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MOLECULAR IDENTIFICATION OF BACTERIAL SPECIES IN STREET VENDED *CHOLAY CHAT* USING 16S rRNA GENE

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ABSTRACT

The contamination of pathogenic microbes in street vended foods is a serious public health concern associated with foodborne illnesses (FBIs). The street vended *Cholay Chat* (SVCC) is a popular savory chickpea-based ready-to-eat food in Pakistan. The microbial quality of 18 SVCC samples was assessed through conventional and molecular identification. A total of three (03) bacterial species (i.e., *Bacillus cereus*, *Macrocooccus caseolyticus* and *Staphylococcus sciuri*) were characterized via phenotypic and biochemical analysis in SVCC. Among them, *B. cereus* remained highly prevalent (88.88%) followed by *M. caseolyticus* (61.11%) and *S. sciuri* (11.11%). The polymerase chain reaction (PCR) based DNA amplification of the 16S rRNA gene and sequencing of isolated bacterial species were performed. The BLASTn analysis revealed that sequences were significantly aligned with *B. cereus* (97.38%), *M. caseolyticus* (93.16%), and *S. sciuri* (97.21%). It is concluded that the microbial quality of SVCC is highly detrimental and thus is liable to cause FBIs among consumers. Therefore, safe food handling and manufacturing practices must be mandatorily assured by street food vendors to lessen the burden of FBIs among consumers.

KEYWORDS: Street vended *Cholay Chat*, molecular identification, 16S rRNA gene

INTRODUCTION

Food safety is a paramount approach to ensure safe food consumption and it subsequently prevents FBIs. According to World Health Organization (WHO), "FBIs are illnesses of toxic or infectious nature triggered by ingesting contaminated foods or water" (Havelaar *et al.*, 2015). Globally, the considerable rates of mortality and morbidity due to FBIs in many developing countries are phenomenal. In Pakistan, food safety is extremely detrimental in street vended foods (SVFs) owing to poor food handling, manufacturing, and storage practices by untrained food vendors (Saranya *et al.*, 2016; Marri *et al.*, 2019). The SVFs are ready-to-eat foods vended by a vendor/hawker in and around the street for an instant or later consumption (FAO, 1989). The Food and Agriculture Organization estimated 2.6 billion daily consumers of SVFs (Imathlu, 2017) since these foods are palatable, affordable (Marri *et al.*, 2019; Addo-Tham *et al.*, 2020) and have a wide range of variety, taste, and form, therefore, are relished by people of all age groups (Mehboob and Abbas, 2019). Conversely, SVFs are also associated with causing FBIs among consumers if are contaminated or have been prepared under poor preparatory conditions (Surujlal and Badrie, 2004).

Around 250 types of FBIs have been identified (Mangal *et al.*, 2016) while according to WHO (1998), 10 to 20 % of outbreaks of FBIs are associated with poor hygienic practices and poor personal hygiene of food workers. According to Havelaar *et al.* (2015), the chief causative agents of FBIs are bacteria followed by chemical agents, viruses, parasites, etc. In Pakistan, SVCC (Table 1, Figure 1) is an exceptionally popular, palatable, and savory snack food prepared from boiled chickpeas, chopped raw vegetables (onion, green chili, tomato, coriander, and mint leaves, etc.), boiled chopped potatoes, yogurt, tamarind paste, salt, and chat masala, etc. According to Hassan *et al.*, (2022), food safety is the major limiting factor during food processing in developing countries. In Pakistan, the food safety of SVFs is not guaranteed (Figure 2). Therefore, food safety risks of SVFs have been rising due to contamination of pathogenic microorganisms or hazardous chemical agents (Proietti *et al.*, 2014; Ankar-Brewoo *et al.*, 2020; Verma *et al.*, 2023). Recently, bacteria such as *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella spp.*, *Shigella spp.*, *Staphylococcus aureus*, etc. have been linked to several foodborne outbreaks. Therefore,

