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Bacterial Biofilm Formation in Milking Equipments in Lilongwe, Malawi

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HIGHLIGHTS

- The biofilm indicator organisms were more prevalent in dairy farmer households compared with the milk bulking centers.
- Fourteen out of 86 (16.3%) microbial isolates formed strong/high biofilms.
- Biofilm formation on milk handling container in Lilongwe, Malawi could serve as source of microbial contamination.

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ABSTRACT

Background: Some microorganisms can adhere to food handling surfaces forming biofilms that pose a safety challenge. This study was done to evaluate bacterial biofilm formation in milking equipments in Lilongwe, Malawi.

Methods: Pooled milk (n=54) and water (n=60) samples were collected from households and milk bulking groups in Lilongwe, Malawi. Swabbing (n=46) and rinsing (n=16) were done on milk handling containers after cleaning. Biofilm determination on the containers was done by detecting biofilm indicator bacteria, including *Bacillus* spp., *Salmonella* spp., and *Pseudomonas* spp. The strength of biofilm was determined by the tube method. Data were analyzed by SAS software version 9.1.3.

Results: Prevalence rates of Gram-negative rods were significantly (p<0.05) higher than the Gram-positive rods and the Gram-positive cocci. Of the 176 cases, contamination rates were 36 (20.4%), 32 (18.2%), and 18 (10.2%) for *Salmonella* spp., *Bacillus* spp., and *Pseudomonas* spp., respectively. The biofilm indicator organisms were significantly (p<0.05) more prevalent in dairy farmer households compared with the milk bulking centers. Fourteen out of 86 (16.3%) microbial isolates formed strong/high biofilms, whereas 18 out of 86 (20.93%) of isolates did not form any biofilm. The rate of isolates forming strong/high biofilms in households (17.6%) was significantly (p<0.05) higher than that of milk bulking centers (11.1%).

Conclusion: Biofilm formation on milk handling container surfaces in Lilongwe, Malawi could serve as a source of microbial pathogens and spoilage organisms.

Introduction

The type and design of equipment coming into contact with food is one of the critical elements in ensuring their consistent quality and safety. Equipments made of materials which are not of food grade with poor hygienic designs result in ineffective cleaning. This will lead to retention of the food residues (soil), which promotes

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survival, multiplication, and attachment of microorganisms. The type of food-contact surfaces influence the level of attachment of microorganisms and effectiveness of cleaning once the attachment occurs (Schmidt and Erickson, 2005; Silva et al., 2010).

Microorganisms such as bacteria can adhere to and colonize food contact surfaces forming layered complex structures known as biofilms, which gives them the ability to respond to and protect themselves against exposure to environmental stresses. The cells in biofilms are embedded in extracellular polymeric substance composed of exopolysaccharides, protein and nucleic acid, exhibiting altered growth, gene transcription, and increased resistance to most antimicrobial agents as compared to unattached cells (Bridier et al., 2011; Marchand et al., 2012). Biofilms are difficult to remove and act as continuous source of contamination, leading to food-borne and nosocomial infections (Abdallah et al., 2014). Some of the microorganisms associated with surface attachment include *Pseudomonas*, *Bacillus*, *Salmonella*, and *Escherichia coli* O157:H7 (Chmielewski and Frank, 2003).

Biofilms are serious quality and safety concern in the food industry owing to their strong tolerance to physical and chemical treatments and sanitation in comparison to the planktonic cells of the same species. The biofilms much of the time are mixed because of numerous bacterial species that exist in a community network and this influences the biofilm structure, action, and sanitizer resistance. Therefore, complete deactivation and elimination of established biofilms formed by or blended in with food-borne microorganisms on processing utensils and contact surfaces is hard to accomplish. Thus, any microbe confined in biofilms may result to cross-contamination of food items (Marchand et al., 2012; Simoes et al., 2010).

