African Journal of Food Science Vol. 6(21) pp. 519-523 15 November, 2012
Available online at http://www.academicjournals.org/AJFS
DOI: 10.5897/AJFS12.132
ISSN 1996-0794 ©2012 Academic Journals

Full Length Research Paper

A comparative microbiological quality assessment of rural and urban milk samples

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Accepted 5 November, 2012

The present study deals with the comparative microbiological quality assessment of raw cow milk samples procured from rural and urban farms of Sambalpur City, Odisha, India. The bacterial load of both the rural and urban milk samples in terms of total viable count was observed to be much higher than the acceptable limits. The average total viable count of urban sample was observed to be 8.756 ± 0.803 log CFU/ml. The preliminary incubation count was significantly higher in urban samples (8.889 ± 0.424 log CFU/ml) indicating un-hygienic milk production / handling practices. However, the laboratory pasteurization count of the rural milk samples was noted to be comparatively higher than the urban samples (8.083 ± 0.081 log CFU/ml). The enteric microorganisms isolated from both rural and urban milk samples were identified to be *Escherichia coli, Enterobacter* sp., *Klebsiella* sp. and *Shigella. Salmonella* and *Pseudomonas* sp. were present in urban samples alone, indicating the urban samples to be highly contaminated in comparison to that of the rural ones.

Key words: Contamination, enteric bacteria, health, quality, raw milk.

INTRODUCTION

Milk, being a wholesome food with high nutritive value is often prone to early contamination and spoilage if not handled properly. Several workers have reported milk to be an ideal growth medium for microorganisms (Ekici et al., 2004; Chatterjee et al., 2006; Muhammad et al., 2009; Lingathurai and Vellathurai, 2010; Mubarack et al., 2010; Ali and Abdelgadir, 2011). Once secreted out of the udder of the cow, the retention of milk quality requires cleanliness, sanitation and cooling. Fresh milk drawn from a healthy cow normally contains a low microbial particularly bacterial load of less than 10^3 CFU per millilitre (Lingathurai et al., 2009; Wallace, 2009), but the load may increase up to 100 fold or more once it is stored for sometime at ambient (30 to 35°C) temperature (Lingathurai et al., 2009). Bacterial spoilage of raw milk depends upon various factors such as health of the animal, cleanliness of the housing area, the nature of feed, the water used at farm, the milk vessels / utensils for storage and essentially the hygiene of the milker / handler (Chatterjee et al., 2006; Ali and Abdelgadir, 2011; Salman and Hamad, 2011). Mubarack et al. (2010) and Lingathurai and Vellathurai (2010) have reported the presence of pathogenic bacteria to be a major threat to public health especially for those individuals who still consume raw milk. Presence of bacteria in raw milk reduces the keeping quality of milk and certain bacteria with their associated enzymes and toxins may even survive pasteurization creating health hazards (Salman and Hamad, 2011).

There have been concerns about the quality of marketable milk samples from different pockets of India. As per the report (Anonymous, 2011), most of the milk samples sampled during the survey from rural as well as urban pockets of India, were adulterated with low nutritional value and were liable to pose a health risk to the consumers. The purpose of the present study was to look into the microbiological quality of the collected milk samples and to identify the microorganisms responsible for the alteration of merchantability and/or health.

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Table 1. Quality of rural and urban milk samples on the basis of MBRT test.

Quality of milk	Methylene blue de-colourization time (hours)	Rural sample (%)	Urban sample (%)
Very poor	0 – ½	0	0
Poor	½ - 2	11	50
Fair	2 – 6	39	50
Good	6 – 8	50	0
Excellent	More than 8	0	0

MATERIALS AND METHODS

Sample collection

A total number of 52 raw cow milk samples (approximately 500 ml each) were randomly collected in the morning from selected ruraland urban farms in Sambalpur (representing approximately 10% of all dairy farms in the district). The definition of urban area adopted is; all statutory places with a municipality, corporation, cantonment board or notified town area committee, etc. or a place with a population density of at least 400 per square km and at least 75% of male working population engaged in non- agricultural pursuits (Census of India, 2011). Milk was sampled aseptically from containers (pails or buckets) of bulk milk from each farm in sterile screw cap glass bottles and transported on ice to the microbiology laboratory, School of Life Sciences, Sambalpur University. Processing was carried out within 2 h of arrival. Samples were analysed for methylene blue reduction time, total viable count, thermoduric count, psychrotropic count and presence of enteric bacteria.

