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Antibiotic Response of *Salmonella spp.* with Biofilm Formation Potential (BFP) Isolated From Street Vended Ready-To-Eat Fried Grasshopper: A Food Safety Concern

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ABSTRACT

Due to their ability to resist antibiotics, bacterial biofilms are a serious global problem for public health. Their resistance to host immunity and other external stress immensely contributes to their chronic infections. The market potential for fried grasshopper consumed by women and children overtakes that of meat in many parts of Northern Nigeria and can serve as reservoir for biofilm forming pathogenic bacteria. The objective of this study was to detect *Salmonella spp.* from exposed fried grasshopper vended in Gombe metropolis, assess their antibiotic resistance and biofilm formation potential (BFP). A total of 250 samples of exposed RTE fried grasshopper were purchased from different locations in Gombe metropolis in sterile containers. They were homogenized in Rappaport Vassiliadis Salmonella enrichment broth and spread plated on selective Salmonella-Shigella agar. Positive samples were Gram stained and further subjected to biochemical identifications. The confirmed isolates were subjected to assessment for biofilm formation potential (BFP) using Congo red agar assay technique and then to antibiotic susceptibility testing to commonly used antibiotics. Of the 250 samples obtained, 36% (n=90) were positive for *Salmonella spp*. out of which 20% (n=18) had biofilm formation potential. A 50% (n=9) of the isolates with BFP were resistant to Amoxycillin; 16.7% (n=3) to Ciprofloxacin, 33.3% (n=6) to Chloramphenicol, 27.8% (n=5) to Streptomycin, 33.3% (n=6) to Ceftriaxone, 44.4% (n=8) to Tetracycline and 22.2% (n=4) to Trimethoprim. Multidrug resistant *Salmonella spp.* with BFP can serve as a serious threat to the health of consumers and generality of public health. Awareness and enforcement of public health laws governing good hygiene practices (GHPs) and good manufacturing practices (GMPs) are thus imperative to achieving food safety.

1.0 INTRODUCTION

Consumption of processed insects to serve as nutrient source to fight hunger and starvation has been a frequent practice in Africa (including but not limited to Nigeria), Australia, America, Thailand and other Asian countries (Milanović et al., 2018).

There are indeed various promising aspects associated with the consumption of insects as they are universally characterized by nutritionally positive profiles for the presence of high-quality amino acids and proteins, vitamins, good lipids, fiber and minerals. They can also breed as easily and soon as possible, and are capable of emitting ammonia and greenhouse gases lower than traditional livestock do (Schlüter et al., 2017; Milanović et al., 2018). As a result of their numerous nutritional, environmental and social benefits, edible insects are recognized as the "food of the future" and are equally categorized as novel foods by Regulation (EU) No 2015/2283 of the European Parliament and Council (Milanović et al., 2018).

Grasshoppers (*Ruspolia differens*) are among the edible insects already commercialized as foods in the countries of the European Union (EU) (Schlüter et al., 2017) including Africa that have not been fully investigated for the presence of pathogenic bacteria of relevance or as a whole, potential microbial pathogens (Ali et al., 2010; Klunder et al., 2012; Stoops et al., 2016; Garofalo et al., 2017).

Foodborne infections and/or intoxications occur due to ingestion of food that is polluted by pathogens, their toxins or poisons, that happens at any stage of food processing or handling from in the food chain. Foodborne diseases or contaminations mostly happen because of eating such polluted, ruined, poisonous or toxic foods. Some of the manifestations associated with food contamination include queasiness, heaving and loose bowels (Gupta et al., 2019).

Salmonella spp. is an important causative agent of foodborne diseases responsible for more than a million cases annually (Beshiru et al., 2017). It is considered the most important foodborne pathogen capable of causing severe infections in humans and global economic losses. Its presence is regularly monitored in various steps of the food chain, and particularly among the finished raw and ready-to-eat (RTE) products, as among the criteria for consumer safety which represents a very important tool for the implementation of efficient systems for food safety (Tîrziu et al., 2020).

