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Exploring the street food bacterial diversity in the city of destiny using the bacterial 16S rRNA genotyping.

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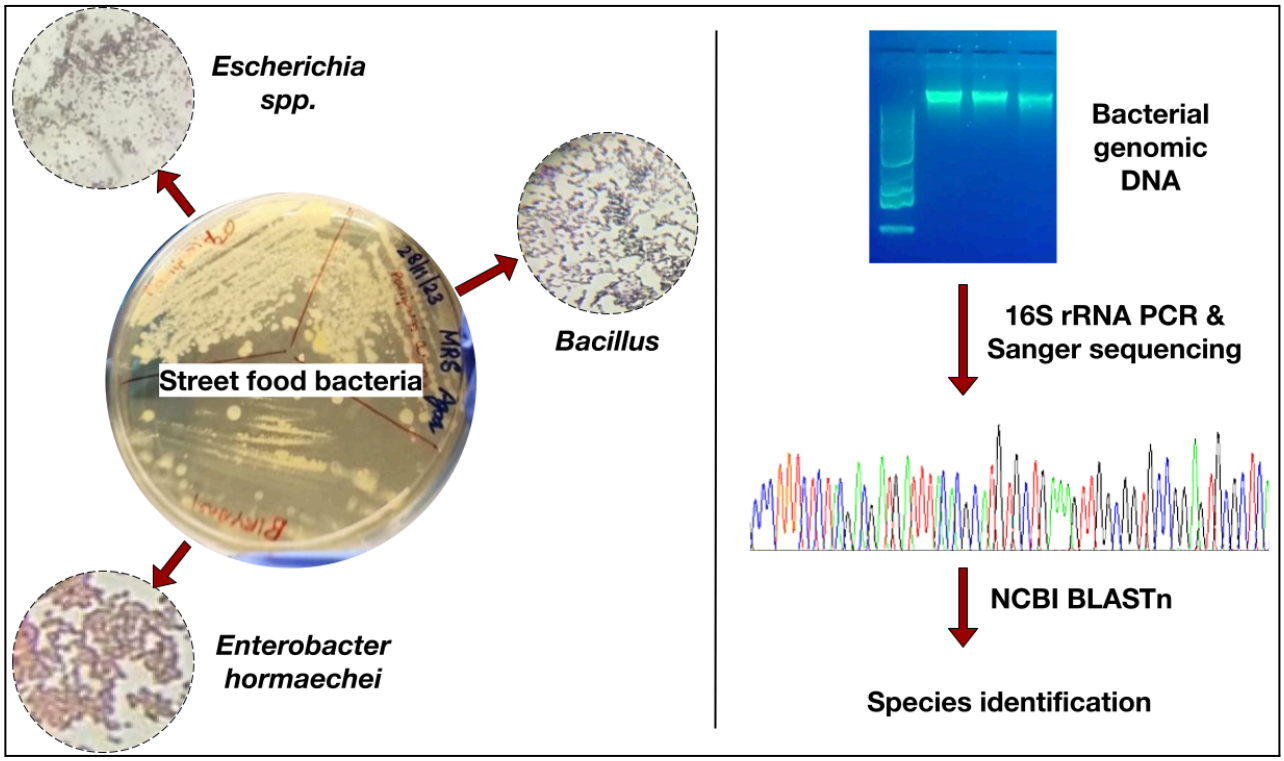
Keywords: Street food, biriyani, panipuri, food microbes, bacterial 16S rRNA, bacterial genotyping, Sanger sequencing.

