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ANTIBIOTIC RESISTANCE OF BACTERIA ISOLATES FROM RAW COW MILK

Resistência antibiótica de bactérias isoladas de leite bovino cru

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ABSTRACT

Milk remains vulnerable to microbial contamination due to favorable microenvironmental conditions. The objective of this study was to assess the antimicrobial susceptibility profiles of bacterial isolates from raw cow milk obtained from four distinct bulk collection centers in Oyo, Southwest Nigeria. A total of 80 samples of raw cow milk were collected in aseptic conditions. A total of 20 samples were collected from each of the four bulk collection centers (Iseyin, Maya, Fashola, and Alaga) at different time intervals. Total viable bacteria count, isolation, and identification of bacteria, as well as antibiotic susceptibility testing by disc diffusion method, were performed. The findings revealed the presence of bacteria from five different genera, namely *Staphylococcus, Enterococcus, Bacillus, Salmonella*, and *Escherichia* in the raw cow milk samples. Antibiotic susceptibility testing demonstrated high sensitivity of the bacterial isolates to ciprofloxacin, gentamicin, and pefloxacin. This study provides additional evidence that raw cow milk serves as a conducive medium for the growth of various bacterial pathogens of medical significance. It is strongly recommended that proper aseptic techniques be implemented at the bulk collection centers to minimize the risk of bacterial contamination.

Keywords: antibiotic susceptibility; bacterial contamination; milk pathogens.

RESUMO

O leite permanece vulnerável à contaminação microbiana devido às condições ambientais favoráveis. O objetivo deste estudo foi avaliar os perfis de suscetibilidade antimicrobiana de isolados bacterianos de leite bovino cru, obtido em quatro centros distintos de coleta a granel em Oyo, sudoeste da Nigéria. Foram coletadas 80 amostras de leite de vaca cru em condições assépticas. Um

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total de 20 amostras foram coletadas de cada um dos quatro centros de coleta em massa (Iseyin, Maya, Fashola e Alaga) em diferentes intervalos de tempo. Foram realizados contagem total de bactérias viáveis, isolamento e identificação de bactérias, bem como testes de sensibilidade a antibióticos pelo método de difusão em disco. Os resultados revelaram a presença de bactérias de cinco gêneros diferentes, nomeadamente *Staphylococcus*, *Enterococcus*, *Bacillus*, *Salmonella* e *Escherichia* nas amostras de leite bovino cru. Os testes de suscetibilidade aos antibióticos demonstraram alta sensibilidade dos isolados bacterianos à ciprofloxacina, gentamicina e pefloxacina. Este estudo fornece evidências adicionais de que o leite bovino cru serve como meio propício para o crescimento de vários patógenos bacterianos de importância médica. É fortemente recomendado que sejam implementadas técnicas assépticas adequadas nos centros de recolha a granel para minimizar o risco de contaminação bacteriana.

Palavras-chave: suscetibilidade a antibióticos; contaminação bacteriana; patógenos do leite.

INTRODUCTION

Milk and dairy products are considered a relevant source of food for humans and animals of all ages (PERIN *et al*., 2019). In most developing countries, milk and its derivatives are produced under poor hygienic conditions and not adequately monitored by relevant authorities (WANJALA *et al*., 2018). These conditions allow the growth of pathogenic microorganisms, which may eventually cause diseases in humans when consumed (WANJALA *et al*., 2018). Thus, the presence of pathogenic bacteria in milk poses a major public health concern, particularly in environments where raw cow milk is consumed (WANJALA *et al*., 2018).

Milk is susceptible to bacterial contamination due to favorable microenvironmental conditions for bacterial growth (WEI *et al*., 2021). Thus, this food serves as an excellent culture medium for many microorganisms, particularly bacterial pathogens, owing to its availability of nutritious components (LEE *et al*., 2019). Milk-borne disease outbreaks have been reported worldwide because of ingesting contaminated milk that is normal in appearance, taste, and smell but is contaminated with a substantial number of pathogenic bacteria (BASHIR, 2021; BOYD *et al*., 2021; JENKINS *et al*., 2022; JONES *et al*., 2019; SINGH *et al*., 2022). Studies have shown that the state of bacterial contamination of milk is affected by the degree of contamination during milking and the cow's udder; however, contamination can still occur after thermal processing during transportation and handling (BORSHCH *et al*., 2017; ORTUZAR *et al*., 2018).

Antibiotic resistance has been reported in recent years in both raw and pasteurized milk samples (ELADLI *et al*., 2019; RANJBAR *et al*., 2018). Resistance to antibiotics such as penicillin, ampicillin, erythromycin, and tetracycline has been reported for several bacteria isolates such as *Escherichia coli, Staphylococcus* spp., and *Streptococcus* spp. (ELADLI *et al*., 2019; LI *et al*., 2019; MASSAWE *et al*., 2019; RANJBAR *et al*., 2018). Molecular studies have also revealed the presence of resistance genes in bacterial isolates from milk samples (LI *et al*., 2019). Although bacteria can acquire antibiotic resistance through other means, inappropriate use of antibiotics and poor hygiene play significant roles in the growth of antibiotic-resistant bacteria in milk and dairy products (MASSAWE *et al*., 2019).

