Acta Scientific Microbiology (ISSN: 2581-3226)

Volume 9 Issue 9 September 2026

Research Article

Unveiling the Pathogen Load and their Multi Drug Resistance in Street-Vended Coconut Water in Kolkata, India: A Pioneering Insight

Souvik Roy[1]*, Salangkrita Roy[2], Sritama Pramanik[2], Srabonti Chattopadhyay[2] and Lopamudra Choudhury[3]

[1]Assistant Professor, Post-Graduate and Research Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata-700 016, West Bengal, India

[2]5th Year Integrated M. Sc. (5 Year Integrated) students, Post-Graduate and Research Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata-700 016, West Bengal, India

[3]State-Aided College Teacher, Department of Microbiology, Sarsuna College (under Calcutta University), 4/HB/A, Ho-Chi-Minh Sarani, Sarsuna Upanagari, Kolkata – 700061, West Bengal, India

***Corresponding Author:** Souvik Roy, Assistant Professor, Post-Graduate and Research Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata-700 016, West Bengal, India.

DOI: 10.31080/ASMI.2026.09.1623

Received: July 01, 2026

Published: August 13, 2026

Abstract

Coconut water is an electrolyte-rich, nutritious natural beverage that is usually considered sterile within the intact exocarp and mesocarp of green coconuts (*Cocos nucifera*). This study, as per the best of knowledge, for the first time, provides a comprehensive insight of the pathogen load in coconut water vended from the streets of Kolkata, India, and their antibiogram profile. Fresh coconut water samples were collected from three randomly-chosen, different geographical locations in and around Kolkata (Central Kolkata, and its northern and southern outskirts), and the aliquots obtained from each were refrigerated for 14 days. Both the fresh and refrigerated sub-samples were then subjected to routine microbiological and biochemical analyses for isolation, identification and confirmation of both bacterial and fungal pathogens. The presence of bacterial (both mesophilic and psychrotrophic) and fungal contaminants were observed in both the fresh and refrigerated coconut water aliquots, the number of proliferating pathogenic mesophilic bacteria outnumbering their fungal counterparts. This study also challenged the idea of safe food preservation by refrigeration, as psychrotrophic bacterial contaminants proliferated profusely in the refrigerated samples with an increased pH of 6.9–7.1 (near-neutral), vastly outnumbering their growth in the fresh samples with a pH of 5.3–5.6 (acidic). A significant coliform count was obtained across all samples tested, with Most Probable Number (MPN) indices being ≥1100 in almost all cases, and subsequent tests confirmed fecal coliform contamination with *E. coli* and their thermotolerant counterparts due to inappropriate hygienic-sanitary conditions. Besides, the presence of notorious foodborne bacteria like Staphylococci*, Shigella, Pseudomonas* and *Bacillus*, and fungi like *Aspergillus*, *Rhizopus* and *Mucor* were confirmed in this study, adding much to the public health concern. The antibiotic susceptibility test of the pathogenic bacteria depicted a mammoth degree of multi-drug resistance, with majority exhibiting 100% resistance to even the newer-generation combinatorial drug Amoxicillin–Clavulanic acid. With a super-swift escalation in antibiotic-resistant pathogens worldwide, these antibiogram analyses thus point towards a potential health hazard. All these key findings signify that the safety of consumption of street-vended coconut water in and around the city of Kolkata has been severely compromised, necessitating immediate authorized intervention to address the casual post-harvest extraction and manual handling, besides improper storage and vending by itinerant roadside sellers.

Keywords: Antibiotic Susceptibility Test; Foodborne Pathogens; Fresh and Refrigerated Sub-Samples; Multi-Drug Resistance; Public Health Concern, Street-Vended Coconut Water

Introduction

Coconut water, a clear, light, and sweet liquid from young green coconuts (*Cocos nucifera*), is a highly popular and refreshing juice in the hot and humid tropical regions of the world, like India, Brazil, South-East Asia and the Carribean, owing to its enormous nutritional benefits, high electrolyte content like potassium, sodium and magnesium, presence of plethora of antioxidants, low calorific value, and superior natural and isotonic hydration abilities [1].

In intact coconuts, the free-nuclear endosperm is naturally sterile due to the presence of the impenetrable outer shell of the fruit [2]. However, due to its high water activity (a_w) and a rich nutrient content in the right kind and proportion required for an effective establishment of a chemoorganotrophic microbial flora, the food-industry recognizes extracted coconut water as a 'perishable food'. Hence, during or after its extraction from the tender coconut, the liquid is highly susceptible to microbial contamination, causing spoilage and posing a potential risk to public health [3]. Besides unhygienic practices during the harvesting, handling, and vending of the fruit by infected roadside vendors, and handling of cut fruit and money by the same person and without intermittent hand washing, the other possible sources of contamination include abiotic stresses like environmental agricultural toxins, repeated post-harvest exposure to dust and soil during transfer and storage, usage of non-sterile cutting tools, and untreated wash water [4]. Therefore, the normal flora microorganisms, as also the contaminating pathogens of the coconuts, vie for the rich nutrients in their endospermic liquids, multiply and predominate there. Bacterial and fungal contamination profiles in coconut water thus serves as an indicator of the sanitary quality in the post-harvest transfer and storage of coconuts, and extraction of the liquid juice.

In hot and humid urban and semi-urban regions like Kolkata and its adjoining areas, drinking raw coconut water is a popular summer delicacy, particularly for the exhausted people out in the sun for long. Unfortunately, changing environmental and handling conditions may influence microbial load in natural beverages, with variations in vendor practices, locality, and surrounding environmental factors contributing to variations in microbial contamination levels. Thus, a number of severe health hazards like stomach upset, abdominal cramps, nausea, vomiting, fever, diarrhea, and dehydration may result from the consumption of these street-vended contaminated coconut water [5].

However, till date, there is no publicized government-initiated study on the hygiene status of street-vended coconut water in Kolkata till, no investigative reports are available on the contamination profile of local street-vended coconut water by the highly notorious food-borne pathogens of public-health significance, like *Staphylococcus, Shigella,* coliforms, including fecal coliforms like *Escherichia coli*, and *Bacillus,* and food scientists have only evaluated the microbial hygienic status of similar fresh beverages. Also, a regular monitoring of the coconut water is necessary due to periodically-changing climatic conditions and hygiene-consciousness in different parts of the city and its surroundings over the years, which may have, in the meantime, altered its usual contamination profile. Besides, as new antimicrobial resistance (AMR) mechanisms are emerging and spreading globally at an alarming rate among the food-borne pathogens in particular. Review of scientific literature is currently presenting reports of around 700,000 people around the world dying annually from antibiotic-resistant infections and, hinting towards a death of around 10 million people per year by 2050 [6]. Hence, whether any such new antibiotic-resistant pathogens infest the contaminated coconut water samples also needs to be immediately checked, and taken care of, as a top priority by the food scientists.

So, to address all these lacunae in peer-reviewed literature, the present study, for the first time, aims to analyze the microbial content of not only fresh and raw coconut water samples collected from three different geographically-distant locations in and around the city of Kolkata, but also of their refrigerated aliquots. By performing the qualitative and quantitative analysis of the microbes, including studying their antibiogram, this novel work seeks to provide an assessment of the hygienic quality of the coconut water, the risks posed by the antibiotic-resistant strains of the harmful food-borne pathogens, if any, to public health, and the importance of proper sanitary practices in storage and handling of coconuts to minimize public health risks.

Materials and Methods

Sample collection

3 coconut water samples, one each from three randomly-chosen different geographical locations in and around Kolkata, namely Sample 1 (from Rajpur, southern outskirts of Kolkata, 22.4382°N/88.4320°E), Sample 2 (from Hazra More, Central Kolkata, 22.5237°N/88.3406°E) and Sample 3 (from Sodepur, northern outskirts of Kolkata, 22.6982°N/88.3895°E), were collected in sterile containers (to represent their actual microbial load), in June 2025, between 9 a.m. and 10 a.m. (Figure 1), and within 2 hours of collection, transported in isothermal boxes containing ice cubes prepared with distilled water, to the laboratory, for analyses [7,8].

Water from freshly-harvested coconut from semi-urban or rural areas as a negative control could not be included in the study, given the urban location of the laboratory that would have required long transportation hours, and hence, compromising with its native sterility.

Sample preparation

From each of the 3 samples, 2 sub-sample aliquots were prepared, making into a total of 6 sub-samples (Figure 1). Out of these, the 3 sub-samples labelled as S1 (Fresh), S2 (Fresh) and S3 (Fresh) (Figure 1), were subjected to immediate physicochemical (colour and pH) and microbiological (mesophilic, and psychrotrophic microorganisms) analyses [8]. For the latter analysis, for each sub-sample, a 10^{-1} working dilution was prepared in 0.1% sterile peptone water (PW) by mixing 2.5 ml sample in 22.5 ml 0.1% of the diluent [8].

On the other hand, 3 sub-samples, labelled as S1 (Ref), S2 (Ref) and S3 (Ref), were stored for 14 days in the refrigerator (Figure 1), prior to which the refrigerators were sanitized completely using 99.9% ethanol (EMSURE® 100983) and acetone (10 mg/ml) (HIMEDIA MB179) [7]. Following refrigeration and routine physicochemical analysis as before, further dilution to a 10^{-5} working solution with 0.1% sterile PW was performed for all the 3 sub-samples before the microbiological analysis (Figure 1).

