Assessment of bacterial contamination in buffalo milk and dairy products and associated factors along the buffalo milk chain in Noakhali district, Bangladesh.

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Roll No: 0119/02

Registration No: 634

Session: 2019-2020

A thesis submitted in the partial fulfillment of the requirements for the degree of Masters in Medicine

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Acknowledgement

All praises go the Almighty Allah who enables the author to accomplish her thesis successfully. The author expresses her deepest and heartfelt gratitude to her respected supervisor Prof. Dr. Pankaj Chakraborty, Department of Medicine and Surgery, CVASU to allow her to work in the buffalo project as research assistant under udder health Bangladesh. The author also wants to show her sincere gratitude to the supervisory team members Prof. Dr. Fabrizio Ceciliani, Department of Veterinary Medicine, University of Milan, Italy, and Prof. Dr. Sofia Boqvist, Department of Biomedical Science and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden and Prof. Dr. Mizanur Rahman and Prof. Dr. Md. Ahasanul Hoque, Department of Medicine and Surgery, CVASU for Their valuable supervision and guidance during the field work, data analysis and thesis writing. The author also expresses her gratitude to Dr. Shuvo Singha (PHD fellow) for his immense support in all sorts of work during thesis. She also wants to give special thanks to Dr. Sanjib Chandra Nath and Dr. Ovirup Bhuson Paul for their help and encouragement during whole research work and Dr. Ajay Deb Nath and Dr. Mohammad Abdul Mannan for their support during field activities. The author gratefully acknowledges Dr. Ylva Persson, Buffalo Project leader, and associate state veterinarian, National Veterinary Institute, Sweden. The author would like to express her deep sense of gratitude to Udder Health Bangladesh, Advanced studies and Research (CASR), CVASU and the Buffalo project "Climate Change mitigation by a sustainable water buffalo dairy chain in Bangladesh" financed by the Swedish Government for providing necessary research funds. Finally, the author expresses her thankfulness to her parents, seniors, juniors and well-wishers.

THE AUTHOR

June 2021

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List of Abbreviations and Symbols

Abbreviation	Elaboration
%	Percent
<	Less than
=	Equal to
>	Greater than
≤	Less than equal
≥	Greater than equal
°C	Degree Celsius
95% CI	95% Confidence Interval
µL	Micro liter
BA	Blood agar
BC	Bacterial culture
BDT	Bangladesh taka
BHI	Brain Heart infusion
BM	Bulk milk
BMPCUL	Bangladesh Milk Producers' Cooperative Union Limited
BMSCC	Bulk milk somatic cell count
BMVC	Buffalo Milk Value Chain
BPA	Baird Parker agar
BRAC	Bangladesh Rural Advancement Committee
CAP	Common agricultural policy
Cfu	Colony forming unit
CM	Clinical mastitis
DCC	DeLaval cell counter
DLS	Department of Livestock Services
EDTA	Ethylenediaminetetra acetic acid
EMB	Eosin Methelene Blue
EU	European Union
GMB	Ganges Brahmaputra Meghna

GMPF	Grameen Motso O Pashusampad Foundation
GSO	Gulf Standardization Organization
ID no	Indentification number
IMI	Intramammary infection
LTL	Lal Teer Livestock Limited
MAC	MacConkey agar
MIC	Minimum Inhibitory Concentration
mL	Mililiter
MR	Methyl red
MSA	Mannitol Salt agar
NGO	Non-Governmental Organization
NMC	National Mastitis Council
NSW	North South Wales
p	Probability
rpm	Rotation per minute
SCC	Somatic cell count
SE	Staphylococcal enterotoxin
SIM	Sulphide Indole Motility
TBC	Total bacterial count
TEC	Total Enterobacteriaceae count
TNAS	Total Non aureus Staphylococcal count
TSA	Total Staphylococcal count
VIF	Variance inflation factor
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose Agar

Abstract

Milk and milk products can harbor a variety of microorganisms, which might have negative impact on human health. Raw milk can be contaminated by microorganisms originating from the udder (either clinical or subclinical mastitis), by zoonotic pathogens from the environment due to poor hygienic status of the teat and udder, unhygienic handling of milk, unclean milking equipment as well as improper cooling during transportation. These are some examples of factors which influence microbial contamination and growth before the milk product reaches to the consumer through the milk chain. The present cross-sectional study was designed to investigate the level of bacterial contamination in buffalo milk and milk products and associated factors along the buffalo milk chain in Noakhali district, Bangladesh. A total of 132 milk samples and 10 milk products from households and semi-bathans (free ranging) milk and from 4 different nodes in the household and semi-bathan milk value chains (farm, middlemen, and collection centre and milk products) were collected during April, 2021. The samples were analyzed qualitatively and quantitatively for various bacterial counts and occurrence of pathogenic micro-organism. Epidemiological analyses were also performed to investigate potential risk factors associated with high bacterial counts. Quantification of bacteria as well as isolation and identification of 5 different bacteria (Non-aureus *Staphylococcus*, *Staphylococcus aureus*, *Streptococcus spp*., *E. coli*., *Klebsiella* spp.) were done following standard bacteriological method. Pearson's correlation coefficient test was performed to identify correlations between bacterial counts while univariable analysis and multivariable regression analysis was performed to investigate risk factors associated with bulk milk somatic cell count (BMSCC) and total bacterial count (TBC). Farm BMSCC varied between 5.09 and 6.08 cells/ ml, whereas the mean BMSCC at the farm milk samples was 5.61 log10 cells/ ml. The mean value of TBC at the farms level was 5.54 log10 cfu/ ml, whereas TBC in milk samples at different nodes (middlemen, collection centres and milk products) were 5.81, 6.80 and 7.24 log10 cfu/ ml, respectively. The progressively increased level of TBC was found significant ($p < 0.001$). However, both BMSCC and TBC have exceeded the European Union (EU) legislative standard limit of milk quality. Other bacterial counts such as total Non-aureus *Staphylococcal* count (TNAS) and total *Enterobacteriaceae* count (TEC) were also found

at significant increasing level ($p<0.001$) across the milk chain except Total *Staphylococcal* count (TSA) which was statistically non-significant ($p=0.48$). The highest mean value of TSA (1.95 to 2.14 log10 cfu/ ml), TNAS (3.44 to 3.88 log10 cfu/ ml) and TEC (3.95 to 4.46 log10 cfu/ ml) were also found at the collection centre and milk product level which was also above the EU legislative standard limit of milk quality. Moreover, significant positive correlations were found between BMSCC and TNAS ($r=0.35$; $p=0.01$), TBC and TNAS($r=0.55$, $p<0.001$) at the farm level, between TBC and TEC ($r= 0.40$, $p =0.05$) at middleman level and between TBC and TNAS ($r=0.31$, $p =0.03$), TBC and TEC ($r=0.39$, $p= 0.02$) at collection centre level. Among the prevalence of different microorganisms, Non-aureus *Staphylococcus* (NAS) had the highest frequency along the nodes in the value chain (71% at farm level, 69% at the middlemen level and 71% at the collection centre) followed by *Streptococcus* spp. (55% at farm level, 51% at the middlemen and 71% at the collection centre). *S. aureus* (26%) and *E. coli*. (29%) were highly prevalent at the collection centre where as a high amount of *Klebsiella* spp. (20%) was found in milk products. The univariable analysis showed different risk factors for BMSCC: “How to sell your milk” ($p= 0.06$) and “How do you clean your container” ($p= 0.06$) and for TBC: farm zones ($p= 0.06$), source of milk ($p= 0.08$), frequency of cleaning milk container/day ($p =0.05$) and the score of milker’s hygiene ($p= 0.10$) at the farm level. However, no risk factor remained in the multivariable linear regression model. The findings of this study indicate that raw milk and milk products in the milk value chain can possess high bacterial contamination that might cause serious public health hazards especially for those communities who still consume raw or improperly treated milk and milk product. So, there is a need to implement appropriate control measures to reduce microbial contamination along the milk value chain to minimize the milk-borne diseases in humans.

Keyword: Bulk milk somatic cell count, total bacterial count, total Non-aureus *Staphylococcal* count, total *Enterobacteriaceae* count, correlation, micro-organisms, risk factor.

Chapter 1: Introduction

Animal-derived foods have gained a growing interest worldwide with increasing population, urbanization and rising income (Kliem and Givens, 2011). Dairy products are unique and complete foods containing essential micro-nutrients contributing to a healthy balanced diet. Although dairy cow milk production dominates worldwide, water buffaloes are the most significant source of non-dairy cow milk (13.2%) (Giovanni et al., 2020). The global buffalo milk production increased by 32% from 2011 to 2018, accounting for 15% of the global fresh milk production in 2018 (Minervino et al., 2020). The total milk production of Bangladesh was about 10.68 Million Metric Ton (MMT) in 2019-2020 (DLS, 2020), of which >90% came from dairy cows (Habib et al., 2017), but only 4% (0.244 metric ton) from water buffalo (DLS, 2015; Habib et al., 2017). The average daily intake of milk in Bangladesh is 175.65 ml/day/person (DLS, 2020) which is lower than the recommendation (250 ml/day/person). Although milk production has increased over the last few years in Bangladesh, it is not enough to meet the demand from the consumers.

Water buffaloes can be an additional source of milk to close the existing demand and supply gap. The water buffalo is sparsely distributed all over the country in Bangladesh and the buffalo population is increasing. The daily milk production of an indigenous water buffalo ranges from 2.70 to 3.43 litres which is comparatively higher than milk production from local indigenous cows (1.9 1itre per day) (Samad, 2020). Moreover, water buffalo produces milk with a fat content ranging from 6.0-8.5% (Habib et al., 2017). The high milk fat content obtains a better price at the milk market than cow milk (Habib et al., 2017). Noakhali district is one of the buffalo farming areas with highest buffalo population in the Chattogram division situated in the Southeastern part of Bangladesh. A large number of farmers rear buffaloes in this district and earn their income by selling milk. A survey on buffalo farming in various region, including Noakhali, showed that 58% of the farmers sold their milk through a middleman where 37% sold directly to the processor and 5% sold their milk directly at the local market (Uddin et al., 2016). Though a large number of farmers are engaged in buffalo farming in Nokakhali district, they have little technical knowledge about buffalo production, and

there is also lack of Government support and training facilities for the development of the buffalo sector.

Milk can get contaminated by various types of microorganisms along the production chain. Previous studies on the microbiological quality of buffalo milk have revealed the presence of Non-aureus *Staphylococcus* (NAS), *Streptococcus agalactiae*, coliform bacteria, pathogenic *Escherichia coli.*, *Bacillus* spp., *Listeria* spp., *Staphylococcus aureus, Campylobacter* spp, *Salmonella* spp., *Klebsiella pneumonia* and *Yersinia enterocolitica* in milk (Jayarao and Henning, 2001; Jayarao et al., 2004; Lorusso et al., 2009; Maniruzzaman et al., 2010; Oliveira et al., 2011; Rahimi et al., 2014; Nobrega et al., 2021). The main bacterial species isolated from water buffalo milk during intramammary infection (IMI) is *Staphylococci* spp. (Pisanu et al., 2019), where the NAS being the most predominant pathogens causing IMI reported from most studies (Moroni et al., 2006; Ali et al., 2011; Guha et al., 2012; Locatelli et al., 2013). In the case of bulk milk, several studies found that the frequency of *S. aureus, S. agalactiae* or *Streptococcus dysgalactiae* was significantly associated with increased levels of bulk milk somatic cell count (BMSCC) (Jayarao et al., 2004; Riekerink et al., 2006; Rysanek et al., 2009). However, milk quality and microbial load status change over time as milk moves along the milk value chain involving multiple personnel, places, and processing steps (Knight-Jones et al., 2016). *Staphylococcus aureus* is of particular importance in the milk value chain as it produces a wide range of virulence factors and antibiotic resistance patterns (Ben Said et al., 2016; Hoque et al., 2018). *Staphylococcus aureus* contamination in milk can be caused by clinical or subclinical mastitis or during unhygienic handling and processing of milk. The bacteria can also be isolated from various types of milk products such as cheese, ice cream, clotted cream, yoghurt, and butter when raw milk is inadequately heated or post-pasteurization contamination during handling, storage, or packaging. Similarly, the presence of *E.coli.* in milk indicates unhygienic milk practices at one or several steps along the milk value chain (Awadallah et al., 2016; Ntuli et al., 2016; Bauzad et al., 2019). The main sources of *E. coli* in raw milk and milk products are faecal contamination during the milking process along with poor hygienic practices (Soomro et al., 2002; Ombarak et al., 2016). *Klebsiella* spp. is another opportunistic gram-negative bacteria isolated from bovine mastitis milk reported in several studies

(Gröhn et al., 2004; Munoz et al., 2007; Gao et al., 2019). Other sources of *Klebsiella spp*. in dairy herd includes faeces, contaminated feed, water and organic bedding material (Munoz et al., 2007). It is thus evident that sale of milk to consumers without maintaining proper hygienic and storage practices, and quality checks can play a critical role in food safety and from public health aspects.

Various tests like bulk milk somatic cell count (BMSCC) can quickly determine the quality of milk, together with total bacteria count, and identification and measuring of specific pathogens, including *Staphylococci* spp. or Enterobacteriaceae. Measuring the BMSCC could provide a strategic indicator that also facilitates monitoring udder health and milk quality (Zecconi et al., 2019). The somatic cells are mainly composed of leucocytes (75%), i.e. neutrophils, macrophages, lymphocytes, erythrocytes and epithelial cells (25%) (Patil et al., 2015). An elevated BMSCC has a negative impact on milk quality and the manufacturing properties of milk and milk products. Somatic cell count (SCC) in milk rises, especially as a second-line defence in response to IMI. Therefore, SCC in milk has been assumed to be the most reliable parameter to determine milk quality and subclinical mastitis (SCM) (Sahin et al., 2017). According to the National Mastitis Council, healthy dairy cows usually have SCC 100,000 cells/ ml, where subclinical mastitis affected cows have SCC ≥200,000 cells/ ml (NMC, 2017). However, there are currently no specific limits provided for water buffalo raw milk. Several other factors influence the milk somatic cell count at individual and herd levels apart from an IMI, including for example number of days in milking, age, breed, parity, season, milk transportation, and stress (Sharma et al., 2011). The SCC is also positively correlated with the total bacterial counts of milk (Jayarao et al., 2004; Koop et al., 2009) and pathogens involved in IMI, as reported in several studies (Jayarao et al., 2004; Sampimon et al., 2010; Condas et al., 2017).

Along with BMSCC, several bacterial counts are also measured in raw milk to measure the hygienic status of the milk during and after production (Pyz-Łukasik et al., 2015). These tests include total bacterial count (TBC), total Enterobacteriaceae count (TEC), total *Staphylococcal* count (TSA) and other pathogen counts. TBC is one of the accepted criteria to define the milk quality and for grading of milk to ensure safe consumption and processing of dairy products (Mhone et al., 2011). TBC is commonly used as an indicator

to evaluate the hygiene of the entire production process (Pasquini et al., 2018). It assesses the bacterial density and estimates the number of bacteria present per millilitre of milk (Cicconi-Hogan et al., 2013). A high bulk tank TBC can also result from bacteria from dirty milking equipment, milk from cows with subclinical or clinical mastitis, or contamination from dirty udders (Murphy and Boor, 2000). The TBC of raw milk can range from less than 10^3 cfu/ ml to more than 10^6 cfu/ ml. In the United States, the legal maximum limit based on the Pasteurized Milk Ordinance is $<10^5$cfu/ ml, which is also following the European Union standards (Cicconi-Hogan et al., 2013; Pyz-Łukasik et al., 2015) although most industry standards require a count $<5\times10^4$ cfu/ ml. A count less than 5×10^3 cfu/ ml indicates proper sanitation and cooling, whereas a value of more than 5×10^5 cfu/ ml is evidence of serious defects in production hygiene (Elmoslemany et al., 2010). The elevated count of TBC in milk has a decisive effect on the quality and safety of dairy products (Szteyn et al., 2005). Similarly, the member of the *Enterobacteriaceae* family and *Staphylococcus* spp. can enter an inadequately handled dairy chain and cause an enzymatic breakdown of proteins or lipids, instigating spoilage contributing to substantial economic losses and waste (Scherrer et al., 2004; Baylis et al., 2011). According to the New South Wales (NSW) food authority, a total *Enterobacteriaceae* count $<10^2$ cfu/ml in ready to eat food is considered as good, whereas 10^2 to $< 10^4$ cfu/ ml is considered an acceptable limit and $\geq10^4$ cfu/ml is regarded as unsatisfactory (NSW). The EU microbiological regulation considers pasteurized milk is satisfactory at TEC of 0 cfu/ g, but it is <3 cfu/ g in GSO (Gulf Standardization Organization) standards (El-Ziney, 2018). Similarly, *S. aureus* count in between 10^6 –10^8 cfu/ ml levels are considered for human food poisoning (Kérouanton et al., 2007; Mhone et al., 2011). Consequently, proper hygienic practices and identifying risk factors associated with elevated bacterial count and microbial contamination are necessary to maintain a good quality of milk along the milk value chain.

Various risk factors have been shown to be associated with bacterial contamination and high bacterial count of milk along the milk value chain such as milkman and udder hygiene, cleanliness of milking equipment, use of the plastic container, transportation, cooling and storage of milk (Chye et al., 2004; Mhone et al., 2011; Ntuli et al., 2016). In the raw milk value chain, milk can be contaminated at any point in the milk value chain

from milk producers to consumers (Lubote et al., 2014), and the level of contamination reflects the general hygienic status of the milk production system. Intramammary infection present in a single udder in the herd can also contaminate the whole bulk milk and introduce unwanted microorganisms into the value chain. Nevertheless, the prevalence of these microorganisms in bulk milk (BM) varies considerably among surveys. They could be influenced by several factors such as geographical area, season, farm size, number of animals on the farm, hygiene, farm management practices and other factors (Jayarao and Henning, 2001). Silage, bedding, soil and manure may also act as sources of contamination of raw milk from the dairy farm environment (Murphy et al., 2019). Unhygienic practices such as milking with dirty hands and using dirty clothes during milking can be potential risk factors for high TBC and Enterobacteriaceae in milk (Rahamtalla et al., 2006; Bereda et al., 2018). Transportation and storage of milk without refrigeration increases the microbial load and decrease the quality of milk (Chye et al., 2004; Muhammad et al., 2009; Manzoor et al., 2012). Milk residue remaining after cleaning of utensils used for milking and storage can also favor microorganism growth (Vissers and Driehuis, 2009). Subsequently, the mode of transportation, the time interval between production and (unhygienic) transportation of milk to the collection centre, and storage temperature of the milk during transport was found positively associated with a TBC and other higher counts of milk (Mhone et al., 2011; Manzoor et al., 2012; Muloi et al., 2018; Paraffin et al., 2018).

With increasing consumption of buffalo milk and milk products, it is needed to reduce milk contamination along the milk value chain and in the final products. To maintain an acceptable level of food safety and minimize contamination of milk and dairy products, milking must be carried out hygienically in a clean environment, ensuring proper udder and teat hygiene, clean and uncontaminated milking equipment, proper storing temperature and transportation through the cool chain. It is also needed to know the actual quality of milk and the presence or absence of microbes in milk and milk products. To the author knowledge, there has been no study reporting findings on bacterial contamination and associated risk along the milk value chain in raw buffalo milk and dairy products in Bangladesh. The present study therefore aimed to assess the various

practices and bacterial contamination in buffalo milk and milk products and to fulfill the following objectives:

1.1. Objectives

i. To identify the occurrence of different types of bacteria at various nodes of the water buffalo milk value chain.
ii. To identify the correlation between the bulk milk somatic cell count (BMSCC) and total bacterial count (TBC) at the production level of the milk value chain.
iii. To assess the level of contamination at various nodes of the water buffalo milk value chain.
iv. To assess the potential risk factor associated with milk contamination along the buffalo milk value chain.

1.2. Outcomes

i. Determine the level of bacterial contamination at various nodes of the milk value chain which will help to identify the leading point of microbial contamination.
ii. Identification of different types of bacteria and the potential risk associated with contamination will help to take proper intervention against that risk factor to minimize the contamination level.
iii. The research findings will help to get an overview of contamination level of informal buffalo milk value chain in the rural areas of Bangladesh.

Chapter 2: Review of Literature

This chapter aims to review the relevant literature that has been published so far to describe the:

- Global features of milk value chain,
- Milk value chain in Bangladesh, with particular focus on the Noakhali district and other buffalo pocket area of Bangladesh,
- Buffalo farming status in Noakhali district including risk factors associated with the microbial contamination along the milk value chain, buffalo milk quality, the public health impact of *Staphylococcus aureus* and the prevention of contamination along the milk value chain.

This review will help to identify the knowledge gaps to justify the present study.

