# ANALYSIS OF MICROBIOLOGICAL QUALITY AND ANTIBIOTIC RESISTANCE PATTERNS IN MILK SUPPLY CHAIN

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## ABSTRACT

The widespread consumption of milk for its nutritional value and health benefits brings the risk of milk-borne diseases due to the presence of various microorganisms, including antibiotic-resistant pathogens. This has led to an increased focus on ensuring the safety of milk products across the supply chain by dairy industries. The study aimed to evaluate microbiological parameters and detect multi-antibiotic-resistant pathogens at three specific supply points and to explore the association between the presence of residual antibiotics and the resistant isolates in milk samples. About 50 milk samples, including raw, soon-after-processed, packaged marketed pasteurized, and UHT milk, were subjected to microbiological analysis. This involved assessing the total bacterial count (TBC) and total coliform count (TCC), conducting antibiotic susceptibility tests through disk and well diffusion assays, detecting virulence genes in multi-antibiotic resistant isolates using gene-specific PCR, and analyzing residual antibiotics by HPLC. The study revealed that the quality of raw milk samples was unacceptable (TBC >$4.5x10^7$ CFU/mL and TCC >$5.6x10^4$ CFU/mL), while pasteurized samples from processing plants had lower counts than those from retail stores (TBC >$5x10^5$ CFU/mL and TCC >$1.6x10^4$ CFU/mL) indicating post-pasteurization contamination. About 70.37% of the isolates were Gram-negative, with *Escherichia coli* (21.4%) and *Vibrio* (18.8%) being the most prevalent. Resistance to antibiotics was substantial, particularly against ampicillin (86.3%), tetracycline (76%), and ciprofloxacin (58.9%). Gene-specific PCR analysis detected *uid*A, *opr*L, and *opr*I virulence genes in multi-drug-resistant *Escherichia coli* and *Pseudomonas sp.* respectively. The study also revealed a direct association between the presence of residual antibiotics and the resistant isolates, emphasizing the need for dairy industry improvements. As high bacterial counts in milk can pose health risks by fostering antibiotic-resistant pathogens, it is essential to mitigate microbiological contamination in the milk supply chain through the implementation of various precautionary measures.

**KEYWORDS:** Milk Supply Chain, Multi-antibiotics Resistance, Virulence Genes, Residual Antibiotics

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## Introduction

Milk, a highly nutritious food, is essential for all ages. It is rich in proteins, carbohydrates, lipids, vitamins (A, D, $B_2$, $B_5$, $B_{12}$), and minerals (calcium, magnesium, sodium, selenium, potassium), making it a valuable dietary source. Its unique nutrient profile has made milk a staple in human diets for its numerous health benefits (Nur *et al.*, 2021). Furthermore, dairy products are essential for health as they enhance bone strength, provide protection against non-communicable diseases, offer antibacterial and anti-inflammatory effects, support immune function, and help maintain a healthy weight (Miciński *et al.*, 2012). However, this beneficial compound is susceptible to contamination at various points along the supply chain, posing a great risk to consumer health.

High moisture, neutral pH, and rich nutrients in milk create an ideal environment for microbial growth, rendering it susceptible to contamination at multiple stages from farms to retail shops. Milk can be contaminated by the udder, skin, animal shed, utensils, milk contact surfaces, milking staff, and the surrounding environment which can destroy the quality of milk (Frazier and Westhoff, 2007). Dairy processing plants prioritize microbial reduction, particularly pathogens, using methods like pasteurization for milk safety. Following sterilization and aseptic packaging, milk is transported to retail shops. Maintaining a constant temperature below 4°C is crucial at this stage, as any fluctuations can lead to milk deterioration and spoilage, designating this temperature as a critical control point. All stakeholders, from farmers to customers, play roles in maintaining milk quality throughout the supply chain (Naganboyina and Kaple, 2022). The perishable and temperature-sensitive nature of milk products

poses challenges, necessitating stringent hygiene and handling measures throughout the supply chain (Howard, 2022).

Milk safety is a common concern in dairy industries due to the link between handling and processing techniques and the occurrence of milk-borne diseases, including *E. coli* enteritis, beta-hemolytic streptococcal infections, shigellosis, typhoid, botulism, listeriosis, diphtheria, etc (Dhanashekar, Akkinepalli and Nellutla, 2012). This can be significantly reduced by pasteurization and enhanced sanitation procedures in dairy production. However, the potential for contamination still exists, either through process lapses or contamination occurring after pasteurization (Arafat *et al.*, 2015). A study conducted in Ethiopia in 2018 reported a range of total bacterial counts, ranging from $5x10^3$ to $3.18x10^8$ CFU/mL in raw milk and $4.4x10^1$ to $4.43x10^5$ CFU/mL in pasteurized milk samples (Tamirat, 2018). The consumption of such inadequately pasteurized milk not only leads to foodborne illnesses but also raises the risk of consuming multi-drug-resistant bacteria (Nowar *et al.*, 2021).

Global public health faces a significant threat from the proliferation of multidrug-resistant (MDR) microorganisms (Hassani *et al.*, 2022). The widespread and unregulated application of antibiotics, including tetracyclines, β-lactams, macrolides, fluoroquinolones, etc (Economou and Gousia, 2015), in both therapeutic and sub-therapeutic contexts for dairy cows, contributes to the escalation of MDR pathogens in milk products (Kamaruzzaman *et al.*, 2020). Moreover, the spread of antimicrobial resistance (AMR) genes among microbes within the dairy environment poses a potential risk, as it may be transmitted to humans through various stages of dairy processing or via the consumption of contaminated dairy goods (Brown *et al.*, 2020). Consequently, raw milk and its products are primary sources of outbreaks of antibiotic-resistant pathogens in developing nations, linked to poor hygienic practices, inadequate food safety regulations, limited resources, and neglected food management systems (Hassani *et al.*, 2022).

In Bangladesh, milk, often informally distributed, faces risks of adulteration and pathogen introduction, necessitating proper treatment for safety. Consumers favor pasteurized and UHT milk for enhanced safety and quality. Growing attention to the microbiological status of milk products in Bangladesh has led to studies on raw, pasteurized, and UHT milk, revealing high bacterial counts and the presence of coliforms in these samples (Hossain, Alam and Sikdar, 2011; Banik, Das and Uddin, 2014; Nur *et al.*, 2021). However, there is only one study carried out to evaluate the microbial quality of the milk samples along the supply chain, focusing on the Northeastern part of Bangladesh (Islam *et al.*, 2018). Despite a limited portion reaching commercial processors, Dhaka has been instrumental in developing crucial dairy zones, supporting the growth of dairy industries in Bangladesh. This study assesses the microbiological condition and identifies multi-antibiotic-resistant pathogens at three supply chain points, comparing pasteurized milk quality between processing plants and retail shops. The investigation aims to investigate the relationship between residual antibiotics and antibiotic-resistant bacteria, aiding in source identification for interventions, and enabling interventions to improve milk safety and quality throughout the supply chain, benefitting both producers and consumers.

