

## Original Article

# Microbiological Analysis of Different Flavoured Milk Samples Collected from Various Areas of Dhaka city, Bangladesh

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**Current study attempted to examine the chances of microbial contamination within the common flavored milk products consumed by the locality of Dhaka city, Bangladesh. All samples, collected from different super shops, revealed the presence of total viable bacterial counts within the range of  $2.5 \times 10^4$  to  $8 \times 10^5$  cfu/mL. *Staphylococcus* spp., *Pseudomonas* spp., and coliforms were noticed to be predominant and were recovered from 6 out of 10 samples with an average load of  $10^3$ - $10^5$  cfu/mL. *Salmonella* spp. was also detected in one sample. Study of the antibiotic susceptibility test further demonstrated that all the bacterial isolates were resistant against most of the commonly used antibiotics with the multi- drug resistant traits in several cases.**

**Key Words: microbial contamination, flavored milk samples, antibiotic susceptibility** INTRODUCTION

Milk and milk products have long been a major part of human food and plays the most important role in maintaining a balanced diet. Milk contains various types of nutrients like proteins and vitamins. The denomination crude protein (CP) includes protein (TP) and non-protein nitrogen (including urea). The protein content is an important quality of milk<sup>1</sup>. Milk includes calcium, magnesium, sodium and potassium for the major cations and inorganic phosphate, citrate and chloride for the main anions as minerals<sup>2</sup>. A number of different types of vitamin are available in milk. Vitamins A, D, B<sub>12</sub>, and riboflavin are common<sup>3</sup>. However, the microbiological propagation into milk and milk products is commonly determined by the facets of composition and hygiene maintenances<sup>4,5</sup>.

Flavored milk is the milk added with various flavoring agents and sweetener. Flavored milk includes both natural sugar (12 gm of lactose per 8 ounce serving) and added sweeteners. Sweeteners can be nutritive (caloric) such as sucrose, generally known as table sugar, or high fructose corn syrup (HFCS), or non-nutritive, depending on the brand. Because each manufacturer has an exclusive formula, including the amount and type of the added sweetener(s), added sugar content may differ among flavored milk products<sup>6</sup>. Flavored milk is offered in traditional flavors such as chocolate as well as inventive flavors including strawberry, vanilla, mocha, root beer and banana, etc<sup>7</sup>. These products have captured a large proportion of the beverage market in recent years globally<sup>8</sup>. To effectively commercialize the products, a dairy industry needs to develop with an increasing variety of flavors and textures while extending the product shelf life both in terms of chemical stability and microbiological quality<sup>9</sup>. By this way the milk industries have been found to

achieve this goal through growing along the market with varieties of flavored milk products, which offers flavor and nutritional benefits over many of its beverage competitors<sup>10,11,12</sup>.

A number of reports have shown that the number and types of microorganisms truly exert quality of milk and milk products<sup>13,14</sup>. Milk aids outstanding medium for the growth and multiplication of various kinds of microorganism, because of its high water activity and enriched nutrition<sup>11</sup>. The majority of microorganisms that significantly affects the quality of dairy products includes *Staphylococcus* spp., *Lactobacillus* spp., *Micrococcus* spp., *Streptococcus* spp. and coliforms. Air, soil, grass, feces, milking equipments and production plant have so far been the prominent reported sources of microbial contamination of milk<sup>12</sup>. It's not unlikely that the milk and milk products would deteriorate quickly as they become biochemically unstable<sup>13</sup>. There are different systems used by the food industry to monitor the quality and the biological safety of milk. These systems including the ISO 9000, the Hazard Analysis and Critical Control Point (HACCP), Total Quality Management (TQM), etc., are often exclusive, expensive and complex but are very effective for extension of the shelf life of milk<sup>14</sup>. Different heat treatments are also applied to remove pathogenic organisms and increased the shelf life of milk<sup>15</sup>. To help the consequent processing, warming before separation and homogenization or as an essential treatment before yoghurt manufacture, production of evaporated and flavored milk products are frequently used<sup>16</sup>. Actually the contamination of milk and milk products can be reduced by proper processing of milk. Hence, it is suggested to maintain appropriate treatment and hygienic packaging of the milk for drinking<sup>14</sup>.

