Isolation and Identification of bacteria associated with spoilage of sweet potatoes during post harvest storage

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Abstract
Post harvest spoilage of sweet potatoes is a major problem in Western Nigeria; this has resulted in losses of the vegetable crop over the years. The present study focused on characterization and identification of bacteria associated with the crop during post harvest storage, using phenotypic and molecular methods. Confirmation of full identities of the bacteria was done using 16S rDNA sequencing. Among the thirty two bacteria identified during post harvest storage, two strains Staphylococcus sciuri (TSADCA1) and Rahnella sp. (TSADCA2) showed pronounced physical symptoms of spoilage of the sweet potatoes. Enzyme study indicated that these two strains produced carbohydrate degrading enzymes, particularly polygalacturonase, cellulose and xylanase which may have contributed to spoilage of the vegetable crop during storage. The study concluded that the two strains of spoilage bacteria should be taken into serious consideration during any proposed preservation methods of post harvest storage of sweet potatoes, especially in the Western part of Nigeria where the crop is mostly cultivated. This is the first report on the isolation and identification of these two strains of bacteria as being associated with spoilage of sweet potatoes, and they are therefore novel spoilage bacteria of the vegetable crop.

Keywords: post harvest spoilage; sweet potatoes; vegetable crop; characterization; spoilage bacteria

INTRODUCTION
Sweet potato (Ipomoea batatas) is one of the most important crops worldwide including wheat, rice, maize, potato and cassava (Truong et al., 2011). Sweet potatoes possess many positive health benefits including sources of anthocyanins, phenolic compounds and other bioactive compounds (Giusti and Wrolstad, 2003; Suda, et al. 2003; KANO et al. 2005; TRUONG et al. 2011). The vegetable crop also possess antioxidant activities (Giusti and Wrolstad, 2003; Suda et al., 2003; Kano et al., 2005). Different species of sweet potatoes have gained popularity in many countries as a result of their health benefits (Lekrisompong et al., 2012). The crop tubers have been consumed in various forms in developing countries of Africa, including eating in cooked form, incorporation with yam during pounded yam preparation, processing into flours along with yams for edible dough (such as amala in Nigeria) etc.

Food spoilage is a metabolic process which may be brought about by microbial action and causes foods to be undesirable or unacceptable for human consumption due to deterioration in quality characteristics (Doyle, 2007). This type of spoilage is commonly associated with sweet potatoes resulting in postharvest losses which are manifested by a loss of quantity and/or quality due to pathological, physiological and mechanical damages (Coursey and Booth, 1972). In most vegetable crops, including yams and sweet potatoes, these factors are interrelated since mechanical injury and physiological stress may greatly influence the susceptibility of the tubers to diseases. Spoilage microorganisms can be introduced to the crop during crop growth in the field, during harvesting and postharvest handling, or during storage and distribution. Therefore, early intervention measures during crop development and harvesting through the use of good agricultural practices will provide dramatic reductions in yield loss due to spoilage at all subsequent steps (Barth et al., 2009).

It has been noted that the largest postharvest losses in vegetable crop tubers result from microbial attacks (Ray et al., 2000). Reports are available on the fungi associated with spoilage of sweet potatoes during post harvest storage (Ray et al., 2000; Doyle, 2007); however, to the best of our knowledge, report on spoilage by bacteria of the vegetable crop has been very scarce. The present paper therefore reports findings on the spoilage bacteria associated with
sweet potatoes during post harvest storage. Investigation on enzyme activities which may have contributed to tissue degradation of the crop by bacterial action also forms part of this report.

MATERIALS AND METHODS

Source of sweet potatoes

Fresh healthy sweet potatoes were obtained from the retail market in Offa, Western Nigeria, from where they were transported in sterile containers to the laboratory.

Post harvest storage of sweet potato samples

Fresh sweet potato samples were distributed into sterile plastic containers and stored at 29ºC (approximate ambient temperature in Nigeria) for a period of four weeks.