Methylene blue reduction time (MBRT) test

The MBRT test was performed according to American Public Health Association (APHA, 1992). 1 ml of methylene blue solution (1 : 25,000) was added to sterilized and labelled test tubes, each containing 10 ml of raw milk sample. The tubes were sealed with rubber stopper and carefully inverted three to four times to mix up the dye with the milk sample. All the tubes were incubated in a water bath at 37°C and examined at intervals of 30 min to 1 h for 8 h. The time taken for the methylene blue dye to decolorize was recorded between last inversion and complete de-colourization when four-fifths of the colour had disappeared (Duangpan and Suriyaphan, 2009).

Total viable count (TVC)

Bacteriological tests for milk quality were performed as described by APHA (1992). Each raw milk sample was diluted up to 10^{-7} dilution in labelled tubes containing 9 ml sterile distilled water using sterilized pipette each time. Samples were mixed well before dilution. A measured aliquot (0.1 ml) of each dilution was transferred onto the surface of labelled nutrient agar plates and spread evenly with a spreader. Replicates of plates were made simultaneously and incubated overnight at 37°C. The colony forming units (CFU) developed after incubation were recorded. The laboratory pasteurization count (LPC) and the preliminary incubation count (PIC), for the enumeration of thermoduric and psychrotrophic bacteria respectively, were also determined by following the similar method of TVC but the milk samples were treated before plating. In case of LPC, each milk sample was pasteurized (62.8°C for 30 min) individually and allowed to cool down before serial dilution and plating and in case of PIC, each raw milk sample was incubated at a temperature of 12.8°C for 18 h. After the temperature time treatment the milk samples were serially diluted and plated in duplicates on nutrient agar plates. CFU developed after incubation was recorded.

Detection of enteric bacteria on selective media

Quantitative analysis for the detection and isolation of enteric microorganisms was done by plating the serially diluted milk samples on selective media. MacConkey agar (LOT no. 0000112122) was used to isolate Gram negative lactose fermenting (coliforms) and non- fermenting microorganisms. The media contains crystal violet dye and bile salts which inhibit Gram positive bacteria. Lactose fermenting (pink) isolates on MacConkey agar were sub- cultured, Gram stained, and confirmed on eosin methylene blue (EMB) agar (LOT no. 0000129529). Lactose fermenters such as *Escherichia coli* and *Enterobacter aerogenes* can be differentiated on this media on the basis of size and the presence of a green metallic sheen (Atlas et al., 1995). *E. coli* colonies in this medium are small and have a metallic sheen, whereas *E. aerogenes* colonies usually lack the sheen and are larger (Chatterjee et al., 2006). The lactose non-fermenting Gram negative non- coliform (colourless) isolates were also sub- cultured and confirmed on selective media. Salmonella Shigella (SS) agar (LOT no. 0000125020) was used for the isolation of *Salmonella* and *Shigella* sp. Representative *Salmonella* colonies with typical black appearance were confirmed using sulphide indole motility (SIM) medium (LOT no. 0000133572). Counts were also made on Cetrimide agar (LOT no. 00001134744) to detect *Pseudomonas* sp. Isolates were biochemically established based on their indole production, methyl red, Voges- Proskauer, citrate utilization (IMViC) pattern, hydrogen sulphide production, urease production, catalase test along with different carbohydrate (lactose, sucrose, glucose, dextrose) metabolism. All medium used were obtained from Himedia Pvt. Ltd, Mumbai, India. The medium were prepared according to the manufacturer's instruction.

Statistical analysis

The microbial counts were converted to logarithm of the number of colony forming units per ml of raw cow milk samples (log CFU/ml). From the data, mean and standard deviation were calculated. Data obtained during the study were analysed by the t- test to determine the level of significance (Snedecor and Cochran, 1967).

RESULTS

The microbiological quality of raw milk samples, determined by MBRT test has been presented in Table 1.

Table 2. Bacterial count of rural and urban milk samples (log CFU/ ml).

Parameter	Bacterial count (log CFU/ml)	
	Rural Sample (Mean ± SD)	Urban Sample (Mean ± SD)
Total viable count	8.257 ± 0.937	8.756 ± 0.803
Preliminary incubation count	8.522 ± 0.929	8.889 ± 0.424
Laboratory pasteurization count	8.083 ± 0.081	7.500 ± 0.739

Table 3. Growth of enteric microorganisms on selective growth media.

Selective growth media	Bacterial load (log CFU /ml)
MacConkey agar	4.133 ± 0.481
Cetrimide agar	3.311 ± 0.388
Salmonella Shigella agar	4.456 ± 0.443

The grading of milk samples into different category was according to Chatterjee et al. (2006). As per the analysis, the percentages of poor quality (reduction time ½ to 2 h) samples from rural and urban areas were 11 and 50%, respectively. Proportion of fair quality (reduction time 2 to 6 h) and good quality (reduction time 6 to 8 h) milk under rural area alone were 39 and 50%, respectively. However, from urban sector no good quality samples were noticed and the fair quality samples represented the rest 50%.

