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Original Research Article

Growth Kinetics of Bacteria Isolated from Laboratory Prepared Cheese Made From Different Milk Sources

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Abstract

***Background*:** Dairy food products constitute a major part of our routine diets and it is fundamental to the food industry globally. However, the need for quality control cannot be over-emphasized. A microbiological guideline must be used to define the differences between unacceptable and acceptable food products as food-borne related diseases have been associated with milk and dairy products. ***Aim of Study*:** The study aims to evaluate the growth kinetics of cheese prepared in the laboratory from different milk types over a 96 hour period in order to determine its bacteriological quality and assess the effect of storage time on the pH of the various cheeses. ***Methods*:** The laboratory cheese was prepared from fresh Goat, cow, sheep and soybeans using the traditional method. Thereafter, 10g of the cheese samples were stored in sterile 250 ml conical flasks at temperature of 28±2 ^{o}C for duration of 96 h. The samples were analyzed every 12-24 h interval using Nutrient Agar, MacConkey Agar, Mannitol Salt Agar and De Mann Rogosa and Sharpe Agar for their bacterial, coliform, staphylococcal and lactobacilli load respectively. ***Results*:** At 96 h the highest heterotrophic bacterial load, coliform, staphylococcal and lactic acid bacteria counts were observed in sheep milk cheese (8.27 ± 0.02 Log_{10}cfu/g), (7.10 ± 0.33 Log_{10}cfu/g), (7.40 ± 0.34 Log_{10}cfu/g) and cow milk cheese (8.19 ± 0.05 Log_{10}cfu/g) respectively. At 0 h the highest pH was recorded in Soybean milk cheese (5.76±0.01) while the least was cow milk cheese (4.97±0.11). The highest pH at 96 h was Soybean milk cheese (6.96±0.01) while the least pH was recorded in sheep milk cheese (6.12±0.00). Comparing results of this present study with that of the European Union microbiological regulations (EUMR) and the Gulf Standards Organization (GSO) for foods, the bacteria load of all cheese samples at 12 h was within the acceptable or permissible limit. ***Conclusion*:** The present study indicates that laboratory prepared cheese contains a repertoire of significant public health microorganisms in numbers above the permissible limit. It is recommended that appropriate aseptic procedures should be strictly upheld by dairy handlers across the entire cheese production value chain.

Keywords: Laboratory prepared cheese, Growth kinetics, Bacteria isolates and Different milk sources.

INTRODUCTION

All over the world, the dairy industry is fundamental to the food sector. In Nigeria today, major part of the routine diets are products from the dairy industry with milk "kunu" and cheese "wara" being key examples (Afolabi and Adetayo, 2022). Cheese is fermented products which serve as good source of proteins, vitamin D, calcium, as well as other essential nutrients. They also provide magnesium, potassium, phosphorus, and other vitamins such as $B_{12,}$ B_2 and A (Murawski *et al.*, 2022).

The low pH value and consequent high acidity of the fermented milk prevents the growth of spoilage and pathogenic bacteria and thus, the shelf-life of milk can be extended while its safety can be guaranteed due to the antagonistic activity of lactic acid bacteria as well as the physicochemical characteristic of cheese (Kumar and Chordia, 2017).

Microorganisms ferment the carbohydrates found in milk, lactose into lactic acid and other products. The protein in milk becomes precipitated due to the presence of the acid thereby making the

fermented products to be thicker than the milk in consistency (Mehra *et al.*, 2022).

In developing countries, traditional milk products like cheese are prepared under crude or primitive conditions (Bamgbose *et al*., 2022). This results in low quality and reduction of quantity of products (Obafemi *et al*., 2022).

The expected high bacteria count in cheese is a function of the increased growth during the process of cheese ripening, but, this however depends on the conditions of processing, the cheese type and the characteristics of the microorganisms used such as salt, heat, acid tolerance, initial population and individual features of the species or strains (Sakaridis *et al.*, 2022).

A plethora of fermented dairy products are readily available for end users (consumers) and the nature of different cheese produced globally depends very much on the used milk type, pre-treatment processes of the milk, climate, fermentation conditions (microbial community which affects the pH) and on the subsequent, technological treatments (Zengin and Recep, 2022).

