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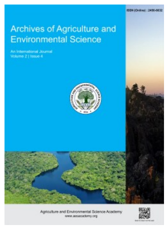

ORIGINAL RESEARCH ARTICLE

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Effects of various methods of milking, container types, and chilling durations on bacterial load of milk

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ABSTRACT

This study was carried out to evaluate the quality of raw milk measured by Standard Plate Count (SPC). Individual raw milk for the Colony Forming Units (CFU) study was carried out in the National Cattle Research Program Rampur Chitwan, Nepal. Milk from Jersey and Holstein cows with two types of milking (hand and machine milking) in collecting three types of containers (Plastic, aluminum, and steel). Milk had different chilling durations (0, 4, 8, 12, 24, 48, and 72 hours). Altogether, 252 milk samples for SPC were examined at farm levels. Results showed significant variability in SPC throughout the study period. The lowest CFU was observed in Holstein cows ($80.49\pm4.83 \times 10^4$), while the highest was found in the Jersey breed ($122.88\pm4.69 \times 10^4$). Similarly, the lowest CFU count was recorded in milk from machine milking ($92.42\pm4.69 \times 10^4$), whereas the highest CFU count was observed in milk from hand milking ($110.95\pm4.83 \times 10^4$). For three milk collecting and transporting containers, the CFU count was lowest in the steel container ($90.09\pm5.82\times10^4$) compared to the aluminum container ($102.42\pm5.82\times10^4$) and plastic container ($112.55\pm5.82\times10^4$). The results of mean CFU for the chilling duration effects at farm 0, 4, 8,12,24,48 and 72 hours were ($114.33\pm8.11\times10^4$, $108.21\pm10.28\times10^4$, $107.71\pm10.28\times10^4$, $106.75\pm10.28 \times10^4$, $104.07\pm6.36 \times10^4$, $94.79\pm8.11\times10^4$, and $75.94\pm8.11\times10^4$). CFU count in hand and machine milking milk differed significantly ($p<0.01$) from each record of the same date at the farm level. The CFU in milk from different containers was significant ($p<0.05$) for the overall experimental period. Steel containers showed a low CFU count compared to Aluminum and plastic containers. The highest number of CFU (114.33×10^4) was observed in the 0-hour chilling, which was significantly ($p <0.05$) different from the rest of the chilling duration. The results obtained from the study indicated that the current situation is critical and needs real improvement from farm to chilling centers. The findings could guide dairy producers in adopting effective strategies to enhance milk quality, minimize bacterial contamination, and ensure safer dairy products for consumers by using these results.