Enumeration and isolation of bacteria during post harvest storage

Sterile swab sticks were used to swab the storage environment during post harvest storage, and these were transferred into sterile containers and brought into the laboratory for analysis. Samples of sweet potatoes were taken weekly during the four week storage for bacterial isolation and enumeration. Microbiological analyses were performed using tryptone soya agar (TSA) and perfringes agar base (PAB). Plates were prepared in replicates and incubated at 37ºC for 36 - 48 h. Emerging colonies on the plates were counted and counts expressed as colony forming units per millilitre (CFU/ml). Some of the distinct colonies were sub-cultured severally until pure cultures were obtained. The pure cultures were subjected to gram staining, catalase and oxidase tests using the methods described by Downes-Keith-Ito (2001).

Identification of bacteria using 16S rDNA genes

Extraction of bacterial genomic DNA: The method of Pitcher et al. (1989) was adopted with modifications. Cells were harvested from an overnight culture plate using sterile swaps into an Eppendorf tube containing sterile reverse osmosis (RO) water. This was mixed well and centrifuged at 13000 g for 2 min in a micro-centrifuge (Biofuge pico Heraeus D-3752O O steroide) and the supernatant was carefully discarded. Cells were washed in a mixture of 100 µl ice cold TE buffer (10mM Tris-HCl, 1mM EDTA pH 8) containing 5 µl lysozyme (50mg/ml; Sigma-Aldrich, United Kingdom), and centrifuged at 13000 g for 2 min. The supernatant was carefully discarded and cells were resuspended in the mixture of fresh TE buffer and lysozyme.

GES solution (5M guanidium thiocyanate, 0.5M EDTA pH 8; 500 µl) was added and the mixture was mixed well by vortexing and then incubated for 10 min at 25ºC. The mixture was chilled on ice for 2 min and 250 µl cold 7.5 M ammonium acetate was added, mixed well and incubated on ice for another 10 min. Thereafter, 500µl of chloroform-isooamylalcohol was added, mixed thoroughly and spun at 13000 g for 10 min. Of the upper phase layer, 850 µl was transferred to a clean Eppendorf tube and 495 µl of ice cold isopropanol was added. This was mixed by repeated reverse-inversion for 1 min and then centrifuged at 13000 g for 10 min to bring down the DNA. The supernatant was removed carefully and the pellet (DNA) was washed three times in 70% ethanol. After the third wash, the ethanol supernatant was removed carefully and the pellet was vacuum-dried for 5 min and stored at -20ºC until use.

Amplification of bacterial 16S rDNA genes: Amplification of the 16S rDNA genes (V3 region) by polymerase chain reaction (PCR) was carried out as described by Danilo et al. (2003) with some modification. A 50 µl (final volume) of the PCR mixture was prepared as follows: 5 µl MgCl2 (2.5 mM) 1.25 µl dNTPs (0.25 mM), 5 µl 10x PCR buffer (750 mM Tris-HCl pH 8, 200 mM (NH4)2SO4, 15 mM MgCl2 (final concentration), 0.1 µl each of V3 forward primer and reverse primer, 0.1 µl DNA template and 1.25 units Taq polymerase (Promega, UK). Volume was made up by addition of sterile RO water. The pair of universal primer used consist of forward primer 5’-AGA GTT TGA TC AC TGG CTC AG-3’ and reverse primer 5’- AAG GAG GTG ATC CA AGCT CC AG CA-3’ (Muyzer et al., 1993). The mixture was subjected to an initial denaturation temperature of 94ºC for 5 min, 40 cycles each of 1 min denaturation at 94ºC, 1 min annealing at 42 ºC, and 1 min elongation at 72 ºC, steps. Reactions were terminated with a final extension step at 72ºC for 10 min. The amplification process was carried out in a Thermal Cycler (Progene, UK). Electrophoresis of the amplified 16S rDNA PCR products was performed on the Bio-Rad Contour-Clamped Homogenous Electric Field DRII electrophoresis cell (Hemel Hempstead, UK), using 1.5% (w/v) agarose gel (general purpose, Biogene, Kimbolton Cambs, UK) in 0.5×TAE buffer at 84 V for 1.5–2 h. A 100-bp (Promega G210A, Madison, WI, USA) ladder was used as a molecular size marker.

