Detection and enumeration of pathogenic microorganisms associated with fresh vegetables and their implication for food safety

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Background: Nowadays, food safety becomes an environmental and public health concern due to the increasing demand of vegetable diets observed from the last decades. This has stipulated research regarding the risks associated with consumption of food stuffs contaminated with pathogenic microorganisms. Objective: The aim of this study was to detect and enumerate pathogenic microorganisms associated with fresh vegetables and their implications for food safety among consumers. Methods: Fresh samples of vegetables including tomato, onion, carrot and spinach were collected immediately after harvest for the bacterial characterization and identification using Bergey’s Manual of Systematic Bacteriology while the fungi were identified by their morphology through examination of their microscopic structures. Results: The results show higher number of Vibrio cholerae in the vegetables, a high mean score of 8.7 × 10⁸ CFU/g for AMB, and 5.6 × 10⁵ for TCB in tomatoes and 3.9 × 10⁶ CFU/g for FCB in carrot sample. There were no statistically significance difference within and between groups in the AMB, FCB and TCB using one-way ANOVA at p = 0.05 and 95% confidence interval (F = 2.065; 0.152 and 4.045 respectively). Spinach contained 495 MPN/g of Aspergillus flavus (ASPf) while onion has 518 MPN/g of Aspergillus niger (ASPn) per each 25 plates of samples. Conclusion: This study concluded that microbial loads on vegetables were above ICMSF limits for vegetables and therefore, consumers should ensure that the raw vegetables are thoroughly washed and adequately processed to prevent chances of food borne diseases.

Keywords: Detection, Enumeration; Microorganisms; Fresh vegetables; Food safety

1. Introduction

Recent shifts in promoting a healthy lifestyle around the globe has contributed to the increased consumption of fruits and vegetables in fresh, raw or undercooked forms (Abadias et al., 2008; and Feroz et al., 2013). Although the health benefits of fresh produce are great, the proportion of foodborne disease outbreaks linked to contaminated produce has increased over the past few decades (Ailes et al., 2008). Contamination of fresh...
vegetables during handling process is a common problem and it is usually ignored the use appropriate techniques of decontamination (Ankita et al., 2014). If consumed, contaminated fruits and vegetables can lead to food poisoning, because of the presence of intestinal infectious microbes on the outer surfaces.

The majority of diseases associated with fresh fruits and vegetables are primarily those transmitted by the fecal-oral route, and this is as a result of contamination at some points in their processing (DeRoever, 1998). Therefore, the endemic of gastroenteritis, giardiasis, hepatitis A, hepatitis E, shigellosis (bacillary dysentery), typhoid fever, vibrio parahaemolyticus infections and vholera is an evident example of fecal-oral route transmitted diseases (Ankita et al., 2014). Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Spoiled foods may be unsafe to eat, and may cause illnesses because of presence of pathogens or toxins. Other spoilage associated changes in texture, smell, taste, or appearance may cause them to be rejected under normal circumstances (Burkepile et al., 2006).

Vegetables, like other food stuffs can become contaminated with pathogenic organisms. This may occur during growth, harvest, postharvest handling, and preservation or during cookery (McMaho and Wilson, 2001; and Beuchat, 1999). The use of untreated wastewater in irrigation represents an important route for transmission of these pathogenic organisms. The common pathogens associated with the use of highly polluted water are the faecal coliforms, E. coli, Klebsiella species, Vibrio cholerae etc. and eggs of some eggs of fungi such as Aspergillus and whose resistant eggs can be found in the wastewater (Amoah et al., 2007). Some of these microbes can cause food poisoning in through infecting the intestines, leading to inflammation, difficulty in absorbing nutrients and water, and may result in diarrhea (Sabiena et al., 2000).

Other microbes produce chemicals in foods (toxins) that are poisonous to the human digestive system and when eaten, these chemicals can lead to nausea and vomiting, kidney failure, and even death (Mara et al., 2007; and Uzeh et al., 2009). Food safety is a major public health concern worldwide (Beuchat, 2002). During the last decades, the increasing demand for food safety has stipulated research regarding the risk associated with consumption of food stuffs contaminated with pathogenic microorganisms and studies have revealed that pathogenic contamination of vegetables increases risk of food borne diseases among the consumers (D’Mello 2003; and Zandstra and De Kryger, 2007). This study was therefore designed to detect and enumerate pathogenic microorganisms associated with fresh vegetables and their implications for food safety among consumers.

