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Research Article

Biochemical and Morphological Determination of Bacteria and Fungi in Spoilt Mango and Watermelon Fruits in Sokoto State, Nigeria

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Abstract

Determination of bacteria and fungi in spoilt watermelon (Citrullus lanatus) and mango (Mangifera indica) sampled from two locations on varied coordinates in Sokoto state was done using standard procedures. The samples were mashed and serially diluted: the third and fourth dilution factors were cultured on Potato Dextrose Agar (PDA) and Nutrient Agar (NA) respectively and incubated aerobically at room temperature for 24 hours for bacteria and 5 days for fungi at 30oC. The pure cultures obtained were identified morphologically and biochemically. Investigations revealed that these fruits were infected with eleven bacterial and four fungal species. Watermelon fruits from Shagari dam site (latitudes 12o37-12o39 north; longitudes 4o59-5o14 east) had more bacterial isolates, out of which M. luteus was the predominant (17.39%). Also, mango fruits from Goronyo dam site (latitudes 13.53844o or 13o32 north; longitudes 5.94823o or 5o57 east) had more bacteria, out of which Bacillus megatarium was predominant one (17.39%). The bacterial composition indicated higher presence of Staphylococcus spp (4 species) and Bacillus spp (3 species) over Pseudomonas, Micrococcus and Escherichia (1 species each). Fungi determination showed that Aspergillus spp were the most prevalent (4 species) with A. niger having greater proportion (40%) and Saccharomyces cerevisiae had the lowest proportion (13.30%). This study recommends improved fruit handling, little or non-exposure of fruits to diseased or contaminated surfaces; and use of disease resistant cultivars in order to reduce spoilage by microorganisms and microbial infections to humans.

Keywords: Determination; Microorganisms; Exposure; Biochemical; Morphological; Mango; Watermelon; Sokoto

Introduction

Microbes are beneficial to biological processes by aiding decomposition, decay and even helping us to digest our food. However, there are some microorganisms which negatively impact on our lives, causing illnesses, bad odours and damaging biological products and surfaces. Fruits play a vital role in human nutrition by supplying necessary growth factors such as vitamins and essential minerals in daily diets which help us to live a healthy life [2]. The succulent nature of fruits and vegetables makes their surfaces easily penetrable by microbes. The high concentration of various sugars, minerals, vitamins and amino acids also provide a good platform for the successful growth and survival of microorganisms [5].

Food Spoilage by Microorganisms

Spoilage refers to any form of deterioration in the condition of food in which the food becomes undesirable or unacceptable for human consumption [1]. Some microbes are capable of colonizing and creating lesions on healthy, undamaged plant tissue. Fungal

spoilage by moulds is responsible for significant spoilage and economic losses in the food chain. In addition, some species belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium* are mycotoxigenic. Mycotoxins are a class of highly toxic chemical compounds produced under specific environmental conditions by several moulds [13].

Origin, distribution and production of watermelon

Watermelon (C*itrullus lanatus*) belongs to the family Cucurbitaceae [21]. Watermelon is grown in more than 96 countries worldwide. Asia Pacific Countries have the highest production figures (in 1000 metric tons), China comes highest with 60,083.39; Vietnam comes medium with 1,456.09 and the least, Brunei with 0.28 [4]. China is the world's leading producer of watermelon, with 63,024,614 tons produced per year followed by Iran with 4,113,711 tons per year. Turkey comes third with 4,031,174 tons per year [12].

In Africa, watermelon is grown not only in dry, low altitude tropical areas like Cape Verde, Mali, Mauritania, Chad, Senegal and Nigeria, but also in equatorial countries like Gabon and Democratic Republic of Congo [8]. In Nigeria, watermelon production has increased significantly in the last one decade with the major production areas being located in the Sahel, Sudan and Guinea agro-ecological zones. In recent times, its cultivation has extended down to the forest belts of south-western Nigeria. However, the northern fringes of the Sudan and Sahel savannah ecological zones and the shores of the Lake Chad remain the major production areas [18].

