INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 13, No. 6, p. 66-73, 2018
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Microbiological quality and shelf life of pasteurized, flavored carabao's *(Bubalus bubalus carabanensis)* milk as influenced by food safety practices

Bryan Rey R. Oliveros[*1], James Arthur V. Robles[2], Maria Teresa M. Perez[3]

[1,2]*Institute of Food Science and Technology, University of the Philippines Los Baños, Laguna, Philippines*

[3]*National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, Laguna, Philippines*

Article published December 15, 2018

Key words: Carabao's milk, Pasteurization, Food safety, Shelf life.

Abstract

Microbiological contamination restricts the shelf life and degrades the quality of milk. Pasteurization usually kills contaminating microorganisms in milk but in the absence of appropriate food safety practices, its safety could be compromised. This study aims to evaluate the influence of food safety practices, aside from pasteurization, on the microbiological quality and shelf life of kitchen-pasteurized, flavored carabao's milk. Careful observation of the pasteurization process and revision of the process flow diagram was made. Milk samples were tested for pathogenic microorganisms *(E. coli* and *Salmonella sp.)* and its the microbiological shelf life was assessed though monitoring of aerobic, coliform, and psychrotrophic plate counts. Results of this study showed that without any improvements on the food safety practices, pasteurized, flavored carabao's milk could be contaminated with *Enterobacter sp.* and *Bacillus sp.,* as identified through 16s rRNA gene amplification. Thus, pasteurized milk was unfit for consumption. On the other hand, the pasteurized, flavored carabao's milk with improvements on the food safety practices came out negative for *E. coli* and its estimated shelf life was set to 8 days. No *Salmonella spp.* was detected in both batches. This study shows that not only pasteurization, but also proper food safety practices are important in order to produce safe milk.

***Corresponding Author:** Bryan Rey R. Oliveros ✉ broliveros@up.edu.ph

Introduction

Milk is a nutritious food consumed by many around the world. Today, highest production of milk comes from cows, but buffalo milk is not far behind. China is the leading producer of buffalo milk and then followed by India (Khedkar *et al.,* 2015) . In the Philippines, buffalo is locally known as carabao and heightened investment on its production, milk, and milk products was seen since the creation of Philippine Carabao Center in 1992 ("Philippine Carabao Center," n.d.) As of July 2018, the total carabao inventory of the country is 2.88 million heads (PSA, 2018). Aside from its meat, carabao's milk is also widely marketed in the Philippines. It is a good alternative to cow's milk especially in tropical countries like the Philippines.

Pasteurization is one way by which carabao milk processors improve the safety and lengthen the shelf life of their product (Smigic *et al.,* 2012). Some processors resort to kitchen-type pasteurization due to financial difficulties. Unlike the conventional methods of pasteurizing milk, this one does not require any machines but only ordinary kitchen equipment and utensils in processing the milk.

The problem with this type of pasteurization is that, without careful attention to proper food safety practices, microbial contamination of the milk is very imminent, and the quality, safety, and shelf life of the product could be compromised.

Food safety practices are essential in any type of food processing activities. In the recent years, increased importance has been given to the effective application of current Good Manufacturing Practices (cGMPs), Sanitation Standard Operating Procedures (SSOP), and Hazard Analysis Critical Control Point (HACCP) (Flores-Miyamoto *et al.,* 2014). For small milk processors, the financial cost of implementing these Food Safety Programs is a hindrance. On the other hand, production of safe food is still possible with careful attention to proper application of food safety practices in the process flow.

This study aims to evaluate the effect of food safety practices on the microbiological quality and shelf life of kitchen-pasteurized, flavored carabao's milk.

Materials and methods

Improvement of kitchen-pasteurized, flavored carabao's milk process flow diagram

The dairy facility for carabao's milk and milk products located in Los Banos, Laguna was visited and a copy of the process flow diagram for the pasteurized, flavored carabao's milk was procured. The entire process for its manufacturing was observed and notes were made on the possible improvements to be made. Thereafter, a meeting with the dairy facility personnel was set and the proposed improvements of the process were discussed and applied (Fig. 1).

