NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF CHEMISTRY

KATIA SOFIA CARDOSO SILVA DA LUZ

BSc in Pharmaceutical Engineering

EVALUATE THE EFFECTIVENESS OF ULTRAVIOLET LIGHT EMITTING DIODES FOR FOOD MICROBIOLOGICAL SAFETY

"A única maneira de fazer com que a mudança faça sentido consiste em mergulhar nela, mover-se com ela, participar da dança"

ALAN WATTS

MASTER IN BIOTECHNOLOGY

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MASTERS IN BIOTECHNOLOGY

NOVA University of Lisbon
September 2023

Evaluate the effectiveness of ultraviolet light-emitting diodes for food microbiological safety

This Dissertation was fully performed at the Laboratory of Membrane Processes in iBET - Instituto de Biologia Experimental e Tecnológica under the supervision of Ana Paula Marques and Vanessa Jorge Pereira.

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Lamentations 3:22-23 - The steadfast love of the Lord never ceases; his mercies never come to an end; they are new every morning; great is your faithfulness.

ABSTRACT

Fresh fruits and vegetables are essential to a healthy diet. However, uncooked products may also be a common source for various microorganisms, including pathogenic and spoilage bacteria.

The development of effective disinfection treatment processes will be crucial to help the food industry cope with the inevitable challenges resulting from the increase in human population and climate change, since food waste can lead to more production and cultivation of food, exploiting more of the earth natural resources.

This work evaluated the inactivation effectiveness of ultraviolet (UV) light emitting diodes (LEDs) that recently emerged as an alternative to traditional UV mercury lamps. UV-C LEDs that emit light at different wavelengths (260 nm, 280 nm and their combination) were tested for inactivation of bacteria associated with foodborne outbreaks (*Salmonella enterica* and *Listeria monocytogenes*) spiked in phosphate-buffered saline solutions and real lettuce samples.

Extremely high inactivation results were obtained after exposure of spiked phosphate buffer solutions and lettuce samples to three single small LEDs, showing that LEDs are a promising alternative disinfection method that the food industry could use to secure their food quality and safety.

Keywords: Ultraviolet light emitting diodes, *Salmonella enterica* Typhimurium and *Listeria monocytogenes*, food disinfection, food safety and quality.

RESUMO

Frutas e vegetais frescos são imperativos para a promoção de uma dieta saudável. Contudo, produtos não submetidos a processos culinários podem constituir uma fonte comum de diversos microrganismos, incluindo bactérias patogênicas responsáveis pela deterioração alimentar.

O desenvolvimento de métodos eficazes de desinfeção se revela crucial para assessorar a indústria alimentar na gestão dos inevitáveis desafios decorrentes do aumento populacional e das mudanças climáticas.

O presente estudo investigou a eficácia de díodos emissores de luz ultravioleta (UV-C) na inativação de determinadas bactérias patogênicas. Os díodos emissores de luz UV-C surgem como uma alternativa viável às lâmpadas ultravioleta de mercúrio. Díodos irradiando luz em diferentes comprimentos de onda (260 nm, 280 nm e combinações) foram submetidos a testes para avaliar a inativação de bactérias associadas a surtos de origem alimentar, como *Salmonella enterica* Typhimurium e *Listeria monocytogenes*, inoculadas em soluções de tampão de fosfato e em amostras de alface.

Resultados de inativação extraordinariamente elevados foram observados com apenas três diminutos díodos emissores de luz após a exposição das soluções de tampão de fosfato e das amostras de alface contaminadas. Esses achados indicam que os díodos emissores de luz representam um método de desinfeção alternativo promissor, com potencial aplicação na indústria alimentar para assegurar a qualidade e segurança dos alimentos.

Palavras-chave: Díodos emissores de luz ultravioleta, *Salmonella enterica* Typhimurium *e Listeria monocytogenes*, desinfeção de alimentos, segurança dos alimentos e qualidade alimentar.

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ACRONYMS

UV Ultraviolet
LED Light Emitting Diodes
WHO World Health Organization
EFSA European Food Safety Authority
ECDC European Centre for Disease Prevention and Control
OD Optical density
DNA Deoxyribonucleic acid
CPDs Cyclobutane pyrimidine dimer
LP Low pressure
MP Medium pressure
TSA Tryptic soy agar
PBS Phosphate-buffered saline

1

INTRODUCTION

1.1 Food safety

Humanity has been dealing with foodborne diseases since the beginning of time. Foodborne diseases have evolved in type, gravity, and impact to many societies all around the world. Still, many regions are still facing the same problems [1]. Many high- and low-income countries report foodborne diseases linked with infectious agents or other food toxins [2].

Quality of life has improved over the years, turning food safety into one of the priorities for many societies. There is an extensive list of challenges related to food safety, including microbiological and chemical safety, personal and environmental hygiene [3].

According to the World Health Organization (WHO) [4], annually, foodborne disease is the origin of 600 million cases of diseases reported in the world, being 1 in 10 people afflicted with the issue. More than 200 agents that lead to human illness are carried out by food [5]. A vulnerable group of people, children with less than five years old, account for 30% of the total lives lost due to foodborne disease, with 120 000 deaths reported annually [6]. Since children are easy targets to foodborne diseases, they endure this burden unjustly, since they have a developing immune system, a small number of pathogens can make them sick due to their small sizes compared to adults [3]. In the European Region, 23 million people get sick from foodborne diseases, while 5000 lose their life's [7].

The WHO indicated that *Salmonella typhi, Taenia solium* and *hepatitis A virus* are the most serious agents responsible for foodborne deaths [1].

Since 2005, the European Food Safety Authority (EFSA) has reported campylobacteriosis as the primary disease responsible for foodborne disease, the same information has been presented in the EFSA report 2022. Furthermore, other common diseases were also reported, salmonellosis, yersiniosis and listeriosis [9].

