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Full Length Research Paper

A novel platform test to detect beta-lactam residues in raw milk

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The mastitis causing microorganisms resist beta-lactam antibiotics by releasing beta-lactamase and the enzyme can be traced in raw milk. This study was aimed at developing a novel platform test to detect beta-lactam antibiotics residues in raw milk based on the Hardy Diagnostic Beta-lactamase Test (HDBT) reagent. The HDBT ingredients modified were penicillin, sodium chloride, trisodium citric acid, trisodium phosphate and phenol red dissolved in distilled water. Pooled raw milk samples were obtained from 3 Friesians and 3 Ayrshires lactating cows identified to have subclinical mastitis and treated using beta-lactam antibiotics. The appropriate mixing ratios were investigated at nine levels. Investigation on the effect of breeds on the test method results was also carried out. Evaluations to determine the colour differences between beta-lactam positive and negative raw milk samples for all the experiments were carried out using trained panelists. The results indicate that gradual addition of trisodium phosphate and phenol red in the reagent showed significant difference (P ≤ 0.05) between a beta-lactam positive and negative raw milk sample. Ratio 5:5 was selected as the best and had significant difference (P ≤ 0.05) from the others. Conversely, the test method indicated no significant difference (P ≤ 0.05) between the Friesians and Ayrshires raw milk samples. This method can be used along the raw milk collection routes to accept, set aside or reject raw milk suspected to have residues. The colour observed for a beta-lactam negative sample was fuchsia purple while peach or pink signified a positive sample.

Key words: Raw milk, antibiotic residues, beta-lactam, beta-lactamase enzyme, trisodium phosphate

INTRODUCTION

In the Kenyan value chain, rapid antibiotic residues testing are not up to standard (Orwa et al., 2017) and a need to improve the quality control procedures is paramount. As an appropriate control and preventive measure at the farm level, a critical control point (Ndungu et al., 2016b) development of highly sensitive detection tools to avoid the false negative results is vital (Sachi et al., 2019). Moreover, antibiotic residues quality control tests that are simple, economically sustainable and suitable for field situations, should be introduced (Prajwal et al., 2017). These platform tests should be simple, rapid and flexible for use in field-based tests to avoid time

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wastage during raw milk collection (Ndungu et al., 2016a). They should serve as a basis for accepting or rejecting raw milk. Milk processors should be encouraged to assess raw milk on residues before acceptance to ensure food safety (Kurjogi et al., 2019; Mwagore et al., 2019) and avoid miss productions when manufacturing fermented products. Factors limiting such quality control tests should be investigated and addressed together with the chain actors.

Worldwide findings have revealed that among other antibiotics, beta-lactam, penicillins and cephalosporins, were the most used antibiotic drugs in disease management at a rate of 36.54% (Sachi et al., 2019). Similar findings have been reported in Kenya and it was associated with management of mastitis (Ahlberg et al., 2016; Ali et al., 2017) which is a prevalent disease. The bacteria associated with causing mastitis include: *Staphylococcus aureus,* Enterobacteriaceae species*, Streptococcus agalacteae* and *Escherichia coli* (Ondiek et al., 2013; Gomes and Henriques, 2016). These bacteria produce beta-lactamase enzyme to counter beta-lactam antibiotics bactericidal effect, which is the most common cause of resistance (Konaklieva, 2014).

The molecular classification for beta-lactamases is based on the amino acid sequence. This classification divides beta-lactamases into class A, C, and D enzymes which utilize serine for beta-lactam hydrolysis and class B metallo-enzymes which require divalent zinc ions for substrate hydrolysis (Palzkill, 2018). Serine beta-lactamases including molecular classes A and D, represent the largest group of beta-lactamases, due to the increasing identification of Extended Spectrum Beta-lactamases (ESBLs). Penicillinases belong to class A and represent a small group of beta-lactamases with a relatively limited spectrum of hydrolytic activity. They are the predominant beta-lactamases in Gram-positive cocci, including the staphylococci and occasionally enterococci. These enzymes preferentially hydrolyze benzylpenicillin and many penicillin derivatives, but have poor hydrolysis on cephalosporins, carbapenems, or monobactams (Bush, 2018).

Beta-lactamases (penicillinases) enzyme can be traced in raw milk and urine after treatment with beta-lactam drugs and thus referred to as endogenous beta-lactamases. Its presence in raw milk indicates presence of beta-lactam antibiotic residues (Wang et al., 2013; Zhang et al., 2015; Canzani and Aldeek, 2017) which is a public health concern. This study aimed at developing a quality control test to detect beta-lactam residues in raw milk. The test development was based on Hardy Diagnostic Beta-lactamase Test (HDBT), an acidimetric test used in detecting production of beta-lactamase enzyme by microorganism to indicate antimicrobial resistance. HDBT reagent aimed at determining the resistance of *Neisseria gonorrhoeae, Haemophilus* and *Staphylococcus* species to β-lactam antibiotics through production of beta-lactamase enzyme which in turn breaks down penicillin drugs to penicilloic acid that is detected by the colour change of phenol red indicator. The ingredients that compose HDBT include penicillin, trisodium phosphate, trisodium citric acid, sodium chloride and phenol red indicator. This test method is based on the ability of mastitis causing microorganisms to resist beta-lactam antibiotics by releasing beta-lactamase enzyme which can be traced in raw milk. The ingredients used in making the HDBT reagent were modified to allow detecting of the enzyme in raw milk.

MATERIALS AND METHODS

Study site

This research was carried out at Olenguruone Dairy Farmers Cooperative Society milk quality control laboratory. Richard's farm located at Olenguruone, Nakuru county was identified and used for this study. The farm had both Friesians and Ayrshire breeds, a requirement that was key in the study.

Selection of experimental animals and treatment

The selected animals had daily milk production per cow of between 5 and 9 kg/day. The criteria used to select the animal that participated in this study involved: the breed type (pure friesians or Ayrshire); lactation stage; must have four functional udder quarters; no visible signs of mastitis. Fourteen cows were selected for milk sampling and analysis on subclinical mastitis in a laboratory. Composite milk samples from all the teats per cow were collected.

The laboratory results indicated that the cows had subclinical mastitis and the causative microorganisms identified were *S. aureus* and Enterobacteriaceae species. Six infected cows (3 Friesians and 3 Ayrshires) were selected for treatment using a qualified veterinary officer. For each cow and in their four teats, intramammary infusions were carried out. The drug used composed of: Procaine Penicillin G (60 mg), Streptomycin Sulphate (100 mg), Neomycin Sulphate (100 mg) and Prednisolone (10 mg). This was done only once in the first day of treatment. In the same day and time, the same cows were injected with 30 ml of a drug made up of 120 mg Procaine benzylpenicillin and 200 mg Dihydrostreptomycin. This was done for 3 consecutive days.

Raw milk sampling

The sampling procedure used was as per ISO 707; IDF 50, 2008. On the first day before treatment, a pooled antimicrobial-free (negative) raw milk sample of 5 L from 3 cows (either Friesians or Ayrshires) was separately obtained. This was to overcome the individual variations in raw milk composition. Similar sampling for the two breeds continued during the 3 days of treatment, 3 days of withdrawal period and 2 days after withdrawal period. Therefore, out of the 18 samples collected, 6 were negative raw milk samples collected during the 1st day before treatment and the 2 days after the withdrawal period was completed. These samples were stored in frozen conditions to retain their integrity.