## Materials and Methods

### *Sample Collection*

From August to October 2022, a study was conducted, involving the random collection of milk samples from four different brands in Dhaka city. This investigation included various milk types, such as raw, soon-after-processed, marketed pasteurized, and UHT (Ultra-High-Temperature) milk samples. Specifically, the raw and soon-after-processed milk samples were sourced from milk processing plants in Joydebpur, Mirpur, Narsingdi, and Narayanganj. On the other hand, various marketed milk samples were randomly collected from retail shops in Dhaka city.

A total of 50 milk samples (n=50) of four different milk brands were obtained from three distinct points within the supply chain, with about 12 samples (n=12) from each brand. Additionally, two UHT milk samples of foreign brands were collected from the markets for the study. Moreover, for each sample, an average of five samples was represented. Before obtaining milk samples, informed permission was obtained from each milk processing facility. To maintain the confidentiality of the local brands, they were anonymized as A, B, C, and D, while the foreign brands as E and F. The samples were collected in sterile falcon conical tubes and were carried in a medical ice box from the place of procurement to the Food Microbiology Laboratory, Institute of Nutrition and Food Science, University of Dhaka.

### *Sample Processing*

The procedure adhered to the methodology outlined by Akter et al. (2021) (Akter *et al.*, 2021). The samples were analyzed within an hour of procurement and were kept at -20°C in the refrigerator. From sterilized conical flasks, 10 mL of samples were extracted and transferred into test tubes, which had been pre-autoclaved and equipped with sterile cotton plugs. Subsequently, these test tubes were agitated to achieve homogenization which was regarded as initial dilution. In a test tube with a sterilized cotton plug, 1 ml of sample milk was mixed with 9 ml of 0.9% sterile sodium chloride solution. Subsequently, the mixture was thoroughly blended through stirring and shaking, resulting in a homogenized solution. This solution was then subjected to additional serial dilution up to $10^{-6}$ according to American Public Health Association (APHA) sample dilution guidelines (Rice, Bridgewater and Association, 2012).

### *Microbiological Analysis*

To isolate bacteria from the milk samples, the spread plate technique was employed (Akter *et al.*, 2021). Various media were used including Plate Count Agar (PCA), MacConkey (MAC) agar, *Salmonella-Shigella* (SS) agar, Eosin Methylene Blue (EMB) agar, Thiosulphate Citrate Bile-Salt Sucrose (TCBS) agar, De Man, Rogosa and Sharpe agar (MRS), Potato Dextrose agar (PDA), and Cooked Meat (CM) media. The preparation of these media strictly followed their respective manuals. From each serially diluted tube, a 100 µL sample suspension was transferred to pre-incubated petri dishes containing the aforementioned agar media. Following even distribution of the suspension by spread plate technique over the agar surface, the dishes were incubated at 37°C for 24-48 hours, resulting in the subsequent visibility of bacterial colonies.

***Enumeration of Total Bacterial Count (TBC) and Total Coliform Count (TCC)***

The count of viable cells present in the samples was determined using the provided formula given by Nowar et al. (2021) (Nowar *et al.*, 2021). Colonies were selectively isolated and preserved on nutrient agar slants.

The calculation of the total bacterial count (TBC) was performed with PCA media and the total coliform count (TCC) was conducted using EMB media. In addition, the acceptable quality standards for raw milk, as set by the Bangladesh Food Safety Authority (BFSA), were established at $<5x10^4$ CFU/mL for TBC and $<5x10^2$ CFU/mL for TCC (BFSA, 2021). For pasteurized and UHT milk quality in terms of TBC, the acceptable thresholds defined by the Bangladesh Standards and Testing Institutions (BSTI) and the Microbiological Criteria for foodstuffs of the European Commission (EC) were employed. These standards stipulate that TBC should be $<2x10^4$ CFU/mL for pasteurized milk (BSTI, 2002). Furthermore, the acceptable limits for TCC in both pasteurized and UHT milk samples were set at <10 CFU/mL by both BFSA and BSTI (BSTI, 2002; BFSA, 2021).

***Identification of Isolates***

Bacterial identification encompassed Gram staining for morphological traits, evaluation of cultural attributes (color, shape, size, margin, elevation, consistency, and opacity), and a range of biochemical tests including the Kliger's Iron Agar (KIA) test, Motility Indole Urease (MIU) test, Methyl Red-Voges-Proskauer (MR-VP), Citrate utilization, Catalase, and Oxidase tests. The procedures were followed as outlined by Akter et al. (2021) (Akter *et al.*, 2021).

***Antibiogram Profiling***

Both the Kirby-Bauer disk diffusion and well diffusion techniques were employed for the antibiotic susceptibility test following the procedure outlined by Akter et al. (2021) (Akter *et al.*, 2021). The antibiotic susceptibility tests of all the isolates were tested against: Tetracycline (TC) 30µg/disc, Oxytetracycline (OTC) 30µg/disc, Doxycycline (DOX) 30µL/disc, Chlortetracycline (CTC) 30µL/disc, Enrofloxacin (ENR) 5µL/disc, Ciprofloxacin (CIP) 5µg/disc, Levofloxacin (LEVO) 5µg/disc, Meropenem (MEM) 10µg/disc, Ampicillin (AMP) 10µg/disc, and Azithromycin (AZM) 30µg/disc. Among these, antibiotics such as tetracyclines (TC, CTC, OTC, and DOX) and fluoroquinolones (ENR) are frequently employed in cattle farming for both disease treatment and growth promotion (Economou and Gousia, 2015; Anika *et al.*, 2019). The results of the inhibition zones were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

The Multiple Antibiotic Resistance Index (MARI), regarded as an effective and justifiable method for detecting and monitoring antibiotic-resistant organisms, is calculated as the ratio of isolated antibiotic-resistant strains to the total number of antibiotics to which the isolates are exposed. A MARI value exceeding 0.2 indicates a significant risk of antibiotic contamination. Additionally, all dairy samples can have their Antibiotic Resistance Index (ARI) calculated following Krumperman's instructions, which involves dividing the total antibiotic-resistant score by the number of isolates and tested antibiotics (Bhaurao, Rajendra and Sarita, 2022).