Watermelon is nutritionally rich in carotenoids. Some of the carotenoids in watermelon include lycopene, phytofluene, phytoene, beta-carotene, lutein and neurosporene. Lycopene makes up the majority of the carotenoids in watermelon. The carotenoid content varies depending on the variety of the watermelon. Based on the variety, carotenoid content in red fleshed watermelon varies from 37 – 121 mg/kg fresh weight, whereas lycopene varies from 35 – 112 mg/kg fresh weight. Watermelon seeds are excellent sources of protein (both essential and non-essential amino acids) and oil. Watermelon seed is about 35% protein, 50% oil, and 5% dietary fibre. Watermelon seed is also rich in micro- and macro-nutrients such as magnesium, calcium, potassium, iron, phosphorous, zinc etc. [11]. The seeds are eaten as a snack or added to other dishes and may be roasted and seasoned. The rind is edible and may be stir-fried, pickled or even grilled.

Origin, distribution and utilization of mango

Mangifera indica, commonly known as mango, is a species of flowering plant in the family Anacardiaceae. It is native to the Indian subcontinent where it is indigenous. Hundreds of cultivated varieties have been introduced to other warm regions of the world. It is a large fruit-tree, capable of a growing to a height and crown width of about 100 feet and trunk circumference of more than twelve feet Mango was brought to East Asia around 400-500 BCE, in the 15th century to the Philippines, and in the 16th century to Africa and Brazil by Portuguese explorers [10]. Mango is the national fruit of India, Pakistan and the Philippines. The mango, in moist Brazilian tropics *M*. *indica* flowers in Sri Lanka Mangiferin (a pharmacologically active hydroxylated xanthone C-glycoside) is extracted from mango at high concentrations from the young leaves (172 g/kg), bark (107 g/kg), and from old leaves (94 g/kg) [23]. Global mango production amounts to almost 35 million metric tons. This quantity is produced on 4.7 million ha of land [9].

Materials and Methods

The study area

The study was carried out in Sokoto state, Nigeria. It is on latitudes 11^0 30$'$N and 14^0 00$'$N, longitude 4^{0}00$'$E and 6^0 40$'$E and altitude 351.0 m above sea level in the Sudan Savannah ecological zone [22]. Sokoto shares boundary with Kebbi State to the south, Zamfara State to the east and the Republic of Niger to the north. The State has an estimated population of about 4,742,459 people as of 2015 with 95.9 persons per square kilometre, and 3% growth rate annually based on 2006 population census [17]. Occupations of city inhabitants include farming, trading, commerce, with a reasonable proportion of the population working in private and public sectors [15].

Samples collection

A total of twenty fruit samples were collected through random sampling method, from the two locations on different geographical coordinates in Sokoto state. Three samples each of watermelon and mango fruits were first collected from Goronyo Dam site (13.53844^o or 13^{o}32 north; 5.94823^o or 5^{o}57 east). Three other samples each of watermelon and mango fruits were collected from Shagari Dam site (12^{o}37-12^{o}39 north; 4^{o}59-5^{o}14 east) [22]. The fruit samples were used for the identification and culturing of microorganisms.

Preparation of samples

Samples preparation and analyses were carried out according to the standard plate count procedures [19]. Serial dilution was carried out on watermelon and mango fruits, which were sliced and dipped in 10 ml of water and a stock solution was obtained, 1 ml was pippeted from the stock tube and transferred to another test tube containing 9 ml of distilled water. This test tube was marked 10^1. Similar procedures were carried out up to 10^5 for watermelon samples and 10^5 for mango samples from Goronyo Dam site. Similar procedure was carried out up to 10^5 for watermelon samples and 10^5 for mango samples from Shagari Dam site as well. From the test tube with dilution of 10^5, media inoculation was carried out.

Media preparation

Nutrient Agar (NA), containing beef extract 3 g, peptone 5 g, sodium chloride 8 g and distilled water 100 ml and potato dextrose agar (PDA), containing potato infusion 4 g, D^+ glucose 20 g, and agar-agar 15 g) were prepared according to the manufacturer's instructions (Difco). The NA and PDA were used in the isolation and enumeration of bacteria and fungi respectively.