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INTRODUCTION

Milk is highly perishable because it is an excellent medium for the growth of microorganisms – particularly bacterial pathogens – that can cause consumer spoilage and diseases. Milk processing allows the preservation of milk for days, weeks, or months and helps to reduce food-borne illness (FAO, 2022). Raw milk contains many nutrients and offers optimal nutritional circumstances for numerous microorganisms (Skeie *et al*., 2019, Cremonesi *et al*., 2020). Under normal conditions, milk should be sterile within the healthy udder cells. However, upon exiting the udder, it typically accumulates small quantities of microorganisms, primarily lactic acid bacteria. Nonetheless, potential exposure to external pollutants contributes to a multifaceted microbiota originating from various sources (Berhanu *et al*., 2021, Machado *et al*., 2017), primarily from the udder and teat surface (Gouranga, 2008, Gleeson *et al*., 2013, Parente *et al*., 2020). Moreover, the quantity and variety of microorganisms present in raw milk are impacted by several factors, including the cleanliness of milking equipment, season, water, diet, and animal well-being (Amagliani *et al*., 2012, Swai & Schoonman, 2011). Understanding the factors that exert positive or negative influence on raw milk micro biota holds significance as they profoundly affect the safety and excellence of the resulting food items. The issue of raw milk quality poses a significant challenge for large-scale production and the consistent creation of products. Farming practice influence the quality of raw milk and is negatively impacted by factors like inadequate knowledge of hygienic methods and the absence of milk-chilling facilities in nearby areas. Processors are concerned that excessive enforcement of quality standards could lead to milk shortages during periods of low production. Microbial contamination of milk can originate from various sources. It can occur within the udder, on the outer surface of the udder, or stem from the milk handling and storage equipment used Ranjit *et al*. (2008). Additionally, contamination can happen during food preparation or due to inadequate hygiene practices by infected personnel (Lore *et al*., 2006). (Tiwari & Paudel 2018) studied that the transporters used various types of transport cans as aluminum cans (52%), stainless steel can (4%), plastic drums (28%), and tanks (16%). The containers made of plastic are of low density and can be easily scratched. These scratches harbor bacteria and decrease milk quality. Aluminum can be introduced into the milk and milk products during production or by contamination from the metal processing equipment (Deeb & Gomaa, 2011). The use of aluminum utensils for processing and storage of milk may increase substantially the level of this metal in milk and milk products (Semwal *et al*., 2006) and leaching of this metal from utensils is influenced by the quality of the containers, pH level, preparation conditions and the presence of complexion agents (Al Juhaiman, 2010). The hygienic milk production poses a significant challenge for the global dairy sector (Li *et al*., 2018). As the demand for dairy products rises, bacterial contamination has become a global apprehension. Milk's microbial composition is the primary determinant of its quality (Naing *et al*., 2019). Implementing effective dairy farming practices, encompassing aspects such as animal health, milking hygiene, nutrition, animal welfare, environmental considerations, and socio-economic management, is imperative to ensure the production of high-quality milk (FAO & IDF, 2011). Establishing and adhering to a Good Manufacturing Practice (GMP) system for providing untreated milk to processors is widely recognized as a foundational measure for upholding the quality of raw milk. In alignment with this necessity, Nepal's Ministry of Livestock Development (MOLD) introduced a set of 40 policy commitments, primarily focusing on enhancing milk quality standards (MOLD, 2016). This research gap underscores the need for a detailed examination of the effects of different factors in the microbial quality of milk. The objective is to record and analyze the microbial quality of milk at various points within the informal value chain, including the production at National Cattle Research Program farm, during transportation, and at the cooling centers.

MATERIALS AND METHODS

Animal selection

The investigation was carried out to assess the influence of breed, milking technique, and chilling duration on the bacterial content of unprocessed milk sourced from individual cows. The conceptual framework of the study is given in Figure 1. A total of 252 milk samples were acquired, comprising raw milk procured from 20 distinct Jersey crossbred and Holstein crossbred cows, spanning three different lactation stages and all the animals were kept under a uniform management system.

Milk sampling and collection

Milk samples were collected twice daily at 6 AM and 3 PM. Containers made of Plastic container (PC), Stainless Steel container (SC), and Aluminum container (AC) were sterilized and dried before use. A 10 ml bulk milk sample was taken from the center of the milking bucket after the initial analysis sample. An icebox was used to maintain the cold chain, and bottles were properly labeled. Samples from 20 cows were collected using both hand and machine milking techniques under aseptic conditions. The chilling durations (CD) of 0, 4, 8, 12, 24, 48, and 72 hours were managed according to the Dairy Development Corporation (DDC) standards in Nepal.

Evaluation of bacterial quality

The bacteriological quality of the raw cow milk was determined by analyzing the 20 milk samples. This assessment was carried out by employing the standard plate count method.

Standard plate count and media preparation

The standard plate count assessment was executed using the pour plate technique in conjunction with Tryptone soya agar as the growth medium. The growth medium underwent sterilization via autoclaving at a pressure of 15 pounds per square inch (psi) for 20 minutes, at a temperature of 121 degrees Celsius. Following autoclaving, the medium was allowed to cool down to a temperature of 45 degrees Celsius, after which it was ready for use in the subsequent testing procedure.

Inoculum preparation

To achieve homogenization and disperse microorganism clusters, the milk samples were shaken vigorously 10 to 15 times. For a 1:10 dilution, 1 milliliter of the milk sample was mixed with 9 milliliters of sterilized normal saline solution (NSS) in a test tube. This dilution was then sequentially transferred to subsequent test tubes, each containing 9 milliliters of NSS, up to a dilution of 1: 1,000,000. From each dilution, 0.1 milliliters were transferred to sterilized Petri plates. Molten plate count agar

(TSA) was added to the plates and mixed by gently rotating them. After solidification, the plates were incubated inverted at 37°C for 24 to 48 hours.

