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Microbial Analysis of Processed Foods Stored in Domestic Refrigerators of Selected Eateries in Ile-Ife, Osun State, Nigeria

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Abstract: Consumption of contaminated refrigerated foods is increasing the incidence of food-borne diseases globally. However, there is paucity of data on prevalence of microbes associated with these foods from Osun State, Nigeria, thus, the need for isolation and characterization of microbes associated with refrigerated foods from this region. A total of 80 processed food samples were collected aseptically and refrigerated for four days. Samples were analyzed for the presence of bacteria and fungi while antibiotic resistance patterns of bacterial isolates were determined using standard methods. Mean bacterial count ranged from 7.0×10^4 to 3.7×10^5 cfu/ml with highest bacterial count of 3.7×10^5 cfu/ml obtained from melon soup sample, while least count of 7×10^4 cfu/ml was obtained from cooked meat. Fungal count ranged from 2.0×10^2 to 5.6×10^3 sfu/ml with cooked beans having the highest count of 5.6×10^3 sfu/ml. The bacteria that were isolated from refrigerated processed food samples include *Corynebacterium kutscheri* (47.05%), *Bacillus* spp (14.70%), *Staphylococcus aureus* (11.76%), *Lactobacillus fermenti* (5.8%), *Aeromonas hydrophila* (5.8%), *Pseudomonas* spp (5.88%), *Lactobacillus casei* (2.94%), *Lactobacillus delbrueckii* (2.94%) *and Citrobacter freundii* (2.94%), while fungi isolated were *Aspergillus fumigatus* (24.56%), *Aspergillus niger* (21.05%), *Aspergillus flavus* (14.04%), *Aspergillus brevipes* (14.04%), *Mucor* spp (7.02%), *Penicillium* spp (8.77%) *and Saccharomyces* spp (10.53%). *Corynebacterium kutscheri* had the highest resistance of 17(37%) while *Lactobacillus casei* showed no resistance (0%) to the antibiotics used. This study showed high bacterial and fungal counts from the analyzed samples, hence, the need to ensure hygienic food processing and refrigerating conditions.

Keywords: Food-borne Diseases, Refrigerated Foods, Antibiotic Resistance, Food Processing

1. Introduction

Foods refrigerated can become vectors for food-borne illness by contamination with food-borne pathogens in retail stores, processing plants, or consumers' homes [1]. These spoilage-causing or pathogenic microorganisms are often able to grow at low temperatures and lead to reduction of shelf life or even affect consumer's health [2-3]. The perishable raw or cooked food that could serve as a growth medium must be stored to prevent the multiplication of

bacteria that have survived during processing or handling [4]. Because most foodborne bacteria and molds that are agents of spoilage or infection can multiply at room temperature, manipulation of the holding temperature is a useful preservation method [4]. Different food requires different places and temperature for storage [5]. For instance, fresh meat, fish and poultry are stored in cold storage such as freezers, and in containers with crack ice just like what they do with the fish sold in the market [6]. Fruits and vegetables are placed in airy and cool places or in the vegetable compartment of the refrigerator [7]. Despite low

temperatures, hygienic designs and cleaning recommendations, refrigerators can be hot spots for bacteria [8]. *Staphylococcus, Pseudomonas, Bacillus,* Enterobacteriaceae and Fungi are primarily found in household refrigerators [9-10]. Studies in Ireland have shown that the concentration of viable cells in refrigerators is around $\log_{10}{}^{7.1}$ cfu cm^{-2} in average. In addition, more than half of the inspected refrigerators contained pathogenic microorganisms [10]. Psychrotolerant microorganisms are more widely distributed in nature than psychrophiles and can be isolated from soils and water in temperate climates, as well as from meat, milk and other dairy products, cider, vegetables, and fruit stored at refrigeration temperatures (4°C). Psychrotropic pathogens include *Yersinia enterocolitica, Listeria monocytogenes,* non-proteolytic strains of *Clostridium botulinum,* some strains of enterotoxigenic *Escherichia coli* and *Aeromonas hydrophila.* The practice of storing food at refrigeration temperature is common for controlling the growth of psychrotrophic microorganisms, some pathogens, and maintaining product quality [11]. Nevertheless, fungi and psychrotrophic bacteria are commonly associated with spoilage of food at refrigerator temperatures. Fungi predominate in refrigerated food spoilage when low water activity, high acidity or packaging conditions selects for their growth over bacteria in foods. Psychrotrophic fungi are also commonly isolated from refrigerated fresh animal products, fruits and vegetables [12] and ready to eat foods [13]. The aim of this study is to isolate, characterize and determine the prevalence of microorganisms associated with refrigerated foods from selected eateries in Ile-Ife, Osun State, Nigeria.

