ABSTRACT

Objective: The present study was carried out to identify the associated Salmonella and Escherichia coli in betel leaves (Piper betle), and to develop an effective method to remove those microbes.

Materials and methods: Betel leaves were collected from local and whole sale markets, and momaj (cultivation place). Salmonella and E. coli were isolated and identified by cultural, morphological, and biochemical tests followed by confirmation by polymerase chain reaction (PCR) targeting the genus specific 16S rRNA genes. Antibiogram of the isolated bacteria was performed by disc diffusion method. Different concentrations of Salmoan-A Soln were used to remediate the contaminating bacteria keeping the quality of betel leaves for longer periods.

Results: Total Salmonella counts in the betel leaves were 3.9×10², 4.9×10⁶, 3.5×10³, 1.1×10³ and 1.5×10⁴ CFU/mL, while E. coli counts were 5.5×10⁷, 6.3×10⁷, 4.4×10⁵, 3.3×10³ and 3.1×10³ CFU/mL in the betel leaves collected from K.R. market, Kewatkhalie Bazaar, whole sale market, momaj in Kushtia and momaj in Natore, respectively. Antibiogram study revealed that the isolated bacteria were sensitive to doxycycline, ciprofloxacin, chloramphenicol and ceftaxime. Application of 0.3% Salmoan-A Soln was found to be the most effective and suitable, where <1 CFU/mL in the betel leaves could be detected after treatment for 10 min.

Conclusion: Results of this study indicated that treatment with 0.3% Salmoan-A Soln for 10 min is capable of removing Salmonella and E. coli from betel leaves keeping the treated leaves fresh up to 7 days of post-treatment. Method developed in this study can be adapted to reduce bacterial contamination of betel leaves more effectively in Bangladesh and other betel leaf exporting countries.

KEYWORDS
Betel leaves; Contamination; Escherichia coli; Salmonella; Treatment
INTRODUCTION

Salmonella and Escherichia coli (E. coli) are Gram-negative, rod-shaped bacteria under Enterobacteriaceae family. Salmonella is the causative agent of typhoid fever, paratyphoid fever, and food poisoning worldwide (Furchtgott et al., 2011). E. coli are considered as a part of normal enteric microflora, and most of them are opportunistic pathogens for animal and human; however, this bacterium may cause serious diarrhea and other systemic diseases in healthy humans and animals (Levine, 1984).

The betel leaf (‘Paan’ in Bengali) is the leaf of a vine belonging to the family Piperaceae. The betel (Piper betle) has no fruit and is cultivated only for its green leaves, which have medicinal properties. The betel plant is an evergreen, perennial and shade loving root climber (Chakraborty, 2011). Paan is available in most south and southeast Asian countries including India, Pakistan, Burma, Vietnam, Malaysia, Singapore, Thailand, Philippines and Bangladesh. Besides, Paan is consumed in some other countries in the world, particularly by the Asian immigrants.

In Bangladesh, the yield of betel leaf varies from region to region. Betel leaf cultivation is one of the major sources of income generation for the farmers in Bangladesh. About 2,825 hectares of land is dedicated for betel leaf production in Bangladesh (Husna et al., 2015). The average production cost for betel leaf production in Bangladesh is USD 4000 per hectare with an average profit of USD 1334 (Annon, 2011).

In Bangladesh, exportable betel leaf is cultivated in Kushtia, Rajshahi, Natore, Jhenaidah, Khulna, Barishal, Satkhira, Bagerhat, Sylhet and Chittagong districts. Bangladesh exports betel leaves mostly to Europe and Middle Eastern countries. Bangladesh exported betel leaves worth USD 31.10 million in the fiscal year 2011-12, which was USD 42.6 million in 2010-11 (Annon, 2011). According to the Export Promotion Bureau (EPB), United Kingdom customs in 2011 had examined 60 consignments of Paan that were imported to the country and found that 44 were infected with Salmonella (Husna et al., 2015). Currently, European Union (EU) has an embargo on the export of Paan from Bangladesh to EU countries due to potential Salmonella contamination (Chowdhury and Kallol, 2013). Husna et al. (2015) could isolate Salmonella from Paan, and used 1.5% vinegar to mitigate the Salmonella contamination.

