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Isolation and identification of bacteria and fungi associated with tomatoes

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Abstract

Lycopersicum esculentum (Tomato) is an important vegetable crop widely grown around the world. Their water content makes them susceptible to spoilage. This study was carried out to isolate, identify and characterize the bacteria and fungi associated with fresh and paste tomatoes collected from Oja-Oba Market in Ado-Ekiti. Standard microbiological protocols were followed in isolation, cultivation and biochemical identification of microorganisms. Bacteria identified included Listeria monocytogenes, Bacillus subtilis, Lactobacillus fermenti, Pseudomonas stutzeri and Rothia sp. While the fungi identified included Penicillum notatum, Saccharomyces sp., Aspergillus niger, Aspergillus flavus, Mucor mucedo, Aspergillus fumigatus and Rhizopus stolonifer. The mineral composition showed that potassium had the highest concentration; 642.230.18 mg/kg while lead showed the least concentration; 0.0010.00 mg/kg. Chemical properties revealed that moisture in fresh tomatoes had the highest concentration of 90.460.14% and fat had the lowest concentration in fresh tomatoes; 0.820.02%. The phytochemical of tomato extracts in qualitative screening showed Alkaloid and phenol are most present while the quantitative screening showed that phenol had the highest concentration; 33.360.00 mg/g and Anthraquinones showed the lowest concentration; 0.020.002 mg/g. Polymerase chain reaction (PCR) amplification was done using gene primers GC-Clamp-EUB f933 and EUB r1389 for bacteria while GMF1 and GMF2 primer were used for fungi with 100bp (base pair) ladder molecular weight marker. This work revealed Rothia sp. as the highest occurring bacteria and Aspergillus fumigatus for fungi. The isolates produce toxins and hereby pose a potential risk to consumers; keeping fresh tomatoes in an uncontrolled environment for about three days or more is not safe for human consumption and this could lead to food poisoning and food-borne illness.

Keywords: *Lycopersicon esculentum* (Tomato), Days, Phytochemical, Human, Bacteria, Fungi.

Introduction

Lycopersicon esculentum (Tomato) is a vegetable that constitute commercially and nutritionally important food commodity. Tomato is one of the most important vegetable worldwide. It has become an important commercial crop so far; as the area, production, industrial values and its contribution to human nutrition is concerned. Tomato plays a vital role in meeting domestic and nutritional food requirements, generation of income, foreign exchange earnings and creation of employment [1]. Mukaminega [2] further suggested that post-harvest losses of tomato fruits also occur on transit due to long distance to markets, poor and inadequate infrastructures and the mode of transportation.

Tomato belongs to the family *Solanaceae*, which includes more than 3000 species. *Solanum* section Lycopersicon includes the cultivated tomato, *Solanum lycopersicum*, the only domesticated species, as well as a dozen other wild relatives [3]. Naturally, Fruits and vegetables carry epiphytic micro flora. During growth, harvest, transportation and further processing and handling the produce can be further contaminated with non-pathogenic and pathogenic organisms from soil, human or animal sources.

Materials and Methods

Samples Collection

Four tomatoes samples (two fresh tomatoes and two tomatoes pastes) were purchased from Oja oba (kings' market) in Ado, Ekiti State, Nigeria. They were transported to the microbiology laboratory of Afe Babalola University, Ado Ekiti in a polythene bag for microbiological analysis. The fresh tomatoes and fresh paste samples were left open for 7 days for spoilage to occur. Studies were performed from day 1 to 7 days.

Materials Sterilization

All apparatus and materials used for the research were sterilized at 121°C for 15 minutes. This was done to avoid contamination during the media preparation as well as the sample processing.

Samples Processing

One gram of each of the spoilt tomatoes was carefully cut with the aid of a sterile scalpel and enriched in sterile sabouraud dextrose broth for twenty-four hours. Ten-fold serial dilutions of the samples were thereafter carried out.

Total Bacteria Counts

The pour-plate method according to Harigan and McCane [4] was adopted. Using standard Microbiological technique (serial dilution), a tenfold dilution of 1g of the sample was carried out in 9 ml of sterile water (this was the aliquot). Precisely, 1 ml of the aliquot (supernatant) was pipetted and mixed in another 9 ml of sterile distilled water in a test-tube. The test-tube was shaken vigorously to homogenize. The exponential dilution continued to the fourth factor (10-4). 1 ml of the fourth factor was aseptically transferred and plated in duplicate sets using sterile molten lukewarm nutrient agar. The poured plates were allowed to set and were incubated at 37°C for 48 hours. Discrete colonies that developed after incubation were counted and enumerated as colony forming unit per gram (cfu/g) after multiplying with the dilution factor 10-4.

Isolation of Fungi

The pour-plate method also was used for the isolation of fungi following the method of Barnett and Hunter [5]. The BECTO Sabouraud Dextrose agar and potato dextrose agar were used. The diluents from the 10-4 test-tube were aseptically transferred to sterile Petri dishes and about 15 to 20 ml of sterile-molten lukewarm SDA/PDA was poured into the plate, allowed to set and incubated at room temperature (282°C). Colonies that developed after incubation were counted, enumerated in colony forming unit per gram (cfu/g) samples.

Purification (subculture) of bacterial isolates Colonies from the primary plates were aseptically picked with a sterile wire loop and transferred onto freshly prepared sterile nutrient agar plate, with a streaking technique such that discrete colonies appear at the ends of streaked lines after incubation. The subculture plates were incubated at 37°C for 24 hours to 48 hours. Discrete colonies from the subculture plates were aseptically transferred and streaked on slant and incubated for another 24 hours at 37°C.

Purification of Fungal Isolates

Colonies from the primary plates were aseptically picked with a sterile inoculation needle and transferred onto a freshly prepared sterile SDA plate with a streaking method and incubated for 5-7days at 28°C-30°C. Discrete colonies were aseptically transferred and stocked on slant and incubated for another 5 days at 28°C-30°C. Pure colonies were stored in the refrigerator at 10°C-15°C until needed for characterization and identification.