In Malawi, 64 747 tons of milk are produced annually and post-harvest losses due to microbial spoilage is estimated at 17% (FAOSTAT, 2014; Sindani, 2012). Economically, these post-harvest losses translate into approximately US$ 606 195 income losses to the dairy farmers per year. The dairy industry in Malawi is composed of formal and informal sectors, with the formal sector selling milk to processors through milk bulking groups. The main role of the milk bulking groups is to gather milk, determine milk quality before acceptance, store the milk in cold chain facilities, and then selling to processors (Sindani, 2012). It seems that milk spoilage is generally as a result of inadequate hygiene at milk production stage and the utilization of unsterile containers to gather and transport milk to milk bulking groups. At the milk bulking groups, there are no routine screening tests for microbial quality. Additionally, animal udder health is not practiced; there are no prior tests for udder infection; the udder and teats are not washed before milking; and the calf is let to suckle before milking. Occasionally, re-used pieces of cloth are used to dry the udder and the teats. The farmers use sand and ash as a scourer to scrub the milk handling containers. The farmers use ash and sand to scour and clean milk holding containers. These practices are likely to contribute to microbial contamination, consequently the high post-harvest milk losses of Malawi's milk production.

These gaps are probably causative factors for microbial contamination and biofilm formation. This study aimed at assessing the formation of bacterial biofilms in milk handling equipments in Lilongwe, Malawi.

Materials and methods

Sampling

Sampling was done from households and milk bulking centers in Lilongwe, Malawi from February to August 2018. All the samples were transported to Community Health Science Unit (CHSU) of the National Public Health (Microbiology) Reference Laboratory in Lilongwe, Malawi within six hours and analytical work started immediately.

-Milk samples

Fifty-four pooled milk samples from milk bulking centers (n=5) and households (n=49) were collected. Prior to sampling, the milk in the container was shaken to mix the milk; then, a 10 ml sample of milk was taken and transferred into a sterile screw-cap tube and placed in a cool box maintained at 8-10 $^{\circ}$C using ice cubes. Sampling was done in the morning at normal milking time (7.00-9.00 AM). The samples were collected from households from six milk bulking groups, including Machite, Nathenje, Lumbadzi, Majiga, Namwiri, and Nkhweza.

Five milk samples were picked from five milk bulking centers in five locations of Lilongwe where milk from households was gathered for transportation to processor. The milk bulking centers included Machite, Nathenje, Lumbadzi, Namwiri, and Majiga. Milk was collected from cooler tanks and milk in each cooler tank was stirred to produce a homogeneous mixture before taking a representative sample.

- Swab and rinse samples

For determining efficacy of the sanitation regime, swabbing and rinsing were done on the milk handling containers after the containers had been cleaned and ready to be used in handling milk. Surface swabs (n=46) for collecting microorganisms were done using a sterile cotton swab buds pre-wetted in peptone water at an area of 25 cm^2 in three replicates (Wafula et al., 2016). Swabs

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were taken after a cleaning regime by rotating the cotton end in contact with the prepared milk handling container surfaces. The farmers cleaned milk handling containers (mostly plastic containers) using untreated, warm borehole water, with bar soap. From the containers that were dried with a towel or sundried, swabs were taken. For containers which were not dried, rinses were taken. The rinse samples (n=16) were obtained by pipetting 1 ml of the final rinsing water in triplicates. The swab and rinse samples were then transferred into the 9 ml 0.1% (w/v) buffered peptone water in a screw-cap tube and stored in a cool box at 4 °C. These were taken to the laboratory and shaken using a vortex for 2 min to dislodge the bacteria.

-Water samples

Sixty water samples from milk production areas, including well (n=12) and borehole (n=48) water samples, were taken from farmer household. The water was collected using a household cup and poured directly into a sterile screw cup glass bottle. The sampling container cup was aseptically replaced by wiping the cup and neck of the container with a paper towel that was soaked in 70% ethanol. The sample was kept in a cool box and transferred to the laboratory for microbial analysis.

Determination of biofilm indicator organisms

-Isolation of Salmonella spp.

Salmonella spp. were isolated and identified according to WHO (2010). Surface swab samples, milk samples, and water samples (10 ml each) were enriched in buffered peptone water (90 ml). The mixture of sample and peptone water was incubated at 37 °C for 24 h. After the incubation, the mixture was shaken gently to mix well; then, using a sterile pipette, 1 ml was transferred into 10 ml Selenite broth (Difco, Thermoscientific, UK). This was incubated in a water bath at 42 °C for 24 h. After incubation, a loopful of the Selenite broth culture was streaked on Xylose Lysine Desoxycolate agar (XLD; Oxoid, Wesel, Germany). These were incubated at 37 °C for 24-48 h. Colonies that appeared dark on XLD were taken to be non-lactose fermenters and were purified on MacConkey agar (Oxoid, Wesel, Germany). The purified colonies on MacConkey agar were inoculated into the Triple Sugar Iron agar (TSI; Oxoid, Wesel, Germany) slants by stubbing the butt and streaking the slant.

