Indian J Dairy Sci 77(3): 236-242
https://doi.org/10.33785/IJDS.2024.v77i03.007

RESEARCH ARTICLE

Aflatoxin M1 and shelf-life analysis of goat and cow milk samples

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Received: 07 July 2023 / Accepted: 24 September 2023 / Published online: 23 June 2024

Abstract: Due to its medicinal benefits in mouth ulcers, dengue fever, etc. raw goat milk is widely used but ingestion of raw goat milk may have severe side effects. Aflatoxin M1 is produced by *Aspergillus* species and under long-term exposure, these are mutagenic, genotoxic, and carcinogenic. This study examined the shelf life of raw goat and cow milk at different storage temperatures (including ambient temperature, 5°C, and -19°C), via analysis of microbiological quality by standard plate count method. Incidence of aflatoxin M1 was also studied in raw goat and cow milk samples by lateral flow system. Cow milk showed a shelf life of 12 days while goat milk had up to 48 days at 5°C. The total plate count, coliform count, and spore count of goat milk was reported 5.89 log cfu/mL, 2.80 log cfu/ml, and 2.3 log cfu/ml in 75 days of storage while in cow milk samples it was 7.93 log cfu/ml, 5.64 log cfu/ml and 3.6 log cfu/ml, respectively in 45 days at -19°C. All the goat milk samples were found negligible for Aflatoxin M1 while cow milk samples showed a 40% incidence at 0.5 ppb. Our study showed that the quality of goat milk is commendable compared to cow milk in terms of aflatoxin M1 and bacterial count but due to less production and other factors goat milk is not widely used at the commercial level.

Keywords: Aflatoxin M1, Shelf-life, Goat, Milk, Microbial count

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Introduction

Goats are a vital component of the livestock business and the socioeconomic framework of India's smallholder farmers. India has the second-largest goat population, with 148.88 million of them (Basic Animal Husbandry Statistics, 2019). India produces 187.75 million tonnes of milk annually, with goat milk accounting for 3% of that total (Basic Animal Husbandry Statistics, 2019). Goat milk has a high protein and fat content of approximately 4.09% and 6.6%, respectively (Cyrillaa et al. 2015), is easy to digest, and is a good source of potassium, a mineral that is crucial for maintaining healthy blood pressure and heart function (Getaneh et al. 2016).

Large-scale industrialization of goat milk is frequently constrained by low individual production, seasonality, and transportation challenges, forcing farmers to keep raw milk refrigerated for longer than one day before processing. This practice affects the industry's quality since psychrotrophic bacteria can proliferate when raw milk is stored at 4 to 7°C for a few days prior to processing. By producing enzymes like lipases and proteases, psychrotrophic bacteria can change the flavor of milk and make it less appealing to customers. Lipases hydrolyze milk fat into smaller molecules known as free fatty acids, giving dairy products their sour and soapy flavors. Both native milk lipases and microbial lipases have the ability to start the lipolytic rancidity of milk (Delacroix and Lamberet. 2000). Psychrotolerants cannot withstand pasteurization, however, the extracellular enzymes mainly proteases and lipases of psychrotolerants continue to function normally and remain active, degrading the product's quality and subsequently shortening its shelf life (Hantsis-Zacharov et al. 2007). A study reported to analyse bacterial composition of goat milk at different storage conditions i.e. 4^0C and -80^0C showed that the milk bacterial diversity differed between different storage conditions (Kamilari et al. 2020).