suitable methods of microbial identification, controlling the spread of pathogenic microbial species, and incidences of FBIs are crucial to safeguard public health (Li and Zhu, 2017). The conventional methods of microbial identification are key factors in evaluating the food safety and quality of SVFs. This provides information about hygiene practices by food handlers during food preparation while in molecular characterization the 16S rRNA gene sequencing has revolutionized bacterial taxonomy and has been increasingly used for identifying pathogenic bacterial species. The 16S rRNA gene sequencing is a time-effective technique that provides rapid identification of pathogens (Barbut *et al.*, 2011). The sequencing of the 16S rRNA gene is applicable for bacterial quantization and engages the hybridization of small-sized ribosomal subunit genetic marker sequences. These sequences contain hypervariable regions with region-specific sites in sequencing units and allow differentiation among bacterial species. Hence, 16S rRNA gene

sequencing is one of the versatile tools to determine bacterial phylogeny, etc. (Větrovský *et al.*, 2013). The present study was therefore conducted to identify the types of presumptive pathogenic bacterial species present in SVCC samples initially by conventional method and for further authentication by molecular characterization i.e., 16S rRNA gene analysis.

MATERIALS AND METHODS

Sample collection: A total of 18 SVCC samples were procured randomly from the main public areas of district Hyderabad during summer (44±2°C). Approximately, 100 grams of each sample was taken in a sterile glass jar, kept in an ice pack cooler, and instantly transported to the Food Microbiology Lab at the Institute of Food Sciences and Technology, Sindh Agriculture University, Tando Jam for microbial isolation and molecular characterization of pathogenic bacterial species.



Figure 1 Street Vended Choley Chat



Figure 2 Food safety condition in and around SVCC stall in Hyderabad, Pakistan

Table 1 Street vended Choley chat, its ingredients, preparation, serving methods with possible routes of contamination

| Ingredients | Method of preparation | Methods of serving practiced by vendors | Possible routes of contamination |
|--|---|--|--|
| Chickpeas, yogurt, onion, green chili, tomato, boiled potato, cucumber, lemon juice, mint, coriander leaves, chat masala (a pounded mixture of different spices), salt, red chili flakes, and tamarind paste, etc. | The chickpeas are soaked in water (4-6 hrs.) and boiled till soft. All vegetables are chopped finely and are added with boiled chickpeas, chat masala, salt, red chili flakes, whipped yogurt, tamarind paste, and lemon juice. | Served on plates with spoons. Served on pieces of magazine, book, or newspaper. Parceled in polyethylene bags. | Improperly washed or sometimes unwashed vegetables Improperly washed serving utensils Poorly cleaned cooking and vending area Poor personal hygiene of vendor /food processor |

Microbial analysis: For microbial analysis, 10g of each SVCC sample was properly homogenized with 90 ml of buffered peptone water. The homogenate was used for preparing serial dilutions (i.e., 1:10⁶, 1:10⁷, and 1:10⁸). Each dilution was poured on NAM (nutrient agar medium) and placed in an incubator at 37°C for 24 hours. The suspected bacterial colonies were selected according to Leisner *et al.* (1997), sub-cultured for purifying isolates on specific media, and incubated for 24 hours at 37°C. The appeared bacterial colonies were examined microscopically according to Shangpliang *et al.* (2016) while preservation of bacterial isolates was done in an appropriate broth mixed with glycerol (20 %) at -20°C as described by Costa *et al.*, (2019).

Phenotypic identification: All bacterial isolates were examined for morphological characteristics, Gram-staining, and motility tests as per the method by Ravelo *et al.* (2001).

Biochemical identification: The biochemical identification was carried out as per the method described in Bergey's manual of determinative bacteriology. The biochemical tests i.e., catalase, coagulase, hydrogen sulfide (HS) production, indole, methyl red (MR), oxidase, Simmons's citrate (SC), triple sugar iron (TSI), urease, and Voges-proskauer (VP) tests were performed.

