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Impact of Hygiene Intervention Practices on Microbial Load in Raw Milk

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An intervention program was designed to show local dairy farmers on Good Agricultural Practices (GAP) to improve the quality of milk produced by the farms. In this current study, sampling of milk and environmental samples were collected pre- and post-intervention program. Analysis of milk quality was done at field to show the microbiological laboratory procedures to the farmers on determination of milk quality. Findings showed a reduction of up to 40% log reduction in the fresh milk sample. Analysis of the environmental samples were carried out using multiplex MPN-PCR method to quantify and detect up to five species of bacteria in milk. Finding showed the persistence of *Salmonella paratyphi* contaminating the milking equipment pre- and post-intervention program. The intervention program will be further improved according to data and findings from this study to be implemented at other farms in the future.

Keywords: Milk zoonoses; food safety; hygiene; dairy farms; exposure assessment; Malaysia.

In Malaysia, consumption of fresh milk has increased over the years. This is mainly attributable to the increased awareness of milk and dairy nutritional benefits coupled with increased consumer preference towards dairy-derived products[1]. With the growing demand of milk and dairy products, food safety becomes of paramount important in ensuring that milk and dairy products are safe to be consumed. As such, various programs such as sustainable dairy farming along with good husbandry practices have been implemented or recommended[1] to further improve the food processing line from farm to fork within the dairy chain, to obtain a higher quality of dairy product for consumption. Detection of foodborne pathogen in food is a critical component in the surveillance system for food safety monitoring.

In Malaysia, agriculture contributed 8.9% to the Gross Domestic Product (GDP) citing oil palm, livestock, fishing, rubber and forestry and logging as the main contributor in 2015[2]. Dairy products for the local demand are often satisfied by the importation and as the country has increased from 5% of self-sustainability of milk demand in 2008[3] to 9.3%[4]. Various policies

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(Malaysia Plans and Agricultural and Food), programs (National Dairy Development Program and strategies (Production, trade and integration) were put in place by the Malaysian government to tackle the low local milk production since the early 1970s[5]. These governmental efforts were geared toward increasing local production of milk for self-sufficiency in the country[6]. The Department of Veterinary Services provided a range of services to assist small-medium dairy producers to increase dairy production and enhance marketing of milk[7]. Milk producers sold their milk to Government established Milk Collection Centers based on quality of milk (3rd and 4th Malaysia Plans)[3] The dairy sector in Malaysia continues to face challenges to meet the demand of growing consumer taste and preference towards milk and dairy products. These challenges include lack of skills and training among small-holder farmers, low breed performance and inadaptability to local environmental conditions, poor dairy farm management and inadequate nutritious feed, and high input and feed costs[1]. Amongst these challenges, poor dairy management deemed to be the most critical component to tackle food safety concerns. With the majority of the farmers involve in managing dairy farms fall into the small-scale holders[8] , tackling the food safety component becomes even more important and necessary as the skills required for efficient milk production and technical knowledge on tropical dairy production are still lacking[9]. At the international level, dairy farming has been competitive and selective breeding of high yielding cows has also led to higher susceptibility to diseases[10]. Issues with farm management by the veterinary services have been reported including issues on disease and production constraints, elaboration and education programs to be part of the integrated services provided by one department. Consequently, the outreach of these services will limited be limited and smallholder farmers -medium dairy producers will be left out. Therefore, the skills required for efficient milk production and technical knowledge on tropical dairy production will be underdeveloped[9], particular for small dairy producers, which was the target of the dairy initiative in the first place.

To address this concern, a program on adopting sustainable hygiene dairy practices was carried out to raise awareness within the small-scale dairy farming community in Malaysia on tproducing safe and quality milk. This paper attempts to report on the impact of this intervention program in the dairy farming community.

MATERIALS AND METHODS

Selection of Farms

Dairy farmers were selected to participate in this program through their feedbacks from interviews and their motivation to improve their practices. Based on the acceptability of the farm owners in allowing the research team to visit the farm, one farm were selected for this study. The selected dairy farm was located in the state of Negeri Sembilan, occupying an area of about 0.3 acres surrounded by oil palm plantation. The dairy farm, herein referred to as "Farm X", housed 50 dairy cows of various breeds and these dairy cows were milked twice in a day (once early in the morning and the next, late in the evening). These dairy cows were visually healthy with no sign of malnutrition and were released for grazing in the morning after milking.

Design of experiment

Collection of milk samples were carried out in Day 1 of the farm visit, observing the routine farm practices of the farm owner and their workers. Swab samples from milk collection bucket, and milk collection were collected. In Day 2, farmers were trained on dairy hygiene practices using appropriate sterilizing method in the pre milking (cleaning of milking equipment), during milking and post milking processes (storage and transportation practices). Sampling of the collected milk were carried out on the same time to minimize the variation in practices and time of the day. During each sampling period, farmers were also interviewed using a structured questionnaire. Samples were processed immediately on site with proper aseptic practices through serial dilution of the milk samples with phosphate buffered saline and plated on 3M Petrifilm for *Staphylococcus aureus*, *Enterobacteriae, E. coli* as well as Yeast and Mold. Petrifilm were immediately transported to laboratory for incubation at 37°C for 24 hours and enumerated the following day. Swab samples of the cleaning equipment were collected for lab

analysis. Farmers and workers were allowed to complete the milking process and a final pooled sample of milk was obtained for microbiological analysis. Swabs were immediately transported back to laboratory under chilled condition and stored in -80°C until further analysis using molecular methods for detection and quantification of microbial contamination.