2. Materials and Methods

2.1. Collection of Samples

A total of 80 samples which comprises of two samples per each of the freshly prepared processed foods which includes cooked rice, melon soup, cooked meat, cooked beans and liquid canned milk were collected aseptically from selected eateries in Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria for 8 weeks between March and May, 2017. The food samples were allowed to cool before refrigerating for 4 days. Afterwards, refrigerated samples were collected aseptically and transported immediately to Microbiology Department laboratory at Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria for microbial analysis.

2.2. Enumeration and Isolation of Organisms

A ten-fold serial dilution was carried out immediately and appropriate dilutions were used for microbial enumeration using standard pour plate method. The microbial load of samples were determined using Nutrient agar (NA) (37°C for 24-48 h) and Malt Extract agar (MEA) containing 0.05 g/L streptomycin (25°C for 5 days) for total bacterial and fungal counts respectively. Isolation of Lactic Acid Bacteria was carried out using De Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK) at 37°C for 24 to 72 hours in microaerophilic

environment. Afterwards, observed colonies were counted and isolated from each refrigerated food samples cultured plates.

2.3. Characterization of Bacterial Isolates

The isolates were characterized based on morphological and biochemical analysis and was compared with standards using Bergey's manual.

2.4. Characterization of Fungal Isolates

The fungal isolates were identified based on their colonial morphology and microscopic examination. Characterization based on size, texture, pigmentation, shape and septation of hyphae were also made. The technique of James and Natalie [14] was adopted for identification of the unknown isolated fungi using lactophenol cotton blue stain. The identification was achieved by placing a drop of the stain on clean slide and a small portion of the mycelium from the fungal cultures was picked and placed on the stain. The mycelium was spread evenly on the slide with the inoculating needle. A cover slip was gently applied with little pressure to eliminate air bubbles and observed using ×10 and × 40 objective lens. The species encountered were identified using Pictorial atlas for identification of seed and soil fungi [15].

2.5. Antibiotics Sensitivity Test

The bacterial isolates were tested for their sensitivity to antibiotics by means of disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI). Sensitivity was carried out on Mueller Hinton Agar (MHA) prepared in accordance to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes before dispensing into sterile Petri dishes. The plates were seeded with standardized inoculums (0.5 McFarland standard) by means of a sterile cotton swab which was used to introduce the organism on entire agar surface horizontally, vertically and around the edges of the plates. The disc was then aseptically applied to the surface of the agar plates with the aid of sterile forceps, incubated in an inverted position at 37°C for 24 hours. After incubation, each zone of inhibition was measured and interpreted by comparing with CLSI standards to determine their resistance patterns.

3. Results

3.1. Bacterial and Fungal Enumeration

Five refrigerated food samples were analyzed for the presence of bacteria and fungi with standard plate count method used for the analysis. The bacterial count ranged from 7×10^4 to 3.7×10^5 cfu/ml with highest bacterial colony count obtained from soup (3.7×10^5 cfu/ml) while the least colony count of 7.0×10^4 cfu/ml gotten from analyzed meat sample. The fungal count ranged from 2.0×10^2 to 5.6×10^3 sfu/ml with beans having the highest counts of 5.6×10^3 sfu/ml as shown in table 1.

Table 1. Mean bacterial and fungal counts in refrigerated food samples.

