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Assessment of airborne bacteria of milk processing unit complex associated environment

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ABSTRACT

Aims: The aim of this study was to identify the sources of airborne contaminants in milk processing units.

Materials and Methods: The aero-bacteriological investigation has been done fortnightly for a period of 1 year extramurally within the premises of milk processing unit complex with the help of modified two-stage Andersen Sampler. The raw milk samples were analyzed for total plate count and total coliform count.

Results: The mean ± standard deviation of bioload of total coliform/mL, total plate count in million/mL, total airborne viable cultivable bacteria, Gram-negative bacteria, and the members of the family *Enterobacteriaceae* recorded were 3193.6 ± 220, 1673.33 ± 229.8, 3117.96 ± 1678.1, 46.33 ± 28.874, and 47.92 ± 33.5, respectively. Seasonal variations in airborne bacterial population were reported for this environment, high humidity and moderate temperature were the major factors for dissemination and distribution of Gram-negative bacilli. The temperature was positively and humidity was negatively significantly correlated with total airborne viable cultivable bacteria of this environment. There was no correlation established between bioload of milk and bioload of airborne bacteria.

Conclusion: The airborne bacterial bioload in milk processing unit complex environment areas were higher than the acceptable limit, with temporal and spatial variations. Mechanical activities were supposed to be the key factor governing aerosolization of potentially harmful bacteria which could contaminate the products. These results could be useful to establish a standard to the small-scale dairy processing units where monitoring of airborne bacteria were rarely adopted by dairy manufacturers in their routine quality control.

Key words: Aerosolization, airborne, bacteria, milk, processes, seasonal variation

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INTRODUCTION

The prevention of microbial airborne contamination in milk processing units is the most significant area of high-care milk production. The duration between processing, packaging, distribution, and consumption of milk in most of the cases is comparatively higher in case of dairies corresponding with

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the classical system of home delivery by the small farmers. The possibility for multiplication of organisms from a small contamination is becoming greater to the extent that outbreak of disease or serious spoilage may occur.[1]

Milk-borne diseases, which may arise due to the contamination during the handling and adulteration, such as typhoid, dysentery, diarrhea, septicemia, sore throat, scarlet fever, diphtheria, cholera, infantile diarrhea, tuberculosis, etc., have been reported.[2] *Campylobacter jejuni*, Shiga toxin-producing *Escherichia coli*, *L. monocytogenes*, *Salmonella* spp. (*Salmonella enterica* serotype Typhimurium and *S. enterica* serotype Newport), and *Yersinia enterocolitica* were detected in the milk samples previously by the researchers.[2,3]

The previous study that has reported the potential sources of aerosolized bacteria in milk processing units are floors, drains, condensate, personnel, outdoor air, and air conditioning systems. Production of aerosols from these sources may result in product contamination when the product is exposed to air.[4] The risk is higher when air is contaminated with eventually foodborne pathogenic microorganisms or spores and even non-spore-forming microorganisms can become aerosolized in water droplets or when they are attached to dust.[5]

Since the members of the family *Enterobacteriaceae* are the good indicators of pollution and contamination in most of the food industries, the presence of species of family *Enterobacteriaceae* in the aerosols of the area under study represents the unhygienic practices and conditions.[6] It is a common conception that the infectious microorganisms must be viable to cause infections, but infectious as well as non-infectious microorganisms may pose other health hazards even if they are dead and disintegrated. Inhalation of non-infectious microorganisms and their constituents can cause inflammation of the respiratory system, while antigens and allergens may activate the immune system and cause allergic and immunotoxic effects.[6-9] Thus, the airborne contamination in a dairy processing unit is not only hazardous to its workers but also the community as a whole. The present studies were therefore conducted at the dairy processing units' complex area at Jabalpur.