However, a detailed study is yet to be done to identify the point(s) from where the contamination is originated. Also, it is crucial to develop an applicable technique to remediate the Salmonella and E. coli contamination from the betel leaves keeping them fresh and consumable for long time. The present study was designed to detect multidrug resistant Salmonella and E. coli in betel leaves originated from different places including cultivation place (commonly known as “baronj” in Bengali), whole sale markets and local/retail markets. In this study, an applicable method was developed to effectively remove contaminating Salmonella and E. coli, thus keeping the leaves in fresh and good condition for long time.

MATERIALS AND METHODS

Study locations: The betel leaves were collected from baronj in Kushtia and Natore districts, whole sale and local markets (K.R. market and Kewatkhali), and were transported to the Bacteriology Laboratory at the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh for bacteriological analysis.

Bacterial cultural and identification: The betel leaves were washed with phosphate buffer solution (PBS) and then aliquots were spread onto Salmonella Shigella (S-S), Xylose Lysine Deoxycholate (XLD) and Eosin Methylene Blue (EMB) agar plates, and incubated at 37°C overnight. Black color colonies formed on S-S and XLD agars, and green metallic sheen colonies formed on EMB agar were screened, as described by Rehman et al. (2014). The black colonies were subjected to subculture on S-S agar to isolate pure culture, which was used for Gram stain, as described by Tanzin et al. (2016) and Rahman et al. (2016). The organisms were also sub-cultured on XLD and EMB agars (Parvej et al., 2016). Pure cultures were used for further testing.

Bacterial biochemical characterization: The isolated pure cultures were subjected to conventional biochemical tests using sugar fermentation, Methyl-Red Voges-Proskauer (MR-VP), Methyl-Red, Voges-Proskauer (VP) and Indole tests. Motility test was done as per the method described by Iheanyichukwu et al. (2016) and Rahman et al. (2016).

DNA extraction and molecular identification: Pure and single bacterial colony was used to isolate bacterial DNA. Bacterial colonies were mixed with 200 µL of distilled water and boiled for 10 min. Immediate after
boiling, the eppendorf tube was kept on ice for another 10 min. Centrifugation at 10,000 rpm for 10 min was done for precipitating the cellular debris. The supernatant was collected and used as DNA template for PCR reactions (Saifullah et al., 2016).

**16S rRNA gene amplification:** PCR reaction (25 μL scale) contained master mixture (12.5 μL), forward and reverse primers (1 μL each), DNA template (4 μL), and nucleic free water (6.5 μL). The 16S rRNA gene from *Salmonella* was amplified using the thermocycler (Eppendorf, Germany). The reaction conditions included an initial denaturation at 94°C for 5 min, followed by a 33 cycles of reaction of denaturation at 94°C for 30 Sec, annealing of primers at 65°C for 30 Sec, elongation at 72°C for 45 Sec, and finally an extension at 72°C for 7 min. Similarly, the 16S rRNA gene of *E. coli* was amplified using an initial denaturation at 95°C for 3 min, followed by a 30 cycles of reaction of denaturation at 94°C for 45 Sec, annealing of primers at 58°C for 45 Sec, elongation at 72°C for 1 min, and a final extension at 72°C for 3 min. All the PCR amplicons were visualized using gel electrophoresis on 2% agar after staining with ethidium bromide under UV transilluminator (UV Solo, Germany).

**Treatment of Salmonella and E. coli contaminations in betel leaves:** The betel leaves were washed using different concentration of Salmosan-A Sohn, as mentioned in the Table 4. Then, the leaves were washed with distilled water. The leaves were then dipped into PBS, and the washings were inoculated onto XLD and EMB agars for evaluating the presence of *Salmonella* and *E. coli*, and the plates were incubated at 37°C overnight. On the other hand, Salmosan-A Sohn treated leaves were stored at room temperature and observed daily for 8-10 days.

### Antibiotic sensitivity test:

The antibiotic sensitivity tests were performed using commercially available antibiotic discs (Oxoid, UK) using disc diffusion method, as described previously (Nazir et al., 2005; Tanzin et al., 2016). The antibiotics were amoxicillin (AML; 10 μg/disc), ampicillin (AMP; 10 μg/disc), ceftazime (CFM; 5 μg/disc), cefotaxime (CTX; 30 μg/disc), chloramphenicol (C; 30 μg/disc), ciprofloxacin (CIP; 5 μg/disc) and Doxycycline (DO; 30 μg/disc). The sizes of the zones of inhibition were interpreted as per the method described by CLSI (2010).

