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ARTICLES

Antimicrobial activity of the extracts of *Albizia masikororum* R.Vig., a Fabaceae from Madagascar


In-vitro antibiotic susceptibility profile of *Salmonella enterica* Serovar Typhi isolated from fecal specimens of humans in Umuahia metropolis, Abia State, Nigeria

Emmanuel O. Ekundayo and Emmanuel Enya

Isolation and identification of *Escherichia coli* and *Edwardsiella tarda* from fish harvested for human consumption from Zeway Lake, Ethiopia

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Antimicrobial activity of the extracts of *Albizia masikororum* R.Vig., a Fabaceae from Madagascar

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This study is aimed at the assessment of antimicrobial potential of *Albizia masikororum*, a Malagasy Fabaceae. Hexanic and methanolic extracts from fruit pods, stem bark, leaves and seeds were tested by disc diffusion and microdilution methods on 10 pathogenic microorganisms including four Gram positive bacteria, five Gram negative bacteria and one yeast. Only the leaf and seed methanolic extracts, LME and SME respectively, were active on some bacteria. LME and SME had a broad-spectrum activity, with SME more effective. The minimum inhibitory concentration (MIC) values of both extracts were <1000 µg/ml of which 30% <100 µg/ml, 20% between 100 and 500 µg/ml and 50% between 500 and 1000 µg/ml. SME MICs ranked from 6.10 µg/ml (*Streptococcus pneumoniae*) to 781.25 µg/ml (*Yersinia enterocolitica*) and those of LME from 97.65 µg/ml (*Streptococcus pneumoniae*) to 781.25 µg/ml (*Bacillus cereus, Streptococcus pneumoniae* and *Yersinia enterocolitica*). LME was bactericidal on all sensitive bacteria whereas SME was bactericidal on some and bacteriostatic on others. Both extracts contained different chemical groups known for their antimicrobial properties, saponins in SME and phenolic compounds in LME.

**Key words:** *Albizia masikororum*, antimicrobial activity, disc diffusion method, microdilution method, minimum inhibitory concentration, minimum bactericidal concentration.

**INTRODUCTION**

Globally, the number of multidrug-resistant strains has increased dramatically over the past 10 years (Roca et al., 2015; Sharifi-Rad, 2016) and new more resistant strains may be expected to emerge in the coming years (McGaw et al., 2008).

This situation may exhaust the chemical defenses at our disposal. Therefore, research programs in alternative therapies including the search for new antimicrobial agents should be encouraged. Natural products still play a major role in the search for new medicines and plants represent a good source for new molecules of interest (Kuete et al., 2009; Sharifi-Rad et al., 2017a, b, c, d, e;
Malagasy plants are good candidates in view of the diversity of Malagasy flora whose originality could promise original molecules. The genus *Albizia* is a group of plants that includes about 150 species and occurs in tropical regions. Worldwide, it has many uses including control of pests such as pathogenic microorganisms (Agyare et al., 2006; Sarki, 2011; Odeyemi et al., 2014). The Malagasy species studied also showed *in vitro* antimicrobial properties (Randriamampianina et al., 2017). In this work, the antimicrobial potential of the endemic species *A. masikororum*, which has already shown appreciable biological properties on mice and chicken (Randrianarivo et al., 2014) was studied.

**MATERIALS AND METHODS**

**Plant materials**

*Albizia masikororum* is a large shrub or tree up to 25 m tall (Figure 1) growing throughout the western part of Madagascar, from the North to the South. It was collected in Belo-sur-Tsiribihina/Betioka (West of Madagascar). Voucher specimens of the plant were deposited in the herbarium of Silo National des Graines Forestières (SNGF) under the reference number SNGF 873.

**Microorganism strains**

The 10 microorganisms used in this study included 4 Gram (+) bacteria, 5 Gram (-) bacteria and 1 yeast (Table 1). They were maintained on agar slants at 4°C and cultured on a fresh appropriate agar plates 24 h prior to any antimicrobial test.

**Chemicals for antimicrobial assay**

Neomycin (30 µg/ml/disc) (Bio-Rad F-92430, Marnes-la-Coquette, France) were used as antibiotic references. Miconazole (500 µg) (Bio-Rad F 92430 Marnes-la-Coquette, France) was used as antifungal reference for yeasts.

**Preparation of hexane and methanolic extracts**

Ground seeds, leaves, pods and stem bark powder (50 g) were delipidated with hexane (3 X 250 ml), then extracted with methanol (3 X 250 ml). After filtration using a Whatman filter paper, extracts were evaporated to dryness under reduced pressure.

**Phytochemical screening**

The reactions of chemical group detection were those developed by Marini-Bettolo et al. (1981).

**Antimicrobial assays**

All the materials and methods used for antimicrobial assay were detailed in a previous study (Randriamampianina et al., 2017). The results were interpreted using the scale of Ponce et al. (2003) and Celikel et al. (2008). With the disc diffusion method, bacteria are not considered sensitive for an inhibition zone diameter (IZD) less than 8 mm, sensitive for IZD of 9 to 14 mm, very
Table 1. List of microorganisms used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 25923</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>ATCC 14579</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>ATCC 6305</td>
<td>+</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>ATCC 19615</td>
<td>+</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>ATCC 13048</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>ATCC 13047</td>
<td>-</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>ATCC 23715</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 10145</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>ATCC 13124</td>
<td>-</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>ATCC 10231</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Extraction yields of the different crude extracts.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Extraction solvent</th>
<th>Designation</th>
<th>Pod</th>
<th>Hexane</th>
<th>Methanol</th>
<th>Bark</th>
<th>Hexane</th>
<th>Methanol</th>
<th>Leaf</th>
<th>Hexane</th>
<th>Methanol</th>
<th>Seed</th>
<th>Hexane</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>solvent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LME</td>
<td>PHE</td>
<td></td>
<td></td>
<td>2.01</td>
<td>4.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SME</td>
<td>PME</td>
<td></td>
<td></td>
<td>0.95</td>
<td>2.38</td>
<td></td>
<td>1.17</td>
<td>4.74</td>
<td></td>
<td>3.36</td>
<td>19.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

sensitive for IZD of 15 to 19 mm and extremely sensitive for IZD larger than 20 mm.

For each extract, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined on susceptible strains only. The standards used to interpret MIC results were those of Dalmarco et al. (2010). For crude extracts and fractions, a MIC lower than 100 µg/mL was considered as an excellent effect, from 100 to 500 µg/ml as moderate, from 500 to 1000 µg/mL as weak, and over 1000 µg/ml as inactive.

The extract action is bactericidal or fungicidal when its ratio MBC/MIC or MFC/MIC is ≤4 and bacteriostatic and fungistatic when the ratio is >4 (Djeussi et al., 2013; Bouharb et al., 2014; Chamandi et al., 2015).

RESULTS

Extraction yields

The different crude extracts were obtained with yields ranging from 0.95% for BHE to 19.01% for SME (Table 2).

Phytochemical analysis

The major secondary metabolites identified in seed methanolic extract (SME) and leaf methanolic extract (LME) are shown in Table 3. In SME, saponins, unsaturated sterols, triterpenes and cardenolides were present, while alkaloids, tannins, flavonoids, leucoanthocyanins, coumarins, iridoids, steroids, and anthraquinones were not detected. LME contained tannins, flavonoids, leucoanthocyanins and triterpenes, while alkaloids, saponins, unsaturated sterols, cardenolids, steroids, coumarins, iridoids, steroids and anthraquinones were absent.

Antimicrobial activity

Disc diffusion

The effects of the various extracts of seeds, pods, bark and leaves at a concentration of 1 mg/disc on the microorganisms are presented in Table 4. LME and SME only displayed activity on some bacteria. Their IZD values ranked from 11.5 to 16.5 mm for LME and from 9 to 15 mm for SME. They were less effective than neomycin which is a pure product. None of the extracts was active against Pseudomonas aeruginosa and Candida albicans.

MIC and MBC values

MIC and MBC values were determined for LME and SME only for active extracts on sensitive bacteria by the disc diffusion method (Table 5).