Figure 1: Coconut Water samples and sub-samples: (a-c) Sample collection; (d-f) Fresh Coconut Water sub-samples: (d) S1 (Fresh) (e) S2 (Fresh) (f) S3 (Fresh); (g-i) Refrigerated Coconut Water sub-samples: (g) S1 (Ref) (h) S2 (Ref) (i) S3 (Ref).

Physicochemical analyses

Physicochemical analyses were performed with three replicates per sample, and the results were expressed in means [8].

Color

The color of each of the fresh and refrigerated sub-samples was visually observed (Table 1) [8].

Sub-sample	Color	pH
S1 (Fresh)	Translucent	5.3
S1 (Ref)	Whitish (+++)	7.0
S2 (Fresh)	Translucent	4.9
S2 (Ref)	Whitish (+)	6.9
S3 (Fresh)	Translucent	5.6
S3 (Ref)	Whitish (++)	7.1

Table 1: Physicochemical characteristics of fresh and refrigerated (14 days) coconut water sub-samples.

+: indicates the intensity of whitish colour.

pH

The pH of each of the fresh and refrigerated sub-samples was determined using a previously calibrated portable digital potentiometer (Table 1).

Total heterotrophic count (THC)

THC of bacteria

The conventional 'Spread plate' method was used to determine the total heterotrophic count (THC) of bacteria. For each sample, 100 µl aliquot from the suitable dilution (10^{-1} from each fresh sample, and 10^{-5} from each refrigerated sample) were evenly distributed on solidified nutrient agar (NA) (HIMEDIA M001) plates, and incubated overnight at 37°C (for mesophilic bacteria), and for 7 days in the refrigerator at 7°C (for psychrotrophic bacteria), following which the THC of bacteria of each group was estimated in CFU/ml [7].

THC of fungi

As for bacteria, the conventional 'Spread plate' method was used to determine the THC of fungi. For each sample, 100 µl aliquot from the suitable dilution (10^{-1} from each fresh sample, and 10^{-5} from each refrigerated sample) were evenly distributed on solidified Yeast Extract Peptone Dextrose agar (YPDA) (HIMEDIA M757) plates containing L-asparagine (2% w/v) (HIMEDIA TC057), amoxicillin (60 µg/ml) (SIGMA – ALDRICH A8523), and tetracycline (20

µg/ml) (SIGMA - ALDRICH T8032), and incubated for 5 days at 28°C, following which the THC was estimated in CFU/ml [7].

Isolation of bacterial pathogens

Detection and isolation of total coliforms, fecal coliforms and thermotolerant coliforms

Presumptive test and MPN index determination

To detect total coliform bacteria by the standard Presumptive test (3x3 MPN Technique), for each undiluted sub-sample, 1 tube containing 10 ml sub-sample, 10 ml 2X Lactose Broth (LB) (HIMEDIA M1003S) and Durham's Tube, and 2 tubes, one containing 1 ml sub-sample, 9 ml 1X LB and Durham's Tube, and another containing 0.1 ml sub-sample, 9.9 ml 1X LB and Durham's Tube, were incubated in a rotary shaker overnight at 37°C in order to check for turbidity, acid (pH), and gas production (%), from which the MPN count/100 ml (MPN Index) was ascertained [9].

Confirmed test

For each undiluted sub-sample, from each 2X 10 ml LB tube that tested positive, a loopful of the growth was streaked onto solidified selective and differential Eosin Methylene Blue (EMB) (HIMEDIA MAP022) agar plates, and incubated overnight at 37^0C, following which the cultural characteristics (colony color and morphology) were checked for [9].

Completed test

For each undiluted sub-sample, 200 µl of culture (2%) from the same 2X 10 ml LB tube (as used in Confirmed test) was inoculated into a tube containing 10 ml 2X *Escherichia coli* (EC) and Durham's tube, and incubated for 48 hours at 45°C for verifying the growth of thermotolerant coliforms, following which turbidity, and acid and gas production were noted down [9].

Isolation of *Staphylococcus* species

100 µl (2%) of the suitable dilution (10^{-1} from each fresh sample, and 10^{-5} from each refrigerated sample) was added to 5 ml of the Tryptone Soya Broth (TSB) (HIMEDIA LQ009A) supplemented with 6.5% sodium chloride (HIMEDIA MB023), and incubated in a rotary shaker overnight at 37^0C. Next day, one loopful of all cultures was streaked onto selective Baird Parker Agar (BPA) (HIMEDIA M1736) plates supplemented with egg yolk–tellurite (HIMEDIA FD046), and incubated overnight at 37^0C, following which the typical pathogen colonies with halo and without halo were noted, and the CFU/ml was determined in each case [10,11]. Subsequently, microscopy and biochemical analyses were performed to observe cellular morphology and to presumptively identify the *Staphylococcus* species, respectively. Species-level confirmation by ribotyping could not be performed due to paucity of time.

Isolation of *Salmonella/Shigella*/other Enterobacteria

100 µl (2%) of the suitable dilution (10^{-1} from each fresh sample, and 10^{-5} from each refrigerated sample) was added to 5 ml of the pre-enrichment Selenite-Cystine Broth (HIMEDIA M025), and incubated in a rotary shaker overnight at 37^0C. Next day, one loopful of all cultures was streaked onto selective Hektoen Enteric Agar (HEA) (HIMEDIA M467) plates, and incubated overnight at 37^0C, following which the typical pathogen colonies were noted, and the CFU/ml was determined in each case [10,11]. Subsequently, microscopy and biochemical analyses were performed to observe cellular morphology and to presumptively identify the genera, respectively. Species-level identification and confirmation by ribotyping could not be performed due to paucity of time.

Isolation of *Bacillus*

100 µl (2%) of the suitable dilution (10^{-1} from each fresh sample, and 10^{-5} from each refrigerated sample) was added to 5 ml of Glucose Free Nutrient Broth (GFNB) (NB prepared without dextrose), and incubated in a rotary shaker for 15 days at 37^0C, following which Gram staining and Endospore staining (by Dorner's Nigrosine method) were performed according to the standard protocols, along with biochemical analyses, to observe cellular and endospore morphology and to presumptively identify the genera, respectively [12]. Species-level identification and confirmation by ribotyping could not be performed due to paucity of time.

Isolation of Fungal pathogens

To isolate fungal pathogens, 11 selective colonies from YPDA plates were sub-cultured in pure-form onto YPDA slants, and incubated for 72 hours at 30°C [12].

Characterization, identification and confirmation of pathogens

Characterization, identification and confirmation of bacterial pathogens

Microscopy (Gram staining and endospore staining)

Gram staining (reagents procured from HIMEDIA) was performed according to the standard protocol, and Gram character and morphology of the cells of the pathogenic bacterial isolates confirmed microscopically [9]. The presence of endospores in the

GFNB isolates was confirmed microscopically after staining by standard Dorner's Nigrosin (HIMEDIA S025) Method [9].

Biochemical tests

The bacterial pathogenic isolates were identified by a range of standard biochemical tests, including starch hydrolysis (SH), urea hydrolysis (UH) and gelatin hydrolysis (GH), nitrate reduction (NR), catalase production (CP), coagulase production (CoP), oxidase production (OP), carbon source (dextrose, sucrose and lactose) utilization [growth in Triple Sugar Iron (TSI) agar] and H_2S production (in TSI agar), and Indole-Methyl RedVoges Proskauer-Citrate (IMViC) tests [9-11,13-18]. All the media were prepared according to the composition prescribed by HIMEDIA.

Characterization, identification and confirmation of fungal pathogens

Lactophenol Cotton Blue (LPCB) (HIMEDIA S016) staining of the selected cultures grown on YPDA slants was performed to microscopically characterize, identify and confirm the pathogenic fungal genera based on their morphological details (hyphal structure and reproductive spores).

Determination of antibiotic resistance in bacteria by antibiotic susceptibility test (AST) and antibiogram analyses

To determine the antibiotic resistance profiles of the pathogenic bacterial isolates, antibiotic susceptibility testing (AST) was performed using the standard Kirby-Bauer Disc Diffusion method [19]. Six antibiotics were selected, including both newer generation and conventional ones, to provide a comprehensive resistance profile. The commercially available antibiotic-impregnated discs used were Ciprofloxacin (CIP, 5 µg/ disc) (HIMEDIA SD060), Amoxicillin (AMX, 30 µg/disc) (HIMEDIA MD002), Tetracycline (TE, 30 µg/ disc) (HIMEDIA MD056), Streptomycin (S, 25 µg/disc) (HIMEDIA MD048), Vancomycin (VA, 30 µg/disc) (HIMEDIA MD060) and Amoxicillin–Clavulanic acid (AMC, 30 µg/disc) (HIMEDIA SD063). For each pathogenic isolate, a fresh bacterial culture was prepared by inoculating into NB, and incubating it overnight in a rotary shaker at 37°C to ensure optimal growth. The turbidity of each culture was then attuned to match an optical density (O.D.) equivalent to a 0.5 McFarland standard at 625 nm. This standardization is critical, as it ensures a consistent bacterial inoculum concentration of approximately 10^8 colony-forming units per milliliter (CFU/ml) for all ASTs, a crucial factor for reproducible and accurate results.100 µl of each bacterial culture was then aseptically spread onto the surface of sterile, solidified Mueller-Hinton Agar (MHA) (HIMEDIA M173) plates [20]. The antibiotic discs were then carefully placed on the surface of the seeded plates, the plates allowed to rest at room temperature for approximately 30 minutes for the antibiotics to diffuse into the agar, and then, the plates were inverted and incubated at 37°C for 24 hours. Post-incubation, the diameter of the zones of inhibition (ZOI) of growth around each antibiotic disc were measured in millimeters (mm) using a ruler, and the results interpreted based on Clinical and Laboratory Standards Institute (CLSI) guidelines to determine whether the bacterial isolates were susceptible or resistant to each antibiotic tested [21-25].