***Detection of Virulence Genes by gene-specific Polymerase Chain Reaction (PCR)***

Isolated and presumed *Escherichia coli* and *Pseudomonas* colonies from nutrient agar plates were cultured overnight in 5 ml of nutrient broth within test tubes at 37ºC with aeration, utilizing a shaking water bath set at 120 rpm. After that, a 1.5 mL Eppendorf tube was filled with a 1 mL aliquot of overnight pure cultures, and the boiling method was used to extract DNA (Dashti A and Dashti H, 2009). In brief, the samples were centrifuged for 5 minutes at 14,000 rpm, the supernatant was discarded and the step was repeated. Again, the supernatant was discarded and pellets were resuspended with 200 µL of PCR-grade water by pipetting. Then, the samples were boiled in a heat block at $100^0$ C for 10 minutes followed by cooling on ice for 10 minutes, and then subjected to another centrifugation for 10 minutes at 14,000 rpm. About 120 to 130 µL of supernatant was collected from each tube and stored in a refrigerator at -20ºC (Rokon-Uz-Zaman *et al.*, 2023). The selected isolates were subjected to the polymerase chain reaction (PCR) for amplifying the target gene. PCR commenced with DNA denaturation at 94 ºC for 1 minute, followed by annealing (Table 1) and extension at 72 ºC for 30 seconds. These steps were repeated 35 times, concluding with a final DNA extension at 72 ºC for 7 minutes.

In this study, presumed *Escherichia coli* and *Pseudomonas* isolates that showed multi-drug resistance were subjected to investigate the three virulence genes namely *uid*A, *opr*I, and *opr*L. Specific primers targeting *uid*A, *opr*L, and *opr*I virulence genes were used to detect these genes (Momtaz *et al.*, 2013; Mokhtari and Amini, 2019). The sequence of the primers is given in Table 1. The expected amplicon size of the *uid*A, *opr*L, and *opr*I were 147, 504, and 249 bp respectively.

**Table 1.** Primer sequences used for the molecular detection of the selected isolates

| Target gene | Primers | Sequence (5′→ 3′) | Amplicon size (bp) | Annealing T (°C) | References |
|---|---|---|---|---|---|
| ***uid*A** | *uid*A F | 5′AAAACGGCAAGAAAAAGCAG3′ | 147 | 48 | (Momtaz *et al.*, 2013) |
| | *uid*A R | 5′ACGCGTGGTTAACAGTCTTGCG3′ | | | |
| ***opr*L** | *opr*L F | 5′ATGGAAATGCTGAAATTCGGC3′ | 504 | 57 | (Mokhtari and Amini, 2019) |
| | *opr*L R | 5′CTTCTTCAGCTCGACGCGACG3′ | | | |
| ***opr*I** | *opr*I F | 5′ATGAACAACGTTCTGAAATTCTCTGCT3′ | 249 | 54 | (Mokhtari and Amini, 2019) |
| | *opr*I R | 5′CTTGCGGCTGGCTTTTTCCAG3′ | | | |

***Detection of Residual Antibiotics by HPLC***

Residual antibiotics in milk samples were detected by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). In accordance with Zahreddine et al. (2021) (Zahreddine *et al.*, 2021), the chromatographic analysis was carried out with minor modifications. Residual antibiotics were separated using a $C_{18}$ Column maintained at 30°C. The separation process was performed under isocratic conditions using a mobile phase consisting of an aqueous solution of oxalic acid (0.05 M) and acetonitrile in a 90:10 (v/v) ratio. The mobile phase flowed through the system at a rate of 1.0 ml/min, and the entire run lasted 25 minutes. To ensure optimal sensitivity, quantitative measurements were made by selecting the appropriate detection wavelengths. Consequently, absorption spectra were examined at 220 and 280 nm (UV), with 280 nm being chosen due to its higher peak intensity and maximum sensitivity.

***Statistical Analysis***

IBM® SPSS® statistical package (version 26.0) and Microsoft Excel were used for the analysis. Descriptive statistics such as mean, standard deviation, prevalence, graphs, MARI, and bivariate analysis (Chi-square test and Pearson's correlation test) were computed. Moreover, to estimate whether the mean values of the microbial parameters and the presence of residues of one or more antibiotics were significantly different, the one-way Analysis of Variance (ANOVA) test was employed.

## Results

***Analysis of Total Bacterial Count (TBC) and Total Coliform Count (TCC) in the Samples***

The mean total bacterial counts (TBC) obtained from all milk samples collected at the collection centers were unacceptable. Sample D had the highest bacterial load of $5.8x10^8$ CFU/mL which was compared with the acceptable level given by BFSA. Meanwhile, the bacterial counts from the processing plants of samples B ($7x10^3$ CFU/mL) and C ($1.2x10^4$ CFU/mL) met the acceptable criteria, while the counts from the other samples were unacceptable based on the regulations set forth by BFSA and BSTI. Additionally, the TBC derived from milk samples obtained from retail shops also exceeded acceptable levels. Moreover, the study findings revealed that the average total coliform counts (TCC) in all milk samples from collection centers were unacceptably high. Sample D ($1.7x10^6$ CFU/mL) had the highest count of coliforms. However, except for sample B ($1x10^3$ CFU/mL), the milk samples from the processing plants showed less coliform growth, which is acceptable according to the aforementioned guidelines. Moreover, the total coliform counts in milk samples from retail shops were likewise unacceptable as per the given acceptable level. From a sanitary perspective, UHT milk samples A, E, and F were entirely free of coliform bacteria. The findings have been compiled in Table 2 which indicated that while there was a reduction in load after subsequent processing of raw milk samples, the loads were observed to increase in retail shops.

