+
Lactose	+	+
Sucrose	+	-
Fructose	+	+

+ = Fermenter, - = Non-fermenter

Table 4: Fermentation test of different carbohydrates by Lactic Acid Bacteria isolates of *Gundruk* samples

Carbohydrates	*Streptococcus* species (5)	*Pediococcus* species(3)
Glucose	+	+
Lactose	+	-
Sucrose	-	+
Fructose	+	+

+ = Fermenter, - = Non-fermenter

Screening of potential Lactic Acid Bacteria for bacteriocin like compound

A total of 26 LAB isolates were screening for bacteriocin against test bacteria in which 7 LAB isolates were positive against all test bacteria.

Table 5: Screening of potential Lactic Acid Bacteria for bacteriocin like compound

Test bacteria	Farm soil isolates			Curd isolates		Gundruk isolates	
	S1	S2	S3	C1	C2	G1	G2
Gram positive bacteria							
S. aureus ATCC 43300	6mm	5mm	10mm	10mm	8mm	5mm	-
S. aureus	7mm	8mm	15mm	8mm	10mm	8mm	-
Bacillus spp	-	6mm	12mm	8mm	-	-	-
Gram negative bacteria							
E. coli ATCC 25922	6mm	6mm	9mm	-	12mm	8mm	7mm
E. coli	10mm	7mm	11mm	10mm	-	7mm	10mm
K. pneumoniae	-	9mm	9mm	6mm	9mm	5mm	-
P. aeruginosa	-	4mm	6mm	-	-	-	9mm

- = no zone of inhibition, S1 = *Lactobacillus* spp, S2 = *Streptococcus* spp, S3 = *Pediococcus* spp C1= *Lactobacillus* spp, C2 = Streptococcus spp, G1 = *Streptococcus* spp, G2 = *Pediococcus* spp

Antibacterial activity of bacteriocin like compound against test bacteria

In this study, bacteriocin like compound extracted from farm soil, curd and *Gundruk* showed different zone of inhibition ranged from 7mm to 20mm against all test bacteria.

Table 6: Antibacterial activity of bacteriocin like compound against test bacteria

Test bacteria	Soil isolates			Curd isolates		*Gundruk* isolates	
	(S1)	(S2)	(S3)	(C1)	(C2)	(G1)	(G2)
Gram positive bacteria							
S. aureus ATCC 43300	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	7mm
Bacillus spp	-	-	-	15mm	13mm	18mm	15mm
Gram negative bacteria							
E. coli ATCC 25922	7mm	-	11mm	12mm	10mm	13mm	12mm
E. coli	13mm	16mm	9mm	20mm	16mm	17mm	15mm
P. aeruginosa	-	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-

- = No zone of inhibition, S1 = *Lactobacillus* spp, S2 = *Streptococcus* spp, S3=*Pediococcus* spp, C1= *Lactobacillus* spp, C2=*Streptococcus* spp, G1= *Streptococcus* spp and G2= *Pediococcus* spp.

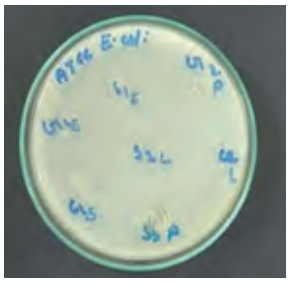
Antibacterial activity of mixed bacteriocin like compound against test bacteria

In this study, mixed bacteriocin like compound (1:1) from farm soil (S1, S2 & S3), curd (C1 &C2) and *Gundruk* (G1 & G2) showed antibacterial activity against all test bacteria except *S. aureus* ATCC 43300.

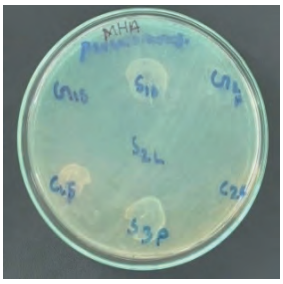
Table 7: Antibacterial activity of mixed bacteriocin like compound against test bacteria

Test Bacteria	Farm Soil (S1, S2 & S3)	Curd (C1 &C2)	*Gundruk* (G1 & G2)
Gram positive bacteria			
S. aureus ATCC 43300	-	-	-
S. aureus	-	-	10mm
Bacillus spp	10mm	20mm	18mm
Gram negative bacteria			
E. coli ATCC 25922	11mm	19mm	21mm
E. coli	15mm	20mm	19mm
P. aeruginosa	-	17mm	18mm
K. pneumoniae	11mm	10mm	15mm

- = No zone of inhibition, S1 = *Lactobacillus* spp, S2 = *Streptococcus* spp, S3 = *Pediococcus* spp, C1 = *Lactobacillus* spp, C2 = *Streptococcus* spp, G1 = *Streptococcus* spp and G2 = *Pediococcus* spp

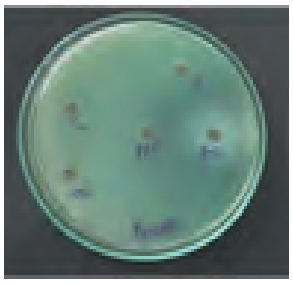


(A)



(B)

Screening of *Lactic Acid Bacteria* for bacteriocin extraction against (A) *Escherichia coli* ATCC 252922 and (B) *Pseudomonas aeruginosa* by dot plate method in MHA plate



(C)

(D)

Antibacterial activity of mixed bacteriocin extraction (1:1) from *Lactic Acid Bacteria* against *Pseudomonas aeruginosa* (C) and *Klebsiella pneumonia* (D) in MHA plate

DISCUSSION

In the present study, 26 (86.67%) isolates were identified as LAB in which three LAB isolates were capable of producing bacteriocin from *Gundruk*, farm soil, and curd. By comparing the morphological, physiological and biochemical tests, the isolates were identified as 14 (46.67%) *Streptococcus* spp, 7(23.33%) *Lactobacillus* spp (23.33%) and 5 (16.67%) *Pediococcus* spp In the study of Diop et al., (2007), 12 strains of LAB that produce bacteriocin were isolated in fermented foods. Ekundayo (2014) isolated 17 isolates of LAB in which 11 isolates were identified as *Lactobacillus* spp The result of the present study is in accordance with the report of Galvez et al., (2007) who revealed that members of LAB could be detected in a variety of habitats including fermented foods.

The isolates of LAB were screened against Gram positive bacteria (*S. aureus*, *S. aureus* ATCC 43300 and *Bacillus* species) and Gram negative bacteria (*E. coli* ATCC 25922, *E. coli*, *P. aeruginosa* and *K. pneumoniae*) by using dot plate technique on MHA plate. In this study, dot plate technique showed the zone of inhibition ranges from 5mm to 15mm against test bacteria. Among 7 LAB isolates, antibacterial activity showed against *E. coli*, *K. pneumoniae* and *S. aureus* but did not show any antibacterial property against *P. aeruginosa* and *Bacillus* species. Similarly, Boguta, et al., (2014) screened 296 strains of *Lactobacillus* and *Pediococcus*.

From farm soil samples, bacteriocin like compound extracted from S1, S2 and S3 samples showed antibacterial activity against Gram negative bacteria *E. coli* ATCC 25922 by 7mm and 11mm and *E. coli*. by 13mm, 6mm and 9mm of zone of inhibition respectively. Similarly, bacteriocin like compound extracted from C1 and C2 showed inhibitory action against Gram positive bacteria *Bacillus* species by 15mm and 13mm of zone of inhibition respectively and Gram negative bacteria *Escherichia coli* ATCC 25922 by 12mm and 10mm of zone

of inhibition respectively and *Escherichia coli* by 20mm and 16mm of zone of inhibition respectively. From *Gundruk* samples, G1 and G2 showed antibacterial activity against Gram positive bacteria *S. aureus* and *Bacillus* species by 7mm and 15mm of zone of inhibition respectively and Gram negative bacteria *Escherichia coli* and *Escherichia coli* ATCC 25922 by 17mm and 15mm of zone of inhibition respectively. Similarly, Collins, et al., (1983) reported that the antibacterial activity of LAB strains against *S. aureus* and *P. fragi*. Elayaraja, et al., (2014) showed bacteriocin as inhibition activity against pathogens and concluded that LAB showed antimicrobial activities of wide range.

Further, bacteriocin like compound extracted from LAB isolates of farm soil (S_1, S_2 and S_3), curd (C_1 and C_2) and *Gundruk* (G_1 and G_2) were mixed (1:1) to analyze the inhibitory activity. The bacteriocin extracted from farm soil was able to inhibit *E. coli* ATCC 25922 (11mm), *E. coli* (15mm), *K. pneumoniae* (11mm) and *Bacillus* species (10mm) with respect to the diameter of inhibition zone. The bacteriocin extracted from curd was able to inhibit *E. coli* ATCC 25922 (19mm), *E. coli* (20mm), *P. aeruginosa* (17mm), *K. pneumoniae* (10mm) and *Bacillus* species (20mm) with respect to the diameter of inhibition zone. The bacteriocin extracted from *Gundruk* was able to inhibit *S. aureus* (10mm), *E. coli* ATCC 25922 (11mm), *E. coli* (15mm), *P. aeruginosa* (18mm), *K. pneumoniae* (11mm), and *Bacillus* species (10mm) with respect to the diameter of the inhibition zone. However, the bacteriocin like compound extracted from LAB isolates of *Gundruk*, curd and farm soil samples in mixed ratio showed the higher zone of inhibition. Bacteriocins like compound extracted from these isolates exhibited varying antibacterial activity, in which bacteriocins like compound extracted from *Gundruk* samples showed the strongest effects.

Combining bacteriocins like compound from different sources in a mixed ratio (1:1:1) increased their antibacterial spectrum, affecting the highest number of test bacteria. However, bacteriocin extracted from all samples did not showed zone of inhibition to *S. aureus* ATCC 43300. Similar result was reported by Sharma et al., (2021), the antimicrobial activity of bacteriocin like compound against *Bacillus* spp, *Shigella* spp and *E. coli* and but didn't show inhibition to *Salmonella* spp, *S. aureus* and *K. pneumoniae*. Perez, et al., (2014) concluded that bacteriocins like compound also use as a next generation antibiotics for inhibiting the multi drug resistant bacteria. However, this study showed antibacterial activity of bacteriocin like compound in limitation number of samples. So, further study can be done in different potential species for the development of novel antibiotic.

CONCLUSION

Diverse species of lactic acid bacteria were isolated from farm soil, curd and *Gundruk* samples however, very few species were able to produce bacteriocin. Bacteriocin extracted from them showed different zone of inhibition to all test bacteria. Overall, this study highlight LAB producing natural antimicrobial agents. So, in this today's world, research on antimicrobial activity of bacteriocin like compound extracted from LAB become a great importance for next generation antibiotics.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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ORIGINAL RESEARCH

Water Quality Assessment: Physicochemical Properties and Microbial Characteristics of Water Across Different Sources

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ABSTRACT

Objectives: To assess the quality of drinking water microbiologically as well as to monitor various physicochemical quality parameters.

Methods: The study was a laboratory-based cross-sectional study. Thirty samples of water were taken without contamination from different sources such as stone spouts, boring wells, pumps, dug wells, tap water and jar water. The samples were transported with a cold chain maintained and analyzed promptly. Physicochemical parameters were identified using methods indicated in APHA (2005). Spread plate technique and membrane filtration technique were conducted for total bacterial load count and total coliform load count respectively. Bacterial pathogens were isolated and identified through selective enrichment and culture on specific media as well as using biochemical characterstics. Kirby-Bauer disk diffusion technique was used to determine the antibiotic susceptibility pattern of the isolates which revealed varying rates of resistance among the isolates with some of them having multi-drug resistance.

Results: The findings indicated that the highest bacteria loads were on pump (1.43 $\times 10^6$ cfu/ml) and stone spout water (1.02 $\times 10^6$cfu/ml), whereas, jar water was not contaminated. In stone spout water counts of coliform were recorded at (70 cfu /ml).

Conclusion: The most frequently isolated pathogens were *E. coli* and *Klebsiella* spp Isolates showed resistance to amoxicillin which could mean there are threats to health and therefore better monitoring and treatments of water quality should be considered.

Keywords: Drinking water quality, physicochemical parameter, Coliform, AST, Kathmandu.

INTRODUCTION

World Health Organization (World Health Organization, 2022a) indicates that pollution of drinking water is responsible for 80% of disease and sickness around world. A study conducted by (Koju et al., 2015) in different water samples in Kathmandu Valley reported 80% of the total water samples to be contaminated with coliforms. In the same study, physicochemical parameters such as pH, conductivity, hardness, turbidity were above WHO permissible limit. Additionally, a study conducted by (Shakya et al., 2013) reported 61.4% distribution of coliforms . *E. Coli* was the most predominant pathogen isolated. Additionally, a study in Tokha, Kathmandu by (Shidiki et al., 2017) reported coliforms in 100% of water samples tested and that exceeding WHO standards.

A study in Bangladesh (Shamimuzzaman et al., 2019) also reported coliforms in 76.25% of the total water sample tested. So this study was conducted with an aim to compare various physiochemical and microbiological aspect of water which has utmost relevance to the context of Kathmandu Valley.

METHODS

Study type: A cross sectional study was conducted between April 2025 to June 2025. The study was carried

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out in the Microbiology Laboratory of Microbiological Research Organization (MiRON), Tinkune, Kathmandu.

Sample type and Size: 30 drinking water samples that consisted of stone spouts (7), Boring water (9), Hand pumps water (3), Tap water (3) and Jar water (3) were collected. Tap water samples, boring water samples and stone sprout samples were directly collected from the source in sterile Biological Oxygen Demand (BOD) bottles.

Sample Collection: For sample collection from well the BOD bottles were immersed inside the well and water sample was collected.

Physiochemical Analysis: pH and temperature was measured on site using standard calibrated pHmeter and thermometer. The samples were transported back to the laboratory using ice box. The conductivity of the water samples were observed using conductivitymeter. Turbidity was measured using nephelometer. Total hardness, ammonia, total iron were calculated using techniques as mentioned in (APHA (2005) Standard Methods for the Examination of Water and Wastewater. 21st Edition, American Public Health Association/American Water Works Association/ Water Environment Federation, Washington DC. - References - Scientific Research Publishing, n.d.)

Microbiological analysis: Enumeration of total bacterial load was carried out using spread plate technique on Plate Count Agar. Total Yeast and Mold Count was carried out using pour plate technique on Potato Dextrose Agar (PDA). Total coliform load was enumerated using pour plate technique on Violet Red Bile Agar (VRBA). For isolation of gram negative organism a loopful of water sample was streaked on McConkey Agar (MA). The McConkey Agar plate was incubated at 37 degree centigrade for 24 hours. The colonies such obtained were subcultured in Nutrient Agar and incubated as mentioned for MA. The isolated were identified using Gram Staining, Biochemical tests such as indole test, Methyl Red test, Voges Prosakaeur test, Citrate utilization test, Triple Sugar Iron agar test, Oxidative Fermantative test, catalase test, oxidase test and urease test. *Staphylococcus aureus* was selectively isolated using Mannitol salt Agar, *Pseudomonas aeuroginosa* was selectively isolated using cetrimide agar.

Antibiotic susceptibility testing: Antibiotic susceptibility testing of the isolates were carried out using Kirby Baeur disc diffusion technique following (CLSI, 2023).

RESULTS

A total 30 water samples were collected across a variety of sources. Boring water 9 (30%), Stone Spout 7 23%), Well 4 (14%), Jar 3 (10%), pump 4 (13%) and, public Tap water 3 (10%)

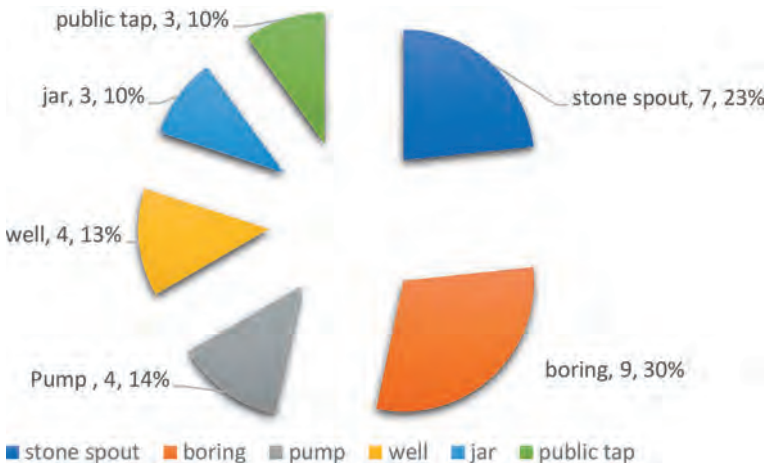

Figure: Distribution of water samples from various sources

Among the collected samples, the highest coliform load was seen on stones spout water samples (70) and least coliform load was seen on jar water (0).

Table 1: Distribution of coliform load in different water samples

S.N.	Types of samples	Total number of samples	Median of coliform load cfu\ml
1	Boring	9	50
2	Stones spout	7	70
3	Well	4	60.5
4	Pump	4	55
5	Tap water	3	16
6	jar	3	0

A total of 65 bacterial isolates were identified from different water sources, including stones spout, boring, well, pump, tap, and jar water samples. Among these, *Escherichia coli* (*E. coli*) which were the isolated species most frequently, 21.5% of the total bacteria detected. This was followed by *Klebsiella* (15.4%) and *Salmonella* (12.3%). Other bacteria such as *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Proteus*, *Vibrio* spp, and *Shigella* were also present, with percentages ranging from (3.1) to (10.8%). The highest number of bacterial isolates was found in boring water (19 isolates), followed by pump water (16 isolates) and well water (12 isolates). Stones spout and tap water had lower bacterial counts, with 11 and 8 isolates respectively. *E. coli* has been considered to be of high health significance and moderate persistence (World Health Organization, 2022b). No bacterial isolates were found in jar water samples. This distribution indicates that water from different sources varies in bacterial contamination, with some pathogens more prevalent in certain types of water sources.

Table 2: Distribution of bacterial pathogens based upon sources of water.

Bacterial species	Stones spout n=(7)	Boring n=(9)	Well n=(4)	Pump n =(4)	tap n =(3)	Jar n=(3)	Total	Percentage (%)
E coli	3	5	2	3	1	0	14	21.5
Pseudomonas aeruginosa	2	1	1	1	1	0	6	9.2
Klebsiella spp	1	3	2	3	2	0	10	15.4
Salmonella spp	1	2	2	1	2	0	8	12.3
Shigella spp	0	1	0	1	0	0	2	3.1
Citrobacter spp	2	1	1	2	1	0	7	10.8
Enterobacter spp	1	1	1	2	1	0	6	9.2
Vibrio spp	0	2	2	1	0	0	5	7.7
Proteus spp	1	3	1	2	0	0	7	10.8
TOTAL	**11**	**19**	**12**	**16**	**8**	**0**	**65**	**100**

The comparative physicochemical analysis reveals that while pH, conductivity, and hardness generally complied with National Drinking Water Quality Standards (NDWQS) across all sources, iron and turbidity levels frequently exceeded safe limits. Boring water and stone spouts exhibited significant turbidity (mean > 4.7 NTU) and iron contamination (mean > 0.5 mg/L), indicative of geogenic leaching and surface runoff. In contrast, the unexpectedly high iron content in commercial jar water (2.03 mg/L) suggests critical failures in processing or storage. Additionally, the detection of elevated ammonia (mean 1.1 mg/l) in tap water serves as a concerning marker for potential sewage infiltration within municipal distribution lines. Collectively, these deviations highlight that despite acceptable mineral balance, the widespread presence of suspended solids and metallic impurities requires robust point-of-use filtration to mitigate aesthetic and health risks.

Table 3: Comparative Assessment of Physicochemical Parameters by Water Source

Parameters	NWQDS Limit	Stone Spout (Mean ± SD)	Boring (Mean ± SD)	Well (Mean ± SD)	Pump (Mean ± SD)	Tap (Mean ± SD)	Jar (Mean ± SD)
Temperature (˚C)	—	22.9	24.1	26.8	26.3	26.4	26.9
pH	6.5 – 8.5	6.57	6.64	6.65	6.63	6.81	6.87
Conductivity (µS/cm)	1500	59.1	103.2	114.8	114.3	155.7	64.3
TDS (mg/l)	1000	36.9	64.5	71.7	71.4	97.3	40.2
Chloride (mg/l)	250	16.7	64.7	28.9	18.0	21.7	30.0
Hardness (mg/l)	500	108	114	226	220	153	184
Iron (mg/l)	0.3	0.52*	0.96*	0.79*	0.51*	0.71*	2.03*
Turbidity (NTU)	5 (10)	4.7	6.8*	6.8	0.8	5.3	6.0
Ammonia (mg/l)	1.5	ND	ND	0.34	0.60	1.1*	ND

ND: Not Detected *: Higher than Limit Value

The water samples collected at higher temperatures (26–30°C) showed a significantly higher median coliform load (72 cfu/ml) compared to samples collected at lower temperatures (20–25°C), This indicates that increased temperature might be associated with elevated microbial contamination in the water sample.

Table 4: Distribution of total coliforms based upon temperature.

S.N	Temperature Range	Total No of samples	Median of coliform load (cfu/ml)
1	20-25	12	35.5
2	26-30	18	72

Among the 30 water samples analyzed, those with pH values within the WHO recommended range of 6.5 to 8.5 (Nepal Standard 2062) (covering pH groups 6-7 and 7-8) showed and a median of coliform load between 46 and 59 cfu/ml, indicating moderate microbial contamination within acceptable pH levels.

Table 5: Distribution of bacterial load and coliform load with respect to pH

S.N.	pH range	Total No. of samples	Median of coliform load (cfu/ml)
1	5-6	1	35
2	6-7	22	59
3	7-8	7	46

Out of 10 isolates of *Klebsiella* spp Ciprofloxacin (CIP) and Gentamicin (GE)were found to be sensitive against *Klebsiella* spp (6) isolates were found to be Sensitive to Cotrimoxazole (COT), 4 isolates were found to be resistant to Cotrimoxazole 7 isolates were found to be resistant to Tetracycline (TE) *Klebsiella* spp (10) were found to be resistant against Amoxicillin.Out of 6 isolates *Pseudomonas aeruginosa*, Amoxicillin was found to be not effective against *Pseudomonas aeruginosa,* 5 isolates were found to be sensitive to Gentamicin. *E coli* was found to be sensitive to Gentamicin and cotrimoxazole.

Table 6: Antibiotic Susceptibility Pattern of *Klebsiella* spp and *Pseudomonas aeruginosa*

Antibiotics	*Klebsiella* spp (n=10)		*Pseudomonas aeruginosa* (n=6)		*E. coli* (n=14)	
	S	R	S	R	S	R
Amoxicillin (AX)	0	10	0	6	4	10
Ciprofloxacin (CIP)	10	0	2	4	9	5
Gentamicin (GEN)	10	0	5	1	14	0
Tetracycline (TE)	3	7	2	4	8	6
Cotrimoxazole (COT)	6	4	5	1	10	4

DISCUSSION

The water samples collected from Kathmandu showed varied reliance on sources, with boring water (30%) and stone spouts (23%) being most common. This reflects the city's dependence on alternative supplies due to irregular municipal water distribution (Pandey et al., 2020). However, studies indicate that both groundwater and traditional sources are often contaminated by sewage leakage and poor protection measures (Shrestha et al., 2014; Khadka and Pathak 2016). The presence of jar, well, pump, and public tap water in smaller proportions highlights mixed usage patterns, each with specific contamination risks (Shrestha et al., 2017). These findings helps to keep emphasize the requirement of regular monitoring and improved water management to ensure safe drinking water in Kathmandu.

The distribution of bacterial load across water sources revealed that pump and stones spout water had the highest microbial contamination, with mean bacterial loads of 1.43×10^6 cfu/ml and 1.02×10^6 cfu/ml, respectively. These values exceed the recommended limits for safe drinking water by WHO, which suggest zero detectable fecal coliforms and very low heterotrophic plate counts to minimize health risks (WHO, 2017). Elevated bacterial counts in these sources may result from inadequate protection of water sources, surface runoff contamination, and poor sanitation infrastructure around the collection sites (Jahn et al., 2009).

Boring water, often sourced from deeper underground aquifers, showed significantly lower bacterial contamination (7.52×10^1 cfu/ml), consistent with the natural filtration provided by soil and rock strata. This aligns with previous findings that deep groundwater sources generally have better microbiological quality than surface or shallow water sources (Ashbolt 2004). Conversely, jar water samples showed no detectable bacterial contamination, likely reflecting post-collection treatment or proper storage conditions, corroborating studies that highlight the importance of water handling

and storage in maintaining microbiological safety (Sobsey 2002).

The coliform bacteria where it is present means of critical indicator of fecal contamination and potential presence of pathogenic microorganisms (Edberg et al., 2000). The highest mean coliform load was found in stones spout water samples (73.71 cfu/ml), indicating substantial fecal contamination, possibly from nearby latrines, open defecation, or animal waste (Levy et al., 2012). Jar water, again, showed no coliform contamination, reinforcing the protective effect of proper storage or treatment.

The presence of coliform bacteria in public tap water (mean 18.67 cfu/ml) raises concerns regarding the efficiency of municipal water treatment and potential contamination in distribution systems. Leaks, backflow, and biofilm formation in pipes can contribute to such contamination even after treatment (Momba and Kaleni 2002).

Water temperature was found to significantly influence microbial loads, with samples collected at 26–30°C showing higher bacterial and coliform counts than those at 20–25°C. Higher temperatures facilitate bacterial replication and increase metabolic activity, leading to accelerated microbial growth (LeChevallier and Au 2004). Seasonal fluctuations in temperature can thus exacerbate water contamination risks during warmer months, emphasizing the need for heightened surveillance and preventive measures in such periods (Borchardt et al., 2003).

Water samples with pH within the recommended range of 6.5–8.5 showed moderate bacterial and coliform contamination. While pH affects microbial survival and chemical stability of water, it is not the sole determinant of microbiological quality (WHO 2017).

Escherichia coli was the most frequently isolated species, accounting for 21.5% of bacterial isolates, confirming the presence of fecal contamination and indicating a high risk for waterborne diseases (WHO, 2017). Other pathogens including Klebsiella, Salmonella, and Pseudomonas were also identified, posing additional health risks ranging from gastrointestinal infections to opportunistic infections in immunocompromised individuals (Momba and Kaleni 2002). The presence of these bacteria in multiple water sources underscores widespread contamination and the need for improved water sanitation and hygiene practices.

The absence of bacterial isolates in jar water samples indicates that water treatment or storage practices may significantly reduce microbial load, supporting interventions focused on household water treatment and safe storage as effective public health measures (Clasen, et al., 2007).

The antibiotic susceptibility patterns revealed multidrug resistance among isolates, particularly *Klebsiella* spp These isolates showed resistance to amoxicillin and tetracycline but remained sensitive to ciprofloxacin and gentamicin. Similarly, *Pseudomonas* spp was resistant to amoxicillin, with variable susceptibility to other antibiotics.

The emergence of antibiotic-resistant bacteria in environmental water sources is an increasing concern globally, as these bacteria can act as reservoirs for resistance genes that may transfer to human pathogens, complicating infection treatment (Berendonk et al., 2015). Kathmandu's water sources, thus, present potential public health risks not only from pathogenic contamination but also from the propagation of antimicrobial resistance.

The findings demonstrate that several water sources in Kathmandu do not meet microbiological safety standards, putting the population at risk of waterborne diseases. The high bacterial and

coliform counts in pump and stones spout water highlight the urgency of source protection measures, including sanitary inspections, fencing, and community education on water handling.

Furthermore, the presence of antibiotic-resistant bacteria stresses the need for surveillance systems to monitor resistance patterns and implement antibiotic stewardship programs.

CONCLUSION

This study confirms that a significant portion of Kathmandu Valley's water sources violate WHO and national standards. The presence of pathogens like *Salmonella* and *E. coli* in traditional spouts and wells exposes a critical lack of source protection. Most alarmingly, the detection of antibiotic-resistant strains identifies these water bodies as active environmental reservoirs for antimicrobial resistance (AMR). This poses a dual threat: an immediate risk of waterborne epidemics and a long-term crisis of untreatable clinical infections. Consequently, urgent remediation is vital to prevent these community water sources from accelerating the spread of drug-resistant pathogens.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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ORIGINAL RESEARCH

Detection of Antimicrobial Activities and Bacteriocin Encoding Genes in Lactic Acid Bacteria Isolated from Fermented Foods in Kirtipur

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ABSTRACT

Objectives: The objective of this study was to isolate and identify Lactic Acid Bacteria (LAB) from fermented foods and evaluate their antimicrobial activities as well as to detect bacteriocin encoding gene and also determine probiotic effect of selective LAB isolates.

Methods: A total of 26 fermented food samples were collected from different small scale dairy shops and community of Kirtipur, Kathmandu and LAB were isolated following standard plate count method. Preliminary identification of isolates was done by staining and biochemical tests. The genes encoding specific bacteriocin were detected using polymerase chain reaction.

Results: Based on the morphological, microscopy and biochemical characteristics, a total of 40 LAB were screened; among them 25 (62.5%) were Gram-positive rod shape LAB A (*Lactobacillus* spp) type and 15 (37.5%) isolates were gram-positive cocci shape LAB B type. Among 40 isolates of Lactic Acid Bacteria, cell free supernatant of 24 (60%) showed antibacterial activity with highest being that of isolates Ca3 and Ca4 LAB against all 6 test bacteria. Isolated LAB were most effective against *Pseudomonas aeruginosa* i.e. out of 24 LAB 20 (83.33%) isolates showed inhibition zone. Enterocin gene was detected in 4 (16.66%) LAB isolates while nisin and pediocin genes were not detected in isolated LAB. All 9 selected LAB isolates were able to resist high acidic condition at pH 3 till 24 hrs, 0.5% and 1% bile salt concentration while isolates C3 (*Enterococcus* spp) and Ca3 (*Lactobacillus* spp) were able to resist pH 3 till 48 hrs of incubation. Therefore, they can survive in stomach high acidic and bile condition indicating their probiotic potentiality.

Conclusion: These findings emphasize LAB's potential as natural antibacterial agents that could be exploited in food preservation and as probiotics to improve human health.

Keywords: LAB, Antibacterial activity, Bacteriocin, Probiotics, Fermented foods

INTRODUCTION

Fermentation of food is one of the oldest processing and preserving methods used all around the world. Varieties of fermented foods has been included in Nepali diet from prehistory. Most commonly used fermented foods are *Dahi, Gundruk, Achaar, Kinema*, although uses of fermented foods vary according to community. These fermented foods harbor wide variety of microorganisms including LAB. LAB is employed for preservation of fermented foods. LAB mainly produce lactic acid as their by-product during their metabolic activities. They play important role in agricultural and

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clinical sectors and also possess therapeutic properties that are vital for human health enhancement (Tamang et al., 2005).

Lactic Acid Bacteria produces different bacteriocins which can be used for the preservation of food and is also safer than other antimicrobials (Clevland et al., 2001). Bacteriocin are ribosomally synthesized, by diverse bacterial groups including LAB, which is biologically antimicrobial in nature, while chemical structure is composed of peptides and proteins (Cotter et al., 2005). Bacteriocins produced by LAB are considerably safe, that's why gaining more attraction on different application. LAB bacteriocin inhibits the growth of closely related bacteria at low concentration in nanomolar level with no side effects on public health which leads to less chances of development of antimicrobial resistance. Bacteriocin acts against the target cell membrane by making a quick pore at very low concentration. Due to its degradable nature in human digestive system, it cannot remain in outer environment also (Zendo et al., 2013).

Probiotics are live microbial food supplements that beneficially affect the host by improving intestinal microbial balance. According to the FAO/WHO (2002), probiotics are defined as "live

microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Most probiotics are bacteria, primarily LAB, though some yeasts (e.g., *Saccharomyces* spp) and molds (e.g., *Aspergillus* spp) are also used. Common probiotic genera include *Lactobacillus, Bifidobacterium, Lactococcus, Enterococcus*, and *Bacillus* (Peng et al., 2022).

The current study has been designed to detect and characterize the anti-bacterial activities, probiotic activities, presence of bacteriocin encoding genes, in lactic acid bacteria isolated from fermented foods used in Nepal.

METHODS

Samples

A total of 26 fermented food (13 fermented dairy product and 13 fermented vegetable products) were collected from Kirtipur from July 2024 to December 2024. Fermented dairy products included yoghurt and cheese while fermented vegetables product included raddish pickle, *Tama*, and *Gundruk*. All of the samples were selected and collected following convenience sampling method, in sterile zip-locked bag and were brought to Central Department of Microbiology, TU, Kirtipur, within 3 hrsof collection, maintaining reverse cold chain temperature (2-8^{0}C). Then samples were further processed for isolation of the lactic acid producing bacteria (LAB) (Bhattrai et al., 2016).

Isolation of LAB

For isolation of LAB, 1gm of each sample (*Gundruk, Tama*, raddish pickle, Yogurt and Cheese) was added to 9ml of phosphate buffer saline to make 1/10 dilution and mixed properly. The 1/10 dilution was then serially diluted up to 10^{-6} dilution. The samples from 10^{-2}, 10^{-4}, 10^{-6} dilution were pipetted out in the Petriplate. Then, de Man, Rogosa and Sharpe (MRS) media incorporated with 1% calcium carbonate was poured properly and incubated at 37^{0}C for 48 h. The isolated colonies with clear zone were sub-cultured on MRS agar.

Preliminary identification was done based on colony morphology on MRS agar, Gram-staining and spore staining. Further confirmation of the isolates were done based on biochemical test (Catalase test, oxidase test, motility test, MR-VP test, TSIA test and citrate utilization tests), sugar fermentation (Glucose, Lactose, Maltose and Sucrose) test results, growth at different range of pH and temperature.

Extraction of cell free supernatant from LAB isolates

The LAB isolates were grown in MRS broth for 48 h at 37^{0}C. Then centrifugation of broth was done at 8000 rpm for 10 minutes. The obtained cell free supernatant (CFS) was further processed for screening of antimicrobial activity.

Antimicrobial activity of cell free supernatant of LAB isolates against test organisms

Five ATCC cultures including *P. aeruginosa* 49619, *S. aureus* 29213, *S. aureus* 43300, *E. coli* 25922, *Klebsiella* spp 700603 and one food borne pathogen *Salmonella* spp isolated from meat sample in the department were used as test organisms for determination of the antibacterial activity of the cell free supernatant from the isolated LAB. Briefly, wells were made on Muller Hinton Agar (MHA) media with the cork-borer of 6mm diameter and test organisms were carpet cultured on MHA. Cell free LAB extract (50 µl) and control (PBS) were loaded on the well and allowed to diffuse at room temperature

for 30 minutes. The loaded plates were incubated at 37^{0}C for 24 hrs and zone of inhibition were observed and recorded (Abdel Tawab et al., 2023).

Probiotic test of selective isolates

Selective isolates with inhibition zones (larger than 16 mm) against the standard ATCC test organisms (*P. aeruginosa* 49619, *S. aureus* 29213, *S. aureus* 43300, *E. coli* 25922, *Klebsiella* spp 700603) were further tested for four different probiotic activities including bile salt tolerance, sodium chloride (NaCl) tolerance, growth on different range of pH and temperature. For bile salt and NaCl tolerance test, total of 9 selected isolates were grown in MRS broth incorporating different concentration (0.5%, 1%, 1.5% and 2% of bile salt) for bile salt tolerance test; and 2% and 4% for NaCl tolerance test; Optical Density (OD) of the incubated tubes were measured using ELISA reader after 24 hrs of incubation at 37^{0}C.

For pH test two acidic pH 3 and 4.4 of MRS broth were adjusted by using Hydrochloric acid (HCl), Then isolates were inoculated in different pH adjusted MRS broth and OD were measured at different incubation time i.e. 0 hr, 24 hrs and 48 hrs (Khushboo et al., 2023). Similarly for temperature, isolates in MRS broth were incubated at different temperature i.e. 28^{0}C, 37^{0}C and 45^{0}C and OD were measured after 24 hrs of incubation at 37^{0}C.

Detection of bacteriocin gene by polymerase chain reaction (PCR)

Bacterial DNA extraction

LAB which showed inhibitory effect against test organism were only proceeded for molecular detection of selected bacteriocin gene. Fresh bacterial LAB culture was used to extract bacterial DNA by phenol-chloroform method. Quantification of Bacterial DNA and quality assessment were performed using nanodrop (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Conventional Polymerase Chain reaction (PCR) to detect Bacteriocin gene

Following the manufacturer's instruction, PCR was used to detect the presence of bacteriocin encoding gene. Briefly, 2 µL of DNA template, 5 µL master-mix (Solis BioDyne), 0.5 µL each of forward and reverse primers, and 17 µL nuclease-free water were added making total volume of reaction mixture 25 µL. Three different reverse and forward primers for Pediocin, Enterocin and Nisin were used to detect bacteriocin gene. The following steps were part of PCR program: 30 cycles of 94^{o}C for 5 min, 94^{o}C for 1 min, 55^{o}C for 30 Sec, 72^{o}C for 45 Sec followed by 72^{o}C for 6 min (Fuente-salcido et al., 2015).

Table 1. Primers used for PCR

Target gene	Primer sequence	PCR product size (bp)	Reference
Pediocin (*ped*)	Forward 5'-GGTAAGGCTACCACTTGCAT-3' Reverse 5'-GGGTACCACTCATAGTGGAA-3	332	Suwanjinda et al., 2007
Enterocin (*entA*)	Forward 5'-GGGTACCACTCATAGTGGAA-3' Reverse 5'-CCAGCAGTTCTTCCAATTTCA-3'	412	
Nisin (*nisR*)	Forward 5'-CTATGAAGTTGCGACGCATCA-3' Reverse 5'-CATGCCACTGATACCCAAGT-3'	608	

Analysis of PCR Results

Amplified PCR products were run on 1.5% agarose gel and the bands were observed on Gel documentation system.

RESULTS

Isolation and Identification of lactic acid bacteria

From 26 samples comprising fermented dairy and fermented vegetable product, 40 LAB isolates were screened based on clear zone around colonies. Among them 25 (62.5%) isolates were gram-positive, non-spore forming rod shaped (LAB A) type and 15 (37.5%) isolates were gram-positive cocci shaped (LAB B) Type. These bacteria fermented all four sugars used and only LAB B type shown growth at 45 ^{o}C.

Distribution of Lactic Acid Bacteria according to samples type

Out of 26 samples collected, 24 samples showed presence of LAB. Among 40 LAB isolates 21(52.5%) were screened from fermented vegetables while 19 (47.5%) were from fermented dairy products ($p > 0.05$).

Distribution of Lactic Acid Bacteria according to sample age

Among 40 LAB isolates, 12 (30%) LAB isolates were from 1-3 days old samples, 15 (37.5%) LAB isolates were from 1-3 weeks old sample and 13 (32.5%) LAB isolates were from more than one month old samples

($p > 0.05$).

Antibacterial activities of cell free extract of LAB isolates against test organisms

Out of total 40 LAB isolates, extract from only 24 (60%) isolates showed inhibition zone against different test organisms. The zone of inhibition ranged between 7 to 28 mm in diameter with maximum being shown by extract from isolate T2. Among these 24 isolates 16 were rod and 8 were cocci.

Probiotic attributes of isolated LAB

Acid tolerance test

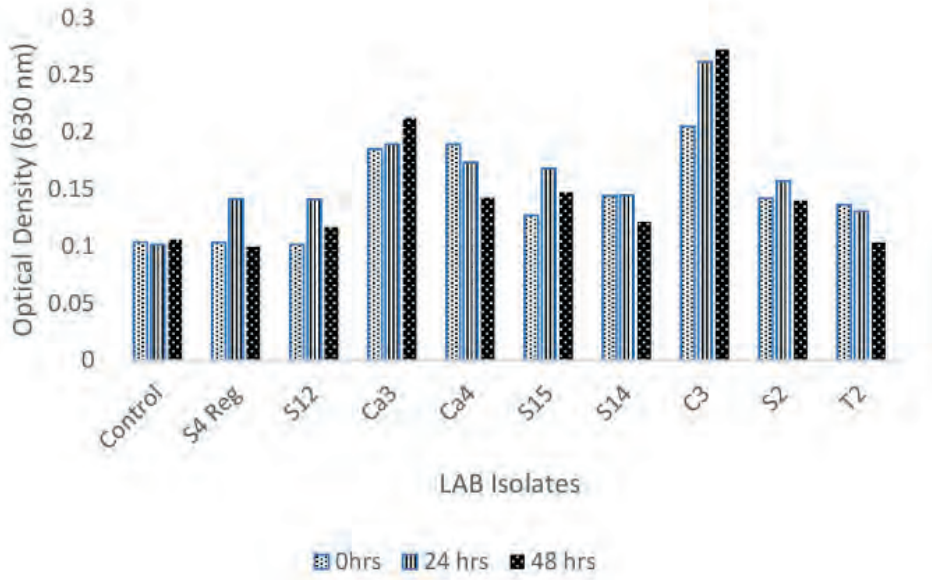

Figure 1: Acid tolerance test of LAB isolates at pH 3

From 24 LAB isolates with inhibitory effect on test organisms, only 9 LAB isolates were selected for probiotic potentiality based on spectrum of antibacterial activity. Two isolates Ca3 and C3 showed increment in OD value at pH 3 till 48 h of incubation.

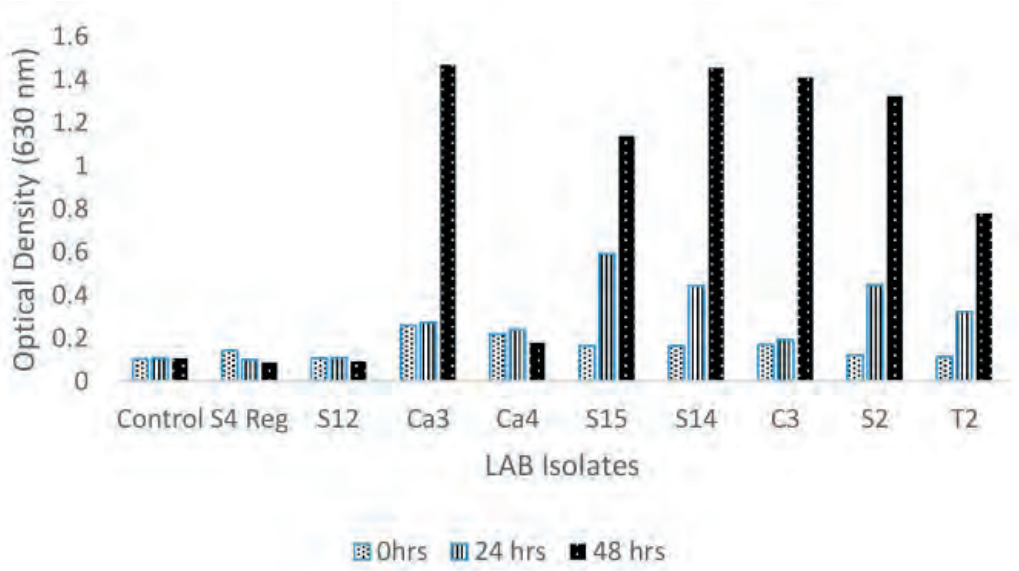

Figure 2: Acid tolerance test of LAB isolates at pH 4.4

In figure 2, LAB isolates Ca3, S15, S14, C3 and T2 showed luxurious growth till 48 h of incubation indicating their potential to tolerate low pH. While Isolates S4 Reg and S12 were unable to resist ph4.4

Bile salt tolerance test

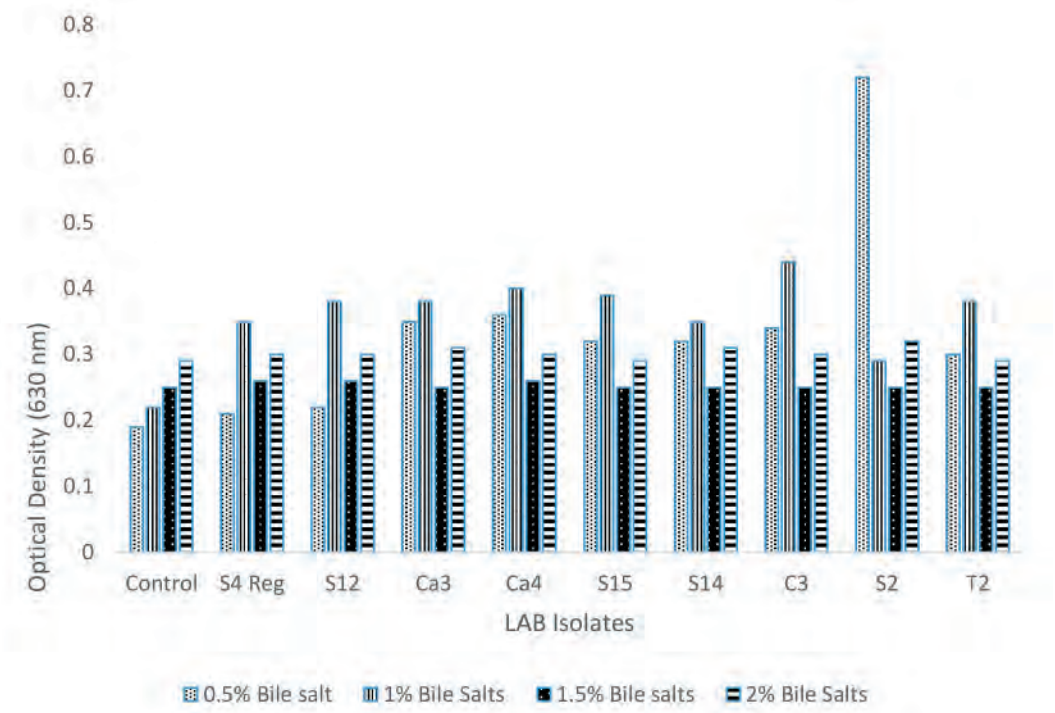

Figure 3: Bile salt tolerance test of LAB Isolates at different concentration of bile salt

Fig. 3 shows the bile salt tolerance pattern of LAB isolates after 24 h of incubation. The results from above figure clearly indicated that all of the 9 LAB isolates survived upto 0.5% and 1% bile bile salt concentration while are unable to survive in higher concentration (1.5% and 2%) of bile salts.

Growth pattern of isolated LAB at various temperature

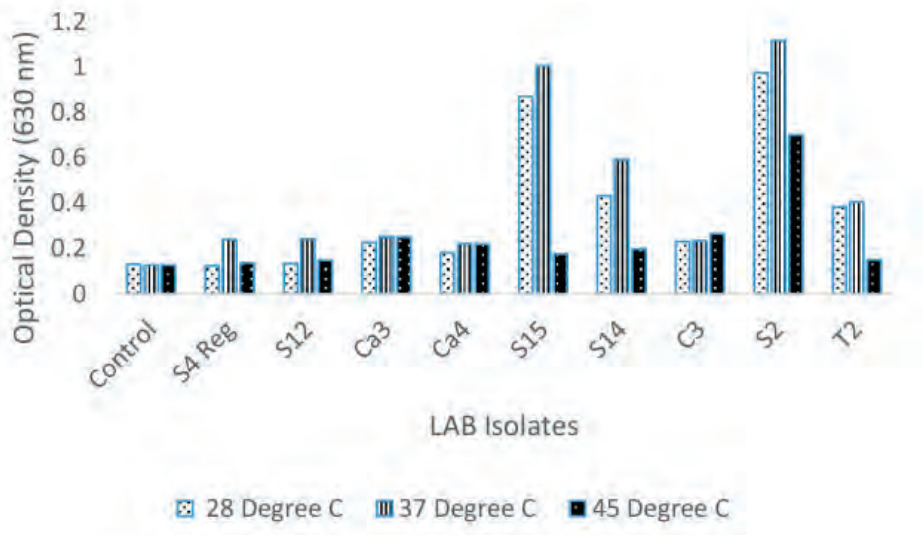

Figure 4: Growth pattern of LAB Isolates at different temperature after 24 h incubation

Figure 4 indicates growth pattern of LAB isolates at three temperature i.e. 28 °C, 37 °C and 45 °C. Most of the isolates showed optimum growth at 37 °C whereas growth of isolate S14, S15 and S2 favored at 28 °C while only isolate C3 showed optimum growth at 45 °C.

Salt (Sodium Chloride) tolerance test

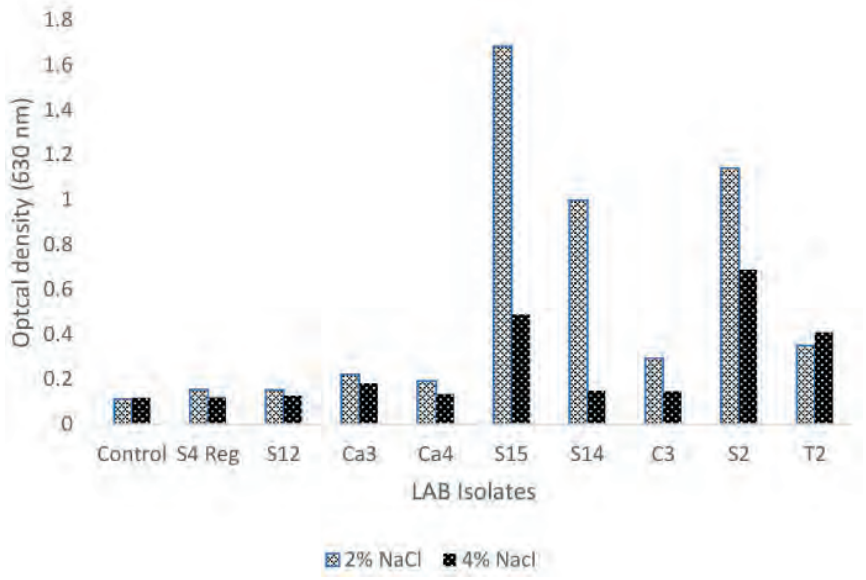

Figure 5. Salt tolerance test at different concentration of NaCl

Figure 5 represents tolerance pattern of isolates towards different concentration of NaCl. Among 9 Isolates, all of the isolates were able to grow at provided salt concentration. Whereas isolate S15, S14 and S2 depicted increment in OD value.

Detection of Bacteriocin Encoding gene (Enterocin, Nisin and Pediocin)

Among 24 isolates of LAB which showed antibacterial properties against test organisms; only 4 (16.66%) isolates showed positive result for enterocin gene. Other two bacteriocin encoding gene i. e. Nisin and Pediocin were not detected in isolates. Enterocin gene were detected in four LAB isolates i.e S_1 1.3, S_{13} 1.2, C3 and Ga.

DISCUSSION

This study aimed to screen LAB from fermented foods with antibacterial activity against proven pathogenic bacteria and detecting selected genes in potent isolates responsible for bacteriocin production. Furthermore, it also investigated on some of the probiotic properties of selected isolates. In total 26 samples were collected; only 24 samples showed presence of Lactic Acid Bacteria. For preliminary identification of isolates; Gram's staining, spore staining, biochemical test and sugar fermentation pattern were done. Among 40 isolates, 25 (62.5%) isolates were Gram positive, non-sporing rod, catalase negative, oxidase negative, and fermented all four sugars tested hence identified as *Lactobacillus* spp The remaining (37.5%) isolates were Gram positive, cocci in short chain, catalase and oxidase negative and fermented all of the four sugars used and shown growth at 45°C. The study conducted in fermented dairy products by Refay et al., 2020 reported 51(75%) rod-shaped, whereas 17 isolates (25%) were cocci shaped LAB.

Among 40 isolates of LAB, cell free supernatant of 24 (60%) showed antibacterial effect against test organisms. The CFS from some isolates i.e Ca3 and Ca4 showed inhibitory effect against all test organisms including *salmonella* spp isolated from meat. Most of the LAB isolates showed antibacterial activity against both Gram positive and Gram-negative bacteria used as test organism. The highest zone of inhibition (28 mm in diameter) was shown by CFS from T2 against *P. aeruginosa* 49619. The study conducted by Adeyemo et al., 2018 was also close to the present study where species of *Lactobacillus* showed inhibition zone against *P. aeruginosa* (20 mm, 15 mm, 19 mm and 4.5 mm) and *S. aureus* (22 mm, 16mm, 19mm and 5 mm). In similar study conducted by Adhikari et a, 2025 revealed the zone of inhibition $\geq$ 15 mm by extract from *Lactobacillus* spp isolated from Nepali fermented foods.

Most probiotics belongs to lactic acid bacteria (LAB). LAB's capacity for fermentation and preservation makes them extremely valuable to the food sector. Probiotic LAB has several health benefits, including modulating the immune system, gut bacteria regulation, antibacterial, cancer fighting, and anti-obesity properties. LAB are utilized as ingredients in functional foods because of their many advantages (Sadiq, 2022).

In present study, 9 LAB isolates with antibacterial activity were selected for screening of probiotic

potential. Probiotic potential was measured through acid and bile tolerance test with varying concentration as these are the major components of gastrointestinal tract hence organisms must resist these hurdles. Out of 9 LAB isolates all isolates resisted pH 3 till 24 hrs, only two isolates (Ca3, C3) resisted pH 3 till 48 hrs. LAB Isolates Ca3 and C3 have potential to be a probiotic as these isolates demonstrated antibacterial activity against test organisms and resist both pH 3 and 4.4 till 48 hrs. Among 9 LAB isolates, all of the candidates survived upto 0.5% and 1% bile salt concentration however some isolates were unable to survive in higher concentration (1.5% and 2%) of bile salts. Among the isolates, 3 were able to survive 2% and 4% NaCl. Amenu and Bacha, 2023 reported that out of 125 possible probiotic LAB isolates, 17 (13.60%) resisted 0.3 % bile salts and low pH 2, 2.5 and 3. A study reported 60 LAB isolates from colostrum of Jordanian camels. *Enterococcus faecium* and *Lactobacillus salivarius* were found to be dominating species. All 60 isolates demonstrated tolerance to varying pH (2,3,4,5,6,7,8,9,10) and additionally, the isolates as a whole exhibited varying bile resistance (%) and tolerance to varying bile salt concentrations (0.2, 0.4, 0.6, 0.8, 1, 2, and 3). Due to the unique characteristics of LAB, they considerably vary level of acid and bile salt tolerance (Pitino et al., 2012).