Colony selection and enumeration

Following the incubation period, Petri plates displaying bacterial colonies ranging between 30 and 300 were singled out for result recording. To determine the count of organisms, present in one milliliter of the milk sample, the number of bacterial colonies on these selected plates was multiplied by the respective dilution factor.

Calculation Coliform Unit (CFU) per ml

The CFU per ml was determined using the standard plate count method by selecting a plate with countable colonies, calculating the total dilution factor (TDF), and applying the equation: CFU/ml = (No. of colonies × TDF) / volume plated.

Statistical analysis

To study the effect of four factors and their interactions on SPC in milk, data were subjected to a linear fixed effect model and analyzed using the R program Version 4.3.2. Significantly different means were separated using Duncan's Multiple Range Test (DMRT). The following statistical model was used for analyzing the data collected in this study:

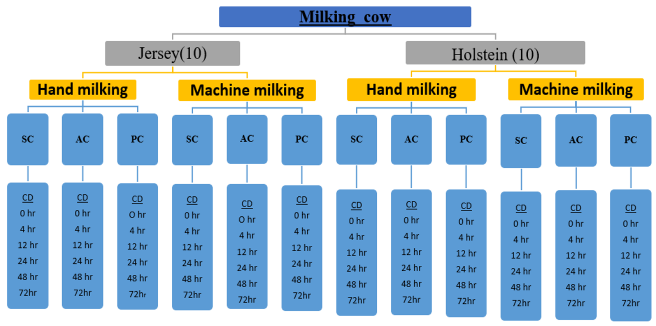

Figure 1. *Conceptual framework of the experiment.*

$$Y_{ijklmn} = \mu + a_i + b_j + c_k + d_l + (ad)_{il} + (bd)_{jl} + (cd)_{kl} + e_{ijklmn}$$

Where,

Y_{ijklm} is the observed mean of SPC (CFU of milk)

a_i is the effect of i^{th} breed (i.e. 1=Jersey, 2=HF)

b_j is the effect of j^{th} method of milking (i.e. 1=machine, 2=hand)

c_k is the effect of k^{th} type of container (i.e. 1=plastic, 2=aluminum, 3=stainless steel)

d_l is the effect of l^{th} chilling duration (i.e. 1=zero hour, 2=four hours, 3=eight hours, 4=twelve hours, 5=twenty-four hours, 6=forty-eight hours, 7=seventy-two hours)

$(ad)_{il}$ is the effect of interaction between i^{th} breed and l^{th} chilling duration

$(bd)_{jl}$ is the effect of interaction between j^{th} milking method and l^{th} chilling duration

$(cd)_{kl}$ is the effect of interaction between k^{th} container type and l^{th} chilling duration

e_{ijklm} is the residual (error) component assumed to be normally and independently distributed.

RESULTS AND DISCUSSION

The study focused on investigating the impact of breed, milking methods, types of containers, and chilling duration on Colony-Forming Unit (CFU) counts (in ×10^4) based on a total of 256 observations. The results of these analyses are presented in the Table 1 and Figures 2-4.

Effect of breed

The CFU counts for Jersey and Holstein breeds were compared, revealing a significant ($p < 0.001$) difference. Jersey cows had a higher mean CFU count (122.88±4.69) than Holstein cows (80.49±4.83). This indicates that breed affects microbial load in raw milk, with Jersey cows showing higher bacterial presence (Figure 2). Peterková (2002) noted that Jersey cows experience a negative energy balance, leading to higher milk fat and protein levels and elevated somatic cell counts (SCC), which promote

Table 1. Effect of breed, milking methods, types of container, and chilling duration on colony formation unit count (×10^4).

