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Metagenomic Profiling and Natural Product-Based Control of Antibiotic-Resistant *Streptococcus* Species in Gut Microbiome of Diarrhea-Associated Children

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ABSTRACT

The threat of antibiotic resistance extends beyond established pathogens. Recent research highlights the concerning role of normal gut bacteria in holding and potentially spreading antibiotic resistance genes. This phenomenon poses a significant risk to human and animal health worldwide. In this study, we aimed to understand the antibiotic resistance profile of gut bacteria and determine the abundance of bacteria at the species level based on metagenomic analysis and investigate control methods. Fecal samples were collected from 150 pediatric diarrhoea patients from Kushtia 250-bed General Hospital, Bangladesh and cultured in anaerobic conditions. The presence of antibiotic resistance was analyzed by the disk diffusion method, and the microbial profiling was plotted by metagenomic analysis. The abundant antibiotic-resistant strain was identified and controlled using natural compounds. Antibiotic-resistant bacteria against the tested 10 antibiotics were prevalent in 0-6-month old children samples and followed by 7-12-month-old children. In 25-30-month old children samples, bacteria were resistant against all the antibiotics except Doxycycline, and Levofloxacin. Based on metagenomic analysis Firmicutes is the most abundant phylum in antibiotic resistance samples. The presence of *Streptococcus* was observed only in antibiotic-resistant samples whereas *Lactobacillus, Lysinibacillus, Vagococcus, Pseudomonas, Lentibacillus,* and *Corynebacterium* have a higher tendency of susceptibility than resistance. Antibiotic-resistant *Streptococcus* was isolated on *Streptococcus* selection agar and controlled by aqueous extract of Cloves. Although human gut microbes perform various health benefits, multiple drug resistance among human gut bacteria will be a great threat to human health. Hence, alternative sources of antibiotics expanded the consciousness of using antibiotics as a treatment. Natural products can be used as therapeutic agents to control multiple drug-resistant bacteria.

INTRODUCTION

Antibiotics are the most effective and life-saving drugs that protect us from infectious diseases and decrease mortality rates (Rahman *et al.*, 2022). However, excessive and irresponsible use of antibiotics is a major cause of antibiotic resistance (Akram *et al.*, 2023). Antibiotic resistance occurs when microbes develop the capability to resist the drugs designed to kill them. Therefore, bacteria become immune to the antibiotics (Chinemerem Nwobodo *et al.*, 2022). As a result, ingestion of antibiotics and other antimicrobial drugs becomes completely ineffective and infections become challenging and even impossible to treat, increasing the risk of disease transmission, severe illness, disability, and death (Geta, 2019; Murugaiyan *et al.*, 2022; Salam *et al.*, 2023; So *et al.*, 2024). Besides, Antibiotic-induced alterations may lead to a decline in the diversity of microorganisms, changes in the functional attributes of the microbiota, and the emergence and proliferation of antibiotic-resistant strains, hence increasing the susceptibility of hosts to pathogen infection (Elvers *et al.*, 2020; Sun *et al.*, 2019; Zhang *et al.*, 2021). It also disrupts the balance of good bacteria in our gut, leading to digestive problems, antibiotic resistance, and potentially chronic diseases (Ramirez *et al.*, 2020; Ribeiro *et al.*, 2020; Wilkins *et al.*, 2019).

The gut microorganisms are those microorganisms that dwell in the gut and play significant roles in many aspects including enhancing nutritional value, improving the immune system; secrete metabolites that affect the brain and behavior (Manaf *et al.*, 2025; Yang *et al.*, 2020; Zhou *et al.*, 2020). Microbes are colonized on the epithelial surfaces, including the alimentary system of infants during birth. This colonization depends on the infant's gestational age at delivery and the method of delivery (Kalbermatter *et al.*, 2021). An imbalance of gut microbes caused by pathogens, chemicals, and drugs is associated with a variety of health problems, including digestive issues like inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), as well as chronic conditions like obesity and even some types of cancer (Singh *et al.*, 2023; Sultan *et al.*, 2021; Vandana *et al.*, 2020). Moreover, emerging evidence suggests a link between

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adverse changes in the gut microbiota composition, or dysbiosis, and the development of neurodegenerative diseases (Sarkar & Banerjee, 2019). This association may be mediated, in part, by the influence of gut microbes on protein folding within the central nervous system (Mahbub *et al.*, 2024). Children are mostly affected by acute infectious enteritis including diarrhea, abdominal cramps, and nausea, which is one of the most prevalent illnesses in the world (Kumari *et al.*, 2022). Diarrheal disease causes 1.3 million deaths globally each year (Manetu *et al.*, 2021). Apart from some viruses like rotaviruses, and adenoviruses, etc., bacterial pathogens like *Escherichia coli (E. coli), Shigella, Salmonella, Campylobacter, Clostridium difficile* (*C. difficile*), and *Aeromonas* are commonly responsible for infectious diarrhea. Moreover, some fecal bacteria are lower during acute diarrhea (Vashisht, 2019).

In most cases, antibiotics are prescribed for the treatment of diarrheal-associated children (Rhee *et al.*, 2019). Recent studies have shown that antibiotic-resistant microbes are established in the infant's gut microbiota within the first week of birth, even in the absence of exposure to antibiotics. (Pärnänen *et al.*, 2022). It may be an alarming issue when the gut microbiota of infants become antibiotic-resistant. Diarrheal-associated child's gut bacteria may have the possibility to be antibiotic-resistant (Roncaioli, 2022). As a result, beneficial bacteria of the gut may be affected by antibiotic therapy and emerge as a health threat as well as causing an imbalance of gut microbiota (AJALA, 2023).