DNA extraction from cultured bacteria: A DNA Purification Kit (GeneJET Genomic, Thermo scientific, USA) was utilized for genomic DNA extraction from cultured bacteria as per instructions of the manufacturer. Briefly, the bacterial cells (up to 2 x 10⁹) were harvested by centrifugating them at 5000xg for 10 minutes. The supernatant was discarded while the pellet was carefully suspended in 180µl of digestion solution. A 20µl of proteinase K solution was also added and the mixture was vortexed to obtain uniform suspension. The samples were subjected to incubation at 56°C in a shaking water bath until bacterial cells were lysed completely. After that, 20µl of RNase A solution was added and the content was incubated for 10 minutes at ambient temperature. Afterward, 200µl of lysis solution was added to the sample, and thoroughly vortexed for 15 seconds, 50% ethanol was added, and the lysate was transferred to GeneJET

Genomic DNA purification column for DNA binding and was centrifuged at 6000xg for 60 seconds. The collection tube containing the flow-through was discarded while the column was placed in a new collection tube. The bound DNA was washed using 500µl of wash buffer II by centrifugation for 1 minute at maximum speed. Finally, DNA was eluted with 200µl elution buffer, and the concentration of the extracted DNA was checked by NanoDrop Spectrophotometer (The Thermo Scientific NanoDrop™ 1000 Spectrophotometer) and was stored at -20 °C.

PCR and sequences analysis

Composition of PCR-mixture: The PCR mixture consisted of 12.5µl master mix (GoTaq1 Green Master Mix, Promega, Madison, WI, USA), 2µl primer 1, 2µl primer 2, 25pmol of each primer, 1µl of DNA template, and 7.5µl nuclease-free water.

PCR amplification and sequences analysis: The PCR amplifications of extracted bacterial genomic DNA were achieved by using the 16S rRNA gene. The 16S rRNA universal primers (16F-ACGCGTCGACAGAGTTTGATCCTGGCT and 16R-GGACTACCAGGTATCTAAT) were utilized for the amplification of the 16S rRNA gene. The PCR amplification was done in a thermal cycler (Applied Biosystem 2720, USA) under these cyclic conditions; initial denaturation at 94°C for 3 minutes followed by cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds whereas final extension was performed at 72°C for 5minutes. The purification of PCR products was performed by using the GeneJET PCR Purification Kit (Thermo scientific, USA) as per the instructions by the manufacturer. The purified PCR product was sent to the Molecular Biology Laboratory at Liaquat University of Medical and Health Sciences (LUMHS), Jamshoro, Pakistan for sequence analysis. The sequencing process was performed using an ABI 3130XL Genetic Analyzer, which is a 16-capillary DNA sequencer manufactured by Applied Biosystems. The reference sequences were retrieved from GenBank with the BLAST system (http://www.ncbi.nlm.nih.gov/BLAST/BLAST_databases.html), all sequences were aligned and were compared with reference sequences retrieved from

GenBank by using tools of bioinformatics (i.e., Clustal X and Mega Version 6.0 program).

Results

All eighteen (18) samples of SVCC were contaminated (100 %) with different pathogenic microorganisms. Three (03) species of bacteria were isolated from SVCC i.e., *B. cereus*, *M. caseolyticus*

and *S. sciuri*. Table 2 indicates that *B. cereus* remained the most prevalent bacterial species in SVCC as it recovered from 16 (88.88 %) samples. However, *M. caseolyticus* was observed in 11 (61.11 %) samples, and *S. sciuri* was merely noted in 02 (11.11 %) samples.