Biochemical Identification of Bacterial and Fungal Isolates

All bacterial and fungal isolates were characterized and identified considering their cultural, morphological, microscopic examination and biochemical characteristics following the methods prescribed by Holt et al. [6]. Biochemical test conducted include the following: Gram stain, Catalase test, Oxidase test, Motility test, Methyl red test, Citrate test, Urease test, Spore formation and Sugar fermentation test. The identity of each fungus was confirmed with the aid of a mycological atlas.

Proximate Analysis of Tomatoes: The proximate analysis of this research was done using the method of Trease and Evans, 2002 [7].

Phytochemicals

Test for Alkaloids (Mayer's test): To a few millilitre of plant sample extract, two drops of Mayer's reagent are added along the sides of test tube. Appearance of white creamy precipitate indicated the presence of alkaloids.

Test for Flavonoids (Lead acetate test): Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavonoids.

Test for Steroids: Two millilitre of acetic anhydride was added to 5 mg of the extracts, each with 2 ml of H2S04. The colour change from violet to blue or green in some samples indicated the presence of steroids.

Test for Anthraquinones: Anthraquinones may be detected by the Borntraeger's reactions. 0.2 gram of leaves and seeds were added to sulfuric acid solution (5 mL, 2N) and boiled for 2 minutes. After cooling, 10ml toluene solvent was added and decanted to stay for 10 minutes. The yellow colour of the toluene layer turned to red in alkaline pH indicated that anthraquinones was present.

Test for Phenols (Ferric chloride test): Ten milligram extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenol.

Test for Saponins: About 0.5 milligram of the extract was shook with five millilitre of distilled water. Formation of frothing (appearance of creamy miss of small bubbles) showed the presence of saponins.

Test for Glycosides: Two millilitre of organic extract was dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added to it. The solution was cooled with ice, sulphuric acid was then added carefully. A colour change from violet to blue to green indicated the presence of glycosides.

Test for Tannins: A small quantity of extract was mixed with water and heated on a water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green colour was formed. It indicated the presence of tannins

Test for Cardiac Glycosides: Crude extract was mixed 2 ml of glacial acetic acid containing 1-2 drops of 2% solution of FeCl3. The mixture was then poured into another test tube containing conc.H_2SO_4. A brown ring at the interphase indicated the presence of Cardiac Glycosides.

Test for Reducing Sugars (Benedict's test): Two (2) gram of the sample is dissolved in distilled water, and a small amount of Benedict reagent is added to it, a positive test was shown by a colour change from clear blue to brick red precipitate.

Digestion of Sample for Mineral Analysis

The samples were homogenized, and oven dried at 45°C-50°C prior to digestion. One gram of sample was weighed in a platinum crucible and placed in a muffle furnace at 450°C-550°C until all carbon contents were removed as evidence by a white ash. The ash was dissolved in 10 ml Nitric acid (5%) and gently warmed on a water bath to speed up dissolution of the ash. The dissolved ash solution was filtered and brought to 50 ml and presented for metal analysis.

The analytical method used for the analysis of metal concentration was spectrophotometry and the equipment used was Atomic Absorption Spectrophotometer (AAS) Buck Scientific model 211 VGP and Flame Photometer FP 902 PG, using the calibration plot method. Three processes were involved; standard preparation, equipment calibration and sample analysis.

For each element, the instrument was auto-zeroed using the blank (distilled water) after which the standards were aspirated into the flame from the lowest to the highest concentration (calibration). The corresponding absorbance was obtained by the instrument and the graph of absorbance against concentration was plotted. The samples were analysed with the concentration of the metals present being displayed in parts per million (ppm) after extrapolation from the standard curve [8].

DNA Extraction

The extraction was carried out using Bio Gene kit and the manufacturer guidelines were followed. A reasonable amount of fungi was picked with took pick deep from the mycelia into eppendorf tube containing 100 microliters of distilled water. Then vortex vigorously and centrifuge for 1 minute. The supernatant was carefully decanted using micropipette. 100 microliters of lysis buffer were added (Autoclave lysis solution at 121°C for 20 minutes before use. Lysis buffer is as follows 50nMole of sodium phosphate at pH 7.4, EDTA and 5% of glycerol). The mixture was then incubated at 85oC in a water bath for 30 minutes. The crude extracts contain genomic DNA.

Polymerase Chain Reaction Amplification

Individual components were mixed prior to use. All reaction components were assembled on ice packs. 25 µl of 2X master mix was pipette and mixed with standard buffer into PCR tubes. 1 µl of 10 µM forward and reverse primers were pipette into the above setup then a variable amount of template DNA was added. 50 µl with Nuclease free water was made up. Primer used are GC-Clamp-EUB f933 (GCACAAGCGGTGGAGCATGTGG) and EUB r1389 (GCCCGGGAACGTATTCACCG) for bacteria while GMF1 (TGTACACACCGCCCGTC) and GMF2 (CTGCGTTCTTCATCGAT) primer. The reactions were gently mixed and collected at the bottom of the tube with a quick spin. The reaction was quickly transferred to a pre heated thermocycler (94°C). The thermocycling conditions for PCR were as follows: for an initial denaturation of 94oC for 30 seconds followed by 30 amplification cycles of 15-30 seconds at 94oC; 15-60 seconds at 45-68°C and 1 minute at 68°C. This was followed by final extention step of 5 minutes at 68°C. Holding temperature was at 4-10°C. The amplification products were separated on a 1g of agarose gel and electrophoresis were carried out at 100V for 45 minutes. 100bp DNA ladders (solis Biodyne) were used as DNA molecular weight marker.