A phylogenetic analysis employing the 16S rRNA gene revealed that infants treated with ampicillin and gentamicin during the first week of life had a lower diversity in their fecal microbiota than those who were not treated. (Morowitz *et al.*, 2022). Metagenomic approaches are an effective and innovative tool to identify bacterial community dynamics in children suffering from diarrhea (De *et al.*, 2020). To take a treatment strategy for diarrhea, the initial step is to know the total antibiotic resistance pattern of gut microbiota (Abdel-Shafi *et al.*, 2019). Isolation and identification of antibiotic-resistant gut bacteria using biochemical assay and 16s rRNA sequencing is a gigantic task because of the large size of the gut microbiome. In this case, Metagenomic analysis can reveal an abundance of bacteria from phylum to species level from a single analysis (Guo *et al.*, 2021). Though antibiotics cannot control antibiotic-resistant bacteria, natural products can inhibit the growth of antibiotic-resistant bacteria (Wu *et al.*, 2019). Natural products, including cloves (Syzygium aromaticum) are found to have an antibacterial effect against several pathogens (Wongsawan *et al.*, 2019).

In our study, feces samples were collected from different-aged diarrheal-associated children and isolated gut bacteria in anaerobic conditions. Then, metagenomic tools evaluated the antibiotic resistance pattern of gut bacteria. Plant extracts were used to control antibiotic-resistant gut pathogens.

MATERIALS AND METHODS

Sample Collection

The fecal microbiome is a significant part of gut microbiota. Fecal samples can represent a microbial profile of gut bacteria. Therefore, fecal samples of 150 diarrheal-associated children were collected to isolate gut bacteria from Kushtia 250 bedded General Hospital with the consent of the parents of the children and ethical clearance was obtained from the dean of the Faculty of Biological Sciences, Islamic University, Kushtia, Bangladesh. Samples were collected from 0 to 30-month-old children. They were divided into five groups according to their ages (group 1: 0-6 months; group 2: 7-12 months; group 3: 13-18 months, group 4: 19-24 months and group 5:25-30 months). Thirty samples were collected from each group. The age and sex of the 150 donors were recorded.

Anaerobic Culture

Fecal samples were cultured in Schaedler broth (HIEMEDIA, M292-500G) in an anaerobic jar (BD BBL GasPak anaerobic jar, 260607) with Anaeropack (Thermo, AN0025A) and incubated overnight. The samples were cultured anaerobically using a spread plate technique. Microbial count was observed and calculated.

Determination of the Presence of Antibiotic-Resistant Bacteria in Fecal Samples

To determine the presence of antibiotic-resistant bacteria in fecal samples 10 commercially available antibiotic discs were introduced to the samples using the Kirby Bauer method. The test was done on the TSA plate (Scharlau, Spain, 01-200-500) in anaerobic and aerobic condition using Ciprofloxacin 5µg/disc (HIMEDIA, SD080-1CT), Gentamicin 10µg/disc (HIMEDIA, SD016-1CT), Erythromycin 15µg/disc (HIMEDIA, SD013-1CT), Streptomycin 10µg/dis (HIMEDIA, SD031-1CT), Tetracycline 30µg/disc (HIMEDIASD037-1CT), Amoxicillin 30µg/disc (HIMEDIA, SD076-1CT), Metronidazole 5µg/disc (HIMEDIA, SD099-1CT), Levofloxacin 5µg/disc (HIMEDIA, SD216-1CT), Azithromycin 30µg/disc (HIMEDIA, SD124-1CT) and Doxycycline 30µg/disc (HIMEDIA, SD012-1CT) following the Kirby-Bauer method (Yang *et al.*, 2019). Samples showing antibiotic sensitivity and resistance against multiple antibiotics were observed and grouped separately.

Metagenomic Analysis

Following the determination of the presence of antibiotic-resistant bacteria, the sample having clear zones around most of the antibiotics (Sample 1) and the sample having no clear zone around all of the introduced antibiotics (Sample 142) were used for metagenomic analysis. 16S rDNA metagenomic libraries were prepared using 16S amplicon PCR with Illumina overhang adapter locus-specific sequences Forward: 5' TCGTCGGCAGCGTCAGATGT

GTATAAGAGACAG and Reverse: 5' G T C T C G T G G G C T C G G A G A T G T GTATAAGAGACAG. Then, samples were run for Illumina sequencing. For this purpose, a combined Denatured and Diluted PhiX control (30 µl) and amplicon library (570 µl) were loaded onto the MiSeq v3 reagent cartridge. By utilizing the MiSeq Reporter software (MSR), the MiSeq system offers a secondary analysis instrument. MSR offers a number of options for data analysis related to MiSeq sequencing. Using a database of 16S rRNA data, the Metagenomics technique classifies organisms from the V3 and V4 amplicon. Using the Greengenes database, the classification was carried out. The result of these operations is a classification of reads at multiple taxonomic levels: kingdom, phylum, class, order, family, genus, and species.

Isolation of Antibiotic-Resistant *Streptococcus* Species

Streptococcus species were isolated from Sample 142, based on metagenomic data indicating that *Streptococcus* species are present in Sample 142 but not in Sample 1. *Streptococcus* Selection Agar (Himedia, M304) containing Neomycin Sulfate and Polymyxin B Sulfate, supplemented with 5% Sheep Blood, was used to isolate *Streptococcus*. was used to isolate *Streptococcus* Species. When single colonies emerged, single colonies from the culture were taken based on their morphological characteristics.