Statistical analysis

All results were reported as mean ± Standard Error of the Mean (SEM), reflecting the precision of the average values. Each assay was performed in triplicate, and the final mean values were calculated from the combined data of these independent replicates to improve accuracy and reproducibility. Wherever applicable, statistical analysis was conducted using Student's t-test to identify significant differences between experimental groups, with a p-value < 0.05 considered statistically significant.

Results and Discussion

Description and Total heterotrophic count (THC)

Description and THC of bacteria

The nature and THC of colonies on NA plates establish that fresh coconut water contains lower mesophilic and psychrotrophic bacteria (Table 2, Figures 2 and 3) in comparison to refrigerated coconut water (Table 2, Figure 4). The refrigerated sub-samples exhibited considerably higher mesophilic counts and an enormous psychrotrophic count. These observations indicate that bacterial contamination of coconut water that has initiated during post-harvest handling (including sale) has only aggravated during refrigeration of the sub-samples. This is because, as the pH turned near-neutral (6.9-7.1) with the coconut water getting spoiled while being refrigerated, the sub-samples had lost their natural acidic protective barrier, only to allow for the luxuriant growth of the bacteria.

Description and THC of fungi

A considerably low fungal count was observed in both fresh (Table 3, Figures 5 and 6) and refrigerated coconut water sub-samples in comparison to the bacterial count (Table 3, Figures 5 and 6).

Sl. No.	Sub-sample	Description of Isolated Mesophilic Bacterial Colonies on NA Plates	THC of Mesophilic bacteria (CFU/ml)	Description of Isolated Psychrotrophic Bacterial Colonies on NA Plates	THC of Psychrotrophic bacteria (CFU/ml)
1	S1 (Fresh)	13 pale-white, raised, large colonies; 11 white, raised, medium-sized colonies; 13 yellow, medium-sized colonies	37×10^2	8 pale-white, raised, medium-sized colonies	8×10^2
2	S1 (Ref)	185 white, raised, medium-sized colonies	185×10^6	Pale-white, medium-sized colonies	Lawn
3	S2 (Fresh)	15 white, large colonies; 8 yellow, medium-sized colonies; 5 white, small colonies	28×10^2	-	0
4	S2 (Ref)	168 pale-white, medium-sized colonies; 40 reddish-pink, medium-sized colonies	208×10^6	Pale-white, medium-sized colonies	Lawn
5	S3 (Fresh)	97 pale-white, medium-sized colonies; 3 pale-white, large colonies; 3 pale-yellow, medium-sized colonies;	103×10^2	10 pale-white, raised, medium-sized colonies; 15 pale-white, small colonies	25×10^2
6	S3 (Ref)	48 white, medium-sized colonies; 20 pale-white, medium-sized colonies	68×10^6	Pale-white, medium-sized colonies	Lawn

Table 2: Description and THC of Bacteria in fresh and refrigerated coconut water sub-samples.

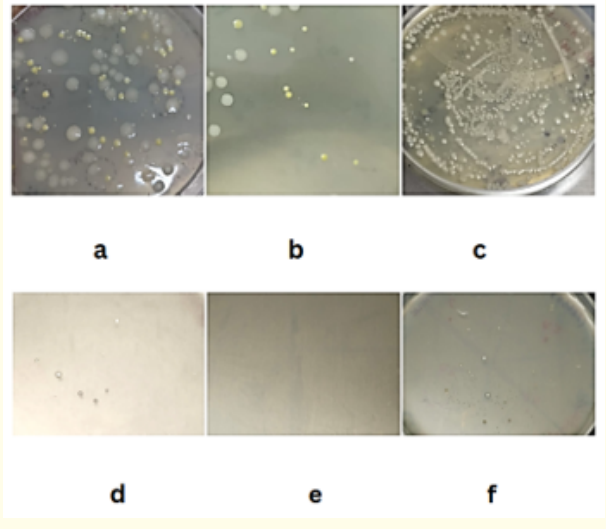


Figure 2: Colonies of bacteria on NA plates: Mesophilic bacteria (a-c): (a) S1 (Fresh) (37 colonies) (b) S2 (Fresh) (28 colonies) and (c) S3 (Fresh) (103 colonies); Psychrotrophic bacteria (d-f): (d) S1 (Fresh) (8 colonies), (e) S2 (Fresh) (0 colony) and (f) S3 (Fresh) (25 colonies).

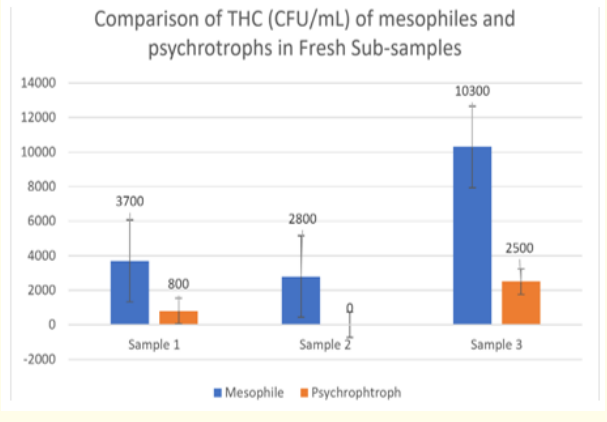


Figure 3: A bar diagram comparative analysis of THC of mesophilic and psychrotrophic bacteria in the 3 fresh coconut water sub-samples.

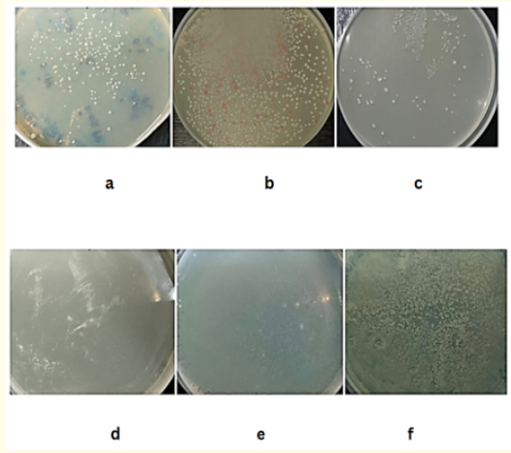


Figure 4: Colonies of bacteria on NA plates: Mesophilic bacteria (a-c): (a) S1 (Ref) (185 colonies) (b) S2 (Ref) (208 colonies) and (c) S3 (Ref) (68 colonies); Psychrotrophic bacteria (d-f): (d) S1 (Ref) (Lawn), (e) S2 (Ref) (Lawn) and (f) S3 (Ref) (Lawn).

These observations indicate that fungal contamination of coconut water which have occurred during post-harvest handling (including sale) have persisted during refrigeration of the sub-samples as well. This is worrisome, as fungi are deteriorating microorganisms and many of them even produce mycotoxins into consumable food and drink, some of which are potentially carcinogenic.

Comparison of the total bacterial and fungal counts across all sub-samples

In all the sub-samples tested, both fresh and refrigerated, the mesophilic bacterial contamination levels were found to be much greater than fungal contaminants (Table 4). The near-neutral pH of the refrigerated sub-samples (6.9-7.1) (Table 1) may be a conducive factor for the selective growth of bacterial contaminants over the fungal ones.

Sl. No.	Sub-sample	Description of Isolated Fungal Colonies on YPDA Plates	THC of Fungi (CFU/ml)	Sl. No.	Sub-sample	Description of Isolated Fungal Colonies on YPDA Plates	THC of Fungi (CFU/ml)
1	S1 (Fresh)	1 large, white, cottony colony and 1 small, black, cottony colony underneath the former	2×10^2	1	S1 (Ref)	1 large, white, cottony colony	1×10^6
2	S2 (Fresh)	1 large, white, cottony colony and 1 small, black cottony colony underneath the former	2×10^2	2	S2 (Ref)	1 large, white, cottony colony and 1 small, black cottony colony	2×10^6
3	S3 (Fresh)	1 large, white, cottony colony and 2 small, black, cottony colonies underneath the first	3×10^2	3	S3 (Ref)	1 large, white, cottony colony, 1 small, black, cottony colony, and 1 small, blue-green, cottony colony	3×10^6

Table 3: Description and THC of Fungi in fresh and refrigerated coconut water sub-samples.

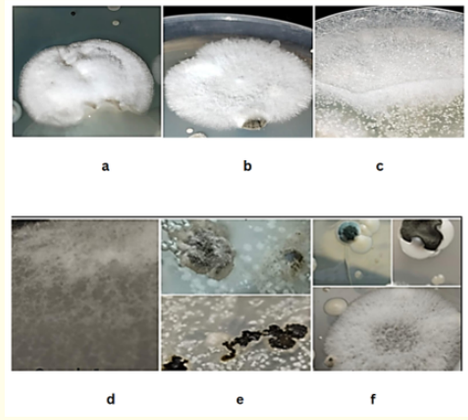


Figure 5: Colonies of fungi on YPDA plates in (left to right): (a) S1 (Fresh) (2 colonies) (b) S2 (Fresh) (2 colonies) (c) S3 (Fresh) (3 colonies; 2 colonies not visible, as underneath 1 large colony) (d) S1 (Ref) (1 colony) (e) S2 (Ref) (2 colonies) and (f) S3 (Ref) (3 colonies).

