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Detection and Molecular Characterization of *Listeria species* in 'Wara', A West African Local Cheese Sold in Ekiti State

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A B S T R A C T

Bacterial analysis of 116 soft cheeses 'wara', a ready-to-eat (RTE) product of fresh milk, bought in major markets of Ekiti State was done. Molecular analysis of isolates was done by coupling PCR to the DNA sequencing of *Listeria* 16S rRNA gene. Results yielded 459 bacteria with 78% positive to *Listeria*. Six different species of *Listeria* were identified with *L. monocytogenes* occurring at 12.4% of total *Listeria*. Ampiclox (ampicillin/cloxacillin) and amoxicillin showed very weak potency against all isolates at 10% and 11% respectively; while ciprofloxacin and septrin (co-trimoxazole) showed high potency at 84.95 and 82.6% respectively. The status of the RTE soft cheese 'wara' as sold in these is non-hygienic, hence microbiologically unsafe for consumption.

Introduction

The average food consumer seeks to improve his or her everyday diet based on personal or health reasons, thus, a desire for a healthy, low in fat, calories and sodium but high in fibre and of great taste are ever on the request in the market places. Ready-to-eat (RTE) foods come in handy with the fast and ever moving nature of man's life, thus, consumers demand for food products that have a more natural content than chemical content. Most of these natural food products require good storage system and a high level of hygiene to prevent spoilage and contamination.

The most common storage system for these foods is by refrigeration, but with the availability of microorganisms capable of surviving the refrigeration system, knowledge of the occurrence and distribution of these microbes in RTE foods and in the environment is needful.

Wara, a local unripened cheese consumed in several parts of West Africa, is prepared by coagulating fresh cow milk with the leaf extract of the Sodom apple (*Calotropis procera)* or pawpaw (*Carica papaya)*. An alternative coagulant, "lemon juice" was

introduced into the processing of 'wara' soft cheese by Adetunji *et al.* (2007) and was found to reduce the microbial load. Several microorganisms have been incriminated to be present in raw milk and milk products (cheese and yoghurt) that are of public health importance. These microorganisms are capable of causing food borne illnesses after ingestion and these organisms range from viruses, rickettsia, bacteria, protozoan and parasitic organisms to their toxins (Kaplan and Bertagna, 1955). Among these bacteria, *Escherichia coli* and *L. monocytogenes* are important indicators of contamination of milk and milk products, thus resulting in coliform infections; which presents as gastroenteritis and listeriosis respectively.

Listeria monocytogenes, the source of the human disease listeriosis, is a ubiquitous food-borne pathogen, identified decades ago to cause food poisoning outbreaks with high mortality rate. These bacteria are short, Gram positive rods, facultative anaerobes, motile by one to five peritrichous flagella, with both psychotropic and mesophilic features (Gray and Killinger, 1966; Seeliger and Jones, 1986). Today, *L. monocytogenes* is considered to be one of the most important agents of food-borne disease. Possible explanations for the emergence of human food-borne listeriosis as a major public health concern include major changes in food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in the eating habits of people, particularly towards convenience and RTE foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) (OIE, 2005). Between the years 2011 and 2014, different outbreaks due *L. monocytogenes* was reported by the Centre for Disease Control and Prevention (CDC) involving multistates in the United States, which recorded mortality rates at 22.5%, 18.2%, 33.3%, and 20% respectively. Outbreak of listeriosis has been linked to the consumption of cheese in many parts of the world (Gellin and Broome, 1989; Goulet *et al*., 2001; Makino *et al*., 2005; Wehr, 1989). In Nigeria, several reports on the occurrence of *L. monocytogenes* have been made in food products such as meat (beef, mutton, pork, poultry), farm produce such as cabbage and lettuce, RTE foods such as vegetable salads, cereal-based beverage called 'kunnu', soil samples from abattoir, smoked fish from market places, critical control points of local cheese and yoghurt production, and faecal samples of some ruminants (Salihu *et al.* 2008; Nwachukwu *et al.*, 2009; Umeh and Okpokwasili, 2009; Ikeh *et al.*, 2010; Adetunji and Arigbede, 2011; Ieren *et al.*, 2013; Eruteyah *et al*., 2014). The contamination of raw and pasteurized milk by these microorganisms suggests the possibility of their transmission to humans through consumption of milk products. Human infection is more devastating in immunosuppressed individuals; pregnant women, infants, geriatrics, HIV-AIDS patients, etc. Pregnant women may experience abortion, stillbirth, premature birth or septicemia in the newborn. The elderly and infants suffer from meningitis, meningoencephalitis or, less frequently, septicaemia (Acha and Szyfres, 2003).

