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# Isolation of Multidrug-Resistant *Acinetobacter* and *Klebsiella* from Fruit Juices and Assessment of their Developmental Toxicity on *Artemia salina*

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**Abstract**

Microbial spoilage of fruit juice poses a significant risk for human infections in Bangladesh. To address this concern, both local and packaged fruit juices were microbiologically assessed for their lactose-fermenting ability. The isolates were evaluated for acute toxicity against the developmental stages of *Artemia salina*, examining the correlation between bacterial dose duration and the abnormalities observed in the brine shrimp. Morphological, physiological, biochemical analyses, and 16S rRNA sequencing identified isolate A as *Acinetobacter* sp. (lactose-non-fermenting, sample-6) and isolate B as *Klebsiella* sp. (lactose-fermenting, sample-9). Both bacteria exhibited developmental toxicity, leading to delayed germination of *Artemia* cysts. After 48 hours, notable abnormalities such as missing antennules, abnormal eyes, contused mandibles, absent swimming legs, and damaged ovaries were recorded. The Duncan Multiple Range Test indicated that *Acinetobacter* sp. was significantly more toxic than *Klebsiella* sp. The median lethal time ($tD_{50}$) was 5 hours for *Acinetobacter* sp. and 6 hours for *Klebsiella* sp., with lethal concentration ($LC_{50}$) values of 6.107±0.19 µL/mL and 6.999±0.21 µL/mL (per 10 mL volume reaction mixture), respectively. Pearson correlation analysis showed a positive relationship between exposure duration to the isolated bacterial strains and the occurrence of defective traits in *Artemia salina*. Furthermore, multidimensional tests confirmed a complete separation between control and treated *Artemia*. Both bacterial isolates were found to be multidrug-resistant. This study emphasizes the need for increased awareness to eliminate microbial health hazards.

## INTRODUCTION

Fruits are an important component of a balanced diet, and fruit juices are widely consumed as a convenient source of vitamins, minerals, antioxidants, and other bioactive compounds [1,2]. Their nutritional composition has been associated with several health-promoting properties owing to the presence of essential vitamins, phytochemicals, and antioxidant compounds [3,4]. However, despite their nutritional value, fruit juices are highly susceptible to microbial contamination during processing, handling, and storage when hygienic practices are not properly maintained [5]. Consequently, contaminated fruit juices may serve as vehicles for foodborne pathogens and pose a significant public health concern, particularly in regions where food safety practices are inadequate [5]. Nevertheless, even if fruit juice has a high nutritional value, the production and processing practices in developing countries may not reach suitable safety levels and put consumers at risk of illness. Foodborne diseases are one of the major reasons for global fatalities, predominantly in developing countries [6]. Several studies have reported foodborne illnesses associated with the consumption of contaminated fruit juices [7,8]. Poor hygienic practices during juice preparation, processing, and handling, including the use of contaminated water and inadequately sanitized equipment, facilitate bacterial contamination of fruit juices and increase the risk of foodborne illness [9,10]. Juices are latent sources of harmful bacteria including *Staphylococcus aureus, Salmonella* spp., *Escherichia coli*, *Shigella* spp., *Klebsiella pneumoniae*,

*Enterobacter* spp., *Acinetobacter* spp., *Pseudomonas* spp., and *Alicyclobacillus* spp. [11]. For instance, *Acinetobacter* spp. are opportunistic bacterial pathogens associated with meningitis, skin and soft tissue infections, bacteremia, respiratory tract infections, and urinary tract infections [12,13]. Furthermore, *Klebsiella pneumoniae* is one of the most clinically important members of the family Enterobacteriaceae and a major cause of healthcare-associated infections, particularly in intensive care, burn, oncology, and neonatal units [14,15]. Prior research has revealed that fruit juices from street vendors as well as those in packs are contaminated with harmful microbes [16].

In Bangladesh, street-vended and packed fruit juices are popular among people because they fulfill nutritional requirements at a reasonable price. Nonetheless, the easy availability of fruit juices is a familiar source of pathogenic bacterial transmission in developing countries like Bangladesh [17,18]. There are several reasons behind this microbial contamination. Firstly, lack of personal hygiene, conventional processes of juice preparation, and inappropriate preserving temperature are the most important factors [19]. Additional justifications include fruit flies, filth, and improperly cleaned tools like cutters and knives, as well as direct contamination from rotting fruits [7,20]. Because of improper handling and serving procedures, street-vended fruit juices are frequently linked to diarrheal illnesses in Bangladesh [21]. Considering the potential public health risks associated with contaminated fruit juices, it is important to evaluate the developmental toxicity of representative bacterial isolates using an appropriate in vivo model.

*Artemia salina* (brine shrimp) is a widely recognized crustacean found in seawater environments (saline lakes and seas) and a vital food source for fish and other aquatic invertebrates [22]. The model organism has been widely used to identify the toxic effects of bacteria and chemicals due to the year-round commercial availability of dried cysts, low cost, short life cycle, and ease of laboratory maintenance under in vivo conditions [23]. In addition, *Artemia salina* is highly sensitive to a wide range of microbial toxins and environmental contaminants, making it a reliable and cost-effective in vivo model for preliminary toxicity assessment [23,44]. This assay is regarded as a reliable way to assess the level of toxicity in plant extracts [24], and to evaluate toxic cyanobacteria [25] and heavy metals [26]. Previous studies have revealed a high association between the cytotoxicity directed against certain tumor cell lines and the toxic activities in the brine shrimp experiment [27] as well as the hepatotoxic activity [28]. Thus, the *Artemia salina* experiment is a more feasible way compared to cell line assays for in vivo acute toxicity evaluation [29].