For the past 10 years, several food-borne related diseases have been proven to be caused by milk and dairy products (El-Ziney, 2018). Some organisms have been documented in various investigations involving the microbial evaluation of mastitis in cows, leading to contamination of milk (Ntuli *et al.*, 2022). Poor hygienic practices in crude cheese production leads to public health problems as a result of the presence of pathogenic microorganisms such as yeast, mold and bacteria (Choi *et al*., 2016).

However, despite the growing popularity of domestic natural cheeses among consumers in Nigeria, information about microbiological safety of these products is limited. Thus, this study is meant to evaluate the growth kinetics of various cheese prepared in the laboratory from different milk types on some bacteriological media, in order to determine its bacteriological quality over a 96 hour period and evaluate the effect of storage time on the pH of the various cheese.

METHODS

Sample Collection from dairy Animals

Goat, cow and sheep milk were purchased in Auchi from the local herdsmen who operates local dairy farms popularly called “Zongo" in a clean container. The various milk samples were immediately transported to the laboratory for further use in an ice pack.

Preparation of Soybean Milk

The soybeans grains were purchased from the local market in Auchi packaged in black cellophane bags and taken to the laboratory. The soybeans (482 g) was weighed into a clean covered bowl, 500 ml of sterile water was added to cover it and left to steep for 8 h. After steeping, the soybean was washed to remove the outer coat. The washed soybeans (959 g) was blended with 1000 ml of water until smooth using a Marlex Electroline bleander (IS 4250; CM/L 7962804). The soybean mixture was filtered using sterile cheesecloth. The liquid filtrate obtained is the raw soy milk (Suleiman *et al., 2022)*

Cheese Preparation

According to the traditional method, 800 ml of the plain raw milk samples obtained from goat, cow, sheep and soybean was heated directly to boil and coagulated immediately with the addition of 0.22g/l lime as thickener at a ratio of 22ml per 100 L of milk. The resulting curds was filtered through sterile muslin cloth and allowed to drain properly by carefully squeezing and pressed into the opening of a 500 ml beaker to help in expression of the remaining whey (Pintado *et al.*, 2008; James *et al*., 2016). After preparation, samples were stored in sterile beakers at temperature 28±2 ^{o}C for duration of 96 hrs. Samples were analysed for microbial load and pH at every 12-24 h interval.

Bacteria Enumeration and Characterization of cheese

Ten (10) gram of each sample was collected aseptically and placed into separate sterile 250 mL conical flasks. Peptone water (90 ml) was added to make 10^{-1} dilution from which sequential decimal dilutions were prepared and plated in triplicate. Nutrient Agar (NA), MacConkey Agar (MCA), Mannitol Salt Agar (MSA) and De Mann, Rogosa and Sharpe Agar (MRS) for the enumeration of total heterotrophic bacteria, coliform, *Staphylococcus* species and Lactic Acid Bacteria respectively (Novella-Rodríguez *et al*., 2004; Abdalla and Omer, 2017). This process was repeatedly done at 0, 12, 24, 48, 72 and 96 h intervals.

In all samples of the cheese, bacterial colonies with varying morphologies were isolated, enumerated and stored at 4°C on Agar slants, for further use. Identification of the various bacterial species was confirmed, using molecular and conventional tests for biochemical and physiological characterization. These tests include: Gram staining, urease, catalase, oxidase, indole, coagulase and sugar fermentation. Further test to identify Lactic Acid Bacteria were bile hydrolysis, haemolysis, phosphate test and growth and the utilization of trehalose, lactose, ribose, sorbitol, raffinose and mannitol at 15 °C, 35 °C and 45 °C. All analyses were performed in triplicate.

Determination of pH

A PyeUnicam pH meter (Model PW9409) was used to determine the pH of each homogenate. Two (2)

grams of each sample was mixed with 100ml of distilled water and homogenized before the meter was used for pH determination.

Statistical Analysis

Data obtained were subjected to descriptive and inferential statistics (ANOVA) using SPSS (version 20 incorporation, Chicago, Illinois, USA).

