



# Phylogeny, Biofilm Production, and Antimicrobial Properties of Fecal Microbial Communities of Adi Tribes of Arunachal Pradesh, India

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## Abstract

The fecal flora consists of trillions of bacteria influencing human health and several host factors. Such population-based fecal flora studies are critical to uplift the health status of ethnic tribes from Arunachal Pradesh. This study aimed to analyze the ethnic tribe's biofilm producing antibiotic resistant bacteria and their phylogenetic analysis in 15 stool samples collected from Adi tribes of Arunachal Pradesh. Of the analyzed samples, 42.85% were *Escherichia*, 20% lactic acid bacteria, 20% *Salmonella*, and 17.14% *Enterococcus*. *Escherichia coli*, lactic acid bacteria, and *Enterococcus* sp. emerged as strong biofilm producers; however, *Salmonella* declined to exhibit characters for a strong biofilm producer. Tetracycline resistance dominated in all the gut bacterial profiles. The 16SrRNA amplified PCR product was used for sequencing, and a phylogenetic tree was constructed exhibiting the relationship between the isolates. The test sequences were compared with the non-redundant Gene bank collection of the database with the Basic Local Alignment Search Tool.

**Keywords** Antibiotic resistance · 16SrRNA sequencing · Adi tribe · Phylogenetic · Arunachal Pradesh

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## Introduction

Antibiotic-resistant bacteria have created problems due to dissemination [14] and possessing high resistance even to newer antimicrobials such as carbapenems and faropenem [13], which has become a severe threat in therapeutic options. In India, the burden of antibiotic-resistant bacteria is relatively high due to the rapid and prolonged use of antibiotics. Antibiotic-resistant bacteria can also produce biofilms, making them resistant to antibiotics and then developing human infections [9]. Biofilm is an accumulation of microorganisms on the surface in which cells are stuck with each other, and a matrix of polymeric substances is produced [43]. This increase in strength within the community is a public health concern with the direct clinical implication in the therapeutic option. Fecal flora such as *Escherichia*, *Salmonella*, lactic acid bacteria, and *Enterococcus* can harbor antibiotic reservoir genes in the human gut. This resistant gene can be transferred from commensal flora to pathogenic microorganisms [7, 34]. Therefore, the urgent need is to understand the prevalence of antimicrobial-resistant bacteria in a healthy community. Many works have been carried out to recover antibiotic-resistant bacteria from different parts of the world [6, 20]. However, tribal people of Arunachal Pradesh of Northeast India (NE) remain uncharacterized. These people live in a remote area and have never been exposed to modern antibiotics. The only possible way to contact antibiotic-resistant bacteria is to ingest soil bacteria accidentally, which may be naturally resistant to these drugs. Therefore, the present study was aimed to determine the frequency, biofilm production, and molecular basis analysis of phylogeny properties of antimicrobial-resistant bacteria from healthy tribal people from Arunachal Pradesh.

## Materials and Method

### Sampling Criteria

A total of 15 stool samples were collected from Adi tribes of Arunachal Pradesh from healthy individuals without any gastrointestinal disorder. Individuals who had not taken any antibiotics for at least 6 months before the sampling period and belonged to the age group of 20–60 years (both male and female) were included in the study. The stool samples were collected in sterile collection tubes, put into an icebox, and transported to the laboratory, where the stool samples were kept at  $-80\text{ }^{\circ}\text{C}$ . Details of the volunteers' dietary habits, age, and physical status were recorded as per their respondents (Table 1).

### Culture-Dependent Isolation

The collected stool samples were inoculated on to lactose broth and brain heart infusion broth and incubated for 24 h at  $37\text{ }^{\circ}\text{C}$  in BOD incubator. To isolate and study the morphological characteristics, samples were streaked on a HiCrome Universal Differential Medium, brain heart infusion agar, MacConkey agar, Salmonella Differential Agar (Twin Pack), Raj Hans Medium, and Lactobacillus MRS Agar. The isolates' phenotypic characters were studied concerning their shape, size, and Gram staining properties followed by a series of standard biochemical tests (IMVIC).