Sample preparation

Two batches of pasteurized, flavored carabao's milk were made. The first batch was prepared employing the procedure before the improvement of food safety practices, herein referred to as pasteurized, flavored carabao's milk-A (PFCM-A). The other batch was prepared employing the procedure with improved food safety practices, herein referred to as pasteurized, flavored carabao's milk-B (PFCM-B). All packaged samples of PFCM-A and PFCM-B were kept in separate coolers filled with ice and were transported immediately to the laboratory. The samples were kept at 4°C in a refrigerator until further use. Two runs of each batch were made.

Sample enrichment and detection of Escherichia coli

Enrichment and detection of *E. coli* in PFCM-A and PFCM-B were done according to the methods of Ombarak *et al.,* 2016. Briefly, 10 mL of the milk was homogenized in a stomacher-blender with 90 mL of tryptic soy broth (TSB) and was incubated at 37°C for 16 h with shaking. After incubation, a loopful of the TSB was streaked onto eosin methylene blue agar (EMBA) plates and these were incubated at 37°C for 18–24 hours. After the said period, presumptive colonies (blue-black with a metallic green sheen) from each EMBA plates were picked, streaked onto tryptic soy agar (TSA) and were incubated at 37°C for another 24 hours.

Gene amplification

16s rRNA gene amplification was done to identify the putative identities of the isolates in EMBA plates. The template was prepared by picking a single colony and resuspending it in 0.2 mL ultrapure distilled water. After resuspension, 24 µL lysing reagent was added and it was allowed to stand for 10 min at ambient temperature. It was then placed in a boiling water bath for 10 min. Then, it was allowed to cool down and was centrifuged for 10 sec in the microfuge at 3000 rpm. Ten-fold dilution was made (10 µL cell lysate + 90 µL ultrapure distilled water) and 5 µL of that dilution was used as PCR template. The primers used were FC27 *(5' AGAGTTTGATCCTGGCTCAG 3')* and RC1492 *(5' TACGGCTACCTTGTTACGACTT 3')*. The PCR tubes were loaded in the thermal cycler (T-Personal, Biometra, Goettingen Germany) programmed with the following parameters: initial denaturation was done at 95°C for 5 min, followed by 30 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec), extension (72°C for 1 min), and final extension at 72°C for 7 min.

16s rRNA gene sequencing

PCR products from the 16s rRNA gene amplification (1.5 kb) were visualized by gel electrophoresis in 1% agarose gel at 100V for 30 min followed by staining in ethidium bromide solution and UV illumination (UVP Inc., Upland CA USA). The molecular weight standard 1 kb plus DNA ladder (Invitrogen Life Technologies, Inc.) was included in the gel. PCR products were sent to 1st BASE Pte Ltd, Singapore for DNA sequencing. Prior to sequencing, the PCR products were cleaned up using Spin Column Purification.

Sample Enrichment and Detection of Salmonella sp.

Enrichment and detection of *Salmonella sp.* in PFCM-A and PFCM-B were done according to Andrews, *et al.,* 2018. Briefly, 25 mL of the milk sample was aseptically transferred into an Erlenmeyer flask containing 225 mL sterilized lactose broth (LB). It was then covered with a cotton plug and was incubated at 35°C for 24 hours. After the incubation period, 0.1 mL of LB was inoculated into 10 mL Rappaport-Vassiliadis (RV) medium and another 1 mL LB was inoculated into 10 mL tetrathionate (TT) broth. RV medium was incubated at 42 ± 0.2°C for 24 ± 2 h while TT broth was incubated at 35 ± 2.0°C for 24 ± 2 h. After the said period, RV medium and TT broth were streaked onto plates of bismuth sulfite agar (BSA), xylose lysine desoxycholate agar (XLDA) and hektoen- enteric agar (HEA). Thereafter, plates were incubated for 24 ± 2 hours at 35°C.