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Md. Aftab Uddin Assistant, Professor Department of Microbiology, Stamford University Bangladesh, 51 Siddeswari Road, Dhaka-1217, Tel: +880 8355626, +880 8355596 ext. 478, Cell: +880 1817581793, E-mail: aftab\_mb12@yahoo.com ABSTRACT

Recently treatment of different kind of diseases is becoming difficult because of the emergence of drug resistant pathogens that may be introduced into the host because of the intake of the microbiologically contaminated milk and milk products<sup>4</sup>. In recent years, many antibiotics have been found to be ineffective due to development of resistant strains as a result of the expression of the resistance genes or spontaneous mutations within the microbial populations<sup>17,18</sup>. Based on these facts, this study was carried out to determine the extent of microorganisms present in different flavored milk products and also to assess the drug resistance properties of these microbial isolates.

## Materials and Method

### Sample collection and processing

Total four categories (3 chocolate milk, 4 mango milk, 2 strawberry milk and 1 banana milk samples) of 10 flavored milk samples (each packet containing 200 ml) were randomly collected from Bashaboo, Shantinagar, and Mailbagh areas of Dhaka city. All the samples were preserved at 4°C before collection. According to the method suggested by American Public Health Association (1998), all the samples were transported to the laboratory as soon as possible<sup>19</sup>. Then 1 mL of each sample was taken very carefully by using a micro pipette from the original samples and transferred to 9 ml of normal saline in sterile test tubes. Prior to the estimation of bacterial load, samples were subjected to serial dilutions up to  $10^{-3}$  (20).

### Microbiological analyses

For the enumeration of total viable bacterial count (TVBC), an aliquot of 0.1 mL of each dilution was introduced onto the nutrient agar (NA) plates by the spread plate technique<sup>4,20</sup>. The nutrient agar plates were incubated at 37°C for 24 hours. For the detection of specific bacteria, from the dilution of  $10^{-2}$  and  $10^{-3}$  of each sample, 0.1 ml was spread onto MacConkey agar, Mannitol salt agar (MSA) and Pseudomonas agar (Himedia) for the enumeration of coliform, *Staphylococcus* spp. and *Pseudomonas* spp., respectively. All the plates were incubated at 37°C for 24 hours<sup>21</sup>.

### Enrichment procedure

For the detection of the viable but non culturable (VBNC) species such as *Salmonella* and *Vibrio*, enrichment technique was followed. In this technique 1 ml of sample was added to 9 ml of selenite cystein broth (SCB) and alkaline peptone water (APW) and incubated at 37°C for 6 hours. Following the incubation, the samples were diluted up to  $10^{-3}$  and then 0.1 ml of samples from  $10^{-2}$  and  $10^{-3}$  dilutions were spread over Salmonella Shigella agar (Himedia) and Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS) from the respective enrichment media. Appearance of small black-centered colonies after incubation for 24 hours at 37°C was indicative of the presence of *Salmonella* spp., while the large (2-

4 mm) and slightly flattened, yellow colonies on the TCBS agar referred to the presence of *Vibrio* spp<sup>30</sup>.

The confirmative biochemical tests were executed to ensure the identification of the isolates found from the flavored milk samples following the standard protocol as described in the 7th edition of Microbiology laboratory manual of Cappuccino (2005)<sup>21</sup>.

### Study of antibiogram

Isolated *E. coli*, *Klebsiella* spp., *Vibrio* spp., *Salmonella* spp., *Pseudomonas* spp. and *Staphylococcus* spp. were subjected to antibiotic susceptibility assay against different groups of antibiotics *in vitro* by the Kirby-Bauer method<sup>22,23</sup>. Drug resistance was observed against Amoxicillin (10 µg), Azithromycin (15 µg), Ampicilin (10 µg), Chloramphenicol (30 µg), Gentamicin (10 µg), Vancomycin (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg) and Imipenem (10 µg). From overnight culture plate, a small portion of a fresh colony was transferred to Muller-Hinton broth and incubated at 37°C for 4 to 5 hours until the growth reached to the equivalent turbidity standard of McFarland (0.5 standards)<sup>24</sup>. Muller-Hinton agar plates were seeded properly by spreading the inoculate using sterile cotton swab<sup>17</sup>. Discs (OXOID, UK) were placed gently at a proportionate distance from each other using a sterile needle. The plates were then incubated overnight at 37°C and zones of inhibition (if any) were measured and interpreted as susceptible, intermediate and resistant categories by referring the recommended interpretative standards<sup>25</sup>.