All the MIC values were <1000 µg/ml: 27.2% were <100 µg/ml, 27.2% between 100 and 500 µg/ml and 45.6% between 500 and 1000 µg/ml. There were more strains sensitive to SME (7 strains) than to LME (4 strains). SME MICs ranked from 6.10 µg/ml (Streptococcus pneumoniae) to 781.25 µg/ml (Yersinia enterocolitica) and those of LME from 97.65 µg/ml (Streptococcus pneumoniae) to 781.25 µg/ml (Bacillus cereus, Streptococcus pneumoniae and Yersinia enterocolitica).

The best activities were observed with SME against Streptococcus pneumoniae (6.10 µg/ml) and Streptococcus pyogenes (24.41 µg/ml).

LME exerted a bactericidal effect against all bacteria
Table 3. Phytochemical screening of seed powder and organ methanolic extracts.

<table>
<thead>
<tr>
<th>Chemical group</th>
<th>Test</th>
<th>Seed powder</th>
<th>Methanolic extracts from</th>
<th>Seeds</th>
<th>Leaves</th>
<th>Pods</th>
<th>Bark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mayer</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wagner</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dragendorff</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gelatin 1%</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenolic compunds</td>
<td>Flavonoids</td>
<td>Flavonols</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unsaturated sterols</td>
<td>Salkowski</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iridoids</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Heterosides</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cardenolides</td>
<td></td>
<td></td>
<td></td>
<td>nd</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Cyanogenic glycosides</td>
<td></td>
<td></td>
<td></td>
<td>nd</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>nd</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

+: Positive test; -: Negative test; nd: Not determined.

Table 4. Effects (IZD in mm) of A. masikororum extracts by disc diffusion method at a concentration of 1 mg/disc.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PHE</th>
<th>PME</th>
<th>BHE</th>
<th>BME</th>
<th>LHE</th>
<th>LME</th>
<th>SHE</th>
<th>SME</th>
<th>Neomycin (30 µg/disc)</th>
<th>Miconazole (500 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (+) bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>11.5</td>
<td>6</td>
<td>6</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>13</td>
<td>6</td>
<td>10</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus pneumonia</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7.5</td>
<td>6</td>
<td>15</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>13</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>12</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>14.5</td>
<td>6</td>
<td>9</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Gram (-) bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>nd</td>
<td>25</td>
<td>-</td>
</tr>
</tbody>
</table>

nd: Not determined.

Sensitive to it whereas SME had a bactericidal effect on 4 bacteria and bacteriostatic effect on 3 others.

**DISCUSSION**

Of all the 8 extracts tested, only methanolic extracts from leaves (LME) and seeds (SME) displayed antimicrobial activity.

The antibacterial compounds in the two extracts did not belong to the same chemical groups: Phenolic compounds for LME and saponins for SME. As these extracts were only semi-purified, the study results did not yet allow the conclusion on the number of active compounds in each one of them.
LME was active against 4 and SME against 7 of the 10 strains tested but unlike other Malagasy *Albizia* species so far studied (Rakoto et al., 2011; Randriamampianina et al., 2017), both extracts were not active against *Candida albicans*. Generally, the strain sensitivity varied significantly with the extract. For example, *Clostridium perfringens* and *Enterobacter cloacae* were sensitive to SME but resistant to LME whereas *Bacillus cereus* was sensitive to LME but not to SME. This difference could lie in the fact that the two extracts contained very different chemical groups. SME was rich in saponins and LME in phenolic compounds. Both chemical groups are known for their antimicrobial properties.

LME and SME displayed an excellent effect against *Streptococcus pneumoniae* and *Streptococcus pyogenes*. Those two bacteria, because of their high prevalence in infectious diseases and their resistance to various antibiotics, have become a serious clinical and health problem in many countries (Nuernberger and Bishai, 2004; Ardanuy et al., 2014; Lu et al., 2017; Sekizuka et al., 2017; Zhao et al., 2017).

In comparison with antimicrobial activity of other malagasy *Albizia* assessed in similar conditions (Randriamampianina et al., 2017), LME and SME gave MIC values much lower than 1000 µg/ml, and therefore far more effective compared to the seed extracts from *A. aurisparsa* against *B. cereus* (MIC = 1980 µg/ml) and from *A. mahalao* (MIC = 3750 µg/ml) and *A. polyphylla* (MIC = 2420 µg/ml) against *Staphylococcus aureus*. However, their activities were more or less similar to those of *A. bernieri* (Randriamampianina et al., 2017) against the same sensitive bacteria.

There is no consensus on the standard scale for interpreting antimicrobial activity of natural products (Benko and Crovella, 2010). According to the scale of Dalmarco et al. (2010), used in this work, 2 bacteria were very sensitive, 3 moderately sensitive and 4 weakly sensitive. With standards used by other authors, plant extracts with MICs values higher than 500 µg/ml (Aligianis et al., 2001) and even much higher than 1000 µg/ml (Maregesi et al., 2008; Abubakar and Majinda, 2016; Touaibia and Chaouch, 2017) were classified as having strong antimicrobial activity. For comparison, LME and SME had excellent activates on all the bacteria tested. Based on the results obtained with the strains tested, LME and SME have a relatively broad activity spectrum. The effect of SME on sensitive bacteria was bactericidal whereas SME exerted a bactericidal effect on some strains and bacteriostatic effect on others.

In conclusion, *Albizia masikororum* exerted antibacterial effect. LME and SME could constitute good alternatives to synthetic antibiotics, particularly against *Streptococcus*. Further studies should be undertaken to determine the number and the identities of active principles responsible of this property and to know whether or not they are new products.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**REFERENCES**


<table>
<thead>
<tr>
<th>Strain</th>
<th>Extract</th>
<th>MIC</th>
<th>MBC or MFC</th>
<th>MBC/MIC or MFC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>LME</td>
<td>781.25</td>
<td>781.25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SME</td>
<td>195.31</td>
<td>&gt;1000</td>
<td>&gt;4</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>LME</td>
<td>781.25</td>
<td>&gt;1000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SME</td>
<td>195.31</td>
<td>&gt;1000</td>
<td>&gt;4</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>LME</td>
<td>97.65</td>
<td>97.65</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SME</td>
<td>6.10</td>
<td>24.41</td>
<td>4</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>SME</td>
<td>24.41</td>
<td>48.82</td>
<td>2</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
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<td>195.31</td>
<td>&gt;1000</td>
<td>&gt;4</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>SME</td>
<td>195.31</td>
<td>&gt;1000</td>
<td>&gt;4</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>SME</td>
<td>781.25</td>
<td>&gt;1000</td>
<td>&gt;4</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>LME</td>
<td>781.25</td>
<td>&gt;1000</td>
<td>&gt;4</td>
</tr>
</tbody>
</table>


In-vitro antibiotic susceptibility profile of *Salmonella enterica* Serovar Typhi isolated from fecal specimens of humans in Umuahia metropolis, Abia State, Nigeria

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Typhoid is routinely diagnosed in Nigeria on clinical grounds or based on Widal serological test result. This approach does not provide information on the antibiotic susceptibility of the bacterium; *Salmonella enterica* Serovar Typhi. This study was done to assess the antimicrobial susceptibility profile of *Salmonella* isolates in Umuahia. In this study, seventy-two (72) *Salmonella* isolates obtained from 135 fecal specimens were tested for their antibiotic susceptibility profile against 13 antimicrobial agents using the disk diffusion method according to the protocol of the Clinical and Laboratory Standards Institute (CLSI). Sixty-two (86.1%) of the isolates were resistant to Amoxicillin, fifty-two (72.2%) to Trimethoprim-sulfamethoxazole (Co-trimoxazole), forty-one (56.9%) to Chloramphenicol and fifty-two (72.2%) to Augmentin. Fifty-nine (81.9%) of the isolates were resistant to Tetracycline, 11 (15.2%) were of intermediate susceptibility and only two (2.7%) were susceptible to the antibiotic. Thirty-five (48.6%) of the isolates were sensitive to Ciprofloxacin, forty-seven (65.2%) to ofloxacin and forty-three (59.7%) to gentamicin. The resistance profile of the isolates to cephalosporin antibiotics was as follows: cefuroxime (70.8%), ceftriaxone (68.0%) and ceftazidime (65.2%). Some of the isolates exhibited resistance to multiple antibiotics ranging from three or more of the antibacterial agents tested. The results obtained suggest that high proportion of *S. Typhi* strains circulating in the study area are resistant to multiple antibiotics and empirical treatment of typhoid fever without antibiotic susceptibility testing is not advisable in this setting.