Statistical Analysis

Data were represented as mean standard error (SE). Significant of differences between tomatoes, minerals and the phytochemical properties and significant results were compared with Duncan's multiple range tests using SPSS version 17 software. For all the tests, the significant was determined at the level of $P<0.05$.

Results

Identification and Characterization of Isolates

In this study, the isolated bacterial and fungal species from the tomatoes sample, both fresh and spoiled tomatoes showed different biochemical and morphological characteristics from day 1 to 7. The cultural, morphological and biochemical characteristics of the bacteria and fungi isolates from fresh and spoiled tomatoes are shown in Tables 1 and 3 respectively. Microorganisms identified include *Listeria monocytogenes, Bacillus subtilis, Lactobacillus fermenti, Pseudomonas stutzeri, Rothia sp, Penicillium notatum, Saccharomyces sp, Aspergillus niger, Mucor mucedo, Aspergillus flavus, Aspergillus fumigatus* and *Rhizopus stolonifer.* A combination of morphological and biochemical reactions were used to identify the isolates based on standard bacteriological manuals (Table 3). The isolates were all gram positive except *Pseudomonas stutzeri* which is the only gram negative. Catalase, urease, methyl red was all positive while coagulase was negative for all the isolates. The isolated bacteria showed different characteristics such as milky, flat, raised, irregular, creamy and translucent and they are all rod shaped while fungi showed different colony colour that range from bluish green, moist milky, brownish, creamish yellow, yellow green, blue green and cotton white (Table 3). Table 2 and 4 showed the distribution of bacterial and fungal isolates. The microbial observed under tomato deterioration for seven days showed the fungal and bacterial present in each day (Table 5 and 6).

Table 1: Morphological, cultural, biochemical characteristic and microscopic examination of the bacteria isolates.

Bacteria isolates	Morphological Appearance	Cell shape	Gram reaction	catalase	oxidase	coagulase	Citrate	Urease	Methyl red	Motility	Spore formation	Lactose	Maltose	Glucose	Sucrose	Manni
Listeria monocytogenes	Milky, flat, Irregular, translucent	Rod	+	+	-	-	+	+	+	+	-	OO	AO	AG	AG	AG
Listeria monocytogenes	Milky, flat, Irregular, translucent	Rod	+	+	-	-	+	+	+	+	-	OO	AO	AG	AG	AG
Bacillus subtilis	Translucent, Milky, Raised, Irregular, translucent	Rod	+	+	-	-	+	+	+	+	+	AG	AG	AG	AG	AG
Lactobacillus fermenti	Milky, flat, Irregular, translucent	Rod	+	+	-	-	+	+	+	-	-	AG	AO	AG	AG	OO
Pseudomonas stutzeri	Creamy, flat, Irregular, translucent	Rod	-	+	+	-	-	+	+	+	-	OO	AO	AO	OO	OO
Rothia sp	Milky, Raised, circular, translucent	Rod	+	+	+	-	+	+	+	-	-	AO	AO	AG	AG	AO

Table 2: Distribution of bacterial isolates.

Bacteria	Total count	Frequency (%)
Listeria monocytogenes	7	5
Bacillus subtiliis	3	2.2
Lactobacillus fermenti	33	23.7
Pseudomonas stutzeri	5	3.6
Rothia sp	91	65.5

Table 3: Morphological and microscopic examination of the fungal isolates.

Fungal vesicle organism shape	Colony colour	Somatic Structure	Nature of byphae	Special vegetative	Asexual spore	Special reproductive	Conidial head	Structure
Penicillium notatum	Bluish green	Filamentous	Septate	Broom like appearance	Globose chained conidia	Brush-like conidiophores	-	-
Saccharomyces sp	Moist milky colony	Unicellular	-	-	Budding cells	-	-	-
Aspergillus niger	Brownish colony	Filamentous	Septate	Footcell	Globose conidia	Smooth walled erect conidia	Globose	Globose
Mucor mucedo	Creamish yellow	Filamentous	Coenocytic	-	Sporangiospore	Sympodially branched sporangiophore, zygospore	-	——
Aspergillus flavus	Yellow green colony	Filamentous	Septate	Footcell	Globose conidia	Phialides borne directly on vesicle, sclerotia	Radiate	Subglobose
Aspergillus fumigatus	Blue green	Filamentous	Septate	Footcell	Globose conidia	Short conidiophores	Dome-shaped broadly clavete	-
Rhizopus stolonifer	Cotton white	Filamentous	Coenocytic	Stolons, rhizoids	Ovoid sporangiospores	Tall sporangiophores in groups	-	-

Table 4: Distribution of fungal isolates

Fungi	Total count	Frequency (%)
Penicillium notatum	71	25
Saccharomyces sp	27	9.5
Aspergillus niger	12	4.2
Mucor mucedo	11	3.9
Aspergillus flavus	7	2.5
Aspergillus fumigatus	128	45
Rhizopus stolonifer	28	9.9

Table 5: Bacteria observed under deterioration process for seven days (cfu/g).

Bacteria	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Listeria monocytogenes	2	5	-	-	-	-	-
Bacillus subtiliis	1	2	-	-	-	-	-
Lactobacillus fermenti	2	7	8	8	5	1	2
Pseudomonas stutzeri	3	2	-	-	-	-	-
Rothia sp	28	15	20	6	8	7	7

Table 6: Fungi observed under deterioration process for seven days (sfu/g).

Bacteria	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Penicillium notatum	16	15	10	10	5	7	8
Saccharomyces sp	14	8	5	-	-	-	-
Aspergillus niger	7	-	-	-	-	-	-
Mucor mucedo	8	3	-	-	-	-	-
Aspergillus flavus	-	5	2	-	-	-	-
Aspergillus fumigatus	25	18	12	15	18	22	18
Rhizopus stolonifer	5	4	5	8	2	2	2

Microbial Counts/Microbial densities

Tables 7 and 8 showed the microbial counts in tomato from day 1 to day 7. Spoilt non-processed tomato had the highest count in bacterial in day 2 ($5.2x10^3$ cfu/g) while Processed and spoilt processed had the lowest count in bacterial ($1.0x10^2$ and $1.0x10^2$ respectively). Table 8 showed non-processed tomato had the highest fungal count in day 3 ($3.5x10^5$ sfu/g) while spoilt processed had the lowest fungal count in day 5 ($1.0x10^3$ sfu/g). There was significant difference at $p<0.05$ between non-processed tomatoes, spoilt non-processed, processed and spoilt processed.

