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Analysis of diversity and antibiotic susceptibility profile of bacterial strains isolated from freshwater canal of Lahore, Pakistan

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Authors' Contribution | **Malik, M.**, conducted the sample collection and isolation of strains; **S. Tanveer** performed 16S rRNA sequencing analysis; **B. Ali** conceived the study, analysed the data, and constructed figures and tables.

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ABSTRACT

The key objective of current study was to analyse the bacterial diversity of canal water of Lahore, Pakistan. Members of Enterobacteriaceae were targeted that may pose serious health issues to human health. Total 50 bacterial strains were isolated by using Eosin Methylene Blue agar and MacConkey agar. The 16S rRNA gene sequencing of 30 strains confirmed the final taxonomic status of bacterial strains. The results manifested that strains belong to genera of *Enterobacter, Escherichia, Klebsiella, Pseudomonas, Acinetobacter, Aeromonas* and *Vogesella*. Antibiotic susceptibility profile of isolates was evaluated against 13 different antibiotics. *E. coli* SCE-1 showed highest resistant against majority of the antibiotics. Whereas all strains were sensitive to Amikacin (30 µg). *Aeromonas hydrophila* SD-6 exhibited highest sensitivity against all antibiotics except ampicillin (10 µg) and amoxicillin (25 µg). None of the bacterial strain was completely resistant or susceptible to all antibiotics. Except *E. coli* all strains were sensitive to Amikacin (30 µg), Gentamycin (30 µg) and Norfloxacin (10 µg). Most of the strains showed intermediate and resistant antibiotic profile towards Nitrofurantoin (300 µg). Latex agglutination test was performed for the screening of *E. coli* O157 that showed negative results for this serotype. The detection of potential human pathogens like *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *A. hydrphila* in canal water indicates that it is not suitable for domestic purposes, recreational activities, and irrigation.

Keywords: Antibiotic susceptibility, bacterial diversity, biochemical tests, canal water, *E. coli* O157, latex agglutination.

INTRODUCTION: About 0.01% of earth's total water is fresh water with reservoirs, lakes and rivers covering approximately 2.3% of global earth's surface. Around 70% of freshwater is consumed for agricultural purposes globally, vs 20% and 10% for industrial and domestic purposes, respectively. Increase in pollution and nutrient loads that has worsened water quality all over the world is due to a growth in human population and socioeconomic activity (Albert *et al.*, 2021). In freshwater, nature of bacterial flora varies. Some are heterotrophic and some are photo or chemotrophic. Oligotrophic surface waters are rich in non-spore forming rods, gram negative bacteria like *Flavorbacterium*. But with increase in eutrophication, other microorganisms become more important like *Pseudomonas*, *Proteus*, *Bacillus, Enterobacter* and *Actinobacter* (Zlatković *et al.*, 2022). There are many sources of water pollution like runoff water surrounding water bodies is an important source of microorganisms. Farm animals are also a potential source of fecal contamination of water. Sediment, residues of herbicides and pesticides, fertilizer runoff, chemical spills, dumping of garbage, thermal pollution and urbanization are serious threats to freshwater sources. Domestic and industrial sewage water containing pathogens is another source of contamination, which results an increase in number of Fecal Indicator Bacteria (FIB) like *Escherichia coli*. Before using water for any purpose, its quality should be analyzed but it is not possible to check the presence of all pathogens in water. With the help of indicator microorganisms, risk of water borne diseases can be reduced. Fecal indicator bacteria such as *Enterococci* and *E. coli* which may predict the presence of pathogens in aquatic environment. According to United States Environmental protection agency, *E. coli* is better indicator of fecal pollution to evaluate the quality of freshwater (Baker *et al.*, 2021). Most of the serotypes of *E. coli* are not pathogenic but some cause serious diseases in humans. Food-borne diseases are mainly due to some virulent strains like *E. coli* O157:H7. It can cause diarrheal infections, septicemia, neonatal meningitis, and urinary tract infections (Offenbaume *et al.*, 2020). Shiga toxin-producing *E. coli* (STEC) O157 has swiftly become a significant issue in the healthcare and food industries since 1982. In the 30 years since the initial report, 740 outbreaks related to STEC O157:H7 have been documented in the United States. It has been reported that total 13,526 cases resulted in in 2,765 hospital admissions (20%), 653 cases of hemolytic uremic syndrome (4.8%), and 73 fatalities (0.5%) (Kim *et al.*, 2020). Almost all *E. coli* O157:H7 organisms are found to have association with production of toxins (Shiga toxin1 and 2), of which 80% produce both toxins, and only some produce Shiga toxin1 (Stx1) (Yang *et al.*, 2020). Pakistan's agronomy relies on irrigation system which comprises reservoirs, barrages, canals, and other water sources over about millions of hectors of land. Available water doesn't fulfill the requirement, so in replacement water of any good or bad quality is used. This water is consumed by livestock which then is responsible for diseases. Crops are also affected which ultimately disturbs human health. Commercial and small-scale farmers irrigate their fruits and vegetables farms with water from nearby streams, rivers, ponds, and wells which usually do not meet the standard required for irrigation (Fida *et al.*, 2022). The Lahore Canal, which passes through the heart of City is mostly used for irrigation and recreation. The Lahore Canal itself takes water from the Bambawali-Ravi-Bedian (BRB) Canal, a branch of the Chenab River. After passing through the city, the water from the Lahore Canal merges with the river Ravi. Lahore canal is an important water source specially to be used for irrigation purposes in parts of districts of Lahore and Kasur. Due to the lack of an appropriate drainage system for the industrial units and housing societies built along the canal's bank, the amount of pollution in the canal is rising day by day. In addition to making the water unfit for irrigation, this poses a major threat to human health (Ulfat *et al.*, 2022).