Considering foodborne outbreaks in 2021, the highest stated cases are due to contaminations with Salmonella, Norovirus and *Campylobacter* [9]. As for higher mortality rates, listeriosis accounts for 13.7 % of all confirmed human cases while salmonellosis 0.18% of all confirmed human cases [9].

Therefore, safe food protocols must be implemented and engaged to avoid economic burden in many aspects [3]. From the microbiological point of view, it is important to remember that the biological origin of the food, favours the growth of microorganisms capable of transmitting human diseases when consumed.

1.1.1 *Salmonella* spp.

Salmonella, a Gram-negative bacterium prevalent in the gastrointestinal tracts of both humans and animals, propels itself using flagella and exhibits remarkable resilience in hostile environments [13]. Following campylobacteriosis, salmonellosis emerges as the most prevalent zoonosis in the European Union (EFSA 2021). *Salmonella* encompasses up to 2600 serovars, classified into typhoidal and non-typhoidal types, with varying degrees of human pathogenicity. Typhoidal serovars cause typhoid fever, primarily transmitted via human-to-human contact, while non-typhoidal serovars mainly spread through animals and contaminated food, causing gastrointestinal illnesses. *Salmonella enterica* serovar Typhimurium contributes to over 70% of human infections [13]. Alarming trends reveal *Salmonella* strains' growing resistance to antimicrobials, escalating human transmission, and prolonged hospitalization periods, thus imposing substantial economic burdens. Addressing this issue is imperative, as it incurs over 3 billion euros in economic costs within the European Union.

Asymptomatic carriers of *Salmonella* serve as vectors for salmonellosis, with symptoms typically manifesting within 6 to 72 hours.

Notably, plants generally do not host *Salmonella*, a human pathogen, owing to their robust surface barriers, primarily composed of galacturonans and pectin. Pectin plays a vital role in bonding cellulose and hemicellulose fibers, reinforcing cell wall integrity. Nevertheless, Enterobacteriaceae organisms are adapting by degrading pectin, warranting further investigation [15]. Gram-negative organisms like

Salmonella possess a periplasmic space, situated between the outer and cytoplasmic membranes, housing enzymes with diverse roles. These enzymes can breach plant cell walls, facilitate interspecies hydrogen transfer via hydrogenase enzymes, and potentially engage with microbiota in plant matrices. Salmonella's presence, especially in poultry, pork, and egg products, poses significant food safety concerns [16] and possibly with a microflora resident on plants matrix [17] [10]. Inadequate hand hygiene and pet-borne infections contribute to bacterial spread [19]. The infection process commences with the ingestion of a contagious dose, leading to intestinal colonization and subsequent disease. Numerous outbreaks linked to infected eggs have been reported in various countries in Europe [20]. France has identified *Salmonella* spp. as major pathogen related to foodborne disease among 13 other infectious agents, leading with 30,598 and 41,139 cases from total of 51,269 and 81,927 cases [21]. In 2020, EFSA in cooperation with ECDC (European Centre for Disease Prevention and Control), reported that *Salmonel*la was the microorganism most regularly detected in foodborne outbreaks within the European Union, with 22.5 % estimated cases of outbreaks [22].

In Portugal, in 2010, there were 205 confirmed cases of salmonellosis [23], while in 2019 were reported 432 cases, being the country in the European Union with the smallest number of reported cases per capita - 4.2 cases per 100,000 of habitants [24]. The limited incidence of reported cases may be attributed to deficiencies in the disease surveillance system, encompassing the entire spectrum from initial diagnosis to subsequent reporting to healthcare authorities. Notably, the clinical manifestations of *Salmonella* infection can readily mimic those of other ailments, consequently obfuscating the accurate identification of *Salmonella* as the causative agent. This, in turn, hinders the initiation of comprehensive investigative procedures to elucidate the disease's origin, leading to delayed recognition of *Salmonella* as the etiological agent.

1.1.2 *Listeria monocytogenes*

Listeria monocytogenes, classified as Gram-positive, non-spore-forming bacilli, typically demonstrates aerobic or facultative anaerobic characteristics. This bacterium exhibits catalase positivity and oxidase negativity and thrives in a psychrotropic niche [25]. *L. monocytogenes* is a widespread foodborne pathogen, often transmitted through contaminated food. Detection of the pathogen in contaminated food with a prolonged incubation period, as in listeriosis, poses challenges for conclusive analysis [26]. Several uncooked and processed foods, and the increasing popularity of ready-to-use and ready-to-eat cold and frozen foods has elevated the incidence of listeriosis in modern society [27]. For the food industry the negative impact is huge because it also brings economic losses.

It displays exceptional resistance to environmental stressors such as heat, salt, nitrite, and acidity, enabling it to thrive on cold surfaces and multiply slowly even at 0°C, bypassing conventional refrigeration defenses [28].

Listeria monocytogenes causes a disease known as listeriosis, a disease with severe implications for vulnerable populations, including pregnant women, newborns, the elderly, and immunocompromised individuals being also a hygiene indicator [29]. The pathogen enters mammalian cells through phagocytosis, evades membrane-bound vacuoles, propagates, and employs actin polymerization to intracellularly disseminate, primarily targeting the liver [30]. Pregnant women can contract listerioses, resulting in intrauterine infections that lead to miscarriage, premature labor, neonatal listeriosis, and maternal complications, often resulting in fatalities [31, 32]. In 2021, 2183 cases of listeriosis were reported, leading to 923 hospitalizations and 196 fatalities in the European Union (EU) [18].

Listeriosis ranks fifth among prevalent zoonoses in the European Union, necessitating heightened vigilance [33].

Global food safety is at risk because there are many foodborne pathogens like *Salmonella* spp. and *L. monocytogenes*. The EFSA report of 2020, accounted with an increase of cases of deaths linked with *L. monocytogenes*, estimated to be 13.0 % and 0.19 % for *Salmonella* spp [34].