Table 7: Total Bacterial Counts (cfu/g).

Bacteria	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A	1.5×10^4	2.8×10^6	2.2×10^4	3.0×10^3	3.1×10^3	3.0×10^2	1.2×10^4
B	2.0×10^3	3.4×10^4	3.7×10^4	3.3×10^3	2.8×10^3	1.0×10^2	1.0×10^2
C	4.5×10^3	5.2×10^3	2.5×10^3	1.0×10^3	2.0×10^3	1.3×10^2	1.0×10^4
D	3.7×10^3	4.8×10^3	2.3×10^5	1.5×10^3	1.3×10^3	1.6×10^3	1.0×10^2
A: Non-processed Tomatoes; B: Processed Tomatoes; C: Spoilt Non-processed Tomatoes; D: Spoilt Processed Tomatoes.							

Table 8: Total Fungi counts (sfu/g).

Bacteria	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
A	2.0×10^5	1.8×10^6	3.5×10^5	2.3×10^4	1.5×10^3	1.8×10^3	1.5×10^3
B	1.0×10^5	1.5×10^6	1.0×10^5	2.1×10^3	2.5×10^2	2.0×10^3	1.6×10^5
C	2.2×10^5	3.0×10^5	2.7×10^5	1.5×10^4	1.7×10^3	2.1×10^3	1.2×10^2
D	1.8×10^5	1.5×10^6	2.1×10^4	3.0×10^5	1.0×10^3	2.0×10	1.3×10^3
A: Non-processed Tomatoes; B: Processed Tomatoes; C: Spoilt Non-processed Tomatoes; D: Spoilt Processed Tomatoes.							

Nutritional Composition of Tomatoes

Metal/Mineral Analysis: This was done using Spectrometry to analyse the nutritional properties of the non-processed tomato. The table shows four different samples of tomatoes that was analysed in triplicates; Non-processed Tomatoes, Processed Tomato, Spoilt Non-Processed Tomato and Spoilt Processed Tomato are represented by A, B,C,D respectively. Eight essential nutrient elements were examined from the tomatoes such as Sodium, Calcium, Potassium, Magnesium, Phosphorous, Iron, Copper, Zinc and Lead. From the graph in Figure 1, potassium shows the highest concentration in sample B; 642.230.18 (mg/kg). Followed by potassium in sample A; 605.030.15 (mg/kg) while Lead shows the least concentration 0.0010.00 (mg/kg) (Table 9)

Table 9: Mineral contents of tomato samples.

Contents	Non-Processed	Processed	Spoilt Non-Processed	Spoilt Processed
Na (mg/kg)	$8.83\pm0.23_a$	$14.60\pm0.15_b$	$5.57\pm0.07_c$	$7.20\pm0.06_{ac}$
Ca (mg/kg)	$101.23\pm0.27_a$	$163.63\pm0.35_b$	$71.07\pm0.52_c$	$68.10\pm0.12_c$
K (mg/kg)	$605.03\pm0.15_a$	$642.23\pm0.18_b$	$373.97\pm0.09_c$	$312.30\pm0.15_d$
Mg (mg/kg)	$12.49\pm0.13_a$	$17.02\pm0.16_b$	$16.70\pm0.62_b$	$16.48\pm0.31_b$
P (mg/kg)	$32.60\pm0.03_a$	$55.67\pm0.49_b$	$31.92\pm0.26_c$	$28.37\pm0.38_a$
Fe (mg/kg)	$0.37\pm0.13_a$	$1.11\pm0.02_b$	$10.28\pm0.07_c$	$7.80\pm0.28_d$
Cu (mg/kg)	$0.37\pm0.01_a$	$0.83\pm0.02_a$	$0.20\pm0.003_a$	$0.22\pm0.01_a$
Zn (mg/kg)	$0.73\pm0.002_a$	$1.11\pm0.08_b$	$0.51\pm0.01_a$	$1.19\pm0.01_b$
Pb (mg/kg)	$0.001\pm0.00_a$	$0.001\pm0.00_a$	$0.003\pm0.001_a$	$0.009\pm0.002_a$
Values are meansSEM of triplicate determinations. Values in the same row having the same subscript are not significantly different at $p<0.05$; A: Non-processed Tomatoes; B: Processed Tomatoes; C: Spoilt Non-processed Tomatoes; D: Spoilt Processed Tomatoes.				

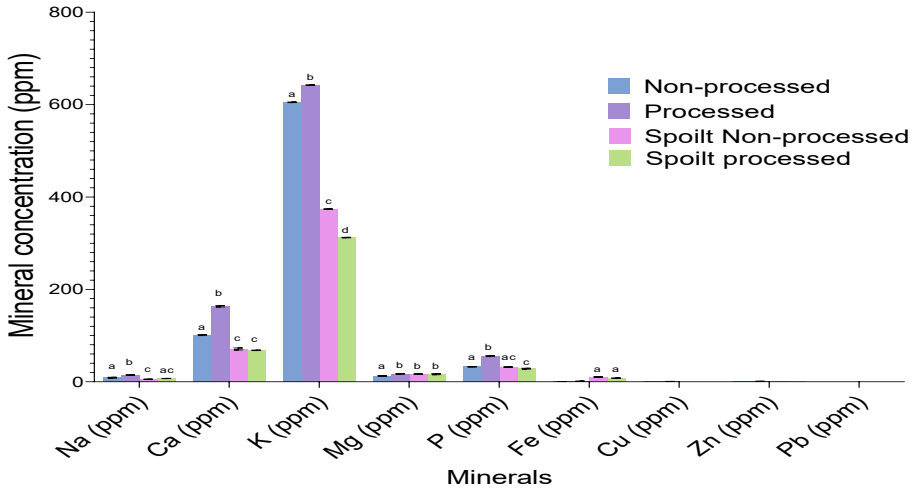

Figure 1: Mineral content of A, B, C D.