Biochemical and Molecular Identification of *Streptococcus* Species

Biochemical Characterization

The biochemical identification of isolated *Streptococcus* species was done following the "Bergey's Manual of Determinative Bacteriology". The isolated antibiotic-resistant bacteria were subjected to biochemical analysis via Gram staining, shape, spore staining, Catalase Test, Oxidase Test, Indole Test, MR-VP test, Citrate Utilization Test, Urease Test, and Motility Test.

DNA Extraction

To extract genomic DNA, overnight broth cultures of isolated *Streptococcus* colonies were used. The 2 mL broth was centrifuged at 5000 rpm for 5 minutes and the supernatant was discarded. 100 µL nuclease-free water was added and kept in a water bath at 95°C for 10 minutes. It was then centrifuged at 5000 rpm for 10 minutes. Then, the supernatant was taken in a fresh Eppendorf's tube, and 0.7 volume absolute ethanol was added which was followed by centrifugation at 14000 rpm for 20 minutes. The supernatant was excluded and 70% ethanol was added and again centrifuged at 14000 rpm for 15 minutes. The liquid was discarded and 50 µL TE buffer was added to nourish the extracted DNA. Finally, the solution was incubated at 37°C overnight to suspend evenly.

PCR Amplification of 16S rRNA Gene

A 50 µL reaction tube containing nuclease-free water, 4X 1.25 µL Dream Taq PCR Master Mix (2X), with Dream Taq DNA Polymerase, 2X Dream Taq buffer, dNTPs, and 4mM $MgCl_2$, DNA template (sample), primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and primer 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR tubes were placed in a thermal cycler (Gene Atlas, Model G02, Japan). Following five minutes of initial denaturation at 95 °C, thirty cycles of denaturing at 95 °C for thirty seconds, annealing at 57 °C for one minute, extension at 72 °C for two minutes, and concluding with a final extension phase of ten minutes at 72 =°C. After that, the PCR tubes were stored at -20 °C.

Sequencing of the PCR Amplicons

Following electrophoresis, the PCR products were cut from the agarose gel, purified using Thermo Fisher Scientific's PureLink® PCR Purification Kit, and partially sequenced using an automated DNA sequencer, the 3500 Genetic Analyzer (Applied Biosystems, USA). CodonCode Aligner was employed for assembly and alignment the nucleotide sequences, and nucleotide BLAST was used to determine the species and strains. All the sequences were uploaded to the National Center for Biotechnology Information (NCBI) database in order to receive accession numbers.

Phylogenetic Tree Construction

The phylogenetic tree was constructed using an online tool Phylogeny.fr.

Control of Multidrug Resistance *Streptococcus* Species by Aqueous Extracts of Cloves *(Syzygium Aromaticum)*

Extraction Method

Cloves was dried and ground to fine powders with a mortar-pestle. 100 g of each powder was dissolved in 1 liter of boiling water in a beaker and stirred continuously to obtain extract. Then, the extract was cooled down to room temperature and filtered with number one Whatman's filter papers. The extracts were diluted to 200 µg/mL, 150 µg/mL, 100 µg/mL, and 50 µg/mL, respectively, to assess the antimicrobial activity against the multidrug-resistant *Streptococcus* species.

Antimicrobial Activity

The overnight culture of multidrug-resistant *Streptococcus* isolates was spread over Muller Hinton Agar Plates (GM 173), and disks impregnated overnight with water extracts of different concentrations of cloves were introduced to the agar plates. The inhibition of *Streptococcus* growth was observed and recorded after 24 hours of incubation at 37°C.

RESULTS AND DISCUSSION

Results

Culture of Human Gut Bacteria from Fecal Samples of Diarrhea-Associated Children

In this study, fecal samples were collected to isolate gut bacteria from 150 diarrheal-associated children. Fecal samples were collected from around 0-30 months aged

diarrheal-associated children. A total of 17% of samples were collected from 0–6-month aged children, 42% sample were collected from 7–12-month aged child, 32% sample were collected from 13–18-month aged child, and 5% samples were collected from 19–24-month aged child, 3% sample were collected from 25–30-month aged child (Figure 1). Fecal samples were cultured in anaerobic conditions by using AnaeropackTM to obtain gut bacteria. Colonies were counted and the bacterial load is demonstrated in CFU/mL (Figure 2).