OBJECTIVES: The objectives of this study were (1) analyze bacterial diversity of freshwater canal in Lahore, Pakistan, (2) characterize isolated strains morphologically and biochemically, (3) analyze phylogenetic relationships among bacterial isolates after sequencing, (4) assess antibiotic sensitivity profile of isolates using standardized antibiotic disc method and (5) confirmation of strains of *E. coli* for the presence of O157 antigen by using Prolex™ latex agglutination kit system.

MATERIALS AND METHODS: Isolation of bacteria: During 2020, a total number of 5 water samples were collected from Khairapul, Mughalpura, Jail Road, Doctors Hospital and Thokar Niaz Baig along the length of canal were selected for sampling. For isolation of microorganisms, samples were immediately processed in the laboratory. Total 50 µL of each sample was plated on EMB and MacConkey agar plates separately. Plates were later incubated for 24 h at 37°C. After incubation, 50 colonies that differed in their morphology were picked and maintained on Luria-Bertani agar (L-agar) for further characterization.

Morphological and biochemical characterization of bacterial strains: The selected bacterial strains were morphologically and biochemically characterized by following the protocol of Cappuccino and Sherman (2002). Initially, strains were subjected to Gram and endospore staining and catalase test was performed to detect catalase production by bacterial strains. Cytochrome oxidase test was executed to detect the presence of the activity of cytochrome oxidase in bacteria. Citrate utilization test was executed to detect bacterial strains that can use citrate as a carbon source. Indole test was performed to identify the ability of bacterial strains to convert L-tryptophan into indolic compounds. Methyl red test was carried out to identify the bacteria that have the ability of producing stable acidic products after glucose fermentation. Based on variations in carbohydrate fermentation patterns and hydrogen sulphide production, the Triple Sugar Iron (TSI) agar test was executed to distinguish between the several genera of the Enterobacteriaceae.

Detection of *E. coli* 0157: Nutritional artificial media: Sorbitol MacConkey agar (SMA) is a differential medium used for *E. coli* O157 identification because of its inability to ferment sorbitol that is present in this media (March and Ratnam, 1986). Most strains of *E.*

coli ferment sorbitol to produce acid while *E. coli* O157 strain utilizes peptone to grow and do not produce acid. This increases the pH of the medium, which allow the O157 to be differentiated from other *E. coli* by formation of white or colourless colonies. SMA agar was prepared, autoclaved, and poured in plates. After solidification, all the strains of *E. coli* were streaked on the plates. The plates were incubated for 24 h at 37°C. Afterward, the plates were observed for white or colourless colonies for presumptive detection of *E. coli* O157.

Latex agglutination test: The colonies screened from the SMA were further screened by Prolex™ *E. coli* Latex Kit (March and Ratnam, 1989). An anti-*E. coli* O157 somatic antigen antibody is coated on the blue polystyrene latex particles utilized in the kit. The *E. coli* serogroup O157 will bind to the antibody when these latex particles are combined with fresh colonies of the bacteria. This causes the latex particles to agglutinate, showing the presence of the positive reaction. Other bacteria cannot bind to the antibody or agglutinate the latex particles because they are not *E. coli* O157 indicating negative reaction. To prevent false or negative results, a quality control procedure was carried out beforehand in accordance with the manufacturer's protocol. To perform the latex agglutination test, thick bacterial suspension of 24 h fresh culture was made in the normal saline to achieve turbidity equivalent to the McFarland Standard 5. One drop of the suspension was mixed with the Prolex™ *E. coli* O157 latex reagent on the card circle, provided with kit, using sterile mixing stick. The card was rocked and then observed for 2 min. for the appearance of agglutination. For further confirmation, mixture was also observed under low power microscope.

Antibiotic sensitivity profiling: To determine the best antibiotic to use against bacteria, isolated strains were tested for sensitivity to several antibiotics according to Hudzicki (2009). Muller-Hinton (MH) agar plates were prepared to test for antibiotic sensitivity. To guarantee the confluent development of the bacteria, plates were inoculated using a cotton swab after solidification. The antibiotic discs for "ampicillin (AM-10 µg), amikacin (AK-30 µg), amoxicillin (AX- 25 µg), cephalexin (CL-30 µg), ciprofloxacine (CIP-5 µg), chloramphenicol (C-30 µg), gentamicin (CN-30 µg), norfloxacin (NOR-10 µg), tobramycin (TOB-10 µg), tetracycline (TE-30 µg), streptomycin (S-10 µg), nalidixic acid (NA-30 µg) and nitrofurantoin (F-300 µg)" were applied aseptically to the surface of agar plate at well-spaced intervals. Afterward, plates were incubated for 24 to 48 h at 37°C. Afterward, the plates were observed to analyze the presence of clear zones. To assess the sensitivity pattern of the bacterial strains, the zones were measured and compared with a standard chart provided by the manufacturer.

