*Asian Journal of Medical Principles and Clinical Practice*

***Volume 7, Issue 1, Page 56-68, 2024; Article no.AJMPCP.112028***

# Phenotypic Characterization and *in vitro* Susceptibility Testing of Fungi Isolated from Madiga Bread (Local Staple Food) in Yenagoa Metropolis, Nigeria

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author OAO designed the study. Authors OAO and EZBO performed the experiments and analyzed the data. Authors EZBO and OAO drafted the manuscript. Both authors read and approved the final manuscript.*

***Article Information***

**Open Peer Review History:**
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***Original Research Article***

***Received: 24/11/2023***
***Accepted: 29/01/2024***
***Published: 07/02/2024***

## ABSTRACT

Local bread known as "madiga" is typically enjoyed by residents of Nigeria's Niger Delta region. The aim of this study is to assess madiga bread for inbuilt pathogenic fungi in evaluating its level of edibility in Yenagoa Metropolis. A total of 42 samples was obtained and analyzed include; Hand swabs from factory workers (8/19%), hand swab of madiga vendors (6/14%), madiga bread gotten from market (12/29%), and madiga bread from factories (16/39%). The samples were processed using the following methods: culturing on Saboraud Dextrose Agar, identifying fungal isolates microscopic with Lacto-Phenol Cotton blue, staining the samples with Grama stain, germ tube test,

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cultivating yeast isolates on CHROMagar Candida (CAC), and standardizing the pure isolates using the 0.5 McFarland turbidity standard. From the samples, 206 fungi in total were isolated. Madiga breads obtained from factories 80 (39%) have the highest isolation rate. *Aspergillus niger* 120(58%), *Aspergillus flavus* 23(11%), *Aspergillus fumigatus* 5(3%), *Candida tropicalis* 21(10%), *Candida parapilosis* 21(10%), *Microsporum auidouini* 14(7%), and *Penicillin spp* 2(1%), are the seven distinct fungal species that was been isolated. The pure fungal isolates were subjected to antifungal susceptibility testing using the following methods: Agar diffusion and Kirby Buer techniques with Clotrimazole (50ug), Econazole (20ug), Caspofungin (5ug), Ketoconazole (15ug), Nystatin (100lu), and Fluconazole (25ug) on Saboraud Dextrose Agar. Clotrimazole was the most effective antifungal agent against all of the fungal isolates, exhibiting a 100% susceptibility rate and 8.3% resistance to fluconazole. Because of the high rate of fungal isolation from the samples that were analyzed, which is a result of the inadequate hygiene practices used in the Madiga bread's manufacturing and distribution. It is suggested that the government implement regulations to ensure appropriate monitoring of Madiga bread production and provide workers at the factory with training on essential hygiene protocols.

*Keywords: Madiga; bread; fungi; amassoma; Yenagoa Metropolis and antifungal agents.*

## 1. INTRODUCTION

People in Nigeria's Niger Delta region, typically eat madiga, a local bread product. Because of its inexpensive price, readily consumable nature, and sweet flavor, this wheat flour product is highly prized by kids [1]. As shown in Image 1.

**Image 1. A sample of a Madiga bread**

In the 1980s, Chief Madiga, founded and initiated the baking of madiga bread in Enekorogha village in the Burutu Local Government Area of Delta State, Nigeria. Compared to regular bread, which is soft, madiga has a significantly denser texture. In the south-south region of Nigeria, more specifically in the states of Bayelsa and Delta, madiga, which is frequently baked in neighborhood bakeries and consumed by rural residents, is increasingly being embraced by urban settlers [2]. Madiga is manufactured and offered for sale in the open market in an unbranded, unlabeled, and nutritionally untagged state (Image 2).

**Image 2. A picture of an individual buying Madiga Bread (sold in an unbranded, unlabeled, and nutritionally untagged state), from a Madiga bread vendor at a Market in Yenagoa**

Fungal organisms are classified as eukaryotes because they digest their food externally and absorb nutrients directly through their cell walls. The majority of fungi are spore-producing organisms with a thallus made up of tiny tubular cells known as hyphae. Fungi are heterotrophs, meaning they get their energy and carbon from other living things, just like animals do. While some fungi, known as biotrophs, get their nourishment from living hosts (plants or animals),

others, known as saprotrophs (saprophytes, saprobes), get their nourishment from dead plants or animals. Certain fungi are known as necrotrophs because they infect live hosts but kill the host cells to feed on their nutrients [3].

The most common food contaminant in the world is fungus. These are common plant pathogens that are also important food and feed spoilage agents [4].

Most countries and cultures consider bakery products to be important staple foods. Breads, buns, cupcakes, cookies, pizza bases, toasts, and other items are the most popular products. The majority of our daily calories and about half of our protein needs come from the cereals used to make bakery goods, making them an important source of nutrients. The nutrients found in bakery goods include energy, calcium, iron, vitamins, lipids, proteins, and carbohydrates [5].

**Image 3. A photo of a Madiga bread production factory worker mixing the raw material (flour) for the baking of the Madiga bread**

Many species of fungi, such as *Aspergillus, Mucors, Penicillin*, and others, contaminate a large amount of the world's food supply, which includes the main feedstocks of low-income countries: maize, rice, sorghum, barley, rye, wheat, peanuts, groundnuts, soy, and cottonseed. Because mycotoxins produced by fungi are nephrotoxic, immunotoxic, teratogenic, and mutagenic, their presence in our food systems and tissues has a significant impact on public health [6]. They can also have both acute and long-term effects on humans and animals, ranging from respiratory and central nervous system disorders to intestinal and cardiovascular diseases. The primary reasons to be concerned are the links between these toxins and human hepatoma and oesophageal cancer, as well as with children's pre-five mortality, shorter life expectancy, and higher susceptibility to illnesses [7,8].