The post-COVID-19 street food culture is on the hike in the city of destiny, a.k.a. Visakhapatnam, India. Among the most popular roadside foods, biriyani and panipuri are consumed highly. Both biriyani and panipuri are usually prepared in bulk and stored by the vendors at room temperature to dispense them easily. Improper handling and/or storage leads to microbial contaminations, primarily with bacteria. In this study we randomly collected biriyani and panipuri samples in Visakhapatnam and determined the bacterial diversity in these samples using the bacterial 16S rRNA genotyping through Sanger sequencing. Our results revealed that the biriyani sample predominantly contained *Enterobacter hormaechei* and the panipuri sample contained *Escherichia* and other *Bacillus* species. In future, bigger sample sizes will be analyzed using NextGen sequencing.



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Indian cuisine is diverse with a lot of options not only in the gourmet section but also in the street food snacks/meals for the consumers [1]. The most common varieties of street food include rice items (e.g. fried rice, biriyani, etc.), noodles, pav bhaji, chat, panipuri, etc. [2]. While the rice items are mostly consumed as meals, the others are more of a snack. Most street foods are prepared in bulk and stored until they are sold in the vending carts or stalls that are usually parked in open air areas close to the traffic on the streets. The tropical weather is a perfect condition for the growth of various food-borne microorganisms [3]. Food mishandling or unhygienic food serving practices can lead to the spread of food-borne microbial contaminations to the consumers that may or may not result in disease outbreaks depending on the host immunity [4]. However, the immunocompromised individuals quickly become a target for such outbreaks [5]. Majority of the street food vendors closed their businesses during the COVID-19 pandemic from early 2020 till late 2021. Reopening in early 2022 the street food business gradually picked up and regained its popularity in the past couple of years.

Identification of bacterial species from various sources is routinely performed using the PCR-based bacterial 16S rRNA genotyping [6]. The advantage of using universal primers to amplify the bacterial 16S rRNA gene makes this technique versatile in various fields of Microbiology and other applied Life Sciences [7]. In this study we collected random street food samples with primary focus on the biriyani and panipuri. The two main components of panipuri, the curry and water, were separately evaluated for their microbial diversity. Bacterial colonies that were obtained from the biriyani, panipuri curry and panipuri water were characterized using the standard bacterial 16S rRNA genotyping technique. Based on the Sanger sequencing results and NCBI BLASTn search, bacterial species diversity was confirmed in all three samples.

Materials & Methods:

Collection of street food samples: The food samples were taken from the carryout parcels of biriyani, panipuri curry and panipuri water that were purchased from the random roadside vendors in the city of Visakhapatnam. Both biriyani and panipuri curry were warm, suitable for eating. The panipuri water was at room temperature as expected. These samples were directly used for the preparation of serial dilutions using freshly autoclaved LB broth.

Bacterial culture: The food samples were serially diluted in sterile LB broth and all the tubes were incubated at 37 ℃ overnight (Figure 1). The cultures were further streaked onto LB agar plates freshly prepared by autoclaving the media. Streaked plates were incubated at 37 ℃ overnight (Figure 2).

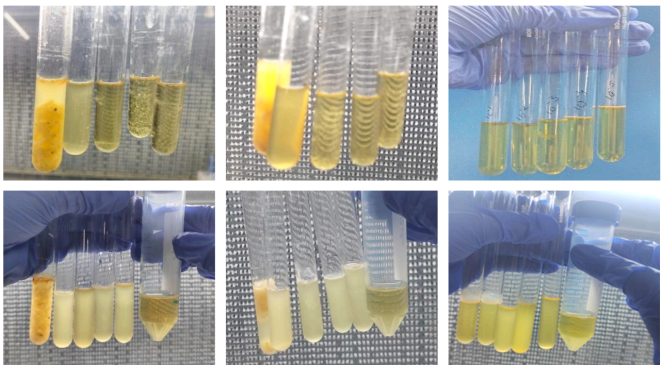

Figure 1. Serial dilution of biriyani (left), panipuri curry (middle) and water (right) samples before (top row) and after (bottom row) incubation.