1.2 Occurrence of foodborne pathogens in ready-to-eat food

Fresh fruits and vegetables are essential to a healthy diet as they are great sources of essential micronutrients, minerals, and phytochemicals which supports our body energy supply [10]. Many government health agencies endorsed their consumption to protect against a range of illnesses such as cancer and cardiovascular diseases. In the last decade, the market of fresh fruits and vegetables had a high consumption increase [11]. However, uncooked ready-to-eat products may also be a common source for various microorganisms, including pathogenic and spoilage bacteria. Moreover, outbreaks of foodborne illnesses associated with the consumption of fresh produce have increased.

Early contamination of pathogens on fruits and vegetables can occur when the food matrices are wounded, cut or damaged when harvesting or during growth. Contamination of ready-to-eat food sold on street markets happen during various phases of food processing, due to inappropriate temperature of conservation and/or indigent hygiene from people who are handling the food directly and indirectly [12].

1.3 Ultraviolet light emitting diodes

Disinfection has been described as the most important processing step to guarantee the quality, safety, and shelf-life of fresh products [35].

Even though chlorine has been widely applied due to its efficacy, relatively low price, and easy application, it is known to produce hazardous disinfection by-products (trihalomethanes and haloacetic acids) [36]. Different biological, chemical, and physical disinfection methods can be proposed to replace or reduce the use of chlorine. Ultraviolet radiation is extremely effective at inactivating a wide range of microorganisms and its use can also degrade organic contaminants [37-39]. It can be used for the inactivation of microorganisms and biofilms present in water, surfaces, and food [40]. As many other types of germicidal technologies, UV irradiation, produced from mercury lamps, has demonstrated to be effective against harmful organisms for surface disinfection in many industries. UV-light application, such as low-pressure (LP) mercury lamps and medium pressure (MP) mercury lamps became an alternative to many other treatments such as chemical and thermal treatments [41]. However, due to the toxicity of mercury, the Governing Council of the United Nations Environment Program created regulations to monitor mercury globally, by signing a Minamata Convention, where the parties acknowledged mercury as a global burden and defended that its use should be moderated. It was also suggested the search for alternatives to the mercury-added products [42].

Given the advantages of the use of light treatment, a more environmentally friendly and promising disinfection technology has been recently proposed, UV light-emitting diodes (UV LEDs) [43]. Comparing UV LEDs to the typical UV mercury lamps, UV LEDs benefit with flexible emission, from invisible to visible wavelengths [44], are non-mercury, have an accessible design, bring small features with higher durability, consuming less energy and lasting longer [45].

Several researchers have shown that the main UV light disinfection mechanism is damage caused to the DNA, due to absorption of the UV light [46], leading to the formation of dimers specially when pyrimidine bases are next to each other in the DNA chain. The two more common dimers formed in the DNA after UV exposure, are the cyclobutane pyrimidine dimer (CPDs) and the 6-4 photoproducts [47]. However, CPDs are the most abundant, comprising around 75% of the DNA damage caused by UV light [48].

In many different studies, authors have demonstrated that for each organism there is a peak absorption of DNA and not all organisms have the highest DNA absorption at 260 nm [47]. For instance, Sara et al [49] observed two different peaks of absorption, being 259 and 265 nm for spores of

adenovirus, while David at el [50] described in his studies that *herpes simplex* marked for different peaks one between 270 and 280 nm. The difference of peak absorbance between organisms could be related to their physiological constitution, such as proteins and cell membrane, therefore this topic should be further explored.

1.4 Aims

Food safety should be everyone´s concern, priority, and business, however one in ten people in the world develop a disease from eating tainted food. It's important to associate food safety with the development of children and productivity of an adult. Thus, it is empirical that in our agenda life becomes sustainable, to protect the public health and the economy from negative impacts. Food safety is often taken for granted, getting poisoned from tainted food is assumed to be a long shot. Unfortunately, food containing infectious agents are the root of more than 200 diseases. *Salmonella* spp. and *L. monocytogenes* are pathogenic bacteria responsible for these diseases [51].

Disinfection is recognized as an important phase in the processing of food, necessary to tackle the world-wide problem of food contamination. To guarantee the safety, quality, and shelf life of food for consumers, new promising disinfection methods are needed and have been proposed.

The aim of this work is to evaluate if UV-C LEDs that emit light at different wavelengths are a promising alternative disinfection method that the food industry could use to secure their food quality and safety. Moreover, the work also focused on understanding the mechanisms of inactivation in terms of morphology and genetic damage by detecting the presence of CPDs.

2

MATERIALS AND METHODS

2.1 Microbial inactivation assays in phosphate buffered saline solutions

2.1.1 Culture and bacteria suspension preparation

The bacteria strains used in this study were *S. enterica* serovar Typhimurium *and L. monocytogenes*, provided by Professor Paula Teixeira from the Centre for Biotechnology and Fine Chemistry (CBQF), Escola Superior de Biotecnologia, Universidade Católica Portuguesa. *S. enterica* serovar Typhimurium and *L. monocytogenes* were stored at -80 ºC in glycerol (50%). *enterica* Typhimurium was grown onto tryptic soy agar (TSA) (VWR Chemicals, USA) at 37ºC for 18 h and *Listeria monocytogenes* was grown onto TSA at 37ºC for 24 h and stored at 4 ºC for no more than a month to be used. At each time when required to do experiments, a single colony from the TSA Petri dishes was inoculated in tryptic soy broth (TSB) (VWR Chemicals, USA) at 37 ºC for 18 or 24 h, for S. *enterica* Typhimurium and *L. monocytogenes*, respectively grown till stationary phase and harvested by centrifugation at 6000× *g* for 10 minutes at 4º C and the supernatant was discarded. The bacterial cells were resuspended in a sterile phosphate buffered saline (PBS) (1x) solution and centrifuged. The process of washing the cells, discarding, and collecting the pellets was performed three times. The resulting pellets from three rounds were resuspended in PBS and diluted to an optical density (OD) of ± 0.45 at 600 nm, using a spectrophotometer (Ultrospec 2100 pro UV/ Visible, UK, Cambridge). This OD value corresponds to a concentration approximately 10^8 CFU/mL.