Antimicrobial-resistant bacteria have been increasingly reported in both clinical and community settings, with multidrug-resistant *Escherichia coli* and other resistant pathogens posing major challenges for effective treatment due to limited therapeutic options (Acharya et al., 2024; Dahal et al., 2025; KC et al., 2019; Pandit et al., 2024; Shrestha et al., 2019; Tiwari et al., 2024). This alarming trend highlights the urgent need to identify and develop novel antibacterial products, such as bacteriocin-producing lactic acid bacteria and other bioactive agents, to counteract resistance and improve public health outcomes.

LAB isolates which showed inhibitory activity against test organisms are subjected to PCR for three bacteriocin encoding gene detection. For bacteriocin encoding gene detection, specific primer sequences used in PCR was taken from as per studies done by Fuente-salcido et al., 2025. In present study 4 (16.66%) LAB isolates showed positive result for enterocin gene in PCR while other two nisin and pediocin gene were not detected in isolated LAB although these isolates demonstrated antibacterial activities against different test bacteria.

CONCLUSION

The purpose of this study was to assess the antibacterial activities and detection of bacteriocin encoding gene in isolated LAB isolated from fermented food. The samples were dominated mostly by *Lactobacillus* spp as compared to cocci. The cell free supernatant of screened isolates demonstrated antimicrobial activities against different test organisms indicating LAB isolated from Nepali fermented foods have potential alternatives as food preservative. Furthermore PCR-based detection confirmed the presence of enterocin encoding gene in 4 LAB isolates. Among 9 selected LAB isolates, all isolates were able to resist high acidic condition of pH 3 while isolates C3 (coccus) and Ca3 (rod) were able to resist pH 3 till 48 hrs of incubations. All 9 selected LAB isolates were able to resist bile salt concentration of 0.5% and 1%. Therefore, they can be able to survive in stomach high acidic and bile condition. These findings emphasize selected LAB's potential as natural antibacterial agents that could be exploited in food preservation and as probiotics to improve human gut health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL RESEARCH

Microbiological Assessment of Selected Vegetables from Kathmandu, Nepal

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ABSTRACT

Objectives: To determine the microbial load and antibiotic susceptibility of bacterial isolates from selected vegetables in Kathmandu.

Method: This study was conducted in the department of microbiology, Tri-Chandra multiple campus from March to July 2025. A total of 30 samples including, moringa, cucumber, spinach, chili, peas, cauliflower, okra, carrot, cabbage, and brinjal, were collected in sterile containers from markets, hotels, and farms and were transported to the laboratory under cold chain. The samples were cultured on Plate Count Agar (PCA), Potato Dextrose Agar (PDA), and Violet Red Bile Agar (VRBA). The PCA and VRBA plates were incubated at 37°C for 24 hours and PDA plates at 28°C for 24-48 hours. Bacterial isolates were identified using Gram staining and various biochemical tests, and antibiotic susceptibility was performed. Fungal presence was detected by using Lactophenol Cotton Blue stain and parasites were identified by Wet Mount method.

Results: Spinach from vendor had the highest mesophilic bacterial load (1.54×10^6CFU/g) and lowest on peas from hotels (1.7×10^3CFU/g). Coliform were highest in vendor cabbage (2.89×10^5CFU/g), while peas consistently showed lowest counts (5×10^2CFU/g) from all sources. Bacteria such as *Pseudomonas* spp, *E. coli*, *Proteus* spp, *Enterobacter* spp, *S. aureus, Klebsiella* spp, and fungi like *Aspergillus* spp, *Fusarium* spp, *Alternaria* spp, *Cladosporium* spp, *Candida* spp, and parasites *G. lamblia*, *E. histolytica*, *Ascaris* were identified. Most of the bacterial isolates were resistant to the antibiotics used, including Ceftriaxone, Cotrimoxazole, Ceftazidime, Amikacin, Erythromycin, and Ciprofloxacin with only few exhibiting susceptibility.

Conclusion: The study revealed a significant presence of pathogens and antibiotic resistant strains highlighting the importance of improved food safety practices, regular microbial monitoring, and public awareness to reduce the risk of foodborne diseases in urban Nepal.

Keywords: Vegetable samples, bacterial count, fungi, parasites, antibiotic susceptibility

INTRODUCTION

Vegetables have a number of benefits to humans and their daily intake has been endorsed as an important aspect of maintaining a healthy life. The World Health Organization (WHO), and the Food and Agriculture Organization (FAO) recommend a daily intake of at least 400 grams of fruits and vegetables to reduce the risk of non-communicable diseases and ensure nutritional wellbeing (WHO and FAO, 2003). Vegetables can serve as vehicles for foodborne pathogens when exposed to unhygienic conditions. Contamination can occur at any stage from farm to the table, including use of untreated irrigation water, manure, processing, distribution, sale and during consumption (Eni et al., 2010), which results

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in foodborne human diseases (Shafa-ul-Haq et al., 2014; Bekele et al., 2017). Pathogenic microbes such as *E. coli*, *Salmonella* spp, *Pseudomonas* spp, *Aspergillus*, *Ascaris* and many others have been frequently associated with fresh production of vegetables. Since vegetables are often consumed raw or sometimes undercooked to preserve vitamin and mineral contents, which may result in various food-borne infections and disease outbreaks like typhoid fever, dysentery, diarrhoea, and cholera (Balali et al., 2020).

Microbial contamination of vegetables has been reported by several researchers inside and outside Nepal. In Brazil, Oliveira et al., (2011) reported that 43% of sampled vegetables were contaminated with fecal coliforms (Oliveira et al., 2011), whereas in Nepal, Sapkota et al., (2019) detected *E. coli* in 13.4% and *Salmonella* in 35.2% of vegetable samples from hotels and restaurants of Bharatpur, Chitwan (Sapkota et al., 2019). The studies conducted in Kathmandu have reported significant microbial contamination in vegetables like spinach, cabbage, tomato, cucumber, and carrots (Ghimire et al., 2020). Vegetables can serve as a major route for the transmission of infections (Rahman et al., 2022). In Saudi Arabia in 2018, consumption of food contaminated with foodborne bacteria resulted in 2,191 cases of illness and 11 deaths (Ministry of Health, 2019). Despite increasing reports of foodborne illness in Nepal, there is inadequate research on fresh produce for microbial contamination. The studies have been focused mostly on total bacterial load or the presence of indicator organisms such as coliforms, rather than identifying specific, disease-causing organisms. The growing global concern over antimicrobial resistance (AMR) has also further complicated the issue as many pathogens have developed resistance to commonly used antibiotics, making treatment and disease management more challenging (Razzaq et al., 2014). The situation is particularly alarming in nations where healthcare infrastructure is strained and advanced diagnostic and treatment options are limited. The prevalence of antibiotic resistance bacteria linked to fresh produce has been well documented and is increasing worldwide (Threlfall et al., 2000).

The importance of this research stems from the real and present threat of foodborne illness. This study aimed to provide an assessment of vegetables from farms, hotels and markets of Kathmandu to detect the presence of foodborne organisms. The study also seeks to address the existing research gaps by isolating and identifying bacteria from selected vegetables in Kathmandu, evaluating their antibiotic susceptibility patterns, and comparing contamination levels between different sources. The results provide deep insight into microbial risks in vegetable consumption and help inform strategies for improved food safety, public health interventions, and policy development in Nepal.

METHODS

Sample collection

A total of 30 fresh vegetable samples were randomly collected from various retail outlets, including local markets, hotels, and vendors across Kathmandu. Each sample was placed in sterile, labeled polyethylene bags and were transported immediately to the microbiology laboratory in the department of microbiology, Tri-Chandra Multiple campus maintaining a cold chain and analyzed.

Sample preparation, enumeration and identification of microorganisms

Twenty-five grams of each sample was homogenized in 225 ml sterile saline solution in a sterile conical flask (Odumeru et al., 1997). Six fold serial dilutions were prepared in different test tubes and the prepared samples of respective dilution were poured on PCA, VRBA plates and incubated for 37°C for 24 hours for bacterial count and on PDA plates at 28°C for 24 to 48 hours for yeast and mold, (Acharya, 2021).

The number of visible colonies were counted, subcultured on NA and calculated by using a standard formula (Ema et al., 2022).

CFU/g = (No. of colonies/ inoculum size) × Dilution factor

Identification of bacteria was done using Gram staining and biochemical tests such as Catalase, Oxidase, IMViC series, Triple Sugar Iron (TSI) test, and motility test (Shoaib et al., 2020). The fungal growth on the PDA plate was subcultured onto fresh PDA plates and incubated at room temperature for 5 days. A piece of cellophane tape was used to slightly lift the fungal colonies, sticky side was placed on a slide containing a drop of Lactophenol Cotton Blue (LCB) and examined under microscope at 10x and 40x (Mohammed, 2024). Antibiotic susceptibility test of bacterial isolates was carried out using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA) plate. The

results were interpreted as sensitive, intermediate, or resistant (Bayot & Bragg, 2024). Parasites were identified using the Wet Mount method, with careful observation for motile trophozoites, cysts, eggs, or larvae, and their morphological characteristics were recorded (Demeke et al., 2021).

RESULTS

Table 1: Total mesophilic count (PCA) and coliform count (VRBA) of different vegetables

Sample	Source	Total mesophilic count	Total coliform count
		Average (CFU/g)	Average (CFU/g)
Moringa	Farm	1.07×10^6	2.52×10^5
	Vendor	1.21×10^6	3.15×10^5
	Hotel	9.22×10^5	2.38×10^5
Cucumber	Farm	1.63×10^5	1.57×10^5
	Vendor	2.24×10^5	2.19×10^5
	Hotel	5.77×10^4	2.7×10^4
Spinach	Farm	1.32×10^6	2.79×10^5
	Vendor	1.54×10^6	2.98×10^5
	Hotel	7.2×10^5	1.57×10^5
Green chilli	Farm	3.83×10^4	3.35×10^4
	Vendor	5.15×10^5	9.65×10^4
	Hotel	3.51×10^4	1.4×10^4
Peas	Farm	1.1×10^4	2.5×10^3
	Vendor	1.35×10^4	3.75×10^3
	Hotel	1.7×10^3	5×10^2
Cauliflower	Farm	4.1×10^5	2.37×10^5
	Vendor	4.69×10^5	2.98×10^5
	Hotel	2.52×10^5	9.6×10^4
Lady's finger	Farm	4.7×10^4	4.2×10^4
	Vendor	1.03×10^5	6.83×10^4
	Hotel	3.9×10^4	2×10^4
Carrot	Farm	1.04×10^5	3.4×10^4
	Vendor	2.67×10^5	4.15×10^4
	Hotel	6.89×10^4	1.75×10^4
Cabbage	Farm	3.17×10^5	9.14×10^4
	Vendor	3.93×10^5	2.89×10^5
	Hotel	1.62×10^5	4.15×10^3
Brinjal	Farm	1.65×10^5	6.17×10^4
	Vendor	1.71×10^5	6.91×10^4
	Hotel	3.33×10^4	3.25×10^4

The average total mesophilic and coliform counts indicate that spinach obtained from vendors had the highest mesophilic load (1.54×10^6CFU/g), while cabbage from vendors showed the highest coliform load (2.89×10^5CFU/g). In contrast, peas consistently exhibited the lowest microbial counts (5×10^2CFU/g) across all sources.

Table 2: Identification of the isolates

Isolates	Gram staining	Catalase	Oxidase	O/F	Indole	Motility	MR	VP	Citrate	TSIA	H_2S	Organism
Isolate 1	-	+	-	Fermentative	-	+	+	-	+	A/A	-	*Enterobacter* spp
Isolate 2	-	+	-	Fermentative	+	+	+	-	-	A/A	-	*E. coli*
Isolate 3	-	+	+	Oxidative	-	+	-	-	+	K/K	-	*P. aeruginosa*
Isolate 4	-	+	-	Fermentative	-	-	-	+	+	A/A	-	*Klebsiella* spp
Isolate 5	-	+	-	Fermentative	-	+	+	-	+	K/A	+	*Proteus* spp
Isolate 6	+	+	-	Fermentative	-	-	+	+	+	A/A	-	*S. aureus*

The isolates were identified as *Enterobacter* spp, *E. coli*, *P. aeruginosa*, *Klebsiella* spp, *Proteus* spp, and *S. aureus*, based on their biochemical characteristics.

Table 3: Fungi identified from vegetables

Vegetable	Source	Colony morphology on PDA	Colony character on bottom side of PDA plate	Microscopic observation	Fungi
Cabbage	vendor	grayish green or black	dark brown	transversely and longitudinally septate, beaked conidia	*Alternaria* spp
Spinach	farm	greenish black and powdery	black	conidia head in mass at the apex of conidiophore	*Cladosporium* spp
Cucumber	farm	white to pink, wooly	dark purple	sickle shaped transversely septate macroconidia produced in sporodochia	*Fusarium* spp
Brinjal	farm	greenish blue, black or green	light yellow	conidiophore arising from foot cell, conidia on phialides	*Aspergillus* spp
Carrot	farm	large round, smooth creamy white	white	small, oval to spherical budding yeast cells, no hyphae	*Candida* spp

The genera of fungal isolates were detected as *Alternaria*, *Cladosporium*, *Fusarium*, *Aspergillus*, and *Candida*, based on their macroscopic and microscopic characters.

Table 4: Identification of parasites on vegetables

Sample	Source	Microscopic observation	Parasite
Cauliflower	Vendor	ovoid, visible nuclei and fibrils	*G. lamblia*
Spinach	Hotel	spherical, nuclei present + ovoid, visible nuclei and fibrils	*E. histolytica* + *G. lamblia*
Soijan	Farm	round, thick shell with an external mammillated layer	*Ascaris*

Parasitological examination revealed the presence of *Giardia lamblia*, *Entamoeba histolytica*, *Giardia lamblia* and *Ascaris*, based on their microscopic observation.

Table 5: Antibiotic susceptibility pattern of *E. coli*, *Klebsiella* spp, *Proteus* spp and *Enterobacter* spp

Antibiotics	Antibiotic Susceptibility Pattern							
	E. coli		*Klebsiella* spp		*Proteus* spp		*Enterobacter* spp	
	Sensitive No. (%)	Resistant No. (%)	Sensitive No. (%)	Resistant No. (%)	Sensitive No. (%)	Resistant No. (%)	Sensitive No. (%)	Resistant No. (%)
Ceftriaxone (CTR 30)	2 (33.3%)	4 (66.7%)	3 (50%)	3 (50%)	3 (50%)	3 (50%)	1 (16.6%)	5 (83.4%)
Ceftazidime (CAZ 30)	0	6 (100%)	3 (50%)	3 (50%)	4 (66.7%)	2 (33.3%)	2 (33.3%)	4 (66.7%)
Cotrimoxazole (COT 30)	4 (66.7%)	2 (33.3%)	6 (100%)	0	2 (33.3%)	4 (66.7%)	2 (33.3%)	4 (66.7%)
Amikacin (AK 30)	5 (83.4%)	1 (16.6%)	4 (66.7%)	2 (33.3%)	6 (100%)	0	1 (16.6%)	5 (83.4%)

The antibiotic pattern of isolates showed that amikacin was the most effective against all isolates, particularly *Proteus* (100% sensitive) and *E. coli* (83.4% sensitive), while ceftazidime exhibited the poorest activity, with *E. coli* showing 100% resistance and high resistance also observed in *Enterobacter* (66.7%), *Klebsiella* (50%), and *Proteus* (33.3%).

Table 6: Antibiotic susceptibility pattern of *Pseudomonas* spp

Antibiotics group	Antibiotics	Susceptibility Pattern	
		Sensitive No. (%)	Resistant No. (%)
Cephalosporin	Ceftriaxone (CTR 30)	3 (50%)	3 (50%)
Cephalosporin	Ceftazidime (CAZ 30)	2 (33.3%)	4 (66.7%)
Trimethoprim	Cotrimoxazole (COT 30)	3 (50%)	3 (50%)
Aminoglycosides	Amikacin (AK 30)	1 (16.6%)	5 (83.4%)
Fluoroquinolone	Ciprofloxacin (CIP 5)	0	6 (100%)

Among the tested antibiotic groups, cotrimoxazole and ceftriaxone showed moderate effectiveness (50% sensitivity), *Pseudomonas* spp was found to be 100% resistant against ciprofloxacin while lowest sensitivity against amikacin.

Table 7: Antibiotic susceptibility pattern of *S. aureus*

Antibiotics group	Antibiotics	Susceptibility Pattern	
		Sensitive No. (%)	Resistant No. (%)
Macrolides	Erythromycin (E 15)	2 (33.3%)	4 (66.7%)
Cephalosporin	Ceftazidime (CAZ 30)	3 (50%)	3 (50%)
Trimethoprim	Cotrimoxazole (COT 30)	1 (16.6%)	5 (83.4%)
Aminoglycosides	Amikacin (AK 30)	2 (33.3%)	4 (66.7%)
Fluoroquinolone	Ciprofloxacin (CIP 5)	3 (50%)	3 (50%)

Across the antibiotic groups, ciprofloxacin and ceftazidime showed the highest effective (50%), erythromycin and amikacin displayed moderate effective (66.7%), and cotrimoxazole was the least effective towards *S.aureus.*

DISCUSSION

Among all the vegetables examined, the highest isolation of bacteria was from vendor samples with a total number of 5 types (*E. coli, Enterobacter, P. aeruginosa, Proteus* spp *and S. aureus*) species. Farm samples yielded 4 types of bacteria (*E. coli, P. aeruginosa, Enterobacter* spp, and *Klebsiella* spp) and hotel samples showed 3 (*E. coli*, *Pseudomonas* spp, and *S. aureus*). These results are consistent with previous study conducted by Falomir, in which *Klebsiella* spp, *Enterobacter* spp, *E. coli* along with other species were present on vegetables (Falomir et al., 2010). Among the total samples, vendor spinach had the highest mesophilic count (1.54×10^{6}CFU/g) and vendor cabbage showed the highest coliform count (2.89×10^{5}CFU/g), while peas had the lowest counts for both bacteria (5×10^{2}CFU/g) from all sources. A study on microbiological safety of raw, freshly consumed vegetables from open markets in Lusaka District found that these vegetables harbor foodborne pathogens, posing potential health risks to consumers (Chakopo, 2017).

Fungi contribute to the spoilage of vegetables because of their pathogenicity to the harvested products as they produce harmful mycotoxins during pathogenesis (Abdulla et al., 2016). Farm samples were more prone to fungal contamination compared to vendor and hotel samples likely due to various factors such as direct exposure to soil, airborne spores, physical damage during harvesting, lack of sanitation, high humidity and moisture. The microbial quality of irrigation water is crucial, as contamination with animal or human waste can introduce pathogens into vegetables during both preharvesting and postharvesting (Suslow, 1997). In this study the identified fungi included *Alternaria* spp (cabbage), *Cladosporium* spp (spinach), *Fusarium* spp (cucumber), *Aspergillus* spp (brinjal), and *Candida* spp (carrot). A study in Nagpur, India showed frequent association of similar organisms in vegetables (Kakde et al., 2001). Parasites were detected only in cauliflower, spinach, and soijan, while the remaining vegetables tested negative. Parasitic examination revealed the presence of *G. lamblia*, *E. histolytica*, and *Ascaris* in the samples. Green leafy vegetables like lettuce have uneven surfaces that may allow parasitic eggs, cysts, and oocysts to adhere more easily, either during cultivation or when washed with contaminated water (El Said Said, 2012). Similarly, the presence of such parasitic species were reported by a study in Ethiopia (Alemu et al., 2020). Vendor samples are exposed to unhygienic handling during transportation, storage, and marketing, where vegetables are often kept in open environments and handled repeatedly without proper hygiene. These conditions allow microorganisms to multiply and persist, leading to higher bacterial counts. Hotel samples often have reduced bacterial diversity due to washing, peeling or proper storage but may still contain some species of bacteria. The variation in contamination level of the vegetables may be due to the differences in shape and surface of each vegetable.

Antibiotic susceptibility testing revealed high resistance among the isolates. All 6 isolates of *E. coli* i.e. 100% were resistant to Ceftazidime, followed by 66.7% to Ceftriaxone, 33.3% to Cotrimoxazole and 16.6% to Amikacin. *Klebsiella* spp showed sensitivity to Cotrimoxazole, and 66.7% susceptibility to Amikacin. *Proteus* spp were sensitive to Amikacin. In contrast, *Enterobacter* spp exhibited low sensitivity overall with only 16.6% susceptibility to Amikacin. A study conducted in Ethiopia has also reported a high burden of antimicrobial resistance among *Klebsiella* and *E. coli* (Kitaba et al., 2024). Several studies have found comparable interpretations on susceptibility patterns of *Proteus* and *Enterobacter* (Basnet et al., 2024). For *Pseudomonas* isolates, Ceftriaxone and Cotrimoxazole were highly effective with 50% sensitivity, followed by Ceftazidime 33.3%, Amikacin 16.6% and complete resistance to Ciprofloxacin. A similar resistance pattern

was reported from a tertiary care hospital in Kathmandu (Shrestha et al., 2023). Research in South East Nigeria has pointed to the possible threat of *S. aureus* and its control measures (Nwankwo & Nasiru, 2011). These results indicate widespread bacterial contamination and variable antibiotic resistance patterns among vegetable associated isolates, suggesting that commonly used antibiotics may not reliably control infections. It also highlights Amikacin as a potentially useful treatment option.

CONCLUSION

The study depicted microbial variety and their burden in vegetable items sold in different markets, hotels and farms. Bacteria such as *S. aureus, E. coli, Proteus* spp, *Pseudomonas* spp, *Enterobacter* spp and *Klebsiella* spp were identified. Fungal species such as *Alternaria* spp, *Cladosporium* spp, *Aspergillus* spp, *Fusarium* spp and *Candida* spp were detected in these vegetables. Additionally, the presence of parasites such as *Ascaris, E. histolytica* and *G. lamblia* was confirmed in the samples during this study. The antibiotic susceptibility patterns showed a concerning level of resistance towards various antibiotics. Most of the isolates were resistant to all the antibiotics used such as ceftriaxone, ceftazidime, amikacin, erythromycin and ciprofloxacin. However, some susceptibility was observed, underscoring the importance of proper antibiotic use and continuous monitoring to combat antimicrobial resistance in foodborne pathogens. These results indicate a prevalence of drug resistance among vegetable associated pathogens.

Thus, the survival of bacteria, fungi and parasites within the fresh vegetables revealed the importance of maintaining proper sanitary conditions during harvesting, processing, storage and handling of the vegetables consumed. It also notified the importance of searching for ways to minimize the risk of getting different diseases. The findings of this research may also help form a policy guideline for safe consumption of raw vegetables based on the capacity of the particular vegetable to resist contaminating pathogens and also contribute to ensure food safety and security.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL RESEARCH

Bacterial Profiling of Fish in Kathmandu Valley Market

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ABSTRACT

Objectives: The purpose of the study was to identify the bacterial species in fishes as well as to enumerate the total viable count and total coliform count in the fish sample from Kathmandu valley market.

Methods: A descriptive study was conducted where two species of fish i.e., Rohu and Bachua were collected from several shops within the Kathmandu valley. Each fish was separated into gills, gut, and skin portions, constituting a total of 18 samples and the samples were then processed according to standard laboratory methods for the isolation and identification of bacteriological species.

Results: In a total sample size of 18 samples, 44 isolates were isolated where 20.46% of the isolates were found to be gram-positive bacteria and 79.54% of the isolates were found to be gram-negative bacteria. *Escherichia coli* accounted for 38.64%, resulting in being the dominant organism. The sample collected from Kalimati in Bachuwa showed the highest bacterial count (1.01×10^7cfu/gm) while the sample from Lalitpur in Rohu showed the lowest bacterial load (9.68×10^4cfu/gm). In Rohu samples, the highest coliform load and lowest coliform load was collected from Kalimati (4.25×10^5cfu/gm) and Lalitpur (3.78×10^3cfu/gm), respectively.

Conclusion: The highest bacterial load, coliform load and isolated pathogens in fishes available in the market of Kathmandu valley from this study concluded that the fishes are highly vulnerable to bacterial contamination, and suggest the potential risk for public's health issues.

Keywords: Fish, Rohu, Bachuwa, Bacterial load, Coliform

INTRODUCTION

Fish and fish products only supply about 34 calories per person per day on average worldwide. Fish does, however, contribute significantly to the diet in terms of high-quality, readily digested animal proteins and particularly in the prevention of micronutrient defi ciencies, in addition to serving as an energy source (FAO, 2018).

Consuming oily fish that is high in long-chain omega-3 fatty acids can help lessen the risk of cardiovascular disease and minimize systemic inflammation(Bowen et al., 2016).In Nepal, aquaculture is one of the agricultural subsectors with the quickest rate of growth. The most popular species cultivated are rainbow trout, pangas catfish, and both native and exotic carp. Although aquaculture's institutional development in Nepal began about seven decades ago, the industry developed at a very modest pace. All the same, this industry has made tremendous strides in the last ten years. In Nepal, people consume comparatively less fish than they do chicken, hog, beef, and mutton. People's growing health consciousness has increased demand for aquaculture sectors and resulted in a surge in fish consumption.

Fish is an essential part of the human diet, and in Nepal, the number of fish consumed per person is rising. However, the rapid expansion of industry and agriculture may contaminate both naturally occurring and artificially created aquatic ecosystems, which could

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have an impact on fish health and raise questions about the safety of fish intended for human consumption (Novoslavskij et al., 2016).

Fish illnesses caused by bacteria are among the most prevalent and challenging medical issues to treat. Fish fingerlings, fry, and eggs have all been known to contract bacterial infections that result in high mortality rates. These microbes are essentially opportunistic pathogens that enter the tissues of fish hosts that have been exposed to stressors that can cause infection (Guzman et al., 1988). Columnariosis, farunuclosis, tail rot/fin rot, bacterial gill diseases, aeromoniasis, edwardsiellosis, vibriosis, eye disease, pseudomonasis, and enteric red mouth disease are among the bacterial infections that are frequently observed. The parasite condition alone results in an 8–12% reduction in productivity when compared to other illnesses. Under favourable circumstances, fish parasites proliferate quickly, impacting fish health and frequently resulting in high mortality. Parasites affect host nutrition by interfering with the metabolism and secretary functions of the alimentary canal and damaging the nervous system (Shrestha et al., 2019).

Fish from the natural environment are known to harbour various species. Regular exposure to contaminated water causes bacterial colonization on fish skin and gills, and contaminated feed or water can harm the digestive tract. When immunological resistance is weakened, fish muscles may also become contaminated (de Cuesta et al., 2011). A study from UK reported that total bacterial count (TBC) on the skin of salmon (*Salmo salar*) varied from 10^2 to 10^3 cfu/cm^3. Meanwhile,a similar study carried out in Turkey revealed a higher number of 10^1 to 10^7 cfu/cm^3 on salmon skin and aerobic microorganisms was detected more often than anaerobic (Minniti et al., 2019). It is commonly known that the bacteria that exist in water that is contaminated are similar to those found on fish skin These bacteria include *Aeromonas* spp, *Flexibacter* spp, *Proteus* spp, *Providencia* spp, *Psychrobacter* spp, *Moraxella* spp, *Pseudomonas fluorescens, Acinetobacter johnsonii, Alcaligenes piechaudii, Enterobacter aerogenes, Escherichia coli, Micrococcus luteus,* and *Vibrio fl uvialis* (Novoslavskij et al., 2016).

METHODS

Research design, duration, and laboratory setting

This study was a descriptive study conducted at KIST College of Management, Kamalpokhari, Kathmandu, Nepal from December, 2023 to May, 2024. A total of six fishes of 2 different species were collected from several shops in Kathmandu Valley. Each fish was separated into gills, gut, and skin portions, constituting a total of 18 samples. These fishes were transported in sterile zip-lock bags along with ice and brought to the laboratory and the samples were then processed according to standard laboratory methods (Cheesbrough, 2006).

Study sites

Sites were selected in various locations: Kathmandu (Kalimati), Bhaktapur (Sukuldhoka), and Lalitpur (Mangalbazar). Parameters such as cleanliness, store location, and general hygiene around the fish shops were recorded. The samples' properties, including temperature, location, date, and time, were appropriately labelled.

Sample collection and Transportation of sample

The fish was placed into a zip-lock, hygienic plastic container with ice box. Surgical gloves and sterile hands cleaned with chloroxylenol were used during the transfer. Each time a sample was to be taken; this process was repeated. With caution to avoid overfilling the container, each sample was moved to a different container. It was sent straight to the lab, where it was handled with care to preserve the sample's integrity by creating an atmosphere that would not change the microbial flora in the fish sample in any manner.

The sample was brought to the lab in minutes or hours in a sterile container. The temperature, amount of sunshine, and other environmental elements was carefully controlled to avoid changing the sample's pre-existing microflora (Cheesebrough, 2006).

Sample processing

Homogenization

Using a sterile blade, the fish sample was cut into tiny pieces. 25 grams of each gill, gut, and skin from the fish samples were weighed and homogenized separately using a sterile mortar and pestle. Then, each homogenized sample was mixed with 250ml of diluent for the microbiota enumeration process (Cheesebrough, 2006).

Enumeration of bacteria and coliform

For enumeration, 1 ml of diluent of the homogenized sample was serially diluted from 10^{-1} to 10^{-6} dilutions. 1 ml of dilution was taken and poured onto sterile petri plates for each dilution. Then, about 25ml of molten Plate count agar (PCA) and Violet red bile agar (VRBA) were poured onto these dilutions for Total Plate Count and Total Coliform Count respectively. Finally, Petri plates

were incubated at 37°C for 24 hours. After 24 hours, the number of colonies on each plate was enumerated.

Enrichment of the Sample

For the isolation of pathogenic bacteria like *Salmonella* and *Vibrio*, the homogenized sample was enriched in their respective enrichment media such as Selenite F broth and Alkaline Peptone water. About 10 ml of each homogenized sample was transferred to 50 ml of Selenite F broth for *Salmonella* as well as Alkaline Peptone water for *Vibrio*. Then, it was incubated at 37°C for 24 hours (Cheesebrough, 2006).

Isolation of pathogenic bacteria

To isolate pathogenic bacteria from the fish samples, different selective and differential media were prepared. For example: Mannitol Salt Agar (MSA), MacConkey Agar (MA), Xylose Lysine Deoxycholate (XLD) Agar, and Thiosulfate Citrate Bile salts Sucrose (TCBS) Agar. MSA is a selective media for the isolation of gram-positive *Staphylococcus* spp MA is commonly used for isolating the Enterobacteriaceae family as well as differentiating lactose fermenters and non-lactose fermenters. XLD agar is used for the isolation of *Salmonella* whereas TCBS agar is used for *Vibrio* spp. A loopful of suspension of diluent was taken in a sterile loop and streaked over the media. This process was repeated for every media prepared. Then, these plates were incubated at 37°C for 24 hours. After 24 hours, the colony characteristics were studied, sub-cultured on the Nutrient Agar (NA), and again incubated at 37°C for 24 hours. The colonies from NA plates were subjected to gram staining and biochemical tests (Cheesebrough, 2006).

Identification

Staining

The gram staining was performed from the isolated pure culture in NA.

Biochemical Tests

Biochemical tests such as Catalase test, Oxidase test, Indole test, Methyl red test, Voges-Proskauer test, Citric acid utilization test, Triple sugar iron agar test, Urease test, Motility test, Sulphide production test, Gas production test, etc. were performed by standard method (Cheesebrough, 2006).

RESULTS

Percentage-wise distribution of bacteria in fish samples

In a total sample size of 18 samples, 44 isolates were isolated where 20.46% of the isolates were found to be gram-positive bacteria and 79.54% of the isolates were found to be gram-negative bacteria. *Escherichia coli* (*E. coli*) accounted for 38.64%, resulting in being the dominant organism. Likewise, *Staphylococcus aureus* accounted for 13.64% of total organisms isolated, while the percentage of *Salmonella* was found to be 11.36%. *Pseudomonas* spp and *Vibrio* spp were both found to be 9.09% and also both *Staphylococcus epidermidis* and *Proteus* were found to be 6.82%. *Klebsiella* was also isolated and found to be 4.55% of the total bacteria isolated from the sample (Figure 1).

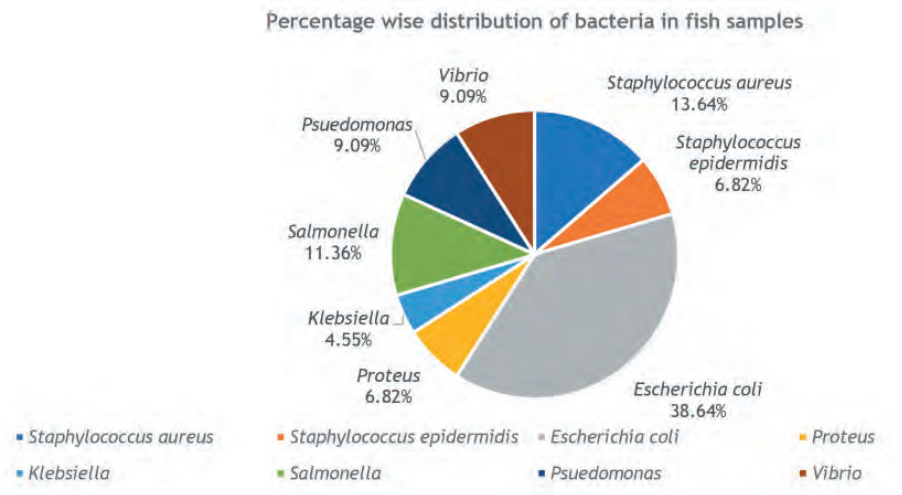

Figure 1: Percentage-wise distribution of bacteria in fish samples

Site-wise distribution of bacterial species in different fish samples

Two species of fish (Rohu and Bachuwa) were collected from above mentioned locations, totalling six fishes. Each fish was separated into gills, gut, and skin portions, constituting a total of 18 samples.

Site-wise distribution of total bacterial load (cfu/gm) in fish sample

The sample collected from Bachuwa in Kalimati showed the highest bacterial count (1.01×10^7cfu/gm) followed by Bachuwa in Lalitpur (2.66×10^6cfu/gm) (Table 1).

Site-wise distribution of total coliform load (cfu/gm) in fish samples

The highest coliform load was found to be in Rohu collected from Kalimati (4.25×10^5cfu/gm) followed by the sample Rohu collected from Bhaktapur (3.65×10^4cfu/gm) (Table 1).

Site-wise distribution of bacterial species in different fish samples

In a total of 18 samples, *Staphylococcus aureus* was present in 6 samples. Among these 6 samples, 3 were from Kalimati, 1 was from Bhaktapur and 2 were from Lalitpur. For *Staphylococcus epidermidis*, 2 samples were from in Kalimati sample and 1 sample from the Lalitpur. From a total of 17 samples, *E. coli* was isolated which was the highest among all the organisms. Likewise, *Proteus* was found in 3 samples, one from Kalimati and two from Lalitpur. *Klebsiella* was seen in each sample from Bhaktapur and Lalitpur. *Salmonella* was present in a total of 5 samples, i.e., 2 samples in Kalimati, 1 sample in Bhaktapur, and 2 samples in Lalitpur. *Pseudomonas* and *Vibrio* were present in 4 samples. Similarly, *Vibrio* was found in 1 sample of both Kalimati and Lalitpur. Kalimati had the highest number of bacterial loads (17 samples) followed by Lalitpur (15 samples) and Bhaktapur (12 samples) (Table 2).

Bacterial species distribution in different parts of fish samples

The skin accounted for the highest number of organisms isolated which was 17 isolates while the gill accounted for the lowest number of organisms which was 12 isolates. *E. coli* was the most dominant bacteria in all three parts of the fish samples (Table 3).

Comparing bacterial species isolated in two different fish samples i.e. Bachuwa and Rohu

Among the 2 species of fish, Rohu accounted for the highest number of isolates which was 23 and Bachuwa accounted for 21 isolates (Table 3). The result provided a statistical basis to conclude that there was no association of bacterial species isolated in two different fish samples i.e., Bachuwa and Rohu ($p>0.05$) (Table 3)

Table 1: Site-wise distribution of total bacterial load (cfu/gm) and total coliform load (cfu/gm) in fish samples

Site	Fish	Parts	Total bacterial load (cfu/gm)	Average bacterial load (cfu/gm)	Total Coliform Load	Average Coliform Load
Kalimati	Rohu	Gill	1.62×10^7		2.42×10^5	
		Gut	3.25×10^6	1.29×10^6	1.57×10^5	4.25×10^5
		Skin	1.93×10^7		8.78×10^5	
	Bachuwa	Gill	6.85×10^5		8×10^3	
		Gut	1.21×10^4	1.01×10^7	4.11×10^3	6.05×10^3
		Skin	2.96×10^7		0	
Bhaktapur	Rohu	Gill	4.02×10^6		8.85×10^5	
		Gut	6.22×10^5	1.4×10^6	1.42×10^5	3.65×10^4
		Skin	8.09×10^4		6.94×10^4	
	Bachuwa	Gill	3.02×10^5		6.7×10^2	
		Gut	1.18×10^5	1.51×10^5	2.58×10^4	1.32×10^4
		Skin	3.45×10^4		0	
Lalitpur	Rohu	Gill	1.94×10^4		1.81×10^3	
		Gut	2.68×10^5	9.68×10^4	9.53×10^3	3.78×10^3
		Skin	2.91×10^3		10	
	Bachuwa	Gill	1.85×10^5		6.7×10^3	
		Gut	7.79×10^6	2.66×10^6	2.16×10^3	4.43×10^3
		Skin	9.4×10^2		0	

Table 2: Site-wise distribution of bacterial species in different fish samples

	No. of samples from				
Microorganism	**Kalimati**	**Bhaktapur**	**Lalitpur**	**Total**	**Total %**
Staphylococcus aureus	3 (17.64%)	1 (8.33%)	2 (13.33%)	6	13.64
Staphylococcus epidermidis	2 (11.76%)	0	1 (6.66%)	3	6.82
Escherichia coli	6 (35.29%)	6 (50.0%)	5 933.33%)	17	38.64
Proteus	1 (5.88%)	0	2 (13.33%)	3	6.82

Microorganism	No. of samples from				
	Kalimati	Bhaktapur	Lalitpur	Total	Total %
Klebsiella	0	1 (8.33%)	1 (6.66%)	2	4.53
Salmonella	2 (11.76%)	1 (8.33%)	2 (13.33%)	5	11.36
Pseudomonas	2 (11.76%)	1 (8.33%)	1 (6.66%)	4	9.09
Vibrio	1 (5.88%)	2 (16.66%)	1 (6.66%)	4	9.09
Total	**17 (38.63%)**	**12 (27.27%)**	**15 (34.1%)**	**44**	**100**

Table 3: Bacterial species distribution in different parts of fish samples

Microorganisms	Bachuwa			Rohu			Total
	Skin	Gill	Gut	Skin	Gill	Gut	
Staphylococcus aureus	1 (11.11%)	1 (16.66%)	0	3 (37.50%)	1 (16.66%)	0	6 (13.63%)
Staphylococcus epidermidis	1 (11.11%)	0	0	1 (12.50%)	1 (16.66%)	0	3 (6.82%)
Escherichia coli	2 (22.22%)	3 (50.0%)	3 (50.0%)	3 (37.50%)	3 (50.0%)	3 (33.33%)	17 (38.63%)
Proteus spp	1 (11.11%)	0	1 (16.66%)	0	0	1 (11.11%)	3 (6.82%)
Klebsiella spp	1 (11.11%)	0	0	0	0	1 (11.11%)	2 (4.54%)
Salmonella spp	1 (11.11%)	1 (16.66%)	1 (16.66%)	0	1 (16.66%)	1 (11.11%)	5 (11.36%)
Pseudomonas spp	0	1 (16.66%)	0	1 (12.50%)	0	2 (22.22%)	4 (9.10%)
Vibrio spp	2 (22.22%)	0	1 (16.66%)	0	0	1 (11.11%)	4 (9.10%)
	9 (20.45%)	6 (13.64%)	6 (13.64%)	8 (18.18%)	6 (13.64%)	9 (20.45%)	44 (100%)

DISCUSSION

A total of 44 isolates were isolated from these 18 samples. Among these 44 isolates, 9 isolates were found to be gram-positive bacteria (20.46%) while 35 isolates were found to be gram-negative bacteria (79.54%). *Staphylococcus aureus* and *Staphylococcus epidermidis* were among the gram-positive bacteria while gram-negative bacteria included *E. coli, Proteus, Klebsiella, Salmonella, Pseudomonas,* and *Vibrio.* Out of 9 gram-positive bacteria, 6 of the isolates were *Staphylococcus aureus* (13.64%) and 3 of the isolates were *Staphylococcus epidermidis* (6.82%). Out of 35 gram-negative bacteria, 17 of the isolates were *E. coli* (38.64%), 3 *Proteus* spp 6.82%), 2 *Klebsiella* spp (4.53%), 5 *Salmonella* spp (11.36%), 4 *Pseudomonas* spp (9.09%) and 4 *Vibrio* spp (9.09%).

Comparing the two species of fish, Rohu had a higher number *of E. coli* than Bachuwa and the bacteria was most frequently isolated from the gut region of the fish. *E. coli* can cause serious complications related to the gastrointestinal tract such as dysentery, urinary tract infections, diarrhoea, meningitis and even pneumonia (Johnson et al., 2009). However, *E. coli* is not a common microflora of gut in fish, which could indicate that the organism came into existence from the faecal contaminated water (Lovell & Barkate, 1969).

After *E. coli, Staphylococcus aureus* and *Salmonella* were the frequently observed bacteria. *Staphylococcus aureus* was present in both skin and gill but absent in the gut. *Staphylococcus aureus* commonly found on human skin and environment can cross-contaminate with fish. They are present in the environment including water and on surfaces where fishes are processed or stored. Poor sanitation practices can contribute to the presence of these bacteria in the fish handling and processing environment (Taylor & Unakal, 2023).

Infections caused by *S. aureus* range from mild to life-threatening producing skin infections, often causing abscesses including bacteraemia, endocarditis, and osteomyelitis. Some strains of *S. aureus* produce toxins that cause staphylococcal food poisoning or toxic syndrome (Taylor & Unakal. 2023).

In the case of *Salmonella*, it was mainly isolated from the gut region of the fish. *Salmonella* is considered unfit for humans to be consumed contaminated with food. *Salmonella* in freshwater fishes has been usually related to the faecal contamination of water from where fish were harvested. *Salmonella* is the causative agent of salmonellosis, a severe form of gastroenteritis which is still a major prevalent considered one of highly common food-borne illness and a major public health problem. It is obvious that consumption of *Salmonella*-infected fishes can increase public health problems. The contamination of fish through aquatic environment, contaminated by humans and poultry itself may create a secondary food reservoir (Bibi et al., 2015).

About 4 isolates of *Vibrio* was found mostly in Bachuwa. This organism was most common in the gut and skin. *Vibrio* spp is key pathogen in many aquacultures system which are part of normal flora of intestine of many aquatic species. Therefore, vibriosis

is a major fish disease among many species of cultured fish (Manchanayake et al., 2023).

Fish hold an important position as a food component for a large section of world population, including Nepal. In Nepal, the consumption of fish and fish products has contributed a remarkable position in the market for many years. So, maintenance of appropriate quality of the products is regarded as vital for achieving desired success in trade of the product and health of the consumer. However, there still exists a huge problem in the fish markets in terms of microbiological view point. As the fish markets lacks in sanitation criteria, it could result in huge economic losses. The main source of contamination in fish usually starts from the fisheries. There might possibility of contaminated water being used in the fisheries. Even when these fish are brought from fisheries to market, they could get contaminated due to a lack of proper transportation facilities. The local cold stores in our locality are not well managed, measuring devices/knives are not fully sanitized and the shopkeeper use barehand to pick the fish and pack it. There can be possibilities of flies flying around the fish if the fish products are not kept inside the glass cover.

CONCLUSION

The highest bacterial load, coliform load and isolated pathogens in fishes available in the market of Kathmandu valley from this study concluded that the fishes are highly vulnerable to bacterial contamination, and suggest the potential risk for public's health issues. The differences in bacteriological load between fish samples highlight how storage, handling, and environmental factors affect the degree of microbial infection. The study emphasized the need for future research to create better methods for preventing bacterial contamination in fish.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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ORIGINAL RESEARCH

Isolation and Characterization of *Bacillus* spp with Potent Proteolytic and Antipathogenic Activities

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ABSTRACT

Objectives: The current study aimed to determine the proteolytic and antipathogenic activity of *Bacillus* spp isolated from soil samples collected across various ecological and land-use categories in multiple locations within the Kathmandu Valley.

Methods: A cross-sectional study was conducted at the Med-Micro Nepal Community Development and Research Centre, Kathmandu. Seventeen different soil samples were collected and processed to isolate *Bacillus* species. The isolates were identified as *Bacillus* spp on the basis of their morphological characteristics, Gram's staining, spore staining and biochemical tests based on Bergey's Manual of Systematics of Archaea and Bacteria. The proteolytic activity was screened and determined by well diffusion method on 1% gelatin agar media for both crude and purified form of enzyme. Similarly, antipathogenic assay was done on Mueller Hinton agar by agar well diffusion method.

Results: All 17 *Bacillus* isolates exhibited proteolytic activity, with enhanced activity observed upon partial purification. Notably, isolate A_M showed the highest increase (25%) in protease activity, while R_S and R_H displayed the largest overall proteolytic zones (32 mm and 31 mm, respectively). Isolates R_H, R_K, F_M, F_N, and F_S demonstrated both strong antipathogenic and proteolytic activities, suggesting a potential correlation between protease production and antimicrobial efficacy.

Conclusion: These findings highlight the therapeutic potential of selected proteolytic *Bacillus* strains, particularly against *Streptococcus* spp and *E. coli*.

Keywords: Protease enzyme, *Bacillus* spp, crude extract, zone of inhibition, antipathogenicity

INTRODUCTION

Bacillus spp are widely recognized for their ecological versatility and biotechnological potential (Contesini et al., 2018; Logan and De Vos 2015; Mokashe et al., 2018). These bacteria thrive in diverse environments including soil, where they contribute to organic matter decomposition and nutrient cycling (Nicholson et al., 2018). Among many attributes, they are prolific producers of extracellular proteases - enzymes that hydrolyze proteins into peptides and amino acids, with applications in industries such as detergents, food processing, and pharmaceuticals (Contesini et al., 2018). The stability of *Bacillus*-derived proteases under extreme conditions, such as high temperatures and varying pH levels, makes them particularly valuable for industrial and therapeutic purposes (Mokashe et al., 2018).

Soil serves as a rich reservoir of diverse microbes, harboring *Bacillus* spp with varying enzymatic capabilities influenced by environmental factors like soil composition and land use (Sharma et al., 2020). The Kathmandu Valley, characterized by diverse ecological niches including residential, agricultural, industrial, and riverine areas, offers a unique opportunity to explore the proteolytic potential of *Bacillus* isolates (Thapa et al., 2019). Recent studies have highlighted the

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ability of soil-derived *Bacillus* spp to have antimicrobial properties, suggesting their potential in combating pathogens such as *Streptococcus* spp and *Escherichia coli* (Saggu & Mishra, 2017; Pant et al., 2021).

Proteases produced from *Bacillus* spp play a critical role in microbial competition by degrading environmental proteins, which may also contribute to antimicrobial activity through the disruption of pathogenic cell structures (Haddar et al., 2021). The agar well diffusion method, a standard technique for assessing enzymatic and antimicrobial activity, has been widely adopted to measure zones of hydrolysis and inhibition (Bauer et al., 2016; Manandhar et al., 2022). These methods enable precise quantification of protease activity and antipathogenic effects, providing insights into the therapeutic potential of *Bacillus* isolates.

This study aims to isolate *Bacillus* spp from soil samples collected across various sites of Kathmandu Valley, characterize their proteolytic activity and evaluate their antipathogenic efficacy against clinical pathogens. By comparing the enzymatic and antimicrobial activities of isolates from different environmental contexts, this research seeks to identify strains with therapeutic potential and investigate potential correlations between protease production and antimicrobial activity. The findings could contribute to the development of novel biotechnological and therapeutic applications byleveraging the natural capabilities of Bacillus spp (Contesini et al., 2018; Pant et al., 2021).

METHODS

Collection of soil samples

Seventeen different soil samples were collected from seventeen different sites of Kathmandu valley. The sites were grouped under six categories viz. residential area- 5, river site- 2, agricultural farm- 3, dumping site- 2, forest area- 3 and industrial area- 2. The five residential samples were taken from Suryabinayak, Mitrapark, Mulpani, Anamnagar, and Koteshwor. Similarly, Bagmati and Dhobikhola were the two river sites. The other three samples were taken from agricultural farms of Suryabinayak, Mulpani and Tarakeshwor. Moreover, two samples were taken from the two dumping sites; Teku and Ratopul. The soil samples from forest areas where taken from three different corners of Kathmandu valley- Suryabinayak, Nagarjun and Mulpani. Finally, two soil samples were obtained from industrial areas of Bhaktapur and Balaju.

The soil samples were collected in sterile aluminum foils. The surface soil was removed and approximately 10-15 grams of soil from depth of 3- 4 inches were collected (Rebecca et al., 2014) with the help of sterilized spatula, kept in a sterile aluminum foil and placed in a zip- lock plastic bags. The soil samples were appropriately labelled mentioning sample code and date and time of collection. The soil samples were brought to laboratory.

Isolation and identification of *Bacillus* species

Heat treatment method was employed to screen heat resistant endospore forming *Bacillus* species as suggested by Logan & De Vos (2015). Five grams of soil sample was added to 45 ml of distilled water and stirred properly. This mixture of soil was heated at 80°C for 15 minutes. The isolation of bacteria was carried out by quadrant streaking method (Lageiro et al., 2025). A loop of processed sample was taken and streaked on a nutrient agar plate. The plate was incubated for 24 hours at 37°C (Lakshmi et al., 2014).

The isolated colonies were identified based on cellular morphology, growth condition, gram staining, endospore staining, growth conditions and biochemical tests according to the guidelines on Bergey's Manual of Systematics of Archaea and Bacteria (Logan & De Vos, 2015). The common biochemical tests to be performed are oxidase test, catalase test and oxidative / fermentative (O/F) test. Schaeffer-Fulton method was used for staining endospores and sub cultured on NA media. The plate was incubated at 37°C for 24 hrs. This was obtained as pure isolated culture of *Bacillus* spp The pure culture was further preserved in a refrigerator at -4°C as master cultures (Pant et al., 2015).

Screening of *Bacillus* isolates for enzyme production

The isolates were screened for protease production by spot assay. The isolated bacteria were point inoculated on 1% gelatin agar plate and incubated for 24 hrs at 30°C. The zone of hydrolysis was checked by flooding the plates with 3% mercuric chloride solution (Rebecca et al., 2014). This is based on gelatin hydrolysis test. The strains producing clear zones were sub cultured on NA and reported as potent isolates.

Production of protease enzyme

The potent isolates from freshly sub cultured NA plates were inoculated in 50mL of gelatin broth. It was fermented in shaking incubator at 30°C for 48hrs.

Enzyme extraction was done by centrifugation process.

At the end of each fermentation period the whole broth culture was centrifuged at 3500 rpm for ten minutes to remove debris. The clear supernatant was recovered by sedimentation and used for further experiments. This is the crude extract of enzyme (Rebecca et al., 2014).

For purification, 5mL of crude extract of enzyme was added with double volume of chilled acetone and refrigerated overnight in a conical flask. The content was then centrifuged at 5000 rpm for five minutes. The supernatant obtained was discarded and the precipitated enzyme pellet was dissolved in small amount of phosphate buffer at pH 7 to obtain partially purified enzyme (Lageiro et al., 2025).

Measurement of enzymatic activity

The enzyme activity was estimated by enzyme assay method based on agar well diffusion procedure (Manandhar & Sharma, 2013). The well was made on 1% gelatin agar plates with the help of 6mm cork borer. 50µl of crude extract of enzyme, 50µl of purified protease and 50µl of autoclaved water were loaded on three different wells bored on a GA media plate and allowed to diffuse at room temperatures for 15 minutes. The plates were incubated at 37°C for 24 hrs. After incubation the plates were flooded by mercury chloride and the zone of hydrolysis was measured (Pant et al., 2014).

Anti-pathogenic Assay

Each of five different clinical isolates - Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus* spp were swabbed on different Mueller Hinton Agar (MHA) plates. Six well were made with 6 mm borer in each plate, where *Bacillus* isolates were filled. The zone of inhibition were measured after incubation for 24 hours.

Data Analysis

The zone of hydrolysis was recorded and analyzed by using Microsoft word and MS- excel. The bar charts and tables were prepared. Similarly, mean of the samples was also calculated as statistical analysis.

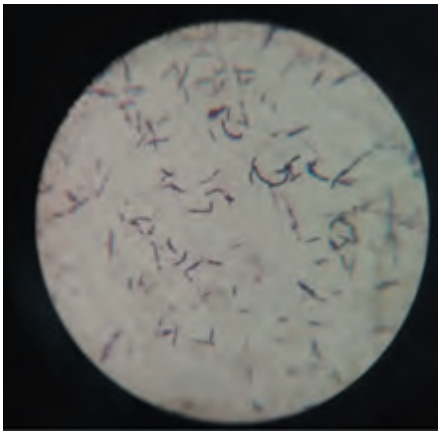

Figure 1: Gram staining of *Bacillus* spp: Gram positive (purple colour) bacteria are seen under light microscopy at 100x magnification

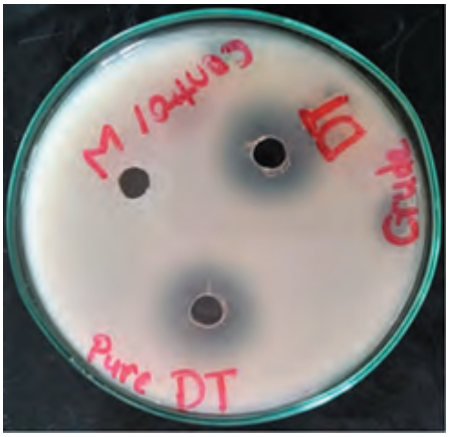

Figure 2: Proteolytic activity of protease extract on gelatin agar: Halo zone around the well shows the zone of hydrolysis of crude (crude DT) and partially purified (pure DT) protease extract from the isolates of dumping sites from Teku. No halo zone was seen around the control (distilled water).