The working places in small-scale milk processing units' areas were rarely characterized microbiologically; the aim of this study was to identify the sources of airborne bacterial contaminants in milk processing units. The objective of this comprehensive study was to evaluate the quantity and quality of potentially hazardous cultivable bacteria of viable types represented in the air of milk processing units with special reference to the members of the family *Enterobacteriaceae*, and to find the inhalable and non-inhalable fraction of bacteria for this environment. The degree of microbial bioload of raw milk used for processing and packaging was also quantified. The effects of environmental factors on the total airborne bacterial bioload were also analyzed using correlation analysis and a regression model for prediction was prepared.

MATERIALS AND METHODS

Jabalpur (Latitude: 23.2; Longitude: 79.95; Altitude: 391.) also known as *Sanskardhani* is the third largest urban agglomeration in the state of Madhya Pradesh, India as per the 2011 census statistics. The climate of Jabalpur is overall pleasant and salubrious except the later part of the summer season. The year may be divided into three main seasons *viz.*, the summer season (from middle of March to middle of June), the monsoon season (from middle of June to the end of September), and the winter season (from October to middle of March).

Sampling site

The dairies chosen for present studies were small-scale milk processing units located at the interior of the city. In these sites, milk was collected from small vendors of nearby villages and processing of milk was performed in small-scale for the purpose of the pasteurization and the production of other milk products.

Isolation of bacteria from environment

In the work environment, culture-based analysis must be performed for the measurements of airborne respirable fraction of microorganisms.[10] Air sampling and the measurements of air contamination were done during morning hours (After 9:00 a.m., soon after the milk collection period) fortnightly for a period of 1 year, as described by Pathak and Verma with some modification,[6] with the help of modified two-stage Andersen Sampler[11,12] at 1 m height from the ground extramurally within the premises of milk processing units complex, using tryptone glucose yeast extract Agar Medium (Hi Media, Mumbai, India) kept on upper stage of the sampler and eosin methylene blue (EMB) Agar Medium (Hi Media, Mumbai, India) and *Lactobacillus* MRS Agar medium (Hi Media, Mumbai, India) kept on lower stage of the sampler according to manufacturer's instruction. Sampling was also done to identify the presence of the bacteria in the discharge and waste of milk processing units' area to compare it with those present in the air.[13] The level of bacterial contamination of air was expressed in terms of number of bacteria-carrying particles per m^3 (bcp/m^3) or the bioload (B).[14] B is calculated from the following equation (eq. 1):

$$B = \frac{1000N}{RT} bcp\, m^{-3} \quad (1)$$

where N is the number of colonies counted on the sample plate after correction using the positive hole conversion table provided by Andersen,[11] T is the duration of the test in min (10 min), and R is the air-sampling rate in L/min (28.3 L/min).[14]

Isolation of bacteria from milk

The raw milk samples were collected from dairies in sterile containers; sampled milk was kept in ice-boxes and processed within 3 h. The milk samples were mixed well before diluting.

The samples were then diluted to 1:1000 and 1:100,000 using sterilized phosphate-buffered solution. The diluted samples were mixed again using sterile pipette each time. 0.1 mL of the milk from diluted samples was transferred into plate count agar medium (HiMedia, Mumbai, India) and EMB Agar Medium (Hi Media) Petri-plates. The milk samples were spread and the Petri-plates were incubated at 35 ± 2°C for 24-48 h. The plates were counted in average in terms of bacteria-carrying particles per m^3 (bcp/m^3) or the bioload (*B*).

Identification of isolates

Bacteria can be identified and grouped according to similar cell and colony morphologies, Gram's staining, growth on specific substances and under special conditions, and production of specific metabolites.[15] After Gram's staining of bacteria, a study by Krahmer *et al.* divided the results into four categories: Actinomycetes, Gram-positive rods, Gram-negative rods, and Gram-positive cocci.[16] In this study, identification of isolates (sources and air) was done using Hi *Enterobacteriaceae* Identification Kit (Hi Media, Mumbai, India) and standard methods and manuals; carbon source utilization profiles were prepared in order to establish source and sink relation.[17-21]