Experimental design

The pooled raw milk samples, known to be beta-lactam antibiotic positive or negative were used in all experiments. Three laboratory

Table 1. The composition of Hardy Diagnostic Beta-Lactamase Test reagent.

Ingredient	Amount (g)/li of H_2O
Penicillin	15.0
Sodium Chloride	5.0
Trisodium Citric Acid	1.5
Trisodium Phosphate	0.3
Phenol Red	0.018

Table 2. The test development experiments, their respective activities and outcomes expected.

Experiment	Activities	Expected outcome
1	Making HDBT reagent in its original form and using it with a beta-lactam positive and a negative raw milk sample	The difference between a beta-lactam positive and negative raw milk sample will be insignificant
2	Making the four different reagents using the ingredients amounts as per HDBT, but excluding one of the ingredient at a time	That exclusion of one of the ingredient will enhance colour differences between a beta-lactam positive and negative raw milk sample will be insignificant
3	Making the reagent using three times more of each ingredient amount at a time.	Increased concentration of one ingredient at a time will show the ingredient that will contribute more to the colour differences
4	Establishing the exact ingredients composition that will exhibit much colour differences at various levels.	To establish the amounts that will compose the reagent concentration that will differentiate between the samples

experiments were carried out including: reagent development through modification of the HDBT ingredients, establishing the appropriate mixing ratios (milk: reagent) for better results and investigating the effect of breed on the test method results. For each experiment, sensory evaluation having trained panelist was used. Randomized Complete Block Design (RCBD) with three replications was used in reagent modification for test method development. Completely Randomized Design (CRD) with three replications was used to establish the mixing ratios (milk: reagent) and the effect of breeds on the test method results.

Modification of HDBT ingredients in test method development

Five ingredients (penicillin, sodium chloride, trisodium citric acid, trisodium phosphate and phenol red) were used as described in the HDBT test. These ingredients were sourced from Chemoquip Limited Nairobi. Their certificate of analysis and seals were verified to ensure integrity before use. Table 1 shows the composition of the reagent as described in the HDBT test. To mix the ingredients, 1 L of distilled water was used.

The first experiment involved using HDBT reagent (reagent 1) in its original composition as described in Table 1. In this experiment, equal portions of raw milk (either beta-lactam positive or negative) and reagent were mixed and colour differences observed.

In the second experiment, four reagents were made using the ingredients amounts as described in Table 1, but excluding one of the ingredient at a time. This yielded four different reagents (reagent 2-5) such that reagent 2 had: trisodium phosphate, sodium chloride, penicillin and phenol red but excluded trisodium citrate; reagent 3 had: trisodium citrate, sodium chloride, penicillin and phenol red but excluded trisodium phosphate; reagent 4 had: trisodium citrate, trisodium phosphate, penicillin and phenol red but excluded sodium chloride; reagent 5 had: trisodium citrate, trisodium phosphate, sodium chloride and phenol red but excluded penicillin. Each reagent was mixed with equal quantities of known beta-lactam positive and negative raw milk samples and the colour differences observed.

In the third experiment, three times more of each ingredient amount at a time was used in making the reagents. This resulted into five different reagents (reagent 6-10). Reagent 6, 7, 8, 9 and 10 had excess of penicillin, trisodium phosphate, sodium chloride, phenol red indicator and trisodium citrate respectively. Each reagent was mixed with equal quantities of known beta-lactam positive and negative raw milk samples to determine the colour differences.

Finally, the exact ingredients composition that will exhibit much change was investigated at various levels. Table 2 shows the experiments involved in the test method development process including the activities and the expected outcomes.

In the four experiments, ten trained panelists were requested to identify the set of samples that gave largest differences between the beta-lactam positive and negative raw milk samples using the ranking as well as a line scale method as indicated by Sharif et al. (2017). For the ranking method, they were supposed to give the highest score (30) to their most preferred set, indicating the greatest difference, and arrange them in a descending order. For the line scale method, they were requested to place a mark on a 15 cm line to indicate how they perceive the difference. The line was marked “no difference” on the extreme left and “different” on the extreme right. A ruler was used to establish the measurement from the “no difference mark” to the point where the analyst had placed a mark. These measurements were used in statistical analysis where the reagent with the highest mean was selected as the most

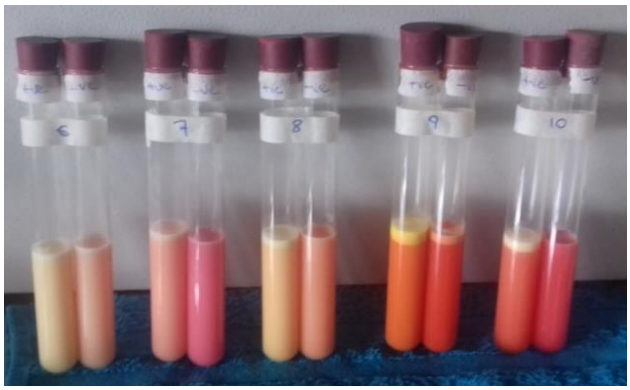

Figure 1. Colour observations when each ingredient amount was three times more of the HDBT reagent.

preferred.

Determination of reagent: Milk sample mixing ratio

The appropriate mixing proportions for reagent and raw milk were determined by mixing equal portions of reagent: milk at 9 levels. The ratios mixed included: 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 independently. For each ratio, a pair consisting of known beta-lactam positive and negative raw milk sample was prepared and respective observations made. Selection of the best ratio was carried out by 12 trained panelists through ranking and line scale method, as described earlier. The ratio ranked first and with the greatest mean indicating the largest difference was considered the best and was applied in subsequent experiments.

Determination of breed's effect on the test method outcome

Investigation on whether the test method was affected by breed differences was carried out. The breeds that were investigated included Friesians and Ayrshires. Pooled raw milk sample from 3 cows from each breed was taken for analysis, along the nine analysis days. The reagents as modified and the mixing ratio established were used in this experiment. The sensory analysis involved 10 trained panelists using the line scale method as described earlier. The measurements were used to analyze the data statistically and the means were used in determining the differences observed.

Statistical analysis

Data analysis was carried out using PROC GLM (general linear model) procedure of the statistical analysis system (SAS) version 9.4M6 (SAS Institute Inc.) for analysis of variance (ANOVA) and least significant difference (LSD) for mean separation where there were significant differences.

RESULTS

Determination of ingredients amounts

When the HDBT reagent in its original composition was used, the colour differences were not distinct and could hardly differentiate between a positive and a negative sample. Similarly, the reagents made in second experiment, where one of the ingredients was eliminated at a time, did not yield distinct differences too.

In the third experiment, where each ingredient was added three times more of the HDBT indicated amount, reagent 7, which had more of trisodium phosphate, gave clear differences between a positive and a negative raw milk sample (Figure 1). This was an expected outcome as described in Table 2. Subsequent experiment to establish the actual amount of trisodium phosphate was carried out.