Aspergillus flavus and *A. parasiticus* are considered to be the principal producers of aflatoxins (AFs), which are furanocoumarin derivative mycotoxins (Frisvad et al. 2019). Aflatoxins can develop in crops before and after harvesting as well as during storage if they are not kept in ideal circumstances (Kiswii et al. 2014). Aflatoxin B1 and B2 are produced by *Aspergillus flavus* which is changed into aflatoxin M1 (AFM1) when animals eat feed

contaminated with aflatoxin B1. The maximum permissible amount of AFM1 in the European Union (EU) for milk and dairy products is 0.050 g/kg. The Codex Alimentarius Commission (CAC) and the Food and Drug Administration (FDA) in the USA both determined an action level for AFM1 in the milk of 500 ng/L (FDA 2005, CAC 2001). Aflatoxins can tolerate pasteurization and they are linked to stunting in children and have been reported to inhibit children's immune systems (Jalili 2015, Raduly et al. 2020). According to the Food and Agriculture Organization of the United Nations (FAO), 25% of the world's food crops are contaminated with mycotoxins. Under long-term exposure, AFs are mutagenic, teratogenic, genotoxic, and carcinogenic (CAC, 2001). AFs can enter into the feed and food chain at any stage from pre-harvest to human consumption. In both animals and humans, these toxins are normally absorbed from the gut and transported to other bodily areas where they can form chemical bonds or undergo chemical modifications (Peleset al. 2019).

Goat milk production is not widely commercialized, despite its compelling prospects. Additionally, it has been discovered that the overall milk output of small-scale dairy farms may not be sufficient to meet the local market's needs for milk. So, the objective of the current study is to evaluate the AFM1 contamination levels and the effect of extended storage on the microbiological quality of raw goat and cow milk in the Udaipur region, India at different storage temperatures for the exploration of goat milk at commercial level.

Materials and Methods

Materials

All the media and reagents were procured from Hi Media Pvt. Ltd. Maharashtra, India. Aflatoxin M1 analysis was done by Rapid one-step assay (ROSA) kit manufactured by Charm Sciences USA. A shelf-life study was done in Biosafety Cabinet-level II, Waiometra, Associated Scientific Technology, Delhi. Incubation was done in MAC BOD incubator (India). Raw goat (n-55) and cow milk samples (n-20) were collected from nearby villages (Amarpura, Kheroda, Khemali, Chinawar, Mudiaphala, Amarpura, and Udaipur city) of Udaipur district, Rajasthan, India. Samples were collected in sterilized bottles and delivered to the laboratory by maintaining the temperature at less than 4°C during transport.

Aflatoxin M1 analysis

Aflatoxin M1 in raw goat (n-55) and cow (n-20) milk samples were analyzed by Rapid one-step assay (ROSA) kit manufactured by Charm Sciences USA which is based on lateral flow mechanism along with positive and negative controls. Dilution buffer (300 µl) was added to the labeled microtube, followed by the addition of raw goat/cow milk sample (300 µl) and mixing. The test strip was labeled with sample identification, placed in Charm EZ system, and incubated at 56℃ for 8 minutes and results were observed.

Shelf-Life analysis

For the shelf-life analysis, raw goat and cow milk samples (n=16) were distributed into three fractions and kept at controlled temperatures i.e. ambient temperature, 5℃, and -19℃ respectively, and analyzed for the total plate count, coliform count, and spore count during storage.

Total plate count

Total plate count was performed on all goat and cow milk samples by the standard plate count method. One ml of each sample was added in 9 ml sterile 0.85% saline and an aliquot of 1 ml of each dilution was then transferred to petriplates followed by the plating of plate count agar medium. The plates were incubated at 37° C for 24±2 hrs and bacterial colonies were counted and expressed in log colony forming unit (CFU) per milliliter.

Coliform count

Coliform count (CC) analysis of all milk samples was done on violet-red bile agar medium following the standard plate count method mentioned above.

Spore count

Isolation of *Bacillus* spores from milk was done by heating the milk at 80°C for 10 minutes in a heating mantle followed by rapid cooling with water for the elimination of vegetative cells. The samples (1ml) were serially diluted by the above method and plated on nutrient agar media. After incubation, a number of colonies were counted and expressed in log colony forming unit (CFU) per ml (Sharma and Singh 2017).

pH analysis

The pH of the samples was determined using Thermo Scientific pH Electrode ECO Testr pH 1 (Eutech USA). The pH meter was calibrated with pH 4.0, 7.0, and 9.0 buffer solution before analysis.