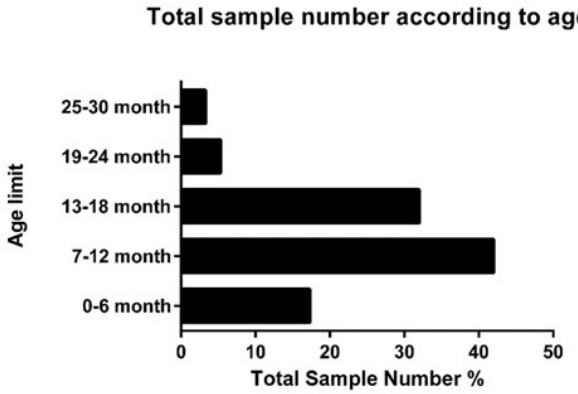

Figure 1: Number of donors at different aged diarrhea patient

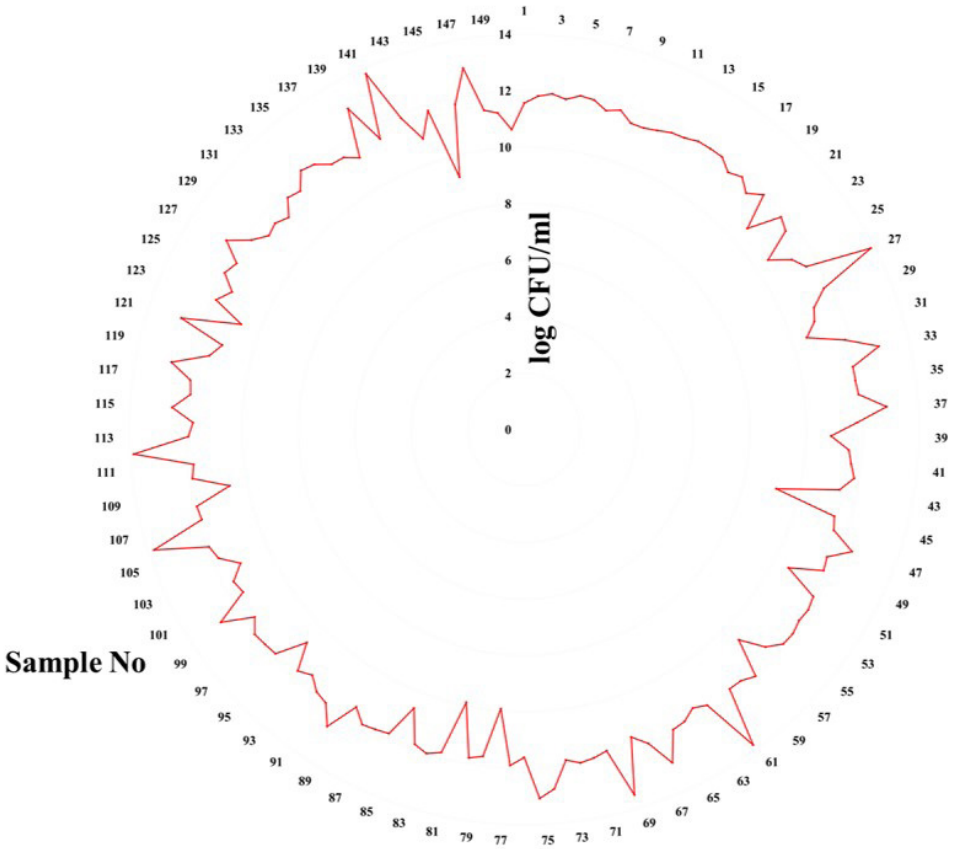

Figure 2: Bacterial count in fecal samples. The edges represent the Log values of CFU per microliter of fecal samples

Determination of Antibiotic-Resistant Bacteria in Fecal Samples of Diarrhea-Associated Children

Gut bacteria samples were collected from the feces of diarrheal-associated children. The Antibiogram profile of these samples indicates an antibiotic resistance pattern against 10 antibiotics. For this purpose, we followed guidelines from the Kirby-Bauer method approved by the Clinical and Laboratory Standards Institute (CLSI). We found samples 75, 142, and 146 containing resistant bacteria against all 10 antibiotics and samples 1, 72, and 89 containing susceptible bacteria against most of the 10 antibiotics (Figure 3).

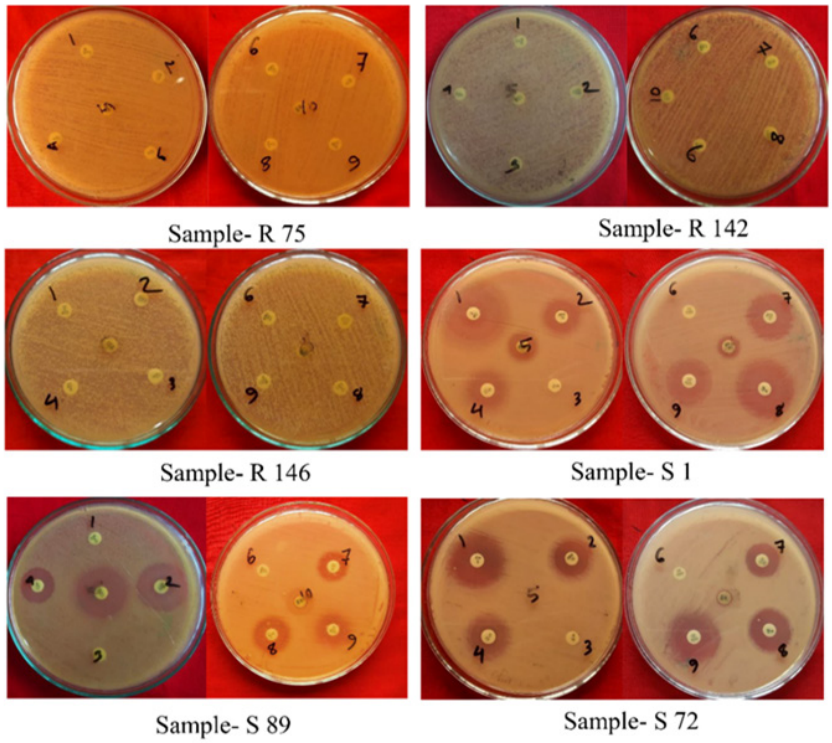

Figure 3: Antibiotic susceptibility test of the fecal samples via Kirby-Bauer disk diffusion method to select the multiple drug-resistant and sensitive fecal samples in human gut microbiota. The samples indicated with R 75, R 142, and R 146 are found resistant against the 10 introduced antibiotics and S 1, S 89 and S 72 are sensitive against most of the antibiotics

Among all the samples, 66% of samples were found resistant against Ciprofloxacin, 54% against Gentamycin, 94% against Erythromycin, 73% against Streptomycin, 71% against Tetracycline, 83% against Amoxicillin, 93% against metronidazole, 65% against Levofloxacin, 84% against Azithromycin and 69 % against Doxycycline. Antimicrobial agents, their disc concentration, and zone interpretative reference was used according to CLSI, 2016.