Although previous studies have documented microbial contamination and antibiotic resistance in fruit juices, limited information is available regarding the developmental toxicity of fruit juice-associated bacterial isolates using *Artemia salina* as an in vivo model. Furthermore, the relationship between bacterial exposure duration and developmental abnormalities has received little attention. Addressing these knowledge gaps formed the basis of the present investigation. Therefore, this study aimed to isolate and characterize multidrug-resistant bacterial contaminants from locally prepared and commercially packaged fruit juices. The developmental toxicity of the isolated bacterial strains was evaluated using *Artemia salina* as an in vivo model, and their antibiotic susceptibility patterns were determined. In addition, the association between bacterial exposure duration and developmental abnormalities was investigated using multivariate statistical analyses. The findings provide valuable insight into the potential public health risks associated with multidrug-resistant bacterial contamination of fruit juices and improve our understanding of bacterial developmental toxicity using *Artemia salina* as an established aquatic model organism.

## MATERIALS AND METHODS

### Sample Collection

Twelve samples of fruit juice were taken from different areas of the Rajshahi Metropolitan Area, Bangladesh (site-1: Laxmipur, site-2: Railway station, site-3: University campus, and site-4: Shaheb Bazar) during May 2022. Among the twelve samples, Samples 1 to 7 were street-vended fruit juice samples, namely papaya juice for Sample 1, orange juice for Sample 2, lemon juice for Sample 3, bael juice for Sample 4, watermelon juice for Sample 5, sugarcane juice for Sample 6, and pineapple juice for Sample 7. The remaining five samples were packed mango juice samples identified as Samples 8 to 12. Subsequently, all of the samples were taken directly to the Microbiology Laboratory of the Department of Genetic Engineering and Biotechnology at the University of Rajshahi to evaluate their microbiological quality. Because the samples were collected from selected locations within a single city during one sampling period, the findings should be interpreted as a preliminary assessment and may not represent the microbiological quality of all fruit juices available in Bangladesh.

### Isolation, Selection, and Growth Optimization of Representative Bacterial Isolates

The samples of fruit juices were collected as a source of inoculum, and droplets of juice were used for plating on MacConkey agar medium, which contains the following substances: (Lactose monohydrate 10.0 g/L, peptones 3.0 g/L, bile salts 1.5 g/L, sodium chloride 5.0 g/L, crystal violet 0.001 g/L, and Agar 13.5 g/L at pH=7.0) separately. Following incubation at 37°C for 24 h, morphologically distinct bacterial colonies were examined. Among all recovered bacterial isolates, two representative isolates (designated isolate A and isolate B) were selected for further investigation based on their distinct colony morphology, lactose fermentation characteristics, and comparatively high bacterial loads. Isolate A and isolate B exhibited the highest viable counts ($8.5 \times 10^9$ CFU/mL and $6.8 \times 10^7$ CFU/mL, respectively), and were therefore selected for subsequent molecular identification, developmental toxicity assessment, and antibiotic susceptibility testing. Pure cultures were maintained for all subsequent experiments.

Different temperatures and pH influence bacterial growth. In this experiment, the pH was varied at 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0, while the temperature was 25, 30, 35, and 40 °C. In both cases, the optical density of the bacterial liquid cultures was recorded with a double-beam spectrophotometer (Analytik Jena AG, Germany) at 660 nm.

### Morphological and Biochemical Characterization of Isolated Bacteria

Morphological characterization included Gram staining and motility testing, whereas biochemical characterization comprised catalase, methyl red, Simmons citrate utilization, urease hydrolysis, and triple sugar iron (TSI) tests following standard microbiological procedures [30]. All media were prepared according to the manufacturer's instructions and standard microbiological protocols.

### Molecular Identification of Isolated Bacteria

The following procedures were used to identify the isolated bacteria at the molecular level: extracting the chromosomal DNA, amplifying the 16S rRNA gene, purifying the PCR product, cycle sequencing with purifying the cycle sequencing product, detecting the nucleotides, and sequence analysis. The CTAB technique was used to extract the bacterial genomic DNA described by Edwards et al. [31]. After extraction, the NanoDrop Spectrophotometer (Thermo Scientific, USA; ND2000 series) was used to quantify DNA. PCR was used to amplify the 16S rRNA gene following the instructions provided by Loffler et al. [32] using 16s F and 16s R (Sigma, USA) universal primers. The primer sequences are provided in the supplementary file (**Table S1**). All PCR reagents were used from the Promega (Catalogue no. M7432, Promega, USA). The composition of the PCR Reaction mix (Total volume is 25 µL) are in supplementary (**Table S2**). The reagents were mixed gently, and short spin was done. PCR amplification was done by Gene Atlas (G2; Astec, Japan). Cycling parameters for PCR analysis in supplementary.

After PCR amplification, samples were run in agarose gel (Catalogue no. V3125; Promega, USA) for visualization of the band. In this process, Promega, USA's 1 kb DNA ladder (Catalogue no. G5711), TAE buffer (Catalogue no. V4251), and ethidium bromide solution (Catalogue no. H5041) were utilized. In order to purify the PCR product, 40 µl of the product was combined with 160 µl of sterile water, resulting in a final amount of 200 µl. Following that, the 200 µl was transferred to the YM-100 rapid spin columns and centrifuged for 10 minutes at 500 × g. The columns were moved into fresh tubes for the DNA elution process, and 25 µl of sterile water was added. Next, for five minutes, the tubes were centrifuged at 1000 × g. Genetic Analyzer (3500, Thermo Scientific, USA) was used for the sequencing of the PCR-purified product. After that, the bioinformatics tool Chromas was used to edit the sequences. Then the BLASTN algorithm (http://www.ncbi.nih.gov) was used to determine the homology between the 16S rRNA gene sequence and the gene sequences of other organisms.

**Evaluating the Toxic Effects of Isolated Bacteria on the Developmental Stages of *Artemia salina***

An acute toxicity assay was conducted to compare the cellular toxicity of bacterial strains obtained from fruit juice at two developmental stages of *Artemia salina*: the stationary and the exponential phases. The experimental protocol was adapted from the method described by Neves et al. [33] with minor modifications.