Salmonellosis has become a major global public health concern and emerged as a significant foodborne disease leading to a significant economic and public health burden. Although direct contact with infected animals was also reported for acquiring Salmonellosis, it is acquired generally via exposure to food, (Majowicz et al., 2010; Kumar et al., 2019).

Biofilm is a stationary microbial community that colonizes and grows on surfaces of substances including foods. This is achieved by the self-production of extracellular polymeric substances (EPS) that help them become firmly attached; thereby causing infections that could only be treated by its removal which in turn leads to unaffordable treatments and mental-illness to the individuals Bacterial biofilms pose a serious health concern worldwide due to their capability for tolerating host defence systems, antibiotics and other external stresses. It thus contributes to persistence with chronic infections (Høiby et al., 2011; Sharma et al., 2019). Biofilm comprises of the crammed bacterial population by extra-cellular matrix (ECM) which possesses bacterial secreted polymers such as exopolysaccharides (EPS), extracellular DNA (e-DNA), proteins and amyloidogenic proteins (Whitchurch et al., 2002).

The protections that biofilms provide to microorganisms within it are not only limited to against nutrients scarcity, altered pH, mechanical and shear forces and osmolarity, but also block the access of host's immunity and antibiotics to the bacterial communities. Thus, the matrix of biofilm provides the additional power of bacterial resistance that renders them tolerable to harsh conditions and antibiotics. This brings about the emergence of infections of the bad bugs such as multidrug resistant (MDR), extensively drug resistant (XDR) and totally drug resistant bacteria (McCarty et al., 2012; Sharma et al., 2019).

An investigation conducted on degutted, washed, spiced, roasted, and sundried *Bunaea alcinoe* (grasshopper) hatchlings revealed the presence of *Pseudomonas sp* and *Proteus sp*. In expansion, there is a danger of microbial contamination of the bug item because of the outside and the street side climates where they are callously showcased just as legitimate food handling practices and information by vendors were absent (Mugo, 2020). Therefore, this study aimed to determine the presence of Salmonella species from uncovered RTE fried grasshopper with the objective of evaluating their biofilm formation capabilities.

The major diarrheal cause is *Salmonella*. It has been investigated that annually 550 million human populations were infected in which millions were under five by 2020. Many cases due to Salmonella infection are fatal. The increasing resistance to antibiotics by *Salmonella* has become a serious threat that leads to severe problems in healthcare settings. According to the World Health Organization (WHO, 2017), pathogens for new antibiotics are urgently needed. One of the highest attention needed pathogens is *Salmonella,* which is resistant to fluoroquinolones (Xiang et al., 2020; Alenazy, 2022). There has been reported resistance by *Salmonella* to various classes such as fluoroquinolones, ampicillin, chloramphenicol, florfenicol, streptomycin, sulfonamides, and tetracycline (Alenazy, 2022). This study therefore aimed to investigate the prevalence of *Salmonella sp.* from uncovered grasshopper with the objective of determining their tendencies for biofilm formation and multiple drug resistance of those isolates with biofilm formation potential.

Antibiotic resistance in *Salmonella* is acquired via efflux pumps thereby forcing out the antibiotics from the bacterial cells. Efflux pumps help bacteria to develop resistance and tolerate antibiotics. A concerted effort of regulators, efflux pumps, other components of the cell help in multiple drug resistance, formation of biofilm and pathogenicity. As a result, efflux pumps have become an important drug target for novel drugs and their inhibitors may help to fight the resistance to existing antibiotics.

Antibiotic resistance by *Salmonella spp.* has been reported.