Ultraviolet light emitting diodes setup

The UV-C LED unit (shown in Figure 1) consists of a PearlLab Beam reactor (AquiSense Tecnologies, USA), comprised of, a control box, a UV homogenizing (collimating) tube, a wavelength selector, an AC-DC adapter (12 V, 90 W) and a UVinaire™ triple-wavelength UV-C LED unit with nine small LEDs (three LEDs that emit light at 260 nm, three LEDs that emit light at 270 nm and three LEDs that emit light at 280 nm). In this study, the inactivation assays were performed with 3 small LEDs that emit light at 260 nm or 280 nm, due to the maximum light absorption of DNA and proteins, respectively.

2.1.2

Figure 1 - UV-C LED reactor used in the inactivation experiments.

Fifty mL of cell suspension in PBS was added to a pre-designed sterile glass dish with refrigeration inlet and outlet able to maintain the temperature of the water circulating at 4º C when exposed to UV-C LEDs (Figure 2). The glass dish has an internal diameter of 5.5 cm. The UV treatment was operated inside a Class II biological safety cabinet, where 50 mL of the bacterial suspension were irradiated, and 50 mL of the same suspension was kept in the dark (dark control sample). The UV-LEDs system was supported by a plastic tube, with 91 mm diameter and 4 cm height, and placed over the samples to be irradiated under three condition 260 nm, 280 nm, and their combination (Figure 2)

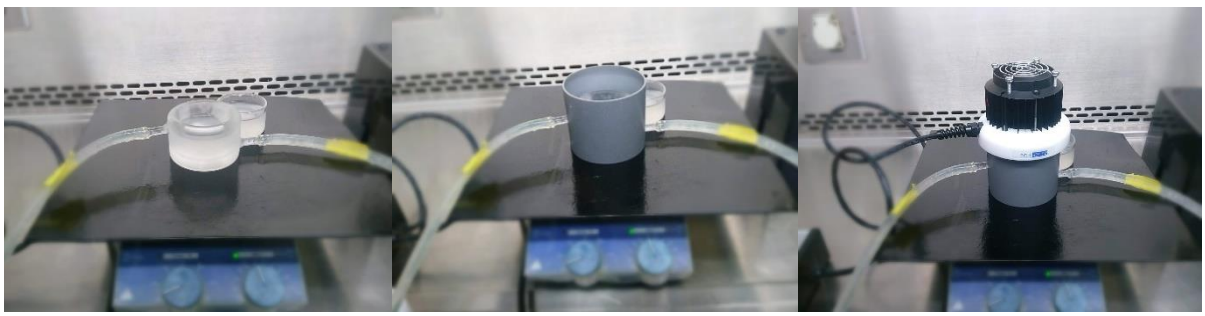


Figure 2 - Inactivation assays conducted in phosphate buffered saline solutions.

At each assay, samples were taken in the beginning and after different exposure times (0, 0.5, 1, 1.5, 2, 3 and 5 minutes), to UV-C LED to further analyse the colony forming units per mL (CFU/mL). A dark control was also done throughout the experiments to verify if the same results could be found for the initial concentration in terms of colony forming units per mL (CFU/mL), which means that the samples didn't suffer any change by an external factor, and the results of the inactivation were reliable. The cell suspensions were kept homogeneous by a magnetic stirrer bar. Samples were taken and serial decimal dilutions were performed using PBS and then plated onto TSA. The incubation was done at 37 ºC during 18 h for *S. enterica* Typhimurium and 24 h for *L. monocytogenes*, where colonies formed were counted to determine the colony forming units per mL (CFU/mL) after each treatment.

To determine the resulting concentration of the bacterial strains in each sample, the number of colonies counted was divided by the volume of sample placed in each Petri dish and multiplied by the dilution factor. The log reduction was then determined.

2.1.2.1 UV fluence determination

In this work, UV fluence (mJ/cm^2) was determined as the product of the irradiance and the time the samples were exposed (in seconds) to be able to compare differences in the results obtained with LEDs that emit light with different intensities and to compare the results obtained with other literature studies which used the UV-LED setup. Bolton and Linden [52] stated that to further determine the average irradiance values in a solution, some correction factors need to be considered to obtain the average irradiance value in the solution, such as divergence, petri dish, water, and reflection. The values for the correction factors were: 0.66 for divergence factor, 0.90 for petri dish factor and 0.98 for reflection factor. The average intensity of the LEDs that emit light at 260 and 280 nm was determined using an ILT 950-UV Spectroradiometer (Massachusetts, USA). The radiometer was placed at 4 cm from the light source (the height used in the PBS inactivation experiments). The setup used to measure the light intensity is depicted in Figure 3. The average intensities measured were: 21.24 ± 0,541 $\mu W/cm^2$ for the LEDs that emit light at 260 nm and 56.02 ± 0.675 $\mu W/cm^2$ for the LEDs that emit light at 280 nm.

Figure 3 - Setup used for the measurement of the light intensity of the LEDs that emit light at different wavelengths.

2.1.2.2 Cell morphology analysis

The samples with *S. enterica* Typhimurium and *L. monocytogenes* were prepared in duplicates combining the red dye FM 4-64 [(N-(3- triethylammoniumpropyl)-4-(p-diethyl-aminopnhenyl-hexa-trienyl) pyridinium dibromide, a lipophilic membrane stain at 10 µg/mL] with cyan dye DAPI (4´,6´-diamidino-2-phenylindole, a DNA stain at 5 ng/mL) to try to visualise morphological damage following exposure to the LEDs.

