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Isolation and Identification of Pathogenic Bacteria in Pasteurized Powdered Milk Sold in Ogun, Lagos and Oyo, Southwest, Nigeria

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Abstract

Milk is a nutritious beverage enjoyed by people of all ages, but it also provides an ideal environment for the growth of various microorganisms. This study aimed to isolate and identify the bacteriological quality of powdered milk sold in Ogun, Lagos, and Oyo, Southwest Nigeria. Three samples from each of five different brands were analyzed by homogenizing 1g of each milk sample with 10ml of nutritional broth, followed by overnight incubation at 37°C. Subsequently, the bacteria were plated on nutrient agar, Mac-Conkey, and EMB, with further characterization through partial sequencing of 16S rRNA genes. The detected species were then evaluated against antibiotics including Acyclovir, Cefotaxime, Augmentin, Cefuroxime, Gentamicin, Erythromycin, Ofloxacin, Nitrofurantoin, Ciprofloxacin, Nalidixic acid, and Azithromycin. The molecular testing revealed the presence of Pseudomonas aeruginosa, Bacillus cereus, Brevibacillus agri, Lysinibacillus sphaericus, and Bacillus thuringiensis as the identified bacterial species. Pseudomonas aeruginosa exhibited sensitivity to Gentamicin and Cefuroxime, with Nitrofurantoin showing the highest sensitivity at 80.0%. Brevibacillus agri (LATC3) demonstrated sensitivity to Cefuroxime and Gentamicin, with a higher sensitivity of 55.00% to Gentamicin. Bacillus cereus (LAKG2 and LAC2) showed higher responsiveness to Ceftriaxone compared to Ofloxacin, Cefotaxime, Augmentin, Acyclovir, and Levofloxacin. These findings indicate the presence of potentially harmful organisms in the microbial populations of pasteurized milk, suggesting inadequate storage practices or insufficient sanitation of facilities and equipment post-production and packaging. Therefore, monitoring the microbial quality of pasteurized milk is essential to ensure safety and extend its shelf life.

INTRODUCTION

People of all ages consume milk due to its nutritional properties, which create an optimal environment for the growth of various microorganisms. Milk contains proteins, vitamins, minerals, and unsaturated fatty acids. Raw milk is pasteurized into a dry powder as it may harbor microbial pathogens that can lead to spoilage (Ahmed *et al.*, 2019). Presence of any bacteria can result in spoilage and impact the flavor and physicochemical attributes of milk (Madika, 2020). Heat treatments like pasteurization and sterilization are commonly used to prolong the shelf life of milk. While pasteurized dry milk powder is more convenient and has a longer shelf life compared to fresh milk, it may not offer the same sensory experience (Ali, 2019). The flavor and texture of reconstituted powdered milk may differ slightly from fresh milk for some individuals. Proper storage in a cool, dry place away from direct sunlight, heat, and moisture is essential to maintain its quality and nutritional value (Kandil *et al.*, 2018). Milk has been a staple food for human nourishment since ancient times, providing essential nutrients but also posing a risk of microbial pathogen contamination. The nutrients in milk can promote the growth of beneficial microorganisms like *Bifidobacteria* and Lactobacilli, enhancing human health (Fernandes, 2019). Pasteurization involves heating milk to a specific temperature for a set duration to eliminate harmful bacteria and enzymes while preserving its nutritional content, extending its shelf life, and ensuring safety for consumption. Proper handling and storage of powdered milk can prevent bacterial growth and contamination, reducing the risk of

foodborne illnesses (Elgadi *et al.*, 2018). Contaminated milk can contain dangerous microorganisms like listeria, *Salmonella*, *E. coli*, and S. aureus, leading to foodborne infections. Introduction of modern equipment and implementation of food safety protocols can mitigate these risks. While pasteurized milk is generally considered safe for consumption, microbial activity during packaging and storage can lead to contamination, altering the milk's flavor and appearance. It is crucial to recognize the importance of assessing the microorganisms present in pasteurized powdered milk and conduct a thorough analysis to identify potentially harmful strains and understand the conditions supporting their survival.

MATERIALS AND METHODS

Samples collection

Three main markets, Oshodi in Lagos State, Obada Market in Ijebu-Igbo, Ogun State, and Orita Challenge Market in Ibadan, Oyo State, were selected to obtain five (5) milk samples. There were three samples for each of the following types: DM, KG, TC, ML, and CB, with each sample weighing 180g. Prior to laboratory analysis, the samples were appropriately labeled based on their type and source. Standard microbiological techniques were employed to examine sample aliquots.