Food samples	Bacterial Counts (Cfu/ml)	Fungal Counts (Sfu/ml)
Cooked Rice	2.6×10^5	3.0×10^3
Cooked Beans	1.8×10^5	5.6×10^3
Cooked Meat	7.0×10^4	3.5×10^3
Melon Soup	3.7×10^5	2.0×10^2
Liquid canned milk	1.1×10^5	4.7×10^3

3.2. Percentage Occurrence of Bacterial Isolates

Table 2 shows the percentage occurrence of bacterial isolated from refrigerated food samples. *Corynebacterium kutscheri* was dominant organism with the percentage frequency of 47.05% followed by *Bacillus* spp with 14.70% while *Lactobacillus casei, Lactobacillus delbrueckii and Citrobacter freundii* had 2.94% respectively as presented on table 2.

Table 2. Percentage occurrence of bacterial isolates.

S/NO	Bacteria	Frequency	% Occurrence
1	*Corynebacterium kutscheri*	32	32(47.05)
2	*Bacillus* spp	10	10(14.70)
3	*Staphylococcus aureus*	8	8(11.76)
4	*Aeromonas hydrophila*	4	4(5.88)
5	*Pseudomonas* spp	4	4(5.88)
6	*Lactobacillus fermenti*	4	4(5.88)
7	*Lactobacillus casei*	2	2(2.94)
8	*Lactobacillus delbrueckii*	2	2(2.94)
9	*Citrobacter freundii*	2	2(2.94)
		Total	100

3.3. Percentage Occurrence of Fungal Isolates

Table 3 shows the percentage frequency of fungi isolated from refrigerated food samples which consist of different species of fungi. The fungal isolates were identified as *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus*, *Mucor* spp, *Penicillium* spp and *Saccharomyces* spp. *Aspergillus fumigatus* (24.56%) was the most predominant fungal isolate followed by *Aspergillus niger* (21.05%). *Mucor* spp was the least fungal isolates with 7.02 %.

Table 3. Percentage occurrence of fungal isolates.

S/N	Fungi	Frequency	% Occurrence
1	*Aspergillus fumigatus*	14	14(24.56)
2	*Aspergillus niger*	12	12(21.05)
3	*Aspergillus flavus*	8	8(14.04)
4	*Aspergillus brevipes*	8	8(14.04)
5	*Saccharomyces* spp	6	6(10.53)
6	*Penicillium* spp	5	5(8.77)
7	*Mucor* spp	4	4(7.02)
		Total	100

3.4. Percentage Resistance of Bacterial Isolates to Various Antibiotics

Table 4 shows the percentage resistance of each bacterial isolates to different antibiotics used in this study. Out of 68 bacteria isolated from refrigerated food samples, 46 bacterial isolates were resistant to different antibiotics used in this study. *Corynebacterium kutscheri* had the highest resistance of 17(37%) while *Lactobacillus casei* showed no resistance (0%) to the antibiotics used.

Table 4. Percentage resistance of bacterial isolates to various antibiotics.

Bacterial isolates	Frequency	Resistant (%)
Aeromonas hydrophila	4	4(8.7)
Bacillus spp	8	8(17.4)
Corynebacterium kutsceri	17	17(37)
Citrobacter freundii	2	2(4.3)
Lactobacillus delbrueckii	2	2(4.3)
Lactobacillus fermenti	4	4(8.7)
Pseudomonas spp	4	4(8.7)
Staphylococcus aureus	5	5(10.9)
Lactobacillus casei	-	- (0%)
	Total	100

3.5. Multiple Antibiotic Resistance Patterns of Isolates

Table 5 shows the multiple antibiotic resistance pattern of bacterial isolates. The bacterial isolates were resistant to different classes of antibiotics. The highest antibiotic resistance pattern (APX, AM, CPX, CN, E, R, S, SXT) was observed in *Corynebacterium kutscheri*.

Table 5. Multiple antibiotic resistance patterns of bacterial isolates.

Bacterial Isolates	Antibiotic Resistance Pattern	Frequency	Resistant (%)
Aeromonas hydrophila	COX, CRO, N, C, SXT, PX	4	4(12.1)
Bacillus spp	APX, CPX, E, R, S, SXT, Z	5	5(15.2)
Citrobacter freundii	COX, N, PX, T	3	3(9.0)
Corynebacterium kutscheri	APX, AM, CPX, CN, E, R, S, SXT	6	6(18.2)
Lactobacillus delbrueckii	APX, E, R, S	4	4(12.1)
Lactobacillus fermenti	AM, CN, CPX, S, E	4	4(12.1)
Pseudomonas spp	COX, C, CN, N, PX, T	5	5(15.2)
Staphylococcus aureus	APX, AM , E	2	2(6.1)
		Total	100

