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Determination of Some Virulence Factors and Antibiogram of Gram-Positive Bacteria Isolated from Vegetables (Spinach, Lettuce, Sorrel)

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Abstract

Human food borne infections traditionally are acquired through the ingestion of foods of animal origin. Fresh fruits and vegetables are major vehicles for the transmission of the food-borne infections. In Adamawa, there is a tradition of consuming raw vegetables, particularly lettuce, spinach and sorrel leaves without adequate treatment. The objective of this study was isolate and identify gram positive bacteria associated with lettuce, spinach and sorrel leaves, to determine their virulence factors and their antibiotic resistant pattern. A total of thirty samples of lettuce, spinach and sorrel leaves were purchased from different outlets in jimeta modern modern market and analyzed. The samples examined yield the growth of staphylococcus aureus and micrococcus which are able to show resistance to at least two class of antibiotics penicillin and cephalosporins. As well as show ability to produce virulence factors such as biofilm, haemolysin and gelatinase.

Keywords: virulence factors, gram positive, infections, bacteria, vegetables.

Introduction

In Nigeria, vegetables are plants that are consumed in little amounts as a side dish or relish with solid cuisine. Their popularity is growing due to the assumption that they provide the body with a lot of nutrients. As a result, Nigerian markets are brimming with fresh veggies. Microorganisms have been found on vegetables in a number of investigations. Poor pre-harvest and post-harvest practices are to blame. (Sperber *et al.*, 2009. Irrigating with low-quality water, which allows pathogenic bacteria to get on the vegetables, is a major source of contamination before harvest. Similarly, utilizing raw animal dung as a fertilizer raises the risk of contamination of the plants.

Contamination may be direct or indirect. Direct contamination relates with the buyers and sellers. Touching of the produce with dirty hands or exposing of produce to flies are typical examples of direct contamination. The unhealthy surroundings of the market may lead to indirect contamination. Very dirty environments may create huge microbial loads on vegetables (Kayode et al., 2018). And merchants strive to maintain the highest level of freshness for their customers. Sprinkleing these fruits and vegetables with water is a frequent way to keep them fresh. Although merchants find that sprinkling vegetables with water is good, there is a considerable risk of microbial infection. (Akogou *et al.*, 2018).

Enteric infections from gastrointestinal microbes have been discovered on a variety of plants, including lettuce, cabbage, and tomatoes. Raw vegetables can be used to isolate a variety of foodborne diseases. Listeria monocytogenes, Micrococcus, Salmonella, Staphylococcus aureus, and Escherichia coli are among the most prevalent isolates (Alaje *and* Owolabi, 2013).

Food's role in human exposure to antimicrobial-resistant bacteria, including zoonotic pathogens and commensal and environmental bacteria that serve as reservoirs for resistance genes, is becoming a rising food safety concern (Campos et al., 2013; Nüesch-Inderbinen et al., 2015).

Microorganisms' innate and acquired resistance to several structurally unrelated antibiotics can be attributed to a variety of adaptations, such as active efflux systems, reduced cell wall permeability, expression of different enzymes, or biofilm formation (Campos et al., 2013). Extracellular and cell-associated virulence agents are produced during pathogenesis. Many virulence factors are expressed in cells with different densities. Hemolysin and gelatinase synthesis are two of the extra virulence factors. Food's role in human exposure to antimicrobial-resistant bacteria, including zoonotic pathogens and commensal and environmental bacteria that serve as reservoirs for resistance genes, is becoming a rising food safety concern (Campos et al., 2013; Nüesch-Inderbinen et al., 2015).

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The core principle of the virulence factor concept is that microbes are endowed with particular components that impart pathogenic capability. Although the definition of a virulence factor is debatable, and the idea has been debunked by the discovery that microorganisms found on vegetables cause disease (Kumar, 2015), the concept has a strong hold on investigators' imaginations and continues to drive much of the intellectual and experimental energy in the field of microbial pathogenesis. The idea of virulence factors has unquestionably led to the discovery of essential microbial virulence features, which has tremendously aided our understanding of microbial pathogenesis. Furthermore, the approach of defining virulence factors by the use of the molecular postulates has provided an experimentally rigorous approach to the study of virulence in certain microbes. (Campos *et al.,* 2013).

Literature Reviews

Fresh fruits and vegetables are well recognized as important parts of a nutritious and healthy diet and many countries, including Nigeria, have undertaken initiatives to encourage consumers to increase their consumption of these products. Consumers demand variety in and availability of these products all year round, which has impacted international trade, particularly for countries such as Nigeria where the growing season is short and many fresh fruits and vegetables are imported. (Berger *et al.,* 2010).