Isolation of bacteria and fungi

Ten grams of the sample was homogenised in a sterile mortar and pestle in 9 ml distilled water. One millilitre (1 ml) of the homogenised sample was added to 9 ml of distilled water and serially diluted. Following serial dilutions, 0.1ml of the 1-2 dilutions were plated aseptically on NA and PDA media using pour plate method [7]. The NA plates were incubated at 37°C for 24h to obtain the total viable bacterial counts and PDA plates were incubated at 28°C for 72h to obtain fungal counts. Resulting colony counts of the isolates were determined in colony forming units per ml of inoculum (CFU/ml). To obtain pure culture of isolates, discrete colonies were streaked unto fresh media incubated at 37°C and 28°C for bacteria and fungi respectively for 24h.

Identification and characterization of fungal and bacterial isolates

Pure cultures of bacterial isolates were maintained in NA slants and stored at 4°C for confirmative tests. Biochemical tests such as Gram staining, catalase, indole, urease, oxidase, citrate utilization, coagulase testing and sugar fermentation tests were used to distinguish the different bacterial isolates (Singh and Lin 2008). Fungal identification was based on macroscopic and microscopic features of colonies as previously described [7]. The macroscopic features were the shape, colour and physical appearance of colonies, while microscopic examination constituted spore staining and subsequent viewing under the microscope. Spore staining was carried out using a lactophenol cotton blue green reagent on a heat fixed smear slide for 1 min. After addition of 95% ethanol for 30 s, the slide was rinsed and viewed under 40× objective lens of a light microscope.

Inoculation and subculture

Exactly 0.1 ml of the diluents from 10^5 tube for bacteria and 10^3 for fungi was transferred to the petri-dishes using syringe. A sterilized bent glass rod (in alcohol) was used to spread the inoculums over the surface of the Petri-dishes. The set up was incubated at 37^0C for 24 hours. Plates showing no growth were further incubated for 48hrs before discarding. The suspected colonies after gram stained were sub-cultured on another fresh nutrient agar plate for pure culture. Confirmed purified isolates (bacteria) were transferred to slants for further biochemical analysis.

Biochemical reactions tests

Investigations include catalase test, coagulase test, reactions on Triple sugar, Iron Agar, Indole, Gram staining, urease, oxidase, citrate and MRVP [19].

Catalase test

A drop of 3% hydrogen peroxide was placed on a glass slide. A bit of growth from the solid medium was removed with a wire loop and placed in a drop of hydrogen peroxide on the glass slide. A positive test was indicated by bubbling whereas a negative one showed no bubbling and/or frothing.

Coagulase test

Two drops of physiological saline were poured into about 2 cm clean glass slide that has been divided into two, one colony was carefully emulsified in each drop of saline. A loopful of citrated human plasma was added to the bacterial suspension on one side and mixed with the wire loop. The slide was held up and tilted back and forth for 1 minute. Clumping of cells was positive.

Triple sugar iron test

Using a sterilized needle, a cultured material was obtained from a broth culture. The surface of the slant was streaked and the butt-stabbed 2 or 3 times. Cap was let loose and incubated at 37^0C for 48 hours. Formation of hydrogen sulfide was determined by the blackening of the whole butt/streak/ring of blackening at the slant junction. Glucose fermentation was indicated by the butt becoming

yellow. When no other sugar was fermented the slant appears red while the butt is yellow. This is referred to as an Alkaline/Acid or K/A reaction. If gas was also formed, it was indicated by the designation K/AG. When lactose, sucrose or both have been fermented, in addition to the glucose, and both the top and bottom of the slant changes to yellow, it indicates acid/acid or A/A reaction. Where no sugar was fermented, either at the butt or slant portions, the reaction was referred to as K/K.

Urease test

Urease agar slant in a bijou bottle was inoculated and incubated for 24 to 48 hours at 37^0C. The development of pink or red colour indicated a positive reaction.

Citrate utilization test

A Simmons citrate agar was slant in a test tube, inoculated and incubated for 24 to 72 hours at 37^0C. The appearance of a deep blue colour indicated a positive reaction.