This study aimed to investigate the antimicrobial susceptibility profiles of bacterial isolates from raw milk obtained from four distinct bulk collection centers in Oyo, Southwest Nigeria.

MATERIALS AND METHODS

Sample collection

A total of 80 samples of raw cow milk were collected in aseptic conditions. A total of 20 samples were collected from each of the four bulk collection centers (Iseyin, Maya, Fashola, and Alaga) at different time intervals. Throughout the transportation process from the point of

sampling to the laboratories, proper temperature control was ensured. An ice cooler and a thermometer were used to maintain a temperature of 5°C, thus preserving the integrity of the samples.

Proximate analysis

Fat, protein, casein, lactose, total solids, solid non-fat, and urea contents were determined for each of the bulk collection centers as described by Meneses *et al*. (2020).

Sample preparation

As described by Vahedi *et al*. (2013), 1 mL of collected raw cow milk at bulk collection centers was transferred aseptically into a sterile tube containing 9 mL of distilled water, followed by serial dilution twice to produce two more dilutions (10^{-3}) of the samples. Using the pour plate technique, 1 mL of the 10^{-3} dilutions of the various samples was inoculated on plate count agar (PCA) and incubated at 30°C for 48h. Bacterial counts were expressed as colony-forming units per milliliter (CFU/mL) (VAHEDI *et al*., 2013).

Isolation and characterization of bacterial isolates

Bacterial isolates were isolated and characterized by subculturing them on specific media using the streaking technique and incubated at 37 °C for 48h. Nutrient agar was used for *Staphylococcus aureus*, eosin methylene blue agar for E. coli, *Salmonella/Shigella* agar for *Salmonella* spp., and MacConkey agar for the other members of the Enterobacteriaceae family.

To identify the isolates based on their morphological characteristics, they were observed under a microscope, and their structural characteristics including shape, arrangement, and size were assessed. As described by Alghamdi (2022), the samples initially underwent a gram staining procedure and were observed using an oil-immersion objective lens at 100x magnification, which allowed classification into Gram-positive (appearing purple) or Gram-negative (appearing pink) categories. Subsequently, a series of biochemical tests, including catalase, coagulase, indole, citrate utilization, and triple sugar iron tests, were conducted. Morphological characteristics and bio-chemical tests were used for identification. The identified organisms were subcultured on nutrient agar slants, incubated at 37 °C for 24h, and stored at 4 °C.

Antibiotics susceptibility test

Each bacterial isolate was grown in nutrient broth at 37°C for 24h. To attain McFarland standard, bacterial isolate from the 24h culture was inoculated in peptone water and placed in a water bath for 2h. Standardized bacteria were inoculated onto sterile plates containing Mueller Hinton agar. Using Kirby-Bauer method, susceptibility tests for antibiotics such as gentamicin, cotrimoxazole, ofloxacin, ciprofloxacin, and pefloxacin, were carefully evaluated by placing discs of these antibiotics on the surface of inoculated agar plates. The plates were subsequently incubated at 37°C for 18h. The diameter of the clear zone surrounding each antibiotic disc, known as the zone of inhibition, was measured using a meter rule. The results were interpreted in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2012).

RESULTS AND DISCUSSIONS

Contamination of milk and dairy products with microorganisms of diverse types has been reported by various researchers (BERHANU *et al*., 2021; CONDOLEO *et al*., 2022; NYOKABI *et al*., 2021). The proximate composition of raw cow milk from samples collected from bulk collection centers such as fat, protein, casein, lactose, total solids, solid non-fat, and urea are presented in Table 1.

The total viable bacteria count of raw cow milk obtained from the four towns varied from 1.2×10^5 to 2.3×10^5 CFU/mL (Table 2). Studies conducted by Serrano *et al*. (2018) and Bohnlein *et al*. (2021) on raw milk samples

reported findings significantly lower than those of this study for total viable bacteria count.

The average *Salmonella* count observed in this study was also reported to be high (Table 2). Younis *et al*. (2018) reported *Salmonella* counts of 4.27 log CFU/mL (approximately 1.9×10^4 CFU/mL), which is lower than the results obtained in this study. The results of this study for coliform count (Table 2) corroborate those of Metz *et al*. (2020) because of the remarkably high coliform count observed and revealed the level of unsatisfactory hygienic-sanitary conditions of bulk collection centers where the raw milk samples were collected. Metz *et al*. (2020) reported that the coliform count in most raw milk studies was below 100 (1.0×10^3 CFU/mL), indicated unsatisfactory hygienic-sanitary conditions during the production of raw milk.

Table 1. Mean proximate composition of raw cow milk samples collected from bulk collection centers.

