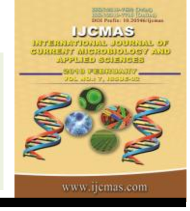
Int.J.Curr.Microbiol.App.Sci (2018) *7*(2): 1711-1718

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 02 (2018)**
Journal homepage: http://www.ijcmas.com



Original Research Article https://doi.org/10.20546/ijcmas.2018.702.207

Microbial Analysis of Buffalo Milk at Various Stages of Supply Chain in and around Parbhani City, India

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Keywords

Microbial analysis
Buffalo milk,
Supply chain,
E. coli

Article Info

Accepted:
17 January 2018
Available Online:
10 February 2018

A B S T R A C T

The TVC average value recorded at farm was Log_{10} 6.13 ± 0.065 cfu/ml at farm, Log_{10} 6.46 ± 0.068 cfu/ ml during transportation and Log_{10} 6.47 ± 0.067 cfu/ ml at retail shops. It is evident that TVC values were the lowest in raw milk at farm which increase significantly (P<0.01) at each stage of supply chain. The weeks and trials did not have effect on TVC counts. A high percentage of (70.83 percent) of raw milk samples were found to be positive for *E. coli* at farm. The percentages rose to 72.91 percent during transportation and 79.16 percent at retail shop. A non-significant effect of stage of supply chain, week and trail was observed. A low incidence (2.08 percent) of *Listeria spp.* isolation was observed from raw milk samples at farm. The organisms could not be isolated subsequently during transportation and retail shop. The percent *Clostridia* recorded at farm was 31.25, during transportation 33.33 and at retail shops 16.66. The ANOVA revealed that stage of supply chain is having highly significant (P<0.01) effect on *Clostridium* spp. isolation. The microbial quality of raw milk sold in the Parbhani city was found to be of fair quality and within the permissible limits. Presence of *E. coli*, *Listeria* spp and *Clostridium* spp. indicate that the milk is contaminated due to external sources, soil and having public health significance.

Introduction

The Indian dairy industry has been going through an advancement right from the British period until today. Being a backbone of the Indian economy, agriculture comprises of animal husbandry as its side business supporting the farmers in terms milk and meat production. India is the world's highest milk producing country for past one decade with total production of 146 million tones of milk and accounted about 18. 5 percent of the world's milk production in 2015 – 2016 (Reddy *et al.*, 2016). The buffalo is an important species for milk production in the country. The 19th Livestock Census, 2012 of India estimated a buffalo population of 108.7 million with a growth rate of 3.13 percent in 12th five year plan (2007-2012). The Maharashtra state is having buffalo population of 55.94 million. India continues to be the largest milk producer in the world. The milk production showed annual growth rate of 6.27 percent. The per capita availability of milk is around 337 gm per day. The average milk yield of buffalo is found to be 5.76 kg per day during 2015-2016. The buffaloes contribute to the 36 percent of total milk production in the country (Annual Report 2015-2016 of Department of Animal Husbandry, GOI). About 10 percent of milk produced goes into organized dairy sector for processing. The milk is processed at dairy plants located in semi urban areas and then transported to cities. The balance of about 90 percent of total production is being handled by private traders. A total of 45 percent of milk production is consumed as fluid milk. Dairy farming is an important source of subsidiary income to small farmers and agricultural labourers (Patil and shinde, 2016). In recent times, a new trend of marketing in small scale dairy units has emerged. The consumers want fresh milk rather than processed and refrigerated milk in semi urban areas. To maximize percentage of profit, a new business model of direct selling of buffalo milk to the consumers through retail marketing has been found to be successful. The supply chain of buffalo retail milk consists of production, transportation and retail selling. The farms are located in vicinity of around 5 kilometers of Parbhani city. The milk is being packed in polythene bags. It is important to evaluate supply chain of buffalo milk from public health point of view. Milk is a perishable commodity and likely to be spoiled during summer season when weather becomes hot. Unfortunately, due to unorganized and non-regulated marketing system quality of milk is hardly maintained at consumer level. It is well established that consumers want clean, wholesome and nutritious milk that is produced and processed in a sound, sanitary manner and is free from pathogens. Micro-organisms tend to be associated with disease; those that cause disease are called pathogens. However, few micro-organisms are pathogens and micro-organisms play a crucial part in life on our planet (C.B. O'Connor). Quality of milk means milk which is free from pathogenic bacteria and harmful toxic substances, adequate in keeping quality and low bacterial counts (Khan *et al.,* 2008). The microbial load of milk is a major factor in determining its quality. It indicates the hygienic level exercised during milking, cleanliness of utensils, condition of storage, manner of transportation and udder hygiene of individual animal (Taseew and Seifu, 2011). The isolation and identification of organisms like *E,coli*, *Listeria*, and *Clostridium* using selective medium helps in hazard analysis of milk. The microbial evaluation of milk at various stages of supply chain gives an assessment of Critical Control Points (CCP).