Statistical Analysis

All collected experimental data were statistically analyzed using the PAST v3.02 software (Hammer et al. 2001). Two-sample t-test was used for analysis of variance for unequal sample size of goat and cow milk. The differences between samples were assessed for effects of storage temperature, and storage time on total viable bacterial count. The mean counts of raw goat and cow milk samples were also calculated considering their storage temperature and storage period in goat farms and compared by Kruskal-Wallis test.

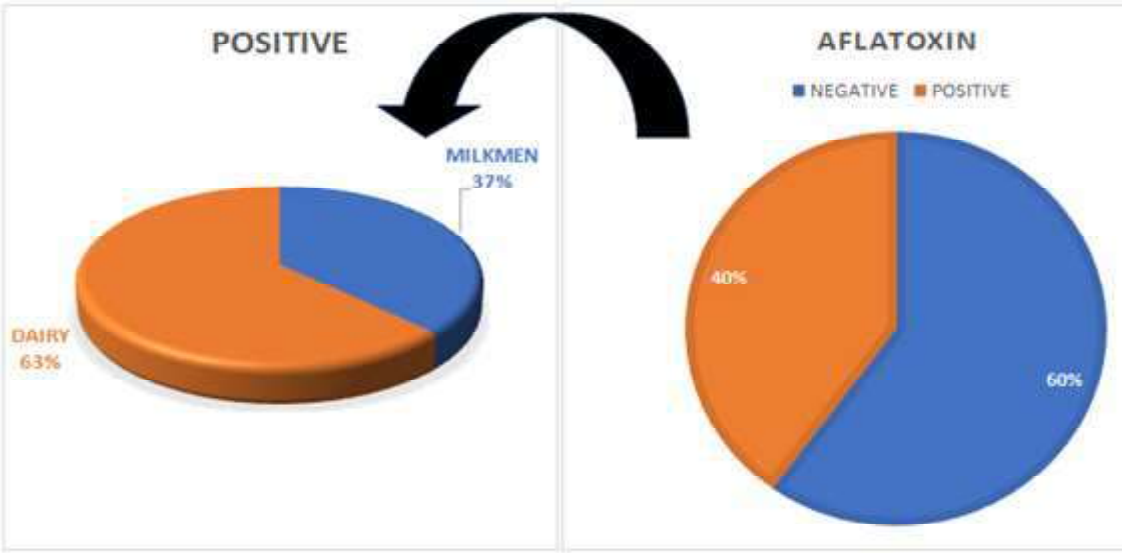


Fig 1. Occurrence of Aflatoxin M1 in raw cow milk samples

Results and Discussion

Aflatoxin M1 analysis

All of the raw goat milk samples (n=55) were found negative for Aflatoxin M1 at a detection limit of 0.5 ppb. In the case of cow milk eight out of twenty samples were found positive showing 40% incidence for Aflatoxin M1 occurrence, while twelve samples were found negative. Among the eight positive samples, 37% samples were from milkmen and the remaining 63% were collected from local dairies as depicted in Fig. 1.

The obtained results for AFM1 in goat milk are in accordance with a survey focused on the incidence of AFM1 in goat milk performed in Lebanon, and all the samples were below the detection limit of the testing methods (Assem et al. 2011). The comparatively high incidence exceeding the EU limit of AFM1 in goat milk was reported by previous studies. In a recent study, 76.0% of goat milk samples were tested AFM1-positive, and 6.7% of them were over the EU limit (Zheng et al. 2022). The concentrations of AFM1in goat milk samples were in a range of 0.0056 to 0.0482 g/L from Brazil (De Matos et al. 2021). AFM1 contamination was positive in 33.3% of 150 goat milk samples in India, of which 10.0% were above the limit of European Union (Nile et al. 2016). The results of the present study revealed lower values of aflatoxin M1 compared to those reported earlier globally however the aflatoxin M1 analysis of the Udaipur region has been done for the first time.