Results shown in Figure 2 represent colour changes after gradual addition of trisodium phosphate and phenol red. After using 9 g of trisodium phosphate in making the reagent (reagent 14), the colour difference was not significant. However, use of 10.5 g in making reagent (reagent 15), better colour distinction between a beta-lactam positive and negative sample was observed, which was similar to reagent 7. Notable was the ceasing of colour differences observed with reagent 16, which had 12 g of trisodium phosphate. Figure 2 shows the observation made between a positive and negative sample with gradual addition of trisodium phosphate and with adjusted phenol red amounts. In Figure 1, each set of samples have a positive raw milk sample on the left test tube and a negative raw milk sample on the right test tube. In Figure 2, each set of samples have a positive raw milk sample on the right test tube and a negative raw milk sample on the left test tube.

The result as shown in Figure 2, reagent number 15, had the greatest mean and was ranked the best with a score of 300 points. Line scale method results similarly indicated that reagent number 15 had the highest mean and was significantly different ($P \leq 0.05$) from the rest. Table 3 indicates the means for every reagent for the line scale method, where reagent number 15 having the highest mean was selected as the best. Reagent numbers 13 and 12 were not significantly different ($P \leq 0.05$), similarly to reagent numbers 12 and 11. Reagent

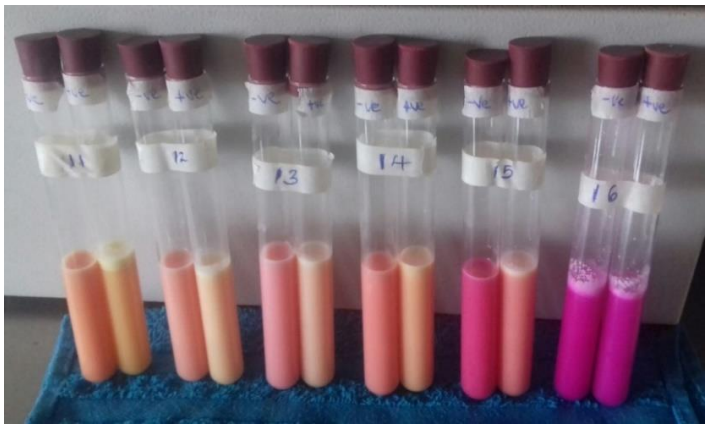

Figure 2. Colour differences with gradual addition of trisodium phosphate.

Table 3. Means for the various reagents having different amounts of phosphate.

Reagents	Line scale method
11	2.10^{d}
12	2.31^{cd}
13	2.75^{c}
14	3.43^{b}
15	14.26^{a}
16	0.34^{d}

Means within a column marked with different letters are significantly different at ($p \leq 0.05$).

Table 4. The composition of the modified test reagent.

Ingredient	Amount (g)/li of H_2O
Penicillin	15.0
Sodium Chloride	5.0
Trisodium Citric Acid	1.5
Trisodium Phosphate	10.48
Phenol Red	0.08

number 16 (Figure 2) had the lowest mean and was also significantly different ($P \leq 0.05$) from the others.

Moreover, the difference between a positive and negative sample for reagent 16 was not distinguishable. Therefore, the experiment concluded the new reagent composition as indicated in Table 4.

Determination of reagent: Milk sample mixing ratio

Out of the nine suggested mixing ratios (milk: reagent), ratios 4:6, 5:5 and 6:4 could show difference between a beta-lactam positive and a negative sample. These three ratios were selected and further analyzed to determine the best out of the three. Figure 3 shows the photo of the results for the three ratios as described. Each set of samples have a positive raw milk sample on the left test tube and a negative raw milk sample on the right test tube.

The panelists were requested to use a line scale and the ranking method to select the best ratio. Ratio 5:5 was ranked as the best having followed by 4:6 and 6:4 was ranked last. Table 5 shows the line scale method results, where ratio 5:5 or otherwise 1:1 had the highest mean and was significantly different ($p \leq 0.05$) from ratios 4:6 and 6:4. This means that for this test method to work efficiently, equal portions of raw milk and reagent should be mixed.

Determination of breed's effect on the test method residue detection

This test was carried out using both beta-lactam positive and negative raw milk samples. Figure 4 shows the test results analysis for the raw milk samples from the two breeds. Each set of samples had the Friesian raw milk sample on the left and Ayrshire raw milk sample on the right. The results indicated no significance difference ($p \leq 0.05$) between the results from Friesians and Ayrshire raw milk samples. Table 6 shows the means for six set of samples.

DISCUSSION

Bacterial resistance may occur if the bacteria can produce beta-lactamase enzyme that is able to inactivate beta-lactam antibiotics. Beta-lactamase (BLs; Enzyme commission (EC) number 3.5.2.6) is an enzyme first identified in *E. coli* and has been described as penicillinase. Beta-lactamase specifically hydrolyzes beta-

Figure 3. The results of the three mixing ratios.

Table 5. Means for different milk and reagent mixing ratio.

Ratio	Means
4:6	6.94[b]
5:5	9.99[a]
6:4	6.52[b]

Means within a column marked with different letters are significantly different at ($p \leq 0.05$).

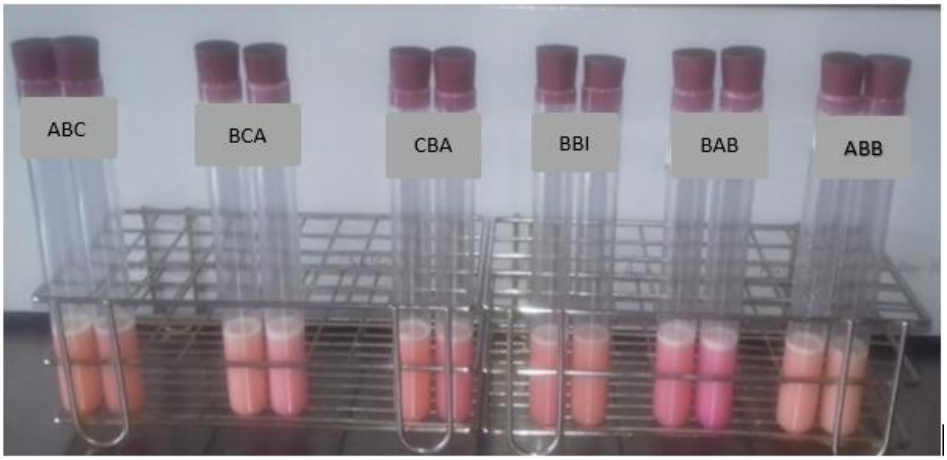

Figure 4. Different set of beta-lactam positive samples using the same reagent but raw milk samples from the different breeds.

lactam ring present in antibiotics such as penicillin, cephalosporins, monobactam, and carbapenem, and confer resistance against these antibiotics (Shaheen, 2013). Penicillinases enzyme is a serine beta-lactamase that is specific for penicillin. In their action, these enzymes first associates non-covalently with the antibiotic to yield the noncovalent complex. The beta-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally

Table 6. Means for different sample sets having Friesians and Ayrshire raw milk samples.

Set code	ABC	BCA	CBA	BBI	BAB	ABB
Means	0.92[a]	1.35[a]	1.29[a]	0.95[a]	1.17[a]	0.98[a]

Means within a row marked with different letters are significantly different at ($p \leq 0.05$).