Table 2 Bacterial species isolated from SVCC samples

| Bacterial species isolated | Percentage (%) | Frequency |
|----------------------------|----------------|-----------|
| <i>B. cereus</i> | 88.88 | 16 |
| <i>M. caseolyticus</i> | 61.11 | 11 |
| <i>S. sciuri</i> | 11.11 | 02 |

Phenotypic Identification: The isolated bacterial species were identified phenotypically, and it was found that these species were closely presumptive to *Bacillus spp.*, *Micrococcus spp.*, and *Staphylococcus spp.* The phenotypic characters of probable bacterial species are presented in Table 3. The *Bacillus spp.* was found as Gram-positive, motile, and exhibited rods of multiple sizes. The cells were single or arranged in chains (short or long). The colonies appeared round with white color, flat or raised elevation, undulated margin, and rough surface. The *Micrococcus spp.* was

Gram-positive, non-motile and cells appeared large cocci arranged singly or in pairs. The colonies appeared circular, and white and medium-sized, with convex elevation and entire margin. The presumptive *Staphylococcus spp.* remained Gram-positive, non-motile, large cocci and were arranged singly, paired, or as tetrads. The cultural properties exhibited large grape-like clusters, with greyish-white colonies having undulate margins, raised elevation, and glistening smooth surfaces (Table 4).

Table 3 Morphological and staining properties of bacterial species isolated from different SVCC

| Bacterial species | Size and shape of bacterial cell | Bacterial cell arrangement | Gram staining | Motility |
|------------------------|---|-----------------------------------|---------------|------------|
| <i>B. cereus</i> | Short and long rods | Chains | +ve | Motile |
| <i>M. caseolyticus</i> | Medium to large cocci, 1.0 to 1.5µm diameter | Single, paired clusters | +ve | Non-motile |
| <i>S. sciuri</i> | Marginally curved cocci, 0.5 to 1.0µm in diameter | Single, paired, irregular cluster | +ve | Non-motile |

Table 4 Cultural characteristics for the identification of bacterial species isolated from different SVCC

| Bacterial species | Colony characteristics on BA/BHIA | Color of colonies |
|------------------------|---|--------------------------|
| <i>B. cereus</i> | Colonies with undulate margins/ irregular and wrinkled colonies | White |
| <i>M. caseolyticus</i> | Undulate and smooth colonies/ circular and irregular colonies | Greyish-white and yellow |
| <i>S. sciuri</i> | Circular colonies/ smooth round colonies with regular edges | White or greyish white |

Biochemical Identification: The biochemical properties of the presumptive organisms are given in Table 4. All three bacterial isolates showed positive results for catalase and urease tests, found non-reactive for VP and SC tests while HS production was not observed in any bacterial isolate. *B. cereus* remained non-reactive for oxidase, indole, and MR tests while for the TSI test both organisms produced an alkaline

slant and alkaline butt. The *M. caseolyticus* remained positive for the oxidase test while it was coagulase negative. The TSI test produced an alkaline slant and acidic butt in the case of *M. caseolyticus*. The *S. sciuri* was coagulase and MR positive, oxidase and indole negative while producing an acidic slant and acidic butt on the TSI test.

Table 5 Biochemical properties of bacterial species isolated from SVCC samples

| Bacterial Species | Biochemical tests | | | | | | | | | |
|------------------------|-------------------|------|------|------|-----|------------------|------|-----|-----|-------|
| | Cat. | Coa. | Oxi. | Ind. | TSI | H ₂ S | Ure. | MR | VP | Scit. |
| <i>B. cereus</i> | +ve | - | -ve | -ve | K/K | -ve | +ve | -ve | -ve | -ve |
| <i>M. caseolyticus</i> | +ve | -ve | +ve | - | K/A | -ve | +ve | - | -ve | -ve |
| <i>S. sciuri</i> | +ve | +ve | -ve | -ve | A/A | -ve | +ve | +ve | -ve | -ve |

Cat.= Catalase test, **Coa.**= Coagulase test, **Oxi.**= Oxidase test, **Ind.**= Indole test, **H₂S**= Hydrogen sulfide production, **Ure.**= Urease test, **MR**= Methyl red test, **VP**= Voges-proskauer test, **Scit.**= Simmons's citrate test, -

= not performed, +ve= Gram positive, -ve= Gram negative, A/A= Acidic slant and acidic butt, K/A= Alkaline slant and acidic butt, K/K= Alkaline slant and alkaline butt

Molecular identification: Amplicon of the 16S rRNA gene was amplified successfully by PCR from genomic DNA isolated from the bacterial culture of SVCC with specific primers as mentioned above and a fragment of the correct size of 766 bp was obtained (Figure 2).

