Molecular characterization of *Listeria monocytogenes* isolated from a ready-to-eat fermented milk and cereal product, *Fura-de-Nunu*

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This study was conducted to determine the occurrence of *Listeria (L.) monocytogenes* in *Fura-de-Nunu*, a ready-to-eat (RTE) fermented milk (*Nunu*) and cereal (*Fura*) blend, the serogroups as well as the virulence of the isolates. A total of 75 *Fura* and 75 *Nunu* samples were examined. *Listeria* species were isolated on PALCAM medium and *Listeria* chromogenic agar, and identified phenotypically according to International Standardization Organization (ISO) procedures. Identification of *L. monocytogenes*, serogrouping and detection of virulence genes were carried out by polymerase chain reaction (PCR). *Listeria* spp. were recovered from 23 (30.67%) and 41 (54.67%) samples of *Fura* and *Nunu*, respectively. The bioloads of *Listeria* ranged from $10^3$ to $10^5$ CFU/ml. Six presumptive species of *Listeria* were identified from the samples, with *L. monocytogenes* accounting for 21.00 and 20.64% of isolates from *Fura* and *Nunu*, respectively. Out of the three major serogroups (1/2a, 1/2b and 4b) associated with human disease, only 1/2a and 4b were identified among the isolates. Some of the isolates tested positive for the presence of virulence genes, *hlyA* and *iap*. Results from this study show that *Fura-de-Nunu*, may represent a risk for transmission of listeriosis to consumers.

Key words: *Listeria monocytogenes*, Fura-de-Nunu, fermented milk, ready-to-eat, Listeriosis.

INTRODUCTION

The genus *Listeria* currently comprises seventeen species of Gram-positive, non-spore forming, and catalase-positive bacteria: *Listeria (L.) aquatica*, *L. booriae*, *L. cornellensis*, *L. fleischmannii*, *L. floridensis*, *L. grandensis*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. newyorkensis*, *L. riparia*, *L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis*, and *L. welshimeri* (Weller et al., 2015). The haemolytic species (*L. monocytogenes*, *L. ivanovii* and *L. seeligeri*) are associated with human disease, although *L. ivanovii* and *L. seeligeri* are more commonly involved in diseases of animals and are rare causes of human disease (Guillet et al., 2010; Jamali et al., 2013; Abdellrazeq et al., 2014).

*L. monocytogenes* has emerged as a very important food-borne pathogen, causing the human disease, listeriosis (Marnissi et al., 2013; Mansouri-Najand et al.,

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2015). It has been detected in various foods, including raw milk and other dairy products, such as cheese and ice cream (Jamali et al., 2013). Infectious doses are reported to range from $10^1$ to $10^5$ CFU/g of food (Majajla et al., 2001; Ooi and Lorber, 2005; Yang et al., 2007; Marnissi et al., 2013; Jamali et al., 2013; Mansouri-Najand et al., 2015; Oyiinloye, 2016). However, according to the European food safety regulations, generally, food samples containing more than 100 CFU/g of L. monocytogenes are considered unsatisfactory. In foods intended for infants or foods where the shelf life is determined without consideration of the organism, there should be complete absence of L. monocytogenes /25 g of the food before the food leaves the immediate control of the producer (Food Standards Agency, 2006; Public Health England, 2014). Listeriosis is a disease of serious public health concern because of the high mortality rate associated with the disease; especially in pregnant women and infants, the elderly and immunocompromised individuals, who are at the greatest risk for the disease (Gillespie et al., 2010; Barton et al., 2011; Abdellrazaq et al., 2014; Oyiinloye, 2016). A survey in 2010, by the Europe PMC Funders Group, estimated that listeriosis resulted in 23,150 illnesses, 5,463 deaths and 172,823 disability adjusted life years (DALYs) globally. The proportion of perinatal cases was 20·7%. This led to a call by the World Health Organization (WHO) for more knowledge of critical control points of food production. 2014). Most of these women are illiterate and have no their women strictly control the processing and marketing RTE food, consumed in various parts of Nigeria, although most of these reports have been repo
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grown overnight on 7% SBA at 37°C and a colony was streaked again on freshly prepared 7% SBA in a manner that the streak allows for streaking of *Listeria* colonies. *Listeria* isolates was streaked at 90° angle and 3 mm apart before incubating them at 37°C for 24 h. A positive reaction was indicated by an enhanced zone of haemolysis at the intersection of the test and indicator strain.