### Results and Discussions

Pathogenic bacteria in milk have been a major factor for public health concern since the early days of the dairy industry<sup>4,20,26,27</sup>. As discussed earlier, the health of dairy herd and milking conditions basically determine the presence of microorganisms in milk. The use of unclean milking and transport equipment also contribute to the poor hygienic quality<sup>28</sup>. Previously, many experiments were conducted on the bases of milk; very limited experiments have been so far specifically done based on the flavored milk products in Bangladesh<sup>20,26,27,29</sup>. So the present study attempted to determine the bacteriological quality of common flavored milk samples collected from various areas of Dhaka city, Bangladesh.

Current investigation showed a scenario of huge microbial contamination in most of the samples (Tables: 1). In this study the highest bacterial load ( $8 \times 10^5$  cfu/ml) was found in sample no. 5 and the lowest bacterial load ( $2.5 \times 10^4$  cfu/ml) was found in sample no. 7. Here total viable bacterial load (TVBC) in sample no. 3, 4, 5, 6, 9 and 10 was almost same, around  $10^5$  cfu/ml, which was not acceptable according to the guidelines set by FDA (2013)<sup>30</sup> (Table: 1).

The presence of coliform bacteria, such as *E. coli* and *Klebsiella* spp. in milk is a common indicator of fecal contamination<sup>29</sup>. In this study, presence of coliform was detected in 4 out of 10 samples. It was unfavorable for consumption because of the presence of more than  $10^3$  cfu/ml coliforms. The range was  $2 \times 10^3$  cfu/ml to  $7 \times 10^3$  cfu/ml (Table: 1). Such prevalence was however lower than the report by Sood *et al.*<sup>11</sup> where average coliform bacterial count was  $2.5 \times 10^4$  cfu/ml.

*Staphylococcus* spp. may cause alarming disease in human through the production of toxins. The formation of effective level of toxin requires a high number of microorganisms, approximately  $10^5$ – $10^6$  microorganisms per ml of food<sup>31</sup>. In this study *Staphylococcus* spp. were found in four out of 10 samples within the range of  $1.8 \times 10^4$  cfu/ml to  $4 \times 10^4$  cfu/ml (Table: 1).

Presence of *Salmonella* spp., *Shigella* spp. is frequently connected with poor sanitary practices and they put a pointer to a potential risk of food borne illness to consumers<sup>26,32,33</sup>. In this

study, *Salmonella* spp. was only found in sample no. 6 ( $9 \times 10^3$  cfu/ml) and *Shigella* spp. were not found in any sample (Table: 1). According to EEC (1992)<sup>34</sup>, *Salmonella* spp. should be completely absent in any food sample. So presence of *Salmonella* spp. in sample no 6 was indicative of potential health hazard for the consumers. This study showed a close link with previous study conducted by Yasmin *et al.*,<sup>26</sup> in Bangladesh.

*Pseudomonas* spp. is a common environmental microorganism and can be found in feces, soil, land water and sewage water<sup>35</sup>. Its presence was considered as an indicator of contamination of the water at source or during the packaging process<sup>36</sup>. In the present study, *Pseudomonas* spp. were detected in the sample no 3, 4, 5 and 6. The range was  $5 \times 10^3$  cfu/ml to  $1.8 \times 10^5$  cfu/ml (Table: 1) indicating the poor hygienic condition of the product.

Presence of *Vibrio* spp. in food is a matter of concern. The ingestion of  $2 \times 10^5$  to  $3 \times 10^7$  cells of *Vibrio* spp. is required to

**Table 1.** Microbial load in different flavored milk samples.

Sample No.	Total Viable Bacterial Count (cfu/ml)	Total Coliform Count (cfu/ml)	<i>Staphylococcus</i> spp. (cfu/ml)	<i>Salmonella</i> spp. (cfu/ml)	<i>Pseudomonas</i> spp. (cfu/ml)	<i>Vibrio</i> spp. (cfu/ml)
1	$3 \times 10^4$	0	0	0	0	0
2	$4 \times 10^4$	0	$3 \times 10^4$	0	0	0
3	$2.8 \times 10^5$	0	$1.8 \times 10^4$	0	$8 \times 10^4$	0
4	$6.8 \times 10^5$	$7 \times 10^3$	0	0	$1.8 \times 10^5$	0
5	$8 \times 10^5$	$2 \times 10^3$	$3.6 \times 10^4$	0	$5 \times 10^3$	0
6	$2.4 \times 10^5$	0	$4 \times 10^4$	$9 \times 10^3$	$6.6 \times 10^4$	0
7	$2.5 \times 10^4$	0	0	0	0	0
8	$3.2 \times 10^4$	0	0	0	0	0
9	$4.8 \times 10^5$	$4 \times 10^3$	0	0	0	0
10	$4.4 \times 10^5$	$6 \times 10^3$	0	0	0	0

