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Haematological and Pathological Effects of Bacteria from Vegetable Wastes in Ilara-Mokin, Ondo State

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Authors' contributions

This work was carried out in collaboration between all authors. Author AOM carried out the study and collected data. Author FOO supervised the study, participated in designing and conducting it. Authors AOM and OSF prepared the original version of the manuscript, participated in designing and conducting it, and revising of the manuscript. All authors studied and approved the content of the present manuscript and participated in revising the paper.

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ABSTRACT

Aims: Different selling points or markets in Ilara-Mokin town were visited after the market session and a day after to collect vegetable wastes. This research aimed to isolate bacteria and fungi from selected waste vegetables, to infect laboratory animals with the bacterial isolates and to study the effect of the isolated bacteria on the laboratory animals.
Methodology: Three major vegetable wastes were commonly found in the markets and they are *Amaranthus cruentus* (Arowojeja), *Senecio biafrae* (Worowo) and *Spinacia oleracea* (Amunututu). Sufficient quantity of these decaying wastes were collected and sterile crucible was used to grind the waste samples. On each sample, serial dilution was done using 2g. Nutrient Agar and potato dextrose agar were used for isolation of bacteria and fungi respectively. Standard Microbiological methods were used for the identification of the microorganisms.

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Results: Five bacteria were isolated from the waste, which are: *Salmonella* spp, *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* and *Proteus mirabilis.* Also six fungi were isolated from the waste vegetable. They are: *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Penicillium chrysogenum, Geotrichum* spp. and *Articularia quercina*. All of the bacteria were sensitive to commercial antibiotics like ciprofloxacin and gentamicin, except *Salmonella* spp that showed resistance to Tetracycline, Augmentin and Ceftriaxone. The bacterial isolates were then used to infect Albino rats and haematogical analysis was performed on the animals' blood. The result showed that *Proteus*, and *Pseudomonas* reduced the PCV from 45.55±1.67% (in control group) to 36.75±0.55% and 37.25±0.33% respectively. The haemoglobin concentration was decreased from 15.00±0.00 (in control group) to 12.33±0.67 in the group infected with *Pseudomonas*. The white blood cell count was highest on the group infected with *Salmonella* with a count of $12.2\pm0.96\times10^{5}$ mm^{-3}. Histopathological Analysis of the liver and intestine of the rats showed that the pathogenic microorganisms cause negative pathological effects such as absence of sinusoid in the liver hepatocytes, hepatocellular drainage and necrosis, while only necrosis and haemorrhage were prominent in their intestine.

Conclusion: The result obtained has shown that pathogenic microorganisms from these vegetable wastes can cause serious health problem for the public if not disposed adequately, especially when these vegetable vendors place freshly harvested ones on the same ground the next market day; and when such is bought and not properly washed or cooked.

Keywords: Waste; Amaranthus cruentus; Senecio biafrae; Spinacia oleracea; bacteria; effect.

1. INTRODUCTION

Vegetable is an edible plant or edible part of a plant. Nearly one thousand species of plants with edible leaves are known. Leafy vegetables most often come from short-lived herbaceous plants such as lettuce and spinach. Vegetables are consumed in relatively small quantities as a side dish or with staple feed. In Africa various nutritious *Amaranthus* is very widely eaten. In Greece, Italy and United states, cusine seasoned with olive oil and lemon is a common side dish eaten hot or cold. Vegetables are often consumed as salads or cooked in swoory or salty dishes [1].

Coridrum sativum, Senecio biafrae and *Amarathus cruentus* popularly known as "Amunututu" "Worowo" and "Arowojeja" in Yoruba respectively. They are all called leafy green or salad green plants. They are plant leaves eaten as a vegetable, sometimes accompanied by the stems. There are common in Africa especially Nigeria. Although share properties with other leaf vegetables in nutrition and cooking methods [2]. In order to keep them from losing their full turgidity after harvest, these vegetables often require low temperature and moist environment during storage. As a result, vegetable in the form of leaves are often sprinkled with water after harvesting and during exposure in the market. The moist conditions of the vegetables fostered by the sprinkling of water together with low temperature conditions encourage the growth of microorganisms [3]. These conditions also facilitate the direct contamination by microorganisms through the handlers (buyers and sellers). Indirect contamination many also occur as a result of poor hygiene environment of the market. Consequently, harvested vegetables so soon begin to spoil if not sold immediately [4].