Statistical analysis

The number of samples collected will influence the precision of the exposure estimate and the associated confidence limits.[22] In order to analyze the effect of various environmental factors on the prevalence of airborne bacterial population and degree of its effectiveness with other environmental factors, Spearman correlation coefficients and stepwise linear regressions analysis were done. The bioload of airborne bacterial population was measured; total viable cultivable bacteria and the members of the family *Enterobacteriaceae* were also correlated with total plate count and total coliform count of raw milk, respectively, by the Spearman correlation coefficients. The means of the factors affecting aerosolization of airborne bacteria and the viable cultivable airborne bacteria of this environment were compared using ANOVA. The coefficient of determinants (R^2) along with eta squared (η^2) were also obtain to measure the association between different variables, using the SPSS Win 12.0 program.[23-25]

RESULTS

From the milk processing units associated environment, 86 types of isolates were identified [Figure 1]. Bioload (*B*) recorded of total viable bacteria was 3117.96 SD ± 1678.099 ranging from 283 to 5725; of inhalable Gram-negative bacteria was 46.33 SD ± 28.874 ranging from 18 to 141; the mean of the atmospheric bioload of members of the family *Enterobacteriaceae* was 47.92. Highest average recorded bioload from milk processing units environment during summer was 4.6×10^3 bcp/m^3, in monsoon was 2.8×10^3 bcp/m^3, and in winter was 2×10^3 bcp/m^3. The variability in the types of airborne bacteria with the seasons

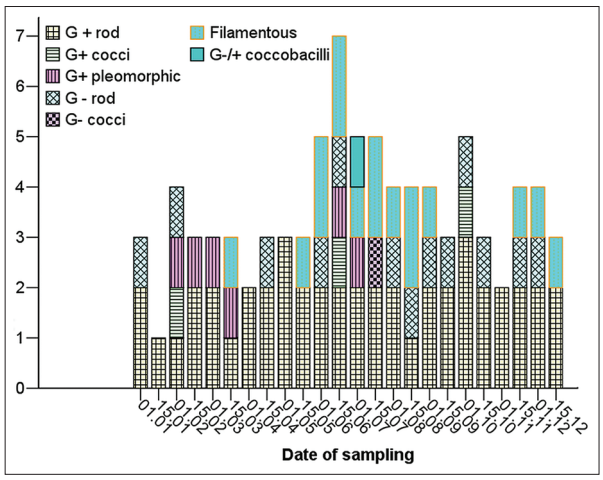

Figure 1: Type of airborne bacterial isolates from milk processing unit environment

was also observed. During winter, the concentrations of Gram-positive bacteria were high (37%), whereas in monsoon, Gram-negative bacteria (42%) were dominant variety. Inhalable fraction of total members of the family *Enterobacteriaceae* [Figure 2] was recorded the highest during monsoon (51%) comparing to winter (39%), and the minimum during the period of summer (34%).

The Gram-positive bacteria were dominant among the total type of viable bacteria (46%) followed by filamentous bacteria (19%) and Gram-negative bacteria (13%). Various species of *Lactobacillus* were isolated using *Lactobacillus* MRS Agar medium. Species of the genera, *Enterobacter*, were dominant; among the group of *Enterobacteriaceae*, *Acinetobacter calcoaceticus* was dominant among Gram-negative bacteria; species of the genera *Pseudomonas*, *Erwinia*, *Actinobacillus*, *Proteus*, and *Escherichia* were also reported.

During the study of sources of these microorganisms, the bacteria reported were *Enterobacter* spp. (*cloacae*) and *Pseudomonas* spp. from both the soil and water samples, whereas *Enterobacter cloacae*, *E. coli*, and *Proteus mirabilis* were reported from the sewage water samples. Highest average recorded total plate count of raw milk during monsoon was 1.83×10^6 cfu/mL, in summer was 1.76×10^6 cfu/mL, and in winter was 1.46×10^6 cfu/mL, whereas average coliform count reported was 3204. The species of the genera *Salmonella*, *Enterobacter*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Vibrio*, *Bacillus*, *Enterococcus*, and *Staphylococcus* were recovered from the raw milk sampled during this study.