-Isolation of Bacillus spp. and Pseudomonas spp.

Bacillus and *Pseudomonas* spp. were isolated using the method described by Roberts and Greenwood (2002). The samples were streaked directly on 5% sheep blood agar (Oxoid, Wesel, Germany) and incubated at 30 °C for 24 h. Growth of direct cultures was examined and subcultured. Selection of colonies from subcultures was done according to their predominance and homogeneity throughout the streak, and type of hemolysis. All blood agar plates that showed none or scarce growth were re-examined after 48 and 72 h of incubation. Hemolytic colonies (presumptive *Bacillus* spp.) were subcultured onto blood agar, whereas fast growing non-hemolytic colonies were subcultured on nutrient agar (Oxoid, Wesel, Germany).

Biochemical tests

The typical colonies were further isolated and identified according to their morphological, physiological, and biochemical tests characteristic for *Salmonella*, *Bacillus*, and *Pseudomonas* spp. The tests such as Gram staining, catalase test, oxidase test, carbohydrate fermentation in TSI agar, Motility, production of H_2S and Indole in Sulfur Indole Motility agar (SIM; Oxoid, Wesel, Germany) were carried out for identification of the organisms based on Roberts and Greenwood (2002).

Biofilm detection

The tube method was used for biofilm detection (Mohamed et al., 2016; Saha et al., 2014). A loopful of test microorganism isolates of *Salmonella*, *Bacillus*, and *Pseudomonas* spp. were inoculated in 10 ml of trypticase soy broth (Oxoid, Wesel, Germany) with 1% glucose in test tubes. The test tubes were incubated at 37 °C for 24 h. After incubation, the tubes were decanted and washed three times with phosphate buffer saline (pH 7.3) and dried in an inverted position. The tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water and then dried in an inverted position. The strength of the stain in the test tube was scored, with reference to a blank test tube. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as none, weak, moderate, and high/strong (Mohamed et al., 2016).

Statistical analysis

The data obtained for the microbial counts in the present investigation was transformed into $\log_{10}$ before analysis. The data means were compared using Analysis of Variance (ANOVA) by the General Linear Model (GLM) of SAS software version 9.1.3 (SAS Institute, Inc.; Cary, NC). Significance levels were determined as $p<0.05$.

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Results

Microbial isolates based on Gram staining

Table 1 shows results of the Gram staining of the microbial isolates from milk, water, rinse, and swab samples. Of the 176 samples, prevalence of Gram-negative rods (83% of samples) were significantly (p<0.05) higher than the Gram-positive rods (21.6% of samples) and the Gram-positive cocci (12.5% of samples). Prevalence of Gram-negative rods and Gram-positive cocci in households samples were significantly (p<0.05) higher than samples obtained from the bulking centers. The rinse samples were the most contaminated with the Gram-negative rods (100%), followed by water samples, surface swabs, and milk samples.

Biofilm indicator microorganisms

Table 2 indicates the incidence of biofilm indicator organisms, including *Salmonella*, *Pseudomonas*, and *Bacillus* spp. from the milk bulking groups and the dairy farms as determined by biochemical tests. Of the 176 cases, contamination rates were 36 (20.4%), 32 (18.2%), and 18 (10.2%) for *Salmonella* spp., *Bacillus* spp., and *Pseudomonas* spp., respectively. Among the samples, *Salmonella* were mostly prevalent isolates in swab samples (14 out of 46; 30.4%). For *Bacillus* spp., the highest incidences were recorded in swab samples. Eight out of 60 (13.3%) water samples were contaminated with *Pseudomonas* spp., whereas it was absent in milk samples. The biofilm indicator organisms were significantly (p<0.05) more prevalent in dairy farmer households samples compared with the milk bulking centers (Table 2).

Biofilm formation capacity

The biofilm formation was categorized into high/strong, moderate, weak, and none. Table 3 presents the incidences of the biofilm forming capacity of isolates from milk, water, swab, and rinse samples. Fourteen out of 86 (16.3%) microbial isolates formed strong/high biofilms, whereas 18 out of 86 (20.93%) of isolates did not form any biofilm. Isolates from water samples had the highest incidences of high/strong biofilms, followed by swab and milk samples, while rinse isolates did not form high/strong biofilms. The rate of isolates forming strong/high biofilms in households (17.6%) was significantly (p<0.05) higher than that of milk bulking centers (11.1%).