RESULTS

Only the most thermotolerant *Bacillus* spp from each sampling site was taken for isolation and characterization. The description of soil samples is presented in table 1.

Table 1: Description of all soil samples

Soil samples	Location	Code name
Residential area	Suryavinayak	R_S
	Umakunda (Mitrapark)	R_{Mu}
	Mulpani	R_M
	Hanumanthan (Anamnagar)	R_H
	Koteshwor	R_K
River site	Bagmati	Ri_B
	Dhobikhola	Ri_D
Agricultural farm	Suryabinayak	A_S
	Mulpani	A_M
	Tarakeshwor	A_T
Dumping site	Teku	D_T
	Ratopul	D_R
Forest area	Suryabinayak	F_S
	Mulpani	F_M
	Nagarjun	F_N
Industrial area	Bhaktapur	I_{Bkt}
	Balaju	I_B

A total of 17 isolates were identified as *Bacillus* spp on the basis of microscopic characteristics and biochemical tests. The characteristics of the isolates is detailed in table 2. All the Gram positive, catalase positive, motile and spore forming isolates were positive in their proteolytic activity.

Table 2: Characterization of isolates

Isolate No.	Catalase	Oxidase	Spore Staining	Motility	Gelatinase
R_S	+	+	+ (central, oval)	+	+
R_{Mu}	+	+	+ (central, oval)	+	+
R_M	+	+	+ (central, oval)	+	+
R_H	+	–	+ (sub-terminal, oval)	+	+
R_K	+	+	+ (central, oval)	+	+
Ri_B	+	+	+ (sub-terminal, oval)	+	+
Ri_D	+	+	+ (sub-terminal, oval)	+	+
A_S	+	–	+ (sub-terminal, oval)	+	+
A_M	+	–	+ (sub-terminal, oval)	+	+
A_T	+	–	+ (sub-terminal, oval)	+	+
D_T	+	–	+ (sub-terminal, oval)	+	+
D_R	+	–	+ (sub-terminal, oval)	+	+
F_S	+	–	+ (sub-terminal, oval)	+	+
F_M	+	+	+ (central, oval)	+	+
F_N	+	+	+ (central, oval)	+	+
I_{Bkt}	+	–	+ (sub-terminal, oval)	+	+
I_B	+	+	+ (sub-terminal, oval)	+	+

The protease enzyme prepared by fermentation with all primarily screened isolates showed increased activity with partially purified extract than the crude extract indicating that the increase in zone of hydrolysis after partial purification is statistically significant. On average, the partially purified samples exhibited 3.18 mm larger zone of hydrolysis than crude samples. (Table 3).

Table 3: Zone of hydrolysis of crude and partially purified protease extract from all samples (t = -9.8182, df = 16, mean difference = -3.18)

Sample	Crude (mm)	Partially_Purified (mm)	Increase (mm)	% Increase	P-value (t-test)
R_S	28	32	4	14.29%	
R_{Mu}	27	30	3	11.11%	
R_M	24	25	1	4.17%	
R_H	27	31	4	14.81%	
R_K	26	30	4	15.38%	
Ri_B	25	30	5	20.00%	
Ri_D	20	22	2	10.00%	
A_S	23	26	3	13.04%	
A_M	24	30	6	25.00%	3.54×10^{-8}
A_T	23	25	2	8.70%	
D_T	18	20	2	11.11%	
D_R	20	24	4	20.00%	
F_S	18	20	2	11.11%	
F_M	20	22	2	10.00%	
F_N	20	22	2	10.00%	
I_{Bkt}	20	24	4	20.00%	
I_B	20	24	4	20.00%	

Similarly, all the *Bacillus* spp isolated from residential area, agricultural area, river bank, industrial area, forest area and dumping site showed a zone of hydrolysis greater than 19 mm (Figure 1).

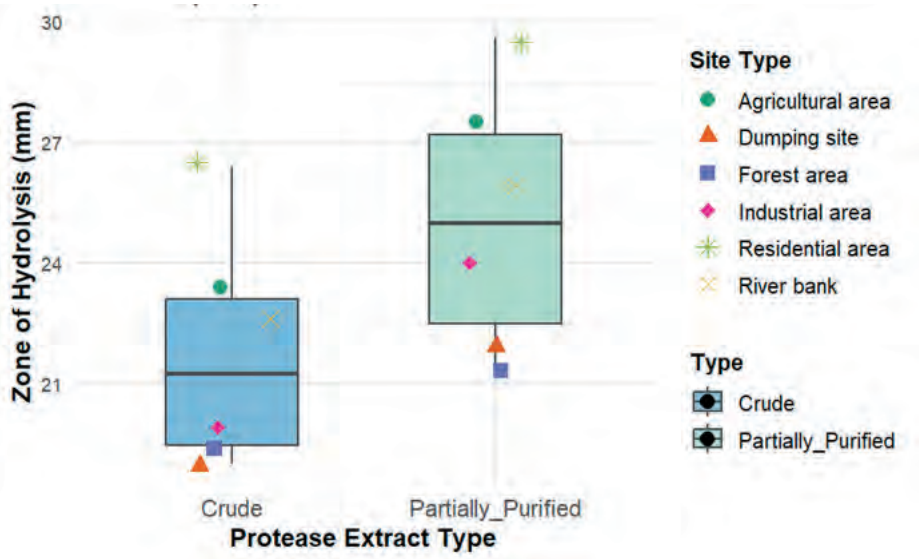

Figure 3: Zone of hydrolysis by crude and partially purified protease enzyme across different sites

All seventeen proteolytic *Bacillus* isolates showed notable anti-pathogenic activity against five different clinical isolates - Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus* spp (Table 4).

Table 4: Antipathogenic activity of different proteolytic isolates

Isolates	Zone of inhibition (in mm)				
	MRSA	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Streptococcus* spp
R_S	20	23	18	19	24
R_{Mu}	18	20	16	17	21
R_M	19	22	17	18	22
R_H	22	25	19	21	25
R_K	21	24	18	20	24
Ri_B	17	19	15	16	20
Ri_D	16	18	14	15	19
A_S	18	21	17	18	22
A_M	19	22	17	19	23
A_T	17	20	16	17	21
D_T	15	17	14	15	18
D_R	14	16	13	14	17
F_S	20	23	18	19	24
F_M	21	24	19	20	25
F_N	22	25	18	21	25
I_{Bkt}	16	18	15	16	20
I_B	17	19	16	17	21

DISCUSSION

The 17 *Bacillus* isolates were selected based on their thermotolerant properties and were identified as Gram-positive, catalase-positive, motile, and spore-forming bacteria. All of them demonstrated proteolytic activity. These characteristics were consistent with *Bacillus* spp, which are widely recognized for their ability to survive in diverse environments, including those with varying temperatures and stress conditions (Parvez et al., 2020). Notably, all isolates were positive for protease production, aligning with findings from previous studies that highlight the proteolytic capabilities of *Bacillus* species, which are essential for various industrial applications, including food processing, detergents, and leather (Bhalerao et al., 2019).

The morphological features of the isolates varied, with some showing central oval spores (e.g., R_S, R_{Mu}, R_M), while others exhibited sub-terminal oval spores (e.g., R_H, R_K, Ri_B), a feature commonly observed in *Bacillus* species (Logan & De Vos, 2015). This variability may reflect the ecological adaptations of these isolates to different environmental conditions across the sampled sites. For instance, differences in spore location could indicate genetic diversity within *Bacillus* populations, a finding that warrants further genomic exploration to better understand their ecological and functional differentiation (Singh et al., 2024).

Proteolytic activity is a key feature of *Bacillus* spp, enhancing their industrial applications. This study showed that all isolates exhibited notable protein hydrolysis, with partially purified protease extracts demonstrating increased activity compared to crude extracts (Table 3). This suggests that the proteases produced by these strains can be effectively concentrated and refined for enhanced industrial utility (Contesini et al., 2018). The increase in zone of hydrolysis upon partial purification ranged from 4% to 25%, with the highest increase observed in isolates from the agricultural farm (A_M) and river bank (Ri_B), indicating that certain environmental factors may enhance protease activity (Razzaq et al., 2019).

The variation in protease activity among isolates could be linked to specific environmental conditions at the sampling sites. For instance, isolates from residential areas exhibited higher proteolytic activity than those from industrial or forest sites, possibly due to differing nutrient availability and microbial competition (Saggu & Mishra, 2019). Such environmental gradients could influence the metabolic pathways of *Bacillus* spp, leading to differential enzyme production (Liu et al., 2020).

In examining site-specific proteolytic activity (Table 4), isolates from residential areas showed the highest average proteolytic activity (29.6 mm for partially purified extracts), followed by agricultural areas (27.6 mm). River bank and industrial areas had moderate activity, while forest and dumping sites exhibited the lowest proteolytic zones. These differences could be attributed to the organic content and microbial diversity in the soil of each sampling site (Radhakrishnan et

al., 2017). Residential and agricultural areas likely have higher organic matter and microbial abundance, enhancing protease production. Conversely, dumping and industrial areas may present polluted environments with limited nutrient availability, restricting *Bacillus* growth and protease production (Saggu & Mishra, 2019).

The antipathogenic activity of the *Bacillus* isolates was evaluated against pathogenic bacteria, including Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus* spp All isolates demonstrated significant antipathogenic activity, with inhibition zones ranging from 14 mm to 25 mm. This is consistent with studies highlighting the antimicrobial potential of *Bacillus* spp, particularly against multi-drug resistant pathogens (Banerjee et al., 2018; Molla et al., 2019). Isolates from residential and agricultural areas, such as R_S, R_H, and F_M, showed the most potent inhibition, possibly due to higher proteolytic activity and the presence of antimicrobial compounds like bacteriocins or lipopeptides (Abriouel et al., 2011).

Interestingly, isolates from industrial areas, which exhibited lower proteolytic activity, also showed reduced antimicrobial activity, suggesting that environmental stressors might impact both proteolytic and antimicrobial potential (LeBlanc et al., 2020). Conversely, isolates from forest areas (F_N, F_S) exhibited strong inhibition against pathogenic bacteria, suggesting production of a broader range of antimicrobial substances (Moldes et al., 2020).

Proteolytic and antimicrobial activities exhibited by the *Bacillus* spp isolated in this study suggest their potential for various biotechnological applications. The enzymes produced could be valuable in industrial processes requiring protein degradation, such as in the production of detergents, leather, or food products (Bhalerao et al., 2019). Moreover, the demonstrated antimicrobial activity, particularly against drug-resistant pathogens like MRSA and *Klebsiella pneumoniae*, highlights the potential of these *Bacillus* strains in developing novel antimicrobial agents (Schillaci et al., 2020). Given the rising concerns about antibiotic resistance, the use of *Bacillus* spp as a source of natural antimicrobial compounds could offer a promising alternative to conventional antibiotics (Gull et al., 2020).

CONCLUSION

This study identified 17 *Bacillus* spp from diverse soils in Kathmandu Valley, demonstrating strong proteolytic and antimicrobial activities. High-performing isolates such as A_M, R_S and R_H showed notable enzyme production and pathogen inhibition. These results highlight the biotechnological potential of soil-derived *Bacillus* spp and warrant further investigation to optimize their use in industrial and antimicrobial applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Tribhuvan University Journal of Microbiology

ORIGINAL RESEARCH

Bacteriological Profile and Antibiotic Subsceptiblity Patterns of Asymptomatic UTI among Female Students of R.R.M Campus Janakpurdham, Madhesh Pradesh, Nepal

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ABSTRACT

Objective: The primary goal of this study was to identify common pathogenic microorganisms and their patterns of antibiotic susceptibility from asymptomatic UTI cases.

Methods: A Total of 151 mid-stream urine samples were collected from female students for bacteriological identification and antimicrobial susceptibility testing. Conventional biochemical tests identified the isolates, and their antibiotic susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Results: Of the 151 samples analyzed, 30.46% exhibited significant bacterial growth, with *Staphylococcus aureus* being the most prevalent (30.43%), followed by *Escherichia coli* (17.39%) and *Klebsiella pneumoniae* (10.86%). Gram-negative bacterial isolates showed a higher level of resistance to amoxicillin (33%-100%), amoxicillin (60%-100%), amoxicillin-clavunic acid (20%-100%), ceftriaxone (60%-100%), cotrimoxazole (40%-100%), nitrofurantoin (40%-100%), and ciprofloxacin (40%-100%). Piperacillin and Tazobactam, levofloxacin, imipenem, and amikacin were less resistant. Gram-positive bacterial uropathogens showed a high level of resistance to amoxicillin (65%-100%), ciprofloxacin (50%-100%), levofloxacin (50%-100%), and clindamycin (64%-100%). Imipenem, gentamicin, piperacillin, and tazobactam have shown reduced resistance.

Conclusion: Uropathogens were found to be highly prevalent, and bacterial isolates were shown to be highly resistant to routinely recommended medications. In order to prevent an asymptomatic infection from developing into a symptomatic UTI, routine UTI screening, regular health education on the risk of asymptomatic infectious diseases for females in the reproductive age group, and antibiotic susceptibility testing should be implemented.

Keywords: UTI, antibiotic resistance, asymptomatic, Janakpur Dham

INTRODUCTION

Urinary tract infections (UTIs) rank among the most prevalent bacterial infections in medical practice, affecting individuals of all ages and posing significant public health challenges due to their widespread occurrence, potential complications, and the growing issue of antimicrobial resistance (Hooton et al., 1996; WHO, 2025). The clinical spectrum of UTIs ranges from symptomatic infections to asymptomatic bacteriuria (ABU), the latter often remaining undetected due to the absence of clear clinical symptoms, yet it can result in adverse outcomes if left untreated (Changizi Maryam et al., 2014). Adult women are particularly susceptible, with 40–50% experiencing at least one UTI episode in their lifetime, with incidence rising with age and during pregnancy (Geerlings, 2016).

Both community-acquired and hospital-acquired UTIs are primarily caused by Gram-negative bacteria

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such as *Escherichia coli, Klebsiella pneumoniae,* and *Proteus species,* with *Staphylococcus saprophyticus* and *Enterococcus faecalis* also contributing to the infection landscape(Foxman, 2014; Geerlings, 2016). Symptoms typically include painful urination and increased frequency, but a significant number of individuals may have substantial bacteriuria without symptoms, known as asymptomatic urinary tract infection (AUTI) or asymptomatic bacteriuria (ABU) (Foxman, 2014). The asymptomatic nature of ABU, particularly among young adults like college students, complicates timely diagnosis and treatment, potentially leading to complications such as pyelonephritis and the spread of antibiotic-resistant bacteria (Changizi Maryam et al., 2014; Nicolle et al., 2019).

Empirical antibiotic treatment is often initiated before culture results are available, contributing to selective pressures that foster the development and spread of multidrug-resistant (MDR) and extended-spectrum beta-lactamase (ESBL) producing organisms in both community and healthcare settings (Flores-Mireles et al., 2015; Fouad & Boraie, 2016). Variations in resistance patterns across regions underscore the need for localized surveillance to guide empirical treatment decisions and stewardship initiatives (Ronald & Pattullo, 1991). Despite the importance of such data, there is a lack of research on the prevalence and resistance patterns of AUTIs among college students in Nepal and similar settings.

METHODS

Study Design and Population

This study employed a cross-sectional design targeting students enrolled at the R.R.M. Campus in Janakpurdham. The study population consisted of female students without signs or symptoms of urinary tract infection, aged 18 to 35 years.

Sample Collection and Laboratory Analysis

Urine samples were collected from participants who provided consent, using the standard midstream clean-catch technique to reduce contamination. Each sample was analyzed with quantitative culture methods to detect significant bacteriuria (≥10^5 CFU/mL), and bacterial species were identified through established biochemical and microbiological techniques (Foxman, 2014; Ipe, 2013).

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method, with results interpreted based on established clinical breakpoints. The antibiotics tested included commonly prescribed agents for UTIs, such as ampicillin, amoxicillin-clavulanic acid, nitrofurantoin, ciprofloxacin, and trimethoprim-sulfamethoxazole (Institute, 2023; Mandal et al., 2012; Odoki et al., 2019).

Data Analysis

Descriptive statistics were used to determine the prevalence of asymptomatic bacteriuria (ABU) in the study population, stratified by sex, age, and academic faculty. The distribution of bacterial isolates and their resistance patterns was analyzed to elucidate the local epidemiological landscape of the disease.

Ethical Considerations

Informed consent was obtained from all participants. Confidentiality and anonymity were maintained throughout data collection and analysis.

RESULTS

Out of 151 urine samples, 46 (just over 30%) showed bacterial growth, indicating a potential asymptomatic UTI. Another 35 samples (about 23%) had some bacteria, but not enough to be considered as significant bacteriuria. Rest of the 70 samples (over 46%) had no detectable bacteria at all Which accounts nearly half of the students.

Among the isolated bacteria, the Gram-negative bacteria were found to be the highest in number, followed by Gram-positive bacteria (**Table. No. 1**).

Table 1: Finding of Gram stain.

Gram Stain	Total No	Percentage (%)
Gram-Negative Bacteria	24	52.17
Gram-Positive Bacteria	22	47.82
Total	**46**	**100**

The analysis of student growth across faculties reveals a total of 46 students, with the following distributions: B.Ed. and BBA 17.39 % each, led by 8 students, and B.Sc. CSIT 13.04% followed by B. Sc. Microbiology, B.Sc. Science and BBA contribute 5 students (10.86%) (Table No. 2).

Table 2: Positive Growth of Participants by Faculty.

Faculty of Significance	Numberof Positive Growth	Percentage (%)
B.Ed. (21)	8	38.09
BBS (31)	8	38.09
BBA (38)	5	13.15
B.Sc. Microbiology (16)	5	31.25
B.sc CSIT (6)	6	100
B.Sc. SCIENCE (15)	5	33.33
M.B.S(Master Finance) (14)	4	28.57
BIT (8)	3	37.5
BICTE (2)	2	100
Total (151)	**46**	**30.46**

Growth According to Marital Status.

The following are the growth results based on marital status (**Table No. 3**). A total of 6 married students (total married, n=22) showed significant growth, while 40 unmarried students' sample didn't show any significant growth.

Table 3: Growth According to Marital Status.

Martial Status	Significant Growth (%)	Insignificant Growth (%)	No Growth (%)
Married (22)	6 (27.27)	6 (27.27)	10 (45.45)
Un Married (129)	40 (31.00)	29 (22.48)	60 (46.51)
Total (151)	**46 (30.46)**	**35 (23.17)**	**70 (46.35)**

Bacteriological Profile

Staphylococcus aureus emerged as the predominant isolate (30.4%), accounting for the majority of positive cultures, followed by *E. coli* (17.3%), *Klebsiella pneumoniae* (10.8%), *Staphylococcus saprophyticus,* and *Enterococcus faecalis* (Table No. 4).

Table 4: The Bacteria Found in Urine Culture.

Culture Result		
Identification of Bacteria	**Numberof Cases**	**Percentage (%)**
Staphylococcus aureus	14	30.43
Escherichia coli (ESBL Positive)	8	17.39
Klebsiella pneumoniae (SBL Producer)	5	10.86
Coagulase-negative *staphylococci (CoNS)*	4	8.69
Acinetobacter spp	3	6.52
Enterococcus faecalis	3	6.52
Morganella spp	2	4.34
Pseudomonas spp	2	4.34
Pseudomonas aeruginosa	2	4.34
Citrobacter koseri	1	2.17
Escherichia coli	1	2.17
Enterococcus spp	1	2.17
Total	**46**	**100**

Antimicrobial Resistance Patterns

The percentage distribution of resistance patterns of Gram-negative bacterial isolates is shown in Table No. 5. The susceptibility test result for *E. coli* shows that more than 50% isolates were resistant to amoxicillin, cotrimoxazole, ceftazidime, ciprofloxacin, and levofloxacin, while none of them were resistant to piperacillin/tazobactam and imipenem. Most of the Gram-negative isolates were sensitive to the piperacillin/tazobactam and imipenem, while moderately sensitive to other antibiotics. Out of the tested antibiotics, Gram-positive bacterial isolates were highly resistant to ampicillin, amoxicillin/clavulanic acid, co-trimoxazole, and clindamycin (Table No. 6).

Table 5: Percentage distribution of Resistance patterns for each Gram-Negative isolate to commonly used antibiotics.

Antimicrobial Agent	*Citrobacter Koseri* Resistance (%) (N=1) (n)	*E. coli* Resistance (%) (N=9) (n)	*K. pneumoniae* Resistance (%) (N= 5) (n)	*P. aeruginosa* Resistance (%) (N=2) (n)	*Acinetobacter* spp Resistance (%) (N=3) (n)	*Morgenella* spps Resistance (%) (N=2) (n)	*Pseudomonas* spp Resistance (%) (N=2) (n)
AK	0	11.11 (1)	0	0	0	100	0
AMP	100	22.22 (2)	20 (1)	-	0	50 (1)	50 (1)
AMX	100	66.66 (6)	60 (3)	100	33.33 (1)	100	100
AMC	100	33.33 (3)	20 (1)	100	33.33 (1)	100	100
CTR	0	44.44 (4)	100	50 (1)	33.33 (1)	100	100
COT	0	66.66 (6)	40 (2)	50 (1)	33.33 (1)	0	100
CPM	0	55.55 (5)	80 (4)	50 (1)	0	100	0
CFM	0	11.11 (1)	-	0	-	-	-
CIP	0	77.77 (7)	40 (2)	50 (1)	100	0	0
GEN	0	22.22 (2)	20 (1)	100	33.33 (1)	100	0
NIT	100	22.22 (2)	40 (2)	50 (1)	100	0	100
LE	0	66.66 (6)	80 (4)	50 (1)	33.33 (1)	100	0
PIT	0	0	0	0	0	50 (1)	0
IPM	0	0	0	0	0	0	0
AZM	-	-	-	-	33.33 (1)	-	-
CX	-	11.11 (1)	-	-	0	-	-
CD	-	11.11 (1)	-	-	-	50 (1)	-
DO	-	-	-	-	-	0	-
MRP	-	11.11 (1)	-	-	33.33 (1)	0	-
E	-	-	-	-	66.66 (2)	-	-
CAZ	-	88.88 (8)	100	100	33.33 (1)	50 (1)	100
CAC	-	55.55 (5)	20 (1)	50 (1)	0	50 (1)	100
TE	-	-	0	-	33.33 (1)	-	-
P	-	11.11 (1)	20 (1)	0	33.33 (1)	50 (1)	0

Amikacin (AK), Ampicillin (AMP), Amoxicillin (AMX), Amoxyclav (AMC), Ceftriaxone (CTR), Co-Trimoxazole (COT), Cefepime (CPM), Cefixime (CFM), Ciprofloxacin (CIP), Gentamicin (GEN), Nitrofurantoin (NIT), Levofloxacin (LE), Piperacillin (PIT), Imipenem (IPM), Azithromycin (AZM), Cefotetan(CX), Clindamycin (CD), Doxycycline Hydrochloride (DO), Meropenem (MRP), Nevobiocin (NV), Erythromycin (E), Ceftazidime(CAZ), Clavulanic acid (CAC), Tetracycline (TE), Penicillin-G (P)

Table 6: Percentage distribution of Resistance patterns for each Gram-positive isolate to commonly used antibiotics.

Antimicrobial Agent	*Staphylococcus aureus* Resistance (%) (N=14) (n)	*Coagulase - negative staphylococci* Resistance (%) (N=4) (n)	*Enterococcus* spp Resistance (%) (N= 1) (n)	*Enterococcus faecalis* Resistance (%) (N=3) (n)
AK	0	-	0	66.66 (2)
AMP	78.57 (11)	100	-	0
AMX	64.28 (9)	100	100	66.66 (2)
AMC	7.14 (1)	-	0	33.33 (1)
CTR	21.42 (3)	0	0	100
COT	28.57 (4)	-	-	33.33 (1)
CPM	21.42 (3)	-	-	66.66 (2)
CFM	0	-	-	-
CIP	57.14 (8)	50 (2)	100	66.66 (2)
GEN	7.14 (1)	-	0	66.66 (2)
NIT	78.57 (11)	75 (3)	100	0
LE	50 (7)	50 (2)	100	66.66 (2)
PIT	0	-	0	0

Antimicrobial Agent	*Staphylococcus aureus* Resistance (%) (N=14) (n)	*Coagulase - negative staphylococci* Resistance (%) (N=4) (n)	*Enterococcus* spp Resistance (%) (N= 1) (n)	*Enterococcus faecalis* Resistance (%) (N=3) (n)
IPM	0	-	-	0
AZM	57.14 (8)	75 (3)	-	33.33 (1)
CX	92.85 (13)	100	-	-
CD	64.28 (9)	100	-	66.66 (2)
DO	0	0	-	0
MRP	0	0	0	33.33 (1)
E	42.85 (6)	-	-	33.33 (1)
CAZ	7.14 (1)	-	100	-
CAC	-	-	-	33.33 (1)
TE	-	-	-	33.33 (1)
P	7.14 (1)	25 (1)	100	66.66 (2)

Amikacin (AK), Ampicillin (AMP), Amoxicillin (AMX), Amoxyclav (AMC), Ceftriaxone (CTR), Co-Trimoxazole (COT), Cefepime (CPM), Cefixime (CFM), Ciprofloxacin (CIP), Gentamicin (GEN), Nitrofurantoin (NIT), Levofloxacin (LE), Piperacillin (PIT), Imipenem (IPM), Azithromycin (AZM), Cefotetan(CX), Clindamycin (CD), Doxycycline Hydrochloride (DO), Meropenem (MRP), Nevobiocin (NV), Erythromycin (E), Ceftazidime(CAZ), Clavulanic acid (CAC), Tetracycline (TE), Penicillin-G (P)

DISCUSSION

Our study at R.R.M campus in Janakpurdham gives us a really in-depth look at asymptomatic Urinary Tract Infections (UTIs) are a significant health concern among students, with this study revealing that nearly one-third (30.46%) of the 151 participants had confirmed bacterial growth in their urine cultures. It's reassuring that the rest of the participants had no detectable bacteria, possibly due to good hygiene. Also, similar results were obtained. But higher than the study done at Pokhara University, Nepal, 4.12% (Sapkota et al., 2020). It might also be due to the variation in the methodology used, and sexual behavior, as individuals with frequent sexual practices are more exposed to UTI, which is due to ascending infection to the urinary tract from the genital area (Gebremariam et al., 2019).

The study found that UTIs are most prevalent among students aged 20-25 years (66.88%), which aligns with the typical university demographic. Second-year students formed the largest group affected (37.08%), and the issue wasn't confined to any specific academic discipline, with Management (BBA) students showing the highest representation (25.16%). However, the observed prevalence of 30.46% signifies a substantial and often undetected health concern, as such asymptomatic infections can serve as a reservoir for the development and dissemination of antimicrobial resistance, a phenomenon increasingly documented among young adults (Kebede et al., 2025). This prevalence aligns with or even exceeds rates reported in other studies involving young adult populations, for instance, a study reporting a 24.6% overall UTI prevalence among college students, with asymptomatic bacteriuria accounting for a substantial proportion of these cases (Kebede et al., 2025). Further research indicates that inadequate water intake and unsatisfactory toilet habits are strong predictors of UTI, especially among female students, who are particularly vulnerable to these infections, with 60% experiencing an infection at some point in their lives (Vyas et al., 2015). This emphasizes the critical need for targeted health education and prophylactic strategies within university settings to mitigate the long-term sequelae of untreated bacteriuria, including potential renal damage and systemic infections (Tabassum et al., 2021). Given that urinary tract infections are among the most common medical conditions globally, with adult women being 30 times more likely to develop them, early detection and management are paramount to prevent severe morbidities (Abadi et al., 2023).

Among forty-six positive isolates, Initial Gram stain results showed a slight majority of Gram-negative bacteria (52.17%) over Gram-positive bacteria (47.82%). This is generally expected in UTIs, as Gram-negative bacteria, often originating from the GUT, are common culprits.

Unexpectedly, *Staphylococcus aureus* was the most common isolate, found in 30.43% of positive cultures. While it can cause UTIs, it's less common than *E. coli* in community-acquired infections. Its prevalence here might suggest alternative infection routes, such as spread from skin or nasal passages due to hygiene

or specific risk factors. *Escherichia coli* accounted for 21.74% of positive cultures. This finding was contradicted with the study conducted in Indonesia (Agustino Purba et al., 2012), Ethiopia (Gebremariam et al., 2019), and Nepal (Sapkota et al., 2020), where *E. coli* was the predominant isolate. While *Klebsiella pneumoniae* (SBL Producer) was identified in 10.86% of cases, its resistance profile is crucial for effective treatment. On *Acinetobacter* spp (6.52%) often seen in healthcare settings, their presence in community-dwelling students warrants attention. However, *3=J,-anella* spp (4.34%) can cause UTIs, especially in those with urinary tract abnormalities. But *Pseudomonas* spp (including *Pseudomonas aeruginosa* 4.34%) can also be the cause of asymptomatic UTI in some cases. These are notorious for their inherent resistance to many antibiotics. And at last, *Citrobacter koseri* (2.17%) can be another opportunistic Gram-negative pathogen.

The high levels of resistance among Gram-negative isolates to both common and advanced antibiotics are a major concern. This emphasizes the critical need for culture-guided therapy to ensure effective treatment and prevent the spread of multidrug-resistant organisms. *E. coli* (N=9) was found to be resistant with the highest percentage to Ciprofloxacin (77.77%), 66.66% for amoxicillin, and Cotrimoxazole 55.55% for Cefepime and 44.44% for Ceftriaxone. Many Gram-negative isolates showed high resistance to commonly used antibiotics. Understanding antibiotic resistance is crucial for effective treatment, and the findings here are concerning. Similar findings were reported by study done in Nigeria (Okafor & Nweze, 2020), Ethiopia (Tigabu et al., 2020), and Saudi Arabia (Al Youssef et al., 2020).

Staphylococcus aureus (N=14) High resistance to Ampicillin (78.57%), Amoxicillin (64.28%) 0% to Amoxyclav, Imipenem, Meropenem. Coagulase-negative *staphylococci* (N=4) 100% resistant to Ampicillin, Amoxicillin, and Amoxyclav 0% to Imipenem, Meropenem, and Erythromycin. *Enterococcus* spp (N=1) & *Enterococcus faecalis* (N=3). Extensive resistance 0% resistant to Imipenem, Meropenem. *Staphylococcus aureus* showed very high resistance to Cloxacillin (92.85%), strongly suggesting the presence of Methicillin-Resistant *Staphylococcus* aureus (MRSA) strains, which significantly limit treatment options. Similarly, Coagulase-negative *staphylococci* showed 100% resistance to Cloxacillin, indicative of MR.-CoNS. *Enterococcus* spp and *Enterococcus faecalis* demonstrated high resistance to various antibiotics. These resistance rates were found to be similar to a previous study conducted in Ethopia (Tigabu et al., 2020) and Saudi Arabia (Al Youssef et al., 2020).

Possible factors contributing to this resistance include inappropriate use and incorrect administration of these antibiotics, as well as other factors such as strain and geographic variation (Walsh et al., 2023).

CONCLUSION

A recent study on Urinary Tract Infections (UTIs) among students at R.R.M College revealed a significant health challenge, with roughly one-third of participants affected by these infections. Unlike typical patterns where *Escherichia coli* is the dominant cause, this study found Staphylococcus aureus to be the most frequently isolated bacterium. Notably, a significant proportion of the isolated bacteria exhibited resistance to ceftazidime, as well as penicillin, ampicillin, and amoxicillin.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TUJM Tribhuvan University Journal of Microbiology

ORIGINAL RESEARCH

Airborne Bacteria and Fungi in the Urban Area of Kathmandu

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ABSTRACT

Objectives: The study was conducted to enumerate and identify the airborne bacteria and fungi in the urban area of Kathmandu using the settle plate method and to determine the antibiotic susceptibility pattern of the identified bacteria.

Methods: The cross-sectional study was done from March to May 2025. A total of 39 air samples were collected from 3 distinct sites: Asan, Kamaladi Ganesh Mandir, and Tri-Chandra Multiple Campus, with 13 samples from each site, using the gravity settle plate method in Nutrient Agar and Potato dextrose agar media at 37˚C for 24 hours for bacteria and 28˚C for 3-5 days for fungi and the research was carried out at the Department of Microbiology, Tri-Chandra Multiple Campus.

Results: The bacterial load ranged from 640 to $4x10^4$ CFU/m^3, while fungal load ranged from $8.4x10^2$ to $4.9x10^3$ CFU/m^3. The dominant bacterial isolates were *Micrococcus* spp (25.17%), followed by *Bacillus* spp (21.79%), *S. aureus* (19.05%), *E. coli* (18.37%), and *Klebsiella* spp (15.65%), whereas *Aspergillus* spp (21.4%) was the most dominant fungi followed by *Fusarium* spp (18.25%), *Penicillium* spp (15.9%), *Cladosporium* spp (15.1%), *Mucor* spp (14.3%), *Rhizopus* spp (8.7%), and *Alternaria* spp (6.4%).

Conclusion: The present study shows that air contains various bacteria and fungi, which can be harmful to human health. It highlights the need to reduce air pollution and raise public awareness.

Keywords: Airborne, Bacteria, Fungi, Kathmandu, Gravity settle plate method

INTRODUCTION

Air is made up of various gases, dust, and droplets of aerosol. Approximately 78% of the various gas types are nitrogen, 21% are oxygen, and 0.04% are carbon dioxide (Manandhar & Sharma, 2018). There are many microscopic organisms in the air, ranging in size from 50nm to 10µm.. Humans and all other living things have survived by developing the ability to effectively manage harmful bioaerosols (Lee, 2011). Humans typically breathe in about 1.5L of air, which means they are consuming roughly 10^6 microbial pieces and cells per day. Hence, bioaerosols are a class of airborne pollutants that include bacteria, fungi, viruses, pollen, and allergens, as well as some secondary metabolites that are mostly linked to particulate matter, including mycotoxins, endotoxins, etc. (Ghosh et al., 2022).

Airborne microorganisms can originate from natural sources such as soil, dust particles, and water droplets, and anthropogenic sources such as human activities, industrial wastes, sewage, overpopulation, and activities of organisms such as birds, animals, and insects. These sources play a significant role in spreading airborne microorganisms and also play a role in environmental and public health (Manandhar & Sharma, 2018). In the atmosphere, microorganisms are common and have a great dispersal range. It is still unclear, therefore, how these airborne microbes differ and what variables affect the microbial dispersal in various areas of anthropogenic activity (Liu et al., 2019). Temperature, relative humidity, light intensity, and wind speed are the four environmental elements that have an impact on outdoor bioaerosol concentrations (Zhu et al., 2003).

Numerous illnesses, including cancer, neurological

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conditions, and infectious and allergic diseases, can result from exposure to these substances. As a result, bio aerosol detection and identification are essential (Rastmanesh et al., 2024). Bioaerosol sampling is a growing but difficult field of study since bioaerosols vary greatly in terms of their sizes, species, biological characteristics, and the conditions needed to detect and measure them (Mainelis, 2019). Even though bioaerosol sampling and analysis techniques have advanced significantly since the late 1800s, the field of bioaerosols is still understudied in comparison to atmospheric chemistry (Xu et al., 2011). We now know very little about the biogeography of the air, despite the potential significance of knowing how life is distributed in the atmosphere. The absence of precise and thorough estimations of numerous crucial aspects of airborne life is one of the research gaps (Womack et al., 2010). Sampling bioaerosols is an interesting and difficult field. Although much progress has been made in recent decades, there is still much to be done, such as creating and modifying tools that will help address the field's challenges (Mainelis, 2019).

In the context of Nepal, many studies were carried out on pathogenic bacteria in indoor areas, dumping sites, and hospital areas, but only a handful of studies were done in outdoor air. Hence, the objective of this study was to examine airborne bacteria and fungi in three different environments of Kathmandu, identify predominant pathogenic bacteria and fungi, and perform an antibiotic susceptibility test of bacterial isolates.

METHODS

This cross-sectional study was conducted in the core areas of Kathmandu during the spring season March-May, 2025. Air samples were collected from three urban areas [Asan area, Kamaladi Ganesh temple, and Tri-Chandra Multiple Campus] of Kathmandu. A total of 39 samples were collected during the course of the study: 13 from the crowded Asan area, 13 from the religious Kamaladi Ganesh temple area and 13 from an academic Tri-Chandra Multiple Campus area. Airborne bacterial and fungal samples were collected using the gravity settle plate method. Here, Petridishes containing culture media such as Nutrient Agar (NA), MacConkey Agar (MA) for bacteria and Potato Dextrose Agar (PDA) for fungi were used as sampling surfaces. Three different culture media were exposed in each of the 3 sampling sites for 15 minutes maintaining the sampling height, i.e., 1m above the ground, to eliminate possible contamination from the surface of the ground to eliminate possible contamination from the surface of the ground. The sampling was conducted twice a week in the afternoon over two months. After collection of samples, the exposed Petridishes were immediately transported to the Microbiology laboratory of Tri-Chandra Multiple Campus inside the ice box maintaining temperature (4°C) and incubated the plates at different temperatures, NA and MA plates at 37°C and PDA plates at 28°C till the bacterial and fungal colonies developed respectively. The number of colonies was counted and converted into CFU/m^3 using Omeliansky's formula. Then, isolated colonies of bacteria and fungi were maintained as pure cultures for further study. Bacteria were identified from colony morphology, Gram staining, Catalase test, Oxidase test, IMVIC test, TSIA, Urease test, and Oxidative/ fermentative tests. Similarly, fungi were identified from colony morphology, Lactophenol cotton blue staining, and microscopic features using reference from Ibrahim et al., 2014.

The Omeliansky formula was used to quantify the airborne bacteria and fungi found on each plate to determine the number of CFU/m^3 (Andriana et al., 2023). Omeliansky's formula is CFU/m^3= $5a\times10^4$ $(bt)^{-1}$

Where,
a= number of colonies on a plate
b= square centimeters of plate size
t= minutes of exposure time

Antibiotic susceptibility test was done by modified kirby bauer method. The antibiotics used were Amikacin (AK 30 mcg), Chloramphenicol (C30), Amoxicillin (AMC 30mcg), Co-trimoxazole (COT 25mcg), Ciprofloxacin (CIP 5 mcg), Cefoxitin (CX 30 mcg), Imipenem (IPM 10mcg), and Ceftriaxone (CTR 30 mcg) (CLSI, 2021).

RESULTS

A total of 39 air samples were collected from 3 different sites in Kathmandu. The highest bacterial load was found in Asan (2.3×10^3-4×10^4 CFU/m^3) with a mean of 2.15×10^4 CFU/m^3, and the lowest was in Tri-Chandra Multiple Campus (6.4×10^2-5.6×10^3 CFU/m^3) with a mean of 2.49×10^3 CFU/m^3. (Table 1). Five genera of bacteria were identified, i.e., *Bacillus* spp, *S. aureus*, *Micrococcus* spp, *E. coli*, and *Klebsiella* spp Among them, *Micrococcus* spp (25.17%) was found dominant, whereas *Klebsiella* spp (15.65%) was least prevalent. (Table 2).

As for fungi, a total of 30 samples were collected from 3 different sites in Kathmandu. Asan has the highest fungal load of 1.4×10^3-4.9×10^3 CFU/m^3 with a mean of 2.6×10^3 CFU/m^3 and the lowest was in Kamaladi Ganesh Mandir (8.9×10^2-2.6×10^3 CFU/m^3) with a mean of 1.9×10^3 CFU/m^3. (Table 3). Seven genera of fungi were identified, i.e., *Penicillium, Aspergillus, Cladosporium, Fusarium, Alternaria, Rhizopus,* and *Mucor*. Among them, *Aspergillus* (21.4%) was found dominant, whereas *Alternaria* (6.4%) was less prevalent. (Table 4).

Table 1: Bacterial load in three different sites

S.N.	Location	Bacterial load	
		Range (CFU/m^3)	Mean (CFU/m^3)
1.	Asan	2.3×10^3 - 4×10^4	2.15×10^4
2.	Kamaladi Ganesh Mandir	7.60×10^2 - 1.2×10^4	3.65×10^3
3.	Tri-Chandra Multiple Campus	6.4×10^2 - 5.6×10^3	2.49×10^3

Table 2: Distribution of total identified bacteria in three sites

S.N.	Bacteria	Kamaladi Ganesh Mandir	Asan	Tri-Chandra Multiple Campus
		Number(%)	Number(%)	Number(%)
1.	*Bacillus* spp	13 (27.66%)	13 (20.97%)	6 (15.79%)
2.	*Staphylococcus aureus*	7 (14.89%)	12 (19.35%)	9 (23.68%)
3.	*Micrococcus* spp	11 (23.40%)	13 (20.97%)	13 (34.21%)
4.	*Escherichia coli*	10 (21.28%)	13 (20.97%)	4 (10.53%)
5.	*Klebsiella* spp	6 (12.77%)	11 (17.74%)	6 (15.79%)
	Identified isolates	**47**	**62**	**38**

Table 3: Fungal load in three different sites

S.N.	Location	Fungal load	
		Range (CFU/m^3)	Mean (CFU/m^3)
1.	Asan	1.4×10^3-4.9×10^3	2.6×10^3
2.	Kamaladi Ganesh Mandir	8.9×10^2-2.6×10^3	1.9×10^3
3.	Tri-Chandra Multiple Campus	8.4×10^2-4.7×10^3	1.96×10^3

Table 4: Distribution of total identified fungi from three sites

S.N.	Fungal genera	Kamaladi Ganesh Mandir	Asan	Tri-Chandra Multiple Campus
1.	*Penicillium*	6(15.38%)	6(13.95%)	8(18.18%)
2.	*Aspergillus*	9(23.08%)	8(18.60%)	10(22.73%)
3.	*Cladosporium*	6(15.38%)	7(16.28%)	6(13.64%)
4.	*Fusarium*	7(17.95%)	8(18.60%)	8(18.18%)
5.	*Alternaria*	3(7.69%)	3(6.98%)	2(4.55%)
6.	*Rhizopus*	2(5.13%)	6(13.95%)	3(6.82%)
7.	*Mucor*	6(15.38%)	5(11.63%)	7(15.91%)
	Identified isolates	**39**	**43**	**44**

Antibiotic Susceptibility Test

Antibiotic susceptibility test was done for *Staphylococcus aureus, E. coli* and *Klebsiella* spp All 9(100 %) *S. aureus* tested was found to be resistant to Cefoxitin (100%). followed by Co-trimoxazole (66.67%), Amoxicillin (45.45%), Chloramphenicol (28.57%), and Amikacin (20%). (Table 5).

Out of total 5 *E. coli*, 60% of *E. coli* were found to be resistance to Ceftiraxone followed by Chloramphenicol (40%). Similarly, 2(50%) isolates of *Klebsiella* were found to be resistant to Ceftriaxone.

Table 5: Antibiotic Susceptibility Test of *Staphylococcus aureus*

Antibiotic discs	Total number of organisms	Sensitive	Intermediate	Resistant
Amikacin (AK30 mcg)	15	11 (73.33%)	1 (6.67)	3(20%)
Chloramphenicol (C 30 mcg)	14	8 (57.14%)	2 (14.29%)	4 (28.57%)
Amoxicillin (AMC 30mcg)	11	3 (27.27%)	3 (27.27%)	5 (45.45%)
Co-trimoxazole (COT 25mcg)	6	2 (33.33%)	-	4 (66.67%)
Ciprofloxacin (CIP5)	9	8 (88.89%)	1 (11.11%)	-
Cefoxitin (CX30)	9	-	-	9 (100%)

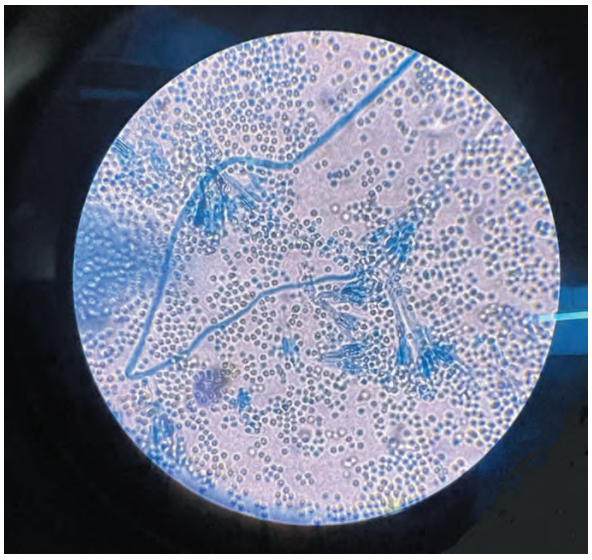

Figure 1: *Penicillium* spp isolated from Kamaldi Ganesh Temple

Figure 2: Fungal load on PDA obtained from Asan

DISCUSSION

For air sampling, three different environments, i.e., crowded Asan, religious Kamaladi Ganesh Mandir, and academic Tri-Chandra Multiple Campus, were chosen and samples were collected from the outdoor air of each site by the gravity settle plate method. The environmental factors, such as temperature and relative humidity, were taken during the sampling period. The temperature ranged from 20-26 °C while the humidity ranged from 32-72%. The lowest bacterial and fungal concentration was found in Tri-Chandra Multiple Campus i.e. ($6.4x10^2$CFU/m^3), ($2.6x10^3$ CFU/m^3) respectively. Similarly, the highest bacterial and fungal concentration ($4×10^4$ CFU/m^3 and $4.9×10^3$ CFU/m^3 respectively) was found from the crowded market Asan site. This is probably due to the market places, frequent movement of people, vehicles, all of which disrupt biological matter and dust. According to a study by Ogah et al., (2023), among the bacterial population in densely populated areas, the marketplaces were found to have a comparatively greater bacterial population. Bariga market had the highest bacterial population, which ranged from 140000 to 440000 CFU/m^3 and the lowest in a garage which ranged from 24600 to 28300 CFU/m^3 indicating that market areas have comparatively higher bacterial count among the public places.

Bacillus spp, *Staphylococcus aureus, Micrococcus* spp, *E. coli,* and *Klebsiella* spp were commonly found bacteria from the outdoor air of Kathmandu during the spring season. According to a study in an urban environment, the most frequently isolated bacteria were *Micrococcus* (41%), *Staphylococcus* (11%), and *Aerococcus* (8%) among the other 19 different genera (Mancinelli & Shulls, 1978). The presence of coliforms like *E. coli* and *Klebsiella* spp is an indicator of faecal contamination (Khan & Gupta, 2019). Here, the high concentration of *E. coli* (21%) and *Klebsiella* spp (13%) were found in religious sites. This suggests the contamination of faeces around religious sites. So, proper sanitation procedures should be implemented to reduce faeces contamination in the religious sites.

S. aureus was found to be 88.89% sensitive to

Ciprofloxacin and 100% resistant to Cefoxitin. Hence, urgent action needs to be taken to improve the quality of the air. According to the study by Kabir et al., (2016), *Staphylococcus aureus* isolates were subjected to antibiotic susceptibility tests and among the isolates, 18.75% showed resistance to Amoxicillin. Whereas none of the isolates showed resistance to Amikacin and Ciprofloxacin. The findings of this study were found to be similar to our study. According to a study in Saudi Arabia, the *E. coli* isolates isolated from outdoor air were 100% sensitive to Imipenem, Amikacin, and resistant to Ceftriaxone (Aabed et al., 2021). These findings are similar to the results of this project.

Median numbers of culturable fungi in Austria varied across environments and ranged from 3.5×10^2 to 4.7×10^3 CFU/m^3, and were usually higher in metropolitan areas than in rural and hilly areas (Haas et al., 2023). Moreover, Sabariego-Ruiz et al., (2000) reported moderate urban atmospheres with greater human activity often exhibited increased spore counts even in the city of southern Spain. In a study by Nageen et al., (2023), airborne fungal diversity was live-tracked over a full year across several urban regions in Tianjin. *Alternaria* (35%) and *Cladosporium* (18%) were the most abundant, and *Penicillium* and *Aspergillus* had low abundances of 5.6% and 2.8%, respectively.

It is common to find *Aspergillus* and *Penicillium* fungal genera in the control of fungal communities through the air because of their resistant and high sporulation nature within the metropolitan regions, as exhibited in various studies done in different parts across the globe. The most frequently found genera in Tianjin, China, proved to be *Alternaria, Cladosporium, Penicillium,* and *Aspergillus*. It was found that *Penicillium* is extensively distributed in most cities and can be hazardous to the respiratory system, such as allergies and asthma (Al-Shaarani et al., 2024).

CONCLUSION

Bacillus spp was the most common bacteria in the air, whereas *Aspergillus* spp was the most common fungi from the three sites we studied. The market's air had a notably higher concentration of microorganisms than the religious and academic sites.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Tribhuvan University Journal of Microbiology

ORIGINAL RESEARCH

Exploring *Bacillus* spp Protease Activity in Slaughterhouse Soils

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ABSTRACT

Objective: To correlate protease-producing *Bacillus* spp with physicochemical parameters from slaughterhouse soils.

Methods: In this study, eight soil samples were collected from three slaughterhouses located in the Kathmandu Valley and designated as $K_{1, 2}$, L_{1-3}, and B_{1-3}. Sterile methods were employed to collect the samples, and their physicochemical properties, including temperature, pH, moisture, electrical conductivity, alkalinity, and nutrients, were analyzed. This was followed by enrichment and isolation of protease-producing *Bacillus* spp.

Results: The collected soils were pale to neutral grey and had alkaline pH. The soils contained moderate water, up to 14%. Even with low organic matter, it had high nitrogen levels. This study isolated *Bacillus* spp that produce proteases and measured the specific protease activity.

Conclusion: Protease-producing *Bacillus* spp found in soils are directly proportional to the nutrients, especially in soils with higher nitrogen levels, which tend to have a greater abundance of protease bacteria. This indicates that soil fertility influences bacterial protease functions.

Keywords: Chemical properties, Nitrogen-rich environments, Physical properties, Protease-producing *Bacillus* spp, Slaughterhouse soil.

INTRODUCTION

Physicochemical properties, including temperature, pH, moisture, nutrient availability, and trace elements, significantly influence bacterial growth, metabolism, and community structure, as these parameters determine enzyme kinetics, membrane stability, and the solubility of nutrients. Optimal temperatures enhance metabolic rates, while extremes will denature enzymes or slow activity. Similarly, pH, electrical conductivity, moisture content, and alkalinity all influence enzyme function and the bioavailability of nutrients and metals (Lu et al., 2025, Yang et al., 2024).

Slaughterhouse soils represent a specialized habitat due to high inputs of animal-derived organic matter (including tissues, blood, and bones) and frequent fresh substrate additions. These soils are often enriched in trace elements, such as copper (Cu^{2+}), iron (Fe^{3+}), and zinc (Zn^{2+}), which act as cofactors for many enzymes; however, these metals could also be inhibitory at high concentrations. The continuous supply of diverse organic substrates supports a wide range of proteolytic, lipolytic, anaerobic, and facultative organisms. It encourages the evolution or enrichment of metabolic pathways for degrading complex animal-derived compounds (Yazdankhah et al., 2014). Microbial interactions in this environment form complex ecological networks that differ from those in typical agricultural soils, producing distinct community structures and functions (Kiprotich et al., 2025, Kracmarova-Farren et al., 2024). Although slaughterhouse soils are rich in microbial diversity, isolating and characterizing these microbes presents

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several research challenges (Alidoosti et al., 2024, Stefanis et al., 2013). The most common bacteria in slaughterhouse soil belong to proteolytic and lipolytic species, which thrive on protein- and fat-rich substrates, such as *Bacillus subtilis*, *Pseudomonas aeruginosa,* and *Clostridium* spp Secondly, anaerobic and facultative anaerobes such as *Bacteroides* spp, *Enterobacter* spp, and *Methanobacterium* spp Thirdly, *Actinomycete* spp and soil-dwelling decomposers contribute to organic matter (OM) cleavage and antibiotic production, including *Streptomyces* spp and *Nocardia* spp Finally, slaughterhouse soils may harbor zoonotic or opportunistic pathogens such as *Listeria monocytogenes, Salmonella* spp, and fecally contaminated *Escherichia coli* (Khan & Rao, 2019).

Extensive research has been conducted on bacterial contamination, patterns of antibacterial resistance, and the associated risks to public health in slaughterhouse soils and meat-handling areas (Bantawa et al., 2018, Singh, 2022). However, exploring isolates that produce proteases with broad industrial applications is an exciting opportunity for further discovery. Therefore, this study aims to isolate protease-producing *Bacillus* spp that can be applied to industries, as slaughterhouse soil is a rich source of nutrients for bacterial growth.

METHODS

Materials

The laboratory experiments used distilled water and analytical-grade chemicals. All chemicals used in this study were purchased from HiMedia Laboratories Pvt. Ltd., India.

Sampling Sites and Slaughterhouse Soil Samples Collection

Eight soil samples ($K_{1, 2,}$ L_{1-3}, and B_{1-3}) were collected from three slaughterhouses in the Kathmandu Valley (27±0.2 °C) using sterile techniques at a depth of 15 cm. The samples were transported to the Environmental Engineering laboratory (Pulchowk Engineering Campus), dried, and passed through 2.5 mm and 2 mm sieves for physical, chemical, and biological analysis (Felde et al., 2020).

Physicochemical Analysis of Soil Samples

Different physicochemical parameters, such as colour and moisture content (Little et al., 1998), were detected. Additionally, chemical analyses were conducted, including pH (Al-Busaidi et al., 2005), electric conductance (EC) (dS/m) (Qi et al., 2020), and total alkalinity (Dhoke, 2023). Similarly, total organic matter (%) (Dewis & Freitas, 1970, WRD, 2009) and total nitrogen (TN) (%) (Bremner & Mulvaney, 1982) were also estimated.