The objectives of this research are to isolate bacteria and fungi from selected waste vegetables, to infect laboratory animals with the bacterial isolates and to study the effect of the isolated bacteria on the laboratory animals.

2. MATERIALS AND METHODS

2.1 Collection of Samples

The samples used are wastes of vegetables: *Amaranthus cruentus* ("Arowojeja''), *Senecio biafrae* ('Worowo'') and *Spinacia oleracea* "(Amunututu") and were collected from different selling points in Ilara-Mokin main market, Nigeria (into sterile polythene bags). The samples were taken to the Microbiology laboratory, Elizade University, Ilara-Mokin for analysis.

2.2 Sterilization of Glass Wares and Chemical Reagents

All the glass wares such as Petri-dishes, Durham tubes, test-tubes, etc. were sterilized using hot oven according [5].

2.3 Media Preparation

The general purpose media, nutrient agar was prepared according to manufacturer's specification that is 28 g of nutrient agar was dissolved in 1 litre of distilled water and 39 g of Potato Dextrose Agar was also dissolved in 1 litre of distilled water and 0.1 mls of the inoculums were introduced into the Petri-dishes before 20 mls of the media were poured into them in triplicates.

2.4 Methods of Identification of Bacteria Isolates

Characterization of bacterial isolates was based on standard microbiological techniques described by [6]. Gram staining, morphological and cultural characteristics was carried out with various biochemical tests which include: catalase test, coagulase test, oxidase test, motility test, and starch hydrolysis, spore test and sugar fermentation [7].

2.5 Antibiotics Susceptibility Test of the Bacteria Isolates

Young broth culture (1 ml) of 24 hours was dispensed into 9 sterile Petri dishes. It was mixed with sterile molten nutrient agar, allowed to cool and a sensitivity disc was placed firmly on the agar with sterilized forceps to ensure complete contact with the agar. These steps were repeated for every test bacterium. The plates were incubated for 24 hours. The susceptibility of each isolate to each antibiotic as indicated by clear zones of inhibition was quantified in mm using metre rule [5].

2.6 Haematological Analysis

Young adult albino rats (3 weeks old) were obtained and allowed to acclimatize for one week before infecting them with each type of bacteria in triplicates after determining the infectivity dose using the method described by Momoh, [8]. Rats that were not infected constituted the control. The infected rats were allowed to suffer from the infection for seven (7) days. At the end of the experiment, blood samples were collected into separate EDTA bottles for analysis at the Elizade Microbiology laboratory. Full blood count analysis and WBC Differential counts were carried out using standard methods described below:

(i) Erythrocyte sedimentation rate (ESR)

A wintrobe tube was filled to the top 0 mark and one end of it blocked with plasticine. It was placed undisturbed in an upright position for 60minutes. The distance of the fall of red cells from 0 mark was read and expressed in mm fall in hour as the ESR.

(ii) Packed cell volume (PCV)

Each blood sample collected into the anticoagulant bottle (EDTA) was mixed and a capillary tube (CT) was filled up to 75% (3/4) of its length and placed in the micro-haematocrit centrifuge with the sealant at the outer end and centrifuged at 12,000 rpm for 5 minutes. The result of the ratio of the packed cells to the separated plasma was read as a percentage of packed red cells to total volume of whole blood using a haematocrit reader.

(iii) Red blood cell count (RBC)

Every blood sample was diluted (1:200) using sodium metabisulphite and mixed properly. The diluted blood (0.02ml) was pipetted into 4ml of diluting fluid in a bijou bottle and washed thoroughly by alternately drawing up and expelling the diluting fluid. A fine Pasteur pipette was used to fill a blood cell counting chamber (haemocytometer) and counted using a counter under a light microscope at × 40 magnification objective.

(iv) White blood cell count (WBC)

The blood was first diluted in ratio 1:20 and 0.05ml of the blood pipette into 0.95 ml of diluting fluid. A little portion was charged into the counting chamber and observed using ×10 objective to count the white cells/cubic mm.

(v) Haemoglobin (Hb)

Using mouthpiece, sucker and a 0.02 ml pipette, blood was withdrawn and expelled into 4ml Drabkin's solution in a tube. The tube was stoppered, mixed and allowed to stand for 5 minutes for full colour development. A standard blood sample of known haemoglobin concentration was prepared. Using a green (624) filter, the calorimeter was set to zero using plain Drabkin's solution as a blank. The readings of the sample and the standard were taken and the result calculated as follows:

$$\text{sample haemoglobin concentration} = \frac{\text{Reading of test} \times \text{standard haemoglobin concentration}}{\text{Reading of standard}}$$

(vi) White blood cell differential (WBC differential) counts

These are divided into granulocytes and agranulocytes. The granulocytes are further divided into three which are neutrophils, eosinophils and basophils. These were counted after staining with Giesma stain and their numbers recorded. The agranulocyte are equally further divided into two, which are lymphocytes and monocytes.