Treatment /Factors	Number of observations (n)	CFU Mean ±SE	Level of significance
Breed			
Jersey	132	122.88±4.69	***(<0.001)
Holstein	120	80.49±4.83	
Milking methods			
Machine Milking	132	92.42±4.69	**(<0.001)
Hand Milking	120	110.95±4.83	
Types of Containers			
Plastic	84	112.55±5.82 ^{a}	* (<0.05)
Aluminum	84	102.42±5.82^{ab}	
Steel	84	90.09±5.82^{b}	
Chilling Duration (hour)			
0 Hour	39	114.33±8.11 ^{a}	* (<0.05)
4 Hour	24	108.21±10.28 ^{b}	
8 Hour	24	107.71±10.28 ^{b}	
12 Hour	24	106.75±10.28 ^{b}	
24 Hour	63	104.07±6.36 ^{b}	
48 Hour	39	94.79±8.11^{bc}	
72 Hour	39	75.94±8.11 ^{c}	

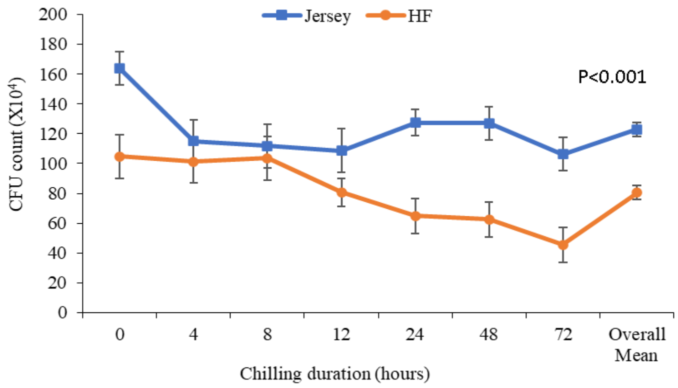

Figure 2. *Interaction between breeds and chilling duration on CFU count (×10⁴).*

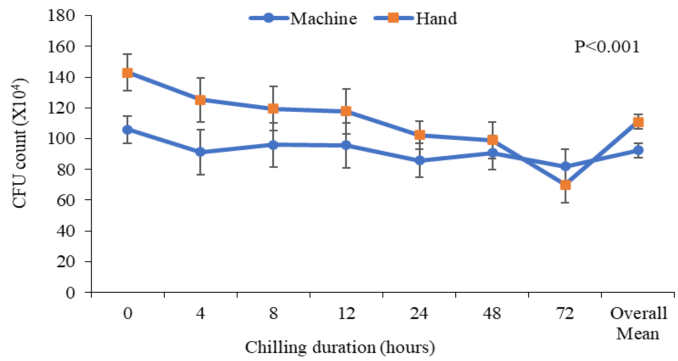

Figure 3. *Interaction between methods of milking and chilling duration on CFU count (×10⁴).*

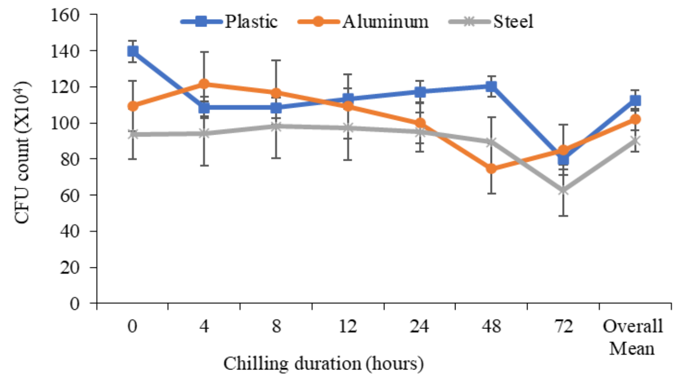

Figure 4. *Interactions between types of containers and chilling duration on CFU count (×10⁴).*

bacterial growth. A similar explanation was done by Petróczki *et al.* (2019) where Jersey was found more prone to negative energy balance, leading to elevated SCC and increased bacterial presence in milk. Also, Soyeurt *et al.* (2006) observed that increased free fatty acids (FFA) in Jersey milk can impact taste and fermentation. Moreover, De Vliegher *et al.* (2005) revealed that breed-specific physiological variation, such as udder morphology and immune response varies the levels of bacterial contamination in milk. Czerniewicz *et al.* (2006) also observed that Milk from Jersey cows was higher in fat, which also had the shape of larger globules when compared to that from Holstein-Friesian cows so temperature effects the favorable condition to grow microorganism in large globules. Calgaro *et al.* (2020) suggested that negative relationships between milk lactose content and SCC score, calving order, and Body condition score for the quality of milk in Jersey cow compare to Holstein cow which causes more bacterial load in jersey cow milk.