2.0 MATERIALS AND METHODS

2.1 Sample Collection and Processing

A total of 250 samples of exposed grasshoppers were purchased randomly from different vendors within Gombe metropolis and aseptically introduced into sterile sampling bottles. They were then transported to the laboratory at the Centre of Excellence for Food Safety Research Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM) for analysis. A 25 g of fried grasshopper sample was added to 225 ml Rappaport Vassiliadis Salmonella enrichment broth and the mixture was homogenized in a stomacher bag for five minutes. One ml aliquot was pipetted into a Petri dish containing salmonella-shigella agar and incubated at 37 ^{o}C for 24 hours. Upon incubation, the black colonies observed on SS agar are typical of Salmonella species.

2.2 Isolation and Identification of the Isolates

The dark colonies obtained on the SS agar were isolated using pure culture isolation technique on nutrient agar and then subjected to Gram staining technique and biochemical tests such as urease, citrate utilization, indole production, glucose fermentation, motility, mannitol fermentation and growth on triple sugar iron (TSI) agar to confirm their identities.

2.3 Standardization of Inoculum for Biofilm Assay

The confirmed isolates were further subcultured on nutrient agar (NA) and incubated for overnight. Thereafter, a portion of the colony was emulsified into sterile buffered peptone water (BPW) to match the 0.5 MacFarland turbidity standard, equivalent to 1.5 x 10^8 CFU/ml.

2.4 Biofilm formation Potential Assay

The assay for biofilm formation potential (BFP) was conducted using phenotypic expression of colonies in Congo Red Agar (CRA). The CRA was prepared by dissolving 37 g Brain Heart Infusion (BHI) agar (Titan Biotech Ltd, INDIA), 36 g sucrose and 0.8 g congo red (BDH Ltd, India) in one liter of distilled water. The solution was sterilized by autoclaving at 121^{o}C for 15 minutes and dispensed in plates. Upon solidification, the medium was inoculated with the Salmonella isolates and incubated at 37 ^{o}C for 18 hours. Isolates with the potential for biofilm formation showed black colonies while those without the BFP remained red.

2.5 Antibiotic Susceptibility Testing

The antibiotic susceptibility testing on the isolated *Salmonella spp.* was conducted against seven commonly used antibiotics; Ciprofloxacin, Streptomycin, Ceftriaxone, Amoxycillin, Chloramphenicol, Tetracycline and Trimethoprim. Agar-disk diffusion technique on Mueller-Hinton agar (MHA) was the procedure employed following the standardization of the inoculum that matched with the 0.5 McFarland turbidity standard equivalent of 1.5 x 10^8 CFU/mL. Standard antibiotic discs were dispensed and the medium incubated at 37 ^oC for 24 hours at an inverted position. Zones of inhibition were recorded following incubation and the results were interpreted in accordance with Clinical and Laboratory Standard Institute (CLSI, 2024). Each isolate was interpreted based on its response against the tested antibiotic as "Resistant, Intermediate or Sensitive."

2.6 Multidrug Resistance Determination

The determination of multidrug resistance (MDR) was done based on the definition that MDR is the resistance by an organism to at least one antibiotic from three or more different antibiotic classes. An organism resistant to many antibiotics from one or two classes is not regarded a multidrug resistant organism.

3.0 RESULTS

Table 1 shows that out of the 250 exposed fried RTE grasshopper samples, 90 were found to be positive following selective isolation on SSA, Gram staining and biochemical tests. This accounts for the 36% of the total samples. Shigella species had an occurrence of 145 samples (58%) while other unidentified organisms accounted for the 6% (n=15) of the samples. Out of the 90 confirmed isolates obtained, it can be observed that 18 were found to have the biofilm formation potential (BFP)

Table 1: Prevalence and biofilm formation potential of *Salmonella spp.* from ready-to-eat fried grasshopper

Bacteria Isolated	Number positive (%)	Number positive for BFP (%)
Salmonella spp.	90(36)	18 (7.2)
Shigella spp.	145(58)	NA (-)
Others	15(06)	NA (-)
Total	250 (100)	

Key: NA = Not Applicable; (-) = NA value

The BFP positive isolates are identified based on the appearance of their colonies on Congo red agar (CRA). They produced black colonies on the CRA, an indication of their potential to produce biofilm (Fig. 1).