2.1. Global features of the milk value chain

The term value chain comprises of 2 concepts: value and chain where the value refers to the incremental value of a product after processing and the chain refers to the supply chain through which a product reaches to its consumer. Thus the term value chain analysis combinedly refers to the production, processing and marketing of a product (Haq, 2012). Several authors define the concept of value chain differently. According to Kaplinsky and Morris (2001), the value chain is defined as the entire chain of production to consumption activities of a product, whereas Birachi et al. (2006) defined the value chain as supply chain consisting of activities and processes including production, processing, trading and consumption. From a production stage, a product goes through various stages of processing through a supply chain. If proper hygienic measures are not taken in every level of the value chain, severe public health hazard can occur.

The value chain differs from country to country or region to region around the world. Table 2.1 shows examples of value chains from different countries on different continents.

Table 2.1: Features of dairy value chain around the world

Country	Features of dairy value chain around the world	References
Dairy value chains in Vietnam	• It is composed of five primary activities such as ingredients, production, processing, marketing and consumptions. • Farmers gain low profit due to monopoly pricing behaviour where large companies generate high profitability. • There is a potential controversy on quality assessment.	Khoi (2013)
	• The formal milk distribution channel starts from dairy farms to milk collector, dairy plants, to wholesalers/retailers and finally to the consumers. • The dairy farmers play the most critical role in the production chain. • But the processors are powerful actors in the value chain and control the whole chain unofficially.	Nga (2017)
Dairy value chain in the US	• It includes four activities: input, production, processing and distribution and marketing. • Inputs refer to the main products and services which needs to run the operation in dairy farms by a farmer. • Production includes calving, cattle raising, milking and pasteurizing of milk in the dairy farm. • The marketing co-operatives are in charge	(Lowe and Gereffi, 2009)

	of the distribution of milk from producer to processors. From there the product goes to the supermarkets and restaurants for selling.	
Dairy value chain in Horogoduruwollega zone, Ethiopia	• Both formal and traditional milk value chains are found in Ethiopia, where the informal (traditional) value chain has remained dominant. • The study revealed that 18% of the respondent produced milk only for home consumption purpose. • 1% of the producers only sold their dairy products to local consumer, and the remaining sold their dairy product to hotels. • The traditional dairy products mostly traditional soured butter which dominate the Ethiopian dairy sector.	(Beyene et al., 2015)
Dairy value chains in Pakistan.	In Punjab, • Two types of milk supply chain were presented. • The informal milk supply chain where traditional collectors, contractors and suppliers supplied the milk to collection centres for processing. • In case of formal milk value chain, cooperative companies played the key roles. • The informal or traditional dairy value chains had high market share due to strong consumer preferences and low prices of	(Shah et al., 2008)

	fresh milk.	
	In peri-urban areas of Pakistan, • A study revealed that milk producers sold their milk mostly to intermediaries (middle men) or nearby neighbours. • Further intermediaries sell the milk to the hotels and sweet shops etc. from where milk and milk by-products are directly supplied to the consumers. • Sometimes adulteration through adding water or using chemicals was done to increase the milk quantity and/or making milk thicker.	(Shah et al., 2015)
Dairy value chain in Bihar, India	• A study revealed that about 21% of marketed milk was sold directly to the consumers. • Despite the presence of modern milk supply chain, the traditional milk marketing supply chain plays a dominant role. • On average, 72% of the farmers market their milk through the traditional milk supply chains and milk marketing agent purchased 60% of the marketed milk.	Kumar (2010)
Dairy value chain in EU	• In 2008, the EU's CAP (Common Agricultural Policy) reform the governance of the EU's dairy sector. • The quota system was introduced in 2003, providing the incentive for production increases. The abolition of the quota	(Krol et al., 2010; Borawski et al., 2019)

	system in 2014 eliminated the support prices for butter and milk powder. • After the abolition of the quota system in 2014, EU prepared new incentives for the development of milk production. • The small dairy farms primarily invest in increasing production and decreasing production costs. • Three stages of the supply chain were found, namely production, wholesale supply and consumption. • Milk either collected via directly from the farm or delivered to a collection centre. • A study revealed that more than 90% of all supplied milk in the Netherlands is processed by cooperatives where in France it was 45%. • In the Netherlands, milk prices paid out to farmers currently are determined by the international market for dairy products and adjusted on a monthly base where in France and Bulgaria milk supply were regulated by long-term contracts.	

Given this background, it is clear that dairy value chains around the world differs from each other depending on economic condition of the people, politics, agricultural development and investments, structures of the farming system and other facilities. However, it is crucial to manage the dairy value chain carefully to maintain high quality standards of the milk and milk products. The lack of effective quality control at various stages of milk production and transportation, lack of cooling facilities and

underdeveloped processing and marketing system can reduce the shelf-life of milk. It also impairs the quality of the milk thereby making poor quality milk products and create public health hazards if potential pathogenic micro-organsims multiply.

2.2. Milk value chain in Bangladesh

The dairy sector of Bangladesh is characterized by various small or medium-sized, scarcely organized and widely dispersed dairy farms. Since a few decades, a lot of improvements have been made in the dairy sector of Bangladesh but still there exists a considerable gap between demand and supply of milk and milk product. In 2018, the supply was only 158.19 ml/day/person against WHO recommendation of 250 ml/day/person with a deficiency of 5.623 million metric ton milk (DLS, 2017-2018). Out of total milk production, 90% of total milk comes from cattle, 6-7% from goats and the remaining 3-4% from buffaloes (Hamid and Hossain, 2014).

Buffaloes are kept in several parts of Bangladesh. The density of buffalo farms is highest in the coastal region of Bangladesh and about 40% of the total buffalo production in Bangladesh is done in this region. Ganges Brahmaputra Meghna (GBM) river system and the Bay of Bengal mostly dominate the Coastal zone of Bangladesh. Natural phenomena such as cyclones, storm surge and rises of the sea level occur regularly in this area with severe negative impacts on the low lying lands (Ahmad, 2019). The coastal area of Bangladesh mainly consists of 19 districts named Patuakhali, Bhola, Lakshmipur, Noakhali, Khulna, Bagerhat, Pirozpur, Jhalakati, Barguna, Jessore, Narail, Gopalganj, Shariatpur, Chandpur, Satkhira, Barisal, Feni, Chittagong, and Cox's Bazar. Among them, the Meghna-anga and Jamuna-Brahamaputra flood plain mainly form the buffalo pocket area of Bangladesh where a large number of buffaloes are reared. In addition, the Sylhet haor (large water body) area and the sugar cane belt of Jamalpur and Kanihari in Trishalupazila of Mymensingh district are also considered as buffalo pocket areas (Sohel and Amin, 2015).

The milk value chains in Bangladesh are not well organized. It is somewhat similar to the Pakistan and Indian dairy value chains. Both traditional or informal milk value chain and formal milk value chain coexist in Bangladesh where the largest share of milk (97%) and milk product marketing occurs through the informal milk value chain. In the case of the

informal milk value chain, farmers directly sell their milk to the consumers or neighbours (Figure 1). In contrast, in the formal milk value chain, farmers mainly sell their milk by contract basis to the co-operatives or middleman or to the nearby market. Mainly three types of production system such as household, semi-intensive and bathan /extensive system are seen here. Among the milk production system, the bathan or extensive production system is mainly controlled by the co-operatives companies. In case of intensive production system, 85% of the total milk is sold by the farmer, and the remaining 15% is used for family consumption (Uddin et al., 2011). Though the formal milk value chain is important as it provides quality milk and market assurance, the informal value chain plays a vital role in employment generation, family nutrition and provides a buffer to trade competition and market access in areas with poor infrastructure (Uddin et al., 2011).

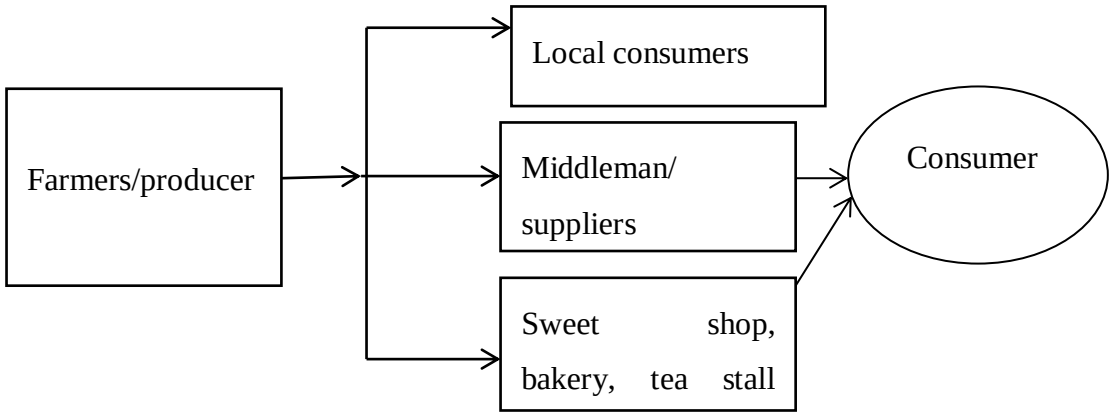


Figure 2.1: Traditional or informal milk marketing model, Source: (Uddin et al., 2011)

The traditional milk marketing system is not uniform in all districts or regions and varies depending on demand, quantity of milk production, processing and market stability. This is exemplified in an analysis of the milk production value chain in Chattogram, Sylhet and Panchagar districts of Bangladesh in which seven different marketing channels were identified (Islam et al., 2016). In that study, the milk from producer to consumer through middlemen was the dominating (39%) channel, followed by sweet sellers (28%), selling of milk directly from farmers to consumers (26%), tea sellers (5%) and milk processors (2%) (Islam et al., 2016).

Another marketing system is the cooperative marketing system. This is an organized marketing system which was developed to help the poor landless and marginal dairy farmers by including them in an infrastructure of milk marketing channels to ensure the quality of milk and milk product and to allow the farmers to obtain fair prices of milk (Ghosh and Maharjan, 2002; Jabbar, 2010). Bangladesh Milk Producers' Cooperative Union Limited (BMPCUL) known as Milk Vita, Bangladesh Rural Advancement Committee (BRAC), Lal Teer Livestock Limited (LTL), Pran Dairy, Amo milk, Bikrampur dairy, Aftab dairy, Akij dairy, Bikrompur dairy, Rangpur dairy, Tulip dairy, Gentech International, EJAB and Grameen Motso O Pashusampad Foundation (GMPF) are well-known dairy cooperatives of Bangladesh (Hamid and Hossain, 2014). Among them, Milk Vita is the pioneer model of formal or co-operative dairy development in Bangladesh. The enterprise (established in 1973) aims to develop the dairy sector of Bangladesh as well as improve the conditions of the rural farmers by paying fair milk prices to the farmers. At present, there are three milk pasteurization plants, eight other production plants and 32 milk chilling plant are being run by BMPCUL in different regions (Milkvita, 2019). The cooperative companies also provides support to improve feeding, breeding, management, veterinary service, AI etc. In the case of Milk Vita, collection of milk is jointly done by the individual producer at the milk collection centre from where collected milk is delivered to the nearby local processing centre for chilling and pasteurization (Figure 2). In the next step, the milk goes to the milk factory in order to process into milk product such as cheese, ice cream, butter and homogenized fresh milk sold in small plastic bags. These milk and milk products are sold to the consumers in the market at a fixed price. As there are no intermediaries involved in this channel, primary producer gets a proper price depending on the fat content in the milk.

BRAC (Building Resources Across Communities) is an another NGO based cooperative company involved in dairy processing and marketing which is known as “Aarang” dairy. Aarang dairy collects milk from various milk-producing areas through their intermediaries or local traders. They have a dairy processing plant, about 70 chilling plants and many collection centres (Jabbar, 2010). They collect milk directly from the supplying milk intermediaries, mainly traditional milk traders and from dairy farmers. Like Milk Vita and BRAC, the other cooperative companies and NGOs, follow the same

procedures of collecting milk from the farmers through their intermediaries. About 15% of the national production of cow milk is currently being procured by Milk Vita, BRAC dairy and PRAN dairy for processing to serve large urban markets (Hamid and Hossain, 2014).

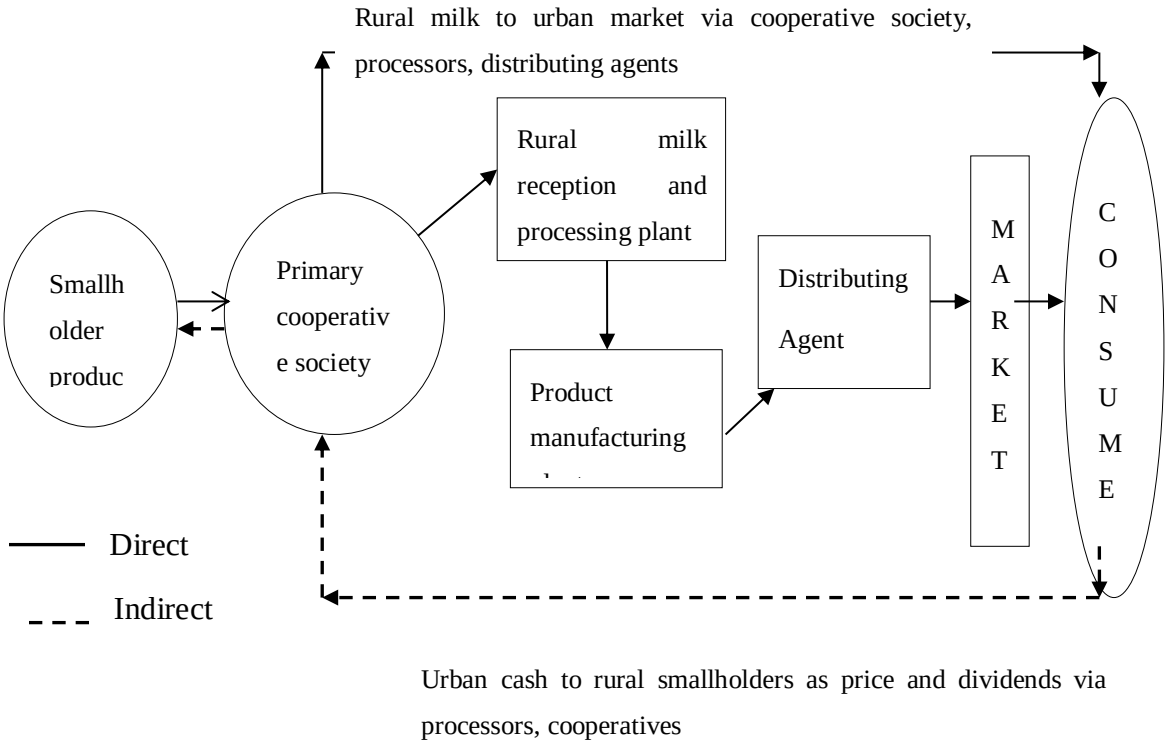


Figure 2.2: The cooperative milk marketing channel (Milk vita) (Uddin et al., 2011)

2.3. Buffalo farming in Noakhali district

Noakhali district is located in the south-eastern part of Bangladesh under Chittagong division with an area of 3685. 87km². It bounds by the Kumilla district on the north, Feni and Chittagong district on the east, Bhola and Lakshmipur on the west and Bay of Bangal on the south.

Figure 2.3: Map of Noakhali district along with Subarnachar upazilla

Subarnachar is an upazila (sub-district) of the Noakhali district which is divided into eight union parisads. The average literacy rate of Subarnachar upazila is around 33%, where 71% of people maintain their livelihood through agriculture. About 68 % of the buffalo farmers are also engaged with agriculture besides farming (Amin et al., 2015). Among the buffalo farmers, the percentage of middle-aged farmer (36-50 years) is higher compared to young and old aged, and the majority of the farmers have only primary level of education (Amin et al., 2015; Rahim et al., 2018).

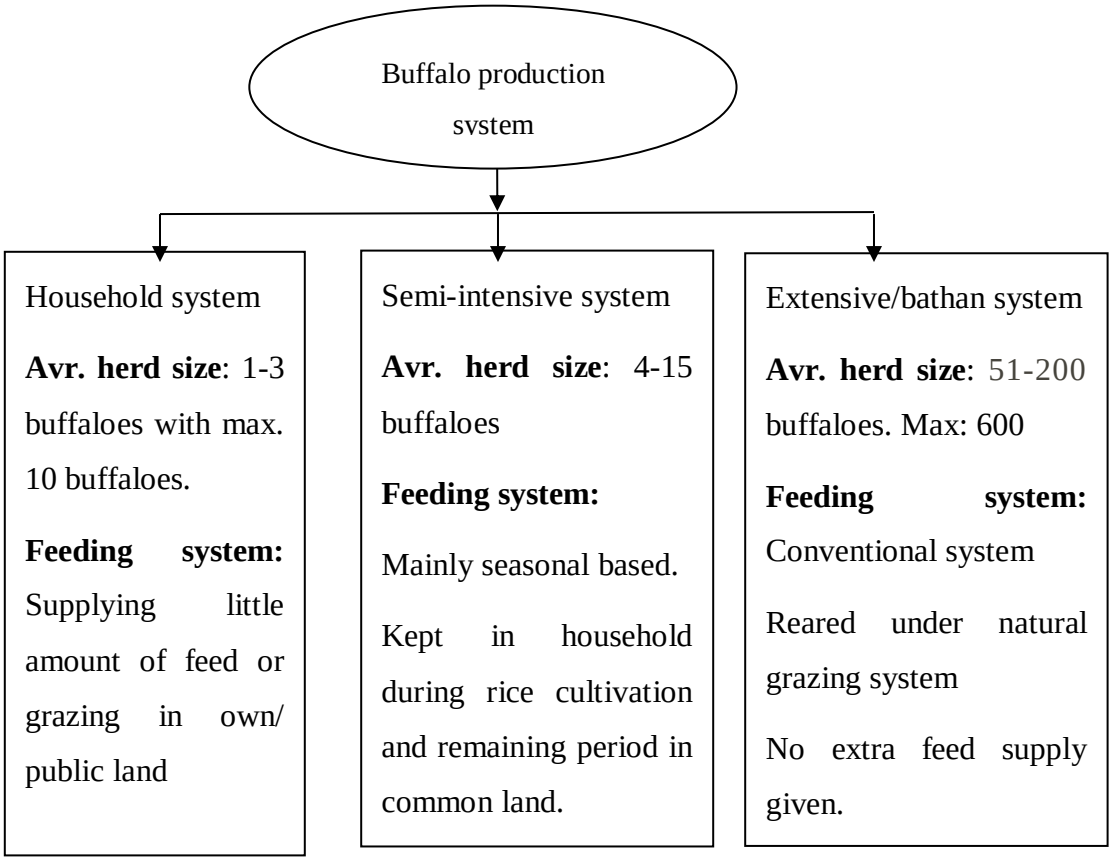


Figure 2.4: Buffalo production systems in Bangladesh

In the case of Noakhali, the buffalo production system is mainly bathan based (80%) where buffaloes are kept in the open field with no housing facilities. A small percentage of farmers practise household and semi-intensive system of production. However, in all rearing systems, people use public land for grazing because only a few farmers own grazing land. The nutritional status of the buffalo is poor with no concentrate feeding, low-quality grass, no vitamin and mineral supplement. Locally available rice straw, pasture grasses and uri-grass were the primary sources of feeds for buffaloes in selected areas. Buffalo farmers do not use concentrate to feed to their buffaloes. The farmers are not familiar with the concept of the balanced ration and this is the main reason behind the low milk production of buffalo in this area. The average milk yield recorded varies from 2.7-3 litre, with an average fat content of 8.2% (Amin et al., 2015; Uddin et al., 2016). Only a few farmers are aware of the importance of deworming and vaccinating their buffaloes. They also have little knowledge about animal reproduction (including artificial

insemination). In both households and bathans, natural breeding is most commonly used. Though a large number of farmers are engaged in buffalo farming in Nokakhali district, they have little technical knowledge about buffalo production, and there is lack of Government support and training facilities for the development of the buffalo sector.

2.4. Buffalo milk value chain in Noakhali

The popularity of buffalo milk is increasing with the rising gap between demand and supply of milk and confectionary items (Uddin et al., 2016). Among the coastal area of Bangladesh, Noakhali is a large buffalo concentrated area of Chittagong division where most of the farmers maintain their livelihood through selling of buffalo milk and milk product. Informal milk value chain is mainly followed here where the farmers sell their milk directly to nearby markets, collection centres or sweet shops or indirectly through the middlemen. At collection centres or sweet shops, milk is transported from different area and then mixed with previously retained milk. The mixed milk is directly sold as milk or by preparing milk products like curd, milk drink and sweet to the consumers. For milk produced on islands, milk from buffaloes of several bathans is carried out in each killas (many bathan forms a killa) and then milk from several killas are mixed together. Then the mixed milk from several islands is transported to the main island by water vehicles (mainly boat) and sold to the nearby market/ collection ccentre/ sweet shop or to middlemen.