In anaerobic condition, the fecal samples of 150 donors showed significantly higher levels of resistance than susceptibility against all tested antibiotics (Figure 4).

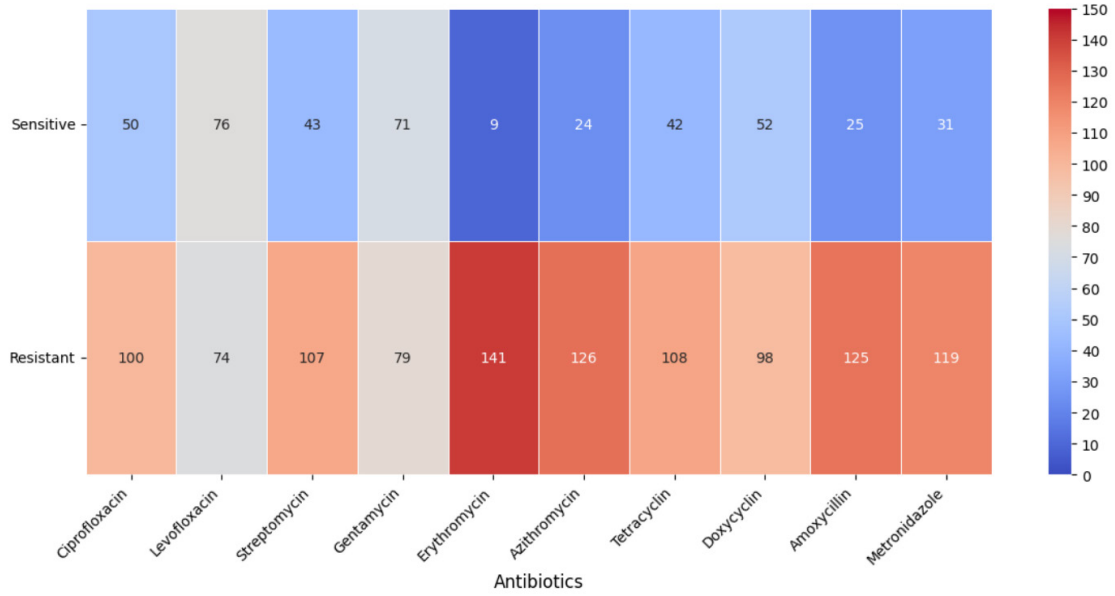

Figure 4: Graphical presentation of Antibiotic Sensitivity in Anaerobic conditions. *P < 0.01, **P < 0.005. ***P<0.0005

Antibiotic resistance pattern was also observed to be different at different age levels (Figure 5). Antibiotic resistance was higher in samples isolated from 0-month-old to 6-month-old patients than sensitivity. Resistance is also higher in samples collected from 7- to 12-month-old patients against all tested antibiotics except Gentamicin and Levofloxacin. However, Bacterial samples isolated from 13-18 months old patients showed higher sensitivity to Ciprofloxacin, Gentamicin, Azithromycin and Levofloxacin and higher resistance against other antibiotics. All samples showed a higher tendency to resistance against all antibiotics except Gentamicin and Levofloxacin. The bacterial community of the fecal sample of 25–30-month-old donor showed a higher level of resistance against almost all antibiotics. They only showed susceptibility against Doxycycline and Levofloxacin.

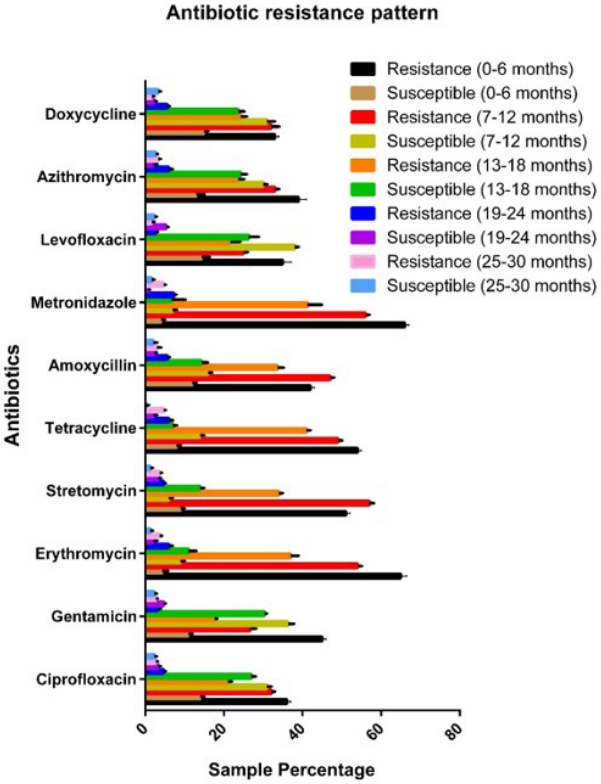

Figure 5: Antibiotic resistance profile of gut bacteria in different age groups of children

Metagenomic Analysis of the Isolated Human Gut Bacteria

After isolating the gut bacteria, the samples of Group 1 and Group 2 were mixed separately based on their antibiotic sensitivity for metagenomic analysis. The genomic DNA was isolated from both Group 1 and Group 2 samples and used for PCR and next-generation sequencing. Metagenomic sequencing was done by Illumina sequencing, and it represents the abundance of bacteria from the phylum to the species level. This data also presents all types of bacteria present in the sample. Therefore, Metagenomic analysis represents fecal and gut microbiota and shows the abundance of various microorganisms in a single assay (Figure 6). According to the metagenomic data, the most abundant bacteria in both samples belong to the order Lactobacillales. The abundance of other bacterial species that belong to the genus *Pseudomonas, Clostridium,* and *Corynebacterium* is lower in both antibiotic-resistant and antibiotic-sensitive samples. Besides, the abundance of the genus *Streptococcus* was found only in antibiotic-resistant samples with a relative abundance of 4.00. Other groups like Enterococcus, Vagococcus, Bacillales, Planococcus, Lysinibacillus, etc., are comparatively abundant in antibiotic-sensitive samples. However, the abundant bacterial group in antibiotic-resistant samples was taken into consideration.