As seen in image 3, because of the unhygienic circumstances that exist throughout the Madiga's manufacturing, distribution, and consumption process. The study of phenotypic characterization and *in vitro* susceptibility testing of fungi isolated from madiga bread is a valuable tool for developing public health policies that support hygienic conditions and promote healthy leaving.

## 2. MATERIALS AND METHODS

### 2.1 Study Area

This study was carried out within Yenagoa Metropolis and Amassoma community. Yenagoa is the capital city of Bayelsa State. Yenagoa is geographically located within Latitude: 4° 55' 36.30" North and Longitude: 6° 16' 3.50" East. It has an area 1,698 $km^2$ and a population of 352,285 at the 2006 census. Amassoma is the host community of Niger Delta University, which is located in Southern Ijaw Local Government Area in Bayelsa State. Geographically, Amassoma is situated in the latitudes of 4° 58' 13.152" North and 6° 6' 32.94" East. The primary occupations of the locals in the Amassoma community are farming and fishing.

### 2.2 Sample Collection

A total of fifty (42) samples was obtained from Madiga factories and major markets in Yenagoa Metropolis and Amassoma community. Samples collected was, Madiga bread and hand swab of works at the factories and sales persons at the market.

### 2.3 Study Design

This is a survey/cross-sectional study on fungi isolated from Madiga breads obtained from markets and factories, as well as hand swabs of workers and vendors in the Madiga bread factories located in Yenagoa City and the Amassoma community.

## 2.4 Methodology

Samples of fungi were acquired from Madiga bread, Madiga factory employees, and Madiga vendors. Using sterile swab sticks, the hands of Madiga factory workers and vendors were swabbed. The samples were then transferred, in less than an hour, to the Department of Medical Laboratory Science at Niger Delta University Wilberforce Island's Microbiology Laboratory. On Sabouraud dextrose agar (SDA) plates, each Madiga bread was streaked after being swabbed with a sterile swab stick. A swab obtained from Madiga vendors and factory workers was streaked onto Sabouraud dextrose agar (SDA) plates and incubated for three days at 37°C. Each culture plate's fungal colonies were sub-cultured on Sabouraud dextrose agar plates, which were then incubated for three days at 37°C. For the purpose of differentiating between Candida species, distinct yeast colonies from each culture plate were sub-cultured on Chrome Candida differential medium plates. These plates were incubated at 37°C for 2 days. The distinctive color of the colonies on the media, as stated by the manufacturers, was used for identification.

An overnight broth was made by autoclaving 2ml of distilled water in a 50 test tubes each, it was then allowed to cool down, and the organism was inoculated into the distilled water. Each test tube was poured on a Sabouraud dextrose agar plate, it was spread with the aid of a sterile spreader, excess was decanted and each of the antifungal agent; Fluconazole, Econazole, Caspofungin, Ketoconazole, Clotrimazole, Nystatin, was applied with the aid of a sterile forcep. It was incubated for 24hours and the zone of inhibition was measured for each of the antifungal agents.

Lactophenol cotton Blue was utilized for the microscopical identification of fungi. A drop of Lactophenol cotton blue was place on a clean grease free slide and with the aid of a sterile wire loop the fungi was picked from the cultured plate and was emulsified on the glass slide and a coverslip was placed on it; and was examined using x10 objective lens and x40 objective lens for confirmation.

## 3. RESULTS

A total of 206 fungi were isolated from the 42 processed samples. The following fungi were isolated from the different clinical samples: *Penicillin spp., Aspergillus niger, Aspergillus flavus*, *Aspergillus fumigatus, Candida tropicalis, Candida parapilosis, and Microsporum auidouini.* Across all samples from the Yenagoa and Amassoma community, *Aspergillus niger* has the highest isolation percentage rate (58%). It is followed by *Aspergillus flavus* (11%), *Aspergillus fumigatus* (3%), *Candida tropicalis* (10%), *Candida parapilosis* (10%), *Microsporum auidouini* 7%, and *Penicillin spp*. 1%. (Fig. 3.).

Table.1 shows that Madiga bread obtained from factories has the highest isolation rate (39%), followed by samples taken from workers in factories (22%) and Madiga bread vendors (22%). Madiga bread obtained from markets has the lowest isolation rate (17%).

Chi-square analysis

> Degrees of freedom (df) = number of categories (N) – 1

Therefore, df = 4-1 = 3

Entering the table at df of 5, the critical value for the chi square value of 22.93 is 7.815 the obtained value, 22.93 is greater than the critical value, 7.815.

