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# Research Article
# Microbiological Quality Assessment of Raw and Commercial Milk Available in the Local Market and its Acceptability

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## Abstract

**Background and Objective:** Milk is an inclusive nutritious food with carbohydrates, fat, proteins, calcium, iodine and potassium etc. and because of these, it is highly vulnerable to bacterial contamination. The present study was carried out to assess the microbial status of processed and raw milk samples collected from different local small farms and markets. **Materials and Methods:** A total set of 45 milk samples, comprising a group of raw, pasteurized and UHT (15 each) milk were collected for microbiological analysis. **Results:** The average means value of total count in raw, pasteurized and UHT milk was $2.6810^3$, $2.8310^2$ and $8.610^1$ CFU $mL^{-1}$, respectively. Both Coliforms and *E. coli* were found in raw and pasteurized milk samples separately. The mean value of Coliforms and *E. coli* was lower in UHT milk (6.53 and 3.11 MPN $mL^{-1}$) whereas comparatively higher in raw (111.7 and 50.49 MPN $mL^{-1}$) and pasteurized milk (46.46 and 21.35 MPN $mL^{-1}$). A significant difference was observed in the comparison of mean count in Coliform ($F = 12.538$, $p = 0.0006$) and *E. coli* ($F = 4.284$, $p = 0.0256$). *E. coli* was identified using the BIOLOG™ identification system and subunit A and B of Shiga-toxin identified near 32 and 7.7 KD using SDS-PAGE. **Conclusion:** The state of contamination in milk samples and the presence of pathogenic bacteria illustrated the alarming situation in the dairy industry. The outcome of the current study leads to enhance awareness concerning public health safety issues and the employment of regulatory agencies of Bangladesh.

Key words: BIOLOG™ Identification, contamination, *E. coli*, milk quality, processed milk

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Milk is known to be the most complete food found in nature with approximate compositions of water (87.1%), lactose (4.6%), fat (4%) and protein (3.3%)[1-3]. Being an enriched source of major nutrients milk and milk-based products has achieved an increased production and consumption rate all over the world. However, milk can be contaminated from various sources such as air, soil, feed, milking equipment, feces, cow's udders, flies and manure, etc[4-6]. Milk is a complex biological fluid, which is highly vulnerable to bacterial contamination as it serves as an excellent growth medium for the majority of microorganisms[7]. The Food industry faces great economic losses by bacterial spoilage, where psychrophilic microorganisms are concerned because of the permissive temperature that exists in dairy products[8,9]. Due to a large number of microorganisms in raw milk and post-pasteurization contamination, pasteurization itself can't guarantee the removal of microorganisms[10,11]. Pasteurized milk has a comparatively short shelf life where UHT milk can be stored for three to six months at ambient temperature as it is heated to 135-140°C for a few seconds[12]. Total viable bacterial count is being considered as one of the acceptance criteria for categorizing milk for human consumption and processing for dairy products and the quality and safety of dairy products directly associated with the bacterial number as the contaminated milk becomes unsuitable for further processing[7,13,14]. The previous results point out that the presence of indicator organisms such as Coliform and *E. coli* is a burning health issue as a very little amount of indicator organisms in both raw and processed milk products can bring out a serious hamper to human health, especially to children as they are vital consumers of milk[13,15].

In most countries, the legal limits for the total viable bacterial count in pasteurized milk range from $5\times10^3$-$5\times10^5$ CFU m $L^{-1}$[7]. Like Zimbabwe as their minimum accepted bacterial limit is $5\times10^5$ CFU $mL^{-1}$, most countries set their accepted bacterial limits for raw milk and milk products[13]. The acceptable limit for viable bacteria and Coliforms in pasteurized milk is $\leq 2\times10^4$ CFU $mL^{-1}$ and <10 CFU $mL^{-1}$, respectively in Bangladesh according to Bangladesh Standards and Testing Institute, (2018)[11]. Besides, coliform is a feasible and cost-effective indicator of fecal contamination of milk and dairy products and the presence of it indicates environmental sources of contamination[13,15,16]. Among the coliform species, *E. coli* usually contaminates raw and processed milk[13]. However, the recovery of Shiga toxin-producing *E. coli* (STEC) from food is a serious public health concern[7]. The STEC is a globally leading and emerging zoonotic pathogen, which causes foodborne illnesses and *E. coli* is the commonest serotype connected with sporadic outbreaks of dysenteric diarrhea and severe hemolytic-uremic syndrome (HUS)[17]. *E. coli* has become an important alarm of dairy industries and cattle are considered its main reservoir[18,19].