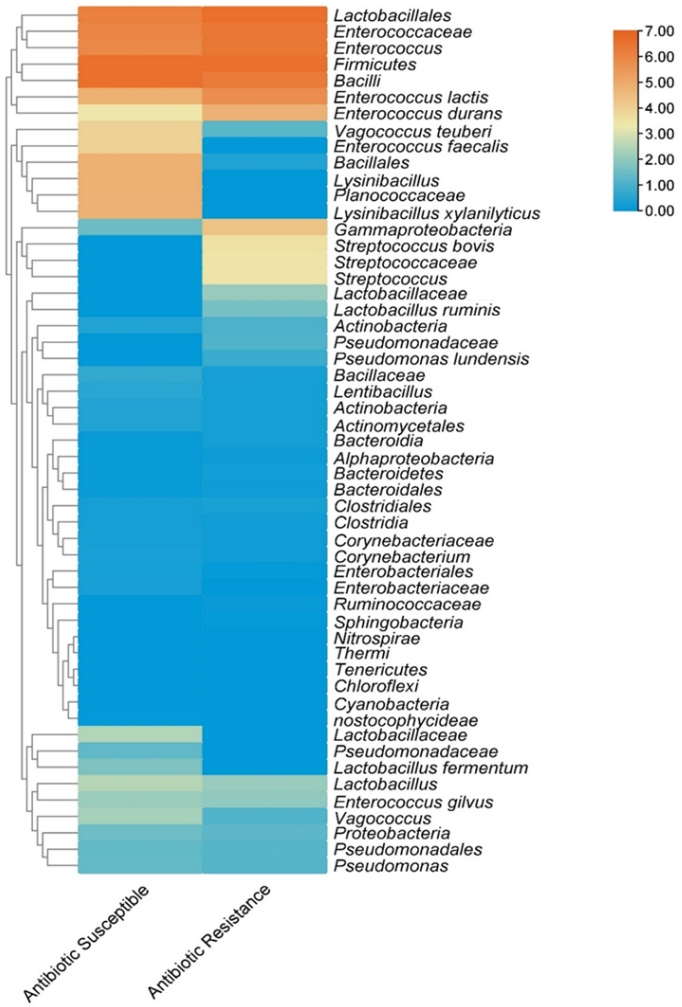

Figure 6: Metagenomics analysis showed the Relative abundance of antibiotic-resistant and antibiotic-sensitive gut microbes. Different relative abundances are indicated by different colors and shades. The columns represent the samples, while the rows specify the microorganisms present

Isolation of *Streptococcus* Species Present in Antibiotic-Resistant Sample

As *Streptococcus* species have been found as dominant in antibiotic resistant samples, it was isolated using *Streptococcus* selection agar (HiMedia M304) (Figure 7A). Three distinct colony morphologies were selected for

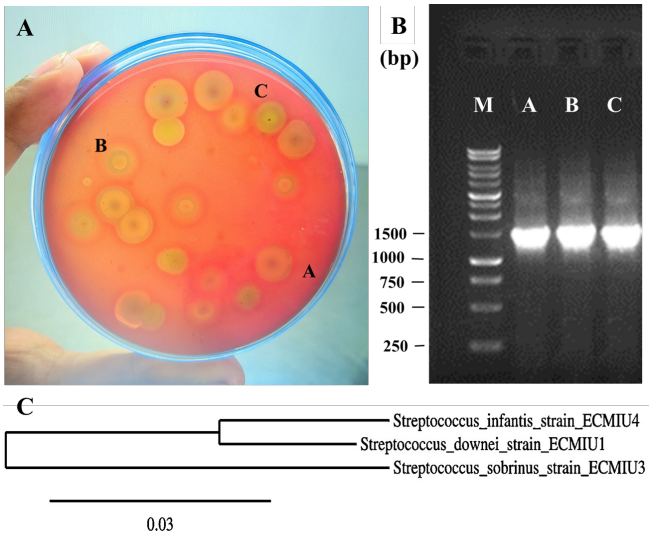

Figure 7: (A) Isolation of *Streptococcus* based on their colony morphology and hemolysis activity on *Streptococcus* Selection Agar. (B) Agarose gel electrophoresis of 16S rRNA gene PCR product. (C) Phylogeny of the isolates

further analysis based on their hemolysis patterns and visual characteristics. Colony A displayed complete beta hemolysis (clear zone surrounding the colony), opacity, a greyish-white color, and a spherical shape. Colony B exhibited similar complete beta hemolysis but was translucent with a white color and maintained a spherical form. Colony C differed by demonstrating partial alpha hemolysis (incomplete zone of clearing), opacity, a white color, and a spherical shape (Table 1). Three types of colonies were taken, identified, and characterized through biochemical assay and 16s rRNA gene sequencing.