Antimicrobial activity of *Streptomyces*: Antimicrobial activity of *Streptomyces* extracts from different strains i.e., "CTF 14, MMG 37, MMG 28, MMG 49, MMG 36, MMG 35, KS 32, KS 30, KS 2, KS 84, KS 14, SCF 25, KS 20, M.Sc 4 and KS 42" against isolated strains was measured by agar well diffusion method. L-agar plates were prepared and inoculated with bacterial strains using cotton swabs. Wells were cut with the help of Pasteur pipette and then 50 µl of each extract was poured in wells against each isolate. Plates were then incubated for 24 h at 37º C. After incubation, plates were observed for clear zones around bacterial growth.

The 16S rRNA sequencing of bacterial strains: Further identification of isolated bacterial strains was accomplished by sequencing 16S rRNA gene. Promega DNA isolation kit was used for genomic DNA isolation from all bacterial strains. The 1.5-kb fragment of 16S rRNA gene was amplified by using 27f forward (5' AGA GTT TGA TCC TGG CTC AG 3') and 1522r reverse primer (3' ACG CC(AG)A CCT AGT GGA GGA A 5') (Johnson and Whitman, 2007). For PCR amplification, Thermo Scientific Dream Taq ™ Green PCR Master Mix (Lot# 01025162) was utilized. The reaction mixture containing tubes were incubated in thermal cycler. Conditions observed during reaction were as follows: initial denaturation (94°C for 5 min.), denaturation (94°C for 1 min.), annealing (55°C for 30 sec, 30 cycles), extension (72 °C for 2 min.) followed by final elongation (5 min. at 72°C). The amplified PCR product was then purified using QIA quick gel elution kit. Later, the purified DNA samples were sent to Centre for Applied Molecular Biology (CAMB) DNA core facility, University of the Punjab, Lahore for sequencing. Sequences obtained from CAMB were then analyzed using Bioinformatics tool CHROMAS lite version 2.01 and National Center for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLAST) for identification of strain species.

Phylogenetic Analysis: Sequences of all strains were aligned with multiple sequence alignment programs by using MEGA 4.1 software (Tamura *et al.*, 2004). Neighbor-Joining method (NJ) was used to construct phylogenetic tree (Saitou and Nei, 1987).

RESULTS: Isolation and identification of *Enterobacteriaceae*: Inoculation of water samples on EMB and MacConkey media resulted in appearance of isolated colonies on the agar surface (figure 1). Then different colonies were selected and re-streaked on EMB agar for further purification. Gram staining of selected strains was performed to confirm microscopic features of all isolates. All the 20 purified strains "SAE-1, SAE-2, SAE-3, SAE-4, SB-3, SB-4, SBE-1, SBE-2, SBE-3, SBE-5, SC-1, SC-2, SCE-1, SDE-1, SCE-3, SCE-6, SD-1, SD-4, SD-6, and SCE-2" were Gram negative rods when observed under the microscope.

Biochemical characterization: All selected isolates i.e., "SAE-1, SAE-2, SAE-3, SAE-4, SB-3, SB-4, SBE-1, SBE-2, SBE-3, SBE-5, SC-1, SC-2, SCE-1, SDE-1, SCE-3, SCE-6, SD-1, SD-4, SD-6, SCE-2" recorded positive results for catalase. Bacterial strains "SB-3, SB-4, SC-1, SC-2, SD-4 and SD-6" showed positive test results for oxidase. Similarly, 9 strains recorded positive test for citrate utilization. For indole production, strains "SAE-1, SAE-2, SAE-3, SAE-4, SBE-1, SBE-2, SBE-3, SCE-1, SCE-2, SCE-3, SCE-6 and SD-6" recorded positive results that was indicated by the appearance of red-colored ring. In methyl red test, MRVP broth tubes showed a color change to pink or red that indicated the positive results for 14 bacterial isolates. Motility of all strains was checked using SIM agar. Most of the strains showed positive result for motility. TSI agar test was performed to differentiate among strains that can ferment carbohydrates which were added in the medium. Results were interpreted based on color change of the slant and butt of TSI agar inoculated test tube. If the slant is red and butt is yellow (R/Y), with or without gas production, it shows that only glucose fermentation has occurred. If slant is yellow and butt is yellow (Y/Y), with or without gas production, it indicates lactose and/or sucrose fermentation has occurred. Red slant and red butt (R/R) mean no fermentation has occurred. Strains "SAE-1, SAE-2, SAE-3, SAE-4, SBE-1, SBE-2, SBE-3, SBE-5, SCE-1, SCE-2, SCE-3, SCE-6, SD-1, SD-6 and SDE-1" showed Y/Y result with gas production while "SB-3, SB-4, SC-1, SC-2 and SD-4" showed R/R slant with no gas production. H_2S production was also checked by observing blackening of media. None of the strains produced black coloration means no H_2S production. All biochemical test results are summarized in table 1.