In Bangladesh, a very large amount of milk is being produced unrecognized ways and because of this, it has been very tough to ensure safety and quality for the consumers[7]. Thus, the present study aimed to provides an indication of the necessity to explore the degree of microbial contamination as well as to elucidate the scenario of microbiological quality of raw, pasteurized and UHT milk samples of Bangladesh.

## MATERIALS AND METHODS

**Study area:** The research was carried out at the Industrial Microbiology Laboratory of the Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR). The sample collection and the investigation were conducted during the period of April to November, 2018.

**Study design and sample collection:** The present research was involved to analyze the microbiological quality of raw, pasteurized and UHT milk samples and the aim were to assess the quality of milk samples. A total number of 45 milk samples (within their expiration date) consisting of raw cow milk (15), pasteurized milk (15) and UHT milk (15) were collected. Pasteurized and UHT milk samples were collected from the prominent band companies of Bangladesh with different batch numbers as prepared for the final consumers and raw milk samples were collected from different local sites including the registered dairy firms. The samples were analyses carefully before the completion of the date of expiry.

**Evaluation of aerobic plate count:** Total aerobic plate count was done by several steps according to aerobic plate count techniques stated in the Bacteriological Analytical Manual[20]. At first, the representative part of the sample was homogenized with diluents to obtain 1:10, 1:100, 1:1000, 1:10000 and others by transferring 10 mL of the previous dilution to 90 mL of sterile diluent. After that, 1 mL from each dilution was pipetted into sterile, duplicate and appropriate mark Petri dishes. Then 12-15 mL of Plate Count Agar (PCA) medium, cooled at 45±1°C, placed on Petri dishes with dilution using pour plate technique with the negative control. Then the sample was serially diluted and plated on agar media and incubated the plates at 37°C for 24±2 hrs.

**Detection and enumeration of Coliform and *E. coli*:** The detection of coliform and *E. coli* was performed by the triplicate tube detection method described by Kumar and Prasad[4]. The enumeration of Coliforms was performed using the Most Probable Number (MPN) technique and MPN per milliliter was calculated of the test sample from the number of tubes showing gas formation in BGB broth. The enumeration of *E. coli* was done using most the probable number technique according to Mhone *et al.*[13]. The most probable number of *E. coli* was determined by means of the MPN table according to the number of tubes of single and double-strength medium whose subcultures had produced gas in the EC broth and indole in the peptone water at 44°C. For further confirmation, a typical colony on EMB agar was predicted as coliform and the *E. coli* was confirmed by Indole production using Kovac's indole reagent.

**Identification by BIOLOG™ system:** After confirmation of coliform and *E. coli*, BIOLOG™ (BIOLOG™, Inc., Hayward, USA) identification assay techniques on GEN III microplate were applied for further precision and species-level identification[21]. This system mainly works based on the utilization of 71 carbon sources and 23 chemical sensitivity assays. The isolates were cultured on Biolog Universal Growth (BUG) agar medium. After 18 hrs incubation of suspected bacterial isolates was added to the inoculating fluid-A (pre-warmed) for getting the (90-98%) turbidity. The cell suspension was then filled into 96 wells of GEN III microplate with perfectly 100 µL and incubates at 37°C for 18-24 hrs. After the incubation period, the microplate was sited into the Micro Station Reader and the result was given through comparing with the database using the software program MicroLog 4.20.05 (BIOLOG™, USA). The possibility of the 96 assay reactions, along with sophisticated interpretation software, conveys a high level of accuracy that is equivalent to molecular techniques.