**Table 2.** Comparative assessment of the TBC and TCC of different samples

| Sample Code | Total Bacterial Count (CFU/mL) * | | | | Total Coliform Count (CFU/mL) * | | | |
|---|---|---|---|---|---|---|---|---|
| | Collection centers (CFU/mL) | Processing plants (CFU/mL) | Retail shops[a] (CFU/mL) | Retail shops[b] (CFU/mL) | Collection centers (CFU/mL) | Processing plants (CFU/mL) | Retail shops[a] (CFU/mL) | Retail shops[b] (CFU/mL) |
| **A** | $4.5 \times 10^7 \pm 1.3 \times 10^7$ | $3.5 \times 10^4 \pm 0.5 \times 10^4$ | $1.8 \times 10^6 \pm 0.27 \times 10^6$ | $5 \times 10^2 \pm 0$ | $1 \times 10^5 \pm 0.35 \times 10^5$ | <10 | $3 \times 10^4 \pm 1.2 \times 10^4$ | ND |
| **B** | $7.5 \times 10^7 \pm 1.0 \times 10^7$ | $7 \times 10^3 \pm 0$ | $1.4 \times 10^6 \pm 0.54 \times 10^6$ | ND | $4 \times 10^5 \pm 1.06 \times 10^5$ | $1 \times 10^3 \pm 0.5 \times 10^3$ | $4.1 \times 10^5 \pm 0.74 \times 10^5$ | ND |
| **C** | $3 \times 10^8 \pm 1.0 \times 10^8$ | $1.2 \times 10^4 \pm 0.45 \times 10^4$ | $5 \times 10^5 \pm 0$ | $4.7 \times 10^1 \pm 0.67 \times 10^1$ | $5.6 \times 10^4 \pm 1.08 \times 10^4$ | <10 | $2 \times 10^2 \pm 0.77 \times 10^2$ | $6 \times 10^2 \pm 0.35 \times 10^2$ |
| **D** | $5.8 \times 10^8 \pm 0.27 \times 10^8$ | $5.5 \times 10^6 \pm 0.35 \times 10^6$ | $2.9 \times 10^7 \pm 0.74 \times 10^7$ | ND | $1.7 \times 10^6 \pm 0.65 \times 10^6$ | <10 | $1.6 \times 10^2 \pm 0.89 \times 10^2$ | ND |
| **E** | NA | NA | NA | $3 \times 10^6 \pm 1.0 \times 10^6$ | NA | NA | NA | ND |
| **F** | NA | NA | NA | $1.5 \times 10^7 \pm 0.5 \times 10^7$ | NA | NA | NA | ND |

***Note.*** ND= Not Detected, NA= Not available, Retail shops[a] indicate pasteurized milk samples, Retail shops[b] indicate UHT milk samples; *The average count of 5 duplicate determinations ± standard deviations (SD) of the same samples

***Identification of Bacterial Isolates***

From 135 bacterial isolates, about 53 isolates were present in milk from collection centers, 41 isolates in milk from the processing plants, and 41 isolates in milk from retail shops. The results after the Chi-square test indicated that the prevalence of isolated microorganisms was significantly higher ($\chi^2$=167.436, p<0.0001) in unpasteurized milk obtained from the collection points (39.26%) compared to processed milk from processing plants (30.37%) and pasteurized milk sold at retail stores (24.44%). In contrast, ultra-high temperature (UHT) treated milk from retail stores had the lowest prevalence of microbial contamination, merely accounting for 5.93% of the total samples analyzed indicating contamination after pasteurization. After Gram Staining, it was found that about 70.37% of isolates were Gram-negative and 29.63% of isolates were Gram-positive bacteria. Based on the results of the biochemical tests, it became evident that the *Enterobacteriaceae* family was the predominant group among the suspected bacterial isolates identified in the milk samples, as detailed in Table 3. The isolated Gram-negative bacterial

strains were presumed, as outlined in the "Manual of Methods for General Bacteriology by the American Society of Microbiology (ASM)" and "Manual for Laboratory Investigations of Acute Enteric Infections by WHO".

**Table 3.** Exploring the biochemical characteristics of Gram-negative isolates

| Presumed bacteria | No. of isolates | KIA | | | | MIU | | | MR | VP | Oxidase | Catalase | Citrate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Slant | Butt | $H_2S$ | Gas | M | I | U | | | | | |
| *Escherichia coli* | 24 | A | A | (-) | (+) | (+) | (+) | (-) | (+) | (-) | (-) | (+) | (+) |
| *Vibrio* | 21 | K | A | (+) | (-) | (+) | (+) | (-) | (-) | (-) | (+) | (+) | (+) |
| *Klebsiella* | 11 | A | A | (-) | (+) | (-) | (+) | (+) | (-) | (+) | (-) | (+) | (+) |
| *Enterobacter* | 8 | A | A | (-) | (+) | (+) | (-) | (-) | (-) | (+) | (-) | (+) | (+) |
| *Proteus* | 7 | K | A | (-) | (+) | (+) | (-) | (+) | (+) | (+) | (-) | (+) | (+) |
| *Yersinia* | 7 | K | A | (-) | (-) | (-) | (+) | (+) | (+) | (-) | (-) | (+) | (-) |
| *Salmonella* | 5 | K | A | (+) | (-) | (+) | (-) | (-) | (+) | (-) | (-) | (+) | (+) |
| *Pseudomonas* | 4 | K | K | (-) | (-) | (+) | (-) | (-) | (-) | (-) | (+) | (+) | (-) |
| *Shigella* | 3 | K | A | (-) | (-) | (-) | (-) | (-) | (+) | (-) | (-) | (+) | (-) |
| *Citrobacter* | 3 | A | A | (+) | (+) | (+) | (-) | (+) | (+) | (-) | (-) | (+) | (+) |
| *Plesiomonas* | 1 | K | A | (-) | (-) | (+) | (+) | (-) | (-) | (-) | (+) | (+) | (+) |
| *Aeromonas* | 1 | K | A | (-) | (+) | (+) | (+) | (-) | (+) | (+) | (+) | (+) | (+) |

***Note.*** In KIA, A = Positive acid reaction (yellow), K= Negative alkaline reaction (red), (-) / (+) =Negative/Positive H2S and Gas production. In MIU, (-) / (+) = Non motile/Motile, (-) / (+) =Negative/Positive Indole production, and (-) / (+) = Negative/Positive Urease activity. In MR, (-) / (+) = Negative/Positive MR test and in VP, (-) / (+) = Negative/Positive VP test, (-) / (+) = Negative/Positive Oxidase test, (-) / (+) = Negative/Positive Catalase test, and (-) / (+) = Negative/Positive Citrate test