A comparative account of the bacterial load in the rural and urban milk samples in terms of TVC, PIC and LPC has been presented in Table 2. It was observed that the average TVC of rural milk samples was 8.257 ± 0.937 log CFU/ml and that of the urban milk samples was 8.756 ± 0.803 log CFU/ml. Similar results were also marked with respect to PIC values of rural and urban milk samples. The rural samples had an average PI count of 8.522 ± 0.929 log CFU/ml and the urban samples had an average value of 8.889 ± 0.424 log CFU/ml. However, in case of LPC, the average bacterial load of rural sample was 8.083 ± 0.081 log CFU/ml and that of the urban milk samples was 7.500 ± 0.739 log CFU/ml. The T- test analysis revealed the rural and urban TVC as well as rural and urban PIC to be non- significant at 0.05 level of confidence. However, the LPC of rural milk sample was significantly higher than the LPC of urban milk samples at 0.05 level of confidence.

The quantitative analysis for the detection and isolation of enteric microorganisms has been presented in Table 3. As revealed from Table 3, the enteric bacterial load of milk samples varied from 3.311 ± 0.388 log CFU/ml to 4.456 ± 0.443 log CFU/ml. Subsequent biochemical analysis confirmed the isolated microorganisms to be *E. coli*, *E. aerogenes*, *Klebsiella* sp., *Salmonella* sp., *Shigella* sp. and *Pseudomonas* sp.

DISCUSSION

MB dye has been employed to check for the overall microbial load and quality control of milk and other liquid foods (Impert et al., 2002). It is assumed that, the greater the number of microorganisms, the more the oxygen demand and lesser the oxygen concentration in the medium resulting in the faster disappearance of the colour. This fact has been used as a broad indicative test of a microbial load representing microbial quality of milk. (Nandy and Venkatesh, 2010). Based on the methylene blue de-colourization time, the urban milk samples were categorized into poor and fair quality indicating high bacterial load, in comparison to rural samples which included poor quality, fair quality as well as good quality (low bacterial load) samples. As reported in the national survey on milk adulteration (Anonymous, 2011), urban milk samples are highly adulterated with fat, solid not fat (SNF), detergent, neutralizers, skim milk powder (SMP) and water as diluent. Such adulteration may contribute to the poor microbiological quality of milk samples from urban areas as observed in the present investigation. The microbial standard for grade “A” raw milk are 100,000 bacteria/ml, for individual producer milk and 300,000 bacteria/ml as commingled milk according to Pasteurized Milk Ordinance (PMO, 2001). Counts higher than the acceptable limits in raw milk are often ascribed to contamination by soil, water and manure (Chatterjee et al., 2006). Hence, pasteurization of raw milk along with proper storage is strongly recommended before consumption, else milk can be a major vehicle for transmission of pathogens (Ekici et al., 2004; Mubarack et al., 2010).

The total bacterial count of milk sample is an index of the quality of milk, herd health, efficacy of farm sanitation, milk handling and storage / transportation temperature

(Muhammad et al., 2009). The acceptable limits of bacterial count in raw milk according to the PMO standard is 10^5 cells per millilitre. The mean value of the TVC in both the rural and urban milk samples was observed to be far above the acceptable limits. Studies carried out by several workers (Lingathurai and Vellathurai, 2010; Muhammad et al., 2009; Hayes et al., 2001) also revealed such high total bacterial count from milk samples. These high counts are linked with un-hygienic milk handling, contamination from animal bedding, mixing of normal milk with the milk collected from the animal suffering from *Streptococcus uberis* induced mastitis, etc. (Muhammad et al., 2009). The PIC is based on the theory, that normal microbial flora of the cow (e.g. skin and teat bacteria) will not grow substantially when held at a temperature and time combination of 12.8°C for 18 h, whereas pathogenic bacteria associated with skin and udder of cows suffering from mastitis, and in cuts, wounds, scratches etc. could grow to significant levels under these conditions if present in milk (Murphy and Carey, 2010; Bodman and Rice, 1993). The PI count provides a better estimate of pyschrophilic (cold-loving) bacteria and the level of on-farm sanitation. It is important that the PIC should always be compared to TVC of the fresh, un-incubated sample before drawing any conclusion. APHA 1992 (17^{th} ed. 2004), suggests a maximum allowable PIC of 200,000 CFU/ml although counts as low as 50,000 CFU/ml is also achievable. In the present investigation, there was significant difference at 0.05 level of confidence between rural TVC/PIC and urban TVC/PIC, respectively, suggesting the presence of psychrotrophs in both rural and urban milk samples. However the difference was significantly higher in case of urban milk samples. The bacteria most commonly associated with high PIC are Gram-negative psychrotrophs such as certain *Pseudomonas* sp. *Clostridium*, *Bacillus* and *Mycobacterium sp.* (Lingathurai and Vellathurai, 2010) which generally do not survive pasteurization but the undesirable effects of their activity (proteins and fat degradation through proteases and lipases) remain. The result is reduced yield of milk products, shortened shelf life, off- flavors, rancidity etc.These types of organisms occur in raw milk due to poor udder preparation, inadequate equipment cleaning and sanitizing procedures and possibly from contaminated water sources.