Materials and Methods

Collection of samples

A total of 4 dairy farms were selected randomly from Parbhani city. A total quantity of 200 ml of raw milk samples were collected at each farm (F1, F2, F3 and F4) during transportation (T1, T2, T3 and T4) and at retail shops (R1, R2, R3 and R4) aseptically in sterile glass bottles. The samples were transported to the laboratory on ice packs maintaining 4˚c. A total of 144 milk samples were collected twice a week for six weeks.

Microbial analysis of milk

Total viable count

Total viable counts were calculated by using standard formula given by AOAC (1997). Tenfold dilution was prepared by transferring 1 ml of milk sample to 9 ml of Normal saline solution (NSS). Dilutions were standardized for further procedure. A quantity of 0.1 ml inoculum from 10^{-3} and 10^{-4} dilutions were used for pour plate technique to which molten agar (Hi-media Laboratories, Mumbai) was poured and mixed thoroughly by rotating the plates. The plates were incubated for 24 hours at temperature of 37˚c. The bacterial colonies were counted with the help of the bacteriological colony counter (MAC) and colony forming unit (CFU) was calculated by using the following formula.

$$\text{Log}_{10}\ \text{CFU/gm} = \frac{\sum C}{[n1+(0.1 \times n2)] \times d}$$

Where,

$\sum C$ = Total number of colonies counted from all plates

n1 = No. of plates of lower dilution

n2 = No. of plates of higher dilution

d = Dilution factor

Isolation of *Escherichia coli spp.*

Isolation of E.coli was done as per the method described by Zinnah *et al.,* (2007). A quantity of 1 ml of milk was mixed with 9 ml of Nutrient broth (1:10) (Hi-media Laboratories, Mumbai) for enrichment and incubated for 24 hours at temperature of 37˚c. A loopful culture was taken from enrichment broth and streaked on Eosine methylene Blue (EMB) Agar plates (Hi-media Laboratories, Mumbai). Plates were incubated for 24 hours at 37˚c. Colonies with greenish metallic sheen were considered as positive for *E.coli*.

Isolation of Listeria spp.

Isolation of *listeria* was done as per the method described by Gasanov *et al.,* (2004). A quantity of 1 ml of milk was mixed 9ml fraser broth (Hi-media laboratory, Mumbai) for enrichment and incubated for 48 hours at 30˚c. A loopful of culture was taken from enrichment broth and streaked on PALCAM agar plates (Hi-media Laboratories, Mumbai). Plates were incubated 35 to 37˚ c for 48 hours. Grey green colonies surrounded by the black zone of esculin hydrolysis were considered as positive for *Listeria.*

Isolation of Clostridium spp.

Isolation of listeria was done as per the method described by Bacteriological Analytical Manual (1998). A quantity of 1 ml of milk was mixed with 9 ml of nutrient broth (Hi-media Laboratory, Mumbai) (1:10) for enrichment and incubated at 44 ˚c for 24-48 hours in the anaerobic jar. 0.1 ml of Inoculum was taken from enrichment broth and placed on sterile petridish. To the petridish sterile molten Sodium Polymixin Sulpha-diazine (SPS) selective agar (Hi-media Laboratory, Mumbai) was poured and mixed well by rotating the plates and allowed to solidify. Plates were incubated at 37 ˚c for 24-48 hours in the anaerobic jar. Typical colonies on SPS agar plates showing black cotton wool like growth were considered as positive for *clostridium spp.*

Results and Discussion

Total Viable Count

All 144 milk samples collected from all farms at various stages of supply chain were assessed for bacterial counts by using TVC. The results are given in Table 1. The TVC average value recorded at farm was Log_{10} 6.13 ± 0.065 cfu/ml at farm, Log_{10} 6.46 ± 0.068 cfu/ ml during transportation and Log_{10} 6.47 ± 0.067 cfu/ ml at retail shops. It is evident that TVC values were the lowest in raw milk at farm which increase significantly ($P<0.01$) at each stage of supply chain. The weeks and trials did not have effect on TVC counts.