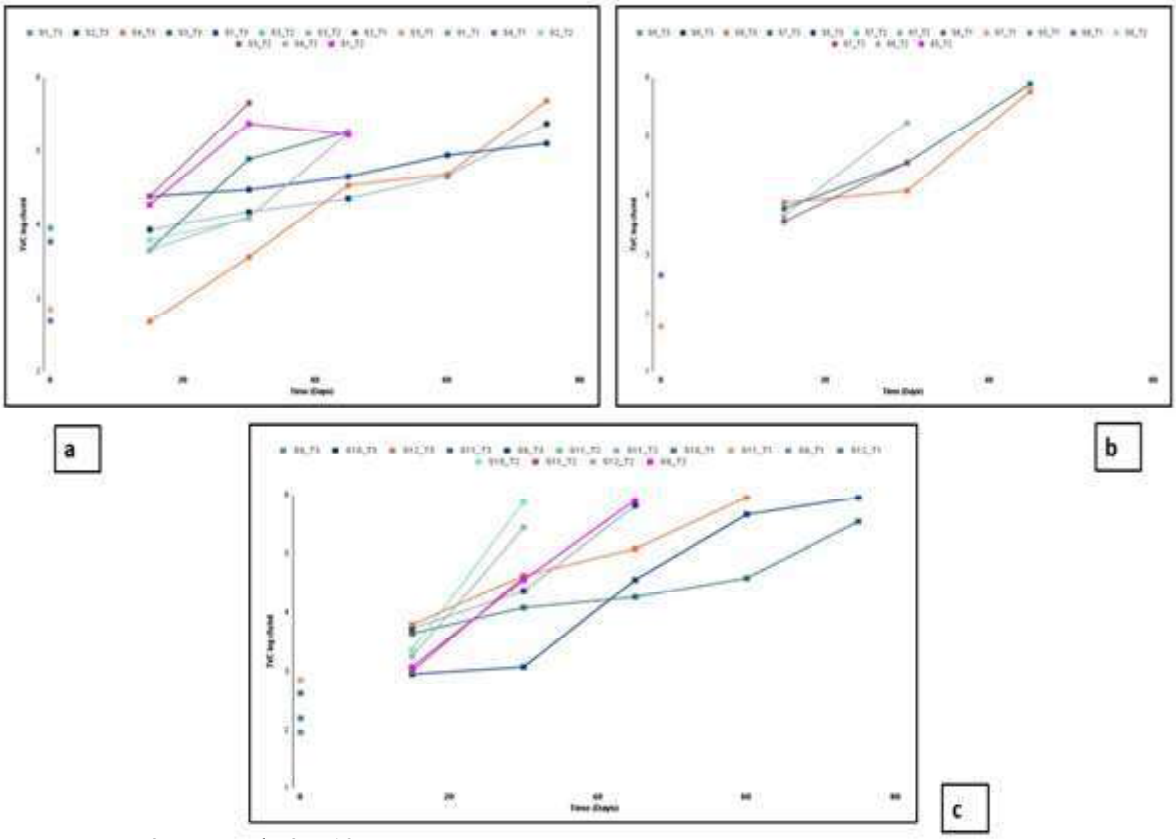
Our study revealed that the percentage of AFM1 contamination found in cow's milk was 40%, which was above the detection limit i.e. 0.5 ppb. Our results were supported by Nile et al. (2016) who reported AFM1 contamination in 45.3% of cow milk samples in India and Zheng et al. (2022) who found AFM1 in 65.7% of cow milk samples from 0.005 to 0.191 g/L. According to an Iranian investigation, 35.9% of cow milk samples were found to be over the EU MRL limit (Bahrami et al. 2016). Mohammedi-Ameur *et al.* reported that 46.42% of samples were positive for AFM1 contamination (toxin levels higher than the 0.050 g/kg EU standard (Mohammedi-Ameur et al. 2020). The cows are maintained in nearby dairy farms and fed compound rations or silage stored in substandard circumstances may be linked to the high prevalence of AFM1 in cow milk samples. This may result in the development of aflatoxin and heavily contaminated areas with toxic *Aspergillus* fungus (Asi et al. 2012).