**Table 1** Demographic characteristic of study population showing the food habits, and consumption of tobacco and alcohol in the Adi tribe of Arunachal Pradesh, India

Sl no.	Sample code	Age	Gender	Fermented, smoke, and dried food	Smokers/alcoholic
1	AdM01	25	Male	Fermented bamboo shoot, smoked fish and meat	Both
2	AdM02	27	Male	Fermented bamboo shoot, smoked fish and meat	Apong
3	AdM03	35	Male	Fermented bamboo shoot, smoked fish and meat	Both
4	AdM04	32	Male	Fermented bamboo shoot, smoked fish and meat	Apong
5	AdM05	40	Male	Fermented bamboo shoot, smoked fish and meat	Apong
6	AdM06	39	Male	Fermented bamboo shoot, smoked fish and meat	Apong
7	AdM07	45	Male	Fermented bamboo shoot, smoked fish and meat	Apong
8	AdF08	25	Female	Fermented bamboo shoot, smoked fish and meat	Apong
9	AdF09	27	Female	Fermented bamboo shoot, smoked fish and meat	Apong
10	AdF10	35	Female	Fermented bamboo shoot, smoked fish and meat	Apong
11	AdF11	32	Female	Fermented bamboo shoot, smoked fish and meat	Apong
12	AdF12	40	Female	Fermented bamboo shoot, smoked fish and meat	Apong
13	AdF13	39	Female	Fermented bamboo shoot, smoked fish and meat	Apong
14	AdF14	45	Female	Fermented bamboo shoot, smoked fish and meat	Apong
15	AdF15	40	Female	Fermented bamboo shoot, smoked fish and meat	Apong

### Antibiotic Susceptibility Test

Kirby-Bauer disc diffusion antibiotic susceptibility profiling was performed on the Muller Hinton agar [36] against commonly used antibiotics (HiMedia, India) towards all the isolated strains from Adi tribes' stool samples. Therefore, to determine the susceptibility patterns [16, 17, 26, 44] of the isolates, ampicillin, ciprofloxacin, tetracycline, gentamicin, aztreonam, meropenem, ertapenem, cefepime, chloramphenicol, ceftazidime, cotrimoxazole, vancomycin, erythromycin, oxacillin, and ceftriaxone were used, respectively. Response to each antibiotic showed by the microbial isolates was categorized as resistant and sensitive to each antibiotic drug. A chi-square test was done for this categorical dataset, and data were considered significantly different at  $p < 0.05$ . All the data were analyzed by using the SPSS 21.0 software package.

### In Vitro Biofilm Assay

All the recovered isolated colonies were subjected to the in vitro biofilm assay by the Congo Red Agar and tube method.

### Congo Red Agar Method

Brain heart infusion agar and sucrose and Congo red stain (aqueous solution) were prepared separately, autoclaved, and mixed with agar as a supplement when the temperature reached 55 °C. The prepared Congo Red Agar (CRA) was then poured into sterile Petri plates and allowed to solidify. The plates were streaked and incubated aerobically at 37 °C for 24 h [42]. Interpretation of the results was recorded as strong, moderate, and weak. The appearance of black dry crystalline colonies on the CRA plates was classified as strong biofilm producer, while red or pink colonies were classified as weak biofilm producer and dark colonies, but the absence of crystalline colonial morphology was classified as intermediate biofilm producer.

## Tube Method

The CRA method's positive isolates were further analyzed by in vitro biofilm production test by tube method as previously described [5] with slight modifications. Test organisms inoculated in Trypticase Soy Broth (TSB) were incubated at 37 °C for 24–48 h. The decanted tubes were then washed with phosphate buffer saline, followed by air-drying at room temperature. Staining with 4% crystal violet was performed. De-ionized water was used to remove excess stain, and the tubes were dried by placing them in an inverted position. The interpretation was recorded according to the visibility of a number of films lining the wall and the bottom of the tube as weak/none, moderate, and high/strong. Those isolate that produced three film linings in the wall of the test tube were classified as strong; two or one film lining in the wall was classified as intermediate, while those isolates that produced film lining in the bottom of the tube was classified as a weak biofilm producer. The experiment was performed in triplicates, and the tubes were compared with control strains.