**Table 1. Percentage of fungi isolated from total samples**

| S/N | Organisms | Frequency | Frequency Percentage (%) |
|---|---|---|---|
| 1 | *Aspergillus niger* | 120 | 58 |
| 2 | *Aspergillus flavus* | 23 | 11 |
| 3 | *Aspergillus fumigatus* | 5 | 3 |
| 4 | *Candida tropicalis* | 21 | 10 |
| 5 | *Candida parapilosis* | 21 | 10 |
| 6 | *Microsporum auidouini* | 14 | 7 |
| 7 | *Penicillin spp* | 2 | 1 |
| | **Total** | **206** | **100** |

**Table 2. Percentage frequency of isolates from samples collected**

| Samples Collected | *A. niger* | *A. flavus* | *A. fumigatus* | *C. tropicalis* | *C. parapilosis* | *M. auidouini* | *Penicillin* | Total fungi isolates | % Frequency |
|---|---|---|---|---|---|---|---|---|---|
| Madiga Bread from Factory | 63 | 11 | 2 | - | - | 3 | 1 | 80 | 39 |
| Hand swabs of Factory Workers | 29 | 9 | 3 | - | - | 4 | 1 | 46 | 22 |
| Madiga Bread from Markerts | 13 | - | - | 15 | - | 6 | - | 34 | 17 |
| Hand Swabs from Madiga Vendors | 15 | 3 | - | 6 | 21 | 1 | - | 46 | 22 |
| **Total** | **120** | **23** | **5** | **21** | **21** | **14** | **2** | **206** | **100** |

**Table 3. Percentage antifungal susceptibility test**

| Isolates | Clotrimazole | Econozole | Caspofungin | Fluconazole | Nystatin | Ketoconazole |
|---|---|---|---|---|---|---|
| *A. niger* | 100 | 96.4 | 60.7 | 0 | 92.9 | 75 |
| *A. fumigatus* | 100 | 100 | 100 | 0 | 100 | 100 |
| *A. flavus* | 100 | 100 | 58 | 8.3 | 83.3 | 58.3 |
| *C. parapilosis* | 100 | 100 | 0 | 100 | 0 | 100 |
| *C. tropicalis* | 100 | 100 | 0 | 100 | 0 | 100 |
| *Penicillin spp* | 100 | 100 | 100 | 100 | 100 | 100 |
| *M. audiouini* | 100 | 9.1 | 9.1 | 0 | 9.1 | 9.1 |

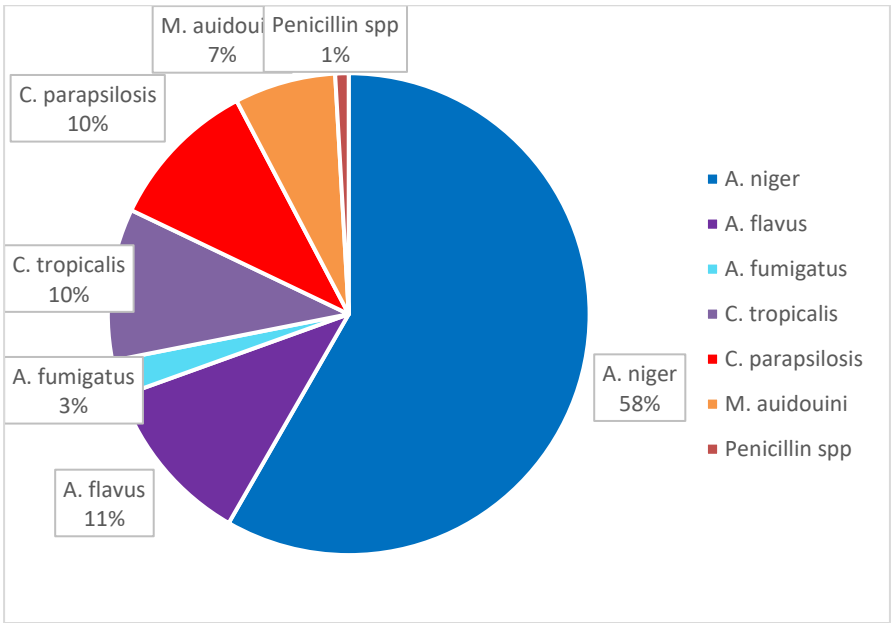


**Fig. 1. Pie chart of % frequency of the total Fungi species isolated from the examined samples**

**Table 4. Chi-square analysis table**

| Category | Observed | Expected | Obs-Exp | (Obs –Exp)$^2$ | (Obs – Exp)$^2$ / Exp |
|---|---|---|---|---|---|
| Factory Bread | 80 | 51.5 | 28.5 | 812.3 | 15.8 |
| Factory workers | 46 | 51.5 | -5.5 | 30.25 | 0.59 |
| Markets | 34 | 51.5 | -17.5 | 306.3 | 5.95 |
| Madiga vendor | 46 | 51.5 | -5.5 | 30.25 | 0.5 |

*£(O - E)2/E = 22.93*

## 4. DISCUSSION

As indicated from the research, the isolated fungi are *Aspergillus niger* 120 (58%), *Aspergillus flavus* 23 (11%), *Aspergillus fumigatus* 5 (3%), *Candida tropicalis* 21 (10%), *Candida parapilosis* 21 (10%), *Microsporum auidouini* 14 (7%), and *Penicillin spp.* 2 (1%); this is in agreement with the work done by Segun et al. [9] in Ibadan, Oyo State which reported 100%, 100%, 50%, 16.67%, 16.6%, 25%, 41.67% and 25% for *Aspergillus niger*, *Aspergillus flavus, Aspergillus fumigatus, Aspergillus tamarii, Fusarium spp., Penicillium notatum* and *Rhizopus nigricans* respectively.

Based on all samples taken from the Amassoma community and Yenagoa, *Aspergillus niger* has the highest percentage of occurrence at 120 (58%). Madiga bread obtained from factories has the highest percentage of fungus isolates (39%) among the samples that were collected. Odetunde et al. [10] reported that *Aspergillus* species were the most common isolates in their investigations. According to the frequency of occurrence (%) of the individual molds recorded in the bakery products, *Aspergillus niger* (100%) and *Aspergillus flavus* (100%) were the most frequent fungi contaminants isolated from the four brands of bakery products, which is based on the study done in 2016 by Segun et al. [9] in Ibadan, Oyo State.

