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Original Research Article

Isolation and Identification of Fungal and Bacterial pathogens associated with consumed skimmed milk in North Western Kano state, Nigeria

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Abstract

Skimmed milk is a fermented milk product primarily consumed by the Hausa and Fulani communities in northern Nigeria. It can easily be contaminated throughout the milking and handling process, which makes it a primary way for milk-borne diseases to enter the human population. Numerous health issues are brought on by the wide diversity of microbial pathogens, which live in practically every environment. The study was carried out to isolate and identify the bacterial and fungal pathogens associated with skimmed milk in the northwest of Kano State, Nigeria. To find out how frequently bacterial and fungal diseases appeared in particular areas where skimmed milk was served, microscopic isolation and identification of these pathogens was done. According to the results of the mycological study, Aspergillus flavus has the lowest percentage of prevalence in skimmed milk at 11.76%, followed by Rhizopus stolonifer at 21.05% in location A and Mucor at 29.41% in location C. In location B, Diplobacilli was the most frequently found bacterium (37.50%), followed by E. coli (33.33%), and staphylococcus (11.76%) which was the least. The result of the study showed that the skim milk sold in the three (3) chosen locations had subpar bacterial and fungal quality. Since the local Fulani people are the main producers of the product (skimmed milk), they should generally be educated in sanitary practices and hygiene. It's also critical to put in place efficient control measures to reduce the possibility of pathogenic microbes contaminating skim milk and other dairy products.

Keywords: *Skimmed milk, Bacteria, Fungi, Kano and Occurrence.*

1.0.Introduction

Milk is the most complete food since it has all the vitamins the body needs for normal growth and development. They provide the most vital amino acid required by humans for body creation and tissue repair (HI, 2019). The Hausa and Fulani tribes in northern Nigeria are the main consumers of skim milk, also known as kindirmo, a locally fermented milk product (Okeke, et. al., 2014). Although the composition of locally fermented milk varies by area, it remains a staple of daily nutrition (Nassarawa & Sulaiman, 2020). Skim milk is also known as fat-free, defatted, or non-fat; if the fat is left in the milk, it can be kept in storage for up to 16 months without spoiling (HI, 2019). The Fulani people produce almost 90% of the skim milk produced in Nigeria; their women exercise strong control over the milk's marketing and processing (Chukwuma, 2009). Skim milk is susceptible to microbiological contamination during processing and marketing if it is not handled carefully. The disregard of hygiene protocols may lead to the development of diseases transmitted by milk, particularly in urban dwellers who consume the fresh or fermented milk that the Fulani women sell (Ijah, et. al., 2002; Chukwuma, 2009). This is because the majority of them lack knowledge in food hygiene and are unaware of the dangers of eating contaminated food.

During the processes of milking, handling, storing, and other pre-processing procedures, microorganisms may contaminate the milk (Addo et al., 2011). Fresh milk from a healthy udder has very few bacteria. There are around 500–1000 bacteria per milliliter; the quantity of bacteria grows dramatically during storage and transit. The milk gets contaminated with germs when it is taken from the udder. The milk's temperature and the kinds of bacteria it contains influence how quickly organisms proliferate. Milk will usually keep for 72 hours when kept at 4°C. The rate of multiplication is also influenced by the milk's bacterial contamination level. Animal droppings, air, feed ingredients, dirt, milk handling equipment, milker's cloth, and other activities are a few potential sources of bacteria in milk (HI, 2019). Skim milk can get contaminated with a range of germs from different sources during the stages of manufacturing, processing, and handling; this makes it dangerous for consumption and puts the public's health at risk. Due to the lack of further processing before consumption, handlers of fermented milk products in local markets may not follow proper hygiene practices, which could lead to spoilage with harmful bacteria, including fungus (Tamine and Robinson, 1988).