Proximate Analysis: Table 10 showed the partitioning of compounds in tomato samples based on the chemical properties of the compounds. The following were determined in triplicate on the four tomato samples; Moisture, Ash, Fat, Crude Fibre, Protein, and Carbohydrate. There is high concentration of moisture in the non-processed sample 90.460.14% and lowest in processed tomatoes 70.410.04% and this was very much visible in Figure 10. There is high concentration in ash content for processed tomatoes 2.780.04% and lowest concentration in non-processed tomatoes 1.170.01%. Fat had the highest concentration in processed tomatoes 2.070.03 % and lowest concentration in non-processed tomatoes 0.820.02%. Crude fibre had the highest concentration in non-processed tomatoes 2.360.03% and lowest concentration in processed tomatoes 2.140.01%.

The percentage concentration of moisture was very high from the graph in figure 10 the different types of tomatoes when compared to the percentage contents of ash, fats, crude fibre, protein and carbohydrate Figure 2 also showed precisely a higher percentage concentration in moisture content of non-processed tomatoes when compared to processed and spoilt processed tomatoes, with the spoilt non-processed tomatoes also showed on the graph to have a higher moisture content when compared to processed and spoilt processed tomatoes.

Protein had the highest concentration in processed tomatoes 3.840.01% and lowest concentration in spoilt non-processed tomatoes 1.130.02%. Carbohydrate had the highest concentration in spoilt processed tomatoes 21.250.03% and lowest concentration in non-processed tomatoes 3.590.14%.

Table 10: Proximate composition of tomato samples.

Contents	Non-Processed	Processed	Spoilt Non-processed	Spoilt Processed
Moisture (%)	$90.46\pm0.14_{a}$	70.41±0.04b	81.63±0.02c	71.21±0.04d
Ash (%)	$1.17\pm0.01_{a}$	2.78±0.04b	1.27±0.02a	2.48±002b
Fat (%)	0.82±0.02a	2.07±0.03b	1.51±0.01c	1.20±0.01d
Crude Fibre (%)	2.36±0.03a	2.14±0.01a	2.33±0.04a	2.17±0.01a
Protein (%)	1.60±0.01a	3.84±0.01b	1.13±0.02c	1.68±0.06a
Carbohydrate (%)	3.59±0.14a	18.76±0.04b	12.14±0.08c	21.25±0.03d
a:Non-processed Tomatoes; b:Processed Tomatoes; c:Spoilt Non-processed Tomatoes; d=Spoilt Processed Tomatoes; Values are means SEM of triplicate determinations. Values in the same row having the same superscript are not significantly different at 5% level.				

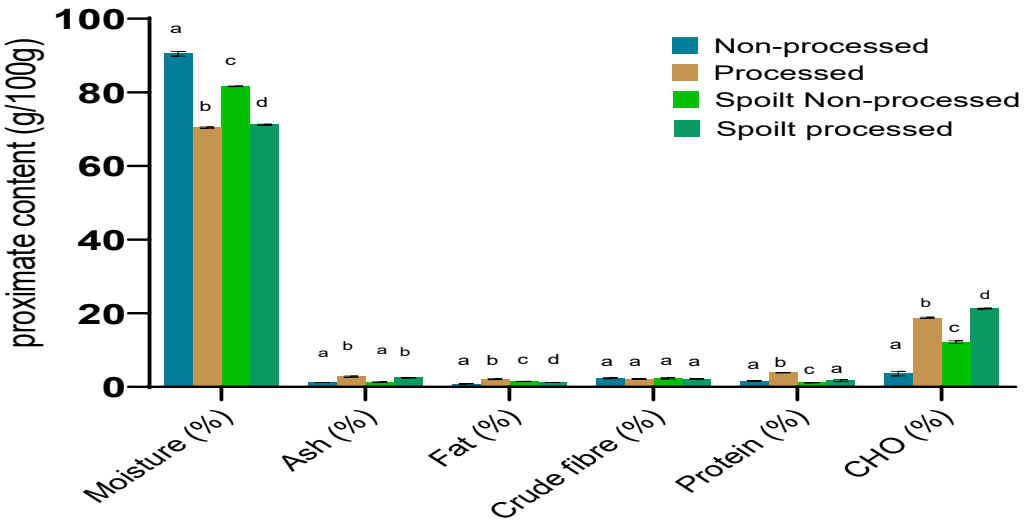

Figure 2: Proximate composition of A,B,C,D.

Phytochemical Screening: Table 11 showed tomato extracts that were subjected to a phytochemical screening to obtain a qualitative criterion of the chemical composition, according to the protocol of [9]. This method helps to detect groups of secondary metabolites present in the extracts using aqueous (aq) and methanol (m) solution, the metabolites that were determined and the solution performed for their identification. The samples were checked for using aqueous and methanol solution for the phytochemical parameters like Alkaloids, Tanins, phenols, Saponins, Anthraquinones, steroids, flavonoids, Glycosides, Cardiac glycosides and reducing sugar. For qualitative screening for sample A in alkaloids which is fresh tomato shows that it is most present (+++) in methanol (m) solution than in aqueous (aq) while it is very present (++) in aqueous solution for alkaloids sample A, B and C. For Tanins, which was present (+) in all the samples; both in methanol and aqueous solution. The Tanins were present (+). For Phenols; sample A(m), D(aq) and D(m) were most present (+++) while A(aq), B(aq), B(m), C(aq), C(m) were very present (++). For Saponins; they were not present (-) in A(aq) but were present in sample A(m), B(aq), B(m), D(aq) and D(m). Steriods is not present (-) for both aqueous and methanol solution for fresh tomatoes which is Sample A as well as in sample C which is the spoiled fresh tomato, then present (+) in both sample B and D (processed and spoilt processed tomatoes). Flavonoids in sample A and sample C were present (+) while they were very present (++) in sample B and D both in aqueous and methanol solution. Glycosides were not present (-) in sample A,B and D while it is not present (-) in sample C. Cardiac glycosides was not present (-) in sample A(aq), they were (-) in sample C(aq) and C(m), along with sample D(aq). It is then present (+) in sample A(m), B(aq), B(m) and D(m). Reducing sugar was present (+) in all the four samples both in aqueous and methanol solution.