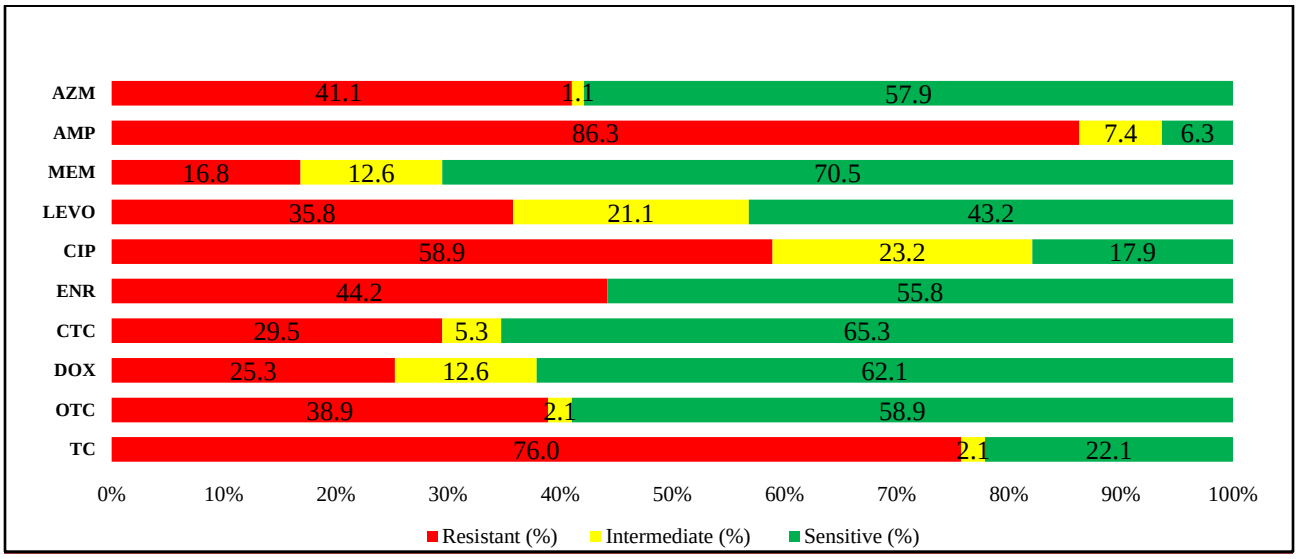
Table 4 depicts the isolated bacterial distribution of milk samples along the supply chain which showed a significant difference ($p<0.0001$) in the prevalence of bacteria among the points where the highest prevalence was seen from the collection centers and the lowest in the UHT milk of retail shops. However, some bacteria were also seen to be only in pasteurized milk of retail shops which included *Plesiomonas*, *Aeromonas*, and *Yersinia*. An in-depth exploration of the supply chain would show that some bacterial species that contaminate the raw samples were subsequently absent in the processed samples. However, due to post-processing contamination, some new bacteria were added to the supply chain.

**Table 4.** Prevalence of bacteria in the supply chain

| Presumed Microorganisms | Sampling Points | | | | Total (n=95) |
|---|---|---|---|---|---|
| | Collection (n=39) | Processed (n=29) | Retail (Pasteurized) (n=27) | Retail (UHT) (n=8) | |
| *Escherichia coli* | 10 (41.7%) | 4 (16.7%) | 9 (37.5%) | 1 (4.2%) | 24 (21.4%) |
| *Vibrio* | 12 (57.1%) | 5 (23.8%) | 4 (19.0%) | 0 (0) | 21 (18.8%) |
| *Klebsiella* | 3 (27.3%) | 4 (36.4%) | 2 (18.2%) | 2 (18.2%) | 11 (9.8%) |
| *Enterobacter* | 3 (37.5%) | 4 (50.0%) | 0 (0) | 1 (12.5%) | 8 (7.1%) |
| *Proteus* | 1 (14.3%) | 6 (85.7%) | 0 (0) | 0 (0) | 7 (6.3%) |
| *Yersinia* | 3 (42.9%) | 1 (14.3%) | 3 (42.9%) | 0 (0) | 7 (6.3%) |
| *Salmonella* | 3 (60.0%) | 2 (40.0%) | 0 (0) | 0 (0) | 5 (4.5%) |
| *Pseudomonas* | 2 (50.0%) | 1 (25.0%) | 0 (0) | 1 (25.0%) | 4 (3.6%) |
| *Shigella* | 0 (0) | 2 (66.7%) | 1 (33.3%) | 0 (0) | 3 (2.7%) |
| *Citrobacter* | 2 (66.7%) | 0 (0) | 0 (0) | 1 (33.3%) | 3 (2.7%) |
| *Aeromonas* | 0 (0) | 0 (0) | 1 (100.0%) | 0 (0) | 1 (0.9%) |
| *Plesiomonas* | 0 (0) | 0 (0) | 1 (100.0%) | 0 (0) | 1 (0.9%) |

***Examination of Antibiotic Resistance Patterns***

The antibiogram profile of the isolates was evaluated and interpreted based on the CLSI guidelines where a considerable number of bacterial isolates showed multi-drug resistance. The profile of each isolate along the supply chain is provided in Table S1 and Table S2 (Supplementary materials) which indicated a high percentage of resistant isolates in raw milk samples, along with a notable presence of resistant isolates in pasteurized milk from retail shops. According to the results in Figure 1, the examined Gram-negative isolates demonstrated different degrees of resistance to the tested antibiotics. About 46% of the antibiotics showed sensitivity, 45.26% of resistance, and 8.75% demonstrated intermediate resistance. The majority of the isolates showed high resistance to AMP (86.3%), which was followed by TC (76%), CIP (58.9%), ENR (44.2%), and AZM (41.1%). Moreover, MEM, DOX, AMP, CTC, OTC, TC, and AZM exhibited significant intermediate resistance, with CIP (23.2%) and LEVO (21.1%) demonstrating the highest levels of such resistance. It also showed that about 22.1% of isolates were resistant to two to three antibiotics, which subsequently increased to about 41.1% for five to eight antibiotics.




***Note.*** AZM (Azithromycin), AMP (Ampicillin), MEM (Meropenem), LEVO (Levofloxacin), CIP (Ciprofloxacin), ENR (Enrofloxacin), CTC (Chlortetracycline), DOX (Doxycycline), OTC (Oxytetracycline), and TC (Tetracycline)

**Figure 1.** Diagrammatic representation of the antibiogram profile of the isolates

The results from Table 5 revealed the mean distribution of the MARI where the index of the bacterial species (except *Proteus sp.*) excelled given the "0.2 limits"(Sebastião *et al.*, 2023). *Pseudomonas*, *Vibrio*, *Salmonella*, and *Escherichia coli* (*E. coli*) *sp.* displayed the highest level of multi-drug resistance. However, there had been no significant difference in MARI among the sampling points.

**Table 5.** Mean distribution of the MARI of isolates

| **Bacterial Species** | **MARI** |
|---|---|
| *Escherichia coli* | 0.53 ± 0. 22 |
| *Vibrio* | 0.63 ± 0.073 |
| *Klebsiella* | 0.35 ± 0.15 |
| *Enterobacter* | 0.29 ± 0.12 |
| *Yersinia* | 0.33 ± 0.28 |
| *Proteus* | 0.13 ± 0.076 |
| *Salmonella* | 0.54 ± 0.21 |
| *Citrobacter* | 0.23 ± 0.057 |
| *Shigella* | 0.23 ± 0.116 |
| *Pseudomonas* | 0.73 ± 0.05 |
| *Plesiomonas* | 0.20 ± 0.0 |
| *Aeromonas* | 0.40 ± 0.0 |

***Note.*** Values are in mean ± SD

Additionally, it is possible to estimate the antibiotic resistance index (ARI) for each milk sample, which will provide a clear and comprehensive view of the extensive exposure of specific isolates in particular dairy samples to certain antibiotics. Figure 2 illustrates the prevalence of %ARI of some of the milk samples which revealed $C_1$, $A_4$, and $B_3$ had the highest percentage as they exceeded the acceptable Krumperman limit suggesting the high use of antibiotics in these samples.