Fig 1: *Salmonella sp.* isolates with biofilm formation potential. The dark swarming colonies indicate BPF and motility, a typical of motile, biofilm-forming Salmonella.

Table 2: Antimicrobial sensitivity of *Salmonella spp.* isolated ready-to-eat fried grasshopper to commonly used antibiotics

Antibiotics (Conc.)	Resistant		Intermediate		Susceptible	
	Positive strain n(%)	Zone diameter (mm)	Positive strain n(%)	Zone diameter (mm)	Positive strain n(%)	Zone diameter (mm)
Ciprofloxacin(10µg)	03(16.7)	≤21	03(16.7)	22-25	06 (33.3)	≥26
Streptomycin(10µg)	05(27.8)	≤11	05(27.8)	12-14	08(44.4)	≥15
Ceftriaxone(30µg)	06(33.3)	≤22	00(0.00)	23-25	12(66.7)	≥26
Trimethoprim(5µg)	04(22.2)	≤17	06(33.3)	18-20	12(66.7)	≥21
Amoxycillin(10µg)	09(50)	≤13	03(16.7)	14-16	06(33.3)	≥17
Tetracycline(30µg)	08(44.4)	≤11	08(44.4)	12-14	02(11.1)	≥15
Chloramphenicol (30µg)	06(33.3)	≤10	00(0.00)	11-13	12(66.7)	≥14

DISCUSSION

Insects are considered to be a 'food for the future' as a result of their positive nutritional characteristics and low environmental impact. The safety associated with their consumption is a growing concern. Such a concern may be, but not limited to their link with contaminating chemical and biological agents. The potential for the pathogenic and toxigenic microbes' presence in ready-to-eat edible insects is among the main biological hazards associated with edible insects (Garofalo et al., 2019). Entomophagy as the consumption of edible insects and has been globally practiced for long by humans (Belluco et al., 2015). It has been investigated that approximately 2000 insect species or more are consumed, with the main consumers in Asia, Africa, Latin America (e.g., Mexico), and Australia. In some of these regions, insects are important and typical part of the diets of humans, with their importantly cultural and gastronomical aspects (van Huis et al., 2016).

However, there are issues associated with the safety of consuming edible insects. In 2015, a Scientific Opinion was issued by the European Food Safety Authority (EFSA) that is concerned with the profile of the risk in relation with the production and consumption of insects as food and feed (EFSA, 2015). The opinion explored the possible issues of safety such as the biological, chemical, and environmental; that are associated with farmed, processed, and non-processed insects along the whole production chain. It was concluded that many factors could have an effect on the edible insects' safety, including the method of production, the substrate, the harvest stage, the species of insect, insect developmental stage and the insect processing. The opinion also highlighted the lack of scientific studies on the possible hazards when insects are used as food and feed, together with a paucity of systematically collected data on animal and human consumption of insects. In addition, there is little or no data on the presence of foodborne potential pathogens like *Salmonella sp* that have the capability of biofilm production isolated from exposed RTE grasshopper vended in the study area. Thus, this study serves as a short communication to enlighten the concerned populace on the potential risks associated with the organisms, which may eventually leads to outbreak that may prove difficult to contain.

Several studies have highlighted a typical microbial profile for grasshoppers, both fresh and processed (boiled and dried), collected from different producers various parts of the world with the prevalence of bacteria and fungi (Garofalo et al., 2019). The microbiota in the grasshopper was characterized by the dominating of LAB (lactic acid bacteria), mainly of the genera *Pediococcus, Weissella, Enterococcus* and *Lactococcus*, and species belonging to the Enterobacteriaceae, such as *Klebsiella*/*Enterobacter* spp. However, *Salmonella sp.* was not detected. *Staphylococcus sp.* and *Bacillus sp.* were prevalent in grasshopper samples in Thailand (Milanović et al., 2018) but with no traces of *Salmonella sp.,* hence the uniqueness of this study.