A total of 80 fungi were isolated from Madiga bread that was obtained from factories located in Amassoma and Yenagoa, as illustrated in Fig. 2, the Madiga breads obtained from Factories in the Amassoma community, 53 fungi were isolated; 43 (81%) of these were *Aspergillus niger*; 7 (13%) were *Aspergillus flavus*; 2 (4%), *Aspergillus fumigatus*; and 1 (2%), *Microsporum auidouini.* However, no isolates of *Candida tropicalis*, *Candida parapilosis*, or *Penicillin spp.* were found in the samples. Three fungal species—*Aspergillus fumigatus*, *Candida tropicalis,* and *Candida parapilosis*—were not isolated from the Madiga bread samples obtained from Factories in Yenagoa. Of the 27 fungal species that were isolated, 20 (74%) were *Aspergillus niger*, 4 (15%) were *Aspergillus flavus*, 2 (7%), and 1 (4%), *Penicillin spp.*

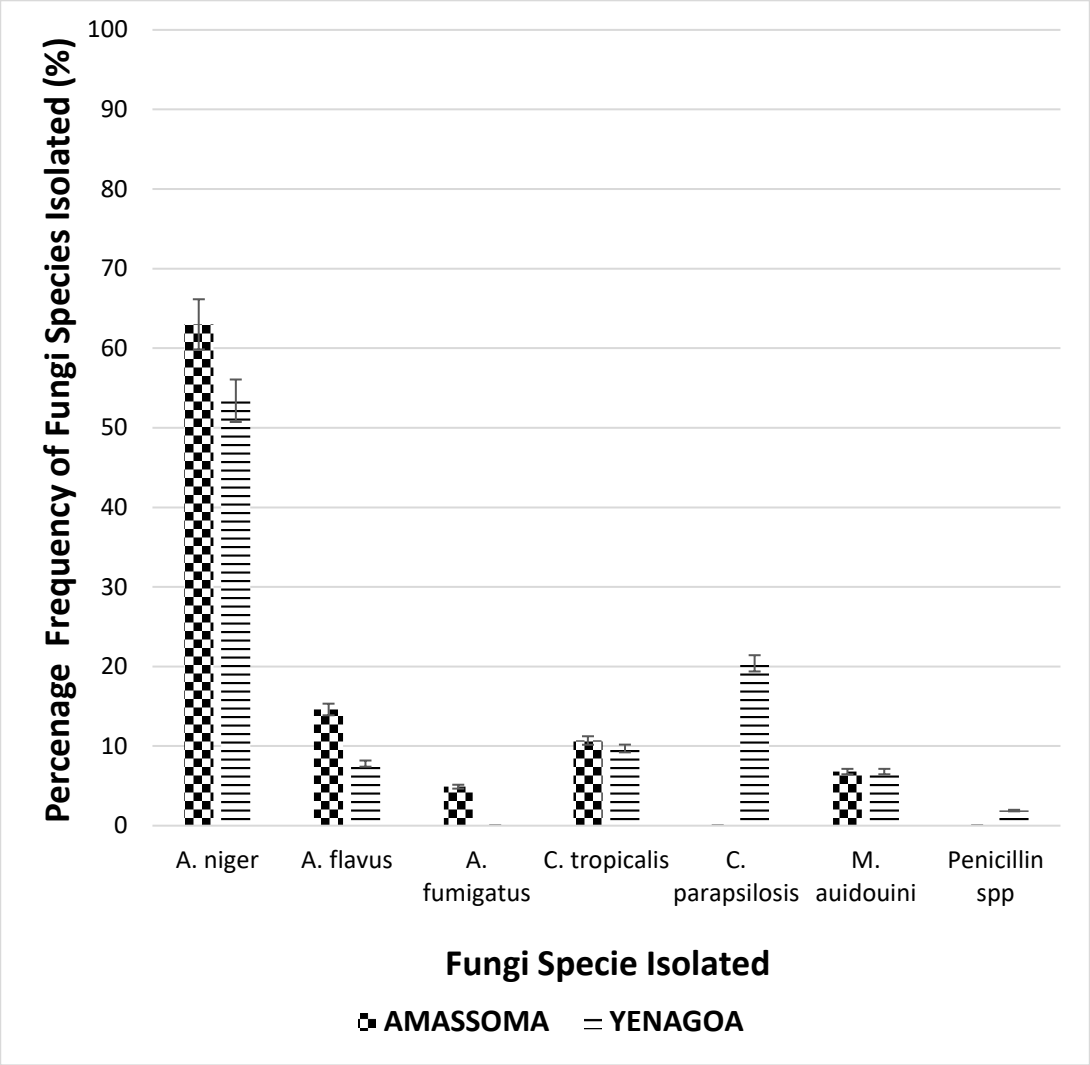


**Fig. 2. Bar chart showing total Fungi species isolated from Madiga breads gotten from Factories, markets and hand swabs of Factory workers and vendors in Amassoma and Yenagoa, Bayelsa State**

46 fungi were isolated from hand swabs taken from Madiga factory workers in Yenagoa and the Amassoma community as illustrated in Fig. 3. 13 (52%) *Aspergillus niger* isolates, 7 (28%) *Aspergillus flavus* isolates, 3 (12%) *Aspergillus fumigatus* isolates, and 2 (8%) *Microsporum auidouini* isolates were obtained from the hand swabs of Madiga factory workers in the Amassoma community. However, no isolates of *Candida tropicalis* or *Candida parapilosis* were found in the samples. From the hand swabs of Madiga factory workers in Yenagoa, 21 fungi were isolated; 16 (76%) of the isolates were *Aspergillus niger*, 2 (10%) were *Aspergillus flavus*, 2 (10%) were *Microsporum auidouini*, and 1 (4%), were *Penicillin spp*. However, the samples did not yield any isolates of *Candida tropicalis* or *Candida parapilosis*.