RESULTS

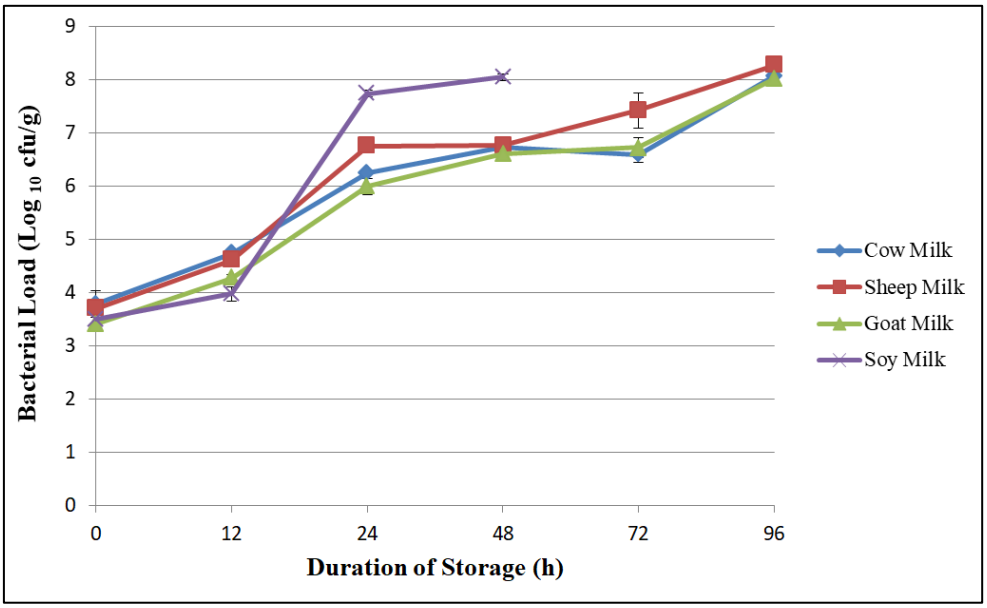

Figure 1: Bacterial load of prepared cheese during storage

Table 1: Coliform count of prepared cheese during storage (Log_{10}cfu/g)

Cheese	Duration of Storage (h)					
	0	**12**	**24**	**48**	**72**	**96**
Cow Milk	0.00±0.00	0.00±0.00	0.00±0.00[a]	5.30±0.32[b]	4.70±1.01[a]	6.39±0.05[b]
Sheep Milk	0.00±0.00	0.00±0.00	4.06±0.18[b]	4.59±0.10[a]	5.22±1.31[b]	7.10±0.33[a]
Goat Milk	0.00±0.00	0.00±0.00	0.00±0.00[a]	5.12±0.04[b]	5.53±0.09[b]	6.70±0.24[b]
Soy Milk	0.00±0.00	0.00±0.00	4.08±0.10[b]	5.20±0.08[b]	ND	ND

**a significantly similar across the milk type based on the time of experimentation*
**b significantly similar across the milk type based on the time of experimentation*
KEY: ND: Not Determined