Enhancement and Selection of Isolates Producing Extracellular Protease

A modified protocol of Laba et al., (2018) was carried out for screening, in which 1 g sample was mixed with 10 mL of sterilized distilled water and spread on skim milk agar plates (7.5% skim milk powder, 1.5% agar, 0.5% peptone, 0.25% yeast extract, and 0.1% glucose). The plates were incubated (32 °C) until visible growth was observed. Single colony with the surrounding highest clear halo zone was selected (Hyseni et al., 2020). The isolated colonies were inoculated on sterile gelatin plates (1% gelatin, 1% peptone, 1% yeast extract, 0.5% sodium chloride, and 1.5% agar) and incubated for 24 h. After flooding with saturated ammonium sulfate, clear zones were detected around the gelatin (Medina & Baresi, 2007). The morphological and cultural characteristics of the subcultured organisms were used for phenotypic identification of colonies (Hyseni et al., 2020), followed by microscopic examination and biochemical tests (Shen et al., 2022).

Specific Activity of Protease

The highest protease-producing isolates were analyzed using a protease-specific assay (Sigma's non-specific protease activity assay) with 0.65% casein as the substrate in potassium phosphate buffer (pH 7.5) (Cupp-Enyard, 2008). The assay conditions were incubated for 10 min at 37 °C, and were terminated with 10% trichloroacetic acid. Tyrosine was measured using Folin and Ciocalteu's phenol reagent (Hyseni et al., 2020). The total protein content was assessed using the Bradford method (Bradford, 1976).

Statistical Analysis of Data

Results were obtained from three independent experiments and expressed as the mean±standard error (SEM). The data were analyzed statistically in GraphPad Prism 8.0.2, and graphs were plotted in Origin 2019 and GraphPad Prism 8.0.2. The ordinary one-way ANOVA ($p<0.05$) was applied to determine a significant difference among intervals in the same groups. Furthermore, Pearson's correlation (r) was used to investigate the relationships between the physicochemical and/or nutritional parameters with protease-producing *Bacillus* spp.

RESULTS

Physicochemical and Nutritional Diversity of Slaughterhouse Soils across the Valley

Soil samples collected from slaughterhouse areas in Kathmandu (K_{1-2}), Lalitpur (L_{1-3}), and Bhaktapur (B_{1-3}) revealed a broad scope of physical and chemical characteristics. These samples showed variation in physical properties such as colour, ranging from pale to neutral grey; alkaline pH levels, as shown in Figure 1a, indicating that the soils tend to favour alkaline conditions; and moisture content (up to 14%), detailed in Figure 1b, which influences microbial activity and soil stability. The study analyzed the chemical parameters, including EC (Figure 2a) and alkalinity (Figure 2b).

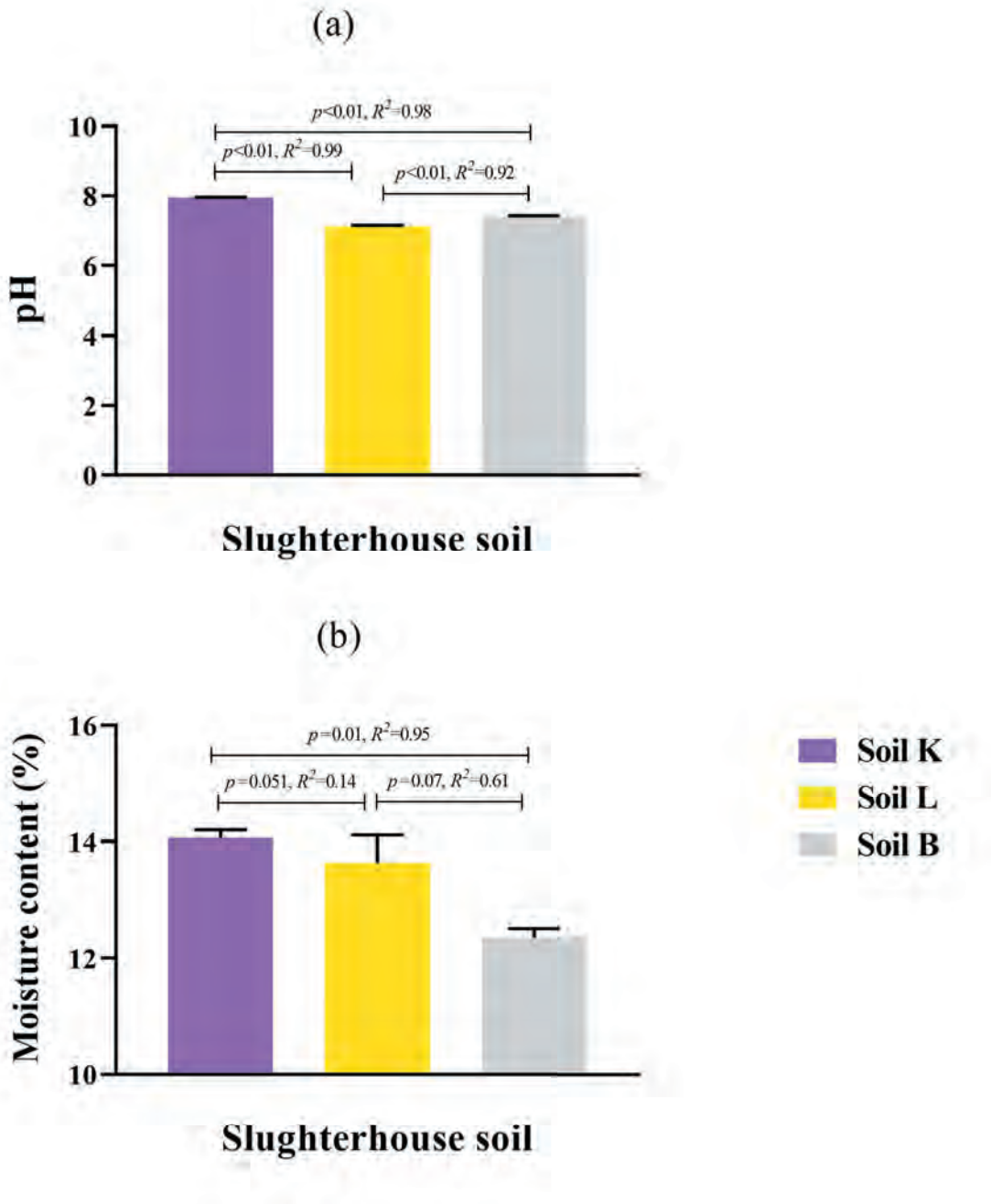

Figure 1: Variation in physical properties of slaughterhouse soil samples. (a) pH and (b) Moisture content. Note: Data represent the mean±standard error (n=3). Statistical significance was determined by ordinary one-way ANOVA ($p<0.05$).

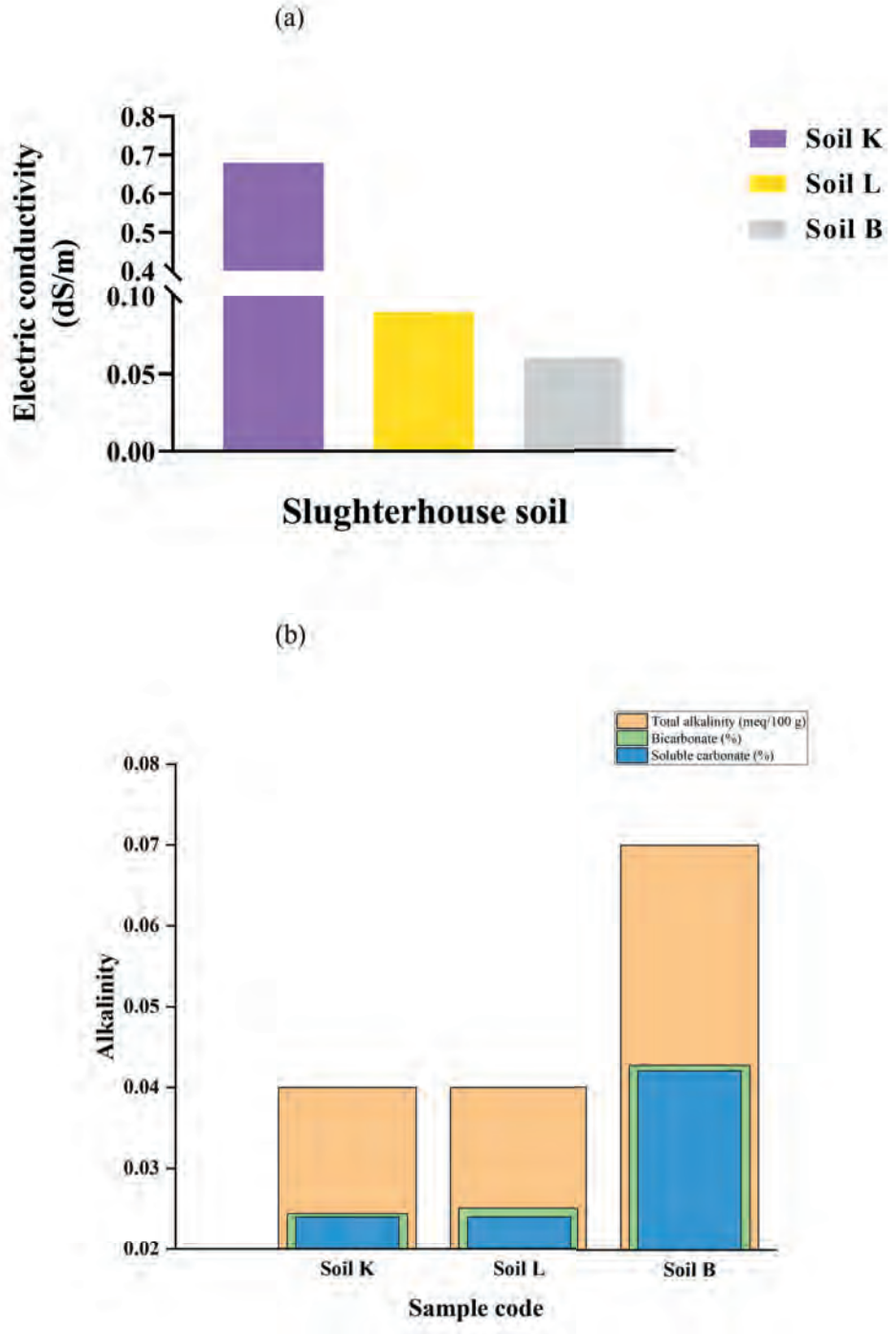

Figure 2: Variation in chemical properties of slaughterhouse soil samples. (a) Electric conductivity and (b) Alkalinity. Note: Data represent the mean±standard error (n=3). Statistical significance was determined by ordinary one-way ANOVA (*p*<0.05).

These variations in physicochemical parameters contributed to the total carbon (TC) (%) and total nitrogen (TN) (%) content, as shown in Figure 3.

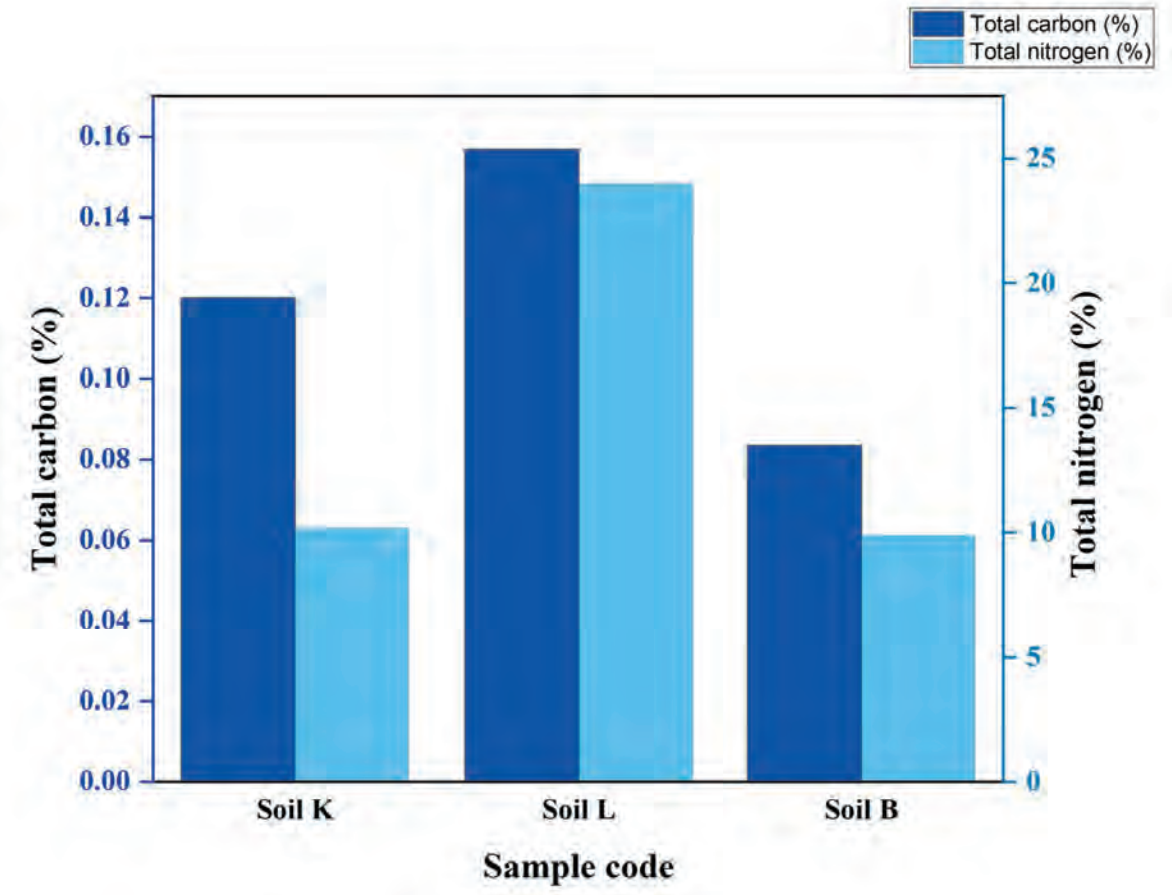

Figure 3: Variation in nutrient contents of slaughterhouse soil samples. Note: Data represent the mean±standard error (n=3). Statistical significance was determined by ordinary one-way ANOVA ($p<0.05$).

Distribution of Gram-Positive Bacterial Isolates Exhibiting Protease Activity

After subculture, 25 bacterial isolates were observed to be Gram-positive and rod-shaped (Figure 4), exhibiting biochemical characteristics consistent with those of *Bacillus* spp. The study included 28% isolates from Kathmandu, 40% from Lalitpur, and 32% from Bhaktapur, as depicted in Figure 5a. The isolates exhibited protease activity (0.25-482.20 U/mL, y = 4000x-182.67, $R^2 = 1$) (Figure 5b) and were subsequently stored in glycerol stock.

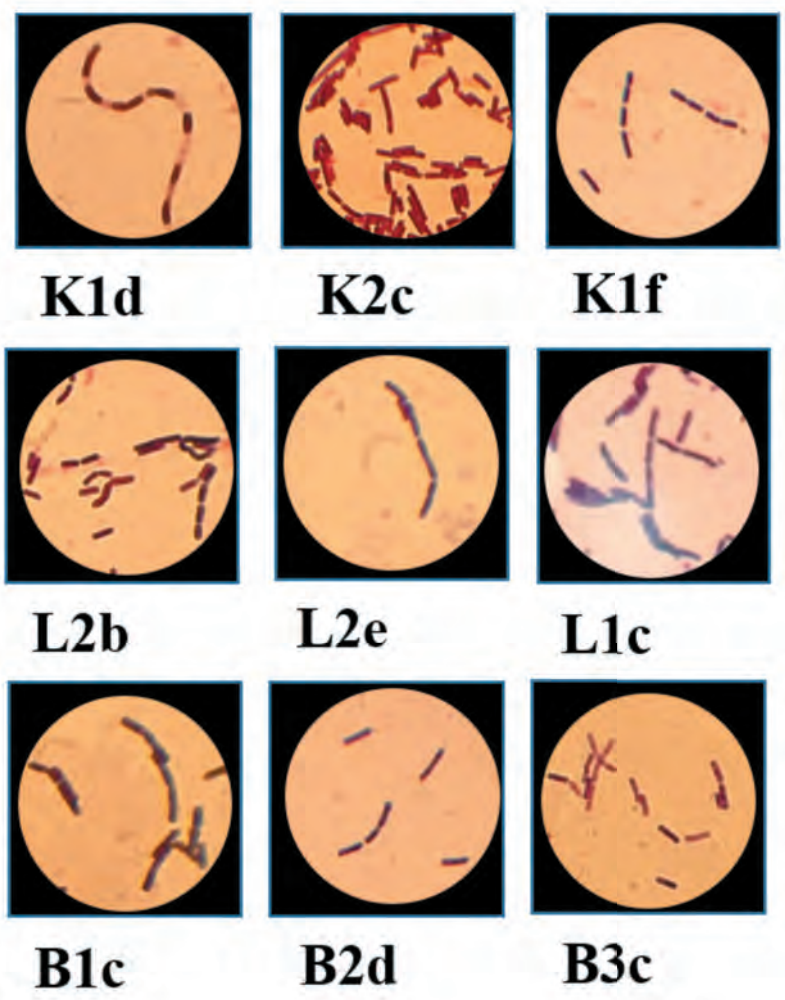

Figure 4: Gram staining of selected protease-producing *Bacillus* spp from slaughterhouse soil samples.

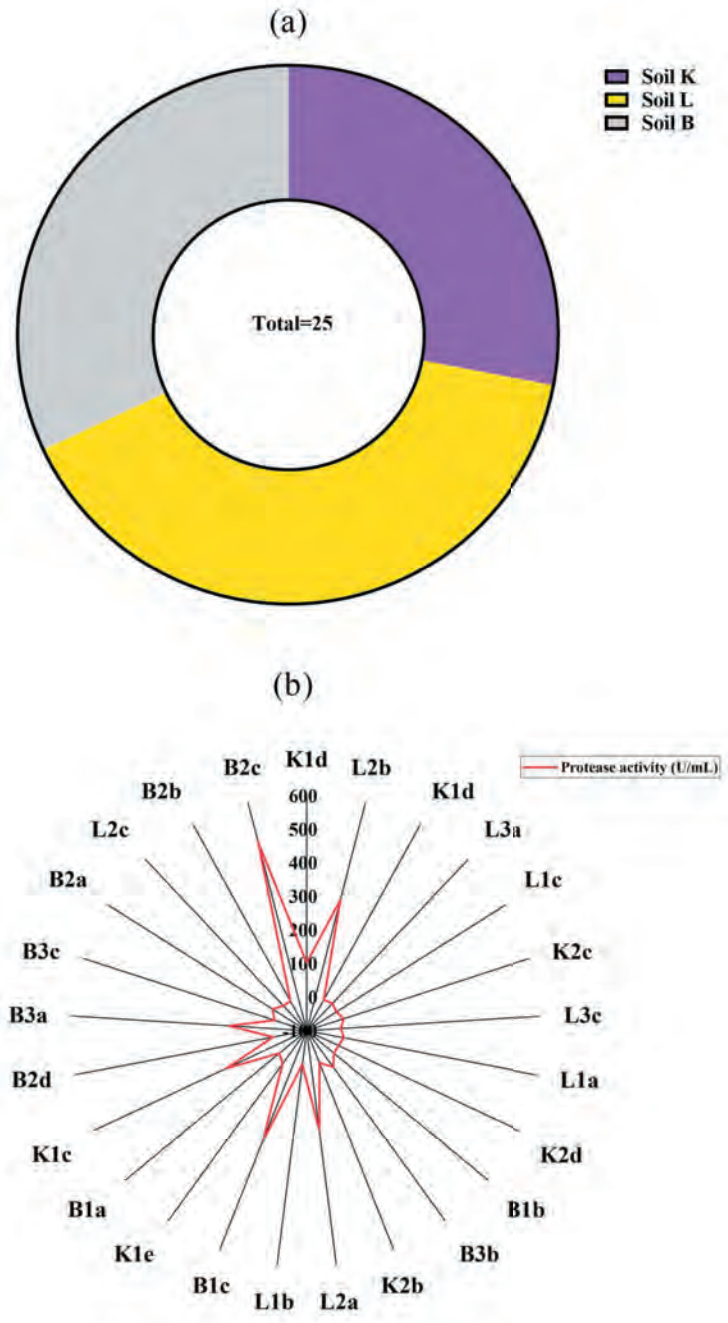

Figure 5: Isolates exhibiting protease activities. (a) Different locations of Kathmandu Valley and (b) Specific protease activity (U/mL).

Association between Slaughterhouse Soil Parameters and Protease-Producing *Bacillus* spp

The study suggested that the individual physical and nutritional parameters have a favourable association; however, the chemical parameters, such as electric conductivity and total alkalinity, have an unfavourable association. The physical and chemical parameters also have a favourable association. Additionally, the moisture content of slaughterhouse soil influences its nutritional content; however, the nutritional content was unaffected by the pH of the slaughterhouse. Electric conductivity exhibits favourable and unfavourable associations with organic carbon and total nitrogen contents. Interestingly, the study observed that all parameters highly influence the protease-producing *Bacillus* spp in the respective soils, as shown in Table 1.

Table 1: Correlation between different parameters of slaughterhouse soil samples

Parameters	pH	MC (%)	EC (dS/m)	TA (meq/ 100 g)	OC (%)	TN (%)	Isolates
pH	1	0.42^{3*}	0.93^{5*}	0.18*	-0.33$^{\#}$	-0.75$^{4\#}$	0.60^{4*}
MC (%)	0.42^{2*}	1	0.73^{4*}	0.97^{5*}	0.72^{4*}	0.29^{2*}	0.98^{5*}
EC (dS/m)	0.93^{5*}	0.73^{4*}	1	-0.54$^{3\#}$	0.04*	-0.44$^{3\#}$	0.86^{5*}
TA (meq/ 100 g)	0.18*	0.97^{5*}	-0.54$^{3\#}$	1	-0.87$^{5\#}$	-0.52$^{3\#}$	0.8^{5*}
OC (%)	-0.33$^{\#}$	0.72^{4*}	0.04*	-0.87$^{5\#}$	1	0.88^{5*}	0.55^{3*}
TN (%)	-0.75$^{4\#}$	0.29^{2*}	-0.44$^{3\#}$	-0.52$^{3\#}$	0.88^{5*}	1	0.89^{5*}
Isolates	0.60^{4*}	0.98^{5*}	0.86^{5*}	0.8^{5*}	0.55^{3*}	0.89^{5*}	1

Note: One-tailed Pearson correlation (p>0.05) where '*': Very low but positive association, '^{2*}': Low but positive association, '^{3*}': Moderately and positive association, '^{4*}': Highly and positive association, and '^{5*}': Very highly and positive association, '$^{3\#}$': Moderately but negative association, '$^{4\#}$': Highly but negative association, and '$^{5\#}$': Very highly but negative association.

DISCUSSION

This study concluded that the slaughterhouse soil has an alkaline pH (Figure 1a), in contrast to the study conducted by Fernández-Calviñoand Baath (2010) that observed the pH range of 4.5 to 7.8 was optimal for bacterial growth in the respective soils. Alter in soil pH by ±1-2 units influence the nitrogen cycling genes, indicating functional consequences beyond bacterial population shifts (Xiong et al., 2024).

Additionally, the moisture content (r=0.98, p>0.05) (Table 1) also directly relates to the bacterial growth, its diversity, and enzymatic activities (Bogati et al., 2025, Iovieno & Baath, 2008). However, reducing alkalinity through microbial amendments enhances microbial diversity and enzymatic activity, whereas higher pH and alkalinity suppress microbial growth by altering soil structure and limiting nutrient availability (Lopes et al., 2021, Tian et al., 2025).

Similarly, to previous studies, this study also concluded that the chemical property, such as a moderate increase in EC, directly relates to microbial biomass by improving soil nutrient availability (Feng et al., 2025). Whereas, higher EC levels may enhance bacterial growth, however induce bacterial stress and shift community composition due to salinity (Kim et al., 2016, Lee et al., 2011) (r=0.86, p>0.05) (Figure 2).

This study also observed that the protease-producing *Bacillus* spp were positively correlated with pH, EC, TC, and TN (Table 1). Protease-producing *Bacillus* spp were enhanced in nutrient-rich soils, suggesting that physicochemical properties directly influence the metabolic potential of bacteria. However, a survey by Meena et al., (2020) indicates that bacterial populations positively correlated with pH, EC, TC, and TN, and inversely with EC. This study also observed that TN availability (r=0.89, p>0.05) had a more substantial impact on microbial communities than pH (r=0.6, p>0.05) and OC (r=0.55, p>0.05). A study conducted by Zhang et al., (2024) also showed that nutrient availability had a more substantial impact on microbial communities than pH, and nitrogen addition promoted the growth of *Proteobacteria* spp.

CONCLUSION

Slaughterhouse soils, the natural habitats of the protease-producing *Bacillus* spp, hold promising potential for various industrial applications, and this is a relatively underexplored field. Exploring these isolates can lead to novel discoveries. However, the physicochemical parameters and high nitrogen levels are essential nutrients that support bacterial growth, affecting the inhabitants of these *Bacillus* spp. A broad range of protease-producing *Bacillus* spp was isolated, which have a wide scope in various industries, such as the pharmaceutical sector (development of new drugs and diagnostics), the leather industry for dehairing, detergent manufacturing, and in the food industry as an eco-friendly alternative. However, this study only focused on the broad-spectrum protease-producing *Bacillus* spp, limiting the other enzyme-producing isolates that can be isolated from this environment. Therefore, the future direction should focus on the isolation, detection of different enzymes, and optimization for the maximal enzyme expression and finally extraction for the respective industries. Hence, conducting comprehensive studies to identify, isolate, and better understanding these microbes is critical, which could lead to industrial microbiology and environmental sustainability breakthroughs.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest pertinent to this work.

AUTHOR CONTRIBUTIONS

Designed the study: PB and MA, collected samples: PB, LM, KS, and MA, Laboratory work: PB and LM, data analysis: PB, first draft of manuscript: PB, final version of manuscript: PB, BKD, IPA, and MA.

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ORIGINAL RESEARCH

Evaluation of Antibiotic Resistance Patterns and Biofilm Formation among the Clinical Isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Objectives: To evaluate the antibiotic resistance pattern of *Pseudomonas aeruginosa* isolated from clinical specimen and to detect Metallo beta lactamase producers as well as to accesses their biofilm forming capacity by both qualitative and quantitative analysis.

Methods: The study was conducted in Shree Birendra Hospital, Chhauni, from June to August 2025. The total of 6444 specimens was cultured and isolates of *P. aeruginosa* were subjected to antibiotic susceptibility tests. Metallo beta lactamase producers were identified by modified Hodge and EDTA synergy tests. Biofilm was detected by the Congo Red Agar and Microtiter Plate Assay method.

Results: Out of 671 positive isolates (15.05%) from pus, urine and wound, 101 isolates of *P. aeruginosa* were obtained. The highest rate of distribution was observed in in-patients as well as in the age group of 61-70 years. Among the isolates, high resistance was observed against Aztreonam (65.59%) whereas isolates were most sensitive against Tobramycin (76%). 37 were found to produce Metallo beta lactamase enzyme and almost 46% were MDR. The biofilm isolates accounted for 34 by CRA but MPA detected 100 biofilm producers. The biofilm producers showed high resistance against Aztreonam (59.41%) and Levofloxacin (56.44%). Furthermore, the MBLS were the most resistant against Levofloxacin (28.7%) followed by Aztreonam (27.7%), Cefepime (27.7%), Ceftazidime (25.7%), Imipenem (25.7%) and Meropenem (25.7%). Out of all the isolates, 36 biofilm isolates were highlighted to produce MBL enzyme as well.

Conclusion: *Pseudomonas aeruginosa* was most frequent in sputum and pus samples from inpatients and older patients, with rising resistance to monobactams, fourth-generation cephalosporins, and fluoroquinolones. High rates of MBL production and biofilm formation contributed to marked β-lactam resistance, emphasizing the need for alternative therapeutic strategies.

Keywords: *Pseudomonas aeruginosa*, Metallo beta lactamase, Microtitre plate, Biofilm

INTRODUCTION

Pseudomonas aeruginosa is one of the opportunistic pathogens, recognized for its significance in clinical settings and thrive most in wet surfaces. It is responsible for number of cases of nosocomial and systemic infections including urinary tract infections, respiratory tract infection, dermatitis, bacteremia, soft tissue, bone and joint infection (Mahaseth et al., 2020, Shrestha et al., 2019). Their remarkable ability to resist antibiotics makes infection proliferate especially

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among inpatients with weakened immune systems. Natural resistance to antibiotics by mechanisms like outer membrane permeability, efflux pumps, antibiotic inactivating enzymes, biofilm production, acquisition of resistance genes makes the entry of antibiotics way more critical (Mahaseth et al., 2020, Pang, et al., 2019). The ability of its genome to encode large amounts of regulatory enzymes causes high morbidity rate in cystic fibrosis patients. MDR Gram negative bacteria are generally resistant to broad spectrum antibiotics which make it burdensome to reduce the relapse of infection (Chaudhary et al., 2024).

Metallo Beta Lactamase (MBL) producing *P. aeruginosa* has been clinically significant with a high mortality rate. MBL enzyme inhibits the action of Carbapenem drugs before the drug reaches the PBP targets. The outer membrane porin protein OprD is responsible for quick uptake of Carbapenems whereas in absence, it is resistant to drug (Pang et al., 2019). Its mechanism is followed by the usage of divalent cation like zinc as cofactor that activates the enzyme to hydrolyze the β-lactam rings (Farajzadeh Sheikh et al., 2014). *IMP, VIM, SPM, GIM, NDM* and *FIM* genes encode enzymes capable of hydrolyzing the β-lactam antibiotics. It is usually inhibited by chelating agents like EDTA but not inhibited by clavulanic acid, sulbactam, or by developmental penicillanic acid sulfones and diazabicyclooctanes (Boyd et al., 2020).

The alarming threat of antibiotic resistant microorganisms poses a significant challenge to public health, as these pathogens can easily spread within the population, doubling the risk for treatment. Infection caused by *P. aeruginosa* is a formidable challenge to treat. MBL enzyme heavily involves the changing of the structural compound of Carbapenems which is usually used as Tier 3-4 drugs. Current key players to treat MBL producers is Colistin (>97% susceptibility) and Aztreonam though its activity weakens if the isolates are known to coproduce extended-spectrum β-lactamases (ESBLs) or AmpC enzymes (Boyd et al., 2020). Though as of currently there are strategies to control MBL producers which includes combination of Carbapenem drugs with Monobactams or direct MBL inhibitors.

Biofilm formation is a multi-step complex process involving the transition of bacteria from free-swimming planktonic to sessile form (Rather et al., 2021). The maturation of biofilm after multiplication and formation of microcolony induces antimicrobial resistance alongside acting as a protection from harsh conditions. Dispersion of biofilm further increases the accumulation of biofilm resulting in severity of the infection. The resistance developed by them at the early stage is less but in the later stage, it is prominent which is about 1000 times foldmore (Krishnasamy & Velmurugan, 2024).

This study addresses that gap by systematically evaluating clinical isolates for MBL production and biofilm formation and correlating these phenotypes with multidrug-resistance profiles and specimen/ clinical source. Understanding the prevalence and co-association of MBLs and biofilms will (1) clarify the microbiological drivers of treatment failure, (2) identify high-risk isolate phenotypes that warrant enhanced infection control and stewardship measures, and (3) inform therapeutic strategies—such as targeted combination therapy, use of MBL inhibitors, or biofilm-disrupting adjuncts. By linking phenotypic resistance mechanisms with clinical specimen data and antibiotic susceptibility patterns, this research work has generated actionable knowledge to improve diagnostics, guide empiric therapy, and reduce relapse and mortality from *P. aeruginosa* infections in healthcare settings.

METHODS

Study design, duration and site

A hospital based descriptive cross-sectional study was conducted in the Microbiology laboratory of Shree Birendra Hospital from June to August 2025. Sample collection, processing and biofilm assessment were done in the hospital laboratory.

Inclusion and exclusion criteria

All age groups of both sexes from inpatient and outpatient departments including immunocompromised patients who gave written consent were enrolled in the study. All kinds of samples were included in the study.

Sample types and size

Non-probability consecutive sampling techniques were used for sample collection. Different clinical samples; sputum, pus, wound swabs, urine, fluids, blood, etc, were taken and processed in the laboratory.

Sample collection and processing

A total of 6444 samples were processed including sputum, pus, wound swabs, urine, aspirates, fluids and blood. All samples were collected aseptically in

sterile containers and sent immediately for processing. They were inoculated aseptically on Mac-Conkey agar, Blood agar and Chocolate agar. For urine samples, Cysteine–Lactose–Electrolyte-Deficient (CLED) agar was used. The isolates were identified by standard Microbiological procedure including colony morphology, Gram staining, and Biochemical tests. Only *P. aeruginosa* were taken in the study. Cetrimide agar was used as a selective media for *P. aeruginosa* (Cheesbrough, 2006).

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out by Modified Kirby Bauer disk diffusion method on Mueller Hinton agar and interpretation was done following Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2024). Antibiotics included in the test were Piperacillin/tazobactam (100/10 µg), Ceftazidime (30 µg), Cefepime (30 µg), Imipenem (10 µg), Meropenem (10 µg), Amikacin (30 µg), Tobramycin (10 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Aztreonam (30 µg). Isolates that showed resistance to at least 3 or more antibiotic categories were considered Multi Drug Resistance (MDR) (Magiorakos et al., 2012).

Detection of Metallo Beta Lactamase (MBL) producers

Metallo beta lactamase (MBL) producers were determined by Imipenem-EDTA Disk Method as described by Yong et al., (2002) with modification. Solution of 0.5 M EDTA was prepared by dissolving 186.1 g of disodium EDTA in 1,000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. One Imipenem disc was taken and 10 µL of the prepared solution was added to the disc. The disc was air-dried. On a lawn culture on Mueller Hinton Agar, 2 Imipenem discs, one with 10 µL of EDTA (750 µg) and the other disc without EDTA were placed and incubated overnight. Zone size of ≥7mm in IMP+EDTA was considered an MBL producer (Shukla et al., 2022).

Biofilm detection by Congo Red Agar method

The screening of biofilm production was done by Congo Red Agar method (Freeman et al., 1989). Congo red was prepared as a concentration solution and autoclaved. It was added to the medium when agar is cooled to 55˚C and poured into petri plates (Harika et al., 2020). The isolates were inoculated in the prepared agar and incubated at 37˚C up to 72 hr. Dark black crystalline colonies were considered as strong biofilm strains, darker colonies without dry and crystalline structure were considered as weak positive and pink or red colonies were considered negative respectively (Abdulhaq et al., 2020, Bhatta et al., 2019).

Biofilm detection by Microtiter Plate Assay

The isolates were quantitatively evaluated by Microtiter plate assay (MPA). The isolate was grown overnight in 2 ml of LB broth and diluted in 1:100 in sterile fresh broth. In a 96-well flat-bottomed plate, 200µl of the diluted culture was inoculated and incubated for 24hr at 37˚C. After incubation, the cells were aspirated out and washed with sterile saline to remove free- floating bacteria. This step was repeated 2 to 3 times. The plate was air-dried for 20-30 min, favoring the fixation of the biofilm. Each well was stained by adding 125µl of crystal violet (0.1%) and incubated at room temperature for 10-15 mins. The plate was rinsed and dried. 200µl of 95% ethanol was added to solubilize the crystal violet. The plate was covered with the lid to minimize evaporation and incubated at room temperature for 15-30 mins. The absorbance or the optical density (OD) was measured at 570 nm using 95% ethanol in water as negative control using an ELISA reader (O'Toole 2011, Stepanović et al., 2007).

To evaluate biofilm formation, the average optical density (OD) of each isolate was calculated, and a cut-off value (ODc) was determined. The ODc was defined as the mean OD of the negative control plus three standard deviations, ensuring a reliable threshold for distinguishing true biofilm production from background noise. Isolates with OD values lower than the ODc were classified as non-biofilm producers. Those with OD values between the ODc and twice the ODc were categorized as weak biofilm producers, while values between two and four times the ODc indicated moderate biofilm formation. Isolates with OD values greater than four times the ODc were identified as strong biofilm producers. For the purpose of this study, all isolates classified as weak, moderate, or strong producers were considered biofilm-positive.

Quality Control

*Pseudomonas aeruginos*a ATCC 27853 was used as a routine quality control for MBL producing strains.

Data analysis

All the analysis of the data was done using Microsoft Excel 2016.

Ethical consideration

The study was approved by the Institutional Review

Committee (IRC) of Nepal Army Institute of Health Science (NAIHS) (Reg. No. 1355, 2025).

RESULTS

Bacterial growth in clinical samples:

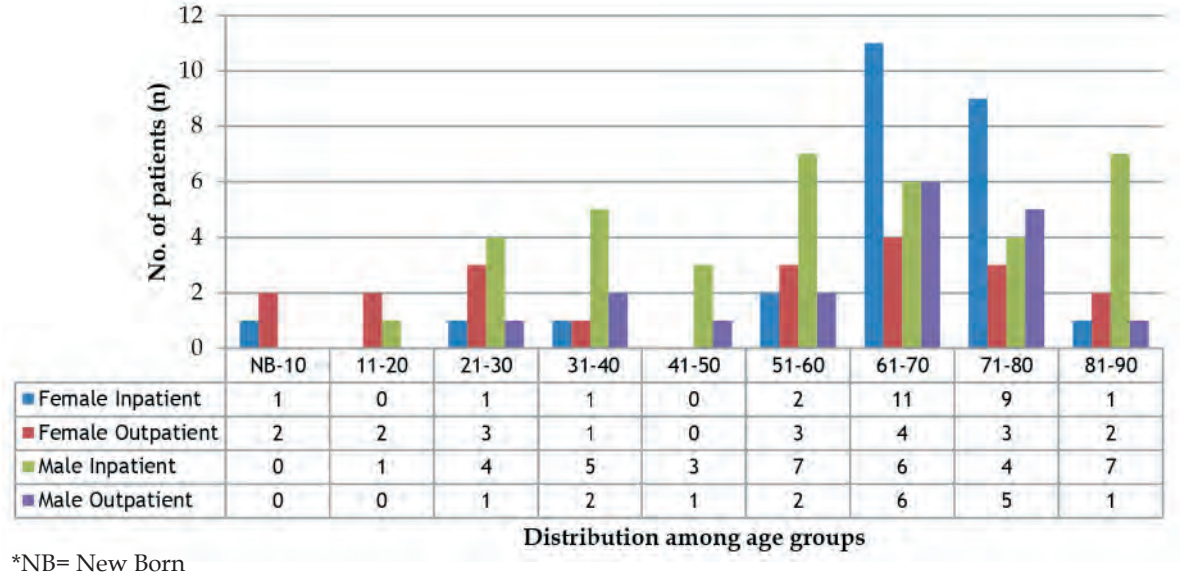
Out of 6444 samples processed, 671 samples were growth positive whereas 101 (15.1%) were positive for *P. aeruginosa*. The maximum number of *P. aeruginosa* was isolated from sputum (n=49), followed by pus (n=19), wound swab and urine (n=9) and other specimens (Table 1).

Table 1: Distribution of *P. aeruginosa* among the clinical specimens

Clinical Specimens	Positive growth	*P. aeruginosa* growth no. (%)
Urine	279	9 (3.2)
Pus	63	17 (27)
Blood	31	1 (3.2)
Sputum	170	49(28.8)
Wound swab	60	9 (15)
BAL fluid	4	1 (25)
Throat swab	1	1 (100)
Tissue	5	2 (40)
Bed sore	5	3 (60)
Aspirated fuid	36	1 (2.8)
Tracheal fluid		7 (19.4)
Intra articular fluid	3	1 (33.3)
EVD tip	4	0
CVP tip	1	0
CSF fluid	1	0
High Vaginal Swab	7	0
Placenta	1	0
Grand Total	**671**	**101(15.1)**

Demographic and Department-Wise Distribution of *P. aeruginosa* among Inpatients and Outpatients

The higher number of *P. aeruginosa* was isolated from inpatients (62.4%) in comparison to outpatients (37.6%). *P. aeruginosa* was isolated more from male inpatients (n=37) than female inpatients (n=26). However, female inpatients of the aged group 61-70 were the largest proportion of all age groups and genders (Figure 1). The statistical analysis demonstrated a significant association in the distribution of isolates between specimen and type of patients ($p<0.05$).



*NB= New Born

Figure 1: Distribution of *P. aeurginosa* among patients of different age groups

Antibiotic susceptibility testing of *P. aeurginosa*

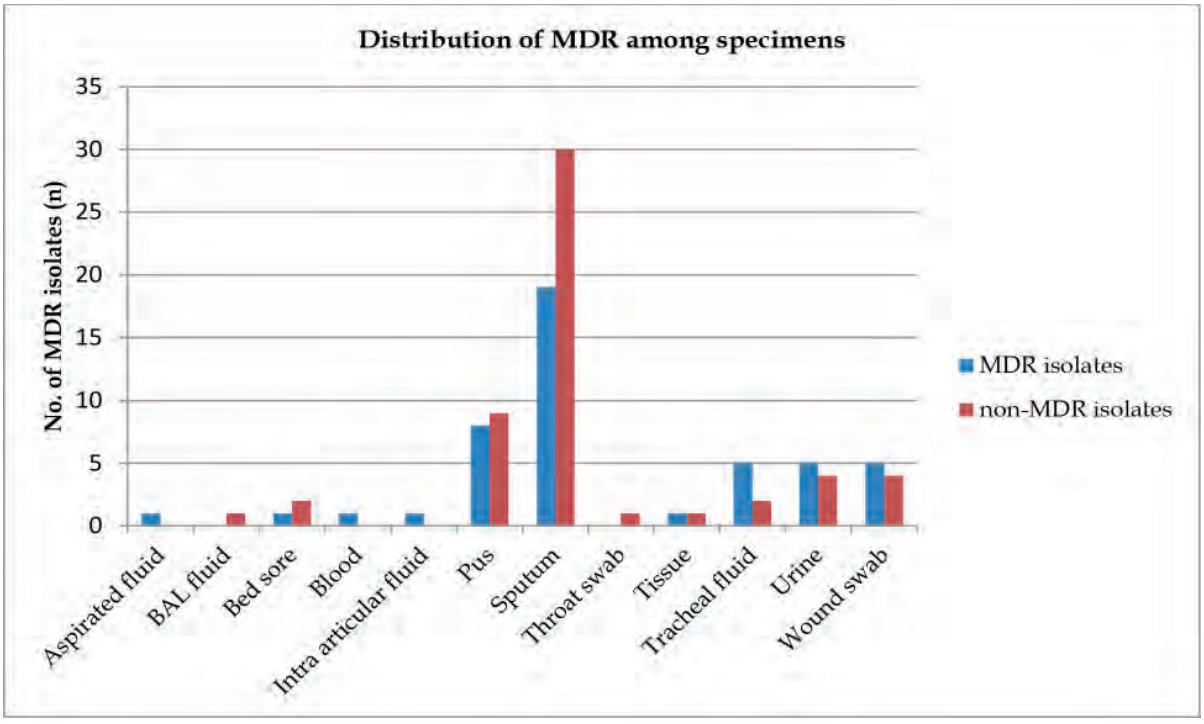
The bacterium was found to be resistant to most of the antibiotics used. Majority of isolates were resistant against Aztreonam, followed by Levofloxacin, Cefepime, Ciprofloxacin and Ceftazidime. However, isolates were sensitive to Tobramycin followed by Imipenem, Meropenem Piperacillin/Tazobactam and Ceftazidime (Table 2).

Table 2: Antibiotic Susceptibility Pattern of *Pseudomonas aeruginosa* (n=101)

Group of Antibiotics	Name of Antibiotics	Antibiotic Susceptibility Pattern	
		Resistant no.(%)	Sensitive no.(%)
Carbapenem	Imipenem	31 (30.7)	70 (69.3)
	Meropenem	38 (37.6)	63 (62.4)
Aminoglycoside	Tobramycin	24 (24)	76 (76)
	Amikacin	23 (31.1)	51 (68.9)
Monobactam	Aztreonam	60 (63.8)	34 (36.2)
B-lactams	Piperacillin + Tazobactam	38 (38.7)	60 (61.2)
Cephalosporins	Cefepime	49 (50)	49 (50)
	Ceftazidime	41 (40.6)	60 (59.4)
Fluoroquinolone	Levofloxacin	54 (54.5)	45 (45.5)
	Ciprofloxacin	42 (42)	58 (58)

Distribution of MDR among specimens

In the study, out of 101 isolates of *P. aeruginosa*, 47 isolates were of MDR strains. The higher number of MDR isolates were obtained from inpatients (32.7%) as compare to outpatients (13.9%). MDR strains were isolated mostly from sputum samples (n=19) followed by pus (n=8), tracheal fluid (n=5), urine (n=5), wound swabs (n=5). (Figure 2).



*MDR= Multi Drug Resistance

Figure 2: Distribution of MDR among different clinical samples

Out of 101 isolates of *P. aeruginosa*, 37 (36.6%) were MBL producers and 64 (63.4%) isolates were MBL negative.

Among the 101 isolates examined, the Congo Red Agar (CRA) method identified 15.8% as strong biofilm producers, 6.9% as moderate producers, and 10.9% as weak producers, while the remaining 66.3% were classified as non-biofilm producers. In contrast, the Microtiter Plate Assay (MPA) demonstrated a substantially higher detection of biofilm formation: 40.6% of isolates were categorized as strong biofilm producers, 48.5% as moderate producers, and 9.9% as weak producers. Only a single isolate (0.99%)

was identified as a non-biofilm producer using this method (Table 3). These findings highlight a marked discrepancy between the two techniques, with MPA showing greater sensitivity in detecting biofilm-producing phenotypes.

Table 3: Biofilm producers by Congo Red Agar Method (CRA) and Microtiter Plate Assay (MPA) method

Isolate of *Pseudomonas aeruginosa*	Congo Red Agar Method (CRA) no.(%)	Microtiter Plate Assay (MPA) no.(%)
Strong Biofilm producer	16 (15.8)	41 (40.6)
Moderate Biofilm producer	7 (6.9)	49 (48.5)
Weak Biofilm producer	11 (10.9)	10 (9.9)
Non-biofilm producer	67 (66.3)	1 (0.9)

(Note: MPA evaluation: Non-biofilm producer = OD < ODc; Weak biofilm producer = ODc < OD < 2×ODc; Moderate biofilm producer = 2×ODc < OD < 4×ODc; Strong biofilm producer = 4×ODc < OD. CRA detection: Non-biofilm producer = pink or red colony; Weak biofilm producer = only dark colony without dryness; Moderate biofilm producer = dark and dry but without crystalline colony; and Strong biofilm producer = dark black dry crystalline colony)

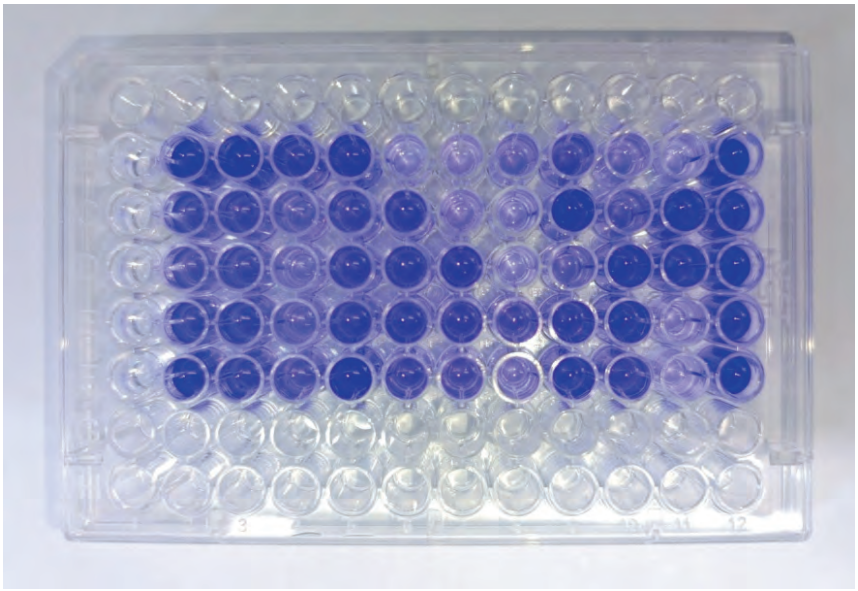


Figure 3: Biofilm formation in 96 well plate

Biofilm detection by CRA and MPA methods:

The CRA method correctly identified 34 biofilm isolates but missed 66 true biofilm producers. This meant that CRA was poor at detecting true biofilm producers. CRA is very specific but poorly sensitive for detecting biofilm in *P. aeruginosa*. The test demonstrated sensitivity of 34%, specificity of 100%, positive predictive value (PPV) of 100%, and negative predictive value (NPV) of 1.49% for CRA method compare with MPA method, indicating low reliability for biofilm detection (Table 4).

Table 7: Comparative study of CRA and MPA method

Biofilm detection techniques / Biofilm formation		CRA no. (%)		Total
		Biofilm Producer	Non-producers	
MPA method (Gold standard)	Biofilm	34	66	100
	Non-Biofilm producer	0	1	1
Total		**34**	**67**	**101**
Sensitivity of CRA		0.34 (34%)		
Specificity of CRA		1 (100%)		
Positive predictive value		1 (100%)		
Negative predictive value		0.0149 (1.49%)		

Comparison of antibiotic susceptibility patterns between biofilm and MBL procedures with non-producers:

Likewise, the investigation also revealed that a higher number of biofilm producers are resistant to antibiotics in comparison to non-biofilm producers. The maximum resistance by the biofilm producers was recorded by Aztreonam (59.4%) in succession with Levofloxacin (56.4%), Cefepime (47.5%), Ceftazidime and Ciprofloxacin (40.6%).

Furthermore, the MBL producers showed maximum resistance towards Levofloxacin (28.7%) followed by Aztreonam (27.7%), Cefepime (27.7%), Ceftazidime (25.7%), Imipenem (25.7%) and Meropenem (25.7%) (Table 5).

Table 5: Antibiotic resistance pattern of biofilm producers and non-biofilm producers

Antibiotic	Resistance Pattern			
	Biofilm producer no.(%)	Non-biofilm producer no.(%)	MBL producer no.(%)	Non-MBL producer no. (%)
Imipenem	29 (28.7)	1 (0.9)	26 (25.7)	4 (3.9)
Meropenem	37 (36.6)	1 (0.9)	26 (25.7)	12 (11.8)
Tobramycin	23 (22.8)	1 (0.9)	18 (17.8)	6 (5.9)
Amikacin	22 (21.8)	1 (0.9)	15 (14.9)	7 (6.9)
Piperacillin/tazobactam	36 (35.6)	1 (0.9)	24 (23.8)	13 (12.8)
Aztreonam	60 (59.4)	1 (0.9)	28 (27.7)	33 (32.7)
Cefepime	48 (47.5)	1 (0.9)	28 (27.7)	21 (20.8)
Ceftazidime	41 (40.6)	1 (0.9)	26 (25.7)	16 (15.8)
Ciprofloxacin	41 (40.6)	1 (0.9)	25 (24.8)	17 (16.8)
Levofloxacin	57 (56.4)	1 (0.9)	29 (28.7)	29 (28.7)

Comparison between biofilm and MBL strains:

The results provided insights into the biofilm producers with comparison to MBL strains. 36 biofilm isolates were determined to produce the MBL enzyme and only one non-biofilm isolate produced the enzyme. The association between biofilm producers and MBL strains were not statistically significant ($p<0.05$) (Table 6).

Table 6: Comparative study between biofilm and MBL strains

Biofilm (MPA)	MBL producers no. (%)	Non-MBL producers no. (%)	Total
Strong biofilm	11 (10.9)	30 (29.7)	41 (40.6)
Moderate biofilm	22 (21.8)	27 (26.7)	49 (48.5)
Weak biofilm	3 (2.9)	7 (6.9)	10 (9.9)
Non-biofilm	1 (0.9)	0	1 (0.9)
Total	37 (36.6)	64 (63.8)	101 (100)

DISCUSSION

Findings from this study showed the increase in distribution of *P. aeruginosa* among clinical samples in comparison to past researches. Bhatta et al., (2019) reported the prevalence rate of 7% which in comparison to this study was less in proportion. The prior publications had observed the less percentage in the prevalence rate of the pathogen in contrast to the present work. The majority of isolates obtained from sputum of patients indicated that *P. aeruginosa* as one of the major bacterium in lower respiratory tract infection. Likewise, the isolates from pus, wound and bed sore samples indicated that it is a common pathogen in hospital or community acquired infections. The sole isolate from a blood sample acquired from inpatient refl ected sepsis, including the severity of the pathogenesis and necessity for proper hospital care. The largest proportion of positive isolates among urine samples of outpatients possibly implies community-acquired infections. The samples from inpatients of both genders (36.6% from male and 25.7% from female) had the highest number of culture positives in comparison to that of male and female outpatients (17.8% and 19.8% respectively) which supported the fact that the occurrence of the pathogen in hospital environment is more common than usual. Furthermore, the study conducted by Chaudhary et al., (2024) from the same hospital earlier reported that had the highest number isolated from sputum samples (33.3%). The comparison of two data shows an increment in the prevalence of the pathogen from the same type of sample. Likewise, Bhatta et al., (2020) reported the highest number of the pathogen was isolated from sputum (n=93), followed by wound (n=35) and pus (n=29) out of 200 isolates over a one-

year study period.