Effect of milking methods

The study compared machine milking and hand milking, revealing a significant ($p<0.001$) difference in CFU counts. Machine milking had a mean CFU count of 92.42±4.69, while hand milking had a higher mean CFU count of 110.95±4.83 (Figure 3). This indicates that the milking method significantly impacts bacterial presence, with hand milking showing higher counts. This results an agreement with Abakar (2021) obtained results indicated highly significant differences between the manual and automatic milking samples of these bacterial types and that ($p < 0.05$) the milking mechanism superior to manual milking Mean level analysis also revealed significant ($P < 0.05$) differences. Reche *et al.* (2015) found a strong influence of initial milk contamination on bacterial counts due to hand milking. Lejeune & Rajala-Schultz (2009) observed that hand milking could lead to increased bacterial contamination because of the higher likelihood of introducing bacteria from the udder, hands, and environment into the milk. On the other hand, Jayarao & Henning (2001) reported that properly maintained machine milking systems are more effective at maintaining lower bacterial counts, provided the equipment is routinely cleaned and sanitized. Factors such as cow health, environment, milking techniques, and equipment cleanliness affect microbial pollution in milk. The hands of the milkers and the milking environment are also potential contamination sources (Filipovic & Kokaj, 2009). Stefan and Baraitareanu (2023) found that whatever method take to milking the cow should be clean utensil for preventing growth of microorganism. This results as per work done by Nyokabia *et al.* (2021) for the small holder dairy farmer in Malawi the challenge of maintain milk quality due to unhealthy practices of milking animals and not following the food safety standard. Tadesse *et al.* (2020) studied in village milk farmer from Ethiopia, in that farmer field hand milking was only types of milking because women are the main worker and they used plastic container for transporting milk, in that place cfu count of hand milked raw milk was 5.9 log10cfu/ml.

Effect of types of containers

The study investigated the impact of different container types (Plastic, Aluminum, and Steel) on CFU counts. Plastic containers had the highest mean CFU count (112.55±5.82), followed by Aluminum containers (102.42±5.82), with Steel containers showing the lowest mean CFU count (90.09±5.82). The significance ($p < 0.05$) level indicates that container material affects bacterial counts, with Plastic containers exhibiting higher microbial load. Plastic is generally less durable than steel, and can become damaged more easily. Some plastics may leach chemicals, especially when heated, which can affect the milk's taste and safety. While plastic can be cleaned, it can be more prone to scratching, which can harbor bacteria. Plastic is less eco-friendly compared to steel and aluminum due to its non-biodegradability and pollution during production. The total bacterial count (TBC) in all raw milk samples was below the maximum level advised by the Indian Standard (BIS, 1992), suggesting that the raw milk quality was satisfactory. The TBC in all raw milk samples fell within a range that was lower than the findings of previous studies conducted in Nepal by Dahal *et al*. (2010) and Phattepuri *et al*. (2020). But these results lined with a study by Acharya *et al.* (2017) conducted in Kathmandu. Research by Ruegg (2003a) indicates that milk stored in stainless steel containers generally has lower SPC levels compared to milk stored in plastic containers. Plastic containers, which are more susceptible to scratches and wear, can harbor bacteria in these imperfections, making them difficult to sanitize effectively (Elmoslemany *et al*., 2010). Consequently, the use of plastic containers often results in higher bacterial counts in milk. In context to our study, the lower bacterial load is attributed to hygienic practices, cow health, high-quality water, clean equipment, and hand washing before milking. Cleanliness of milking equipment significantly affects bacterial contamination, with proper cleaning and disinfection practices, such as using a 0.1% sodium hypochlorite solution, reducing CFU counts from 10^2–10^8 to 10^1–10^4 (Oie & Kamiya, 2001). Wafula (2016) also studies that Milk handling plastic jerry can containers were collected from dairy actors analyzed for Total Viable Count (TVC), Total Coliform Count (TCC) and Lactic Acid Bacteria (LAB) whereby LAB was isolated from the plastic detergent bottles as human colon at numbers, posing high risk to human health after consumption of contaminated milk that had been packaged in the same handling vessels. Aluminum containers can risk microbial proliferation due to residual milk, and narrow-necked plastic containers are linked to higher microbial loads (Shija, 2013). This highlights the importance of effective cleaning and appropriate container selection for maintaining milk quality. Steel containers are stronger and can endure rough handling during transportation. Stainless steel is especially inert with milk, meaning no chemical action will take place that may affect quality and flavor. The smooth quality of steel makes it easier to clean and sanitize, leading to a lower chance of bacterial contamination. But if the milk storage container is double-walled, steel work– not only provides durability and ease of care, but also functions well in thermal insulation for our sake to have the temperature more easily maintained. Steel is much more sustainable; it can be recycled again and again without any loss in quality. Owusu *et al*. (2020) studied in Africa that the hygiene condition and proper handling of milk minimize the bacterial growth by adopting the good management practices i.e., proper use of milking machine and chilling methods.

