

# Isolation and Characterization of Microbial Biofilms from Domestic Refrigerators

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**Abstract:** Domestic refrigerators are designed to maintain temperatures around 4-5°C to preserve food and prevent microbial growth. However, biofilms forming on refrigerator surfaces pose significant risks to food safety and public health by harboring pathogenic and spoilage-causing microorganisms. This study isolated and characterized bacterial and fungal biofilms from five domestic refrigerators in Abeokuta, Nigeria. Samples were collected using sterile swab sticks and cultured on nutrient agar (for bacteria) and Potato Dextrose Agar (for fungi). Bacterial isolates were identified using Gram staining, biochemical tests, and 16S rRNA sequencing; fungal isolates were identified using microscopy and ITS region sequencing. Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Results showed 100% contamination rate. Bacterial isolates phenotypically included *Streptococcus spp.* (60%), *Escherichia coli* (20%), and *Bacillus spp.* (20%). Molecular identification of the dominant bacterium revealed *Bacillus velezensis* strain CUAB-AOS02 (PP657698). Fungal isolates phenotypically included *Penicillium spp.* (60%), *Cryptococcus spp.* (20%), and *Aspergillus spp.* (20%). Molecular identification revealed the dominant fungus as *Aspergillus niger* strain CUAB-AOS05 (PP657690). Phylogenetic and plasmid analyses demonstrated genetic diversity and adaptive potential. Antimicrobial susceptibility testing revealed that all bacterial isolates were multidrug-resistant, showing resistance to gentamicin, pefloxacin, ofloxacin, chloramphenicol, streptomycin, ciprofloxacin, trimethoprim/sulfamethoxazole, and erythromycin. The presence of *Bacillus velezensis* CUAB-AOS02, *Aspergillus niger* CUAB-AOS05, and multidrug-resistant organisms in domestic refrigerators is concerning. Regular cleaning, proper food storage, and consumer education are essential to mitigate health risks.

**Keywords:** Biofilm; Domestic Refrigerator; *Bacillus Velezensis*; *Aspergillus Niger*; Multidrug Resistance; Food Safety.

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## I. INTRODUCTION

Bacterial and fungal biofilms are intricate communities of microorganisms that adhere to surfaces, creating organized clusters encased in a self-produced extracellular polymeric substance (EPS) matrix. The EPS matrix provides structural support and protection, allowing biofilms to thrive in diverse environments, including the cold surfaces of domestic refrigerators [1], [2]. The presence of these biofilms in residential refrigerators poses serious challenges to food safety and public health, as they can harbor dangerous pathogens and facilitate cross-contamination of stored food products [3], [4].

The domestic refrigerator is a staple in modern households, designed to combat food spoilage by maintaining an internal temperature between 4-5°C. This temperature

range is critical; it is low enough to inhibit microbial activity but not so low as to freeze food [5]. However, refrigeration is not a panacea. Food can still spoil due to the activity of psychrotrophic microorganisms, enzymatic reactions, and oxidative processes [6], [7]. Many consumers are unwilling or unaware of the need to clean their domestic refrigerators regularly, which can lead to hazardous microbial cross-contamination, allowing bacteria and fungi to spread from one food to another via the refrigerator's contact surfaces [8], [9].

Microorganisms from contaminated, unwashed raw foods, leaking packages, or human hands can transfer to internal refrigerator surfaces, where they may directly contaminate other stored foods or attach and persist. Although the environment inside a refrigerator is cold, many microorganisms, including bacteria, molds, and yeasts, can

grow or survive at 4°C. These include *Pseudomonas spp.*, *Aeromonas spp.*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Penicillium spp.*, and *Cladosporium spp.* [10].

Fungal biofilms are particularly problematic. Medically important fungi such as *Candida*, *Aspergillus*, and *Cryptococcus* form biofilms that exhibit increased resistance to antifungal treatments [11], [12]. The formation involves adhesion, colony formation, and production of an extracellular matrix that protects against drugs and host immunity. Within the biofilm, metabolic heterogeneity and the presence of persister cells contribute to treatment failure [13].