Microbial Shelf Life Determination of Pasteurized, Flavored Carabao's Milk

In determining the microbial shelf life of PFCM-A and PFCM-B, monitoring of the aerobic plate count (APC), psychotropic count (PC), and coliform count (CC) was made every 2 days for 14 days. For APC and PC, 1 mL of the milk sample was serially diluted up to 10^{-4} into 9 mL peptone water. All dilutions were pour-plated using plate count agar (PCA). After pour plating, APC plates were incubated at 37°C for 48 hours while PC plates were incubated at 4°C for 7 days. Quantification of coliforms count (CC) was similar with APC and PC but violet red bile agar (VRBA) was used. After pour plating, CC plates were incubated at 37°C for 24 hours. Five samples per day, per run, per batch were tested.

Statistical Analysis

The colony forming unit (CFU) data were expressed as mean ± standard deviation.

Results and discussion

Conventional Detection of Salmonella spp. and Escherichia coli in Pasteurized, Flavored Carabao's Milk

Salmonella contamination could be introduced in various ways such us through infected cows, contaminated milking equipment, and even through contaminated water (Poppe, 2011).

Table 1. Detection of pathogenic microorganisms in pasteurized, flavored carabao's milk at day 0.

Carabao's milk	Microbial parameters	
	Presumptive *E. coli*	Presumptive *Salmonella sp.*
PFCM-A	+	-
PFCM-B	-	-

(+) present; (-) absent.

Table 2. Putative identities of the isolates from PFCM-A for the forward primer (27F) aided by sequence analysis using BLASTN.

Isolate	Putative identity (species level)	% Homology
2	*Enterobacter cloacae*	97
3	*Enterobacter cloacae*	98
	Enterobacter xiangfangensis	
	Enterobacter hormarchei	
5	*Bacillus cereus*	99
	Bacillus thuringiensis	

Fortunately, for PFCM-A and PFCM-B, no *Salmonella sp.* isolates were found in BSA, XLDA, and HEA (Table 1). Hence, no further tests were made to confirm the presence of this pathogen. On the other hand, for *E. coli* detection, samples from PFCM-A had positive EMBA plates as indicated by the presence of green metallic sheen growths (Table 1).

Table 3. Putative identities of the isolates from PFCM-A for the reverse primer (1492R) aided by sequence analysis using BLASTN.

Isolate	Putative identity (species level)	% Homology
2	*Enterobacter cloacae*	98
3	*Enterobacter cloacae*	99
	Enterobacter xiangfangensis	
	Enterobacter hormarchei	
5	*Bacillus cereus*	95

Thus, typical *E. coli* colonies were isolated and were subjected to DNA isolation and 16s rRNA gene amplification. Of the 5 typical *E. coli* isolates, 4 isolates had 16s rRNA amplicons of about 1.5 kbp (Fig. 2).

Molecular Identification of Presumptive Escherichia coli in Pasteurized, Flavored Carabao's Milk

Of the 4 isolates, isolate 1 had very low yield. Because of this, only isolates 2, 3, and 5 were sequenced. Homology search using BLASTN (National Center for Biotechnology Information, USA) was performed to analyze the sequences of these isolates. The putative identities of the three isolates are presented in Tables 2 and 3. According to Table 2 and 3, isolates 2 and 3 are putatively *Enterobacter sp.*.

The putative identity of isolate 2 is *Enterobacter cloacae* since it has 97% sequence homology for the forward primer and 98% sequence homology for the reverse primer. Meanwhile, three different species were identified as putative identities of isolate 3.

It has a percent homology of 98% for the forward primer and 99% for the reverse primer for the following species: *Enterobacter xiangfangensis, Enterobacter hormaechei*, and *Enterobacter cloacae*. The presence of these microorganisms in pasteurized, flavored carabao's milk poses health risks.