For bioload of inhalable cultivable bacteria, no significant correlations were found with any of the analyzed variables, whereas temperature significantly positively correlated ($r = 0.67$; $P = 0.00$) and humidity negatively correlated ($r = -0.376$; $P = 0.035$) with total viable cultivable bacteria and humidity is significantly positively correlated ($r = 0.396$; $P = 0.028$) with airborne members of the family *Enterobacteriaceae* for this

environment. These correlations were further reiterated by the test of analysis of variance [Table 1]. There is no correlation established between the bioload of airborne bacterial population of total viable cultivable bacteria, Gram-negative bacteria and the members of the family *Enterobacteriaceae*, with total plate count and total coliform count of raw milk, respectively [Table 2].

To compare the means for the different groups, analysis of variance (ANOVA) was performed; the significance level for all types of airborne bacteria in relation to milk-borne bacteria exceeds 0.05, indicating that the airborne bacterial population and bioload of milk do not differ; however, there is no linear relationship exists between these parameters [Table 1].

The stepwise multiple regression models showed that temperature was statistically significant predictors of total type of airborne viable bacteria of this environment. An increase of 180.38-unit in bacterial bioload accounted for about 45% of the variance by increasing one unit in temperature in total types of viable bacteria in milk processing units' environments was obtained. The constant was-1356.912 and standard error (SE) of the estimate 1272.412 showing the bioload at any temperature range.

Using multiple regressions (stepwise), the model prepared for this atmosphere was as follows (temp. = temperature; ave. = average) (Eq. 2):

Estimated model of total type of airborne viable bacteria (R^2 45%) = −1356.912 + 180.38 × ave. temp. ±1272.412 (2)

From the ANOVA under degree of freedom of V1 = 1, V2 = 22, the test statistic was the *F* value of 18.004 (Sig. =0.000) for the model. Using the significance level of 0.05 implies that critical value (F_{cv}) was 4.30 from the *F* distribution table. Thus, we could reject null hypothesis (Ho) (there is no linear relationship between the total type of airborne viable bacteria and the average temperature of ambient environment) in favor of alternate hypothesis (Ha) (there is a significant linear relationship between the total type of airborne viable bacteria and the average temperature of ambient environment). This means that the linear regression model that had been estimated was not a mere theoretical construct; indeed, it did exist and was substantially significant. Square root of mean square error for model was 1272.412; bioload could vary by ± 1272.412 about the estimated regression equation for the value of average temperature.

DISCUSSION

Raw milk has been a known vehicle for pathogens for more than 100 years.[26] Outbreaks associated with the consumption of raw milk and raw milk products were the major causes of foodborne morbidity and mortality.[27] Raw milk normally contains very low numbers of microorganisms;

