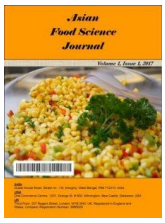

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Inactivation of Antibiotic-Resistant *Listeria monocytogenes* Isolated from Vegetables Using 405nm Ultraviolet Laser Light

K. T. Mumuney[1*], A. A. Farouq[1], R. M. Aliyu[2], B. A. Kabiru[1], H. Salisu[1] and A. D. Ibrahim[1]

[1]Department of Microbiology, Faculty of Science, Usmanu Danfiodiyio University, Sokoto State, Nigeria.
[2]Department of Veterinary Microbiology, Faculty of Vertinary Medicine, Usmanu Danfiodiyio University, Sokoto State, Nigeria.

Authors' contributions

This work was carried out in collaboration between the authors. Author KTM carried out the sample collection, designed the study template. Author AAF managed the literature searches Author RMA carried out the inactivation procedure. Authors BAK and HS managed the discussion of results. Author ADI prepared the manuscripts for publication. All authors read and approved the final manuscript.

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ABSTRACT

The photo-inactivation property of light is an area of increasing interest. The invisible C region of the ultra-violet band (UVC) has been employed extensively in decontamination and sterilization processes, but the A and B (UVA, UVB) have not received such considerations. This study investigates the susceptibility of food-borne pathogen *Listeria monocytogenes* to selected dosages of 405nm UVA irradiation. Listeriosis ranks third in mortalities caused by food-borne bacterial pathogens. This research study focused on the inactivation of *L. monocytogenes* previously isolated from vegetables sold within Sokoto metropolis, Sokoto State, Nigeria. *L. monocytogenes*

**Corresponding author: Email: temitopekafilat@gmail.com;*

was exposed to different irradiations of doses of 90uWcm^{-2}, 120uWcm^{-2}, 150uWcm^{-2}, 180uWcm^{-2}, and 210uWcm^{-2}, at durations of 10, 20, 30, 40, and 50 minutes. The results showed that 210uWcm^{-2} irradiation for 30 minutes was effective in completely inactivating the bacteria. Partial inactivation was observed at 150uWcm^{-2} and 180uWcm^{-2}. The research study lends support to the potentials of photo-inactivation mechanisms as viable alternatives to established processes utilized in food industries, hospital, medical environments, etc., for disinfection and sterilization.

Keywords: UVA; UVB; UVC; Listeria supplement; Listeria monocytogenes; time interval.

1. INTRODUCTION

The resistance of human pathogens to established treatment regimens using antibiotics has become a source of global concern. In April 2014 World Health Organization release warned of the "post-antibiotic" era, in which infections previously-treatable by antibiotics can become fatal with increasing mortality. The so-designated "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) have clearly exhibited resistance to almost all types or classes of antibiotics [1], suggesting the need for a paradigm shift from traditional medical practices of combating pathogens with antibiotics against which these pathogens are now rapidly developing resistance.

Antibacterial photodynamic therapy (APDT) is a promising approach to treat bacterial infections that are recalcitrant to antibiotics. APDT issued on the photosensitization of bacteria with exogenous compounds referred to as photosensitizers (PSs). Cell death is subsequently triggered by lethal oxidative stress that is induced by irradiation of the infected area with light of a resonant wavelength, typically in the visible wavelength range (400-700 nm) [1].

Light-based inactivation of microorganisms is a field of revived attention, not least due to the emergence of resistance to a number of traditional control methods like the use of disinfectants and antibiotics [2]. Till date, studies of these energies in human subjects are still on-going, with publications reporting positive germicidal effectiveness of UV light on human pathogens in vivo [3,4,5]. Eric successfully photo-inactivated *Staphylococcus aureus, Escherichia coli, Trichophyton rubrum,* and *Candida albicans,* in vitro, with a blended mix of 870 and 930 nm laser light [6]. In photodynamic inactivation, bacteria exposure to visible-light energy, typically wavelengths in the visible region, causes the excitation of photosensitizer molecules (either exogenous or endogenous), which results in the production of singlet oxygen ($^1O_2$) and other reactive species that react with intracellular components, and consequently induce bacterial cell inactivation [7]. Visible light, in combination with a wide range of exogenous photosensitizing molecules such as methylene blue, the cationic thiazine dye toluidine blue, cationic porphyrins, phthalocyanines and chlorins, is well established as being an effective antimicrobial treatment, successfully demonstrated to inactivate bacteria, yeast, fungi, protozoan, parasites and viruses [8]. Until relatively recently light within the visible spectrum (400–700nm) was considered to have little biocidal effect compared to UVC light due to the lower photon energy of these wavelengths. Wavelengths of violet-blue light, particularly around 405 nm, have, however, been shown to possess antimicrobial capabilities, and there is scope for exploiting these wavelengths for the control of problematic microorganisms in many areas of application including the disinfection of air and exposed surfaces in the clinical environment. The following section provides an overview of the antimicrobial inactivation mechanism, and antimicrobial efficacy of high intensity 405nm violet blue light [9].