Table 1: Morphology of *Streptococcus*

Characteristics	A	B	C
Shape	Mucoid	Spherical	Oval
Color	White	Milky White	Greyish White
Opacity	Opaque	Opaque	Opaque
Hemolysis	β	β	α

Characterization and Identification of *Streptococcus* Species

Several biochemical tests have been performed to identify *Streptococcus* species following "Bergey's Manual of Determinative Bacteriology" (Table 2). Three isolates that had similar results were taken for DNA Extraction and Purification, 16s rRNA gene amplification, and Sanger sequencing. Phylogenetic analysis revealed all three *Streptococcus* species (S. infantis, S. downei, and S. sobrinus) cluster closely, indicating a shared evolutionary history (Figure 7B, 7C).

Table 2: Biochemical assay of *Streptococcus*

Test Name	Result
Gram Staining	+ve
Shape	cocci
Spore Forming	Non-sporing
Catalase	-ve
Oxidase	-ve
Indole	-ve
Methyl Red	+ve
Vogues Proskauer	-ve
Citrate Utilization	+ve
Urease	-ve
Motility	Non-motile
Acid Tolerance	+ve
Bile Salt Tolerance	+ve

Table 3: Result of 16S rRNA gene sequencing

Sample ID	Bacterial Name with strain	Accession Number
A	*Streptococcus downei* ECMIU1	OP030715
B	*Streptococcus sobrinus* ECMIU3	OP045501
C	*Streptococcus infantis* ECMIU4	OP045595

All sequences were uploaded and given an accession number to the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/) database (Table 3). The phylogenetic relationship is also displayed (Figure 5D). Sequencing results identify the isolates as *Streptococcus* downei ECMIU1, *Streptococcus* sobrinus ECMIU3, and *Streptococcus* infantis ECMIU4. They are presented as follows: their sample ID and accession number.

Control of Multidrug-Resistant *Streptococcus* Species

Multidrug-resistant *Streptococcus* sp. could disturb the native microbial balance, making individuals more vulnerable to infections by bacteria with stronger defenses, impairing immune system performance, and accelerating the onset of metabolic diseases. Therefore, in order to prevent this infection, novel approaches are critically needed, and utilizing natural resources is certainly an alternative. After identification and biochemical characterization of antibiotic-resistant *Streptococcus* species, the mixture of the *Streptococcus* isolates was controlled with water extracts of natural compounds.

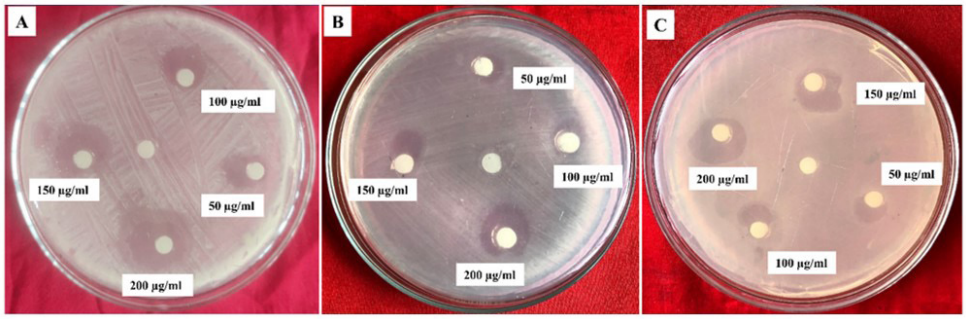

Figure 8: Natural compound-mediated control of antibiotic-resistant *Streptococcus* species. Control by water extract of cloves (A) *Streptococcus* downei ECMIU1 (B) *Streptococcus* sobrinus ECMIU3 (C) *Streptococcus* infantis ECMIU4. Extract was introduced in different concentrations to the plates, and the central discs of each plate were used as the negative control

The evaluation of water extracts from cloves (Syzygium aromaticum) against multidrug-resistant *Streptococcus* species yielded promising findings (Figure 8). The extract exhibited dose-dependent inhibitory activity against the tested strains. Water controls (negative control) did not exhibit any inhibitory activity against the *Streptococcus* species.

Discussion

The use of antibiotics is the most efficient way to prevent bacterial infection (Cook & Wright, 2022). However, during the past few years, the increasing number of antibiotic-resistant strains and treatment failures of bacterial infections have increased public concern about the use of antibiotics (Band & Weiss, 2019). Treatment with broad-spectrum antibiotics generates an evolutionary force that stimulates the emergence of multidrug-resistant bacteria resulting in a chain of unsuccessful treatments and new antibiotic-resistant bacterial strains (Chinemerem Nwobodo *et al.*, 2022). The human gut microbiome is a gigantic storehouse of bacteria with the capacity for both accepting and transmitting antibiotic resistance genes (Loftus *et al.*, 2021). Consequently, this enhances the risk of antibiotic-resistant genes transferring across the normal flora and, even worse, spreading to other pathogenic bacteria (Schjørring & Krogfelt, 2011). This study investigated the patterns of antibiotic resistance within the gut microbiota and employed metagenomic analysis to explore the relative abundance of microbes in antibiotic-resistant and sensitive samples. Additionally, we evaluated the potential of various plant extracts to control the abundance of specific species enriched in the antibiotic-resistant samples. Fecal samples that imitate the gut microbiome, were collected and the microbial count was determined via the spread plate culture technique. The Kirby-Bauer disk diffusion method is a standardized laboratory technique that was used to determine bacterial susceptibility of the bacteria present in fecal samples to various antibiotics (Segawa *et al.*, 2020). The bacteria in 0-6-month old children's feces were observed as higher resistant than the 7-12-month old children and other groups against most of the tested antibiotics. This early emergence of antibiotic resistance in newborns could be linked to the immature gut microbiome. Upon exposure, antibiotic-resistant pathogens can colonize easily due to the underdeveloped gut microbiome (Klassert *et al.*, 2020; Saturio *et al.*, 2023). The resistant bacteria can also be transmitted to children during birth or breastfeeding (Matok *et al.*, 2021; Samarra *et al.*, 2023). However, in the gut microbiome of 25-30-month-old children, bacteria are resistant against all antibiotics except Levofloxacin and Doxycycline. Levofloxacin is the third generation of fluoroquinolones, whereas Doxycycline is the second generation tetracycline antibiotics. These drugs are relatively new and therefore their exposure is less than other older antibiotics, resulting in the susceptibility of the bacteria in 25-30-month-old children gut microbiome (Muteeb *et al.*, 2023).