Isolation and Identification

One gram of each milk sample was taken under aseptic conditions, homogenized with ten milliliters of nutrient broth, and then incubated at 37°C overnight. After the incubation period, the bacteria were inoculated onto nutrient agar (PCA) from Oxoid Ltd., Basingstoke, Hampshire, UK, as well as selective agar media such as MacConkey and EMB agar. The plates were then incubated in an aerobic environment for up to twenty-four hours at 37ºC. Morphologically distinct colonies were sub-cultured onto new Nutrient Agar (NA) plates (Oxoid Ltd., Basingstoke, Hampshire, UK). A variety of colonies with different morphologies were selected from each plate. Isolates were streaked for purity up to three times. Subsequently, pure colonies of the isolated bacterial strains were preserved on Nutrient Agar slants at 4ºC (Wassie and Wassie, 2016).

DNA extraction

The Zymo kit was utilized to extract DNA following the manufacturer's instructions. The genomic DNA isolation technique established by Anadika (2013) was employed for molecular characterization. The Mehrotra *et al.* (2000) protocol for PCR amplification of 16S rRNA-Genome DNA was followed with slight modifications as suggested by Ezeamagu *et* al. (2018). The approach by Mehrotra et al. (2000) was adopted for the amplification of 18S rRNA Genomic DNA. Subsequent to amplification, a 10.0 µl result was examined using 1.5% agarose gel electrophoresis as per the instructions of Ezeamagu *et* al. (2018). For DNA sequencing, extraction was carried out following the Zymo research Mini Kit, USA, guidelines and quantified using the NanoDROP 3300 spectrometer (Thermo Fisher Scientific Inc., USA). Agarose gel electrophoresis was conducted following the method described by Ezeamagu *et al.* (2018). The Maximum Likelihood approach, based on the Tamura-Nei model, was used to deduce the evolutionary history. Evolutionary analyses were executed in MEGA6 as outlined by Tamura *et* al. (2013). The proportion of trees where related taxa clustered together was indicated next to the branches.

Antibiotic Sensitivity Test

The CLSI, 2021 protocol was adhered to for the antibiotic sensitivity test. The British Society for Antimicrobial Chemotherapy (Andrew, 2007) and the Clinical Laboratory Standard Institute issued instructions for the analysis of the data. The following antibiotics were used, along with their corresponding concentrations: cefuroxime (30µg), gentamicin (10µg), erythromycin (15µg), ofloxacin (5µg), augmentin (30µg), ceftazidime (30µg), acyclovir (1.3µg), cefotaxime (25µg), imipenem (10µg), ofloxacin (5µg), nalidixic acid (30µg), cefixime (5µg), ceftriaxone (45µg), sulbactam (10µg), levofloxacin (5µg), nitrofurantoin (300µg), Ciprofloxacin (5µg), and azithromycin (15µg). Using sterile forceps, antibiotic discs were positioned on the agar plates with uniform spacing between them. The widths of the clear zones of inhibition were measured in millimeters (mm) after incubation.