KEY

COX→Cephalexin, CRO→ Ceftriaxone, PX→ Ampicillin, CPX→ Ciprofloxacin,
N→ Nitrofuratoin, SXT→ Cotrimoxazole, T→ Tetracycline, C→ Chloramphenicol,
OFX→ Ofloxacin, CN→Gentamicin, PEF→ Pefloxacin, APX→ Ampiclox,
R→Rocephin (Ceftriaxone), E→Erythromycin, Z→Cefuroxime, S→Streptomycin, AM→Amoxicillin

4. Discussion

The high microbial load (3.7 x 10^5 cfu/ml and 5.6 x 10^3 sfu/ml) observed in the refrigerated processed food samples as shown in table 1 are likely to be caused by unhygienic conditions and temperature abuse of the domestic refrigerators used for this study. Since most microorganisms are likely destroyed by the high temperature used for cooking, the high microbial load is likely to be post cooking. According to Oluwafemi *et al.* [16], the pathogens found in domestic refrigerators could contaminate food directly or indirectly and pose a major health risk to consumers in terms of food poisoning. Even at proper storage temperatures, some spoilage-causing or pathogenic microorganisms grow at low temperatures and lead to reduction of shelf life or affect consumer's health [2, 17]. The high fungal count (5.6 x 10^3 sfu/ml) detected in the refrigerated food samples as shown in table 1 may be due to the fact that fungi grow well in an environment that has adequate nutrients and moisture [18]. Mold growth on refrigerated foods depend on several factors such as temperature (being the most important one), time of storage, moisture content, and concentration of carbohydrates on food source etc. Psychrotrophic bacteria and fungi are commonly associated with spoilage of food at refrigerator temperatures [19]. Low water activity, high acidity or packaging conditions selects for the growth of fungi over bacteria in refrigerated food spoilage. Mold genera that have psychrotropic species *include Alternaria, Aspergillus, Botrytis, Cladosporium, Fusarium, Geothricum, Monascus, Mucor, Penicillium, Rhizopus, and Trichothecium*. Among the yeast genera involved are *Candida, Debaryomyces, Saccharomyces* and *Torulopsis* [20].

Food kept for too long or that has been left out in humid conditions provides a good substrate for fungal growth [5]. Some fungi are cold tolerant and can even grow on old food in a refrigerator [5]. Some foods with high sugar or salt concentration will be difficult for most fungi to colonize, but most kinds of breads, fruits and vegetables are ideal [5]. The following organisms were isolated in descending order, *Corynebacterium kutscheri* (47.05%), *Bacillus* spp (14.70%), *Staphylococcus aureus* (11.76%), *Lactobacillus fermenti* (5.13%), *Aeromonas hydrophila* (5.13%), *Pseudomonas* spp (5.13%), *Lactobacillus casei* (2.56%), *Lactobacillus delbrueckii* (2.56%) and *Citrobacter freundii* (2.56%) as shown in table 2. Several researchers have reported the frequency of *Pseudomonas* spp, *Lactobacillus delbrueckii, Bacillus cereus, Aeromonas* spp, *Citrobacter* spp, and *Staphylococcus aureus* in domestic refrigerators [19, 21, 22, 23]. The percentage occurrence of *Staphylococcus aureus* recovered in this study was 11.76%. This result is higher than some previous reported detection, for example, 5.5% [24], but lower than the 20% reported by Ojima *et al.* [10], or the 27.3% reported by Otu-Bassey *et al.* [25]. *Staphylococcus aureus,* as a common inhabitant (up to 50%) of the human nose, throat, and skin [26] is perhaps more likely to contaminate foods stored inside domestic refrigerators by

direct or indirect human contact during domestic food handling and storage. Many domestic refrigerators are incorrectly adjusted, operating above the recommended temperature and are therefore capable of supporting sub-optimum but significant growth of mesophilic organisms such as *Staphylococcus aureus, Escherichia coli* and *Salmonella* spp [27].