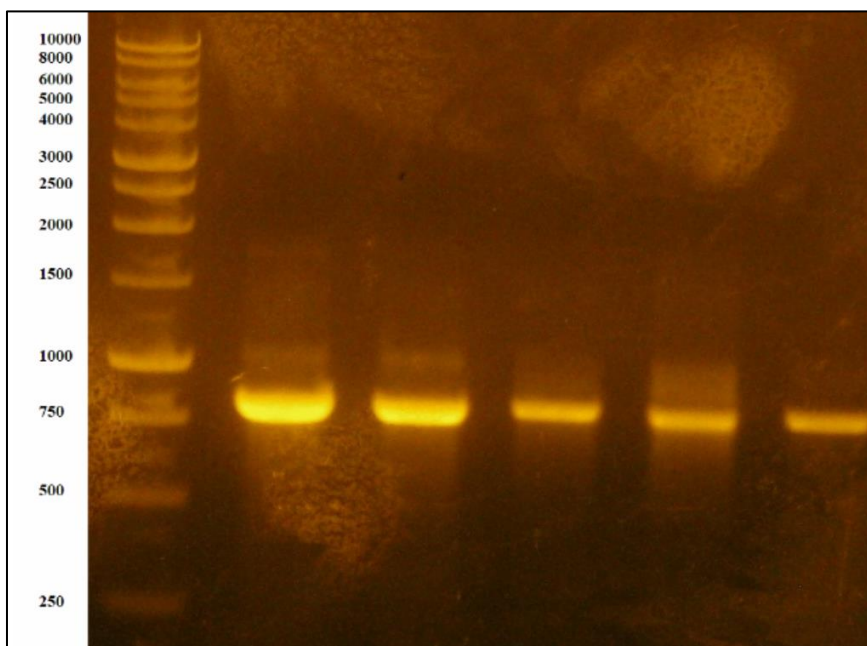


Figure 3 Amplified PCR product of bacterial 16SrRNA gene isolated from bacterial culture of SVCC

Sequence analysis: The purified PCR products were sent to the Molecular Biology Laboratory, LUMHS for sequence analysis and confirmed as 16S rRNA of *B. cereus*, *M. caseolyticus*, and *S. sciuri*. The recovered information is presented below:

***B. cereus*:** The sequence of 16S rRNA was confirmed as *B. cereus* by BLASTn analysis and was aligned significantly (97.38 %) to *B. cereus* 16S rRNA genes (Figure 4A). The lineage report (LR) from BLASTn analysis exhibited 89 hits that were associated with the 16S rRNA gene of Bacillus whereas 47 hits out of 89

hits were associated with *B. cereus* (Figure 4B). The results for multiple sequence alignment (MSA) and phylogenetic tree (PT) in Figure 4C and 4D indicated that 16S rRNA genes of isolated *B. cereus* are closely associated with MK045766.1 (Malaysian strain), KC867315.1 (Brazilian strain), MH778050.1, MH778042.1, MG705999.1, KF387719.1 (Indian strains), MH368501.1 (Chinese strain), MF767513.1 (Moroccan strain), and KP050499.1 (Swiss strain). Silico analysis (SA) of the 16S rRNA sequence confirmed and validated the data obtained from phenotypic and biochemical analysis.