**Outer membrane protein analysis:** Outer membrane protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the use of 10% separating gel and 5% stacking gel. The 10 µL of molecular weight standard of Benchmark TM Pre-Stained Ladder was used as a marker. 0.1% Coomassie Brilliant Blue R 25 (0.1%) and 10% Acetic acid were used as staining and destaining of the gel.

**Statistical analysis:** The data was entered and analyzed using SPSS (IBM Corporation, USA) version 23 statistical package software. All experiments were conducted in triplicate and presented as the Mean±Standard Deviation (SD). Statistical significance was set at a P-value of less than 0.05 ($p<0.05$) by using a One-way Analysis of Variance (ANOVAs).

## RESULTS

**Enumeration of total aerobic plate count:** An investigation on recognition of contamination in raw, pasteurized and UHT milk samples were designed to be done by total plate count technique[20]. ANOVA was used to compare the level of contamination in various types of milk samples. The total mean of aerobic bacteria was $2.6810^3$, $2.8310^2$ and $8.610^1$ CFU $mL^{-1}$ in raw, pasteurized and UHT milk samples, respectively. In specific interpretation, the F-value was found to be 1.243 and P-value as 0.306 (Table 1). The mean aerobic counts indicate that UHT milk samples have a lower burden of microbial loads compared to raw and pasteurized milk samples. The experiments were repeated three times.

**Recognition of total coliform and *E. coli*:** A total of 45 milk samples were examined and the results were given away in Table 2. Out of 45 samples, Coliforms and *E. coli* were detected from the 21 and 20 samples, respectively. Both Coliforms and *E. coli* were found in 10 (out of 15, >70%) pasteurized milk samples and one (out of 15) UHT milk samples. Raw milk was entirely contaminated with Coliforms. The result from the present study stated that the presence of Coliforms and *E. coli* was much higher in raw cow milk as compared to pasteurized and UHT milk samples that showed similarity with previous studies where Coliforms and *E. coli* was found dominant in raw milk and moderately in pasteurized milk as well as comparatively lower in UHT milk samples respectively[7,22,23].

**Enumeration of coliform and *E. coli*:** The total mean value of Coliform and *E. coli* in raw milk was 111.7 and 50.49, 46.46 and 21.35 in pasteurized milk, 6.53 and 3.11 MPN $mL^{-1}$ in UHT milk samples, respectively. A comparison of mean counts of three types of milk samples showed a statistically significant difference ($F = 12.538$, $p<0.01$) in total Coliform counts as well as *E. coli* counts ($F = 4.284$,