Purification and sequencing of amplified 16S rDNA PCR products: The QIAquick PCR purification kit (Promega, USA) was used, following the manufacturer’s instructions. An addition of 5 volumes of buffer PB (5 M Guanidinium-HCl, 10 mM Tris-HCl, pH 6.6, 30% ethanol) was made to 1 volume of the PCR sample and mixed. The mixture was then applied to the QIAquick column and centrifuged for 60 seconds, after which the overflow was discarded. The underflow containing the DNA was washed with 0.75 ml of buffer PE (25 mM NaCl, 5 mM Tris-HCl pH 7.5, 75% ethanol) and centrifuged for 60 sec. The supernatant was carefully removed and the remaining content was further centrifuged for 1 min after which 50 µl of Buffer EB (50 mM Tri-Cl, 1.4 M NaCl, 15% (w/v) ethanol) was added and centrifuged for an additional 1 min to elute the DNA. The purified DNA was sent to Germany (MWG-Biotech, Ebersberg, Germany) for sequencing. The specific nucleotide sequences obtained were subjected to Basic Local Alignment Search Tool (BLAST) search programme at GenBank (http://www.ncbi.nlm.nih.gov/blast/) to determine the closest known relatives of the isolates.

Test for spoilage potential of the bacterial isolates

The potential of the bacterial isolates to bring about spoilage of sweet potatoes was tested by employing reported methods, with modifications (Ekundayo and Daniels, 1973; Ikediegwu and Ejale, 1980). Fresh sweet potato tubers were surface sterilized in 10% (v/v) Sodium hypochlorite solution for 1 h. They were rinsed with six changes of sterile RO water to remove the residual effect of sodium hypochlorite. A sterile 5mm cork borer was used to remove cylindrical cores (1 cm) from the middle portion of the tuber. Each well was inoculated with 100 µl

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(-1x10⁹ cfu/ml) and cores were immediately replaced and sealed off with sterile petroleum jelly. Some of the cores were inoculated with same amount of sterile maximum recovery diluent (MRD) to serve as control. Additionally, tubers were cut into slices and surface sterilized as above. The sterilized surfaces were inoculated with 100 µl of each bacterial isolates. All inoculated tubers were distributed into sterile plastic containers and incubated at 29°C for 4 weeks. The sweet potato tubers were inspected weekly to detect spoilage.

**Evaluation of enzyme activities of bacterial isolates**

The abilities of the bacterial isolates to produce different enzymes capable of degrading sweet potato tissues were evaluated. Selected bacterial isolates were grown in tryptone soya broth medium, supplemented with 2% (w/v) mashed sweet potato, in a shaking incubator for 9 days at 37°C. Daily, 1 ml of the culture medium was collected and centrifuged at 13000 rpm for 3 min; the culture supernatant (CS) was carefully withdrawn and the sediment discarded. The CS represents the enzymes secreted in response to the sweet potato substrate. As a control, a similar volume of the CS was boiled, to inactivate any enzyme present, in a water bath for 20 min to obtain boiled culture supernatant (BCS). The enzymes analysed include cellulase, amylase, xylanase, polygalacturonase, glucanase, xylosidase, arabinofuranosidase and ferulic acid esterase, using the modified method of Bernfield (1955). The substrates used for the respective enzymes were carboxy-methyl cellulose (Acros Organics, UK), starch (Fisher Scientific, UK), birch wood xylan (Sigma-Aldrich, UK), polygalacturonic acid (Alfa Aesar, UK), glucon (Sigma Aldrich, UK), p-nitrophenol xylopyranoside (Fisher Scientific, UK), p-nitrophenol α-L- arabinofuranoside (Fisher Scientific, UK) and methyl ferulate (Sigma Aldrich, UK). One molar potassium hydrogen phosphate (pH 6.5) was used as buffer. Two millilitre of the CS was mixed with 1 ml of 1% substrate solution and incubated at 40°C for 1 h. The reaction was stopped by the addition of 3 ml dinitrosalicylic acid (DNSA) reagent. The mixture was boiled in a water bath for 5 min, cooled in cold water and then diluted with 18 ml water; the absorbance of the resultant solution was measured at 550 nm. The control (BCS) was subjected to similar treatment. The amount of the reducing sugars formed was calculated from a standard curve prepared with known concentrations of substrates.