Detection and Quantification of Contamination from Environment

Bacterial strains used in this study was *E.coli* ATCC 25922, ETEC (confirmed environmental strain), *B. cereus* (environmental strain), *Salmonella paratyphi* (environmental strain), and *Vibrio parahaemolyticus* (food isolate) that was confirmed using PCR. Swab samples were taken from surface of milking equipments (clusters, cups and milk churns of pooled milk) pre and post intervention. Approximately 10cm^2 of surface area was swabbed at the farm, and transported back to the laboratory and kept in -80°C until further analyses.

Swab samples were mixed and vortexed in 5ml of PBS buffer, and divided to 3 dilution MPN-tubes containing 1ml each tube. A total of 9-MPN tubes were used for each sample. A simple DNA extraction was conducted using crude cell lysis[11]. Briefly, bacterial cultures were streaked on nutrient agar and purified by selection of single colony in brain heart infusion broth followed by an incubation period at 37°C for 12-16 hours in the shaker. Post incubation, the culture were harvested by transferring 1ml of the culture into microcentrifuge tubes, and centrifuged at 12,000 ×g for 1 min. Supernatant was discarded and the cultures resuspended in 1ml of 1× TBE buffer (pH 8.0) and vortexed for 30 seconds. Tubes were subjected to rapid heat (100°C) and freeze (-20°C) treatment for 20 minutes each, and continued with the final centrifugation at 12,000×g for 2 minutes. The supernatant from the extracted cell lysis was used as DNA template for the study. Control cultures were extracted using crude cell lysis and measured for absorbance reading and quantification of DNA using UV-VIS spectrophotometer.

Primers used in this study were previously used in Wang, Cao[12]. The initial nucleic acid amplification protocol for the detection of 13 bacterial species was modified to five species. Five sets of primers targeting ETEC, *Salmonella spp., B. cereus, V. parahaemolyticus* and *Escherichia coli* were selected and optimized for multiplex MPN-PCR detection and enumeration. The total concentration of primers, magnesium chloride and annealing temperature was optimized for this study. The optimized PCR profile used was pre-denaturation at 94°C, 15 s, a total of 35 cycles of denaturation at 94°C, 30s; annealing 56°C for 15s; extension of 72°C for 35s, and followed by post-extension 72°C for 2 mins with a final extension 45°C at 2 mins. Reaction mixture of each PCR reaction tube contained 1× GoTaq buffer (PROMEGA, USA), 3mM of $MgCl_2$, 0.5mM of dNTP, 300 nm of each primers, 2.5U of Taq Polymerase (PROMEGA, USA) and 2.5ul of crude DNA lysate in a total of 25ul reaction tube. Visualization of the PCR product was carried out using an AATI fragment analyzer (Advanced Analytical AATI, USA) according to the manufacturer's protocol.

Analysis of Intervention Improvement using Relative Exposure

Improvement to the Good Agricultural Practice from Farm X was assessed using the relative risk associated to the analytical results of the samples analyzed from the farms.

In this analysis, baseline exposure was defined as the representative level of toxins produced by the concentration of bacteria (log cfu/ml) in the milk sample, which is the pre-intervention data. Adapting from[13] from using risk relative estimates in *Campylobacter* in broiler meat as microbiological criterion, this study adapted the relative exposure estimates of *Staphylococcus aureus* enterotoxin in milk based on the concentration data results in the present study. To obtain the estimated level of toxins in the milk samples in relation to the concentration of *S. aureus,* a constant relation between toxin production model and cell numbers developed using milk data by[14], the following equation was used:

$$Tox = 0.9300751 \times C - 6.662092$$

Where Tox, is the toxin production (log ng/ml) and C is the number of cells (log cfu/ml).

The relative exposure indicate the exposure level of the intervention procedure pre- and post-intervention to the quality of milk using the toxin level produced by *S. aureus* enterotoxin. The model was described in[15] and modified to

assess the toxin level reduction as a result of the intervention.

$$Relative\ exposure = \frac{post\ intervention\ toxin\ level}{pre\ intervention\ toxin\ level}$$

RESULTS AND DISCUSSION

Observation of the farm practice before the implementation of the intervention program occurred at the first day. The medium- size dairy farm have 25 milking cows with the number of workers corresponding to one to five workers at point of visit. The farm milked the cows twice daily, using calves to suckle the cows briefly, followed by the milking process. The farm workers milked the cows using vacuum milking clusters. Equipment was washed and scrubbed prior to milking using tap water without soap or sanitizer, and after milking with disinfectant. The teat and hose were not washed between each milking and milk was collected into one steel churn for the entire farm, and was covered throughout the milking process. The milk collection was then pooled in a refrigerated bulk tank with a temperature monitor of 2-4°C. Sample was collected from this pool of milk as pre-intervention process.