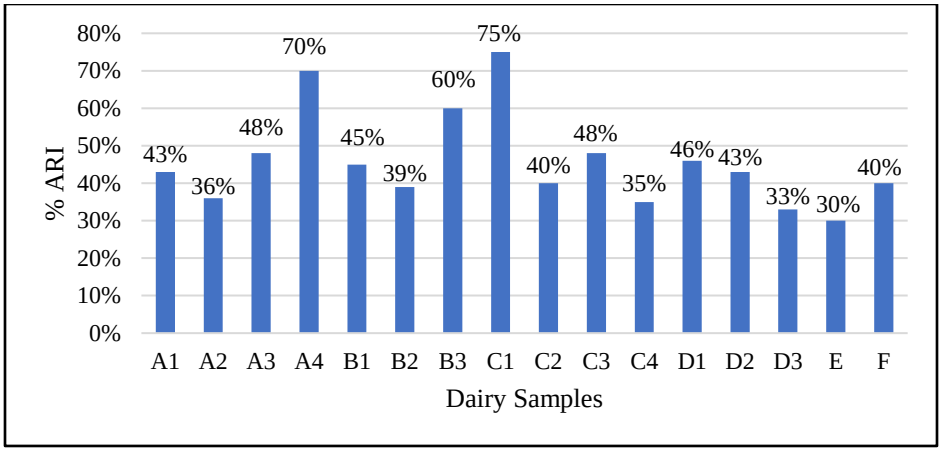
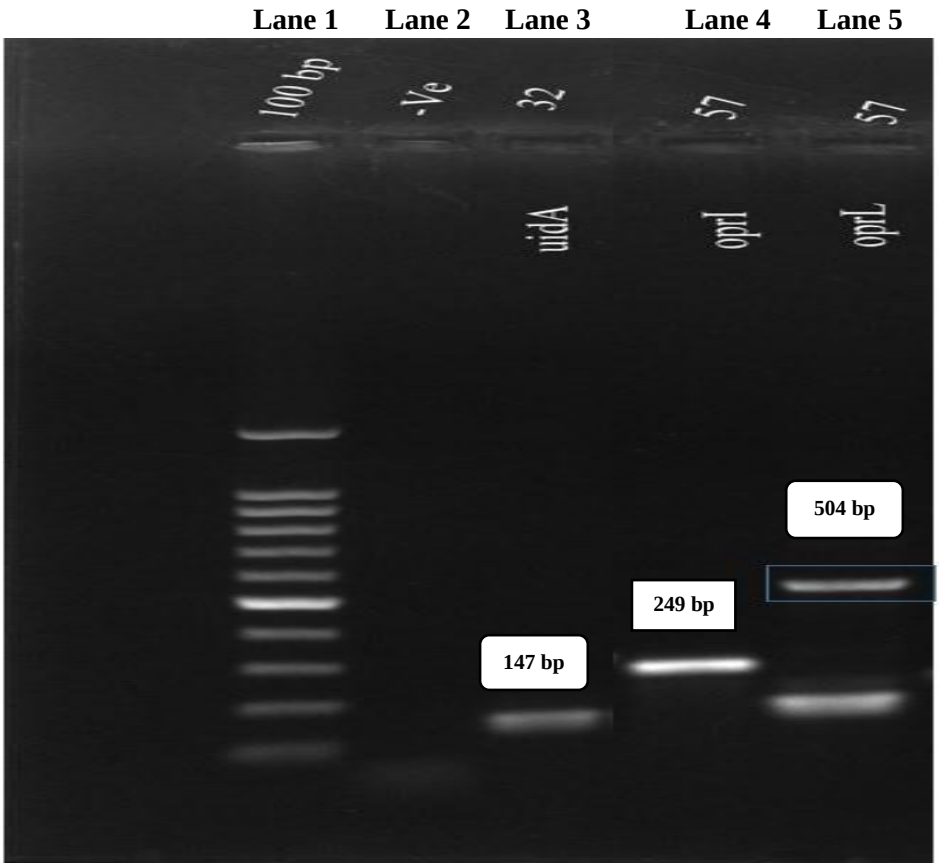



**Figure 2.** Visual representation of %ARI of the dairy samples

***Detection of Virulence Genes***

Based on the antibiogram profile and availability of primers, isolates that were presumed as *Escherichia coli* and *Pseudomonas* were selected to detect their pathogenicity. To detect the presence of *uid*A, *opr*L, and *opr*I virulence genes in the selected isolates, a gene-specific polymerase chain reaction (PCR) was done. The findings in Figure 3 revealed that the *Escherichia coli* was *uid*A positive and the *Pseudomonas sp*. was positive for both *opr*I and *opr*L genes.




***Note.*** Here, 100-basepair Ladder (Promega, USA)**: Lane 1**; Negative control**: Lane 2**; *uid*A for *Escherichia coli***: Lane 3**; *opr*I and *opr*L for *Pseudomonas sp*. respectively**: Lane 4 & 5**

**Figure 3.** Detection of virulence genes

***Comparison of Microbial Parameters Stratified by Residual Antibiotics***

To estimate whether the mean values of the microbial parameters and the presence of residues of one or more antibiotics were significantly different, a one-way ANOVA test was employed. The findings showing the comparisons are in Table 6 which revealed the mean total bacterial counts (TBC) and total coliform counts (TCC) for the milk samples that were stratified by the presence of the number of residual antibiotic agents showed significant differences in coliform count ($p=0.03$) and standard plate count ($p=0.02$) which could be interpreted that when the level of antibiotic residue increases, a decrease in the counts were seen.

**Table 6.** Comparison of microbial quality of milk with the presence of antibiotic residues

| Parameters | Mean | 95% CI |
|---|---|---|
| ***At least 1 antibiotic residue*** | | |
| TBC ($x10^8$ CFU/ mL) | 4.83 | 5.08, 14.7 |
| TCC ($x10^5$ CFU/ mL) | 3.62 | 2.09, 9.35 |
| ***2-3 antibiotic residues*** | | |
| TBC ($x10^6$ CFU/ mL) | 1.35 | 0.71, 3.42 |
| TCC ($x10^4$ CFU/ mL) | 2.23 | 1.47, 5.9 |
| ***4-5 antibiotic residues*** | | |
| TBC ($x10^5$ CFU/ mL) | 3.7 | -4.3, 5.1 |
| TCC ($x10^3$ CFU/ mL) | 0.8 | -1.7, 3.3 |

***Note***. The mean value and 95% confidence interval (CI) for the one-way ANOVA were used to assess the samples based on the number of antibiotic residues

Moreover, the linear correlation between residual antibiotic agents and resistant organisms was seen by Pearson's correlation test which is shown in Table 7. The findings indicated a moderate to a strong significant relationship ($p<0.05$) between the presence of tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) residues in samples and the occurrence of resistant isolates.