Table.1 Results of Total viable count of milk samples at various stage of supply chain

		*Farms				
Sr. no	**Stage of supply chain**	**F1**	**F2**	**F3**	**F4**	**Average (Log$_{10}$) cfu/ml ± SE**
1	Farm	6.24	5.97	6.11	6.19	6.13 ± 0.065
2	Transportation	6.48	6.49	6.22	6.63	6.46 ± 0.068
3	Retail shop	6.52	6.47	6.31	6.58	6.47 ± 0.067

* Farms- F1, F2, F3 and F4

ANOVA for Total viable count in milk

Sr. no	Source	df	Sum of square	Mean square	F value	P
1	Stage of supply chain	2	3.625	1.812	8.499**	0.000
2	Week	5	0.947	0.189	0.888 N	0.491
3	Trial	1	0.007	0.007	0.032 N	0.859
4	Error	135	28.787	0.213		

N- Non significant, **Highly significant

Table.2 Results of *E. coli* isolation in milk samples at various stage of supply chain

		*Farms (No. of positive samples / No. of samples tested)				Positive (%)
Sr. no	**Stage of supply chain**	**F1**	**F2**	**F3**	**F4**	
		Positive	**Positive**	**Positive**	**Positive**	
1	Farm	9/12 (75%)	10/12 (83.30%)	8/12 (58.30%)	7/12 (58.30%)	34/48 (70.83%)
2	Transportation	9/12 (75%)	10/12 (83.30%)	8/12 (66.60%)	8/12 (66.60%)	35/48 (72.91%)
3	Retail shop	9/12 (75%)	10/12 (83.30%)	11/12 (91.60%)	8/12 (66.60%)	38/48 (79.16%)

*Farms- F1, F2, F3 and F4

ANOVA for *E. coli* isolation in milk

Sr. no	Source	df	Sum of square	Mean square	F value	P
1	Stage of supply chain	2	0.120	0.060	0.298 N	0.743
2	Week	5	2.296	0.459	2.272 N	0.051
3	Trial	1	0.097	0.097	0.481 N	0.489
4	Error	135	27.286	0.202		

N- Non significant

Table.3 Results of *Listeria* spp. isolation in milk samples at various stage of supply chain

		*Farms (No. of positive samples / No. of samples tested)				Positive (%)
Sr.no	**Stage of supply chain**	**F1**	**F2**	**F3**	**F4**	
		Positive	**Positive**	**Positive**	**Positive**	
1	Farm	1/12 (75%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	1/48 (2.08%)
2	Transportation	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/48 (0%)
3	Retail shop	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/48 (0%)

*Farms- F1, F2, F3 and F4

ANOVA for *Listeria* spp. isolation in milk

Sr. no	Source	df	Sum of square	Mean square	F value	P
1	Stage of supply chain	2	0.011	0.005	0.766 N	0.467
2	Week	5	0.032	0.006	0.928 N	0.465
3	Trial	1	0.007	0.007	1.019 N	0.315
4	Error	135	0.941	0.007		

N- Non significant

Table.4 Results of *Clostridium* spp. isolation in milk samples at various stage of supply chain

		*Farms (No. of positive samples / No. of samples tested)				Positive (%)
Sr. no	**Stage of supply chain**	**F1**	**F2**	**F3**	**F4**	
		Positive	**Positive**	**Positive**	**Positive**	
1	Farm	4/12 (33.30%)	4/12 (33.30%)	4/12 (33.30%)	3/12 (25.30%)	15/48 (31.25%)
2	Transportation	4/12 (33.30%)	4/12 (33.30%)	4/12 (33.30%)	4/12 (33.30%)	16/48 (33.33%)
3	Retail shop	3/12 (25%)	2/12 (16.60%)	1/12 (8.30%)	2/12 (16.60%)	8/48 (16.66%)