The LPC is a simple test, often used as an indicator of the effectiveness of farm sanitation and hygiene procedures, providing a good benchmark of the relative number of organisms that may survive and be present in a pasteurized milk sample. LPC's are much lower than standard plate count (SPC's) of unheated milk. Counts > 300/ml are considered indicative of some source of contamination. High LPC's are often associated with chronic/persistent cleaning failures. The initial microflora of freshly pasteurized milk usually reflects the Gram-positive thermoduric organisms present in the raw milk. Gram-negative psychrophiles generally do not survive pasteurization. Even though the bacteria detected in the LPC are capable of surviving pasteurization, most are incapable of growing under refrigeration storage; they remain dormant, some even die off (Murphy and Carey, 2007). In the present study, the rural and urban LP count indicates the presence of thermoduric organisms, with high count in rural samples.

Enteric bacteria, mostly coliforms are important mastitis pathogens (Hogan and Smith, 2003), and are widely distributed in the farm environment. Bovine bedding, faeces and water used for cleaning the farm are explained to be factors for the presence of mastitis coliforms in milk samples. There have been many reports about the presence of coliform especially *E. coli* from milk samples (Chatterjee et al., 2006; Ekici et al., 2004; Mubarack et al., 2010; Salman and Hamad, 2011). The coliform limits in the raw milk accepted internationally are >100 cell/ml. Salman and Hamad (2011) have reported seasonal and geographical variation of coliform in milk samples. Detection of *E. coli* in milk often reflects faecal contamination although environmental coliforms which have also been detected in milk (Shehu and Adeysiun, 1990). Coliform bacteria have minimum generation time (Muhammad et al., 2009), and multiply at a rapid rate to reach its number to un-hygienic levels. Their very occurrence in milk sample has to be taken up seriously in context of the maintenance of the quality of the milk. Improper sanitation around the bovine shed, improper handling and contamination of the sample by un-hygienic water may be few factors for the occurrence of coliform bacteria in the milk samples (Sanderson et al., 2005). The natural flora of the cow generally does not influence the TVC, PIC or the LPC. Thus, it is the additional bacteria which are extra and happen to enter during un-hygienic milking and handling procedures which affect the keeping quality of milk.

Studies concerning the microbiological quality of milk that is, TVC, PIC and LPC are being undertaken at different regions in India. Lingathurai and Vellathurai (2010) have reported TVC (7.096 log CFU/ml), psychrophilic count (3.698 log CFU/ml) and thermoduric count (3.835) in Madurai, South India. Sharma et al. (2005) have reported a TVC (6.618 log CFU/ml), psychrophilic count (2.654 log CFU/ml) and thermoduric count (4.822 log CFU/ml) in Indore, central India. Lingathurai et al. (2009) have reported a TVC (5.84 log CFU/ml), coliform (2.76 log CFU/ml) and *E. coli* count (1.63 log CFU/ml) in Tamil Nadu. The purpose of presenting these data was to compare the average TVC, PIC and LPC values with those obtained in the present study. It is observed that the counts of TVC, PIC and LPC (Tables 2 and 3) are relatively higher than the average counts mentioned in other studies. This is in accordance

with the survey report (Anonymous, 2011) which revealed 100% raw milk adulteration in Odisha state, affecting public health as well as the merchantability of the product.

The present study dealing with comparative evaluation of microbiological quality of rural and urban raw milk samples concluded that all the milk samples were microbiologically contaminated, specifically the urban samples being more contaminated than the rural ones. Enteric bacteria isolated from rural milk samples included *E. coli, Enterobacter sp., Klebsiella sp.* and *Shigella*. In addition to these, *Pseudomonas* and *Salmonella sp*. were also detected in the urban milk samples confirming the poor microbiological quality of the urban samples. This necessitates that raw milk should be properly pasteurized before consumption and stored at the right temperature. Further the urban farms as well as the rural farms should maintain hygienic environment and adhere to good manufacturing practices.

ACKNOWLEDGEMENT

The authors thank the U.G.C., New Delhi for financial support to one of the authors (A.K.M) in the form of Rajiv Gandhi National Fellowship and The Head, School of Life Sciences, Sambalpur University for providing the required resources.

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