Isolation of bacterial genomic DNA: The colonies obtained on the plates were further cultured for the bacterial genomic DNA extraction. Five ml of bacterial culture from each of the food samples was used as the source of genomic DNA. Harvested pellets were lysed and processed further using the HiMedia kit (Cat. No. MB505) as per the manufacturer's protocol. Extracted DNA was purified using the HiElute Miniprep Spin Column. The purified genomic DNA samples were qualitatively analyzed on a 1% agarose gel by electrophoresis. Bands were visualized using SYBR safe fluorescent dye purchased from HiMedia.

Polymerase chain reaction: The genomic DNA samples were used as templates for amplifying the bacterial 16S rRNA gene using universal primers. The PCR was performed using a kit purchased from HiMedia (Cat. No. MBPCR094). The PCR products were qualitatively analyzed on a 1% agarose gel by electrophoresis and bands were visualized using SYBR safe fluorescent dye. These PCR samples were further purified using spin columns and the pure PCR products were outsourced for sequencing to the Barcode Biosciences, Bangalore.

DNA sequence analysis, NCBI BLAST search and Genbank deposition: Chromatograms were analyzed for base calling and the final sequence was confirmed without ambiguity by checking manually. Sequences were searched against the NCBI database using the BLASTn search algorithm. The three sequences from biriyani, panipuri curry and panipuri water were deposited in the GenBank with accession IDs: PP812682.1, PP812681.1 and PP812683.1, respectively.

Gram staining: Gram staining was performed using the kit purchased from HiMedia (Cat. No. K001) as per the manufacturer's protocol. Briefly, the crystal violet staining and the safranin counter staining were performed with a wash step in between the 2 steps. Results given in Figure 3.

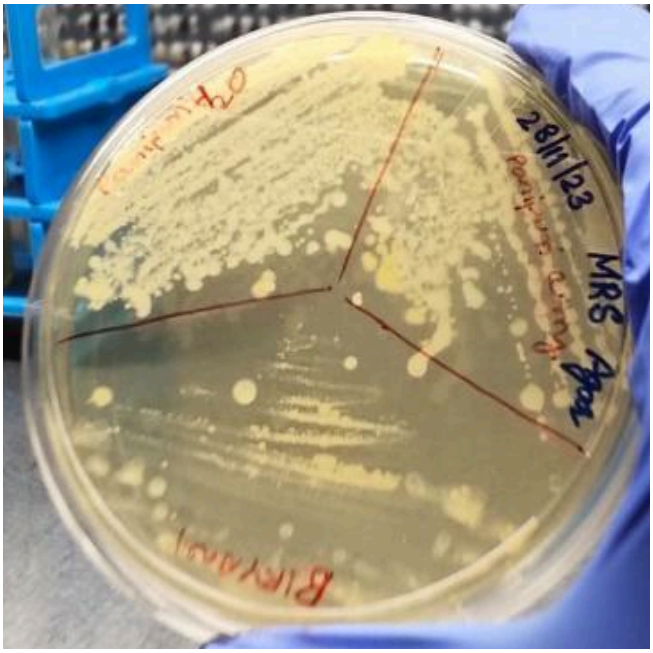

Figure 2. Bacterial colonies obtained from street food samples.

Cells were heat-fixed and finally observed under microscope to confirm the species.

Results and Discussion:

Qualitative analysis of the bacterial genomic DNA and the 16S rRNA PCR products: Agarose gel electrophoresis of the bacterial genomic DNA samples show clear and high molecular weight bands with negligible degradation suggesting that the bacterial genomic DNA in each of the three samples was intact and is suitable for serving as template to amplify the 16S rRNA gene. As shown in Figure 4, the genomic DNA from three samples was extracted and purified using both the Gram positive and Gram negative protocols provided by the manufacturer. Qualitatively no apparent differences are seen in the two protocols. Quantitative differences could be due to experimental errors.