After the preparation of the cell suspensions with an average optical density of 0.450, 1 mL of both *S. enterica* Typhimurium and *L. monocytogenes* that were exposed to UV-C LEDs that emitting light at 260 and 280 nm and samples that were not exposed, were pipetted into 1.5 mL tube in duplicates. The samples were centrifuged at 4000×*g* for 2 minutes for pellet formation. Then, the liquid was discarded, and the pellet was slowly resuspended with 1 mL of PBS to avoid cell disruption. The dyes were then mixed in the tubes, FM 4-64 and DAPI and let the rest in the dark for 2 minutes. Finally, the samples were once again centrifuged, and resuspended with 40 µL of PBS.

An agarose solution was prepared to coat the glass slides to support the samples to be analyzed in the microscope. The solution was prepared by mixing 100 mL of distilled water and 1.7 g of agarose. Then, it was heated in a microwave to slowly dissolve the agarose. Each glass slide was coated 800 µL of the agarose solution and labelled. To finish, 4 µL of each sample was pipetted into the glass slides (two samples per slide) and spread with a thin cover glass. The samples were analyzed within 30 minutes for a viable result interpretation.

Images were acquired on a Leica DM 6000B upright microscope equipped with an Andor iXon 885 EMCCD camera and controlled with the MetaMorph V5.8 software, using the 100x 1.4 NA oil immersion objective plus a 1.6x optovar, the fluorescence filter sets FITC, TX2, DAPI and Contrast Phase optics.

2.1.2.3 DNA damage analysis

When exposed to UV, the formation of photoproducts in the DNA chain occurs. The most common damage caused by UV light is the formation of CPDs, when two pyrimidines next to each other join by a covalent bond in the same DNA chain. In this work DNA damage caused by the UV-C light was analyzed by quantifying the CPDs formed. Bacteria suspensions were exposed to the same UV dose 5.43 mJ/cm^2 at two different wavelengths (260 and 280 nm).

The formation of CPDs was determined as described by Oliveira et al (2021) [53]. Briefly, the DNA from the non-exposed and UV exposed samples was extracted using the DNeasy® UltraClean® Microbial Kit (Qiagen, USA). After extraction, DNA was quantified using a NanoDrop ND-1000 Spectrophotometer(Thermo fisher scientific, USA). The concentration of the DNA samples is presented in the appendix A, table A1). The DNA samples were diluted to 4 µg/mL and an enzyme-linked immunosorbent assay (OxiSelectTM UV-Induced DNA Damage ELISA Kit, CPD Quantification, Cell Biolabs, Inc, USA) was used to quantify the presence of CPDs in the DNA samples by comparing its absorbance with the absorbance of a CPD-DNA standard calibration curve (see appendix A, figure A1).

2.2 Microbial inactivation of *Salmonella enterica* Typhimurium on lettuce

Lettuce, bought from a local supermarket, was cut using a circular shape, with 12.6 cm^2 of total area used to adjust the circular size cut, maintaining the uniform size for all samples. Ten freshly cut pieces were then placed in sterile Petri dishes inside the Class II biological safety cabinet, spiked with 5 drops of 100 µL of the bacteria cell suspension (1.29×10^8 CFU/mL) on its surface and left to air dry for 2h30m (Figure 4). The concentration of *S. enterica* serovar Typhimurium in the leaves was determined in 40 pieces of lettuce.

Figure 4 - Lettuce leaves spiked with *Salmonella enterica* Typhimurium.

Of the ten lettuce leaves spiked, five dried pieces were subject to irradiation and the other five were reserved to determine the initial concentration of *Salmonella enterica* Typhimurium present in lettuce samples (not irradiated).

The inactivation assay was conducted with one piece of lettuce at the time, placed inside a sterile lid of a glass Petri dish on top of ice to keep the temperature low and avoid the possible heat of the LEDs (Figure 5). Both sides of each lettuce sample received the light emitted from the diodes at 260, 280 nm and their combination for 10 minutes. The time of exposure in this assay is supported by Kim et al [54] which described in his study 10 minute of exposure were settled to test the conditions of the UV treatment, achieving a log reduction of 4.45 and 4.21 for *S. enterica* Typhimurium and *L. monocytogenes*, respectively, on both sides. The irradiated and non-irradiated samples were then transferred to sterile stomacher bags, 5 mL of peptone water (Frilabo, Lisbon, Portugal) was added, and the bags were placed in a stomacher (Stomacher 400 Circulator, Seward, England) for 2 min at 260 rpm to extract the

microorganisms from the leaves. The solution obtained was transferred to a sterile plastic tube, decimal dilutions were prepared with a PBS solution and plated onto chromogenic agar (RAPID 'Salmonella Medium, Bio-Rad). The incubation was done at 37 ºC during at 18 h and then the CFUs were counted.

UV fluence determination

2.2.1

The setup depicted in Figure 3 was used to measure the light intensity of the LEDs that emit light at 260 and 280 nm when placed at a 2 cm distance from the light source (the same height used in the lettuce inactivation experiments). When placed at 2 cm, the average intensity of light measured using an ILT 950-UV Spectroradiometer (Massachusetts, USA) was 391.85 ± 0.80 μW/cm^2 for the three small LEDs that emit light at 260 nm and 914.42 ± 52.64 μW/cm^2 for the three small LEDs that emit light at 280 nm.

The UV fluence (mJ/cm^2) was determined as the product of irradiance and the exposure time as previously described in section 2.1.2.1.