Table 12 showed quantitative screening of the tomatoes in aqueous (aq) and methanol (m) solution. The presence of alkaloids were high in sample A(m) 10.550.04 (mg/100g) while sample D(aq) shows the lowest 2.280.090 (mg/100g). For Tanins; sample A(m) shows the highest concentration 2.670.291 (mg/g) while sample C(aq) shows the lowest with 0.20 ± 0.007 (mg/g). Phenols; sample B(m) shows the highest concentration 33.36 0.00 (mg/g) while sample C(aq) shows the lowest 12.490.05 (mg/g). Saponins; sample D(aq) shows the highest concentration 0.850.003 (mg/100g) while sample C(aq) shows the lowest 0.350.006 (mg/100g). Steroids; sample D(aq) and D(m) shows the highest 0.160.000 (mg/g) and sample A(aq) shows the lowest 0.040.003 (mg/g). Anthraquinones; sample B(m) shows highest concentration 0.21 ± 0.001 (mg/g) and sample C(aq) shows lowest with 0.020.002 (mg/g). Flavonoids; sample B(m) shows highest concentration 12.740.00 (mg/g) while sample A(aq) shows the lowest 5.010.002 (mg/g). Glycosides; sample D(m) shows highest with 2.610.00 (mg/100g) and sample C(aq) shows lowest with 0.490.004 (mg/100g). Cardiac glycosides; sample B(m) shows highest concentration 0.68±0.001 (mg/g) while sample C(m) shows lowest concentration with 0.040.003 (mg/g). Reducing sugar; sample D(m) shows highest concentration with 2.480.04 (mg/g) while sample A(aq) shows lowest with 1.110.004 (mg/g).

Table 11: Phytochemical Screening (QUALITATIVE) of aqueous and methanol extracts of the samples.

Phytochemical screening (Qualitative parameters)	A(aq)	A(m)	B(aq)	B(m)	C(aq)	C(m)	D(aq)	D(m)
Contents	**A(aq)**	**A(m)**	**B(aq)**	**B(m)**	**C(aq)**	**C(m)**	**D(aq)**	**D(m)**
Alkaloids	++	+++	++	++	++	+	+	+
Tanins	+	+	+	+	+	+	+	+
Phenols	++	+++	++	++	++	++	+++	+++
Saponins	-	+	+	+	+	+	+	+
Anthraquinones	-	+	+	+	+	+	+	+
Steroids	-	-	+	+	-	-	+	+
Flavonoids	+	+	++	++	+	+	++	++
Glycosides	+	+	+	+	-	-	+	+
Cardiac Glycosides	-	+	+	+	-	-	-	+
Reducing sugar	+	+	+	+	+	+	+	+

Table 12: Phytochemical Screening (QUANTITATIVE) of aqueous and methanol extracts of the samples.

Contents	A(aq)	A(m)	B(aq)	B(m)	C(aq)	C(m)	D(aq)	D(m)
Alkaloids (mg/100g)	$7.16\pm0.022_a$	$10.55\pm0.04_b$	$5.83\pm0.033_c$	$5.40\pm0.250_d$	$4.18\pm0.024_e$	$4.73\pm0.103_f$	$2.28\pm0.090_g$	$3.53\pm0.00_h$
Tanins (mg/g)	$0.31\pm0.009_a$	$2.67\pm0.291_b$	$1.53\pm0.088_b$	$1.82\pm0.016_b$	$0.20\pm0.007_c$	$0.29\pm0.002_c$	$0.24\pm0.004_c$	$0.31\pm0.00_d$
Phenols (mg/g)	$20.13\pm0.004_a$	$28.28\pm0.00_b$	$30.16\pm0.00_c$	$33.36\pm0.00_d$	$12.49\pm0.05_e$	$14.77\pm0.07_f$	$22.12\pm0.011_g$	$30.54\pm0.0_h$
Saponins (mg/100g)	$0.53\pm0.004_a$	$1.12\pm0.001_b$	$1.36\pm0.004_c$	$1.81\pm0.005_d$	$0.35\pm0.006_e$	$0.70\pm0.006_f$	$0.85\pm0.003_g$	$1.26\pm0.00_h$
Steroids (mg/100g)	$0.04\pm0.003_a$	$0.05\pm0.002_b$	$0.14\pm0.002_b$	$0.15\pm0.001_b$	$0.07\pm0.001_a$	$0.08\pm0.004_a$	$0.16\pm0.000_b$	$0.16\pm0.00_b$
Anthraquinones (mg/g)	$0.04\pm0.002_a$	$0.14\pm0.003_b$	$0.14\pm0.035_b$	$0.21\pm0.001_b$	$0.02\pm0.002_a$	$0.09\pm0.001_c$	$0.12\pm0.002_c$	$0.17\pm0.00_d$
Flavonoids (mg/g)	$5.01\pm0.002_a$	$7.06\pm0.001_b$	12.15±0.00c	$12.74\pm0.00_d$	$4.47\pm0.020_e$	$5.32\pm0.007_f$	$9.14\pm0.008_g$	$9.60\pm0.00_h$
Glycosides (mg/100g)	$1.04\pm0.003_a$	$1.13\pm0.002_b$	$1.14\pm0.001_b$	$2.38\pm0.001_c$	$0.49\pm0.004_d$	$0.76\pm0.010_e$	$1.34\pm0.004_f$	$2.61\pm0.00_f$
Cardiac Glycosides (mg/g)	$0.06\pm0.003_a$	$0.14\pm0.012_b$	$0.22\pm0.004_b$	$0.68\pm0.001_c$	$0.05\pm0.003_d$	$0.04\pm0.003_e$	$0.05\pm0.000_e$	$0.11\pm0.00_f$
Reducing Sugars (mg/g)	$1.11\pm0.004_a$	$1.87\pm0.027_b$	$2.25\pm0.002_c$	$2.28\pm0.002_d$	$1.76\pm0.009_b$	$1.35\pm0.003_e$	$2.31\pm0.003_f$	$2.48\pm0.04_f$