Enterobacter hormaechei was identified as the major contaminant in biriyani samples: DNA sequence analysis using the chromatograms (Figure 5) followed by the NCBI BLASTn search was performed for the bacterial species identification. Out of the 100 hits in BLASTn search with an E-value of "0.0", 83% of the hits were *E. hormaechei* suggesting that this particular bacterium is the major contaminant in the biriyani samples. Among the remaining 17% of the hits were, *E. bugandensis*, *E. cloacae*, *E. quasihormaechei*, *E. chuandaensis* and other *Enterobacter sp.* It is noteworthy that all 100 hits belong to the *Enterobacter* genus that consists of bacteria which are capable of opportunistic pathogenicity primarily in immuno-compromised people. *Enterobacter sp.*

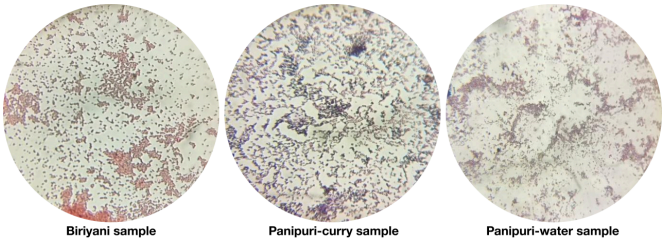

Figure 3. Gram staining results of bacteria isolated from street food samples.

have been known to cause urinary tract infections (UTIs) in humans. Summary diagram given in Figure 6.

Bacillus sp. were identified as the major contaminants in panipuri curry samples: The NCBI BLASTn results revealed that *Bacillus cereus* was the major contaminant of panipuri curry sample. Out of the 100 hits in BLASTn with an E-value of "0.0", *B. cereus* was 39% while the remaining 61% contained *B. albus* (9%), *B. thuringiensis* (8%), *B. paramycoides* (8%), *Bacillus sp. (firmicutes)* (7%), *B. paranthracis* (6%), *B. tropicus* (6%) and other species such as *B. nitratireducens*, *B. toyonensis*, *B. anthracis*, *Enterobacter cloacae*, *B. clarus* & *B. wiedmannii* (Figure 7). B. cereus has been known to cause diarrhea, vomitings and beta-hemolysis in humans that are pathogenic.

Escherichia sp. were identified as the major contaminants in panipuri water samples: Based on the NCBI BLASTn search we evaluated a list of 100 hits with an E-value of "0.0". Among these hits 80% was found to be *Escherichia coli* strains suggesting that the panipuri water was contaminated mainly with *E. coli* strains. The remaining 20% of the hits contained *Escherichia marmotae*, *Salmonella enterica*, *Aeromonas hydrophila*, *Enterobacter sp.*, *Shigella flexneri*, *Shigella sonnei*, *Atlantibacter hermannii* and other unknown bacterial species that have not been sequence-characterized by the NCBI database (Figure 8).

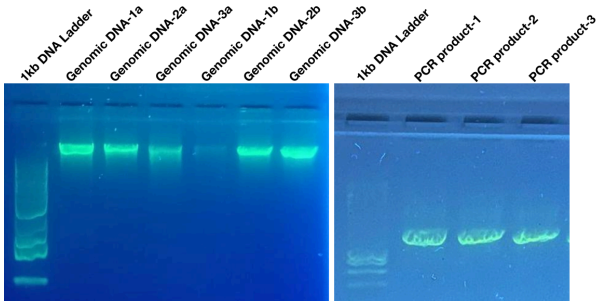

Figure 4. Qualitative analysis of bacterial genomic DNA (left panel) and the 16S rRNA PCR products (right panel).