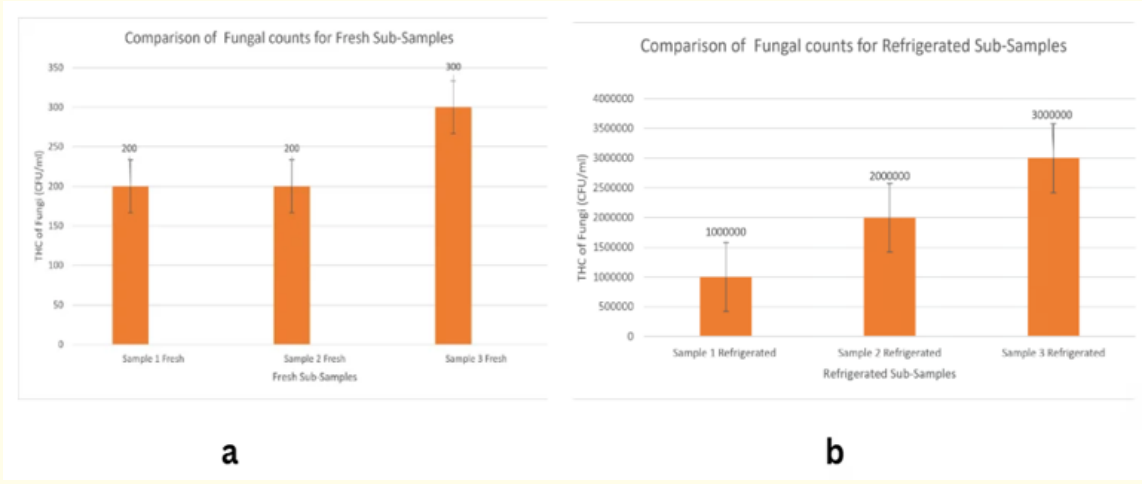


Figure 6: (a) A bar diagram comparative analysis of THC of fungi in the 3 fresh coconut water sub-samples; (b) A bar diagram comparative analysis of THC of fungi in the 3 refrigerated coconut water sub-samples.

Sl. No.	Sub-sample	THC of Mesophilic bacteria (CFU/ml)	THC of Fungi (CFU/ml)	Sl. No.	Sub-sample	THC of Mesophilic bacteria (CFU/ml)	THC of Fungi (CFU/ml)
1	S1 (Fresh)	37×10^2	2×10^2	4	S1 (Ref)	185×10^6	1×10^6
2	S2 (Fresh)	28×10^2	2×10^2	5	S2 (Ref)	208×10^6	2×10^6
3	S3 (Fresh)	103×10^2	3×10^2	6	S3 (Ref)	68×10^6	3×10^6

Table 4: A Comparison of the THC of Mesophilic Bacteria and Fungi across all fresh and refrigerated coconut water sub-samples.

Isolation of bacterial pathogens

Detection and isolation of total coliforms, fecal coliforms and thermotolerant coliforms

Presumptive test and MPN index determination

As evident from the Presumptive test (3x3 MPN Technique) results, both fresh and refrigerated coconut water sub-samples had exceptionally high coliform loads, with 83% exhibiting MPN/100 ml values (MPN Index) above 1100, hinting towards a massive bacterial contamination post-harvest (including sale) and its continuation during refrigeration (Table 5, Figure 7) [7,9].

Sl. No.	Sub-samples	10 ml 2X LB + 10 ml sub-sample	9 ml 1X LB + 1 ml sub-sample	9.9 ml 1X LB + 0.1 ml sub-sample	MPN/100 ml (MPN Index)
1	S1 (Fresh)	3	3	3	>1100
2	S1 (Ref)	3	3	3	>1100
3	S2 (Fresh)	3	3	3	>1100
4	S2 (Ref)	3	3	3	>1100
5	S3 (Fresh)	3	3	2	1100
6	S3 (Ref)	3	3	3	>1100

Table 5: MPN Index of fresh and refrigerated coconut water sub-samples in Presumptive Test.

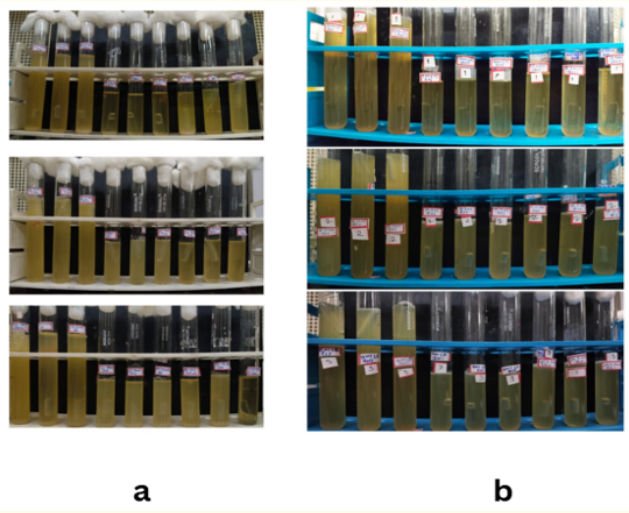


Figure 7: A representative set of Presumptive Tests for: (a) Fresh Coconut Water sub-samples (top to bottom): S1 (Fresh), S2 (Fresh) and S3 (Fresh); (b) Refrigerated coconut water sub-samples (top to bottom): S1 (Ref), S2 (Ref) and S3 (Ref).

Confirmed test

Coliform contamination, both fecal (*E. coli*) and non-fecal, was further confirmed by observation of their typical colonies on the EMB plates in the Confirmed Test (Table 6, Figure 8), especially in refrigerated sub-samples that showed much higher fecal (*E. coli*) contaminations, indicative of the persistence of the bacterial load during cold storage also [9].

Sl. No.	Sub-samples	No. of Isolated Colonies	Colony Characteristics	Coliform Type
1	S1 (Fresh)	14	Small, purple colonies with deep-purple center	Fecal Coliform
		10	Large, mucoid, raised, pinkish-purple colonies	Non-Fecal Coliform
2	S1 (Ref)	178	Small, purple colonies with deep-purple center	Fecal Coliform
3	S2 (Fresh)	0*	-	-
4	S2 (Ref)	64	Small, purple colonies with deep-purple center	Fecal Coliform
5	S3 (Fresh)	9	Small, purple colonies with deep-purple center	Fecal Coliform
		5	Large, mucoid, raised, pinkish-purple colonies	Non-Fecal Coliform
6	S3 (Ref)	13	Small, purple colonies with deep-purple center	Fecal Coliform
		7	Large, mucoid, raised, pinkish-purple colonies	Non-Fecal Coliform

Table 6: Results of Confirmed Test.

* 82 small, rough, and pale convex colonies were instead isolated, indicative of the presence of non-coliform, Gram-negative *Pseudomonas.*

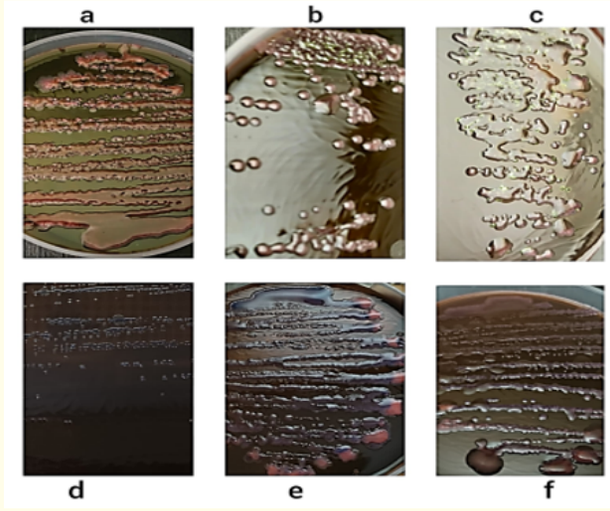


Figure 8: Results of Confirmed Test: (a-c): Fecal coliform, non-fecal coliform and non-coliform colonies on EMB agar plates from fresh Coconut Water sub-samples: (a) S1 (Fresh) (b) S2 (Fresh) and (c) S3 (Fresh); (d-f): Fecal coliform and non-fecal coliform colonies on EMB agar plates from refrigerated coconut water sub-samples: (a) S1 (Ref) (b) S2 (Ref) and (c) S3 (Ref).

Completed test

The presence of thermotolerant fecal coliforms (*E. coli*) was confirmed by observation of the EC broth tubes incubated at 45^{0}C, which showed moderate-to-dense turbidity, moderate-to-huge gas production in the Durham's tubes, and a slight decrease in pH (from an original pH of 6.9 of the EC broth) (Table 7, Figure 9), indicative of a massive coliform contamination [9].

Sl. No.	Sub-samples	Turbidity	Gas production (%)	pH
1	S1 (Fresh)	+	15	6.8
2	S1 (Ref)	+	15	6.3
3	S2 (Fresh)	++	35	6.3
4	S2 (Ref)	++	100	6.1
5	S3 (Fresh)	+++	100	6.3
6	S3 (Ref)	+++	100	6.2

Table 7: Results of Completed Test.