**Key words**: *Salmonella typhi*, antimicrobial resistance, typhoid fever, antibiotic susceptibility profile, Umuahia

**INTRODUCTION**

Typhoid fever is a life-threatening systemic febrile illness caused by the bacterium *Salmonella enterica* Serovar Typhi (*S. Typhi*). Typhoid fever is a global public health problem with an estimated 21.7 million illnesses and...
217,000 deaths worldwide in 2000 (Crump and Mintz, 2004). Antibiotic therapy has been the mainstay of management of typhoid fever but strains resistant to commonly used antibiotics have emerged and are spreading around the world (Zaki and Karande, 2011). Nigeria falls within the region of estimated high incidence of typhoid fever in Sub-Saharan Africa (Von Kalckreuth et al., 2016). The co- endemicity of typhoid fever along with other non-bacterial febrile infections like malaria and viral diseases makes direct clinical diagnosis difficult (Enabulele and Awunor, 2016).

For confirmation of diagnosis of typhoid fever and for effective antibiotic therapy, there is a need to isolate S. Typhi and carry out antibiotic susceptibility testing. Isolation of S. Typhi from culture of bone marrow aspirate is regarded as the gold standard for confirmation of typhoid fever but blood culture is preferred because obtaining blood specimen is easier and less invasive (Bhutta, 2006). Blood culture is slightly less sensitive than bone marrow aspirate culture (Tanyigna et al., 2001). Stool culture can be done as an alternative to blood culture. However, culture for isolation of S. Typhi is rarely done in low income countries due to high cost, lack of equipment and technical expertise. As a result, data on the prevalence and pattern of resistance to antimicrobials among strains of S. Typhi are limited in low income countries (Von Kalckreuth et al., 2016). Available data indicate that strains of S. Typhi resistant to multiple antibiotics have been isolated in some parts of Nigeria (Akinyemi et al., 2014).

In Nigeria, typhoid fever is typically diagnosed on clinical grounds or based on the results of Widal serological test and antibiotic treatment is done empirically with no certainty about the sensitivity of the infecting pathogen. Widal test has the problem of low sensitivity and specificity with positive predictive values (PPV) around 17-20% (Tanyigna et al., 2001; Enabulele and Awunor, 2016). In view of the low PPV of Widal test and the uncertainty associated with the antibiotic susceptibility profile of S. Typhi strains, many patients are likely to receive inappropriate antibiotic therapy in the absence of culture and antibiotic susceptibility testing.

Increasing rates of reduced susceptibility to ciprofloxacin commonly used in cases of suspected resistance to first line antimicrobials have been reported (Harrois et al., 2014; Lunguya et al., 2013; Kariuki et al., 2010, Akinyemi et al., 2007). Antibiotic susceptibility surveillance systems can provide insights into the areas where resistance is most prevalent and where the prevalence of resistance is increasing the fastest. Determination of antimicrobial susceptibility patterns not only helps shape successful treatment plans for individual patients but also assists with the development of public health policy for control of antimicrobial resistance. The aim of this study was to determine the antibiotic susceptibility profile of strains of Salmonella isolated from fecal specimens of patients in Umuahia.

MATERIALS AND METHODS

Specimen collection and inoculation

Fecal specimens were collected from patients attending four different clinics in Umuahia metropolis, the Capital of Abia State. Each of the patients that submitted specimens gave an informed consent. Only one fecal specimen was collected per participant. The management of each of the clinics gave approval for the collection of specimens. The specimens were inoculated within 24 h of collection into Selenite Faece broth (Titan Biotech Ltd, India) for enrichment and incubated at 37°C for 18 - 24 h and subsequently plated on Salmonella-Shigella agar (Titan Biotech Ltd, India) for primary isolation. The culture plates were incubated aerobically at 37°C for 24 h and observed for growth through the formation of colonies. Colonies growing on the plates were purified by streak plate method on nutrient agar and subsequently maintained on nutrient agar slants.

Biochemical characterization of isolates

Standard microbiological techniques were performed for biochemical characterization of the isolates. Biochemical tests carried out according to standard procedures as described in Cheesbrough (2006), included Indole, Citrate, Urease, Methyl Red, Voges Proskauer and Triple Sugar Iron tests.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk agar diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI, 2015) on Mueller Hinton agar (Hardy Diagnostics, USA). Discrete colonies from a 24 h nutrient agar plates were suspended in sterile normal saline in a test tube to achieve a bacterial suspension equivalent to 0.5 McFarland Turbidity Standard. A cotton swab was dipped into the bacterial suspension and used to inoculate the entire surface of a Mueller Hinton agar plate, rotating the plate to ensure confluent growth of the bacterium. The antimicrobial susceptibility disks were placed on the surface of the inoculated plate with flame sterilized forceps. The plates were incubated in an inverted position for 16-18 h at 35-37°C. The diameter of the zone of inhibition produced by the antibiotic disks were measured to the nearest millimeter (mm) using a transparent ruler.

Data and statistical analysis

The criteria for categorizing the diameter of zones of inhibition into sensitive (S) or intermediate (I) or resistant (R) were based on the interpretive charts of the Clinical and Laboratory Standards Institute (CLSI, 2015). Proportions of males and females and resistant isolates were compared using the Pearson’s approximation to the Chi-squared test. The significance level was tested at α = 0.05.

RESULTS

Fecal specimens were collected from a total of 135 study participants, made of 59 (43.7%) males and 76 (56.3%) females. The demographic characteristics of the study participants and the number of Salmonella isolates recovered according to gender and age groups are shown in Table 1. Difference between the number of
Table 1. Demographic characteristics of study participants and isolation of *Salmonella* from fecal specimens.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. (%) of fecal specimens</th>
<th>No. yielding <em>Salmonella</em> isolates</th>
<th>Isolation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>59 (43.70)</td>
<td>30</td>
<td>22.20</td>
</tr>
<tr>
<td>Female</td>
<td>76 (56.30)</td>
<td>42</td>
<td>31.10</td>
</tr>
<tr>
<td>Total</td>
<td>135 (100)</td>
<td>72</td>
<td>53.30</td>
</tr>
<tr>
<td><strong>Age groups (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>14 (10.40)</td>
<td>9</td>
<td>6.67</td>
</tr>
<tr>
<td>21-30</td>
<td>63 (46.70)</td>
<td>39</td>
<td>28.9</td>
</tr>
<tr>
<td>31-40</td>
<td>30 (22.20)</td>
<td>12</td>
<td>8.99</td>
</tr>
<tr>
<td>41-50</td>
<td>15 (11.10)</td>
<td>9</td>
<td>6.67</td>
</tr>
<tr>
<td>51-60</td>
<td>11 (8.00)</td>
<td>3</td>
<td>2.22</td>
</tr>
<tr>
<td>&gt;61</td>
<td>2 (1.50)</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 2. Antimicrobial agents tested and criteria for assessment of susceptibility of *Salmonella* isolates.