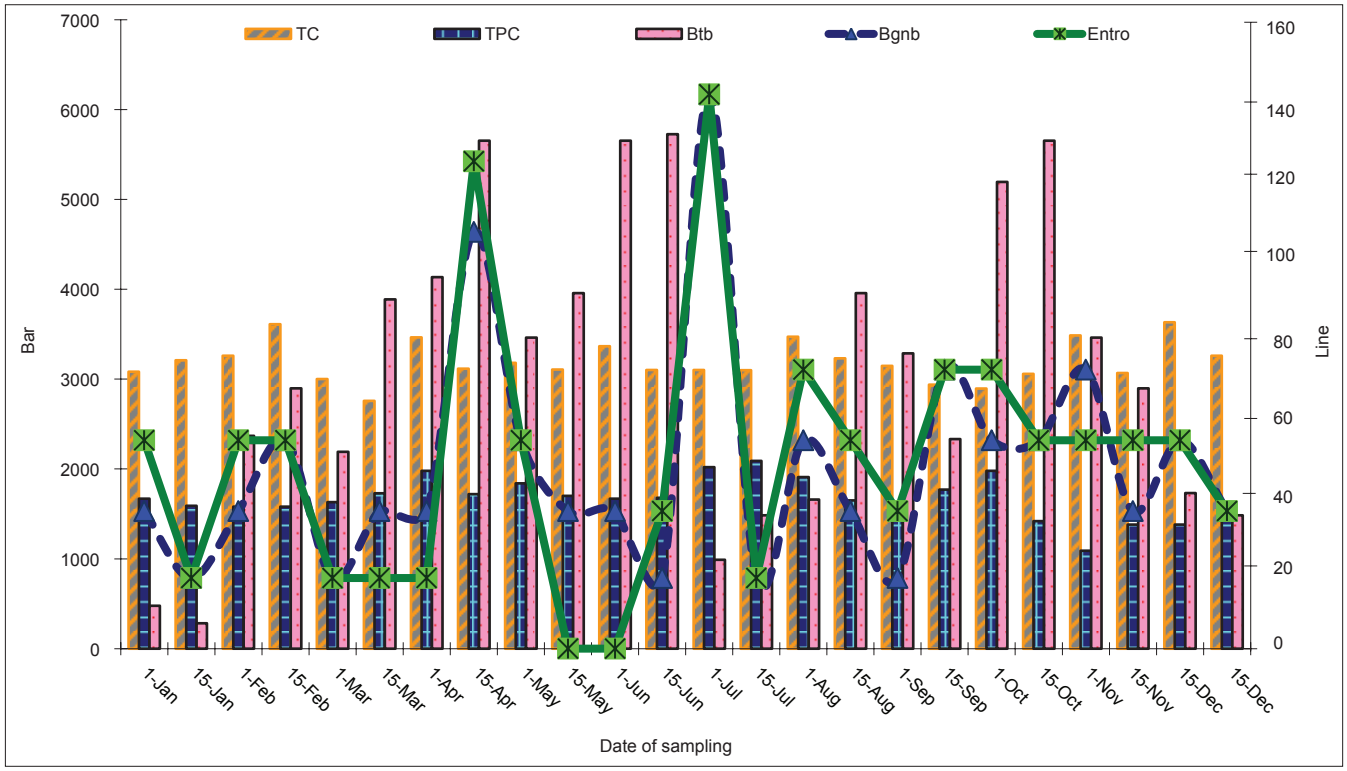

Figure 2: Total plate count, total coliform count, and respirable and non-respirable fraction of viable bacteria at milk processing unit environment (cfu/m3). (Notes: TC – Total coliform; TPC – Total plate count in thousand; Btb – Bioload of total bacteria at milk processing unit environment; Bgnb – Bioload of Gram-negative bacteria at milk processing unit environment; Entro – Bioload of members of the family Enterobacteriaceae at milk processing unit environment)

Table 1: F-value and the test of association between the microorganisms isolated from milk and air

Pairs	Dependent variable-independent variable	R squared	Eta squared	F (ANOVA)	Significance	Decision (α=0.05)
1	Total type of airborne viable bacteria-Total coliform	0.021	0.758	0.531	0.494	NS
2	Total type of airborne viable bacteria-Total plate count	0.003	0.593	0.046	0.838	NS
3	Total respirable Gram-negative bacteria-Total coliform	0.001	0.117	0.027	0.871	NS
4	Total respirable Gram-negative bacteria-Total plate count	0.019	0.205	0.429	0.521	NS
5	Total airborne Enterobacteriaceae population-Total coliform	0.002	0.150	0.030	0.864	NS
6	Total airborne Enterobacteriaceae population-Total plate count	0.021	0.497	0.703	0.414	NS
7	Total type of airborne viable bacteria-Average Humidity	0.141	0.646	2.392	0.173	NS
8	Total type of airborne viable bacteria-Average temperature	0.450	0.758	11.14	0.016	S
9	Total respirable Gram-negative bacteria-Average humidity	0.02	0.88	0.94	0.38	NS
10	Total respirable Gram-negative bacteria-Average temperature	0.008	0.79	0.07	0.81	NS
11	Total airborne Enterobacteriaceae population-Average humidity	0.16	0.94	12.43	0.017	S
12	Total airborne Enterobacteriaceae population-Average temperature	0.022	0.89	0.43	0.58	NS