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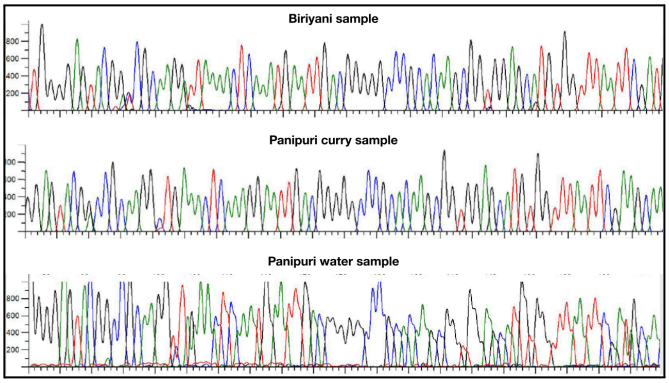

Figure 5. DNA chromatograms of the 16S rRNA genes isolated from street food samples.

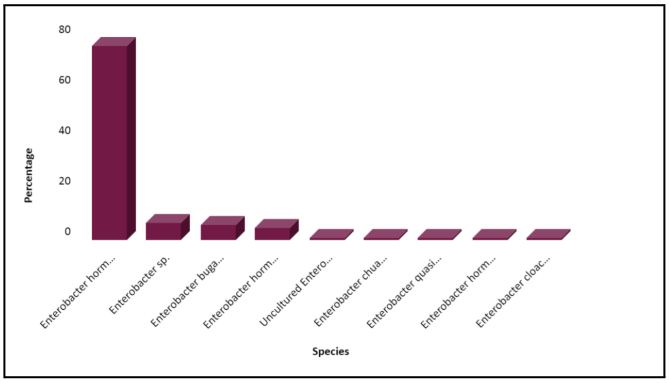

Figure 6. Percentage of identified species present in biriyani sample.

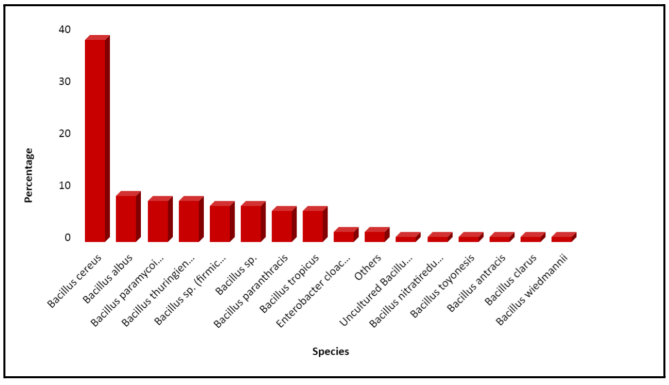

Figure 7. Percentage of identified species present in panipuri curry sample.

Conclusion and Future directions:

The current study explored the bacterial diversity in the street food samples, biriyani, panipuri curry and panipuri water, collected randomly in the city of Visakhapatnam, India and concludes that *Enterobacter hormaechei*, *Bacillus cereus* and *Escherichia sp.* as the major contaminants of street food samples, biriyani, panipuri curry and panipuri water, respectively. These bacteria will further be investigated for their antibiotic-resistance (if any) in future that will shed light on any future possible bacterial endemic/pandemic breakouts. Standard antibiotics such as amoxicillin, tetracycline, ciprofloxacin, etc. will be used for screening. This information is valuable for the government agencies to issue warnings to the citizens to protect themselves.

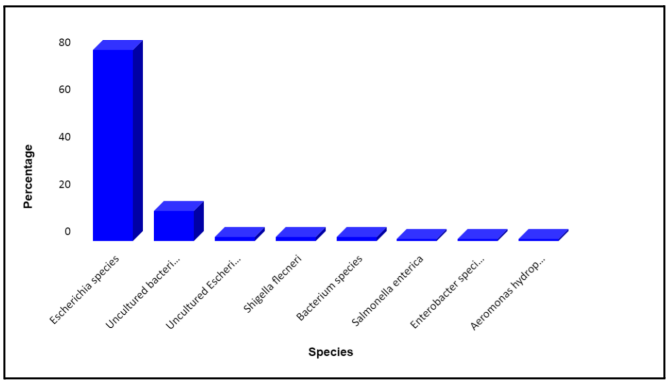

Figure 8. Percentage of identified species present in panipuri water sample.