Determining the types of microorganisms present, examining their structural characteristics, and evaluating their antimicrobial resistance profiles are necessary steps in characterizing biofilms in residential refrigerators. Researchers employ various techniques, including microscopy, biochemical testing, molecular identification (e.g., next-generation sequencing), and antibiotic susceptibility testing [4], [14]. This study aimed to isolate and characterize both bacterial and fungal biofilms from domestic refrigerators, providing a more complete picture of the microbial ecology of these essential appliances.

## II. MATERIALS AND METHODS

### ➤ Sample Collection

Samples were collected from five randomly selected domestic refrigerators within Abeokuta metropolis, Ogun State, Nigeria. Using sterile swab sticks moistened in sterile peptone water, samples were taken from the base, shelves, sides, and door seals of each refrigerator. The swabs were transported to the laboratory within one hour of collection. The refrigerators operated at temperatures between -10°C and 4°C.

### ➤ Culture Media Preparation

Nutrient Agar (NA) and Potato Dextrose Agar (PDA) were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes. Chloramphenicol (50 µg/mL) was added to PDA after sterilization to inhibit bacterial growth.

### ➤ Microbiological Analysis and Isolation

Each swab stick was aseptically inoculated onto NA plates (for bacteria) and PDA plates (for fungi). For bacterial culture, NA plates were incubated aerobically at 37°C for 24-48 hours. For fungal culture, PDA plates were incubated at 25°C for 3-7 days. Distinct microbial colonies were sub-cultured onto fresh media until pure cultures were obtained. Bacterial isolates were stored on NA slants at 4°C, while fungal isolates were stored on PDA slants at 4°C.

### ➤ Characterization and Identification

#### • Gram Staining and Microscopy (Bacteria)

Gram staining was performed to differentiate Gram-positive and Gram-negative bacteria. Smears of pure cultures were heat-fixed, stained with crystal violet, treated with

Lugol's iodine, decolorized with alcohol, and counterstained with safranin. Gram-positive bacteria appeared purple, and Gram-negative bacteria appeared pink.

#### • Biochemical Tests (Bacteria)

Biochemical characterization was performed according to Fawole and Oso [15]. Tests included catalase, coagulase, motility, oxidase, sugar fermentation (glucose, lactose, mannitol), citrate utilization, indole production, and Voges-Proskauer (VP).

#### • Fungal Microscopy

Fungal isolates were characterized based on colonial morphology (color, texture, reverse color, growth rate) and microscopic features. A lactophenol cotton blue stain was used to observe hyphae, mycelium, and spore structures under a microscope at x10 and x40 magnifications.

### ➤ Molecular Identification

#### • DNA Extraction

Genomic DNA was extracted from pure cultures. Briefly, 1.5 ml of broth culture was centrifuged at 10,000 rpm for 2 minutes, and the pellet was washed twice with distilled water. The final pellet was resuspended in 100 µl of Tris-EDTA buffer, and the supernatant containing DNA was stored at -20°C. DNA concentration and purity were measured using a spectrophotometer [16].

#### • PCR Amplification

The 16S rRNA gene for bacteria and the ITS region for fungi were amplified using universal primers. For bacteria, primers 27F and 1492R were used. For fungi, primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used. PCR master mix contained buffer, dNTPs, primers, Taq polymerase, and template DNA. Thermal cycling conditions included initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C for 30s), annealing (55°C for 45s), extension (72°C for 1 min), and a final extension at 72°C for 5 min [16].

#### • Agarose Gel Electrophoresis

PCR products were visualized on a 1.5% agarose gel prepared in 1X TBE buffer. Gels were stained with ethidium bromide, run at 100 volts for 30 minutes, and visualized under UV transillumination.

