Journal of Advances in Biology & Biotechnology
11(2): 1-7, 2017; Article no.JABB.30655
ISSN: 2394-1081

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Proximate Composition and Antibiotic Resistant *Listeria* Species in Fura-de-nunu Sold in Port Harcourt

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Authors' contributions

This work was carried out in collaboration between both authors. Author OCE handled the proximate composition and wrote the draft. Author COE handled the isolation, characterization and antibiotic sensitivity. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2017/30655
Editor(s):
(1) Fernando José Cebola Lidon, Universidade Nova de Lisboa, Campus da Caparica, Portugal.
(2) James W. Lee, Department of Chemistry and Biochemistry, Old Dominion University, USA.
Reviewers:
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(3) Niramol Punbusayakul, Burapha University, Thailand.
Complete Peer review History: http://www.sciencedomain.org/review-history/17366

Original Research Article

Received 22nd November 2016
Accepted 9th December 2016
Published 28th December 2016

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 acriflavin 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,

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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 millet 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 *Listreria* 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 polymixin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar (Oxoid, England). Ten milliliter of each ready-to-drink fura-de-nunu sample were transferred to a conical flask containing 90 mL of sterile half-

strength Fraser broth and shaken for proper mixing. The test portion was incubated at 30℃ for 24 h. From the pre-enrichment culture (half-strength Fraser broth), 0.1 mL was transferred into 10 mL of full-strength Fraser broth and was incubated at 35℃ for 24-48 h. From the culture obtained in Fraser broth showing evidence of darkening due to aesculin hydrolysis by *Listeria* spp., 0.1 mL was transferred onto duplicate PALCAM plates. After spreading, plates were incubated at 37℃ for 24- 48 h. The plates were examined for the presence of characteristic colonies presumed to be *Listeria* sp. – 2 mm 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℃ for 18 to 24 h, 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 [16,17].

2.4 Antibiotic Sensitivity

Antibiotic susceptibility of the isolates was determined by the disk diffusion method involving: tetracycline (30 µg), amoxicillin (20 µg), augmentin (20 µg), 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 (LAB M, UK), a 10^8 cell/mL (0.5 McFarland turbidity standards) bacterial culture was prepared in sterile saline, from which 0.1 mL was inoculated onto Mueller Hinton agar. Thereafter, antibiotic discs were aseptically placed on the surface of the agar and plates incubated at 37℃ for 24 h. 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

Parameter	% composition (range)	Average composition (%)
Moisture content	79.42-89.91	84.92
Ash	0.42-0.54	0.48
Carbohydrate	4.88-11.13	7.70
Protein	2.36-3.91	3.32
Lipid	2.11-4.48	3.09
Fibre	0.25-0.64	0.49

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 pumillus, 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.

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 [34]. 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

Table 2. Physiological and biochemical characteristics of *Listeria* species

Listeria	Gram reaction/shape	Catalase	Oxidase	Haemolysis	Xylose	Rhamnose	Mannitol
Listeria monocytogenes PCM 2191 serovar 01/2	+ve/rod	+	-	+	-	+	-
L. grayi	+ve/rod	+	-	-	-	-	+
L. welshimeri	+ve/rod	+	-	-	+	V	-
L. seelegeri	+ve/rod	+	-	+	+	-	-

+=positive; -=negative; v= variable

Table 3. Distribution of antibiotic resistant *Listeria* isolates against eight antibiotics according to species

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

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, augumentin, 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].

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-borne 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 of healthy animals, adequate cleaning of milking equipment and proper storage of products since they do not undergo further processing before consumption.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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