Few studies report on buffalo milk value chain of Noakhali district. A survey on bathan farming in Bhola, Noakhali, Lakshmipur and Patuakhali showed that 58% of the farmers sold their milk through a middleman where 37% sold directly to the processor and 5% sold their milk directly at the local market (Uddin et al., 2016). To the authors' knowledge, no study has been done about the quality control of milk between production and consumption. As bathan farming is mostly done in remote areas long transoportation time are often required to reach the collection centres or nearby markerts for selling. Thus, the long transportation time without any cooling facilitiies may lead to spoiling the quality of milk.

In case of other buffalo pocket areas, the milk marketing approaches differ according to the agro-climatic conditions. A study on milk marketing approaches of dairy buffalo in

different agro-climatic districts (Bhola, Mymensingh and Dinazpur) of Bangladesh reported that the highest share of milk trading (92%) was found in the coastal region (Bhola) and the second-highest in the river basin region (Mymensingh) (Rahman et al., 2019). In the coastal area, farmers prefer to sell their milk through middlemen where in case of river basin and semiarid area, farmer sell their milk to the local market or sweet shop by themselves (Rahman et al., 2019). None of these studies described any formal value chain.

2.5. Bacterial contamination of buffalo milk and risk factors in buffalo milk value chain

2.5.1. Contamination from udder infection

Bacterial contamination of milk due to udder infection is commonly seen in dairy herds. A single buffalo suffering can thereby contaminate the bulk milk. The bacterial contamination in the milk can increase throughout the milk value chain if proper cooling facilities and hygiene measures are not maintained, thereby enabling multiplication of bacteria. Milk from a healthy individual can contain a wide range of bacterial species (the microbiota) including potential pathogens (Catozzi et al., 2017). Any microbial imbalance in this microbiota can induce the onset of mastitis. Mastitis organisms enter the teat canal, causes inflammation of the udder and thus reduces the milk yield and the milk quality as well as induce animal sufferings (de Medeiros et al., 2011; Cicconi-Hogan et al., 2013) . During milking, high concentrations of the infectious organisms can be transmitted to milk (Vissers and Driehuis, 2009). Among the organisms that can cause mastitis, non-aureus staphylococcus (NAS) was the most frequently isolated group of pathogens found during mastitis in both cows and buffalo (Pitkälä et al., 2004; Moroni et al., 2006; Tenhagen et al., 2006; Dhakal et al., 2007; Chavoshi and Husaini, 2012; Tremblay et al., 2013). Other pathogenic bacteria that are related to mastitis are *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, disgalactiae, Coliforms (E. coli, Klebsiella, Enterobacter), Pseudomonas, Prototheca,* yeasts and *Escherichia coli* etc. (Fagiolo and Lai, 2007). However, a study on subclinical mastitis in dairy cows found that *S. aureus*, *Strep. uberis* or *Strep. dysgalactiae* was higher in chronically infected cows than the newly infected ones (Persson et al., 2011). Although

clinical mastitis can be easily diagnosed by observing clinical signs, it is difficult to diagnose subclinical mastitis. Using the bulk milk somatic cell count (BMSCC) is one way to detect subclinical mastitis at herd level. Apart from mastitis-causing organisms, other bacteria that are pathogenic to human may also be present in the milk, for example *Mycobacterium* spp. (Leghari et al., 2016), *Brucella abortus, (Capparelli et al., 2009), Coxiella burnetii (Kim et al., 2005)* and *Salmonella* spp. *(Ogilvie, 1986)*. A three year study on *Coxiella burnetii* in bulk milk samples in U.S. dairy herds found that more than the 90% milk samples were contaminated with the bacteria (Kim et al., 2005). In another study, using ELISA, *Mycobacterium avium* subsp. paratuberculosis was detected in 8.3% of investigated raw buffalo's milk samples (Hafiz et al., 2016).

2.5.2. Contamination from the external surface of the udder

The external surface of the udder is a great source of microbial contamination of milk. Contaminants such as dirt, dung, mud, soil can readily stick to the udder surface and contaminate the milk during milking. Udder and teat can be soiled when they come in contact with the dirty stalls and muddy barnyards. Similarly, the udder, if not dried properly after washing,wiped using contaminated or single cloth or contaminated during urination and defecation, can also contaminate the milk during milking (Islam et al., 2018; Regasa et al., 2019). The washing procedures and the extent of soiling on the teat and udder surface directly influence the total bacterial count of milk. Milking of heavily soiled animals may result in bulk tank milk counts of more than 10^4 bacteria per ml of milk (Afzal et al., 2011). Similarly, microorganisms attached to the exterior surface of the teats can enter the teat canal and cause mastitis (Makovec and Ruegg, 2003). Consequently, the application of pre milking and post milking teat disinfection is critical to reduce bacterial milk contamination, thereby lowering the bacterial count of milk (Kelly et al., 2009; Piepers et al., 2014; Regasa et al., 2019). Along with the udder and teat hygiene, the hygienic condition of the hind limb of the animal is also a risk factor for milk contamination. A study on the effect of management practices in mastitis of Nili Ravi buffalo found a significant ($p<0.05$) effects between the type of farm and washing of the udder, and washing of the udder and milking methods (Ali et al., 2014). On the other hand, the udder and teat morphometric traits are also related to the mastitis in animal (Okano et al., 2015). For instance, a study on risk factor associated with sub-

clinical mastitis in buffalo revealed a significant association between mastitis and teat conformation (cylindrical and round teats than pointed), pendulous udder and dirty hind leg (Kaur et al., 2018). Therefore, a suitable teat morphology is important to reduce the risk of pathogenic organisms invading the udder (Kaur et al., 2018). Similarly, it is necessary to maintain good udder hygiene practices during milking to prevent milk contamination.

2.5.3. Environmental sources of contamination

Safety of milk and dairy product starts at the farm level, which continues throughout the milk chain during processing continuum (Oliver and Murinda, 2011). The environmental contamination of milk and milk product is one of the leading causes of food spoilage worldwide. Environmental pathogens are a natural part of the farm environment, and they can be introduced into the milk by various means. Silage, bedding, soil, manure can be sources of bacterial contamination of raw milk (Murphy et al., 2019). Poor silage, grass, surface water and sewage are the main source of presence of *L. monocytogens* in raw milk (Hassan et al., 2001). Other bacterial pathogens such as *Salmonella spp., E. coli., Campylobacter* spp. can also be present in milk from dairy farm environments (Nam et al., 2004; Cerva et al., 2014). The hygienic status of the milking environment directly affect the bacteriological quality and safety of milk and milk products (Griffiths, 2010).

2.5.3.1. Personnel

The hygiene status of the milkman is a crucial factor for milk contamination. Unhygienic practices such as milking with dirty hands, use of dirty clothes during milking can be potential risk factors for high levels of total bacterial counts in milk (Rahamtalla et al., 2006; Bereda et al., 2018). The presence of coliforms in milk reflects poor hygienic practices while the presence of *E. coli*. reflect faecal contamination of milk and milk product (Chye et al., 2004; Altalhi and Hassan, 2009; Mhone et al., 2011). It is also important how the milkmen wash their hands and it has been shown that 29% of included milk samples had higher aerobic plate count where the milkmen washed their hands with only water in comparison to the milkmen who washed their hands with soap and water (15%) (Islam et al., 2018). A study along the milk value chain reported that about 32% of the *S. aureus* isolates were coming from the milkman's hand (Ayele et al., 2017).

Another study showed a significant correlation between personal hygiene and sanitation with *E. coli* contamination in cow milk (Perwira et al., 2019). The educational status of the milkman may play a role as it has been shown that the incidence of mastitis decreased with higher level of education of the milkman (Suresh et al., 2017; Islam et al., 2018). Another study on postharvest loss of milk found that the most critical risk factor for possible milk contamination is the herdsmen who both milk and handle the animal at the same time (Odongo et al., 2016).

2.5.3.2. Aerial contamination

Aerial contamination is an insignificant microbial contamination of raw milk (Griffiths, 2010). Bacteria present in the air can contaminate milk, so the farm environment of the animal and the milking parlour should be cleaned enough to prevent the milk contamination. The air of the farm premises can contain a large number of bacteria (Zhao et al., 2014). A study on the occurrence of *Salmonella* in dairy cattle and in the environments found that the air of the milking parlour can be a potential source of *Salmonella* spp. The numbers of *Salmonella* in the air was higher during winter and spring (60-62%) than during summer (25%) which may be due to the use of a fan during summer increased air velocity (Pangloli et al., 2008). Therefore, the farm environment should be cleaned enough, and the ventilation should be appropriate to reduce the aerial contamination of milk (Chege and Ndungu, 2016).

2.5.3.3. Water

Water can be a source of microbial contamination of milk and dairy products when it is used during cleaning of the milk container and during handling and processing of milk (Amenu et al., 2014; Amenu et al., 2016). Contaminated water can carry several species of microorganisms, including coliforms, *Pseudomonas*, *Bacillus* spp. and other types of bacteria (Griffiths, 2010). When contaminated water is used to rinse and wash dairy equipment bacteria may adhere to the surfaces. Thus, seemingly clean equipment can accumulate large numbers of bacteria. The water mostly used by the dairy farmers has been long time stored. In most cases, the water source remains open so that the external dirt containing feed, faecal material can pollute the water, thereby contributing to microbial contamination of milk (Mhone et al., 2011). When these types of unclean

container are used in milk production, transport and storage of milk, the level of contamination increases at every point of the milk value chain as milk transfers from one container to another until it reaches the consumers (Mhone et al., 2011). A study on the relationship between bulk tank milk quality and wash water quality on dairy farms indicated that the presence of *E. coli* in equipment wash water samples was significantly associated with elevated bacteria counts in raw milk sample (Perkins et al., 2009). Another similar study revealed that the water used for washing milk equipment in farm contained different types of bacteria such as *E. coli, Bacillus spp., Pseudomonas spp., Citrobacter spp., Enterobacter spp., Klebsiella spp., Micrococcus spp., S. aureus, S. epidermidis, Streptococcus spp*. which could create potential food safety and health risks (Amenu et al., 2016). So, it is necessary to clean the dairy equipment with clean and uncontaminated water storage to decrease the contamination level of milk along the value chain.

2.5.3.4. Transportation

Milk is a highly perishable product which is vulnerable to various environmental conditions along the milk value chain. Transportation and storage of milk at ambient temperature for extended periods can lead to multiplication of pathogenic bacteria as well as increase the microbial load and decrease the quality of milk due to multiplication of spoilage bacteria (Chye et al., 2004; Muhammad et al., 2009; Manzoor et al., 2012). In the informal milk value chain, milk transportation occurs most often without any cooling facilities. The small scale producers also do not maintain any cooling facilities after milking which may be due to considerable investment cost. The unprocessed and unchilled raw milk has a very short shelf life and usually gets sour within 4 to 6 hours due to bacterial growth (Afzal et al., 2011). Subsequently, the mode of transportation, the time interval between the production and transportation of milk to the collection centre and the storage temperature of the milk during transport were found positively associated with total bacterial count of milk (Mhone et al., 2011; Manzoor et al., 2012; Muloi et al., 2018; Paraffin et al., 2018). Extended time of transportation of the milk to the collection centre and long holding time at ambient temperature or lack of cooling facilities during transportation favors the exponential growth of previously introduced microorganisms and further microbial contamination of milk along the milk value chain (Beyene, 2015;

Islam et al., 2018; Muloi et al., 2018). Studies on risk factor associated with milk contamination and *S. aureus* findings revealed that samples from the dairy farmers that had more than 30 min travel time to the collection centres had a 5. 6 times higher risk of contamination with *S. aureus* compared to farmers that had <30 min of travel time to the collection centres (Tigabu et al., 2015). Another perception study reported that the small-scale farmers were 4 times more likely to consider that the transportation is a main contributor to poor milk quality compared to the commercial farmers (Paraffin et al., 2018). Ideally, milk should be cooled below 4℃ to ensure safe and high quality of milk to prevent further microbial growth (Owusu-Kwarteng et al., 2020).

2.5.4. Contamination of equipment used for milking and storage of milk

Milking equipment can be contaminated during milking and storage of milk. Microorganism can multiply and form biofilm to the surface of the milking equipment that may make it hard to get rid of the bacteria (Latorre et al., 2010). Similarly, after each cleaning, milk residue can remain in the milking and storage equipment which can favour growth of microorganism as milk serves as an ideal medium for microbial growth (Vissers and Driehuis, 2009). Several studies have identified presence of *S. aureus* from surfaces of milking equipment (Zadoks et al., 2002; Ayele et al., 2017; Regasa et al., 2019). In a study on risk factor of milk contamination, it was found that the plastic bucket used for milking/ milk transport/ storage can be a risk factor. Plastic containers are cheap, easy to carry (Regasa et al., 2019) and allow multiplication of bacteria on milk bucket. Due to their porous nature, it becomes challenging to remove milk residues. Another study from Ghana found that the use of plastic milk containers was a potential risk factor associated with coliform bacteria contamination of milk (Donkor et al., 2007). When milk containers are not adequately cleaned, coliform bacteria can rapidly build up in moist milky residues. The method of cleaning milk container is also an important factor. Sometimes cleaning with only water cannot remove all bacteria properly. In this case, the use of detergent and good quality water can decrease the level of contamination (Chye et al., 2004). A risk factor assessment on milk contamination revealed that cleaning milk container using cold water increased risk of contamination with *S. aureus* three times compared with using cold water with detergent, whereas using detergent with hot water lowered the risk by 30% than using detergent and cold water (Tigabu et al., 2015).

2.6. Quality of buffalo milk

2.6.1. Nutritional composition

Buffalo milk is the 2nd most widely available milk source around the world after cow milk (Li et al., 2008; Guo and Hendricks, 2010; Han et al., 2012). The composition of buffalo milk, it's nutritional importance and bioactive properties have received much attention (Ahmad et al., 2013). Buffalo milk is rich in protein, fat, lactose, minerals such as calcium, phosphorous and vitamins A and C but has a lower levels of vitamin E, riboflavin and cholesterol compared to cows' milk (Ahmad et al., 2008; El-Salam and El-Shibiny, 2011; Han et al., 2012; Ahmad et al., 2013; Kapadiya et al., 2016; Pisanu et al., 2019). However, the nutritional composition of buffalo milk is affected by several factors such as region, climate, animal genotype, feeding practices, breeds and lactation stage etc. (El-Salam and El-Shibiny, 2011; Hashmi and Saleem, 2015). Many studies have been published worldwide to describe the nutritional composition of buffalo milk, of which some are presented below (Table 2.2).

Table 2.2: Buffalo milk composition in different countries

Fat%	Protein%	Lactose%	Ash %	Total solid %	Country and References
7.97±0.44	4.36	5.41	0.81	18.45	Pakistan (Mahmood and Usman, 2010)
7±0.6	4.4	5.2	8.4	17.4	France (Ahmad et al., 2008) in Murrah buffalo
7.3	4.6	5.6	-	17.6	France (Ménard et al., 2010)
6.57-7.97	4.9-5.37	4.49-4.73	0.91-0.92	16.39-18.48	USA (Han et al., 2012) in water buffalo
7.59±1. 31	4. 86 ± 0. 4	4.74 ± 0.20	0.85±0.05	18.4±1.6	China (Han et al., 2007)

8.30±0. 37	4. 11- 4.74	4.67- 5.27	0.69-0.89	18.45	India (Kapadiya et al., 2016)
7.84	3.8	4.7	0.71	17.17	Bangladesh (Khan et al., 2007) in swamp and water buffalo
7.4-8.8	4.3-4.5	-	-	-	Italy (Varricchio et al., 2007) in Mediterranean buffalo
8.26	4.73	4.72	-	-	Italy (Costa et al., 2020)in water buffalo
7.48, 7.31	4.76, 4. 38	4.76, 4.73	0.80, 0.80	17.80, 17.2	China (Wang et al., 2019) in Murrah and Niliravi respectively
7.52	4.02	5.02	0.80	17.65	Egypt (Soliman, 2005)
6.40± 0.17	3.80±0.1	5.11± 0.07		16.24± 0.3	Brazil (Sales et al., 2018)

Water buffalo milk contains a larger percentage of fat than milk from other livestock species. Also, the average size of fat globules in buffalo milk (5µm) is larger than that in cow, goat and sheep milk, being 3.2, 2.6 and 3.0 µm respectively (Ahmad et al., 2013). Water buffalo milk also features a higher melting point, density, specific gravity and saponification value properties but lower refractive index, acid and iodine values than cow milk fat. The 2nd major component in buffalo milk is lactose; in average 5.05±0.53 g per 1000g as recorded in published studies (Han et al., 2007; Ahmad et al., 2008; Kapadiya et al., 2016). This higher lactose percentage is beneficial for the brain function and regulates hormonal activities (Ahmad et al., 2013). But it can cause digestive disturbances for those suffering from lactose intolerance (Ahmad et al., 2013). The fatty acid content of buffalo milk is also higher than that in cow milk. A study on Italian Mediterranean buffalo found that the five most important fatty acids in quantitative terms

were C16:0, C18:1c, C18:0, C14:0 and C4 where saturated fatty acids (65.5%) predominated, followed by monounsaturated (27.0%) and polyunsaturated (4.5%) fats, respectively (Varricchio et al., 2007). Another study which compared fatty acid content in milk from different buffalo breeds in Pakistani found that the saturated fatty acid content of milk from Kundi buffalo was significantly lower ($P < 0.05$) than in milk from the Nili-Ravi buffaloes (67 and 69% respectively) (Talpur et al., 2007). Buffalo milk also has a higher buffering capacity, rennet coagulation, higher curd tension, higher amounts of total solids and casein which helps to form thick creamy curd (Tripaldi, 2005; Ahmad et al., 2008; Han et al., 2012). It also contains some bioactive gangliosides which have anti-inflammatory activity as well as it has a GM1-specific binding to cholera toxin subunit B (Colarow et al., 2003). However, a comparative study on humoral and cell-mediated immune responses associated with allergenicity of the major milk proteins (casein and β-lactoglobulin) of cow, buffalo and goat revealed that the cow milk proteins (caseins and β-lactoglobulin) have high protein-specific IgE sensitization and lymphocyte proliferation index significantly than buffalo and goat milk protein (Kapila et al., 2013).

2.6.2. Total bacterial count

Milk free from pathogenic and harmful bacteria is defined as quality milk. Quality of milk can be easily determined by using several bacteriological parameters, including total bacterial counts, total *Staphylococcal* count, total *Enterobacteriaceae* count and coliform counts. Among them, the TBC is considered one of the acceptance criteria for grading of milk quality which partly indicates the safety of milk for human consumption and processing of dairy product (Mhone et al., 2011). It is a good indicator for the hygienic quality of milk, which can be influenced by, for example, herd health, farm hygiene, milk handling and storage condition and also cooling facilities (Minj and Behera, 2012). Many countries have set legal limits for TBC in milk and milk product. The limit values for TBC at 30°C established by the European Union Regulation (EC) No (EU) are: 1×10^5cfu/mL for raw cow milk; 1.5×10^5cfu/mL for raw milk from species other than dairy cows; in addition, 5×10^5cfu/mL limit for raw milk from species other than cows used for manufacturing cheese products without heat treatments (Pasquini et al., 2018). All countries within the European Union (EU) follow the European legislation criteria for

TBC in raw milk (Piepers et al., 2014; Pyz-Łukasik et al., 2015; Abera et al., 2016; Lan et al., 2017; Pasquini et al., 2018). In China, the national standard requires that the raw milk should have a TBC of $<2 \times 10^6$ cfu/mL (Lan et al., 2017) whereas the criteria in Zimbabwe is 5×10^5 cfu/ ml (Mhone et al., 2011). In Bangladesh, the acceptable limit for TBC in pasteurized milk is $\leq2\times10^4$ cfu/ml (Islam et al., 2018), but there are no microbiological standards concerning raw milk in Bangladesh. Various studies have been conducted to determine the milk quality by determining TBC in raw milk (Table 2.3).