Methyl red test

Ten milliliters (10 ml) of MR-Vp medium in a test tube was inoculated with the test organism and incubated at 35^0C for 48 hours, five drops of methyl red indicator solution was added. The appearance of a red color indicates a positive result while yellow indicates negative.

Voges – proskauer test

Five milliliters of MR – Vp medium was inoculated with the test organisms and incubated at 35^0C for 48 hours. Then 0.6 ml of alpha naphtha solution and 0.2 ml of 40% potassium hydroxide solution were added to the mixture. It was kept for 3 hours and observed. The appearance of a red color indicates positive while absence of red color indicates negative results.

Indole test

The isolates were inoculated in peptone water medium and incubated at 37^0C for 24 hours. After the incubation period, a drop of Kovac's reagent was added and observed. The appearance of a red color indicates positive result.

Fungal analysis

Fungal cultures were made with Sabouraud Dextrose Agar (SDA), impregnated with antibiotics and incubated for 5 days. The isolates were identified by staining with lacto-phenol cotton blue on glass slide in which a portion of the fungi was collected using sterile needle. The fungal isolates were identified on the basis of their colonial morphology on the SDA medium and the types of hyphae after subculture was identified.

Data analysis

All treatments were replicated three times and the data obtained were presented as mean ± SE of the means. The data were subjected to Analysis of Variance (ANOVA) using Genstat$^{(R)}$ 18th edition. Where treatments were significantly different, mean separation was carried out using Duncan's New Multiple Range Test (DNMRT) at 5% level.

Sample		Location	Bacterial Load
Watermelon	A	Goronyo Dam Site	47 x 10^4
	B		16 x 10^4
	C		82 x 10^4
Total			145 x 10^4
Mean			48.33 x 10^4
Watermelon	A	Shagari Dam Site	85 x 10^4
	B		80 x 10^4
	C		83 x 10^4
Total			248 x 10^4
Mean			82.67 x 10^4
Mango	A	Goronyo Dam Site	62 x 104
	B		14 x 104
	C		37 x 104
			113.10 x 10^4
			37.67 x 10^4
Mango	A	Shagari Dam Site	24 x 10^4
	B		28 x 10^4
	C		46 x 10^4
			98 x 10^4
			32.67 x 10^4

Table 1: Bacterial Load of Watermelon and Mango fruits.

SAM	GRAM	MOT	SHAPE	URE	SUC	GLU	LAC	H_2S	GAS	CIT	IND	MR	VP	CAT	DENT
GM_1A	+	+	Rod	+	+	+	-	+	+	+	+	+	-	+	*B. megatarium*
GM_1B	+	-	rod	+	+	+	+	+	-	+	+	+	-	+	*B. megatarium*
GM_2	-	+	rod	+	+	+	+	-	+	+	-	+	-	+	*E. coli*
GM_3	+	+	Cocci	+	+	+	+	-	-	+	+	+	-	+	*S. aureus*
GWA_1	+	+	Short rod	-	+	-	+	+	+	+	+	+	-	+	*B. megatarium*
GWA_2	+	+	Long rod	-	+	-	+	+	+	+	+	+	-	+	*B. cereus*
GWA_3	+	-	Cocci	+	-	+	-	-	+	+	+	+	-	+	*M. luteus*
GWB_1	-	+	Short rod	+	-	+	-	-	+	+	+	+	-	+	*P. mirabilis*
GWB_2	+	-	Cocci	+	-	-	-	-	-	+	-	-	+	+	*M. luteus*
GWB_3	+	-	rod	+	-	+	-	-	-	+	+	-	+	+	*B. subtilis*
GWC_1	+	+	Rod	+	-	+	-	-	+	+	+	-	+	+	*B. subtilis*
GWC_2	+	+	Cocci	+	+	+	-	-	+	+	+	-	+	+	*S. ureae*
GWC_3	-	+	rod	+	-	+	-	-	-	+	-	+	-	+	*P. fluorescens*
SM_1	+	+	Cocci	+	+	+	+	-	-	-	-	+	-	+	*S. epiderm*
SM_2	+	+	rod	-	+	+	+	+	-	-	+	+	-	+	*B. megatarium*
SM_3	+	-	Cocci	+	+	+	+	-	-	+	+	+	-	+	*S. aureus*
SWA_1	+	-	Cocci	+	-	-	-	-	+	+	+	-	+	+	*M. luteus*
SWA_2	+	+	Long rod	-	+	-	+	+	+	+	+	+	-	+	*B. subtilis*
SWA_3	+	-	rod	+	-	+	-	-	+	+	+	+	-	+	*S. ureae*
SWA_4	-	+	rod	+	-	+	-	-	+	+	+	+	-	+	*P. vulgaris*
SWB_1	-	+	rod	-	-	+	-	-	+	-	+	+	-	+	S. *flexneria*
SWC_1	+	+	rod	+	-	+	-	-	+	+	+	-	+	+	*B. cereus*
SWC_2	+	-	cocci	+	-	+	-	-	+	+	+	-	+	+	*M. luteus*