NS = Non-significant, ANOVA = Analysis of variance

Table 2: Correlation coefficient environmental parameters with bacterial isolates

combinations of parameters	Total coli form	Total plate count	Bioload total bacteria	Bioload total gram-negative bacteria	Bioload total enterobacteriaceae	Average humidity
Total plate count						
Sig	0.082					
D	NS					
Bioload total bacteria						
Sig	0.248	0.398				
D	NS	NS				
Bioload total Gram-negative bacteria						
Sig	0.433	0.261	0.471			
D	NS	NS	NS			
Bioload total Enterobacteriaceae						
Sig	0.428	0.251	0.382	0		
D	NS	NS	NS	S		
Average humidity						
Sig	0.358	0.415	0.035	0.239	0.028	
D	NS	NS	S	NS	S	
Average temperature						
Sig	0.331	0.056	0	0.343	0.245	0.024
	NS	NS	S	NS	NS	S

D = Decision (α=0.05), Sig: Significant (1-tailed), S = Significant, NS = Non-significant

microbes from exogenous sources contribute significantly to the total numbers of microorganisms in the milk.[28] Air is not considered a significant source for microbial contamination in raw milk, though the air can transfer aerosolized microorganism from a microbial-laden source into exposed milk surface. Airborne contamination in milk processing facilities not only resulted in recontamination of milk products which could reduce shelf life of the products and to make it the vehicles of pathogens but also the inhalation of microorganisms or their constituents can evoke inflammatory reaction leading to respiratory disease.[9,29]

Extramural airborne bacteriological investigations, carried out at the milk processing unit complex in order to analyze the quality and quantity of airborne bacteria and its variations in course of seasonality, revealed that both pathogenic and saprophytic bacterial forms were prevalent in the area of study. The bioload of total viable bacteria, inhalable (respirable) Gram-negative bacteria, and total enteric bacteria were recorded in the ranges from 283, 18, and 0 to 5725, 141, and 141 accounted in humidity ranging from 33 to 84 and temperature ranging from 19 to 31°C, respectively.

The Gram-negative bacteria accounted less than 2% of total viable cultivable bacteria for this atmosphere. From the inhalable amount of viable bacteria, those bacteria that can be deposited on the lower airway of respiratory system of human beings were the highest in the month of July, whereas the viable bacterial bioload was highest in the month of June. According to Kelly and Pady, the dry weather favors bacteria to get into the air,[30] the soilborne bacteria of air were greatest in number during spring and autumn, and this finding is similar to this study. Highest average bioload was recorded in milk processing unit complex environment during the winter, followed by monsoon and summer. During the winter season, low temperature and moderate humidity favor the survival of most of the airborne bacteria; higher fraction of Gram-negative bacteria represented during the early monsoon was probably as a result of mechanical activities including splashing.

The correlation of the atmospheric bioload of members of the family *Enterobacteriaceae* with total viable cultivable

Gram-negative bacteria was explicable because the coliforms represented in air were a part of the total viable cultivable Gram-negative bacteria of this environment. There was no correlation established between the bioload of total viable cultivated bacteria and total enteric bacteria, which was probably due to the diverse effect of environmental factors on tenacity of these groups. In a study made by Tham and Zuraimi, the percentages of particles that were viable airborne bacteria at different sizes were all found to be very low at higher temperatures (<1%), which is comparable to the this finding.[31] The survivability pattern shows that humidity had pronounced effect on airborne survival of most of the bacteria, since these airborne bacteria comprises the group of soilborne actinomycetes, cocci, and other Gram-positive bacteria which have the mechanism to resist the desiccation factors. Temperature is a major factor which governs the viability of airborne Gram-negative bacteria. Gram-negative bacteria and members of the group enteric bacteria could only survive in low temperatures and moderate humidity. Lower value of coefficient of determinant ($R^2 = 0.008$-0.2) illustrates that, though the temperature governs the distribution, dissemination, and tenacity of airborne bacteria, yet this is not the major factors for generation and aerosolization of airborne microorganisms for this environment.