**Determination of pH of samples**

For the *Fura* samples, 10 g of each sample was homogenized with 20 mL of distilled water in a beaker, for 1 min and the pH of the homogenate determined using digital pH meter calibrated with standard buffer solutions. For the *Nunu* samples, the samples were mixed properly and 25 mL of each sample was poured into a beaker and the pH determined using a digital pH meter.

**Determination of titratable acidity of the samples (using phenolphthalein indicator)**

Ten millilitres (*Nunu* or 10 g (*Fura*)) of each sample was added to 50 mL of distilled water in a 250 mL beaker and homogenized properly. Then, 3 drops of phenolphthalein were added to the mixture, while carefully swirling the beaker to ensure thorough mixing. The point of neutrality was reached when the indicator changed colour from colourless to pink, which must remain stable for at least 30 s. The amount of NaOH used on the burette was recorded and result expressed as percentage acid, using the following formula:

\[
\text{Percent acid} = \frac{\text{Titre} \times \text{acid factor} \times 100}{10 \text{ mL of sample}}
\]

Acid factor for Lactic acid = 0.0090

**Determination of NaCl (salt content) with standard silver nitrate solution**

To each sample neutralized as described above, about 1 mL of 5% aqueous potassium chromate solution was added and titrated with 0.1 N AgNO₃ solution to produce red-brown end point.

\[
\text{NaCl} \% = \frac{\text{Titre value} \times \text{Normality of AgNO₃} \times 58.4 \times 100}{\text{Weight of the sample} \times 1000}
\]

**Determination of moisture content**

About 5 mL and 5 g of well mixed samples (*Nunu* and *Fura*) were weighed in a well dried oven sterilized glass tray. The tray was placed in a hot air oven maintained at 105 ± 2°C and dried for at least 2 h. It was then cooled in a dessicator and weighed. The process of heating, cooling and weighing was repeated until the difference between two successive weighing was less than 1 mg and the lowest weight recorded.

\[
\text{% Moisture content} = \frac{100 \times (M₁ - M₂)}{M₁ - M}
\]

Where; M₁ = Weight in gram of dish with the dried material, and M = Weight in gram of empty dish

**Determination of water activity (a<sub>w</sub>)**

This was done using water activity meter (Buchi, Switzerland). About 10 g of each *Fura* and 10 mL of each *Nunu* sample was placed on the sample scale pan and readings recorded.

**DNA extraction**

Genomic DNA was extracted from the isolates using an extraction kit (Zymo Research, U.S.A.) following the procedure of the manufacturer. A 72- h tryptoy soya broth culture of the isolates was transferred in an Eppendorf tube and centrifuged for 5 minutes to pellet the cells. A 200 µL volume of nuclease free water was added to the cell pellet and vortexed for 1 min in a vortex mixer to wash the cells, it was then centrifuged for 1 min and the supernatant was decanted out. A 50 to 100 mg weight of the bacterial cells was resuspended in 200 µL of nuclease free water and transferred to a ZR Bashing Bead Lysis tube. Then, 750 µL of lysis solution was added to the tube. The tube was then vortexed in a vortex mixer vigorously for 5 to 10 min to lyse the cells. The ZR Bashing Bead Lysis tube was then centrifuged for 1 min at 10,000 × g. About 400 µL of supernatant was transferred to a ZR Spin IIC column in a collection tube and centrifuged at 7,000 × g for 1 min. To the filtrate in the collection tube, 1,200 µL of bacterial DNA Binding Buffer was added. About 800 µL of the mixture was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 × g for 1 min. The flow through the collection tube was discarded and the process repeated. Then, 200 µL of DNA Pre-wash buffer was added to the Zymo-Spin IIC column in a new collection tube and centrifuged at 10,000 × g for 1 min. Next, 500 µL of bacterial DNA Wash Buffer was added to the Zymo-Spin IIC column and centrifuged at 10,000 × g for 1 min. The Zymo-Spin IIC column was finally transferred to a clean Eppendorf tube and 50 µL of DNA Elution Buffer was added directly into the column matrix and centrifuged at 10,000 × g for 30 s to elute the DNA.