In the intervention program, workers were trained on how to prepare sanitizing solution in hot water (65-70°C) to firstly rinse the interior and exterior parts of all equipment, followed by a second rinse with plain hot water. The udders and teats were also cleaned with sanitizing solution and clean towel, with replacement of the solution once it is dirty. Clean towel was used and workers were advised to wash their hands with sanitizing solution and wipe dry prior to milking the cows. After the milking process, additional disinfectant on the teat such as Alfadin™ was recommended before allowing the cows to go out for grazing. Storage of milk should be at cold temperature, or delivered to vendor within 1 hour post-milking.

The results of the microbiological quality of milk were shown in Table 1. The aerobic plate count did not exceed the limits set by the Department of Veterinary Services, Malaysia for price incentive[16]. Log reduction in *S. aureus, Enterobacteriae,* total coliform and mold were observed in the study post-intervention. Referring to ICMSF guideline, Mesophilic Aerobic Microorganisms are generally used as an index of utility, or indicators of general contamination, shelf life or spoilage and are not usually related to a health hazard. Although the product is not meant for international trade purpose, it can be used for verification of hygiene programs. The observed increase in the log percentage post-intervention (17%) showed that there may be other contributing factors, i.e., environment to the contamination during the milking process that was beyond the control of the trainers. Using *Enterobacteriaeae* as indicators of the history of the hygiene of the food production process[17], the 4% reduction shows that the hygiene practices per se that were emphasized in this intervention program could not deter contamination of the milk.

Table 1. Quantification of microbial analyses using Petrifilm™ of milk s amples collected pre- and post- intervention procedure at Farm X

	Concentration (log cfu/ml)		
Microbiological Analyses	Pre-Intervention	Post-Intervention	Percent log reduction (% cfu/ml)
Staphylococcus aureus	5.51	3.19	-42.1
Escherichia coli	2.48	2.86	+15.3
Total coliform count	4.70	4.54	-3.4
Enterobactericaea	4.74	4.55	-4.0
Aerobic count	5.18	6.10	+17.8
Mold	4.34	1.21	-72.1
Yeast	ND	ND	-

ND- Not detected

In order to identify the source of contamination, quantification of the swab samples collected from the milking equipment were tested semi-quantitatively using MPN-PCR. The detection limit for each targeted pathogen for this multiplex assay was 88.5 ng/µl of DNA template from *Salmonella spp.,* 56 ng/µl for *V. parahaemolyticus,* and 0.24 ng/µl of template for *B. cereus, E.coli ETEC* and *E. coli*. Swab samples collected from the equipments, milk and clusters showed absence of ETEC, *Bacillus cereus* and *V. parahaemolyticus*. However, two swab samples were shown positive for *Salmonella spp.*, located at the cluster of the milking equipment pre and post-intervention showing low level contamination of less than 100 cfu/ml (8 and 23 MPN/ml respectively). This finding also suggests the same as what was reported earlier, that the hygiene practices per se that were emphasized in this intervention program could not deter contamination of the milk . The persistence of *S. paratyphi* in the clusters after the intervention process, i.e., using sanitizing solution and hot water at the interior and exterior and rinsing again with hot water may imply a possibility of biofilm adherence to the interior part of the equipment where the milk flows. Therefore, future training component related to hygiene practices should include extending the soaking and rinsing period particularly for the interior part of the clustersto tackle the contamination matter.

From the aspect of food safety, *S. aureus* log reduction at 40% was then used for further characterization of the intervention program for the relative exposure based on the toxin levels that may occur in the milk sample. Simple analysis showed that the hygiene practices emphasized in the training were able to reduce the exposure of the toxin levels in milk samples up to 2.4× compared to the pre-intervention samples. During the study, all collected milk were pooled and stored in a refrigerated tank with a temperature monitor between 2 to 8°C at all times. Since predictive studies of *S. aureus* has shown that low temperature has a strong inhibitory effect on growth rate[18], the increase and growth of nonpathogenic and pathogenic bacteria as well as toxin production from *S.aureus* was assumed to be negligible during storage.

CONCLUSION

The effectiveness of the intervention program focusing on hygiene dairy practices coupled with science-based evidence can be considered good. This is demonstrated on the low contamination of milk detected in the microbiological analyses, which can be overcome by increasing the soaking and rinsing period of the cluster on the milking equipment pre and post intervention. Meanwhile, the intervention program was effective on reducing risk factors concerning the growth of nonpathogenic and pathogenic bacteria. The findings of this study demonstrate that dairy hygiene practices should be stressed upon in training programs for dairy farmers in order to tackle food safety concerns while increasing local milk production. Additionally, similar to other training programs, future dairy hygiene training programs must take into consideration of enhancing the training scope of the program so as to increase the efficacy and effectiveness of it, i.e., imparting science based evidence and solution.

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