2. Materials and methods

One hundred and sixty samples of fresh vegetables were collected immediately after harvest at River Getsi irrigation site, in Kano metropolis, Nigeria. The vegetable include of 40 tomatoes, 40 onions, 40 carrots and 40 spinaches respectively. These samples were collected using sterile hand gloves in order to avoid cross contamination, put into new polythene bags and transported to laboratory. The samples (30 g each) were washed thoroughly with fresh water in order to remove the adhering dirt and finally washed with deionized water. Each of the vegetable samples was dried separately at room temperature in a sun free environment. The dried samples were grinded into homogenous fine powder mass with the aid of ES-314 blender (220 V, 50 HZ and 300 W) of SONCAP approved, put into airtight polythene bags and kept in a refrigerator for further analysis.

The vegetable homogenate was prepared by mixing 15g of each vegetable sample with 225ml of buffered peptone water (BPW) in a sterile beaker and blended for about 2-3 min. Vegetable homogenates in three test tubes each containing 9 ml MacConkey broth with an inverted Durham were used for coliforms presumptive test (FAO/WHO, 2008a). Bacterial colonies in pure form were collected using streak plate method. Based on phenotypic colony characters on media plates, total 127 different bacterial colonies were isolated in pure forms, which were further identified. This detection was done by visual colony characters, microscopy (Gram and Endospore staining) and some other tests like motility test, oxidase test, catalase test, indole test, Gram’s reaction, citrate utilization test, and Voges-Proskauer test, while, the secondary identification of the isolates was carried out on the basis of biochemical tests (IMViC tests) and carbohydrate utilization test.

For the detection of fungi, a drop of methyl blue was placed on a clean slide; a sterile needle was used to introduce a small amount of the growth from incubated plate into the stain. A cover slip was gently applied with little pressure to prevent air bubbles. The slide was mounted and observed under × 40 objective lens (Fawole, 1988).
The isolation of fungi was done with Potato Dextrose Agar media, after isolation of all fungal colonies in pure culture, all of them were visually examined for phenotypic characters like color, texture, exudates, growth zones, aerial/submerged hyphae, and macroscopic structures such as ascocarps, pycnidia, sclerotia, sporodochia, and synnemata.

The final identification of fungi was performed by morphological examination of microscopic structures, particularly the spores and the conidia, using lactophenol and cotton blue. The data obtained were analyzed using frequency distribution tables including number and percentages for the identified bacteria, descriptive statistics using mean based on the bacterial colony forming units per gram as well as one-way analysis of variance (ANOVA) for between and within group differences in bacterial counts per samples of the vegetables. For the fungal species, descriptive statistics using mean was employed based on the fungal most probable number per gram (MPN/g) in 25 plates of each sample of the vegetables. All the statistical analyses were performed using SPSS software version 25 at significance level of $p = 0.05$ and 95% confidence interval.

3. Results and discussion

This experimental study was conducted to find any pathogenic microbes (bacteria and fungi) on vegetables collected from river Getsi irrigational farm site, which could be responsible for contamination of these vegetables and ascertain their potential implications for food safety. Different techniques were used for isolation and characterization was done with help of phenotypic and biochemical characters. The total number of the identified bacterial colonies from all vegetables (Spinach, Tomato, Onion and carrot) were 127 as shown in Table 1. The strains of the identified bacterial species were counted and *Vibrio cholerae* was found to be the highest specie (31 stains) while *Klebsiella* (6 strains) and *Pseudomonas* (3 stains) were the least species among all the detected bacteria as shown in Table 2.

| Table 1: Cultural, biochemical and morphological characteristics of the isolated bacteria |
|---|---|---|---|
| Bacterial culture | Reactive tests (+) | Cell type | Inference |
| NA: Small slightly raise area colonies | Gram's reaction Catalase test, Oxidase test | Cocci | Staph. Aureus |
| MSA: Yellowish colonies | Catalase test Citrate utilization Voges proskauer | Cocci | Klebsiella species |
| EMB: Pink-blue mucoid with a metallic sheen | Catalase test Motility, Indole | Cocci | E.Coli, Pseudomonas species |
| BA: Large flat colonies greenish blue colorSmall gray translucent drop like colonies | Gram's reaction Catalase test, Motility Vogesproskauer | Rod | Listeria species |
| KIA: Red pink slope and yellow butt. | Motility Voges proskauerIndole Citrate utilization Oxidase test | Bacilli | Vibrio cholera |
| KIA: Red pink slope and yellow butt. | Catalase test Citrate utilization | Rod | Citrobacter species |