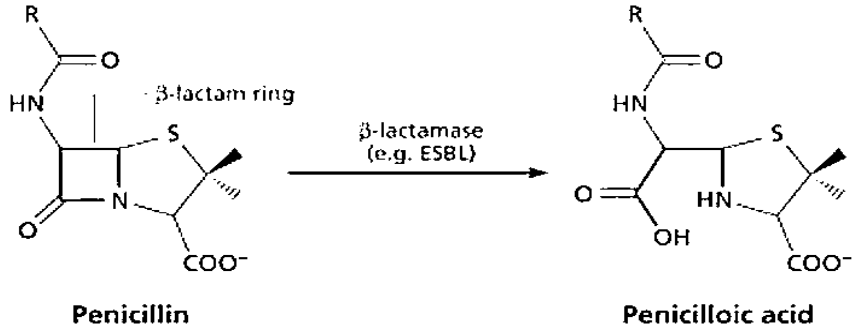

Figure 5. Hydrolysis of beta-lactam antibiotics by beta-lactamase enzymes (Harris, 2015).

liberates active enzyme and the hydrolyzed inactive drug. This mechanism is followed by beta-lactamases of molecular classes A, C, and D, but class B enzymes utilize a zinc ion to attack the beta-lactam ring (Bonomo, 2017).

Li et al. (2014) carried out research on degradation of penicillin in raw milk by beta-lactamase enzyme using ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry. In their study, beta-lactamase enzyme and penicillin were spiked in raw milk samples. The study indicated that in enzymatic degradation, two products are formed (penicilloic acid and penilloic acid). In another study carried out by Shaheen (2013), beta-lactamases was found to specifically hydrolyze beta-lactam rings present in beta-lactam antibiotics and confer resistance against the beta-lactam antibiotics. They indicated that the amide bond in a beta-lactam ring can be hydrolyzed by this enzyme to a corresponding penicilloic acid. Similarly, Harris (2015) indicated that when Beta-Lactam ring is broken by penicillinase, the resultant product is penicilloic acid without bactericidal effect. Canzani and Aldeek (2017) investigated the stability of penicillin G in various conditions including acidic, alkaline, natural acidic matrices after treatment of citrus trees infected with citrus greening disease. Penillic acid, penicilloic acid, and penilloic acid were found to be the most abundant metabolites of penicillin G. They indicated that iodometric method to determine penicilloic acids based on the decolourization of a chromophore has also been developed. Figure 5 shows the hydrolysis of beta-lactam antibiotics by beta-lactamase enzymes to yield penicilloic acid.

HDBT is an acidometric method recommended for use in testing beta-lactamase production by *N. gonorrhoeae, Haemophilus* and *Staphylococcus* spp. In HDBT test, the microorganisms were introduced into the reagent. With penicillin being one of the ingredient, and as the bacterial strive to resist its bactericidal effect by producing beta-lactamase enzyme, hydrolyses of penicillin by the enzyme occurs. This phenomenon, exhibited by phenol red indicator, showed colour changes from fuchsia purple to yellow (Hardy Diagnostic Catalogue, 2018). In this novel test method, when HDBT reagent was modified by increasing the quantity of trisodium phosphate, better results were observed. The beta-lactamase enzyme endogenically present in raw milk samples was utilized. Therefore, hydrolysis of the penicillin ring, available as an ingredient, yielding penicilloic acid was expected for beta-lactam positive raw milk samples. Production of penicilloic acid in the hydrolysis reaction causes a pH drop in the medium.

Subsequently, colour change occurs which is exhibited by an acid-base phenol red indicator, also available as an ingredient. This study agrees with previous findings that penicilloic acid is formed after hydrolysis of penicillin and this causes a drop in pH in the reaction as exhibited by phenol red indicator. This is because, the results of the experiment indicated the colour change from fuchsia purple for a beta-lactam negative test to peach/pink for a positive test. Although yellow colour expected for beta-lactam positive raw milk was not achieved, the colour difference observed between a beta-lactam negative and positive raw milk sample are enough to make judgement on the presence of residues in raw milk. These results could have been influenced by the amount of enzyme

present in raw milk. As indicated by Li et al. (2014), the dosage of beta-lactamase influences the production of penicilloic acid. Hence, if the enzyme is deficient, more penicilloic acid will not be formed limiting anticipated the colour (yellow).

Beta-lactamase, from its action of breaking penicillin, can be categorized as a hydrolase enzyme that operates optimally under neutral to alkaline pH conditions. The major modification of the HDBT reagent is the change in trisodium phosphate quantity which makes it possible to observe the differences between a positive and a negative raw milk sample. Trisodium phosphate and trisodium citrate make up the McIlvaine buffer which is used in colorimetric comparison and is prepared between a pH of 2.2 to 8.0. With increased trisodium phosphate (a strong alkali) quantity, the pH will tend towards alkaline (McIlvaine, 1921). In addition, phenol red indicator, also known as phenolsulfonphthalein, is a pH indicator dye that exhibits a gradual transition from yellow to red over a pH range of 6.2 to 8.2. Above 8.2 the dye turns to a bright fuchsia colour (Held, 2018). Therefore, when penicilloic acid results, the pH drops and phenol red exhibits the colour changes. Li et al. (2014) also established that penicillin decompose in aqueous solution into penicilloic acid by the action of either an alkali or the bacterial enzyme. This could be another reason why better colour observation was made when the concentration of trisodium phosphate was increased as the medium became more basic. With continued increase of trisodium phosphate, the pH of the medium became more basic. Since with a pH of more than 8.2 the dye turns to bright fuchsia purple, the difference between a positive and negative raw milk sample could not be realized. As indicated in this study, at ratio 1:1, optimum pH conditions for the enzyme and the indicator was achieved contributing to favorable outcomes. Li et al. (2014) considered several factors influencing beta-lactam enzymatic degradation including beta-lactamase dosage, temperature, time and pH. They established that the enzyme dosage and pH are the greatest determinant in the hydrolysis reaction. When the pH was increased from 2 to 6 and in presence of beta-lactamase enzyme, response of penicilloic acid increased. Moreover, the degradation of penicillin was enhanced by increased quantities of the enzyme.

A similar test has been developed to detect the enzyme using phenol red indicator. Nordmann et al. (2012) developed a test for rapid identification of Extended-Spectrum-Beta-Lactamase (ESBLs) in Enterobacteriaceae. This test was based on detection of beta-lactam (cephalosporin) hydrolysis which can be reversed by adding tazobactam. According to their study, ESBL activity was evidenced by change in color change from red to yellow, exhibited by phenol red indicator which is similar to this study. Also, presence of penicilloic acid has been used to detect presence of penicillin in milk. As an example, Liu et al. (2011) developed a rapid, sensitive, and specific method for the determination of penicillin G, benzylpenicilloic acid, benzylpenilloic acid, and benzylpenillic acid in bovine milk using ultra-high performance liquid chromatography-tandem mass spectrometry. Their established method was successfully applied in the determination of penicillin and their major metabolites in bovine milk samples. They detected penicilloic acid in 20% of the bovine milk samples at an average concentration 320 ng/ml. Gaare et al. (2012), detected beta-lactam antibiotics in milk using indicator strain *Bacillus cereus* producing beta-lactamase enzyme through induction. The test ampoules containing spore were tested for induction with penicillin G in spiked milk and their findings indicated complete correlation with other reference methods. They indicated that the induction method can be applied in detection of beta-lactam antibiotic residues in dairy products.