On the basis of this study, bacterium showed maximum resistance against Monobactam followed by Cephalosporin and Fluoroquinolone. Kamali et al., (2020) also reported that *P. aeruginosa* isolated from ICU samples showed resistance against Amikacin and Piperacillin/tazobactam (12.5%) to Levofloxacin (23.7%). However, our study revealed higher rate of resistance indicating the progressive increase in resistance over time. In the contrary, the data presented by Soni et al., (2024) revealed that the bacterium exhibit the significant resistance (93.3%) against Ceftazidime. The present study has concordant fi nding with the study of Krishnasamy &Velmurugan (2024) in terms of antibiotic resistance against classes of antibiotics. Likewise, isolates of *P. aeruginosa* were highly sensitive towards Tobramycin followed by Imipenem and Meropenem. However, sensitivity towards Amikacin and Meropenem was high in the similar study performed by Bhatta et al., (2020). This can be interpreted as the change in the sensitivity of drugs over time which requires thorough investigation. Overall, the resistance pattern seemed to vary along the years among different researches indicating demand for in-depth evaluations.

The rate of MDR has exceeded as reported in the past that was 34.5% from Bhatta et al., (2020). Sharma et al., (2021) reported the pus sample contained the highest MDR (n=18) which is a similar to this study (n=17). Likewise, many papers have drawn attention to a higher number of ICU isolates containing possible MDR strains. This might be due to the prolonged stay in hospital rooms which might have proliferated the survival and the resistance pattern. The trend of the MDR seemed to be progressively rising with almost no sign of possible downward trend if there is no availability of standard control of antibiotic distribution (Kamali et al., 2020, Soni et al., 2024).

In our study, 36.6% (37/101) of *P. aeruginosa* isolates were MBL producers. Maharjan (2022) stated that only 6 out of 68 were MBL producers, Shukla et al., (2022) depicted 22 out of 115 and Yadav et al., (2024) reports 58 out of 205 (28%) produced this enzyme. A similar study conducted in Brazil reported crucial prevalence of MBLs among Imipenem resistant *P. aeruginosa* (30.4%) obtained from blood samples (Franco et al., 2010). Such Beta-lactamase enzyme inactivates Carbapenems, Cephalosporins and Penicillins, and very often not effective by use of Beta-lactamase inhibitors. As of now, the phenotypic method for its detection has not been standardized nationally and internationally but the number of the resistance caused by the enzyme is still prevalent (Farajzadeh Sheikh et al., 2014). Hence, requiring a guideline for proper diagnosis is vital in clinical laboratories. The manual technique in lab detection usually involved the use of chelating agent like EDTA in combination with Imipenem disc (Shukla et al., 2022, Yong et al., 2002).

Congo Red Agar method is a qualitative method for biofilm detection where the presence of dark crystalline colonies is considered a certain level of biofilm producers. A past research paper detected a total 57 biofilm positive isolates from UTI patients in which out of 72 isolates of *P. aeruginosa* produced biofilm (Bhatta et al., 2019). While the results were comparable, the analysis done by Baniya et al., (2017) revealed only 13 out of 85 were biofi lm producers from the CRA method. It is worth mentioning that a lot of past literatures in CRA method had ruled out false-negative as biofilm producer as well. As for the data observed by the Microtiter Plate Assay, 100 isolates were calculated to produce a certain level of biofi lm out of 101 isolates in our study. The data was taken and analyzed after the absorbance reading of Crystal Violet at 570 nm where visually the biofi lm that stained darker was corresponding to the biofi lm isolates. The ratio of biofilm to non-biofilm isolates were found to be parallel to Kamali et al., (2020) where 70/80 isolates were related to biofilm related genes. Superior outcomes highlighted the relation of biofi lm phenotype and genotype where 176 detected as biofi lm formers while 29 did not form biofilm. Other literature also highlights closely related findings similar to the trend of the above data. The current study detected a higher rate of biofilm production from the Microtiter Plate Assay cancelling out false positive results from the CRA method. A similar trend was observed as CRA detected 44.2% while MPA detected 94.2% biofilm formers (Abdulhaq et al., 2020) and likewise, from findings of Harika et al., (2020). This investigation differed from the fi ndings of Bhatta et al., (2019) where their findings contained a lesser proportion of biofi lm producers. The CRA method correctly identified 34 biofilm isolates but missed 66 true biofilm producers in this study. This meant that CRA was poor at detecting

true biofi lm producers. CRA is very specific but poorly sensitive (sensitivity 34%) for detecting biofi lm in *P. aeruginosa*.

Maximum number of the biofilm isolates was resistant to the antibiotics as opposed to non-biofilm isolates. While almost all isolates were resistant to Aztreonam, Levofloxacin, Cefepime and Ciprofloxacin, Tobramycin and Amikacin were the least resistant. This ratio of resistance by biofi lm producers was consistent with those reported by Chhunju et al., (2021) whereas Imipenem and Meropenem resistant isolates were more resistant from findings of Saha et al., (2018). The fi ndings suggest that the ineffective treatment with antibiotics alone was due to the result of biofi lm secretion leading to promotion of chronic and recurrent infections.

This study has also shown correlation between antibiotic resistance and Metallo beta lactamase (MBL) producers, especially against Carbapenem drugs where each 26 isolates were resistant to Imipenem and Carbapenem. The MBL production were shown to be directly associated with Carbapenem resistance. The findings suggest that the MBL enzyme were able to break down the β-lactam antibiotics making them unaffected against pathogen. MBL genes are often carried on plasmids that also carry additional resistance genes leading towards MDR. Overall, the prevalent number of MBL isolates displaying resistance to various antibiotics showed MBL enzyme as one of the crucial factor behind antibiotic resistance against the broad spectrum β-lactam drugs. The study by Baniya et al., (2017) was not able to statistically associate biofilm with MBL producers which is in agreement with our findings. However, there have been reports where the isolates coproduced strong biofilms as well as MBL (Heydari & Eftekhar, 2015, Singhai et al., 2013).

CONCLUSION

This study shows a higher prevalence of *Pseudomonas aeruginosa* infections in older individuals, with MDR isolates occurring more frequently in inpatients, likely due to prolonged hospital exposure and cross-contamination. The strong biofilm-forming ability of the isolates may contribute to persistent or recurrent infections, especially in immunocompromised patients. Resistance was highest to monobactams, cephalosporins, and fluoroquinolones, indicating these antibiotics are unsuitable for treatment in this setting. The prominence of biofilm and MBL producers suggests the presence of related resistance genes, supporting the need for molecular confirmation through methods such as PCR. Given the increasing carbapenem resistance, routine phenotypic detection of MBLs and improved diagnostic strategies are essential for effective management and control of *P. aeruginosa* infections.

ABBREVIATIONS

AMR: Antimicrobial Resistance, AST: Antibiotic Susceptibility Pattern, BHI: Brain Heart Infusion, CFU/ml: Colony Forming Unit per Milliliter, CIP:, CLED: Cysteine Lactose Electrolyte Deficient, CLSI: Clinical and Laboratory Standards Institute, CSF: Cerebrospinal Fluid, EDTA: Ethylenediamine tetraacetic acid, ELISA= Enzyme linked Immunosorbent Assay, LB: Luria Bertani/ Lysogeny Broth, MBL: Metallo Beta Lactamase, MDR: Multi Drug Resistance, MHA: Mueller Hinton Agar, MPA: Microtiter Plate Assay, OD: Optical Density, ODc: cut-off value of Optical Density, PBP: Penicillin- binding protein, PBS: phosphate-buffered saline, XDR: Extensively established drug resistance

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL RESEARCH

Determination of Antibacterial Efficacy of Different Soaps Found in the Local Market against Common Pathogenic Bacteria

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ABSTRACT

Objective: This study aimed to evaluate the antibacterial efficacy of different type of soaps against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus* species.

Methods: A laboratory-based experimental study was conducted from February 2024 to July 2024. Eight commonly used soaps (Herbal, Non-medicated, Medicated, Liquid and Laundry) were randomly collected from the local market of Kathmandu valley. The bacterial cultures used for test were standardized to 0.5McFarland. The soap solutions were prepared by dissolving each soap in sterile distilled water (100mg/ml), and two-fold dilution made for MIC testing. Antibacterial activity was assessed by Agar Well Diffusion method using Mueller-Hinton Agar. Wells were filled with 1ml of each prepared soap solution and incubated at 37°C for 24 hours. Zones of Inhibition were measured, and MIC was determined as the lowest dilution showing no visible growth.

Results: Non-medicated soaps showed mean ZOI of 16.7mm (NM1 brand being effective with mean ZOI of 21 mm) and medicated soap showed mean ZOI of 16.1mm. Handwash showed ZOI range between 16-19mm with mean of 17.3mm. Herbal soap has a strong 17mm of mean ZOI with range of 8-23mm. The brand NM1 soap showed most effective against *E. coli* (20mm) and least against *S. aureus* (15mm) with MIC value 1.56mg/ml. Herbal soap showed maximum effect against *E. coli* (20mm) with MIC value 6.25mg/ml and of 3.125mg/ml against *S. aureus*. Other soaps showed MIC in the range from 3.125mg/ml to 50mg/ml.

Conclusion: Non-medicated soap showed the strongest antibacterial activity against *Bacillus* and *E. coli*, while Herbal soap was most effective against *S. aureus*. Difference in efficacy among soaps are attributed to variation in their antimicrobial compound. The finding challenges the idea that medicated soaps are always more effective than non-medicated ones. Soap formulation should prioritize proven antimicrobial efficacy while remaining affordable for widespread use.

Keywords: Soap, Antibacterial efficacy, Zone of inhibition, MIC, Pathogenic bacteria

INTRODUCTION

Soap is the cleaning agent that is available in various forms like bars, liquid and powders. It is the chemical product that has been used by the human since ancient time (Achaw & Danso-Boateng, 2021). Soap is the product made from the mixture of fat, water and alkali such as sodium hydroxide or potassium hydroxide (Jariah et al., 2021). As a common household item, soap has been utilized for thousands of years, with the earlier evidences of and hygiene is one of the most effective measures for preventing the transmission of infectious diseases (Kagan et al., 2002). Soap plays a key role in handwashing because its surfactant properties help remove dirt, oils, and microorganisms from the skin surface (Salager, 2002.). When soap molecules interact with water, they form

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micelles that trap and detach microbes, thereby reducing the risk of infection. In addition to basic cleansing action, some commercial soaps contain antimicrobial agents or herbal extracts that may enhance their ability to inhibit pathogenic bacteria (Collett et al., 2023).

Hand hygiene is considered one of the most effective and affordable public-health measures for reducing the transmission of infectious diseases (Singh et al., 2020). Hands frequently come in contact with contaminated surfaces and serve as a major vehicle for the spread of pathogenic microorganisms. Proper handwashing with soap significantly lowers the burden of enteric and respiratory pathogens by removing transient flora from the skin surface (Caioni et al., 2023). Soap remains the most widely used cleansing agent because of its ability to emulsify oils, dirt, and microorganisms (Huang et al., 2014).

From a theoretical standpoint, the cleansing ability of soap is due to the amphiphilic nature of surfactant molecules produced through the saponification of fats and alkali (Dunn, 2010). These molecules form micelles, which encapsulate lipids and microbes, allowing their removal from the skin during rinsing. In addition to basic surfactants, commercial soaps often incorporate antimicrobial agents, essential oils, herbal extracts, or antiseptic compounds that may enhance the inhibition of bacterial growth (Chaudhary et al., 2020). The presence, concentration, and stability of these additives play an important role in determining the antibacterial efficacy of different soap formulations

Pathogenic organisms such as *E. coli*, *S. aureus*, and *Bacillus* spp are commonly associated with community and environmental contamination (Hoang et al., 2021). These organisms are frequently found on hands, household surfaces, and everyday items, and can cause gastrointestinal, respiratory, and skin infections (Kagan et al., 2002). These organisms represent different structural categories: Gram-negative rods, Gram-positive cocci, and spore-forming bacilli that makes them useful indicators of the broad antimicrobial spectrum of soaps (Srain et al., 2021). Several studies have shown that the inhibitory activity of soaps varies greatly depending on pH, fatty acid composition, active antimicrobial ingredients, and the manufacturing process (Matta et al., 2022). The effectiveness of soap in reducing microbial load depends on its formulation, pH, concentration of active ingredients, and the presence of antimicrobial compounds. Studies have shown that different commercial soaps vary widely in their antibacterial activity, making comparative evaluation important for public health (Bin Abdulrahman et al., 2019).

In Nepal, a wide range of commercial, herbal, antiseptic, and medicated soaps are available, but there is limited scientific information validating their antibacterial claims (Chaudhary et al., 2020). Evaluating the antibacterial activity of commonly used soaps is important for guiding consumer choice, ensuring product quality, and supporting hygiene-related public-health recommendations. Therefore, the present study investigated the antibacterial efficacy of selected commercial soaps available in the Kathmandu Valley against common pathogenic bacteria using standard microbiological techniques.

METHODS

Research type, Study site and duration

This laboratory based experimental study was conducted in the Microbiology Laboratory of Kist college of Management, from February 2024 to July 2024.

Sample type and sampling

A total of eight commercially available soaps of different categories were collected randomly from local markets inside Kathmandu valley using convenient sampling method. All soaps were unused, stored at room temperature and tested within their expiry period.

Inclusion and Exclusion criteria

The soap sample that were purchased from different local shops of Kathmandu valley inside ring road with labeled manufacturing date, expiry date and presence of manufacturer's seal and unused were included in the study. While those soap with broken seal, no labeling of date of manufacture, expiry and used were excluded.

Preparation of selected soaps for Antimicrobial testing

The obtained soaps were taken out aseptically and were scrapped by using a sterile blade. After scrapping, 1gm of each soap sample was weighed and dissolved into 10ml of sterile water to prepare a stock solution of 100mg/ml concentration. Three bacterial species: *E. coli*, *S. aureus* and *Bacillus* spp were selected for evaluating antibacterial activity. These organisms were chosen because they commonly represent Gram

negative rods, Gram positive cocci and spore-forming bacilli, respectively. Pure culture was maintained on Nutrient Agar and sub-cultured for 24 hours at 37°C before testing. The bacterial suspension was standardized to 0.5McFarland turbidity. Antibacterial activity was determined using Mueller–Hinton Agar (MHA) and the agar well diffusion method. The MHA plates were lawn-cultured with standardized bacterial inoculum using sterile cotton swabs. Wells of 4 mm diameter were punched aseptically by borer, and 1ml of each soap solution was added to the wells. Plates were allowed to diffuse for 30 minutes at room temperature and were incubated at 37°C for 24 hours. Zones of inhibition were measured in millimeters. The antibacterial efficacy of each soap extract against common pathogenic organisms was compared.

Determination of MIC

The prepared soap solutions with highest antibacterial efficacy was diluted up to seven times (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128) for the purpose of calculating MIC which was determined by examining the zone of inhibition using several dilutions. Proper sterilized plates, utensils, and equipment are used. Data were recorded manually, and descriptive analysis was performed. Results were expressed as mean zone diameters and MIC values.

RESULTS

All tested soaps exhibited variable inhibitory effects, and the results demonstrated marked differences according to soap formulation and bacterial species. The antibacterial activity of different soap showed that NM1 and medicated soaps were most effective overall. Handwash, laundry soap, and MS2 brand demonstrated moderate antibacterial activity, while NM2, and NM3 were generally less effective. Herbal soap has a strong but selective activity, being highly effective against *E. coli* and *S. aureus* but weak against *Bacillus* spp. For all soaps, higher concentration produced larger zones of inhibition, indicating strong antibacterial effects. NM1 soap showed increasing effectiveness with concentration, being most effective against *E. coli* and least against *S. aureus*. Herbal soap showed concentration dependent activity, with maximum effect against *E. coli*. NM2 and NM3 exhibited higher antibacterial activity particularly against *E. coli*. Handwash became effective with strong effect on *S. aureus*. Medicated soap shows strong activity against *E. coli*, weak against *S. aureus* and least effective on *Bacillus* spp.

Antibacterial efficacy of working solution of different soaps against selected pathogens

Table 1 represents the antibacterial activity of different soaps based on their zone of inhibition (ZOI) against *Bacillus* spp, *S. aureus*, and *E. coli*. Among all, NM1 showed the highest mean ZOI of 21 mm, and MS2 also had strong inhibition mean of 18 mm that follows the activity of HW (17.3 mm ZOI), laundry soap (15.6mm) and MS1 (14.3mm) show moderate activity. NM2 and NM3 brand (14.6 mm) were lesser effective which followed the brand MS1 which had least activity of 14.3mm mean ZOI. The MS2 brand soap showed highest effectivity against *E. coli* with ZOI 25 mm, NM1 was almost equally effective with ZOI 24 mm; while against *S. aureus*, the brand HS was highly effective with ZOI 23 mm and NM1 showed effectivity of 19mm ZOI against *Bacillus* species.

Table 1: Antibacterial efficacy of different soap against selected pathogens

S.N.	Soap	Concentration	Zone of inhibition (ZOI) in mm			Mean ZOI
			Bacillus spp	*S. aureus*	*E. coli*	
1	NM1	100 mg/ml	19	20	24	21
2	NM2		12	13	19	14.6
3	NM3		12	12	20	14.6
4	LS		14	17	16	15.6
5	HW		17	19	16	17.3
6	HS		8	23	20	17
7	Control (CO)		0	0	0	0
8	MS1		10	15	18	14.3
9	MS2		15	14	25	18

NM= Non-medicated soap (NM1= Pears, NM2= Lifebuoy, NM3=Lux, MS= Medicated soap(MS1=Dettol, MS2= Acnoshine), LS = Laundry Soap (Dhoni), HW= Handwash (Savlon), HS= Herbal Soap (Okhati)

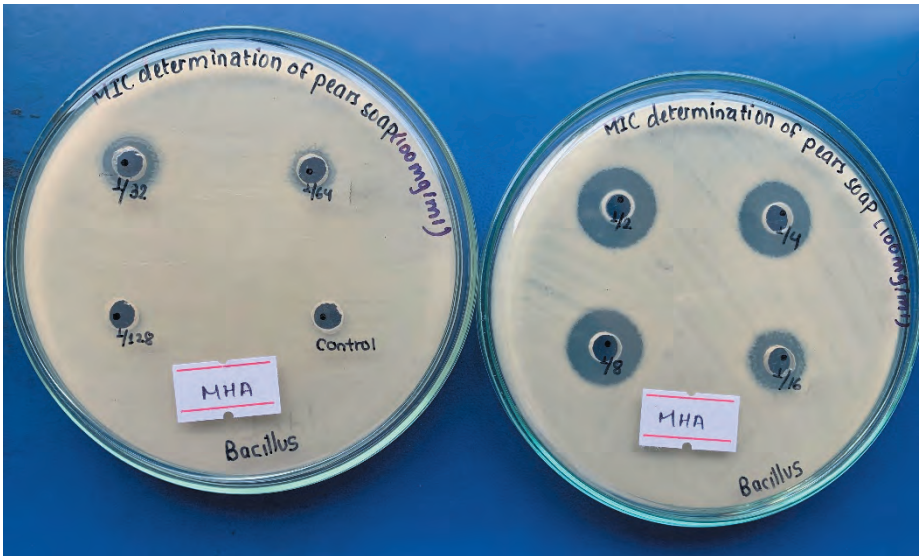

Figure 1: Showing MIC determination of NM1 brand soap against *Bacillus* spp (Right to left dilutions 1:2 to 1:128 MIC= 1.56mg/ml at 1:64 dilution)

Minimum Inhibitory Concentration of Non-medicated Soap (NM1, NM2 and NM3) brand against selected pathogens

Table 2 displayed data on antibacterial properties of Non-medicated soap which showed no antibacterial activity at the most diluted concentration (1:128). Inhibition began at 1:64 for all three bacteria, making the minimum inhibitory concentration for NM1 to be 1.56mg/ml. Likewise, NM2 had MIC against *Bacillus* spp to be 25mg/ml, and 12.5mg/ml against *S. aureus* and *E. coli*. Similarly, NM3 brand showed no antibacterial activity against *Bacillus* spp or *S. aureus* at any dilution. However, it was effective against *E. coli*, with inhibition starting at 1:8 (12mm) denoting the MIC to be 12.5mg/ml against it. As the concentration increased toward 1:2, the ZOI increased across all bacteria. *E. coli* showed the largest ZOI (20mm), making it most susceptible. *Bacillus* spp showed moderate inhibition (17mm), while *S. aureus* had the smallest inhibition zone (15mm) and was least susceptible. Overall, NM1 soap was most effective at higher concentration and showed strongest activity against *E. coli*.

Table 2: Minimum Inhibitory Concentration of Non-medicated Soap (NM1, NM2 and NM3) brand against Common Pathogenic Bacteria

Sample	Concentration	Zone of inhibition (ZOI) in mm		
		Bacillus spp	*S. aureus*	*E. coli*
NM1	1:128	0	0	0
	1:64	9	8	12
	1:32	11	10	14
	1:16	12	11	17
	1:8	14	12	18
	1:4	16	13	19
	1:2	17	15	20
NM2	1:128	0	0	0
	1:64	0	0	0
	1:32	0	0	0
	1:16	0	0	0
	1:8	0	10	12
	1:4	10	12	15
	1:2	11	13	17

Sample	Concentration	Zone of inhibition (ZOI) in mm		
		Bacillus spp	*S. aureus*	*E. coli*
NM3	1:128	0	0	0
	1:64	0	0	0
	1:32	0	0	0
	1:16	0	0	0
	1:8	0	0	12
	1:4	0	0	15
	1:2	0	0	16

Minimum Inhibitory Concentration of Herbal soap (HS) and Hand Wash (HW) against selected pathogens

Table 3 showed the antibacterial properties of Herbal soap (HS) that showed no antibacterial activity at the most diluted concentration (1:128). Inhibition began at 1:16 for *Bacillus* spp, and *E. coli*, and at 1:32 for *S. aureus*, indicating the minimum inhibitory concentration to be 6.25mg/ml for *E. coli* and *Bacillus* spp but 3.125 mg/ml for *S. aureus*. As the concentration increased up to 1:2, the ZOI increased for all bacteria. *E. coli* showed the largest ZOI (up to 20mm), making it the most susceptible. *Bacillus* spp show moderate inhibition (up to 18mm), while *S. aureus* showed the smallest ZOI (16mm) and was the least susceptible. For antibacterial properties of Handwash (HW), it showed no antibacterial activity at high dilutions (1:128 to 1:8). Inhibition began only at 1:4, with ZOIs of 10mm against *Bacillus* spp, 9mm for *S. aureus*, and 12mm for *E. coli* making 25mg/ml as MIC value. At the strongest concentration (1:2), the ZOI increased to 12mm, 17mm and 14mm respectively. The handwash was therefore effective only at higher concentrations (1:4 and 1:2), showing the greatest activity at 1:2. *S. aureus* was the most sensitive, while *Bacillus* spp and *E. coli* showed moderate susceptibility.

Table 3: Minimum Inhibitory Concentration of Herbal soap (HS) and Hand Wash (HW) against common pathogenic bacteria

Sample	Concentration	Zone of inhibition (ZOI) in mm		
		Bacillus spp	*S. aureus*	*E. coli*
HS	1:128	0	0	0
	1:64	0	0	0
	1:32	0	9	0
	1:16	12	11	13
	1:8	12	3	18
	1:4	16	14	19
	1:2	18	16	20
HW	1:128	0	0	0
	1:64	0	0	0
	1:32	0	0	0
	1:16	0	0	0
	1:8	0	0	0
	1:4	10	9	12
	1:2	12	17	14

Minimum Inhibitory Concentration of medicated soap against common pathogenic bacteria

Table 4 represent the antibacterial activity of medicated soap (MS1 and MS2) against *Bacillus* spp, *S. aureus*, *E. coli*, that showed no antibacterial activity against *Bacillus* spp at any dilution. For *S. aureus*, inhibition of MS2 brand began only at 1:8 (5mm) and increased slightly to 7mm at 1:2, showing the weaker effectiveness of MIC being 12.5mg/ml. For *E. coli*, activity started at 1:16 (7mm) and increased steadily to 11mm at 1:2, making MIC value 6.25mg/ml showing the strongest response among the three bacteria. But for MS1 brand, the MIC against *S. aureus* was 50mg/ml and against *E. coli* was 25mg/ml. Overall, medicated soap was most effective against *E. coli*, weakly effectively against *S. aureus*, and ineffective against *Bacillus* spp.

Table 4: Minimum Inhibitory Concentration of medicated soap against common pathogenic bacteria

Sample	Concentration	Zone of inhibition (ZOI) in mm		
		Bacillus spp	*S. aureus*	*E. coli*
MS1	1:128	0	0	0
	1:64	0	0	0
	1:32	0	0	0
	1:16	0	0	0
	1:8	0	0	0
	1:4	0	0	8
	1:2	0	7	9
MS2	1:128	0	0	0
	1:64	0	0	0
	1:32	0	0	0
	1:16	0	0	7
	1:8	0	5	9
	1:4	0	6	10
	1:2	0	7	11

DISCUSSION

Soaps are commonly used for cleaning skin and removing germs, mainly by disrupting the microbial cell membrane and protein (Schaffner et al., 2018). Although people choose soaps based on preference, an effective soap should be able to fight disease-causing bacteria on the skin (Odoyo et al., 2021; Schaffner et al., 2018). According to table 1, Non-medicated soap 1 had shown higher effectiveness with mean ZOI of 21mm, conversely, Medicated soap brand 2 had mean ZOI of 18mm that followed other Herbal soap, Handwash and Laundry soap (Range of mean ZOI:14.3-17.3mm).

Generally, the medicated soaps had higher antibacterial activity due to its antimicrobial ingredients like triclosan, chlorhexidine, povidine iodine and Sulphur that help in killing the bacteria or inhibit their growth by disrupting cell membranes, denaturating proteins or sometimes inhibiting fatty acid synthesis and altering metabolic pathways (Madigan et al., 2018). The result is also consistent with the study done in Nigeria, where different medicated soaps have shown greater activity towards common pathogens like *S. aureus* and *E. coli*. (Jesumirhewe &Timothy, 2024). However, it cannot also be neglected that the brand NM1 with the ingredients like thymol, terpinol, sodium palmitate and sodium cocoate (Fotsing & Kezetas, 2020) had shown antibacterial activity against tested bacterial pathogens in vitro. It may have correspondence to high pH and release of free fatty acids like lauric acid and palmitic acid for disrupting the bacterial membrane.

Non-medicated soaps and handwash are very useful in dealing with microbes since they act using strong physical abrasion which does not require any antimicrobial chemical. The surfactants in soap dissolve skin oils, dirt, and organic residues where microbes adhere on them, then they can be removed off the skin (Madigan et al., 2018). Oils and contaminants being surrounded by the soap molecules create so-called micelles that capture bacteria, fungi, and viruses and then are washed off with water (CDC, 2020). Moreover, soap has the potential to interfere with the lipid shell of most viruses including influenza and coronaviruses rendering them inactive (WHO, 2009).

The mechanical friction of washing hands also increases the microbial flora to a greater extent, as it removes living beings hidden in the folds of skin and by removing the nails. Since non-medicated soaps do not kill microbes, but only eliminate them, they cause less antimicrobial resistance, and can be used daily (Ananthanarayan and Paniker, 2017). As such, handwashing and non-medicated soap is among the most effective and evidence-based ways of infection prevention.

In another hand, the herbal soaps tend to be antibacterial as the products contain plant-based ingredients like neem, tulsi, turmeric and essential oils, which all contain natural antibacterials. Such phytochemicals as azadirachtin in neem, curcumin in turmeric, and eugenol in tulsi have the ability to inhibit the synthesis of cell walls, disrupt cell membranes, or disrupt the metabolic processes, and they thus reduce the survival of bacteria on the skin (Madigan et al., 2018). Secondly, the surfactant activity of soap aids in erasing dirt and microorganisms and this makes the herbal soaps have a combined chemical and physical microbial control mechanism.

Research on the use of herbal preparations has always demonstrated a great effect of antibacterial activities in comparison to controls which are not treated, and the efficacy of plant-based soaps is confirmed in daily practices of hygiene (WHO, 2009). This is in consistent with the result of this study which demonstrated the mean ZOI of 17mm against selected pathogens. The Medicated soap brand 2 showed no effectiveness against *Bacillus* spp, weak activity against *S. aureus* (MIC= 12.5mg/ml), and moderate effectiveness against *E. coli* (MIC=6.25 mg/ml) as the concentration increased (Kutol, 2019) that is higher in comparison to MIC of 1.56mg/ml of Non-medicated soap brand 1. These findings challenge the belief that medicated soaps are always superior, highlighting the need for choosing soaps based on proven antimicrobial performance rather than marketing claims (Lawan & Idris, 2021).

Overall the study emphasizes that different soaps work differently against specific bacteria, further research could help develop formulations with broader antibacterial coverage(Hoang et al., 2021). Using effective antimicrobial soaps can help reduce the risk of infections, especially in places where hygiene is critical, such as hospitals, kitchens, and public areas (Kampf & Kramer, 2004). Most soaps in this study worked best against *E. coli*, likely because its Gram-negative, lipid- rich outer membrane is easily disrupted by soap molecules. In contrast, *S. aureus* and *Bacillus* spp, being gram positive, have thick peptidoglycan layers that provide greater resistance. This explains why *E. col*i was more sensitive to soap action, while the other two bacterial species showed comparatively lower susceptibility (Matta et al., 2022).

CONCLUSION

Non-medicated soap showed the strongest antibacterial activity against *Bacillus*, while Herbal soap was most effective against *S. aureus* and Medicated soap was effective against *E. coli.* Other samples including the liquid hand wash (HW) and laundry soap exhibited comparatively lower inhibition, likely due to difference in their antibacterial components, although hand wash still performed moderately well, that is ascribed to better lathering and mechanical removal of microbes. These findings challenge the assumption that medicated soaps are always superior, highlighting the need to choose soaps based on validated microbial efficacy rather than marketing claims. Difference in efficacy among soaps are attributed to variation in their antimicrobial compound. Handwash also demonstrated notable activity, likely due to better lathering and mechanical removal of microbes. The finding challenges the idea that medicated soaps are always more effective than non-medicated ones. Soap formulation should prioritize proven antimicrobial efficacy while remaining affordable for widespread use.

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CONFLICT OF INTEREST

The authors declared no conflict of interests.

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ORIGINAL RESEARCH

Antibiofilm Activity of Bacteriophage Isolated from Sewage-Polluted Water against *Escherichia coli*

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ABSTRACT

Objectives: This study aimed to explore bacteriophages, viruses that infect bacteria, as an alternative antibiofilm agent.

Methods: A laboratory-based, cross-sectional study was conducted at the Sainik Awasiya Mahavidyalaya Laboratory from February to July 2025. The water samples that were contaminated with effluents were collected from eight rivers and ponds across the Kathmandu valley. *E. coli* isolates were used as the host strain after being confirmed by biochemical tests. Phages were isolated and enriched from wastewater using centrifugation, filtration, and multiple cycles of incubation with log-phage host bacteria to gain high titres. The plaque assay, host range by spot assay, and Efficacy of Plating (EOP) were performed. Antibiofilm activity was evaluated using the microtiter plate crystal violet assay. The study compared biofilm formation in the isolated *E. coli* (E.C 8) with that of the standard *E. coli* (ATCC 8739). Statistical significance was determined using the t-test ($p \leq 0.05$).

Results: Bacteriophages were found in six of eight samples. Only Mulpani had a lytic phage with a titre of 7.5 PFU/ml, which was used for further testing. The phage exhibited moderate EOP, ranging from 0.28 to 0.60, and a moderate host range. The isolated phage showed selected antibiofilm activity, as it effectively reduced the biofilm of the isolated *E. coli* (22.8%).

Conclusion: This emphasizes the ability of lytic phages as antibiofilm agents.

Keywords: Effluent, Antibiotic Resistance, Biofilm, Extracellular Polymeric Substances, Bacteriophage

INTRODUCTION

Bacterial biofilms are a major contributor to antibiotic resistance, posing a major health concern. Biofilms are found in environmental settings, processing facilities, industrial settings, hospital settings, and natural environments. Most bacteria produce biofilms (Zhao et al., 2023). Biofilm is the accumulation of eukaryotic or prokaryotic cells, surrounded by the matrix of extracellular polymeric substances (EPS). EPS consists of a long chain of sugars, DNA, and other biological molecules (Harper et al., 2014). Biofilm formation is a five-stage developmental cycle determined by biochemical and mechanical adaptations. The first step is the initial attachment of planktonic cells to a surface, followed by adhesion and aggregation, which establishes the irreversible connection. Then, micro-colonies formation occurs by cell division and multiplication, causing the maturation stage where EPS matrix is produced, and structure is finalized via signalling. The cycle completes with dispersion, where cells are released into the environment to colonize new sites (Azeem et al., 2025). The biofilm matrix forms resistance to agents like antibiotics by creating impermeable barriers, which is the major cause of

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antibiotic resistance (Harper et al., 2014).

Antimicrobial resistance (AMR) currently causes the death of 700,000 deaths annually and is expected to increase to 10 million by 2050 (WHO, 2025). This growing challenge has driven interest in identifying alternative antimicrobial strategies. Bacteriophages show promising potential as antibiofilm agents. They are the viruses that affect the bacteria. They might be lysogenic phages, which coexist with hosts by inserting themselves into a bacterial genome, or lytic phages, which destroy themselves by replicating inside their hosts and releasing new phages to infect more bacteria (Harper et al., 2014). They produce enzymes such as depolymerase and lysin that break down EPS, disrupting the biofilms more effectively than the conventional antibiotics (Wiguna et al., 2022).

They can be used as biotechnological tools to combat harmful bacteria, including MDR organisms, *Shigella* spp, *Salmonella* spp, *Escherichia coli, Staphylococcus* spp, and *Proteus* spp. Because of their ability to infiltrate and destroy bacterial cells, lytic bacteriophages are primarily employed in phage therapy (Rogovski et al., 2021). Despite growing interest and its urgency, a significant gap in research and development. The challenge of effectively targeting mature and multispecies biofilms in common clinical and environmental settings remains largely unaddressed. The interaction between the phage and biofilm matrix is not understood. This gap limits the translation of phage therapy for biofilm-associated infections (Harper et al., 2014). Therefore, this study aimed to isolate the bacteriophages from river and pond waters collected from around Kathmandu Valley and evaluate their antibiofilm activity, exploring their potential to disrupt biofilms as an alternative to antibiotics.

METHODS

Study Design

The study was a laboratory-based, cross-sectional, analytical study. The numerous rivers and ponds across the Kathmandu valley, polluted with household and industrial effluents, served as the study location and were the sites of the sample collection. The water samples were collected from Jhaukhel, Siddhapokhari, Jorpati, Bramayani, Basbari, Kasan, Guhesowori, and Mulpani.

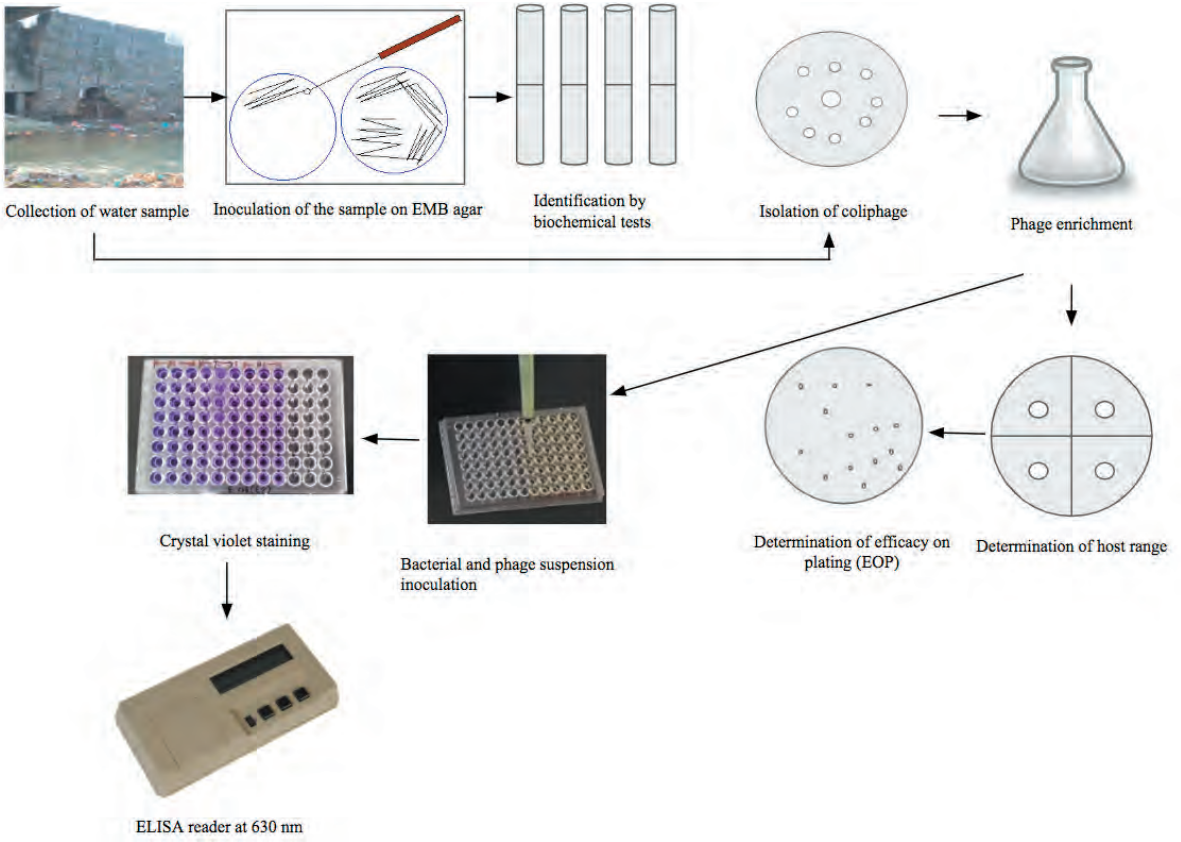


Figure 1: Showing the detailed method of phage screening and Antibiofilm Activity

Sample Collection

The contaminated waters were directly collected in plastic or glass containers. The sample bottles were closed until the time of the collection. While collecting the sample, the bottle was inserted into the water with its neck slightly below the surface and tilted upward. The opening was pointed in the direction of the water flow. The sample was stored at 4°C (US EPA, 2015).

Bacterial Isolation

Bacterial isolates were obtained from the processed polluted water sample in the laboratory. One ml of each sample was inoculated into the Nutrient Broth (NB) and incubated at 37°C for 24 hours. A loopful of enriched culture was inoculated on Eosin Blue Methylene (EMB) using the quadrant streaking technique and incubated at 37°C for 24 hours. Colonies exhibiting a distinctive metallic sheen were selected and subjected to a series of biochemical tests to confirm the presence of the bacteria (Fonteh, 2015). The isolated and biochemically identified *E. coli* was used as the host strain for the phage isolation (APHA, 1998; Cheesebrough, 2006).

Three clinical isolates and one from poultry faeces, available in the Sainik Awasiya Mahavidyalaya Microbiology laboratory, were also biochemically tested, which confirmed to be *E. coli* (APHA, 1998; Cheesebrough, 2006).

Plaque Assay

The samples were centrifuged at 8000 rpm for 10 minutes and followed by syringe filtration with a 0.22 µm filter to collect the supernatant (Wang et al., 2024). To increase the phage concentration, 20 ml of phage solution was added to 100ml of log-phase host bacteria. The mixture was incubated at 37°C in the shaking incubator at 200 rpm for 10 hours. Following incubation, it was centrifuged at 12000 rpm for 5 minutes to collect the supernatant, which was then filtered through a 0.2 µm syringe filter. The cycle was repeated three times to obtain a high-titre phage preparation. Phage detection was carried out using a plaque assay. The 50 µl host bacteria and 2 ml phage titre were mixed with 4 ml of top agar prepared and poured onto double-layer plates. It was incubated at 37°C, and plaque formation was observed (Wang et al., 2024).

Elution of Bacteriophage from the Plaque

The double-layer agar plates with plaques were chosen to recover bacteriophage. It was covered with a Salt of Magnesium (SM) buffer. After 30 minutes of incubation at room temperature, the top agar surface was scraped using the scraping tools. The top agar, along with the SM buffer, was collected in the sterile Falcon tube (Wang et al., 2024). Vortexing was used to mix it. The phage lysate suspension was centrifuged at 6000 rpm for 5 minutes. The supernatant was filtered through a 0.22µm filter and stored at 4ºC for further use (Phage Purification, 2023).

Phage Enrichment

The phage enrichment was performed with a slight modification of Quinones-Olvera. The 20µl bacteria were inoculated in the LB broth and incubated at 37ºC for 2 hours to obtain log phase bacteria. Subsequently, 2ml of phage suspension was added to it and incubated at 37ºC overnight for enrichment. After incubation, it was centrifuged at 12,000 rpm for 5 minutes. The resulting supernatant was syringe filtered without disturbing the pellets, and the filtrate was transformed into the pre-labelled sterilised tubes. The enriched samples were stored at 4°C for further tests (Quinones-Olvera, 2023).

Determination of Host Range

For determining the host range, spot assay was performed using different bacterial strains, i.e., host bacteria (environmental isolates and clinical isolates) and reference bacteria *E. coli* (ATCC 8739 and ATCC 35218). The lawn was made on the LB agar using sterile cotton after being moistened with broth culture. In the marked area, 5 µl of each phage was spotted and allowed to dry before incubation at 37°C for 18-24 hours. The lysis zones were observed areas of the plates to note the effectiveness of the phage (Bhetwal et al., 2017).

Efficiency of Plating (EOP)

To assess the EOP, the plaque assay was conducted with a few modifications. The two sets of phage bacteria mixtures were prepared by combining 50µl of phage stocks with either 50µl of bacteria culture, i.e., host bacteria and reference bacteria *E. coli* (ATCC 8739), respectively, and incubated at room temperature for 10 minutes. The mixture was added to 4ml of soft agar and vortexed for a short period of time. The soft agar, maintained at around 50°C, was poured onto the bottom agar plates and allowed to solidify at room temperature. It was incubated at 37°C for 24 hours. Zone of lysis was observed, the number of plaques was counted, and EOP was calculated (Khan Mirzaei

& Nilsson, 2015).

It is calculated as the ratio of PFU/ml of test host to PFU/ml of reference host. EOP values were interpreted as >0.5 (high efficiency), 0.1-0.5 (moderate), 0.001-0.1 (low), <0.001 (no infection) (Khan Mirzaei & Nilsson, 2015).

Antibiofilm Activity of Phage

The biofilm-forming capacity of the host bacteria strain was tested in triplicate using the crystal violet assay with a minor modification. The test was done using the Microtiter Plate method. One set of the test contained three controls, viz., positive control, negative control, and phage-treated. The positive control test tube contained only bacterial isolates, the negative control test tube contained only LB broth, and the treated test tube contained bacterial isolates with phage. Three to five similar colonies were sub-cultured in 5 ml LB broth and incubated for 24 hours. After vortexing, 2 µl of the suspension was inoculated into 200 µl LB broth. For the phage activity, 2 µl bacterial suspension with 50 µl stock phage suspension were inoculated in 150 µl LB broth. They were aerobically incubated at 37°C for 24 hours ± 30 minutes. After washing each tube with 300 µl sterile saline, they were heat-fixed at 60°C for 1 hour. Staining was performed with 200 µl 0.1% crystal violet for 15 minutes, followed by washing with tap water and air drying. After homogenous resolubilization of the dye with 1 ml of 95% ethanol, the optical density (OD) of the microtiter plate was measured at 630 nm using an ELISA reader (Plota et al., 2021). The same set of tests was performed for *E. coli* ATCC 8739.

Statistical Analysis

The statistical analysis was performed using Excel. To interpret the effectiveness, the Mean OD, Standard Deviation, and % biofilm inhibition were calculated. Two-sample t-test was performed to compare the dependent variable, Mean OD, between two specific groups (the independent variable). Group 1 (control) was the mean OD of the *E. coli* without phage, and group 2 (treatment) was the mean OD of the *E. coli* with phage. The values were interpreted as "Significant" or "Not Significant" by comparing the p-value, such that if the p-value ≤ 0.05, the value is significant, otherwise not significant.

RESULTS

The *E. coli* (E.C 8) that was isolated from sample no. 8 was used as the host strain to isolate bacteriophage. Among the water samples collected from eight rivers and ponds, bacteriophage presence was detected in six samples. Notably, samples from Jorpati, Mulpani, and Siddhapokhari exhibited the highest plaque counts. No plaque formation was detected in samples from Jhaukhel and Bramayani, suggesting the absence of detectable phage.

The water samples collected from Mulpani exhibited lytic phage activity, forming 15 plaques with a calculated titer of 7.5 PFU/ml, as shown in *Table* 2. The plaques were spherical, smooth, transparent, colorless, and lytic in nature. In contrast, plaques observed from the remaining five sites were lysogenic in nature.

Table 1: Distribution of water samples used for screening of Coliphage

S.N.	Sample No.	Sample Site	Plaque Formation	No. of Plaque
1	E8	Mulpani	+	15
2	E7	Guheswori	+	7
3	E6	Kasan	+	10
4	E5	Basbari	+	13
5	E4	Bramayani	-	0
6	E3	Jorpati	+	30
7	E2	Siddhapokhari	+	18
8	E1	Jhaukhel	-	0

(+): Plaque formation (-): No plaque formation

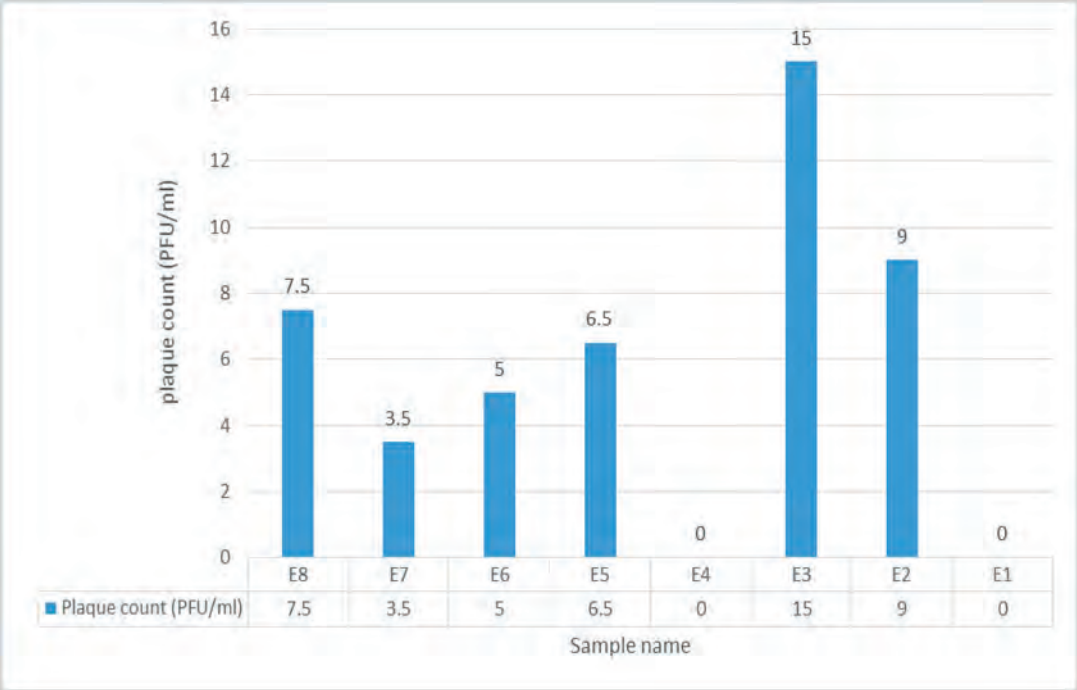


Figure 2: Bar Graph showing PFU/ml of the Phage Isolated from Different Sample Sites

Table 2: Plaque Morphology Table

SN	Sample	Name of Bacteria	Plaque Morphology					
			Size (in mm)	Shape	Margin	Opacity	Color	Remarks
1	E8	*E. coli*	3	Sphere	Smooth	Transparent	Colorless	Lytic
2	E7		1	Sphere	Smooth	Opaque	Colorless	Lysogenic
3	E6		3	Irregular	Smooth	Opaque	Colorless	Lysogenic
4	E5		5	Sphere	Smooth	Opaque	Colorless	Lysogenic
5	E4		-	-	-	-	-	-
6	E3		1	Sphere	Smooth	Opaque	Colorless	Lysogenic
7	E2		3	Irrigular	Smooth	Opaque	Colorless	Lysogenic
8	E1		-	-	-	-	-	-

(-): No plaque formation

The determination of the host range was performed via spot assay. Moderate lysis was observed on the isolated E. *coli* (E.C 8), but no zone was observed in E.C 2 by the coliphage E8 in Table 3. By comparing the EOP value (0.1≤ EOP ≤ 0.5), the strains showed a moderate host range on average. *Table 4* shows the host range of *E. coli* that were clinical and poultry fecal isolates via spot assay. Zone formation was not observed on any of the strains. Similarly, *Table 5* shows the host range of standard *E. coli*. ATCC 8739 showed a clear lysis, whereas ATCC 35218 didn't show zone formation.

Table 3: Determination of Host Range of *E. coli* (Environmental isolates)

SN	Method	Volume Spotted	Bacterial Strain (EC2)	Bacterial Strain (EC8)
1	Spot Assay	10µl	-	+
2			-	+
3			-	+
4			-	+
5			-	+
6			-	+

(++): Clear zone formation; (+): Moderate zone formation; (-): No zone formation

Table 4: Determination of Host Range of *E. coli* (Clinical and Poultry feces isolates)

SN	Method	Volume Spotted	Bacterial Strain (EC.I)	Bacterial Strain (EC.II)	Bacterial Strain (EC.III)	Bacterial Strain (EC.IV)
1	Spot Assay	10µl	-	-	-	-
2			-	-	-	-
3			-	-	-	-
4			-	-	-	-
5			-	-	-	-
6			-	-	-	-

(-): No zone formation

EC.I, EC.II and EC.III= Clinical isolate

EC.IV=Poultry faeces isolate

Table 5: Determination of Host Range of Standard *E. coli*

SN	Method	Volume Spotted	Bacterial Strain E.coli ATCC 8739	Bacterial Strain E.coli ATCC 3518
1	Spot Assay	10µl	++	-
2			++	-
3			++	-
4			++	-
5			++	-
6			++	-

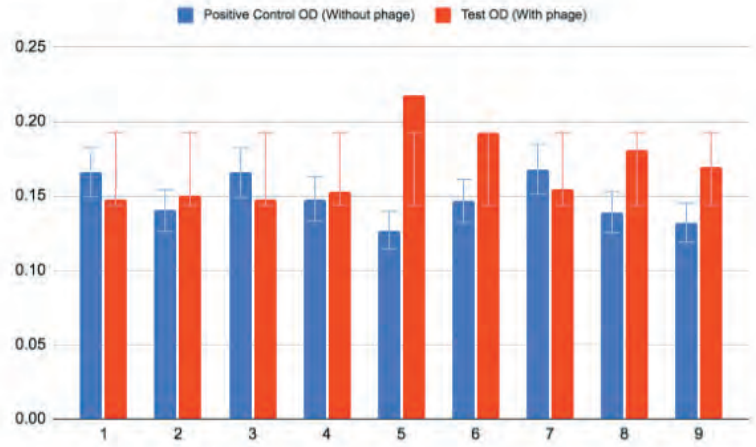
(++): Clear zone formation; (-): No zone formation

The EOP value ranged from 0.28 to 0.60, as shown in *Table 6*, indicating that the strain exhibits moderate phage infectivity related to the standard host.

Table 6: Efficacy of Plating (EOP) Coliphage against test isolates and ATCC reference strains.

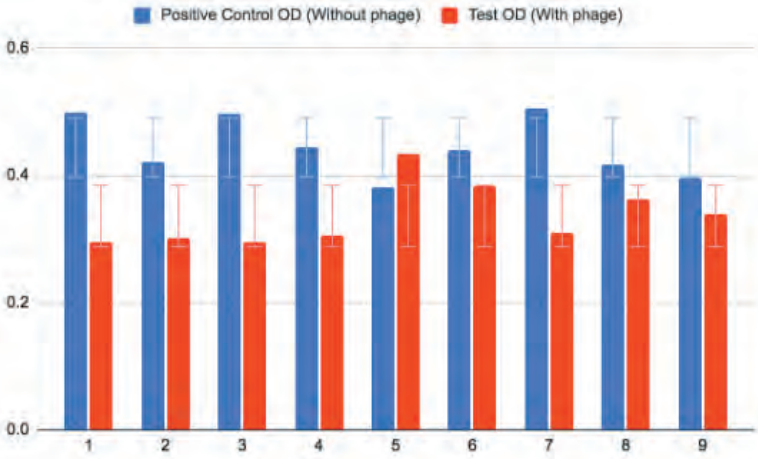
SN	Host Strain (Test Host)	Host Strain (Standard Host)	Titer (PFU/ml) of Test strain	Titer (PFU/ml) of Standard strain	EOP Value	Interpretation
1	*E. coli*	*E. coli* ATCC 8739	2000	6000	0.33	Moderate
2			1466.666	2466.66	0.59	High
3			1200	2000	0.60	High
4			2000	4400	0.45	Moderate
5			1333.33	4666.66	0.28	Moderate
6			1200	3200	0.37	Moderate

Antibiofilm activity of the bacteriophage was assessed using a microtiter plate reader. The mean Optical Density (OD) values for the positive control (untreated bacteria) and the test control (phage-treated bacteria) for the isolated and the standard *E. coli* were calculated. The respective mean OD of the isolated *E. coli* was found to be 0.444 and 0.336 (p-value < 0.05), whereas that of the standard *E. coli* was found to be 0.148 and 0.168 (p-value >0.05). These OD values were then used to calculate the percentage of biofilm inhibition. The outcome suggests that the bacteriophage has a reducing effect on biofilm formation by isolated *E. coli*, as indicated by an average inhibition percentage of 22.8%. However, the standard *E. coli* (ATCC 8739) displayed a negative inhibition value of -15.73%.