Detection of *E. coli* O157: Nutritional artificial media: Among 30 identified bacterial isolates, 11 were confirmed as *E. coli* strains after biochemical tests. Strains "SAE-1, SAE-2, SAE-3, SAE-4, SBE-1, SBE-2, SBE-3, SCE-1, SCE-3, SCE-6, and SCE-2" were streaked on Sorbitol MacConkey (SMA) agar to check the presence of O157 serotype. All strains showed pink colored colonies on SMA indicating their ability to ferment sorbitol present in the medium. None of the strains showed colorless colonies which indicated the absence of O157 serotype of *E. coli*.

Latex agglutination test: For further confirmation of *E. coli* strains for the presence of serotype O157, Prolex™ latex agglutination kit was used. The bacterial suspension for each *E. coli* strain was mixed with Prolex™ *E. coli* O157 latex reagent and observed for presence of agglutination. None of the *E. coli* strains showed agglutination which is a proof for absence of O157 serotype and negative result (figure 1). Microscopic examination was also conducted for positive and negative control and for latex agglutination negative strains (figure 1).

Antibiotic susceptibility profiling: Antibiotic susceptibility pattern of selected strains was analyzed by disc diffusion method. Strains "SAE-1, SAE-2, SAE-3, SAE-4, SB-3, SB-4, SBE-1, SBE-2, SBE-3, SBE-5, SC-1, SC-2, SCE-1, SDE-1, SCE-3, SCE-6, SD-1, SD-4, SD-6 and SCE-2" were tested for their sensitivity against 13 different antibiotics of different concentrations and zones around colonies were measured (figure 1). Amikacin (30 µg) showed highest activity against all the bacterial isolates and showed an inhibition zone size of 18-24 mm. Next to amikacin, highest activity was shown by "ciprofloxacin (5 µg), gentamicin (10 µg) and norfloxacin (10 µg)". Except for one strain, all strains were sensitive to these antibiotics. "Ampicillin (10 µg), amoxicillin (25 µg) and cephalexin (30 µg)" showed no promising activity against the isolates. None of the bacterial strains were completely sensitive or resistant to all the antibiotics. Strain SCE-1 showed highest level of resistance against the tested antibiotics. It was resistant to 10 antibiotics including "ampicillin (10 µg), amoxicillin (25 µg), amoxicillin (25 µg), cephalexin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg),

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	Biochemical Tests							
Strain	**Catalase**	**Oxidase**	**Citrate**	**Indole**	**MR**	**Motility**	**TSI**	**H_2S**
SAE-1	+	_	_	+	+	+	Y/YG	_
SAE-2	+	_	_	+	+	+	Y/YG	_
SAE-3	+	_	_	+	+	+	Y/YG	_
SAE-4	+	_	_	+	+	+	Y/YG	_
SB-3	+	+	+	_	_	+	R/R	_
SB-4	+	+	+	_	_	+	R/R	_
SBE-1	+	_	_	+	+	+	Y/YG	_
SBE-2	+	_	_	+	+	+	Y/YG	_
SBE-3	+	_	_	+	+	+	Y/YG	_
SBE-5	+	+	+	_	+	_	Y/YG	_
SC-1	+	+	+	_	_	+	R/R	_
SC-2	+	+	+	_	_	+	R/R	_
SCE-1	+	_	_	+	+	+	Y/YG	_
SCE-2	+	_	_	+	+	+	Y/YG	_
SCE-3	+	_	_	+	+	+	Y/YG	_
SCE-6	+	_	_	+	+	+	Y/YG	_
SD-1	+	_	+	_	+	_	Y/YG	_
SD-4	+	+	+	_	_	+	R/R	_
SD-6	+	+	+	+	_	+	R/R	_
SDE-1	+	_	+	_	+	+	Y/Y	_

Table 1: Biochemical characterization of different bacterial isolates.
+ = Positive test**, - =** Negative test**,** R/Y= Slant red and butt yellow (glucose fermentation occurred), Y/Y= Slant yellow and butt yellow (lactose and/or sucrose fermentation occurred), R/R= Red slant and red butt (no fermentation) and G= Gas production.