Conclusion

This study aimed at developing a test that can be applied in detecting beta-lactam residues. The novel colour reaction test described will aid in raw milk acceptance, segregating raw milk suspected with residues as well as rejecting raw milk evidently established to have residues. It can be used as a preliminary test to detect presence of beta-lactam residues at the raw milk reception platform and along the collection chain. The test results are obtained immediately which facilitates faster milk collection. The testing procedure involves preparing the reagent as described earlier and completely mixing equal portions of the reagent and fresh raw milk, making observations and recording. The colours expected for a beta-lactam positive sample are peach or pink while for a beta-lactam negative sample is fuchsia purple. Advanced research aimed at improving its applicability in detecting other residues is recommended.

Research permit and Ethical approval

This study was approved by Egerton University Board of Post Graduate Studies. Ethical clearance approval was obtained from Egerton University Ethics Review Committee (EUREC) (approval number; EUREC/APP/ 097/2020) and the research license obtained from National Commission for Science, Technology and Innovation (NACOSTI) (license number; NACOSTI/P/20/ 5861).

The aim and procedures of the study were explained to the study participants who were required to give written informed consent prior to their voluntary participation in the study. Confidentiality of research information and data was observed and maintained through forms issued and signed before research commenced, password protected computers and observing good professional conduct.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Uses and microbiological quality of fresh cow's milk sold in three markets in South Benin

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The aim of this study was to determine the common uses and evaluate the microbiological safety of raw milk sold in southern Benin. To determine the different uses of raw milk, 345 individuals were surveyed in three locations (Allada, Ouidah, and Zongo). Per location, 115 individuals were randomly sampled. For the microbial analysis, milk samples were collected from the three targeted locality in southern Benin (Allada, Ouidah, and Zongo). Our data shows that the mean values (CFU/ml) of the analyzed samples vary according to the research organisms. Thus, it is recorded 1.8×10^8 for total aerobic mesophilic flora (TMC), 4.0×10^7 for fecal coliforms (FC), 3.5×10^7 for *Escherichia coli*, 2.8×10^7 for total coliforms (TC), 2.1×10^7 for Fecal *Streptococci* (FS), 1.6×10^7 for yeasts and molds (YM), 1.7×10^7 for sulfur reducing anaerobic bacteria (SRA) and 1.2×10^7 for *Staphylococcus* spp. None of the milk samples contained *Salmonella* spp. Globally, milk samples had important bacterial load with the highest values for those collected from Zongo and Ouidah. It can be said that raw milk sold in the Southern Benin's markets does not comply with good hygienic practice rules in milking, storage, transportation and sale. Thus, raw cow milk sold presents a serious health risk for potential consumers.

Key words: Raw milk, microbiological quality, food safety, pathogens, Benin.

INTRODUCTION

The consumption of fresh milk in Benin is still relatively moderate and the government's strategies for the

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Figure 1. Map of south Benin showing the study area (dotted line).

development of the dairy sector have not succeeded in boosting it considerably. Thus, imported milk occupies an important part of the dairy market (Anihouvi et al., 2019). However, due to the relatively high price of industrial fresh milk and the lack of storage facilities, most consumers buy fresh milk, generally milked in the morning, the same day. While it is up to the manufacturer to ensure the safety and suitability of milk produced on factory farms and dairy industries, local ranchers are not making this a priority. Fresh milk as drawn from a healthy cow may contains a low microbial load which may increase in some conditions between milking and selling in local markets (Knight-Jones et al., 2016; Velázquez-Ordoñez et al., 2019). It is important that the government's strategies for the development of the dairy sector include direct collaboration with traditional producers in order to give them basic training in good hygiene practices and *in situ* quality control. To this end, data on the quality of fresh milk, for which urban demand continues to grow, are important to alert the authorities and producers and help them to set up guides to good hygiene practices, in terms of collection and storage. To our knowledge, the most recent data on the microbiological quality of fresh milk are those of Farougou et al. (2011). These authors carried out their studies in northern Benin targeting two municipalities with large dairy production.

Although, the microbial load of freshly milked milk can be decreased and the multiplication of the bacteria prevented by using clean containers and refrigerating (Bekuma and Galmessa, 2018), this is not customary in Benin. Milk is not refrigerated and barely heated between milking zones and transportation to the markets. This study was undertaken to examine the conditions on which local raw milk is stored, transported, sold and consumed and its microbiological quality as it is currently sold to consumers in local markets in South Benin.

MATERIALS AND METHODS

Survey on milk uses

A questionnaire has been addressed to milk consumers to assess milk supply, transport, storage, preservation and treatment before consumption. Stratified simple random sampling has been adopted where the study area (Figure 1) was divided into three zones: Zongo (local market at Cotonou), Allada and Ouidah at West and north of Cotonou respectively. The three zones have been selected for their importance (relative) of milk marketing. Simple random sampling was then applied to each zone (Taherdoost, 2016). A total of 345 individuals were surveyed at the rate of 115 individuals per zone.

Sample collection

A total of 387 raw cow milk samples were collected and analyzed from 3 local dairy markets between January 2019 and December 2020. Sample were randomly collected at three zones: Zongo (local market at Cotonou), Allada and Ouidah at west and north of Cotonou respectively (Figure 1). Samples were collected in the late

Table 1. Media and conditions used for the isolation of germs in milk.

Targeted microorganisms	Medium	Temperature (°C)	Incubation time (h)
Total aerobic mesophilic flora	Plate count agar (Oxoid)	30	72
Total coliforms	Brilliant Green Lactose Bile Broth (BioRad)	30	72
Fecal coliforms		44	48
Salmonella spp.	Rappaport-Vassiliadis (Oxoid),	37	24
	Salmonella-Shigella (Oxoid)	37	24
Yeasts and molds	Dichloran Rose Bengale Chloram-phénicol (Oxoid)	30	72
Sulfur reducing anaerobic bacteria	Trypticase Sulfite Neomycin (BioRad)	37	48
Staphylococcus spp.	Baird Parker (Oxoid)	37	48
Escherichia Coli	Eosin methylene blue (Oxoid)	37	24

morning and performed to reflect the relative quality of individual zones, based on data. They were transported as bought in cans to the laboratory. Approximately 100 - 200 ml milk was aseptically sampled from milk cans bought from each individual zone into a sterile bottle. Samples were directly used after withdrawal and the rest cooled at 4°C (within 30 min). Milk is collected by farmers in small plastics and brought together into 25 L water buckets (usually). The retailers then bring their 1.5 or 1 L plastics cans where they buy the fresh milk. The milk is brought to markets in this final container and sold as is to the final consumer.

Microbiological analysis

All samples and media preparation and germs counting were performed according to international standards (ISO 7218, 2007; Lahou et al., 2012; Centre for Food Safety Food and Environmental Hygiene Department, 2014). Decimal dilutions of the milk samples up to 10^{-7} have been made using buffered peptone water. The main selective media used for the isolation and enumeration of colonies are described in Table 1. The isolation and enumeration of total aerobic mesophilic flora (TMC), total coliforms (TC), fecal coliforms (TC), sulfite-reducing anaerobic bacteria (SRA), *Staphylococcus* ssp., fecal *Streptococci* (FS), yeasts and molds (YM) were performed according to international standards.