A total of 34 fungi were isolated from Madiga breads obtained from markets in Yenagoa and the Amassoma community. The Madiga breads obtained from the markets in the Amassoma community were found to contain 15 different types of fungi. Of these, 7 (47%) were *Aspergillus niger*, 5 (33%) were *Candida tropicalis*, and 3 (20%) were *Microsporum auidouinis*. However, the samples did not contain any *Aspergillus flavus, Aspergillus fumigatus*, *Candida parapilosis*, or *Penicillin spp*. From Madiga breads purchased from Yenagoa markets, 19 fungi were isolated; 6 (32%) of these were *Aspergillus niger*, 10 (53%) were *Candida tropicalis*, and 3 (15%) were *Microsporum auidouinis*. However, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida parapilosis*, and

*Penicillin spp.* were not isolated from the samples (Fig. 4).

From Fig. 5 which shows that, from the hand swabs of Madiga vendors, 46 fungi were isolated from the samples gotten from both Amassoma community and Yenagoa. The hand swabs of Madiga vendors in the Amassoma community yielded 10 total fungi isolates: 2 (20%) were *Aspergillus niger*; 1 (10%) was *Aspergillus flavus*; 6 (60%) were *Candida tropicalis*; and 1 (10%) *was Microsporum auidouinis*. However, the samples did not yield isolates of *Aspergillus fumigatus*, *Candida parapilosis*, or *Penicillin spp*. 13 (36%) of the 36 fungi that were isolated from the hand swabs of Madiga vendors in Yenagoa were *Aspergillus niger,* 2 (6%) were *Aspergillus flavus*, and 21 (58%) were *Candida parapilosis*. The samples did not contain any isolates of *Aspergillus fumigatus*, *Candida tropicalis,* or *Microsporum auidouinis*.

The raw materials used in the production of Madiga bread is flour which is applicable to other bread products, popl[pcorn, granola, cheese balls and biscuits; fungi contaminate these ready-to-eat foods either by their whole cell or by secreting their metabolites, “mycotoxins" especially aflatoxins as stated by dos Santos et al. [11] who worked on granola bread in Teresina, Brazil and reported 100% aflatoxins contaminants. Meanwhile, Moreira et al. [12] reported 0% aflatoxin in the work done on granola bread in northeastern Brazil. As a result, regular monitoring is necessary for both these commercial foods and other RTE foods or snacks that children in Nigeria frequently eat [13,14].