Table 1: Prevalence of microbial isolates by Grams staining reaction from different samples from milk bulking centers and households in Lilongwe, Malawi

Sample	N	No. of positive cocci (%)	No. of positive rods (%)	No. of negative rods (%)
Milk	54	18 (33.3)	10 (18.5)	34 (63.0)
Rinse	16	0 (0.0)	4 (25.0)	16 (100)
Swab	46	2 (4.3)	14 (30.4)	40 (87.0)
Water	60	2 (3.3)	10 (16.7)	56 (93.3)
Total	176	22 (12.5)	38 (21.6)	146 (82.95)
Origin				
Bulking center	34	2 (5.9)	18 (52.9)	24 (70.6)
Households	142	20 (14.1)	20 (14.1)	122 (85.9)

Table 2: Prevalence of biofilm indicator microorganisms in different samples from milk bulking centers and households in Lilongwe, Malawi

Sample	N	No. of *Bacillus* spp. (%)	No. of *Pseudomonas* spp. (%)	No. of *Salmonella* spp. (%)
Milk	54	10 (16.7)	0 (0.0)	8 (13.3)
Rinse	16	4 (25.0)	4 (25.0)	2 (12.5)
Swab	46	12 (26.1)	6 (13.0)	14 (30.4)
Water	60	6 (10.0)	8 (13.3)	12 (20.0)
Total	176	32(18.2)	18 (10.2)	36 (20.4)
Origin				
Milk bulking centers	34	16 (47.0)	0 (0.0)	6 (17.6)
Farm gate	142	16 (11.3)	18 (12.7)	30 (21.1)

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Table 3: Number (%) of biofilm forming capacity of microbial isolates from different samples from milk bulking centers and households in Lilongwe, Malawi

Sample	N	None	Weak	Moderate	High/Strong
Milk	18	2 (11.1)	8 (44.4)	6 (33.3)	2 (11.1)
Rinse	10	0 (0.0)	0 (0.0)	10 (100)	0 (0.0)
Swab	32	8 (25.0)	10 (31.3)	8 (25.0)	6 (18.8)
Water	26	8 (30.8)	2 (7.7)	10 (38.5)	6 (23.1)
Total	86	18 (20.93)	20 (23.3)	34 (39.5)	14 (16.3)
Origin					
Milk bulking centers	18	6 (33.3)	2 (11.1)	8 (44.4)	2 (11.1)
Households	68	12 (17.6)	18 (26.5)	26 (38.2)	12 (17.6)

Discussion

In this survey, the microbial isolates from the milk, water, and milk handling contact surfaces comprised mostly of Gram-negative rods. This group includes pathogenic microorganisms such as *Salmonella* spp. and *Pseudomonas* spp. that could have emanated from the soil (excreta in the barn), water, and the personnel milking the cows. Coliforms show the hygienic conditions under which the milk was produced and also further signify the impact of the hygiene practices at the farm level on the microbial quality of milk (Pantoja et al., 2011; Wanjala et al., 2018). *Pseudomonas* spp. produce heat stable proteases, lipases, and lecithinases which are responsible for off-flavours in milk as well as sweet curdling after pasteurization (Boor et al., 2017; Cruz and Da Motta, 2019). In agreement with our finding, *Pseudomonas* spp. were found to predominate bacteria found in 31% of raw milk produced in USA (Jindal et al., 2018) and are normally isolated in milk which has undergone poor refrigeration conditions. In another study by Cruz and Da Motta (2019), it was found that *Pseudomonas fluorescens* strains isolated from raw buffalo milk in a dairy farm located in the municipality of Glorinha, Brazil were able to produce biofilm at 7 °C for 72 h. These authors stated that for the biofilm production test on stainless steel, adherent cell count was up to 7.1 log Colony Forming Unit/cm^2 at 7 °C.

As detected in this study, *Bacillus* spp. have been found to be common in dairy farms and processing plants (Oliveira et al., 2019). Similarly, Jindal et al. (2018) reported that 25% of isolates from raw milk in USA were belonged to the *Bacillus* spp. These bacteria are available in nature; soil, air, water, and animal feeds. The animals' tail, udder, and hind legs are normally soiled with dung and mud. Thus, *Bacillus* spp. are attached to the skin of the animal, and are transferred from the skin and hair of the animal during milking to the milk, which could have been a probable source in this study. Vegetative *Bacillus* spp. also produce stable extracellular protease and lipase enzymes which cause spoilage to milk and dairy products (Pasvolsky et al., 2014). *Bacilli* were also found to be predominant in the biofilms of milk and stainless steel milk handling equipment (Ksontini et al., 2013; Oliveira et al., 2019), hence their contribution to biofilm formation and eventual spoilage of milk and dairy products.