<table>
<thead>
<tr>
<th>Antimicrobial agents (code)*</th>
<th>Potency (µg)</th>
<th>Inhibition zone (mm) cut-off values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(S)</td>
</tr>
<tr>
<td>Gentamicin (GEN)</td>
<td>10</td>
<td>≥15</td>
</tr>
<tr>
<td>Chloramphenicol (CHL)</td>
<td>30</td>
<td>≥18</td>
</tr>
<tr>
<td>Oftloxacin (OFL)</td>
<td>30</td>
<td>≥16</td>
</tr>
<tr>
<td>Augmentin (AUG)</td>
<td>30</td>
<td>≥18</td>
</tr>
<tr>
<td>Ciprofloxacin (CPR)</td>
<td>5</td>
<td>≥21</td>
</tr>
<tr>
<td>Amoxicillin (AMX)</td>
<td>25</td>
<td>≥18</td>
</tr>
<tr>
<td>Cefuroxime (CXM)</td>
<td>30</td>
<td>≥18</td>
</tr>
<tr>
<td>Cotrimoxazole (COT)</td>
<td>25</td>
<td>≥16</td>
</tr>
<tr>
<td>Cefazidime (CAZ)</td>
<td>30</td>
<td>≥21</td>
</tr>
<tr>
<td>Nitrofurantoin (NIT)</td>
<td>100</td>
<td>≥17</td>
</tr>
<tr>
<td>Ceftriaxone (CRO)</td>
<td>30</td>
<td>≥23</td>
</tr>
<tr>
<td>Tetracycline (TET)</td>
<td>30</td>
<td>≥15</td>
</tr>
<tr>
<td>Streptomycin (STR)</td>
<td>25</td>
<td>≥15</td>
</tr>
</tbody>
</table>

*Interpretative break points according to Clinical Laboratory Standards Institute, CLSI (2015). a, Abtek™ Biologicals, UK.

males and females that submitted fecal specimens was not significantly different (p>0.05). The difference between the number of *Salmonella* isolates recovered from males and females was however, significantly different (p<0.01). *Salmonella* was isolated from 53.3% of the total number of participants who submitted fecal specimens.

The antimicrobial agents tested and the interpretative criteria for assessment of the susceptibility of *Salmonella* based on the diameter of zones of inhibition of each antimicrobial agent are presented in Table 2. The antibiotic susceptibility profile showing the proportions of Sensitive, Intermediate and Resistant categories is presented in Figure 1. The isolates exhibited high resistance level to amoxicillin, augmentin, trimethoprim sulfamethoxazole (co-trimoxazole) tetracycline, chloramphenicol and the second and third generations cephalosporins. Sensitivity of the isolates to ofloxacin and gentamicin was moderate. Greater than 30% of the isolates have reduced sensitivity (intermediate category) to Chloramphenicol and Ciprofloxacin.

The isolates were assessed for resistance to multiple antimicrobial agents, from combination of three agents to seven. The results are shown in Table 3. Six patterns of resistance to combination of three antimicrobial agents were identified. About 52% of the isolates were resistant to combinations of three different types of antimicrobial agents and about 35% were resistant to combinations of four antimicrobial agents. The commonest multidrug resistant (MDR) phenotypes are amoxicillin, chloramphenicol and tetracycline (14 isolates), followed by amoxicillin, chloramphenicol, tetracycline and co-trimoxazole (12 isolates). The classical *Salmonella* MDR phenotype defined by resistance to amoxicillin,
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Figure 1. Antimicrobial susceptibility profile of salmonella isolates in Umuahia. Gentamicin (Gen), chloramphenicol (Chl), ofloxacin (Ofl), augmentin (Aug), ciprofloxacin (Cpr), amoxicillin (Amx), cefuroxime (Cxm), ceftriaxone (Ctx), tetracycline (Tet), streptomycin (Str), ceftazidime (Caz), cotrimoxazole (Crx), nitrofurantoin (Nit).

Table 3. Multiple antibiotic resistance profile of the isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>No. of antibiotics</th>
<th>No. of antibiotic resistant isolates</th>
<th>Overall (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMX, CHL, COT</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>AMX, CHL, TET</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>AMX, TET, GEN</td>
<td>3</td>
<td>1</td>
<td>38 (52.8)</td>
</tr>
<tr>
<td>AMX, TET, CPR</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>AMX, CHL, CPR</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, COT</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>AMX, CHL, COT, TET</td>
<td>4</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, GEN, CAZ</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, OFL, COT</td>
<td>4</td>
<td>1</td>
<td>23 (31.9)</td>
</tr>
<tr>
<td>AMX, CHL, COT, CPR</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, GEN, CAZ</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, OFL, COT</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, GEN, OFL, CHL</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AMX, TET, CRO, OFL, CHL</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AMX, CPR, CRO, OFL, NIT</td>
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<td>1</td>
<td>4 (6.15)</td>
</tr>
<tr>
<td>AMX, CPR, COT, TET, NIT, GEN</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AMX, COT, CAZ, CRO, AUG, CPR</td>
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<td>5</td>
<td>8 (12.30)</td>
</tr>
<tr>
<td>AMX, AUG, CRO, OFL, NIT, GEN</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AMX, AUG, CRO, CPR, NIT, STR, CHL</td>
<td>7</td>
<td>1</td>
<td>1 (1.53)</td>
</tr>
</tbody>
</table>

Number of MDR Patterns | 18 | 72 | 90.3% |

Gentamicin (GEN), chloramphenicol (CHL), ofloxacin (OFL), augmentin (AUG), ciprofloxacin (CPR), amoxicillin (AMX), cefuroxime (CXM), ceftriaxone (CRO), tetracycline (TET), streptomycin (STR), ceftazidime (CAZ), cotrimoxazole (COT), nitrofurantoin (NIT).
chloramphenicol and co-trimoxazole was observed in nine isolates. The number of isolates resistant to amoxicillin, chloramphenicol and ciprofloxacin was relatively small (only two isolates).

DISCUSSION

From the 135 fecal specimens cultured, 72 yielded *Salmonella* organisms representing an isolation rate of 53.3%. This is similar to that of Duthie and French (1990) who reported an isolation rate of 59%. The sensitivity of stool culture is believed to be lower than that of blood culture which is recommended as the gold standard for confirmation of diagnosis of typhoid (Tanyigna et al., 2001; Ameya et al., 2017). However, blood culture is more difficult and requires more expensive laboratory infrastructure and greater technical expertise (Von Kalckreuth et al., 2016). Although, stool culture may not give accurate incidence rate, the results of this study suggest that stool culture can yield adequate number of isolates for monitoring antimicrobial susceptibility profile of *Salmonella* from humans. In this study, we found a significant difference between number of isolates from male and female study participants. This is consistent with the findings of others (Ja’afar et al., 2013; Yaseen et al., 2017). The explanation for this is beyond the scope of the present work. It could be that the females are more susceptible to infection with *Salmonella* or more exposed to sources of infection. This requires further epidemiological study.

In this study, the antibiotic susceptibility profile of *Salmonella* isolates from fecal specimens of males and females indicate that strains of *Salmonella* resistant to first line drugs are common in the study area. However, we did not find strains that were completely resistant to all the antimicrobial agents. The isolates with multidrug resistant (MDR) phenotype were still highly susceptible to ciprofloxacin although considerable number of strains has developed resistance to ciprofloxacin without the MDR phenotype. The prevalence of isolates with reduced susceptibility to ciprofloxacin is also high. Resistance to the fluoroquinolones typically evolves in a stepwise fashion (Rahman et al., 2014). This means that prolonged and continuous abuse of this antibiotic could lead to full resistance to the antibiotic. This will greatly limit the treatment options for the isolates that have developed resistance to the first line drugs. The susceptibility of the isolates to ofloxacin and gentamicin stood at 65.2 and 59.7%, respectively. These antibacterial agents are not commonly used for treatment of typhoid but in an environment where self-medication is rampant and abuse of antibiotics is common, the organisms might have acquired resistance to these agents under antibiotic pressure or through horizontal transfer of resistance genes.

The results of this study indicate that *Salmonella* isolates from humans have developed resistance to common antibiotics such as amoxicillin, chloramphenicol, co-trimoxazole and this resistance has also spread to the fluoroquinolones. The decrease in the susceptibility of the *Salmonella* isolates to ofloxacin and ciprofloxacin has been reported in other parts of Nigeria (Akinyemi et al., 2014). Increased MDR has been reported among *Salmonella* isolates in many countries including Iran (Butaye et al., 2006) and Ethiopia (Zewdu and Cornelius, 2009). A previous study in Nigeria by Akinyemi et al. (2007) had reported a multi-drug resistance rate of 61.0%. The result of this study, however, revealed a comparatively high proportion of multidrug resistance with a prevalence rate of up to 90.3%.