Effect of chilling duration

The impact of chilling duration (0, 4, 8, 12, 24, 48, and 72 hours) on CFU counts was noted significantly ($p < 0.05$) different. CFU counts decreased from 114.33±8.11 at 0 hours to 75.94±8.11 at 72 hours, indicating that longer chilling durations reduce bacterial presence in raw milk. The study revealed that Jersey cows consistently had higher CFU counts than Holstein Friesians across different chilling durations. At 0 hours, Jersey cows showed 163.76±11.05 CFU compared to 104.75±14.54 CFU in Holstein Friesians. By 72 hours, these counts decreased to 106.39±11.05 CFU for Jersey and 45.50±11.87 CFU for Holstein Friesians. The overall mean CFU counts were 122.88±4.69 for Jersey and 80.49±4.83 for Holstein Friesians, indicating a significant ($p < 0.001$) interaction between breed and chilling duration. However, O'Brien *et al*. (2016) found that Holstein-Friesian milk typically has higher bacterial counts than Jersey milk, likely due to differences in milk composition. Chilling duration is key in controlling bacterial growth, as shown by Smith & Jones (2018), who reported that immediate chilling reduces CFU counts across all breeds. Nevertheless, Holstein milk needs faster chilling than Jersey milk to achieve similar microbial control, indicating breed-specific interactions with chilling practices. Gurunathan (2023) studied the shelf-life of hygienically processed yak milk stored at refrigeration temperature (4±1°C) was found to be 9 days, during which there was no significant deterioration in its physicochemical, microbial, and sensory quality. Gume *et al*. (2023) found in their study that from the total of 150 raw milk sample contaminated 61.3% along the dairy value chain with one or more pathogen were reported, the highest and lowest bacterial counts recorded were 4.88 log 10 cfu/ml and 3.4log10 cfu /ml due to less time stay in chilling centers. The study observed variations in CFU (Colony Forming Unit) counts in milk across different chilling durations (0 to 72 hours) for both machine and hand milking methods. Initially, hand milking showed higher CFU counts (143.00 × 10^4) compared to machine milking (105.85 × 10^4) at 0 hours, indicating higher microbial contamination. As the chilling duration increased, CFU counts decreased for both methods. After 72 hours, hand milking, which initially had higher contamination, exhibited the lowest CFU count (70.00 × 10^4) compared to machine milking (81.89 × 10^4). The result shows that the extended chilling effectively reduces microbial contamination in milk, with both milking methods showing lower CFU counts over time. While machine milking generally maintained lower contamination levels, hand milking eventually resulted in the lowest CFU counts after 72 hours of chilling. The method of milking—hand versus machine—significantly impacts the initial microbial load in milk, affecting the effectiveness of chilling in reducing CFU counts was also observed by (Boor *et al*., 1998) where hand milking resulted in higher initial CFU counts due to contamination from the milker's hands and environment while machine milking generally had lower counts due to a more controlled environment (Ruegg, 2003b). DeVries & Putnam (2019) found that machine-milked samples showed more consistent CFU reduction with chilling compared to hand-milked samples, which required longer chilling to achieve similar microbial reductions due to their higher initial contamination. Gunasena & Sriwardhana (2021) found in their reports that the high load of bacterial count indicates the passage of all points harboring milk in the entire process. Maintaining good health status of animals, milking practices, Storage and transportation should be hygienic so as to minimize the microbial contamination in milk which helps in producing good quality milk. Regular checks of bulk milk quality. Figure 4 illustrates the effects of chilling duration and container type on CFU counts in milk. At 0 hours, plastic containers had the highest CFU counts (139.71 × 10^4), while aluminum and steel had lower counts (109.56 × 10^4 and 93.71 × 10^4, respectively), indicating plastic's higher susceptibility to bacterial growth. CFU counts decreased across all containers with extended chilling. After 4 hours, aluminum showed the highest count (121.75 × 10^4), while plastic and steel had lower counts. This trend persisted through 8 and 12 hours, with aluminum maintaining the highest counts and steel the lowest. By 24 hours, plastic still had higher counts (117.33 × 10^4), though all containers showed reduced contamination. At 48 hours, plastic had the highest CFU count (120.30 × 10^4), but all containers saw a significant decrease in counts by 72 hours. Plastic consistently showed higher CFU counts, with aluminum and steel showing lower counts. This highlights that both container material and chilling duration are critical for managing microbial contamination in milk. (Chen *et al*., 2014) also revealed that plastic containers are more prone to bacterial growth due to their porous nature, which can retain bacteria even after cleaning. Research by Fox *et al*. (2017) found higher CFU counts in milk stored in plastic compared to aluminum or steel, attributing this to plastic's poor thermal properties, which slow chilling and allow more bacterial growth. Conversely, steel containers, with better thermal conductivity, achieved more effective microbial control through faster cooling and consistent CFU reductions during extended chilling (Jang & Lee, 2020).