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RESULTS

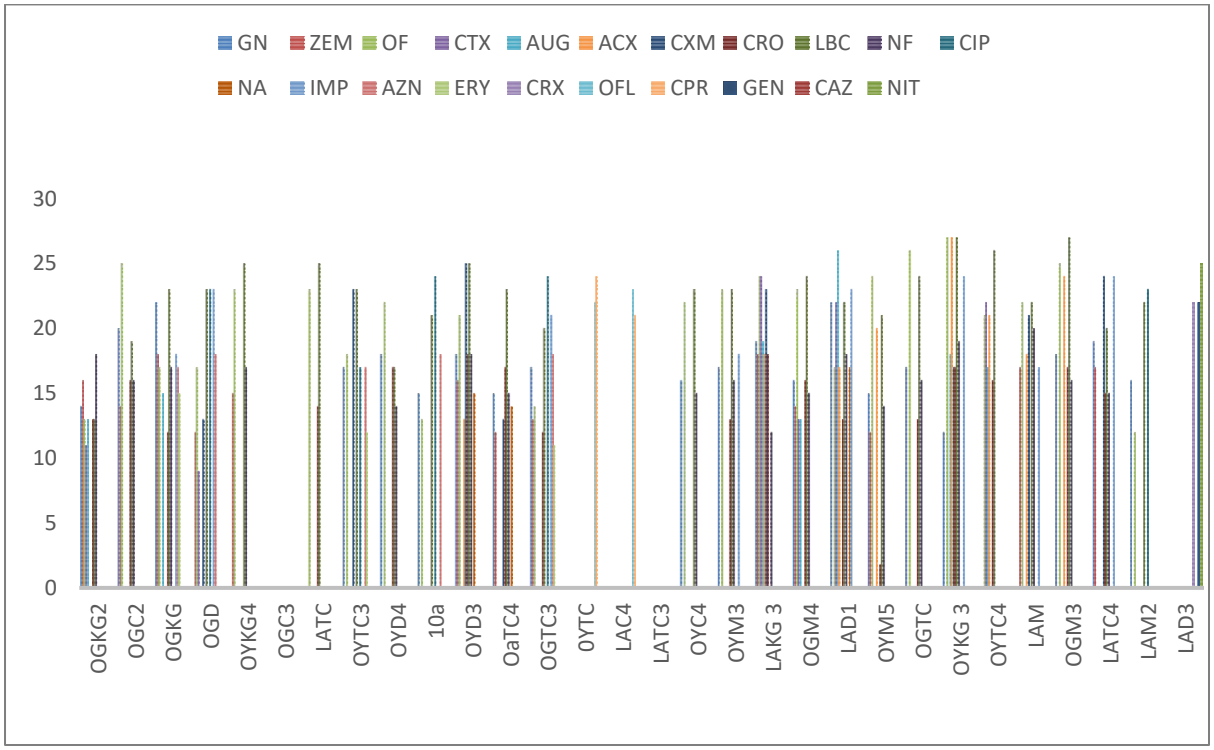

Figure 1: Antibiotic susceptibility test

Keynotes:CRX- Cefuroxime, GEN- Gentamicin, ERY- Erythromycin, OF- Ofloxacin, AUG Augmentin,CAZ- Ceftazidime, CXM- Cefuroxime, ACX-Acyclovir,CTX-Cefotaxime, IMP-Imipenem, OFX- Ofloxacin, GN- Gentamicin, NA- Nalidixic Acid, ZEM- Cefixime, CRO- Ceftriaxone Suibactam,LBC- Levofloxacin, NF-Nitrofurantoin, CPR- Ciprofloxacin,NIT -Nitrofurantoin,AZN-Azthromycin,. LAD3-*Pseudomonas aeruginosa., OYC4 -Bacillus cereus*, LAKG2-*Bacillus cereus*,LAC2-*Bacillus cereus*,LATC3- *Brevibacillusagri,LAD1- Lysinbacillussphaericus*and OYKG4-*Bacillus thuringiensis,OGTC4-Bacillus thuringiensis.*