Table 2: Summary of Morphology and Biochemical Characteristics of Bacteria Isolated from Mango and Watermelon fruits.

Key: SAM = Sample, Gram = Gramstain Reaction, MOT = Motility, Ure = Urease, Suc = Sucrose, Glu = Glucose, Lac = Lactose, H_2S = Dihydrogen Sulphate, Cit = Citrate, Ind = Indole, MR = Methyl Red, VP = Voges Proscauer, Cat = Catalase, Ident = Identified Organism.

Table 3: Bacterial Isolates in Spoilt Watermelon and Mango Fruits.

Isolates	Frequency	Percentage
Micrcoccus luteus	4	17.39
Bacillus megatarium	4	17.39
Bacillus cereus	3	13.06
Bacillus subtilis	2	8.69
Staphylococcus ureae	2	8.69
Pseudomonas vulgaris	2	8.69
Staphylococcus aureus	2	8.69
Stahylococcus lexneria	1	4.34
Pseudomonas fluorescens	1	4.34
Escherichia coli	1	4.34
Staphylococcus epiderm	1	4.34
Total	23	100

Table 4: Frequenc/y of Fungal Isolates in Spoilt Watermelon and Mango Fruits.

Fungi isolates	Number of colonies	Frequency (%)
Aspergillus niger	6	40.00
Aspergillus fumigatus	4	26.70
Aspergillus flavus	3	20.00
Saccharomyces cerevisiae	2	13.30
Total	15	100

Results

Result for bacterial load of watermelon and mango is presented in Table 1. It shows that watermelon fruit from Shagari dam site had higher total bacterial load of 248×10^4 with a mean of 82.67×10^4 than that of Goronyo dam site, which had a total bacterial load of 145×10^4 with a mean of 48.33×10^4. Mango fruit from Goronyo dam site had higher total bacterial load of 113×10^4 with a mean of 37.67×10^4 than that from Shagari dam site, which had a total bacterial load of 98×10^4 with a mean of 32.67×10^4 and 100% positive samples in one or more bacterial species.

The result of biochemical tests which comprises of: gram staining, indole test, citrate test, urease test, catalase test, MRVP and TSI of bacteria isolated from the fruit pulp of spoilt watermelon and mango is presented in Table 2. The result indicated that these spoilt fruits were contaminated with *S. aureus, B. megatarium, S. ureae, M. luteus, B. cereus, S. flexneria, P. fluorescens, E. coli, S. epiderm, B. Subtilis P. vulgaris*.

The frequency and percentages bacteria in spoilt watermelon and mango fruits in this study ~~is~~ are presented in Table 3. The result shows that *B. megatarium* and *M. luteus* have the highest percentage of occurrence with 17.39% each, followed by *B. cereus* with 13.04% and *S. aureus, S. ureae, B. subtilis,* and *P. vulgaria~~s~~* with 8.69% each. The least bacterial pathogens encountered were *E. coli, S. epiderm, P. fluorescens and S. flexneria* with 4.34% each.