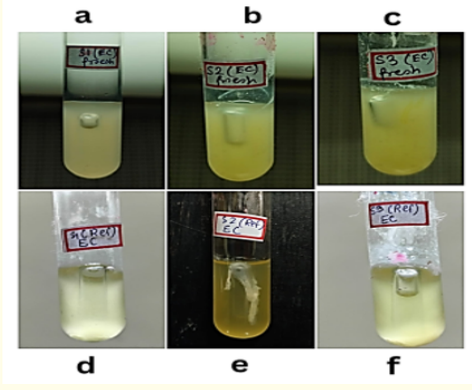


Figure 9: Results of Completed Test for: (a) S1 (Fresh) (b) S2 (Fresh) (c) S3 (Fresh) (d) S1 (Ref) (e) S2 (Ref) and (f) S3 (Ref) coconut water sub-samples.

Isolation of *Staphylococcus* species

The presence of typical greyish-black to black colonies, some of which were surrounded by distinct halos on the BPA plates, indicated the presence of *Staphylococcus* species in both fresh (Table 8; Figure 10) and refrigerated sub-samples, excepting the S1 (Ref) sub-sample (Table 8, Figure 10) [7,9]. The S2 (Ref) sub-sample was found to have the maximum number (230 x 10^6 CFU/ml) of *Staphylococcus aureus* (colonies with halo), as well the maximum number (20 x 10^6 CFU/ml) of other *Staphylococcus* species (colonies without halo).

Sl. No.	Sub-sample	CFU/ml (halo)	CFU/ml (non-halo)
1	S1 (Fresh)	67 x 10^2	3 x 10^2
2	S1 (Ref)	0	0
23	S2 (Fresh)	0	3 x 10^2
4	S2 (Ref)	230 x 10^6	20 x 10^6
35	S3 (Fresh)	27 x 10^2	75 x 10^2
6	S3 (Ref)	0	7 x 10^6

Table 8: Colony Count of *Staphylococcus* species on BPA Plates for fresh and refrigerated coconut water sub-samples.

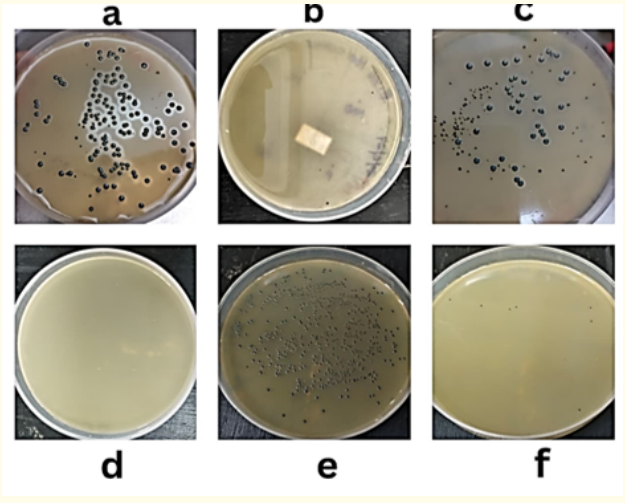


Figure 10: Colonies of *Staphylococcus* species growing on BPA plates from: (a) S1 (Fresh) (b) S2 (Fresh) (c) S3 (Fresh) (d) S1 (Ref) (e) S2 (Ref) and (f) S3 (Ref) coconut water sub-samples.

Isolation of *Salmonella/Shigella*/other Enterobacteria

Green and raised colonies without black center, typical of *Shigella*, were observed in two of the sub-samples, S1 (Fresh) (Table 9, Figure 11) and S2 (Ref) (Table 9, Figure 11), indicating persistent survival of these pathogens even under refrigeration conditions [7,9]. However, no contamination by species of *Salmonella* was observed in any of the sub-samples.

Sl. No.	Sub-sample	CFU/ml
1	S1 (Fresh)	79 x 10^2
2	S1 (Ref)	0
23	S2 (Fresh)	0
4	S2 (Ref)	25 x 10^6
35	S3 (Fresh)	0
6	S3 (Ref)	0

Table 9: Colony Count of *Shigella* species on HEA Plates for fresh and refrigerated coconut-water sub-samples.

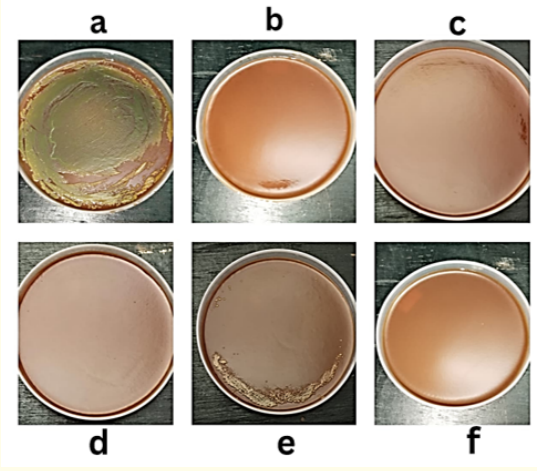


Figure 11: Colonies of *Shigella* species growing on HEA plates from: (a) S1 (Fresh) (b) S2 (Fresh) (c) S3 (Fresh) (d) S1 (Ref) (e) S2 (Ref) and (f) S3 (Ref) coconut water sub-samples.

4 Isolation of *Bacillus* species

For both the fresh and refrigerated coconut water sub-samples, growth occurred in GFNB, except in the S1 (Ref) sub-sample (Figure 12).

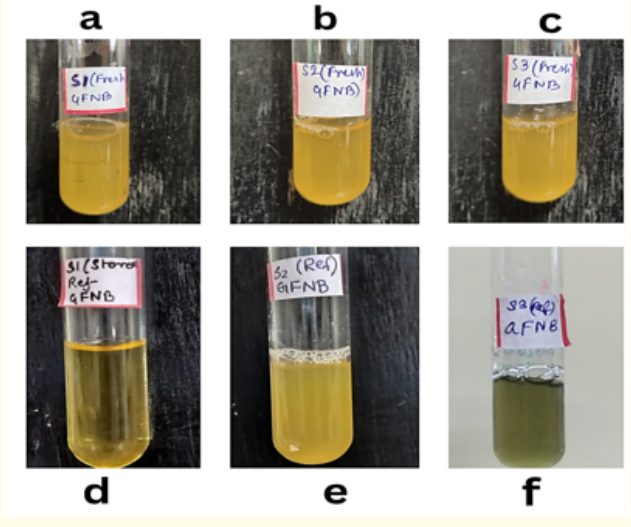


Figure 12: Growth of *Bacillus* species in GFNB tubes from: (a) S1 (Fresh) (b) S2 (Fresh) (c) S3 (Fresh) (d) S1 (Ref) (e) S2 (Ref) and (f) S3 (Ref) coconut water sub-samples.

Isolation of fungal pathogens

Fungal colonies selected from YPDA plates were sub-cultured in pure form onto YPDA slants (Table 10).

Characterization, identification and confirmation of pathogens

Characterization, identification and confirmation of bacterial pathogens

Microscopy (Gram Staining)

The isolated bacterial pathogens were identified by their Gram-character and morphology through microscopic observations (400X) following Gram-staining.

Coliforms

Cells from both fecal- and non-fecal coliform colonies isolated on EMB plates appeared as Gram negative, short rods, in small clusters (Figure 13).

Pseudomonas species

Cells from the non-coliform colonies isolated on EMB plates appeared as Gram negative, straight and slightly-curved short rods, appearing singly (Figure 13).

Staphylococcus species

From the BPA plate colonies (with and without halo), characteristic cells of *Staphylococcus* species (including *S. aureus*) were observed in the form of Gram positive, medium- sized cocci in clusters and a bunch of grape-like arrangements (Figure 13).

Sl. No.	Coconut Water Sample	Sub-sample	Type of Colony chosen from YPDA plate	Nature of Growth on YPDA slant
1	Fresh	S1 (Fresh)	A small, black, cottony colony	Luxuriant black, cottony (velvety) growth
2		S1 (Fresh)	A large, white, cottony colony	Very fast growing, white, fluffy growth
3		S2 (Fresh)	A large, white, cottony colony	Very fast growing, white, cottony (velvety) growth
4		S2 (Fresh)	A small, black, cottony colony	Luxuriant black, cottony (velvety) growth
5		S3 (Fresh)	A large, white, cottony colony	Very fast growing, white, dense and cottony (candy floss-like) growth
6		S3 (Fresh)	A small, black, cottony colony	Luxuriant black, cottony (velvety) growth
7	Refrigerated	S1 (Ref)	A large, white, cottony colony	Very fast growing, white, dense and cottony (candy floss-like) growth
8		S2 (Ref)	A small, black cottony colony	Luxuriant black, cottony (velvety) growth
9		S2 (Ref)	A large, white, cottony colony	Very fast growing, white, dense and cottony (candy floss-like) growth
10		S3 (Ref)	A small, black, cottony colony	Luxuriant black, cottony (velvety) growth
11		S3 (Ref)	A small, blue-green, cottony colony	Dense, white, cottony (velvety), growth with a powdery, bluish-green central part.

Table 10: Description of Fungal Colonies Chosen from YPDA Plates and Isolated on YPDA Slants.

Shigella species

From the HEA plate colonies, characteristic cells of *Shigella* species were observed in the form of Gram negative, short rods, singly or in small clusters (Figure 13).

Bacillus species

From the GFNB tubes, characteristic cells of *Bacillus* species were observed in the form of Gram positive, medium-sized rods in chain arrangement (Figure 13).