Presence of yeasts and molds cause the product to deteriorate, which is why it is not good for dairy products. Furthermore, counts of yeast and mould are recognized as the industry standard in a number of countries for assessing factory sanitation since they are indicators of poor production and packaging standards in yoghurt. Fungal deterioration in fermented skim milk products is indicated by a variety of metabolic by-products that produce off flavors and odors in addition to noticeable color and texture changes. The most common spoiling species are spore formers, which include *Aspergillus, Penicillium, Rhizopus, Fusarium*, and *Trichoderma* as many authors; Abeer and Dardir (2009); El-Malt et al. (2013); Ibrahim (2005) (ElAnsary, 2014); and (Gebril et al., 2021) have investigated the microbiological quality of fermented milks. This study was originally conducted to detect the pathogenic bacteria and fungi associated with skimmed milk in northwest Nigeria, Kano state, because skim milk is commonly consumed in that region.

2.0. MATERIAL AND METHOD

2.1. Study area

The study was conducted in the northwest Nigerian state of Kano, which has 4,491,000 inhabitants as of 2024 (World Population Prospects). The Kano metropolitan area is composed of the following eight local governments: Kumbotso, Ungogo, Tarauni, Nassarawa, Gwale, Fagge, and Kano Municipal. It is the third-largest state in Nigeria, after Lagos and Ibadan (Nassarawa & Sulaiman, 2020).

2.2. Sample Collection

In this study, nine (9) samples of locally fermented skim milk (kindirmo) were used. The samples were collected at random from various locations within Kano metropolitan area, including the Yankaba market in Nassarawa metropolitan area, the Kofar wambai market in Fagge metropolitan area, and the Kano State Ministry of Land, where the majority of Hausa/Fulani women hawk the product. The samples were aseptically collected at random from three (3) separate dealers in each hamlet at these various points. They were all coded as samples 1 through 9 for identification purposes and kept in a refrigerated box with ice blocks while the fieldwork was being conducted. The sample was transported to biological science laboratory of Yusuf Maitama Sule University in Kano.

2.3. Isolation and Identification of Bacterial Pathogens of Skimmed Milk

2.3.1. Serial Dilution and Plate Count

Six (6) test tubes, each containing nine milliliters of sterile distilled water, were used to serially dilute the samples. The numbers 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} were written on the test tubes. One milliliter of the sample was pipetted into the first test tube, yielding a 10^{-6} dilution and also same throughout the other tubes and all the three samples. Petri-dish plates were arranged on the work bench following serial dilution, and transferred in 1 mL of the dilution product to an appropriate Petri dish plate. Aseptically, 20ml of nutritional agar was added to each Petri dish holding the sample. After then, it was stirred and given time to solidify. Following that, the Petri dishes were inverted and incubated for 24 hours at 37 ºC (Cheesebrough, 2006). Following incubation, the average number of bacterial colonies in each plates was determined.

2.3.2. Gram's staining and Microscopy

After 24 hours of incubation, the colonies' growth was observed, and the samples were ready for microscopy using Gram's staining techniques as conducted by (Smith & Hussey, 2016). Using a wire loop to select a subset of the colonies, a tiny smear was created on a glass slide. The smear was heated over flames after being let to air dry. The fix smear was stained with crystal violet for sixty seconds. The discoloration was swiftly gone, and it was then cleaned once again after being covered for 60 seconds with Lugol's iodine. The smear was immediately washed after being rapidly recolored for a few seconds with acid alcohol. Methyl red stain was then applied for two minutes before being removed with water. The smear was placed on a draining rack to air dry after the slide's back had been cleaned. The smear was first examined under a microscope with 40x lenses to verify the staining and material distribution. After that, oil immersion objectives were used to identify the bacteria. In order to determine the organism's physical characteristics, slides were inspected using a 100x objective lens (Cheesebrough, 2006).