Investigations into the mechanism of action of 405 nm violet-blue light indicate that photodynamic inactivation occurs as a result of the photo-excitation of intracellular porphyrin molecules within the exposed bacterial cells. Laboratory studies have shown that a range of violet-blue light wavelengths in the region 400-425 nm can be used for bacterial inactivation, [10]. However, optimal antimicrobial activity has been found at 405 nm [11]. This peak in activity correlates with the absorption maximum of porphyrin molecules, termed the *soret* band, being in this wavelength region [12]. Exposure to light of this wavelength induces an oxygen dependent photo-excitation reaction within exposed microorganisms, where excited porphyrins react with oxygen or cell components to produce reactive oxygen species (ROS) causing oxidative damage and microbial cell death [13]. Cell death has been accredited to

oxidative damage to the cell membrane, with a recent study demonstrating disruption of the cytoplasmic content and cell walls of exposed *S. aureus*, and it is likely that, due to the non-selective nature of ROS, multi target will be induced in the microbial cell [14,15].

In response to the urgency posed by mounting antibiotic resistance, [16] have called for the investigation of non-antibiotic approaches for the prevention of and protection against infectious diseases [16] Light energy has been demonstrated to have potential benefit in terms of microbial inhibition. Various wavelengths from ultraviolet (UV), through the visible range, and into the infrared (IR) spectrum have been shown to produce from moderate to significant inhibition. Unfortunately, energies in the UV spectrum can produce unwanted changes in the host tissues [17].

2. MATERIALS AND METHODS

Different vegetables: spring onion, cabbage, lettuce, tomatoes, and ready-to-eat salad were collected from Kasuwar Daji, Kasuwar Kure and Gawo Nama areas of Sokoto State. The vegetables mentioned above were each collected in 10 lots in a polythene bag, and kept in an ice box prior to transportation to the research laboratory of the Microbiology Department of Usmanu Danfodiyo University, Sokoto State, Nigeria, where the analyses were carried out.

2.1 Isolation of *Listeria monocytogenes*

L. monocytogenes from the vegetable samples was isolated in line with U.S. Food and Drug Administration BAM/CFSAN method, described by Tiwari [18]. Ten grams of each vegetable was weighed and macerated with a sterile mortar and pestle. A gram of the crushed sample was taken and serial dilution carried out on this amount. 0.1ml of diluent 10^4 and 10^5 was plated on *Listeria* Oxford agar containing selective agent [0.5% (w/v) acriflavin and nalidixic acid and 1.0% (w/v) cycloheximide) and incubated at 37°C for 24 hours. The standard methods described by Cruickshank [19] were adopted for the identification of *L. monocytogenes*.

2.2 Macroscopic Examination of Culture Plates

Attention was paid to microorganisms that showed the cultural and morphological evidence of aesculin hydrolysis or black – halo formation on Oxford *Listeria* agar based incorporated with *Listeria* agar supplement plates. The colonial morphology of the *Listeria* species on the solid media (Oxford Listeria agar) was observed after periods of 24, 48, and 72 hours incubation at 37°C. The presumptive identification of Listeria species was based on colors, sizes, growth temperatures, colonial morphology (macroscopic) patterns, and the appearance on the solid media compared with reference existing stock cultures of *Listeria* species. However, other microorganisms that grew on the agar plates were also identified and characterized as described by Cruickshank [19]. The details of presumptive identification of *Listeria* species on the culture plates were recorded accordingly.

2.3 UVA Laser Light Source

A Class IV ultraviolet laser generator model FA02 was used for irradiation in the research work. The laser generator ran off a power supply of 12V DC, with an integrated laser LED driver. It emits at a wavelength of 405nm. The quoted optical power for the unit was 5mW. Output power control was via the power source, using a DC-DC converter. A variable focus capability using the focusing ring on its neck enabled precision focusing of the generated laser beam. A cooling fan and heatsink attached directly to the aluminum body provided the required thermal control of the heat generated in operation.