Positive Control OD (without phage)	0.1661	0.1401	0.1659	0.1479	0.1269	0.1464	0.168	0.139	0.132
Test OD (with phage)	0.1477	0.1505	0.1475	0.1531	0.2172	0.192	0.1545	0.1808	0.1698

(i)



Positive Control OD	0.4983	0.4203	0.4977	0.4437	0.3807	0.4392	0.5043	0.4176	0.3954
Test OD	0.2954	0.301	0.295	0.3062	0.4344	0.384	0.309	0.3616	0.3396

(ii)

Figure 3: Bar graph showing the antibiofilm activity of E8 against (i): *E. coli* ATCC 8739, and (ii): isolated *E. coli* by the microtiter plate method

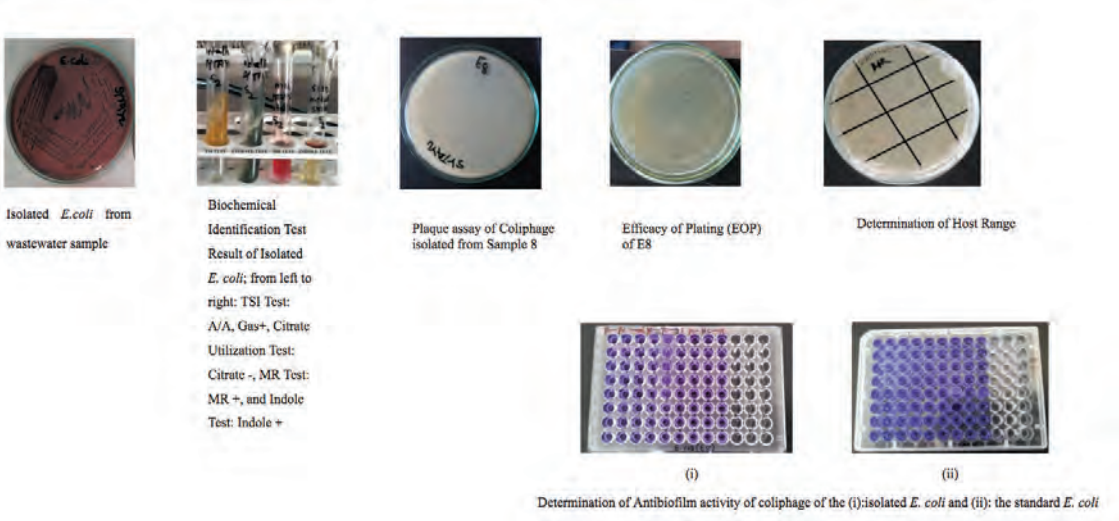


Figure 4: Photographs show the results of Coliphage isolates and Microtiter assay for Antibiofilm Activity

DISCUSSION

This study found the lytic bacteriophage was recovered only from Mulpani, which is because the relationship between phage and bacteria is host-dependent. This agrees with Ballesté et al., (2022), who noted that if the wastewater has a low population of bacteria or fewer phage-susceptible strains, the numbers of that phage can also be low or absent. The isolation of lytic bacteriophage from the wastewater was confirmed by the formation of round plaques, which are capable of infecting and lysing the host-specific bacteria immediately, consistent with the observation of Doekes et al., (2021). In a study done by Fathy et al., (2024) and Sivakumar et al., (2025), lytic phages were found in their respective wastewater samples, indicating that the wastewater acts as a reservoir of phages. The lysogenic phages should be excluded because of their low bactericidal effect (Gordillo & Barr, 2021). They can integrate their genome into a bacterial cell chromosome, which is replicated and passed on to the daughter cell without killing them (Kasman & Porter, 2022). This underscores that they are not useful as a biocontrol or therapeutic means as a lytic phage.

The spot assay of the phage showed a moderate host range and when compared with the average value of EOP (average EOP = 0.436), the strain was found to have moderate host range as well. The moderate host range was observed only in the isolated *E. coli* (E.C 8), while the clinical isolates and poultry faeces isolate, *E. coli*, had no host range, which shows that the coliphage had only intraspecific host range, but not interspecific. The EOP values in our study range from 0.28 to 0.6, which fall under the moderate range; they indicate reduced infectivity in the host strain compared to the standard strain. These values are lower in comparison to previous findings (1.2-1.4) by Sada & Tessema (2024). In the experiment conducted by Fathy et al., (2024), the isolated phages, particularly S3 and F3 coliphages, exhibited significant lytic potential against diverse *E. coli* strains of 66.6% and 41.6% respectively. Khan Mirzaei & Nilsson (2015) found that the bacterial strain in their study had high productive infection (EOP ≥ 0.5), which was lower than the results of the spot tests. They compared both the spot test result and EOP value for determining host range, as the spot assay is a qualitative test while EOP is a quantitative measure. In the study done by Bhetwal et al., (2017), almost all the coliphages showed very high host range against the *E. coli* strain, suggesting it is highly effective against a variety of *E. coli* strains. This finding is consistent with our result. Even the same bacterial strain can have a wide range in phage activity due to bacterial mutation, which causes the variation in Efficacy of Plating (EOP) levels (Bull et al., 2014).

The antibiofilm activity against isolated *E. coli* by the coliphage showed 22% biofilm inhibition. The biofilm's EPS consists of exopolysaccharide, extracellular DNA, and protein (Archell et al., 2025). Bacteria produce biofilms to protect themselves from external pressures (Archell et al., 2025). The bacteriophage encodes enzymes like lipase, depolymerase, or hydrolase to disrupt the EPS (Archell et al., 2025). These enzymes

are produced after bacteriophages infect bacterial cells within the biofilm, and when phages are released from the lysed bacteria, they degrade the biofilm (Archell et al., 2025). Our result can be supported by the experiment conducted by González-Gómez et al., (2021), which found that certain bacteriophages had an adhesion ability of 10% and 20%. They suggested that less biofilm inhibition may be due to EPS producing strains and a lower adhesion ability.

Our results on antibiofilm activity were consistent with Shivakumar et al., (2025) and Pérez et al., (2024). In the study conducted by Shivakumar et al., (2025), the phage was an efficient antibiofilm agent against *E. coli* (ATCC 25922), with 1.0 MOI, which was the optimal concentration for gaining maximum initial inhibition. The greatest dramatic change in biofilm-forming ability was seen in the phage-sensitive *E. coli* WG5, as reported by Pérez et al., (2024). *E. coli* WG5 showed a significant reduction in biofilm formation (53.8%, $p < 0.05$) at a phage titre of 105 PFU/mL, reaching 100% suppression when the cells were treated with 109 PFU/mL of SOM7 coliphage (Pérez et al., 2024).

In the study conducted by Bràs et al., (2024), a decrease in biofilm after phage treatment. Phage may be unable to inhibit the biofilm of the standard ATCC strain due to a stronger polysaccharide layer or the formation of more biofilm layers as a defence mechanism (Adeyemo et al., 2022). These findings align with those showing that standard *E. coli* ATCC 8739 exhibited -15.73% inhibition. The production of biofilm in the presence of bacteriophage has also been observed in the experiment done by Mangieri et al., (2021). They suggested that phage predation can be the cause of an increase in the biofilm levels in bacteria.

A limitation of the study is that only a few *E. coli* isolates and ATCC strains were used. This limits the ability to assess the phage's lytic range and specificity. The host range determination did not include the other members of Enterobacteriaceae, restricting the broader applicability of the phage. Further studies should include diverse bacterial isolates, multiple phage isolates and high-quality replicates to determine the phage lytic potential and bacterial host specificity.

CONCLUSION

In this study, lytic and lysogenic phages were isolated from the 8 wastewater samples; however, lytic phages were recovered only from Mulpani and were further processed for the study. This suggested a varying distribution of phages due to ecological and microbial factors such as the availability of specific hosts, pH, temperature, and nutrient levels. The antibiofilm activity of coliphage was studied by using a crystal violet assay in a 96-well microtiter plate. The coliphage showed selective efficacy, demonstrating a good biofilm inhibition against the isolated *E. coli* but no inhibition of biofilm in the standard *E. coli* (ATCC 8739).

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CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest regarding the publication of this paper.

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ORIGINAL RESEARCH

Assessment of Antibiotic Resistance of *Escherichia coli* Isolated from Poultry Droppings in the Kathmandu Valley

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ABSTRACT

Objective: To assess antibiotic resistance in *Escherichia coli* isolated from chicken droppings from selected poultry farms within Kathmandu valley.

Methods: Stool samples of chicken from 21 different farms were collected aseptically and transported to Central Department of Microbiology laboratory. Samples were cultured in MacConkey and M-Endo agar to isolate *E. coli* and was confirmed phenotypically using biochemical tests. The isolates were tested for antimicrobial susceptibility by modified Kirby-Bauer disk diffusion method following CLSI guidelines.

Results: *E. coli* were isolated from all 104 dropping samples. *E. coli* isolates were resistant to ampicillin/sulbactam (91.34%), cefoxitin (98.07%), chloramphenicol (77.88%), ciprofloxacin (83.65%), contrimoxazole (79.81%), gentamicin (65.38%), levofloxacin (83.65%), and tetracycline (100%). While less proportions of *E. coli* were resistant to ceftriaxone (16.35%), colistin (3.85%) and imipenem (25%). Out of 104 isolates, 94 (90.38%) were multidrug resistant (MDR). Frequency of cleaning of the coop ($p = 0.0017$), farm size ($p < 0.001$), farm operation ($p<0.001$), water source ($p < 0.001$) and common diseases of poultry ($p < 0.001$) were found significantly associated with MDR *E. coli*.

Conclusion: High prevalence of MDR *E. coli* was found on chicken within Kathmandu valley, which might be introduced into humans through food chain. Therefore, adherence of biosecurity measures for reducing the use of antibiotics in the poultry farms is suggested.

Keywords: *Escherichia coli,* antibiotic susceptibility, poultry, stool samples

INTRODUCTION

Antimicrobial resistance is a critical global health problem wherein, over time, pathogens develop the ability to evade treatment, making infections increasingly difficult to manage. Responsible for 700,000 deaths globally in 2019, this figure is projected to rise to 10 million by 2050, surpassing cancer mortality. AMR arises through either intrinsic resistance, whereby it forms a part of bacterial physiology, or through acquired resistance due to genetic modifications. Horizontal gene transfer-enabling conjugation, transformation, and transduction-allows for the spread of resistance genes through mobile genetic elements such as plasmids, phages, and genomic islands, significantly accelerating the global spread and impact of resistance (Luiken et al., 2022).

Gram-negative bacterium of the Enterobacteriaceae family, *Escherichia coli* is widely recognized as an indicator for antimicrobial resistance due to its wide dissemination and extraordinary ability to carry resistance genes on mobile genetic elements, which can then be transferred to pathogenic strains. In the context of poultry, APEC acts as the primary etiological agent of colibacillosis, further solidifying its dual role as both a disease-causing microorganism and a reservoir of resistance-associated genetic determinants (de Mesquita Souza Saraiva et al., 2021).

Poultry meat is one of the fastest-growing agricultural

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sub-sectors in Nepal, sharing around 4% in the nation's GDP. It stands globally in the position of 112nd in the production of chicken meat and 92nd in egg production. The production of poultry meat, as recorded in 2021/22, has reached 65,387 tons with an average growth rate of 6.6% annually (Adhikari et al., 2023). In Nepal, 14 antibiotic types are available for veterinary use, among which tetracycline, enrofloxacin, neomycin, doxycycline, levofloxacin, colistin, and tylosin are primarily used for treating colibacillosis (Bhattarai et al., 2024). Nepal is among the 30 countries with a high burden of AMR. The amount spent on veterinary medicine and feed supplement sales is very high, and almost 13% of veterinary expenditure goes towards antibiotics. The most frequently used antibiotics are tetracycline, enrofloxacin, neomycin-doxycycline, levofloxacin, colistin, and tylosin. Ampicillin, amoxicillin, ceftriaxone, and gentamicin are commonly misused in clinical and veterinary practices (Bhattarai et al. 2024).

Poultry and their production environments are reservoirs for both resistant bacteria and genes for health consequence to humans. Fresh broiler litter sample stored at room temperature for about two months reduced the potential pathogens to less than 83% (Kyakuwaire et al., 2019). Poor hygiene and biosecurity in poultry favor pathogen emergence and excessive use of antimicrobials. Antimicrobial residues, derived from either administration or excretion by chickens, often concentrate in litter, with associated environmental and health risks. Non-standardized treatment practices and a lack of professional training among farm personnel further increase the inappropriate use of antimicrobials, which are often used not only for therapeutic reasons but also as growth promoters and for prophylaxis. Poor selection of antimicrobials, inappropriate dosages, and prolonged duration of use enhance AMR development, increasing the growing global threat (Moffo et al., 2022; Salam et al., 2023). The monitoring of antimicrobial resistance in poultry could provide the information of AMR that could potentially transmit to human. Further, this can provide AMR status on poultry which is one of the biggest meat industry in the country.

METHODS

Study design

This was a field and laboratory based cross-sectional study. Stool samples from different breeds of chicken were taken from farms within and out of Kathmandu valley area and were processed at the laboratory of Central Department of Microbiology, Tribhuvan University, Kritipur, Kathmandu. These stool samples were collected from 5 different sites within the farm where the activity of the chicken was most.

Study sites and duration

There were 21 different farms within inside and outside of Kathmandu valley, these locations included Changunagar, Chandragiri and Kritipur area. The study was conducted from July to August 2025.

Sample collection

A total of 5 sites within a farm was taken as a point for sample collection. Fresh feces of the chicken was collected in sterile swab and brought to the Central Department of Microbiology on Stuart transport medium.

Isolation and identification of *E. coli* from samples

Fresh fecal swab was brought in the laboratory and cultured on MacConkey agar and M-Endo agar to isolate *E. coli* which were confirmed through biochemical series of test including catalase test, oxidase test, sulphur indole motility (SIM) test, triple sugar iron agar (TSIA) test, urease, citrate utilization, and methyl-red and Voges Proskauer (MR and VP) test (Cheesbrough, 2006).

Antibiotic susceptibility test

Depending on farms antimicrobial treatments were applied at chick, all *E.coli* isolates were subjected to AST using the disc diffusion test on Mueller-Hinton agar, against 12 antibiotics (Hi-media) and inhibition zones were interpreted following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2024). The following antibiotics discs were used: ampicillin sulbactam (10/10µg), gentamicin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), tetracycline (30 µg), colistin (25 µg), imipenem (10 µg), levofloxacin (5 µg), cefoxitin (30 µg), ceftriaxone (30 µg), and co-trimoxazole (25 µg).

Statistical analysis

All the data were entered using Epicollect5 and analysis and graphical representation was done with R with tidyverse package. Antibiotic resistance of *E. coli* was presented using percentages and comparison was done using chi-square test. p-value less than 0.05 was considered significant.

RESULTS

A total of 21 farms were chosen at random, 16 (76.2%) from Kathmandu whereas 5 (23.8%) from Bhaktapur. These farm included farmers who were in this business for 10 years having flocks of 0-6 weeks (85.72%) with many farm having 1000 plus chickens. These farms were properly fenced with mostly (95.24%) having proper flooring for the chicken. Though chicken are changed every 45 days (life cycle in broiler), these coops are often cleaned or whenever new batch of chicks to be introduced. It was observed that most of the farms sell the manure as fertilizer with few using it themselves too. These farms were supplied with tap water from local tap water where most of the house using the same water supply as the one in the coops for their day to day use. Chronic respiratory diseases (CRD) (61.90%) and *E. coli* infection (71.43%) seemed to be common infection around these farms and these farms were mostly vaccinated with rani-khet (Newcastle Disease) and gambaro (Infectious Bursal Disease/IBD) vaccine which are usually recommended through veterinarian or through the dealer who supplies these farm with chicks. Among these farms, there seemed to have decent biosecurity whereas only 5 farms with local breed of chicken and few with broiler were found to follow the maximum biosecurity practices which includes visitors control, use of proper gears, pest and rodents control and isolation of sick chicken.

Out of 104 isolates from stool samples for the chicken, 94 (90.38%) were MDR. There was no association between biosecurity of the chicken ($p = 0.218$) and vaccine use ($p = 0.755$) with MDR *E. coli*. Frequency of cleaning of the coop ($p = 0.0017$), farm size ($p < 0.001$), farm operation ($p<0.001$), water source ($p < 0.001$) and common diseases of poultry ($p < 0.001$) were found significantly associated with MDR *E. coli* (Table 1).

Table 1: Poultry farm characteristics of poultry and isolation of MDR *E. coli*

Factors	Variables	MDR (%) (n=94)	Non-MDR (%) (n=10)	p-value
Farm size				
	100-1000	5 (5.3)	10 (100.0)	< 0.001
	1000-2000	39 (41.4)	0	
	2000-4000	50 (53.2)	0	
Farm operation				
	Fenced	0	5 (50.0)	<0.001
	Fenced with proper flooring	94 (100.0)	5 (50.0)	
Frequency of coop cleaned				
	Sometimes	30 (31.9)	10 (100.0)	0.0017
	Every flock change (>45 days later)	64 (68.1)	0	
Water source				
	Tap water	64 (68.1)	5 (50.0)	< 0.001
	Hand pump	5 (5.3)	5 (50.0)	
	Others	25 (26.5)	0	
Most common diseases				
	Rani khet	25 (26.5)	10 (100.0)	< 0.001
	E. coli	25 (26.5)	10 (100.0)	
	Gambaro	69 (73.4)	5 (50.0)	
	CRD	59 (62.7)	5 (50.0)	
	Others	39 (41.5)	0	
Vaccine use				
	Rani khet	89 (94.6)	10 (100.0)	0.7557
	Gambaro	89 (94.6)	10 (100.0)	
	Others	5 (5.3)	0	
Bio-security				
	Use of gumboot	40 (42.5)	10 (100.0)	0.2186
	Visitor control	84 (89.3)	10 (100.0)	
	Pesticide use	89 (94.6)	10 (100.0)	
	Isolation of sick chicken	20 (21.3)	5 (50.0)	

The antibiotics used in poultry as reported by farmers included levofloxacin (66.6%), enrofloxacin (42.8%), doxycycline (42.8%), ciprofloxacin (38.1%) (Table 2).

Table 2: Antibiotics used on chicken as reported by farmers

Antibiotics	Number (%) (n=21)
Neomycin	2 (9.5)
Enrofloxacin	9 (42.8)
Levofloxacin	14 (66.6)
Tetraycline	3 (14.2)
Doxycyline	9 (42.8)
Colistin	6 (28.5)
Ciprofloxacin	8 (38.1)
Streptopenicillin	1 (4.7)
Others	10 (47.6)

A total of 104 *E. coli* were isolated from 21 different farms consisting of 3 different breeds of chicken i.e., broiler, local and giriraj. High percentages of *E. coli* were resistant towards ampicillin/sulbactam (91.34%), cefoxitin (98.07%), chloramphenicol (77.88%), ciprofloxacin (83.65%), co-trimoxazole (79.81%), gentamicin (65.38%), levofloxacin (83.65%) and tetracycline (100%). While less proportions of *E. coli* were resistant to ceftriaxone (16.35%), colistin (3.85%) and imipenem (25%) (Table 3).

Table 3: Antibiotic susceptibility pattern of *E. coli* (n=104)

Antibiotics	Sensitive (%)	Resistant (%)
Ampicillin/Sulbactam	9 (8.66)	95 (91.34)
Cefoxitin	2 (1.93)	102 (98.07)
Ceftriaxone	87 (83.65)	17 (16.35)
Chloramphenicol	23 (22.12)	81 (77.88)
Ciprofloxacin	17 (16.35)	87 (83.65)
Co-trimoxazole	21 (20.19)	83 (79.81)
Colistin	100 (96.15)	4 (3.85)
Doxycycline	63 (60.58)	41 (39.42)
Gentamicin	36 (43.62)	68 (65.38)
Imipenem	78 (75)	26 (25)
Levofloxacin	17 (16.35)	87 (83.65)
Tetracycline	0 (0)	104 (100)

Comparing antibiotic resistance pattern of isolates according to types of chicken, it was observed MDR *E. coli* were isolated from broiler and giriraj breed of chicken, whereas there were no MDR isolate among local breed of chicken stool. Biosecurity measures were more commonly practiced on broiler farm than giriraj and local breed farms (Figure 1).

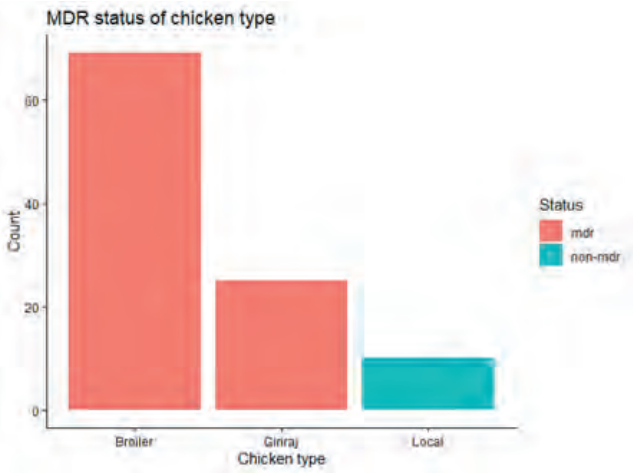

Figure 1: MDR status among different breeds of chicken

DISCUSSION

The results of this survey showed that antibiotics were commonly administered to broilers as compared to local and giriraj breeds of poultry. The antibiotics commonly used in the farms were levofloxacin, enrofloxacin, doxycycline, and ciprofloxacin. This over-reliance on antibiotic further consolidates AMR, particularly in the absence of stringent regulatory and biosecurity measures (Poudel et al., 2024).

This study revealed diverse antimicrobial sensitivity of *E. coli* isolated from chicken's droppings. In particular, it was found that none of the antibiotics were seen to be 100% effective. The resistance rates were 100% for tetracycline, over 90% for ampicillin/sulbactam and Cefoxitin, and above 80% for ciprofloxacin, levofloxacin, and co-trimoxazole showing similar trends as reflected in previous study from Nepal (Khanal et al., 2017). The resistance to antibiotics and used antibiotics were found correlated and antibiotics pressure could result resistance of *E. coli*. A universal trend in the resistance to tetracycline, a common antibiotic used both for therapeutic and prophylactic purposes in Nepalese poultry, represents a particularly alarming situation that calls for immediate action.

Considering the resistance for each antibiotic, resistance to ceftriaxone and colistin were relatively low comparing with other antibiotics as well as reports of the previous studies (Shrestha et al., 2017; Joshi et al., 2019; Dawadi et al., 2021), reflecting their limited use in poultry or their limited use only to critical human infections. The national active surveillance protocol specifically identifies colistin as a highest priority antimicrobial requiring immediate monitoring in animal health sector (Ministry of Agriculture and Livestock Development, 2024). Imipenem resistance significantly exceeds the sporadic detection rates in Nepal of less than 5% in poultry. However, despite low application in Nepalese veterinary medicine, a 25% resistance rate to imipenem might point toward co-selection or horizontal gene transfer of resistance determinants from other commonly used same class of antibiotics, as increasingly reported in poultry.

From the survey, most of the farms seem to emphasize biosecurity, visitor restriction, pesticides use for rodent control but isolation of sick chicken and gumboot use received less attention. WOAH's Terrestrial Animal Health Code recommends a weekly spot-cleaning and full litter removal/disinfection between broiler flocks-every 4-6 weeks-to keep moisture below 20%, thus minimizing ammonia levels and persistence of pathogens (WOAH, 2025). Frequency of cleaning coop shows reduction rate of respiratory and gastrointestinal pathogen outbreaks decreasing antimicrobial use. Untreated surface water harbors fecal coliforms and *Salmonella*, causing gastrointestinal/respiratory pathogens outbreaks. Ranikhet (ND) and gambaro (IBD) were most prevalent viral diseases pointing as one of the reason for frequent use of antimicrobials.

Although broiler farms tend to have better biosecurity but it did not substantially help in explaining their association with MDR occurrence. While the precise data on the use of antibiotics in such poultry is lacking, over-reliance on these antibiotics accelerates the selection of resistant strains and thus poses a serious risk to human and animal health (Mana et al., 2022). The implication is that overuse of antimicrobials outweighs the effects of a good biosecurity system, a pattern that is very similar to other intensive poultry production system (Khanal et al., 2017).

The data showed that around 90% tested *E. coli* isolates were MDR, indicating an extensive presence of strains with high resistance in commercial poultry. The rate of MDR was higher compared to previously reported study from Nepal (Bhattarai et al., 2024). The widespread presence of MDR *E. coli* in poultry droppings poses a significant public health challenge. Its application as organic fertilizer and the high consumption of poultry meat pose a risk of resistance transfer through bacteria and mobile genetic elements to the human food chain and the environment, alarming AMR in Nepal. Therefore, immediate actions of enhanced antimicrobial stewardship, regular AMR surveillance, restricted use of critical antibiotics in poultry, vaccination, and better farm management/ biosecurity are required to minimize this threat for the protection of both human and poultry health.

This study was limited to antibiotic resistance in single bacteria *E. coli* from limited number of poultry farms in Kathmandu valley. We did not explore antibiotic resistant genes that can transfer to bacterial communities.

CONCLUSION

E. coli isolated from chicken droppings were resistant to most commonly used antibiotics. Broiler breed of

chicken with poor treatment may lead to development of antimicrobial resistant flora within them, which through food chain might be transmitted to human. Hence, a proper monitoring of antibiotic resistance of bacterial isolates and appropriate treatment of chicken are important for protecting poultry and human health. Adherence of biosecurity measures for reducing the use of antibiotics in the poultry farms is suggested.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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ORIGINAL RESEARCH

Molecular Detection of CTX-M Type ESBL Genes in Clinical Isolates of *Klebsiella* Species

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ABSTRACT

Objective: The objective of this study is to determine the prevalence of Extended-Spectrum β-Lactamase (ESBL) production and CTX-M genes among *Klebsiella* species isolated from clinical specimens.

Methods: A total of 1,815 clinical samples—including urine, blood, sputum, pus, and body fluids were collected at Himal Hospital Kathmandu, during 2019–2020. Standard microbiological techniques were used for isolation and identification of bacterial pathogens. Antimicrobial susceptibility testing was performed using the modified Kirby–Bauer disk diffusion method following CLSI (2019) guidelines. ESBL screening was conducted using third-generation cephalosporins, and confirmation was done via the Double Disk Synergy Test (DDST). Molecular detection of the CTX-M gene was performed using PCR with specific primers targeting a 544 bp amplicon.

Results: Among 1,815 clinical samples, urine constituted the majority (65.8%), followed by blood (25.1%). *Escherichia coli* was the predominant isolate (89.1%), while *Klebsiella pneumoniae* (6.2%) and *Klebsiella oxytoca* (0.74%) comprised a smaller proportion. Of the 28 *Klebsiella* spp isolates, the highest antibiotic sensitivity was observed toward Amikacin (60.7%) and Meropenem (57.1%), whereas complete resistance to Amoxicillin (100%) and high resistance to Cefixime (89.3%) and Cefotaxime (75.0%) were recorded. ESBL screening identified 22 (78.6%) potential ESBL producers, of which 18 (64.3%) were confirmed phenotypically. PCR analysis revealed the CTX-M gene in 7 of the 18 ESBL-positive isolates, demonstrating a notable presence of CTX-M–mediated resistance among *Klebsiella* spp.

Conclusion: The findings highlight a concerning prevalence of ESBL production and CTX-M genes in *Klebsiella* species in the study population, underscoring the need for continuous surveillance, rational antibiotic use, and strengthened antimicrobial stewardship programs to limit the spread of multidrug-resistant strains.

Keywords: *Klebsiella* spp, ESBL producer and CTX-M

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) possess a major global antimicrobial resistance threat because of their ability to hydrolyze extended-spectrum cephalosporins. Over the past two decades, the CTX-M family has become the predominant ESBL type worldwide, surpassing earlier TEM- and SHV-derived ESBLs in prevalence and clinical impact (Edelstein et al., 2003; Pitout, et al., 2019). CTX-M enzymes confer high-level resistance to cefotaxime and are frequently

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co-located with additional resistance determinants on mobile genetic elements, particularly conjugative plasmids, facilitating rapid dissemination among Enterobacterales, including *Klebsiella* species (Zhao & Hu, 2013).

The CTX-M family originated from the chromosomal β-lactamases of environmental *Kluyvera* species, later mobilized into clinically significant bacteria through insertion sequences such as ISEcp1 and ISCR1 (Zhao & Hu, 2013). More than 200 CTX-M variants have now been identified and are classified into distinct phylogenetic groups (e.g., CTX-M-1, CTX-M-2, CTX-M-9 groups), with several—most notably CTX-M-15—demonstrating rapid global spread and strong association with epidemic clones (Vignoli et al., 2016). The widespread dissemination of plasmid-borne *bla* $_{CTX-M}$ gene has been particularly concerning among *Klebsiella pneumoniae*, a major healthcare-associated pathogen implicated in urinary tract infections, pneumonia, septicemia and surgical-site infections. ESBL-producing *K. pneumoniae* infections are associated with prolonged hospital stays, increased morbidity, and limited therapeutic options (Peymani et al., 2017).

In Nepal, various laboratory-based studies and systematic reviews have documented a high burden of ESBL producers among clinical Enterobacteriaceae, with CTX-M–type genes repeatedly reported as a predominant genetic determinant. A national systematic review and meta-analysis and several hospital-based studies have highlighted widespread detection of CTX-M (including CTX-M-15 alleles) among *Klebsiella pneumoniae* across diverse clinical sources and geographic regions of Nepal, underscoring a continuing and possibly expanding reservoir of CTX-M in human clinical isolates (Shyaula et al., 2023).

Although phenotypic methods such as the Kirby–Bauer disc diffusion ESBL screen and combined disc tests remain essential for routine detection, molecular assays are required for definitive confirmation, epidemiologic surveillance and variant characterization (Xie et al., 2025). Polymerase chain reaction (PCR) targeting *bla* $_{CTX-M}$ gene is the most widely employed approach because of its sensitivity, specificity and rapid turnaround time (Edelstein et al., 2003).

Given the increasing global burden of ESBL-mediated resistance and the clinical significance of *Klebsiella* species as reservoirs and disseminators of *bla* $_{CTX-M}$, molecular detection of CTX-M genes remains critical for guiding antimicrobial therapy, informing infection prevention strategies, and strengthening surveillance systems. This study focuses on the molecular detection of CTX-M–type ESBL genes among clinical isolates of *Klebsiella* species, contributing to the understanding of their distribution and epidemiological patterns in the local clinical setting.

METHODS

Materials

All the laboratory experiments were conducted using distilled water and analytical grade chemicals. The culture media, biochemical tests media, stains and antibiotic discs were purchased from HI media Laboratories Pvt. Ltd., India.

Sample collection and processing

This study was conducted at Himal Hospital and Kantipur College of Medical Science, Kathmandu during the year 2019-2020 and analyzed 1815 clinical samples (blood, urine, sputum, and wound swabs) from patients of all ages, including both inpatients and outpatients. The clinical samples were collected using aseptic techniques and were processed following standard microbiological techniques (Forbes et al., 2012). The clinical samples were subjected to Gram's staining, culture on Blood Agar and MacConkey Agar plates for the isolation and were further subjected to biochemical tests for the identification of the isolates.

Antimicrobial susceptibility testing

The antibiotic susceptibility test was performed by modified Kirby–Bauer method of disk diffusion within the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2019).

ESBLs Screening and Confirmation

Screening of ESBLs

Isolates were screened for Extended Spectrum Beta-Lactamase (ESBL) production using the disc diffusion method based on CLSI (2019) guidelines. Antibiotics used for initial screening included Ceftazidime (30µg), Cefotaxime (30µg), and Ceftriaxone (30µg). Isolates showing inhibition zones of ≤22mm (Ceftazidime), ≤27mm (Cefotaxime), or ≤25mm (Ceftriaxone) were considered potential ESBL producers.

Confirmation of ESBLs

Suspected ESBL-producing isolates were confirmed using the Double Disk Synergy Test (DDST) per CLSI, 2019. Mueller-Hinton agar was inoculated with

a 0.5 McFarland suspension of the test organism. Discs of Cefotaxime (30µg), Ceftazidime (30µg), Ceftriaxone ((30µg)) and Cefotaxime-Clavulanic acid (30/10µg), Ceftazidime-Clavulanic acid(30/10µg), and Ceftriaxone-Clavulanic acid (30/10µg) were placed 16–20mm apart. After incubation at 37°C for 18–24 hours, a ≥5mm increase in the inhibition zone for the combination disc compared to the single antibiotic disc confirmed ESBL production. *E. coli* ATCC 25922 was used as the control strain.

Molecular Detection of CTX-M gene

Klebsiella spp was cultured in Luria Bertani (LB) broth at 37°C for 24 hours using an orbital shaker at 120rpm. Plasmid DNA was extracted from 1.5ml of culture via the alkaline lysis method and suspended in TE buffer, then labeled and stored at -20°C. PCR amplification was carried out using 3µl of plasmid DNA, 21µl of master mix, and 0.5µl each of forward and reverse primers in a total volume of 25µl. Detection of the CTX-M gene utilized primers CTX-M F (5′-TTTGCGATGTGCAGTACCAGTAA-3′) and CTX-M R (5′-CTCCGCTGCCGGTTTTTATC-3′), yielding a 544bp product (Edelstein et al., 2003).

Thermal cycling for CTX-M included initial denaturation at 94°C for 15min, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1.5min, extension at 72°C for 1min, and a final extension at 72°C for 7min. PCR products were analyzed via 2% agarose gel electrophoresis stained with ethidium bromide, run at 100V for 70 minutes. A 100bp DNA ladder was used for molecular size estimation, and bands were visualized using a gel documentation system (Edelstein et al., 2003).

Data Analysis

Collected data were entered into Microsoft Excel and analyzed using SPSS version 23.0 and analyzed accordingly.

RESULTS

Out of 1,815 clinical specimens analyzed, the majority were urine samples (65.8%, n=1,194), followed by blood (25.1%, n=455), sputum (5.2%, n=94), pus (3%, n=55), body fluids (0.5%, n=10), and other specimen types (0.4%, n=7). Among the isolates, *Escherichia coli* was the most predominant species, accounting for 89.1%, followed by *Klebsiella pneumoniae* (6.2%), *Proteus* spp (3.96%), and *Klebsiella oxytoca* (0.74%).

Table 1: Distribution pattern of Gram-Negative bacteria in clinical samples

Organism isolated	No. of isolates	Total percentage (%)
Escherchia coli	360	89.1
Klebsiella pneumoniae	25	6.2
Proteus spp	16	3.96
Klebsiella oxytoca	3	0.74
Total	404	100

Antimicrobial susceptibility pattern of *Klebsiella* spp

Among 28 *Klebsiella* spp isolates, the highest sensitivity was detected against Amikacin (60.7%), followed by Meropenem (57.1%) and Gentamicin (46.4%). Moderate sensitivity was detected against Cotrimoxazole (39.3%), Ceftriaxone (35.7%), and fluoroquinolones—Norfloxacin, Levofloxacin, and Ciprofloxacin (32.1% each). The isolates were highly resistant against Amoxicillin (100%), Cefixime (89.3%), Cefotaxime (75.0%), Ofloxacin (71.4%) and Ceftazidime (64.3%) as shown in table 2.

Table 2: Antimicrobial susceptibility pattern of *Klebsiella* spp

Antibiotic used	Sensitive N(%)	Intermediate N(%)	Resistance N(%)
Amikacin	17(60.7)	4(14.3)	7(25)
Amoxicillin	0(0)	0(0)	28(100)
Cefixime	3(10.7)	0(0)	25(89.3)
Ciprofloxacin	9(32.1)	0(0)	19(67.9)
Ceftriaxone	10(35.7)	3(10.7)	15(53.6)
Cotrimoxazole	11(39.3)	1(3.6)	16(57.1)
Ceftazidime	4(14.3)	6(21.4)	18(64.3)
Cefotaxime	5(17.9)	2(7.1)	21(75)
Doxycycline	8(28.6)	4(14.3)	16(57.1)
Gentamycin	13(46.4)	4(14.3)	11(39.3)
Levofloxacin	9(32.1)	4(14.3)	15(53.6)

Antibiotic used	Sensitive N(%)	Intermediate N(%)	Resistance N(%)
Meropenem	16(57.1)	4(14.3)	8(28.6)
Nalidixic acid	5(17.9)	6(21.4)	17(60.7)
Norfloxacin	9(32.1)	1(3.6)	18(64.3)
Nitrofurantoin	5(17.9)	7(25)	16(57.1)
Ofloxacin	6(21.4)	2(7.3)	20(71.4)
Piperacillin/tazobactam	11(39.3)	13(46.4%)	4(14.3)

Screening and confirmation of ESBL producing *Klebsiella* spp

ESBL production was screened using the Kirby-Bauer disc diffusion method in accordance with CLSI guidelines, employing 30µg discs of third-generation cephalosporins (Ceftazidime, Cefotaxime, and Ceftriaxone). Of the 28 *Klebsiella* spp isolates, 22 (78.6%) were screened positive for ESBL production whereas 18 (64.3%) were confirmed as ESBL producers by at least one of the three antibiotics.

Prevalence of CTX-M gene among *Klebsiella* spp

Among 28 *Klebsiella* spp isolates, 18 (64.3%) were confirmed as ESBL producers, with the CTX-M gene detected in 7(38.9%) of these isolates, indicating its prevalence among ESBL-producing strains.

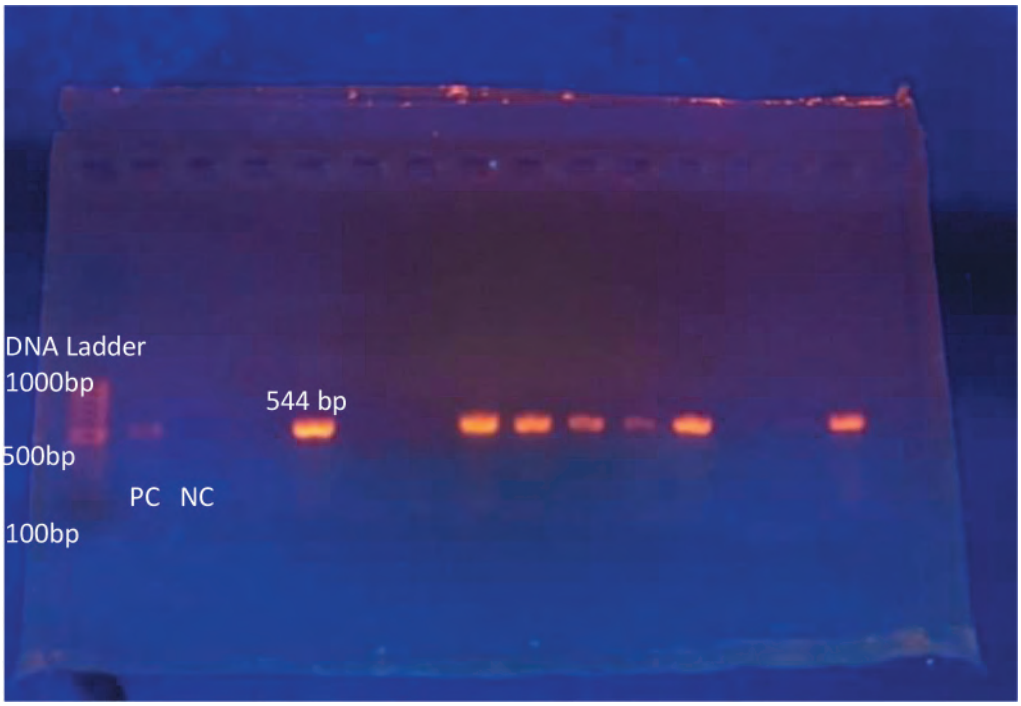

Figure 1: Gel electrophoresis patterns of CTX-M gene producing *Klebsiella* spp

DISCUSSION

In this study, urine was the predominant specimen type (65.8%), followed by blood (25.1%) and sputum (5.2%). This pattern reflects the high burden of urinary tract infections (UTIs) as reported globally and in Nepal, where urine samples typically account for the majority of clinical submissions (Flores-Mireles et al., 2015; Shaikh et al., 2008; Shrestha et al., 2019).

E. coli was the most frequently isolated organism (89.1%), consistent with its established role as the leading cause of UTIs and bloodstream infections worldwide (Tandogdu & Wagenlehner, 2016) and in Nepalese hospitals (Khanal et al., 2022; Mahaseth et al., 2019). Its dominance is likely due to virulence factors such as adhesins and biofilm formation which is responsible for colonization of the urinary tract (Flores-Mireles et al., 2015).

Klebsiella pneumoniae was the second most common isolate (6.2%). Though less prevalent than *E. coli*, it remains clinically significant due to its frequent association with ESBL and carbapenemase production, as reported by previous investigators in Nepal (Mahaseth et al., 2019; Pyakurel et al., 2021; Sharma et al., 2024). Lower prevalence of *Proteus* spp (3.96%)

and *Klebsiella oxytoca* (0.74%) is consistent with prior studies from Nepal where these species contribute less frequently to UTIs but may harbor multidrug resistance (Khanal et al., 2022; Shrestha et al., 2019).

In this study, the 28 *Klebsiella* spp isolates demonstrated the highest sensitivity to Amikacin (60.7%), followed by Meropenem (57.1%) and Gentamicin (46.4%). Moderate sensitivity was observed against Cotrimoxazole (39.3%), Ceftriaxone (35.7%), and fluoroquinolones (32.1%). In contrast, the isolates showed high resistance to Amoxicillin (100%), Cefixime (89.3%), Cefotaxime (75.0%), Ofloxacin (71.4%), and Ceftazidime (64.3%). This susceptibility profile aligns with trends reported in Nepal, where Amikacin and carbapenems remain the most effective agents against *Klebsiella* urinary isolates, whereas third-generation cephalosporins and fluoroquinolones are largely ineffective due to widespread ESBL production (Khanal et al., 2022; Mahaseth et al., 2019; Sharma et al., 2024).

Similar patterns have been reported elsewhere in the world. Studies from India, Europe, and the Middle East countries demonstrate high resistance of *Klebsiella pneumoniae* to β-lactams and fluoroquinolones, with preserved activity of aminoglycosides and carbapenems (Lahlaoui, et al., 2014; Logan & Weinstein, 2017; Pitout et al., 2019). The high resistance to cephalosporins and fluoroquinolones in our study likely reflects the dissemination of ESBL genes such as *bla* $_{CTX-M}$, which hydrolyze third-generation cephalosporins and confer cross-resistance to multiple antibiotic classes (Cantón et al., 2012).

Although Meropenem retained over 50% sensitivity, the emergence of carbapenem-resistant *Klebsiella* strains is a growing concern in Nepal (Mahaseth et al., 2019; Pyakurel et al., 2021). This underscores the importance of routine antimicrobial susceptibility testing and judicious use of broad-spectrum antibiotics to limit further resistance. The moderate susceptibility to Piperacillin/Tazobactam (39.3%) and Gentamicin (46.4%) suggests that these agents could be considered for empirical therapy where carbapenem-sparing strategies are required, but susceptibility-guided therapy remains essential.

Screening for ESBL production using the Kirby-Bauer disc diffusion method revealed that 22 of 28 isolates (78.6%) were screen-positive, and 18 isolates (64.3%) were confirmed as ESBL producers. Molecular characterization demonstrated the presence of the *bla* $_{CTX-M}$ gene in 7 of these confirmed ESBL-producing isolates.

The dominance of CTX-M–type ESBLs among ESBL producers is well documented elsewhere in the world. For instance, studies from Ethiopia reported 95.8% of ESBL-producing isolates harboring CTX-M–type genes, with CTX-M-1 group (particularly CTX-M-15) being most common (Worku et al., 2025). In Iran, 92% of ESBL-producing *Klebsiella pneumoniae* urinary isolates carried *bla* $_{CTX-M}$, highlighting the widespread distribution of this genotype (Maleki et al., 2018). Similarly, recent investigations demonstrate high CTX-M prevalence, with many isolates also co-carrying resistance determinants to aminoglycosides, fluoroquinolones, and sulfonamides (Park et al., 2024).

In Nepal, the predominance of CTX-M–type ESBLs has been reported in multiple studies. Khanal et al., (2022) found that 89.6% of phenotypically confirmed ESBL producers carried blaCTX-M (Khanal et al., 2022), while Mahaseth et al., (2019) reported CTX-M prevalence of 30–50% among ESBL-positive uropathogens (Mahaseth et al., 2019). Pantha et al., (2024) observed ESBL production in 78% of pediatric urinary isolates, with a significant proportion harboring CTX-M genes (Pantha et al., 2024). These findings align with our results and confirm the widespread dissemination of CTX-M–type ESBLs among *Klebsiella* spp in Nepal.

Detection of CTX-M in only 7 of 18 phenotypic ESBL isolates suggests that other ESBL genes, such as blaTEM and blaSHV, may be present in the remaining isolates, a phenomenon reported by previous investigators in Nepal (Chander & Shrestha, 2013; Mahaseth et al., 2019) . The global and local dominance of CTX-M-type ESBLs likely reflects several factors: high transmissibility via plasmids, co-carriage of resistance genes to other antibiotic classes, and selective pressure from widespread cephalosporin use (Cantón et al., 2012).

CONCLUSION

The antimicrobial susceptibility profile of the 28 *Klebsiella* spp isolates revealed limited treatment options, with the highest sensitivity observed for Amikacin, Meropenem, and Gentamicin. Moderate susceptibility to cotrimoxazole, ceftriaxone, and fluoroquinolones further reflects emerging resistance trends. Notably, the isolates exhibited

very high resistance to amoxicillin, third-generation cephalosporins, and ofloxacin underscoring the continued spread of multidrug-resistant *Klebsiella* spp and the need for vigilant antibiotic stewardship and routine resistance monitoring. Similarly, A high proportion of *Klebsiella* spp isolates demonstrated extended-spectrum β-lactamase activity, with 64.3% confirmed as ESBL producers. Molecular analysis further revealed that the CTX-M gene was present in a notable subset of these isolates, underscoring its role in mediating resistance among ESBL-producing *Klebsiella* spp. These findings highlight the growing significance of CTX-M–type β-lactamases and emphasize the need for continued surveillance and effective antimicrobial stewardship.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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ORIGINAL RESEARCH

Integrative Analysis of Community-Level Determinants of Pediatric Diarrhea in the Peri-Urban area of Kathmandu

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ABSTRACT

Objectives: This study aimed to identify community-level environmental and behavioral determinants associated with acute pediatric diarrhea among children under five years of age in peri-urban areas of Kathmandu Valley, Nepal.

Methods: A community-based cross-sectional study was conducted between May 2024 and October 2025 across three peri-urban community hospitals in Kathmandu Valley. A total of 550 children under five years of age presenting with acute diarrheal symptoms were enrolled following informed consent from guardians. Data on clinical presentation, environmental exposures, child-specific behaviors were collected using pretested semi-structured questionnaires and analyzed.

Results: Abdominal cramps (66.7%) and mucus in stool (64.2%) were the most commonly reported symptoms, while blood in stool (8.5%) and blood in urine (1.6%) were rare. Jar water was the predominant drinking water source (65.8%) and showed a strong association with vomiting and watery stool (χ^2, $p < 0.001$ for both). Multivariate logistic regression identified increasing age (adjusted OR = 1.32, 95% CI = 1.15–1.52) and putting toys in the mouth (adjusted OR = 2.02, 95% CI = 1.29–3.15) as significant, independent predictors of fever ($p \leq 0.002$). The model demonstrated a classification accuracy of 69.8% with marginal goodness-of-fit (Hosmer – Lemeshow, p = 0.045). Receiver operating characteristic (ROC) analysis indicated that age had the highest individual discriminatory power, whereas stool consistency and mucus in stool were poor predictors.

Conclusions: Pediatric diarrhea in peri-urban Kathmandu remains strongly influenced by child-specific behaviors and household water safety. Targeted interventions addressing hygiene practices, safe water handling and caregiver awareness are essential to reduce diarrheal morbidity in rapidly urbanizing settings.

Keywords: Pediatric diarrhea, Kathmandu Valley, community level determinants, hygiene behaviors

INTRODUCTION

Pediatric diarrhea remains a major global public health challenge, particularly among children under five years of age. Despite advances in sanitation and health care, diarrheal diseases are responsible for nearly half a million deaths annually, predominantly in low- and middle-income countries especially in South Asia and sub-Saharan Africa (World Health Organization, 2024). Besides mortality, recurrent diarrheal episodes lead to chronic malnutrition, growth delays, and cognitive

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impairment, perpetuating cycles of poverty and poor health across generations. In Nepal, diarrheal disease is ranked among the top five disease of childhood morbidity, with the highest incidence observed in children aged 6-23 months. The burden is further intensified during the monsoon season, when water quality and sanitation are compromised, leading to seasonal peaks in disease incidence (Ministry of Health and Population, 2023; Nepal Demographic and Health Survey, 2022). The persistence of diarrhea, which extends beyond pathogens, involves a complex environmental and behavioral determinant. Understanding these interconnected factors is essential for designing effective and context-specific interventions.

Kathmandu Valley, with its rapidly evolving mix of urban, peri-urban and rural lifestyles, highlights profound disparities in water access, sanitation facilities, and hygiene practices. These challenges are particularly pronounced in rapidly growing informal settlements and peri-urban areas, where high population density and inadequate infrastructure increase the vulnerability to diarrheal diseases. Although governmental and non-governmental initiatives have aimed to improve water, sanitation, and hygiene (WASH) infrastructure and promote healthy behaviors, progress has been uneven across regions and socioeconomic groups (Getahun and Adane 2021; UNICEF Nepal, 2021). Continued reliance on conventional drinking water sources that are frequently contaminated, coupled with irregular piped water supply in many regions, further exacerbates the risk of childhood diarrhea, especially during periods of heavy rainfall.

Despite the availability of effective, low-cost interventions such as oral rehydration therapy, acute diarrheal disease remains a leading cause of childhood morbidity and mortality in Nepal, with prevalence ranging from 11.9% to over 50%. The burden is exacerbated by significant geographic and socioeconomic disparities, with children from poorer households, those living in provinces such as Sindhuli, Karnali and Sudurpaschim (Far Western Nepal), and whose caregivers lack adequate knowledge or access to improved sanitation are affected (Ansari et al., 2011; KC et al., 2025). While community-based programs, have contributed to reductions in severe disease mortality, significant gaps persist, including limited maternal knowledge of dehydration signs and home management, treatment costs, and inconsistent access to preventive and curative services in remote areas (Ghimire et al., 2010, Thapa et al., 2023; Mishra et al., 2022).

Optimistically, reported diarrheal cases in Nepal have declined threefold over the past decade, largely attributed to improvements in WASH practices and reductions in open defecation (MOHP, 2022). However, evidence suggests that contamination of water sources remains widespread, and children's behaviors, and feeding practices continue to play a critical role in the incidence of diarrhea (Bhandari et al., 2020; Dhimal et al., 2021). Continued efforts to ensure safe drinking water and public awareness are essential to sustain this progress.

Existing research in Nepal has predominantly relied on hospital-based surveillance data and pathogen identification, offering limited insights into community level determinants within rapidly urbanizing settings like Kathmandu valley. Consequently, there is a lack of robust evidence on the interplay between household hygiene practices, animal exposure, childcare practices, and environmental conditions at the community level. Addressing this knowledge gap is vital for informing locally tailored and effective prevention strategies. This study aims to assess community-level determinants of pediatric diarrhea in Kathmandu Valley, generating evidence to support targeted public health interventions and contribute to Nepal's ongoing efforts to reduce childhood morbidity and mortality from diarrheal diseases.

METHODS

Ethical Approval: Ethical clearance was obtained from the Institutional Review Committee of the Nepal Health Research Council (NHRC), Kathmandu, Nepal (Ref. No. 1504). Written informed consent was obtained from the parents or legal guardians of all participating children prior to enrollment, ensuring voluntary participation.

Study design and setting: A community-based cross-sectional study was conducted across three strategically selected community hospitals located in the peri-urban areas of the Kathmandu Metropolitan City ring road: Gokarneshwar Hospital, Gokarna; Tokha Chandeshwori Hospital, Tokha; and Shankharapur Hospital, Jorpati. These hospitals primarily serve semi-rural and peri-urban populations and were selected to

capture diarrheal disease determinants in communities transitioning from rural to urban settings. Although participant recruitment occurred in hospitals, the catchment populations of these facilities represent defined community settings, allowing assessment of community-level environmental and behavioral factors associated with pediatric diarrhea. Data and samples collection were conducted over an 18-month period from May 2024 to October 2025.

Sample size: The sample size was calculated using Cochran's formula, assuming a 40% prevalence of pediatric diarrhea based on prior study (Sangma and Rasania, 2025), a 5% margin of error, and a 95% confidence level, with a 50% inflation factor (design effect [DEFF] = 1.5) to account for non-response and to ensure adequate statistical power for subgroup analyses, resulting in a final target sample size of 550 children.

Study participants and sample collection: Children under 60 months of age presenting clinically suspected acute diarrhea were enrolled from the outpatient departments or emergency units of the participating hospitals. Eligible cases were identified by attending pediatricians, who introduced the study to the guardians. After obtaining written informed consent, participants were enrolled consecutively. A pretested semi-structured questionnaire was administered by trained pediatricians to ensure uniformity and data reliability.

Data collection: Pediatricians collected data at the time of clinical evaluation and sample collection. The questionnaire collected detailed information on sociodemographic factors (age, gender), clinical features of diarrhea and medical history, vaccination status, environmental and behavioral factors, including household conditions, child-specific habits and hygiene practices at home and in the community, and recent travel history.

Data management and statistical analysis: Data from the semi-structured questionnaires were entered into Microsoft Excel, followed by data cleaning, validation, and consistency checks. Descriptive statistics were used to summarize participant characteristics, while chi-square tests and multivariable logistic regression were applied to assess associations between community-level factors and diarrheal outcomes. A p-value of <0.05 was considered statistically significant. All analyses were conducted using Linux-based R software (version 4.3.2).

