ORIGINAL ARTICLE

MICROBIOLOGICAL QUALITY AND SAFETY ASSESSMENT OF FRESHLY PREPARED FRUIT JUICES SOLD IN DIFFERENT AREAS OF LAHORE, PAKISTAN

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Abstract

Fruit juices are among the best food products with respect to the nutritional applications. However, mishaps can come along the way, as bacteria can cause spoiling of fruit juices through contaminated raw materials, extraction equipment and malpractice of handlers and results into the deleterious health effects including skin infections, allergies, diarrhea and chronic inflammation. Fruit juices can be susceptible to contamination by various bacterial strains, such as Streptococcus spp., Escherichia coli, Candida albicans, Staphylococcus aureus, Vibrio cholera, etc., leading to potential health risks. In the present study, a total of 164 bacterial strains were isolated from nine samples of fresh fruit juices (Carrot, Orange and sugarcane) from different areas in Lahore. Three media i.e., Nutrient agar, MacConkey agar and Eosin Methylene Blue agar (EMB) were used for enumeration and isolation of various bacterial genera. Among the total 164 isolated bacterial strains, 36 were recovered from sugarcane juice samples, 91 from carrot juice samples and 37 from orange juice samples. The most frequent strains were E. coli and Enterobacter spp. which were most likely due to the contamination from water. A panel of 10 antibiotics including Erythromycin, Ceftizoxime, Ciprofloxacin, Tobramycin, Vancomycin, Ampicillin, Streptomycin, Polymyxin B, Levofloxacin, and Cephalexin was used to determine the antibiotic sensitivity (AST) status of the isolated strains. The sensitivity pattern was recorded by measuring the inhibition zone size in millimeters (mm). It was observed that Levofloxacin showed highest zones of inhibition against all the tested strains and overall, strains didn’t have significant resistance against the tested antibiotics.

Keywords
Antibacterial Susceptibility Testing;
Bacterial strains; Fruit juice; Lahore;
Microbial quality

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The practice of proper hand and fruit washing can minimize the potential threat of bacterial contamination in food products.

1. INTRODUCTION

Fruit juice is a drink made from the extraction or pressing of the natural liquid contained in fruit and vegetables (Naseem et al., 2023). Fruit and vegetable juices are consumed for their high nutritional value, revitalizing properties, and medicinal importance (Pavlović et al., 2023). Due to their high vitamin C content, antioxidants, and polyphenol content as well as their low sodium, fat, and cholesterol levels, consumer demand has increased in the past 10 years (Khazaei et al., 2023). The use of fruit juices boosts the economy, but it also raises problems like rotting and disease interruptions. Fruit juices consumption positively impacts the economy but also poses issues like spoilage and disease disruptions (Mandha et al., 2023; Moulin, 2023).

The disease-causing microorganisms come into contact with fruit juice due to any inappropriate handling of fruits, as when food is prepared with moist hands, the physical contact facilitates the spread of microorganisms (Kowalska, 2023; Tenea et al., 2023). During the development of fruit, pathogenic microbes may penetrate them and cause disease thus, these juices could potentially harbor infections acquired from raw materials, juice-extraction equipment, handlers, and contaminated environments (Hassan et al., 2023). Though Salmonella, Staphylococcus, and entire coliform bacteria are abundant in the raw, unprocessed fruit juices sold on the street but the main pathogenic adulterants in fruit juices are strains of Streptococcus, Escherichia coli, Candida albicans, Staphylococcus aureus, and Vibrio cholera (Noumavo et al., 2023). Moreover, juices have now become the most common means of transmission of microbes like E. coli, Cryptosporidium spp. and Salmonella spp. (Mousanejadi et al., 2023).

Several spoilage bacteria are significantly interested in the fruit juice industry (Karanth et al., 2023). A microbe known as Alicyclobacillus acidoterrestris has been isolated from certain juices and the product of juices with the described incidence are in the range of 14 to 18%. Fruit juice spoilage has been attributed to temperature resistant microbes such as Neosartorya fischeri, Byssochlamys fulva, and Propionibacterium cyclohexanicum (Walker & Phillips, 2007). Propionibacterium cyclohexanicum, a Gram positive bacteria was isolated from orange juice for the first time in 1993 (Lim & Ha, 2021).

The public health has been severely damaged by microorganisms that are resistant to antibiotics. World Health Organization (WHO) has categorized bacteria based on their ability to resist antibiotics which includes E. coli, K. pneumonia, S. aureus, S. pneumonia, A. baumannii, P. aeruginosa etc. Contrary to the rising prevalence of bacterial resistance, the development of some advanced antibiotics remains constrained. The current demand is to develop substitutional methods that counter the activity of microorganisms (Choi et al., 2023; Numan et al., 2022).