2.4. Isolation and Identification of Fungal Pathogens of Skimmed Milk

Serial dilution was also carried out in order to isolate the fungal pathogens linked to skim milk, using the procedure previously described by (Cheesebrough, 2006). The plates were serially diluted and then incubated for seven days at room temperature (28 ± 3°C) in order to obtain pure cultures on PDA slants, each kind of fungal colony was subcultured using a new medium (PDA) after the colonies on each plate were counted and recorded. Cotton blue and lactophenol stain were used in the (Balali and Malakout, 2002) method to identify the unidentified isolated fungus. To aid in identification, a drop of the lactophenol stain was added to the clean slide after a tiny portion of the mycelium from the fungal cultures was removed using a mounting needle and placed in a drop of lactophenol. The mycelium was uniformly spread out on the slide with the use of the needle. A cover-slip was carefully put with minimal pressure in order to eliminate air bubbles. Subsequently, the slide was mounted and examined using ×10 and ×40 objective lenses, respectively (Cheesbrough, 2000). Using the following formula, the number of bacteria or fungal colony forming units per gramme of the samples was determined:

N= n/vd

Where; N= the number of bacterial colonies per ml of sample.

n= number of colonies counted.

V= volume of sample (inoculums) used.

D= dilution factor.

2.5. Experimental Designs and Statistical Analysis

Three replications of the completely randomized design (CRD) were employed. The SAS software was used to statistically analyze the data, and Analysis of Variance (ANOVA) was used to look at the inhibitions of radial mycelial growth, the protected Fisher's Least Significance Difference test (LSD) was used to distinguish the means that were significant at p = 0.05.

3.0. RESULTS AND DISCUSSION

3.1. Isolation and identification of bacterial pathogens of skimmed milk

Occurrence of Bacterial Isolate In the three locations (Sample A, B, and C).

The Table 1 below showed the occurrence of bacterial isolates in the three different locations carried out in the current study; the result revealed the occurrence and presence of *Bacillus spp* in moderate amount in location (A and B), but found to be in higher amount in the samples collected from sample (C) *Staphylococcus spp* was also found in sample taken from location (A) in moderate amount and in higher amount in location (B) samples; but not detected in location (C) sample. *Micrococcus spp* was found in location (A) sample in a moderate amount but absent in the two samples of location (B and C); Additionally, the presence study revealed the presence of *E. coli spp* in both the samples gotten in all the locations, Location (A and B) in higher amount and location (C) in low amount. The current study also noticed the occurrence of *Micrococcus spp* in Location (A) and no any trace of it the samples of location (B and C). Interestingly *Streptobacilli spp* and *Diplobacilli spp* were also noticed in Location (C) sample and no any trace of it in location (A and B) samples.

Table 1: Occurrence of Bacterial Isolate In three 3 Locations (A, B, and C)

	Bacillus spps	***E.coli spp***	***Micrococcus spp***	***Staphylococcus spp***	***Streptococcus spp***	***Streptobacilli spp***	***Diplobacilli spp***
Location A	++	+++ +	++	+	++	-	-
Location B	++	+++ +	-	++	++++	-	-
Location C	++++	+	-	-	-	+	++

Key: higher (++++), Moderate (++); Low (+), and (-) absence

3.2. Percentage occurrence of Bacterial Isolates in Location (A, B & C)

The result of bacterial percentage occurrence in location (A, B & C) were presented in table 2, the result showed that *Bacillus spp* has percentage occurrence of (17.84%) in location A, (20%) in location B and (31.25%). *E. coli* occurred with (29.41%) in location A, (33.33%) in location B and (18.50%) in location C. *Micrococcus* with percentage occurrence of (17.64%) in location A, but absence in location B & C. *Staphylococcus* occurred with (11.76%) in location A, (20%) in location B but absence in location C. *Streptococcus* has percentage occurrence of (23.52%) in location A, (26.66%) in location B and absence in location C. *Streptobacilli* is absent in location A & B but presence in location C

with (12.50%) percentage occurrence. *Diplococci* spp does not occur in location A & C but presence in location C with (17.50%) percentage occurrence.