The global production of fresh vegetables and fruits has increased by 30% over the last few years. It has increased from 30 million tons to 60 million metric tons. This increment has been gradual and hence the increase in exports is at pace with the growth of fruit and vegetable production worldwide

(Gadafi *et al.,* 2020). However, the value of the European countries in the export of fruit and vegetable trade is gradually declining (Czy˙zewski *et al.,* 2017)

Fruits and vegetables may be contaminated at any point in time during the production chain. Sources of contamination can be grouped into two broader groups, namely, pre-harvest and postharvest sources of contamination (Gil *et al.,* 2015). With regard to pre-harvest sources of contamination, studies have shown that the soil in which fruits and vegetables are cultivated may be a source, and also water used for irrigation, water used to apply insecticides and fungicides, faeces, dust, improperly composted manure, and finally human interaction with these vegetables at various points during the production period.

Dry season farming and its associated microbial contamination of fresh fruits and vegetables in poor regions of the world need to be researched. The use of irrigation method of farming during the dry season is a major practice in Africa. However, in Sub-Saharan Africa, many vegetable crops are produced in fresh forms using the irrigation method. They mostly use dirty water or wastewater in the watering of the crops (Amoah, 2014)

Several pathogenic bacterial species are primarily responsible for the contamination of fruits and vegetables as having been evidenced by the isolation of these species from various fruits and vegetables, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., and others from many fruits and vegetables including lettuce, cabbage, and cucumbers. Also, in the year 2011, Germany recorded one of the highest outbreak of EAEC O104, amounting to over 2220 cases (WHO, 2013).

The introduction of antibiotics over half a century ago revolutionized medicine. Although antibiotics are fundamentally effective against bacteria (Czy˙zewski and Czakowski, 2017).

Statement of the Problem

In Nigeria, information on microbial food safety is limited. There are very few reports explaining the microbial quality of fresh vegetables. The consumption of contaminated vegetables may pose risk to consumers. However, antibiotic resistance may result from the use of antibiotics as growth promoters in agriculture. This resistance may also arise from a variety of mechanisms, particularly horizontal gene transfer of virulence and antibiotic resistances gene, which is often facilitated by biofilm formation as well as production of gelatinase. The determination of antibiotic susceptibility patterns, the concurrence of antibiotic resistance bacteria, and the increasing occurrence of foodborne pathogens on vegetable is worrying.

Research Questions

How do we determine virulence factors and antibiogram of Gram-Positive Bacteria Isolated from Vegetables?

Research Objectives

To isolate and identify gram positive bacteria on Lettuce, spinach and sorrel leaves.

To determine the virulence factors of the isolates.

To determine the antibiotic resistant pattern of the isolates.

Materials and Methods

Three different vendors were selected for the study at three different locations in the market, and three different composite vegetable samples (Spinach, Lettuce, and Sorrel) were collected from each vendor. A total of 30 samples of vegetables were collected i.e. 10 for each vegetable (10 sorrels, 10 Lettuces and 10spinach). The vegetables were labeled appropriately and package in resalable bags, and then transported immediately to the laboratory for processing.

Gram positive bacteria was isolated from the fresh vegetables according to the method of Obieze et al., 2011. The vegetables were aseptically cut into bits using a sterile blade. One gram (1g) of each vegetable were dispensed into 9ml of sterile distilled water inside a beaker and shaken at room temperature. The stock solution was serially diluted in sterile distilled water to 10^8. Inoculum (1ml) were taken from 10^2 and 10^4 dilutions respectively and pour plated on nutrient agar plates, all in replicates and labeled. The nutrient agar plates were incubated at 37°C for 24-48hrs. Combinations of cultural characteristics, cellular morphology, and biochemical characterization were employed in identifying the isolates.

After 24-48hours of incubation, the NA plates were carefully examined for visible growth. The resultant colonies were counted and the cultural characteristics carefully studied and recorded.

Preparation of pure culture was done by preparing a fresh medium of Nutrient agar. For each of the distinct colonies formed, sterile inoculation loop was used to streak the colonies on the sterile plates of the fresh medium and incubation was done at 37°C for 24hours. The colonies were selected in a way that separate colonies, were picked up to avoid picking more than one colony at a time. Inoculating loop was flamed red hot to become sterilized and swung for 20s to allow for cooling before use. The cellular morphology of the pure isolates were determined by gram staining and recorded. All isolates were preserved and maintained on NA for further characterization and full identification

The identification of the colonies of the isolates were done by performing the following biochemical tests: Catalase Test, Coagulase test, and Oxidase test

According to standard microbiological methods, the antibiotic susceptibility was determined by disk diffusion method. In this, the bacterial suspension was seeded on Mueller-Hinton agar and the antibiotic containing disks were placed on the surface. For gram positive, antibiotics to be examined include: Erythromycin 5µg, cloxacillin 5µg, gentamycin 10µg, cotrimoxazole 25µg, tetracycline 30µg, augmentin 30µg, chloramohenicol 10µg and Streptomycin 10µg. All the plates were incubated at 37˚c for 24 hours. The antibiotic susceptibility/resistant pattern were conducted at regular intervals of 24hours until there is a noticeable decline in the potency of the antibiotics to inhibit the growth of the test organism. Zones of clearance round each test antibiotics were noted and the diameters of the zones were calculated. The zones of inhibition were used to determine the clinical behavior of the isolates in term of resistance, intermediate resistance and susceptible to antibiotics.