2.7 Histopathological Studies

The animals were sacrificed using cardio-vascular puncture as approved by "The Care and Use of Animals for Scientific Purposes of the National Veterinary Research Institute (VOM) of Nigeria. Histopathological examination was carried out on the liver and intestine of the albino rats used for haematological analysis. The organs (liver and intestine) were removed, grossly examined and stained with haematoxylin-eosin before examining under the light microscope if the treatment has any effect on them. The organs were compared with that of the control rats. The histological processing was carried out and interpreted at the Histopathology Department of Obafemi Awolowo University Teaching Hospital, Ile Ife, Osun State, Nigeria. Histopathological tests were carried out as follows: the organs of the animals were collected and fixed in 10% formalin to prevent decay. They were dehydrated in different percentage (50%, 70%, 80% and 100%) of alcohol 1 ½ hours each. After dehydration they were cleared with 100% xylene and left for 2hours to remove any remnant alcohol and impregnated in liquid wax for 2 hours for embedding. The embedded organs were sectioned using microtome and were stained with haematoxylin-eosin. Excess stain was removed with tap water. After clearing in xylene, Canada balsam was added and cover slips placed on the slides. The preparations were left in the oven at 40^{o}C and then placed under the microscope with a digital camera connected to a computer system to be examined by a histopathologist that took the photographs and interpreted the results [9].

2.8 Statistical Analysis of Result

Numerical values obtained were subjected to descriptive one way analysis of variance, BMI SPSS version 21 Microsoft Windows 10 and Duncan Multiple Range Test were used.

3. RESULTS

The microbial load analysis result showed that *Senecio biafrae* had the highest microbial load and *Amaranthus cruentus* has the least microbial load as shown in Table 1. The isolation and identification results showed that *Pseudomonas aeruginosa* was oxidase positive, *Staphylococcus aureus* was gram positive and coagulase positive while others were gram negatives. All were catalase positive and motile except *Staphylococcus aureus.* No spore was seen. They were all able to produce acid and gas in glucose except *Staphylococcus aureus* and *Pseudomonas aeruginosa.* They all fermented arabinose and maltose except *Escherichia coli.* Starch hydrolysis was negative except for *Escherichia coli* that was positive.

Table 2 shows antibiotics susceptibility of gram positive bacterial isolate, all the isolate were susceptible to antibiotic except *Proteus mirabilis* that has low susceptibility in Augumentin with zones of inhibition of 4mm. *Salmonella* was resistant to tetracycline, Augumentin, and Amoxillin. Each of the assay was done in triplicates to see possible variations in the results using statistical analysis.

The fungal isolates are: *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Articulospora inflata, Penicillum chrysogenum* and *Geotrichum* spp.

3.1 Result of Infection

Various symptoms were observed on the rats after 3days of infection. Generally the bacteria caused weakness, sluggish movement, falling fur and loss of appetite. Table 3 shows some of the physical signs that were observed in the experimental rats after infecting them with the isolated bacteria. The group infected with *Escherichia coli* showed weakness and loss of appetite; those infected with *P. aeruginosa* showed weakness with sores on the skin. The group infected with *S. aureus* showed weakness, discharge from the eyes and falling fur while the group infected with *Salmonella* showed in addition to weakness loss of appetite and unformed stool. There was however no visible signs of infection in groups infected with *Proteus.*

Table 1. Microbial load form the various wastes

Vegetable waste	*C. sativum*	*S. biafrae*	*A. cruentus*
Microbial load (cfu/g)	4.0×10^5	6.9×10^5	2.3×10^5

Key: cfu/g =colony forming unit /gram.