These results showed that milk contamination levels with AFM1 differ between nations. These variations could be attributed to variations in forage and feed quality, cow diet, geographic location, climatic and seasonal variations, genetic variations in dairy cows, farming systems, and feed storage (Eskandari and Pakfetrat 2014; Sahin et al. 2016). According to Paterson and Lima, the high temperature brought on by climate change promotes mycotoxin contamination (Paterson and Lima 2010). The fact that goats in India are mostly fed by grazing and are only fed on stored grains for three to four months is likely the cause of the reduced presence of AFM1 in goat milk as compared to buffalo and cow milk (Nile et al. 2016).

Microbiological analysis

Total plate count

The initial total plate count ranged from 1.77-3.95 log cfu/ml for goat milk and 4.65-4.94 log cfu/ml for cow milk respectively at zero days. The samples kept at ambient temperature got spoiled within 24 hours in the case of cow milk, while for goat milk the spoilage time was 24-48 hours. The total plate count for the goat milk samples increased up to 5.94 log cfu/ml at -19°Cduring prolonged storage of 75 days and exceeded the EU limits at the end of the shelf-life. The initial total plate count noted ranged from 3.0-4.38 log cfu/ml at 5°C. The samples kept at 5°C showed

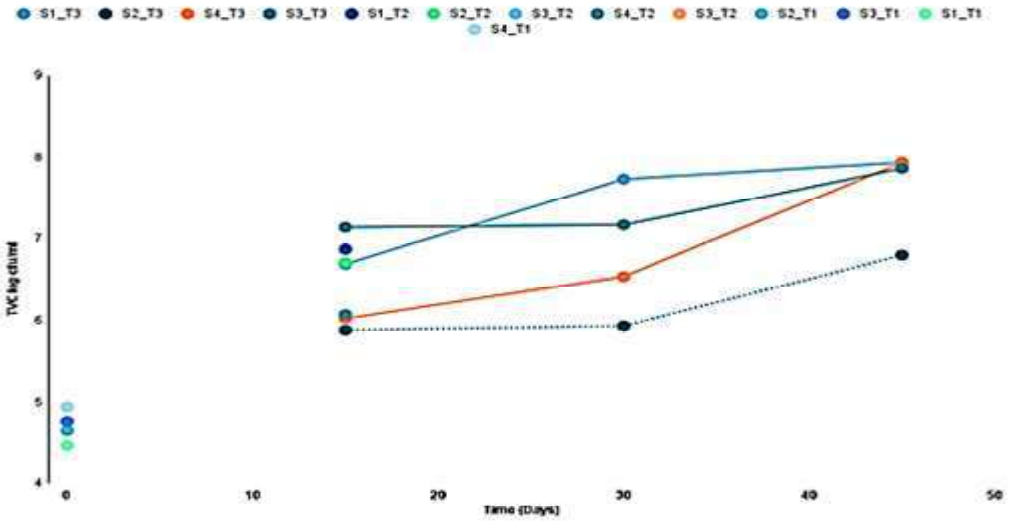


Where T1=ambient temperature, T2= 5°C and T3= -19°C
S1=Sample 1, S2= Sample 2, S3=Sample 3, and S4= Sample 4, onwards upto S12

Fig 2. Total plate count of raw goat milk during storage at different temperatures. a) Sample no.1-4, b) Sample no.5-8, c) Sample no.9-12.

Fig 3. Total plate count of raw cow milk during storage at different temperatures

Where T1=ambient temperature, T2= 5°C and T3= -19°C
S1=Sample 1, S2= Sample 2, S3=Sample 3, and S4= Sample 4



maximum variability in spoilage time where few samples got spoiled at 32 days only, some samples were safe up to 48 days with TPC value 5.86 log cfu/ml and later got spoiled (Fig.2). The total plate count of cow milk samples kept at 5°C was observed ranging from 6.07-6.87 log cfu/ml with a shelf-life of 10-15 days.