The TAM count was carried out after appropriate dilutions of the sample in the buffered peptone water broth then inoculation on Plate Count Agar (PCA) medium and incubated at 30°C for 72 h (ISO 4833, 2013). The count of total coliforms is carried out on Brilliant Green Lactose Bile medium and incubated at 30°C for total coliforms and at 44°C for fecal coliforms, the count of red colonies is carried out after 24 h of incubation (AFNOR NF V08-060, 2009).

For the detection of *Salmonella*, a pre-enrichment stock suspension (10 ml of milk in 90 ml of sterile diluent) was incubated for 18 h at 37°C. The resulting culture was inoculated with Rappaport-Vassiliadis broth at 41.5°C for 24 h. These cultures are then incubated in XLD agar at 37°C for 24 h (ISO 6579-1, 2017).

For the enumeration of yeasts and molds, 0.1 ml of the decimal dilutions is placed on the surface of the petri dishes pre-cast with DRBC agar and incubated at 25°C for three days in the dark (ISO 21527-1, 2008).

For the detection of *Staphylococcus* spp., Baird - Parker agar, a partially selective medium which exploits the capacity of staphylococci to reduce tellurite to tellurium and to detect the presence of lecithinase from the lecithin of the egg, was inoculated with 0.1 ml of dilution then incubated at 37°C for 48 h (ISO 6888-1, 1999).

For *Escherichia coli*, EMB plates were streaked by 0.1 ml sample then the plates were incubated at 37°C for 24 h. Large, blue-black colonies with a shiny metallic green appearance are counted.

Statistical analysis

Contingency tables and independence test of the χ^2 have been performed on the survey data. A significance level of 0.05 were used. Analysis of variance (ANOVA) for the comparison of means on log-transformed data was performed on microbiological parameters. Correlations analyses between different microbiological parameters were also performed on log-transformed data. All statistical analyses were performed using R software version 4.0.1 (R Core Team, 2017) and Rstudio version 1.3.959 (RStudio Team, 2020). A significance level of 0.05 were used.

RESULTS

Milk uses

The diagram of Figure 2 represents the market chain of fresh milk in the study area. Fresh and fermented milk are provided by areas around Cotonou, namely, Ouidah (~ 28 km at west Cotonou) and Allada (~ 50 km at north Cotonou). The herders collect the milk in 25 L cans in the Allada and Ouidah zones. Early in the morning, the resellers go from one herd to the other to buy the milk which they load in 1 or 1.5 L plastics cans. The milk is then transported as is from these areas to the points of sale in Allada, Ouidah and Zongo in taxi (in motorcycle or car). The milk sold in Zongo therefore comes from Ouidah and/or Allada. Some Ouidah resellers, especially those who buy fresh milk for cheese making, also get supplies from Allada where milk is apparently more available. The end consumers will buy the milk in the market exclusively.

Throughout the milk marketing chain, particular attention has been paid to the hygiene of the containers used for storage and transport. At the level of the breeders, the milking takes place with utensils that only undergo a simple washing and not each time before milking. The cans and bowls used for storage are also simply washed. The milk is stored at room temperature. The resellers bring the cans where they transfer the milk,

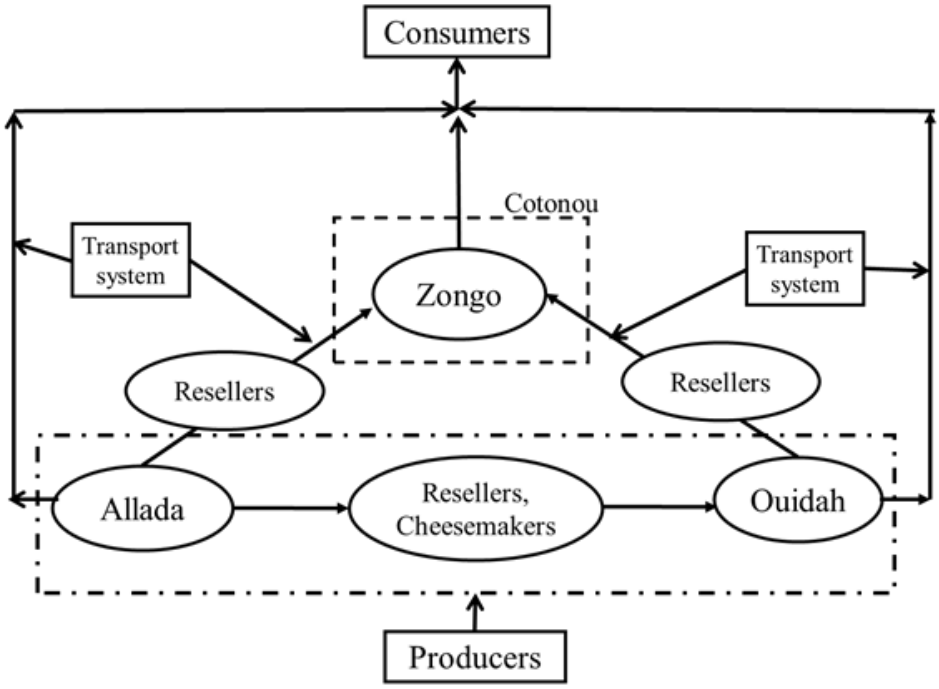

Figure 2. Diagram of the milk marketing chain in the study area.

Table 2. Cross tabulation of Zone vs. Form on which milk is sold. Cheese means sold for cheesemaking.

	Cheese	Fermented	Fresh	Total
Allada	48	10	57	115
%*r*	41.74	8.70	49.57	100.00
%*c*	59.26	9.62	35.63	33.33
Ouidah	33	18	64	115
%*r*	28.70	15.65	55.65	100.00
%*c*	40.74	17.31	40.00	33.33
Zongo	0	76	39	115
%*r*	0.00	66.09	33.91	100.00
%*c*	0.00	73.08	24.38	33.33
Total	**81**	**104**	**160**	**345**
	23.48	30.14	46.38	100.00
	100.00	100.00	100.00	100.00
	23.48	30.14	46.38	100.00

% *c* = column percent; % *r* = row percent.

Table 3. Cross tabulation of user vs. treatment.

	Cold	Heat	None	Total
Cheese maker	0	82	0	82
%*r*	0.00	100.00	0.00	100.00
%*c*	0.00	51.57	0.00	23.77
Consumer	19	53	108	180
%*r*	10.56	29.44	60.00	100.00
%*c*	100.00	33.33	64.67	52.17
Reseller	0	24	59	83
%*r*	0.00	28.92	71.08	100.00
%*c*	0.00	15.09	35.33	24.06
Total	19	159	167	345
%*r*	5.51	46.09	48.41	100.00
%*c*	100.00	100.00	100.00	100.00

% *c* = column percent; % *r* = row percent.

cans also only undergoing a simple washing. At the point of sale, the milk remains at room temperature.