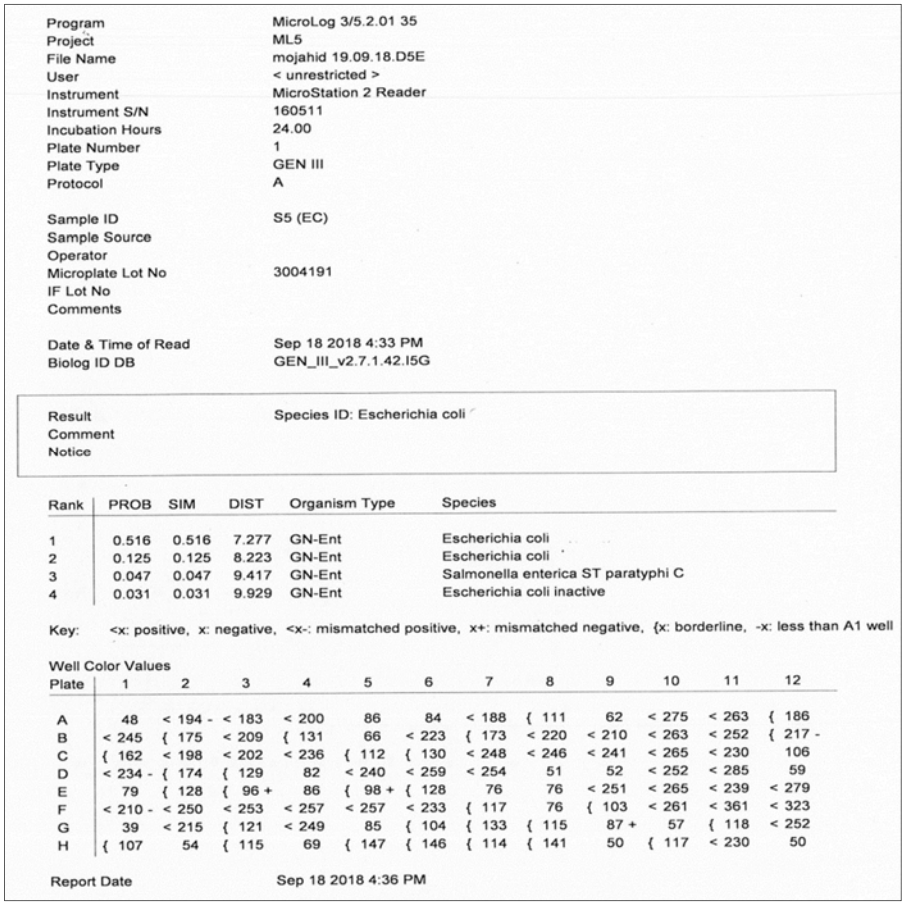
Table 1: Mean value of aerobic bacteria in raw, pasteurized and UHT milk samples in per milliliter

| | | Total aerobic bacteria/milliliter | | |
|---|---|---|---|---|
| Sample category | Sample (N) | Mean | ANOVA (F-value) | p-value |
| Raw milk | 15 | 26840.00 | | |
| Pasteurized milk | 15 | 2836.00 | 1.243 | 0.306 |
| UHT milk | 15 | 86.00 | | |

Table 2: Detection status of Coliforms and *E. coli* in milk samples

| | | Total coliforms | | *E. coli* | | |
|---|---|---|---|---|---|---|
| Sample ID | Sample category | Gas in LT broth | Typical colony on EMB | Gas in EC broth | Typical colony on EMB | Indole production |
| 1.1.E | Pasteurized | + | + | + | + | + |
| 2.1.A | Pasteurized | + | + | + | + | + |
| 3.1.P | Pasteurized | - | n/a | - | n/a | n/a |
| 4.1.V | Pasteurized | - | n/a | - | n/a | n/a |
| 5.2.A | Pasteurized | + | + | + | + | + |
| 6.2.V | Pasteurized | + | + | + | + | + |
| 7.2.U | Pasteurized | + | + | + | + | + |
| 8.3.V | Pasteurized | + | + | + | + | + |
| 9.4.A | Pasteurized | + | + | + | + | + |
| 10.4.F | Pasteurized | - | n/a | - | n/a | n/a |
| 11.5.F | Pasteurized | + | + | + | + | + |
| 12.5.V | Pasteurized | + | + | + | + | + |
| 13.7.V | Pasteurized | + | + | + | + | + |
| 14.7.F | Pasteurized | - | n/a | - | n/a | n/a |
| 15.3.A | Pasteurized | - | n/a | - | n/a | n/a |
| 16.6.R | UHT | - | n/a | - | n/a | n/a |
| 17.6.A | UHT | - | n/a | - | n/a | n/a |
| 18.2.R | UHT | - | n/a | - | n/a | n/a |
| 19.3.V | UHT | - | n/a | - | n/a | n/a |
| 20.5.R | UHT | - | n/a | - | n/a | n/a |
| 21.6.A | UHT | - | n/a | - | n/a | n/a |
| 22.1.F | UHT | - | n/a | - | n/a | n/a |
| 23.3.F | UHT | - | n/a | - | n/a | n/a |
| 24.4.A | UHT | - | n/a | - | n/a | n/a |
| 25.3.R | UHT | - | n/a | - | n/a | n/a |
| 26.2.F | UHT | - | n/a | - | n/a | n/a |
| 27.3.A | UHT | - | n/a | - | n/a | n/a |
| 28.7.A | UHT | + | + | + | + | + |
| 29.7.P | UHT | - | n/a | - | n/a | n/a |
| 30.7.S | UHT | - | n/a | - | n/a | n/a |
| 31.8.GT | Raw | + | + | + | + | + |
| 32.9.GA | Raw | + | + | - | n/a | n/a |
| 33.10.KD | Raw | + | + | + | + | + |
| 34.10.AD | Raw | + | + | + | + | + |
| 35.11.LA | Raw | + | + | + | + | + |
| 36.12.LM | Raw | + | + | + | + | + |
| 37.12.LM | Raw | + | + | + | + | + |
| 38.12.LM | Raw | + | + | + | + | + |
| 39.12.LM | Raw | + | + | + | + | + |
| 40.12.LM | Raw | + | + | + | + | + |
| 41.11.LA | Raw | + | + | + | + | + |
| 42.11.LA | Raw | + | + | + | + | + |
| 43.12.LM | Raw | + | + | + | + | + |
| 44.9.GA | Raw | + | + | - | n/a | n/a |
| 45.9.GA | Raw | + | + | - | n/a | n/a |