The prevalence of multi-drug resistance reported in this study reveals a limitation of an effective treatment of human *Salmonella* infection with commonly used antibiotics. The antimicrobial susceptibility profile of *Salmonella* isolates from humans reported in this study suggests that effective treatment of typhoid fever cannot likely be achieved without antibiotic susceptibility testing of individual isolates. Therefore, diagnosis of typhoid fever by non-cultural methods and empirical antibiotic therapy without information on the antibiotic susceptibility of the causative agents are no longer appropriate. In addition, this study indicates a need for continuous monitoring of antibiotic susceptibility pattern of *Salmonella* isolates in Umuahia and the neighborhood.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

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., 2014).
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 (Maijala et al., 2001; Ooi and Lorber, 2005; Yang et al., 2007; Marnissi et al., 2013; Jamali et al., 2013; Mansouri-Najand et al., 2015; Oyinloye, 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; Abdellrazeq et al., 2014; Oyinloye, 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 studies, particularly in developing countries (de Noordhout et al., 2014).

The presence of *L. monocytogenes* in ready-to-eat (RTE) foods and the low infectious dose of the organism are a cause for concern as such foods could constitute health hazards to consumers. *L. monocytogenes* has been reported from humans, environment and various foods in Nigeria, although most of these reports have come from studies based on phenotypic and biochemical characterization of the organism (Nwaiwu, 2015). *Fura-de-Nunu*, a fermented milk-cereal mix, is a street-vended, RTE food, consumed in various parts of Nigeria, especially in Northern Nigeria. In Nigeria, about 90% of the dairy cattle belong to the Fulani agro-pastoralists and their women strictly control the processing and marketing of their milk products (Chukwuma, 2009; Okeke et al., 2014). Most of these women are illiterate and have no knowledge of critical control points of food production. *Fura* is a semi-solid dumpling millet-based meal. The millet is blended with spices and water, molded into dough balls and then cooked. To make the complete meal, *Fura-de-Nunu*, the cooked dough balls are broken up and mixed into the fermented milk (*Nunu*), to form porridge (Kordylasi, 1990; Jideani et al., 2001).

*Fura-de-Nunu*, like many other indigenous fermented foods, depends on spontaneous fermentation initiated by natural microorganisms on raw materials and equipment, the hands of producers and the local environment. In addition, some aspects of the production of this widely consumed product, such as use of leftover products as starter for fresh fermentation of the *Nunu* and molding of the *Fura* by hand, all increase the chances of contamination of the product. This study therefore aimed to determine the prevalence of *L. monocytogenes* in RTE *Fura-de-Nunu* samples, the PCR serogroups as well as virulence genes of the isolates.

**MATERIALS AND METHODS**

**Samples collection**

A total of 150 samples, comprising 75 *Fura* and 75 *Nunu*, were purchased from vendors in different communities within Nsukka metropolis. *Nunu* samples were collected in a sterile large screw capped bottles while *Fura* balls were placed in sterile polyethylene bags and transported in ice-packed containers to the Microbiology Laboratory, University of Nigeria, Nsukka for analysis.

**Isolation and enumeration of organisms**

The procedure recommended by the International Organization for Standardization (ISO) 11290 (2004) was used as follows: A 25 g representative portion from each *Fura* sample, mashed into a semi-powdery paste or 25 mL representative portion of each *Nunu* sample was introduced aseptically into a sterile conical flask containing 225 mL of Half Fraser Broth to obtain a 1:10 sample dilution. The samples were homogenized for 1 min and incubated for 24 h at 37°C. After incubation, a 0.1 mL aliquot from each Half Fraser Broth culture was added to 9.9 mL of Fraser Broth and incubated for 24 to 48 h at 37°C. Following incubation, the culture was serially diluted ten-fold and 0.1 mL from $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions was spread on *Listeria* selective agar (PALCAM; Oxoid, UK). Samples were also inoculated onto plates of Brilliance *Listeria* chromogenic agar (Oxoid, UK), selective for *Listeria* spp. The plates were incubated for 24 to 48 h at 37°C and examined for grayish colonies, typical of *Listeria*. Total viable counts were taken from plates containing countable colonies.

**Biochemical confirmation of the isolates**

Colonies showing typical morphology of *Listeria* were streaked onto Tryptone Soy Agar supplemented with 0.6% yeast extract powder (TSYE; Oxoid, UK) and incubated at 37°C for 24 h to further obtain pure cultures for identification and characterization (Alsheikh et al., 2013). The isolates were identified and characterized using the following tests: Gram staining, oxidase, catalase, motility test, sugar fermentation (Glucose, Rhamnose, Mannitol, methyl-D-mannose, xylose), and haemolysis test. Results from suspected *L. monocytogenes* isolates were compared to those of a reference *L. monocytogenes* strain ATCC13932.

**Haemolysis test**

The isolates were inoculated onto 7% Sheep Blood Agar (SBA) by piercing into the medium and incubated at 37°C for 24 h. The haemolytic zones around the colonies were examined after incubation (Alsheikh et al., 2013; Momtaz and Yadollahi, 2013).

**Christie, Atkins, Munch-Petersen (CAMP) test**

This was performed according to the procedure described by Yadav et al. (2010). A β-haemolytic strain of *Staphylococcus aureus* was
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:

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

\[
\text{NaCl \%} = \frac{\text{NaCl \%}}{\text{Weight of the sample} \times 1000}
\]

**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.

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

Where; \(M₁ = \) Weight in gram of dish with the dried material, and \(M₂ = \) Weight in gram of empty dish

**Determination of water activity (aw)**

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 Zymo-Spin IV Spin Filter 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 IIIC 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 IIIC 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 IIIC column and centrifuged at 10,000 × g for 1 min. The Zymo-Spin IIIC 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 amplicon of the prs and hlyA genes, respectively.

**Serogrouping of L. monocytogenes and detection of virulence genes**

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><em>prs</em></td>
<td>F: GCTGAGAGATTGCGAAAGAAG R: CAAAGAACCTTGATTGC GG</td>
<td>370</td>
<td>Genus <em>Listeria</em></td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td><em>lm00737</em></td>
<td>F: AGGGCTCAAGGACCTTACC R: ACGATTCTGCTTGGCATTC</td>
<td>691</td>
<td><em>Lm</em> 1/2a</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td><strong>ORF 2819</strong></td>
<td>F: AGGAAAAATGCCAAAATCGGT R: CATCACTAAAGCCTC CATTG</td>
<td>471</td>
<td>Lm 1/2b</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td><strong>ORF 2110</strong></td>
<td>F: AGTGGACAAATTTGATTGTGAA R: CATCCATCCCTTACTTTGGAC</td>
<td>597</td>
<td>Lm 4b</td>
<td>Doumith et al., 2004</td>
</tr>
<tr>
<td><strong>plcA</strong></td>
<td>F: CTGGTTGATCCTGTTCCATCCCC R: CATGGGTTCCTACTCTTCTAC</td>
<td>1484</td>
<td>plcA gene</td>
<td>Motmaz &amp; Yadollahi, 2013</td>
</tr>
<tr>
<td><strong>hlyA</strong></td>
<td>F: GCAGTTGGCAAGGCTTGGATGGAA R: GCCAGGTATCCTCCAGGTAGT CG</td>
<td>456</td>
<td>hlyA gene</td>
<td>Swetha et al., 2012</td>
</tr>
<tr>
<td><strong>iap</strong></td>
<td>F: ACAAACTGCACCTTGTGGAC R: TGACAGCGTGATGTA AGCA</td>
<td>131</td>
<td>iap gene</td>
<td>Swetha et al., 2012</td>
</tr>
</tbody>
</table>

* Lm = *L. monocytogenes*.

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., 2013; Marnissi et al., 2013; Mansouri-Najand et al., 2015; Shamloo et al., 2016), but lower than 78% reported from *Nunu* and some other African countries. The mean *Listeria* counts for the samples in this study were $7.7 \times 10^4$ CFU/g and $8.6 \times 10^4$ 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; Ikeh 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 serogroups 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).