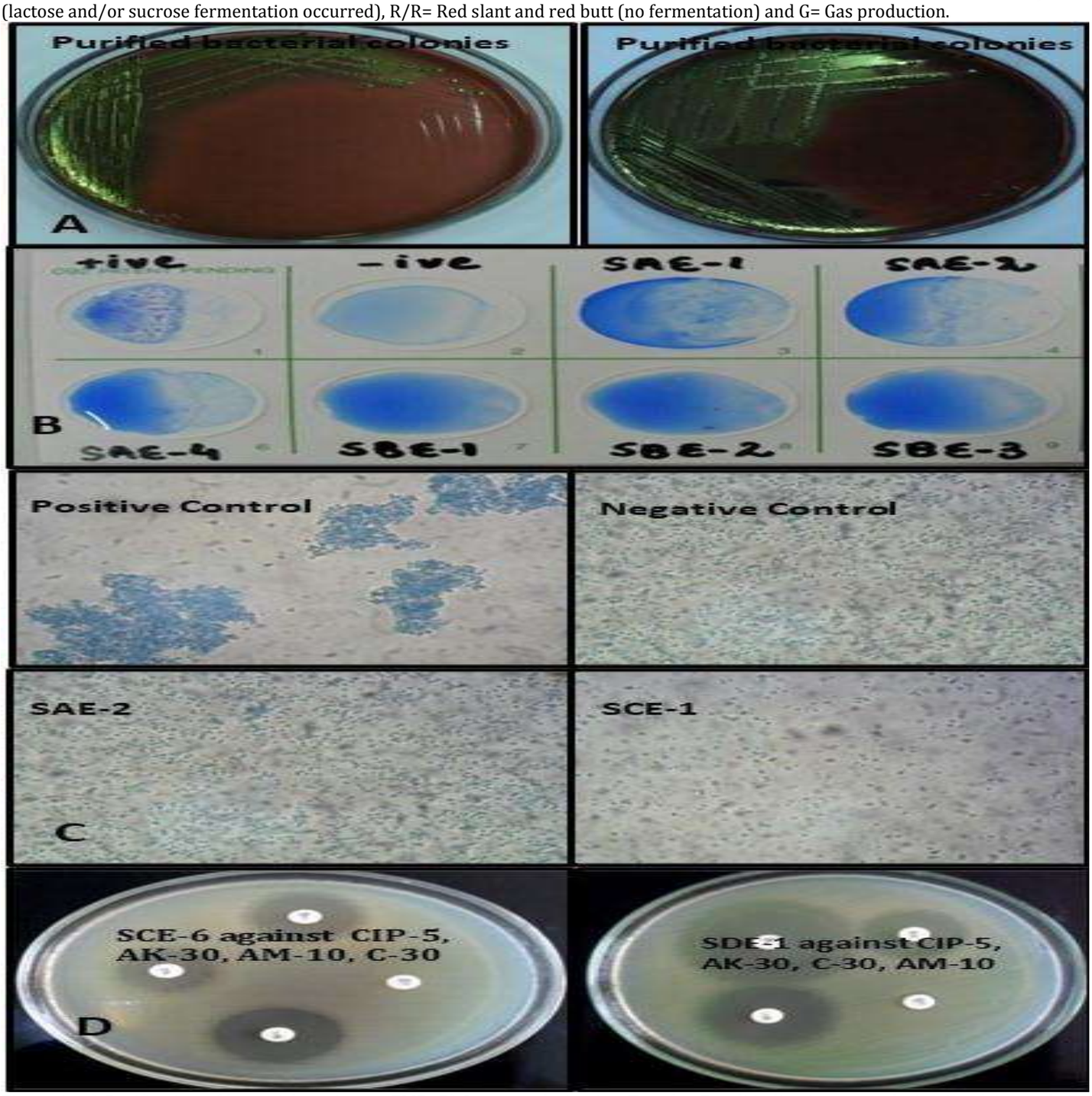

Figure 1: A) Purification of bacterial strains on EMB, B) Latex Agglutination Test for *E. coli* indicating no agglutination (+ive and -ive showing positive and negative control respectively, C) Microscopy of Agglutination Reaction and D) Antibiotic susceptibility Pattern of bacterial isolates.

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gentamicin (10 µg), norfloxacin (10 µg), tobramycin (10 µg), tetracycline (30 µg), and chloramphenicol (30 µg)". SD-6 showed highest sensitivity against all antibiotics except "ampicillin (10 µg) and amoxicillin (25 µg)". On average, *E. coli* isolates showed more resistance against antibiotics as compared to all other strains. Antibiotics resistance profile for all strains is shown in figure 2.

Antimicrobial activity of *Streptomyces* strains: The strains exhibiting the more resistance against tested antibiotics were evaluated against antimicrobial activity of 15 *Streptomyces* extracts i.e., "CTF 14, MMG 37, MMG 28, MMG 49, MMG 36, MMG 35, KS 32, KS 30, KS 2, KS 84, KS 14, KS 42, KS 20, M.Sc 4 and SCF 25". A total number of 12 bacterial strains SAE-3, SB-3, SB-4, SBE-2, SBE-5, SC-2, SCE-1, SCE-2, SCE-6, SDE-1, SD-1, SD-6 were used for this assay. Maximum strains were exhibiting the resistance against *Streptomyces* extracts. The SBE-2 were found resistance against all extracts, while SD-6 were exhibiting the minute sensitivity against all *Streptomyces* (table 2).

Bacterial Strains	Antibiotics Resistance Profile												
	AK	AM	AX	CL	CIP	C	CN	NOR	TOB	TE	S	NA	F
SAE-1													
SAE-2													
SAE-3													
SAE-4													
SB-3													
SB-4													
SBE-1													
SBE-2													
SBE-3													
SBE-5													
SC-1													
SC-2													
SCE-1													
SCE-2													
SCE-3													
SCE-6													
SD-1													
SD-4													
SD-6													
SDE-1													

Figure 2: Antibiotic susceptibility profiles of bacterial strains.
AK=Amikacin, AM= Ampicillin, AX= Amoxicillin, CL=Cephalexin, CIP= Ciprofloxacin, C= Chloramphenicol, CN= Gentamicin, NOR= Norfloxacin, TOB= Tobramycin, TE= Tetracycline, S= Streptomycin, NA= Nalidixic acid, F= Nitrofurantoin", Green= Susceptible, Yellow= Intermediate, Red= Resistant.