Table 2.3: Total bacterial count in raw milk in different countries

	Recorded value	**Species**	**Country and references**
Total bacterial count in raw milk	4.96–7.56log10 Cfu/ml	Cow milk	Poland (Pyz-Łukasik et al., 2015)
	5.29 log10 Cfu/ml	Buffalo milk	Pakistan (Soomro et al., 2016)
	5.87±4.81log10 Cfu/ml	Cow milk	Morocco (Sraïri et al., 2009)
	5.59±0.11log10 Cfu/ml 5.10 log10 Cfu/ml	Buffalo milk Cow milk	China (Han et al., 2007) (Lan et al., 2017)
	6.4 ± 5.6 log10 Cfu/ml	Cow milk	Zimbabwe (Mhone et al., 2011),
	6.36±0.28log10 Cfu/ml	Buffalo milk	Turkey (Kuyucuoğlu and Pamuk, 2013)
	3.96 log10 Cfu/ml	Cow milk	Belgium (Piepers et al., 2014)
	6.36±0.28log10 Cfu/ml	Buffalo milk	India (Hashmi and Saleem, 2014)

	4.11 log10 Cfu/ml	Cow milk	Finland (Ruusunen et al., 2013)
	5.09 log10 Cfu /mL 4.75±0.17log10 Cfu/ml	Cow milk Camel milk	Ethiopia (Tolosa et al., 2016) (Abera et al., 2016)
	1.99 log10 Cfu/mL	Buffalo milk	Italy (Pasquini et al., 2018)
	7.08 log10 Cfu/ml	Cow milk	Malaysia (Chye et al., 2004)
	5.37±0.78log10 Cfu/ml	Buffalo milk	Egypt (Elshaghabee et al., 2017)
	8.756±0.803 log Cfu/ml	Cow milk	India (Minj and Behera, 2012)

In many cases, the average TBCs' recorded in raw milk in various studies were above the recommended level for human consumption, which emphasise the need of pasteurization before human consumption. Pasteurization has a positive effect on milk quality and shelf life of dairy product as it reduces the TBC, coliform bacterial count and other pathogens in milk (Abd Elrahman et al., 2013). The higher bacterial count can be found due to infected udder or contaminated udder and teat, contamination from animal bedding, mixing of normal milk with the mastitis milk, unhygienic practices of milking, contaminated milking equipment and wash water as well as transportation and storage condition. As the level of contamination depends on so many factors and the likelihood for bacterial contamination increases in every step of milk value chain, the TBC of the bulk milk in the final stage of the milk value chain also increases. Similarly, the quality of dairy products, such as yogurt/dahi, can be evaluated by counting the total bacteria. However, there is no well documented criteria for levels of TBC in this product (Younus et al., 2002; Islam et al., 2018) even if several studies have been carried out to check the quality of marketed dahi by enumerating TBC (Younus et al., 2002; Nahar et al., 2007; Chowdhury et al., 2011; Bhattarai and Das, 2015; Matin et al., 2018). A study on TBC in

buffalo dahi found levels of 5.99 ± 0.05 log10 Cfu/ml (Nahar et al., 2007). The high TBC in dahi may be the result from poor starter culture, poor milk quality or contaminated or unhygienic processing practices involved such as contaminated utensils, storage temperature etc.

2.6.3. Total Enterobacteriaceae count

Total *Enterobacteriaceae* count is another mandatory index which is considered as a reliable index of food safety for all kinds of food products. Number of *Enterobacteriaceae* in food product indicates the quality of processed foods, especially with regard to good manufacturing and processing practices (Mullane et al., 2006). *Enterobacteriaceae* includes many genera such as *Escherichia coli, Shigella, Salmonella, Enterobacter, Klebsiella, Serratia* and *Proteus.* The presence of this group of bacteria in food of animal origin indicates the environmental sources of contamination since these micro-organisms are abundant in the environment (Mhone et al., 2011). These genera are found widespread in the environment and can contaminate milk the milk duriong milking, storage, refrigeration, milking machine sanitation and premilking udder hygiene (Murphy and Boor, 2000; Davidson, 2004; Pantoja et al., 2011; Cerva et al., 2014). *Enterobacteriaceae* can cause enzymatic breakdown of proteins or lipids, instigating spoilage that contributes to substantial economic losses and waste (Baylis et al., 2011).Thus the presence of *Enterobacteriaceae* in dairy products induces undesirable changes that render the product of inferior quality, unmarketable, and unfit for human consumption. Raw milk is also a potential source of exposure of *Enterobaacteriaceae* for the consumer in farmer-to-consumer direct marketing trend (Odenthal et al., 2016). So the Total *Enterobacteriaceae* count in milk should be routinely assessed to determine the hygienic quality of foods, particularly dairy products (Martín et al., 2010). According to NSW food authority the total *Enterobactericea*e count $<10^2$ cfu/ml in ready to eat food is considered as good whereas 10^2 to $< 10^4$ cfu/ml is considered as acceptable limit and $\geq 10^4$ cfu/ml is regarded as unsatisfactory (NSW). The EU microbiological regulation considers pasteurized milk is satisfactory at TEC of 0 cfu/g (El-Ziney, 2018). Various studies have been conducted to determine the milk quality by determining total *Enterobacteriacea* count in raw milk (Table 2.4 below). The presence of these organisms

certainly constitutes a food safety problem (Martín et al., 2010) and induces undesirable changes that render the product of inferior quality, unmarketable, and unfit for human consumption.

Table 2.4: Total Enterobacteriaceae count in raw milk in different countries

	Recorded value	**Species**	**Country and references**
Total Enterobacteriaceae count	1.80 – 6.23 log10 Cfu/mL	Cow milk	Poland (Pyz-Łukasik et al., 2015)
	6.0 log10 cfu/ml	Chain milk NS	Egypt (Sobeih et al., 2020)
	6.08 log10 Cfu/ml	Chain milk NS	Kenya (Wanjala et al., 2017)
	5.68 log10 Cfu/ml	Chain milk NS	Brazil (Ferraz et al., 2010)
	5.30 – 9.55 log10 Cfu/ ml	Buffalo milk	Egypt (EL-Tantawy et al., 2018)
	4.47 log10 Cfu/ml	Chain milk NS	Turkey (Tasci, 2011)
	2 - 4.94 log10 Cfu/ ml	Cheese	Switzerland (Serrano et al., 2018)
	7.91 log10 Cfu/ml.	Chain milk NS	Egypt (Saad et al., 2017)

#NS: Not specified in study

2.6.4. Total Staphylococcal count

Along with other food safety indexes such as TBC, TEC; total *Staphylococcal* count is another reliable index considered in many countries. Staphylococcal food poising is often caused by intoxication of a sufficient amount of Staphylococcal enterotoxins (Fetsch and Johler, 2018). Staphylococcal food poisoning in human typically occurs after ingestion of

different foods, particularly processed meat and dairy products. Foods can be contaminated with *S. aureus* due to improper handling and subsequent storage at elevated temperatures and produce toxin in the food (Argudín et al., 2010; Fetsch et al., 2014). People colonized with *S. aureus* can also introduce the bacteria into the food chain during handling and processing. Animal affected with mastitis, air, dust, and food contact surfaces also serve as vehicles in the transfer of Staphylococcus into the milk chain (Argudín et al., 2010). However, both *S. aureus* and Non-aureus *Staphylococcus* can produce toxins and act as a reservoir of antimicrobial resistance genes and biofilm producer (Khoramrooz et al., 2016; Saka and Terzi Gulel, 2018; Pizauro et al., 2019). But a certain amout of *Staphylococcus* toxin need to produce public health impact. It is found that food containing *S. aureus* becomes potentially hazardous when the count becomes $>10^4$ cfu/ml (Han et al., 2007). However, *S. aureus* count in between 10^6 –10^8 cfu/ ml levels are considered as significant for human food poisoning (Kérouanton et al., 2007; Mhone et al., 2011). Various Studies reported higher total *Staphylococcal* count in milk (Table 2.5). However, an increase in their numbers in bulk milk and milk product is suggestive of problems related to farm management, udder hygiene and milking practices such as proper transportation, storage proper hygiene practice should be followed in all steps of milk chain to minimize the contamination level and occurrence of public health hazard.

Table 2.5: Total Staphylococcal count in raw milk in different countries

	Recorded value	**Species**	**Country and references**
Total Staphylococcal count	1.87-4.47 log10 Cfu/ml	Cow milk	USA (Gillespie et al., 2012)
	3.20-4.70log10 Cfu/mL	Cow milk	Poland (Pyz-Łukasik et al., 2015)

	2.34-2.87 log10 Cfu/ml	Cow milk	Ethiopia (Tegegne and Tesfaye, 2017)
	5.83 log10 Cfu/ml	Chain milk NS	Kenya (Wanjala et al., 2017)
	1.68 log10 Cfu/ml	Buffalo milk	China (Han et al., 2007)
	2.62 log10 Cfu/ml	Buffalo milk	Turkey (Kuyucuoğlu and Pamuk, 2013)
	1.92 log10 Cfu/ml.	Cow milk	India (Lingathurai et al., 2009)
	6.37 log10 Cfu/ml	Chain milk NS	Egypt (Saad et al., 2017)
	5.32 log10 Cfu/ml	Buffalo milk	Egypt (EL-Tantawy et al., 2018)
	2.89 log10 Cfu/ml	Cow milk	Bangladesh (Khaton et al., 2014)

#NS: Not specified in study

2.6.5. Somatic cell count

Somatic cell count (SSC) represents the inflammatory response of the mammary gland of an individual animal or quarter (Schukken et al., 2003). BMSCC is a strong indicator for herd udder health as well as milk quality (Zecconi et al., 2019). The somatic cells are mainly composed of leucocytes (75%) i.e. neutrophils, macrophages, lymphocytes, erythrocytes and epithelial cells (25%) (Patil et al., 2015). The SSC is increased during intramammary infection and thereby SCC in milk has been assumed to be the most reliable parameter to determine milk quality and levels of subclinical mastitis in herds or individual cows (Patil et al., 2015; Sahin et al., 2017). It is also related to decreased milk

yield, marked compositional changes and shorter shelf life of milk and can thereby cause considerable economic losses for dairy farmers (Sahin et al., 2017). Elevation of SSC can also be caused by various factor such as herd size, age, breed, parity, stage of lactation, length of the dry period, foremilking, season, stress, diurnal variation, etc. (Skrzypek et al., 2004; Bytyqi et al., 2010; Sharma et al., 2011). In high-income countries, milk sold for human consumption is highly regulated by estimating SCC of milk to ensure safe milk for dairy product manufacturing (Middleton et al., 2014). According to the Regulation (EC) No (EU) of the European Parliament and of the Council of 29 April 2004, the threshold for SCC in bovine raw milk within the European Union is 400,000 cells/ml. However, different countries use different thresholds of SCC to categorize the milk quality. In the US, the legal maximum somatic cell count for Grade A farm cow bulk milk is 750,000 cells/ml, whereas in Canada the limit is 500,000 cells/ml for raw milk and in Norway, Switzerland, Australia, and New Zealand, the maximum BMSCC limit is 400,000 cells/ml (Norman et al., 2000)). According to the National Mastitis Council, cows having no infection have SCC 100,000 cells/ml where subclinical mastitis affected cows have SCC $\geq 200{,}000$ cells/ml (NMC, 2017). In one study, the SCC in buffaloes was found to be lower than the commonly observed SCC of dairy cattle (Moroni et al., 2006). In this study they found that 100% of quarters from buffalo having intramammary infection had SCC>200,000 cell/mL whereas 98% of quarters below the threshold level were uninfected. But there is no standard or regulation for SCC in buffalo milk yet. Recently, National Dairy Research Institute (NDRI), India has proposed SCC 100,000 cells/mL of milk in Murrah buffalo where >100,000 cell/ml is considered as subclinincal mastitis (Pers. Comm: Prof. Abdul Samad, 2020). Several studies have been conducted in buffalo considering the bovine threshold level 200,000 cells/ ml as standard to define the subclinical and clinical mastitis. In a study on Murrah buffaloes, milk SCC threshold level 200,000 cells/ ml was used as the cut-off value to define subclinical mastitis in buffaloes (Dhakal, 2006). However, it is also associated with the productive traits. A study on relationship between milk somatic cell count with milk yield and quality traits in Italian water buffaloes found that SCC was negatively correlated with the milk yield and lactose percentage of the buffalo milk whereas positive correlations were obtained with fat and protein percentage (Costa et al., 2020). An association between the

SCC and pathogen involved with IMI was also observed in a study (Jayarao et al., 2004). The study showed that an increase in the frequency of isolation of *S. aureus* and *Strep. agalactiae* were significantly associated with the increased BMSCC and TBC. Other similar studies revealed that quarters infected with NAS had higher SCC counts than quarters affected with the major pathogens (Sampimon et al., 2010; Condas et al., 2017). However, high SCC in milk can exert adverse effects during cheese making, with a direct negative impact on curd firmness, cheese yield and sensory characteristics of milk (Pasquini et al., 2018). It has also impact on human health as with the SCC increases, the neutrophils percentage and microorganism also increases.

2.6.6. Zoonotic bacteria in buffalo milk and milk products

Microorganisms can be introduced into the milk at any stage in the milk value chain, for example during production, processing, handling, transport or preservation. But the food borne illness mainly occurs through consumption of raw milk and raw milk product (dahi, cheese etc.) as most of the bacteria can be destroyed by heat treatment. Some of the most significant zoonotic pathogens of food safety concern are: Shiga toxin-producing *E. coli* (STEC) O157, *S. aureus, Salmonella spp., Mycobacterium spp., Brucella abortus, Coxiella burnetii, Campylobacter jejuni, Yersinia enterolitica, Listeria monocytogenes* etc. (Jayarao et al., 2006; Morandi et al., 2007; Junior et al., 2009). People can become infected with theses bacteria by consuming unpasteurized raw milk and dairy products. Among them, STEC O157 is of major concern in dairy industry due to its high pathogenicity to the consumers. Only 5-50 cell of STEC O157 can cause human disease (Farrokh et al., 2013). Among the other zoonotic pathogens, *L. monocytogenes* constitutes a serious public health threat due to its ability to multiply and survive at low refrigerator temperatures. Mastitis due to *Listeria spp*. is not so common but the bacteria can contaminate the milk from milking equipment and through the environment (silage, milking parlor, unhygienic handling etc.) (Cortesi and Murru, 2007). Another zoonotic disease is salmonellosis which is caused by *Salmonella spp*. Salmonella contamination of bulk milk mainly occurs through fecal contamination and the potential for this organism to grow in improperly stored raw milk and milk product can cause public health hazard (Karns et al., 2005). A study on risk factors associated with *Salmonella* spp. in bulk milk and milk product found association with consumption of

raw milk directly from the udder of cows (P<0.001, OR = 8.7), use of water from open source for processing (P= 0.001, OR = 3.7) and absence of udder washing before milking (P= 0. 0041, OR = 0.21) (Karshima et al., 2013). A study on zoonotic pathogens in bulk tank milk (BTM) and milk filters in dairy farms in the United States found that the prevalence of virulent *E. coli* in BTM was 30.5% at farm level where *Salmonella enterica* was isolated from 18.0% and *L. monocytogenes* from 3.0% of the included farms (Sonnier et al., 2018).

Staphylococcal food poisoning caused by *S. aureus* is another important pathogen in dairy industry. Staphylococcal infection occurs through ingestion of contaminated food containing staphylococcal enterotoxins. It is one of the major pathogens found in ruminants during mastitis. *S. aureus* starts to produce the enterotoxin when they occur in number above $10^{6.5}$cfu/ml in milk (Fujikawa and Morozumi, 2006) and can produce illness with a very small amount of staphylococcal enterotoxin (ranging from 100 to 200 ng) (Makita et al., 2012). Another bactieria of importance is *Brucella abortus* causing brucellosis which can occur via direct contact with infectious excretions caused by abortions, via ingestion (for example milk), inhalation and by the venereal route. A large numbers of *Brucella* spp. can be excreted in fetal fluids and mammary secretions (Marianelli et al., 2008). *Mycobacterium bovis*, the causative organism of tuberculosis can also be present in milk and milk products (Jha et al., 2007; Sgarioni et al., 2014). *C. burnetii* in milk is a rising health concern which can cause Q fever in human when unpasteurized raw milk is consumed (Owusu-Kwarteng et al., 2020).

2.6.7. Mastitis in buffalo

Mastitis is one of the most prevalent production diseases of dairy buffalo, causing economic losses and animal health and welfare implication (Manimaran et al., 2014; Guccione and Ciaramella, 2017). With the growing interest in consuming buffalo milk around the world, several studies have been done on buffalo mastitis. Some authors have described that buffaloes are less susceptible to mastitis than cows (Khan and Muhammad, 2005; Mustafa et al., 2013). This is likely due to it's long narrow teat canal and tight teat sphincter which prevent microbial invasion, however the buffalo may also have some characteristics, such as more pendulous udder and longer teat, that can contribute to

greater risk of mastitis (Moroni et al., 2006; Fagiolo and Lai, 2007; Hussain et al., 2013). In buffalo, the most common mastitis-causing bacteria are: *Strep. agalactiae, S. aureus, Arcanobacter pyogenes, Micoplasma.* Some of the mastits causing bacteria are environmental, such as *Strep. uberis, and Strep. dysgalactiae*, *E. coli., Enterobacteriacea*, yeasts and moulds and opportunistic bacteria such as NAS (Fagiolo and Lai, 2007). NAS was found to be the most predominant pathogens causing IMI in most studies (Moroni et al., 2006; Ali et al., 2011; Guha et al., 2012; Locatelli et al., 2013). However, a recent study on microbiota of water buffalo milk during mastitis revealed that the microbiota diversity was richer in healthy quarters of buffalo than in quarters suffering from sub-clinical mastitis or clinical mastitis. A study showed that the healthy milk microbiota is dominated by Firmicutes (58%), followed by Proteobacteria (23%), Actinobacteria (12%), Bacteroidetes (6%) and Fusobacteria (1%) whereas subclinical mastitis milk presents a decrease of Firmicutes (48%) and Actinobacteria (6%), and a relative increase in Bacteroidetes (11%) and Proteobacteria (33%). In clinical mastitis milk, the relative abundance of Bacteroidetes increases to 24% and Fusobacteria to 8%, whereas Proteobacteria, Tenericutes and Actinobacteria decreased (Catozzi et al., 2017). However, another study on variability of intra- and inter-individual milk microbiota in both healthy and infected water buffalo revealed that the intra-individual variability of milk microbiota was lower in both healthy and subclinical mastitis-affected quarters than the inter-individual variability and the healthy quarters within the same mammary gland exhibit a higher range of shared ASV (Amplicon sequence variant). It has also been found that the most stable phylum and family across healthy or subclinical mastitis affected quarters was Bacteroidetes and Propionibacteriaceae (Catozzi et al., 2019). Similar findings were also reported in another study on microbiological profiles in clinical and subclinical cases of mastitis in Jafarabadi buffalo milk where the subclinical mastitis samples comprised a more complex bacteria diversity as compared to clinical samples where out of 168 and 222 genera observed, 44 and 98 genera were present in abundance (>0. 1%) in clinical and subclinical samples, respectively (Patel et al., 2019). In another study an abundance of *Staphylococcus* (25. 9%, 10. 1% and 0. 03%), *Enterococcus* (10. 8%, 8. 72% and 0. 36%), *Escherichia* (8. 88%, 0. 38% and 0. 00%), *Streptococcus* (3. 97%, 0. 42% and 0. 00%), *Lactococcus* (3.

73%, 23. 9% and 0. 01%), and *Ralstonia* (0. 54%, 12. 7% and 0. 00%) genera in clinical, subclinical and healthy samples, respectively have been reported. However, an infected quarter can also influence the infection chance of other neighbouring uninfected quarter (Jensen et al., 2013).

2.7. *Staphylococcus aureus* and its public health impact

S. aureus is considered as an important pathogen and a leading cause of foodborne illness around the world (Fetsch et al., 2014). 'Aureus' means golden in latin and the name indicates a pigmented color colony producing bacteria. *S. aureus* is mainly found in mastitis affected dairy animal. It can be also found in animal skin, contaminated udder and teat, milking equipment, milkman's hand etc. The bacteria can also be isolated from various types of milk products such as cheese, ice cream, clotted cream, yogurt and butter when raw milk is inadequately heated or post pasteurization contamination during handling, storage or packaging occurred. But the bacteria don't produce disease itself. It produces various types of virulence factor such as enterotoxins (SEs), exfoliative toxin (ET), toxic shock syndrome toxin (TSST-1), thermonuclease, hemolysins (induced by the hla, hlb, hld, and hlg genes), hyaluronidase, deoxyribonuclease, catalase, lipases and coagulase and leukocidin (Sandel and McKillip, 2004; Saka and Terzi Gulel, 2018; Thongratsakul et al., 2019). These secreted proteins hampers the immune function of the host by interfering with many critical components of innate and adaptive immune system (Koymans et al., 2015). SEs have been classified into five classical serological types (SEA, SEB, SEC, SED, and SEE) which have been reported to be responsible for 95% of staphylococcal food poisoning outbreaks (Saka and Terzi Gulel, 2018). Staphylococcal enterotoxin A (SEA) is most frequently reported as the causative agent of staphylococcal food poisoning outbreaks followed by SEB, SEC, and SED. SEs are heat-stable and cannot be destroyed by cooking. LukMF is a another potent toxin specifically killing bovine neutrophils and contribute in the severity of IMI infection (Hoekstra et al., 2018). Therefore, they may retain their biological activity even after pasteurization and various processing steps (Sharma et al., 2017). The severity of the illness depends on the amount of enterotoxin produced by the organism, but a small amount of enterotoxin 100-200ng in food is enough to cause disease (Makita et al., 2012). The bacteria can produce enterotoxins under a wide range of temperature, pH, sodium chloride concentration and

generally the accepted limits for SE production are temperatures between 10 and 48°C, a pH range of 4 to 9.6, and NaCl concentrations of 0-10% (Zeaki et al., 2019). In most cases, β-lactam antibiotics are the drug of choice for the treatment of Staphylococcal infection (Marques et al., 2017; Shah et al., 2019; Thongratsakul et al., 2019). The beta-lactam group of antimicrobials is also widely used in the treatment of bovine mastitis (Chandrasekaran et al., 2014; El-Ashker et al., 2015). But due to indiscriminate use of this group of antibiotic in the livestock and human (Mohanta and Mazumder, 2015; Elmonir et al., 2019), *S. aureus* has become resistance to this group of antibiotics (El-Ashker et al., 2015). However, public awareness about good milk handling practices and monitoring rational use of drugs and periodic assessment of the antimicrobial sensitivity of drugs prior use are very much important to reduce *S. aureus* contamination and antibiotic resistance (Ayele et al., 2017). Therefore, consuming raw milk and low heat-treated milk products should be avoided (Saka and Terzi Gulel, 2018). Hygienic practice in milking, handling and transportation of milk and milk product, use of detergents and good quality water for cleaning the equipment, can reduce the microbial load in milk (Chye et al., 2004) .