Values are meansSEM of triplicate determinations. Values in the same row sharing the same letters are not significantly different at $p<0.05$. A: Non-Processed Tomatoes; B: Processed Tomatoes; C: Spoilt Non-Processed Tomatoes; D: Spoilt Processed Tomatoes.

DNA Fingerprints

The DNA fingerprints of bacteria and fungi were represented on the gel from lane1-12. The fingerprints for bacteria had the same allelic representation and base pair from lane 1-5.of the agarose gel and the fungi DNA were very visible as well running from LANE 6-12, this was well represented in Plate 1.

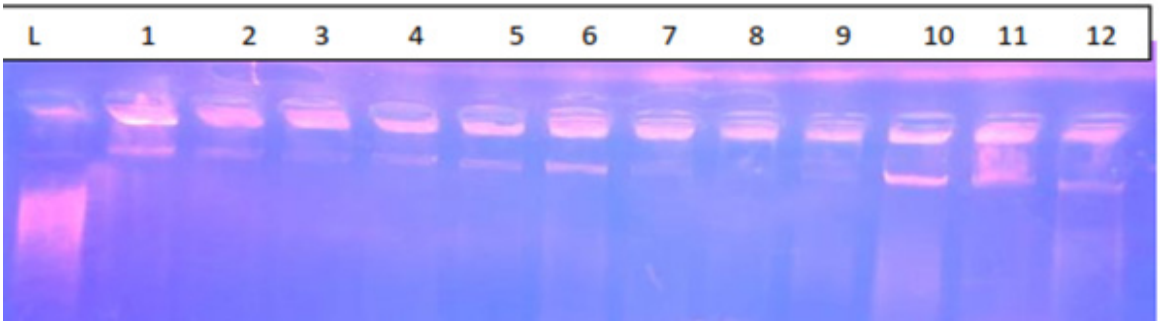

Plate 1: Amplification of DNA fingerprints for bacterial and fungal isolates.

The letter L represents 100bp ladder molecular weight marker. Samples 1 to 5 were bacteria while samples 6 to 12 were fungi.

Discussion

In this study, some microorganisms have been identified with the major cause of spoilage in tomatoes. Microorganisms identified include; *Listeria monocytogenes, Bacillus subtilis, Lactobacillus fermenti, Pseudomonas stutzeri, Rothia sp, Penicillium notatum, Saccharomyces sp, Aspergillus niger, Mucor mucedo, Aspergillus flavus, Aspergillus fumigatus* and *Rhizopus stolonifer.* In a previous study by Ibrahim (2011) showed that several fungi at different frequency of occurrences were found to be associated with contamination of tomato commonly sold at Dutse Ultra-Modern market. The most commonly encountered fungi associated with tomato contamination were Aspergillus *flavus* (18.18%), *A. niger* (36.36%), *Mucor spp.* (18.18%), *Penicillium spp.* (9.10%) and *Rhizopus stolinifer* (27.27%). This is in agreement with the finding of Akintobi *et al.* [10] who isolated A. niger, R. stolonifer, Mucor spp. and A. flavus from microorganisms associated with deterioration of tomato at Umuahia market, Abia State, Nigeria. This could probably be due to mechanical injuries such as cuts that occur during harvesting, postharvesting or storage periods which could provide infection sites for spoilage fungi, improper handling and lack of good storage facilities, ability of the fungi to produce spores and their ubiquitous nature, environmental conditions such as temperature and relative humidity etc. This is closely linked to the fact that spores of these organisms are easily transmitted via the air which could lead to spoilage. Some of the fungi isolated from this research have been reported to produce secondary metabolites which are potentially harmful to humans and other animals [11].

The microbial counts in tomato from day 1 to day 7. Spoiled fresh tomato had the highest count in bacterial in day 2 ($5.2x10^3$ Cfu/g) while fresh paste and spoiled paste had the lowest count in bacterial ($1.0x10^2$ and $1.0x10^2$ respectively). Fresh tomato had the highest fungal count in day 3 ($3.5x10^5$ Sfu/g) while spoiled paste had the lowest fungal count in day 5 ($1.0x10^3$ Sfu/g). There was significant difference at $p<0.05$ between fresh tomato, spoiled fresh, fresh paste and spoiled paste. *Listeria monocytogenes, Bacillus subtilis, Lactobacillus fermenti, Pseudomonas stutzeri, Rothia sp, Penicillium notatum, Saccharomyces sp, Aspergillus niger, Mucor*

mucedo, Aspergillus fumigatus and *Rhizopus stolonifer* was present in the raw sample. *Lactobacillus fermenti, Rothia sp, Penicillium notatum, Aspergillus fumigatus* and *Rhizopus stolonifer* were present throughout the deterioration process. *Rothia sp* showed the most occurring bacteria while *Aspergillus fumigatus* showed the most occuring fungi with 91 and 128 unit counts respectively.