#### • Gene Sequencing and BLAST Analysis

PCR products were sequenced using Sanger dideoxy sequencing. Obtained sequences were compared with the NCBI GenBank database using the Basic Local Alignment Search Tool (BLASTn) algorithm to identify the closest related species [16].

#### • Phylogenetic Analysis

Phylogenetic trees were constructed using the Neighbor-Joining method [17] in MEGA11 software. Bootstrap analysis with 1000 replicates was used to assess

tree stability [18]. Evolutionary distances were computed using the p-distance method [19].

• *Plasmid Mapping*

Plasmid sequences were assembled using Plasmid SPAdes and annotated using NCBI PGAP. Plasmid maps were visualized using BLAST tools [20].

• *Antibiotic Susceptibility Testing (Bacteria)*

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [21]. A 24-hour pure culture was suspended in sterile saline to a 0.5 McFarland standard and swabbed onto Mueller-Hinton agar plates. Antibiotic discs were placed on the agar, and plates

were incubated at 37°C for 24 hours. Zones of inhibition were measured, and results were interpreted as sensitive (S), intermediate (I), or resistant (R) according to CLSI standards [21]. Antibiotics tested included Gentamicin (CN, 10µg), Pefloxacin (PEF, 5µg), Ofloxacin (OFX, 5µg), Chloramphenicol (CH, 30µg), Streptomycin (SP, 10µg), Ciprofloxacin (CPX, 5µg), Trimethoprim/Sulfamethoxazole (SXT, 25µg), and Erythromycin (E, 15µg).

**III. RESULTS**

➤ *Cultural Characteristics of Isolates*

The cultural characteristics of bacterial isolates are presented in Table 1. All bacterial isolates showed regular shapes, creamy colors, and entire margins. Isolates varied in elevation (flat or raised) and opacity (wet or dry).

Table 1 Cultural Characteristics of Bacterial Isolates from Domestic Refrigerators

Isolate Code	Shape	Colour	Edge	Elevation	Opacity	Margin
BF1	Regular	Creamy	Smooth	Flat	Wet	Entire
BF2	Regular	Creamy	Wavy	Raised	Wet	Entire
BF3	Regular	Creamy	Wavy	Raised	Dry	Entire
BF4	Regular	Creamy	Smooth	Raised	Dry	Entire
BF5	Regular	Creamy	Smooth	Raised	Dry	Entire

Table 2 Cultural Characteristics of Fungi Isolated from Domestic Refrigerators

Isolate code	Colour	Texture	Reverse	Growth type	Suspected organisms
FF1	Dark green	Effuse powdery	Pale yellow	Fast	<i>Penicillium spp</i>
FF3a	Dark green	Effuse powdery	Pale yellow	Fast	<i>Penicillium spp</i>
FF3b	pink vinaceous	Cotton	Creamy	Fast	<i>Cryptococcus sp</i>
FF2a	Dark green	Effuse powdery	Pale yellow	Fast	<i>Penicillium spp</i>
FF2b	Green	Powdery	White	Fast	<i>Aspergillus spp</i>

The cultural characteristics of fungal isolates are presented in Table 2. *Penicillium-like* isolates (FF1, FF3a, FF2a) showed dark green, effuse powdery colonies with pale yellow reverse and fast growth. *Cryptococcus-like* isolate (FF3b) showed pink vinaceous, cotton-like texture with creamy reverse. *Aspergillus-like* isolate (FF2b) showed green, powdery colonies with white reverse.

➤ *Biochemical Characterization of Bacterial Isolates*

The results of biochemical tests performed on bacterial isolates are shown in Table 3. Isolates BF1, BF2, and BF3 were Gram-negative cocci; BF4 was Gram-positive rod; BF5 was Gram-positive cocci. Based on these results, isolates were presumptively identified as *Streptococcus spp.* (BF1, BF2, BF5), *Escherichia coli* (BF3), and *Bacillus sp.* (BF4).