Table 2: Percentage Occurrence of Bacteria in Location (A, B & C)

	LOCATION A	LOCATION B	LOCATION C
Bacillus spp	17.84%	20%	31.25%
E.coli spp	29.41%	33.33%	18.50%
Micrococcus spp	17.64%	_	_
Staphylococcus spp	11.76%	20%	_
Streptococcus spp	23.52%	26%	_
Streotobacilli spp	_	_	12.50%
Diplobacilli spp	_	_	17.50%

3.3. Isolation of pathogenic fungal isolates across the three locations

The results of the pathogenic fungi associated with locally fermented skimmed milk in three different location in Kano state was highlighted in Table 3: the result revealed the presence of *Aspergillus flavips, Aspergillus niger, Aspergillus flavus, Mucor, and Rhizopus stolonifer* across the three (3) locations samples analyzed in higher and moderate amount, only *Mucor* and *Rhizopus stolonifer* of location (C) were found in low amount.

Table 3: Occurrence of Fungal Isolate in the three locations (Location A, B, and C)

	Aspergillus flavips	***Aspergillus flavus***	***Aspergillus niger***	***Mucor spp***	***Rhizopus stolonifer***
Location A	+ + + +	+ +	+ +	+ + + +	+ + + +
Location B	+ + + +	+ + + +	+ +	+ + + +	+ + + +
Location C	+ + + +	+ +	+ + + +	+	+

Key: higher (+ + + +); Moderate (+ +); Low (+); and (-) absence

3.4. Percentage Occurrence of Fungi in Location (A, B & C)

The results of the percentage occurrence of isolated Pathogenetic fungi from the three (4) different locations of the current study was showed in the Table 4 below, the result showed *Aspergillus niger* occurrence of (15.79%) in location A, (15%) in location B and (23.52%) in location C. *Aspergillus flavips* occurred with (21.05%) in location A, (20%) in location B and (17.64%) in location C. *Aspergillus flavus* occurred with (15.79%) in location A, (20%) in location B and (11.76%) in location C. *Mucor* occurred with (26.31%) in location A, (25%) in location B and (29.41%) in location C. *Rhizopus stolonifer* occurred (21.05%) in location A, (20%) in location B and (17.64%) in location C.

Table 4: Percentage Occurrence of Fungi in Location (A, B & C)

FUNGI	LOCATION A	LOCATION B	LOCATION C
Aspergillus niger spp	15.79%	15%	23.52%
Aspergillus flavips spp	21.05%	20%	17.64%
Aspergillus flavus spp	15.79%	20%	11.76%
Mucor spp	26.31%	25%	29.41%
Rhizopus stolonifer spp	21.05%	20%	17.64%

3.5. Total Coliform Count of the three samples

Table 4 Represent the total fungal Coliform counts (CFU/ml) of the three samples of fermented skimmed milk (Nono) analyzed collected from the three different locations. The total bacterial count ranges from $11x10^5$ to $89x10^5$ CFU/ml in the samples collected from location A. and $25x10^5$ to $51x10^5$ CFU/ml in the samples collected from location B; and samples collected from location C, ranges from $14x10^5$ to $96x10^5$ CFU/ml.

Table 4: Total Coliform Count across the three Sample

No of plates	Total fungal count in location A	Total fungal count in location B	Total fungal count in location C
1	$32x10^5$	$51x10^5$	$96x10^5$
2	$19x10^5$	$32x10^5$	$14x10^5$
3	$21x10^5$	$27x10^5$	$30x10^5$
4	$11x10^5$	$41x10^5$	$52x10^5$
5	$89x10^5$	$36x10^5$	$17x10^5$
6	$49x10^5$	$25x10^5$	$21x10^5$