**Stationary Phase**

Assays for intoxication were conducted using sterile 6-well plates, with 10 mL of filtered seawater in each well. Ten *Artemia salina* cysts were introduced into each well and co-incubated with overnight bacterial cultures. Prior to inoculation, bacterial suspensions were standardized to $OD_{660}$ = 0.5, and the inoculum concentration was adjusted to 200 cells $mL^{-1}$, following the protocol of Neves et al. [33]. A control containing brine shrimp without isolated bacteria was also implemented in three times. In this case, a mineral salt (MS) liquid medium was used for bacterial culture. Every six hours during the 48-hour incubation period, the germination of the cysts was inspected, and any significant anomalies were noted using an inverted microscope (USA: LABOMED CXL).

**Exponential Phase**

The exponential-phase toxicity assay was performed using sterile 6-well plates, with each well containing 10 mL of filtered seawater, and this phase was observed for up to 7 hours. Six actively swimming *Artemia salina* nauplii were exposed to overnight bacterial cultures standardized to $OD_{660}$ = 0.5 at an inoculum concentration of 200 cells $mL^{-1}$ following Neves et al. [33]. All treatments, including the control, were performed in triplicate. Every hour after incubation, the survival of individuals and significant anomalies were examined under an inverted microscope (LABOMED CXL, USA).

**Determination of $tD_{50}$**

In the case of the exponential phase, the $tD_{50}$ (the median lethal time when 50% *of Artemia salina* died) value was calculated following the protocol outlined by Neves et al. [33].

**Determination of $LC_{50}$**

The lethality bioassay of brine shrimp is considered a helpful method for conducting an initial toxicity assessment [24]. Nauplii were obtained by hatching *Artemia salina* eggs in simulated seawater. To evaluate the cytotoxic activity of the isolated bacteria on brine shrimp, six autoclaved test tubes were prepared, and 10 mL of artificial seawater containing 10 nauplii were introduced into each tube. Bacterial cultures were grown overnight and standardized to $OD_{660}$ = 0.5 prior to exposure to ensure a uniform bacterial suspension throughout the experiment. Finally, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 µL/mL of the previously cultured bacterial samples were sequentially added to the test tubes using a micropipette. The test tubes were exposed to light and incubated at ambient temperature for 24 hours to monitor the cytotoxic effects. After the 24-hour period, the number of survivors was recorded. This mortality data was then analyzed using a basic probit analysis tool to calculate the $LC_{50}$ value together with its 95% confidence interval. Because all bacterial cultures were standardized to the same optical density ($OD_{660}$ = 0.5) prior to exposure, $LC_{50}$ values are reported as the volume (µL/mL) of the standardized bacterial suspension required to produce 50% mortality.

**Statistical Analysis of Developmental Toxicity and Correlation between Bacterial Exposure and *Artemia salina* Abnormalities**

For statistical analysis, the Duncan Multiple Range Test (DMRT) was conducted using Statistical Analysis Software (SAS, version 9.1.3) to compare the means of three replicates between groups. To visualize the significant changes through multidimensional scaling (MDS), the data of *Artemia* cysts treated with bacterial isolate A and isolate B were normalized and analyzed separately for the stationary and exponential phases. ANOSIM (Analysis of Similarity) was performed to determine significant differences in abnormalities between coliform-treated *Artemia* and untreated *Artemia* using Primer E (Version 7). To measure the Pearson correlations between the abnormal features of treated *Artemia* and the dose duration, heatmaps were generated using the heatmaply package in R (version 4.1.3).

**Antibiotic Sensitivity Test**

The antibiotic sensitivity profile of the isolated bacterial strains was assessed using the Kirby-Bauer disc diffusion technique [34]. In this study, both Luria broth (LB) liquid and LB agar were utilized as culture media to facilitate bacterial growth. Commercially available and commonly recommended antibiotics such as penicillin (10 µg per disc), amoxicillin (10 µg per disc), erythromycin (15 µg per disc), ampicillin (10 µg per disc), kanamycin (30 µg per disc), tetracycline (30 µg per disc), ceftazidime (30 µg per disc), gentamycin (10 µg per disc), ciprofloxacin (5 µg per disc), cefuroxime (30 µg per disc), cefixime (5 µg per disc), Doxycycline (10 µg per disc) were used as antibiotic discs, placed in the center of the corresponding plates, and incubated overnight at 37 °C. Following overnight incubation at 37°C, the diameters of the inhibition zones were

measured in millimeters (mm). The susceptibility of each isolate (susceptible, intermediate, or resistant) was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) Performance Standards for Antimicrobial Susceptibility Testing (latest applicable edition), using the appropriate interpretive criteria for the tested bacterial species and antimicrobial agents [35].

## RESULTS

### Isolation and Optimization of Growth Characteristics of Isolated Bacteria

Bacterial strains were isolated from the collected fruit juices through screening on MacConkey agar media and the highest number of colonies was found in the following pattern: Sample 6> Sample 9> Sample 4> Sample 2> Sample 1 > Sample 12> Sample 7>Sample 5> Sample 10>Sample 3 (**Fig. 1**). Out of the twelve samples, Sample 6 (street sugarcane juice) yielded the highest number of bacterial colonies, while Sample 9 (packed mango juice) yielded the second highest number. Based on their colony abundance and distinct colony morphology on MacConkey agar, representative isolates from these two samples (designated isolate A and isolate B) were selected for subsequent physiological, molecular, toxicity, and antibiotic susceptibility analyses. However, Samples 8 and 11, both packed juices, showed no growth on MacConkey agar media (**Fig. 1**). According to our findings, the maximum growth of bacteria was observed in isolates A (**Fig. S1**) and B (**Fig. S2**) at pH 7.0 and 7.2, respectively. The optimal temperature for bacterial growth was 35°C for isolate A (**Fig. S3**) and 37 °C for isolate B (**Fig. S4**).