*Farms- F1, F2, F3 and F4

ANOVA for *Clostridium* spp. isolation in milk

Sr. no	Source	df	Sum of square	Mean square	F value	P
1	Stage of supply chain	2	3.685	1.842	14.471**	0.000
2	Week	5	6.451	1.290	10.133**	0.000
3	Trial	1	0.455	0.455	3.577 N	0.061
4	Error	135	17.188	0.127		

N- Non significant, **P<0.01

In the presents study the milk was found to be of fair quality as per BIS at each stage of supply chain. The TVC counts of $\log_{10}$ 6.13 ± 0.065 cfu/ml at farm (Table 1) might be due to external contamination of raw milk. Whereas, subsequent increase in TVC counts during transportation (Log_{10} 6.46 ±0.068 cfu/ml) might be due to contamination from cans used for transportation. The farms are located in the vicinity of Parbhani city within perimeter of 5 kms. The transportation is usually done without refrigeration. The milk is sold as freshly at retails shops. The TVC counts of $\log_{10}$ 6.47 ± 0.067 cfu/ml at retail shop might be due to effect of environmental temperature and time on microflora for facilitation of their growth.

E.coli spp. isolation

All the 144 milk samples collected from all farm at various stages of supply chain were screened for presence of *E.coli*. The results are shown in Table 2. It is evident from results (Table 2) that a high percentage of (70.83 percent) of raw milk samples were found to be positive for *E.coli* at farm. The percentages rose to 72.91 percent during transportation and 79.16 percent at retail shop. A non-significant effect of stage of supply chain, week and trail was observed. The *E.coli* isolated from milk is the indicator of poor hygienic condition (Zubeir and Ahmed, 2007). The coliforms may be associated with unclean udder, contamination from various sources such as manure, soil, feed, personnel and water (Bille *et al.,* 2009). In present study also the results of *E.coli* isolation indicate contamination of raw milk from unclean udder and external sources such as water, soil, utensils and personnel.

Listeria spp. Isolation

In the present study a low incidence (2.08 percent) of *Listeria spp.* isolation was observed from raw milk samples at farm. The organisms could not be isolated subsequently during transportation and retail shop. The presence of Listeria *spp.* in the raw milk sample indicates that the milk is having public health significance. The incidence of 4.2 percent *L. monocytogenes* was reported in USA by Lovett *et al.,* (1987). Incidence of *Listeria* spp. in raw milk was reported by many other workers (Michael and Mark, 1988; Marrakchi *et al.,* 1993). The source of contamination might be from soil (Table 3).

Clostridium spp. Isolation

In present study all 144 milk samples were screened for presence of *Clostridium* spp. by using SPS agar unaerobically. The results are depicted in Table 4. The presence of *Clostridia* recorded at farm was 31.25 percent, during transportation 33.33 percent and at retail shops 16.66 percent. The ANOVA revealed that stage of supply chain is having highly significant ($p<0.01$) effect on *Clostridium* spp. isolation. Earlier, Julien *et al.,* (2008) studied isolation of *Clostridium* spp. at 4 dairy farms for a period of 2 years. The study made in various components of milk production chain. A total of 87 percent raw milk samples were found to be positive for *Clostridial* spp isolation at two farms. Incidence of *Clostridial* spp. isolation in raw milk was also reported by Bhadsavle *et al.,* (1972). *Clostridia* are the ubiquitous in terrestrial environments. Spores of organisms are responsible for spoilage of milk and milk products. They are soil born and able to convert lactic acid to butyric acid, carbon dioxide and hydrogen at relatively low pH (Julien *et al.,* 2008).

It was concluded that microbial quality of raw milk sold in the Parbhani city was found to be of fair quality and within the permissible limits. Presence of *E.coli* spp, *Listeria* spp and *Clostridium* spp. indicate that the milk is contaminated due to external sources, soil and having public health significance.

Acknowledgement

We are extremely thankful to the College of Veterinary and Animal Sciences, Parbhani, MAFSU, Nagpur for providing such a good opportunity and working platform and also for all those who helped us during critical situation.

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How to cite this article:

Pudale, P.S. and Deshmukh, V.V. 2018. Microbial Analysis of Buffalo Milk at Various Stages of Supply Chain in and Around Parbhani City, India. *Int.J.Curr.Microbiol.App.Sci.* 7(02): 1711-1718. doi: https://doi.org/10.20546/ijcmas.2018.702.207