Furthermore, large discrepancies between η^2 and R^2 value indicated that there is no linear relationship established between total coliform bacteria of milk with airborne gram negative and the members of the family *Enterobacteriaceae* of the dairy environments; reiterated that the airborne Gram-negative bacteria of this environment was not originated from the milk subjected to processing, other factors such as cleaning, transportation, services, water spray, machineries, and putrefactions are major contributors, and this finding is similar to the previous study.[6,32] Dispersion of human microflora cannot be ruled out as a contributor of airborne microorganism.

During this study, wide varieties of Gram-negative bacteria were reported from milk processing unit complex environment. The species of the genera *Pseudomonas*, *Erwinia*, *Actinobacillus*, *Proteus*, and *Escherichia* were reported from the air of this environment. In air, among *Enterobacteriaceae*, *Enterobacter* spp. was dominant and *Acinetobacter* spp. was dominant among Gram-negative bacteria. The previous researchers while studying similar environment also reported species of *Enterobacter*, *Pseudomonas*, and *Acinetobacter*.[5,33-36] The species of *Proteus* and *Escherichia* probably get into the air during the process of rinsing the floor. It is suggestive that the composition of aerosols generated during the floor cleaning must be evaluated; as tenacity of airborne microorganism is largely depended upon the nature of associated particles, these associated particles may contain milk proteins or fats having protective natures. The bioload of total viable bacteria and total enteric bacteria of present environment was reported higher than the results reported for any other dairy processing units' areas using similar methods of sampling.[32-36]

Microorganisms detected in raw milk by previous researchers were *Enterococccus*, *Proteus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Microbacterium*, *Propionibacterium*, *Micrococcus*, *Bacillus*, *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium*, and *Enterobacter*.[37,38] According to Ryser, during storage and transport of the raw milk, microbiota of milk changes from predominantly Gram-positive to predominantly Gram-negative organisms as they grow.[39] The species of the genera *Salmonella*, *Enterobacter*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Bacillus*, *Enterococcus*, and *Staphylococcus* were recovered from the raw milk sampled during this study which shows higher hold-up time before deliveries for milk.

According to Food and Drug Administration, the bacteriological count of commingled raw milk prior to pasteurization should not exceed 3×10^5 cfu/mL, for milk from an individual producer, it should not exceed 1×10^5 cfu/mL prior to commingling of milk , and for coliform, it should not exceed 10 cfu/mL.[40] During this study, the average bacteriological count of raw milk recorded was exceeded the limit set up by most of the authorities. If we include the rate of recontamination from air that is 1.5% of total airborne viable bacteria,[29] that make thing worse for small-scale milk processing units where they are rarely following the sanitary norms set up by the authorities.

CONCLUSION

Bioaerosols are undoubtedly important constituents of aerosols generated in occupational environment; furthermore, the airborne bacteria are directly or indirectly associated with myriad of health effects in human and hence it must be included during the practices of air quality analysis. For this environment, the average presence of viable airborne bacteria seems to be higher and not only requires protection of milk from airborne contamination by keeping under properly shielded condition from outside contamination but also requires a tolerable breathing protection for dairy workers. Higher degree of seasonal variation in bacterial bioload both in terms of quality and quantity was reported in air and milk sampled from this environment. Both the temperature and humidity were responsible for tenacity and dissemination of airborne microorganisms; however, the aerosolization is governed by the mechanical activities other than handling of milk. The result of this study would not only aid in designing the air quality parameters, but would also contribute to a better understanding of the probable origin and fate of airborne contaminates in small-scale dairy industries.

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