ABSTRACT

**Aims:** The study evaluated the proximate composition and the occurrence of antibiotic resistant *Listeria* species from fura-de-nunu sold in Port Harcourt metropolis.

**Study design:** The experiment was conducted in duplicate and mean values calculated.

**Place and Duration of Study:** Department of Microbiology, University of Port Harcourt, Nigeria, between October 2013 and May, 2014.

**Methodology:** A total of 101 samples were examined for the presence of *Listeria* using standard methods employing Fraser broth and polymixin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) agar. Characteristic colonies were identified on the basis of Gram staining, catalase reaction, oxidase reaction, beta haemolysis on sheep blood agar and carbohydrate fermentation using mannitol, rhamnose and xylose. Antibiotic susceptibility of the isolates was determined by the disk diffusion method on Mueller-Hinton Agar, according to CLSI guidelines using the breakpoints of *Staphylococcus* species resistance since no resistance criteria exist for *Listeria* susceptibility testing in the CLSI guidelines. The moisture, crude protein, crude fibre, crude fat, carbohydrate and total ash contents of the fura-de-nunu samples were analysed using the method described by Association of Official Analytical Chemists.

**Results:** The average proximate chemical analysis shows the following: moisture (84.92%), ash (0.48%), carbohydrate (7.70%), protein (3.32%), lipid (3.09%) and fibre (0.49%). Out of the 101 samples examined, *Listeria* spp. was present in 38 (37.60%) samples. The resulting isolates were confirmed to be *L. grayi* 29.63% (40 of 135 isolates), *L. seeligeri* 18.52% (25 of 135 isolates) and *L. welshimeri* 51.85% (70 of 135 isolates). The results of the antibiotic sensitivity testing showed varying degrees of resistance against the selected antibiotics with the highest resistance against erythromycin (73.53%), followed by cloxacillin (61.76%) and least was against chloramphenicol (16.91%).

**Conclusion:** The presence of antibiotic resistant *Listeria* spp. portends danger for consumers of this product hence efforts should be intensified in ensuring the safety of consumers.

**Keywords:** Antibiotic sensitivity, fura-de-nunu, *Listeria* spp., millet, yoghurt

1. INTRODUCTION

Fura-de-nunu, is a highly nutritious beverage which is a two in one product consisting of a cereal “fura” made from millet (*Pennisetum glaucum*) and “nunu” a fermented unpasteurized milk product similar to yoghurt [1, 2, 3]. The product is traditionally produced and consumed particularly by the Hausa and Fulanis of Northern Nigeria and parts of the Saharan West Africa where its production and consumption derives much food security and economic benefits to the rural people in the region [4, 5].

Depending on the consistency, the product is used as complete food, with milk serving as a source of protein while the milk provides energy, snack, an appetizer, a thirst quencher, a weaning food for infants or as having a therapeutic [1, 6]. Fura-de-nunu contains proteins, carbohydrates, fats, vitamins, minerals, enzymes, cholesterol and beneficial bacteria (probiotic) [5].

It, however, does not appeal to majority of the people because of the apparent unhygienic conditions in which it is prepared, and also its short shelf-life [3, 4, 7]

A number of authors have isolated potential pathogenic bacteria such as *Staphylococcus* sp., *Escherichia coli*, *Salmonella* sp., *Campylobacter* sp., *Pseudomonas* sp., *Listeria* sp., *Acinetobacter mallei*, *Alcaligenes faecalis*, *Bacillus cereus*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Yersinia* sp., from nunu and raw milk products [5, 8, 9, 10, 11, 12] indicating the possibility of these product serving as source of microbial food poisoning.
Currently, *Listeria monocytogenes* is considered one of the most important pathogens responsible for food-borne infection [13]. In dairy industry, *Listeria* can contaminate directly or indirectly the products and the environment through contaminated raw milk, resulting in huge losses both in terms of public health and economy [13].

The interest given to *L. monocytogenes* as emerging pathogens and paucity of information on the occurrence of *Listeria* in fura-de-nunu necessitated the desire to determine proximate composition and the presence of *Listeria* and antibiotic susceptibility of *Listeria* in fura-de-nunu sold in Port Harcourt, Nigeria.

### 2. MATERIALS AND METHODS

#### 2.1 Sample source

The 101 samples of ready-to-drink fura-de-nunu were purchased from retailers at the Slaughter market in Trans-Amadi area of Port Harcourt and transported to the Microbiology Laboratory of University of Port Harcourt for analysis. The *Listeria monocytogenes* PCM 2191 serovar 01/2 which served as positive control was obtained from the Polish Collection of Microorganisms, Poland.