RESULTS

Study population

A total of 550 children aged 1-60 months with clinically suspected acute diarrhea were enrolled from peri-urban community hospital settings at outside ring-road in Kathmandu Valley. Any one of the following key symptoms: acute diarrhea lasting at least one day, lose or watery stool, or a frequency of three or more defecations within a 24-hour period, abdominal cramps and mucus and/or blood in stool, was considered during enrollment. Males were predominant (60.4%) and the most represented age group was 13 - 24 months (30.7%), followed by 7 – 12 months (19.8%). The majority of participants (70.5%) were from Gokarneshwar Hospital **(Table 1).**

Table 1: Sociodemographic characteristics of the study population

Categories		Gender		Total (%)
		M	F	
Study centers	Gokarneshwar Hospital	220	168	388 (70.5)
	Shankharapur Hospital	57	20	77 (14)
	Tokha Hospital	55	30	85 (15.5)
Age group (months)	below 6	35	22	57 (10.4)
	7 to 12	66	43	109 (19.8)
	13 to 24	110	59	169 (30.7)
	25 to 36	48	43	91 (16.5)
	37 to 48	41	22	63 (11.5)
	48 to 60	32	29	61 (11.1)
Total		**332 (60.4%)**	**218 (39.6%)**	**550 (100)**

Clinical presentation and vaccination status

Semi-formed stool was the most frequently reported consistency (57.5%), followed by watery stool (35.3%) **(Figure 1)**.

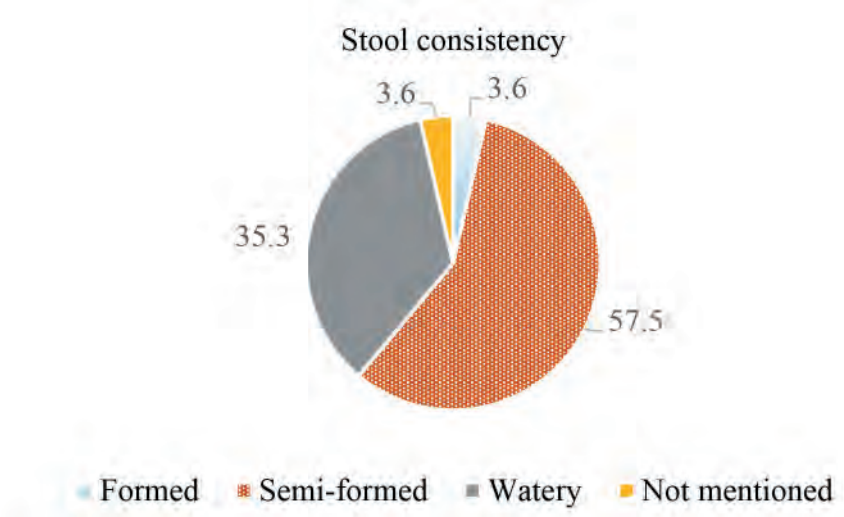

Figure 1: Stool consistency

Abdominal cramp was the most common clinical symptom (66.7%), followed by mucus in stool (64.2%). Fever and vomiting were reported in 27.8% and 20.5% of cases respectively. Blood in stool was uncommon, with only 8.5%, and blood in urine was rare (1.6%) (**Table 2a**). Regarding vaccination status, rotavirus vaccine coverage was the highest (73.2%), followed by typhoid (43.5%) and cholera vaccines (24.9%) (**Table 2b**).

Table 2a: Patients clinical symptoms and medical history

Clinical Symptoms and Medical History	Yes (%)	No (%)	Not mentioned (%)
Blood in stool	47 (8.5)	481 (87.5)	22 (4)
Mucus in stool	353 (64.2)	174 (31.6)	35 (4.2)
Fever	153 (27.8)	362 (65.8)	35 (6.4)
Nausea	82 (14.9)	413 (75.1)	55 (10)
Vomiting	113 (20.5)	409 (74.4)	28 (5.1)
Muscle ache	113 (20.1)	415 (75.5)	24 (4.4)
Abdominal cramp	367 (66.7)	159 (28.9)	24 (4.4)
Blood in urine	9 (1.6)	516 (93.8)	25 (4.6)
Prior antibiotic therapy	46 (8.4)	468 (85.1)	36 (6.5)

Table 2b: Vaccination Status

Vaccine taken	Yes (%)	No (%)	Not mentioned (%)	Didn't Know (%)
Typhoid vaccine	239 (43.5)	271 (49.2)	39 (7.1)	1 (0.2)
Rota vaccine	403 (73.2)	106 (19.3)	39 (7.1)	2 (0.4)
Cholera vaccine	137 (24.9)	365 (66.3)	40 (7.3)	8 (1.5)

Diarrhea Onset and Defecation Frequency

Most children (60.2%) presented within 1-2 days of symptoms onset, indicating predominantly acute cases. Defecation frequency was highest at 2 episodes per day (34.4%), while 11.8% reported more than 5 daily episodes. Only 4.4% experienced symptoms lasting over 5 days, suggesting most cases were self-limiting **(Table 3)**.

Table 3: Diarrhea onset and frequency of defecation

Factors	Response in questionnaires	Frequency (%)
Symptoms/Diarrhea appeared since (days)	1 Day	129 (23.5)
	2 Days	202 (36.7)
	3 Days	113 (20.5)
	4 Days	44 (8)
	5 Days	18 (3.3)
	More than 5 Days	24 (4.4)
	Not mentioned	20 (3.6)

Factors	Response in questionnaires	Frequency (%)
Frequency of defecation	1 time a day	40 (7.3)
	2 times a day	189 (34.4)
	3 times a day	114 (20.7)
	4 times a day	77 (14)
	5 times a day	42 (7.6)
	More than 5 times a day	65 (11.8)
	Not mentioned	23 (4.2)

Behavioral and environmental determinants

Frequent oral exploratory behavior was observed, with 63.3% of children putting fingers in their mouth and 32.7% placing toys in their mouth. Hand hygiene practices were suboptimal, as only 20% of caregivers reported that children always washed their hands after returning home. Environmental exposures risks were relatively low: 85.3% of households reported no pets or cattle, and reports of animal diarrhea (0.7%), recent travel to endemic areas (2.2%), diarrhea among family members (2.9%), and diarrhea in neighbors (0.4%) were uncommon (**Table 4** and **Table 5**).

Table 4: Child behaviors and hygiene practices

Child-specific behaviors and hygiene practices				
Child behaviors	**Yes (%)**	**No (%)**	**Not mentioned (%)**	**Sometimes (%)**
Finger in mouth	348 (63.3)	140 (25.5)	53 (9.6)	9 (1.6)
Crawling of child	144 (26.2)	296 (53.8)	106 (19.3)	9 (1.6)
Toys in mouth	180 (32.7)	314 (57.1)	55 (10)	1 (.02)
Hand washing when back to home from out	110 (20)	244 (44.4)	54 (9.8)	142 (25.8)

Table 5: Potential source exposure history

Potential source exposure history	Yes (%)	No (%)	Not mentioned (%)	Not noticed (%)
Pets/cattle at home	25 (4.5)	469 (85.3)	56 (10.2)	--
Diarrhea in pets/cattle	4 (0.7)	490 (89.1)	56 (10.2)	--
Recent travel history	12 (2.2)	483 (87.8)	55 (10)	--
Diarrhea in family	16 (2.9)	480 (87.3)	54 (9.8)	
Diarrhea in neighbors	2 (0.4)	483 (87.8)	54 (9.8)	11 (2)

Household Environment and behavioral risk factors

More than half of households had cemented floors (57.2%), while jar water was the predominant drinking source (65.8%). Approximately one-third of children (34.4%) consumed no food outside the home during the preceding week, whereas kindergarten canteens were a common source of outside food (20.4%). Missing responses ranged from 10 to 15.8% across environmental variables **(Table 6).**

Table 6: Distribution of household environment and behavioral risk factors

Risk Factors	Response in questionnaires	Frequency (%)
Floor of house	Carpeted	114 (26.2)
	Cemented	315 (57.2)
	Muddy	34 (6.2)
	Others	2 (0.4)
	Not mentioned	55 (10)
Child food from outside (within a week)	Home only	93 (16.9)
	Breastfeeding only	8 (1.5)
	Hotel	17 (3.1)
	Kindergarten Canteen	112 (20.4)
	Junk foods	4 (0.6)
	Sometimes	35 (6.4)
	No	189 (34.4)
	Others	5 (0.9)
	Not mentioned	87 (15.8)

Risk Factors	Response in questionnaires	Frequency (%)
Drinking water type	Boiled water	78 (14.2)
	Breastfeeding only	12 (2.2)
	Filtered water	12 (2.2)
	Jar water	362 (65.8)
	Natural resource	3 (0.5)
	Tap water	27 (4.9)
	Deep well water	1 (0.2)
	Not mentioned	55 (10)

Association between stool consistency with gender and fever

Fever prevalence increased with worsening stool consistency, from formed stool (15.0%) to semisolid (25.3%) to watery stool (36.1%). This association was stronger in males. The relationship between stool consistency and fever was statistically significant across gender (p<0.0001), with moderate-to-large effect sizes (Cramer's V= 0.535). Missing stool data corresponded with missing fever data, indicating a documentation gap (**Table 7**).

Table 7: Association of stool consistency with genders and fevers

Stool consistency	Fever in Female			Fever in Male			p-value
	Yes	No	Not mentioned	Yes	No	Not mentioned	
Formed	2	5	--	1	12	--	Both genders and overall (0.0001)
Semisolid	31	95	6	49	129	6	
Watery	23	46	--	47	75	3	
Not mentioned	--	--	10	--	--	10	
Total	**56**	**146**	**16**	**97**	**216**	**19**	

Fecal characteristics and abdominal cramp

Abdominal cramps were significantly more common among children with blood in stool (70.2%) and mucus in stool (75.6%) compared to those without these features. Strong associations were observed between abdominal cramp and blood in stool (χ^2 = 525.2, p = 0.0001), as well as mucus in stool (χ^2 = 522.940, p = 0.0001) (**Table 8**).

Table 8: Association between blood and mucus in stool and abdominal cramp

Fecal characteristics		Abdominal Cramp				Total	p-Value
		Yes	No	Not mentioned	Not noticed		
Blood in stool	Yes	33	14	--	--	47	x^2 = 525.2 p-Value = 0.0001
	No	334	145	1	1	481	
	Not mentioned	--	--	22	--	22	
Mucus in stool	Yes	267	84	1	1	353	x^2 = 522.940 p-Value = 0.0001
	No	99	75	--	--	174	
	Not mentioned	1	--	22	--	23	

Vaccination status by age group

Vaccination coverage increased with age, peaking in the 13-24 month age group. Significant associations were observed between age and rotavirus vaccines (χ^2 = 57.257, p < 0.001) and typhoid vaccines (χ^2 = 102.635, p < 0.001). No significant age-based association was observed for cholera vaccination (χ^2 = 17.580, p=.0.285), with remained low (19.3%-32.3%) across all ages (**Table 9**).

Table 9: Association between age group and vaccination status

Age (months)	Rota vaccine		Typhoid vaccine		Cholera vaccine	
	Yes	No/don't know/others	Yes	No/don't know/others	Yes	No/don't know/others
below 6	26	31	7	50	11	46
7 to 12	79	30	24	85	28	81
13 to 24	130	39	79	90	35	134
25 to 36	71	20	46	45	27	64
37 to 48	50	13	36	27	18	45
48 to 60	47	14	47	14	18	43
Total	**403**	**147**	**239**	**311**	**137**	**413**
	x^2 = 57.257, p = .0001		x^2 = 102.635, p < .001		x^2 = 17.580, p = 0.285	

Defecation frequency and pet animal exposure

Most households reported no pets or cattle (84.5%), and animal diarrhea was rare. Although statistically significant associations were observed between defecation frequency and pet ownership (χ^2 = 206.71, p < 0.001, Cramer's V = 0.43) and animal diarrhea (χ^2 = 207.03, p < 0.001, Cramer's V = 0.43), interpretation is limited due to moderate effect sizes (Cramer's V = 0.43) and a high proportion of cells with expected counts below five. The significance appears largely driven by missing data pattern rather than biologically meaningful relationships (**Table 10**).

Table 10: Child defecation frequency in relation to household animal presence and animal diarrheal illness

Frequency of defecation per day (times)	Pets/cattle at home			Diarrhea in pets/cattle		
	Yes	No	Not mentioned	Yes	No	Not mentioned
1	4	32	4	1	35	4
2	6	165	18	2	169	18
3	5	98	11	--	103	11
4	4	72	1	--	76	1
5	3	39	--	--	42	--
More than 5	3	62	--	1	64	--
Not mentioned	--	1	22	--	1	22
p-Value: 0.0001				*p*-Value: 0.0001		

Drinking water source as a determinant of vomiting and watery diarrhea

Type of drinking water is strongly associated with vomiting (χ^2 = 217.74, p = 0.0001) and stool consistency (χ^2 = 350.11, p = 0.0001). Jar water and boiled water accounted for the majority of vomiting and watery stool cases, suggesting potential contamination or handling related risks (**Table 11**).

Table 11: Association between drinking water type, vomiting and stool type

Types of drinking water	Vomiting			Types of stool			
	Yes	No	Not mentioned	Formed	Semisolid	Watery	Not mentioned
Boiled Water	31	47	--	1	13	64	--
Breastfeeding	1	11	--	--	8	4	--
Deep well	1	--	--	--	--	1	--
Filtered Water	5	7	--	--	2	10	--
Jar Water	58	299	5	9	266	87	--
Natural Resource	3	--	--	1	--	2	--
Tap Water	10	17	--	--	11	16	--
Not mentioned	4	28	23	9	16	10	20
***p*-Value**	**x^2 = 217.74, *p*-value= 0.0001**			**x^2 = 350.11, *p*-value= 0.0001**			

Predictors of fever

Multivariable logistic regression analysis was conducted among 148 complete cases to identify predictors of fever among children with diarrhea. The model was statistically significant (χ^2 (4) = 34.621, p < 0.001) and explained approximately 9.8% of the variance in fever occurrence (Nagelkerke R^2 = 0.098). The Hosmer–Lemeshow test indicated a borderline poor model fit (χ^2 (7) = 14.375, p = 0.045), suggesting that the model's predictions deviated from the observed outcomes. The overall classification accuracy was 69.8%. Age and putting toys in the mouth were strong, significant predictors of fever (p < .001 and p = 0.002, respectively). A one-unit increase in age category increased the odds of fever by 32.1%, and children who placed toys in their mouth had twice the odds of developing fever (aOR = 2.02). Vomiting showed a marginal association (p = 0.079), while stool consistency was not a significant predictor (**Table 12**).

Table 12. Logistic regression analysis of factors associated with fever

Predictor variable	B	SE	Wald	df	*p*-value	Adjusted OR	95% CI for OR
Age Category	0.279	0.072	14.854	1	0.0001	1.321	1.147 – 1.523
Toys in Mouth	0.701	0.227	9.526	1	0.002	2.017	1.292 – 3.148
Stool Consistency	0.111	0.232	0.231	1	0.631	1.118	0.710 – 1.760
Vomiting	0.434	0.247	3.085	1	0.079	1.544	0.951 – 2.505
Constant	-1.916	0.243	62.112	1	0.0001	0.147	—

Model χ^2 (df = 4) = 34.621, Cox & Snell R^2 = 0.070, Nagelkerke R^2 = 0.098, Overall Classification Accuracy = 69.8%, $p < .001$, Hosmer–Lemeshow p-value = 0.045

B = Regression coefficient, SE = standard error of the regression coefficient, df: degree of freedom, CI: Confidence Interval, OR: Odds Ratio

ROC curve analysis confirmed age as the strongest single predictor of fever, followed by putting toys in the mouth. Vomiting demonstrated moderate predictive value, whereas stool consistency and mucus in stool showed minimal discrimination (AUC ≈ 0.50), aligning with their non-significance in the adjusted model (**Figure 2**).

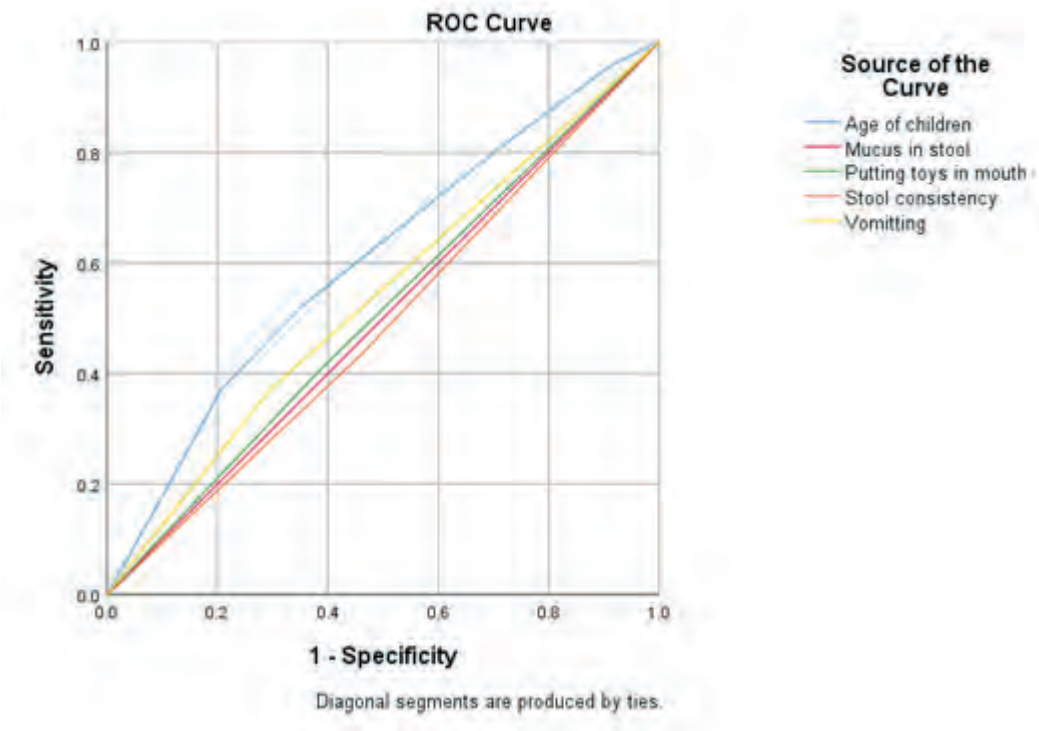

Figure 2: Receiver operating characteristic (ROC) curves comparing the diagnostic accuracy of individual clinical and behavioral predictors for fever

DISCUSSION

The study focused on a high-risk pediatric population, with children aged 13–24 months constituting the largest proportion of cases. This age group is widely recognized as vulnerable to diarrheal disease due to increased mobility, and frequent oral exploratory behaviors. Although a substantial proportion of participants were recruited from a single, potentially limiting generalizability across diverse geographic settings, the use of stringent clinical inclusion criteria ensured enrollment of children with active, symptomatic diarrheal illness, enhancing the clinical relevance of the findings. Despite a reported 70.7% decline in under-five diarrheal mortality in Nepal between 2005 and 2015, diarrhea remains a significant public health burden, with millions of cases and thousands of deaths annually. While expanded use of ORS and zinc supplementation has contributed to reductions in mortality, persistent gaps in preventative WASH infrastructure, child-specific exposure risks, and food safety continue to drive high disease incidence (GBD 2017; Getahun and Adane 2021).

This study highlights critical community-level determinants of pediatric diarrhea in peri-urban Kathmandu, a setting increasingly vulnerable to climate-sensitive health threats. The high prevalence of oral exploratory behaviors, combined with suboptimal hand hygiene practices, reflects well established fecal-oral transmission pathways that are exacerbated by inadequate WASH conditions. The strong associations between commonly used drinking water sources (particularly jar and boiled water), and clinical outcomes such as watery stool and vomiting raise concerns regarding post-treatment contamination during water storage and distribution. These findings

emphasize that access to improved water source alone is insufficient without concurrent attention to safe handling practices and household hygiene, particularly in rapidly urbanizing peri-urban environments.

Vaccination coverage patterns further support critical preventive gaps. While rotavirus vaccination coverage was relatively high, coverage for typhoid and cholera vaccines remained low. In Nepal, rotavirus vaccine is routinely administered in early infancy, whereas typhoid vaccine is typically given at 15 months and cholera vaccines mainly during outbreaks. Low typhoid and cholera vaccine uptake likely reflects a combination of supply constraints, access barriers, and limited parental awareness (Lawrence et al., 2025). Reports of early rotavirus vaccination may reflect documentation errors, misattribution of sibling vaccination status, or reporting inaccuracies under high clinical workloads (Paul et al., 2022). These findings highlight the need for improved vaccination record-keeping and caregiver education to ensure effective immunization coverage.

Although direct exposure to pets was reported infrequenty in this study, the role of zoonotic transmission warrants careful consideration. Numerous studies from Nepal and South Asia have demonstrated that domestic animals can serve as reservoirs for enteric pathogens such as *Campylobacter*, *Salmonella*, *Cryptosporidium*, *Giardia*, and diarrheagenic *E. coli*, which are transmitted to children, with evidence of genetically similar strains circulating between humans and animals (Heyworth et al., 2006; Klous et al., 2016; Delahoy et al., 2018; Shrivastava et al., 2020; Khan et al., 2023; Tumwebaze et al., 2025). Approximately 10% of caregivers in this study reported uncertainty regarding animal exposure or animal diarrhea, indicating limited awareness and surveillance at the community level. This knowledge gap likely leads to underestimation of zoonotic transmission risks and obscures the true contribution of animal fecal contamination to diarrheal disease burden in peri-urban settings.

Clinically, the strong association between blood or mucus in stool and abdominal cramps, observed in this study highlights the frequent co-occurrence of these symptoms in acute diarrheal illness. Compared with reports from rural Nepal, India and Kenya, the overall prevalence of severe symptoms such as abdominal cramping appeared lower, potentially reflecting improved healthcare access and health-seeking behavior in peri-urban Kathmandu. Consistent with previous studies, pathogens such as *Shigella* spp and *Entamoeba histolytica* have been commonly implicated in similar clinical presentations, supporting the importance of hand hygiene, safe water handling, and sanitation as key preventive measures against acute diarrhea (Aggarwal et al., 2016; Njuguna et al., 2016; Taneja and Mewara 2016; Rai et al., 2018).

The association between drinking water sources and gastrointestinal symptoms observed in this study further emphasizes the role of environmental contamination in pediatric diarrhea. The higher frequency of vomiting and watery stool among children consuming boiled and jar water was suggested contamination during storage or handling. However, breastfeeding and use of filtered water were associated with better outcomes. These findings are consistent with studies from Nepal, India, and Ethiopia, demonstrating that unsafe water and poor handling significantly increase diarrheal risk (Lakew et al., 2016; Mitkari et al., 2019; Getahun and Adane, 2021; Shrestha et al., 2021; Zulfiana et al., 2021; Mebrahtom et al., 2022; Merid et al., 2023). Ensuring safe water, proper handling, and strengthening caregiver education on water safety and hygiene practices remains essential for reducing diarrhea transmission.

Multivariable regression analysis identified increasing age and putting toys in mouth as significant predictors of fever among children with diarrhea. The strong effect of age corroborates national evidence identifying children aged 12–24 month as the most vulnerable group. A key behavioral predictor putting toys in the mouth highlights a direct transmission pathway often overlooked in large demographic surveys that emphasize broad determinants like sanitation and household wealth. These findings suggest that while socioeconomic conditions establish baseline risk, immediate child behaviors play a critical role in triggering febrile illness in high-risk age groups (Pathak et al., 2019; Li et al., 2020; Thapa et al., 2023).

CONCLUSION

This study provides a comprehensive characterization of acute diarrheal illness among children under five years of age in peri-urban Kathmandu Valley. The burden is highest among children aged 13–24-month and is driven by a combination of developmental behaviors, inadequate hygiene practices, unsafe water handling, and gaps in vaccination coverage.

While rotavirus vaccination uptake is encouraging, persistently low typhoid and cholera coverage represents a missed opportunity for prevention. Contamination of commonly used water sources, including jar and boiled water, remains a significant risk factor. Although reported direct animal exposure was low, limited awareness suggests that zoonotic transmission risks may be underestimated. Addressing pediatric diarrhea in peri-urban Nepal will require integrated interventions that combine WASH improvements, strengthened immunization programs, and targeted caregiver education focused on child-specific behaviors and household hygiene.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Tribhuvan University Journal of Microbiology

ORIGINAL RESEARCH

Comparative Study of Surgical Site Infections and Antimicrobial Resistance with Focus on Carbapenem Resistant *Klebsiella pneumoniae*

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ABSTRACT

Objectives: The aim of this study evaluates the trends in surgical site infections (SSIs), bacterial distribution, antimicrobial resistance, and molecular detection of resistance genes in two different phases.

Methods: The clinical specimens were collected from SSIs patients in both phases. The bacteria were isolated using standard microbiological techniques and further identified by the VITEK 2 system. Phenotypic screening for carbapenemase was conducted through Modified carbapenem inactivation method (mCIM) while presence of bla_{KPC}, *acrA*, and *acrB* genes were detected by PCR.

Results: In phase II, the SSIs rate reduced by 33% particularly due to improvement in prophylactic practices. The number of isolated bacteria decreased by 73.13%, with Gram-negative bacteria remaining predominant. The study reported significant increase in resistance in *Klebsiella pneumoniae* particularly to amikacin (100%), colistin (80%), and tigecycline (20%) whereas found 100% susceptible to doripenem. All carbapenem resistant *K. pneumoniae* were found mCIM positive but bla_{KPC} negative, while *acrA* and *acrB* genes were detected in all the isolates.

Conclusion: Implementation of improved antimicrobial prophylaxis guidelines resulted in a significant reduction in infection rates. However, an increase in antimicrobial resistance among *K. pneumoniae* was observed. Although the *blaKPC* gene was not detected in carbapenem-resistant isolates, the presence of efflux pump genes in all resistant strains suggests their contributory role in resistance. These findings underscore the urgent need for strengthened antibiotic stewardship and continuous surveillance of resistance mechanisms to curb antimicrobial resistance in surgical settings.

Keywords: Antimicrobial resistance, Carbapenem, Comparative, Efflux pump, Surgical site infections

INTRODUCTION

Surgical site infections (SSIs) and antimicrobial resistance (AMR) are the global health crisis which are linked with each other. According to the survey of 2019, AMR has led to deaths of 1.27 million people worldwide (Murray et al., 2022). The emergence of AMR in the surgical settings is more critical as post-operative infections are common complication. The risk of multidrug resistant bacteria in surgeries have increased leading to difficulty in the treatment of infections (Murray et al., 2022). *Escherichia coli* and *Staphylococcus aureus* are the most common bacteria for morbidity in the context of both post-operative infection and AMR (Chaudhary et al., 2017).

According to World Health Organization (WHO), the global incidence rate of SSIs ranges from 3-50% which

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varies according to the type of surgeries (Leaper et al., 2017). The SSIs rate is significantly higher in low and middle income countries (Leaper et al., 2017). Among patients undergoing caesarean sections, the overall infection rate was estimated at 5.6%, with a substantially higher rate of 11.9% reported in African regions. Following the onset of the COVID-19 pandemic, this infection rate has increased steadily (Farid et al., 2023).

In the hospital settings, administration of antibiotics plays a crucial role in preventing infections during surgery (Leaper et al., 2017). Patients with SSIs receive antibiotics seven times higher than other patients (Aiken et al., 2013). During hospitalization, 60% of patients undergoing surgery receive antibiotics and 50% of patients continue taking antibiotics after discharged (Charani et al., 2023). Overuse of antibiotics as prophylaxis and treatment play a significant role in the development of AMR, threatening healthcare system (Nayan et al., 2023). A rise in the AMR has contributed to an increase in healthcare cost and economic burden. Extended spectrum beta-lactamases (ESBLs) and carbapenem resistant Gram negative organisms have concerned the surgeons for the significant impact on SSIs (Gashaw et al., 2018). The lack of effective therapeutic options has made the treatment of infections caused by carbapenem-resistant Gram-negative bacteria-particularly *Enterobacterales*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*-increasingly difficult. As a result, these infections are associated with higher rates of treatment failure, prolonged hospital stays, increased healthcare costs, and elevated morbidity and mortality, thereby posing a serious challenge to clinical management worldwide (Dossim et al., 2019).

Carbapenem resistance is mediated by multiple mechanisms, most notably the production of carbapenemases, including Ambler class A enzymes such as *K. pneumoniae* carbapenemase (KPC), class B metallo-β-lactamases (NDM, VIM, and IMP types), and class D oxacillinases, particularly OXA-48 (Lee et al., 2014; Kanj et al., 2011). Besides, resistance to carbapenem is also mediated by a non-enzymatic method, involving efflux pumps (Dinh et al., 1994). It is necessary to diagnose carbapenem resistant enterobacterales (CRE), particularly carbapenemase producing enterobacterales (CPE) accurately on time to ensure effective treatment for prevention of SSI infections (Cui et al., 2019).

This study analyzed the SSIs rate, negative bacteria, trends in antimicrobial resistance, change in pattern of molecular detection of bla_{KPC} gene and efflux gene in carbapenem resistant *K. pneumoniae* between phase I and phase II studies. The outcome will help the health professionals make decisions regarding selection of antibacterial agent, to reduce antibiotics misuse and emergence of AMR.

METHODS

Study design

The study was conducted in two different time periods: phase I study (October 2021 to October 2022) and phase II study (April 2023 to July 2023) at Birat Medical College Teaching Hospital (BMCTH), Biratnagar. Total SSIs observed was 48.52% in phase I therefore to improve the preoperative prophylaxis antibiotics change was implemented where overall SSIs decreased to 12.27% in phase II.

Isolation and identification of bacterial pathogens

Specimens such as pus, tissue and body fluids were collected from patients showing symptoms of SSIs, under aseptic conditions. Specimens were cultured on MacConkey agar and incubated at 37°C for 18-24h. The identification and antibiotic susceptibility test (AST) were conducted using a VITEK 2 compact system (BioMérieux, USA).

Phenotypic confirmation test for carbapenemase production

The *K. pneumoniae* resistant to at least one carbapenem were selected and further subjected to confirmatory test of carbapenemase production (Pyakure et al., 2021) Confirmation of carbapenemase production was conducted by the Modified Carbapenem Inactivation Method (mCIM) test (Van Der et al., 2015).

Extraction of DNA and detection of bla_{KPC} and efflux pump genes

Bacterial DNA was extracted by the boiling method and extracted DNA was quantitated. Then 25µL of reaction mixture was prepared by mixing 2µL of template DNA, 5µL of 5X Master Mix (FIREPol), 1µL of 10ρmol of each primer (Table 1) and 16µL of ddH_2O nuclease free water. The bla_{KPC} and efflux pump genes (*acrA and acrB*) were detected by amplification using conventional PCR (Omar et al., 2014; Poirel et al., 2011). Primers used for the amplification are summarized in (Table 1).

Table 1: Specific primer sequence used for the amplification of target genes

Gene name	Primers	Primer sequence (5' - 3')	Amplicon size (bp)	Reference
bla_{KPC}	Primer F	CGTCTAGTTCTGCTGTCTTG	798	(Omar et al., 2014)
	Primer R	CTTGTCATCCTTGTTAGGCG		
acrA	Primer F	TGATGCTCTCAGGCAGCTTA	226	(Poirel et al., 2011)
	Primer R	GCCTGGATATCGCTACCTTC		
acrB	Primer F	CGTCTCCATCAGCGACATTAAC	219	Poirel et al., 2011)
	Primer R	GAACCGTATTCCCAACGCGA		

Statistical analysis

The collected data were entered in excel spreadsheet (2011) and subsequently into SPSS for analysis (Version 26.0). Descriptive statistics were calculated to summarize the relevant variables, and the results were presented using text, figures, and tables. Statistical significance difference is considered at $p<0.05$.

Ethics approval and consent to participate

This study was approved by the Nepal Health Research Council (NHRC) (Ref. No. NHRC 234/2020 for phase I and 160/2023 for phase II data collection). Participants were informed about the study's purpose, potential risks, confidentiality of personal information, and their right to voluntarily participate. Written informed consent was obtained from adult participants, while assent was obtained from participants under 18 years of age, along with consent from their parents or legal guardians. Specimens were then collected from all study participants and analyzed according to the study protocol.

RESULTS

In the phase I study, 251 patients were included among which 134(46%) were found culture positive. In phase II study, the infection rate dropped by 33% (Figure 1).

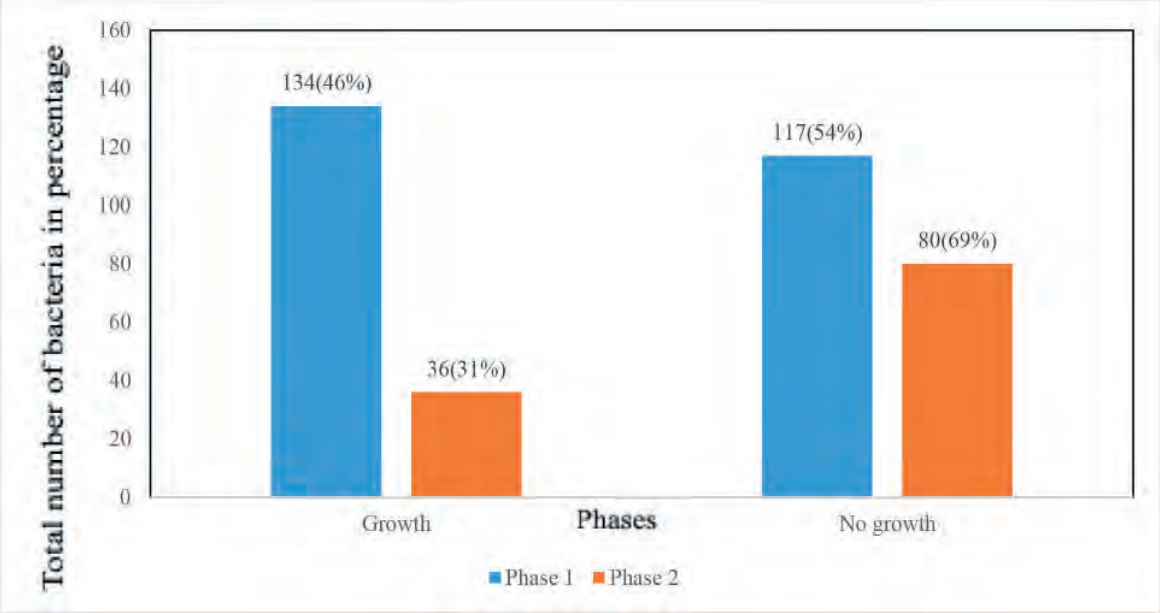

Figure 1: Comparison of prevalence of SSIs in Phases I and II

Distribution of different types of specimen

In phase II only 36 bacteria were isolated. A decrease in 73.13% was observed in phase II compared to phase I. The samples were mainly pus 31(86%), body fluid 4(11%), tissue 1(3%) (Table 2).

Table 2: Distribution of different types of specimen in phases I and II

Specimen	Number of bacteria in phase I N(%)	Number of bacteria in phase II N(%)	Variation rate (%)
Pus	100(75)	31(36)	-69
Body fluid	20(15)	4(11)	-80
Tissue	14(10)	1(3)	-92.85
Total	**134**	**36**	**-73.13**

Prophylactic antibiotics use in two different study period

In phase I, ceftriaxone was the most frequently used antibiotic for preoperative, intraoperative, and postoperative prophylaxis. Other antibiotics administered included cephalexin, azithromycin, ampicillin, clindamycin, amoxicillin–clavulanate, and cefazolin, as illustrated in (Figures 2-4). During this phase, the SSIs rate was 46%. Owing to the high incidence of infections, the findings were communicated to the hospital, and the adoption of an improved antimicrobial prophylaxis regimen was recommended. In phase II, in addition to the antibiotics used in phase I, patients received broader-spectrum agents, including cefixime, piperacillin–tazobactam, ciprofloxacin, amikacin, cefuroxime, and levofloxacin. Following this modification in antibiotic prophylaxis, the SSIs rate decreased by 33%.

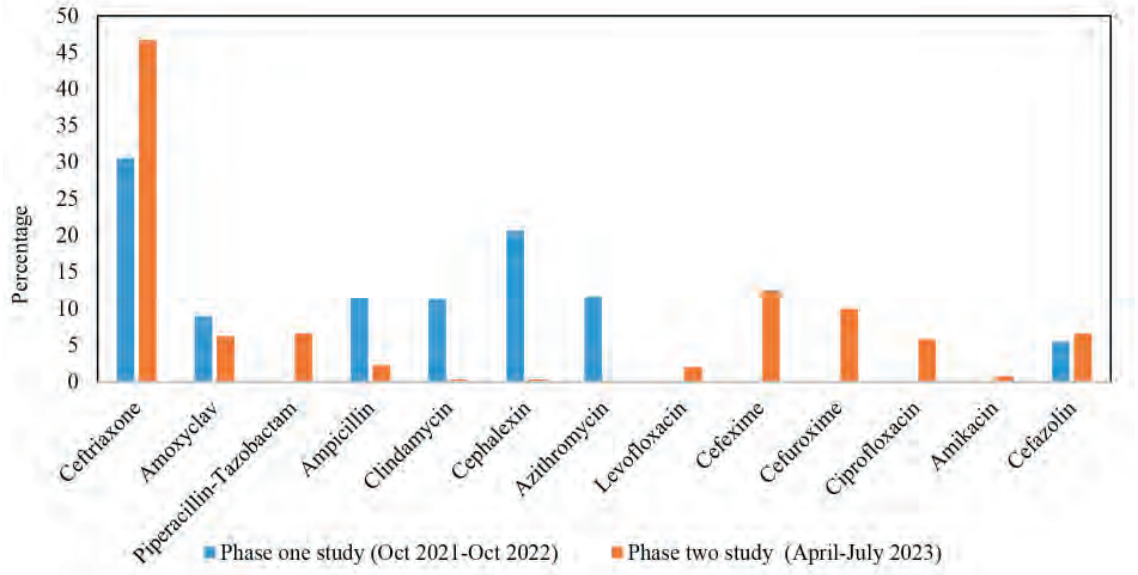

Figure 2: Comparative bar diagram on the pattern of preoperative antimicrobial prophylactic agent used in the patient undergoing surgery

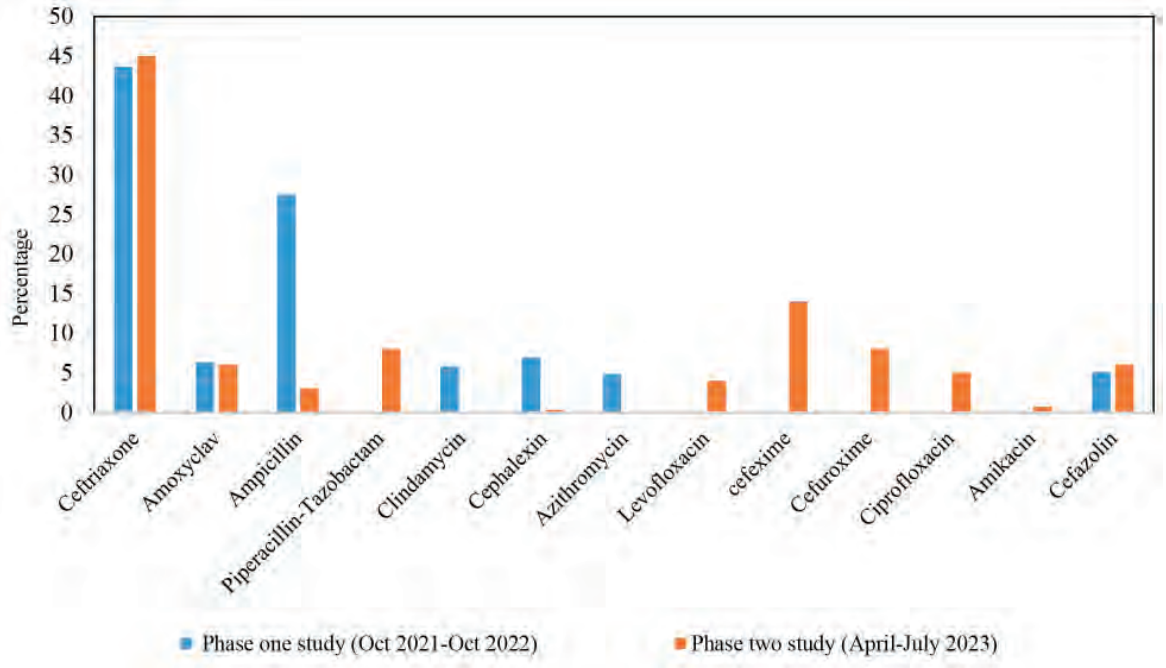

Figure 3: Comparative bar diagram on the pattern of intraoperative antimicrobial prophylactic agent used in the patient undergoing surgery

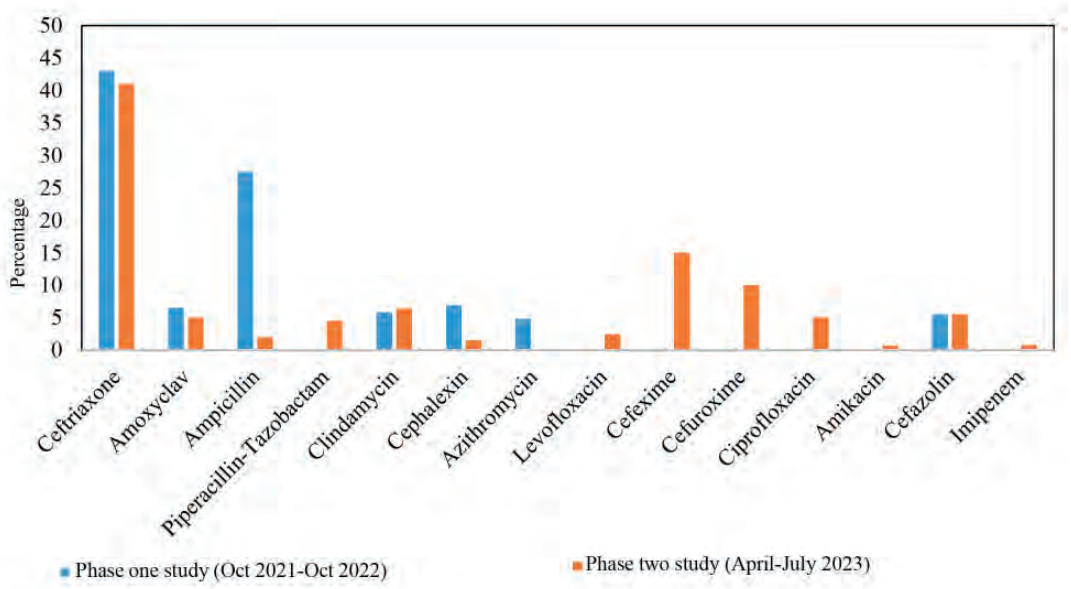

Figure 4: Comparative bar diagram on the pattern of postoperative antimicrobial prophylactic agent used in the patient undergoing surgery

Frequency and distribution of Gram negative bacteria

In phase I, the overall prevalence rate of Gram negative bacteria was 60.44% which included *E. coli* 31(38%), *K. pneumoniae* 24(30%), *P. aeruginosa* 21(26%), *Acinetobacter* spp 4(5%) and *Proteus* spp 1(1%). In phase II, the overall detection rate of Gram negative bacteria was 58.33% accounting *E. coli* 7(33%), *Acinetobacter* spp 4(29%), *K. pneumoniae* 5(24%), and *P. aeruginosa* 5(24%) (Table 3).

Table 3: Comparison of Gram negative bacteria isolated from surgical wounds

Species	Phase I N(%)	Phase II N(%)	Variation rate (%)
E. coli	31(38)	7(33)	-77
K. pneumoniae	24(30)	5(24)	-79
P. aeruginosa	21(26)	5(24)	-76
Acinetobacter spp	4(5)	4(19)	0
Proteus spp	1	0	-100

Antibiotic susceptibility test

In phase II, the resistance rate of *K. pneumoniae* to the majority of the antibiotics had increased significantly compared to phase I study. The resistance rate to amikacin was significantly higher than phase I, with resistance rate of 100% and MIC value of ≥128µg/mL. Compared to phase I, the resistance rate of *K. pneumoniae* to tigecycline and colistin increased from 0% to 20% and 80% respectively. In phase II, doripenem was found 100% sensitive to *K. pneumoniae* (Table 4).

Table 4: Change in antibiotic susceptibility pattern and MIC values

Antibiotics	Phase I		Phase II	
	Resistant (%)	MIC (µg/mL)	Resistant (%)	MIC (µg/mL)
Piperacillin-Tazobactam	45.8	≥128	80	≥128
Ciprofloxacin	45.8	≥2	80	≥4
Gentamicin	29	≥16	80	≥16
Tigecycline	0	-	20	8
Trimethoprime/Sulfamethoxazole	37.5	≥320	80	≥320
Amikacin	37.5	≥64	100	≥128
Cefoperazone/Sulbactam	45.8	≥64	80	≥64
Cefepime	45.8	≥64	80	≥64
Imipenem	45.8	≥16	80	≥16
Meropenem	45.8	≥32	80	≥16
Doripenem	-	-	0	-
Colistin	0	0	80	≥64

Molecular detection of bla_{KPC}, *acrA* and *acrB* genes

During Phase I, a total of 11 carbapenem-resistant *K. pneumoniae* isolates were identified, of which 7 (63.6%) were positive by the modified carbapenem inactivation method (mCIM). In Phase II, the number of carbapenem-resistant *K. pneumoniae* isolates decreased to 4 where all four isolates (100%) were mCIM positive. Detection of the blaKPC gene was not performed in either phase (ND) (Table 5).

Table 5: Phenotypic carbapenemase positive and bla_{KPC} gene in *K. pneumoniae* isolates of phases I and II

Study period	Carbapenem resistant *K. pneumoniae*	mCIM positive *K. pneumoniae*	bla_{KPC}
Phase I	11	7	*ND
Phase II	4	4	*ND

ND: Not detected

All carbapenem resistant isolates were tested for the presence of efflux pump genes (*acrA* and *acrB*). In both phases I and II, efflux pump genes were present in all the carbapenem resistant isolates (Table 6, Figure 5).

Table 6: *acrA* and *acrB* genes in carbapenem resistant *K. pneumoniae* in phases I and II

Study period	Carbapenem resistant *K. pneumoniae*	*acrA*	Percentage (%)	*acrB*	Percentage (%)
Phase I	11	11	100	11	100
Phase II	4	4	100	4	100

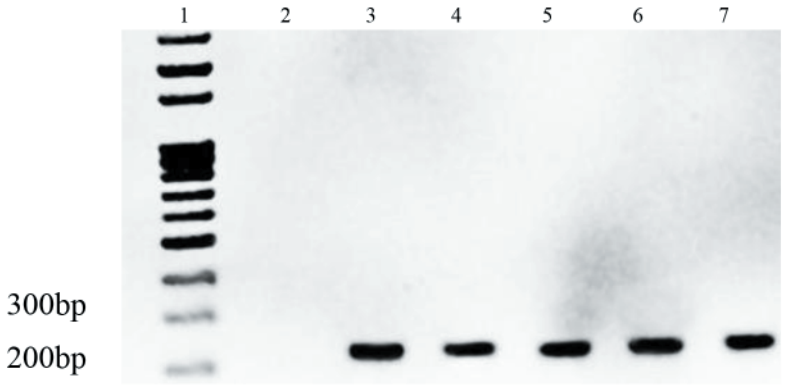

Figure 5: Agarose gel 8% (w/v) image showing efflux pump gene (*acrA* gene-226bp) amplified from carbapenem resistant *K. pneumoniae* isolated from SSIs in phase I. Lane 1: DNA marker, Lane 2: negative control, Lane 3: positive control, Lane 4-7: *acrA* gene of *K. pneumoniae* from SSIs

DISCUSSION

Comparative analysis of SSIs between two study periods revealed that infection rate dropped iin phase II by 33%, highlighting that the prophylactic measures had been effectively implemented and the infection control measures had been enhanced.

In both phases, specimens such as pus, body fluid and tissue were collected from SSIs patients. In phase II study, 36 different bacteria were isolated, which was a significant reduction of 73.13% compared to phase I. In both studies, majority of bacteria were Gram negative bacteria. This finding was consistent with a study conducted in Ethiopia (57.9%) (Razavi et al., 2020; Worku et al., 2023). Furthermore, the prevalence rate of Gram negative bacteria had dropped from 60.44% in phase I to 48.33% in phase II. However there was also a change in the composition of bacteria. In phase I study, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were predominant bacteria. In phase II study, the rate of *E. coli* and *K. pneumoniae* decreased by 77% and 79% respectively.

In this study, the outcome of the changes made in antimicrobial prophylaxis due to high infection rate observed during phase I was analyzed. In phase I, majority of the patients were administered ceftriaxone across all the stages of surgery. Reporting of high infection rate resulted in introduction of more effective antimicrobial prophylaxis. During phase I study, additional broad spectrum antibiotics such as; cefixime, piperacillin-tazobactam, and levofloxacin were introduced, which reduced the infection rate by 33%. A study conducted by Bratzler et al., (2013)

have shown the importance of selecting antimicrobial agents and the time of administration in preventing SSIs. The study suggested cefazolin or cefuroxime for clean surgeries and broader-spectrum agents for surgery with the high risk of contamination (Bratzler et al., 2013). Furthermore, a meta-analysis conducted by Allegranzi et al., (2016) showed that infection rate can be reduced significantly through application of updated version of prophylaxis protocols.

All other bacteria except *K. pneumoniae* were excluded from the study. In phase II, there was a marked increase in antibiotic resistance in *K. pneumoniae* compared to phase I. In phase II study, the resistance to amikacin increased to 100%. An earlier study reported only 23.1% of *K. pneumoniae* were resistant to amikacin (Nepal et al., 2017). The resistant rate to tigecycline had risen to 20%, which is of concern since this antibiotic is often used for the treatment of infection with resistant Gram negative pathogens. Recent study have also reported an increase in resistance to tigecycline, particularly in *K. pneumoniae* (Elgendy et al., 2018). Similarly, a marked increase in colistin resistance was observed between phase I (0%) and phase II (80%). Although colistin is considered a last-line agent for the treatment of multidrug-resistant Gram-negative infections, such a sharp rise in resistance is unexpected and warrants careful interpretation. Several factors may have contributed to this observation. First, the expanded use of broad-spectrum antibiotics in phase II, including agents such as piperacillin–tazobactam, fluoroquinolones, and aminoglycosides, may have exerted indirect selective pressure favouring the emergence or enrichment of intrinsically resistant or heteroresistant *B. fragilis* subpopulations. Second, increased empirical or off-label use of colistin in critically ill patients during Phase II cannot be excluded and may have contributed to resistance selection. Third, anaerobic bacteria such as *B. fragilis* exhibit variable and poorly characterized susceptibility patterns to colistin, and phenotypic resistance may be influenced by methodological limitations of in vitro testing, including inoculum size, growth conditions, and breakpoint interpretation. Importantly, the small sample size in phase II and the absence of molecular characterization of colistin resistance mechanisms (e.g., lipid A modification pathways or *mcr* genes) limit definitive conclusions. Therefore, this finding should be interpreted cautiously and highlights the need for further investigation using standardized susceptibility testing methods and molecular confirmation. Nevertheless, the observed increase raises concern regarding the potential impact of changing antimicrobial practices on resistance patterns and underscores the importance of antimicrobial stewardship. A study conducted in Italy reported 43% of colistin resistance in carbapenem resistant *K. pneumoniae* collected from different hospitals and stated the possibility of evolution of colistin resistance in surroundings with a high prevalence of *K. pneumoniae* producing *K. pneumoniae* carbapenemase (KPC) (Monaco et al., 2014). In phase II study, doripenem was found 100% effective. A dramatic increase in resistance to commonly used antibiotics such as amikacin, tigecycline and colistin emphasizes a critical need of strong antibiotic stewardship initiatives.

Furthermore all the *K. pneumoniae* isolates resistant to at least one type of carbapenem were screened for carbapenemase production phenotypically. In phase II, all the 4 carbapenem resistant isolates were found mCIM positive indicating 100% phenotypic positivity whereas in phase I only 63.6% positivity was obtained. All mCIM positive isolates however were found negative for bla_{KPC} gene, in both phases I and II. The mCIM test is highly sensitive and specific phenotypic method for the detection of carbapenemase production (Li et al., 2019). The 100% mCIM positivity observed in this study is consistent with findings reported by Lee et al., (2019) supporting the high sensitivity of mCIM for the detection of resistant strains.

The isolates exhibiting positive results in the mCIM test being negative for bla_{KPC} gene, highlights that carbapenem resistance in this study was not due to bla_{KPC} gene.

Both *acrA* and *acrB* genes were present in all the carbapenem resistant *K. pneumoniae* in both phases. This findings is similar with a study conducted in Iraq which showed presence of *acrA* and *acrB* genes in all the multidrug resistant *K. pneumoniae* (Abid et al., 2022). The absence of bla_{KPC} gene but presence of efflux genes in all the mCIM positive isolates in this study suggest the common resistance mechanisms, such as efflux pumps to be important contributor for AMR. The lack of the bla_{KPC} gene in all mCIM positive isolates however requires further investigation to delineate other possible mechanisms too. Although bla_{KPC} gene is common genetic element contributing for carbapenem

resistance other mechanisms besides efflux pumps, such as changes in porin channels, and modified beta-lactamases can also be driving factor co-existing to confer resistance. This study involves a comparative analysis of SSIs and AMR during two different study periods to assess the impact of implementing improved antimicrobial prophylactics on SSIs rates and emergence of resistance among bacteria. Phenotyic and genetypic test contributed to a comprehensive investigation of factors contributing to resistance in *K. pneumoniae*. Reduction in infection rates and alteration of resistance profile underscores the importance of antibiotic stewardship strategies. Furthermore, the study focused only on detection of bla_{KPC} and efflux pump genes but other carbapenemase genes such as $bla_{NDM,}$ bla_{OXA-48} and bla_{VIM} were not investigated and porin loss was not considered either. Moreover, various clinical information such as patient mortality, readmission rates, and length of hospital stay were not analysed. Lastly, the short duration of phase II may have limited the observation of seasonal trends or longer-term changes.