LT: Lauryl tryptose broth, EC: *E. coli*, EMB: Eosin methylene broth



| | |
|---|---|
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| Project | ML5 |
| File Name | mojahid 19.09.18.D5E |
| User | < unrestricted > |
| Instrument | MicroStation 2 Reader |
| Instrument S/N | 160511 |
| Incubation Hours | 24.00 |
| Plate Number | 1 |
| Plate Type | GEN III |
| Protocol | A |
| Sample ID | S5 (EC) |
| Sample Source | |
| Operator | |
| Microplate Lot No | 3004191 |
| IF Lot No | |
| Comments | |
| Date & Time of Read | Sep 18 2018 4:33 PM |
| Biolog ID DB | GEN_III_v2.7.1.42.I5G |
| Result | Species ID: Escherichia coli |
| Comment | |
| Notice | |

| Rank | PROB | SIM | DIST | Organism Type | Species |
|---|---|---|---|---|---|
| 1 | 0.516 | 0.516 | 7.277 | GN-Ent | Escherichia coli |
| 2 | 0.125 | 0.125 | 8.223 | GN-Ent | Escherichia coli |
| 3 | 0.047 | 0.047 | 9.417 | GN-Ent | Salmonella enterica ST paratyphi C |
| 4 | 0.031 | 0.031 | 9.929 | GN-Ent | Escherichia coli inactive |

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

| Plate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 48 | < 194 - | < 183 | < 200 | 86 | 84 | < 188 | { 111 | 62 | < 275 | < 263 | { 186 |
| B | < 245 | { 175 | < 209 | { 131 | 66 | < 223 | { 173 | < 220 | < 210 | < 263 | < 252 | { 217 - |
| C | { 162 | < 198 | < 202 | < 236 | { 112 | { 130 | < 248 | < 246 | < 241 | < 265 | < 230 | 106 |
| D | < 234 - | { 174 | { 129 | 82 | < 240 | < 259 | < 254 | 51 | 52 | < 252 | < 285 | 59 |
| E | 79 | { 128 | { 96 + | 86 | { 98 + | { 128 | 76 | 76 | < 251 | < 265 | < 239 | < 279 |
| F | < 210 - | < 250 | < 253 | < 257 | < 257 | < 233 | { 117 | 76 | { 103 | < 261 | < 361 | < 323 |
| G | 39 | < 215 | { 121 | < 249 | 85 | { 104 | { 133 | { 115 | 87 + | 57 | { 118 | < 252 |
| H | { 107 | 54 | { 115 | 69 | { 147 | { 146 | { 114 | { 141 | 50 | { 117 | < 230 | 50 |