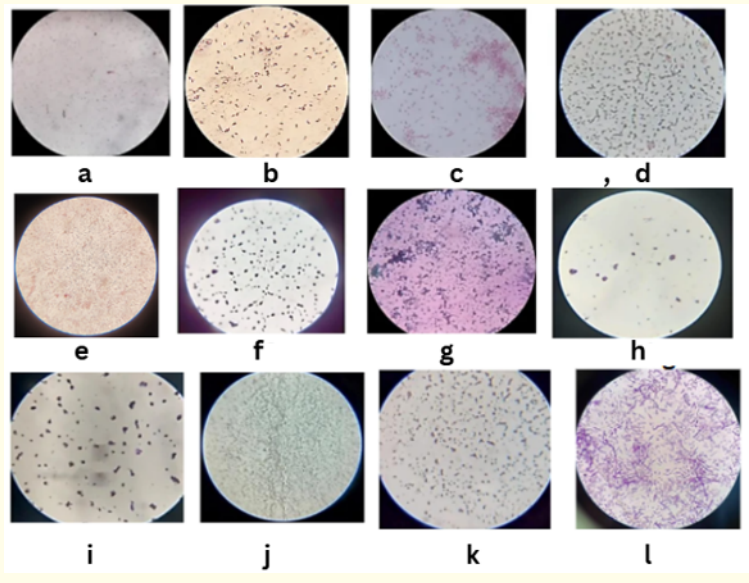


Figure 13: Microscopic observation (400X) of Gram-stained cells of: (a-d): Fecal- and Non-Fecal coliforms from EMB plates: (a) Fecal fresh (b) Fecal refrigerated (c) Non-Fecal fresh (d) Non-Fecal refrigerated; (e): *Pseudomonas* species from EMB plate; (f-i): *Staphylococcus* species from BPA plates: (f) Halo fresh (S. aureus) (g) Halo refrigerated (*S. aureus*) (h) Non-halo fresh (other *Staphylococcus* species) (i) Non-halo refrigerated (other *Staphylococcus* species); (j-k): *Shigella* species from HEA plates: (j) fresh (k) refrigerated; (l) *Bacillus* species from a GFNB Tube.

Microscopy (Endospore Staining)

The conventional Dorner's Nigrosin Method of Endospore staining identified endospore-forming *Bacillus* species as the contaminant of the coconut water sub-samples. The endospores released free appeared vibrant pink [stained by Carbol Fuchsin (HIMEDIA S006)], whereas the medium-sized, rod - shaped vegetative cells appeared colorless against the dark background of Dorner's Nigrosin (Figure 14).

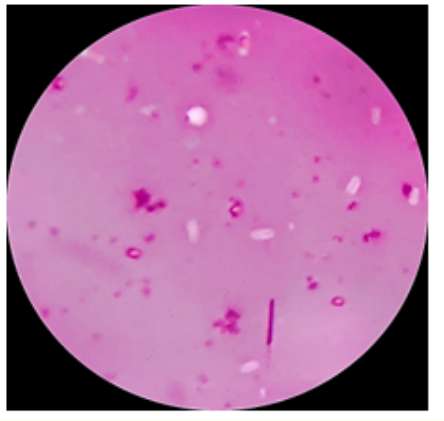


Figure 14: Microscopic observation (400X) of endospores of Bacillus species from a GFNB Tube.

Biochemical tests

The pathogenic bacterial isolates were characterized and identified by the following set of standard biochemical tests (Table 11, Figure 15). Apart from the highly notorious food-borne pathogenic species belonging to genera like *Staphylococcus, Shigella, Bacillus* and fecal coliforms (*Escherichia coli*), *Pseudomonas* was also detected in one of the sub-samples, indicating huge microbial contamination of the coconut water samples collected from different geographical locations in and around Kolkata, during post-harvest handling of coconuts, sale of coconut water by itinerant vendors, and their refrigeration thereafter, and their role as a potential risk factor towards public health and hygiene.

Characterization, identification and confirmation of fungal pathogens

From the microscopic (400X) observations of their morphological details (hyphal structure and reproductive spores) following fungus staining with the usual staining reagents, the different pathogenic fungal isolates from both fresh and refrigerated coconut water sub-samples (Table 12) were characterized, identified and confirmed [26-29].

Sl. No.	Isolate	IT	MR	VP	CUT	CP	OP	NR	UH	CoP	SH	GH	TSI	Bacterial Pathogen confirmed
S1 (Fresh)														
1	EMB Faecal	+	+	-	-	+	-	+	-	-	-	-	Y/Y	*Escherichia coli*
2	EMB Non-Faecal	-	-	+	+	+	-	+	-	-	-	-	Y/Y	*Enterobacter* sp.
3	BPA Halo	-	+	+	+	+	-	+	-	+	-	+ (Slow)	Y/Y	*Staphylococcus aureus*
4	BPA Non-Halo	-	-	+	-	+	-	+	-	-	-	-	Y/Y	*Staphylococcus epidermidis*
5	HEA	-	+	-	-	+	-	+	-	-	-	-	R/Y	*Shigella* sp.
6	GFNB	-	-	+	+	+	-	+	-	-	+	+ (Slow)	R/Y	*Bacillus* sp.
S1 (Ref)														
1	EMB Faecal	+	+	-	-	+	-	+	-	-	-	-	Y/Y	*Escherichia coli*

2	GFNB	-	-	+	+	+	-	+	-	-	+	+ (Slow)	Y/Y	*Bacillus* sp.
S2 (Fresh)														
1	EMB Faecal	+	+	-	-	+	-	+	-	-	-	-	Y/Y	*Escherichia coli*
2	EMB Non-Faecal	-	-	-	+	+	+	+	-	-	-	+	R/NC	*Pseudomonas* sp.
3	BPA Non-Halo	-	-	+	-	+	-	+	-	-	-	-	Y/Y	*Staphylococcus epidermidis*
4	GFNB	-	-	+	+	+	-	+	-	-	+	+ (Slow)	Y/Y	*Bacillus* sp.
S2 (Ref)														
1	EMB Faecal	+	+	-	-	+	-	+	-	-	-	-	Y/Y	*Escherichia coli*
2	EMB Non-Faecal	-	-	+	+	+	-	+	-	-	-	-	Y/Y	*Enterobacter* sp.
3	BPA Halo	-	+	+	+	+	-	+	-	+	-	+ (Slow)	Y/Y	*Staphylococcus aureus*
4	BPA Non-Halo	-	-	+	-	+	-	+	-	-	-	-	Y/Y	*Staphylococcus epidermidis*
5	HEA	-	+	-	-	+	-	+	-	-	-	-	R/Y	*Shigella* sp.
6	GFNB	-	-	+	+	+	-	+	-	-	+	+ (Slow)	R/Y	*Bacillus* sp.
S3 (Fresh)														
1	EMB Faecal	+	+	-	-	+	-	+	-	-	-	-	Y/Y	*Escherichia coli*
2	EMB Non-Faecal	-	-	+	+	+	-	+	-	-	-	-	Y/Y	*Enterobacter sp.*
3	BPA Halo	-	+	+	+	+	-	+	-	+	-	+ (Slow)	Y/Y	*Staphylococcus aureus*
4	BPA Non-Halo	-	-	+	-	+	-	+	-	-	-	-	Y/Y	*Staphylococcus epidermidis*
5	GFNB	-	-	+	+	+	-	+	-	-	+	+ (Slow)	R/Y	*Bacillus* sp.
S3 (Ref)														
1	EMB Faecal	+	+	-	-	+	-	+	-	-	-	-	Y/Y	*Escherichia coli*
2	EMB Non-Faecal	-	-	+	+	+	-	+	-	-	-	-	Y/Y	*Enterobacter* sp.

3	BPA Non-Halo	-	-	+	-	+	-	+	-	-	-	-	Y/Y	*Staphylococcus epidermidis*
4	GFNB	-	-	+	+	+	-	+	-	-	+	+ (Slow)	Y/Y	*Bacillus* sp.

Table 11: Summary of Biochemical tests and their results for fresh and refrigerated coconut-water sub-samples.

IT = Indole Test; MR = Methyl red Test; VP = Voges Proskauer Test; CUT = Citrate Utilization Test; CP = Catalase Production test; OP = Oxidase Production test; NR = Nitrate Reduction test; UP = Urease Production test; CoP = Coagulase Production test; SH = Starch Hydrolysis test; GH = Gelatin Hydrolysis; TSI = Triple Sugar Iron Test; + = Positive; – = Negative; In TSI test: slant/butt, Y = Yellow, R = Red, NC = no change.