## DNA Extraction

Extraction of DNA was performed for only those isolates expressing their resistance towards antibiotics by boiling method [22]. A colony of overnight grown culture was taken into a microcentrifuge tube containing TE (Tris-EDTA) buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA, 0.1 ml), vortexed and incubated in a water bath at 95 °C for 10 min followed by centrifugation for 10 min at 10,000 pm.

## PCR Amplification of the 16SrRNA Gene

PCR reactions were carried out for antibiotic resistance *Escherichia* sp., lactic acid bacteria, and *Enterococcus* sp. in a gradient thermocycler (Eppendorf, USA). The primers used were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTAC GACTT-3') [37]. A reaction mixture of 12.5 µl Taq polymerase, 1 µl of each forward and reverse primer, 8.5 µl of nuclease-free water, and 2 µl of DNA template was taken in each tube. The PCR reaction conditions were set in as initial DNA denaturation for 5 min at 94 °C, 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and elongation for 1 min at 72 °C with a final extension for 5 min at 72 °C.

A 5 µL of the PCR product was then electrophoreses in 1% agarose gel, at 100 V for 30 min, followed by staining with 1% solution of ethidium bromide (50 µL/L) and destaining with TBE (Tris-borate-EDTA ) 1× for 10 min. UV trans-illumination then visualized the band sizes, and the image was captured in Gel Doc.

## Sequence Analysis

The 16SrRNA amplified PCR product was used for sequencing with 27F and U1492R primer. The sequenced data were then analyzed using NCBI (National Centre for Biotechnology Information) and BLAST (Basic local alignment search tool) for highly significant matches. MEGA 7 software was used to construct the phylogenetic tree.

## Strains Used for Quality Control:

For quality control, *E. coli* ATCC 25922 was used as a control strain for **antibiotic** susceptibility profiling. A *Staphylococcus aureus* ATCC25923 and *Staphylococcus epidermis* 12228 were used as reference strains for positive biofilm and negative biofilm control strain.

## Statistical Analysis

The statistical software package SPSS version was used to analyze the antibiotic resistance shown by different isolates. A chi-square test was applied where  $p < 0.05$  was considered statistically significant.

## Results

### Prevalence of Aerobic Culture-Dependent Isolates

Based on microscopic observations and cultural characteristics, a total of 35 isolates were recovered aerobically from stool samples of healthy individuals. Of these 35 isolates, 15 (42.85%) confirmed to be *Escherichia* sp., 6 (17.14%) *Enterococcus* sp., 7 (20%) *Salmonella* sp., and 7 (20%) isolates as lactic acid bacteria. The above isolates' biochemical characterization revealed that *Escherichia* spp. expressed themselves as gram-negative rods and were catalase, indole, methyl red, motility test-positive and shown in oxidase Voges Proskauer and citrate utilization tests as negative. *Enterococcus* spp. expressed themselves as gram-positive cocci and Voges Proskauer test-positive; *Salmonella* spp. expressed as gram-negative rods and catalase and methyl red test-positive while lactic acid bacteria expressed themselves as gram-positive rods and methyl red test-positive.

Since the Adi tribe is distinct and unique in its tradition and food habits, it mainly consumed fermented bamboo shoots, smoked fish and meat, and home-prepared alcohol, popularly known as Apong.