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Fig. 1: Identification of *E. coli* by BIOLOG™ microarray system

Table 3: Enumeration total coliforms and *E. coli* in per mL milk sample

| | | Coliform, MPN mL$^{-1}$ | | | *E. coli*, MPN mL$^{-1}$ | | |
|---|---|---|---|---|---|---|---|
| Sample category | Sample (N) | Mean | ANOVA (F-value) | p-value | Mean | ANOVA (F-value) | p-value |
| Raw milk | 15 | 111.7 | 12.538 | 0.0006 | 50.49 | 4.284 | 0.0256 |
| Pasteurized milk | 15 | 46.46 | | | 21.35 | | |
| UHT milk | 15 | 6.53 | | | 3.11 | | |

p = 0.025) in 1 mL sample. This indicates the UHT milk samples contain the significantly lowered amounts of microbial load compared to raw milk and pasteurized milk (Table 3).

**Identification of isolates using the BIOLOG™ system:** The BIOLOG™ system aimed to provide a rapid, convenient approach of identification of bacteria with a database of 3000 species. In the BIOLOG™ identification system, Coliform and *E. coli* strains were correctly identified up to the species level. The result specifies that *E. coli* was identified from the bacteria isolated from milk samples (Fig. 1). For further confirmation, these isolates examined for three replications. Figure 1 demonstrates the result of BIOLOG™ identification system.

**Identification of Shiga toxin from *E. coli*:** The outer protein of *E. coli* was determined by SDS-PAGE (10% gel) and was compared with the standard protein marker obtained from Promega, USA. The result of the present study states that Shiga toxin, an etiologic agent of STEC, identified from the *E. coli*. The molecular mass of subunit-A of Shiga toxin identified near 32 KD and the subunit-B of this toxin identified near 7.7 KD. The STEC associated Outer Membrane Protein (OMP) was identified near 35 KD in separating gel of SDS-PAGE (Fig. 2).

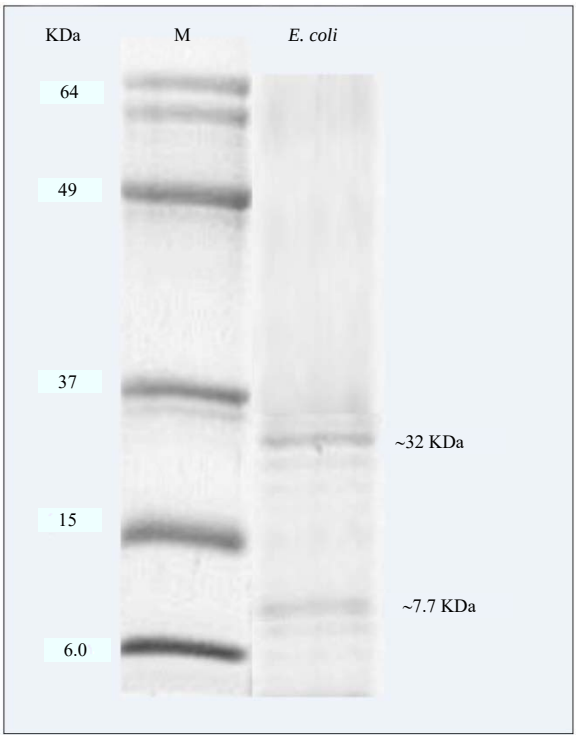


Fig 2: Identification of Shiga toxin, the left lane (M) indicates the band patterns of the molecular marker, the right lane shows the band pattern of Shiga toxin where subunit-A at ~32 KD and subunit-B at ~7.7 KD