### RESULTS AND DISCUSSION

*Salmonella* were detected in the betel leaves. On S-S agar, all of the isolates produced translucent, black, smooth and small round colonies which were suspected for *Salmonella*. All the suspected *Salmonella* isolates produced pink color colony with black center after incubated at 37°C overnight on XLD agar. Gram stain revealed the presence of Gram-negative, pink colored, short rod bacteria, arranged in single and paired. The bacteria were motile seen by hanging drop slide technique.

The *E. coli* isolates detected in betel leaves showed greenish-black colonies with metallic sheen on EMB agar and bright pink or red colonies on McConkey agar plates. Gram stain of *E. coli* revealed as Gram-negative, pink color, short rod, arranged in single or paired.

### Table 1. Primers used for molecular detection of *E. coli* and *Salmonella*

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Targeted Gene (primer)</th>
<th>Primer Sequence (5’-3’)</th>
<th>PCR amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC16SrRNA F</td>
<td>16S rRNA (E. coli genus specific)</td>
<td>GAGCCGCTTTAAGTTCACAGGA</td>
<td>585</td>
<td>Seidavi et al. (2010), Serena et al. (2010), Hassan et al. (2014).</td>
</tr>
<tr>
<td>EC16SrRNA R</td>
<td></td>
<td>CACAGCTGAGGCTGACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F strand</td>
<td><em>Salmonella</em> genus specific</td>
<td>ACTGGCGTTATCCCTTTGCTGTGTG</td>
<td>496</td>
<td>Noah et al. (1993).</td>
</tr>
<tr>
<td>R strand</td>
<td></td>
<td>ATGTGTCCTCCTGCTGCTGAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Total *Salmonella* and *E. coli* counts in betel leaves before and after treatment with 0.3% Salmosan-A solution

<table>
<thead>
<tr>
<th>Sources betel leaf</th>
<th>TVC/mL before treatment</th>
<th>TVC after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borujj</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) Kushia</td>
<td>1.1×10³</td>
<td>3.3×10³</td>
</tr>
<tr>
<td>ii) Natore</td>
<td>1.5×10³</td>
<td>3.1×10³</td>
</tr>
<tr>
<td>Whole sale market</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hathiaboro bazaar,Kushia</td>
<td>3.5 x 10⁴</td>
<td>4.4×10³</td>
</tr>
<tr>
<td>Local market</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) K. R. market, BAU</td>
<td>3.9×10⁵</td>
<td>5.5×10⁵</td>
</tr>
<tr>
<td>ii) Kewarkhal, Mymensingh</td>
<td>4.9×10⁶</td>
<td>6.3×10⁵</td>
</tr>
</tbody>
</table>
positive for Methyl-Red (M-R) test and negative for V-P test. *E. coli* isolates were catalase positive, V-P test negative, M-R positive, citrate negative and Indole test positive. The isolated *E. coli* were found to be motile using hanging drop slide technique.

PCR using *Salmonella* genus specific primer for *Salmonella* and 16SrRNA detects primers for *E. coli*, showed positive band at 496-bp and 585-bp, respectively (Figure 1).

All the isolated *Salmonella* were found to be sensitive to CIP, C, DO and CTX, whereas resistant to AML, CFM and AMP. On the other hand, the isolated *E. coli* isolates were sensitive to CIP, C, DO and CTX, and resistant to AML, CFM and AMP (Table 4).

We detected *Salmonella* contamination in local (Table 3) market samples to be more than the *borouj* samples in non-treated group. In addition, *E. coli* contamination was detected (Table 3) in local market sample more than the *borouj* samples in non-treated group.

### Experiments to remove *Salmonella* and *E. coli*

Different experiments were conducted to remove *Salmonella* and *E. coli* from betel leaves keeping them in good condition for 7 days. The experiments were shown in Table 4.

Various concentrations of Salmosan-A Soln were used to remove *Salmonella* and *E. coli* from betel leaves. We found that 0.3% Salmosan-A Soln was the most effective to protect the leaves. With this concentration of Salmosan-A Soln, not only *Salmonella* and *E. coli* were removed successfully but also the leaves were remained in good condition up to 7 days (Figure 2).