*Note:* NA: Nutrient agar; MAC: MacConkey agar; MSA: Mannitol salt agar; BA: Blood agar; KIA: Kligler iron agar; E.coli: Escherichia coli; Staph. Aureus; and Staphylococcus aureus.
From Table 3, the bacteria species were further grouped into aerobic mesophilic bacteria (AMB), total coliform bacteria (TCB) and faecal coliform bacteria (FCB) based on their morphological features and the numeric index of each was determined using the colony forming units per gram (CFU/g). Table 4 shows the results of the one-way ANOVA for statistical difference between and within the groups for the AMB, FCB and TCB. These were found to be insignificant for AMB ($F = 2.065, \ P = 0.05$ at 95% confidence interval), TCB ($F = 4.045, \ P = 0.05$ at 95% confidence interval) and FCB ($F = 0.151, \ P = 0.05$ at 95% confidence interval) respectively.

### Table 2: Number of bacterial species found to be present on vegetables

| Bacterial species       | No. of identified strains (%) |
|-------------------------|-------------------------------|
| Citrobacter spp         | 13 (10.2)                     |
| Eschericia coli         | 9 (07.1)                      |
| Faecal coliforms        | 26 (20.5)                     |
| Staphylococcus aureus   | 20 (15.7)                     |
| Klebsiella spp.         | 6 (04.7)                      |
| Listeria monocytogene   | 19 (14.7)                     |
| Pseudomonas spp         | 3 (02.4)                      |
| Vibrio cholera          | 31 (24.4)                     |

### Table 3: Mean number of isolated bacteria in the tested vegetables

| Sample ID | Indicator bacteria | Vegetable types (CFU/g) |
|-----------|--------------------|-------------------------|
|           |                    | Tomato | Onion | Spinach | Carrot |
| 1         |                    |        |       |         |        |
| 2         | AMB                | 9.3 x 10^5 | 1.7 x 10^6 | 2.2 x 10^4 | 1.6 x 10^3 |
| 3         |                    | 8.0 x 10^5 | 1.6 x 10^6 | 2.0 x 10^4 | 1.4 x 10^3 |
| Mean veg  |                    | 8.7 x 10^5 | 1.7 x 10^6 | 2.1 x 10^4 | 1.6 x 10^3 |
| 1         |                    | 5.8 x 10^5 | 3.7 x 10^6 | 6.6 x 10^5 | 5.2 x 10^5 |
| 2         | TCB                | 5.3 x 10^5 | 4.0 x 10^6 | 5.4 x 10^5 | 3.1 x 10^5 |
| 3         |                    | 5.6 x 10^5 | 3.4 x 10^6 | 4.1 x 10^5 | 4.1 x 10^5 |
| Mean veg  |                    | 5.6 x 10^5 | 3.7 x 10^6 | 5.4 x 10^5 | 4.1 x 10^5 |
| 1         |                    | 3.1 x 10^5 | 3.7 x 10^6 | 5.6 x 10^5 | 4.5 x 10^5 |
| 2         | FCB                | 3.2 x 10^5 | 3.2 x 10^6 | 3.8 x 10^5 | 4.1 x 10^5 |
| 3         |                    | 3.0 x 10^5 | 3.0 x 10^6 | 3.0 x 10^5 | 3.2 x 10^5 |
| Mean veg  |                    | 3.1 x 10^5 | 2.9 x 10^6 | 3.3 x 10^5 | 3.9 x 10^5 |

**Note:** 1, 2 and 3 = Sampling in first, second and third months respectively, AMB = Aerobic mesophilic bacteria, TCC = Total coliform count, FC = Faecal coliform and CFU/g = Colony forming unit per gram.
Table 4: One-way ANOVA of isolated bacteria in the tested vegetables

| Isolated bacteria | Sum of Squares | df  | Mean square | F        | Sig. |
|-------------------|----------------|-----|-------------|----------|------|
| Aerobic mesophilic| Between Groups | 73457.450 | 57 | 4179.856  | 1.065  | 0.376 |
| Bacteria          | Within Groups  | 4049.000  | 2  | 3354.500  |        |      |
| **Total**         |                | **78966.250** | **59** |          |      |      |
|                   | Between Groups | 74768.050 | 47 | 4398.121  | 3.045  | 0.256 |
| Total coliform     | Within Groups  | 2174.500  | 2  | 1087.250  |        |      |
| **Total**         |                | **76942.550** | **49** |          |      |      |
|                   | Between Groups | 31134.050 | 57 | 1762.238  | 0.151  | 0.732 |
| Feacal coliform    | Within Groups  | 26356.800 | 2  | 13458.250 |        |      |
| **Total**         |                | **67371.350** | **59** |          |      |      |

Note: df = degree of freedom, p = 0.05 at 95% confidence interval.