Strain	Streptomyces Extract														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
	Zone of inhibition (mm)														
SAE-3	15	R	14	15	25	R	R	12	R	R	R	R	R	15	R
SB-3	16	15	13	R	18	R	R	R	17	14	11	R	R	R	R
SB-4	13	11	13	12	18	13	12	R	13	R	R	13	13	12	11
SBE-2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SBE-5	13	12	14	12	19	R	R	12	14	11	12	R	R	13	R
SC-2	R	R	R	R	16	R	R	R	R	R	R	R	R	R	R
SCE-1	R	R	R	R	14	R	R	R	R	R	R	12	R	R	R
SCE-2	R	R	14	R	15	R	R	R	R	R	R	R	R	R	R
SCE-6	R	R	R	R	16	R	R	13	12	R	R	R	R	R	R
SD-1	R	12	R	R	17	R	R	R	16	R	R	R	R	R	R
SD-6	11	11	11	10	19	9	11	11	13	11	11	12	10	9	10
SDE-1	R	R	R	R	R	R	R	R	R	R	R	R	15	R	R

Table 2: Antimicrobial activity measurement of Streptomyces extracts against isolates.
R= Resistant, A= CTF 14, B= MMG 37, C= MMG 28, D= MMG 49, E= MMG 36, F= MMG 35, G= KS 32, H= KS 30, I= KS 2, J= KS 84, K= KS 14, L= KS 42, M= KS 20, N= M.Sc 4, O= SCF 25.

The 16S rRNA gene sequencing: Around 30 bacterial strains that showed good concentrations of purified DNA were sent to Centre of Advanced and Molecular Biology (CAMB) for sequencing. Bacterial strains" SAE-1, SAE-2, SAE-3, SAE-4, SBE-1, SBE-2, SBE-3, SCE-1, SCE-2 and SCE-3 and SCE-4" showed 99% homology with *Escherichia coli.* Strains SA-3 and SC-4 showed 99% and 98% homology with *Acinetobacter soli* and *Ac. junii* respectively. Whereas "SB-1, SB-2, SB-3 and SB-4" showed homology with *Pseudomonas aeruginosa.* Similarly, SB-5 showed similarity with *P. otitidis.* On the other hand, SC-1, SC-2 and SD-4 showed 99% homology with *P. putida* and SE-1 recorded 97% homology with *P. alcaligenes*. Strains SCE-4, SD-6 and SDE-4 showed homology with *Aeromonas hydrophila.* Bacterial strains SD-3 and SDE-1 manifested homology with *Vogesella mureinivorans* and *Enterobacter cloacae,* respectively. A strain SBE-5 showed homology with *Klebsiella oxytoca* whereas SD-1 and SD-2 with *K. pneumoniae* (table 3). Analysis showed that the locality near Doctor's hospital underpass recorded the highest diversity of 6 bacterial strains in water samples. Similarly, water samples collected from Jail road and Mughalpura locality also recorded the presence of 4 different species of bacterial strains.

Phylogenetic analysis: The MEGA 11 software was used to assess the phylogenetic relationships among the bacterial strains (figure 3). In this phylogenetic tree, different clusters were formed which represent different groups of closely related strains. Overall, three major clusters were formed in this phylogenetic tree. The upper cluster showed all strains that belong to *E. coli*. Middle cluster assembled the strains of *Enterobacter*, *Klebsiella* and *Aeromonas.* Whereas the cluster formed at bottom showed close relation between *Vogesella*, *Pseudomonas*, and *Acinetobacter.*

DISCUSSION: In the present study, bacterial diversity associated with the freshwater canal of Lahore, Pakistan was reported by a combination of selective media and 16S rRNA gene sequencing. Overall, 50 isolates with variable morphological characteristics were selected for further screening. Finally, 16S rRNA gene sequencing confirmed the taxonomic status of 30 bacterial strains. The results of sequence analysis depict that bacterial strains belong to *Escherichia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Aeromonas*, *Acinetobacter* and *Vogesella* bacterial genera. This is the first detailed report of bacterial diversity associated with freshwater canal of Lahore, Pakistan. Batrich *et al.* (2019) has isolated *Pseudomonas* diversity including *P. aeruginosa* and *P. putida* from

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Strain	Location	Identification	GenBank Accessions Numbers
SA-3	Khairapul	Acinetobacter Soli	KF994928
SAE-1	Khairapul	Escherichia coli	KF994929
SAE-2	Khairapul	E. coli	KF994930
SAE-3	Khairapul	E. coli	KF994931
SAE-4	Khairapul	E. coli	KF994932
SB-1	Mughalpura	Pseudomonas aeruginosa	KF994933
SB-2	Mughalpura	P. aeruginosa	KF994934
SB-3	Mughalpura	P. aeruginosa	KF994935
SB-4	Mughalpura	P. aeruginosa	KF994936
SB-5	Mughalpura	P. otitidis	KF994937
SBE-1	Mughalpura	E. coli	KF994938
SBE-2	Mughalpura	E. coli	KF994939
SBE-3	Mughalpura	E. coli	KF994940
SBE-5	Mughalpura	Klebsiella oxytoca	KF994941
SC-1	Jail Road	P. putida	KF994942
SC-2	Jail Road	P. putida	KF994943
SC-4	Jail Road	A. junii	KF994944
SCE-1	Jail Road	E. coli	KF994945
SCE-2	Jail Road	E. coli	KF994946
SCE-3	Jail Road	E. coli	KF994947
SCE-4	Jail Road	Aeromonas hydrophila	KF994948
SCE-6	Jail Road	E. coli	KF994949
SD-1	Doctors Hospital U.pass	K. pneumoniae	KF994950
SD-2	Doctors Hospital U.pass	K. pneumoniae	KF994951
SD-3	Doctors Hospital U.pass	Vogesella mureinivorans	KF994952
SD-4	Doctors Hospital U.pass	P. putida	KF994953
SD-6	Doctors Hospital U.pass	A. hydrophila	KF994954
SDE-1	Doctors Hospital U.pass	Enterobacter cloacae	KF994955
SDE-3	Doctors Hospital U.pass	A. hydrophila	KF994956
SE-1	Thokar Niaz Baig	P. alcaligenes	KF994957