In cow milk samples the total plate count values reached up to highest 7.93 log cfu/ml at the end of shelf-life i.e. 45 days at -19°C (Fig. 3).

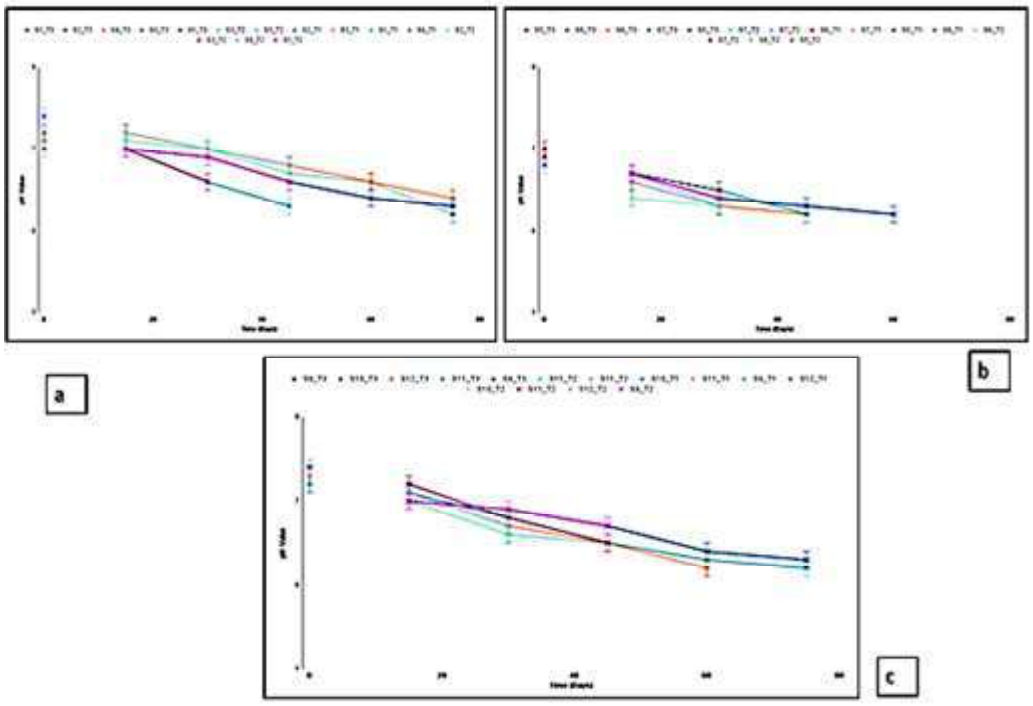


Fig 4. pH analysis of goat milk during storage at various temperatures. a) Sample no.1-4, b) Sample no.5-8, c) Sample no.9-12
Where T1=ambient temperature, T2= 5°C and T3= -19°C
S1=Sample 1, S2= Sample 2, S3=Sample 3, and S4= Sample 4, onwards upto S12