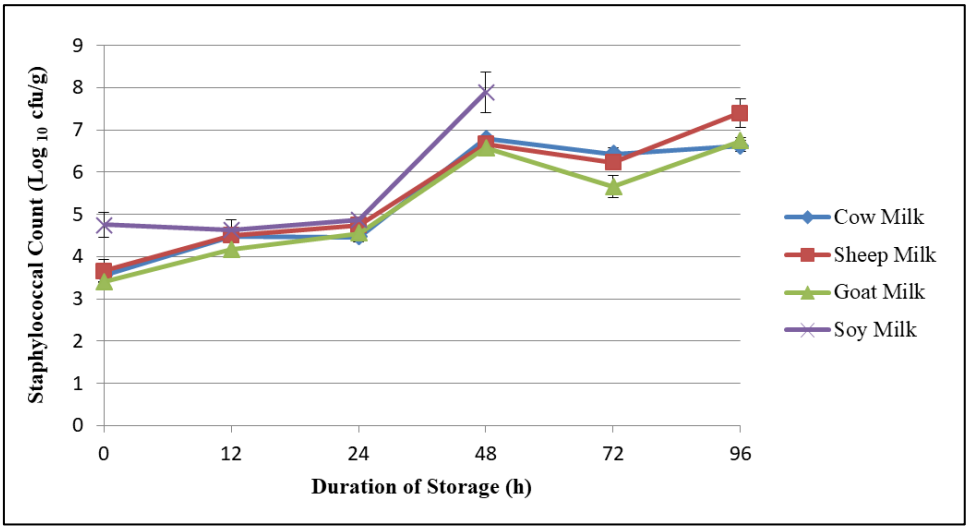

Figure 2: Staphylococcal count of prepared cheese during storage

Table 2: Lactic acid bacteria count of prepared cheese during storage (Log_{10}cfu/g)

Cheese	Duration of Storage (h)					
	0	**12**	**24**	**48**	**72**	**96**
Cow Milk	6.53±0.07[b]	6.75±0.06 [b]	6.93±0.08 [b]	7.19±0.04[b]	7.71±0.13[a]	8.19±0.05[a]
Sheep Milk	6.61±0.07[b]	6.88±0.03 [b]	6.82±0.31 [b]	6.97±0.03[a]	7.58±0.09[b]	7.60±0.15[b]
Goat Milk	6.40±0.03[a]	6.24±0.33 [a]	6.93±0.07 [b]	7.25±0.02[b]	7.69±0.08[b]	7.55±0.12[b]
Soy Milk	6.28±0.14[a]	6.87±0.52 [b]	7.16±0.02 [a]	7.51±0.02[c]	ND	ND

**a significantly similar across the milk type based on the time of experimentation*
**b significantly similar across the milk type based on the time of experimentation*
**c significant across the milk type based on the time of experimentation*
KEY: ND: Not Determined