Pseudomonas are aerobic, Gram-negative soil bacteria, some of which can degrade a wide variety of unusual compounds [28]. They generally require a high water activity for growth (0.95 or higher) and are inhibited by pH values less than 5.4 [28]. Some species grow at refrigeration temperatures (psychrophilic) while other are adapted for growth at warmer, ambient temperatures. *Pseudomonas* spp was isolated from the refrigerated food samples and this was consistent with Doyle, [28] which stated that some strains are adapted for growth at cold temperatures and spoil the foods in the refrigerator. Rawat, [29] also stated that some species of *Pseudomonas* can attack refrigerated and processed foods. *Salmonella* spp, *Campylobacter* spp and *Escherichia coli* were not detected in any of the refrigerated processed food samples. This was in agreement with Spiers *et al.* [30] who reported failure to detect these pathogens, not only in refrigerators but in a wide range of sites examined in domestic kitchens. *Citrobacter freundii*, the least bacterial isolated from refrigerated processed food samples was also reported by Osborne *et al.* [23].

Aspergillus fumigatus, *Aspergillus niger*, *Aspergillus flavus, Aspergllus brevipes, Saccharomyces* spp, *Penicillium* spp and *Mucor* spp were recovered from the refrigerated processed food samples. This was consistent with the work of Altunatmaz *et al.* [19] who reported the isolation of *Aspergillus flavus, Aspergillus niger, Mucor* spp, *Penicillium* spp and yeast in different types of ready-to-eat (RTE) foods stored in refrigerators. Pitt and Hocking, [31] also reported that different mold species have different optimal growth temperatures, with some able to grow in refrigerators.

Aspergillus fumigatus, Aspergillus niger, *Aspergillus flavus, Aspergillus brevipes* were the species of Aspergillus genera isolated from refrigerated food samples and these can be a possible source of aspergillosis transmission among consumer of the refrigerated foods. *Aspergillus* and related molds generally grow faster and are more resistant to high temperatures and low water activity than *Penicillium* spp and tend to dominate spoilage in warmer climates [28]. *Mucor* spp (6.06%), the least fungi isolated was also reported in ready to eat foods stored in the refrigerator [19].

Out of the nine (9) bacterial genera isolated from refrigerated food samples, eight (8) were resistant (*Aeromonas hydrophila, Bacillus* spp, *Corynebacterium kutscheri, Citrobacter freundii, Lactobacillus delbrueckii, Lactobacillus fermenti, Pseudomonas* spp, *Staphylococcus aureus*) to different antibiotics used in this study while one (1) was susceptible (*Lactobacillus casei*) as shown in table 4. Also, high antibiotics resistance profile by *Corynebacterium kutscheri* 17(37 %) was observed followed by *Bacillus* spp

8(17.4%). As shown in table 5, the bacterial isolates were resistant to more than one class of antibiotics. This was in agreement with the findings of Lowy, [32]. Out of all the bacterial isolates recovered from the refrigerated processed food samples, *Corynebacterium kutscheri* had the highest antibiotics resistant (APX, AM, CPX, CN, E, R, S and SXT) pattern. This was consistent with the work of Olender, [33] who reported the resistance of *Corynebacterium* spp to different classes of antibiotics such as macrolides, lincosamides, fluoroquinolones, tetracyclines, beta-lactam and glycopeptides. Previously, multiple resistance to antibiotics by microorganisms were found mostly in hospitals where antimicrobial agents are used most frequently but resistance is found in refrigerated processed food materials as observed in this study. The clinical management of a number of food-borne infections have been complicated by antimicrobial resistant bacteria [34].

5. Conclusion

This study has shown that the pathogenic bacteria and fungi can survive in refrigerated food samples due to the high bacterial and fungal counts observed on the food samples and could therefore pose a consumer health risk. Refrigerated foods can become vectors for food-borne pathogens through temperature abuse and inadequate cleaning of the domestic refrigerators. It is crucial that the public be made aware that the refrigerator can represent a significant niche for the persistence and dissemination of food borne pathogens due to their reliance on refrigeration as a means of food preservation. Care must be taken in the consumption of refrigerated foods most especially after a long period of time. This study also suggests the need for surveillance and public health practitioners centre to intensify their effort in monitoring the refrigerated and processed food materials so as to prevent the emergence of antibiotic resistance by microorganisms.

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