Table 2. Antibiotics susceptibility of bacterial isolates to commercial antibiotics

Samples	Isolates	Zone of Inhibition by the Antibiotics(mm)									
		CPX	TET	PFX	AUG	CRO	NIT	GEN	COT	OFL	AMX
A	*Proteus mirabilis*	10.4±0.5	11.2±0.2	10.1±0.1	4.0±0.0	9.2±0.25	10.5±0.1	7.2±0.8	10.3±0.3	5.8±0.4	5.7±0.3
	Proteus mirabilis	11.6±0.4	10.0±0.0	9.7±0.3	4.2±0.2	11.1±0.7	10.0±0.0	6.5±0.1	10.4±0.8	5.0±0.0	5.3±0.7
B	*Salmonella* spp.	10.2±0.5	0.0±0.0	11.3±0.7	0.0±0.0	0.0±0.0	8.4±0.1	10.2±0.4	10.4±0.4	10.0±0.0	0.0±0.0
A	*Salmonella* spp.	10.0±0.0	0.0±0.0	10.2±0.2	0.0±0.0	0.0±0.0	8.2±0.4	10.5±0.8	10.3±0.7	10.7±0.1	0.0±0.0
B	*Salmonella* spp.	10.8±0.2	0.0±0.0	10.4±0.6	0.0±0.0	0.0±0.0	8.6±0.2	10.0±0.0	10.0±0.0	9.6±0.8	0.0±0.0
B	*E. coli*	10.7±0.3	10.0±0.0	10.2±0.4	4.3±0.7	10.5±0.3	10.2±0.1	10.7±0.9	6.0±0.0	5.3±0.1	5.0±0.0
B	*Pseudomona aureginosa*	10.2±0.0	10.5±0.5	10.0±0.0	8.3±0.7	10.3±0.9	10.2±0.5	8.0±0.0	10.2±06	10.0±0.0	10.1±0.3
C	*Pseudomona aureginosa*	10.4±0.8	10.0±0.0	10.0±0.0	8.6±0.9	10.0±0.0	10.2±0.0	8.5±0.2	10.5±0.1	10.6±0.2	10.5±0.2
B	*Staph aureus*	10.8±0.6	0.0±0.0	11.2±0.4	0.0±0.0	10.0±0.0	0.0±0.0	10.6±0.2	10.0±0.0	10.0±0.0	10.9±0.6
C	*Staph aureus*	10.5±0.2	0.0±0.0	11.0±0.4	0.0±0.0	10.5±0.1	0.0±0.0	10.0±0.0	10.0±0.0	10.0±0.0	10.5±0.3

Keys: Sample A-Coriandrum sativum Sample B-Senecio brafrae Sample C-Amarantus cruentus

CHL- Chloramphenicol (30 µg), CRO- Ceftriazone (30 µg), GEN- Gentamycin (10 µg), PFX- Pefloxacin (5 µg), COT- Cotrimazole (30 µg), CPX- Ciprofloxacin (10 µg), ERY- Erythromycin (5 µg), AMX- Amoxicillin (25 µg), OFL-Ofloxacin (5 µg), TET – Tetracyline (10 µg), AUG- Augmentin (30 µg)

Table 3. Physical signs presented by the rats after infection with different bacteria

Escherichia coli	Weak, loss of appétit
Pseudomonas aeruginosa	Weak and presence of sores on the skin.
Staphylococcus aureus	Weak, discharge from the eye, falling of fur.
Salmonella spp	Weak, loss of appetite, unformed stool
Proteus mirabilis	No visible signs.

Fig. 1 shows that the result of the haematological analysis carried out on the blood of the infected rats showed that the infections caused a reduction in the PCV from 45.55±1.67% (in control group) to 36.75±0.55% and 37.25±0.33% of the rats infected with *Proteus* and *Pseudomonas*, ESR values were generally high in all infected animals with different isolates. Comparatively, the PCV of the control was almost 50% higher than that of the rats infected with *E. coli* and *Proteus mirabilis*.

Fig. 2 shows the result of the white blood cell differential count analysis carried out on the blood of the infected rats. The infection caused reduction in some of the WBC differential count. Lymphocytes reduced to about 72% while the control was about 78%. The neutrophil was high in the animals infected with *Staphylococcus aureus* while the monocyte increased in the group infected with *E. coli, Proteus mirabilis, Pseudomonas aeruginosa and Salmonella* spp. Except *Staphylococcus aureus* which had no significant increment. The Basophil alone increased in the group infected with *Staphylococcus aureus.*

Others are in same level with the control. Monocyte was high in all the group of animal infected. There was increase in Eosinophil in all the groups except in the animal group infected with *Staphylococcus aureus.* Only the group infected with *E. coli* and some other species has increase in Basophil.