In case of large scale experiments, 0.3% Salmosan-A Soln was found as effective. We found that all the treated betel leaves were free from *Salmonella* and *E. coli*, whereas presence of *Salmonella* and *E. coli* could be detected in untreated betel leaves, as well as all the treated leaves were in good quality as consumable even after 7th day of treatment (Table 4).

<table>
<thead>
<tr>
<th>Concentration of Salmosan-A Soln</th>
<th>Time (min)</th>
<th>Non-treated</th>
<th>Treated</th>
<th>Condition of treated leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Salmonella</td>
<td><em>E. coli</em></td>
<td>Salmonella</td>
</tr>
<tr>
<td>2%</td>
<td>10</td>
<td>HG</td>
<td>HG</td>
<td>NG</td>
</tr>
<tr>
<td>1.5%</td>
<td>10</td>
<td>HG</td>
<td>HG</td>
<td>NG</td>
</tr>
<tr>
<td>0.5%</td>
<td>5</td>
<td>HG</td>
<td>HG</td>
<td>NG</td>
</tr>
<tr>
<td>0.5%</td>
<td>5</td>
<td>HG</td>
<td>HG</td>
<td>few colonies</td>
</tr>
<tr>
<td>0.5%</td>
<td>12</td>
<td>HG</td>
<td>HG</td>
<td>Few colonies</td>
</tr>
<tr>
<td>0.3%</td>
<td>5</td>
<td>HG</td>
<td>HG</td>
<td>NG</td>
</tr>
<tr>
<td>0.3%</td>
<td>10</td>
<td>HG</td>
<td>HG</td>
<td>NG</td>
</tr>
</tbody>
</table>

Table 4: Treatment of betel leaves with salmosan-A soln and observation of bacterial growth and stability of the leaves

\(HG=\) Huge growth, \(NG=\) No growth

**Figure 1.** (A) PCR image of *Salmonella* spp. Lane M: 100-bp DNA ladder, Lane 1: Positive control, Lane 2-5: Isolated positive *Salmonella*, Lane 6: Negative control; (B) PCR image of *E. coli* Lane L: 1000-bp DNA ladder, Lane 1: Positive control, Lane 2-3: Isolated positive *E. coli*, Lane 4: Negative control.

**Table 3: Antibiotic sensitivity pattern of *Salmonella* spp. and *E. coli* isolated from betel leaves.**

<table>
<thead>
<tr>
<th>Name of the antibiotics</th>
<th><em>E. coli</em></th>
<th>Salmonella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Amoxycillin (AML)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Chloramphenicol (C)</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Cefixime (CFM)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Doxycycline (DO)</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Ampicillin (AMP)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cefotaxime (CTX)</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

\(S=\) Sensitive, \(R=\) Resistance

In this study, PCR was used to confirm the *Salmonella* and *E. coli* (Saifullah et al., 2016; Parvej et al., 2016). PCR with *Salmonella* genus specific primer showed positive band at 496-bp confirming that the isolates were *Salmonella* spp. (Noah et al., 1993; Saifullah et al., 2016). PCR with *E. coli* genus specific 16SrRNA primer showed positive band at 585-bp confirming that the isolates were *E. coli* (Seidavi et al., 2010; Serena et al., 2010).

The sugar fermentation test profile of *Salmonella* spp. in the present study was similar to previous reports (Rahman et al., 2016). In motility test, the organism showed swinging movement as reported previously (Merchant and Packer, 1967; Buxton and Fraser, 1977). The genus *Salmonella* usually includes motile rods of peritrichous flagella with the exception of *Salmonella gallinarum* and *S. pullorum* (Blood et al., 2003). Therefore, the result of the motility test of this study indicates that the isolates are belonging to the species of *Salmonella* other than *S. gallinarum* and *S. pullorum*. The isolates might be any of the followings namely *S. typhimurium*, *S. enteritidis*, *S. beidelberg*, *S. agona*, *S. indiana*, *S. badar*, *S. virchow*, *S. newport* etc. (Perilla, 2003; Cheesbrough, 2006).