### Antibiotic Susceptibility Test

Phenotypically confirmed isolates were further screened against commonly used antibiotics, and it was observed that 13% (2/15) of *Escherichia* spp. were resistant towards tetracycline and 100% susceptible towards aztreonam, meropenem, cefepime, gentamicin, ceftriaxone, ertapenem, chloramphenicol, and ceftazidime. Similarly, *Salmonella* sp. was 100% susceptible towards cotrimoxazole, gentamicin, ampicillin, tetracycline, and ciprofloxacin; lactic acid bacteria were 100% susceptible towards erythromycin, gentamicin, ampicillin, and 28.57% (2/7) resistant towards tetracycline and 14.28% (1/7) towards vancomycin, while *Enterococcus* sp. was 50% (3/6) resistant towards tetracycline followed by 33.33% (2/6) towards vancomycin and 16.67% (1/6) towards erythromycin, and 100% susceptible towards ampicillin, oxacillin, and gentamicin. After statistical analysis, it was observed that *Escherichia* sp. and lactic acid bacteria were significantly different at  $p < 0.05$ . *Enterococcus* sp. did not show any significance ( $p = 0.087$ ). However, no statistical analysis was performed against *Salmonella*

**Table 2** Antibiogram profiling of the isolated *Escherichia* sp., lactic acid bacteria, *Enterococcus* sp., and *Salmonella* sp.

Organisms	Antibiotics	Number of isolates	Sensitive		Resistance		p-value
			n	%	n	%	
<i>Escherichia</i> sp.	Tetracycline	15	13	86.7	2	13.3	p = 0.039
	Aztreonam	15	15	100	0	0	
	Meropenem	15	15	100	0	0	
	Cefepime	15	15	100	0	0	
	Gentamicin	15	15	100	0	0	
	Ceftriaxone	15	15	100	0	0	
	Ertapenem	15	15	100	0	0	
	Chloramphenicol	15	15	100	0	0	
	Ceftazidime	15	15	100	0	0	
Lactic acid bacteria	Tetracycline	7	4	57.1	3	42.9	p = 0.048
	Gentamicin	7	7	100	0	0	
	Erythromycin	7	7	100	0	0	
	Ampicillin	7	7	100	0	0	
	Vancomycin	7	6	85.7	1	14.3	
<i>Enterococcus</i> sp.	Tetracycline	6	3	50	3	50	p = 0.087
	Gentamicin	6	6	100	0	0	
	Erythromycin	6	6	100	0	0	
	Ampicillin	6	6	100	0	0	
	Vancomycin	6	4	66.7	2	33.3	
	Oxacillin	6	6	100	0	0	
<i>Salmonella</i> sp.#	Cotrimoxazole	7	7	100	0	0	-
	Gentamicin	7	7	100	0	0	
	Ampicillin	7	7	100	0	0	
	Tetracycline	7	7	100	0	0	
	Ciprofloxacin	7	7	100	0	0	

Each microbial isolates were categorized as sensitive and resistant based on their response to each antibiotic provided and are presented as number and percentage. Statistical analysis: Chi-square test was performed to assess the level of significance

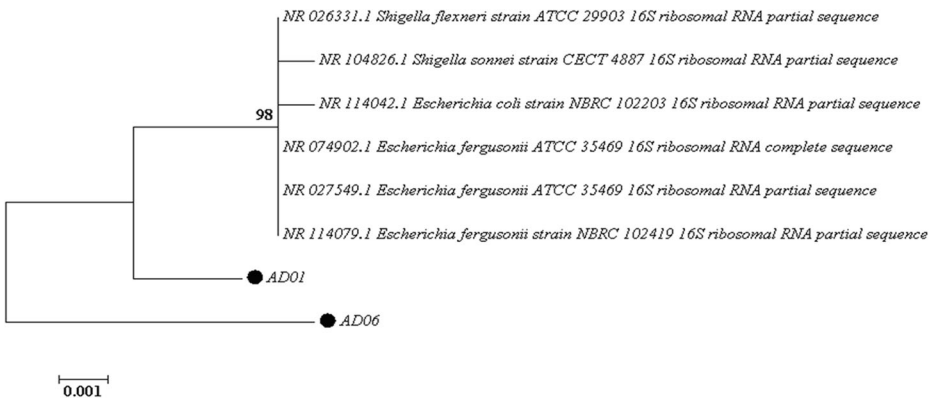
#No statistical analysis was performed against *Salmonella* sp. since it did not show any resistance to antibiotics

sp. since it did not show any resistance. Antibiotics susceptibility profiling for all the isolates is summarized in Table 2.