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Several factors of microbial origin are responsible for bacterial virulence properties. A few of these factors were tested to identify the ones present on the vegetables. The virulence tests that were carried out on the isolates were biofilms formation test, gelatinous and detection of hemolysin.

Results and Discussions

A total of two bacterial sp were isolated from the vegetables sampled. The result obtained show that all the lettuce and spinach yielded the growth of *staphylococcus aureus* and *micrococcus*. One of the sorrel sample, do not yield *micrococcus*. As presented in Table 1.

For the virulence factors of gram-negative bacteria isolated from vegetables, 25 isolates of *staphylococcus aureus* were confirmed to have a number of virulence factors, such as biofilm producing ability, haemolysin and production of gelatinase. While 10 isolates of *micrococcus species* showed biofilm and haemolysin producing ability. As presented in Table 2.

9 *staphylococcus aureus* that were isolated from spinach, show resistance to at least two class of antibiotics, 3 micrococcus isolates showed resistance to at least two class of antibiotics. As well as on lettuce, where out of the 10 *staphylococcus aureus* isolated from lettuce, 10 isolates were able to show resistance to at least two class of antibiotics, 5micrococcus isolates showed resistance to at least 2 class of antibiotics. And Out of the 6 *staphylococcus aureus* isolated from sorrel leaves, 6 isolates were able to show resistance to at least two class of antibiotics, 2 micrococcus isolates showed resistance to at least two class of antibiotics. As presented in table 3

Table 1. Total Bacteria isolated from vegetable samples

Samples	*Staphulococcus*	*Micrococcus*
Lettuce N=10	*10*	5
Spinach N=10	*9*	3
Sorrel N=10	*6*	2

Table 2. Virulence factors of gram-positive bacteria isolated from vegetable samples

Microorganisms	Biofilm	Hemolysin	Gelatinase
Staphylococcus	25	25	25
Micrococcus	10	10	0

Table 3: antibiotic resistant pattern of gram positive bacteria isolated from vegetables

Class of antibiotics	Antibiotics	Lettuce		spinach		Sorrel leaves	
		S.aureus n=10	*Micricoccus* n=5	*S.aureus* n=9	*Micricoccus* n=3	*S.aureus* n=6	*Micricoccus* n=2
Aminoglycoside	Gentamycin	0	0	0	0	0	0
	Streptomycin	0	0	0	0	0	0
Macrolides	Erythromycin	0	0	0	0	0	0
Penicillin	Amoxicillin	10	5	9	3	6	2
	Ampiclox	10	5	9	3	6	2
Fluoroquinolones	Ciprofloxacin	0	0	0	0	0	0
	Pefloxacin	0	0	0	0	0	0
Cephalosporins	Zinnacef	10	5	9	3	6	2
	Rocephin	10	0	9	0	6	0
Sulfonamides	cotrimoxazole	0	0	0	0	0	0

Conclusion

This study assessed the Anti-biogram and Virulence factors of gram negative bacteria associated with vegetables majorly eaten raw. A total of 30 vegetables, 10 Sorrel, 10 Spinach and 10 Lettuce from three different vendors in Jimeta Modern market were examined. Bacteria were isolated from all the vegetables sampled. Their resistance to standard antibiotics was investigated. The investigation revealed that *micrococcus* has only *Biofilm* and *haemolysin* as its virulence mechanism, whereas *staphylococcus aureus* had Biofilm producing ability, production of geletinase and the ability to lyse blood (Hemolysis). The tendencies of *S. aureus* to have a variety of mechanisms of disease causation is worrisome. Thus, there is need to sensitize the community of the safety hazard associated with consumption of contaminated vegetables and to educate them on how to de-contaminate such vegetables before consumption. This study will also give Physicians a guide on how to tackle foodborne illnesses associated with strains of *staphylococcus aureus* and *micrococuss.* The gram-positive bacteria isolated from lettuce, spinach, and sorrel leaves in this study were *staphylococcus aureus* and *micrococcus.* The virulence factors of bacteria isolated from the vegetables were biofilm, haemolysin and gelatinase.The results of the determination of the antibiotic resistance pattern show that the isolates were resistant at least two class of antibiotics which include Penicillin and Cephalosporins.

Recommendations

The results established in this research may be of use to farmers, retailers, food safety educators, and policy makers in improving the microbiological quality and safety of vegetables in our markets and in preventing the occurrence of diseases associated with it. To achieve the above, it is recommended that

1. Government and other stakeholders in fresh vegetables farming should provide adequate facilities and conducive environment to enable vegetables free from bacteria's.

2. Steps must be taken to control the overuse of antibiotics in the region and Nigeria in general, as well as in other developing countries.
3. The present study however challenge scientists on the need for development of new antibiotics to combat the infections caused by these antibiotics.

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