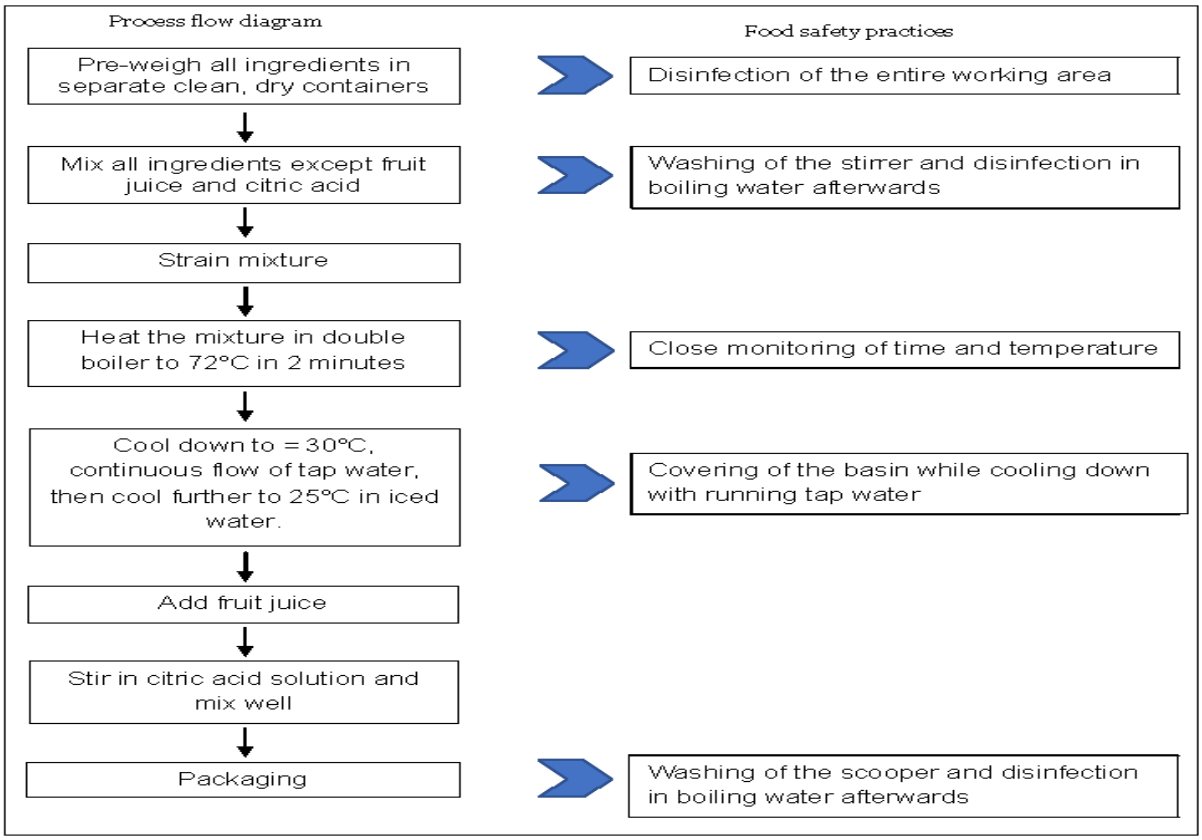

Fig. 1. Improvements on the processing of pasteurized, flavored carabao's milk through food safety practices.

This genus is known as an important foodborne pathogen since it can dominate the gastrointestinal tract of humans (Nascimento *et al.,* 2016). In addition, *Enterobacter hormaechei* is a nosocomial pathogen which can cause an outbreak among premature infants while *Enterobacter cloacae* is a known opportunistic, nosocomial pathogen which can be isolated from animal and human feces (Muensritharam *et al.,* 2016). However, no recent studies revealed that *Enterobacter xiangfangensis* is considered a pathogen. Lastly, isolate 5 is putatively *Bacillus sp*. This isolate has a 99 % sequence homology to *B. cereus* and *B. thuringiensis* for the forward primer and 95% sequence homology to *B. cereus* for the reverse primer. These microorganisms are phenotypically and genotypically related that in some criteria, they could be considered as single species (Griffiths and Schraft, 2017).

The post-pasteurization presence of this species is also an important issue because these species also pose some health risks. While *Bacillus thuriengiensis* is a known soil-borne microorganism that can produce bioinsecticide, *Bacillus cereus* is a known foodborne pathogen. It is a human pathogen that causes diarrheal and emetic diseases (Griffiths and Schraft, 2017).

Spoilage of PFCM-A samples is due to poor food safety practices. Accordingly, enterobacter species are usually from extrinsic sources; hence, spoilage of milk by enterobacters could be due to post pasteurization contamination as these microorganisms are easily destroyed by proper heating (Nascimento *et al.,* 2016). As for *Bacillus spp.,* these microorganisms can come from the milk and from improperly cleaned equipment.