## Biofilm Assay

Using the biofilm assay, both antibiotic-resistant and resistant isolates were screened using the tube method and the Congo Red Agar method. It was observed that in the CRA method, out of 15 *Escherichia* isolates, 13.33% (2/15) were strong biofilm producers, 40% (6/15) were weak biofilm producers, and 46.66 (7/15) were non-biofilm producers. *Salmonella* spp. were observed as 14.28% (1/7) intermediate biofilm producers and 85.71% (6/7) as non-biofilm producers; lactic acid bacteria were observed as 28.57% (2/7) strong biofilm producers, 42.85% (3/7) as intermediate biofilm producers, and 14.28% as weak biofilm producers, while *Enterococcus* spp. were observed as 33.33% (2/6) strong biofilm producers, 50% (3/6) as intermediate biofilm producers, and 16.66%(1/6) as weak biofilm producers (Fig. 1).

In the tube method, all the isolates expressed themselves as highly strong biofilm producers compared to those in the CRA method. *Escherichia* spp. represented themselves as 20% (3/15) strong biofilm producers, 6.66% (1/15) as intermediate biofilm producers, and 73.33% (11/15)

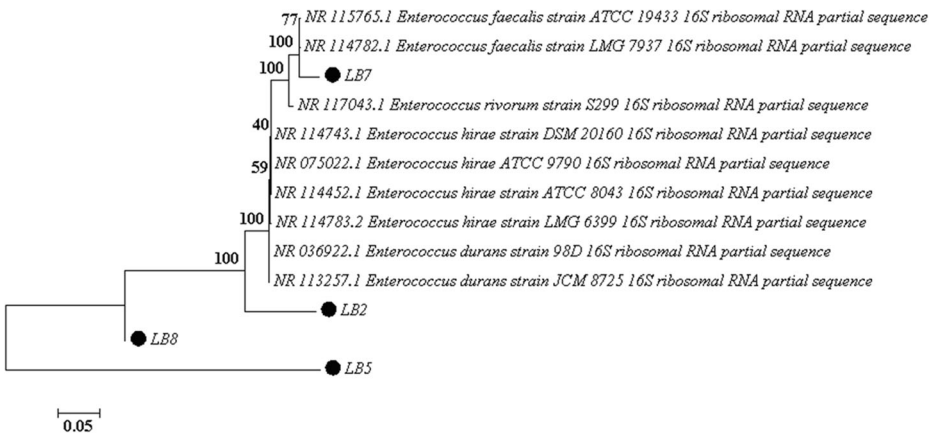


**Fig. 1** Neighbor-joining tree of selected 16S rRNA gene sequence from a BLAST search of the strain Ad06 and Ad01 sequence

as weak biofilm producers; lactic acid bacteria showed 71.42% (5/7) as strong biofilm producers and 28.57% (2/7) as intermediate biofilm producers; *Enterococcus* spp. showed 66.66% (4/6) as strong biofilm producers and 33.33% (2/6) as weak biofilm producers, while in *Salmonella* spp., only 14.28% (1/7) were weak biofilm producers and the rest of them are non-biofilm producers (Fig. 2).