**PCR identification of *L. monocytogenes***

A 25 µL PCR supermix comprising 12.5 µL One Taq Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs, U.S.A.), 0.5 µL of each primer (10 µM), 4.5 µL of nuclease free water and 5 µL of bacterial genomic DNA solution was subjected to thermocycling conditions, in a TECHNE thermocycler (Bibby Scientific, UK), according to the following schedule: Initial denaturation, 95°C for 5 min; 35 thermal cycles of 95°C, 30 s; 58°C, 30 s; 72°C, 45 s; final extension, 72°C for 5 min; and hold, 4°C. The PCR products were analysed on a 1.5% agarose gel containing ethidium bromide (10 mg/mL) and specific DNA bands were visualized using a bench top UV transilluminator (Upland, U.S.A.). Primers used for PCR are shown in Table 1. Identification of *L. monocytogenes* was by detection of the 370 and 456 bp ampiclon of the *prs* and *hlyA* genes, respectively.

**Serogrouping of *L. monocytogenes***

Serogroups of the isolates were determined by PCR using specific primers (Table 1) for the three major serogroups (1/2a, 1/2b and 4b) associated with human listeriosis. The PCR conditions were: Initial denaturation, 94°C for 5 min; 35 thermal cycles of (94°C, 40 s; 53°C, 75 s; 72°C, 75 s); final extension, 72°C for 7 min; and hold,
Table 1. Nucleotide sequences of primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>Product size (bp)</th>
<th>Primer target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>prs</td>
<td>F: GCTGAAAGAGATTGGCAAAAGAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CAAAGAAACCTTGGAATTTCGGG</td>
<td>370</td>
<td>Genus Listeria</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>lmo0737</td>
<td>F: AGGGCTCCAAGGAATACCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: ACGATTCTGCTTGGAATTTC</td>
<td>691</td>
<td>*Lm 1/2a</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>ORF 2819</td>
<td>F: AGCAAAATGGCCAAAATCTGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CATCCTAAAGCCTCCCCATTG</td>
<td>471</td>
<td>Lm 1/2b</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td>ORF 2110</td>
<td>F: AGTGGACAAATTGGATGGTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CATCCATCCCTTACTTTGGAC</td>
<td>597</td>
<td>Lm 4b</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td></td>
<td>R: CATGGGTTTCACTCTCTCTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hlyA</td>
<td>F: GCAAGTTGGCAGCTTGATGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCAACGTATGCTCCAGATGTCG</td>
<td>456</td>
<td>hlyA gene</td>
<td>Swetha et al., 2012</td>
</tr>
<tr>
<td>iap</td>
<td>F: ACAAGCTGCACCTTTGCGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TGACAGGCTGTGTAGTGCA</td>
<td>131</td>
<td>iap gene</td>
<td>Swetha et al., 2012</td>
</tr>
</tbody>
</table>

4°C. The isolates were also screened for the presence of virulence genes. hlyA and iap by PCR, under the same conditions described under DNA amplification above.