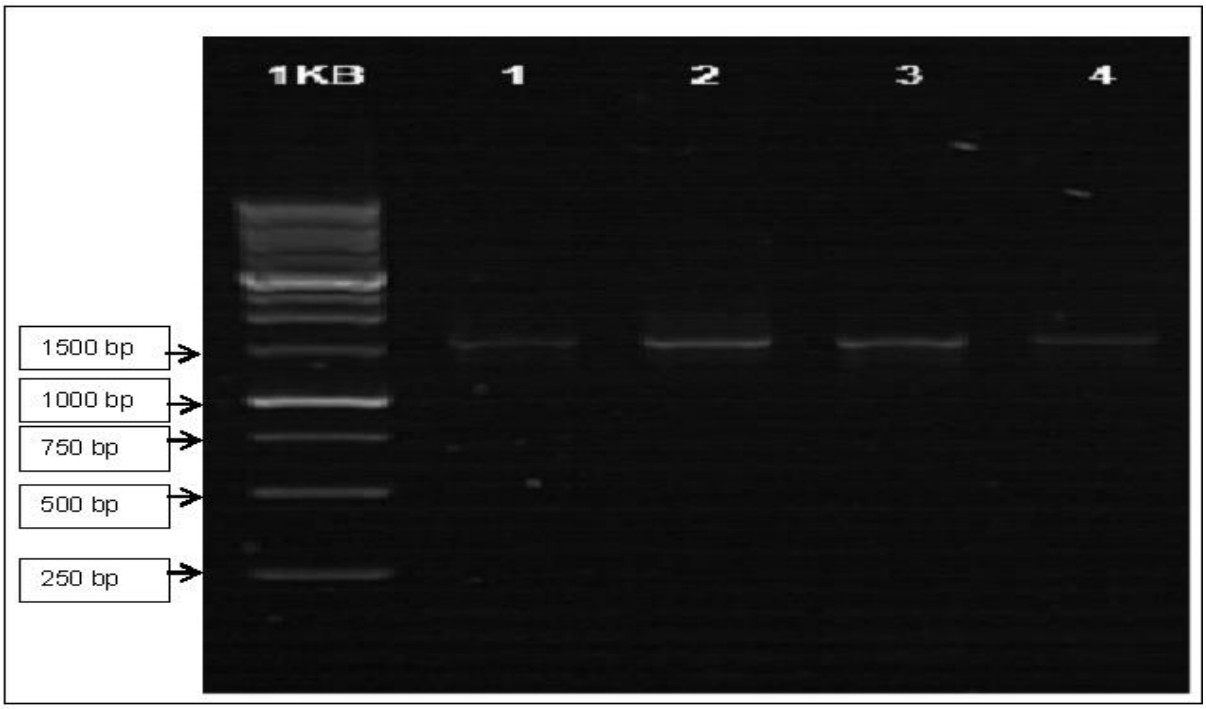

Fig. 2. Purified PCR products of isolates from PFCM-A as visualized through gel electrophoresis.

This species can overcome pasteurization through their heat resistant spores that could lead to either food poisoning or sweet curdling of milk (Batt, 2014). In another study, the presence of *Paenibacillus sp.* in pasteurized buffalo milk was reported to predominate after 21 days of storage (Li *et al.,* 2016).

Monitoring of the APC, CC, and PC of Pasteurized, Flavored Carabao's Milk for Microbial Shelf Life

Since milk samples from PFCM-A had high initial coliform count, it was no longer subjected to microbial shelf life determination. This result is coherent with the observed physical appearance of the samples after one day of incubation wherein there was already bubbling, thus making it unfit for further testing. For PFCM-B, no positive result was seen in the enrichment for *E. coli* and *Salmonella sp.* (Table 2), hence it was tested further for microbial shelf life.