2.8. Prevention of bacterial milk contamination along the milk value chain

Safe milk is a global health concern. Milk can be contaminated at any stage in the value chain through poor hygiene and sanitation practices. According to the EU (Regulation:178/2002), unsafe food which is unfit for human consumption and constitutes health hazards should not be placed on the market. In order to determine the safety of food, a value chain approach should be used to identify critical points for contamination. Milk is an ideal substrate for growth of microbial populations and the quality and safety regarding milk and milk products should be a primary concern for the dairy industry. In the informal milk value chain contamination is higher than in the formal milk value chain in most of the cases due to poorer handling practices as well as inadequate hygienic measures (Kunadu et al., 2018). Milk can be contaminated due to clinical or sub-clinical mastitis and/or environmental contaminations at different steps in the milk value chain (Cortesi and Murru, 2007). In addition, some social factors such as culture, economic status, education status, lack of food safety awareness, lack of inadequate infrastructure can facilitate the entry of enteropathogens in milk along the

milk value chain (Kunadu et al., 2018). To maintain an acceptable level of food safety and to minimize contamination of milk and dairy products, milking must be carried out hygienically in clean environment ensuring proper udder and teat hygiene, clean and uncontaminated milking equipment, proper storing temperature, transportation through cool chain etc. Low somatic cell count, total bacterial counts and early diagnosis of clinical and subclinical mastitis are important for quality milk production which can be reduced through proper disinfection and inspection in dairy herd (Dhakal, 2006). Government should impose a strict legislation to improve the milk and milk product's quality and guarantee its safety to the consumers. Governments and regulatory agencies should engage with appropriate stakeholders in the milk value chain to establish national and regional controls and standards, including inspection and surveillance and to ensure safe production, transportation and storage of milk and dairy products (Owusu-Kwarteng et al., 2020). Analysis monitoring the quality of milk also needs to be conducted along the production and marketing system (Perwira et al., 2019). However, setting up an efficient, hygienic and economic dairy chain is a serious challenge in many low-income countries as most of farmers are smallholder which deliver small amounts of milk (Ndungu et al., 2016). Therefore, lack of hygienic awareness, poor infrastructure, poor transport facility, lack of technology, lack of knowledge of milk processing, storage and cooling, lack of government training and facilities may also create difficulties in proper value chain settings. In order to minimize the contamination, the critical control points should be identified in line with HACCP principles identifying the deteriorating factors at the various level of value chain and their subsequent monitoring would enhance milk quality in all the steps (Chege and Ndungu, 2016; Ndungu et al., 2016). In addition, milk must be pasteurized before it reaches to the consumers. If milk is fermented, the temperature must be kept as low as possible to avoid contamination.

Conclusion

In this review, global feature of milk value chain along with buffalo milk value chain in Bangladesh, sources of contamination and various practices along the milk value chain, microbial contamination with total bacterial count, somatic cell count of milk and microbial contamination at different level of milk value chain were reviewed meticulously. There are only few studies that have been reported in buffalo in Bangladesh

and there are lack of evidences of sources of contamination and etiological agents along the milk value chain. Therefore, this investigation will focus on the knowledge gap related to the risk factor associated with bacterial contamination and prevalence of microorganisms along the milk value chain. By conducting a cross-sectional study along the buffalo milk value chain can abate and fulfill one of several knowledge gaps.

Chapter III: Materials and Methods

3.1. Description of the study site and study population

Noakhali district is a part of the Chittagong division situated in the Southeastern part of Bangladesh at the fringe of the Bay of Bengal. The district lies between 22º07` and 23º08` North latitudes and between 90º53` and 91º27` East longitudes. It is bounded on the North by Cumilla district, on the East by Feni and Chittagong districts, on the South by the Bay of Bengal and on the West by Bhola and Lakshimpur districts (BBS, 2011). The total area of the district is 3.685.87 sq. km. (1423.12 sq. miles) with the annual average temperature ranging from a maximum of 34.3 ℃ to a minimum of 14.4 ℃. The average annual rainfall recorded in the district is 3302 mm (BBS, 2011). Forty percent of the GDP in this district comes from agriculture and 80% of the population is employed in the agriculture sector (LGED, 2011). The Noakhali district consists of 9 upazilas (sub-district), 91 unions and 967 villages. Among the upazilas, Subarnachar is one with a large buffalo population. At Subarnachar upazila, 68% of the farmers were engaged in buffalo farming and the remaining 32% were engaged in non-agricultural businesses (Amin et al., 2015). The buffalo breeds in this area were mostly of indigenous types and the average milk yield recorded varied from 2.7 - 3 litres per day (Amin et al., 2015; Uddin et al., 2016). A few percentages of the farmers practised household farming systems in this region (Faruque et al., 2019). The buffalo production system was mainly (80%) Bathan based (free-range on the island) (Amin et al., 2015) where buffaloes were kept in the open field with no housing facilities. Semi-Bathan is a type of rearing system where buffaloes are reared several months inland during the rice and watermelon season followed by several months on the island for grazing. In this case, buffaloes are brought from the bathan to the inland during the end of the winter due to less availability of grass and drinkable water in the bathan. Among the buffalo farmers, most farmers have been reported to be middle-aged (36-50 years) and the majority of the farmers had only primary education (Amin et al., 2015; Rahim et al., 2018).

The milk value chain in the Noakhali district is mainly informal where the farmers sell their milk directly to nearby markets, collection centres or sweet shops, or indirectly through middlemen. The milk value chain in household and semi-bathan rearing systems

are almost the same, whereas the bathan system milk value chain can vary, which is shown below (Figure 3.1):

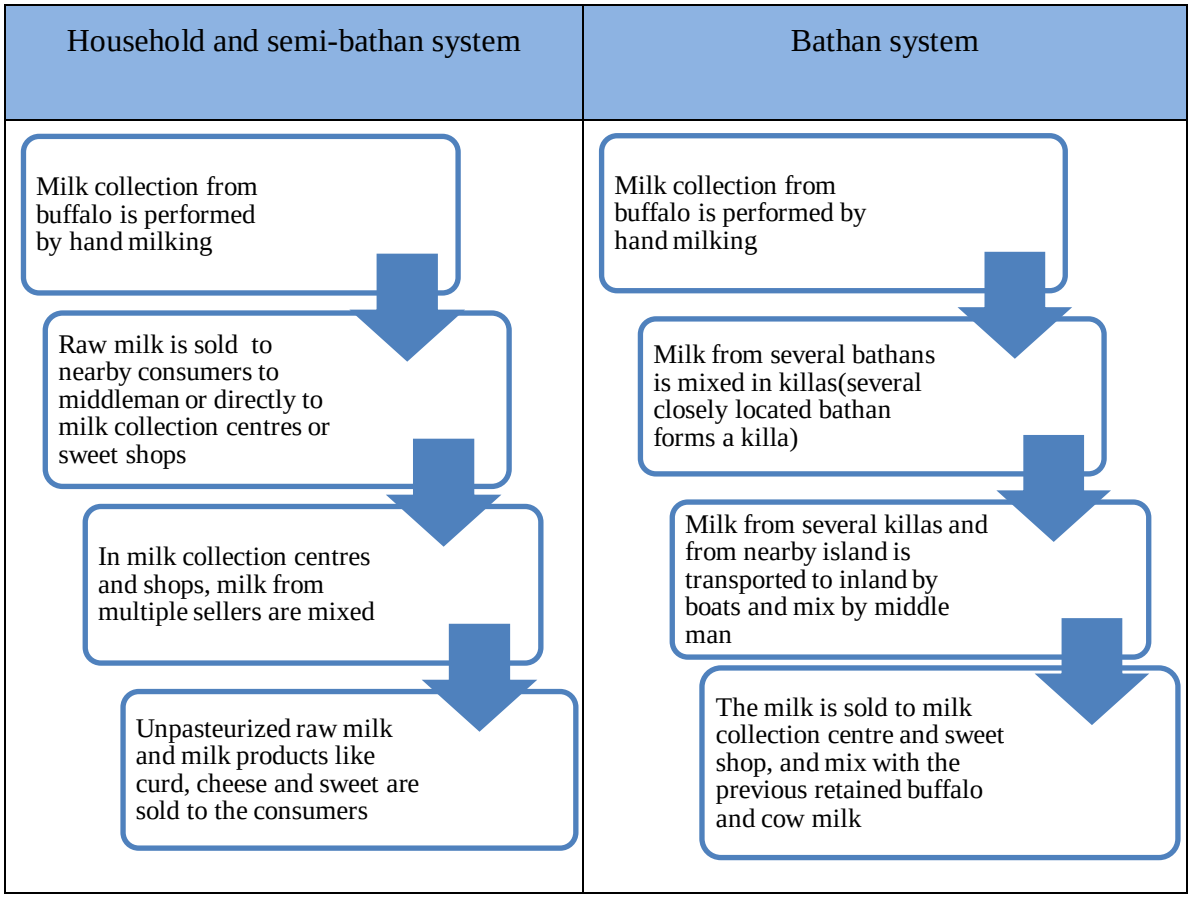


Figure 3.1: Milk value chain in different buffaloes rearing system in Noakhali district

3.2. Study design, sample size and sampling

3.2.1. Study design and samples size calculation

A cross-sectional study was conducted at different nodes of the Buffalo Milk Value Chain (BMVC) in Shubornochar upazila in the Noakhali district of Bangladesh during April 2021. Milk value chain farms and different nodes were selected by making a comprehensive list of farmers who had at least 3 lactating buffalo with the help of regional livestock officers, local farmers and owners of the different milk collection centres. After that, a convenient selection procedure was performed by using the list of the farmers as well as considering the concentrated buffalo areas.

Only semi-bathans and household farms were included in the study. The reason for this was that most of the buffaloes were brought from the bathan area to the inland due to less availability of feed-in bathan at the time of sampling.

a) Semi-bathan - In the case of semi-bathan, the sample size was calculated assuming the expected prevalence of *Staphylococcus* spp. in bulk milk at semi-bathan level was 70%, (Jørgensen et al., 2005; Riekerink et al., 2006; Francoz et al., 2012), with 15% precision and 95% confidence interval. Therefore, the sample size was estimated at 36 at the semi-bathan level. For practical reasons and assuming similar kind of expected prevalence of study, the sample size was estimated to be the same for middlemen and collection centres in the semi-bathan buffalo milk chain.

b) Household – Fewer farmers were practising the household buffalo rearing system compared with the semi-bathan system and most of the household farmers had only 1 or 2 lactating buffaloes. That is the reason why fewer household farms were included in the study. Therefore, 10 samples from each node (10 samples from 10 household farm, 10 from 10 middlemen and 10 from 10 collection centres) were considered, thus, a total of 30 samples were considered at the household milk value chain.

In the case of dairy products, 10 dairy products from 10 individual shops were considered in the study. The estimated number of samples at different nodes is shown in Table 3.1.

Table 3.1: Sample collection point and estimated numbers of samples from different nodes

Milk value chain	**Samples at different nodes**				
	Household (n)	**Middlemen** (n)	**Collection centre** (n)	**Dairy shop** (n)	**N**
Household level	10	10	10		30
Semi-bathan level	36	36	36		108

Dairy product	-	-	-	10	10

N= Total number of samples collected

3.3. Epidemiological data collection

A questionnaire was developed to collect data from various nodes of the buffalo milk value chains. The questionnaire was pre-tested before the start of the main study to identify gaps and assess the required time. The questionnaire was administered through face to face interviews. Farmer's participation in the study was voluntary and oral consent was taken in each case. Each questionnaire was given a specific ID matched with the milk samples ID taken from each node.

The questionnaire was divided into four sections; section A was designed to capture general background information of household farms and semi-bathans while the other sections (B, C, D) were intended to collect information on the milk value chain (middlemen, collection centre and milk product shop). There were 45 questions in the questionnaire section A, where the other section (B, C, D) contained around 10-15 questions. It took around 15-20 minutes to complete section A where the other section (B, C, D) took around 5-10 minutes.

In section A, data included information such as the total number of lactating buffaloes, number of dry buffaloes and average milk yield per day. Section A also collected data on milk containers (cleanliness of milking equipment, how the containers were cleaned, drying of milk can before milking, using a lid or not, keeping the bulk milk container open during each milking, a score of milker's hygiene and udder hygiene, milking procedure, storage time at household). Some generic data on milk storage, transportation and farmers obtaining a good price when selling the milk were also collected from farmers in this section. Section B and C collected information from the middlemen and collection centre. Data on milk transportation (means of transportation, types of container used during transportation, use of covering material, cleanliness of milk container) and type of containers using at the shop, milk storage time at the shop, use of cold storage or not, duration of milk kept at the shop were collected in these two sections. Section D was

aimed at gathering information on milk products (composition, storage time and containers).

The full questionnaire is attached in **Appendix I.**

3.4. Sample collection, preservation and transportation

3.4.1. Bulk milk samples from household farms/ semi-bathans- An amount of 50 mL bulk milk sample was collected from each household farm/semi-bathan in sterile 50 mL screw-capped falcon tubes following aseptic measures using 70% alcohol. Each tube was labelled using a permanent ink pen with date, household/semi-bathan ID and time. Milk samples were immediately stored inside the icebox (4℃) containing an ice pack.

3.4.2. Bulk milk samples from middlemen: Samples were collected from the middleman within 1 hour from the collection of the milk from the household/ semi-bathan. An amount of 10 mL milk samples was taken at this point and kept in the icebox (4°C) immediately after collection.

3.4.3. Bulk milk samples from collection centres*:* An amount of 10 mL of mixed bulk milk was collected in a sterile falcon tube from each collection centre/shop approximately 1 hour after delivery by farmers or middlemen. The milk samples were stored immediately in the icebox.

3.4.4. Milk products from food shops*:* Milk products such as curd and cheese were collected from the different shops by using a sterile spoon. The samples were put in 10 mL falcon tubes and stored in the icebox immediately after collection.

After collection of all samples each day, the samples were transferred to the freezer (-10 to -15 ℃) available in the field visit area. Within seven days after collection, the frozen milk and milk product samples were transported to the Udder Health Bangladesh laboratory at Chattogram Veterinary and Animal Sciences University. The milk samples were stored at −20 ℃ and bacteriological culture was performed within 24 hours after thawing at room temperature.

3.5. Bulk milk somatic cell count

Bulk milk somatic cell count was performed by using a somatic cell counter (DeLaval Group, Stockholm, Sweden; Sensitivity 88% and Specificity 80%) immediately after

collecting the milk samples. The DeLaval cell counter (DCC) displays the BMSCC results as cells/ mL milk. Analyses were performed according to manufacturer instructions, but are described briefly below. A small amount of milk (around 60 µL) was loaded into the cassette and inserted into the DCC. The DCC determines SCC optically on a cassette by staining with a DNA-specific fluorescent reagent. With the help of a digital camera, pictures of the cell nuclei are taken one by one and the SCC results are displayed immediately on the screen. It took only 45 seconds to display the results after the cassette was inserted.

3.6. Isolation and identification of bacteria

A total of 132 bulk milk value chain samples and 10 milk products were undertaken for bacteriological evaluation following the National Mastitis Council (NMC) bulk milk isolation procedure by using Blood agar, Baird-Parker agar and MacConkey agar for *Non-aureus Staphylococcus (NAS), Staphylococcus aureus, Streptococcus* spp., *Escherichia. coli* and *Klebsiella* spp. Staphylococcal counts were done on Baird Parker agar (BPA) (Oxoid Ltd, Basingstoke, Hampshire, UK) and *Enterobacteriaceae* counts on Violet Red Bile Glucose Agar (VRBG) (Liofilchem ltd, Roseto Degli Abruzzi, Italy) from diluted milk samples (10^{-2} to 10^{-5}) (Hyera, 2015; Islam et al., 2018). Reasons for including these dilutions were to reduce the chance of missing bacteria from the bulk milk and chain milk samples. The detailed isolation procedures and further biochemical identifications are given in **Appendix II.**

As milk products are thick in consistency (curd) and solid dry (cheese) 1 g of curd and cheese samples were put into a test tube containing 0.9% 9 mL sterile saline and mixed thoroughly by using a vortex machine and then transferred 1 mL to the 2nd test tube from the 1st test tube. After that, 100 µL of diluted curd/cheese samples were further transferred from the first dilution (10^{-1}). This allowed a detection limit of 100 CFU/g. The dilutions were spread evenly to the surface of the same types of agar medium as for the milk product samples.

Specific bacteria were also isolated during the quantification procedure from BPA in *Staphylococcal* count and VRBG during *Enterobacteriaceae* count from the diluted curd samples (10^{-2} to 10^{-5}).

3.7. Preservation of isolates

All isolates were inoculated in 700 µL Brain Heart infusion (BHI) broth (Oxoid Ltd, Basingstoke, Hampshire, UK) in a 1.5 mL cryovial for each isolate with unique identity numbers from a pure culture of blood agar. The cryovials were incubated at 37°C for 24 hours. A quantity of 300 µL of 50% glycerol was added to the culture containing broth in the cryovials and stored at - 80°C until further investigation.

3.8. Quantification of bacteria

3.8.1 Total bacterial count

The TBC was performed by using the pour plate method (ISO, 2013). Milk samples were serially diluted up to 10^{-7} by transferring 1 mL of the milk (In case of curd 1g) from the original sample into test tube no.1 containing 9 mL 0.9% sterile normal saline and mixed thoroughly by using a vortex. Then 1 mL of the diluted milk samples was transferred to the next test tube until the final dilution was obtained from the last tube, 1 mL diluted sample was discarded. From each sample, 1 mL aliquots of each of ten-fold dilutions were mixed with 15-20 mL of molten plate count agar (PCA) (Oxoid Ltd, Basingstoke, Hampshire, UK) and the petri dish was rotated for 5 - 10 seconds to mix thoroughly with the media and left to solidify. After that, the plates were incubated aerobically at 30 °C for 72 h. Bacterial counts were made on up to five inoculated dilutions on the plates containing between 30 and 300 colonies and the last countable dilution was considered as a result and expressed as Cfu/ mL by using the following formula:

Colony count in the final countable dilution x dilution factor/ mL of milk × 1.1 = CFU/ mL of original culture.

3.8.2. Total Staphylococcal count

The TSA was determined by using the surface spread plate method as described by Viçosa et al. (2010). Milk/curd samples were serially diluted up to 10^{-5} as described for TBC. For each sample, 0.1 mL was vortexed and diluted milk/curd sample from each 10 fold dilutions of the sample were spread evenly over the solidified Baird-parker agar (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 37 °C for 48 hours. Counts were made for *S. aureus* (TSA) and Non-aureus *Staphylococcus* (TNAS) on the plates

with < 300 colonies to enable counting. The counts were converted into cfu/mL by following the formula.

Colony count in the final countable dilution x dilution factor/ mL of milk sample= CFU/ mL of the original culture

3.8.3 Total Enterobactericeae count

The TEC was determined by using the pour plate method (ISO, 2017). Milk/ curd samples were serially diluted up to 10^{-5} dilution as described above. From each dilution, 1.0 mL vortexed diluted milk/curd sample from each 10 fold dilution was mixed with 10 – 15 mL VRBG agar in a petri dish and rotated for 5 - 10 seconds for proper mixing. The agar plate was left to solidify and then an overlay of 5 - 10 mL VRBG was added to it. The agar plates were incubated at 37°C for 48 hours. Counts were made on up to four inoculated dilutions (10^{-2}- 10^{-5}) on the plates with counts < 300 and the last countable dilution was considered as a result and converted into cfu/mL using the below formula.