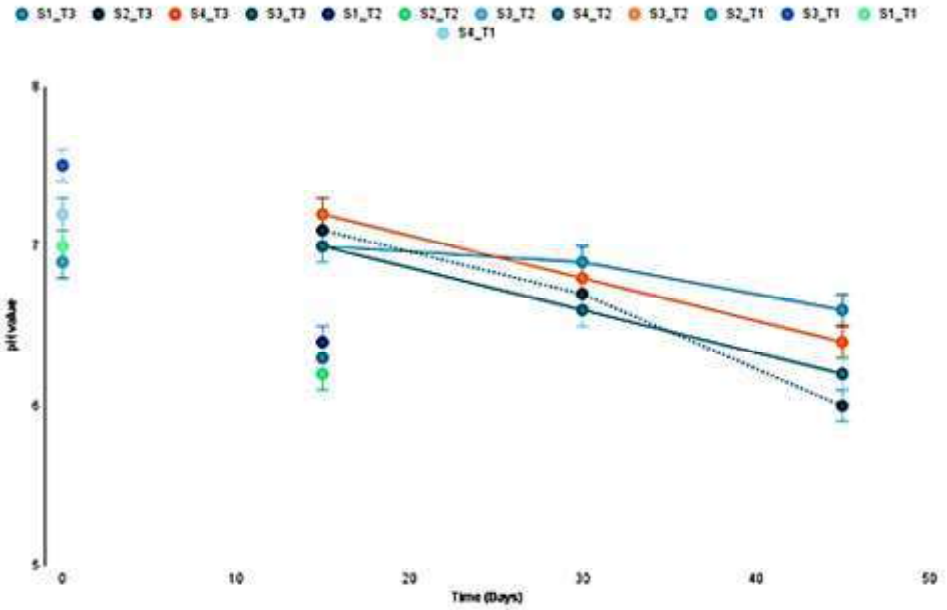


Fig 5. pH analysis of cow milk during storage at various temperatures
Where T1=ambient temperature, T2= 5°C and T3= -19°C
S1=Sample 1, S2= Sample 2, S3=Sample 3, and S4= Sample 4

In our study, the TPCs for the goat milk samples reached up to 5.94 log cfu/ml at -19°C within 75 days while cow milk samples showed the same trend of rise but count was more i.e. 7.93 log cfu/ml in 45 days of storage. These results are in accordance with many previous studies. According to a study by Nile et al. (2016) the bulk tank goat milk's 2007 SPCs kept rising and by day 6 had surpassed the grade A goat milk's limit of 100,000 CFU/ml. A preliminary bacteria count indicates the degree of contamination and quality of the milk samples. The total concentration of aerobic bacteria was 3.44 log cfu/ml (Lai et al. 2016). According to the Malaysian Food Act 1983 and Food Regulations 1985, the total aerobic bacteria concentration in milk, which is safe for consumption should not exceed 5.0 log cfu/ml (USFDA 2005).

In most of the samples including goat and cow milk, the spore count was found nil. During the prolonged storage at -19°C, the spore count in raw goat and cow milk samples grew steadily and reached 2.3 log cfu/ml and 3.6 log cfu/ml respectively. In all three

categories, bacterial concentration showed an increasing trend throughout storage. The longer shelf-life of goat's milk as observed in this experiment (more than 60-70 days as compared to 30-45 days for cow's milk) may be explained by the presence of natural bacterial inhibitor in goat's milk.

Evidently, when milk samples were stored at low temperatures (5°C and -19°C), the growth of aerobic bacteria was considerably slower. Lafarge et al. discovered that 24 hours of cold storage dramatically increases the number of psychrotrophic bacteria (Lafarge et al. 2004). One of the main groups of spoilage bacteria found in raw goat milk samples or other dairy products is the psychrotrophic bacteria. *Pseudomonas fluorescens* is the most often isolated species from milk and is the dominant member of the bacterial community at the time of spoiling, making up the majority of the psychrotrophic microorganisms. One of the useful markers for the shelf life and degree of deterioration of raw goat milk samples might be the proteolytic count (Mcphee and Griffiths, 2006). The impact of variables on the composition of the raw milk microbiota and spoilage activity has already been described, mostly in cow milk (Ercolini et al. 2009; Perin et al. 2009).

Coliform Count

In most of the samples of both raw goat and cow milk, the coliform count was found nil. The coliform count (CC) in goat milk continuously increased with time spent in storage. On day zero, the CC was 2.44 log CFU/ml at ambient temperature and at the end of storage, it had risen to 2.80 log CFU/ml at -19°Cwhile CC noted 2.56 log cfu/ml at 5°C. The initial coliform count determined from the raw cow milk sample at zero-day was 1.36 log cfu/ ml at ambient temperature and 4.84 log cfu/ml at 5°C at 15 days storage period. The coliform count in raw cow milk reached 5.64 log cfu/ ml with the end of shelf-life of 45 days -19°C.