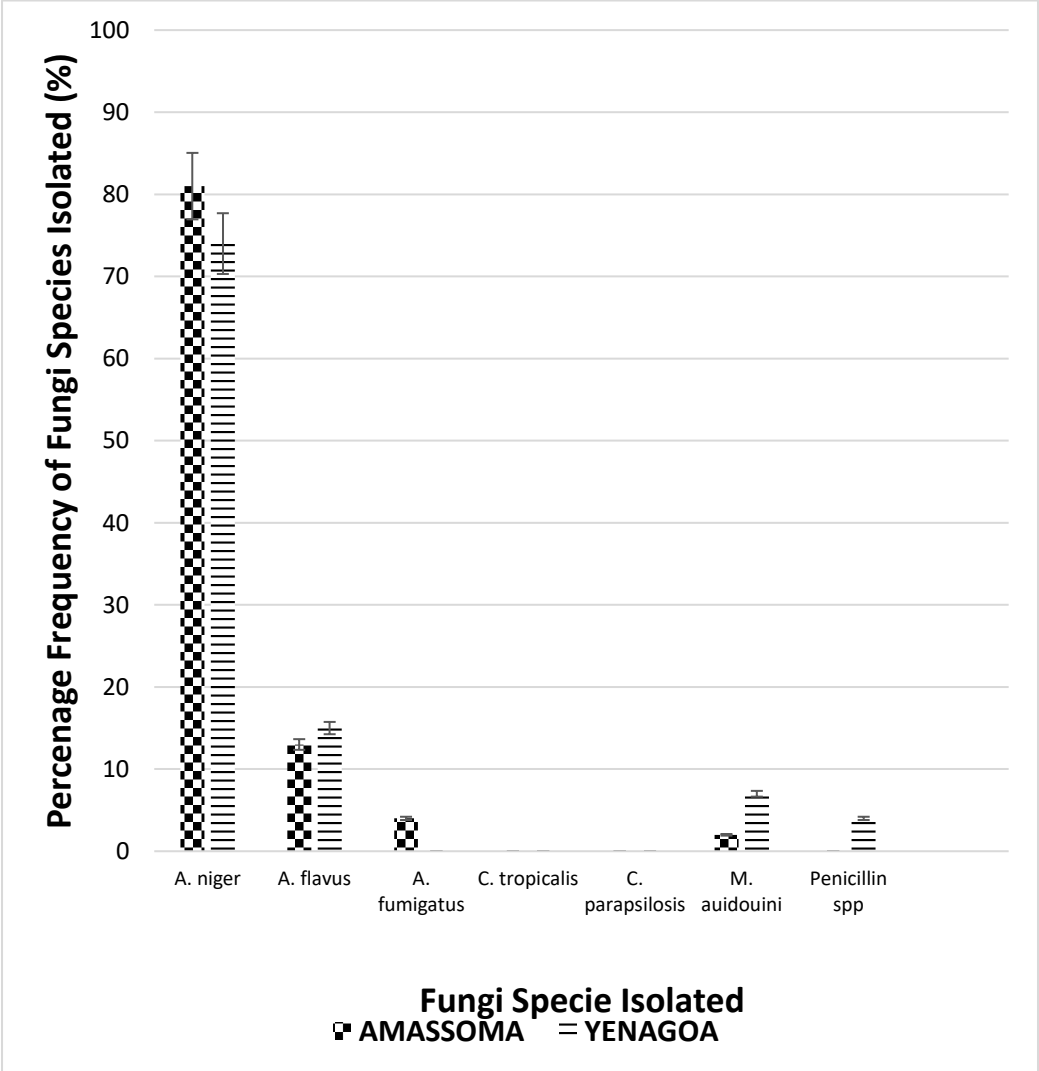


**Fig. 3. Bar chart showing total Fungi species isolated from Madiga breads gotten from Factories in Amassoma and Yenagoa, Bayelsa State**

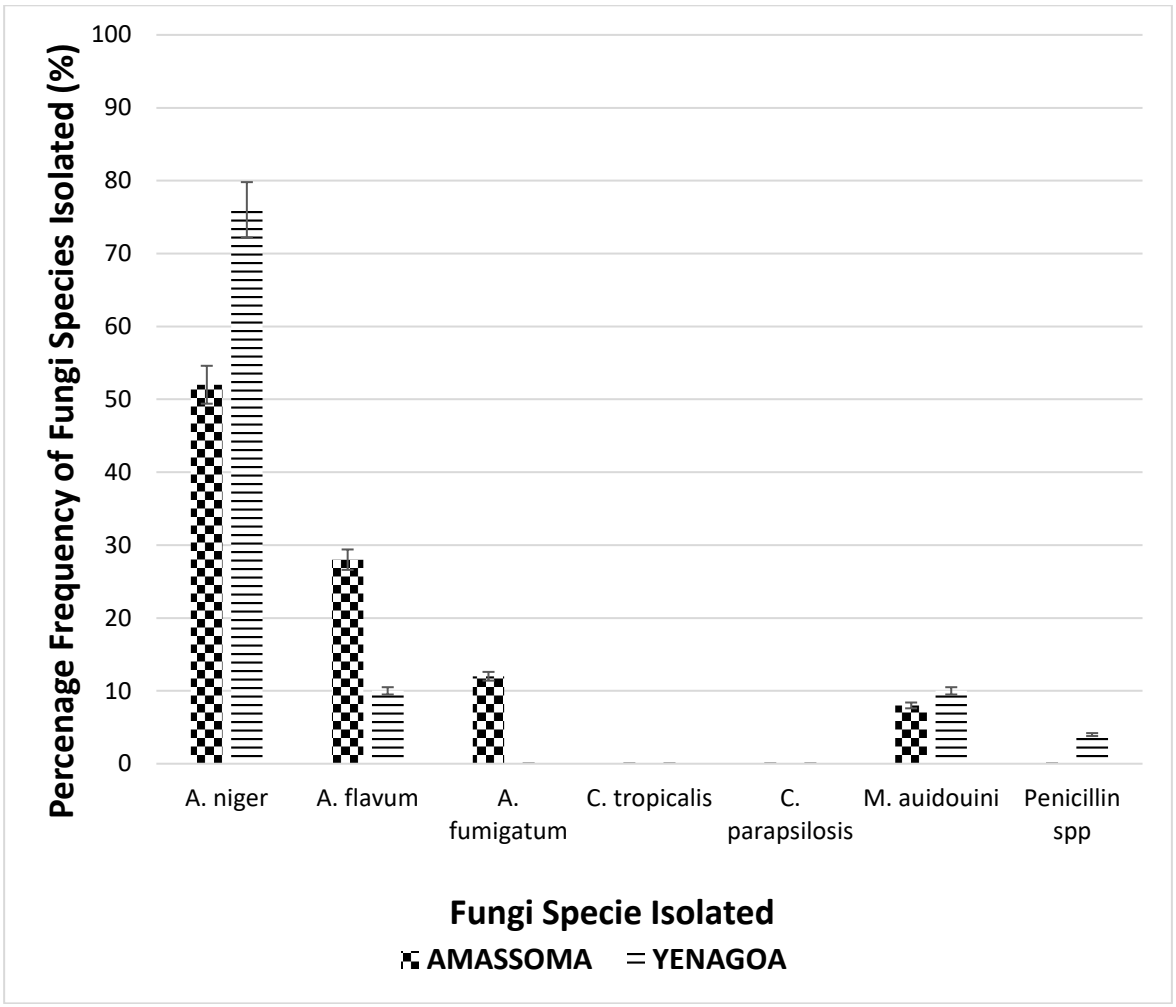


**Fig. 4. Bar chart showing total Fungi species isolated from hand swab of Madiga Factories workers in Amassoma and Yenagoa, Bayelsa State**

Moulds growth in the study, Madiga bread could have originated from the air, the bakery surface, tools, operational temperatures and humidity; this is in agreement with the work of Susanti et al. [15] in Indonesia on bread; the authors reported 33.3% and 66.6% fungi contaminants after 5th and 7th days respectively as a result of high temperature and humidity and stated that any of the flour products store at room temperature ($27^0$C to $30^0$C) couple steam exposure are bound to have fungi contaminants. These present findings are also in correlation with the research conducted in Brazil by Morassi et al. [16] on industrialized cake products and reported *Aspergillus flavus* (28.15%); *Penicillium citrinum* (18.45%); *Penicillium paxilli* (14.56%) and *Aspergillus niger* (6.8%) and the same fungi were also isolated from the air around the study industries, their raw materials and the environments; the authors stated that air and raw materials could be sources of fungi contaminants in food industries.