In a previous study by Onuorah and Orji [12] showed that the fungi associated with the spoilage of post-harvest tomato fruits sold in major markets in Awka, Nigeria were studied, and the result revealed the presence of a teeming population of fungi. The average fungal counts ranged from 1.3 x 10^3 to 2.0 x 10^3cfu/ml. The fruits from Eke-Awka market had the highest count of 2.0 x 10^3cfu/ml while those from Nodu market had the lowest count of 1.3 x 10^3cfu/ml. The fungal isolates from the fruits were *Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Saccharomyces cerevisiae, Alternaria alternata, Penicillium digitatum* and *Geotrichum candidum.* A report of Ibrahim, [13] indicates *Aspergillus niger* as one of the major fungi responsible for the production of volatile compounds in spoilt tomatoes. A report of Baker, [14] also isolated *Aspergillus niger* from rotten tomato fruits and reported that they are pathogenic on tomato fruits. Report that *Rhizopus spp* were associated with the spoilage of tomatoes [15]. *Aspegillius spp, Penicillum spp, Fusarium spp* and *Saccharomyces* spp was isolated from spoilt tomato fruits [16]. *Aspergillius spp, Penicillum spp* and *Saccharomyces cerevisiae* was isolated from spoilt tomatoes [17] while [18] reported the presence of Alternaria alternata and *Fusarium oxysporum* in the spoilt tomato fruits they studied. Fusarium oxysporum, *Aspergillius niger* and *Rhizopus stolonifer* was isolated from the spoilt tomato fruits studied [19]. The result agreed with the work of [15,20]. They reported that Aspergillus niger had the highest rate of occurrence in the tomato fruits they studied and concluded that the fungus may be the major organism responsible for the spoilage of tomato fruits. The result of the pathogenicity test showed that the fungi inoculated into the healthy tomato fruits had the same features as the ones re-isolated from them, indicating that the fungi were responsible for the spoilage of the tomato fruits. *Aspergillus niger* produced the highest rot in the tomato fruits, with a rot diameter of 30mm while *Geotrichum candidum* produced the lowest rot diameter of 10 mm in the tomato fruits.

The mineral analysis was used to analyze the nutritional properties of both fresh and spoiled tomatoes using Spectrometry. The four different samples of the tomatoes were analyzed with eight essential nutrient element from the tomatoes such as Sodium, Calcium, Potassium, Magnesium, Phosphorous, Iron, Copper, Zinc and Lead. For all the four samples, potassium had the highest concentration in sample B; 642.23 ± 0.18 (mg/kg). Followed by potassium in sample A; 605.03 ± 0.15 (mg/kg). While Lead had the least concentration 0.001 ± 0.00 (mg/kg) in all the samples. In the proximate composition, there is high percentage concentration of moisture in the fresh sample 90.46 ± 0.14 % and lowest in paste tomatoes 70.41±0.04 %. Ash had highest concentration in paste tomatoes 2.78 ± 0.04 % and lowest concentration in fresh tomatoes 1.17 ± 0.01 %. Fat had highest concentration in paste tomatoes 2.07 ± 0.03 % and lowest concentration in fresh tomatoes 0.82±0.02 %. Crude fibre had highest concentration in fresh tomatoes 2.36 ± 0.03 % and lowest concentration in paste tomatoes 2.14 ± 0.01 %. Protein had highest concentration in paste tomatoes 3.84 ± 0.01 % and lowest concentration in spoiled fresh tomatoes 1.13 ± 0.02 %. Carbohydrate had highest concentration in spoiled paste tomatoes 21.25 ± 0.03 % and lowest concentration in fresh tomatoes 3.59 ± 0.14 %. Several factors could account for such a difference. The moisture content of the fresh tomato is in conformity with the finding of Romain [21] and Harry [22-24]. The phytochemical screening showed tomato extracts to obtain a qualitative criterion of the chemical composition, according to the protocol of [9]. This help to detect groups of secondary metabolites present in the tomatoes using aqueous (aq) and methanol (m) solution. The tomato samples were checked for using aqueous and methanol solution for the phytochemical parameters like Alkaloids, Tanins, phenols, Saponins, Anthraquinones, steroids, flavonoids, Glycosides, Cardiac glycosides and reducing sugar. In the qualitative screening, Alkaloids (methanol extract) in fresh tomatoes, phenols (methanol extract) in fresh tomatoes, phenols (methanol and aqueous extract) in spoiled paste tomatoes had the highest concentration i.e. they are most present in the tomato samples. In the quantitative screening, the phenols (methanol extract) had the highest concentration 33.36 ± 0.00 (mg/g) in paste tomatoes while anthraquinone had the lowest concentration 0.02 ± 0.002 (mg/g) in spoiled fresh tomatoes. In a previous study, the phytochemical screening showed that the aqueous extracts prepared from the fruits of *Solanum lycopersicum L* irrigated with the water treated with static magnetic field between 20 and 200 mT, revealed a marked presence of carotenoids, phenols, tannins, carbohydrates and flavonoids. There are no other previous studies that show phytochemical screening of tomatoes fruits and paste using aqueous (aq) and methanol (m) extracts.

Polymerase chain reaction (PCR) Amplification were done using gene primers GC-Clamp-EUB f933 and EUB r1389 for bacteria while GMF1 and GMF2 primer were used for fungi with 100bp (base pair) ladder molecular weight marker.

Conclusion

This work has revealed the highest occurring organisms isolated from tomatoes which are Rothia sp for bacteria and *Aspergillus fumigatus* for fungi produce toxins and hereby pose a potential risk to consumer. Keeping fresh tomatoes in an uncontrolled environment for about three days or more is not safe for human consumption and this could lead to food poisoning and food-borne illness.

Conflict of Interest

The Authors hereby declare there is no conflict of interest associated with this study or any of the procedures and materials used for the purpose of this study.

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