The frequency of fungi isolates from spoilt watermelon and mango fruits is presented in Table 4. The result shows that *Aspergillus niger* had the frequency with 40%, followed by *Aspergillus fumigatus* with 26.7% and *Aspergillus flavus* with 20%. The least occurring fungus was *Saccharomyces cerevisiae* with 13.3%.

Discussion

Mango and watermelon are among the favoured edible fruit crops in Nigeria and many other countries worldwide. They are well known for their rich taste, flavour, colours and their distinct usage. Eleven bacteria species have been identified in the fruit samples. They include, *M. luteus, B. megatarium, B. cereus, B. subtilis, S. ureae, P. vulgaris, S. aureus, S. flexneria, P. fluorescens, E. coli* and *S. epiderm.* This finding presents a more diverse composition than that in which eight bacteria species were isolated from seven different types of fruits in Gwagwalada market, Abuja, Nigeria [16]. This indicates occurrence of a relatively higher bacterial contamination on fruit samples in the present study.

The result also shows higher bacterial contamination in watermelon than in mango fruits from Shagari and Goronyo dam areas. This indicates higher susceptibility of the watermelon fruits to bacterial contamination more than the mango fruits in general. The result further shows that watermelon fruits from Shagari dam had higher bacterial contamination than those from Goronyo dam sites. But on the contrary, mango fruits from Goronyo had more bacterial contamination than those from Shagari dam sites. This may indicate higher presence of watermelon-related bacteria species (*M. luteus*) than the mango-related bacteria (*B. megatarium*) ones in Shagari dam site and vice versa.

The bacteria in this study have shown variable morphological structures ranging from simple, short and long rods to cocci shapes with positive or negative motility. The bacteria exhibited varying levels of positive or negative responses to biochemical tests such as Gram staining, Urease, glucose, lactose, hydrogen sulphate, citrate, indole, methyl red (MR), Voges proscaure (VP) and catalase.

Improper handling of fruits from field during harvesting to the final consumer may be responsible for fruit contamination with the bacteria species. The bacterial isolates may have originated from fields and transported on surfaces of fruits to fruit stalls and stores [13].

The investigations on mango fruits indicated the occurrence of higher bacterial load in fruits from Goronyo dam site with a mean of 37.67 as compared to fruits from Shagari dam site, having a mean of 32.67. All the mango fruit samples from the two locations show positive infestations with one or more of the identified bacterial species. High bacterial diseases incidence of up to 100% was reported on mango fruits [20].

The results also indicated that four fungal species are associated with the spoilage of these fruits samples. The species are *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus* and *Saccharomyces cerevisiae.* All these species with the exception of *A. fumigatus* have been reported to cause varying levels of deterioration in watermelon fruits [3]. The *Aspergillus* spp, mostly identified in this study, have been reported to inflict greater damage to watermelon than other fruits and vegetables. *Aspergillus* sp. produced the highest tissue rot diameter on watermelon fruit (1.93 cm) than *Colletotrichum* sp (1.30 cm) and *Curcuvularia* sp (1.20 cm) [14]. In addition to the above mentioned species, *Aspergillus wentii, Debaryomyces hansenii, Kluyveromyces maxicanus and Scopularyopsis brevacaulis* have been found to be pathogenic to watermelon. Most of them cause rot to watermelon fruit especially freshly cut ready to eat [13].

Conclusion

The confirmation of many bacteria and fungi species on watermelon and mango fruits samples was an indication of high microbial contamination of fruits in the study area. More bacteria (11 species) than fungi (4 species) have been isolated from fruit samples in this study. Both organisms (bacteria and fungi) have been reported to inflict greater damage to watermelon than the mango fruits, an indication of greater susceptibility to microbial contamination of the watermelon fruit tissues, as well as presence of more watermelon-related species of microbes than mango-related ones in the study locations. Eco-friendly approaches like the use of disease resistant varieties, proper fruit handling from the crop field during harvest up to the final consumer as well as application of bio-formulations against the isolated disease agents could help reduce incidences of crop losses and health risks to humans and other animals.

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