#### 2.2 Isolation procedure

The techniques recommended by the United States Department of Agriculture (USDA) [14] and the Health Products and Food Methods of the Government of Canada [15] were employed using Fraser broth (Oxoid, England) and polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) agar (Oxoid, England). Ten milliliter of each ready-to-drink fura-de-nunu sample was transferred to a conical flask containing 90ml of sterile half-strength Fraser broth and shaken for proper mixing. The test portion was incubated at 30°C for 24h. From the pre-enrichment culture (half-strength Fraser broth), 0.1ml was transferred into 10ml of full-strength Fraser broth and was incubated at 35°C for 24-48h. From the culture obtained in Fraser broth showing evidence of darkening due to aesculin hydrolysis by *Listeria* spp., 0.1ml was transferred onto duplicate PALCAM plates. After spreading, plates were incubated at 37°C for 24-48h. The plates were examined for the presence of characteristic colonies presumed to be *Listeria* sp. 2mm greyish-green colonies with a black sunken centre and a black halo on a cherry-red background, following aesculin hydrolysis and mannitol fermentation. Five typical colonies were selected randomly from a pair of PALCAM plates for confirmation and identification.

#### 2.3 Biochemical test

Colonies suspected to be *Listeria* were transferred onto trypticase soy agar (Becton, Dickinson & Company, France) with 0.6% yeast extract (LAB M, UK) and incubated at 37°C for 18 to 24h, before being subjected to the following standard biochemical tests: Gram staining, catalase reaction, oxidase reaction, beta haemolysis on sheep blood agar and carbohydrate fermentation using mannitol, rhamnose and xylose as described by Janzten et al [16] and Jemmi and Stephan [17].

#### 2.4 Antibiotic sensitivity

Antibiotic susceptibility of the isolates was determined by the disk diffusion method involving: tetracycline (30 µg), amoxicillin (20 µg), augmentin (20µg), gentamycin (10 µg), erythromycin (15 µg), cloxacillin (5 µg), chloramphenicol (30 µg) and cotrimoxazole (20 µg) (Abtek biological,UK) on Mueller-Hinton Agar (MHA, Oxoid, Basingstoke, UK), according to CLSI guidelines using the breakpoints of *Staphylococcus* species resistance since no resistance criteria exist for *Listeria* susceptibility testing in the CLSI guidelines [18, 19, 20]. From an overnight culture in brain heart infusion broth, a 10^8 cell/ml (0.5 McFarland turbidity standards) bacterial culture was prepared in sterile saline, which 0.1ml was inoculated onto Mueller Hinton agar. Thereafter, antibiotic discs were aseptically placed on the surface of the agar and plates incubated at 37°C for 24h. Zone of inhibition was measured in millimeter.

#### 2.5 Proximate composition
The moisture, crude protein, crude fibre, crude fat, carbohydrate and total ash contents of the fura-de-nunu samples were analysed using the method described by Association of Official Analytical Chemists [21].

3. RESULTS AND DISCUSSION

3.1 Proximate analysis

Proximate analysis involves the determination of the major components of food as moisture, ash, crude fat, crude protein, crude fiber, and carbohydrate [22, 23]. The percentage proximate composition of randomly selected samples showed a range and average of the parameters evaluated as presented in Table 1. There are no available records of previous composition of these samples, but there exist reports for individual component. Pearl millet had proximate composition for ash, fibre, lipid, protein and carbohydrate contents ranging from 1.60 to 2.40%, 2.00 to 4.00%, 2.70 to 7.10%, 8.50 to 17.40% and 58.00 to 76.30% respectively [24, 25, 26, 27] while nunu had proximate composition for ash, lipid, protein, carbohydrate and moisture contents ranging from 0.23 to 0.79%, 2.18 to 3.68%, 2.62 to 6.45%, 1.52 to 2.90% and 86.03 to 93.45% respectively [10, 28]. The results of the study, except for carbohydrate in the pearl millet is comparable to proximate composition for the individual component and supplies basic nutrient needs for humans.

Table 1. Proximate composition of fura-de-nunu

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% Composition (range)</th>
<th>Average composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>79.42-89.91</td>
<td>84.92</td>
</tr>
<tr>
<td>Ash</td>
<td>0.42-0.54</td>
<td>0.48</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>4.88-11.13</td>
<td>7.70</td>
</tr>
<tr>
<td>Protein</td>
<td>2.36-3.91</td>
<td>3.32</td>
</tr>
<tr>
<td>Lipid</td>
<td>2.11-4.48</td>
<td>3.09</td>
</tr>
<tr>
<td>Fibre</td>
<td>0.25-0.64</td>
<td>0.49</td>
</tr>
</tbody>
</table>