4.0. Discussion

In the dairy sector, pathogenic microorganisms are a major worry since they can seriously endanger customers' health, especially when present in skim milk. Skim milk is a byproduct of skimming milk, which is made by separating the fat from the milk, leaving behind a nutrient-rich liquid that fosters the growth of a variety of microorganisms. These microorganisms include *Salmonella species*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Campylobacter spp*. (Claeys et al., 2013; Oliver et al., 2005; Vivant et al., 2013; Le Loir et al., 2003; and Whyte et al., 2004) as well as *Aspergillus* species, specifically *Aspergillus flavus* and *Aspergillus parasiticus*. This study found the presence of *Staphylococcus spp*., *Bacillus spp*., *Micrococcus spp., Streptococcus spp., Diplobacilli; Streptobacilli* and *E. coli.* The results revealed that the presence of *Staphylococcus spp*., *Bacillus spp*., *Micrococcus spp., Streptococcus spp* and *E. coli* were present in both Location A and B is in consistent with the findings of Nassarawa and Sulaiman (2020) which showed the presence of these three pathogenic bacterial isolates (*Bacillus spp., Staphylococcus spp.,* and *Micrococcus spp*.) in the three sites. The results of this study show that *E. coli* has the highest occurrence of (33.33%) in location B and (29.41%) in location A; followed by *Streptococcus* (23.52%) in location B, and *staphylococcus* which has the lowest occurrence of (11.76%) in location A and. The present study is similar to the findings of Nassarawa & Sulaiman; but their findings indicated the presence of *Staphylococcus* species has the highest occurrence of (34%), followed by *Bacillus* species (14.3%), *Micrococcus* species (14.3%), *Streptococcus* species (20%), and revealed that *E. coli* has the least occurrence of (17.1%) (Nassarawa & Sulaiman, 2020). Taking milk contaminated with any of the isolated pathogens of the current study can pose a severe health effect because they were producers of enterotoxins which are heat-stable toxins (Bukuku 2013).

E. *coli* was found in this study in a rather high detection rate, which showed it extreme potentiality to causes disease to human when looking into the effect of *E. coli* bacterium, the observation cannot be disregarded. Therefore, in order to protect the public's health, *E. coli* and other pathogenic microorganisms in milk should be addressed seriously. Lactic acid bacteria such as *Bacillus species* and certain *Streptococcus specie*, thrive in milk and facilitate fermentation. Nevertheless, is dependent upon the native micro flora that grows there, which in turn represents the local climate. As the study revealed the presence of *Streptococcus* bacterium in a moderate amount, Pharyngitis, tonsillitis, arthritis, bone infections, bacterial pneumonia, and rheumatic fever have all been linked to *Streptococcus species* (Okeke et al., 2014). As per other findings, bacteria exhibited the maximum proportion of incidence; the dairy industry is faced with a devastating farm animal ailment called mastitis, which is linked to *Staphylococcus aureus* (Mdegela et al., 2009). Therefore, strains that are coagulase positive alone are deemed possibly enterotoxic. Strict sanitation and hygiene regulations must be followed at every stage of the production and processing chain to further reduce the risk of pathogenic microorganisms in skimmed milk. These procedures include correct pasteurization or other efficient heat treatments, as well as maintaining the right storage temperatures. Implementing efficient control measures is also essential to reducing the danger of aflatoxins generation and pathogenic microbe contamination in skimmed milk and other dairy products. These methods include acceptable manufacturing processes, suitable storage conditions, and routine monitoring.

The current study demonstrated the presence of the three fungal isolates (*Aspergillus niger, Aspergillus flavus, Rhizopus stolonifer,* and *Mucor*) in all of the samples that were taken from the three locations. *Mucor* is the highest occurrence, in all the three locations with (A, B, and C 26.31%, 25%, and 2941%, respectively). Followed by *Rhizopus stolonifer* with (21.05%) in location A; (20%) in location B and (17.64%) in location C, while *Aspergillus flavus* is the least with percentage of occurrence of (11.76%) in location C. These results are comparable to those of Okeke et al. (2014), who isolated the aforementioned fungi and discovered that *Aspergillus niger* had the highest percentage, followed by *Rhizopus stolonifer* (27.3%), and *Aspergillus flavus* with the least percentage, 6%. The existence of fungi in milk products may be caused by air pollution, polluted earthenware, or improper hygienic practices on the part of local producers. Polluted cattle feed may also be a source of fungal spread. Because some strains of Aspergillus flavus produce aflatoxins, a strong toxin linked to hepatoxicity and cancer in mammals, including humans, consuming cow milk

products could be dangerous due to the presence of this fungus (Okeke, 2014). The presence of the isolated fungi may be a consequence of the low level of hygiene maintained during processing and handling of the product, this includes the handlers, the quality of water used and the utensils.