Detection of *Salmonella sp.* in grasshopper has been rare as Stoop et al., (2016) also was able to detect only *Enterobacter sp.*, *Klebsiella sp.* and *Yersinia sp.* among the members of Enterobacteriaceae. Similar research conducted by Grabowski and Klein (2017) on fried grasshopper also did not reveal the presence of *Salmonella sp., Staphylococcus aureus, E. coli* and *Listeria monocytogenes*. The detection of Salmonellae in this study is therefore is imperative to the food safety of the populace, as consumption of the RTE grasshopper in northern Nigeria and Gombe metropolis has been on the increase. No data so far has been published to the knowledge of the authors on the detection of *Salmonella sp.* from fried grasshopper in Nigeria and elsewhere where the grasshopper is grossly consumed.

About 80% of chronic and recurrent human infections due to microorganisms are due to bacterial biofilms. The cells of microorganisms within a biofilm have been shown to have 10–1000 times more resistant to mechanical stress and antibiotics than their planktonic counterparts. The uptake of resistance gene by the horizontal gene transfer is among mechanisms of resistance to antibiotics by bacterial. Moreover, increased genetic competence, high cell density, accumulation of genetic elements or resistance genes uptake and increased genetic competence are compatible conditions provided by biofilms for their survival (Mah, 2012).

Salmonella sp. possesses the capability of biofilm formation on several surfaces, and this favours its survival in hostile environments such as fried foods, utensils and slaughter houses (Borges et al., 2018). The presence of biofilm formation potential of *Salmonella sp.* on various surfaces has been reported by several studies (Agarwal et al., 2006; Tondo et al., 2010; Steenackers et al., 2012; Borges et al., 2018). In this study, the report of the BFP of 77.8% of the *Salmonella sp.* isolated from exposed vended RTE grasshopper is an indication of risks associated with the consumption of the grasshopper. This if left unchecked can eventually lead to outbreak due to this organism and because of the BFP associated with it, which may consequently lead to loss lives and properties.

The results of the antibiotic resistance indicate that the *Salmonella* spp. strains isolated from meat can be categorized as resistant to MDR: that is, bacteria exhibiting resistance to one or more antibiotics from three or more classes of antibiotics. These bacteria are resistant to β-lactams, aminoglycosides, cephalosporins, fluoroquinolones, sulfonamides, and tetracyclines. Resistance to third generation cephalosporins exhibited by the strains isolated from meats represents a concern, because these antibiotics are used for salmonellosis treatment in human, thus rendering the transmission of resistant bacteria a public health problem (Mengistu *et al.,* 2020).

CONCLUSION

This study provides an insight on the risks associated with the exposed RTE fried grasshopper vended in the streets of Gombe metropolis, Gombe State Nigeria. A substantial number of the samples were found to harbor *Salmonella sp.* out of which approximately 20% were of biofilm formation potential (BFP). Consequently, this may be the reason for some of them having the ability to resist antibiotics such as Amoxycillin, Chloramphenicol, Streptomycin, Ceftriaxone, Trimethoprim, Ciprofloxacin and Tetracycline from classes of Penicillins, Amphenicols, Aminoglycosides, Cephalosporins, Sulphonamides, Fluoroquinolones and Tetracyclines respectively. Thus, this increases their survival rate within the human host.

RECOMMENDATION

This study is a short communication; highlighting the potential presence of *Salmonella sp.* from fried grasshopper. More studies are therefore needed for the presence of antibiotic resistance as well as the use of various methods for the assessment of biofilm formation. Further studies are also encouraged to ascertain the cause of the presence of *Salmonella sp.* in the street vended exposed fried grasshopper so as to avoid imminent outbreaks due to this organism for the avoidance of loss of lives and properties due to the organism.

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CONFLICT OF INTEREST

There was not a conflict of interest observed among the authors during the conduct of the study

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