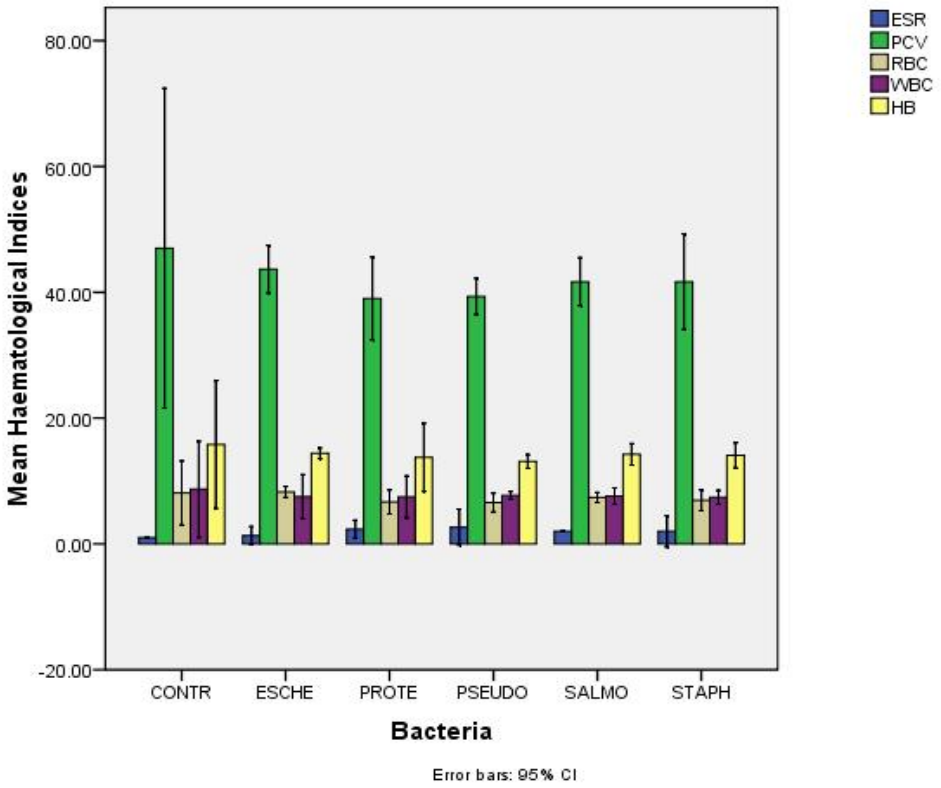


Fig. 1. Haematological results of infected albino rats and their control

Keys: CONTR-control, ESCHE - Escherichia coli, PROTE - Proteus mirabilis PSEUDO - Pseudomonas aeruginosa, SALMO - Salmonella spp, STAPH – Staphylococcus aureus.
PCV- Packed Cell Volume, ESR- Erythrocyte Sedimentation Rate, RBC- Red Blood Cell, WBC- White Blood Cell, HB- Haemoglobin concentration.