Table 3 Biochemical Test Results of the Bacterial Biofilm Isolated from Domestic Refrigerator

Sample Code	Gram Type	Shape	Urease	Citrate	Motility	Sulphide	Indole	Sugar fermentation
BF1	-ve	Cocci	+	+	+	-	-	-
BF2	-ve	cocci	+	-	+	-	-	+
BF3	-ve	cocci	+	+	+	+	+	-
BF4	+ve	Rod	-	-	+	-	-	+
BF5	+ve	Cocci	+	+	+	-	-	-
Sample Code	Gram Type	Shape	Urease	Citrate	Motility	Sulphide	Indole	Sugar fermentation

➤ *Prevalence of Isolates*

Table 4 shows the prevalence of bacterial isolates. *Streptococcus spp.* was the most prevalent (60%), followed by *E. coli* and *Bacillus spp.* (20% each). Table 5

shows the prevalence of fungal isolates. *Penicillium spp.* was the most prevalent (60%), followed by *Cryptococcus spp.* and *Aspergillus spp.* (20% each).

Table 4 Percentage Occurrence Bacteria Biofilm Isolated from domestic refrigerator

Isolates	Occurrence (%)
<i>Streptococcus sp.</i>	20
<i>Streptococcus sp.</i>	20
<i>E coli</i>	20
<i>Bacillus sp.</i>	20
<i>Streptococcus sp.</i>	20

Table 5 Prevalence of Fungi Isolated from Domestic Refrigerators

Sample Code	Colony Count	Occurrence (%)
<i>Penicillium spp</i>	3	60
<i>Cryptococcus spp</i>	1	20
<i>Aspergillus spp</i>	1	20

➤ Molecular Identification of Isolates

The most prevalent bacterial isolate (BF5, suspected *Streptococcus sp.*) and the most prevalent fungal isolate (FF, suspected *Penicillium spp.*) were subjected to molecular identification. The results are presented in Table 6. The bacterial isolate was molecularly identified as *Bacillus*

*velezensis* strain CUAB-AOS02 (Accession No. PP657698). The fungal isolate was molecularly identified as *Aspergillus niger* strain CUAB-AOS05 (Accession No. PP657690). Fig. 1 shows the agarose gel electrophoresis results confirming successful PCR amplification.

Table 6 Molecular Identification of Isolates

Isolate Code	Suspected Organism	Molecularly Identified Organism	Accession Number
BF5	<i>Streptococcus sp.</i>	<i>Bacillus velezensis</i> strain CUAB-AOS02	PP657698
FF	<i>Penicillium spp.</i>	<i>Aspergillus niger</i> strain CUAB-AOS05	PP657690