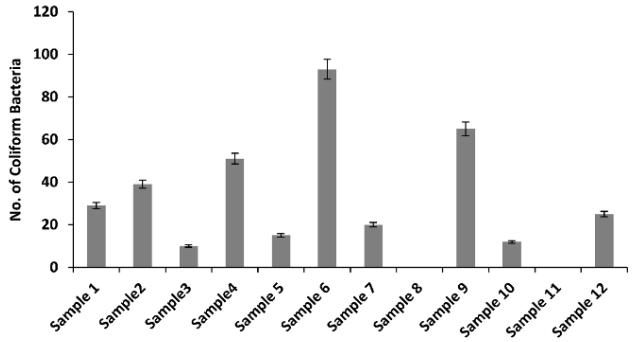


**Fig. 1**. The presence of isolated bacterial strains in twelve different samples (local street juice = S1 to S7 and packed juice = S8 to S12) isolated from different packed and local juices. All samples were screened in triplicate using the MacConkey medium to count the bacterial CFU numbers.

### Molecular Profiling of Isolated Bacteria

Following sequencing of the amplified 16S rRNA gene, the obtained nucleotide sequences were deposited in the GenBank database. Isolate A was assigned GenBank accession number LC437022, whereas isolate B was assigned LC552682**.** BLASTN analysis showed that isolate A shared 97% sequence similarity with *Acinetobacter* sp. (reference sequence accession no. NR_117677.1), while isolate B shared 98% sequence similarity with *Klebsiella* sp. (reference sequence accession no. NR_117683.1). The PCR amplification products, DNA quality assessment, and sequencing analyses are presented in **Figs. S5-S7**.

### Evaluating the Toxicity of Isolated Bacteria on the Developmental Stages of *Artemia salina*

#### Stationary Phase

After 16 hours, *Artemia* cysts treated with both isolates (*Acinetobacter* sp. and *Klebsiella* sp.) showed no germination (**Fig. 2**). After 24 hours, both bacteria-treated cysts showed delayed germination (10% germination rate) compared to the control (above 65% germination rate). Following the recorded intervals at 36 hr and 48 hr, the germination rate of treated groups was lower than 30% and 40%, respectively, while the control group showed more than 80% germination rate in both intervals. Major developmental abnormalities were recorded separately, including missing antennules, abnormal eyes, absent swimming legs, and a damaged ovary under a LABOMED CXL microscope (USA), as depicted in **Fig. 3**.

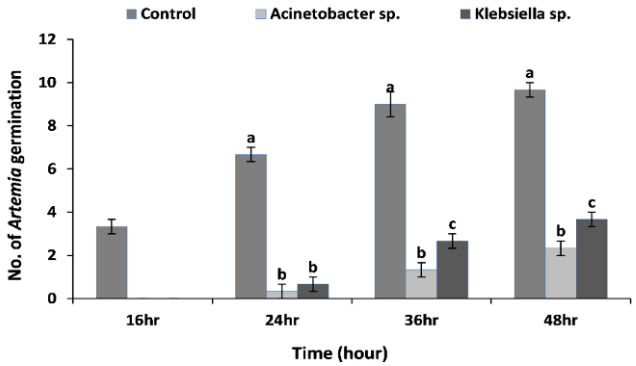


**Fig. 2.** The effect of *Acinetobacter* sp. (isolate A) and *Klebsiella* sp. (isolate B) on the germination of *Artemia salina* cysts during the stationary phase at different exposure times. Data represent mean ± SEM (n = 3 biological replicates). Bars sharing different lowercase letters indicate statistically significant differences according to Duncan's Multiple Range Test (DMRT) ($P < 0.05$).

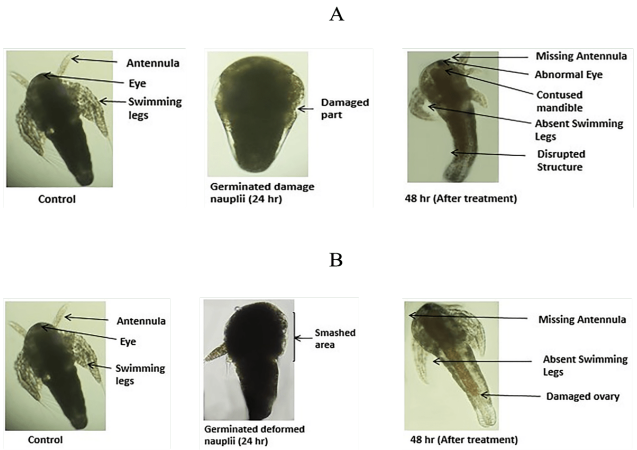


**Fig. 3.** The toxic impacts of isolated bacteria *Acinetobacter* sp. (A) and *Klebsiella* sp. (B) on the stationary phase of *Artemia salina*. The stationary phase denotes the treatment of the isolated bacterial strains prior to the germination of cysts in order to observe defects in the germinated nauplii, such as damaged nauplii in the case of *Acinetobacter* sp. and deformed nauplii observed in the case of *Klebsiella* sp.

#### Exponential Phase

The toxic effects of the two isolated bacterial strains were examined on *Artemia salina* after germination. Treatments were applied in triplicate for each bacterium, along with controlled maintenance. All of the isolates exhibited the highest level of survival after an hour, and no significant variation was observed between the treatments and the control group (**Fig. 4**). *Acinetobacter* sp. exhibited lower survival after 2 hours compared to *Klebsiella* sp., although the difference between the two isolates was not statistically significant when compared to the control (**Fig. 4**). We observed that, between 3 to 7 hours, the survival rate of *Artemia* decreased over time, resulting in a variation that differed significantly from the control (**Fig. 4**).

After 8 h of exposure, *Acinetobacter* sp. produced the lowest survival among all treatments, with survival approaching zero, and this reduction was significantly different from the control (**Fig. 4**). Significant variations were also noticed using a LABOMED CXL microscope (USA), as illustrated in **Fig. 5**. Therefore, it is evident that the toxicity level of *Acinetobacter* sp. was higher than that of *Klebsiella* sp. in both phases of *Artemia salina*. The complete raw numerical data (three biological replicates) used for the stationary- and exponential-phase developmental toxicity assays and for generating Figures 2 and 4 are provided in Supplementary File 2.