Today, life stock farming (especially ruminants), is a promising venture which can provide income, employment, food, farm energy, manure, fuel and transport to the average Nigerian, and it is gaining more support by government policies. These animals may shed these bacteria in milk over long periods without showing any symptoms of the disease (Low and Donachie, 1997), and spoiled silage has been identified as the principal source of

infection in these animals (Vazquez-Bolland *et al.,* 1990; Low and Donachie, 1997; Weidmann, 2003). Most bacterial pathogens associated with human food poisoning often originate from farm, vegetation, soil, or animal products.

This study therefore investigated the occurrence of *L. monocytogenes* and other species and their antimicrobial profiles in RTE locally marketed 'wara' in five known towns of Ekiti State, South-Western Nigeria.

Materials and Methods

Sample Collection and Laboratory Analysis

A total of 116 soft cheese products were bought from Fulani women marketers in the RTE state from five different towns of Ekiti State; Ifaki (23), Otun (25), Ado (30), Ikere (22) and Ijan (16). These samples were stored on ice and transported to the laboratory for further analysis.

The FDA bacteriological and analytical method (BAM) (Hitchins, 2001) was employed; Analytical portions (25g) was enriched for *Listeria* species in 225ml of selective enrichment broth, University of Vermont Medium Modified *Listeria* Enrichment Broth (Alpha Biosciences, USA), with selective agent (Oxoid, UK) after 4 hours of incubation, and then subsequent incubation at 35°C for 44 hours. The enrichment culture was streaked at 24 and 48 hours on Brilliance *Listeria* agar with differential and selective supplements (Oxoid, UK). Blue-green colonies on media that expressed Gram positive coccobacilli on Gram staining were presumed to be *Listeria* species.

Biochemical Differentiation

This was done in reference to Collins *et al.,* (1991). More presumptive identification was done using the Oxoid Biochemical Identification System, OBIS mono kit (ID0600) (Oxoid, UK) in which isolates showing no colouration (purple colour) in reaction with the reagents is presumed *L. monocytogenes*.

Molecular Studies

This study was done by coupling PCR to the DNA sequencing analysis of *Listeria* 16S rRNA genes (Wang *et al.,* 1992; Manzano *et al.,* 2000). Genomic DNA was extracted using QIAamp DNA mini kit (250) cat no 51306. Polymerase Chain Reaction (PCR) was performed using 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1525R (5'- AAGGAGGTGWTCC ARCCGCA -3') universal primers and PCR protocols were performed as described by Bubert *et al.* (1992). PCR procedures were as follows: 36cycles, each at 94^{o}C for 30 seconds, 56^{o}C for 30 seconds, and 72^{o}C for 7 minutes.

The end products were separated on a 1.5% agarose gel which was stained with ethidium bromide. PCR products were visualized under UV light. The amplicons were subjected to sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Kit. The products were loaded unto 3130xl Genetic Analyzer (Applied Biosystems), and molecular sequences were identified by a combination of BLAST and FASTA (Donkor *et al.,* 2014).

Phenotypic Expression of Pathogenic Traits

The production of lecithinase was demonstrated on the medium (Brilliance *Listeria* agar with the differential supplements). Production of an opaque white halo around the colony confirms the presence of the enzyme. The production of listeriolysin was demonstrated on 5% sheep blood agar.

Antibiotic Susceptibility Testing

Using the Kirby-Bauer disc diffusion method as recommended by the Clinical Laboratory standards Institute (CLSI, 2013). The prevailing common antibiotics were employed; Pefloxacin (10g), Gentamycin (10g), Ampiclox (Ampicillin/cloxacillin, 30g), Zinnacef (cefuroxime, 20g), Amoxacillin (30g), Rocephin (ceftriaxone, 25g), Ciprofloxacin (10g), Streptomycin (30g), Septrin (cotrimoxazole, 30g), and Erythromycin (10g).