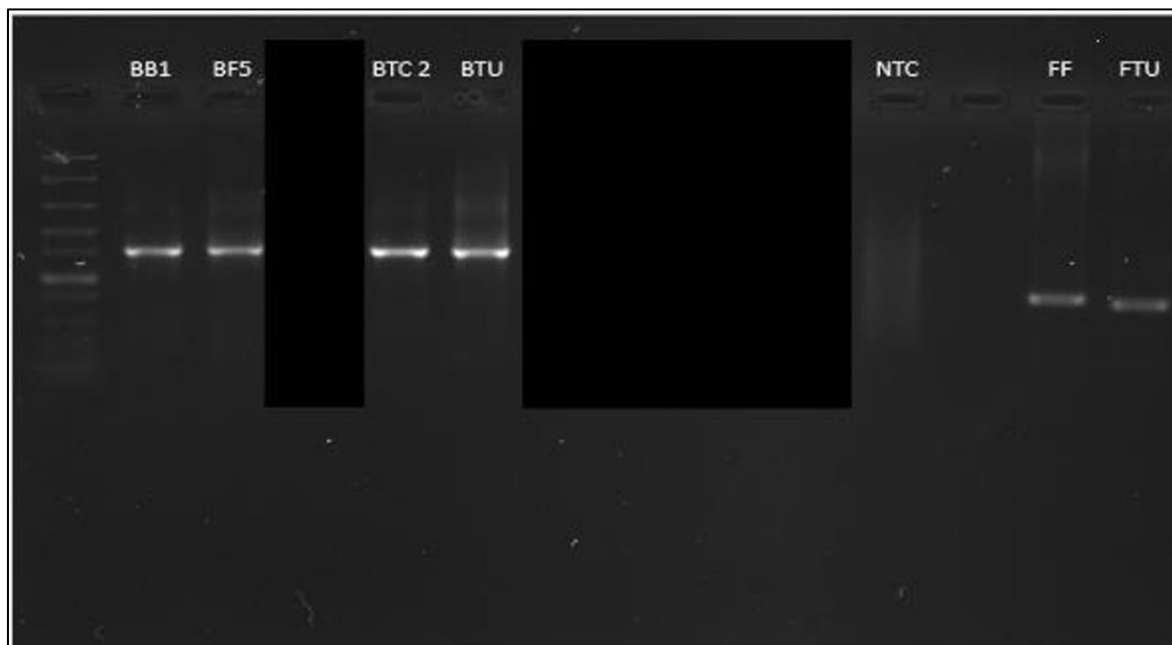


Fig 1 Agarose Electrophoresis Gel Plate

KEY

NTC- Negative control

BF5: *Bacillus velezensis* strain CUAB-AOS02

FF = *Aspergillus niger* strain CUAB-AOS05

➤ Phylogenetic Analysis

Fig. 1 shows the phylogenetic relationships of *Bacillus velezensis* strain CUAB-AOS02 (PP657698) with other

isolates from the NCBI database. The tree showed close relationships with *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus siamensis*.

Fig. 2 shows the phylogenetic relationships of *Aspergillus niger* strain CUAB-AOS05 (PP657690). The tree showed the closest relationship with *Aspergillus niger* strain RRFO4 (bootstrap value of 34), indicating a recent common ancestor.