In all sampling days for PFCM-B, no PCs were found (Fig. 3). Psychrotrophic bacteria are common milk spoilage microorganisms and the increasing population of psychotolerant microorganisms like *Pseudomonas* and *Acinetobacter* during cold storage of raw milk has been reported (Li *et al.,* 2016). In Thailand, the level of psychrotrophic microorganisms in raw buffalo milk ranged from 2.52–5.76 $\log_{10}$ CFU/mL (Alexandraki *et al.,* 2016). After pasteurization, it should be reduced to a minimum as these microorganisms can be easily killed by heat. The result of this study is coherent with a previous report wherein psychrotrophs were not observed until 21 days of storage (Li *et al.,* 2016). In PFCM-B, proper monitoring of the pasteurization temperature and time was closely implemented. In addition, all equipment that were used in mixing and monitoring of the temperature of carabao's milk during pasteurization were sterilized in boiling water (Fig. 1). With these improved food safety practices, psychrotrophs were successfully eliminated (Fig. 3).

APC is a parameter of the hygienic quality of the food and a good basis for the measurement of the shelf life of food (Koushki *et al.,* 2016). The acceptable level of APC determined by conventional plating in milk is set at 5×10^4 CFU/mL while the level which when exceeded in one or more samples that would cause the lot to be rejected as it indicates potential health hazard is set at 1×10^5 CFU/mL (FDA, 2013). PFCM-B was only considered unacceptable on Day 14 as it had reached 5 $\log_{10}$ APC (Fig. 3).

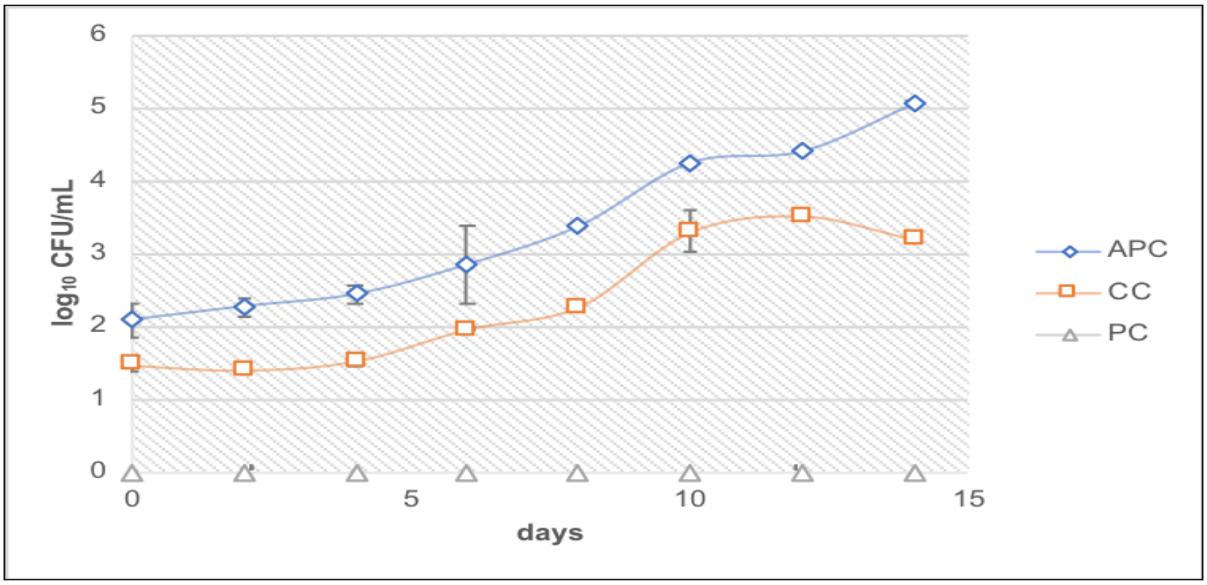

Fig. 3. Mean + SD APC, CC, and PC (log10 CFU/mL) of PFCM-B during 14 days of monitoring stored at 4°C.

At this level of APC, the milk samples are already unfit for consumption as it can cause potential health risks.