Colony count in the final countable dilution x dilution factor/ mL of milk sample= CFU/ mL of the original culture

Oxidase test was performed to differentiate the non-*Enterobacteriaceae* from the *Enterobacteriaceae* family. In this case, randomly selected five prominent colonies were undertaken for oxidase test during the counting of bacteria from each agar plate. Detailed procedure of the test was given in Appendix II.

3.9. Statistical evaluation

Collected data and results from bacteriological analyses were entered into the spreadsheet of Microsoft Excel 2013. Data cleaning, coding and integrity of the data were checked in MS Excel before exporting to STATA-IC-13 (StataCorp, Texas, USA) for statistical analysis. A p-value of ≤ 0.10 in univariable analysis was selected for multivariable logistic regression analysis. A p value less than or equal **0.05** was considered statistically significant in the final multivariable model.

3.9. 1. Descriptive statistics

Box plot analysis was performed for BMSCC at the farm level and the other bacterial counts (TBC, TSA, TNAS, TEC) to display minimum, first quartile, median, third quartile, and maximum values for every node of the milk value chain (farm level, middlemen, collection centre and shop level).

The prevalences of NAS, *S. aureus, Streptococcus* spp., *E. coli, Klebsiella* spp. were estimated by dividing the number of the samples positive of each type of pathogens with the total number of samples tested. The results were presented as the frequency, percentage and 95% confidence interval (CI). The prevalence was determined for each node of the milk value chain and frequencies of different bacteria along the nodes were compared descriptively.

Summary statistics were produced for some quantitative data such as the total number of animals, number of lactating animals, average milk yield etc.

3.9.2. Correlation between bacterial count

A Pearson correlation test was performed to investigate potential correlations between bacterial counts for the included bacteria. This test was performed using the log 10 values of BMSCC, TBC, TSA, TNAS and TEC. The r-value and p values were interpreted to assess potential significances.

3.9.3. Risk factor analysis

Risk factors were analyzed for mean log10 BMSCC and mean log10 TBC at farm level, middlemen, collection centre and milk production levels.

3.9.3.1. Univariate regression analysis: All factors were categorized into 2 to 3 categories based on a moderate frequency (30- 40%) in each case. The factors which didn't have a moderate frequency in each category were excluded from the association test. For other variables, either a t-test or 1-way ANOVA was carried out to test potential associations between the factors at the various nodes. Some quantitative factors were converted into categorical factors based on their frequency and percentage. Some categorical factors were also regrouped into fewer groups to ensure that the groups would be big enough to allow for analyses of association.

3.9.3.2. Multivariable linear regression analysis: Farm-level factors with a p-value of 0.1 were included in separate multivariable linear regression models (one for BMSCC and one for TBC). The models were constructed by applying the maximum likelihood estimation procedure following backward selection and the statistical significance of the contribution of the individual or group of factors. Variance inflation factor (VIF) and the Cook Weisberg test were also performed to check the multicollinearity and homogeneity of the variance respectively.

Chapter IV: Results

4.1. Farm characteristics

Most of the farmers included in the study had a primary level of education (up to grade V) (63.4%). The age of the farmers ranged from 18 to 58 years and all farmers were male. Farm size varied from 3 to 156 buffaloes (mean: 30.8; median: 24) of which there were 3 to 35 lactating animals per farm (mean: 9; median: 6). The milk production per farm ranged from 2-60 litres (mean: 11.8; median: 7). In most cases (81%) milk was sold within 30-120 minutes after milking, 12% within 30 minutes and the rest 7% was sold their milk more than 120 minutes after. About 57% of the farmers sold their milk to middlemen and 42% of farmers sold it directly to food shops.

97% of the farmers did not wash their hands and udder of their lactating animals before milking. The milking process was usually initiated by the farmer tying up the feet of the buffalo by rope and letting the calf suck. Milking was done by farmers (49%) or workers (51%) at an open field. The milk was kept at room temperature (96%) with no cooling facilities before being handed over to the middlemen/shops/market. Middlemen also reported that they transported milk without cooling. They had also no knowledge about hygienic milk transportation. Both farmers and middlemen thought that the milk quality remained intact at room temperature during transportation. As per farmers' response, the price of milk per litre at the farm was 45-100 BDT (from 0.45 to 1 €) (mean: 69; median: 60) which was lower than the retail price of milk in the market

4.2 Assessment of bacterial contamination

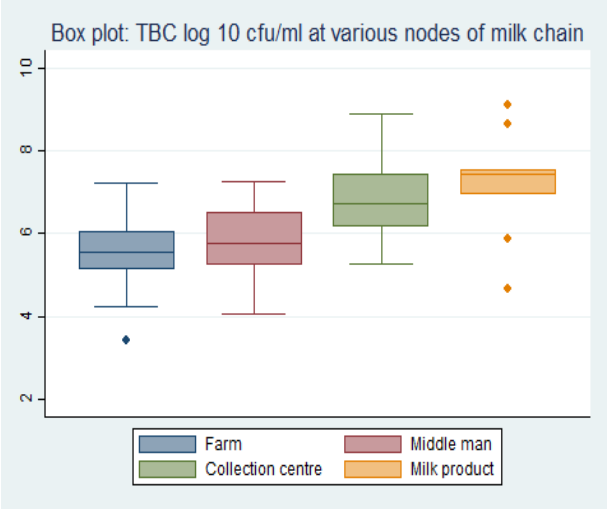
A total of 132 bulk milk value chain samples and 10 milk products were taken for assessment of bacterial contamination. In the case of the semi-bathan milk value chain, a total of 105 samples (36 samples from semi-bathan, 33 from middlemen and 36 from collection centre) and a total 27 samples (9 samples from household level, 9 from middlemen and 9 from collection centre) were collected under the household milk value chain.

Farm bulk milk sample (both household and semi-bathan level) were collected at the on spot from the farm. Middlemen samples were collected 1 hour later from the sample

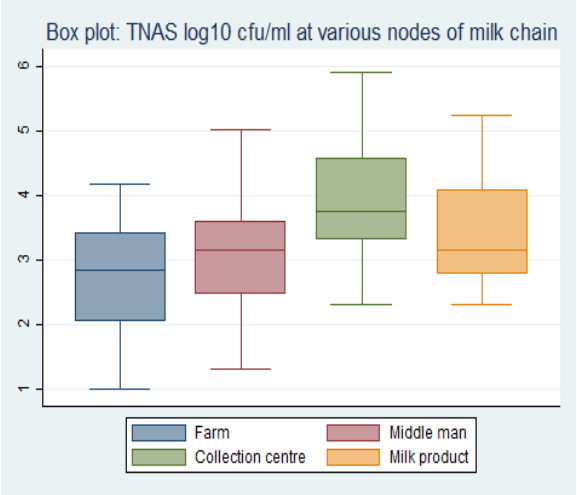
collected from the farm whereas samples from the collection centre were collected 1 hour later from the sample collected from the middlemen.

The level of bacterial contamination in sampled milk at the various nodes of the milk value chain is presented in the box plot below (Figure 4.1). Each box plot displayed the median, minimum, maximum and interquartile range of different bacterial counts. A progressive level of bacterial counts was found along the milk value chain at different nodes. A significant increasing level of bacterial contamination has been found for all the bacterial counts such as TBC ($p<0.001$), TNAS ($p<0.001$) and TEC ($p<0.001$) along the milk chain except for TSA ($p=0.48$).

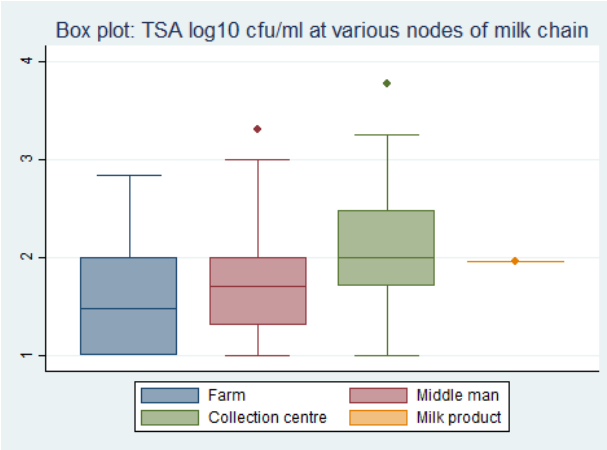
a. Total bacterial count (TBC)



b. Total Non-aureus *Staphylocooccuus* count (TNAS)



c. Total *Staphylococcal* count (TSA)



d. Total *Enterobacteriaceae* count (TEC)

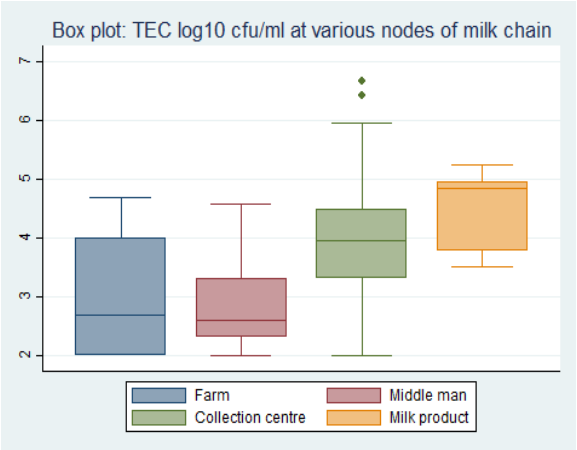


Figure 4.1: The median, minimum, maximum, interquartile range of different bacterial counts at various nodes of the milk chain

4.2.1. Bulk milk somatic cell count

The BMSCC was estimated at the included farms and is shown in the box plot below (figure 4.2). Among the 45 farms, the highest value of BMSCC was observed 6.08 log10 cell/ml with the mean value of 5.61 log10 cells/ml.

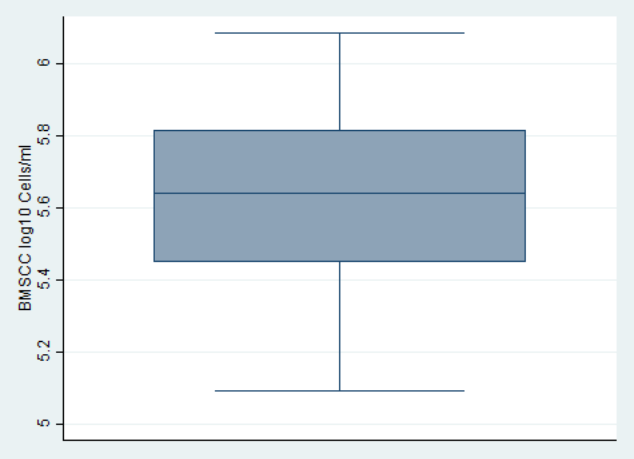


Figure 4.2: Bulk milk somatic cell counts at the farm level

4.2.2. Distribution of pathogens isolated from milk sample

The frequency of different pathogens in the different nodes of the milk value chain is displayed in Table 4.1. Non-aureus *Staphylococcus* had the highest frequency followed by *Streptococcus* spp. and *E. coli* across different nodes.

Table 4.1: Pathogen distribution at different nodes of buffalo milk value chain

Pathogens	**Farm (N=45)**	**Middleman (N=42)**	**Collection centre (N=45)**	**Shop (Milk products) (N=10)**
	% positive (n) *95% CI*	% positive (n) *95% CI*	% positive(n) *95% CI*	% positive (n) *95% CI*
Non-aureus Staphylococcus	78 (35) *65-90*	69 (31) *51-80*	71(32) *57-84*	0

Staphylococcus aureus	15 (7) *4-26*	26 (12) *13-40*	26 (12) *13-40*	20 (2) *-1-5*
Streptococcus spp.	57 (26) *42-72*	55 (25) *40-70*	71 (32) *57-84*	50 (5) *12-87*
E coli	18(8) *6-29*	18(8) *6-29*	29(13) 15-42	10 (1) *-12-32*
Klebsiella spp.	18 (8) *6-29*	11(5) *1-2*	4 (2) *-1-1*	20 (2) *-1-5*

CI: Confidence interval, N=Total number of samples

4.3: Pair-wise correlation of bacterial contamination at different nodes of the value chain

Pearson's correlation was performed to identify the significant correlations among different bacterial counts at various nodes of the milk value chain (Table 4.2). Significant positive correlations were found for TNAS and TEC with BMSCC (at farm level) and TBC at different nodes of the milk value chain.

Table 4.2: Pair-wise correlation matrix (Pearson's correlation) of bacterial contamination at various nodes of buffalo milk value chain

(Upper value denotes the r and lower value: italic bold front denotes the p-value)

	BMSCC	TBC
Farm		
TNAS	0.35 ***0.01***	0.55 ***0.00***
Middlemen		

TEC		0.40 ***0.05***
Collection centre		
TNAS		0.31 ***0.03***
TEC		0.39 ***0.02***

BMSCC: Bulk milk somatic cell count, TBC: Total bacterial count, TNAS: Total Non-aureus *Staphylococcus* count, TEC: Total *Enterobacteriaceae* count.

4.4. Risk factor analysis for bulk milk somatic cell count and total bacterial count at different nodes of the milk value chain

Univariable analyses were performed for the various risk factors and BMSCC, TBC at various nodes of the milk value chain to identify the significant association among these variables. Significant associated factors with BMSCC and TBC are presented in the table below for the farm level (Table 4.3) and shop level (Table 4.4). No significant factors were found for TBC at the middlemen and the collection centre level at univariable analysis.

The detailed analysis for the risk factor at various nodes is attached in **Appendix III.**

Table 4.3: Univariable analysis for bulk milk somatic cell count (BMSCC) and total bacterial count (TBC) at farm level

Variable name	**Categories**	**N**	**BMSCC (log Mean)**	**N**	**TBC (log Mean)**	**N**
Farm zone	Coastal	4	5.42	4	4.79	4

	Semi-coastal	30	5.63	30	5.58	30
	River basin	1	5.38	1	5.26	1
	Inland	10	5.66	10	5.92	10
		P	0.25		**0.06**	
How sell your milk	Contact basis to middleman	24	5.54	24	5.48	24
	Contract basis to shop	18	5.69	18	5.66	18
		P	**0.05**		0.4	
Source of milk	Household	9	5.59	9	5.96	9
	Semi-bathan	36	5.62	36	5.49	36
		P	0.7		**0.08**	
How do you clean your container	Hot water	2	5.83	2	5.40	2
	Tube well	7	5.46	7	5.42	7
	Both tube well water and pond water	2	5.86	2	6.25	2

	Tube well water with detergent	8	5.61	8	5.46	8
	Pond water	25	5.64	25	5.66	25
		P	**0.06**		0.63	
Frequency of cleaning milk container/day	Once	30	5.63	30	5.73	30
	Twice	15	5.58	15	5.29	15
		P	0.54		**0.05**	
The score of milker's hygiene	Excellent (Antiseptic and wash hand)	0	0	0		0
	Good (only wash hand)	2	5.71	2	4.77	2
	Poor (Never wash hand)	43	5.61	43	5.62	43
		P	0.55		**0.10**	

BMSCC: Bulk milk somatic cell count; TBC: Total bacterial count

Table 4.4: Univariable analysis for the total bacterial count at the shop level (dairy product)

Variables Names	**Categories**	**N**	**TBC (Log Mean)**
Place where made?	Shop	7	7.67

	Household	1	4.69
	Other	3	7.23
		P	**0.06**

#TBC: Total bacterial count

4.5 Multivariable linear regression model

The significant variables from univariable analysis for BMSCC, "How to sell your milk" (p= 0.06) and "How do you clean your container" (p= 0.06) (Table 4.3) and for TBC such as farm zones (p= 0.06), source of milk (p= 0.08), frequency of cleaning milk container/day (p =0.05) and a score of milker's hygiene (p= 0.10) (Table 4.3), were forwarded to multivariable linear regression analysis to identify the association between the significant risk factor and bacterial counts. However, none of the factors was determined as significant risk factors for either BMSCC or TBC.

Chapter V: Discussion

The present cross-sectional study was conducted to assess the microbial load of raw buffalo milk along the value chain and associated risk factors to give an overview of food safety and recommending preventive measures for this. Liquid milk for consumption is the main product in the informal milk value chain, while traditional sweets, yoghurt and ghee are locally processed dairy products (Jabbar, 2010). Milk and milk products can be contaminated by various types of microorganisms. The ability of these microorganisms to cause spoilage and disease mainly depends on the load of microbial contamination of the milk, milkers hygiene and post-harvest handling of the milk (Abera et al., 2016). However, it is crucial to managing the milk value chain carefully to maintain the quality of the milk and milk products. In the Noakhali district of Bangladesh, milk is produced mostly in unorganized and informal ways. Even though a very small proportion of milk is going to commercial processors, the biggest hurdle is to ensure the safety and quality of milk from the producers to the consumers. However, information on microbial quality and safety of buffalo milk and research outputs available on microbial load across the milk chain is lacking in the Noakhali region.

5.1. Bacterial counts along the milk chain

5.1.1. Total Bacterial count

The total bacterial count provides a reliable key indicator of milk quality and reflects the quality standard of primary production, collection, transportation and storage of milk (Hassainya et al., 2006; Islam et al., 2018). The mean TBC observed in the current study at the farm level was 5.54 log 10 cfu/ml which was slightly higher than several previous study findings on-farm bulk milk in Pakistan (5.29 log10 Cfu/ml) (Soomro et al., 2016) and in Egypt (5.37 log10 Cfu/ml) (Elshaghabee et al., 2017) but somewhat similar with study findings from China (5.59 ± 0.11 log10 Cfu/ml) (Han et al., 2007). Moreover, the value of TBC was found significantly increased across the milk value chain ($p < 0.001$) and the highest TBC was observed at the collection centre (6.80 log10 Cfu/ml) and milk product (7.24 log10 Cfu/ml). This increasing TBC level may indicate unsatisfactory cleaning and disinfection of the equipment or improper storage and transportation of raw milk in the markets (Kalmus et al., 2015). Similar findings of increasing TBC counts at

successive stages through the supply chain has also been documented previously (Pyz-Łukasik et al., 2015; Shivaji, 2017; Islam et al., 2018). Inadequate hygiene practice during harvesting of milk might be attributed to high levels of TBC as well as for presence of pathogenic bacteria in milk samples (Kalmus et al., 2015). However, many countries have set legal limits for TBC in milk and milk products. The limit values for TBC at 30°C established by the European Union Regulation (EC) No(EU) are 5 log10 cfu/mL for raw cow milk; 5.17 log10 cfu/mL for raw milk from species other than dairy cows; in addition, 5.6 log10 cfu/mL limit for raw milk from species other than cows used for manufacturing cheese products without heat treatments (Pasquini et al., 2018). All countries within the European Union (EU) have to follow the European legislation criteria for TBC in raw milk. The higher TBC in the present study compared with that in the EU legislation could be contributed by poor animal husbandry practices, unclean udder and teats, personnel, unhygienic milking, and/or use of inferior water for washing and drinking as well as poor storage conditions (Khan et al., 2011). Similarly, transportation time of the milk to the collection point and use of inadequately cleaned instruments may also be attributed to the excessive TBC as well as for the presence of pathogenic bacteria in milk samples (Kalmus et al., 2015; Islam et al., 2018). Another reason for high levels of TBC and the presence of pathogenic bacteria may be that the buffaloes are mostly predilected for water and muddy places and they consistently sit in dirty and unhygienic milking places. They may also be in close contact with diseased animals in common grazing and wallowing places (Dhakal, 2006) which could be an another reason for high TBC level and presence of pathogens. However, to improve the hygiene quality of milk offered through direct sale points, it is necessary to increase compliance with good hygienic practices of milk production and processing.

5.1.2. Total *Staphylococcal* count

Milk and milk products have frequently been implicated in staphylococcal food poisoning where contaminated raw milk is the major player (De Buyser et al., 2001). Total Staphylococcal count in raw milk is also an indicator of the safety and quality of raw milk. However, both *S. aureus* and NAS can produce toxins and be reservoirs of antimicrobial resistance genes and biofilm producers (Khoramrooz et al., 2016; Saka and Terzi Gulel, 2018; Pizauro et al., 2019). The present study found an increasing level of

Staphylococcal count from producer to consumers. The mean *S. aureus* count observed at farm level was 1.7 log10 cfu/ml which increased to 2.14 log10 cfu/ ml at the collection centre, though the level of increase was not statistically significant (p=0.48). In the case of NAS counts, a significant increase ($p< 0.001$) was observed from the farm level (mean NAS count 2.67 log10 cfu/ml) to the milk product (mean NAS count 3.44 log10 cfu/ml). Similar results were also documented by other study (Han et al., 2007; Kuyucuoğlu and Pamuk, 2013) at the farm level and also in collection centre (Saad et al., 2017). According to the EU criteria, the acceptable limit for *S. aureus* count in raw bovine milk from marginally acceptable quality is 2.70 log10 CFU/ml; and for defective quality is 3.30 log10 CFU/ml (EC, 1992). However, *S. aureus* count between 6-8 log10 cfu/ml) are considered significant for human food poisoning (Kérouanton et al., 2007; Mhone et al., 2011). The current study finding was lower than that limit but still, from a public health perspective, the general hygienic practices aimed at minimizing bacterial contamination of milk post-pasteurization should be emphasized (Mhone et al., 2011). *S. aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing of raw milk (Scherrer et al., 2004; Jørgensen et al., 2005). The high *Staphylococcal* counts may be in part the consequence of improper storage and poor hygiene according to the nature of surfaces and number of people handling the food (Chen et al., 2001).