Further, we are also going to focus on various methods of food handling/serving that may or may not be hygienic especially in the case of street foods because cooking involves boiling to high temperatures but mishandling the cooked food may introduce microbes into the food. Majority of people may be under the impression that cooked foods are safe to eat. However, mishandling the cooked food may cause contaminations. Additionally the air quality should also be checked in the future studies because bacterial spores from the street (open air) may contaminate the cooked food and the warm temperature of cooked food would provide perfect growth conditions for the bacterial spores to germinate and propagate.

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Conflict of interest: The authors declare no conflict of interest in this study. However, this research article is based on an ongoing project currently at TCABS-E, Visakhapatnam, India. We are open to collaborations.

Author contributions: B.S., L.A., M.V.S.A., H.T.M., A.S. and L.N.M. performed all wet lab experiments. J.A. and K.A. assisted in wet lab experiments. M.S. performed NCBI-BLASTn search and mentored J.A. and K.A. M.A. performed chromatogram analysis. M.S., S.A. and M.V. mentored B.S., L.A., M.V.S.A., H.T.M., A.S. and L.N.M. R.S.Y. is the principal investigator who designed the project, trained all students, secured required material for the project, provided the laboratory space and facilities needed. B.S., L.A. and M.V.S.A. wrote the draft of the manuscript with all the results. R.S.Y. wrote and edited the final version of the manuscript.

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Full figure legends:

Figure 1. Serial dilution of biriyani (left), panipuri curry (middle) and water (right) samples before (top row) and after (bottom row) incubation. Not much turbidity was seen before incubation but noticeable turbidity is seen after incubation at 37 °C overnight. The control sterile LB broth in all three cases did not show turbidity after incubation.

Figure 2. Bacterial colonies obtained from street food samples. Luxurious growth of colonies is seen for the panipuri curry and water (top right and top left sectors) while lower number of colonies were seen for biriyani sample (bottom sector).

Figure 3. Gram staining results of bacteria isolated from street food samples. Bacteria from the biriyani sample were predominantly Gram negative displaying pink color (left panel). Bacteria from the panipuri curry predominantly showed purple colored rod shaped Gram positive cells (middle panel) and the bacteria from the panipuri water sample showed a mix of both Gram positive and Gram negative with a dominant pink color (right panel).

Figure 4. Qualitative analysis of bacterial genomic DNA (left panel) and the 16S rRNA PCR products (right panel). One percent agarose gel was used for electrophoresis and bands are visualized using SYBR safe fluorescent dye. The genomic DNA samples were prepared using Gram negative (1a, 2a and 3a) and Gram positive (1b, 2b and 3b) protocols. Samples 1a/2a, 1b/2b and 1c/2c were isolated from biriyani, panipuri curry and panipuri water samples, respectively. Similarly, the 16S rRNA gene amplification PCR products 1, 2 and 3 represent biriyani, panipuri curry and panipuri water, respectively.

Figure 5. DNA chromatograms of the 16S rRNA genes isolated from street food samples. The top, middle and the bottom chromatograms belong to the biriyani, panipuri curry and panipuri water samples, respectively. The overall quality of all chromatograms is good with clear peak resolutions without much ambiguity.

Figure 6. Percentage of identified species present in biriyani sample. *E. hormaechei* was found to be the predominant species (>80%) among the other species out of the top 100 hits obtained in the NCBI-BLASTn search results.

Figure 7. Percentage of identified species present in panipuri curry sample. *Bacillus cereus* was found to be the predominant species (almost 40%) among other species out of the top 100 hits obtained in the NCBI-BLASTn search results.

Figure 8. Percentage of identified species present in panipuri water sample. *Escherichia sp.* was found to be the predominant species (80%) among other species out of the top 100 hits obtained from the NCBI-BLASTn search results.