<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.
(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 Ciccio 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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Emphasis on functional properties of cocoa-specific acidifying lactic acid bacteria for cocoa beans fermentation improvement

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Lactic acid bacteria (LAB) strains isolated from six main Ivorian cocoa producer regions were investigated based on their biochemical properties in order to select the best one as potential starter. Three main technological and useful properties for good cocoa beans fermentation were monitored among the 568 isolated LAB strains. Thus, between the 408 cocoa-specific acidifying LAB strains identified, 05.88% (24 isolates) of them were able to maintain this activity in pH and temperature conditions as same as fermentation heap with an acidifying rate ranged within 0.03±0.00 to 1.19±0.070%. All these 24 cocoa-specific acidifying LAB strains displayed the ability to degrade the citrate while six (25%) of them were able to produce acetoin. These six LAB strains namely T1Gb8, T6C5, T6C12, T7C8, T9C9 and T11C9 were identified as Lactobacillus plantarum. They were the best able to continuously produce lactic acid in the required standards. Firstly under varying condition of temperature (30 à 45°C) and pH (3 à 6) with sharp acid (acetic, lactic and citric acids) influence. Secondly beyond 8% of ethanol content. The highlighted properties in this study and the performance of these six strains may make them best candidate as starters for cocoa fermentation control.

Key words: Lactic acid bacteria, cocoa-specific acidifying, technological properties, starter, Ivorian cocoa, fermentation.

INTRODUCTION

Cocoa beans constitute a basic export product for many tropical countries in America (Central and South), Asia (South and Southeast) and mainly West Africa. Export of raw cocoa beans is of great economic importance for West African countries which supply two thirds of the world's cocoa crop. Côte d'Ivoire is the world's leading producer of cocoa beans since 1978 (Deheuvels, 2003). Today, this country is highly dependent on this crop.

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which accounts for 50% of national export income and 22% of the Gross Domestic Product (ICCO, 2016). Cocoa beans are an important source of cocoa powder and the principal raw material of chocolate. The sensory and physical properties of West African cocoa make it a much sought-after product, although overall production quality seems to be declining (Afoakwa et al., 2013). Unfortunately, ensuring the marketable quality of cocoa beans remains difficult due to variability in the organoleptic quality of this product. This inconsistency in cocoa quality is mainly linked to post-harvest processing including fermentation, drying and roasting of the crude and fresh cocoa beans. Fermentation is considered as the most important processing step influencing the quality of cocoa beans. Indeed, during this process, there is initiation of flavor precursors, color development and a significant reduction in bitterness and astrignency of the beans (Afoakwa et al., 2013). These changes involve the action of diverse microorganisms naturally found in the cocoa pulp. The succession of these microbial groups has been clearly established and lactic acid bacteria (LAB) population is considered as the second microbial group growing in mucilaginous pulp of cocoa beans after yeast population (Crafack et al., 2013). This micro-biota group is mainly dominating until 24 to 48 h of fermentation (Kouame et al., 2015). The most important roles of LAB are the degradation of the citric acid in the pulp, leading to increase in pH, the production of ethanol and both volatile and non-volatile organic acids such as acetic and lactic acids (Penia et al. 2013). Organic acid will diffuse into the beans cotelydon and induce the enzymatic reactions to form precursor of flavor, aroma and color (Afoakwa et al., 2013). The diffusion of certain amount of acid into the bean during fermentation is essential for the development of flavor precursors. Indeed, the presence of excessive acidity usually correlates with poor development of chocolate flavor (Serra et al., 1997). Thus, acidity is a critical factor in defining the quality of cocoa used in chocolate manufacturing. The control of cocoa fermentation process is very challenging for farmers in cocoa producing countries because cocoa fermentation has always been a natural process which sometimes results in poorly fermented cocoa. Therefore, studies suggest the use of starter microbial culture as the best approach to improve the fermentation process. In this perspective, LAB strains as monoculture or co-culture would be essential component of starter culture aimed at the control of cocoa beans fermentation process. Studies conducted by different authors, such as Papalexendratou et al. (2013), Penia et al. (2013) etc., on cocoa fermentation showed that the maximum rate of lactic acid produced ranged between 0.5 and 1.2%. As excessive acidity, mainly lactic acid, produces unsuitable raw cocoa for chocolate making, it appears more judicious to isolate, screen and characterize LAB strains with cocoa-specific acidifying potential included in this limit of 1.2%. These strains may positively and durably influence the quality of a controlled cocoa fermentation especially if they provide useful additional properties such as citrate degradation and acetoin production (Rodriguez-Campos et al., 2012). Information on cocoa-specific acidifying LAB strains with adequate lactic acidifying potential, citrate degradation and acetoin production may be a starting point for the development of starter culture. Thus, the aim of the research was to select strains of LAB that best meet these different criteria in order to optimize and standardize the cocoa fermentation process.

**MATERIALS AND METHODS**

**Fermentation condition**

Cocoa pods were harvested on farms from six cocoa producing regions of Ivory Coast: Cavally (6° 25’ 0” North 7° 33’ 14” West), Gôh (6° 05’ 19” West), Haut-Sassandra (7° 0’ 0” North 6° 34’ 59 West), San-Pedro (4° 44’ 54 North 6° 38’ 10 West) and Tonkpi (7° 24’ 45” North 7° 33’ 14” West). Spontaneous cocoa bean fermentation was performed using traditional conditions, with temperature and humidity ranging respectively, from 28 to 30°C, and 60 to 65% for 6 days. The fermenting mass (50 kg) set on banana leaves and covered with banana leaves were constituted of mixed genotypes (Forastero, Trinitario and Criollo cultivars).

**Sampling**

The fermenting heap was mixed and 100 g beans were collected, at the beginning and each 12 h of fermentation, in sterile Stomacher bag. A total, 13 samples of each fermenting cocoa according to the locality were collected for physico-chemical and microbial analysis.

**Isolation of bacterial strains**

Isolation of LAB was performed according to the standard method described by Pereira et al. (2012). An amount of 25 g of fermenting cocoa beans was homogenized in 225 mL sterile peptone water in a Stomacher bag (Seward, Worthington, United Kingdom) for 5 min at room temperature. After appropriate dilution of samples in sterile saline solution, 0.1 mL from each dilution was inoculated onto duplicate plates of MRS agar (Oxoid) supplemented with 50 μg/mL of nystatin to inhibit the fungal growth. Plates were incubated at 30°C for 48 to 72 h, under anaerobic conditions.

**Preliminary biochemical test of lactic acid bacteria**

LAB isolates, identified using conventional colonial morphology, gram staining and biochemical reactions (oxidase and catalase tests), were stored in cryotubes at -20°C in MRS broth supplemented with 20% glycerol for further investigations.

**Screening of cocoa-specific acidifying LAB strains**

Cocoa-specific acidifying LAB strains were screening based on their acidification capacity at 30°C according to the protocol of Guha et al. (2013). 100 μL of a preculture recording an optical
density (OD) of 1 at 600 nm against a control (MRS broth) were used to inoculate 5 mL of MRS broth. The seeded broths were incubated at 30°C in a water bath (Julabo TWB 12) for 48 h. Then, fermented broths were centrifuged at 4500 trs/min for 10 min to pellet out the bacterial growth. Supernatant was used to estimate lactic acid content by titrimetric method with 0.1 NaOH. The percentage of lactic acid was calculated using the formula in equation 1. Each treatment was tested in triplicate.

\[ % \text{Lactic acid} = \frac{V(\text{NaOH}) \times N \times 0.09 \times 100}{V(\text{test})} \]  

(1)

\[ V(\text{NaOH}) : \text{Volume of NaOH solution used for titration} \]
\[ N : \text{Normality of the NaOH solution (0.1)} \]
\[ V(\text{test}) : \text{Sample Volume} \]

**Analysis of additional properties of cocoa-specific acidifying lactic acid bacteria isolates**

**Screening of LAB strains for citrate degradation**

The ability of lactic acid bacteria to degrade citrate was investigated using the method described by Kempler and McKay (1980). A basal medium containing 1% milk powder, 0.25% casein peptone, 0.5% glucose and 1.5% agar was first prepared and then 1L of the medium, was supplemented with 10 mL of potassium ferricyanide (10%) solution and 10mL of mixed iron citrate and sodium citrate (2.5%) solution. The medium was inoculated with pure 24 h preculture of LAB strains and incubated in an anaerobic jar at 30 °C for 48 to 72 h. The citrate metabolism was assessed by the formation of a blue complex surrounding the colonies.