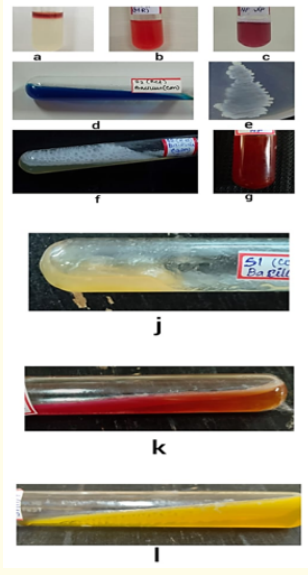
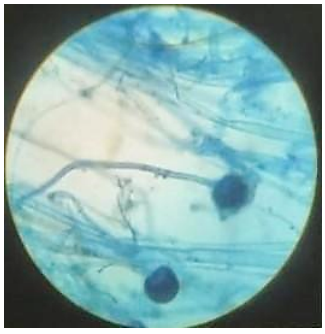
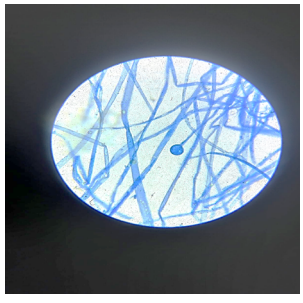
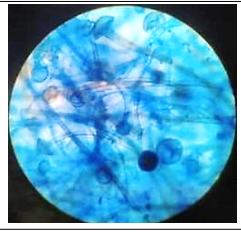
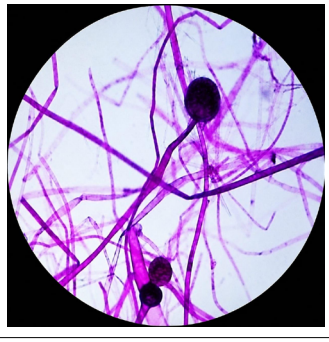
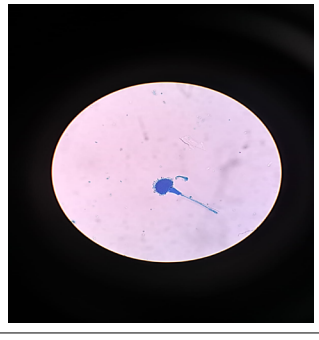


Figure 15: A representative set of Biochemical Test results as a confirmation of different pathogenic bacterial isolates. a. Positive result of Indole test [a) cherry red ring appeared at the top upon addition of Kovac's reagent (HIMEDIA R008) to the Tryptone broth with bacterial culture]; b. Positive result of Methyl Red test [a uniform red coloration appeared upon addition of Methyl Red reagent (HIMEDIA I007) to the MR-VP broth (HIMEDIA LQ082) with bacterial culture]; c. Positive result of VP test [a greyish-pink colouration appeared upon sequential addition of, and shaking for 15 minutes, Barritt's Reagents A (HIMEDIA R029) and B (HIMEDIA R030) to the MR-VP broth with bacterial culture]; d. Positive result for Citrate Utilization test [bacterial growth and change in colour of Simmon's Citrate Agar (HIMEDIA M411) from green to deep prussian blue upon overnight incubation at 37°C]; e. Positive result for Oxidase Test [appearance of bluish colour on NA plates within 30 seconds of application of colourless N,N,N',N' - tetramethyl-p-phenylenediamine dihydrochloride (HIMEDIA GRM445)]; f. Positive result for Catalase Test [evolution of copious oxygen bubbles from a NA slant culture upon addition of 3 drops of 3% H2O2 (EMSURE ® 88597)]; g. Positive result for Nitrate Reduction Test [red colouration in nitrate broth (HIMEDIA M439) culture immediately upon sequential addition of sulfanilic acid (HIMEDIA GRM428) and α-naphthylamine (HIMEDIA R009) solutions to the to the Nitrate broth with bacterial culture]. h. Negative result for Urease Test [no pinkish red colouration in the Urea broth (HIMEDIA M111) culture upon overnight incubation at 37°C]; i. Positive result for Starch Hydrolysis [clear halo zone surrounding bacterial growth on starch media (HIMEDIA M107S) plate observed after addition of Gram's iodine (HIMEDIA S013 solution)]; j. Positive result for Gelatin hydrolysis [rapid hydrolysis of gelatin at the top of the nutrient-gelatin (HIMEDIA M060) medium by gelatinase of the GFNB isolate observed after 48 hours of incubation at 37°C and 30 minutes chilling at 4°C, in comparison to BPA isolate unable to hydrolyze gelatin)]; k. – l. Results for Triple Sugar Iron [TSI agar (HIMEDIA M0211); k. red slant/yellow butt: indicates only dextrose fermentation: dextrose runs out quickly, slant reverts to red (alkaline), while the butt stays yellow (acidic), l. yellow slant/yellow butt: indicates the fermentation of dextrose, lactose and/or sucrose; both slant and butt remain acidic (yellow)].

Sl. No.	Sub-sample	Growth characteristics on YPDA Slant	Microscopic Observation after LPCB Staining	Fungal Pathogen confirmed	Microscopic Image (400X)
1	S1 (Fresh)	Luxuriant black, cottony (velvety) growth	Presence of a tubular, septate, hyaline, and branched stalk called a conidiophore that enlarges at the apical part, giving rise to a spherical structure with a layer of radiating phialides, called a vesicle, and clustered chains of exogenous reproductive structures developed from sterigmata called conidia [26]	*Aspergillus* sp.	
2	S1 (Fresh)	Very fast growing, white, fluffy growth	Presence of broad, non-septate (coenocytic) hyphae producing tall, branched sporangiophores; globose sporangia supported by a columella. [27].	*Mucor* sp.	
3	S1 (Ref)	Very fast growing, white, dense and cottony (candy floss-like) growth	Presence of filamentous, branching, coenocytic hyphae with a spherical sporangium, supported by a large apophysate columella on the top of a long stalk called the sporangiophore [28].	*Rhizopus* sp.	
4	S2 (Fresh)	Very fast growing, white, cottony (velvety) growth	Presence of broad, non-septate (coenocytic) hyphae producing tall, branched sporangiophores; globose sporangia supported by a columella. [27].	*Mucor* sp.	
5	S2 (Fresh)	Luxuriant black, cottony (velvety) growth	Presence of a tubular, septate, hyaline, and branched stalk called a conidiophore that enlarges at the apical part, giving rise to a spherical structure with a layer of radiating phialides, called a vesicle, and clustered chains of exogenous reproductive structures developed from sterigmata called conidia [26]	*Aspergillus* sp.	

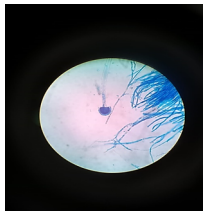
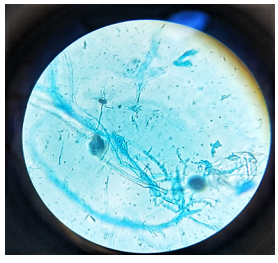
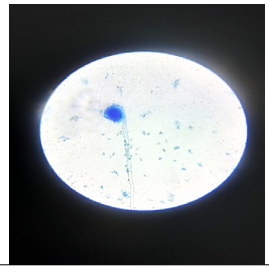
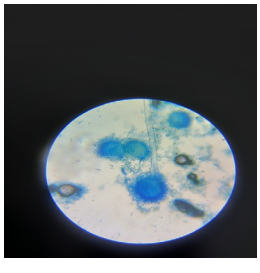
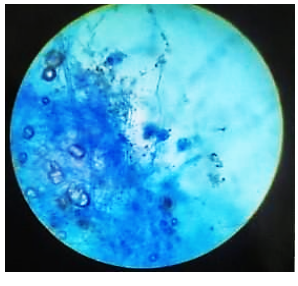
6	S2 (Ref)	Luxuriant black, cottony (velvety) growth	Presence of a tubular, septate, hyaline, and branched stalk called a conidiophore that enlarges at the apical part, giving rise to a spherical structure with a layer of radiating phialides, called a vesicle, and clustered chains of exogenous reproductive structures developed from sterigmata called conidia [26]	*Aspergillus* sp.	
7	S2 (Ref)	Very fast growing, white, dense and cottony (candy floss-like) growth	Presence of filamentous, branching, coenocytic hyphae with a spherical sporangium, supported by a large apophysate columella on the top of a long stalk called the sporangiophore [28].	*Rhizopus* sp.	
8	S3 (Fresh)	Very fast growing, white, dense and cottony (candy floss-like) growth	Presence of filamentous, branching, coenocytic hyphae with a spherical sporangium, supported by a large apophysate columella, on the top of a long stalk called the sporangiophore [28].	*Rhizopus* sp.	
9	S3 (Fresh)	Luxuriant black, cottony (velvety) growth	Presence of a tubular, septate, hyaline, and branched stalk called a conidiophore that enlarges at the apical part, giving rise to a spherical structure with a layer of radiating phialides, called a vesicle, and clustered chains of exogenous reproductive structures developed from sterigmata called conidia; many free conidia were also observed [26]	*Aspergillus* sp.	
10	S3 (Ref)	Luxuriant black, cottony (velvety) growth	Presence of a tubular, septate, hyaline, and branched stalk called a conidiophore that enlarges at the apical part, giving rise to a spherical structure with a layer of radiating phialides, called a vesicle, and clustered chains of exogenous reproductive structures developed from sterigmata called conidia; many free conidia were also observed [26]	*Aspergillus* sp.	
11	S3 (Ref)	Dense, white, cottony (velvety), growth with a powdery, bluish-green central part.	Broom-like appearance, with elongated, branching stalks called conidiophores, and phialides, that produce reproductive structures called conidia, chains of which extended outward in a broom-like fashion [29].	*Penicillium* sp.	

Table 12: Characterization, Identification and Confirmation of Fungal Pathogens from fresh and refrigerated coconut water sub-samples.