**RESULTS AND DISCUSSION**

There is very scarce information on the identification of bacteria associated with post harvest storage of sweet potatoes. Most of the reported cases have only identified fungi (Ray et al., 2000; Doyle, 2007). In the present report, bacteria were isolated from sweet potato tubers during post harvest storage. Fresh sweet potatoes were stored at 29°C to mimic ambient temperature in Nigeria for four weeks. During this period, samples were taken for microbiological analysis – bacterial characterization and identification. Identification was initially based on their phenotypic characteristics while full identities were obtained molecularly by 16S rDNA gene sequencing. Fully identified isolates were tested for their abilities to bring about spoilage in the tuber crop. During spoilage test experiments, probable production of various enzymes that could be responsible for degradation of the tuber crop tissues was evaluated. Overall, thirty two bacteria were isolated from the sweet potatoes through phenotypic methods; some of the isolates shared similar biochemical characteristics, implying that they could be same species of organisms, as later revealed by result of molecular methods. The results of the biochemical tests carried out on them are shown in Table 1. The confirmation and full identification of the bacteria, using their 16S rDNA sequences in the GenBank database, revealed that the different isolates belong to eight genera *Staphylococcus*, *Rahnella*, *Klebsiella*, *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Lysinibacillus* and *Listeria* (Table 2). Among the genera of the bacteria identified, the genus *Arthrobacter* was most predominant, followed by *Staphylococcus* and *Klebsiella* respectively. The specific representative species of the different genera identified in the sweet potato tubers during storage include *S. kloosii*, *S. sciuri*, *S. gallinarium*, *K. pneumoniae*, *A. woluwensis*, *A. nicotiana*, *A. methylotrophs*, *P. syringae*, *Lys. fusiformis* and *L. monocytogenes*; some isolates among the genera could not be identified to species level. All identified bacterial isolates had a minimum identity of 97% to the closest relatives, using BLAST in the geneBank database. Typical PCR amplimers of the 16S rDNA genes in some of the bacterial isolates are represented in Figure 1; each amplimer was approximately 1.500bp in size.

**Figure 1.** Typical PCR amplimers of the 16S rDNA genes in bacterial isolates obtained from sweet potatoes

Legend

1, 1kb marker; 2, DNA negative control; 3, DNA positive control; 4, isolate PABSP21; 5, isolate PABSS3B; 6, isolate PABSP2; 7, isolate PABSS3A; 8, isolate PABSS3C; 9, isolate PABSS2A; 10, isolate TSASS2C; 11, isolate TSASA; 12, isolate PABSPB

**Figure 2.** Sweet potato tuber showing lesions at the point of inoculation with *Staphylococcus sciuri* TSADCA1 (A) and control (B) during the four week storage at 29°C.
The result of the test for spoilage potentials of the bacterial isolates on the sweet potatoes revealed that only two bacteria, *Staphylococcus sciuri* (TSADCA1) and *Rahnella* sp. (TSADCA2), showed clear presumptive physical evidence of spoilage. The two bacteria were observed to have created lesions around the areas of inoculation (Figure 2) and off odours and this is characteristic of most spoilage microbes that infect the tissue of plants (Tournas 2005; Ray and Ravi 2005). These research workers asserted that microbes that infect the tissue of plants normally accelerate tissue degradation, before spoilage by the two bacteria could be ascertained. Studies have shown that spoilage organisms of tuber crops produce enzymes which help degrade surrounding tissues for eventual assimilation by the invading organisms (Barth et al., 2009).

The enzymes are usually found at the zones of microbial infection where the organisms are presumed to have secreted them (Doyle, 2007; Barth et al., 2009). The foregoing reasons could be responsible for the physical evidence recorded for the two bacterial isolates, *S. sciuri* (TSADCA1) and *Rahnella* sp. (TSADCA2), in the possible spoilage of the sweet potatoes under study.