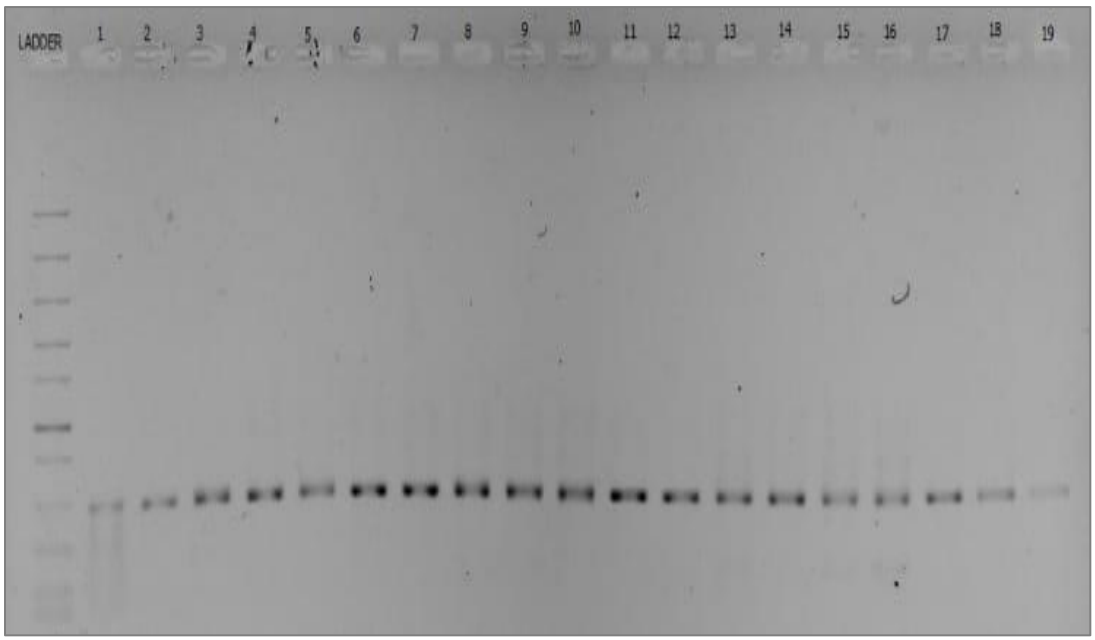

Plate 1: Gel picture of the bacteria gene from pasteurized dry milk powder

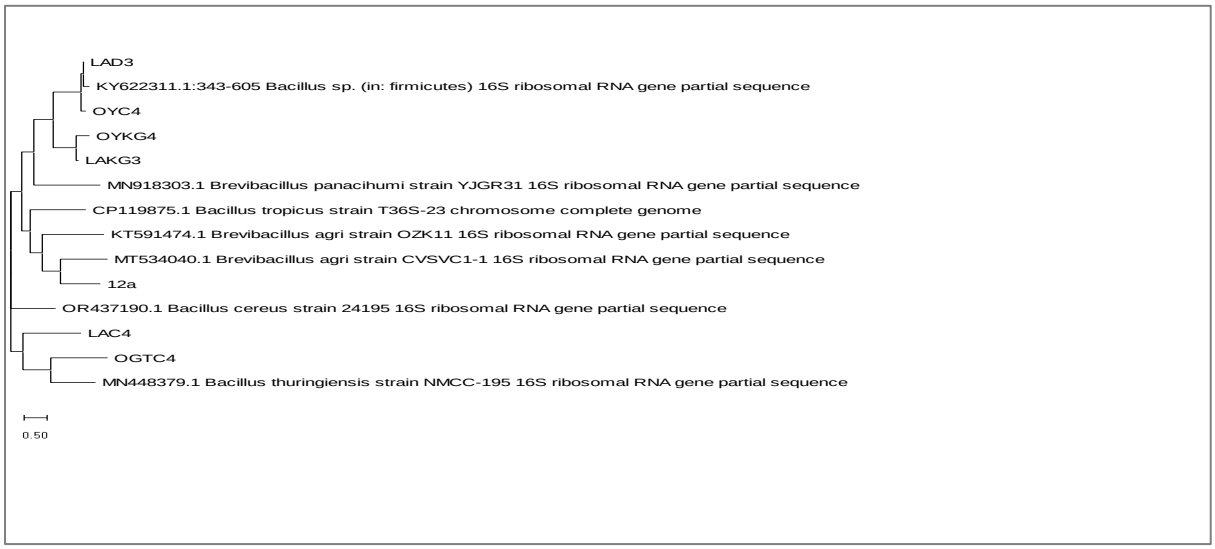

Figure 2: Phylogenetic tree of the bacteria isolates using their closest relativeness in Genbank.

DISCUSSION

Milk samples from Oshodi market in Lagos state, Obada market in Ijebu-Igbo, Ogun state, and Orita Challenge market in Ibadan, Oyo state were used to isolate approximately thirty (30) microorganisms. The identification of the isolated species was conducted using molecular markers based on 16S rRNA. The sequencing of the DNA chromatogram was corroborated using the NCBI database. Based on the phylogenetic tree created using MEGA 6, the isolates were identified as *Pseudomonas aeruginosa, Bacillus cereus*, *Brevibacillus agri, Lysinibacillus sphaericus*, and *Bacillus* thuringiensis. This is illustrated in Figure 2. In microbial taxonomy and phylogenetics, the application of 16S rRNA molecular markers for the identification of isolated organisms is a widely accepted and reliable technique. The 16S rRNA gene, which is conserved in all bacteria but contains variable regions that aid in species identification, is the specific gene region sequenced in this method. An integral part of the identification process involves validating the DNA chromatogram sequencing with the National Center for Biotechnology Information (NCBI) database. The NCBI database contains a wealth of genetic information, including 16S rRNA sequences of known bacterial species, which researchers can use to confirm the identity of the isolated organisms by comparing the obtained sequences. The phylogenetic tree depicts the evolutionary relatedness and genetic distances between the identified isolates. For example, KY622311.1:343-605 represents a partial sequence of the 16S ribosomal RNA gene in Bacillus sp. (family: Firmicutes). MN918303 shows a partial sequencing of the 16S ribosomal RNA gene for strain YJGR 31 of *Brevibacillus* panachumi. KT591474.1 displays an incomplete sequence of the 16S ribosomal RNA gene for *Brevibacillus agri* strain O ZK11. MT534040.1 presents a partial sequence of the 16S ribosomal RNA gene for *Brevibacillus agri* strain CVSVC 1-1. OR437190.1 shows a partial sequence of the 16S ribosomal RNA gene for Bacillus cereus strain 24195. The complete genome of *Bacillus tropicus* strain T36S-23 chromosome is represented by CP119875.1, showcasing the entire genomic sequence of the 16S rRNA gene from *Bacillus tropicus* strain T36S-23.