Table 3: The 16S rRNA gene sequencing of bacterial strains isolates from Lahore canal, Pakistan.

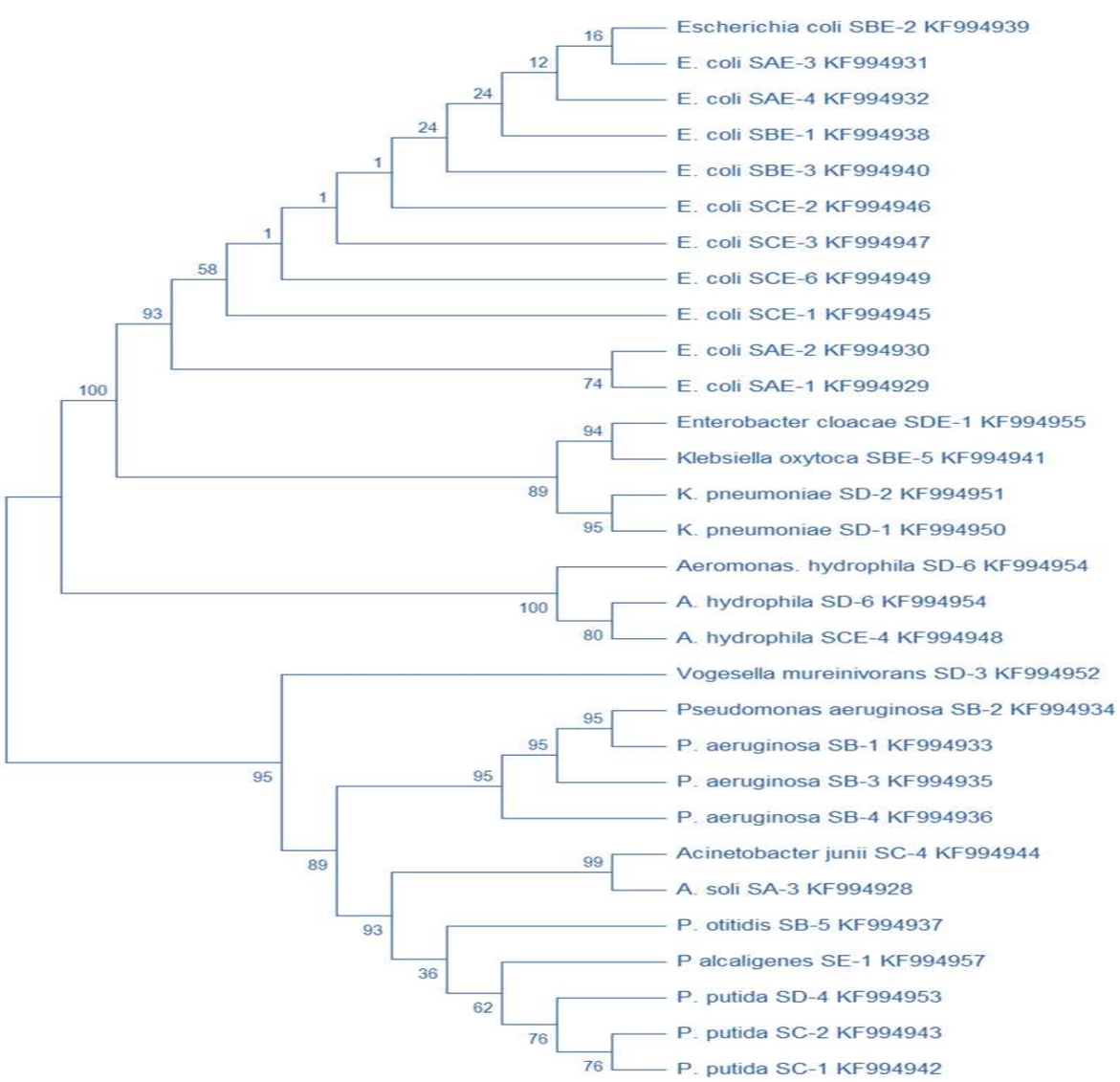

Figure 3. Phylogenetic analysis of 30 local bacterial strains isolated from freshwater canal of Lahore, Pakistan.

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Chicago nearshore waters of Lake Michigan. It was confirmed that freshwater environment act as a reservoir of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae especially; *E. coli* and *K. pnuemoniae* (Cho *et al.*, 2023).