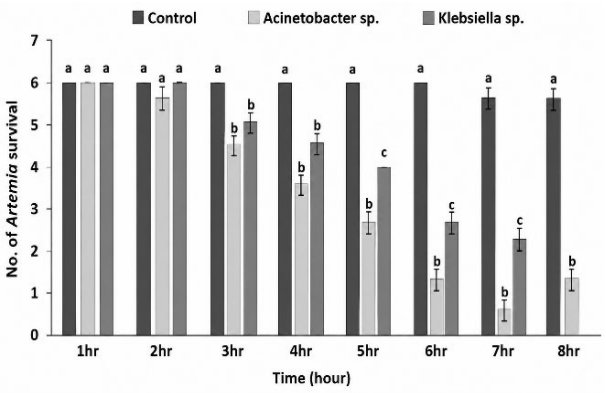


**Fig. 4.** The effect of *Acinetobacter* sp. (isolate A) and *Klebsiella* sp. (isolate B) on the survival of *Artemia salina* during the exponential phase at different exposure times. Data represent mean ± SEM (n = 3 biological replicates). Bars sharing different lowercase letters indicate statistically significant differences according to Duncan's Multiple Range Test (DMRT) ($P < 0.05$).

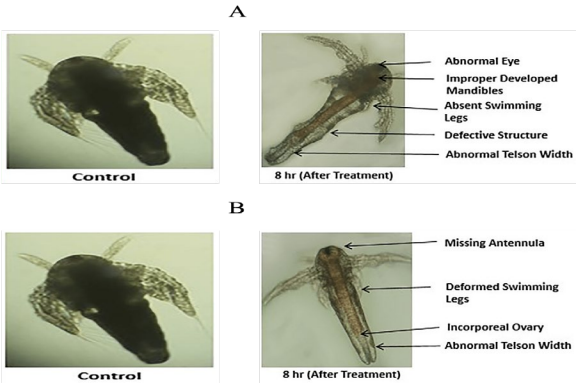


**Fig. 5.** The toxic impacts of isolated bacteria *Acinetobacter* sp. (A) and *Klebsiella* sp. (B) on the exponential phase of *Artemia salina*. The exponential phase indicates the bacterial treatment after the germination of cysts to observe and count the defects in the developmental nauplii, such as the defective structure of mandibles, eyes, swimming legs, and telson in the case of both *Acinetobacter* sp. and also in *Klebsiella* sp.

**Determination of $tD_{50}$**

The $tD_{50}$ value for both *Acinetobacter* sp. and *Klebsiella* sp. was recorded during the exponential phase, which lasted 5 hours for *Acinetobacter* sp. and 6 hours for *Klebsiella* sp.

**Determination of $LC_{50}$**

The cytotoxic effect of isolated bacteria was examined at different concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 µL/mL in this current investigation. The $LC_{50}$ value for *Acinetobacter* sp., determined through the brine shrimp lethality bioassay, was 6.107±0.19 µL/mL, with the regression equation Y =2.532x±0.477. The 95% confidence interval was between 4.373 and 8.529 µL/mL after 24 hours of exposure (**Table 1.** and **Fig. S8**). In contrast, the value for *Klebsiella* sp. was 6.999±0.21 µL/mL, with a regression equation of Y = 2.390x±0.589. The 95% confidence limits ranged from 5.011 to 9.779 µL/mL after 24 hours of exposure (**Table 1** and **Figure S10**). Additionally, the mortality rates of *Artemia salina* induced by the isolated bacteria are presented in **Figs. S9 and S11**, respectively. The above results indicate that *Acinetobacter* sp. exhibited comparatively greater toxicity toward *Artemia salina* than *Klebsiella* sp.

**Table 1.** After a 24-hour exposure, the LC50 values, 95% confidence limits, regression equations, and Chi-square values for the isolated bacteria tested against brine shrimp nauplii.

| Test sample | $LC_{50}$ (µL/mL) | 95% Confidence limits (µL/mL) | Regression equation | $\chi^2$ value (Degrees of freedom) |
|---|---|---|---|---|
| *Acinetobacter* sp. | 6.107±0.19 | 4.373 to 8.529 | Y =2.532x±0.477 | 0.585 (4) |
| *Klebsiella* sp. | 6.999±0.21 | 5.011 to 9.779 | Y =2.390x±0.589 | 1.283 |

**Pearson correlation Between Exposure Duration and Developmental Abnormalities of *Artemia salina***

Using Pearson correlation, anomalies of treated *Artemia* after 48 hours were found to be negatively correlated with controls in the case-stationary phase (**Fig. 6a**). Similar significant correlations were also detected during the exponential phase (**Fig. 6b**). Moreover, the ANOSIM and multidimensional scaling (MDS) identified the significant changes and separation between controls and treated *Artemia's* abnormalities (**Fig. 7a and 7b**; **Table S3** for ANOSIM).

**Antibiotic Sensitivity Test**

The antibiotic susceptibility patterns of the bacterial isolates were interpreted according to CLSI performance standards. *Acinetobacter* sp. exhibited resistance to ciprofloxacin, doxycycline, tetracycline, cefixime, ceftazidime, and penicillin (**Table 2)** but was susceptible to cefuroxime, kanamycin, gentamycin, amoxicillin, and erythromycin. On the contrary, *Klebsiella* sp. was resistant to penicillin, tetracycline, doxycycline, and cefixime while susceptible to amoxicillin, kanamycin, ceftazidime, gentamycin, ciprofloxacin and cefuroxime (**Table 2**).

## DISCUSSION

Fruit juices are a popular source of nutrients among people in developing countries like Bangladesh. The reason behind this is that fruit juices are delicious, accessible at low prices, and convenient for the consumers [36]. Notably, the detection of coliform bacteria in fruit juice is not acceptable according to food safety standards and serves as an indicator of inadequate hygienic practices and possible contamination by other pathogenic microorganisms [37]. Therefore, coliform is a significant marker as it helps raise awareness and determine the source of other pathogenic bacteria. Based on this approach, we isolated representative bacteria from twelve fruit juice samples (local and

packed) available in Rajshahi City. Among these samples, a lactose non-fermenting white-colored colony (isolate A) from sugarcane juice (Sample 6) and a lactose-fermenting pink-colored colony (isolate B) from packed mango drinks (Sample 9) were chosen for further research based on colony number and morphology.