### 16S rRNA Gene Amplification by PCR and Sequence Analysis

The phylogenetic analysis was performed only for antibiotic-resistant *Escherichia* sp., lactic acid bacteria, and *Enterococcus* sp. The results revealed that *Escherichia* sp. belongs to the genera *Escherichia coli*; lactic acid bacteria belong to *Enterococcus hirae*, *Enterococcus faecalis*, and *Enterococcus durans*. *Enterococcus* sp. belongs to *Enterococcus faecalis* and *Enterococcus faecium*. The 16S rRNA gene sequence–based phylogenetic tree for the isolates Ad06 and Ad01 was constructed, exhibiting the relationship between the isolates. The evolutionary history was inferred using the neighbor-joining method [29]. The optimal tree



**Fig. 2** Neighbor-joining tree of selected 16S rRNA gene sequence from a BLAST search of the strain LAB8, LAB7, LAB5 and LAB2 sequence for phylogenetic inference

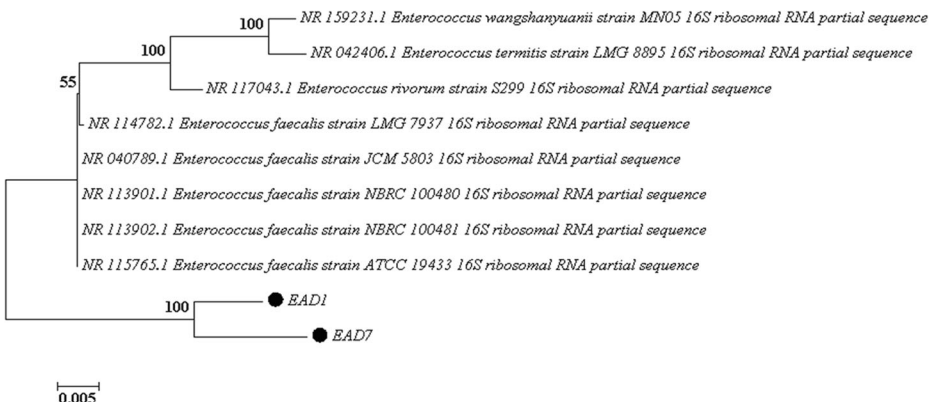
with the sum of branch length = 0.01537371 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown above the branches [12]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method [38] and are in the units of the number of base substitutions per site. The analysis involved eight nucleotide sequences. Codon positions included were the 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1376 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 [18] (Fig. 3).

Similarly, the evolutionary history of the isolates of lactic acid bacteria, LAB8, LAB7, LAB5, and LAB 2, was prepared by using the neighbor-joining method [29]. The sum of the branch length = 0.84222741 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown above the branches [12]. The isolates LAB8, LAB7, LAB5, and LAB2, which were isolates from Adi tribe stool samples in the present study, were closely related to the source of semolina and intestinal tracts of young people. The results revealed that LAB7 belongs to *Enterococcus faecalis*, and LAB2, LAB5, and LAB8 belong to *Enterococcus durans* (Fig. 4).

The evolutionary history was also prepared for *Enterococcus* isolates [29]. The optimal tree with the sum of branch length = 0.08889978 was shown. The strain *Enterococcus* sp. belonged to the genera *Enterococcus faecalis*, which was closely related from the source of the fish intestine and murine cecal content (Fig. 5).

## Discussion

The emergence of antibiotics-resistant bacteria among the healthy population has become a serious condition in therapeutic options. Therefore, the present study aimed to study the Adi tribal people of Arunachal Pradesh. Since this, people follow the ancient lifestyle. The study recorded a total of 35 isolates that were dominated by different groups of bacteria belonging to *Escherichia* sp. 15/35 (42.85%), lactic acid bacteria 7/35 (20%), *Salmonella* sp. 7/35 (20%), and *Enterococcus* sp. 6/35 (17.14%). High recovery of *Escherichia* sp. observed in the present



**Fig. 3** Neighbor-joining tree of selected 16SrRNA gene sequence from a BLAST search of the strain *Enterococcus* EAD1, *Enterococcus* EAD7 sequence for phylogenetic inference