**Table 7.** Linear association between the presence of antibiotic residues and resistant isolates

| Parameters | | Resistant isolates |
|---|---|---|
| **TC** | Correlation coefficient (r) | 0.554 |
| | p-value | 0.049* |
| **OTC** | Correlation coefficient (r) | 0.721 |
| | p-value | 0.016* |
| **DOX** | Correlation coefficient (r) | 0.126 |
| | p-value | 0.22 |
| **CTC** | Correlation coefficient (r) | 0.67 |
| | p-value | 0.021* |
| **ENR** | Correlation coefficient (r) | 0.412 |
| | p-value | 0.57 |

* Correlation is significant at the 0.05 level (2-tailed)

## Discussion

Dairy products are one of the most popularly consumed foods worldwide among people of every age because of their high amount of nutrients and health benefits. However, this array of nutrients can harbor a wide range of microorganisms, especially multidrug-resistant (MDR) bacteria due to the improper use of antibacterial treatments in dairy farming which has the potential to cause milk-borne diseases. Taking this into concern, the main objective of the study was to assess the microbiological quality and to identify the multi-antibiotic-resistant pathogens in the milk supply chain.

The total bacterial count not only reflects the microbiological quality of milk but also signifies the performance of dairy farmers, milk processing companies, and retail shops in meeting quality standards. As per the guidelines, the microbial quality of the raw milk samples from the collection centers in the study was poor indicating the possibility of fecal contamination, poor personal hygiene, filthy udder, polluted milking machine, unclean living conditions, inappropriate

cooling and refrigeration conditions (Islam *et al.*, 2018; Sobeih *et al.*, 2020). Furthermore, maintaining proper herd management is crucial for preserving the sterility of raw milk, as cow mastitis is a frequent contributor to milk contamination (Nirwal, Pant, and Rai, 2013). A study conducted with 22 raw milk samples gathered from various dairy farms in Dhaka city yielded analogous results, indicating that the milk samples did not meet the standards concerning total bacterial counts (TBC) and total coliform counts (TCC) (Banik, Das and Uddin, 2014). Moreover, the pasteurized milk samples obtained from milk processing plants before packaging displayed a relatively high total bacterial count and some presence of coliform count suggesting potential issues with manufacturing practices and treatment procedures. Since appropriate pasteurization effectively eliminates pathogens, the majority of milk-borne disease outbreaks in humans have been linked to raw or insufficiently pasteurized milk, or milk that becomes contaminated post-pasteurization (Jamal, Akter and Uddin, 2018).

To assess the microbiological quality of milk consumed in Dhaka city, we analyzed packaged pasteurized milk from retail shops. Our findings revealed that all milk samples, despite being within their expiry dates, exhibited high TBC ($>5x10^5$ CFU/mL) and TCC ($>1.6x10^2$ CFU/mL) suggesting proper aseptic conditions and refrigeration temperatures were not maintained in the retail stores and resulted in post-pasteurization contamination (Arafat *et al.*, 2015). These findings may result from various factors, including inadequate freezer temperatures at retail shops influenced by load shedding and power outages, insufficiently sterilized packaging materials, and cross-contamination due to packaging defects or exposure to external materials during transport. Long-term storage of milk at low temperatures can also promote the growth of many proteolytic and psychotropic bacteria, with *Pseudomonas sp.* being the most common type found (Nur *et al.*, 2021). A similar study conducted with 60 milk samples in Ethiopia found a substantial bacterial load in the milk from retail shops (Kumar, Tolossa and Abdisa, 2015). Furthermore, another study was conducted in Dhaka with five renowned pasteurized and UHT milk samples that showed a high load in pasteurized samples and an absence of growth in UHT (Nur *et al.*, 2021). However, in this study, a high load of TBC but low TCC was found in the UHT milk samples. This can be attributed to various factors, including milk quality, sanitation practices in the processing plant, the condition of packaging materials, inadequate heat sterilization, post-pasteurization contamination, and the methods employed during handling (Tekinsen, Elmali and Ulukanli, 2007).

About 70.37% of isolates were found to be Gram-negative. The study mainly focused on the Gram-negative rods because it was seen that Gram-negative pathogens developed antibiotic resistance, are highly virulent, potential bioweapons, and have severe disease burdens with high costs (Oliveira and Reygaert, 2022). CDC has also explored and investigated many outbreaks and found many significant epidemics caused by Gram-negative pathogens such as *Klebsiella*, *Escherichia coli*, *Pseudomonas, Salmonella*, *Shigella, Enterobacter*, *Vibrio sp.*, and many more which can cause many foodborne and waterborne illnesses. The study analysis found that the majority of the isolates belong to the *Enterobacteriaceae* family. Among them, fecal coliforms like *Escherichia coli* (21.4%), *Klebsiella* (9.8%), and *Enterobacter* (7.1%) were the most prevalent. Furthermore, *Vibrio, Proteus, Yersinia, Salmonella, Shigella, Citrobacter, Plesiomonas,* and *Aeromonas* were also present. The presence of these isolates suggests unhygienic manufacturing practices, insufficient pasteurization, or contamination that occurred after pasteurization. Similar results were found in a study conducted in Egypt which detected isolates of *Klebsiella, Escherichia coli, Enterobacter, Proteus, Citrobacter, Shigella,* and *Yersinia* from 200 samples of raw milk and milk products (Sobeih *et al.*, 2020). The high prevalence of *E. coli* is a reliable index of fecal contamination and reflects poor processing techniques as coliform bacteria cannot survive the pasteurization process (Hossain, Alam and Sikdar, 2011).