In Nigeria, skimmed milk is a widely consumed dairy product, and its safety is of utmost concern due to the potential for fungal contamination and subsequent mycotoxin production. Several studies have been conducted to isolate and identify the fungal pathogens associated with skimmed milk in various regions of the country as carried out in the current study. The most commonly isolated fungal genera from skimmed milk samples in Nigeria include *Aspergillus,* it is known to produce various mycotoxins, such as aflatoxins, ochratoxins, fumonisins, and patulin, which can have severe health implications for consumers (Oyebanji et al., 2021; Adebesin et al., 2001.) Rhizopus is a genus of filamentous fungi that belongs to the order Mucorales and is known to cause spoilage in various food products, including dairy products. *Rhizopus species* has been isolated as a fungal pathogen from skimmed milk samples in Nigeria as isolated in the current study. (Adebesin et al., 2001) isolated various fungal species, including *Rhizopus species*, from skimmed milk samples collected in Bauchi, Nigeria; (Nwachukwu et al., 2013) also investigated the fungal contamination of various milk products, including skimmed milk, in Warri, Nigeria, *Rhizopus species* were among the isolated fungi from skimmed milk samples. The study conducted by (Oyedeji et al., 2013) also reported the presence of *Rhizopus species* as one of the fungal contaminants isolated in Ilorin, Nigeria. Rhizopus species are known to produce various metabolites, including mycotoxins like rhizoxin, which can be harmful to human health if consumed in significant quantities. The presence of *Rhizopus* in skimmed milk samples indicates the need for proper handling, storage, and processing practices to prevent fungal contamination and potential mycotoxins production in dairy products. Poor storage conditions, including high temperature and humidity, as well as inadequate processing and handling practices, are major contributing factors to fungal growth and mycotoxins contamination in skimmed milk in Nigeria (Bankole and Adebanjo 2003) To mitigate the risks associated with fungal pathogens in skimmed milk, it is crucial to implement strict quality control measures throughout the production, processing, and distribution chain. This includes maintaining proper storage conditions, implementing effective heat treatments (pasteurization or sterilization), and regularly monitoring skimmed milk samples for the presence of fungi and mycotoxins. Additionally, raising awareness among producers, handlers, and consumers about the potential risks and preventive measures is essential to ensure the safety and quality of skimmed milk consumed in Nigeria.

5.0. Concluding remarks

A dairy product high in nutrients, skimmed milk can be the perfect growing medium for bacteria fungi and other harmful germs. It is critical to identify and manage harmful bacteria and fungus in skimmed milk in order to protect the general public's health. By combining preventive measures at every stage of the production and distribution process with efficient isolation and identification procedures, the risk of foodborne illnesses linked to skimmed milk consumption can be greatly decreased. The current investigation found that the skim milk sold in the three chosen locations had subpar fungal and bacteriological quality. This was ascribed to the existence of indicator and pathogenic bacteria and fungi, including *Aspergillus flavus, mucor, and E. coli,* and *S. aureus*, It is quite concerning for human health that these bacteria and fungi are present in skimmed milk. Poor hygienic standards in the production of skimmed milk are reflected in the rise of these pathogenic organisms and their toxins in local dairy cow products. However, it should be mentioned that the kinds of organisms and their density in the milk products from the study areas should worry the health authorities much because these provide major risks to the public's health.

Declarations

Consent for publication: All the authors read and are aware of publishing of the manuscript

Conflict of interest: The authors declare that they do not have any conflict of interest.

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