Determination of antibiotic resistance in bacteria by antibiotic susceptibility test (AST) and antibiogram analyses

Presently, in this era of mammoth drug-resistance, it is of utmost importance to gain knowledge on the profiles of the contaminating pathogens and their antibiotic resistance in coconut water samples collected from different geographical sites in and around the city of Kolkata. The results of the AST (Figure 16) and subsequent antibiogram analyses of the 27 typical pathogenic bacterial isolates revealed much variations in their drug-resistance patterns. Very alarmingly, out of 27 isolates, 23 (85.2% of the isolates) from across all the sub-samples (fresh and refrigerated) exhibited complete resistance to the conventional Amoxicillin (AMX), 19 (70.4% of the isolates) exhibited complete resistance to the newer-generation Amoxycillin–Clavulanic acid (AMC), and 18 (66.67% of the isolates) exhibited complete resistance to the conventional Vancomycin (VA), including the much notorious *Pseudomonas, Staphylococcus aureus* and fecal coliform *E. coli*. These results hint that the majority of the pathogenic bacterial isolates from the contaminated coconut water samples have acquired the AMX^r, AMC^r and VA^r genes, either through horizontal gene transfer (HGT) or by spontaneous mutations, thus evolving as multi-drug resistant (MDR)-pathogens.

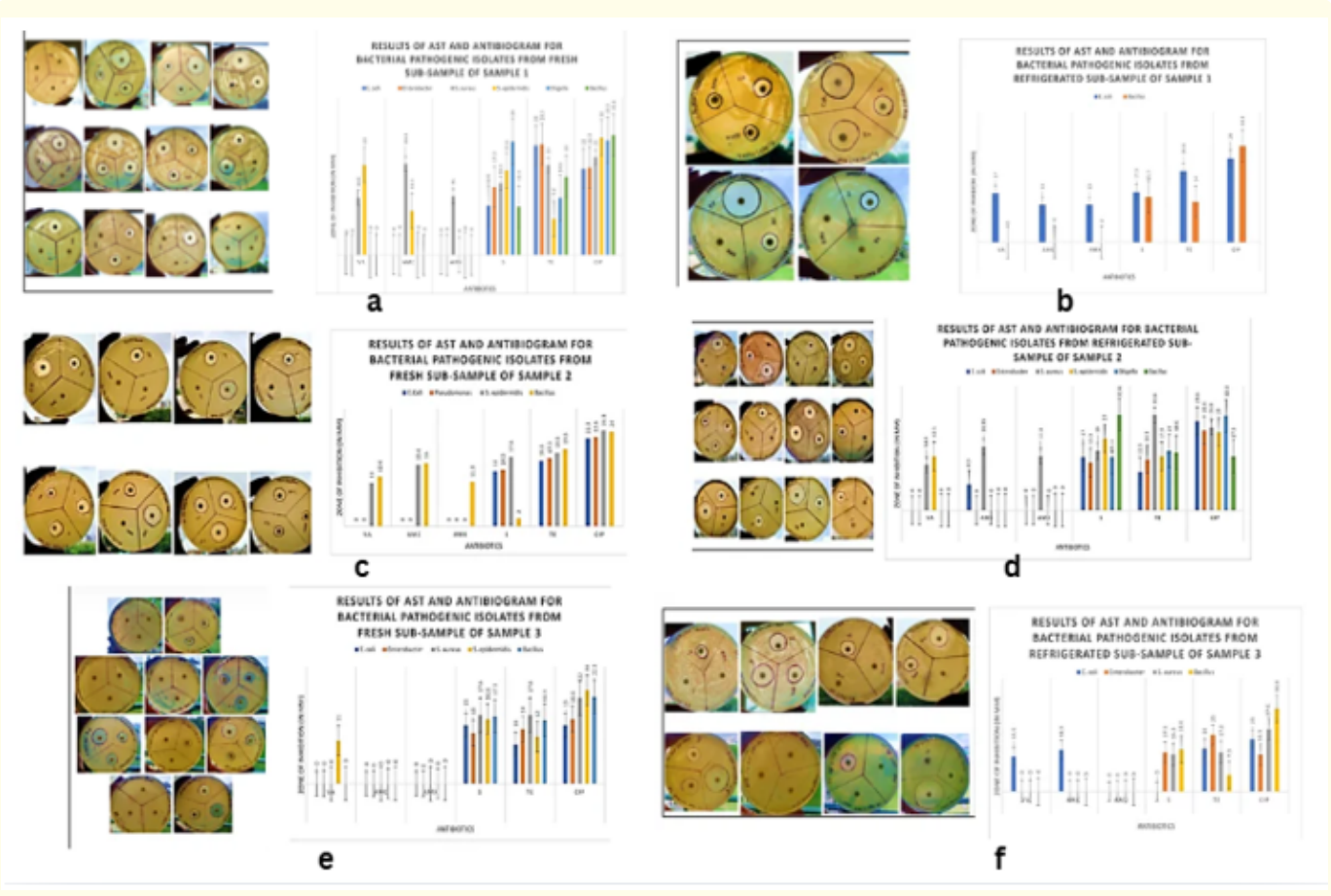


Figure 16: Results of AST and antibiogram analysis through Bar Diagram for the 6 different bacterial pathogenic isolates from: (a) S1 (Fresh) (b) S1 (Ref) (c) S2 (Fresh) (d) S2 (Ref) (e) S3 (Fresh) (f) S3 (Ref) coconut water sub-samples.

Conclusion

This study, possibly a novel one from this part of the country, has tried to shed light on the contamination status of coconut water vended by itinerant roadside sellers from three geographically distinct regions of Kolkata, representing its central, northern and southern parts. The findings demonstrate that anthropogenic activities might have played a significant role in shaping the pathogen profile of these samples. Furthermore, the study reveals the

occurrence and dissemination of MDR-pathogens, highlighting the potential public health risks associated with the consumption of contaminated coconut water sold under less-than ideal hygienic conditions, and raising concerns regarding the rampant use of medicines, including the over-the-counter (OTC) ones.

Coconut water is often considered by food microbiologists as a safe and healthy choice of beverage, because the liquid endosperm is virtually sterile inside an undamaged, mature green fruit. The hard outer covering, the exocarp and mesocarp, and the inner husk, together act as an impenetrable mechanical barrier against the entry of environmental pathogens. The present study findings, however showed that processing, cutting, and vending operations might often breach this natural barrier, making the liquid unsafe to be consumed. This study showed the presence of bacterial (both mesophilic and psychrotrophic) and fungal contaminants in both the fresh and refrigerated coconut water sub-samples, with the number of proliferating pathogenic mesophilic bacteria higher than their fungal counterparts. This study also challenged the idea that refrigeration ensures product safety, as psychrotrophic bacterial contaminants seeded in the freshly-collected samples grew and proliferated further even when stored under standard refrigeration conditions, vastly outnumbering their growth in the fresh samples.

The microbial profile might have been heavily influenced by unhygienic post-harvest handling, storage, and vending. A high MPN index across all tested samples indicated a huge coliform load, including fecal coliforms like *E. coli,* highlighting severe fecal contamination probably due to inappropriate hygienic-sanitary conditions, either when the fruits are stored or cut open, or when the endospermic liquid is sold by the roadside vendors. Even thermotolerant *E. coli* were detected in the samples as an alarming threat to public health and safety. Besides, the presence of pathogenic species of notorious foodborne bacteria like Staphylococci*, Shigella* and *Bacillus* were indicated in this study. A highly pathogenic Pseudomonas isolate was detected in one of the samples, adding much to the concern.

Furthermore, our antibiogram analyses of these coconut water pathogens revealed an alarmingly high degree of multi-drug resistance to even the newer-generation combinatorial drug like Amoxicillin–Clavulanic acid. With the rapid surge in antibiotic-resistant pathogens worldwide, this study demands considerable public health significance. Due to the unregulated use of broad-spectrum antibiotics as human medicine, such antibiotic-resistant pathogenic bacteria have probably evolved as a challenge to a threatened survival in a natural environment full of antibiotics, originated either from other antibiotic-resistant bacteria, actinobacteria and fungi that may secrete related antimicrobials into their surrounding medium, and/or unused pharmaceutical discharges. Such antibiotic-resistant bacteria generally thrive in polluted environments, with resistance rapidly spreading in nature through the HGT of resistance genes [6].

Hence, the present research underscores the urgent need to improve the hygiene and cleanliness in handling and storage of the fruits, and in extraction and storage of coconut water, along with consumer awareness regarding antibiotic use, and routine and periodic pathogenic profile monitoring in a hot and tropical country like India, including this part of the country. It is hoped that the awareness will call for a more intense inspection by the regulatory authorities, will accordingly modulate the existing policies of the concerned authorities (Government or NGOs), and will implement stringent food-safety protocols very soon, as the associated health risk to every people in every stratum of the society is profound and real.

However, a major limitation of the study was the small sample size, restricted to only three geographical regions, which might not perfectly represent the city-wide and seasonal fluctuations. Furthermore, the identification of pathogenic organisms was performed based on culture characteristics, microscopy and biochemical tests, as molecular confirmation methods like ribotyping and other advanced ones could not be performed due to paucity of time. Future studies may include a larger number of samples collected over a year, taking into consideration seasonal fluctuations, geographical variations and differences in socio-economic-anthropogenic influences on the contamination profiles, which would provide a more comprehensive understanding of the public health risks associated with street-vended coconut water.

Ethical Approval and Consent to Participate

Not applicable.

Funding

The authors did not receive any funding for this study.

Conflict of Interest

The authors have no competing interests to disclose.

Acknowledgment

The authors express their deepest gratitude to Rev. Dr. Dominic Savio, SJ, Principal, St. Xavier's College (Autonomous), Kolkata and Dr. Gopal Chandra Mandal, Principal, Sarsuna College, Kolkata, India.

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