This study aimed to assess bacterial count, isolate and identify bacteria from contaminated fruit juices, and evaluate antibiotic sensitivity. The research systematically examined microbial abundance, identified prevalent strains, and explored antibiotic resistance profiles. The findings significantly contribute to understanding the microbiological integrity of fruit juices, informing public health implications. These insights not only enhance our understanding but also serve as a basis for implementing specific measures to reinforce consumer health and adhere to stringent food safety standards, emphasizing the importance of microbiological aspects in fruit juice safety.

2. METHODOLOGY

Sample Collection

The samples of sugarcane, carrot and orange juices were collected in triplets in sterile bottles from the different areas of Lahore including Model Town, Samanabad and Gulberg. The samples were transported to the laboratory within two hours of collection and subjected to further processing.

Isolation of Bacteria from Juice Samples

Serial dilution method was used in which six-fold dilution of each sample (i.e., sugarcane, carrot and orange juice)
were prepared (10<sup>-1</sup> to 10<sup>-6</sup>) and 25µl of sample from the dilutions 10<sup>-3</sup> and 10<sup>-4</sup> were spread on different agar media. The agar media used included Nutrient agar (1.2% agar, 0.5% sucrose, 4 mM KNO<sub>3</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.3 mM MgSO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 89 µM iron citrate, 46.3 µM H<sub>2</sub>BO<sub>3</sub>, 9.1 µM MnCl<sub>2</sub>, 0.77 µM ZnSO<sub>4</sub>, 0.31 µM CuSO<sub>4</sub>, and 0.11 µM MoO<sub>3</sub>, pH 7.1) (Van Der Weele <i>et al.</i>, 2000), MacConkey agar (g/l: peptone 17, protease peptone 3, lactose 10, NaCl 5, crystal violet 1, neutral red 30, bile salts 1.5, distilled water up to 1000 mL, agar 13.5, pH 7.1) and Eosin Methylene Blue agar (g/l: agar 13.50, Eosin-Y 0.40, Methylene blue 0.065, lactose 5.0, sucrose 5.0, dipotassium phosphate 2.0, peptic digest of animal tissue 10.0). All the plates were incubated for 24 hours at 37°C.

**Determining Colony Forming Units (CFU)**

A 25 µl sample from dilutions 10<sup>-3</sup> and 10<sup>-4</sup> was spread over different media, and CFUs per ml were counted using a colony counter on the spread plates.

**Morphological, Physiological, and Biochemical Characterization of The Isolated Strains**

For taxonomic identification, isolated bacteria were subjected to various morphological, physiological, and biochemical analysis. Both microscopic and macroscopic characters were observed in morphological characterization. Colony size, color, texture, shape, margins, and pigment production were observed macroscopically. While microscopically, colonies were stained with Gram-staining reagent to distinguish between Gram-negative and Gram-positive bacteria. The biochemical characteristics were determined by various tests including oxidase test, citrate test, indole test, methyl red, Voges-Proskauer test and catalase test.

**Determination of Antibiotics Sensitivity (AST)**

The antibiotics sensitivity status of the isolated strains was determined by using representative antibiotics of almost all the major classes, following the Kirby-Bauer disc diffusion assay. In the Petri plates, the Muller-Hinton (MH) agar was poured (g/l: Casein Hydrolysate 17.5, beef extract 2.0, starch 1.5, agar 17.0, pH 7.3±0.2) (Barry & Effinger, 1974). The 24-hours fresh bacterial culture (adjusted to 0.5 McFarland) was swabbed on media plates with the help of sterile cotton swab. With the help of sterile forceps, selected antibiotic discs were then placed on the agar at appropriate distance to avoid overlapping and the plates were incubated for 24 hours at 37°C. The plates were examined after the incubation period. A lack of a clear zone around the discs indicated antibiotic resistance, while the presence of a zone suggested sensitivity.

3. RESULTS

**Morphological and Physiological Characteristics**

The colonies, collected from various media such as N-agar, MacConkey agar, and EMB agar, exhibited diverse morphological properties. Morphological observations included the shape (irregular, circular, rhizoid, filamentous), elevation (flat, raised, concave, convex), margins (undulate, entire, lobate, curled, filiform), color, and size of the colony. The size variations were categorized as pinpoint, small, moderate, and large colonies as shown in Figure 1.

![Figure 1: Bacterial colonies grown on (A) EMB agar (B) MacConkey agar (C) Nutrient agar.](image-url)
Colony Forming Units per ml (CFUs)

Colony forming units of each sample was determined by spreading 25 µl sample on different media by serial dilution of 10⁻¹ and 10⁻³. The minimum CFUs from sugarcane juice samples with serial dilution of 10⁻⁴ obtained were 07 on nutrient agar while maximum CFU/ml was 220 on MacConkey agar. The minimum CFU/ml from carrot juice samples were 59 while maximum were 254 whereas from orange juice samples the minimum and maximum CFU/ml were 87 and 228, respectively.