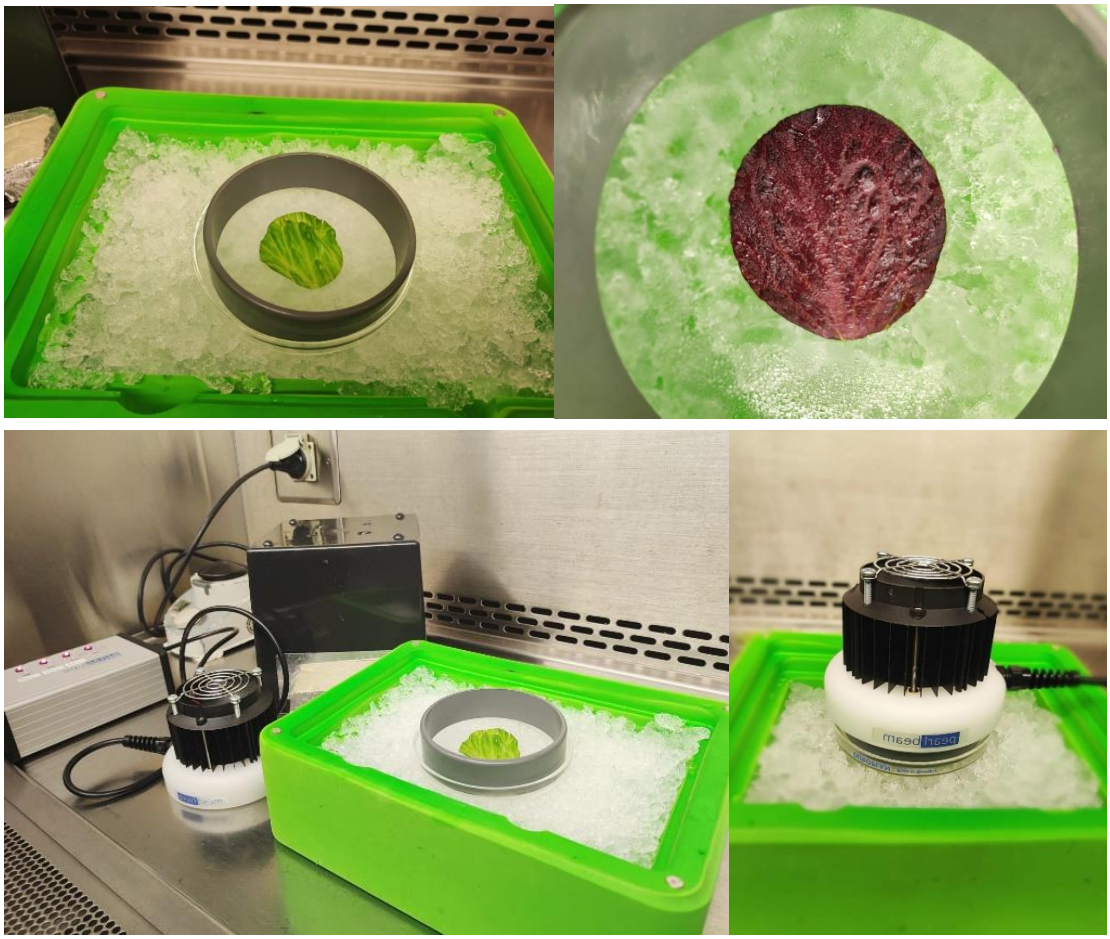


Figure 5 - Setup for the irradiation of lettuce leaves with UV-V LEDs.

3

RESULTS AND DISCUSSION

3.1 Microbial inactivation in phosphate buffered saline solutions

In this study, the inactivation of *S. enterica* Typhimurium and *L. monocytogenes* spiked independently in PBS solution was tested. The inactivation by UV-C LEDs that emit light at different wavelengths (260, 280, 260 + 280 nm) was tested after different exposure times: 0, 0.5, 1, 1.5, 2, 3 and 5 minutes (Figure 6). The LEDs inactivation efficiency was tested for longer times than 5 minutes of exposure, but no greater results were observed, thus, in the inactivation assays, the maximum UV-C LEDs exposure time tested was 5 minutes.