CONCLUSION

The study showed a significant reduction in infection rate after implementation of enhanced prophylactic measures. However, increase in the antibiotic resistance, especially in *K. pneumoniae*, highlights the need for continuous surveillance, strong antimicrobial stewardship, and continued exploration of alternative treatment options. Furthermore absence of bla_{KPC} gene and presence of efflux gene in our isolates highlights the role of non carbapenemase based resistance mechanisms, such as efflux pump in carbapenem-resistant *K. pneumoniae* infections.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TUJM Tribhuvan University Journal of Microbiology

SHORT COMMUNICATION

Assessment of Oxygen Demand and Antibiotic Resistant *Citrobacter* spp in the Bagmati River

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AMR *Citrobacter* in Bagmati River

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ABSTRACT

Objectives: To correlate the Oxygen demand with antibiotic resistant *Citrobacter* species in Bagmati river.

Methods: A Cross-sectional study was conducted from May to August 2025 at the Department of Microbiology, Tri-Chandra Multiple Campus, Kathmandu. Fifteen water samples collected in duplicate were examined for biological oxygen demand (BOD), dissolved oxygen (DO), most probable number (MPN) counts and presence of antibiotic resistant *Citrobacter* species. The correlation analysis was conducted using Exploratory software version 6.10.3.1.

Results: The MPN count of total coliforms was lowest at the upstream site (4 MPN/100 mL), and peaked downstream (35,000 MPN/100 mL). This was accompanied by a decline in DO (8.7, 4.2, and 1.2 mg/L) and an increase in BOD (0.1, 250.8, and 425.4 mg/L). *Citrobacter* appeared as pink, non-metallic colonies on EMB agar, and 394 isolates were confirmed biochemically. Among these, 100% were resistant to ampicillin (penicillin) and 99 % (390/394) toward erythromycin (macrolide), while resistance to cefotaxime (68%), amikacin (53%,aminoglycoside), ciprofloxacin (49%, fluoroquinolone), and chloramphenicol (44%, phenicol) was also recorded. Positive correlation ($r = 0.835$, $p < 0.05$) was observed for presence of *Citrobacter* and BOD and whereas negative correlation ($r = -0.85$, $p<0.05$) was observed for presence of *Citrobacter* and level of DO.

Conclusion: The increase in BOD and decrease of DO in the downstream region and its correlation with the presence of antibiotic resistant *Citrobacter* species signifies polluted water as contributor to antibiotic resistant coliform.

Key words: Bagmati River, *Citrobacter* spp, antimicrobial resistance, correlation

INTRODUCTION

The Bagmati River, traversing the Kathmandu Valley, represents a vital component of Nepal's cultural, religious, and ecological landscape (Yonzon & K.c, 2019). Despite its significance, the river has experienced substantial deterioration in water quality due to rapid urbanization, uncontrolled sewage discharge, and inadequate regulatory oversight, particularly in downstream segments (Shultana & Khan, 2022). The dissolved oxygen and biochemical oxygen demand of water signifies the pollution level in surface water (Tamrakar & Parajuli, 2019). Polluted aquatic environments serve as reservoirs for diverse microbial communities, including opportunistic pathogens capable of acquiring and disseminating antimicrobial resistance (AMR) determinants (Meradji et al., 2025).

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Citrobacter species, Gram-negative coliform bacteria widely distributed in soil, water, and the intestinal tracts of humans and animals, exemplify such pathogens (Jabeen et al., 2023). This study examines the presence, antibiotic susceptibility patterns of *Citrobacter* spp and its correlation with the physicochemical parameters of the isolates from different stretches of the Bagmati River, providing critical insight into the contribution of polluted water to the dissemination of antibiotic-resistant bacteria.

METHODS

Study design

A cross-sectional study was carried out from May to August 2025, at the Department of Microbiology, Tri-Chandra Multiple Campus, Kathmandu.

Study area

Bagmati river flowing from Baghdwar to Chobar inside Kathmandu valley.

Sampling sites

Fifteen sampling sites were stratified through google maps with equal distance between all the sampling points.

Sample collection and transportation

Fifteen water samples were collected in duplicate from three major segments of the Bagmati River-upstream (relatively pristine areas), midstream (urbanized sections), and downstream (heavily polluted stretches). Grab samples were obtained from subsurface water using sterile 300 mL BOD bottles, transported in an ice box to the laboratory.

Sample processing

Physicochemical parameters, including dissolved oxygen (DO) and biochemical oxygen demand (BOD) were analyzed by using the titration method (Ma et al., 2020), and total coliform counts were determined using five tubes Most Probable Number (MPN) technique, with positive tubes confirmed in Brilliant Green Lactose Bile Broth (BGLB) following APHA (2017) standards. Samples showing gas production and color change were further cultured on Eosin Methylene Blue (EMB) agar, and presumptive isolates were subculture on nutrient agar and characterized using Gram staining, catalase and oxidase tests, and a panel of biochemical tests including IMViC, Triple Sugar Iron (TSI) agar reactions, oxidative-fermentative tests, and urease activity (Jabeen et al., 2023b). *Citrobacter* spp were subjected to antimicrobial susceptibility testing (AST) using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar for ampicillin (10 µg), erythromycin (15 µg), amikacin (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), and chloramphenicol (30 µg), and zones of inhibition were measured based on CLSI (2023). Quality control was maintained using standard *E. coli* ATCC 25922 (Ghimire et al., 2023).

Ethical consideration

Ethical approval was obtained from the Institutional Review Committee, IOST, TU (IRC Ref. No.: 26-081/82) and sampling permission was granted by the Department of National Parks and Wildlife Reserve, Kathmandu (Ref no.: 3065).

RESULTS

Correlation between *Citrobacter* isolates and Oxygen demand value of Bagmati river

The correlation analysis revealed a strong positive relationship between BOD and *Citrobacter* spp The MPN per 100 mL showed a moderate positive correlation with BOD and a moderate, yet significant, positive correlation with *Citrobacter* spp ($r = 0.59$, $p = 0.00057$, $t = 3.8$). In contrast, a negative correlation was observed between MPN/100 mL and dissolved oxygen (DO) levels. BOD and DO were strongly inversely correlated and a similarly strong negative association were found between *Citrobacter* spp and DO (Table 1).

Table 1: Correlation analysis of different physiological and microbial parameters

SN	Parameters	r	p	t
1	BOD & *Citrobacter* spp	0.8345	9.9 e $^{-9}$	8.01
2	MPN/100 mL & BOD	0.683	0.0003	4.95
3	MPN/100 mL & *Citrobacter* spp	0.59	0.00057	3.8
4	MPN/100 mL & DO	-0.513	0.036	-3.16
5	BOD & DO	-0.84	5.6 $^{e-9}$	-8.24
6	*Citrobacter* spp & DO	-0.85	2.38 -9	-8.6

Chi square test of significance for antimicrobial resistance pattern in *Citrobacter* spp along river streams

Among 394 isolates, all were resistant to ampicillin (penicillin class) and 99% (392/394) towards erythromycin (macrolide class), while resistance to cefotaxime (68%, cephalosporin class), amikacin (53%, aminoglycoside class), ciprofloxacin (49%, fluoroquinolones class), and chloramphenicol (44%, phenicol class) was also recorded. The chi-square test showed statistically significant differences in the distribution of antibiotic resistance among isolates across the river sections. Specifically, the p-values for AK (amikacin, 0.015), E (erythromycin, 0.033), C (chloramphenicol, <0.001), CIP (ciprofloxacin, 0.005), and CTX (cefotaxime, 0.001) indicate that the presence of antibiotic resistant *Citrobacter* spp to these antibiotics varies significantly along the river.

DISCUSSION

The isolation of antibiotic-resistant and multidrug-resistant *Citrobacter* spp from the Bagmati River highlights the impact of unchecked wastewater discharge on river sources. The progressive deterioration of river water quality downstream is a direct consequence of accumulating anthropogenic influence (Castro et al., 2021). As the river receives increasing loads of domestic sewage, industrial effluent, and organic waste, its biochemical oxygen demand (BOD) rises substantially. This surge in organic matter fuels microbial metabolism, a process that consumes dissolved oxygen (DO) and leads to a marked decline in oxygen available for aquatic life (Yuliati et al., 2023). Our correlation analysis reveals a tightly coupled relationship between organic pollution and microbial contamination in the river system. We observed a strong positive association between BOD and *Citrobacter* spp ($r = 0.8345$), indicating that increasing organic load directly promotes the proliferation of these enteric bacteria. The results show a strong inverse link between dissolved oxygen and *Citrobacter* bacteria ($r = -0.85$), suggesting *Citrobacter* species flourish in oxygen-poor, organically rich conditions. Consequently, the resulting hypoxic conditions selectively favor the growth of facultative anaerobic bacteria like *Citrobacter*. This pattern of contamination is systemic, as shown by the positive correlations between MPN (a measure of fecal bacteria) and both BOD ($r = 0.683$) and *Citrobacter* spp ($r = 0.59$) (Holcomb & Stewart, 2020). The corresponding negative relationship between MPN and DO reinforces that fecal pollution intensifies as oxygen levels drop. Collectively, these results demonstrate that the decline in water quality driven by high organic load and oxygen depletion creates a favorable environment for the survival and growth of fecal indicator bacteria and specific pathogens, directly reflecting the cumulative impact of sewage inputs downstream (Bisimwa et al., 2022).

As anticipated, BOD exerted a strong positive effect on bacterial counts, underscoring the role of organic pollution in promoting *Citrobacter* proliferation. Conversely, DO levels showed a pronounced negative relationship, where higher oxygen concentrations corresponded with a substantial reduction in bacteria (Mutai et al., 2024). The statistical significance of both variables confirms that the interplay between organic nutrient availability and oxygen concentration are central determinants shaping the spatial distribution of *Citrobacter* spp in the river (Li et al., 2025).

The analysis of antibiotic resistance along the river showed a clear and statistically significant pattern for all five antibiotics tested. The consistently low p-values ($p < 0.000$ to $p = 0.033$) across the upstream, midstream, and downstream sections indicate that the differences in resistance levels are unlikely to have occurred by chance. These findings suggest that the position along the river is an important factor shaping the distribution of antibiotic-resistant bacteria. As the river flows downstream, the prevalence of resistance changes in a consistent and notable way. This pattern reflects the cumulative impact of human activities such as untreated wastewater discharge and agricultural runoff on the river environment (Sidrach-Cardona et al., 2014). Increasing pollutant input downstream likely creates greater selective pressure, allowing resistant bacterial strains to become more dominant across multiple antibiotic classes (Tello et al., 2012).

Our findings serve as a warning to people residing near the downstream and midstream areas of Bagmati river. Curbing the spread of antibiotic-resistant bacteria in this river system requires immediate and targeted interventions. Specifically, mitigating organic pollution through improved wastewater treatment and strict control of sewage discharge is not just beneficial but critical for public health.

CONCLUSION

This study demonstrates that the Bagmati River harbors antibiotic-resistant *Citrobacter* spp with resistance patterns intensifying in downstream stretches characterized by deteriorating Oxygen demand quality. The combination of elevated coliform loads, reduced dissolved oxygen, high biochemical oxygen demand are prominent marker for elevated drug resistant *Citrobacter* in Bagmati river.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SHORT COMMUNICATION

Biofilm Forming Antibiotic Resistant Enteric Bacteria from Drinking Jar Water in Kathmandu Valley, Nepal

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ABSTRACT

Objectives: To determine bacteriological quality of drinking jar water from Kathmandu valley, Nepal and detect biofilm forming antibiotic resistant enteric bacteria.

Methods: A cross-sectional study was conducted during February-March 2023. Drinking jar water samples (n=36) were collected from Kathmandu valley. Isolation and enumeration of coliform were done by membrane filtration method. Bacterial isolates were characterized by biotyping, antibiotic susceptibility pattern was assessed and biofilm formation was detected by tube adhesion method.

Results: Bacteriological analysis revealed the contamination of 97.2% of water samples with total coliform and 11.1% of water samples with thermotolerant coliform. Among 78 bacterial isolates, *Klebsiella* spp (32.1%) was the most common bacteria followed by *Escherichia coli* (15.4%). Most of *E. coli* and *Klebsiella* spp isolates were resistant to antibiotics. Multiple antibiotic resistant (MAR) *Klebsiella* spp and thermotolerant *Klebsiella* spp were detected. Biofilm forming *E. coli* (38.5%) and *Klebsiella* spp (37.9%) were more resistant to antibiotics tested.

Conclusion: The higher number of drinking jar water in Kathmandu valley were contaminated with coliform and presence of biofilm forming antibiotic resistant enteric bacteria in water showed a high risk of waterborne infections to consumers. Quality control of drinking jar water is essential to improve water quality and prevent waterborne diseases.

Keywords: Antibiotic resistant, biofilm, coliform, drinking jar water, MAR

INTRODUCTION

Water pollution is a worldwide problem and poses a serious threat to human life. Nepal has been facing challenges on both the accessibility and quality of drinking water (Adhikari et al. 2021). Kathmandu valley is the most densely populated area in Nepal including the capital city and affected by rapid and unplanned population growth, lack of sustainable water sources and a poor water management system that resulted in low availability of safe water (Udmale et al. 2016; CBS 2022). Kathmandu Upatyaka Khanepani Limited (KUKL) which manages the water supply in Kathmandu valley reported water demand of 506 million liters per day (MLD) in 2025. However, there is an average supply of 240 MLD of water including existing water sources and Melamchi water to fulfill the water requirement of consumers (KUKL 2025). Many countries with water crises and poor quality drinking water depend on packaged/bottled drinking water instead of public water supply and bottled water is considered as safe (Dindarloo et al. 2016). In Kathmandu valley, because of the scarcity of adequate public water supplies, many people use jar water as an alternative drinking water source. Jar water in Kathmandu valley was highly contaminated with coliform and *Escherichia coli* which is a public health issue (Bhandari et al. 2009; Burlakoti et al. 2020; Gautam 2021). Contamination of drinking water with faeces and poor sanitation are linked to diarrhoeal disease transmission and is estimated to cause approximately 505,000 diarrhoeal deaths each year (WHO 2023a).

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Antimicrobial resistance (AMR) is a global health concern and bacterial pathogens are becoming highly resistant to most of the first and some second-line antibiotics in Nepal (Acharya et al. 2019). Antibiotic resistant bacteria have the potential to contaminate drinking water sources that intensify health risks associated with antimicrobial resistance (Dhengesu et al. 2022). Coliform from drinking water sources of Kathmandu valley showed high resistance to antibiotics and are more often multiple drug resistance (MDR) (Gautam 2021; Chaudhary et al. 2011). Biofilm formation in drinking water is a potential source of bacterial contamination including pathogens (Liu et al. 2016) and plays a crucial role in increasing antibiotic resistance. In packaged/bottled water, biofilm forming bacteria were reported previously (Effiok et al. 2019; Shrestha et al. 2024). Therefore, this study was conducted to assess the bacteriological quality of different brands of drinking jar water marketed in Kathmandu valley, Nepal and detect biofilm forming antibiotic resistant enteric bacteria from water.

METHODS

This cross-sectional study was conducted for the bacteriological quality analysis of drinking jar (processed) water during February-March 2023. The different brand of sealed drinking jar water samples (n=36) were collected randomly from Kathmandu valley including Kathmandu (Thamel, Samakhusi, Swoyambhu, Raniban), Lalitpur (Lagankhel) and Bhaktapur (Madhyapur Thimi). Water samples were collected and transported to the laboratory of Department of Microbiology, Amrit Campus, Thamel, Kathmandu according to standard methods of American Public Health Association (APHA), 1998.

Isolation, enumeration and identification of coliform

Isolation and enumeration of total coliform and fecal coliform from drinking jar water samples were done by membrane filtration (MF) method. The measured volume of water sample was vacuum filtered through 0.45 µm membrane filter, the filter paper was transferred on eosin methylene blue (EMB) agar plates and plates were incubated one at 35±0.5°C for total coliform and another at 44±0.2°C for thermotolerant coliform, for 24-48 hours (APHA 1998; Aneja 2023). Then, the number of colony was counted and colony forming units (CFU)/100 mL was calculated. Enteric bacterial isolates from jar water on EMB agar plate were subculture on MacConkey agar and nutrient agar plates. From pure culture of bacteria, colony morphology was studied and then Gram staining and various biochemical tests were done for identification of bacteria (Cheesbrough 2019; Forbes et al. 2007).

Antibiotic susceptibility testing

Antibiotic susceptibility pattern of enteric bacteria from drinking jar water was assessed by modified Kirby-Bauer disc diffusion method. The isolated colony was inoculated into nutrient broth and incubated at 35±0.5°C for 4 hours. The turbidity of inoculum was adjusted to 0.5 McFarland standard. Then, bacterial suspension was inoculated on Mueller Hinton agar (MHA) plate using a sterile cotton swab and the surface of agar was allowed to dry for 5 minutes. Different antibiotic discs, amikacin (30mcg), ampicillin (10mcg), azithromycin (15mcg), ceftriaxone (30mcg), chloramphenicol (30mcg), ciprofloxacin (5mcg), cotrimoxazole (25mcg), gentamycin (10 mcg), nalidixic acid (30 mcg) and tetracycline (30 mcg), were placed on MHA plate inoculated with bacteria and the plate was incubated at 35±2°C for 16-18 hours. The diameter of zone of inhibition was measured after incubation and results were interpreted according to zone size interpretation chart (CLSI 2022, Cheesbrough 2019).

Detection of biofilm forming Bacteria

Biofilm formation by *E. coli* and *Klebsiella* spp from drinking jar water was detected by tube adhesion method. The bacterial isolate from overnight culture was inoculated into 5 mL of tryptone soya broth (TSB) contained glass test tube and incubated at 37°C for 24 hours. The test tube was decanted, washed with phosphate buffer saline (PBS) (pH 7.2) and air dried. Then, test tube was stained with 0.1% crystal violet solution for 10 minutes and washed with distilled water. In inverted position, test tube was left for air dry and the visible biofilm formation on the wall and at bottom of test tube was observed. The assay was performed in triplicates at three different times. Bacterial isolates were classified based on biofilm formation as strong positive, moderate positive and weak positive/negative. *Staphylococcus aureus* ATCC 25923 was used as reference bacterial culture for biofilm assay (Christensen et al. 1985; Harika et al. 2020).

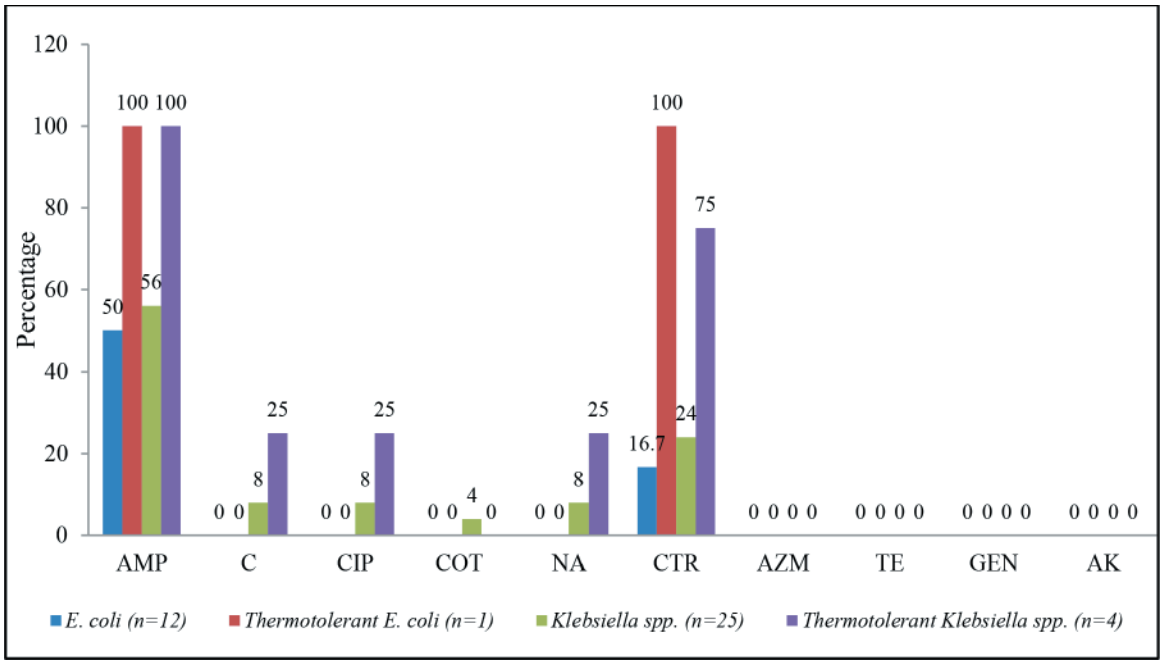
RESULTS

Out of 36 drinking jar water samples from Kathmandu valley, 97.2% (n=35) of samples were contaminated with total coliform and 11.1% (n=4) of samples were

contaminated with thermotolerant coliform which exceeded the World Health Organization (WHO) guidelines as well as National Drinking Water Quality Standards (NDWQS) of zero CFU/100 mL. The maximum total coliform count and thermotolerant coliform count was found to be 632 CFU/100 mL and 12 CFU/100 mL respectively. All drinking jar water samples from Samakhusi, Swoyanbhu, Raniban, Lagankhel and Madhyapur Thimi were found to be contaminated with total coliform whereas 83.3% and 16.7% of water samples from Thamel showed the contamination with total coliform and thermotolerant coliform respectively. Among 78 bacterial isolates from drinking jar water, *Klebsiella* spp (32.1%) was the most common bacteria followed by *E. coli* (15.4%), *Pseudomonas* spp (9.0%), *Enterobacter* spp (6.4%), *Citrobacter* spp (3.8%) and *Proteus* spp (3.8%). Thermotolerant *E. coli* (n=1, 20%) and *Klebsiella* spp (n=4, 80%) were also detected from jar water.

Antibiotic resistance pattern of *E. coli* and *Klebsiella* spp

The most of *E. coli* isolates showed resistance to ampicillin (50%) and ceftriaxone (16.7%). Similarly, the higher number of *Klebsiella* spp were resistant to ampicillin (56%) and ceftriaxone (24%). Thermotolerant *Klebsiella* spp showed resistance to most of antibiotics tested. Multiple antibiotic resistant (MAR) *Klebsiella* spp (12%) and thermotolerant *Klebsiella* spp (25%) were also detected (Figure 1).



AMP- Ampicillin, C- Chloramphenicol, CIP- Ciprofloxacin, COT- Cotrimoxazole, NA- Nalidixic acid, CTR- Ceftriaxone, AZM- Azithromycin, TE- Tetracycline, GEN- Gentamycin and AK-Amikacin

Figure 1: Antibiotic resistance pattern of *E. coli* and *Klebsiella* spp from drinking jar water in Kathmandu valley

Biofilm formation and antibiotic resistance pattern of biofilm forming bacteria

Of *E. coli* isolates (n=13), 15.4% were strong positive, 23.1% were moderate positive and 61.5% were weak positive/negative for biofilm formation. Similarly, among *Klebsiella* spp isolates (n=29), 13.8% were strong positive, 24.1% were moderate positive and 62% were weak positive/negative for biofilm formation. Biofilm forming *E. coli* isolates were resistant to ampicillin and ceftriaxone while majority of biofilm forming *Klebsiella* spp isolates were more resistant to antibiotics (Table 1).

Table 1: Antibiotic resistance pattern of biofilm forming *E. coli* and *Klebsiella* spp from drinking jar water

Antibiotics	Biofilm forming *E. coli* (n=5)		Biofilm forming *Klebsiella* spp (n=11)	
	Resistance		Resistance	
	Number	Percentage	Number	Percentage
Ampicillin	4	80	11	100
Ceftriaxone	3	60	9	81.8
Chloramphenicol	0	0	3	27.3
Ciprofloxacin	0	0	3	27.3
Nalidixic acid	0	0	3	27.3

DISCUSSION

Contamination of drinking water with coliform and *E. coli* indicates poor water quality and lack of sanitation as well as a serious health risk. In this study, 97.2% of drinking jar water from Kathmandu valley were contaminated with total coliform that exceeded the WHO guideline value. Burlakoti et al. (2020) and Gautam et al. (2021) reported the contamination of 92% and 52% of jar water in Kathmandu valley with total coliform respectively. Majority of water samples contaminated with total coliform and thermotolerant coliform showed a high risk category of coliform contamination according to WHO that has increased the risk of waterborne diseases to consumers in Kathmandu valley and also indicated low quality of jar water as well as lack of appropriate treatment and proper sanitation during production of drinking water. The most common enteric bacteria isolated from drinking jar water was *Klebsiella* spp followed by *E. coli*. The presence of thermotolerant *Klebsiella* spp and *E. coli*. in jar water revealed the faecal contamination of water. Contamination of jar water in Kathmandu valley with *E. coli*, *Klebsiella* spp, *Citrobacter* spp and *Salmonella* spp along with thermotolerant *E. coli* was reported previously (Bhandari et al. 2009; Subedi et al. 2010). The presence of these indicator bacteria showed that human pathogenic bacteria may be present in jar water which cause waterborne diseases. In present study, *E. coli* and *Klebsiella* spp from jar water were mostly resistant to ampicillin and ceftriaxone. In addition, *Klebsiella* spp and thermotolerant *Klebsiella* spp from jar water were MAR bacteria. Gautam (2021) has reported the resistance of fecal *E. coli* to ampicillin and chloramphenicol. This existence of antibiotic resistant and MAR enteric bacteria in drinking water sources are of great concern. The misuse and overuse of antimicrobials in humans, animals and plants as well as prolonged exposure of antimicrobials have lead to the emergence and spread of AMR in microorganisms which is a global public health threat (WHO 2023b).

E. coli (38.5%) and *Klebsiella* spp (37.9%) isolated from drinking jar water were biofilm producers of which 15.4% of *E. coli* and 13.8% of *Klebsiella* spp were strong biofilm producer in our study. Effiok et al. (2019) reported 32% of *E. coli* isolates from packaged water as strong biofilm producing bacteria. Long term use of plastic bottles like jar for marketing of drinking water increases the risk of biofilm formation (Kim & Lee 2022). Antibiotic resistance and its link to biofilm forming bacteria from bottled drinking water is posing a significant health problem. There was a positive correlation between antibiotic resistant profiles and biofilm forming capability in extensively drug-resistant *K. pneumoniae* (Santiago et al. 2020). Shrestha et al. (2024) reported that *E. coli* and *Klebsiella* spp in bottled water were biofilm-producing and drug-resistant bacteria. In present study, biofilm forming *Klebsiella* spp were more resistant to antibiotics, mostly MAR bacteria, than biofilm forming *E. coli*. These findings showed that the bacteria with higher rate of antibiotic resistance were biofilm forming as biofilm provides a protective environment for bacteria.

CONCLUSION

The higher number of drinking jar water was contaminated with coliform which indicated the poor quality of jar water in Kathmandu valley. The common bacteria in drinking jar water was *Klebsiella* spp and *E. coli* which showed biofilm forming ability as well as antibiotic resistance and MAR. Biofilm forming enteric bacteria were more resistant to antibiotics tested, mostly ampicillin and ceftriaxone. The presence of biofilm forming antibiotic resistant bacteria in jar water showed a high risk of waterborne diseases to consumers. Quality control of drinking jar water is essential to improve water quality and prevent waterborne diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SHORT COMMUNICATION

Bioactive Potential of Guava Leaves against Bacterial Pathogens

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ABSTRACT

Objectives : The study was conducted to analyze antimicrobial activities of leaf extract of Guava against Gram positive and Gram negative bacteria.

Methods: The leaf samples of Guava were collected from different parts of Lalitpur district and were processed to analyze the antimicrobial property of different solvent extract of guava leaf by using Agar well diffusion method.

Results: The results of the study indicated that three out of three of the crude solvent extract prepared from the leaves of guava, Methanol, Ethanol and Distilled water showed inhibitory activity against bacteria. Only Gram-positive bacteria, *Staphylococcus aureus* were susceptible to the all the extract, while *Escherichia coli* did not show any inhibitory action. Among the four sample extract of guava leaf from different solvent, the distilled water showed highest zone of inhibition by 25mm, followed by methanol extract with 20mm zone of inhibition and ethanol extract by 19mm zone of inhibition. At 10mg/50µL, the distilled water extract showed more antimicrobial activity with mean zones of inhibition 23.66mm than Methanol and Ethanol extracts with mean zone of inhibition 18mm and 16.33mm respectively.

Conclusion: Leaf extract of Guava showed the antibacterial activity against *Staphylococcus aureus*.

Keywords: Guava leaves, Medicinal properties, Agar well diffusion method, solvents extract, Antimicrobial activity

INTRODUCTION

From the ancient times different plant based herbal medicine are used to cure the diseases. Medicinal plants are used for the ailment of several microbial and non-microbial originated diseases due to their valuable effects in health care (Akroum, et al., 2010). The affordable, reliability, and low toxicity of medicinal plants in therapeutic use has made them popular and acceptable by all religion for implementation in medicinal health care all over the world. According to WHO about 80% of the developing world's population rely on traditional herbal medicine for their primary health care (WHO, 1993). The wide spread antibiotic resistance observed is now posing a serious health concern with medical scholars warning a return of pre-antibiotic era (Davies, et al., 2010). Due to increase in drug resistance the use of alternative therapies and natural remedies has rapidly increased in present context.The bioactive compounds are type of chemical found in small amounts in plants and promotes good health. They are being studied in the prevention of cancer, heart, disease etc. Example are: Flavonoids, tannins, terpenoids, glycoside. Among all plant organs, leaves are the largest accumulators of bioactive compounds, such as secondary metabolites. Several recent studies reported phytochemical profiles and biological activities of leaf extracts of various cultivated plants (Kumar, et al., (2021). Hence, although plant leaves are considered as agricultural waste, they are a rich source of high-value nutra-pharmaceutical compounds.

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The guava (*Psidium guajava* L.) tree, belonging to the Myrtaceae family, is a very unique and traditional plant which is grown due to its diverse medicinal and nutritive properties. Guava has been grown and utilized as an important fruit in tropical areas like India, Nepal, Indonesia, Pakistan, Bangladesh, and South America etc. Different parts of the guava tree, i.e., roots, leaves, bark, stem, and fruits, have been employed for treating stomachache, diabetes, diarrhea, and other health ailments in many countries. Guava leaves, along with the pulp and seeds, are used to treat certain respiratory and gastrointestinal disorders, and to increase platelets in patients suffering from dengue fever (Mateus, et al., 2020).

METHODS

Study duration and sample sites

The study was carried from October 2021 to December 2021 at the Microbiology Laboratory, Department of Microbiology, D.A.V. College. The total of four Guava leaves samples were collected from different part of Lalitpur district.

Preparation of Guava leaves extract

The leaf samples of Guava were collected from different areas of Lalitpur district. The leaves were first washed thoroughly with tap water to remove dirt and later washed with distill water. The samples were sun dried 2-3 days, after the leaves were dried it was grind in the grinder to make a powder of leaves and preserved at room temperature (RT) for future use. The three solvent were taken which Alcohol, Methanol and Distill water. The leaf powder was added to each of solvents to make a 20% concentration i.e. 20gm of leaf extract in a 100ml of solvents. Then it was kept for 3 days in a room temperature and filtered by Whattman No.1 filter paper. The extract was then centrifuged at 4000rpm for 10 min. The supernatant was collected and stored at 4oC until further use. Standard reference is missing. Add references in each method section

Phytochemical Analysis

Chemical test for the screening and identification of bioactive chemical constituents in the guava leaf were carried out with the extract using the standard procedure as described by Chapman and Hall (1973). In a test tube 2ml solvent extract was taken and shaken vigorously, the formation of stable foam was taken as an indication for the presence of saponins. In another test tube 1ml solvent extract was taken and added 2% of FeCl3. The black coloration indicated the presence of phenols and tannins. Similarly, 1ml extract was mixed with chloroform and added 2ml of concentrated sulfuric acid and was shaken gently. A reddish brown coloration of the interphase was formed to show presence of terpenoids. Same amount of extract was taken in another test tube and mixed with magnesium ribbon fragments. The concentrated hydrochloric acid was added dropwise. Orange red coloration indicated the presence of flavonoids. Similarly, 1ml extract was taken and mixed with 2ml glacial acetic acid containing 2 drops of 2% FeCl3. The mixture was poured into another tube containing 2ml of concentrated sulfuric acid. A brown ring at interphase indicated the presence of glycosides reference ????.

Antimicrobial susceptibility assay

The agar well diffusion method was performed to analyze the antimicrobial activity of guava leaf extract of different solvent on *S. aureus* and *E. coli*. All these test organisms were obtained from the Microbiology Laboratory of D.A.V. College, Lalitpur. The inoculums of test organisms were prepared by inoculating 3-4 bacterial colonies to 5ml Nutrient broth and incubated at 370C for 4 hour, after which it was adjusted to the 0.5 McFarland standard solution. The agar plates were inoculated with test organism using sterile cotton swab and was allowed to dry for 5 minutes. With the help of sterile 6 mm cork borer, a well was made on all agar plate. Then 50 microliters of each respective sample extract and neat (negative control) in separate wells respectively. It was allowed to rest for 15 minutes for diffusion of enzymes in media and incubated at 370C for 24 hours. After the observation was made for clear zone around the extract well and zone of inhibition was measured and noted.

RESULTS

Phytochemical Analysis

The results showed the presence of active compounds in guava leaf sample in three different solvents extracts. As the table shows, the methanol and ethanol extracts indicate the presence of tannins, Phenols, flavonoids, terpenoids, and glycosides, but absence of saponins. Distilled water is the only that showed the presence of all the phytochemicals in all the four sample of guava leaves.

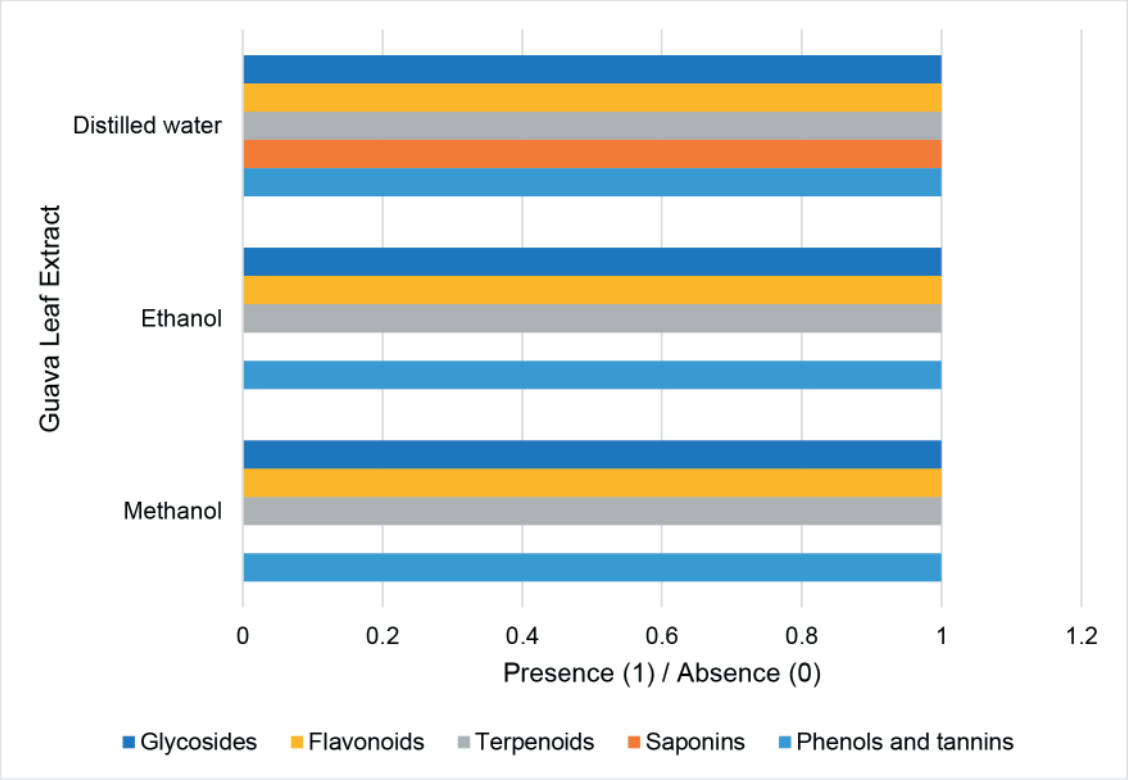

Figure 1: Phytochemical constituents of guava leaf extracts

Antibacterial activities of guava leaf extracts against Gram positive and Gram negative bacteria

All of the solvent extract showed highly effective against Gram-positive bacteria (*Staphylococcus aureus)* but the Gram-negative bacteria (*E. coli*), did not show any zone of inhibition against all three solvent extract.

Table No: 1 Comparison of antibacterial activities of guava leaves extract on bacteria.

Guava Extract	Organism	Mean Zone of inhibition
Methanol	*Staphylococcus aureus*	18mm
	Escherichia coli	-
Ethanol	*Staphylococcus aureus*	16mm
	Escherichia coli	-
Distilled water	*Staphylococcus aureus*	23.25mm
	Escherichia coli	-

DISCUSSION

About four samples were collected from different parts of Lalitpur district. The bacteria was provided by the Microbiology laboratory of DAV College, this was further re-confirmed by identifying colony characteristics, gram staining and biochemical testing.

The result showed that the methanol and ethanol extract contained phenol and tannins, flavonoids, terpenoids and glycosides but lacked saponins, while distilled water showed the presence of saponin, phenols and tannin, flavonoids, terpenoids and glycosides in all the four samples of guava leaves. This analysis has led the presence of phytochemical that has antimicrobial property. For example, tannins are polyphenolic compounds that bind to proline rich protein that interferes with protein synthesis and has shown antimicrobial property (Ulubelen, 2003). Flavonoids are hydroxylated polyphenolic compounds known to be produced by plants in response to microbial infection to which this aspects has been studied and found to have antimicrobial activity (Cowan, 1999). Terpenoids are mainly used for the aromatic purpose but it has also been found to have potential inhibitory action against bacteria (Tsuchiya, et al., 1996). Saponins which are glycosides have been found to have inhibitory effects on Gram-positive organism (*Staphylococcus aureus).*

Therefore, the phytochemical analysis showed that the methanol, ethanol and distilled water extract have chemical compounds that have been found to possess the antimicrobial activities.

The results of the study indicated that three out of three of the crude solvent extract prepared from the leaves of guava, Methanol, Ethanol and Distilled water showed inhibitory activity against bacteria. Only Gram-positive bacteria, *Staphylococcus aureus* were susceptible to the all the extract, while Gram-negative i.e *Escherichia coli* did not show any inhibitory action. Among the four sample extract of guava leaf from different solvent, the distilled water showed highest zone of inhibition by 25mm, followed by methanol extract with 20mm zone of inhibition and ethanol extract by 19mm zone of inhibition. At 10mg/50µL, the Distilled water extract showed more antimicrobial activity with mean zones of inhibition 23.66 than Methanol and Ethanol with mean zone of inhibition 18 and 16.33 respectively. Distilled water showing more antimicrobial activity might be the reason that it contained all the phytochemical mentioned (table 4) while other solvent lacked saponin. Saponins possess detergent-like properties and might increase the permeability of bacterial cell membranes without destroying them (Jacob, et al., 1991). This may have facilitated the influx of other phytochemical into the cell of bacteria which might be the reason that distilled water containing saponin showed highest zone of inhibition. Sanches, et al. (2005), found that the aqueous extract of guava was effective against *Staphylococcus.* Beside distilled water, methanol showed the second highest zone of inhibition with mean zone of inhibition of 18mm while the ethanol extract showed least of the zone of inhibition with mean zone of inhibition 16.33. Biswas, et al. (2013) conducted similar study where they found out that methanol extract showed highest zone of inhibition followed by ethanol extract. But the distilled water did not show any zone of inhibition throughout the study. In case of bacteria used, gram-positive *Staphylococcus aureus* only showed the zone of inhibition while the gram-negative *Escherichia coli* did not show any zone of inhibition.

The resistance of the Gram-negative bacteria could be due to its cell wall structure. Gram-negative bacteria have an effective permeability barrier, comprised of thin lipopolysaccharide exterior membrane, which could restrict the penetration of the plant extract. It has been reported earlier that Gram-negative bacteria are usually more resistant to the plant antimicrobial property and even show no effect, compared to gram-positive bacteria (Tajkarimi, et al., 2010). Gram-positive bacteria have a mesh like peptidoglycan layer which is more accessible to permeation by the extract (Rameshkumar et al., 2007).

Nascimento, et al. (2000) conducted a study which supports the finding of the present study in which the guava extract was able to have inhibitory effects against *Staphylococcus aureus* and no effect on *Escherichia coli.* Mahfuzul Hoque, et al. (2007) found no effect on antibacterial activity of ethanolic extract of guava against *E. coli;* however, Viera, et al. (2001) found that the guava sprout extracts were effective against inhibiting *E. coli.*

CONCLUSION

The current study demonstrate the antimicrobial activity of guava leaves extract using various solvent. The result showed that the distilled water was better than methanol and ethanol respectively. It also showed that the guava leaves extract has no antimicrobial effect on the gram-negative bacteria (*Escherichia coli).* The observed inhibition of gram-positive bacteria, *Staphylococcus aureus,* suggests that guava possess compounds containing antimicrobial properties that can effectively suppress the gram positive bacterial growth.

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SHORT COMMUNICATION

Analysis of Bacteriological Quality and Antibiogram of *Escherichia coli* and *Staphylococcus aureus* in Raw Milk Sold in Janakpurdham

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ABSTRACT

Objectives: To assess the microbiological quality of raw milk sold in Janakpurdham, Nepal, by determining the Total Viable Count (TVC) and Total Coliform Count (TCC), isolating *Escherichia coli* and *Staphylococcus aureus*, and evaluating its antibiotic susceptibility pattern.

Methods: A total of 74 raw milk samples were aseptically collected from dairies and farmers in Janakpurdham from March to June 2025. TVC was determined using the pour plate method on Nutrient Agar, and TCC/*E. coli* isolation on Eosin Methylene Blue Agar. *S. aureus* isolation was done on mannitol salt agar after enrichment. Conventional biochemical tests were used to confirm the isolates. Antimicrobial susceptibility of the isolates was performed using the Kirby-Bauer disk diffusion method.

Results: Mean TVC was 1.13×10^9 CFU/ml (range: 2.075×10^8–2.76×10^9), and mean TCC was 7.40×10^7 CFU/ml (range: 2.0×10^5–5.28×10^8). *E. coli* was isolated from all seventy-four samples. Susceptibility was highest to gentamicin (100%) and chloramphenicol (85.13%), but 100% resistance was observed to ampicillin and amoxicillin-clavulanate. 60.81% isolates were multidrug-resistant. *S. aureus* was detected in 48 samples (65%). Antibiotic susceptibility testing revealed 100% resistance to ampicillin and 72.9% to cefoxitin, while varying resistance was observed for linezolid, erythromycin, clindamycin, and others.

Conclusion: Raw milk in Janakpurdham exhibits high microbial contamination and antibiotic-resistant bacteria, posing public health risks; therefore, improved hygiene and antibiotic stewardship are essential.

Keywords: Raw milk, *Escherichia coli*, *Staphylococcus aureus*, Total Viable Count, Coliform Count, Antimicrobial resistance, Janakpurdham.

INTRODUCTION

Raw milk contamination occurs during milking, handling, storage, or transportation from sources such as udder infections (mastitis), dirty equipment, or fecal matter (Jay et al., 2005). Poor pre-milking hygiene and lack of pasteurization increases the health risk in developing countries (Dhungel et al., 2019; Rahmatalla et al., 2016). Raw milk, which is unpasteurized milk from animals such as cows or buffaloes, can contain pathogens like *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* if not handled properly (Pal et al., 2016). The presence of pathogenic microorganisms in raw milk poses significant public health risks, including foodborne disease.

Escherichia coli indicates fecal contamination and poor sanitation. While most strains are harmless, pathogenic and resistant variants pose a risk. *S. aureus* can cause a variety of illnesses. Presence of both these

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organisms represents a significant burden on public health globally, particularly in low- and middle-income countries. Antimicrobial resistance (AMR) exacerbates the issues caused by antibiotic misuse in veterinary practices (Oliver et al., 2005). This study evaluated raw milk quality sold in Janakpur and isolated two important milk-contaminating bacteria to assess antibiotic resistance levels for public health implications.

METHODS

Study Site and Duration: Raw milk samples (50 mL each) were collected aseptically in sterile glass bottles from dairies, farmers, and shops across 25 wards of Janakpurdham Sub-Metropolitan City during the morning hours (8-10 AM) from March to June 2025. Samples were transported in an ice box and processed within 3-4 hours.

Microbial Analysis: Serial ten-fold dilutions (10^{-1} to 10^{-7}) were prepared. Total Viable Count (TVC) was determined by pour plate method on Nutrient Agar (NA), incubated at 37°C for 24 hours (NDDB, 2001). Total Coliform Count (TCC) and *E. coli* isolation were performed using spread plates on Eosin Methylene Blue Agar (EMBA), incubated at 37°C for 24-48 hours. Colonies with a greenish metallic sheen were subcultured for purity. For *S. aureus* isolation, 1 ml of milk sample was inoculated into 9 ml of sterile peptone water. After enrichment, a loopful was streaked onto Mannitol Salt Agar to get golden yellow colonies of *S. aureus*.

Biochemical tests: Confirmation of *E. coli* and *S. aureus* was done by biochemical tests using methods described elsewhere (Isenberg, 2007; Cheesbrough, 2006).

Antibiotic Susceptibility Test: Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (MHA) following the CLSI guidelines (2023). Antibiotics tested were Ampicillin (10 µg), Amoxycillin-Clavulanate (30 µg), Gentamicin (10 µg), Ceftazidime (30 µg), Tetracycline (30 µg), Chloramphenicol (30 µg) and Ciprofloxacin (5 µg) for *E. coli*. For *S. aureus*, ampicillin, cefoxitin, linezolid, erythromycin, clindamycin, chloramphenicol, tetracycline and gentamycin were used. Multidrug resistance (MDR) was defined as resistance to ≥3 classes of antibiotics (Magiorakos et al., 2012).

Data Analysis: Data were entered into MS Excel 2016 and analyzed descriptively (mean, standard deviation, range, quartiles).

RESULTS

All 74 samples exhibited microbial growth. The mean TVC was 1.13×10^9 CFU/ml (SD: 5.62×10^8), ranging from 2.075×10^8 to 2.76×10^9 CFU/ml. While the Mean TCC was 7.40×10^7 CFU/ml (SD: 7.72×10^7), ranging from 2.0×10^5 to 5.28×10^8 CFU/ml.

E. coli was isolated from all samples, confirmed biochemically. AST showed 100% sensitivity to gentamicin (74/74) and 85.13% to chloramphenicol (63/74); 79.23% to ceftazidime (59/74); 78.38% to tetracycline (58/74); 71.62% to ciprofloxacin (53/74). There was 100% resistance to ampicillin and amoxicillin-clavulanate while 28.38% to Ciprofloxacin, 21.62% to tetracycline, 20.77% to ceftazidime, 14.87% to chloramphenicol (Table 1). 60.81% (45/74) isolates are multi drug resistant showing resistance to ≥3 classes of antibiotics.

Table 1. Antimicrobial Susceptibility pattern of *Escherichia coli* Isolates

Antimicrobial Agents	Concentration (µg)	Zone Diameter (mm)		
		Sensitive (n, %)	Intermediate (n, %)	Resistant (n, %)
Ampicillin (AMP/AP)	10	≥ 17 (0, 0%)	14–16 (0, 0%)	≤ 13 (74, 100%)
Amoxycillin clavulanic (AMC/AUG)	30	≥ 18 (0, 0%)	14–17 (0, 0%)	≤ 13 (74, 100%)
Tetracycline (T/TE)	30	≥ 15 (58, 78.38%)	12–14 (0, 0%)	≤ 11 (16, 21.62%)
Ceftazidime (CAZ/C)	30	≥ 21 (59, 79.23%)	18–20 (0, 0%)	≤ 17 (15, 20.77%)
Chloramphenicol (CMP/CAC)	30	≥ 18 (63, 85.13%)	13–17 (0, 0%)	≤ 12 (11, 14.87%)
Gentamicin (GEN)	10	≥ 15 (74, 100%)	13–14 (0, 0%)	≤ 12 (0, 0%)
Ciprofloxacin (CIP)	5	≥ 26 (53, 71.62%)	22–25 (0, 0%)	≤ 21 (21, 28.38%)

A total of 48 *S. aureus*-positive strains (65%, 48/74) were isolated and identified. The highest resistance was found to Ampicillin (100%) and cefoxitin (72.9%), followed by linezolid (31.25%), erythromycin (25%), clindamycin (25%), chloramphenicol (18.75%), tetracycline (14.5%) and gentamycin (12.5%) (Fig. 1). Out of the 48 verified S. aureus isolates, 18 (37.5%) were determined to be MDR.

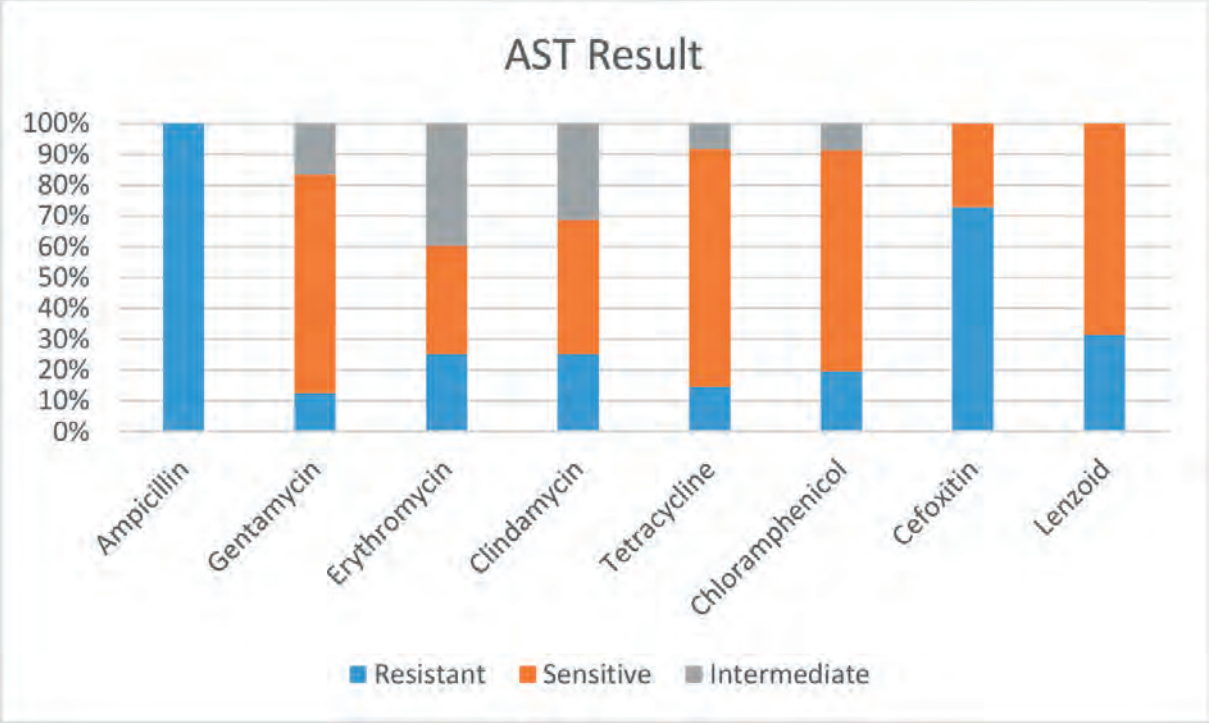

Figure 1. Antimicrobial Susceptibility of *S. aureus* isolates

DISCUSSION

The nutrient-rich composition of raw milk makes it susceptible to contamination. The high TVC (1.13×10^9 CFU/mL) and TCC (7.40×10^7 CFU/mL) in this study exceeded the WHO/FAO limits ($<10^5$ CFU/mL TVC, $<10^2$ CFU/mL coliforms), indicating poor hygiene. Similar findings in Kathmandu (Bhattarai et al., 2017:10^6–10^8 CFU/mL) and Chitwan (Koirala & Joshi, 2019:8.5×10^8 CFU/mL) suggest widespread issues in Nepal due to inadequate refrigeration and poor sanitation. In India, Sarkar (2015) reported 10^7–10^9 CFU/mL, emphasizing the need for pasteurization.

E. coli detection signals fecal contamination. The antibiogram showed 100% resistance to beta-lactams, aligning with Sharma et al. (2021) in Nepal's Terai (100% ampicillin resistance). Hasan et al. (2015) in Bangladesh reported high resistance to Ampicillin (95%) and Tetracycline (60%). Lower resistance to gentamicin (0%) offers treatment options, but the 28.40% MDR rate is concerning and linked to veterinary antibiotic misuse.

Out of 74 milk samples, *Staphylococcus aureus* was detected in 48 samples, representing an overall prevalence of 65% (48/74). The isolation rate of *S. aureus* varies widely across different studies and regions.

Comparable results were reported by Alnakip (2009), El-Jakee et al. (2008), Jakeen et al. (2010), and Nassar (2013), who documented prevalence rates of 16% to 22.7% in cow milk. In contrast, significantly higher prevalence rates were reported by El-Gendy (2015), Ralls et al. (2008), and Wafy (2006), ranging from 60% to 90.4%. On the other hand, relatively lower isolation rates were found in studies by Amer et al. (2007) and Kivaria et al. (2006), which reported prevalence rates between 6.3% and 14.5%.

Liu et al. (2017) reported a 27.7% isolation rate of *Staphylococcus aureus* in 195 raw milk samples collected from northern China. Similarly, Zhao et al. (2020) found a 28.9% contamination rate in bulk tank milk samples from dairy farms in Shandong Province—both lower than the prevalence found in the current study. Contamination of raw milk with *S. aureus* commonly originates from mastitis-infected animals or human carriers. Poor hygiene practices during milking and processing significantly increase the risk of contamination (Schmidt et al., 2017).

CONCLUSION

The present study has shown that *Staphylococcus aureus* and *E. coli* is widely prevalent in milk in Janakpur city. The high rate of isolation indicates the higher

public health risk among this region. The results also emphasize the importance of regular microbiological examination of milk and milk products for the production of quality and safe products.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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