Segun et al. [9] also reported *Aspergillus niger* (100%) from bakery product in Ibadan, just like *Aspergillus niger* was reported in this study; the author stated that improper handling, packaging and inappropriate storage can aid fungi growth in any ready-to-eat flour products. likewise, over exposure of these products in open trays during hot climatic condition could lead to moisture settlement on the foods which in turn could serve as a good environment for fungi growth and proliferation.

The Fungi were isolated and treated with antifungal agents, as Table 2 demonstrates. Based on testing, all *Aspergillus* species were found to be susceptible to Clotrimazole (100%), Econozole (98.8%), Caspofungin (72.9%),

Nystatin (92.1%), Ketoconazole (77.8%), and Fluconazole (8.3%). Candida species demonstrated susceptibility to 100% for Clotrimazole, 100% for Econozole, 100% for Ketoconazole, and 100% for Fluconazole; however, resistance was observed to 0% for Caspofungin and 0% for Nystatin. The antifungal agents used, namely 100% Clotrimazole, 100% Econozole, 100% Caspofungin, 100% Nystatin, 100% Ketoconazole, and 100% Fluconazole, all showed susceptibility to Penicillin spp. 100%). *Microsporum auidouini* was susceptible to Clotrimazole (100%), Econozole (100%), Caspofungin (100%), Nystatin (100%), Ketoconazole (100%) but Fluconazole (0%) showed resistance.

Due to the unhygienic conditions associated with Madiga bread production, distribution, and consumption, this study was conducted.

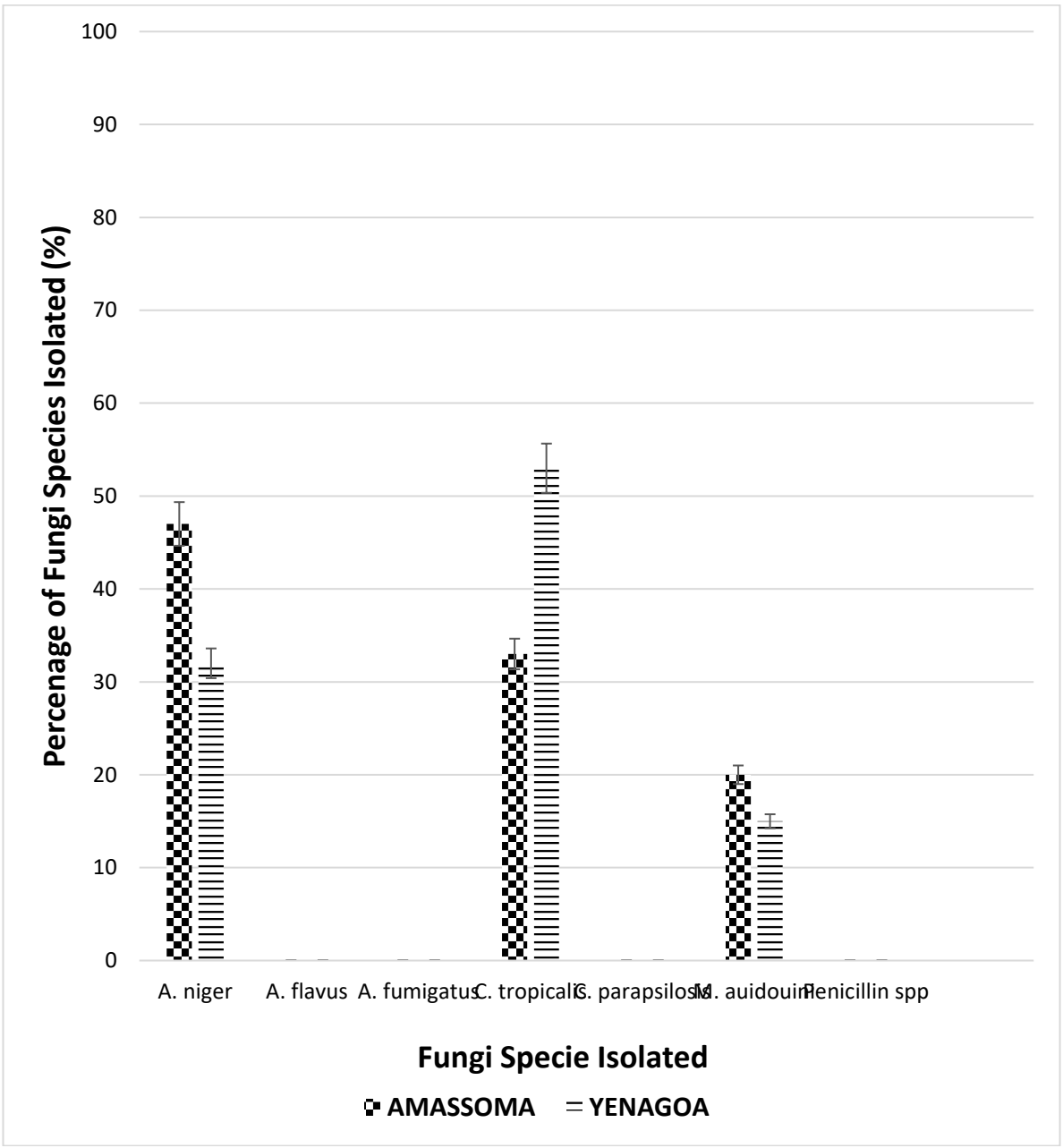


**Fig. 5. Bar chart showing total Fungi species isolated from Madiga breads gotten from Markets in Amassoma and Yenagoa, Bayelsa State**