Results and Discussion

A total of four hundred and fifty nine (459) isolates were gotten from 116 wara samples sold in the markets in the RTE state. The prevalence of positive samples to *Listeria* was 78%. *Listeria* species identified are *L. innocua* (19.4%), *L. rocourtiae* (12.2%), *L. monocytogenes* (12.4%), *L. grayi* (8.5%), *L. fleischmannii* (17.7%), and *Listeria* species (7.8%) (Figure 1). The distribution of the isolates per towns of samples collection is detailed in Table 1. *Listeria ivanovii* was not isolated in this study. Of the *Listeria* species isolated, *Listeria innocua* had the highest occurrence at 19.4%, followed by *L. fleischmannii, L. monocytogenes, L. rocourtiae, L. grayi* and *Listeria* species at 17.7%, 12.4%, 12.2%, 8.5% and 7.8% respectively. In all bacterial isolates however, *Brochothrix* species occurred the most at 22.0%. Samples from Ifaki town market recorded the highest occurrence of *Listeria* at 91 (25.4%), while the least was recorded in samples from Ijan town market at 34 (9.5%). The known pathogenic isolate, *L. monocytogenes*, was observed in samples from other towns except in Ijan town market and was observed to be highest in Otun town market at 19 (22.9%).

The expression of lecithinase production was observed in all isolates of *L. monocytogenes*, while 56 (98.2%) expressed a thin line of haemolysis on blood agar. Other isolates were negative to both tests except *L. innocua*, showing a wide line of haemolysis on blood agar (Table 2).

Antibiotic susceptibility test results reveals a 100% resistance in all isolates against ampiclox (ampicillin/cloxacillin) and amoxicillin, 90% and 89% resistance in all isolates against zinnacef (cefuroxime) and rocephin (ceftriaxone) respectively. In all isolates, ciprofloxacin showed the highest potency at 84.9%, closely followed by septrin (co-trimoxazole) at 82.6% (Figure 2).

Table.1 Distribution of Isolates per Town

Towns (n)	*Brochothrix* sp.	*L. innocua*	*L. rocourtiae*	*L. monocytogenes*	*L. grayi*	*L. fleischmanni*	Uncultured *Listeria* spp
Ado (30)	30	22	13	16	0	18	07
Ijan (16)	12	07	07	0	0	09	11
Otun (25)	24	21	16	19	11	16	0
Ikere (22)	12	21	07	11	08	22	05
Ifaki (23)	23	18	13	11	20	16	13
Total (116)	**101**	**89**	**56**	**57**	**39**	**81**	**36**

Table.2 Pathogenic Trait Expression

Isolates	Lecithinase	Haemolysis
Brochothrix sp.	-	-
L. innocua	-	+++
L. rocourtiae	-	-
L. monocytogenes	++	+
L. grayi	-	-
L. fleischmannii	-	-
Uncultured *Listeria* spp	-	-

Figure.1 Overall Occurrence of Isolates

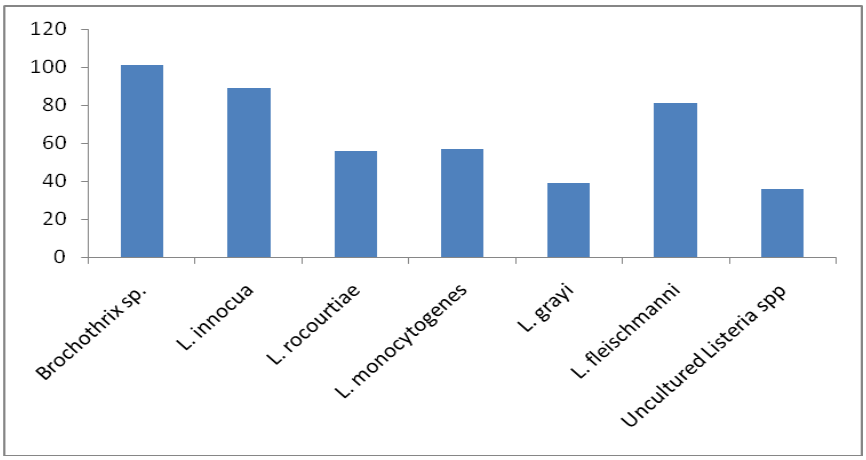
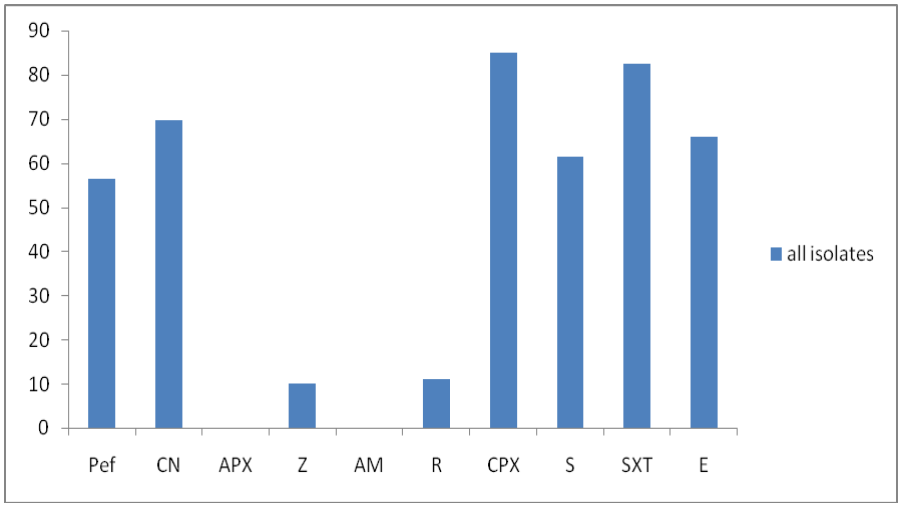