Microorganisms found in milk samples suggest possible sources of contamination during manufacturing, handling, processing, and storage. Additionally, several studies have highlighted the contamination of powdered milk by bacteria, with Bacillus species being a significant contaminant due to their spore-forming ability (Checinska *et al.*, 2015). Following pasteurization, the milk samples contained *Pseudomonas aeruginosa* (LAD3), *Bacillus cereus* (OYC4, LAKG2, LAC2), *Brevibacillus agri* (LATC3), *Lysinibacillus sphaericus* (LAD1), and *Bacillus thuringiensis* (OYKG4, OGTC4), as observed in this study. This finding aligns with research by Zhengyuan Zhao *et al.* (2022), which discussed antibiotic susceptibility and resistance gene transfer in Bacillus strains isolated from pasteurized milk. Additionally, rasheda and Nazia (2018) reported similar results in their examination of dry powdered milk in Bangladeshi markets, identifying *Staphylococcus aureus*, *Bacillus sp.*, *Pseudomonas sp.*, and *Enterobacter sp*. The use of rRNA region comparison has proven valuable in distinguishing between genera and species,

demonstrating the reliability of molecular characterization in bacterial identification. Moreover, a comprehensive database of 16S rRNA sequences from diverse microorganisms facilitates this identification process. Another study by Zhengyuan Zhao *et al*. (2022) focused on Bacillus strains in pasteurized milk, revealing the presence of 16 *Bacillus* strains and emphasizing the importance of proper handling and storage to ensure safety despite the resistance of spore-forming bacteria to pasteurization methods. Furthermore, Yeshambel et al. (2021) investigated lactic acid bacteria (LAB) in milk products, identifying various genera like *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Bifidobacteria*, and *Pediococcus* spp. Hager et al. (2019) studied the microbial composition of different milk samples, indicating the presence of *Escherichia coli* and *Staphylococcus aureus* in commonly consumed milk types. Strict adherence to hygienic practices during milk production, packaging, and distribution is crucial to minimize contamination risks due to the favorable growth conditions milk provides for bacteria. The bacterial species isolated in this study, such as *Pseudomonas aeruginosa* (LAD3), *Bacillus cereus* (OYC4, LAKG2, LAC2), *Brevibacillus agri* (LATC3), *Lysinibacillus sp*. (LAD1), and *Bacillus thuringiensis* (OYKG4, OGTC4), were subjected to antibacterial sensitivity tests, revealing susceptibility to various antibiotics. Notably, *Bacillus cereus* strains showed differing levels of sensitivity to different antibiotics. Similarly, a study in southwestern China on *Bacillus cereus* in buffalo milk reported comparable findings regarding antibiotic susceptibility, essential for managing diseases caused by these bacteria (Yunhe *et al*., 2021).

CONCLUSION

Pseudomonas aeruginosa, *Bacillus cereus*, *Brevibacillus agri*, lysinibacillus sphaericus, and *Bacillus thuringiensis* are among the five bacterial species found in this study on the isolation and characterization of bacteria associated with specific pasteurized dry milk. This study highlights the persistence of food spoilage microorganisms and the presence of some pathogenic organisms, particularly *Bacillus* spp., that resist heat due to the presence of spores despite pasteurization. This may occur as a result of retailers storing milk products improperly or because facilities and equipment were not adequately sanitized during the production and packaging process. Therefore, to ensure the safety and shelf life of pasteurized milk, it is crucial to monitor its microbiological quality. Additionally, individuals consuming contaminated milk should use antibiotics that are sensitive to the tested organism.

RECOMMENDATION

It might be advised to do the following:

(i) Consistently checking the quality of milk during processing and packing to ensure the finished product meets specifications.
(ii) (ii) ensuring that storage conditions are maintained to prevent the growth of harmful bacteria and the occurrence of off-flavors.
(iii) (iii) Strict hygienic procedures, such as thorough equipment and facility sanitization and adherence to good manufacturing practices, should be implemented throughout the production process to reduce the risk of contamination from external sources and enhance the quality and safety of pasteurized milk.

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