2.4 Exposure of *L. monocytogenes* on Agar Surface

For exposure on agar surface, the laser source was fixed 20cm directly above the petri dish. The bacterial suspension incubated for 24 hours in a normal saline was inoculated onto Muller Hinton agar (MHA) using the spread plate method. Plain white paper was used to cover a half of the Petri dish, this being the control for the analyses.

2.5 Dose Delivery Experiments

To establish if inactivation of the bacteria was dose-dependent, suspensions of *L. monocytogenes* were exposed to UVA at 90uWcm^{-2}, 120uWcm^{-2},150uWcm^{-2}, 180uWcm^{-2}, and 210uWcm^{-2} for time regimes of 10, 20, 30, 40, and 50 minutes.

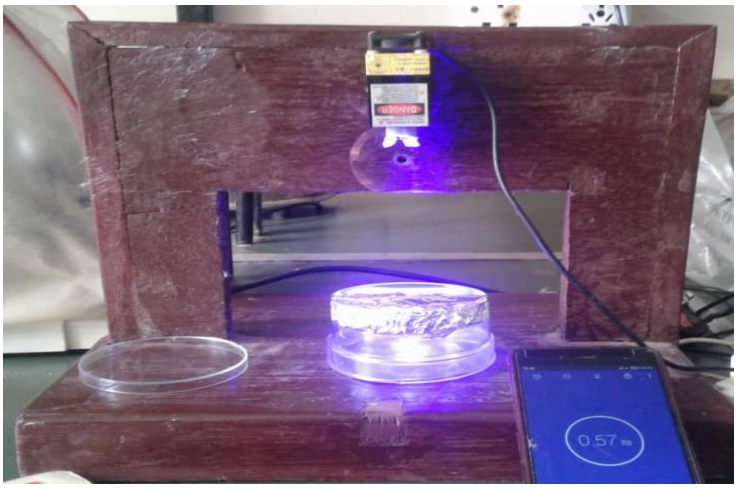

Fig. 1. Laser generator mounted as used in the research work

Measurement of the intensities was effected with an UV meter placed underneath the laser source. The magnitude of the desired intensity was then adjusted using a variable DC-DC power converter. Following this procedure, the petri dishes were then placed under the UV generator for subsequent exposures.

3. RESULTS

The following expressions evaluate the amount of dosage administered to the different isolates as a function of the exposure window and the magnitude of irradiance.

$$E = P * T * 60$$

Where

E = Dosage administered to isolate over a prescribed time window, in J/m^2

P = Irradiance measured in W/m^2, being the same as the dose

T = Time of exposure (measured in minutes)

60 = Minute-to-seconds conversion constant

However, due to small magnitudes of the variables involved in the research work, E will be presented in mJ/cm^2, and P in uW/cm^2.

In Table 3, a detectable inactivation of *Listeria monocytogenes* was observed at a dosage of 360 mJ/cm^2. Due to the granularity of the exposure timestep, the dosage at the instant of which inactivation was initiated could not be determined. Left-over (more than 100) colonies were still observable, indicating only partial inactivation.

From Table 4, exposure to UVA at 180uW/cm^2 produced very significant effects on the population of *Listeria monocytogenes*, leaving only a few colonies active. This is in a marked contrast to previous results presented in Tables 1, 2, and 3.

From the results provided in Table 5, total inactivation of *L. monocytogene*s was observed at 210uW/cm^2 irradiation, at the 30 minute mark. UVA completely eradicated the isolates previously existent in the agar, proving that UVA possesses anti-microbial properties.

Table 1. Exposure of *L. monocytogenes* to UVA at 90uw/cm^2

Isolate	Exposure (minutes)	Dosage (mJ/cm^2)	Observation
LM1	10	54	+++
LM3	20	108	+++
LM7	30	162	+++
LM10	40	216	+++
LM17	50	270	+++

Key: +++ Heavy colonies (uncountable); The evidence presented in Table 1 shows no observable inactivation at the indicated dosages.

Table 2. Exposure of *L. monocytogenes* to UVA at 120uw/cm^2

Isolate	Exposure (minutes)	Dosage (mJ/cm^2)	Observation
LM1	10	72	+++
LM3	20	144	+++
LM7	30	216	+++
LM10	40	288	+++
LM17	50	360	+++

Key:+++ Heavy colonies (uncountable); Like the results presented in Table 1, irradiation at 120uW/cm^2 produced no observable inactivation.