**Screening of LAB strains for acetoin production**

The production of acetoin was investigated using the method described by King (1948). Acetoin production from citrate was checked in the basal liquid medium. Clark and Lubs medium was seeded with pure 24 h preculture of LAB strains and incubated at 30°C for 48 h. After incubation time, acetoin production was revealed in 1mL of cell free supernatant by adding two drops of an alcoholic solution of alpha-naphthol (6%) and two drops of sodium hydroxide (16%) solution. The presence of acetoin was assessed by appearance of red color.

**Effect of environmental stress on lactic acid production by cocoa-specific acidifying LAB strains**

**Influence of temperature and pH**

The influence of temperature and pH on the acidification capacity of cocoa-specific acidifying lactic acid bacteria strains was evaluated by Guha et al. (2013) method. Seeded broths were incubated at 30, 35, 40, 45 and 50 °C to assess the influence of temperature and at pH 3; 4; 5 and 6 for pH influence, in a water bath (Julabo TWB 12) for 48 h.

**Influence of alcohol and organic acids on lactic acid production by cocoa-specific acidifying LAB strains**

To analyze the acidification potential of cocoa-specific LAB strains in alcoholic and acidic conditions, a MRS liquid medium was prepared. After autoclaving, this medium was cooled and maintained in liquid state at 45 °C and supplemented with alcohol (2, 4, 6 and 8%), acetic and lactic acid (0.2; 0.4; 0.6; 0.8; 1; 1.2%), citric acid (0.5; 1; 1.5; 2; 2.5 and 3%). The standard inoculum was prepared as follow: a 18 h preculture was suspended in a saline solution to obtain an absorbance OD 1 at 600 nm. 100 μL were used to inoculate 5 mL of MRS broth. The seeded broths were incubated at 30°C in a water bath for 48 h. After incubation time, the amount of total acid produced by each strain was determined by titration as previously described.

**Molecular characterization of lactic acid bacteria starters**

**16S rRNA gene PCR and sequence analysis**

To perform PCR reactions, The isolates of LAB strains were grown for 24 h at 30°C on agar plates. A loopful of pure culture was suspended in 100 μL of sterile distilled water for colony PCR.

**PCR amplification of ribosomal 16S gene**

The hypervariable regions (V1, V2 and V6) of the different LAB strains were amplified by using universal primers F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R520 (5'-ACGGCGGCTGCTGGC-3') (Ouattara et al., 2011) for colony PCR. Each mixture (final volume 50 μL) contained about 1 μL of sample 1.25 U of Taq DNA polymerase (Biolabs, Lyon France), 5 μL of 10x Taq Buffer (10 mM TrisHCl, 50 mM KCl, 1,5 mM MgCl2), 1 μL of 10mM dNTP, 2 μL of each primer (10 μM) (Eurofins, Genomics, Germany) supplemented by sterile water Milli-Q. PCR amplification was carried out in a thermocycler (Sensoquest Labcycler) as described previously (Ouattara et al., 2011). After an initial denaturation at 95 °C for 4 min, reactions were run for 35 cycles. Each cycle comprising denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1min; and final extension at 72 °C for 10 min.

**Gel electrophoresis**

After migration of the products realised at 70 V for 2 h in a tank, amplicons were revealed by incubating on 0.8% agarose gel electrophoresis in a 1x phosphate TAE buffer containing bromide of ethidium. The molecular weight of the products was estimated in reference to a molecular weight marker (Biolabs, France) of 500 bp.

**Sequencing and analysis of hypervariable sequence of 16S RNA gene amplified**

The PCR products were purified using the nucleospinR Gel and PCR Clean-up kit (MachereyNagel, Germany). Then, they were sequenced by MWG Eurofins using the primer F27. The basic local alignment search tool (BLAST, blast N) from the NCBI database site (blast.ncbi.nlm.nih.gov/ BLAST/) was used to find the closest sequences relative to the amplified 16S RNA genes in order to identify our LAB strains.

**RESULTS AND DISCUSSION**

**Acidification capacity of LAB strains isolates**

A total of 568 strains characterized as gram positive, oxydase negative and catalase negative identified as
LAB were isolated from six Ivorian regions. Thus, these LAB strains were analysed for their capacity to acidify the fermentative medium. The results showed that the number of isolated LAB strains have unequal distribution depending on the region. San Pédro recording the highest (21.47%) number of isolates (Table 1) unlike Gôh, which has the lowest number (08.45%) of strains. This variability indicated the influence of the local geographical area on the composition of the microflora involved in cocoa fermentation (Schwan and Wheals, 2004). Therefore, this factor could impact on the distribution of their different activities. One of the important activities attributed to LAB strains is to acidify the fermentative medium. As this property is susceptible to play a key role in the fermentative process (Afoakwa et al., 2013), it appears important to screen them based on this ability. Most importantly, this type of metabolic pathway should be determined to identify potential starters among these isolates.

All the 568 isolated LAB microflora which were screened for their acidification capacity showed naturally acidification capacity with different levels of acid production (Table 1). Indeed, this natural capacity for acidification of the fermentative medium by LAB is related to the fact that they are able to exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is lactic acid which is the main metabolite of two broad metabolic categories of LAB. The first metabolic category is represented by the homofermentative LAB. They convert glucose into lactic acid more than 90% by the Embden-Meyerhof (E-M) pathway. The second metabolic group is represented by the heterofermentative LAB producing about 50% of lactic acid as the principle by-product of sugar fermentations (Raimbiault 1995).

It should be noted that a acidification scale has been established based on the acidification capacity of LAB from different fermentation medium such as milk, cheese, wine, fish, meat, sausages, butter and cream (Behannis and Kayanush, 2012; Joko et al., 2014). The recorded lactic acid values (around twenty) ranged between 0.05% and 1.6% and allowed to identify three types of LAB strains according to their capacity of acidification: LAB strains with low acidity capacity (acidification rate (AR) < 0.45%); LAB strains with medium acidity capacity (0.45 < AR ≤ 1.35%) and those with high acidity capacity (AR ≥ 1.35%).

The results obtained are depicted in Table 1. Based on this classification, three types of LAB strains were observed in the different regions except in Cavally and Tonkpi with respect to the low lactic acid producing strains and the regions of Haut Sassandra and San Pédro with respect to the high producers. In addition, medium acidifying LAB strains dominated with 57.57% compared to 24.12% for high acidifying LAB strains and only 18.31% for low acidifying LAB strains. This diversity in acidifying capacity of LAB strains suggesting a difference in acidification potential and therefore efficiency in acidification by these strains. This could be the main determining factor in the variability of cocoa bean quality as cocoa beans acidification often has negative impact on the final quality of the market cocoa.

Therefore, the cocoa market could ultimately be related to cocoa bean quality which is dependant on selective LAB strains with satisfactory level of acidification. This approach could make it possible to ensure the control of the acid level of marketable beans and thus the control of their quality. With regard to lactic acid, the satisfactory level of acidification can only be obtained with LAB strains that do not have high acidification capacity. Indeed, the low volatility of this acid promotes its accumulation in beans. If this acid is produced in large quantities, it can hardly be eliminated. This will irremediably lead to acidic beans and therefore of poor quality. Thereby, LAB strains with low and medium acidification capacity (431) retained our attention,
approximately two-thirds of LAB strains isolated.

Then, a second level of selection has been established to better refine our selection. This selection takes into account the maximum rate of lactic acid produced during the fermentation of cocoa. Studies conducted by Afokawa (2013), Kouame et al. (2015) and Papalexendratou et al. (2013; 2015) on cocoa fermentations, around the world, lead to the identification of a maximum rate of lactic acid produced of 1.2%. Thus, in order to mimic the natural conditions of fermentation, it is important that the selected LAB strains do not produce lactic acid beyond this limit (1.2%). The results of this selection are shown in Table 1. Among the 568 strains of LAB previously isolated, approximately two-thirds (71.83%) produced an amount of lactic acid that not exceed 1.2%. This total of 408 LAB strains applies to all the strains with low acidifying capacity (104) and part of the medium acidifying strains (304 of 327). These strains are considered to be "Cocoa-specific acidifying LAB strains". As high acidity, mainly lactic acid in cocoa beans produces unsuitable raw cocoa, these 408 Cocoa-specific acidifying LAB strains appear to be the best candidates as potential starter culture to control cocoa bean fermentation.