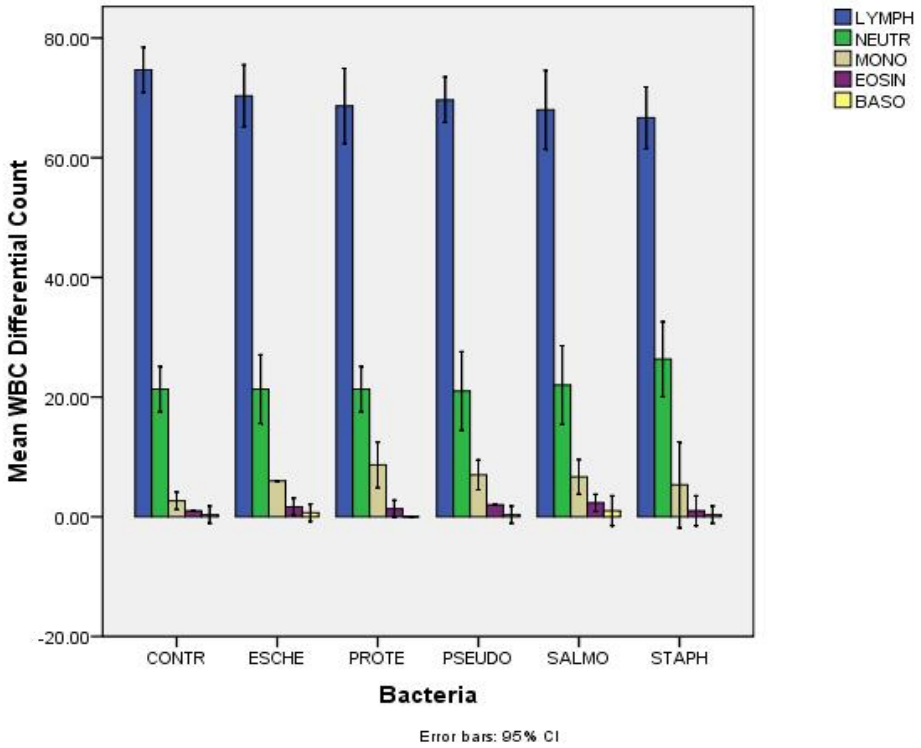
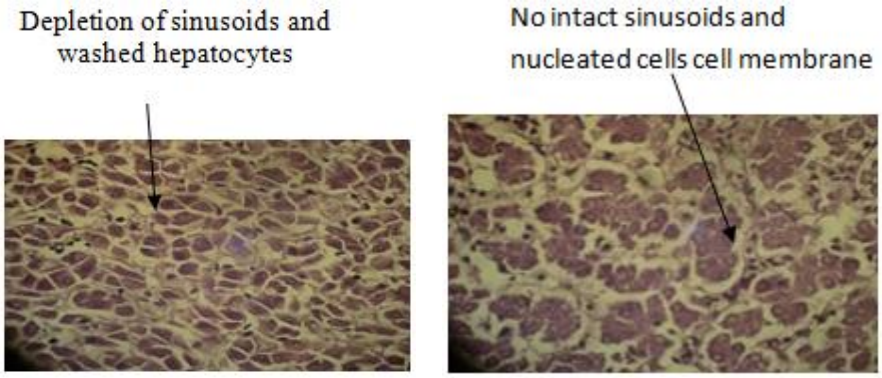


Fig. 2. White blood cell differential count of infected albino rats and their control

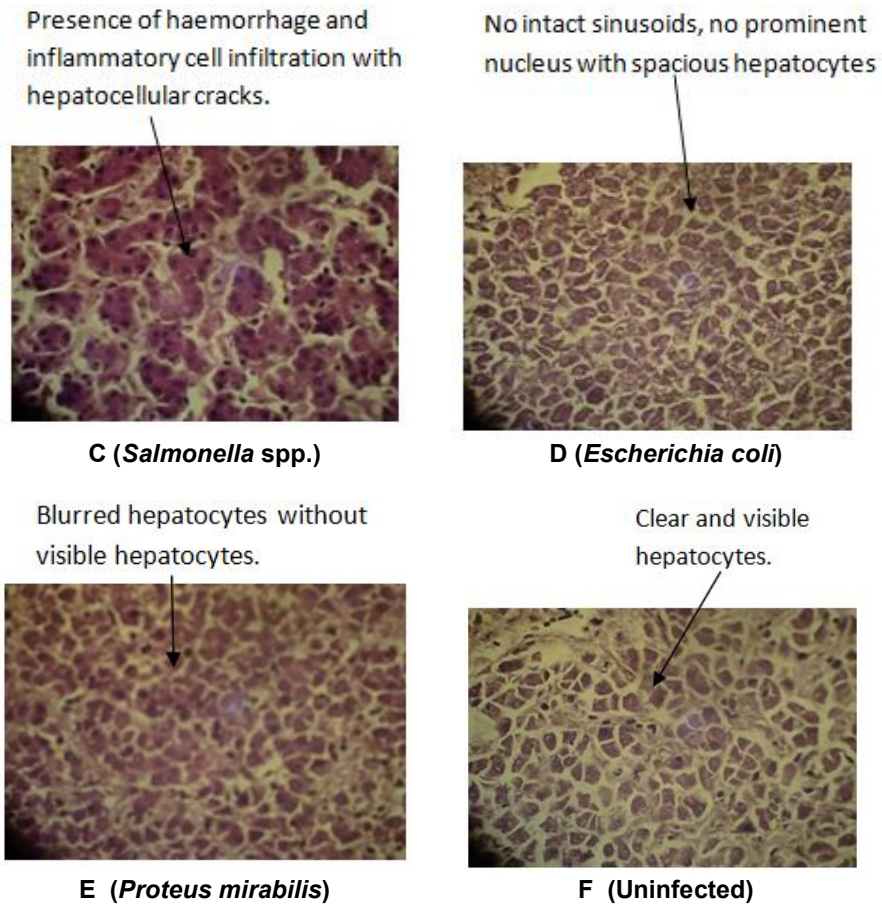
Keys: CONTR-control, ESCHE - Escherichia coli, PROTE - Proteus mirabilis PSEUDO - Pseudomonas aeruginosa, SALMO - Salmonella spp, STAPH – Staphylococcus aureus. LYMPH=Lymphocyte, NEUTR=Neutrophil, MONO=Monocyte, EOSINE=Eosinophil.