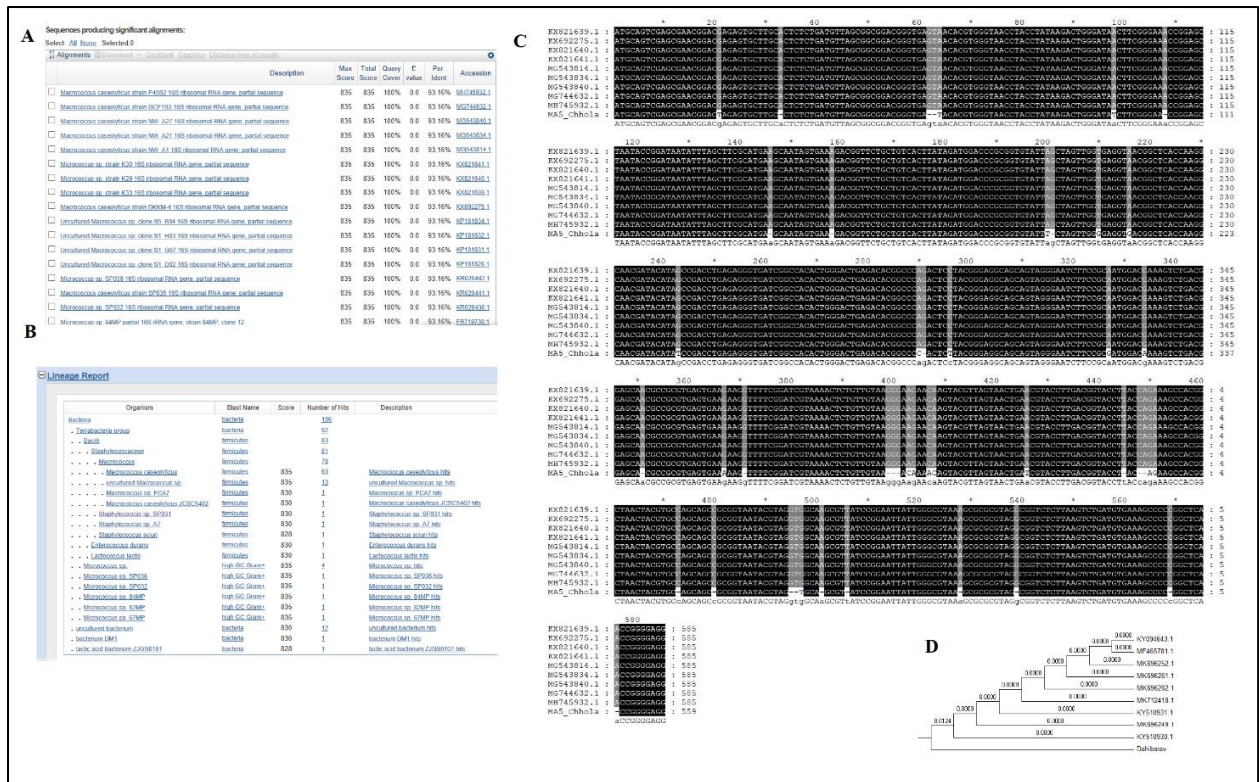


Figure 5 Sequence analysis of *M. caseolyticus* recovered from SVCC. (A) BLASTn analysis showing alignment (93.16%) with *M. caseolyticus* (B) LR indicating 63 hits with *M. caseolyticus* (C) MSA of isolated 16S rRNA sequence with reference sequences (D) Taxonomic relationships of *M. caseolyticus* based on PT