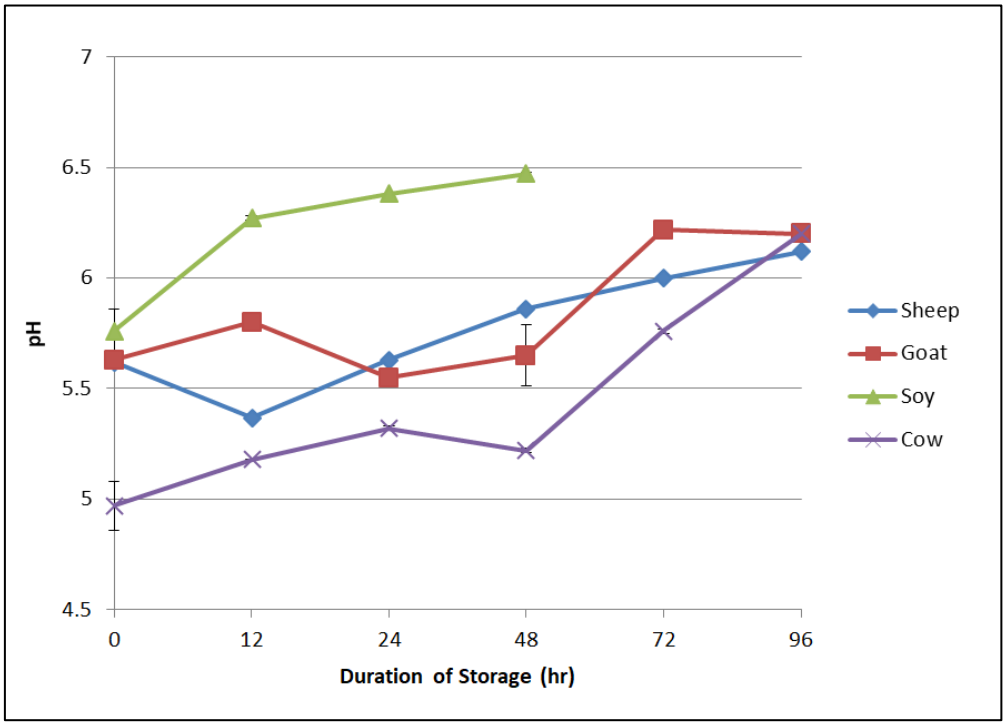

Figure 3: pH of prepared cheese during storage

DISCUSSION

In this present study, laboratory prepared cheese made from cow, sheep, goat and soybean milk were analyzed for total aerobic bacteria, coliform (*Enterobacteriaceae*), staphylococci and lactic acid bacteria. Figure 1 shows the bacterial load of prepared cheese during storage. There was progressive increase in bacterial load of the cheese samples throughout the period of study with significant difference ($p>0.05$) observable from 12 h till the end of the study. The highest bacterial load was observed in sheep milk cheese at 96 h (8.27 ± 0.02 Log_{10}cfu/g) while the least count was observed in goat milk cheese at 0 h (3.40 ± 0.10 Log_{10}cfu/g). Complete deterioration leading to foul-smelling reddish discolouration was observed in soymilk cheese at 72 h resulting in bacterial load too numerous to count.

Table 1 shows the coliform count of prepared cheese during storage. There was progressive increase in bacterial load of the cheese samples throughout the period of study with significant difference ($p>0.05$) observable from 24 h till the end of the study. The highest coliform count was observed in sheep milk cheese at 96 h (7.10 ± 0.33 Log_{10}cfu/g) while the least count was observed in all cheese samples at 0 and 12h as well as cow and goat milk 24 h (0.00 ± 0.00 Log_{10}cfu/g). Complete deterioration leading to foul-smelling reddish discolouration was observed in soymilk cheese at 72h resulting in bacterial load too numerous to count.

Figure 2 shows the Staphylococcal count of prepared cheese during storage. There was also progressive increase in Staphylococcal count of the cheese samples throughout the period of study with significant difference ($p>0.05$) observable from 24 h till the end of the study. The highest Staphylococcal count was observed in soybean milk cheese at 48 h (7.88 ± 0.48 Log_{10}cfu/g) although the highest at the end of storage was sheep milk cheese (7.40 ± 0.34 Log_{10}cfu/g) while the least count was observed in goat milk cheese at 0 h (3.40 ± 0.00 Log_{10}cfu/g). Complete deterioration

leading to foul-smelling reddish discolouration was observed in soymilk cheese at 72 h resulting in bacterial load too numerous to count.

Table 2 shows the Lactic acid bacteria count of prepared cheese during storage. There was also progressive increase in Lactic Acid Bacterial count of the cheese samples throughout the period of study with significant difference ($p>0.05$) observable at 0, 48, 72 and 96 h. The highest Lactic Acid Bacterial count was observed in cow milk cheese at 96 h (8.19 ± 0.05 Log_{10}cfu/g) while the least count was observed in goat milk cheese at 12 h (6.24 ± 0.33 Log_{10}cfu/g). Complete deterioration leading to foul-smelling reddish discolouration was observed in soymilk cheese at 72 h resulting in bacterial load too numerous to count.

The morphological, biochemical and molecular characterization of bacterial species isolated from the cow, sheep, goat and soybean milk cheese in this study has already been reported by (Oleghe, *et al.*, 2020). The bacterial isolates were identified as *Enterobacter cloacae, Lactobacillus plantarum, Myroides odorantimimus, Bacillus cereus, Escherichia coli, Lactobacillus reuteri, Enterobacter hormaechei, Kosakonia cowanii, Klebsiella quasipnuemonia, Lactobacillus delbrueckii, Staphylococcus aureus, Staphylococcus sciuri* and *Lactobacillus fermentum*.

Figure 3 shows the effect of fermentation on the pH of the cheese samples during storage. There was progressive increase in the pH of all cheese samples throughout the period of storage with the highest value observed at the end of the study preceding complete spoilage of the samples. There was observable and significant difference in the pH of all cheese samples amongst the samples and throughout the period of study. At 0 h the highest pH was recorded in Soybean milk cheese (5.76±0.01) while the least at 0 h also was cow milk cheese (4.97±0.11). The highest pH at 48 h was Soybean milk cheese (6.47±0.01) while the least pH was recorded in sheep milk cheese (6.12±0.00).