Conclusion

This study demonstrated that breed, milking methods, types of containers, and chilling duration significantly impact CFU counts in raw milk. This implies that selection of the breed might be an important factor affecting milk quality, and also affecting the microbial load in raw milk. This observation highlights the importance of milking practices for microbial contamination control to ensure milk quality. The results indicate that container material selection may have an effect on microbial load and dairy operations should carefully select equipment. This emphasizes the importance of rapid and efficient chilling to maintain the quality of milk and limit bacterial growth. The large CFU count differences over chilling times also underscore the importance of minimizing time to effective chilling by dairy producers after milking. The results emphasize the importance of these factors in maintaining microbial quality and safety of raw milk. Percentage of samples exceeding limit of CFU, Previous research indicated that animal having history of mastitis is likely having higher CFU value. So, this can recommend the association study for the same. The study also reflects the udder health and nature of the dairy animal milk secretion. The findings could guide dairy producers in adopting effective strategies to enhance milk quality, minimize bacterial contamination, and ensure safer dairy products for consumers.

DECLARATIONS

Author contribution statement

Conceptualization: S.P. and N.B.., Methodology: H.B.B and S.P., Software and validation: N.A.G., N.B. and S.P., Formal analysis and investigation: S.P and N.B., Resources: P.H. and S.P., Data curation: S.P, P.H. and N.B., Writing—original draft preparation: S.P., N.A.G. and N.B., Writing—review and editing: S.P., N.B, H.B.B and N.A.G., Visualization: S.P., Supervision: S.P., Project administration: S.P. and P.H. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Ethics approval: Ethical approval was taken from the Nepal Veterinary Council, Agriculture and Forestry University, and the National Cattle Research Program (NCRP) to conduct all the research activities.

Consent for publication: All co-authors gave their consent to publish this paper in AAES.

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