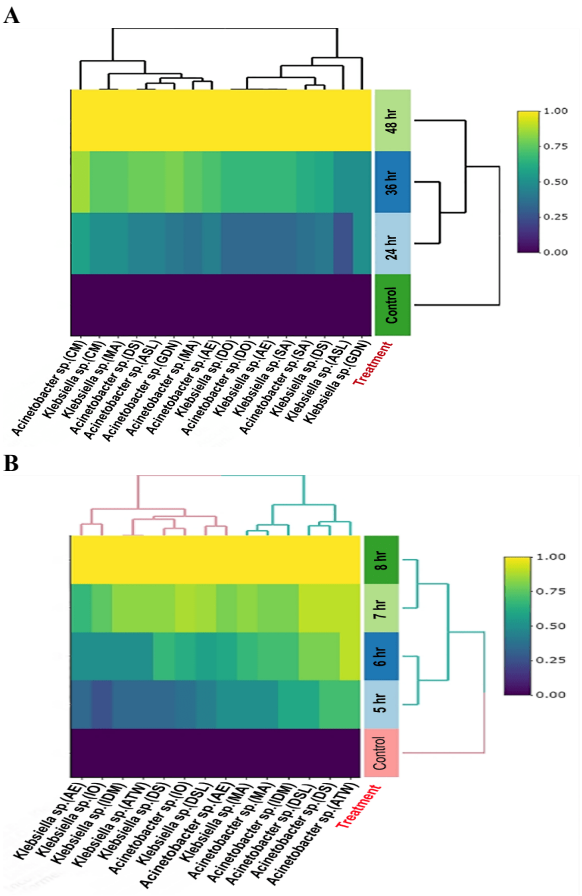


**Fig. 6.** Pearson correlation based heatmaps between bacterial dose and abnormal traits of *Artemia salina* treated with *Acinetobacter* sp. and *Klebsiella* sp. (A) Stationary-phase cultures assessed at 24, 36, and 48 h. (B) Exponential-phase cultures assessed up to 8 h. Abnormalities included morphological and developmental defects. Experiments were performed in triplicate. Yellow indicates higher and blue lower Pearson correlation coefficients (*r*).

**Table 2.** Antibiotic resistance profile of isolated bacteria.

| Name of Antibiotic | Zone of inhibition (mm) | | Resistant pattern | |
|---|---|---|---|---|
| | *Acinetobacter* sp. | *Klebsiella* sp. | *Acinetobacter* sp. | *Klebsiella* sp. |
| Penicillin | 8 mm | 9 mm | Resistant | Resistant |
| Amoxicillin | 19 mm | 17 mm | Susceptible | Susceptible |
| Erythromycin | 22 mm | 14 mm | Susceptible | Intermediate resistant |
| Ampicillin | 11 mm | 15 mm | Intermediate resistant | Intermediate resistant |
| Kanamycin | 16 mm | 20 mm | Susceptible | Susceptible |
| Ceftazidime | 10 mm | 18 mm | Resistant | Susceptible |
| Gentamycin | 17 mm | 22 mm | Susceptible | Susceptible |
| Tetracycline | 9 mm | 10 mm | Resistant | Resistant |
| Doxycycline | 7 mm | 8 mm | Resistant | Resistant |
| Ciprofloxacin | 8 mm | 23 mm | Resistant | Susceptible |
| Cefuroxime | 16 mm | 17 mm | Susceptible | Susceptible |
| Cefixime | 6 mm | 9 mm | Resistant | Resistant |

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm

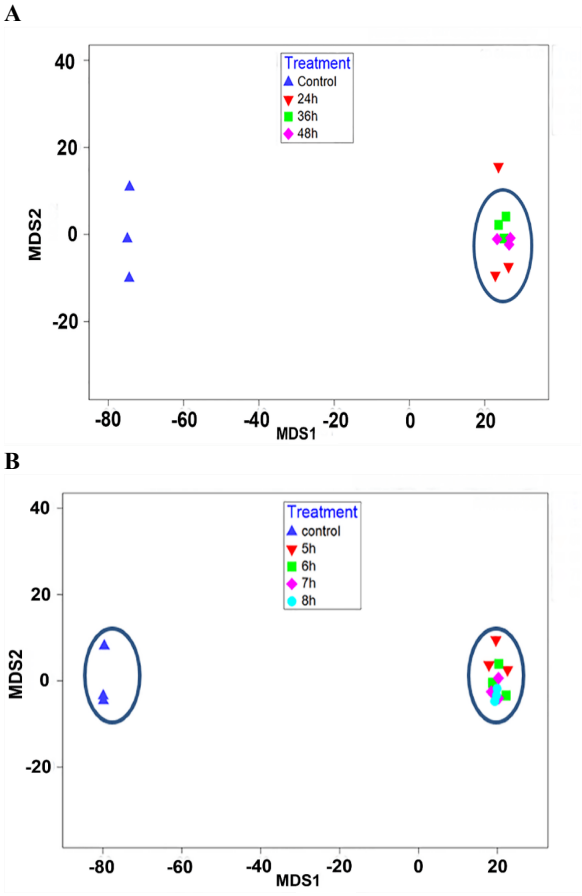


**Fig. 7.** Multidimensional scaling (MDS) ordination of abnormalities observed in *Artemia* following bacterial treatment during the stationary and exponential phases. **(a)** MDS ordination of the stationary-phase abnormalities dataset, with observations grouped according to sampling time; deformities in germinated *Artemia* were assessed at 24, 36, and 48 h. **(b)** MDS ordination of the exponential-phase abnormalities dataset, with developmental deformities assessed at 5, 6, 7, and 8 h. Different colors represent the respective observation times, with the untreated control indicated in blue.