Antibiotic resistance, driven by horizontal and vertical gene transfer as well as intrinsic chromosomal genes, has led to a global surge in multi-drug resistant Gram-negative pathogens, posing a significant public health threat (Wall *et al.*, 2016). Thus, we assessed antibiotic susceptibility tests of Gram-negative bacterial isolates only. The study findings revealed that about 45.26% of isolates were resistant, 46% were susceptible, and 8.75% of them developed intermediate resistance. This study also showed that the majority of the isolates were highly resistant to AMP, followed by TC, CIP, ENR, and AZM. The highest level of intermediate resistance was shown by CIP and LEVO implying that some routinely used antibiotics are becoming ineffective by the examined bacterial isolates, which may have significant effects on the treatment of such bacterial illnesses. The prevalence of these pathogenic-resistant strains may be attributed to inadequate hygiene standards and the uncontrolled utilization of antimicrobials (Peters *et al.*, 2019). In addition, our study demonstrated that the majority of the antibiotic-resistant isolates were from the collection points and retail shops reflecting the possibility of health risks associated with the newly developing resistant isolates calling for proper farming and processing techniques to reduce the prevalence of such resistant pathogens.

In our study, *Pseudomona*s exhibited a Multiple Antibiotic Resistance Index (MARI) of 73%, *Vibrio* demonstrated a MARI of 63%, and *E. coli* and *Salmonella* both showed a MARI of 54% signifying that these bacteria are developing resistance to multiple antibiotics. A previous study indicated that around 60% of the antibiotic-resistant isolates displayed resistance to multiple drugs, signifying a multidrug-resistant (MDR) profile (Ntuli, Njage and Buys, 2016). Our study findings also indicated that about 41.1% of isolates were resistant to 5-8 antibiotics depicting the emergence of multi-antibiotic-resistant pathogens. After calculating the Antibiotic Resistance Index (ARI) for each sample, it was found that all the samples contained antibiotic-resistant pathogens, particularly, $A_4$, $C_1$, and $B_3$ had the highest level.

Through gene-specific PCR assay, the pathogenicity of multi-antibiotic resistant *Escherichia coli* and *Pseudomonas* were identified by the presence of their virulence genes which were *uid*A, *opr*L, and *opr*I respectively. These virulence genes were selected based on their availability, their ability to invade tissues, and their public health impact. Despite exhibiting high multi-antibiotic resistance, *Vibrio sp.*, *Salmonella sp*., and other suspected microorganisms could not have their virulence genes detected due to the absence of necessary primers and other resources. The study was able to detect the virulence genes of *Escherichia coli* and *Pseudomonas*, particularly,

*Pseudomonas aeruginosa*. The virulence gene found in *Escherichia coli* is prevalent in extraintestinal pathogenic *E. coli* strains (Aslam *et al.*, 2014), which are responsible for a range of infections such as urinary tract infections, neonatal meningitis, sepsis, pneumonia, surgical site infections, and extraintestinal infections (Smith, Fratamico and Gunther, 2007), as they produce adhesins, toxins, lipopolysaccharides, and other virulence-related factors (Sarowska *et al.*, 2019). Moreover, *Pseudomonas* virulence genes lead to spoilage of milk and chronic respiratory infections in humans (Sainz-Mejías, Jurado-Martín and McClean, 2020) by producing virulence factors known as I lipoprotein (*opr*I) and L lipoprotein (*opr*L) (Nikbin *et al.*, 2012) highlighting the significant impact of these genes on their pathogenicity. A similar study in Ghana also detected *uid*A (147 bp fragments) species and gene-specific *E. coli* in 250 milk samples (Adzitey et al., 2022). Moreover, using the same PCR technique a study in Egypt identified *Pseudomonas sp.*, especially *P. aeruginosa* (product size of 504 bp) (Atia et al., 2022).

One of the main concerns of this study was to see the relationship between the presence of residual antibiotic agents and the prevalence of resistant organisms. Thus, Pearson's correlation test was performed to investigate any linear association between antibiotic agents and resistant isolates (Jayarao and Wang, 1999; Brown *et al.*, 2020; Buczkowska *et al.*, 2021). The test demonstrated that there existed a significant positive moderate association between resistant isolates and TC, OTC, and CTC residues. Several factors may be responsible for these findings. Firstly, the frequent use of TC and OTC antibiotics in cattle treatment for clinical mastitis is a significant source of residual antibiotics in milk samples, potentially inducing antibiotic-resistant pathogens (Siljanoski *et al.*, 2018). Apart from the overuse of antibiotics in cattle farming, antibiotic resistance may develop from excessive usage of antibiotics by humans, shortage of safe water and hygiene, inadequate medical management, lack of access to medications and vaccines, poor knowledge, inaccurate drug prescription, ignorance about antibiotics dosage and course, and scarcity of novel antibiotics. In South Asia, antibiotic misuse has been a major driver of antibiotic resistance leading to prolonged disease progression, extensive medications, higher levels of illness and death rates, and lastly increased economic burden at the national level (Ventola, 2015; Hussain *et al.*, 2023). Furthermore, antibiotics are actively eliminated in cattle feces, contaminating the environment and facilitating the spread of antibiotic-resistant pathogens (Rahman, Hassan and Chowdhury, 2021). Finally, bacteria itself can quickly develop adaptive resistance through mutations in their genomes under the selective pressure of antibiotics and their environment (Skalet *et al.*, 2010). Therefore, while residual antibiotics in dairy products are a significant contributor to the occurrence of antibiotic-resistant pathogens, they are just one of several factors at play in this complex issue.

## Strengths and Limitations

The primary strength of the study lies in its novelty. The study found antibiotic-resistant Gram-negative bacteria along the supply chain. The study also identified significant differences in the presence of microbial parameters and residual antibiotics highlighting a positive linear correlation between resistant isolates and residual antibiotics, calling for urgent actions to protect public health and enhance safety measures. However, time and resource constraints limited the exploration of Gram-positive bacteria and their virulence, highlighting the need for further research. Moreover, the relatively small sample size constrained the ability of the study to draw definitive conclusions. Due to resource limitations, virulence genes in *Vibrio*, *Salmonella*, and other isolates could not be assessed.

## Conclusion

The study findings emphasize the microbiological quality of the milk supply chain and provide a substantial contribution to the scientific knowledge of Gram-negative bacteria and their involvement in multi-drug resistance. Since the findings were not that acceptable, some precautionary approaches can be recommended to maintain the quality of milk which include, proper farm management, the application of quality management protocols by the dairy industries, stringent prohibition of random and misuse of antibiotics, and implementation of proper guidelines at both farm and industry levels. Moreover, the study also established a correlation between residual antibiotics and multi-drug resistance, highlighting a growing concern for public health. The findings from this study would also help the food safety authorities to conduct regular and vigilant monitoring of both milk processing companies and retail shop sellers.

## Ethical Statement

Ethical approval was unnecessary for this study since it did not involve any human subjects or animals. All milk samples were obtained from collection centers, processing plants, and retail shops, eliminating the need to handle animals.

## Conflict of Interest

The authors have no conflict of interest.

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