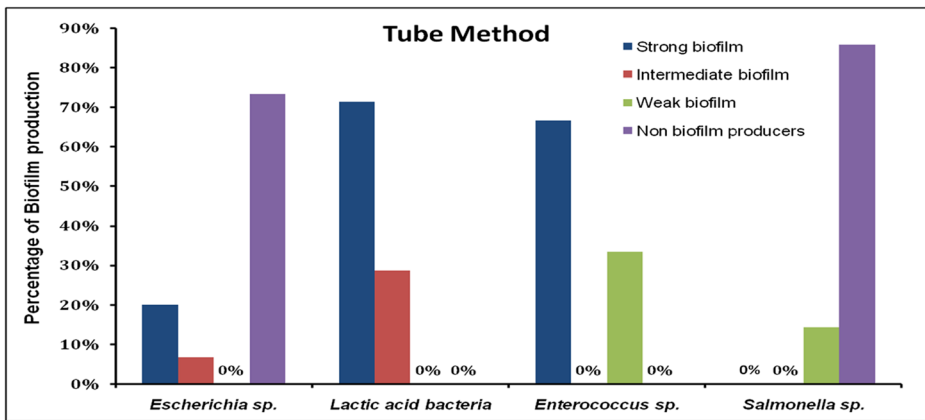


Fig. 4 Graphical representation of biofilm production in Tube Method

study expressed the close contacts of the Adi tribes with domestic animals and consuming their raw milk from the collection container that may be contaminated with poor quality of water [3]. Lactic acid bacteria that dominate the human stool are believed to elucidate beneficial effects in the human host and are regarded as transient strains that originate in food and pass through the intestinal tract [39], but the present study recorded only 20% (7/35) prevalence rate of these strains in the Adi tribes [26]. The low prevalence rate for *Salmonella sp.* 7/35 (20%) was also recorded among this tribe, and such low occurrences of *Salmonella sp.* in the fecal microbiota of Adi tribes may be due to the consumption of raw vegetables or aquatic environment that acts as a source [2, 23]. *Enterococci* are the most common gastrointestinal organisms which have been documented as the vast majority of healthy individuals, but the present study recorded a low prevalence of *Enterococcus sp.* 6/35 (17.14%). The current results are in contrast to the reports [1, 15] that mention the record of all the isolates was positive for *Enterococcus sp.* recovered from stool samples of healthy humans.

Phenotypic resistance profiles of *Escherichia sp.*, lactic acid bacteria, *Salmonella*, and *Enterococcus sp.* obtained from healthy individuals from Adi tribes of Arunachal Pradesh were studied where *Escherichia sp.* exhibited high resistance (13%) towards tetracycline and