Morphological characteristics are important for the preliminary identification of bacteria. Kabler and Clark [38] described coliforms as rod-shaped, Gram-negative, non-spore-forming bacteria capable of fermenting lactose within 24 h at 37°C. In the present study, the isolates were Gram-negative, non-motile, and rod-shaped, with optimum growth observed at pH 7.0 and 35°C for isolate A and pH 7.2 and 37°C for isolate B. Biochemical characterization further supported their identification. Overall, the observed physiological and biochemical characteristics were generally consistent with descriptions reported in Bergey's Manual [39]. The 16S rRNA gene sequencing is regarded as a favored genetic method for identification at the molecular level [40]. Molecular identification using 16S rRNA gene sequencing confirmed that the sequences generated in this study were deposited in GenBank under accession numbers LC437022 (isolate A) and LC552682 (isolate B).

BLASTN analysis showed 97% sequence identity between isolate A and the reference sequence of *Acinetobacter baumannii* (GenBank accession no. NR_117677.1) and 98% sequence identity between isolate B and the reference sequence of *Klebsiella pneumoniae* (GenBank accession no. NR_117683.1). Although these results support assignment of the isolates to the

genera *Acinetobacter* and *Klebsiella*, the observed sequence similarities are insufficient for confident species-level identification based solely on the 16S rRNA gene. Therefore, the isolates were conservatively reported at the genus level throughout this study, and additional approaches such as multilocus sequence analysis or whole-genome sequencing would be required for definitive species identification. Asif et al. reported *Acinetobacter baumannii* as a persistent infectious agent [41], while Martin and Bachman classified *Klebsiella pneumoniae as* an opportunistic, hypervirulent, and multidrug-resistant pathogen based on a genome study [42].

Neves et al. assessed acute dinoflagellate toxicity at a concentration of 200 cells $mL^{-1}$, which affected the germination and survival rates of *Artemia salina* during the stationary and exponential phases, respectively [33]. They also observed the highest $tD_{50}$ value for *G. excentricus* within 4 hours in the case of the exponential phase. In our present study, we evaluated the toxicity of isolated bacteria, and developmental abnormalities were also identified in both the stationary and exponential phases of *Artemia salina* at 200 cells $mL^{-1}$. Arulvasu et al. reported the impact of silver nanoparticles on both live animals and cysts. In their findings, brine shrimp guts filled with nanoparticles showed a significant mortality level within 24 hours of exposure [43].

Pinheiro et al. reported the accumulation of $MTiO_2$ in *Artemia* sp. after 24 hours and 48 hours without any morphological damage observed in the control group [44]. In their experiment, $MTiO_2$ was found in the gut of *Artemia* sp., and the cephalothorax showed swelling at concentrations of 50 ppm and 100 ppm, respectively. Additionally, the positive control $K_2Cr_2O_7$ exhibited underdeveloped body growth within 24 hours of exposure. However, $MTiO_2$ dispersed throughout the body of *Artemia* sp., resulting in morphological changes such as tissue degradation at the highest concentration of 100 ppm, while individuals exposed to $K_2Cr_2O_7$ showed improper development with deformities in the appendages and abdomen [44]. In the present study, during the stationary phase, isolated bacteria had a toxic effect on germination of *Artemia salina,* as both isolates showed delayed germination after 24 hours, while the control group showed germination after 16 hours.

The variation between the treatment and control group was significant. In the case of the stationary phase, damaged germinated nauplii were found after 24 hours of *Acinetobacter* sp. treatment, exhibiting missing antennule, abnormal eyes, contused mandibles, absent swimming legs, and disrupted structure. After 48 hours of treatment with *Acinetobacter* sp., missing antennule, absent swimming legs, and damaged ovaries were observed. Similarly, germinated deformed nauplii were observed after 24 hours of treatment with *Klebsiella* sp., while missing antennule, absent swimming legs, and damaged ovaries were observed after 48 hours. *Klebsiella pneumoniae* was reported to cause mortality in Nile tilapia [45]. Bi et al. described disease-causing *Acinetobacter johnsonii* in rainbow trout [46]. Again, during the exponential phase, the treatment with *Acinetobacter* sp. exhibited the highest level of toxicity. After 8 hours, it showed no survival with significant variation compared to the control group. Developmental abnormalities such as abnormal eye, improperly developed mandibles, absent swimming legs, defective structure, and abnormal telson width were also identified after 8 hours of treatment. Moreover, *Artemia* treated with *Klebsiella* sp. exhibited low survival after 8 hours compared to the previous hour, and the variation was significant. Developmental abnormalities, including missing antennule, deformed swimming legs, incorporeal ovary, and abnormal telson width, were also observed. In addition, the $tD_{50}$ was found to be within 5 hours for *Acinetobacter* sp. and 6 hours for *Klebsiella* sp., in the exponential phase. Similar abnormalities were found in a previous study by Talukder et al [47], and our findings aligned with their data. Therefore, it is evident that the toxicity level of *Acinetobacter* sp. was higher than that of *Klebsiella* sp. in both phases of *Artemia salina*. Overall, the toxicity of the isolated bacterial strains at the developmental level was significant and analyzed through DMRT. Furthermore, the ANOSIM result confirmed significant changes compared to the control group in both the stationary ($R=0.646$; $P<0.001$) phase and exponential phase ($R=0.52$; $P<0.002$). Moreover, the Pearson correlation validated the negative correlation between the control group and the treated *Artemia* in both cases.

However, because the present study did not include heat-killed bacteria, sterile culture supernatants, or medium-only controls, the observed developmental effects cannot be attributed specifically to bacterial infection, secreted toxins, alterations in the culture medium, oxygen depletion, or other bacterial metabolites. Therefore, although exposure to the bacterial cultures clearly impaired the development and survival of *Artemia salina*, the precise mechanism underlying the observed toxicity remains uncertain. Future studies incorporating these additional controls, together with analyses of bacterial virulence factors and secreted toxins, will be necessary to elucidate the mechanisms responsible for the developmental abnormalities. Future investigations should also evaluate bacterial virulence determinants, toxin production, and whole-genome sequencing to better understand the relationship between antimicrobial resistance and developmental toxicity.