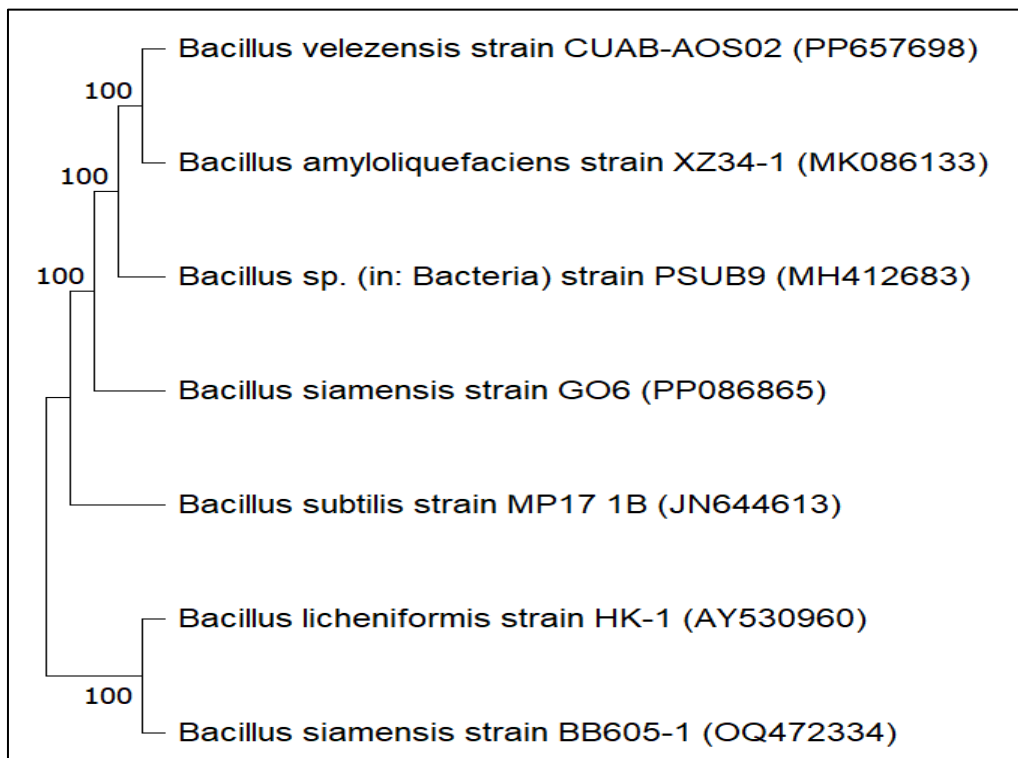


Fig 2 Phylogenetic Tree Showing the Relationship of the Bacteria Isolates with Isolates from NCBI

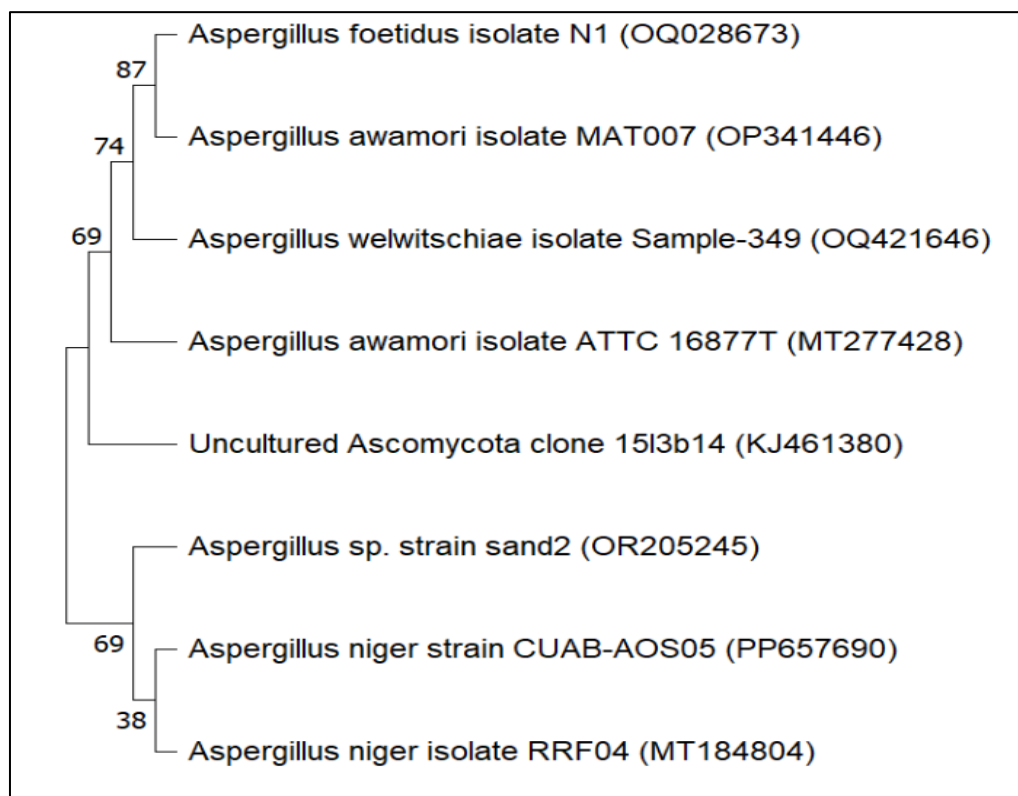


Fig 3 Phylogenetic Tree Showing the Relationship of the Fungal Isolates with Isolates from NCBI

➤ *Plasmid Analysis*

Plasmid analysis of *Bacillus velezensis* CUAB-AOS02 (Fig. 4) revealed a circular DNA plasmid of 867 base pairs with 15 identified restriction sites. Restriction enzymes Cfr421 and EcoRI were found on multiple sites.