The European Union microbiological regulation (EUMR) considers dairy products as satisfactory at aerobic plate count of $<5 \times 10^4$cfu/g and *Enterobacteriaceae* of 0 cfu/g while fresh, soft, semi-hard and hard cheese are documented to be recalled at counts $\geq 10^5$, while the Gulf Standards Organization (GSO) standards for aerobic plate count and *Enterobacteriaceae* in dairy products is $<3 \times 10^4$cfu/g and <3 cfu/g respectively while fresh, soft, semi-hard and hard cheese are recalled at $\geq 10^3$. Comparing results of this present study with that of the EUMR and GSO revealed that higher counts which exceeded the standard limits were recorded. According to these standards, *Listeria monocytogenes* and *Salmonella* species should not be detected (0 cfu/g) in all types of cheese. Comparing the bacterial load of all cheese samples in this study with the EUMR and the GSO, revealed that all cheese met the standard at 12 h but had counts ≥5 Log_{10}cfu/g at 24 h which made them unacceptable for consumption at that time. For the coliform count (*Enterobacteriaceae*), cow and goat milk cheese met the required standards of 0 cfu/g even at 48 h while sheep and soybean milk cheese became unacceptable after 12 h. Similarly, for the counts at 48 h, the staphylococci count exceeded the acceptable limit of ≥5 Log_{10}cfu/g. *Staphylococcus aureus* can produce toxin when it reaches 10^5 to 10^6cfu per gram of food (Prates *et al*., 2017). Hence, storage of cheese at temperatures which may permit the growth of *S. aureus*, to reach enough cell concentrations to produce staphylococcal toxins should be avoided.

The implication of the results from this study is that the various laboratory prepared cheeses from the four different milk sources are only safe for consumption within 0 – 12 h at room temperature. Similar observation was reported by El-Ziney, (2018). The occurrence of these microbial species in the samples indicates cross-contamination, which can only occur from poor hygiene of the milk farmers, unsterilized milking equipment and unhygienic environment containing polluted air and water (Suleiman *et al.*, 2022). El-Ziney, (2018) reported that high moisture content of soft cheese enhances the microbial survival and reproduction.

Coagulase positive *Staphylococcus aureus* is one of the most widespread pathogens infecting dairy cows and a major causative agent of mastitis (Verraes *et al*., 2015 and Prates *et al*., 2017). It is one of the most frequently isolated microorganisms from raw milk and represents a probable risk for public health due to the possibility of staphylococcal toxin production (Arqués *et al*., 2015 and Verraes *et al*., 2015). Occurrence of *E. coli, Klebsiella* and *Enterobacter* species are indicative of fecal contamination and are major organisms involved in human gastroenteritis (El-Ziney, 2018).

Data from this study revealed that *L. monocytogens* and *Salmonella* species were not recovered from any of the cheese samples. This result significantly shows the efficiency of the laboratory cheese preparation method and the good hygienic practice followed during the cheese preparation to avoid cross-contamination and inability of *L. monocytogenes* and *Salmonella* to survive the cheese processing conditions. According to Kessel *et al*., (2011), high frequency of *Salmonella* sp. in bulk-tank raw-milk samples amounted up to 10.5% as was reported by the United States Department of Agriculture (USDA) in 2007 while; 3.8% United States dairies tested positive for *L. monocytogenes*.

Most fermented foods are generally known to be safe and nutritious, but consumption of a number of

them such as cheese has been documented in many countries to be associated with foodborne illnesses (Cancino-Padilla *et al*., 2017 and Ntuli *et al.,* 2022). Many fermented milk products which are consumed as they are, contain large volumes of microorganisms especially bacteria (Ntuli *et al.,* 2022). The rising demand for health promoting foods with high-value such as cheese has encouraged many research fields and food industries to investigate the microbiological safety both in the organism present and their population in these foods which could result to reduction of their shelf-life (Choi *et al.,* 2016).

The origin of the organisms could be the microflora of the animals from which the milk samples were collected and that of individuals who tender these animals. They could also have originated from the unhygienic market environment and the containers used for the milk extraction (Suleiman *et al*., 2022). Similar bacterial genera (*E. coli, En. aerogenes, S. aureus* and *K. pneumonia*) were reported from soybean milk by Agboke *et al*., (2012) and from cow milk by Ogbolu *et al.*, (2014).