The biofilm indicator organisms isolated from the water, swab/rinse, and milk were tested for the ability to form biofilm using tube method, which is a simple and cheap method able to do phenotypic identification of biofilm-forming microorganisms (Mohamed et al., 2016). The isolates formed high/strong biofilm, moderate, weak and some of the isolates had no capacity to form biofilms. Water had the highest number of biofilm forming isolates with higher percentage of high/strong biofilm forming capacity, followed by isolates from swabs and lastly isolates from milk. The high number of biofilm forming microorganisms in water could be attributed to use of contaminated water that was not treated prior to use for cleaning by about half of farmers in Lilongwe. The cleaning process of the containers did not meet the standards as an earlier study in which more than 80% of the dairy farmers did not disinfect their milk handling containers after cleaning (Banda et al., 2019). Consequently, the poorly cleaned surfaces that are not disinfected have high concentration of organic substances to serve as nutrient for the microbes and attachment for microbes to form biofilms (Orwa et al., 2017). In a similar study, Paz-Méndez et al. (2017) found that *Salmonella* spp. could produce biofilm on polystyrene and stainless steel containing food residues. The occurrence of *Salmonella* spp. in our samples confirms the possible faecal contamination of the milk, water, and milk handling containers during milking (Banda et al., 2019; Wanjala et al., 2018). It should be noted that the other hazardous pathogenic bacteria may be present in the

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biofilms existed in milk. For instance, during a research carried out on a single 330-cow dairy farm in New York State, USA; it was showed that *Listeria monocytogenes*-containing biofilm in milking equipments was a potential source of tank milk contamination (Latorre et al., 2010). Also, Osman et al. (2014) detected pathogenic *Listeria* spp. in 5.6% of goat and 3.9% sheep milk samples from dairy farms in Cairo, Egypt. These researchers found a strong ability for biofilm formation in *L. monocytogenes* isolates.

In the free-floating organisms/planktonic culture, bacterium possesses one flagellum (Ksontini et al., 2013). However, upon surface attachment several lateral flagella sprout. The resultant daughter cell from the sessile cells develops lateral flagella that enable its easy surface attachment. The bacteria use multigenerational memory coupled on genes to adaptively adhere to surfaces. Similarly, *Bacillus* spp. and *Pseudomonas* spp. develop flagella which enhances the ability of the microbes to swim to, and attach to a surface as the microorganisms in the biofilm share genes (Guilhen et al., 2017).

This study identified *Salmonella* spp., *Bacillus* spp., and *Pseudomonas* spp. as some of the organisms contributing to biofilm formation on milk handling containers in Lilongwe, Malawi. This indicates that multispecies organisms are involved in biofilm formation as reported previously by Oliveira et al. (2019). In particular, proteolytic psychrotrophic bacteria can reduce the quality of milk and dairy products. Proper sanitation has been shown to prevent and eliminate biofilms in the dairy industry (Meesilp and Mesil, 2019). However, there are other strategies used to reduce biofilm formation, which include container construction material modification such as modified stainless steel (Jindal et al., 2018; Liu et al., 2017), and application of ultrasonic waves and enzymes (Oulahal-Lagsir et al., 2003). However, in low-income areas like Lilongwe, training, proper cleaning and disinfection practices should be enhanced to reduce bacterial contamination and minimize milk spoilage and losses.

Conclusion

Bacterial biofilm formation on milk handling container surfaces in Lilongwe, Malawi could serve as source of microbial pathogens and spoilage organisms. Therefore, it is very critical that the milk handling containers surface are thoroughly cleaned and disinfected and the water used to clean the milk handling equipment should be treated before use. The farmers and the personnel handling milk need regular training on hygiene practices and the curriculum needs to be reviewed and up-scaled in order to produce safe milk.

Author contributions

R.B., J.N., and J.M. designed the study, analyzed the data, and wrote the manuscript; R.B. conducted the experimental work. All authors read and approved the final manuscript.

Conflicts of interest

The authors had no conflict of interest.

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