Plasmid analysis of *Aspergillus niger* CUAB-AOS05 (Fig. 5) revealed 20 identified restriction enzymes within 521 base pairs, indicating significant genetic complexity and potential for genetic manipulation.



- Keys: CN: Gentamicin (10µg), PEF: Pefloxacin (5µg), OFX: Ofloxacin (5µg), CH: Chloramphenicol (30µg), SP: Streptomycin (10µg), CPX: Ciprofloxacin (5µg), SXT: Trimethoprim/Sulfamethoxazole (25µg), E: Erythromycin (15µg). (-) indicates no zone of inhibition.

Table 8 presents the interpretation of susceptibility results according to CLSI standards [21]. All bacterial isolates, except *Streptococcus* isolate 1 (which showed intermediate resistance to chloramphenicol and sensitivity to pefloxacin, ofloxacin, streptomycin, and ciprofloxacin), were resistant to all antibiotics tested. This classifies them as multidrug-resistant (MDR).

Table 8 Interpretation of Antimicrobial Susceptibility (CLSI Standard 2024)

Bacteria Isolate	CN	PEF	OFX	CH	SP	CPX	SXT	E	Interpretation
<i>Streptococcus sp. 1</i>	R	S	S	I	S	S	R	R	MDR
<i>Streptococcus sp. 2</i>	R	R	R	R	R	R	R	R	MDR
<i>E. coli</i>	R	R	R	R	R	R	R	R	MDR
<i>Bacillus sp.</i>	R	R	R	R	R	R	R	R	MDR
Bacteria Isolate	CN	PEF	OFX	CH	SP	CPX	SXT	E	MDR

#### IV. DISCUSSION

This study successfully isolated and characterized both bacterial and fungal biofilms from domestic refrigerators in Abeokuta, Nigeria. The results demonstrate that these appliances, despite their intended function of food preservation, harbor a diverse array of potentially pathogenic and spoilage-causing microorganisms. The 100% contamination rate is consistent with previous studies by Otu-Bassey et al. [5], Fazelipour et al. [22], and Sarabi et al. [23], confirming that domestic refrigerators are frequently contaminated with a variety of microbes.

Phenotypic characterization of bacterial isolates suggested a predominance of *Streptococcus spp.* (60%), followed by *E. coli* and *Bacillus spp.* (20% each). However, molecular identification of the most prevalent isolate (BF5) revealed it to be *Bacillus velezensis* strain CUAB-AOS02 (PP657698). This discrepancy between phenotypic and molecular methods underscores the critical importance of molecular techniques for accurate species-level identification, a finding consistent with Shehu et al. [24] and Nuangmek et al. [25]. The presence of *E. coli*, a widely accepted indicator of fecal contamination, suggests that refrigerator surfaces are frequently contaminated by raw foods or poor personal hygiene [26], [27].

The *Bacillus velezensis* strain identified in this study appears well-adapted to the refrigerator environment, possibly due to its ability to thrive at lower temperatures and resist desiccation. This is concerning as *Bacillus* species can produce heat-resistant endospores and some strains harbor virulence factors or antibiotic resistance genes [28].

Phenotypic analysis of fungal isolates indicated a dominance of *Penicillium spp.* (60%), with *Cryptococcus spp.* and *Aspergillus spp.* each at 20%. Molecular identification of the dominant fungus, however, identified it as *Aspergillus niger* strain CUAB-AOS05 (PP657690). This finding aligns with Otu-Bassey et al. [5] and Fazelipour et al. [22], who also reported *Aspergillus* species in refrigerators. The high prevalence of *A. niger* can be attributed to its fast growth rate, versatile metabolism, and prolific spore production, allowing it to outcompete other fungi in the

refrigerator's confined environment. The presence of *Cryptococcus spp.*, though less prevalent, is noteworthy as some species are opportunistic pathogens, particularly for immunocompromised individuals [29].