The presence of *Staphylococcus aureus* could be due to poor handling; this is because humans are the primary reservoir of *Staphylococcus aureus* and is found in the nasal region, hand and skin. The symptoms of staphylococcal gastroenteritis may include vomiting, abdominal cramps, fatigue, headache and weakness (Esho *et al*., 2013).

The presence of *Escherichia coli* indicates the contamination by faecal material of the cheese which may be from the milking process, inappropriate washing of hands or during preparation. *Escherichia coli* cause varying degrees of intestinal disorders which include diarrhea which is sometimes bloody, urinary tract infection, abdominal cramps and dysentery (Agholor *et al.*, 2018).

The presence of *Myroides odorantimimus*a ubiquitous environmental organism could be from the soil and water since they are not components of the normal human microflora. Although, a rare clinical organism, it has been isolated from urine, blood, wounds and respiratory secretions causing infections in severely immunocompromised persons (Beharrysingh, 2017).

In several national standards, coliform group are suggested to be replaced by *Enterobacteriaceae* as more accurate indicators of faecal contamination (Hervert *et al.,* 2016). The present quantity of this family of bacteria provides an insight into the efficiency of personal hygiene, Good Manufacturing practice (GMP) and sanitation process (Sakaridis *et al*., 2022).

The occurrence of lactic acid bacteria in all variants of cheese used in this study could be attributed to documented facts that they originate from raw or fresh milk as high counts were observed throughout the period of this study. However, occurrence and increase of lactobacilli in cheese are beneficial, as they are members of lactic acid bacteria (LAB) which are relevant probiotics, as well as biotechnological microbes in dairy industry (Mehra *et al*., 2022), they are often Generally Regarded As Safe (GRAS) organisms. The result of this work for *Lactobacillus* agrees with the reports of Luiz *et al*., (2017) who documented that the average count of Lactic acid bacteria in cheese was 7.5 log units when made with raw milk. They play a major part in the ripening of traditional and industrial cheeses in which they synthesize volatile flavor compounds that add to the sensory profile of the cheese (Sgarbi *et al.,* 2013). Amongst the lactic acid bacteria identified, *L. plantarum* was the most frequently isolated. Bamgbose *et al*., (2022) documented that *L. plantarum* and a few other *Lactobacillus* species are advantageous in production of cheese as they are connected to the synthesis of exceptional sensory properties as well as exhibition of probiotic and antibiotic potential through production of secondary metabolites such as bacteriocins. Also, *Lactobacillus plantarum* DR7 alleviates stress and anxiety in adults (Chong *et al*., 2019a), as well as improved upper respiratory tract infections via enhancing immune and inflammatory parameters (Chong *et al*., 2019b).

Increase in pH from acidity to neutral was observed throughout the period of study. Studies by various authors have shown that change in pH of cheese during storage could be as a result of proteolysis which is responsible for the changes in rheological properties of cheese. The pH of cheese during fermentation is affected by various factors which include temperature, water activity, and microbial population in the cheese (Bamgbose *et al*., 2022).

The differences between the results obtained here from several studies reflects the diversity and differences among the production techniques, post-contamination after production, storage and handling conditions.

CONCLUSION

The present study thus indicates that laboratory prepared cheese contains various microorganisms which may have resulted from the milking process, cheese preparation, environment, and materials used. The presence of some of these bacteria strains in the cheese suggests that the intake of this dairy product could pose a potential health hazard to consumers when consumed in such contaminated state. As a results, it is therefore recommended that prior to cheese making, all standard milking procedures that ensures the quality of milk conform to the standards

should be observed. The udder of the animal should be sterilized before milking, and the milking process should be done with machines for easier milking and to avoid contamination from handlers, but where such is unavailable, hand washing and wearing of latex gloves by the workers during manual milking will minimize the spread of mastitis-causing organisms as well as protect the workers skin. The materials and equipment to be used for the preparation of the cheese should be adequately sterilized. Finally, because milk can be a very useful substrate for the growth of various bacteria, the environment to be used for the preparation should be hygienic.

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