Table 5 shows the morphological features of the identified fungal species. These were obtained from the potato dextrose agar medium and were of typical *Aspergillus flavus* (ASPf) and *Aspergillus niger* (ASPn) respectively. Table 6 shows the MPN/g of the ASPf and ASPn in 30 plates of each vegetable (tomato, onion, onion, and spinach).

Table 5: Cultural and morphological characteristics of the isolated fungi

| Cultural appearance | Microscopic characteristics | Inference     |
|---------------------|-----------------------------|---------------|
| PDA: Irregular size, shape, black colonies | Conidia arise from stigma and bore in chain | *Aspergillus niger* |
| PDA: Grey to green irregular colonies | Conidiophore bore laterally on the hyphae | *Aspergillus flavus* |

Note: PDA = Potato Dextrose Agar

Table 6: Mean number of isolated fungi in the tested vegetables

| Sample ID | No. of plates (N) | Indicator Fungi | Vegetable types (MPN/g) |
|-----------|-------------------|-----------------|-------------------------|
|           |                   |                 | Tomato | Onion | Spinach | Carrot |
| 1         | 30                |                 | 244    | 401   | 621     | 543    |
| 2         | 30                | ASPf            | 514    | 437   | 543     | 332    |
| 3         | 30                |                 | 500    | 399   | 321     | 514    |
| Mean value|                   |                 | 419.3  | 412.3 | 495     | 463    |
| 1         | 30                |                 | 442    | 633   | 399     | 484    |
| 2         | 30                | ASPf            | 387    | 592   | 408     | 365    |
| 3         | 30                |                 | 542    | 328   | 537     | 501    |
| Mean value|                   |                 | 457    | 517.7 | 448     | 450    |

Note: 1, 2 and 3 = Number of sampling in first, second and third month respectively, ASPf = *Aspergillus flavus* ASPn = *Aspergillus niger*, MPN = Most probable number and N = No. of plates.
spinach, carrot) in three samples. The mean value of ASPf was higher in spinach (495 MPN/g) while ASPn was found to be higher in onion sample. We detected and enumerated an array of pathogenic bacteria and selected fungi associated with post-harvest fresh vegetables that are irrigated with wastewater in river Getsi irrigation site.

The objective of our study is to determine the contaminating microbes, their implications for food safety and effect on food borne illnesses or infections outbreak. Presence of microorganisms in vegetables is directly related to the water used and the hygienic conditions practiced during their cultivation, harvesting, postharvest handling, processing and distribution of the produce (Halablab et al., 2011). Consequently, these microorganisms on vegetables may act as a reservoir which may be responsible for further post-harvest contamination, if not reduced or eliminated (Barth et al., 2010; and Joint FAO/WHO Codex Alimentarius Commission, 1994). Eight different bacteria were identified which include Eschericia coli, Listeria monocytogene, Staphylococcus aureus, Klebsidia spp, Citrobacter spp, Faecal coliforms, Pseudomonas spp and Vibrio cholera respectively. In this study, nine cell of Eschericia coli were detected in the 25 plates of the vegetables.

Although, this number is lower than the infectious dose of 10-1000 cells per plate, it is still a concern, as it may cause symptomatic food-borne infection (Beuchat, 1996). Enterotoxigenic E. coli is a common cause of travelers’ diarrhea, an illness sometimes experienced when visiting developing countries. Raw vegetables are thought to be a common cause of travelers’ diarrhea. A prospective study comprising of 121 participants that were attending a conference in Mexico City revealed that enterotoxigenic E. coli caused illness in 44% of the participants as a result of eating salads containing raw vegetables. The typical incubation of E. coli is 2-5 days, causing symptoms comprising of bloody diarrhea, abdominal pain, leading hemolytic uremic syndrome and kidney failure especially in children and the elderly (Merson et al., 1976).