Antibiogram of the isolated bacteria was determined and antibiotic sensitivity test revealed that the isolated *Salmonella* and *E. coli* showed same resistance pattern where, all of the isolates were sensitive to CIP, C, DO, CTX while resistant to AML, CFM, and AMP.

In the present study, total *Salmonella* count was $3.9 \times 10^5$ CFU/mL and total *E. coli* count was $5.5 \times 10^6$ CFU/mL in K.R. market betel leaf, whereas total *Salmonella* count was $4.9 \times 10^6$ CFU/mL, and *E. coli* was $6.3 \times 10^7$ CFU/mL in Kewathkali betel leaf. Samples collected from the whole sale market from Hathiboro bazaar, Khusthia had a total *Salmonella* count of $3.5 \times 10^4$ CFU/mL in control group and found <1 colony in treated group. While *E. coli* count was $4.4 \times 10^5$ CFU/mL in control group. On the other hand, samples which were brought from boraj were also examined and the total *Salmonella* count was $1.1 \times 10^5$ CFU/mL and total *E. coli* count was $3.3 \times 10^3$ CFU/mL in
Kushtia and had a total *Salmonella* count was $1.5 \times 10^6$ CFU/mL and total *E. coli* count was $3.1 \times 10^3$ CFU/mL in the sample brought from Natore in control group. However, after application of Salmosan A Soln, we found <1 colony in treated group.

Husna et al. (2015) also studied the occurrence of *Salmonella* in betel leaf. It was found that *Salmonella* in the betel leaf sold at local markets and different *baroj* in Mymensingh. In addition, it was found that vinegar was an effective agent to mitigate *Salmonella*. Another study was conducted on fruits and vegetables purchased from street vendors to determine microbial load and the bacterial load ranged from $1.3 \times 10^4$ to $1.8 \times 10^6$ CFU/gm (Nwachukwu and Chukwu, 2013). Another study was on restaurant salad vegetables in Chittagong city recorded TVC ranged from log 5.20 to 6.87 CFU/gm (Nawas et al., 2012). In Nigeria, TVC of RTE salad vegetables was ranged from log 6.20 to 8.47 CFU/gm (Mercy et al., 2011).

As we found fractional result between local market sample and *boroj* sample that the *Salmonella* and *E. coli* load were much higher in local market *Paan* than the *boroj*, and it gradually increased from *boroj* to whole sale market to local markets. It might be because of the unhygienic transportation of the betel leaves after picking from the trees to sell in the market. Though betel leaves cultivators trades are not much aware about producing and selling *Paan* at market in unhygienic condition. They use unhygienic water on *Paan* to keep those fresh. And, the water may cause high level of contamination.

Singla et al. (2009) artificially inoculated betel leaves with *Aeromonas*, *Salmonella* and *Yersinia* followed by the application of Salmosan-A Soln on that leaves and found effective result to remove these bacteria. In the present study, we also used 2% Salmosan-A Soln and it can remove *Salmonella* and *E. coli* but the betel leaves were found to be rotten within 2 days. However, when we used 0.3% Salmosan-A Soln, results were good and the leaves remained in original condition as like non-treated one.

**CONCLUSION**

The betel leaves examined in this study are contaminated with multidrug resistant *Salmonella* and *E. coli*. Load of these bacteria gradually increases from *boroj* to retail markets. The cultivators and traders use contaminated water on the betel leaves to keep them fresh, from where the contaminating bacteria originate. Here, we optimize the concentration of Salmosan-A Soln suitable not only to keep the betel leaves free from *Salmonella* and *E. coli*, but also to keep the leaves in good condition for long time. The best result was found with 0.3% Salmosan-A Soln for 10 min, in which the leaves were remained as consumable for 7 days. This technique may help in promoting export business for betel leaves. Measures should be taken to improve the general knowledge of hygiene and sanitation of the farmers cultivating *Paan* so that consumers can access contamination free safe *Paan*.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflicting interest with regards to the publication of this manuscript.

**AUTHORS’ CONTRIBUTION**

TK and WKA conducted the research work. SNP and MMA helped in collection of samples. MSP, SA and MLH did the necessary analysis. TK made the primary draft of this manuscript. KHMNHN, MTR, MR and MFRK critically reviewed and improved the manuscript. MBR supervised the total team and finally approved the manuscript for publication. Before submission, all the authors approved the final version having no conflict of interest.

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