Table 3. Exposure of *L. monocytogenes* to UVA at 150uw/cm^2

Isolate	Exposure (minutes)	Dosage (mJ/cm^2)	Observation
LM1	10	90	+++
LM3	20	180	+++
LM7	30	270	+++
LM10	40	360	++
LM17	50	450	++

Key:+++ Heavy colonies (uncountable); ++ Minimal Colonies (more than 100colonies); + Few colonies (less than 20 colonies); - No colonies

Table 4. Exposure of *L. monocytogenes* to UVA at 180uw/cm^2

Isolate	Exposure (minutes)	Dosage (mJ/cm^2)	Observation
LM1	10	108	++
LM3	20	216	++
LM7	30	324	+
LM10	40	432	+
LM17	50	540	+

Key: +++ Heavy colonies (uncountable); ++ Minimal Colonies (more than 100 colonies); + Few colonies (less than 20 colonies); - No colonies

Table 5. Exposure of *L. monocytogenes* to UVA at 210uw/cm^2

Isolate	Exposure (minutes)	Dosage (mJ/cm^2)	Observation
LM1	10	126	+
LM3	20	252	+
LM7	30	378	-
LM10	40	504	-
LM17	50	630	-

Key:+++ Heavy colonies (uncountable); + Few colonies (less than 20 colonies); - No colonies

4. DISCUSSION

Results obtained, and presented in the previous sections establish the bactericidal property of 405nm UVA light on antibiotic-resistant *L. monocytogenes* on media surfaces.

The bacteria entity was most susceptible to UVA at an irradiance of 210uW/cm^2. Susceptibility was also established at the lower irradiance of 180uW/cm^2. Based on the irradiance and time of exposure, it was observed that inactivation is directly related to the intensity of radiation. At lower intensities and high dosages, (Table 1, E = 216 and 270mj/cm^2), no visible effect was observed. However these same dosages, and lower, produced significant antimicrobial effects at higher intensities (Tables 4 and 5) which is generally consistent with the results obtained in the current study is in agreement with the observations of Bush et al. [20] in which bacteria susceptibility to UVA was at an irradiance of 200uW/cm^2. It can therefore be argued that the susceptibility of *Listeria monocytogenes* to photo-inactivation is dependent primarily on the dose of the radiation, and to a lesser extent on the dosage. However, Maclean et al. [21] agreed that light, administered in a similar manner, could significantly inactivate bacterial pathogens following exposure to 405 nm radiations. It does

appear there is potential for light application to inactivate bacterial growth while leaving the host unharmed.

During the process of photo-inactivation, two adjacent Thymine (T) bases in the DNA bond with each other to form a thymine dimer. This dimer prevents DNA from being decoded correctly. If a cell develops too many thymine dimers, the cell dies the inactivation data for bacteria exposure in the present study can be compared with the 405 nm inactivation data obtained in other studies. This is in agreement with Dai et al. [22] who utilized blue 405nm light to rescue mice from potentially fatal *Pseudomonas aeruginosa* burn infection. These results offer the prospect of radical anti-microbial engagements employing visible UV-A radiation. The primary advantage of UV-A compared to UV-B and UV-C is its non-carcinogenicity as human calls are much less sensitive to 405 nm than it is to UV-B and UV-C violet 405 nm light has already been tested to eliminate *Helicobacter pylori* in human patients [23].

This study has demonstrated that exposure to high-intensity 405 nm light is capable of inactivating bacterial pathogen without the requirement for exogenous photosensitizer molecules. The bactericidal effect was demonstrated quantitatively and qualitatively. The inactivation process has been shown to be dose-dependent; therefore, higher-intensity UVA could achieve lethal doses in shorter time periods which agrees with the demonstration of Murdoch et al. [23] achieving inactivation of *Campylobacter jejuni* by exposure to high-intensity visible light at 180uW/cm^2 in a shorter period. This antibacterial characteristic of UVA is particularly significant for potential practical applications within the food and healthcare industry, where cross-contamination from environmental contact surfaces and equipment is a problem. The fact that UVA falls within the visible light range and does not require the containment conditions of harmful UVC light potentially permits the continuous treatment of food contact areas in the presence of operator personnel, a uniquely advantageous feature. In order to evaluate the effectiveness of UVA for practical applications, such as the continuous treatment of large surface areas, custom-designed light sources are required that can achieve a more uniform power density distribution, perhaps through the use of lenses. Future works to assess the treatment potential of UVA, as well as further studies on the inactivation of bacterial biofilms, will also be important in order to fully assess the potential of this inactivation technology for applications within the food industry alongside other safety control methods.

5. CONCLUSIONS

The ability of UVA to inactivate *L. monocytogenes* has been demonstrated. Because it is safe on the skin and other human surfaces, it is a potential substitute for processes that are being currently handled using cancer-causing UVC. It can easily find niche applications in food sterilization and disinfection.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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