3.2 Occurrence of Listeria species

Milk obtained from healthy animal’s udder is free from pathogenic bacteria, but some of the animals in field condition may be suffering from sub-clinical mastitis and are excreting the causative agents in milk [29]. Out of the 101 samples examined, Listeria spp. was present in 38 (37.60%) samples. The resulting isolates were confirmed to be L. grayi, 29.63% (40 of 135 isolates), L. seeligeri 18.52% (25 of 135 isolates) and L. welshimeri 51.85% (70 of 135 isolates) (Table 2). Listeria monocytogenes, the pathogenic strain for humans was not identified among the randomly picked colonies. There are no reports of the isolation of Listeria species from fura-de-nunu or millet seeds/flour in available articles. However, Listeria has been detected in nunu and raw milk, a component of fura-de-nunu [12, 13, 30]. A number of authors have reported the detection of Pseudomonas aeruginosa, Serratia mercesens, Bacillus pumilus, Staphylococcus aureus, Salmonella spp., Escherichia coli, Enterococcus sp, Streptococcus sp, Micrococcus sp, Corynebacterium sp, Lactobacillus sp, Enterobacter aerogenes, Lactobacillus sp, Leuconostoc sp Bacillus cereus and Bacillus alvies in fura-de-nunu examined [3, 31, 32, 33,34]. The inability of these authors detecting Listeria species may not be unconnected with the isolating media employed for the enumeration of bacteria in the samples examined.
Table 2. Physiological and biochemical characteristics of *Listeria* species

<table>
<thead>
<tr>
<th><em>Listeria</em></th>
<th>Gram reaction/shape</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Haemolysis</th>
<th>Xylose</th>
<th>Rhamnose</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>+ve/rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PCM 2191 serovar 01/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>+ve/rod</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>L. welshimeri</em></td>
<td>+ve/rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>V</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>L. seelegeri</em></td>
<td>+ve/rod</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+=positive; -=negative; v= variable

3.3 Antibiotic susceptibility testing

The availability of antibiotics to treat infectious diseases has radically improved human and animal well-being, and also to a minor degree in plant health. They are often used as antimicrobial agents in treatment of animal diseases and as growth promoters in the rearing animals for food and this use among others leads to the creation of resistant strains of bacteria [34, 35]. The results of the antibiotic sensitivity testing showed varying degrees of resistance against the selected antibiotics (Table 3). Varying resistance was observed against the antibiotics employed for this study with the highest resistance against erythromycin (73.53%), followed by cloxacillin (61.76%) and least was against chloramphenicol (16.91%). A number of authors have reported varying levels of resistance of *Listeria* spp. and *L. monocytogenes* to tetracycline, oxacillin, gentamycin, chloramphenicol, augmentin, cotrimoxazole and erythromycin [36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46] comparable to the result of this study. The findings of this study were however, not in agreement with reports that 100% *Listeria* isolates were sensitive to gentamicin [41, 47, 48, 49, 50].
### Table 3. Distribution of antibiotic resistant *Listeria* isolates against eight antibiotics according to species.

<table>
<thead>
<tr>
<th><em>Listeria</em> species</th>
<th>No. tested</th>
<th>AUG 30µg/disc</th>
<th>AMX 25µg/disc</th>
<th>ERY 5µg/disc</th>
<th>TET 10µg/disc</th>
<th>CXC 5µg/disc</th>
<th>GEN 10µg/disc</th>
<th>COT 25µg/disc</th>
<th>CHL 30µg/disc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>2191</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PCM 2191 serovar 01/2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>L. seeligeri</em></td>
<td>25</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>40</td>
<td>22</td>
<td>11</td>
<td>29</td>
<td>29</td>
<td>34</td>
<td>23</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td><em>L. welshimeri</em></td>
<td>70</td>
<td>29</td>
<td>33</td>
<td>70</td>
<td>12</td>
<td>37</td>
<td>12</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>136</td>
<td>64</td>
<td>57</td>
<td>100</td>
<td>55</td>
<td>84</td>
<td>48</td>
<td>70</td>
<td>23</td>
</tr>
</tbody>
</table>

% resistance

|             | 47.06 | 41.91 | 73.53 | 40.44 | 61.76 | 35.29 | 51.47 | 16.91 |

AUG, augumentin; AMX, amoxacylin; ERY, erythromycin; TET, tetracycline; CXC, cloxacillin; GEN, gentamycin; COT, cotrimoxazole; CHL, chloramphenicol.

### 4. CONCLUSION

This present study has shown the nutritive nature of this beverage and the occurrence of *Listeria* species exhibiting multi resistance to antibiotics in fura-de-nunu examined. This is a pointer to the fact that raw milk, even in the form of fura-de-nunu, represents a considerable risk for consumers, who may experience severe food-born diseases, including listeriosis that can be transmitted through unpasteurized milk and milk products. There is a very high chance of passing the antibiotics resistant bacteria into the human ecosystem. This portends a great danger to human health hence, the need to ensure hygienic milking methods, adequate cleaning of milking equipment and proper storage of products since they do not undergo further processing before consumption.

### REFERENCES

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