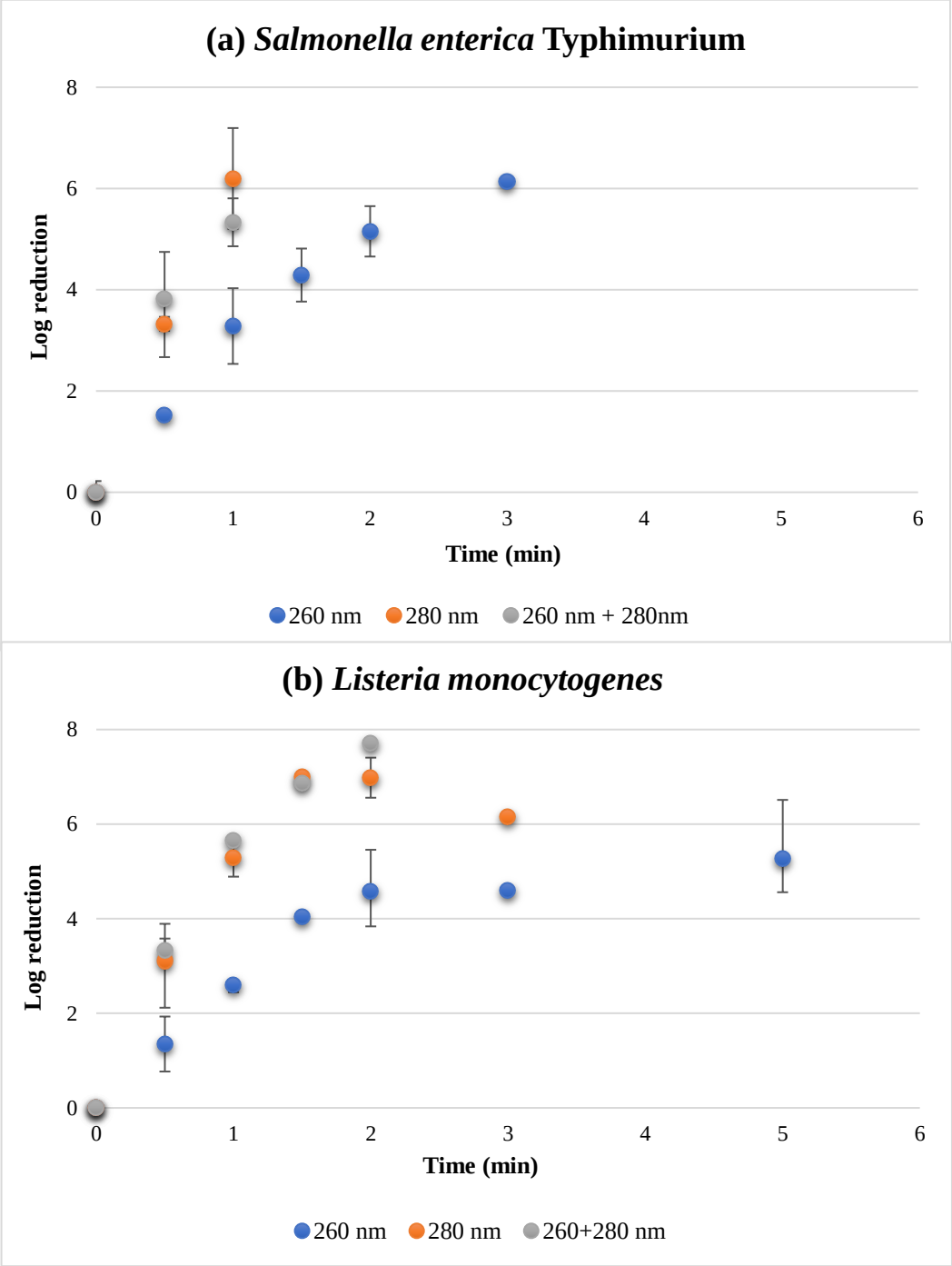


Figure 6 - UV-C LEDs inactivation (Log (C0/C)) of spiked phosphate buffered saline solutions as a function of time: (a) *Salmonella enterica* Typhimurium (b) *Listeria monocytogenes*. The error bars correspond to the results obtained for two independents experiments.

It was observed that three small LEDs that emit light at 260 and 280 nm were extremely effective to achieve inactivation of *S. enterica* Typhimurium and *L. monocytogenes* spiked in phosphate buffered saline solutions (Figure 6). After 1.5 minutes of inactivation with the LEDs that emit at the different wavelengths tested, log reductions higher than 4 were obtained for both *S. enterica* Typhimurium and *L. monocytogenes*. After 2 minutes of exposure to the LEDs, the inactivation reached a

plateau for *S. enterica* Typhimurium and *L. monocytogenes*, respectively. Figure 6 also shows that there isn't a notorious advantage in combining the two wavelengths compared to using the LEDs that emit light at 280 nm. To evaluate the potential efficacy of the synergy of 260 and 280 nm irradiation, the sum of both log reduction was analyzed and concluded that the combination of both wavelengths did not lead to synergy inactivation, however it is observed a higher log reduction of the bacterial strains when applied the UV-C LEDs that emit light at 280 nm to the cell suspensions.

Since the LEDs that emit light at 280 nm have a much higher intensity than the LEDs that emit light at 260 nm (Figure 7), to enable the comparison of the efficiency of LEDs that emit light at the different wavelengths, the inactivation results were plotted as a function of the UV fluence (Figure 8).

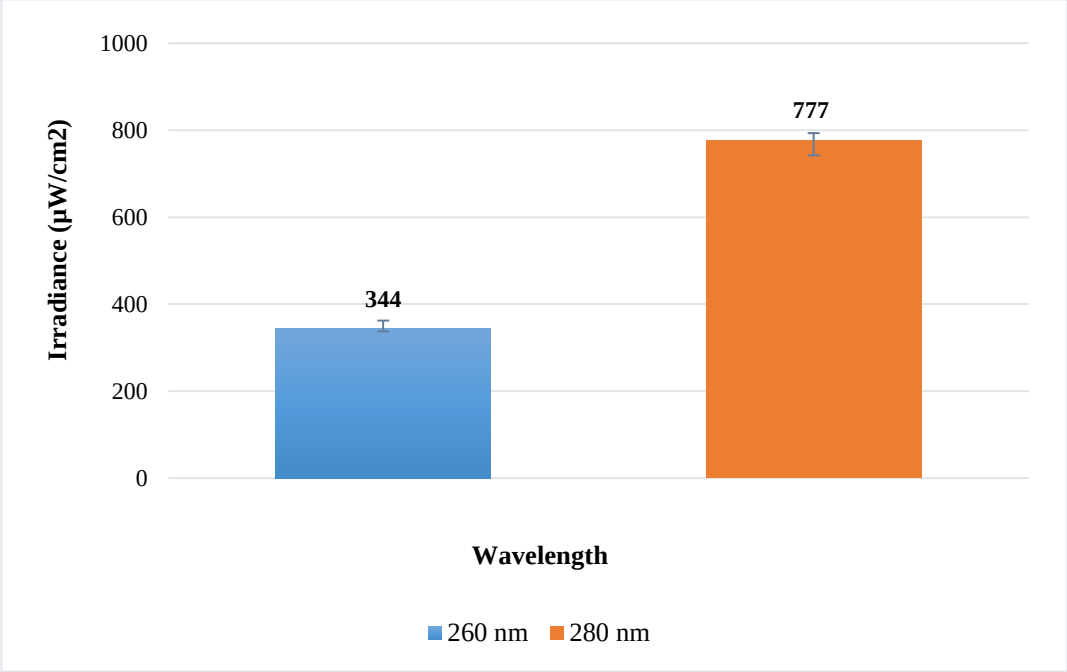


Figure 7 - Light intensity emitted by the UV-C LEDs used in this study. The error bars correspond to the results obtained from 5 technical replicates.