Biochemical and Physiological Characteristics

Every single isolated strain was subjected to various morphological and physiological features (Table 1) and biochemical tests including Catalase, Oxidase, Methyl red, Voges-Proskauer, Starch hydrolysis and Citrate test and the biochemical profiles of strains were recorded (Table 3). As three types of fruit juices—sugarcane juice, carrot juice, and orange juice—were utilized, a total of 164 bacterial strains were isolated. From the sugarcane juice, the total of 36 purified strains were isolated with predominant bacteria Enterobacter, as 13 (35.1%) strains were identified as Enterobacter. In carrot juice, E. coli outnumbered other bacterial strains, accounting for 20 (22%) out of the total 91 strains. Whereas, in the case of orange juice, 37 purified strains were isolated and Enterobacter (27.7%) was predominant bacteria (Table 2).

Table 1: Morphological characteristics of isolated strains.

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>Colony Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus</td>
<td>Shape: Circular, Elevation: Convex, Margin: Undulate, Color: Creamy yellow, Size: Small</td>
</tr>
<tr>
<td>E. coli</td>
<td>Shape: Rods, Elevation: Flat, Margin: Entire, Color: Metallic green sheen, Size: Small</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>Shape: Rods, Elevation: Flat, Margin: Irregular, Color: Off-white, Size: Large</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>Shape: Rods, Elevation: Raised, Margin: Smooth, Color: Pink, Size: Medium</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>Shape: Rods, Elevation: Flat, Margin: Irregular, Color: Yellow, Size: Small</td>
</tr>
</tbody>
</table>

Table 2: Frequency of selected bacterial strains in various juice samples.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Sugarcane juice (n=36)</th>
<th>Carrot juice (n=91)</th>
<th>Orange juice (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus</td>
<td>10</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>E. coli</td>
<td>3</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>2</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacteriaceae spp</td>
<td>13</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 3: Biochemical characteristics of selected isolated strains.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Biochemical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS</td>
</tr>
<tr>
<td>Bacillus</td>
<td>+</td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacteriaceae spp</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: Gram Staining, Catalase Test, Oxidase test, Starch Hydrolysis, Methyl Red, Citrate Utilization, Indole Production.
Gram-positive bacteria exhibited violet color while Gram-negative bacteria showed red color in the Gram staining protocol. In the oxidase test, the bacteria which showed blue color after oxidase production was represented as positive while no color was declared as a negative result. In the Citrate utilization test, the color of the medium changes from green to blue exhibited positive results and vice versa. The formation of the red ring in the test tube showed a positive indole test and the formation of the yellow ring in the test tube indicated a negative indole test. A change of color from yellow to red indicated a positive MRVP test and vice versa. In the catalase test, bubble formation with the addition of H₂O₂ indicated a positive catalase test and no bubble formation was announced as a negative catalase test (Figure 2).

**Figure 2:** Biochemical characteristics of the bacterial strains isolated from three different juices.

**Antibiotic Sensitivity of Strains Isolated from Different Juice Sample**

The antibiotics sensitivity and resistance of isolated strains of sugar cane, carrot and orange juice samples were checked on different agar media including MacConkey, nutrient and EMB agar by using various antibiotics including Vancomycin, Ampicillin, Streptomycin, Polymyxin B, Levofloxacin, Cephalexin, Erythromycin, Ceftizoxime, Ciprofloxacin and Tobramycin on different media. During antibiotic sensitivity testing, 16 representative strains were obtained from sugarcane, carrot, and orange juice samples were grown on Nutrient agar and showed Gram-positive characteristics. 8 of these strains showed resistance to vancomycin (VA), eight to ampicillin (AM), and just one to streptomycin (S). Additionally, 23 strains from aforementioned samples were grown on MacConkey's agar at the same time, displaying Gram-negative characteristics. Only one strain in this group was resistant to cephalexin (CN), but two strains were resistant to erythromycin (E), three to polymyxin (PB), eleven to ceftizoxime (CZ), and ten to erythromycin (E).

Furthermore, 26 strains that were grown on Eosin Methylene Blue agar (EMB) underwent Antibiotic Susceptibility Testing (AST), and each of them were Gram-negative. Remarkably, no strain showed resistance to Cephalexin (CN), only one strain showed resistance to Ciprofloxacin (CIP), no strain showed resistance to Tobramycin (TOB), two strains showed resistance to Polymyxin (PB), and no strain showed resistance to Levofloxacin (LEV). The sensitivity and resistance pattern of selected antibiotics against the isolated strains is shown in Figure 3 and 4.
Figure 3: Antibiotics sensitivity pattern of selected the isolated strains against the panel of tested antibiotics.

Figure 4: Antibiotics sensitivity and resistance pattern of isolated strains.