RESULTS AND DISCUSSION

Out of a total of 75 Fura and 75 Nunu samples examined in this study, 23 (30.7%) and 41 (54.7%) samples, respectively, yielded Listeria spp., based on growth on PALCAM Listeria agar, Listeria chromogenic agar and biochemical characterization. These values indicate that both the cereal and milk used in preparation of the RTE Fura-de-Nunu, were contaminated with Listeria spp. The occurrence recorded in this study are very much higher than the values of about 5 to 7% reported by some authors for raw milk and dairy products (Jamali et al., 2004; Mansouri et al., 2013; Mansouri-Najand et al., 2015; Shamloo et al., 2016), but lower than 78% reported from a study in south western Nigeria on an RTE soft cheese product (Oyinloye, 2016). Six presumptive Listeria species were identified from the samples. The distribution of the presumptive Listeria spp. in the Fura was as follows: L. ivanovii (36.7%) > L. monocytogenes (30.0%) > L. seeligeri (20.0%) > L. grayi (10.0%) and L. welshimeri (3.3%), while for Nunu, it was L. ivanovii (46.4%) > L. monocytogenes (26.8%) > L. seeligeri (16.1%) > L. innocua (5.4%) > L. grayi (3.6%) and L. welshimeri (1.8%). Another study in Nigeria had also reported these species to be the most predominant Listeria species in raw milk and attributed their presence in milk to unhygienic milking practices (Yakubu et al., 2012). Among the Listeria species, L. monocytogenes is the most commonly associated with human listeriosis. Although the illness is relatively rare, it can have mortality rates as high as 30%, especially among vulnerable groups such as infants, pregnant women and the elderly (Mansouri-Najand et al., 2015). For this reason, most western countries have developed set-down policies and guidelines to monitor and control L. monocytogenes in foods. Unfortunately, the same cannot be said for Nigeria and some other African countries. The mean Listeria counts for the samples in this study were 7.7 × 10^4 CFU/g and 8.6 × 10^4 CFU/mL, for Fura and Nunu, respectively. Although these values represent total Listeria counts and not L. monocytogenes counts, they are still a cause for concern, considering that L. monocytogenes was the second most predominant species isolated from the food samples. According to international standards for acceptable levels of L. monocytogenes in foods, foods with a shelf life of less than five days or foods with the potential to support the growth of L. monocytogenes should not contain more than 100 CFU/g of food; and when the food is intended for infants, there should be complete absence of L. monocytogenes per 25 g of the food (EC 2073/2005; FSA UK, 2010; Health Canada, 2011; FOOD Standards Australia, 2014). The high frequency of L. monocytogenes in this study and the high total Listeria counts suggest a possibility that the L. monocytogenes counts in these products could well exceed the acceptable limit of 100 cfu/g of food. Moreover, as Fura-de-Nunu is a street-vended food,
there is no specification for the groups of individuals that can consume the product. It is available to all groups of consumers including infants, pregnant women and the elderly; it is in fact considered a complete food, suitable for weaning babies and should therefore meet the standard of 'complete absence of L. monocytogenes / 25 g of food'. Other studies conducted in Nigeria have also reported high loads of L. monocytogenes from different foods, including RTE foods (Chukwu et al., 2006; Ikehe et al., 2010; Nwaiwu, 2015).

According to Food Safety guidelines, methods that have been recommended to control the growth of Listeria in foods including the use of antibacterial treatments, pasteurization, reduction of pH, refrigeration and consumption within safe periods (FSA UK, 2010; Health Canada, 2011; FOOD Standards Australia, 2014). However, for the RTE food studied here, none of these measures are usually applied. The products are made crudely by local Fulani women and then hawked in pans and containers on the streets, under conditions which would surely support growth of the Listeria organisms.

Evaluation of the physico-chemical properties of the samples showed that the pH and water activity (aw) values of the Fura and Nunu samples were 4.5±0.1, 0.76±0.0 and 3.6 ±0.1, 0.94±0.0, respectively (Table 2). Although these values appear to be within the safe ranges (pH < 4.4 and aw <0.92), the lack of specified shelf life and the conditions under which the foods are vended still leave reasons for concern. According to the Canadian Bureau of Microbial Hazards policy on L. monocytogenes in RTE foods (2011), growth of L. monocytogenes is assumed to occur in RTE foods if the pH and aw values fall outside the safe range, unless the RTE food processor is able to present data, to be reviewed by regulatory authorities which demonstrates that the growth of L. monocytogenes will not occur within the specified shelf life (Health Canada, 2011). Sadly, for the RTE Fura de Nunu, these criteria are neither considered nor met, before the street-hawking of the products.

Representative L. monocytogenes isolates (n=23) identified by phenotypic and biochemical methods were subjected to PCR, which has proved to be a very useful and rapid method for detection of Listeria (Mansouri-Najand et al., 2015). Screening of the presumptive L. monocytogenes isolates by PCR showed that only about 77% of the isolates could be confirmed as L. monocytogenes, by detection of the 370 bp amplicon and 456 bp amplicon of the prs and hlyA genes, for identification of genus Listeria and L. monocytogenes, respectively (Figures 1 and 2). This result suggests that non-molecular methods of detection of these organisms may not be very specific and may lead to artificially high prevalence values. Thus, more specific molecular methods such as PCR must be used when evaluating L. monocytogenes contamination of food samples. The value of molecular methods of identification and typing has also been emphasized by other authors. For instance, it has been suggested that molecular methods are useful not just for accurate identification, epidemiological and trace-back investigations, but also for understanding the diversity and evolution of the organism (Doumith et al., 2004; Laksanalamai et al., 2014). It has also been suggested that although the classical biochemical methods of identification are standard, they can sometimes give false positives (Nwaiwu, 2015).