3.2 Histopathology Results

The histopathology result of the organs of albino rats analyzed showed that there were negative pathological changes noticed in the liver of all the infected rats except those infected with *Proteus mirabilis* whose liver did not show any pathological escalation. The liver sinusoids of the control group were intact without any dilation, necrosis or haemorrhage. There was necrosis and haemorrhage noticed in other groups infected with other bacteria with some cracks leading to hepatocellular drainage. The intestines were equally affected without prominent finger-like projections of the villi. Only the group infected with *Proteus* had no visible haemorrhage even on its villi projections.



A (*Pseudomonas aeruginosa*) **B (*Staphylococcus aureus*)**



C (*Salmonella* spp.) **D (*Escherichia coli*)**

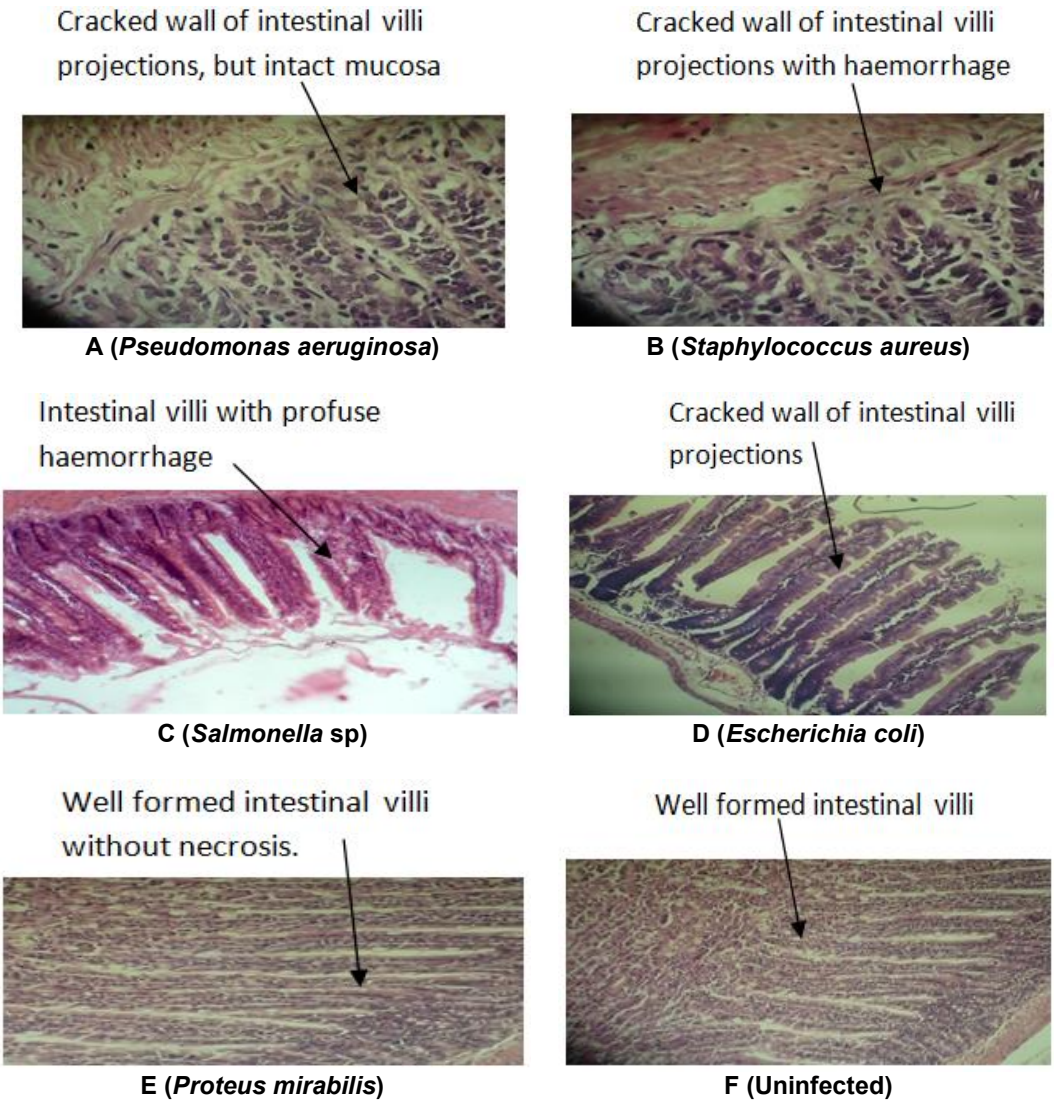
E (*Proteus mirabilis*) **F (Uninfected)**