Brine shrimp bioassay is also used for cytotoxicity tests of microbial toxins and bioactive compounds [48]. We also tested the toxicity of the isolates against *Artemia salina* and the $LC_{50}$ values for *Acinetobacter* sp. and *Klebsiella* sp. were 6.107±0.19 µL/mL and 6.999±0.21 µL/mL, respectively after 24 hours of exposure. From the results, it was clear that *Acinetobacter* sp. exhibited higher toxicity than *Klebsiella* sp. against aquatic organisms. We identified several multidrug-resistant (MDR) isolates in the current investigation. Our results showed that *Acinetobacter* sp. (isolate A) was resistant to tetracycline, ciprofloxacin, penicillin, ceftazidime, cefixime, and doxycycline, while susceptible to kanamycin, gentamycin, erythromycin, amoxicillin, and cefuroxime, and had intermediate resistance only against ampicillin. In hospitalized patients, Dortet et al. isolated *Acinetobacter ursingii* and *Acinetobacter schindleri* that were resistant to cefixime and susceptible to amoxicillin, kanamycin, and gentamycin [49].

The previous report found multidrug-resistant *Acinetobacter* sp. from fruits, which exhibited 60% resistance against ceftazidime and 84% resistance against ciprofloxacin [50]. Another investigation carried out by Pal et al. reported that *Acinetobacter baumannii* was 100% resistant against penicillin [51], followed by Ferdous et al. who showed a resistance pattern of *Acinetobacter* sp. isolated from the hospital exhibiting 68.5% resistance against tetracycline, 92% resistance against ampicillin, and 84.9% resistance against ciprofloxacin [52]. These findings are generally consistent with previous reports. On the other hand, our results depicted that *Klebsiella* sp. (isolate B) was resistant against penicillin, tetracycline, doxycycline, and cefixime, intermediately resistant against ampicillin and erythromycin, but susceptible to amoxicillin, kanamycin, ceftazidime, gentamycin, ciprofloxacin, and cefuroxime.

According to Khalif et al. an antibiogram study of pathogenic bacteria from street-vended food showed that

*Klebsiella* sp. was susceptible to kanamycin, gentamycin, and ciprofloxacin, but resistant to erythromycin [53]. Another study revealed that multidrug-resistant *Klebsiella pneumonae* isolated from powdered infant formula showed 15.4% resistance against cefixime, 53.8% resistance against ampicillin, 38.5% susceptibility to cefuroxime, 84.6% susceptibility to ceftazidime, and 100% susceptibility to ciprofloxacin, gentamycin, and kanamycin [54]. In addition, Padamadan et al. reported an antibiogram of street-vended and packed fruit juices in which *Klebsiella* sp. was resistant to ampicillin, cefixime, and penicillin [8]. Collectively, these findings suggest that the antimicrobial resistance patterns observed in the present study are broadly consistent with previous reports of food-associated isolates. From the results, it is clear that *Acinetobacter* sp. was more resistant to antibiotics in contrast to *Klebsiella* sp.

Overall, the isolation of multidrug-resistant bacteria belonging to the genera *Acinetobacter* and *Klebsiella* from fruit juices highlights the importance of maintaining appropriate hygienic practices during juice preparation, processing, storage, and distribution. Although the present study was limited to isolates collected from a single geographical region and did not investigate bacterial virulence determinants or toxicity mechanisms, the findings emphasize the potential public health risks associated with contaminated fruit juices and provide a foundation for future investigations into bacterial developmental toxicity and antimicrobial resistance in foodborne bacteria.

## CONCLUSIONS

From the results of the current study, it has been confirmed that fruit juices (both street-vended and packaged) show higher levels of contamination with pathogens, including *Acinetobacter* sp. and *Klebsiella* sp. Additionally, these indicative pathogens have shown resistance to multiple antibiotics. The DMRT analysis revealed notable abnormalities in *Artemia salina* due to multidrug-resistant isolated bacterial strains in terms of bacterial developmental toxicity. Furthermore, ANOSIM and ordination MDS indicated significant abnormalities among bacteria-treated *Artemia* compared to the control group. Pearson correlation analysis showed that longer exposure times of the isolated bacterial strains to *Artemia salina* were significantly positively correlated with abnormalities when compared to the control group. Since fruit juices are a popular beverage, their quality is of utmost importance to human health. The microbial contamination of these juices poses a serious threat as it continues to be a significant source of illness, particularly among children, and affects the health of the population in both developed and developing regions. Therefore, storage strategies should be implemented to ensure the microbial standards of street-vended and commercially available fruit juices, in order to guarantee food security and safety. This way, the spread of antibiotic-resistant infections within the population can be addressed and prevented.

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## AUTHOR CONTRIBUTIONS

Conceptualization, M.N.H., and M.A.E.; Methodology, M.N.H., M.A.E., and A.P.T.; Data analysis, M.A.E., R.Z., and M.N.H.; Data curation, M.N.H., M.A.E., A.P.T., and S.S.D.; Writing-original draft, M.N.H., A.P.T., and S.S.D.; Writing-reviewing and editing, M.A. S., M.A. E.; Visualization, M.A.S. R.Z., and S.Z.; Supervision, M.S.U., and M.A.E.; Laboratory facilities, M.A.E., M.A.S., M.S.U., and S.Z. All of the authors read and approved the manuscript.

## CONFLICTS OF INTEREST STATEMENT

"The authors declare no conflict of interest."

## FUNDING STATEMENT

Not applicable.

## DATA AVAILABILITY STATEMENT

The supplementary files and raw dataset supporting the findings of this study titled "Isolation of Multidrug-Resistant *Acinetobacter* and *Klebsiella* from Fruit Juices and Assessment of their Developmental Toxicity on *Artemia salina*" are openly available in Figshare at https://doi.org/10.6084/m9.figshare.33389629.

## ETHICAL APPROVAL AND CONSENT

Not applicable

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