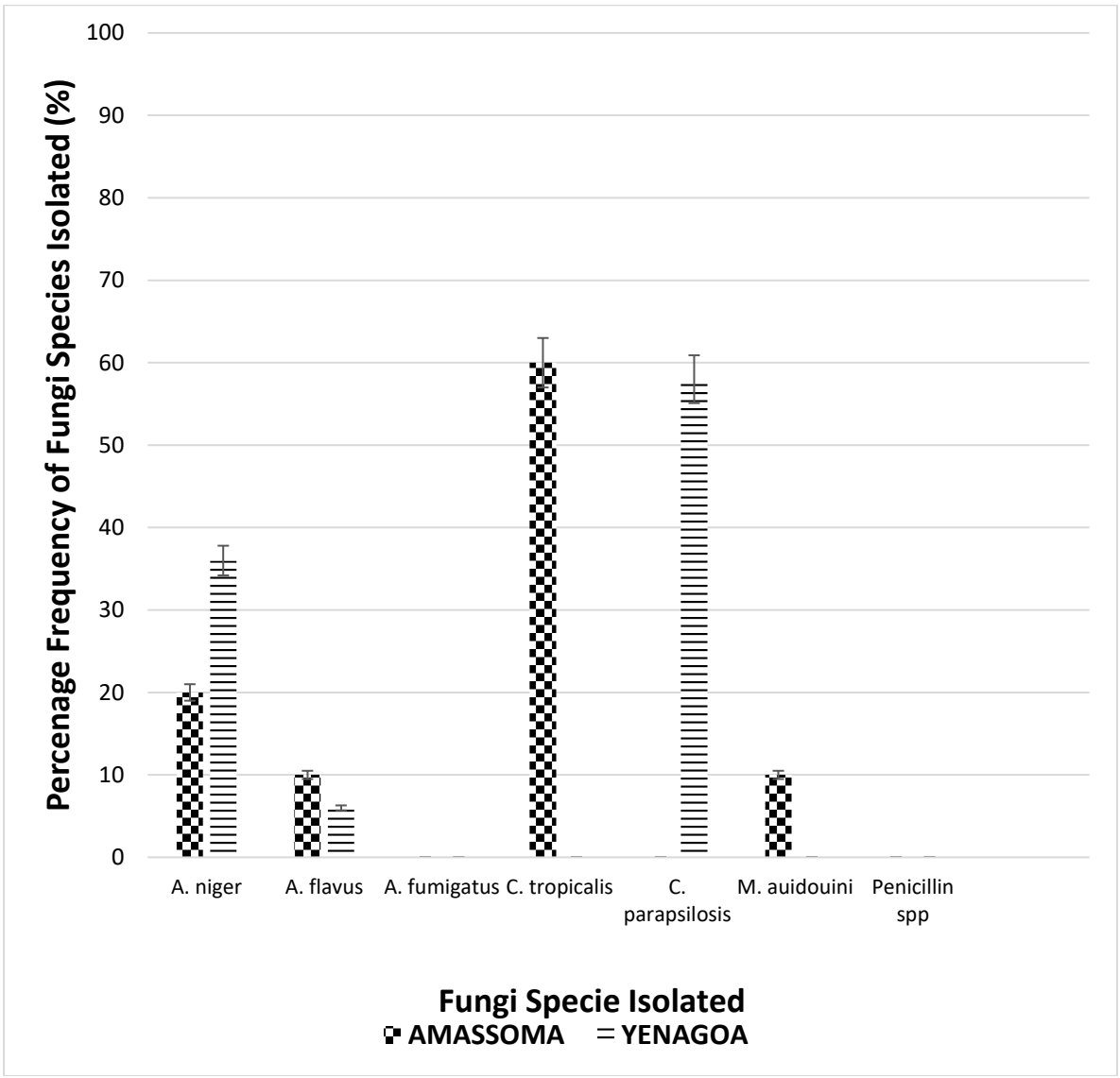


**Fig. 6. Bar chart showing total Fungi species isolated from hand swabs of Madiga Vendors in Amassoma and Yenagoa, Bayelsa State**

## 5. CONCLUSION

Although madiga is a popular ready-to-eat food in Bayelsa State, there are unhygienic practices in place from the point of production to the point of distribution to customers. It can be inferred that the inadequate hygiene practices led to a high number of fungi being isolated from the different samples collected.

With a 58% frequency of occurrence, *Aspergillus niger* was the most isolated fungus found in all the samples. Madiga breads obtained from factories had the highest percentage of fungus isolates (39%) among the samples that were collected.

## 6. RECOMMENDATION

1. It is necessary to take hygienic precautions to prevent the Madiga from becoming contaminated by fungi.
2. The Madiga bread needs to be packaged properly.
3. Government should enact policies for proper supervision of Madiga bread production.
4. Training of factory workers in aspect of necessary hygiene measures.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENT

Our sincere appreciation goes to God Almighty for HIS inflicting love and grace in making this work a success.

We appreciate the Management of Niger Delta University, for creating an enabling environment, for research works to be carried out.

## COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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