Another good indicator of hygienic quality and shelf life of milk is the level of coliforms. The presence or absence of coliforms is usually an indication of fecal contamination caused by pasteurization deficiency and/or secondary contamination (Koushki *et al.,* 2016). The standard limit for coliforms in processed milk is set at $1x10^3$ CFU/mL (FDA, 2013). In Fig. 3, it shows that the PFCM-B is only good until the 8th day where the level of coliforms was at 2.27 $\log_{10}$ CFU/mL. By the 10th day, the coliform level of PFCM-B was already at 3.3 $\log_{10}$ CFU/mL and this value exceeds the standard limit for coliforms. Thus, the estimated shelf life of PFCM-B was marked until day 8.

Conclusion

This study found out that with the implementation of improved food safety practices, PFCM-B has a better microbiological quality and longer shelf life than PFCM-A. The former has no putative *Enterobacter sp*. and *Bacillus sp*. contaminants while the latter has. In addition, PFCM-B also has longer shelf life than PFCM-A as it was good for consumption until the 8th day. Without the improvements on the food safety practices, PFCM-A was already spoiled and/or unfit for consumption at day 0. This study recommends that small scale processors implement proper food safety practices. Hit-and-miss practices will only lead to microbiological contamination that leads to limited shelf life and financial losses. Thus, proper food safety practices in every stage of the process are highly needed.

Acknowledgements

The proponents would like to thank the Philippine Carabao Center-University of the Philippines Los Baños (PCC-UPLB) for the samples used in the entire study.

References

Alexandraki V, Kazou M, Angelopoulou A, Arena MP, Capozzi V, Russo P, Tsakalidou E. 2016. The Microbiota of Non-cow Milk and Products. Non-Bovine Milk and Milk Products.

Andrews WH, Wang H, Jacobson A, Hammack T. 2018. Chapter 5: Salmonella.

ABatt CA. 2014. Bacillus: Bacillus cereus. In Encyclopedia of Food Microbiology (Second Ed, **1,** p 124–128). Academic Press.

FDA. 2013. FDA Circular 2013-010.

Flores-Miyamoto A, Reij MW, Velthuis AJ. 2014. Do farm audits improve milk quality? Journal of Dairy Science **97,** 1–9.

Griffiths MW, Schraft H. 2017. Bacillus cereus: Food Poisoning. In H. P. R. Christine E.R. Dodd, Tim Aldsworth, Richard A. Stein, Dean O. Cliver (Ed.), Foodborne Diseases (Third Ed, p 395–405). Academic Press.

Khedkar CD, Kalyankar SD, Deosarkar SS. 2015. Buffalo Milk. Encyclopedia of Food and Health (3rd ed.). Elsevier Ltd.

Koushki M, Koohy-Kamaly P, Azizkhani M, Hadinia N. 2016. Microbiological quality of pasteurized milk on expiration date in Tehran, Iran. Journal of Dairy Science **99,** 1796–1801.

Li L, Renye JA, Feng L, Zeng Q, Tang Y, Huang L, Ren D, Yang P. 2016**.** Characterization of the indigenous microflora in raw and pasteurized buffalo milk during storage at refrigeration temperature by high-throughput sequencing. Journal of Dairy Science **99,** 7016–7024.

Muensritharam L, Fanning S, Meharg C. 2016**.** Pathogens in Milk: Enterobacter Species. In Reference Module in Food Science. Elsevier.

Nascimento WGR, Moraes MS, Souza BV, Farias JOR, Melo DH, Nascimento JS. 2016. Enterobacter cloacae isolated from utensils used in baby feeding in a milk dispensary in rio. In XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos.

Ombarak RA, Hinenoya A, Awasthi SP, Iguchi A, Shima A, Elbagory ARM, Yamasaki S. 2016. Prevalence and pathogenic potential of Escherichia coli isolates from raw milk and raw milk cheese in Egypt. International Journal of Food Microbiology **221,** 69–76.

Philippine Carabao Center. n.d.. Retrieved October 19, 2018, from www.pcc.gov.ph

Poppe C. 2011. Pathogens in Milk: Salmonella spp. In Encyclopedia of Dairy Sciences (Second Ed, p 93–98). Academic Press.

PSA. 2018. Carabao Situation Report, April-June 2018.

Smigic N, Djekic I, Tomasevic I, Miocinovic J, Gvozdenovic R. 2012**.** Implication of food safety measures on microbiological quality of raw and pasteurized milk. Food Control **25,** 728–731.