Parameter	Mean ± SD			
	Iseyin town	**Maya town**	**Fashola town**	**Alaga town**
Fat (%)	4.94 ± 0.52	3.99 ± 0.11	4.06 ± 0.01	3.36 ± 0.04
Protein (%)	3.86 ± 0.25	4.01 ± 0.21	3.99 ± 0.11	3.89 ± 0.01
Casein (%)	3.80 ± 0.34	3.91 ± 0.16	3.81 ± 0.12	3.81 ±0.11
Lactose (%)	5.30 ± 0.32	5.16 ± 0.11	5.00 ± 0.00	5.06 ± 0.21
Total solids (%)	12.40 ± 1.00	11.90 ± 0.91	12.10 ± 0.81	12.30 ± 0.39
Solid non-fat (%)	9.18 ± 0.73	10.10 ± 0.51	11.80 ± 0.43	11.10 ± 0.21
Urea (mg/L)	129 ± 3.82	127 ± 2.41	120 ± 0.39	121 ± 0.09

Table 2. Microbiological quality of raw cow milk samples.

Parameter	(Mean ± SD) × 10^5 CFU/mL			
	Iseyin town	**Maya town**	**Fashola town**	**Alaga town**
Total viable bacteria count	2.1 ± 0.07	2.3 ± 0.15	1.2 ± 0.20	1.4 ± 0.20
Salmonella count	1.6 ± 0.15	1.6 ± 0.12	0.8 ± 0.13	1.0 ± 0.19
Coliform count	2.3 ± 0.18	1.3 ± 0.15	0.7 ± 0.13	1.0 ± 0.16

In this study, 77 bacterial contaminants belonging to five different genera, *Staphylococcus, Enterococcus, Bacillus, Salmonella*, and *Escherichia*, were isolated from raw cow milk collected from bulk collection centers of dairy farms in towns in Oyo State, Nigeria (Table 3). These genera included the following species*: S. aureus, Staphylococcus epidermis, Enterococcus faecalis, Bacillus subtilis, Salmonella enterica serovar*. Typhimurium, and *E. coli* (Table 3). Of all the species *E. coli* has the highest percentage of occurrence 36.4% followed by *S. epidermis* 22.1%, *Salmonella* Typhimurium 18.2%, *S. aureus* 14.3%, *E. faecalis* 6.5%, and *B. subtilis* had the lowest percentage with 2.6% as is shown in Table 3.

For all the bacterial isolates, *S. aureus* was fully susceptible to gentamicin and ciprofloxacin, *S. epidermidis* showed the greatest sensitivity to ofloxacin, *E. faecalis* was susceptible to all the antibiotics used in the study, and *B. subtilis* was least sensitive to ofloxacin (Table 4). *S. epidermidis* isolates were the group that characteristically displayed low susceptibility, studies such as Eladli *et al*. (2019) and Chajęcka-Wierzchowska (2019) have proved that resistant strains can be found in milk and dairy products.

Table 3. Distribution of bacterial species isolated from raw milk samples following identification using morphological and biochemical characteristics.

Bacterial species	Number of isolates	Percentage (%)
E. coli	28	36.4
S. epidermidis	17	22.1
S. Typhimurium	14	18.2
S. aureus	11	14.3
E. faecalis	5	6.5
B. subtilis	2	2.6
Total	77	100

Table 4. Antibiotic susceptibility patterns of bacteria isolated from raw milk samples.

Bacterial species	Number of isolates	Gentamicin	Cotrimoxazole	Ofloxacin	Ciprofloxacin	Pefloxacin
S. aureus	11	100	82	82	100	91
S. epidermidis	17	59	59	65	59	17
E. faecalis	5	100	100	100	100	100
B. subtilis	2	100	100	50	100	100
S. Typhimurium	14	50	50	50	57	57
E. coli	28	36	29	29	71	86

S. Typhimurium showed the highest sensitivity to ciprofloxacin and pefloxacin, and *E. coli* showed the least sensitivity to gentamicin (Table 4). *E. coli* characteristically showed low susceptibility to the antibiotics used in this study, which was also observed in a study carried out by Singh *et al*. (2021) in milk and dairy products, but the susceptibility level of this current study was significantly lower. Recent studies carried out by Algammal *et al*. (2020) and Yu *et al*. (2020) investigating raw milk samples from mastitis cases in Egypt and China, respectively showed that *S. aureus* and *E. coli* were resistant to antibiotics. Antibiotic resistance could arise from indiscriminate antibiotic use for cows to prevent diseases such as mastitis.

CONCLUSION

This study showed that raw cow milk remains a suitable food for microbial growth because of the variety of nutrients it contains. The diversity of isolates found in this study raises concerns about the environmental conditions of bulk collection centers, as well as the overall hygiene of the handlers. Antimicrobial susceptibility also suggests that antibiotics may have been misused. To avoid disease outbreaks in these bulk collection centers, it is mandatory to maintain a high standard of hygiene in these facilities. It is also recommended that appropriate aseptic techniques be used at bulk collection centers to reduce the risk of bacterial contamination.

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