### Table 1. Biochemical characteristics of the bacterial isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram reaction</th>
<th>Catalase test</th>
<th>Oxidase test</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSASP1</td>
<td>+ve cocci in clusters</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSADCA1</td>
<td>+ve cocci</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSADCA2</td>
<td>+ve rods</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASP3</td>
<td>+ve cocci</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSP2</td>
<td>+ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSP15</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP1</td>
<td>-ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASP15</td>
<td>+ve cocci</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASS3A</td>
<td>+ve rods in chains</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>TSASS2C</td>
<td>+ve cocci</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASS2B</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>TSASP6</td>
<td>+ve rods</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP19</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP18</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP17</td>
<td>+ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSS3C</td>
<td>+ve rods in chains</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>TSASP4</td>
<td>+ve cocci</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP13</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSS3B</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>TSAS</td>
<td>-ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASP2</td>
<td>+ve cocci in clusters</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASS1B</td>
<td>+ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSS2A</td>
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<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSP12</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSPB</td>
<td>+ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSP6</td>
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<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP21</td>
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<td>-ve</td>
</tr>
<tr>
<td>PABSP7</td>
<td>+ve rods</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>TSASA</td>
<td>+ve rods in chains</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>PABSP8</td>
<td>+ve rods</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>PABSS3A</td>
<td>+ve rods</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>TSASS2A</td>
<td>+ve cocci</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

The abilities of the two bacteria being implicated in the current study to secrete extracellular enzymes which may help in tissue degradation during spoilage of the sweet potatoes were tested. The potential of microorganisms to cause spoilage in vegetable crops has been linked with their ability to secrete extracellular enzymes, particularly those that can degrade the plant cell wall (Warren, 1996; Doyle, 2007). The results of the enzyme activities in the bacteria isolates are presented in Table 1.
after inoculated on sweet potatoes and incubated for nine days are shown in Figures 3 and 4. It was found that the activities of carbohydrate degrading enzymes (particularly polygalacturonase, cellulose and xylanase) in these two organisms were comparatively high, suggesting that the category of enzymes may be actively involved in complementing tissue damage during the growth of the producer bacteria on sweet potato tubers. Activities of other enzymes were found to be lower when compared to those of the carbohydrate degrading enzymes. The *S. sciuri* (TSADCA1) and *Rahnella* sp. (TSADCA2) have therefore been shown as spoilage agents of sweet potatoes during post harvest storage. The bacteria are capable of causing decay in the vegetable crop, and this may pave way for growth of additional opportunistic microorganisms which may contribute to spoilage. Most spoilage microorganisms find their ways into tissues of vegetable crops through wounded areas. Wounded areas on plant tissue provide a better substrate for microbiological growth by providing nutrients (Zagory, 1999). There is therefore the need to prevent or minimise bruises or wounds which are normally associated with crops by mechanical action during harvest. This will help limit microbial infection, and hence spoilage of the crops.

In conclusion, results of this study have confirmed *S. sciuri* (TSADCA1) and *Rahnella* sp. (TSADCA2) as part of the microflora of sweet potatoes that contribute to spoilage of the crop. It is worth mentioning, to the best of our knowledge, that this is the first report on the association of the two organisms with spoilage of sweet potatoes, and could therefore be of great significance towards improving methods of the crops’ preservation.

**Table 2.** Bacterial isolates identified based on their 16S rDNA gene sequences.

<table>
<thead>
<tr>
<th>SN</th>
<th>Organism Identified</th>
<th>Min % Identity</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Arthrobacter</em> wolinensis</td>
<td>97</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td><em>A. nicotianae</em></td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td><em>A. methylotrophs</em></td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td><em>Arthrobacter</em> sp.</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td><em>Staphylococcus</em> kloosii</td>
<td>99</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td><em>S. sciuri</em></td>
<td>99</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td><em>S. gallinarium</em></td>
<td>99</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td><em>Staphylococcus</em> sp.</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td><em>Klebsiella</em> pneumoniae</td>
<td>99</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Klebsiela</em> sp.</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td><em>Bacillus</em> sp.</td>
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<td>4</td>
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<td>12</td>
<td><em>Rahnella</em> sp.</td>
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<td>13</td>
<td><em>Pseudomonas</em> syringae</td>
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<td><em>Lysinibacillus</em> fusiformis</td>
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<td>15</td>
<td><em>Listeria</em> monocytogenes</td>
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</table>

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**Reference**


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