Figure.2 Overall Percentage Susceptibility to Antibiotics in all Isolates



Key: Pef – pefloxacin, CN – gentamycin, APX – ampiclox, Z – zinnacef, AMX – amoxicillin, R – rocephin, CPX – ciprofloxacin, S – streptomycin, SXT – septrin, E – erythromycin

From the study, the local cheese in its RTE state, sold in the markets sampled is contaminated with indicator organisms, which makes it unhealthy for food, especially by those classified to be at high risk of infection. It has been reported by different works that *L. monocytogenes* has the ability to survive the manufacture and storage conditions of several cheeses (Adetunji and Arigbede, 2011; Anonymous, 2006; Manfreda *et al*., 2005; Carminati *et al*., 2004; Erkmen, 2001; Buazzi *et al*., 1992; Yousef and Marth, 1990). Emphasis have been made that, the contamination of cheese or end-products with *L. monocytogenes* is most likely due to; contamination during the ripening stage (Pak *et al*., 2002), post process contamination from environmental sources, cross-contamination in the dairy plant and/or retail stores, inadequate processing (FAO/WHO, 2004; Pak *et al*., 2002; Rudolf and Seigfried, 2001; Sagun *et al*., 2001) and colonization in retail stores (Sergelus *et al*., 1997). The high occurrence of these organisms in wara depicts the hygiene level of either the source (milk), process of production, storage facility or even the marketers. Adetunji and Arigbede (2011), reported the occurrence of *Listeria* through the processing line and in the finished product at significant levels ($p<0.05$) above international standards. Identified *Listeria* species were 71% of all isolates in this study, which corroborates reports from other countries about foods of animal origin (Ikeh *et al.*, 2010; Choi *et al*., 2001; Miettinen *et al*., 2001; Hassan *et al*., 2001).

The antibiotic susceptibility test of the isolates showed that *Listeria monocytogenes* is susceptible to ciprofloxacin, septrin (cotrimoxazole), gentamycin, erythromycin, streptomycin and pefloxacin but resistant to ampicillin/cloxacillin, amoxicillin, ceftriaxone and cefuroxime. This finding is similar to reports of Yakubu *et al.,* (2012) in Sokoto State and Nwachukwu *et al.,* (2009) in Abia State. However, conflicting reports to that of this study was reported by Umeh and Okpokwasili (2009), showing *Listeria monocytogenes* to be sensitive to erythromycin, ampicillin, cotrimoxazole and amoxicillin. The resistance to the third generation cephalosporin may be due to its use in the selective isolation of species in this genus. Resistance could also be attributed to the irrational use of the antibiotics in veterinary medicine in the study area.

In conclusion, this study established the occurrence of *L. monocytogenes* and other species in wara in the RTE state from markets in Ekiti State. This brings to light the poor handling and unhygienic level of this product for consumption, especially by persons classified as high risk.

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