Listeria monocytogene was found to be 19 cells in the 25 plates of the vegetables, this pathogenic microorganism is implicated in febrile gastroenteritis in healthy adults, may lead to spontaneous abortion or stillbirth pregnant women; severe septicaemia and meningitis in neonates and immune compromised adults and mortality may be 20 to 40. Infectious dose of Listeria monocytogene is unknown, it depend upon the health of individual at an incubation period of 1 day to 5 or more weeks. It is widely distributed on raw fruits and vegetables and on plant material (Beuchat, 1996). Factors affecting its presence or persistence have yet to be determined. Vegetables and plant parts used as vegetables recipes may play an indirect role in disseminating the pathogen from natural habitats to the human food supply.

Moreover, 20 cells of staphylococcus aureus have been detected on these fresh and ready-to-eat vegetables. This organism is known to be carried by food handlers and survives in low temperature between 4 to 8 °C (39.2 to 46.4 °F). Therefore, survival of this organism may cause disease outbreak. This is because vegetables are mostly processed at lower temperature which impinges on their growth and proliferation. Similarly, Klebsidia spp was also detect and differentially confirmed. This organism is implicated in the aetiology of gastroenteritis with secondary abdominal cramp, discomfort, systemic fever and local septicemia is well established (WHO 2009), but exact ratio of its flora loads is debatable despite its well-known association with food borne disease.

Citrobacter spp, faecal coliform, pseudomonas spp and vibrio cholerae were also isolated. These entroinvasive microbes are implicated in the aetiology of urinary tract infection, cholera, bloody and non-bloody diarrhoea, acute abdomen, food poisoning and listeriosis (WHO, 2009). More many decades, food borne disease outbreaks specifically associated with leafy vegetables is been on records in USA and a recent study indicates that between 1973 and 2006, 502 (4.8%) outbreaks, 18.242 (6.5%) illnesses and 15 (4.0%) deaths were associated with “leafy greens”, describe as lettuce, cabbage, onions, Spanish or a salad item containing one or more of these leafy vegetables (Herman et al., 2008). Obviously, there is an observed paucity of data in Nigerian based public health consequences and reliable statistics on microbes induced food borne diseases out-brakes.

The bacteria species were further grouped into AMB, TCB and FCB base on their morphological features and the numeric index of each was determined using the colony forming units per gram (CFU/g). Table 3 below showed that, spinach contained the least CUF/g mean index of AMB (2.1 × 10⁵) and higher for TCB (5.4 × 10⁴), tomato was found to contain higher CUF/g for AMB (8.7 × 10⁴) and FCB (3.1 × 10⁴), while, onion contained the least CFU/g index of TCB (3.7 × 10⁴) and FCB (2.9 × 10⁴) respectively. The AMB, TCB and FCB levels were above the International Commission on Microbiological Specifications for Foods (ICMSF) recommended level of 3 log CFU g⁻¹ fresh weight. Carrots, tomato, onion and spinach are while harvested from the farm, and may become contaminated by pathogenic organisms that were harbored in the soil. The
high bacterial load in the vegetables can be attributed to the large surface area of the leaves suitable for water contact, making them susceptible to bacterial contamination or could be due to poor hygiene practices by handlers.

Furthermore, ASPf and ASPn were isolated from the vegetables. This outcome is concordant with other findings (Akinmusire, 2011), which also isolated ASPf and Phytopthora sp. from irrigated products. These are fungi of grey to green morphological appearance and conidiopore bore on microscopic outlook which proliferate in a favorable pH, water activity, atmospheric composition. In this study, we determined the most probable number (MPN) of the fungi. We obtained moderately higher values and imply that there are chances that the contaminated vegetables can harbor pathogens potential of causing foodborne communicable diseases associated with fungal contaminants.

4. Conclusion

Fresh vegetables obtained from farm site can be associated with pathogenic microbial load due to use of wastewater irrigational practice and should be which should be thoroughly wash before consuming them. This study shows that microbial loads on vegetables in the river Getsi irrigation farm were well above ICMSF recommended limits for vegetables. Consumers of these vegetables stand a chance of contacting food-borne diseases if the vegetables are not thoroughly wash and adequately processed. To prevent an endemic outbreak, efforts have to be made by the concern bodies in ensuring that standard of microbiological guidelines are stick to by both the farmers and marketers, and to promote campaigns, awareness and enlightenment on general hygiene and public food safety.

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