The milk is bought fresh from breeders. However, in times of scarcity, the breeders collect the milk in the cans for 2 days (maximum 3 days) for the resellers who therefore recover it fermented. Table 2 shows the forms in which milk is sold to the consumers in the three zones. The columns (% *c*) and row (% *r*) percent are also shown. 41.74, 8.70 and 49.57% of milk are sold for cheesemaking, fermented and fresh respectively. The reason for separating milk sold fresh and for cheesemaking is the treatment it undergoes before consumption. At Ouidah, the proportions are 28.70, 15.65 and 55.65% respectively; while at Zongo they represent 0.00, 66.09 and 33.91% respectively. At Zongo, the milk is exclusively sold for direct consumption. As shown in Table 2, 30.14% of the milk is sold fermented and 46.38% is sold fresh in the three zones.

Table 3 shows the milk treatment by the different users in the three zones. The columns (% *c*) and row (% *r*)

Table 4. Mean values for microbiological parameters (CFU/ml)*.

Zone	Allada	Ouidah	Zongo	Mean
Total mesophilic aerobic bacteria	$9.3 \times 10^{7\,a}$	$1.7 \times 10^{8\,b}$	$2.7 \times 10^{8\,c}$	1.8×10^{8}
Total coliforms	$2.6 \times 10^{7\,a}$	$2.8 \times 10^{7\,a}$	$3.0 \times 10^{7\,c}$	2.8×10^{7}
Fecal coliforms	$< 10^{6}$ a	$8.6 \times 10^{7\,b}$	$3.4 \times 10^{7\,c}$	4.0×10^{7}
Fecal streptococci	$< 10^{6\,a}$	$4.9 \times 10^{6\,b}$	$5.9 \times 10^{7\,c}$	2.1×10^{7}
Sulfur reducing bacteria	$< 10^{6\,a}$	$2.4 \times 10^{7\,b}$	$2.8 \times 10^{7\,c}$	1.7×10^{7}
Escherichia coli	$5.3 \times 10^{7\,a}$	$5.4 \times 10^{7\,b}$	$< 10^{6\,c}$	3.5×10^{7}
Yeast and molds	$2.1 \times 10^{7\,a}$	$1.1 \times 10^{7\,b}$	$1.7 \times 10^{7\,c}$	1.6×10^{7}
Staphylococcus spp	$< 10^{6\,a}$	$5.2 \times 10^{6\,b}$	$3.1 \times 10^{7\,c}$	1.2×10^{7}
Salmonella	$< 10^{6\,a}$	$< 10^{6\,b}$	$< 10^{6\,c}$	-

*In the rows of the table, means with different letters (a, b, c) are significantly different at the level considered (0.05).

Table 5. Correlations coefficients between the different microbiological milk quality parameters.

	TMC	TC	FC	FS	SRA
TC	0.835				
FC	0.938	0.593			
FS	0.953	0.630	0.999		
SRA	0.955	0.635	0.999	1.000	
STAPH	0.993	0.896	0.889	0.909	0.912

TMAF = Total aerobic mesophilic flora; TC = total coliforms; FC = Fecal coliforms; SRA = Sulfur reducing anaerobic bacteria; STAPH = *Staphylococcus* spp.

percent are also shown. Due to the process of traditional cheese making which involves heating, 100% of cheesemakers heat their milk; while 29.44% of the direct consumers heat up the milk before use; 60.00% use it as they bought it and 10.56% refrigerate it. None of the resellers refrigerate their milk before selling or the unsold milk; while 28.92% heat it up before selling and 71.08% sell it as they buy from the farms.

Microbiological quality

The results of the microbiological analyses of the raw milk samples are summarized in Table 4. In general, the mean value of total mesophilic aerobic bacteria is very high in the three zones. However, the value is higher in central Cotonou market (Zongo, 2.7×10^{8} CFU/ml) than in Ouidah (1.7×10^{8} CFU/ml), which in turn is higher than Allada (9.3×10^{7} CFU/ml). The means are statistically different ($p < 0.001$). A similar mean total coliform was found in Allada and Ouidah ($p = 34.6\%$). Higher count was found for samples from Zongo (3.0×10^{7} CFU/mL). Fecal coliforms were not found in sample from Allada but their count is high in samples from Ouidah (8.6×10^{7} CFU/ml) and Zongo (3.4×10^{7} CFU/ml), Ouidah having a higher count. Fecal *Streptococci* were not found in sample from Allada but their count is high in samples from Ouidah (4.9×10^{6} CFU/ml) and Zongo (5.9×10^{7} CFU/ml), Zongo having a higher count. Sulfur reducing bacteria were not found in sample from Allada but in samples from Ouidah (2.4×10^{7} CFU/ml) and Zongo (2.8×10^{7} CFU/ml), Zongo having a higher count.

E. coli were not detected in samples from Zongo but in samples from Ouidah (5.4×10^{7} CFU/ml) and Allada (5.3×10^{7} CFU/ml), Ouidah having a slightly higher count. Yeast and molds have been detected in the samples from the three zones. Higher count was obtained at Allada (2.1×10^{7} CFU/ml) followed by Zongo (1.7×10^{7} CFU/ml) and then by Ouidah (1.1×10^{7} CFU/ml). *Staphylococcus* spp. were not detected in samples from Allada but in samples from Ouidah (3.1×10^{7} CFU/ml) and Zongo (5.2×10^{6} CFU/ml), the later having a higher count. *Salmonella* was absent in all samples (Table 4).

The results of the correlation analyses between pairs for the different microbiological parameters are summarized in Table 5. Results for *salmonella* were excluded from the correlation analyses because they were not present in the samples. High correlations were found between TMC and all other microorganisms except YM. There was also a high correlation between sulfur reducing bacteria and fecal coliforms (0.999) and fecal streptococci (1.000), the late correlation being the highest. Note that, also, yeast and molds and *E. coli* were not included in the correlation analysis because their presence was not constant among the three sampled zones. *E. coli* were not detected in samples from Zongo. Fecal coliforms, fecal Streptococci, sulfur reducing bacteria and *Staphylococcus* spp. were not present in sample from Allada.

DISCUSSION

The ‘‘traditional’’ milk market chain in the studied area lacks proper planning, collection and distribution facilities.

The milking conditions, the means of storage, transport and marketing do not make it possible to ensure good control of the hygienic quality of the milk sold in the studied local markets. A chi-square test of independence was performed to examine the relation between the zones and the form in which milk is sold and between the use made of the milk and the treatment before that use.

The relation between the form and the zone was significant, χ^2 (4, N = 345) = 125.75, p < 0.001. The form in which milk is sold (mainly in Zongo) is more likely to depend on to where it comes from. In the same way, there were a significant relationship between the zones and milk preservation technics. Consumers in Zongo were more likely than the others to heat or refrigerate their milk, χ^2 (4, N = 345) = 41.67, p <0.001. The small portion of users (5.51%, Table 2) who use refrigeration to preserve their milk could be explained by the fact that most milk consumers cannot afford a refrigerator and that consumers who can would buy industrial milk in supermarkets as an important part of milk is imported in Benin (Sombo, 2013). Naturally fermented milk can't be heated as this would affect its taste and cook the curd. Traditionally, milk is consumed mixed to other foods (porridge and other local meals). The time it takes to go through the sales chain without any treatment should allow microorganisms to grow rapidly in the milk. This subjects the milk consumed in Zongo to a lower microbiological quality. This is proved by the second part of this study.