The coliform count (CC) was observed as nil in most of the goat and cow milk samples while the increasing trend was followed by the remaining samples. When the storage duration was extended, there was a progressive increase in the concentration of coliform bacteria. The microbiological standard's limit for the concentration of coliform bacteria was exceeded in samples of raw cow milk and reached up to 5.64 log cfu/ml. Although coliform bacteria may grow more slowly in colder temperatures, many of them, including *E. coli* and *Klebsiella* spp., are temperature tolerant and quickly recover under warm conditions. As a result, these coliform groups provide a special risk for the spoiling and contamination of a sample of raw goat milk stored at a low temperature (Lai et al. 2016).

Spore Count

Spore count was found nil in most of the samples of both goat and cow milk. The initial spore count noted at ambient temperature was 1.97 log cfu/ml and 1.51 log cfu/ml for goat and cow milk respectively at zero days. The count was observed as nil at 5°C as the samples got curdled on heating in both the cases of goat and cow milk. The final spore count reached up to 2.3 log cfu/ml for goat milk and 3.6 log cfu/ml for cow milk at -19°C till the final spoilage of samples.

There was a significant difference found between goat and cow milk sample medians in reference to temperature variance by Kruskal-Wallis test and sample variance by t-test. The $H\ (chi^2)$ values for goat and cow milk samples were found as 11 and 6.038 respectively. The p -values ($p<0.005$) for goat and cow milk were noted 0.003 and 0.048 respectively, represented a significant value.

pH analysis

The initial pH for raw goat and cow milk samples ranged from 7.4±1 to 6.8±1 and 7.5±1 to 6.9±1 respectively at zero days. However, the pH of the milk samples decreased during the storage time and fall up to 6.3±1 for goat milk and 6.2±1 in the case of cow milk at 5°C. The final pH observed at -19°C was 6.2±1 and 6.0±1 for goat and cow milk respectively with the end of the shelf-life of samples (Fig. 4 & 5).

The initial value of pH which ranged from 7.4-6.8 at ambient temperature decreased significantly, and the final pH noted was 6.2 for goat milk while for cow milk it is 6.0 at -19°C. Both lactose fermentation and/or microbial alterations in the product during storage can cause a reduction in the pH values of raw milk. Growing casein and phosphate concentrations during late lactation increased the milk's intrinsic acidity and may be the source of a pH reduction Singh and Sengar 1990). The recent study by Kamilari et al. (2020) provided the information that keeping goat milk overnight in the refrigerator may lead to alterations in the bacterial composition.

Conclusions

Based on our findings, a direct relationship between raw goat milk storage practices and its microbiological quality was found. The study represented that goat milk has a low count and high/ more shelf-life in comparison to cow milk. The shelf-life of raw goat milk was observed more (75 days) in comparison to cow milk (45 days) at -19°C. The total bacterial count, coliform, and spore count in raw goat and cow milk samples increased gradually after prolonged storage at various temperatures. The high bacterial count in local raw goat and cow milk samples highlights the tendency of the bacteria to grow and multiply when stored at low temperatures. A high total plate count and the coliform count were not advisable for raw milk consumption proceedings due to food contamination concerned. Goat milk was found safe for consumption in reference to Aflatoxin M1 while it is a matter of concern that in cow milk incidence is 40%. These findings on aflatoxin M1 level and shelf-life analysis on raw goat and cow

milk samples from the Udaipur region, India will be helpful for dairy plants during taking decisions regarding product development using goat milk.

Acknowledgments

The authors are thankful to the authorities of Mohanlal Sukhadia University for providing the infrastructure facilities. Namita Ashish Singh is thankful to the University Grants Commission (UGC), New Delhi for providing the UGC-BSR Research Start-Up-grant letter No.F.30-476/2019 (BSR).

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