### Table 2. Influence of temperature on lactic acid production by Cocoa-specific acidifying LAB strains per region.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Number of LAB strains with acidifying potential ≤1.2% at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C (%)</td>
</tr>
<tr>
<td>Cavally</td>
<td>036 (08.82)</td>
</tr>
<tr>
<td>Gbôkli</td>
<td>078 (19.12)</td>
</tr>
<tr>
<td>Gôh</td>
<td>028 (06.86)</td>
</tr>
<tr>
<td>H.S.</td>
<td>107 (26.23)</td>
</tr>
<tr>
<td>San-Pedro</td>
<td>121 (29.66)</td>
</tr>
<tr>
<td>Tonkpi</td>
<td>038(09.31)</td>
</tr>
<tr>
<td>Total</td>
<td>408 (100)</td>
</tr>
</tbody>
</table>

**Influence of temperature and pH on lactic acid production by Cocoa-specific acidifying LAB strains**

The pH of cocoa beans during fermentation is critical with regard to the biochemical reactions that take place in the beans (Afokawa et al., 2013). The ability of the 408 Cocoa-specific acidifying LAB strains to maintain their acidifying potential ≤1.2% at temperatures up to 45°C is depicted in Table 2. Only 104 strains maintained their acidifying potential under 45°C of temperature among the 408 strains previously selected.

Cocoa fermentation is generally characterized by high temperature up to 45°C occurring between 48 and 72 h of fermentation (Ho et al., 2014). In view of these results, we can assess that the thermo-tolerance exhibited by these 104 heat-resistant strains should allow them to maintain a satisfactory level of lactic acid production under thermic stress conditions during cocoa fermentation.

On the other hand, the effect of pH variations on these 104 heat-resistant strains, showed that only 24 of them were able to continuously maintain their acid production for pH variations ranging from 3 to 6 (data not showed). These pH values are those prevailing in cocoa beans fermentation within 24 to 72 h of fermentation (Kouame et al., 2015). Table 3 highlights the amounts of lactic acid produced by these 24 cocoa-specific acidifying LAB strains under pH fluctuation.

Regarding the rate of lactic acid produced by these Cocoa-specific acidifying LAB strains, values are ranged from a minimum of 0.03 to a maximum of 1.19 for pH values between 3 and 6 (Table 3). These findings pointed out the fact that these Cocoa-specific acidifying LAB strains did not have the same capacity of adaptation to temperature and pH fluctuations of the fermentative heaps even if they are able to maintain their lactic acid production in the required standards.

**Additional activity of isolated Cocoa-specific acidifying LAB strains: citrate degradation and acetoin production**

Citrate degradation and acetoin production are important and relevant properties desirable for a good cocoa fermentation (Schwan and Wheals, 2004). Thus, biochemical characterization of the 24 Cocoa-specific acidifying LAB strains showed that they exhibited the property to break down citric acid. Additionally, only six (25%) of them displayed both the ability to degrade citrate and to produce acetoin.

The screening of citrate consumers among these cocoa-specific acidifying LAB strains indicates that all of them are able to breakdown citrate. Citrate metabolism constitutes, an important and particular property, since LAB strains are not usually able to utilize citric acid as carbon source (Kouame et al., 2015). As citrate metabolism is an essential factor for modulating the pH
and promoting the growth of desirable bacterial flora in fermenting cocoa (Schwan and Wheals, 2004) all these 24 LAB strains should be responsible for the raising of the pH recorded during cocoa beans fermentation.

In addition, acetoin production shows that these six cocoa-specific acidifying LAB strains (TgGb8, TgC5, TgC12, TgC8, TgC9 and T11C9) were able to metabolized citric acid via pathways pH producing acetoin from citrate. This ketone carboxylic compound (such as acetoin) is crucial for the development of good cocoa flavor. Indeed, Rodriguez-Campos et al. (2012) indicated that acetoin appears to be a precursor of tetramethylpyrazine, an important odor-active component of cocoa flavor. Therefore, these six LAB strains are of interest for the fermentation process as potential starter.

16S rRNA gene PCR and identification of isolates

Amplification of hypervariable regions (V1, V2 and V6) of the 16S gene of six potential starters LAB by colony PCR yielded a DNA fragment with respect to the 500bp (Figure 1). Analysis of these DNA fragments in the NCBI data bank led to the identification of these six LAB (TgGb8, TgC5, TgC12, TgC8, TgC9 and T11C9) isolates as belonging to Lactobacillus plantarum with 99% of similarity. L. plantarum has been isolated from many cocoa fermentations around the world (Camu et al., 2007; Pereira et al., 2012). This observation indicates that these strains play key roles in cocoa bean fermentation. L. plantarum species has also been used as starter for cocoa fermentation (Joko et al., 2014; Penia et al., 2013) and are widely used in food industries to make other fermented foods (Joko et al., 2014).

Influence of additional stress on lactic acid production by isolated Cocoa-specific acidifying LAB strains.

In addition to pH and temperature, other factors such as acids (acetic, lactic and citric) and alcohols (ethanol) are key parameters that may strongly influence the performance of the LAB strains in lactic acid production. Thus, the six selected LAB strains (TgGb8, TgC5, TgC12, TgC8, TgC9 and T11C9) and identified as Lactobacillus plantarum were tested to assess the influence of these key parameters on their ability to maintain lactic acid production.

Effect of acids (acetic and lactic acids) on lactic acid production

In acid conditions induced by lactic, acetic and citric acids, the six selected LAB strains proved to be able to maintain their potential of lactate acid production despite the observed decline. Lactic acid production occurred in medium containing lactic and acetic acid in the same range 0-1.4% for the six strains belonging to Lactobacillus plantarum (Figure 2 A and B) while this acid production continues up to 1.6% of acetic acid in the medium. The influence of citric acid on lactic acid production was less than the other tested acids (Figure 2C). Lactic acid production was slowly decreased with increasing of citric acid concentrations in a range of 0-3%. All Lactobacillus
plantarum tested strains maintained their ability to produce lactic acid in proportions ranging from 35 to 66.96% of the initial level with a maximum of lactic acid (between 1.12 and 1.35%) produced under 0.5% of citric acid in the medium. The effect of acids results in a reduction of cytoplasmic pH that is likely to impair lactic acid production (Lopez and Dimick, 1995). This indicates that, compounds present at variable concentrations in the cocoa pulp such acids may be limiting factors for lactic acid production in LAB strains during cocoa fermentation. However, with their ability to maintain lactic acid production despite pH fluctuations between 3 and 6 in cocoa beans fermentation, all the six selected LAB strains should be particularly interesting as starters.

**Effect of ethanol on lactic acid production**

The influence of ethanol concentration on lactic acid production is shown in Figure 2D. The results suggested
that, all these six studied strains maintained a good level of lactic acid production until 8% of ethanol concentration. This lactic acid production ranged between 43.50 and 91.67% of their initial potential production. These results are similar to those described by Behannis et al. (2012) with a decrease in acid produced when ethanol concentration increased from 2.5 to 7.5%. These results are interesting because ethanol is a major metabolite of cocoa pulp fermentation and the maximum rate of ethanol produced by yeasts is around 8% during this process (Lefebre et al., 2012). High concentrations of ethanol reduce cellular vitality and increase cell death resulting in a gradual decline in lactic acid production (Birch et al., 2000). Thus, these six ethanol tolerant isolates (T₅G₅b, T₆C₅, T₇C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) at high ethanol concentration, could both maintain their citrate and acetoin metabolism and their lactic acid production in the required standards to improve the final fermenting cocoa bean quality.

Conclusion

In this study, six LAB strains (T₅G₅b, T₆C₅, T₇C₁₂, T₇C₈, T₉C₉ and T₁₁C₅) involved in Ivorian cocoa fermentation were identified as Lactobacillus plantarum. These particular cocoa-specific acidifying bacteria presented both the required acidifying capacity and in a lesser extent, citrate degradation and acetoin production. All of them were able to maintain their lactic acid production under fermentative stress conditions in the required standards. That makes them suit candidate as starter for cocoa fermentation control.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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