## DISCUSSION

Food safety is a critical issue for all over the earth, especially in dairy chains where most of the operations depend on human handing. Among the dairy products demand for direct consumption milk is very high, keeping this issue in mind, the research was designed to explore the contamination level in raw milk and processed milk samples from the local market of Bangladesh. The Aerobic Plate Count (APC) is the indicator of the level of microbial contamination in a product[20]. The analysis showed that the average value of the total viable count from nearly all samples (45) does not comply with the standard value, which was set by BSTI in Bangladesh[11]. The mean microbial load in UHT milk ($>10^1$ CFU mL$^{-1}$) was lower compared to raw ($>10^3$ CFU mL$^{-1}$) and pasteurized milk ($>10^2$ CFU mL$^{-1}$). In a recent study at ICDDR'B where they found that around 77% of the pasteurized milk samples were high in aerobic plate count ($> 10^4$CFU mL$^{-1}$) from the standard value. The result indicated the defects in dairy processing as viable count reflects the standards of primary production operations, collection, transportation and storage[7]. The contaminated milk reduces the nutritional quality as well as threatens the health of the society[24]. The study revealed that all raw milk samples were contaminated by Coliforms and >70% of pasteurized milk samples were contaminated by both Coliforms and *E. coli* respectively, which was extremely higher than the previous findings in Bangladesh where pasteurized milk contained >31 and >2% of this microorganisms[7]. The averages mean value of Coliforms and *E. coli* in raw and pasteurized milk were higher from a standard value (>10 CFU mL$^{-1}$). However, the mean value of Coliforms and *E. coli* in UHT milk samples had drastically lower (<10 CFU mL$^{-1}$) than raw and pasteurized milk. Coliform bacteria is usually regarded as an indicator of unhygienic condition and post-processing contamination, while fecal contamination is considered due to the presence of *E. coli* in the samples and probably connected with human enteric pathogens[7,22,23].

The presence of *E. coli* in the milk sample suggested the milk contaminated with wastewater and fecal kinds of stuff[24]. Although, most of the strains of *E. coli* in noxious, some can cause diarrhea, Urinary Tract Infections (UTI), respiratory illness, pneumonia and other illnesses. Among coliform, enterohemorrhagic *Escherichia coli* has become an effective biological weapon[25]. From random identification, the study found that the milk sample was contaminated with Shiga toxin-producing *E. coli* and the toxin was identified through the determination of molecular mass by SDS-PAGE. Both types of Shiga toxins (St×1 and St×2) have an AB structure, where A-subunit has a molecular weight of 32 KD and B-subunit has a molecular weight of 7.7 KD[26]. A subunit and B subunit of Shiga toxin was identified near 32 KD and 7.7 KD respectively. Moreover, near 35 KD another band was identified. A previous study found that STEC associated, the most abundant molecular masses of Outer Membrane Proteins (OMPs) were close to 35 KD[17]. In a previous study from Bangladesh, around 10% of raw milk samples were found to be positive for STEC[27]. The presence of pathogenic bacteria in milk can cause several symptoms such as nausea, fever, vomiting, loose stools and even death in severe cases[28]. If the contaminated milk is consumed without proper treatment and/or sterilization this can conduct the outbreak of serious foodborne infection in society[7].

## CONCLUSION

Food safety is paramount for healthcare management in Bangladesh. This becomes very critical when food supply sectors, for instance, dairy chains and their operations, remain unorganized and are not automatically regulated and largely dependent on human handling. Besides economic loss, milk

spoilage can cause severe life-threatening diseases. The study revealed that raw and pasteurized milk was equally contaminated as well as UHT milk was also infected. In general, raw and pasteurized milk are consumed after heat treatment by consumers. However, UHT milk is consumed directly without any treatment process. The presence of pathogenic bacteria with their virulence factors in milk is very alarming for the milk industry of Bangladesh. An emergency need for good hygiene practice for the milk processing industry is a prerequisite to avoid this situation.

## SIGNIFICANCE STATEMENT

This study finding indicated that the available commercial pasteurized and UHT milk was contaminated by pathogenic bacteria which are a threat to public health. This study will help the researchers to analyze the critical areas of milk processing on a large scale that many researchers were not able to explore.

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