**Table 2.** Biochemical identification of the isolates found in the different flavored milk samples.

Pathogenic Microorganism	TSI (Triple Sugar Iron Test)			H <sub>2</sub> S Gas Production	Indole Production	MR Test	VP Test	Citrate Utilization Test	Motility Indole Test
	Slant	Butt	Gas						
<i>E. coli</i>	Y	Y	+	-	+	+	-	-	+
<i>Klebsiella</i> Spp.	Y	Y	+	-	-	-	+	+	-
<i>Vibrio</i> Spp.	R	Y	+	-	-	-	-	+	+
<i>Salmonella</i> Spp.	R	Y	+	+	-	+	-	-	+
<i>Staphylococcus</i> spp.	Y	Y	-	-	-	+	+	+	-
<i>Pseudomonas</i> spp.	R	Y	-	-	-	-	-	+	+

cause disease in human<sup>37</sup>. An attempt was taken to isolate *Vibrio* spp. from ten different flavored milk samples. No *Vibrio* spp. was found in any of those samples.

Food contamination with antibiotic-resistant bacteria is a major threat to public health<sup>15,38</sup>. Recently treatment of various diseases is becoming difficult due to the emergence of drug resistant pathogens that may be introduced into the host<sup>16,27,34</sup>. In this experiment the Kirby- Bauer disk diffusion test was used to make a decision whether the isolated organisms were susceptible or resistant to a selected pool of antimicrobial agents<sup>22</sup>. The current study showed that, most of the isolates exhibited multi-drug resistance (MDR) phenotype (Table 3). All

the isolates were found to be highly resistant against Vancomycin (100%) and Erythromycin (70%). *Salmonella* spp. isolates were found to be resistant against 5 different antibiotics namely Amoxicillin, Ampicilin, Vancomycin, Erythromycin, and Imipenem, while sensitive against Chloramphenicol, Gentamicin, Tetracycline, Ciprofloxacin and Intermediate to Azithromycin. In this antibiogram study, although some of the isolates were susceptible towards some antibiotics, several other antibiotics were proved ineffective; indicating the risk of the emerging resistant isolates causing health hazards. All of the isolates were resistant for three or more antibiotics. This study was quite similar with the results of Marjan *et al.*<sup>4</sup>.

**Table: 3.** *Antibiogram of the pathogenic isolates found in the different flavored milk.*

Pathogenic Microorganisms	Sample No.	Amoxycilin (AML 10)	Azithromycin (AZM 15)	Ampicilin (Amp 10)	Chloramphenicol (C 30)	Gentamicin (CN 10)	Vancomycin (VA 30)	Tetracycline (TE 30)	Erythromycin (E 15)	Ciprofloxacin (CIP 5)	Imipenem (IMP 10)
<i>E. coli</i>	Sample 9	R	I	R	S	I	R	S	R	S	I
	Sample 10	I	S	S	S	S	R	S	R	S	R
<i>Klebsiella</i> spp.	Sample 4	R	R	R	S	S	R	S	R	S	I
	Sample 5	R	I	R	S	S	R	S	R	S	S
<i>Vibrio</i> spp.	Sample 5	R	S	R	S	S	R	R	R	R	R
	Sample 10	R	S	R	S	S	R	R	R	R	R
<i>Salmonella</i> spp.	Sample 6	R	I	R	S	S	R	S	R	S	R
<i>Staphylococcus</i> spp.	Sample 2	I	S	I	S	I	R	R	R	S	I
	Sample 3	R	R	R	S	S	R	S	R	S	S
	Sample 5	R	S	R	S	I	R	S	R	S	R
	Sample 6	R	S	R	S	S	R	R	R	S	I
<i>Pseudomonas</i> spp.	Sample 3	R	S	R	S	S	R	S	S	S	S
	Sample 4	R	S	R	S	R	R	R	S	R	I
	Sample 5	R	S	R	S	S	R	S	S	S	S
	Sample 6	R	S	R	S	S	R	S	S	S	S

*Antibiogram profiling (R=Resistance, S=Sensitive or I=Intermediate) was determined according to NCCLS (2000)<sup>25</sup>.*

## Conclusions

According to the results of the current study results, the presence of microorganisms in the studied samples is adequately indicative of uncompromising health risk upon consumption of the different flavored milk samples tested unless appropriate microbiological control measures are taken. Proper hygiene maintenance is required during handling and processing of flavored milk products, as well as proper function of sterilization procedure which could ensure quality of these foods.

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