L. monocytogenes has 13 serotypes (1/2a,1/2b,1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7), but serotypes 1/2a, 1/2b and 4b are believed to be responsible for about 95% of human listeriosis (Kathariou, 2002; Jamali et al., 2013). Out of 24 L. monocytogenes (23 isolates and reference strain) screened by PCR-serogrouping in this study, only seven (including the reference strain) could be placed in any of the three serogroups tested for; and of these, only 1/2a and 4b were identified (Table 3). The 1/2a isolates were positive for lmo0737 gene alone while the 4b isolates showed the presence of ORF 2110. Therefore, even though it has been suggested that variant strains of one serotype can sometimes acquire the gene clusters of another as in the reports of Laksanalamai et al., (2014), no such variants were detected in this study. Due to the epidemiological and clinical relevance of serotypes of the organism, serotyping is usually recommended for suspected L. monocytogenes isolates, particularly since the development of a PCR-based grouping scheme by Doumith et al. (2004). This molecular serogroup-related PCR typing has been endorsed by other researchers (Huang et al., 2011; Laksanalamai et al., 2014).

It has been suggested that pathogenic L. monocytogenes may be identified based on detection of some virulence markers, including internalins (inlA, inlC, inlI), listeriolysin O (hlyA), actin polymerization protein

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### Table 2. Intrinsic parameters of Fura and Nunu samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>aw</th>
<th>%LA</th>
<th>%NaCl</th>
<th>%Wc</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fura</td>
<td>0.76±0.02</td>
<td>0.31±0.04</td>
<td>2.6±0.17</td>
<td>58.11±8.59</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>Nunu</td>
<td>0.94±0.01</td>
<td>1.17±0.02</td>
<td>3.3±0.12</td>
<td>91.13±1.87</td>
<td>3.6±0.1</td>
</tr>
</tbody>
</table>

Values represent means ± standard deviation of triplicate experiments; aw = Water activity; LA = lactic acid; NaCl = Sodium chloride; Wc = Water content.
Figure 1. PCR detection of 370 bp amplicon of prs gene for identification of Listeria species. Lane M, 100 bp DNA ladder; lane 1, positive control (L. monocytogenes ATCC13932); lanes 2-23, presumptive L. monocytogenes isolates; lane 24 negative control. Lanes 3, 4, 5, 6, 7, 9, 11, 12, 18, 20, 21 and 23 are positive.

Figure 2. PCR detection of 456 bp amplicon of hlyA gene for identification of L. monocytogenes. Lane M, 100 bp DNA ladder; lane 1, positive control (L. monocytogenes ATCC13932); lanes 2-23, presumptive L. monocytogenes isolates; lane 25 negative control. Lanes 2-12, 16-18, 20, 21 and 23 are positive.

(actA), phosphatidyl-inositol-phospholipase C (plcA), invasive associated protein (iap) and virulence regulator (prfA), which are believed to be important in L. monocytogenes infection and pathogenesis (Liu et al., 2007; Di Cicco et al., 2012). PCR-based methods have also been used to identify pathogenic L. monocytogenes strains possessing these genes (Huang et al., 2011; Laksanalamai et al., 2014; Swetha et al., 2015). The
isolates in this study were screened for the presence of two virulence genes: hlyA and iap by PCR and both genes were detected in some of the isolates (Table 3). Detection of these virulence genes suggests that the L. monocytogenes isolates from this study are pathogenic. Further studies will aim to carry out full genome sequencing on these isolates, in order to further characterize and document the strains. The isolation of virulent L. monocytogenes from the RTE product, Fura-de-Nunu, in this study is a significant cause for concern. Fura de Nunu is considered to be a complete food and is relatively cheap. Therefore, in a country with a large population of low-income earners, it comes highly recommended as a means of reducing malnutrition and is widely consumed across demographic groups, including pregnant women, infants and the elderly, who are among the groups most at risk for listeriosis. The results from this study highlight a need for the development and implementation of food safety policies and standards to guide the production and distribution of RTE foods in Nigeria. Producers and consumers of RTE products should also be made aware of the need to observe the highest possible standards of hygiene during production of RTE and street-vended foods to reduce the risk of listeriosis and other food-borne diseases.

### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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### REFERENCES


### Table 3. Genetic characteristics of some L. monocytogenes isolates from Fura and Nunu.

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