16S rRNA gene metagenomic analysis offers an effective way to explore the relative abundance and composition of microbes within the gut microbiota in various human health conditions (Peterson *et al.*, 2021). In our study, 16S rRNA gene metagenomic analysis was performed on both the antibiotic-resistant and sensitive samples to classify the microbial communities present, ranging from kingdom down to species level (Garg *et al.*, 2021). *Streptococcus* was relatively abundant in antibiotic-resistant sample. The abundance of *Streptococcus* in antibiotic-resistant gut sample suggests a potential link between the genus and resistance phenotypes. In addition, the genus *Streptococcus* was found to have a natural tendency to develop resistance against a wide range of antibiotics (Gergova *et al.*, 2024; Jin *et al.*, 2022). *Streptococcus* selection agar was employed to isolate *Streptococcus* species from the antibiotic-resistant gut bacterial sample, allowing their selective enumeration and identification. *Streptococcus* selection agar contains polymixin B and neomycin, which act as selective agents (Singh *et al.*, 2023). These antibiotics inhibit the growth of a wide range of Gram-negative bacteria and some Gram-positive bacteria other than *Streptococcus*, allowing *Streptococcus* species to grow preferentially (Kadhim *et al.*). After culturing on this media, colonies are selected for further analysis based on their hemolysis activity and morphology (shape, color, and opacity) (Pinatih *et al.*, 2022). Colonies exhibiting complete or partial hemolysis were selected for further investigation as potential pathogens for humans (Tagg *et al.*, 2019). Following colony selection, biochemical tests based on Bergey's Manual of Determinative Bacteriology were performed to identify the isolates as *Streptococcus* species (Batubara *et al.*, 2021). To ensure the identification, genomic DNA from the isolates was extracted and subjected to 16S rRNA gene amplification by PCR and subsequent Sanger sequencing. This analysis confirmed the isolates as S. downei, S. sobrinus, and S. infantis (Church *et al.*, 2020).

These antibiotic-resistant bacteria within the gut normal flora might disrupt the delicate microbial balance, potentially increasing susceptibility to infections with resistant pathogens, compromising immune function, and contributing to the development of metabolic disorders (Shreiner *et al.*, 2015). While *Streptococcus* downei, *Streptococcus* sobrinus, and *Streptococcus* infantis are found in the normal gut microbiota, they are also opportunistic pathogens (Chamat-Hedemand *et al.*, 2020; Conrads *et al.*, 2014; Fukunaga *et al.*, 2023). However, Antibiotic-resistant *Streptococcus* downei, S. sobrinus, and S. infantis in the gut can become reservoirs for antibiotic resistance genes, potentially spreading them to other bacteria through horizontal gene transfer (Ahsan *et al.*, 2024; Jeżak *et al.*, 2022; Lamberte & van Schaik, 2022; Maeusli *et al.*, 2020; McInnes *et al.*, 2020). This creates an alarming circumstance where even non-pathogenic gut bacteria can lead to the development of multidrug-resistant pathogens (Ducarmon *et al.*, 2019). Therefore, the control of these species is imperative to minimize the

spread of antibiotic resistance. To minimize the potential for these *Streptococcus* species to act as carriers for antibiotic resistance genes, exploring natural sources for treatments focused on preventing infections caused by them is necessary (Kumar *et al.*, 2021). Aqueous extracts of Cloves *(Syzygium aromaticum)* offer therapeutic activities against the isolated *Streptococcus* species (Wongsawan *et al.*, 2019). The aqueous extract in 200 µg/ml concentration exhibits the highest zone of inhibition against the mixture of *Streptococcus* species, which indicates the effectiveness of the extracts as a controlling agent for the antibiotic-resistant species (Abdel-Shafi *et al.*, 2019).

CONCLUSION

The rise of antibiotic-resistant bacteria in the gut is a critical issue demanding immediate attention. It not only increases the risk of infections but also facilitates the spread of resistance among all types of bacteria. Therefore, strategies should be developed to control and combat these antibiotic-resistant bacteria. This study investigated the patterns of antibiotic resistance within the gut microbiota using metagenomic analysis. The findings highlight the concerning trend of antibiotic resistance of *Streptococcus* downei, *Streptococcus* sobrinus, and *Streptococcus* infantis and their potential to disrupt the gut ecosystem and increase susceptibility to infections with resistant pathogens. Our evaluation of aqueous extracts of Cloves *(Syzygium aromaticum)* demonstrates the promising potential as a natural approach to control the abundance of specific antibiotic-resistant bacterial species identified in the gut. While further in vivo evaluation is compulsory to validate effectiveness and safety, these results suggest plant extracts may offer a new and valuable strategies for managing antibiotic-resistant bacteria within the complex gut environment.

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