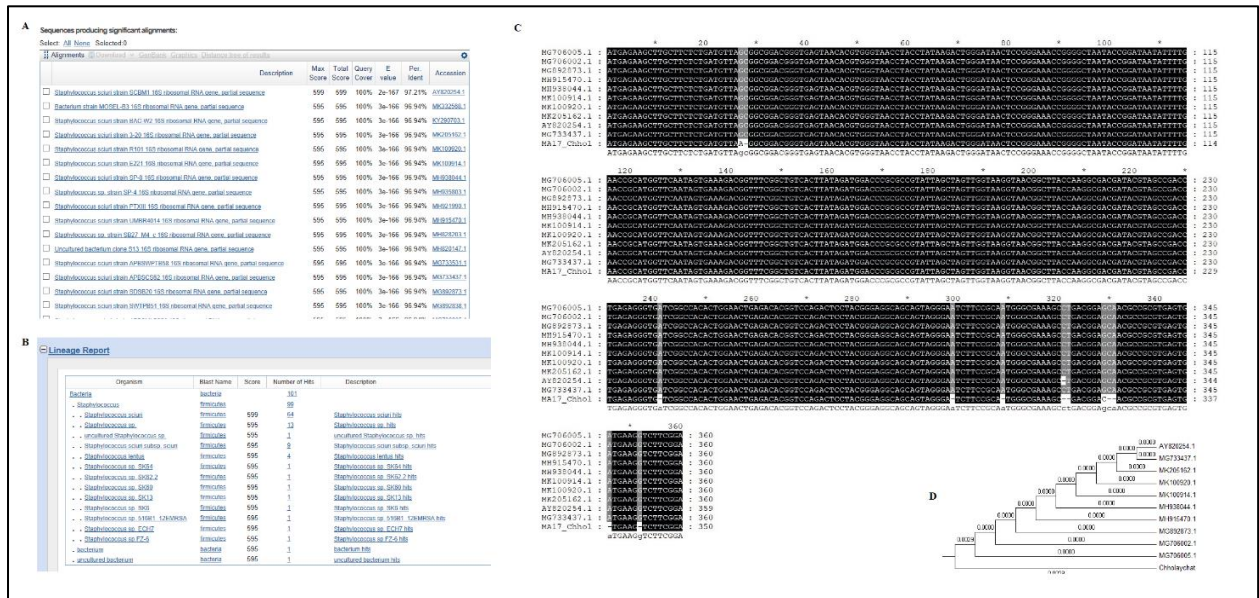


Figure 6 Sequence analysis of *S. sciuri* recovered from SVCC. (A) BLASTn analysis showing alignment (97.21%) with *S. sciuri* (B) LR indicating 64 hits with *S. sciuri* (C) MSA of isolated 16S rRNA sequence with reference sequences (D) Taxonomic relationships of *S. sciuri* based on PT

DISCUSSION

The scientific literature for the isolation and molecular characterization of pathogenic bacteria from SVCC samples is scanty. The SVCC is a traditional savory snack food sold by vendors on the streets and is extensively popular among the dwellers of Pakistan

owing to its palatability. It is a fact that the safety practices for handling, preparing, and serving the SVCC are poorly implemented by the street food vendors (Marri *et al.*, 2019) which may lead to microbial contamination and subsequently the outbreaks of FBIs among the consumers. The SVCC is

a minimally cooked snack merely chickpeas are boiled in this food while other ingredients are added without any cooking treatment which favors the growth of problematic microorganisms. Khasnabis *et al.* (2017) state that factors involved in enhancing microbial activity may be intrinsic (water activity, pH, and nutrient content) and extrinsic (temperature and humidity). The unsafe food handling and manufacturing practices coupled with the unhygienic personal condition of street vendors are also a deal of concern in microbial contamination.