4. DISCUSSION

Fruits are an essential nutrient in the diet, but as they are harvested, watered, and transported to the market, they come into contact with microbial flora that is present on their surfaces. The consumption of raw fruits and vegetables, especially those that are consumed unwashed, can spread a variety of surface-associated bacteria that can lead to outbreaks of human-related diseases.

In this study, nine juice samples (sugarcane, carrot and orange juice) were collected from various sites in Lahore and subjected to bacterial isolation and enumeration by using three media including Eosin Methylene Blue (EMB) agar, MacConkey's agar, and Nutrient agar. By careful aseptic techniques, 164 bacterial strains were isolated from these juice samples. All strains were subjected for Gram staining and various biochemical tests. For catalase test, 32/36 (88%) strains of sugar-cane juice, 91 (100%) strains from carrot juice and 11/14 (78.5%) strains from orange juice exhibited positive results. For oxidase test, 12/36 (33.3%) strains from Sugar-cane juice samples, 55/91 (60.4%) strains
from carrot juice samples and 6/14 (42.8%) strains showed positive results. For Methyl Red test, 15/36 (41.6%) strains from Sugar-cane juice samples, 22/42 (52.3%) strains from carrot juice and 5/14 (35.7%) strains from orange juice samples showed positive results. For Starch agar test, 10/36 (27.7%) strains from Sugar-cane juice samples, 9/36 (25%) strains from carrot juice samples and no strain from orange juice samples exhibited positive result. Overall, the study offered important new understandings of the bacterial diversity and microbial development in fruit juice samples.

During harvest and postharvest procedures like storage, processing, and transportation microflora come on the surface of fruit juices (Babiye, 2017). Fresh fruit juice contamination is mostly caused by soil contamination and exposure to the environment, both of which pose substantial risks due to the widespread consumption of fresh juices. A similar study was conducted by Jabin et al. (2022) in Bangladesh, in which they assessed the microbial load in fresh fruit juices and also detected the antibiogram of the isolated strains. They obtained E. coli, Klebsiella, Salmonella, and Vibrio, whereas in our study, E. coli and Klebsiella were isolated, but pathogenic strains of Salmonella and Vibrio were not present in our samples (Jabin et al., 2022).

For the antibiogram of our isolated strains, we applied 10 antibiotics including Vancomycin, Ampicillin, Streptomycin, Polymyxin B, Levoﬂoxacin, Cephalexin, Erythromycin, Ceftizoxime Ciprofloxacin and Tobramycin. In comparison to our study, conducted by Sultana et al., (2019) conducted a study for microbiological quality assessment of commercially packed fruit juices. They isolated Vibrio cholerae, Klebsiella pneumoniae, and S. aureus from their samples in compared to our study in which Klebsiella and S. aureus were also isolated. They analyzed their isolates against 5 antibiotics including Amoxicillin, Ciprofloxacin, Imipenem, Gentamicin & Levoﬂoxacin and found that all isolates were resistant against amoxicillin while sensitive against Levoﬂoxacin. As compared to our study, in which no sample was resistant against Levoﬂoxacin and the maximum resistivity was exhibited against Ampicillin as 8 isolates were resistant from 16 selected isolated strains. (Sultana et al., 2019).

Antibiotic resistance in bacteria is facilitated by mechanisms such as enzymatic degradation, efflux pumps, target modification, and biofilm formation. To control antibiotic resistance, approaches like stewardship programs, development of new antibiotics, combination therapy, and public education on responsible antibiotic use are essential. When consuming juices, safety precautions involve choosing pasteurized options, maintaining hygienic practices during preparation, sourcing from reputable suppliers with stringent quality controls, and ensuring proper refrigeration to minimize the risk of bacterial contamination. These measures collectively contribute to safeguarding public health and promoting responsible antibiotic use in the context of fruit juice consumption.

5. CONCLUSION

In order to avoid any health risks related to microbial contamination, it is important to make sure that the microbiological quality of fruit juice’s satisfies acceptable standards. For this purpose, the samples of different fruit juices in triplicates were obtained from nine different locations of Lahore, Pakistan. The fruit juices contained these bacterial genera Bacillus, E. coli, Pseudomonas, Klebsiella and Enterobacteriaceae spp. which according to the antibiotic susceptibility pattern were not significantly resistant to the tested antibiotics. Levoﬂoxacin (LEV) exhibited the highest zone of inhibition against strains selected from these fruit samples. The highest frequency of E. coli and Enterobacteriaceae spp. most probably was due to the water contamination. Bacillus spp. most probably comes in contact with these fruits during transport. So proper washing of these fruits before usage can reduce their microbiological count.

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Author contributions

All authors have made a substantial direct and intellectual contribution to the work and approved it for publication.
Conflict of interest

The author declares that there is no conflict of interest.

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