The dairy policy is not a priority for the Beninese government, the implementation of the policy for the promotion and valuation of livestock products being based on livestock development programs (Dutilly et al., 2020). However, with growing interest, these policies should include collaboration with traditional marketers of milk and dairy products. This collaboration should be based on training aimed at improving the quality of milking, storage and transport of milk and publicizing the dangers that can be represented by the consumption of poor-quality milk. Unsanitary conditions or practices during the pre- and post-pasteurization processes and/or a deficiency in pasteurization have been found to conduct to high level of contamination (Martin et al., 2018). Furthermore, a study conducted in pasteurization centers in Africa (Owusu-Kwarteng et al., 2020) and in Brazil (Silva et al., 2010), suggest that pasteurization is not the only critical step for improving the microbiological quality of milk products (Pappa et al., 2019; Enayaty-Ahangar et al., 2021). Hence, the importance to integrate all the market chain in the promotion of the quality of milk and dairy products.

The European Union requirements for raw milk include an upper limit of 5 log CFU/ml (10^5 CFU/ml) for the total plate count (European Commission, 2004; Marri et al., 2020) for satisfactory quality of raw milk. In the three analyzed zones, the Total Mesophilic Count (TMC) load exceeds this microbiological criterion applicable to milks, although the ANOVA showed a significant difference between them ($p < 0.05$). The mean value for TMC (1.8×10^8 CFU/ml) is higher than that obtained by Kamana et al. (2014) in Rwanda's farms and collection centers. However, Aaku et al. (2004) in Botswana and Bonfoh (2002) in Bamako obtained a TMC range from 5.3 to 7.8 log CFU/ml (2×10^5 to 1.6×10^7 CFU/ml) and 8.1 log CFU/ml (1.3×10^8 CFU/ml) respectively. The results obtained for the TMC for Ouidah and Zongo are similar to those obtained by Farougou et al. (2011) in northern Benin. The total aerobic mesophilic flora being an indicator of the hygienic quality of raw milk, its presence in such large numbers indicates poor hygiene control during milking or of the equipment used in the transport and storage of raw milk (Clarence et al., 2009; Chorfi et al., 2020).

The results obtained for the total coliforms (TC) and fecal coliforms (FC) in raw milk from Ouidah and Zongo are higher than those (6.17×10^2 CFU/ml and 9.24×10^2 CFU/ml respectively) obtained by Farougou et al. (2011) in northern Benin. However, at Allada these germs were absent. Moreover, Kouamé-Sina et al. (2010) found in the Côte d'Ivoire that raw milk contained, on average, 5.5 log CFU/ml coliforms in milk storage tanks at farms and 6.0 log CFU/ml during sale, while Swai and Schoonman (2011) found between 6.2 and 6.6 log CFU/ml in retail raw milk in Tanzania.

Total coliforms were found in all milk samples analyzed. In addition, *E. coli* was detected in Allada and Ouidah; fecal coliforms and fecal streptococci were found in samples from Ouidah and Zongo. All values present are higher than those found by Farougou et al. (2011) in Northern Benin. El-Leboudy et al. (2014) reported mean total coliform counts of 3.28×10^2 - 1.4×10^3 CFU/ml in Egypt with the dominant isolated coliforms of 8% *E. coli*. In Zimbabwe, *E. coli* counts of 1.78- 2.21 log10 CFU/ml were reported in milk samples by Chimuti et al. (2016).

Total coliforms and fecal coliforms are considered technical indicators for the general control of fecal contamination and cleaning systems, but it is above all fecal coliforms and *E. coli* which are the most effective indicators of the direct fecal contamination. Moreover, the existence of total coliforms would be an indicator of poor hygiene practices but not necessarily direct fecal contamination of milk (Meshref, 2013). The presence of these germs in milk could be of environmental origin (Martin et al., 2016).

Sulfur reducing bacteria were found in samples from Ouidah and Zongo (2.4×10^7 and 2.8×10^7 CFU/ml respectively) but not Allada. Our counts are higher than those found by Farougou et al. (2011) in northern Benin (3.8 CFU/ml) and lower than those found by Edward and Inya (2003) in Nigeria. Contamination by these bacteria may come from cow's intestine microflora, soil or from milkers themselves as carriers (Underwood et al., 2015; Owusu-Kwarteng et al., 2020). In milk analysis, sulfur reducing bacteria are also used as fecal contamination

indicators. However, contamination by these bacteria can be minimized efficiently by good manufacturing practices (EFSA, 2005; Virpiranta et al., 2019).

Yeasts and molds were present in all samples at values higher than those found by Farougou et al. (2011) in northern Benin but much lower than those found by Edward and Inya (2003) in Nigeria. Yeasts and molds contamination is due mainly to milking and packing tools (Frank, 2007; Sørhaug, 2011). Yeasts and molds in milk may represent a potential health risk (Garnier et al., 2017) as they have been related to some disease due to milk (Fernández et al., 2015). However, they usually do not survive pasteurization (Elshrawy et al., 2019). *Salmonella* spp. were absent in all samples analyzed.

Staphylococcus spp. were found in samples from Ouidah and Zongo but not Allada. Our counts are lower than those found by Edward and Inya (2003) in Nigeria. *S. aureus* lives naturally in skin and mucous membranes of the nose and oropharynx of warm-blooded animals (Otto, 2010; Hanssen et al., 2017). It can be eliminated by simple pasteurization after which, nonetheless, some stains can keep their biological activity (Gayà and Calvo, 2018; Yu et al., 2020). Care must be taken regarding *Staphylococcus* particularly *S. aureus* because it has been known to be responsible for food poisoning outbreaks (De Buyser et al., 2001; Kadariya et al., 2014).

A correlation analysis has been performed between the microbiological parameters assessed (Table 3). Except for total coliforms, high correlation has been found between all the microbiological parameters, specially between total aerobic mesophilic count and the others parameters. Given that the total aerobic mesophilic indicate the global bacterial load in the milk, this correlation confirms the overall results obtained. Mainly, most of bacteria were present at high numbers. Furthermore, the high correlation between the fecal coliforms and fecal streptococci (0.999) and sulfur reducing bacteria (0.999) confirms the fecal contamination. On other hand, the relatively medium and high correlations between total coliforms (0.593) and fecal streptococci (0.630) and sulfur reducing bacteria (0.635) and *S. aureus* (0.896) would be due to the fact that these four bacterial groups were not present in samples from Allada. Note that however, the values for *S. aureus* obtained at Zongo and Ouidah are high, which could explain the relatively high correlation coefficient (0.896) observed.

Conclusion

Results of the study clearly indicated the low microbiological quality of raw milk sold in local markets. The milk samples analyzed from Zongo and Ouidah are generally unfit for human consumption. This is directly linked to the storage, preservation and transport steps in the milk market chain in the studied area. The presence of bacteria such as *S.aureus*, *E. coli*, yeasts and molds and some sulfur reducing bacteria (*Clostridia* for example) in raw milk is potentially dangerous and become a public health concern. Therefore, it is recommended that the dairy sector development plan involves training and guiding farmers and local milk sellers in good hygiene practices. Meanwhile, information on health hazards associated with contaminated raw milk should be extended to the public to allow the consumers to know the risks associated with the consumption of unhygienic raw milk.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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