5.1.3. Total Enterobacteriaceae Count

The presence of *Enterobacteriaceae* is often considered to be an indicator of quality for processed foods, especially concerning good manufacturing and processing practices (Mullane et al., 2006). The presence of *Enterobacteriaceae* has been steadily adopted by the European Union as an index of food safety. The present study found that the mean TEC at the farm level was 2.94 log10 cfu/ ml which was lower than the observed value in bulk milk of buffalo in Egypt (EL-Tantawy et al., 2018). The mean TEC across the nodes were also lower than the other study findings (Ferraz et al., 2010; Wanjala et al., 2017; Sobeih et al., 2020), however, the level of increasing TEC was found statistically significant in the current study ($p< 0.001$). According to the NSW food authority, the total *Enterobactericea* count of 2 log10 cfu/ml in ready to eat food is considered as good

whereas 2 to < 4 log10 cfu/ml is considered as acceptable limit and ≥ 4 log10 cfu/ml is regarded as unsatisfactory (NSW,2009). However, the presence of *Enterobacteriaceae* might be due to the unhygienic milk handling practices which in turn may cause an enzymatic breakdown of proteins or lipids, instigating spoilage that contributes to substantial economic losses and waste (Baylis et al., 2011). *Enterobacteriaceae* is also a good indicator of contamination of equipment caused by for example environmental sources. However, members of this family are sensitive to thermal treatments and sanitisers. So proper heat treatment of milk, either by pasteurization or by ultra-high temperature safely inactivates *Enterobacteriaceae* (Odenthal et al., 2016). Therefore, TEC in milk should be routinely assessed to determine the hygienic quality of foods, particularly dairy products (Martín et al., 2010).

5.2. Bulk milk somatic cell count at herd level

Bulk milk somatic cell count is the key indicator of milk quality, udder health and prevalence of mastitis at herd level (More, 2009). It is monitored at the producer level to ensure the supply of high-quality milk to the consumers. The current study found the mean BMSCC was 5.61 log10 cells/ ml which was higher than reported in some previous studies of buffalo milk (Sharif et al., 2007; Costa et al., 2020; Singha et al., 2021). There are regulations on levels of BMSCC in bovine milk in many developed countries, for example in Italy, Sweden (5.60 log10 cell/ml), Norway (5.06 log10 cells/ml), Ireland (5.39 log10 cells/ml) and Canada (5.69 log10 cells/ml) (Norman et al., 2000), but there is no standard or regulation limit set for BMSCC in buffalo milk yet. However, most of the countries have adopted the European standard level (5.60 log10celll/ ml) as the international export standard for milk. But a decreasing trend is observed in the coming years since the national BMSCC scores in the Scandinavian countries ranged from 5.30-5.39 log10 cell/mL (More, 2009; Mesquita et al., 2019). *Several factors* such as the number of days in milking, age, breed, parity, season, milk transportation, and stress (Sharma et al., 2011) as well as milking technique, housing conditions, disease prevalence and breed effects might be associated with high levels of BMSCC in bulk milk (Koop et al., 2009). Elevated levels of BMSCC have a negative impact on milk quality and the manufacturing properties of milk and milk products. High BMSCC has been shown to reduce the quality and shelf life of pasteurized milk as well as causes

sensory defects, mainly included rancidity and bitterness, and higher levels of lipolysis and proteolysis (Ma et al., 2000). An elevated BMSCC is also associated with a higher prevalence of *S. aureus, Streptococcus* spp. and NAS *(Riekerink et al., 2006; Schukken et al., 2009). So, it is necessary to* maintain the standard limit of BMSCC intended for direct sale to ensure safe milk for consumers and safe production of dairy products (Kalmus et al., 2015).

5.3. Correlation with BMSCC and different bacterial counts

Significant positive correlations among the different bacterial counts and BMSCC at different nodes of the buffalo milk value chain were found in the current study. Several studies have reported a positive correlation between BMSCC and TBC in different livestock species, such as a positive correlation between BMSCC and TBC of r = 0.23 (Gonzalo et al., 2006) in sheep, r = 0.32 in cows (Jayarao et al., 2004), r = 0.4 in goat (Koop et al., 2009). The current study found a weak correlation (r=0.14) between BMSCC and TBC which was statistically non-significant. Similar findings were also observed in a milk chain study (Pyz-Łukasik et al., 2015). This result might be due to the small sample size at various nodes. However, a positive significant correlation between TBC and TEC was found at the levels of middlemen and collection centres which was also reported in several studies (Jayarao et al., 2004; Pyz-Łukasik et al., 2015; Marshall et al., 2016). This finding suggests that TBC is a suitable proxy for faecal contamination of bulk milk (Marshall et al., 2016). Statistically significant positive correlations were also found between TBC and TNAS count (r=0.55, p<0.001) at farm level and collection centres (r=0.31, *p* =0.03) (r = 0.44; P < 0.05), which were also observed by Pyz-Łukasik et al. (2015). All other correlations between bacterial counts were non-significant in the current study.

5.4. Risk factors associated with bacterial contamination

Bacterial count and BMSCC can be associated with several risk factors. In the current study the risk factors “How farmers sell their milk” (p= 0.06) and “How they clean the milk container” (p= 0.06) were found significant factors associated with the bulk milk somatic cell count and farm zones (p= 0.06), source of milk (p= 0.08), frequency of

cleaning milk container/day (p =0.05) and a score of milker's hygiene (p= 0.10) were found significant factors for TBC in the present study.

Milk in the informal markets is normally transported to collection centers or shops. Once the milk is received it is directly sold to the consumers. The means of transportation are mainly motorbikes, bicycles, or using public transports. The composition of several nutrients and other features of the milk, such as high water activity make the milk an outstanding medium for bacterial growth. However, milk is usually transported in plastic buckets, in drums or milk cans without cooling facilities. The conditions of the milk cans used in the process of transportation largely affect the quality of milk (Chege and Ndungu, 2016). Moreover, in most cases, the middlemen don't follow adequate hygienic procedures during the transportation of milk. The milk contamination can be increased at any point during milk transportation because the cleanliness of equipment during milk transportation is highly crucial to control microbial contamination of milk (Amenu et al., 2016). Poor road network systems increase the time for transportation and distribution of raw milk, and coupled with poor cold chain facilities, allows the rapid growth of pathogens in raw milk (Owusu-Kwarteng et al., 2020). However, the use of narrow mouth storage containers with valves (Clasen and Bastable, 2003) can minimize microbial contamination. It is also needed to maintain a cool chain from milking and throughout transportation (Li et al., 2016)

How farmers clean the milk containers were also found to be a risk factor for increasing contamination level. Tigabu et al. (2015) found that dairy farms that cleaned milk containers only with cold water had doubled the risk of contamination with *S. aureus* compared to those who cleaned the milk containers with detergent and cold water. He also found that the risk of contamination of the milk with *S. aureus* was reduced by 66% when hot water used with detergent to clean the milk container compared. However, the use of detergents and good quality water for cleaning the equipment could be expected to remove milk dirt including micro-organisms and thereby improve the microbiological quality of milk (Bonfoh et al., 2006; Piepers et al., 2014; Tegegne and Tesfaye, 2017). Thus, farmers must use clean, safe water free from any type of bacterial contamination that may affect milk quality (Chye et al., 2004)

Different farm zones (coastal, semi-coastal, river basin and inland) and buffalo farming systems (household and semi-bathan) were found to be significant factors for TBC in the present study. Most of the milk samples were collected from the semi-coastal area where farmers mainly followed the semi-bathan farming system. Large numbers of buffaloes were kept together in the semi-bathan area in the water and in the muddy places, which easily contribute to unhygienic milking conditions. Habib et al. (2017) reported that the management practices adopted by buffalo farmers usually depends on the type of production in which they are involved. Unhygienic conditions of the milking area may lead to the increasing level of intramammary infection which result in high bacterial contamination in the bulk milk (Sharma et al., 2011). Other management factors such as the type of flooring, conditions of the surrounding, urination of the animal during milking may also increase the bacterial contamination in milk (Sharma et al., 2011; Islam et al., 2018).

The cleanliness of milking equipment influences the total bulk milk bacterial count more than any other factor (Afzal et al., 2011). Milk drops left on the surface of milking equipment act as excellent media for the growth of a variety of bacteria. The current study reported the frequency of cleaning milk containers as a borderline risk factor for TBC which was also documented in other studies where it was significantly associated with TBC in raw milk (Piepers et al., 2014; Lan et al., 2017). The cleanliness and hygiene of milking equipment surfaces act as a triggering factor affecting the TBC in bulk-tank milk (Vilar et al., 2008). Hence effective systems for ensuring the cleanliness of milking equipment are essential to keep low levels of TBC in milk.

A score of milker’s hygiene was also found significantly associated with levels of TBC. Dirt and pathogens may also emanate from the milker's hand into the milk (Chege and Ndungu, 2016). In the study area, all the farmer's milk by hand and milking was initiated by the teat suckling of the calf which could be the reason for increasing bacterial count in milk due to residual effect after calf sucking. Similar findings were also documented by (Islam et al., 2018; Regasa et al., 2019) where hand washing practices were found to be significantly associated with microbial contamination. It is also observed that washing hands with soap and water reduces the contamination level more compared with those

producers who wash their hands with only water before milking (Islam et al., 2018) . So, it is necessary to maintain proper milker's hygiene to reduce the TBC in milk.

Even when better hygiene is applied at one single point of the value chain, limited hygiene at other steps allowed microbial contamination with subsequent bacterial multiplication, resulting in similar microbial quality of the end product regardless of variation in upstream hygienic practices. However, no significant risk factors in the multivariable model were detected in the current study. Therefore, it is needed to ensure end-product microbial safety in all aspects of production and a final kill-step to remove upstream contamination, with subsequent hygienic handling and refrigeration (Knight-Jones et al., 2016).

5.5. Pathogens level at various nodes of the milk value chain

Non-aureus *Staphylococcus* was the most prevalent micro-organism found in the present study (78%) at the farm level which was comparable to findings documented in some previous studies (Ali et al., 2011; Guha et al., 2012; Pisanu et al., 2019). In the present study, the prevalence was similar at the middlemen and collection centre levels. NAS has been reported as a main bacterial species isolated from water buffalo milk causing IMI in several studies (Moroni et al., 2006; Ali et al., 2011; Guha et al., 2012; Locatelli et al., 2013; Singha et al., 2021). Their presence on human and animal skin, mucosae and from environmental samples easily introduced them into the milk (Otto, 2004; Seo et al., 2008; Piette and Verschraegen, 2009) that might explain the high frequency of their presence in the milk chain. But, NAS are rarely involved in foodborne illnesses, despite the high incidence of enterotoxin genes detected in their genomes (Bertelloni et al., 2015). However, the high frequency of *Staphylococcal* enterotoxin genes suggests a potential role of NAS in spreading enterotoxigenic determinants among other microorganisms; thus, the concern about the presence of NAS in milk and dairy products remains.

The 2nd most prevalent organism found in the current study was *Streptococcus* spp. (57%) at the farm level which increased to 71% at the collection centre level. A high prevalence of *Streptococcus* spp. was also found in several studies (Phuektes et al., 2003; Zadoks et al., 2004; Kalmus et al., 2015). This can be explained by the fact that *Streptococcus* spp. usually remain in the teat and on the skin of the animal, and some species are found in the

environment (especially floors, cow beds, water troughs) (Jørgensen et al., 2005). Also, cows that are affected by *S. agalactiae* infections can shed very high levels of the bacteria into the bulk tank and cause elevated plate counts (Maroney, 2005). Some species act as a potential zoonotic pathogen and might cause significant morbidity and mortality in both infant and adult humans when present in milk (Kalmus et al., 2015).

The prevalence of *S. aureus* and *E. coli* were lower (compared with NAS and *Streptococcus* spp.) at farm level (15% and 18%, respectively) but their counts also increased along the milk chain and were also detected at the milk product. Various studies have also documented the presence of *S. aureus* and *E. coli*. at the bulk milk and chain milk (Phuektes et al., 2003; Ali et al., 2011; Kalmus et al., 2015; Yang et al., 2020). However, *S. aureus* is of particular importance in the milk value chain as it produces a wide range of virulence factors and antibiotic resistance patterns (Ben Said et al., 2016; Hoque et al., 2018). The presence of *S. aureus* in raw milk might be due to clinical or subclinical or clinical mastitis or during unhygienic handling and processing of milk which possess a potential public health hazard for humans (Pyz-Łukasik et al., 2015). Similarly, the presence of *E. coli* in milk indicate possible contamination by manure, soil, equipment and water. *E. coli* can also contaminate the milk through infected food handlers who practice poor personal hygiene or by water containing human discharges (Chye et al., 2004). However, increasing contamination levels at other nodes in the current study may be the outcome of poor hygienic conditions practiced by middlemen and collection centre during handling of milk and an insufficient cold chain that favors exponential growth of previously introduced microorganisms. Other factors contributing to bacterial growth may be an extended time of transportation of the milk to chilling plants and possibly also due to dirty instruments employed in the milk chain (Islam et al., 2018).

Klebsiella spp. is another opportunistic gram-negative bacteria isolated from the current study in bulk milk (18% at the farm level) and milk product (20%) which was higher than in some previously documented studies (Ali et al., 2011; Yang et al., 2020). *Klebsiella* spp. has been isolated from bovine mastitis in several studies (Gröhn et al., 2004; Munoz et al., 2007; Gao et al., 2019) as well as from bedding material, faeces, manure splash, water, milk, mattresses, or milking machine (Munoz et al., 2006; Munoz et al., 2008).

This could explain why *Klebsiella* spp. was found in the milk chain in the present study. However, the presence of *Klebsiella* spp. in feed refusals, water troughs, and oral cavities of cows is most likely the result of faecal contamination (Munoz et al., 2007). So, proper hygienic management to reduce faecal contamination is required here.

However, the generally high prevalence of microorganisms in the current study suggests proper hygienic management in all steps to reduce the milk contamination along the milk chain as well as at the farm level.

5.6. Farm characteristics and farmer's perception

Most of the households in the current study had 1-3 buffaloes, which reflects that mainly household farms were included. People were mainly illiterate (30%) while some farmers had primary (3%), secondary (3%) and graduation (3%) levels of education. The study showed that 80% of the farmer's age varied between 18 to 45 years of old while 20% of the farmer's age was above 46 years old which was similar to the findings of Amin et al. (2015). Farmers in the study area didn't wash their hands before milking (97%) and also didn't wash the udder as well as didn't use any pre and post dipping of the lactating buffalo. This could be at the background of high BMSCC and other bacterial counts in the study area. About 57% of the farmers sold their milk to middlemen and 42% of farmers sold it through contact basis shop. Similar study findings have been reported from Noakhali in which 58% of the farmers sold their milk through middlemen and 42% sold directly to the processor and at the local market (Uddin et al., 2016). The milk was kept at room temperature (96%) with no cooling facilities before being handed over to the middlemen/shops/market. This practice favors an exponential growth of previously introduced microorganisms in the milk chain.

5.7. Limitations of the study

1. Sample size: The study was conducted on a small scale with limited numbers of samples based on certain assumptions for each node of the value chain. Besides, the sudden death of buffalo at one sampling area due to less availability of feed and the corona pandemic situation were also the reason behind smaller small sample size.

2. Diagnostic error: Due to the remote area from the bacteriology lab, it was not possible to culture the milk samples immediately after collection. This reason could be attributed to the mild alteration of the microbial population in the collected samples. Besides, the sensitivity and specificity of the BMSCC test was 88% and 80% respectively. This might also intend some false positive and false negetive error.
3. Statistical assumption and selection bias: The farms and households were mostly selected by the convenient selection method. A little number of household and milk product samples were introduced into the statistical evaluation that can underestimate or overestimate the risk factors results.

Chapter VI: Conclusion, Recommendations and Future Directions

6.1. Conclusions

The study findings indicate that the milk quality is lower than the standard level in the study area, which might put people living there at a great risk of illnesses from food-borne pathogens as a result of poor milk handling along the informal milk value chain.

The study revealed significant increasing numbers of bacterial counts along the milk value chain. The microbial contamination increased gradually at the successive nodes of the value chain. In all cases, it has been observed that the bacterial counts were above the legislative standard limits of milk quality. Similarly pathogens were found at all nodes investigated at the milk value chain and the presence of zoonotic pathogens, such as *S. aureus, E. coli, Klebsiella* spp., were the highest at the collection centres and in the milk products. This indicates that the contamination of milk may increase due to lack of temperature control which favors exponential growth of previously introduced microorganisms. Other factors may include long transportation of the milk to the collection centre and possibly also use of dirty containers to for transportation and storage of milk and milk product.

Understanding the level of contamination and associated risk factors is crucial first step for design of control strategies to combat this public health issue. An unmet need for good hygienic practices required to be urgently introduced at each level of milk production starting from producers to the consumer level. It is also necessary to develop the infrastructures of the buffalo milk value chain and standard quality check program should be introduced before the milk reaches to the final consumer. Paying attention to the mentioned actions can help to improve milk safety and quality and thereby reduce the risk of food-borne illnesses.

6.2. Recommendations

I. BMSCC should be monitored regularly to identify subclinical mastitis at the farm level. A single animal affected with subclinical mastitis can contaminate the whole bulk milk which can have negative effects on milk quality and safety at all subsequent steps in the milk value chain. The microorganisms which have been

introduced into the bulk milk at the farm level also get the longest time for growth and multiplication.

II. Milk quality parameter such as TBC, TSA, TNAS and TEC should be checked before the milk reaches the final consumers to ensure the hygienic status and quality of milk. Therefore, it is needed to develop a standard limit of BMSCC and other bacterial count for buffalo milk. Elevated bacterial counts in milk have decisive effects on the quality and safety of dairy products and cause enzymatic breakdown of proteins or lipids, instigating spoilage contributing to substantial economic losses. Thus, the quality of the locally produced milk must be monitored carefully on a regular basis.

III. Various risk practices along the milk value chain should be analyzed and proper action should be taken to minimize the bacterial contamination of milk.

IV. Farmers should be provided with basic training on hygienic milk production and transportation. Also, least cost cooling facilities should be created on-farm which will help them to improve their insight microbiological quality of milk.

V. The government should establish a buffalo dairy policy framework. Such policy should include some strategic measures aiming to increase quality milk production targeting buffalo farmers.

6.3. Future Directions

I. The study area was limited to the Noakhali district. In order to investigate the features of the milk value chains at different production system in different regions as well as to determine the status of milk quality, similar studies should be conducted across the buffalo concentrated zones of Bangladesh.

II. A longitudinal study should be conducted by including a small number of farms to investigate risk factors for microbial contamination and obtain long term data on BMSCC and other bacterial counts. This will allow estimating the BMSCC and other bacterial count at different rearing system as well as seasonal fluctuation can be observed.

III. Future studies should be conducted to describe isolated bacterial organisms at species level together with their antibiogram and biofilm production.

Chapter VII: Appendices

Appendix I: Questionnaire survey

Survey questionnaire for assessing risk factors associated with contamination level of buffalo milk chain at Noakhali, Bangladesh.