In the present findings, microbial contamination was observed in SVCC, which is indicative of ultimate poor safety practices followed by the vendors. The results of morphological and biochemical characterization exhibited the contamination of *Bacillus spp.*, *Micrococcus spp.*, and *Staphylococcus spp.* in SVCC. The conventional methods of microbial identification are not adequately sufficient since are not rapid, less sensitive, and lack specificity (Lui *et al.*, 2019). Instead, among molecular methods of identification the nucleic acid-based method i.e. PCR signifies a powerful tool owing to its high throughput analysis (Kralik and Ricchi, 2017). The conventional/traditional biochemical tests are not accurate for bacterial identification at the species level in comparison to molecular methods. Using 16S rRNA gene sequence analysis is more reliable than other ribosomal regions for distinguishing species and sub-species levels of bacterial pathogens (Rohinishree and Negi, 2011). During molecular identification of bacterial isolates, DNA extraction is the initial phase, and extraction of high-quality DNA is the foremost prerequisite to assure successive DNA amplification (Cremonesi *et al.*, 2014). Primers and probes confirm sensitive and specific microbial detection via amplification. For the identification of bacteria, the 16S rRNA gene has been used at the bacterial species level. Therefore, the presumptive bacterial species were further subjected to PCR-based molecular identification and were characterized as *B. cereus*, *M. caseolyticus*, and *S. sciuri*. In a similar study, Lui *et al.*, 2019 recovered 12 food-borne bacterial pathogens from different food and drinking water samples including *β-streptococcus hemolyticus*, *C. jejuni*, *E. coli O157:H7*, *E. faecalis*, *L. monocytogenes/ivanovii*, *P. mirabilis*, *S. aureus*, *S. enterica*, *Shigella spp.*, *V. fluvialis*, *V. parahaemolyticus* and *Y. enterocolitica* using TaqMan real-time PCR assay.

The molecular identification based on 16S rDNA revealed that among the isolated species, *B. cereus* remained the most prevalent bacterial species in SVCC followed by *M. caseolyticus* and *S. sciuri*. The *Bacillus* species are food spoilage organisms that have great efficiency in exhibiting outbreaks of FBIs (Tshipamba *et al.*, 2018). Genetically, *B. cereus* exhibits highly variable pathogenic potential (Ehling-Schulz *et al.*, 2019) and is a main foodborne pathogen that may provoke food poisoning. *B. cereus* is a potent pathogen and has implicated many food poisoning outbreaks

globally. Several studies have reported the occurrence of *B. cereus* from different snacks and street-vended foods (Das *et al.*, 2009; Suneetha *et al.*, 2011; Hafeez *et al.*, 2012; Tshipamba *et al.*, 2018). Khasnabis *et al.* (2017) ascertained the presence of *B. cereus* in three popular Indian pulse-based traditional snack foods i.e. *soan papdi*, *laddu*, and *ghugni* above the permissible limit. The SVCC samples were found contaminated with *M. caseolyticus*. Generally, *M. caseolyticus* is considered non-pathogenic for humans, however, a recent study by Li *et al.*, (2018) suggests that a strain of *M. caseolyticus* isolated from commercial broiler chicken in China i.e., *M. caseolyticus SDLY* can cause high mortality rate. According to Tshipamba *et al.*, 2018, the presence of *M. caseolyticus* in food samples is linked to poor handling practices of vendors and exposure of food items to dust.

S. sciuri is emerging as a human pathogen and it widely occurs in dairy industries. It has been isolated from the body of farm animals, pets, and humans, cow milk, and various food products (Piessens *et al.*, 2012). Its presence in SVCC samples indicates its contamination possibly due to i) improper food handling and manufacturing practices, ii) transmission through vendors, or iii) soil particles. Goyal *et al.* (2019) also isolated *S. sciuri* strain *KM16* from the soil via genome analysis while Rohinishree and Negi (2011) also recovered *S. sciuri* from different food samples.

Conclusion

It is concluded that SVCC is detrimental owing to the limitation of safety practices by vendors, poorly sanitized vending areas, and personal hygiene of food personnel since the PCR-based molecular assay confirmed the presence of some problematic bacterial species in SVCC i.e., *B. cereus*, *M. caseolyticus*, and *S. sciuri*. The occurrence of these bacterial isolates is an ultimate sign of the frequent epidemics of FBIs among the consumers of SVCC. Therefore, the governmental regulatory bodies should play a pivotal role in ensuring the well-being of consumers by enforcing and overseeing the implementation of proper handling, manufacturing, and storage practices among street food vendors.

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