To identify isolates, biochemical characterization was done, and different tests were performed like catalase test, oxidase test, indole test, methyl red test, citrate test, motility test and TSI agar test. Bacterial strains "SAE-1, SAE-2, SAE-3, SAE-4, SBE-1, SBE-2, SBE-3, SCE-1, SCE-2, SCE-3, SCE-6" showed positive results for catalase, indole, MR and motility test while negative for H_2S, oxidase and citrate, and potentially identified as *E. coli.* Some strains were negative for MR while positive for citrate test and were identified as Klebsiella species. Kalasseril *et al.* (2020) also reported similar biochemicals tests results for *E. coli, K. pneumonia, K. oxytoca* and *A. baumannii* isolated from water bodies adjacent to hospitals in India. In this study, around 11 bacterial strains were identified as *E. coli.* Initially, *E. coli* strains were screened on Sorbitol MacConkey agar (SMA) that recorded negative result on this culture media by showing pink color growth. Overall, none of the *E. coli* strains showed colorless colonies because they fermented sorbitol that is the major feature of non O157 serotypes. March and Ratnam (1989). also used Sorbitol MacConkey agar for the detection of *E. coli* O157 isolates that were involved in hemorrhagic colitis. Darkazanli *et al.* (2019) also confirmed the presence of *E. coli* O157 by using Sorbitol MacConkey agar while identifying the *E. coli* O157: H7 genetic diversity isolated from Aleppo River water samples. Further detection and confirmation of this serotype was accomplished by using Prolex™ E. coli O157 Latex Kit. Latex agglutination test for the detection of serotype O157: H7 is rapid and more sensitive (March and Ratnam, 1989). During screening, none of the isolates recorded positive result for agglutination (figure 1). (Osuolale, 2023)checked the presence of O157 by using latex agglutination reaction and got the negative results. Previously, we also reported that non-sorbitol fermenters strains isolated from portable water samples recorded positive results for O157 by showing positive agglutination test (Zareen *et al.*, 2014).

Antibiotic sensitivity testing of selected isolates "*E. coli* SAE-1, *E. coli* SAE-2, *E. coli* SAE-3, *E. coli* SAE-4, *P. aeruginosa* SB-3, *P. aeruginosa* SB-4, *E. coli* SBE-1, *E. coli* SBE-2, *E. coli* SBE-3, *K. oxytoca* SBE-5, *E. coli* SCE-1, *E. coli* SCE-2, *E. coli* SCE-3, *E. coli* SCE-6, *P. putida* SC-1, *P. putida* SC-2, *K. pneumoniae* SD-1, *P. putida* SD-4, *A. hydrophila* SD-6 and *E. cloacae* SDE-1" was done by disc diffusion method. Antibiotic Amikacin (30 µg) showed highest activity against all stains. All strains were sensitive to this antibiotic. Next highest activity was shown by "Ciprofloxacin (5 µg), Gentamicin (10 µg) and Norfloxacin (10 µg)". All strains except one were sensitive against these antibiotics. And the interesting point is that the strain that showed resistance to these antibiotics is same for all three antibiotics, that is *E. coli* SCE-1. And the third highest antibacterial activity was shown by Chloramphenicol (30 µg). Ampicillin (10 µg) and Cephalexin (30 µg) showed very less activity against isolates. Most of the bacterial isolates were resistant to these antibiotics. The strain that showed highest resistance against most of the antibiotics was *E. coli* SCE-1. Américo-Pinheiro *et al.* (2021) studied antibiotic susceptibility pattern of *E. coli* isolated from surface water of urban park in southeastern Brazil and found that isolates were more resistant to erythromycin, amoxicillin and tetracycline while the isolates showed sensitivity against ciprofloxacin, levofloxacin and norfloxacin. (Bleichenbacher *et al.*, 2020) also assessed the antimicrobial-susceptibility of Enterobacteriaceae from freshwaters of Switzerland and found *Escherichia* and *Klebsiella* strains to be resistant to ampicillin, amoxicillin, gentamycin, and chloramphenicol. Antimicrobial activity of 15 Streptomyces extracts against 12 bacterial isolates was also evaluated. The screening results showed that most bacterial strains recorded resistance against Streptomyces extracts. For instance, strain *E. coli* SBE-2 and *E cloacae* SDE-1 were resistant to all *Streptomyces* extracts. Rammali *et al.* (2022) has similar findings of moderate to strong the antimicrobial potential of *Streptomyces sp*. against *E. coli*, *Staphyloccus aureus*, *Bacillus. cereus* and *Candida albicans*. It has been evaluated that isolates tested were more active against Gram-positive bacteria as compared to Gram-negative bacteria (*E. coli*).

CONCLUSION: It can be concluded that canal water contained members of *Enterobacteriaceae* family like *E. coli* and *Klebsiella* which are serious threats to human health. In addition, it has also indicated the presence of different bacterial genera like *Escherichia, Klebsiella, Enterobacter, Vogesella, Pseudomonas, Acinetobacter* and *Aeromonas*. However, for *E. coli*, none of the strain gave positive indication for O157 antigen as confirmed by Prolex™ Latex agglutination test. Nevertheless, presence of *E. coli* indicated the contamination of canal water by animal and human originated pollutants. It can be speculated that in addition to *E. coli* other pathogenic microbes may also be present in canal water that make it unfit for human and agricultural purposes.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

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