The phylogenetic analysis showed that *B. velezensis* CUAB-AOS02 shares close nodes with several *Bacillus* strains from the NCBI database, suggesting a recent common ancestor and potential for shared genetic traits, including those related to environmental adaptability and possibly antimicrobial resistance [25]. Similarly, *A. niger* CUAB-AOS05 showed closest relationship with strain RRFO4, indicating a recent common ancestor and potential for shared genetic traits related to mycotoxin production or antifungal resistance [30].

The plasmid mapping of *B. velezensis* CUAB-AOS02 revealed a circular DNA of 867 bp with 15 restriction sites, indicating genetic complexity and potential for horizontal gene transfer [31]. Plasmids often carry genes that confer advantageous traits, such as antibiotic resistance or metabolic capabilities. The plasmid of *A. niger* CUAB-AOS05 contained 20 restriction enzymes within 521 bp, suggesting significant genetic potential for adaptation and survival, though plasmids are less common in fungi [32].

The antibiotic susceptibility testing yielded concerning results. According to CLSI standards [21], all bacterial isolates except one *Streptococcus* isolate were resistant to all antibiotics tested. Even the partially susceptible *Streptococcus* isolate showed resistance to gentamicin, trimethoprim/sulfamethoxazole, and erythromycin, and intermediate resistance to chloramphenicol. This classifies the majority as multidrug-resistant (MDR). The isolation of MDR bacteria from domestic refrigerators is alarming, as it implies that common household appliances could act as reservoirs and conduits for the spread of resistant genes. This finding is consistent with Oluwole and Olumuyiwa [33] but contrasts with Bello et al. [34], who found 85% of isolates from refrigerators were susceptible to tested antibiotics. The high level of resistance observed here may reflect the widespread use of antibiotics in the environment and community.

The presence of *Aspergillus niger* is significant, as this species can produce mycotoxins (ochratoxin A) that are harmful to human health [5]. Ochratoxin A is nephrotoxic, immunotoxic, and carcinogenic. The presence of *Cryptococcus spp.*, some of which are opportunistic pathogens causing cryptococcosis, is also worrisome [29]. These findings, combined with the MDR bacteria, highlight the critical need for improved hygiene practices. While food pathogens cannot be entirely eliminated from kitchens, their growth, spread, and survival can be significantly reduced by proper food preparation, storage, regular cleaning, and routine disinfection of food contact surfaces [35], [36].

## V. CONCLUSION

This study effectively isolated, characterized, and identified robust bacterial and fungal biofilms within domestic refrigerators. The key findings are: (1) Domestic refrigerators in Abeokuta harbor a diverse microbial community, including potentially pathogenic bacteria and fungi; (2) Molecular identification revealed the dominant bacterial strain to be *Bacillus velezensis* CUAB-AOS02 and the dominant fungal strain to be *Aspergillus niger* CUAB-AOS05, both representing novel strains; (3) The majority of bacterial isolates exhibited multidrug resistance, raising significant public health concerns; (4) Phylogenetic and plasmid analyses provided insights into the genetic diversity, adaptability, and evolutionary potential of these strains. These findings advance our knowledge of the intricate microbial communities found in residential refrigerators and emphasize the necessity of ongoing research and public health interventions to guarantee the safety and quality of stored food.

## RECOMMENDATIONS

Based on the findings of this study, the following recommendations are made: (1) Consumers should implement regular cleaning and maintenance protocols for domestic refrigerators, including wiping surfaces with disinfectants and promptly cleaning spills; (2) Proper food storage practices should be followed: wrap foods adequately, store raw meats on lower shelves, and avoid overpacking to ensure proper air circulation; (3) Public health authorities should launch education campaigns emphasizing the importance of refrigerator hygiene and safe food handling; (4) Future research should examine the molecular mechanisms behind biofilm production, evaluate new antimicrobial agents against these novel strains, and explore innovative approaches for biofilm control such as antimicrobial surface coatings.

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