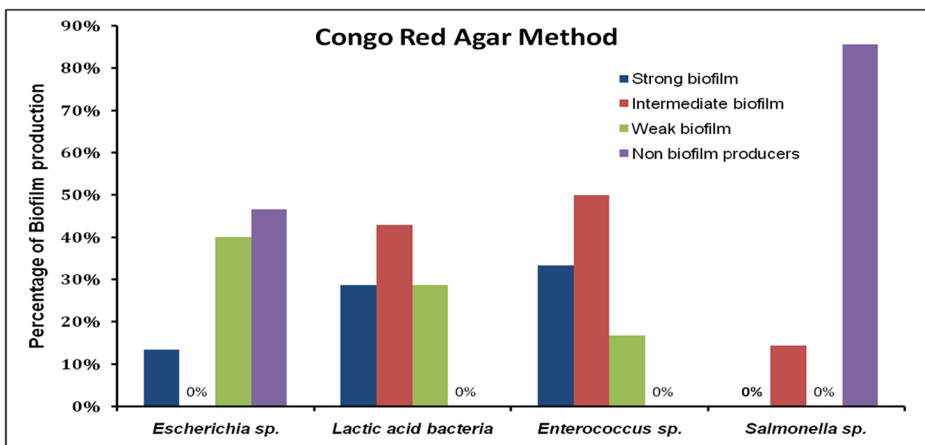


Fig. 5 Graphical representation of biofilm production in Congo Red Agar Method

100% susceptibility towards the antibiotics aztreonam, meropenem, cefepime, gentamicin, ceftriaxone, ertapenem, chloramphenicol, and ceftazidime. Though differences in resistant rates in our results and other coworkers are observed in *Escherichia coli* isolates studied in Ujjain (37%), southern India, and eastern India (35%) [28, 31, 33], a similar resistance pattern was noted [3] mentioning that the occurrence of 16.8% of *E. coli* resistance towards tetracycline is in agreement to the present study. Furthermore, it was observed that high doses of tetracycline antibiotics within the Adi tribes were due to their frequent uses in backyard animal husbandry practices, aquaculture, and poultry farming. Lactic acid bacteria were observed to be highly susceptible (100%) towards erythromycin, gentamicin, and ampicillin and showed its resistance towards tetracycline at 28.57% and resistivity towards vancomycin at 14.28%. In contrast to the above findings, reports have shown that lactic acid bacteria are susceptible towards erythromycin, tetracycline, and vancomycin [17], but there are also few reports documenting *Lactobacilli* to possess a high natural resistance towards vancomycin, a property that is useful in distinguishing them from other Gram-positive bacteria [11, 17, 24]. *Enterococcus* sp. was observed to be highly resistant towards tetracycline at 50%, followed by vancomycin at 33.33% and erythromycin at 16.67%. All the isolates exhibited 100% susceptibility towards ampicillin, oxacillin, and gentamicin [10, 25]. Furthermore, *Salmonella* sp. did not exhibit any resistance towards any of the above antibiotics. Such variations observed in antibiotics resistance patterns may be due to geographical differences [4, 40].

The human gut microbiome constitutes various nutritional and physiochemical environment [19], which may contribute to the formation and survival of biofilms. Microbial biofilms pose multiple serious infections by protecting the constitute bacteria from antibiotics and host immune effects. The present study revealed that *Escherichia coli*, *Salmonella*, lactic acid bacteria, and *Enterococcus* sp. had the ability to form biofilms from the Adi tribe stool samples [21, 30, 32, 41]. The isolates that were observed as strong biofilm producers colonized and exhibited their resistance properties towards antibiotics [27, 43].

16SrRNA sequencing is regarded as the standard gold method for the identification of bacteria. When compared with the non-redundant gene bank collection of the database with BLAST, the test sequences revealed that antibiotic resistance isolates from the Adi tribe of Arunachal Pradesh belonged to genera *Enterococcus hirae*, *Enterococcus durans*, *Enterococcus faecalis*, and *Escherichia coli*. The present study findings agree with the previous reports in which *Enterococcus faecalis* and *Enterococcus hirae* were the most prevalent isolates from feces of Thai newborns [8, 35]. Subsequently, coworkers [37] have also reported that *Escherichia coli* are the most common strains isolated from human stools. The present study data also revealed that antibiotic-resistant isolates from Adi fecal microbiota tribe possessed a similarity to that of the goat milk and fish intestine microbiota. Such similarity observed may be due to the consumption of goat milk and fermented fish or contamination of drinking water fecal contents.

## Conclusion

For the first time, the study provided interesting data on the fecal microbial communities from Adi tribes of Arunachal Pradesh, where *Escherichia coli* was the most abundant microbiota observed, constituting the bulk of the fecal flora. Antibiotics resistance and biofilm formation

observed in *E. coli*, *E. hirae*, *E. faecalis*, and *E. durans* in the healthy Adi tribe of Arunachal Pradesh have raised the alarm as this may have direct implications in therapeutic options.

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**Code Availability** Not applicable.

**Author Contribution** IS, IC, and YC prepared the study design and analysis tools; PH performed experiments; and MU helped in sampling. IS and PH wrote the manuscript, and all the authors read and approved the final manuscript.

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**Data Availability** The datasets generated during/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics Approval** The present study was approved by the Human Ethical Committee, Assam University, Silchar, Assam, India. All the experiments were performed following relevant guidelines and regulations.

**Consent to Participate** The samples were collected with the participants' consent, and the participants duly filled the consent forms.

**Consent for Publication** The participants gave their consent for publication.

**Conflict of Interest** The authors declare no competing interests.

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