Plate 1a-1f. Histopathology of liver of rats infected with different bacteria from vegetable wastes

4. DISCUSSION

The bacteria isolated from the three leafy vegetable wastes were both Gram negative and Gram positive bacteria, they include *Escherichia coli*, *Salmonella* spp. *Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively. Most of these bacteria may have had contact with these vegetables from the soil or are often either in the container used in conveying the vegetables to the market [10]. *Senecio biafrae* has the highest bacteria load and the *Amaranthus cruentus* has the least bacterial load. This may be due to their water content which may also facilitate microbial process on the vegetable [11]. In reference to Lacey, [10] that vegetable wastes can be hazardous to animal health, if they are used for animal feed without proper treatment, then this statement is further supported by the level of microbial load obtained within 24 hrs. A similar studies conducted by [12] and [13] gave similar results. These studies concluded that pre and post-harvest handling of vegetables from farm via market to retailer as well as preserving its freshness with untreated water increased the chances of contamination. Therefore, these bacteria isolated from these vegetable wastes may have come from pre and post-harvest handling via farmers-whole sellers and market retailers.

All the microorganisms were sensitive to available antibiotics except *Salmonella* spp. that was resistant to tetracycline, augumentin and cetfriazone. This result indicates that most of the available antibiotics may not be effective in treating the diseases caused by these bacteria when these contaminated vegetables are eaten by animals or man. According Trias et al. [3] prevention of contamination of vegetables is the best way to avert epidemiological outbreak of diseases that can be transmitted via contaminated vegetables.



A (*Pseudomonas aeruginosa*) **B (*Staphylococcus aureus*)**

C (*Salmonella* sp) **D (*Escherichia coli*)**

E (*Proteus mirabilis*) **F (Uninfected)**

Plate 2a-2f. Histopathology of intestine of rats infected with different bacteria from vegetable wastes

The result of haematology showed that erythrocyte sedimentation rate (ESR) was highest in the group of rats infected with *Pseudomonas aeruginosa*, followed by the one infected with *Staphylococcus aureus* while lowest in the control group infected with *E. coli*. This may be as a result of the activities of these bacteria which have can cause bacteremia and septicemia in the blood. Packed cell volume (PCV) results shows that the control group had PCV of 50% while the once infected with *Pseudomonas aeruginosa* and *Proteus mirabilis* had the PCV valve of less than 40%. The laboratory investigations on the PCV is in accordance with the investigation by Naturo et al. [12] who reported the drop in the PCV of laboratory animals as a result of haemolysis in the blood caused by the bacteria causing the infection. Therefore, the difference in the PCV level of the infected and the none infected control may be caused by haemolysis induced in the blood stream of the infected rats.

The result of the histopathology of liver and intestine showed that the entire pathogenic microorganisms caused negative pathological effects. The absence of liver sinusoids noticed in the animals infected with bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* spp. and *Escherichia coli* are

in accordance with the result obtained by Oladunmoye et al. [14] who got similar pathological result when they infected albino rats with *E. coli*. The pathological effects obtained in the intestine of infected rats are also in accordance with the result obtained by Baker et al. [9]. *Proteus mirabilis* is not pathogenic to the Gastro-intestinal tract and are not implicated of any diseases in man. This probably may be responsible for absence of any negative pathological effects in organs of rats infected with the organism.

The results obtained justified the pathology of the microorganisms used in infecting these animals and the effects they can have on vital organs when they infect man and animals.

5. CONCLUSION

This research has shown that the consumption of waste vegetables by animals can be dangerous to their health. Vegetables have been associated with outbreak of food borne disease. It is therefore recommended that wastes from raw leafy vegetable from market be treated before being fed to domestic animals.

ETHICAL APPROVAL

The study was approved by Elizade University and FUTA. All the members were fully informed of the purpose of the investigation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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