Objectives:

1) Identification of different practices at various nodes of buffalo milk value chain in Noakhali, Bangladesh
2) Assessing the level of contamination of milk/milk products associated with various practices along the buffalo milk value chain in Noakhali, Bangladesh

Sample ID:	Date

A) Study unit: Household/ Bathan

1. General information:

1.1. Name of the interviewee:			1.2 Mobile no:	
1.3. Gender and Age ☐ Male ☐ Female			☐ Age:	
1.4. Location	Village:	Union:	Upazilla:	
1.5. GPS coordinates : Latitude (Degree): Longitude (Degree): Elevation from the sea level:				
1.6. Title of Interviewee:	☐ Owner	☐ Manager	☐ Milk collector	☐ Other ……..
1.7. Educational status:	☐ Illiterate	☐ Primary	☐ Secondary	☐ Graduation

1.8: Composition of household/bathan	No. of Lactating	No of dry	No of heifers	No of calves	Average milk yield (Farm) per day (Litres)
1.9. Selling price of milk(BDT/Litre)					
1.10. Where do you sell your milk?	On spot sell from farm/ middle man/ milk collection centre				
1.11. How you sell your milk?	☐By own basis to shop ☐ Contract basis to middle man ☐ Contract				

2. Source and type of sample

2.1. Source of milk	☐ Household	☐ Bathan	☐ Semi-bathan
2.2 Nodes type of value chain	☐ Farm/household /Semi-bathan/Bathan	☐ Trader shipment (at entry)	☐Selling point (Couple of hrs later)
2.3.Farm bulk milk somatic Cell Count (BMSCC)			
2.4. Type of Sample		☐ Milk	☐ Milk Product: curd/ghee/Sweet/milk drink/……
2.5 Time of sample collection			

3. Milk Container

3.1. Types of container use	☐ Aluminum ☐ Plastic ☐ Others…

3.2. What do you use to clean the container?	☐Hot water ☐Tube well water ☐ Tube well water with detergent ☐ Pond water ☐Others
3.3. Frequency of cleaning of milk container (per day)?	☐ Once ☐ Twice ☐ Thrice
3.4. Cleanliness score of milk container	☐ Excellent (no greasiness and dirt inside and outside the container) ☐ Good(No greasiness and dirt inside the container) ☐ Poor(greasiness and dirt present)
3.5. Do you use brush or something like that during cleaning container	☐ Yes ☐ No
3.6. Do you dry the container after cleaning	☐ Yes ☐ No
3.7. If yes, how you dry the container?	☐ Sun/air dry ☐ Using cloth ☐ Using tissue paper
3.8. Do you keep the container upside down during drying?	☐ Yes ☐ No
3.9. Do you keep the bulk milk container open during each milking?	☐ Yes ☐ No
3.10. If no, what do you use for covering?	
3.11. Do you use anything for sieving milk after milking into the BM container?	☐Yes ☐No
3.12. If yes, what do you use?	☐ Cloth ☐ Plastic sieve ☐ Other…

4. Milker's and Buffalo hygiene

4.1. Who does milk the buffalo cows?	☐ Owner ☐ worker ☐

	Others……..
4.2. Do you wash buffalo before milking?	☐ Yes ☐ No
4.3. If yes, which type of water do you use for bathing?	☐ River ☐ Pond ☐ Tube well
4.4. Does the milker wash udder before milking?	☐ Yes ☐ No
4.5. Does the milker dry the udder before milking?	☐ Yes ☐ No
4.6. If yes, how they dry the udder?	☐ Wait for air dry ☐ Using individual cloth ☐ Using common cloth ☐ Others……
4.7. Does the milker wash hand before milking?	☐ Yes ☐ No
4.8. Score of milker's hygiene?	☐ 1=Excellent=Milkers use antiseptic and wash hand ☐ 2=Good=Milkers only wash hand ☐ 3=Poor=Milkers don't wash
4.9. Score of udder hygiene	☐1=Excellent=Udder is clean and dried ☐2=Good=Udder is clean but not dry ☐ 3=Poor=Udder is not clean enough
4.10. Does any buffalo affected with clinical mastitis (either changes in milk, udder, systematic weakness)?	☐ Yes ☐ No
4.11. Do you mix the mastitis milk with normal milk?	☐ Yes ☐ No

5. Storage:

5.1. Milk storage at home	☐ Using lid ☐ Without lid
5.2. Storage time before shifting from home?	☐……. day ☐……Min ☐……. Hour
5.3. How do you store milk at home?	☐ Room temp ☐ Cold storage ☐Freezer

5.Some generic question to farmers:

9.1 Do you face any problem during milking of your animal?	☐ Yes ☐ No
9.2. Do you face any difficulty to store milk at home before shifting?	☐Yes ☐ No
9.3Do you have proper transportation facility to shift the milk to shop?	☐Yes ☐ No
9.4. Do you get the proper price by selling milk?	☐Yes ☐No

Declaration: I have answered all the questions in the interview sheet and I have full consent about the information given. Best of my Knowledge, the information given by me is correct and can be used in research. If necessary the researcher can contact me for further information or vice versa in future.

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Interviewer signature Interviewee signature

B) Middlemen

6. Milk transportation

Sample ID:	Date

Name of person: …… Mobile No: ……..	Type of trader: Whole seller curd producer/ Occasional retail curd producer/ Middleman/ Family consumer/ Other…….
Latitude:	Longitude:
6.1. Transport milk sample through(by boat/bicycle/both/others….)	
6.2. Types of container use	☐ Aluminum ☐ Plastic ☐ Others…
6.3. Covering material during transport	☐ Cloth ☐ Plastic plate ☐ Aluminum plate ☐ Banana leaves
6.4. Travel time to collection point	☐…….min ☐……hour
6.5. Do you use any material inside the container to prevent milk spoilage during transportation?	☐Yes ☐No
6.6. If yes, what you use?	☐ Leaves ☐ Ice cubes ☐ Others…
6.7 Trading experience of milk transporter	☐ Yes ☐ No
6.8. Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
6.9. Frequency of cleaning milk container per day?	☐ Once ☐ Twice ☐ Thrice
6.10. Do you clean the milk container after each shipment?	☐Yes ☐No
6.11. What do you use to clean the milk container?	☐Hot water ☐Tube well water ☐ Tube well water with detergent ☐ Pond water ☐Others

6.12. Cleanliness score of milk container	☐ Excellent (no greasiness and dirt inside and outside the container) ☐ Good(No greasiness and dirt inside the container) ☐ Poor(greasiness and dirt present)
6.13. Do you use brush or something like that during cleaning container?	☐ Yes ☐ No
6.14. Do you dry the container after cleaning?	☐ Yes ☐ No
6.15.If yes, how you dry the container?	☐ Sun/air dry ☐ Using cloth ☐ Using tissue paper
6.16. Do you keep the container upside down during drying?	☐ Yes ☐ No

C) Collection centre

Sample ID:	Date

Name of person: …… Mobile No: ……..	Type of trader: Whole seller curd producer/ Occasional retail curd producer/ Middleman/ Family consumer/ Other……
Latitude:	Longitude:
Time (Milking to arrival to market/ hand over to middle man)…… min/ hour	Travel time to collection point (Walking time/ time by vehicle)…… min/ hour
7.1 Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
7.2. Types of container use at selling point	☐ Aluminum ☐ Plastic ☐ Others……
7.3. Milk storage at shop	☐ Using lid ☐ Without lid
7.4. Milk kept in cold storage/freeze	☐ Yes ☐ No
7.5. Duration of milk kept at shop	☐…….min ☐……. Hour ☐……. Day

D) Milk product

Sample ID:	Date

Name of person: …… Mobile No: ……	
Latitude:	Longitude:
8.1. Types of the product	☐ Curd ☐ Ghee ☐ Sweet ☐ Other
8.2. Where it is made?	☐ At shop ☐ At households ☐ Other
8.3. Storage time	☐……. day ☐……Min ☐……. Hour
8.4. Containers used	☐ Earthen pot ☐ Plastic container ☐ Other
8.5 Type of seller?	☐ Retail seller ☐ Whole seller
8.6 Source of milk purchase	☐ Own shop ☐ Buy milk from others
8.7 Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
8.8 Milk used	☐ Boiled ☐ Unboiled

Appendix II: Isolation and Identification of Bacteria

Isolation of bacteria from Blood agar:

A total of 132 bulk milk value chain samples were undertaken for bacteriological evaluation in accordance with National mastitis council bulk milk isolation protocol (NMC, 2017)

Blood agar inoculation:

Milk samples stored at -20°C was thawed at room temperature and inoculated into the freshy prepared blood agar (Oxoid, Basingstoke, Hampshire, UK). 0.01 ml of milk sample after properly vortex was streaked vertically the diameter of blood agar plate (BA) and incubated 37°C for 24- 48 hour. All dominant colonies were selected for further inoculation into blood agar to get pure isolate and for each samples, colony characteristics such as size, color and appearance were recorded. Further identification was done by using selective media and biochemical test.

Manitol Salt agar inoculation:

The bacteria that were properly grown on blood agar and also those who produced characteristics hemolysis properties on blood agar were further inoculated into Mannitol Salt agar (MSA) (Oxoid, Basingstoke, Hampshire, UK) and incubated for 24 hr at 37°C. Those who produce ß- hemolysis on blood agar and bright yellow coloured on MSA and coagulase positive were considered as *S. aureus*. Those who are MSA positive (pink color) and coagulase negative were consider for Non-aureus *Staphylococcus* and those who are MSA and MAC negative but produce minute transparent growth was observed in blood agar were considered as *Streptococcus* spp. (Persson et al., 2011).

MacConkey agar inoculation:

Bacteria which give opaque transparent colonies in blood agar were further inoculated into MacConkey agar (MAC) (Oxoid, Basingstoke, Hampshire, UK). *E. coli*. produce large pink color colony due to lactose fermentation after incubation of 24 hrs at 37°C where non-lactose fermentative bacteria produces colorless colonies (Persson et al., 2011). The suspected pink color colony from MAC agar were further inoculated into Eosin Methelene Blue (EMB) (Oxoid ltd, Basingstoke, Hampshire, UK) agar and

incubated for 24 hours at 37ºC. *E. coli.* produces characteristics metallic sheen on EMB. Colony which give mucoid large shiny and dark pink colony were identified as *Klebsiella* spp. (Dasohari et al., 2017)

Isolation of bacteria from Baird-parker agar:

0. 01 ml of milk sample after properly vortexed was streaked in the Baird-Parker Agar (Oxoid, Basingstoke, Hampshire, UK) enriched with Egg Yolk Tellurite Emulsion and incubated at 37°C for 48 hour. *Staphylococci* spp. can reduce tellurite to telluride, which results in grey to black coloration of the colonies. A clear halo develops around colonies were considered as positive for *S. aureus* and further inoculated on MSA and Blood agar colonies to observe the bright yellow in MSA and B- hemolysis on blood agar and undergo for biochemical test

Isolation from the MacConkey agar:

After enrichment the milk sample in MacConkey broth, 0.01 milk samples were streaked into the MAC agar and incubated for 24 hr 37°C. Further identification was performed for *E. coli.* and *Klebsiella* spp. following previous procedure.

For all types of bacteria further confirmation was done by Grams staining and biochemical test.

Grams staining:

Bacteria which were difficult to identify by their growth appearance were undergone for gram staining to identify the gram positive or negative bacteria.

Biochemical test

For Gram positive bacteria:

Tube coagulase test:

Horse plasma collection: Whole blood from horse was collected into commercially available sterile tubes containing EDTA to perform the test. Then blood was centrifuged at 2600 rpm for 10 minutes using a refrigerated centrifuge device. The resulting supernatant, the plasma, was then immediately transferred to a sterile 1.5 ml eppendorf tube using sterile tips and stored at -20ºC for future use.

Coagulase test was done by using horse plasma. All the positive samples were subjected to coagulase tests for biochemical confirmation of Staphylococcus sp. For this, few colonies were picked up and transferred to a 10 ml test tube containing 5 ml of BHIB which was prepared according to the instructions of manufacturer (Oxoid ltd, Basingstoke, Hampshire, UK), incubated at 37 °C for 6 h. From each tube cultivated in BHIB, 50 µL was transferred to sterile tubes containing 50 µL of horse plasma. The incubation was done at a temperature of 37°C for 24hours. The presence of coagulates was justified, considering large organized coagulation and coagulation of all the contents of the tube which do not come off when inverted. A control tube also is placed to validate the result. *S. aureus* gives positive coagulase test whereas Non-aureus *Staphylococcus* gives negative reaction.

Gram negetive bacteria identification

Indole test

Pure bacterial culture was grown in sterile peptone broth/ Brain Heart Infusion Broth (BHIB) for 24 hours. Following incubation, 4-5 drops of Kovac's reagent was added to the culture broth. A positive indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy (MacWilliams, 2009). A variable result can also occur, showing an orange color as a result. *E. coli.* gives positives indole reaction whereas *Klebsiella* spp. and other gram negative bacteria give negative reaction.

Oxidase test

A piece of filter paper was placed in a clean petri dish and 2 drops of oxidase reagent was added to filter paper. A colony of test organism was removed using a wire loop (not an oxidized wire loop) and rubbed onto treated filter paper. The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzyme. A color change to blue/ dark purple within 5 to 10 seconds was considered as positive test. Microorganisms were oxidase negative if the color did not change. *Pseudomonas* spp. gives positive reaction in this test where *E. coli.* and *Klebsiella* spp. gives negative reaction

Methyl red (MR) test

Using a light inoculum, tubes of MR-VP media were inoculated with 24-hour pure cultures of test organisms. Then tubes were incubated aerobically at 37°C for a minimum of 48 hours. After incubation, 2.5 ml of culture was transferred into a new sterile culture tube and 5 drops of the methyl red reagent was added to the tube. Red color at surface of the medium as a result of high acid production and a decrease in the pH of the culture medium was indicated as positive test. Yellow color at surface of the medium was indicated negative test. *Klebsiella* spp. gives negative result for MR test and E. coli gives positive result in this test (McDevitt, 2009).

Voges-Proskauer (VP) test

0.6 ml (or 12 drops) of Barritt's reagent A was added to the remaining 2.5 nil (after MR test) of culture grown in MR-VP broth. Then 0.2 ml (or 4 drops) Of Barrit's reagent B was added and carefully shaken the tube for 30 seconds to expose the medium to atmospheric oxygen. Then the tube was allowed to stand for at least 30 minutes. Red coloration on top of the culture was considered as VP positive. Yellowish color at surface of the medium was considered as VP positive. *Klebsiella* spp. gives positive result for VP test while E. coli. gives negative result (McDevitt, 2009)

Sulphide Indole Motility test (Sim)

Freshly growing culture by using a straight needle was given on SIM agar medium containing tube. Stab once to a depth of only $^{1/3}$ to ½ inch in the middle of the tube and it was make sure to keep the needle in the same line it entered as it is removed from the medium and incubate at 37°C for 48 hours. Positive result indicates diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. Negative results indicates growth that is confined to the stab-line, with sharply defined margins and leaving the surrounding medium clearly transparent. *Klebsiella* spp. gives negative motility result and *E. coli.* gives positive motility test (Alves et al., 2006)

Appendix III: Risk factors analysis at various nodes

Table I: Univariable analysis for bulk milk somatic cell count (BMSCC) and total bacterial count (TBC) at farm level

Variable name	Categories	N	BMSCC (log Mean)	N	TBC (log Mean)	N
Farm zone	Coastal	4	5.42	4	4.79	4
	Semi-coastal	30	5.63	30	5.58	30
	River basin	1	5.38	1	5.26	1
	Inland	10	5.66	10	5.92	10
		P	0.25		0.06	
Education level	Illiterate	14	5.62	14	5.55	14
	Primary and above	30	5.60	30	5.59	30
		P	0.82		0.87	
How sell your milk	Contact basis to middleman	24	5.54	24	5.48	24
	Contract basis to shop	18	5.69	18	5.66	18
		P	0.05		0.4	
Source of milk	Household	9	5.59	9	5.96	9
	Semi-bathan	36	5.62	36	5.49	36

		P	0.7		0.08	
Type of container used?	Aluminum	24	5.58	24	5.72	24
	Plastic	15	5.62	15	5.45	15
	Others(Bamboo mainly)	6	5.69	6	5.32	6
		P	0.63		0.35	
How do you clean your container	Hot water	2	5.83	2	5.40	2
	Tube well	7	5.46	7	5.42	7
	Both tube well water and pond water	2	5.86	2	6.25	2
	Tube well water with detergent	8	5.61	8	5.46	8
	Pond water	25	5.64	25	5.66	25
		P	0.06		0.63	
Frequency of cleaning milk container/day	Once	30	5.63	30	5.73	30
	Twice	15	5.58	15	5.29	15
		P	0.54		0.05	
Cleanliness score of container	Excellent (Clean, inside and outside)	11	5.61	11	5.47	11
	Good (Clean inside)	20	5.57	20	5.69	20

	Poor	14	5.67	14	5.51	14
		P	0.53		0.66	
Use of brush to clean	Yes	37	5.64	37	5.61	37
	No	8	5.60	8	5.43	8
		P	0.71		0.53	
Do you dry the container	Yes	41	5.60	41	5.54	41
	No	4	5.74	4	5.95	4
		P	0.26		0.29	
Do you keep upside down to dry?	Yes	34	5.61	34	5.51	34
	No	11	5.61	11	5.81	11
		P	0.99		0.24	
Do you use a sieve after pouring milk into the BM container	Yes	4	5.57	4	5.27	4
	No	41	5.61	41	5.61	11
		P	0.73		0.37	
Who does milking?	Owner	26	5.64	26	5.59	26
	Worker	17	5.55	17	5.57	17
	Both owner and worker	2	5.65	2	5.57	2

		P	0.49		0.99	
Do you dry udder before milking?	Yes	2	5.74	2	5.20	2
	No	43	5.60	43	5.60	43
		P	0.44		0.45	
The score of milker's hygiene	Excellent (Antiseptic and wash hand)	0	0	0		0
	Good (only wash hand)	2	5.71	2	4.77	2
	Poor (Never wash hand)	43	5.61	43	5.62	43
		P	0.55		0.10	
The score of udder hygiene	Excellent (Udder is clean and dry)	31	5.60	31	5.54	31
	Good (Udder is clean but not dry)	2	5.85	2	5.37	2
	Udder is not clean enough	10	5.61	10	5.56	10
		P	0.36		0.31	
Do you mix mastitis milk with normal	Yes	9	5.66	9	5.57	9
	No	36	5.60	36	5.62	36
		P	0.47		0.87	

Milk storing at home	Using lid	9	5.65	9	5.55	9
	Without lid	36	5.60	36	5.59	36
		P	0.59		0.89	

BMSCC: Bulk milk somatic cell count; TBC: Total bacterial count

Table II : Univariable analysis for total bacterial count (TBC) at middleman level

Variable Names	**Categories**	**N**	**TBC(Log Mean)**
How do you transport?	Walk	6	6.12
	Bicycle	9	6.09
	Others	13	5.79
	Do not transport /on farm	5	5.79
		P	0.81
Container type	Aluminum	20	5.71
	Plastic	23	5.96
		P	0.34
Covering material during transport	Plastic plate	15	5.77
	Aluminium plate	1	5.54
	Banana leaves	3	6. 18

	Plastic screw cap	3	6.21
		P	0.70
Nature of milk composition	Mixed milk (cow-buffalo)	21	5.78
	Buffalo milk	22	5.91
		P	0.62
Frequency of cleaning milk container	Once	23	5.92
	Twice	19	5.79
		P	0.63
Clean after each shipment?	Yes	34	5.82
	No	9	5.94
		P	0.69
How do you clean your container?	Tube well water	4	5.59
	Tube well water with detergent	15	5.61
	Pond water	15	5.90
	Pond water with detergent	9	6.26
		P	0.31
Cleanliness score of container	Excellent (Clean, inside and outside	13	6.05

	Good (Clean inside)	24	5.66
	Poor	5	5.99
		P	0.36
Use of brush to clean	Yes	35	5.78
	No	7	6.06
		P	0.45
Do you dry the container	Yes	38	5.78
	No	5	6.29
		P	0.19
Do you keep upside down to dry?	Yes	37	5.82
	No	6	5.98
		P	0.66

#TBC: Total bacterial count

Table III : Univariable analysis for total bacterial count (TBC) at collection centre level

Variable Names	**Categories**	**N**	**TBC(Log mean)**
Nature of milk composition	Mixed milk (cow-buffalo)	38	6.84
	Buffalo milk	6	6.92

		P	0.83
Container type	Aluminum	16	6.85
	Plastic	27	6.81
		P	0.86
Milk storing at the shop	Using lid	37	6.93
	Without lid	5	6.38
	Using net	1	6.14
		P	0.33
Milk kept at cold storage/freezer	Yes	2	7.38
	No	41	6.82
		P	0.40

#TBC: Total bacterial count

Table IV: Univariable analysis for the total bacterial count at the shop level

Variables Names	**Categories**	**N**	**TBC (Log Mean)**
Place where made?	Shop	7	7.67
	Household	1	4.69
	Other	3	7.23

		P	0.06
Container	Earthen pot	1	8.68
	Plastic container	7	7.03
	Other	2	7.47
		P	0.51
Type of seller	Retailer	3	7.09
	Whole seller	2	7.47
	Both	3	7.39
	Self- consumer	2	7.23
		P	0.99
Source of milk purchase	Own shop	4	7.38
	Buy milk from other	6	7.23
		P	0.86
Nature of milk composition	Mixed milk (both cow-buffalo)	8	7.30
	Buffalo milk	2	7.23
		P	0.95

#TBC: Total bacterial count

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Biography

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