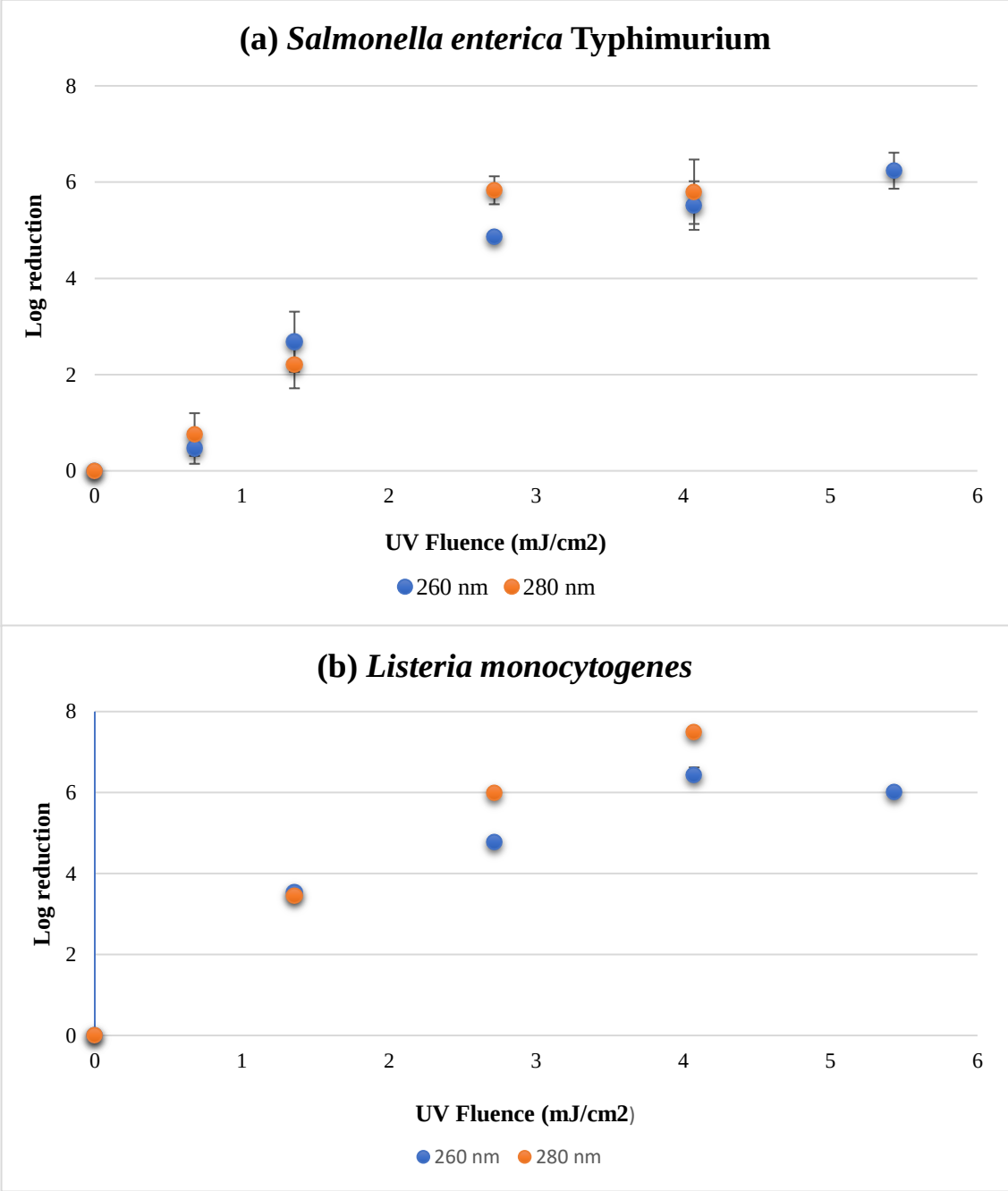


Figure 8 - UV-C LEDs inactivation (Log (C0/C)) represented as a function of UV fluence (mJ/cm^2) for *Salmonella enterica* Typhimurium (a) and *Listeria monocytogenes* (b) using LEDs that emit light at two different wavelengths (260 and 280 nm). The error bars correspond to the results obtained for two independent experiments.

With a small UV fluence of 4.07 mJ/cm^2, much lower than what is normally applied on water treatment plants to achieve inactivation [55], a log reduction higher than 5.5 was achieved for the target bacteria using the UV-C LEDs that emit light at 260 and 280 nm.

In the interval from 0 to 2.72 mJ/cm^2 it is observed a linear increase of the log reduction for both bacterial strains. The fluence based inactivation rate constants (k_D) by direct photolysis were therefore determined by linear regressions with high coefficients of determination (Table 1). The results presented in Figure 8 and Table 1 show that the LEDs that emit light at 280 nm are more effective at achieving inactivation compared to the LEDs that emit light at 260 nm.

Moreover, *L. monocytogenes* was found to be more sensitive to irradiation than *S. enterica* Typhimurium. Kim et al [56] reported a higher inactivation for *Salmonella* as a gram-negative bacteria and lower inactivation for *Listeria* a gram-positive bacterium justifying these results based on the cell wall properties such as the thickness [57]. However, in this study the same results were not observed, so further studies need to be conducted to understand the obtained results.

Table 1 - Fluence based inactivation rate constants (k_D) determined by linear regression and coefficients of determination (R^2)

		260 nm	**280 nm**
***Salmonella enterica* Typhimurium**	**k_D* (cm^2/mJ)**	1.772	1.998
	R^2	0.980	0.980
Listeria monocytogenes	**k_D* (cm^2/mJ)**	1.931	2.272
	R^2	0.969	0.996

* Determined by the linear regression between 0 and 2.72 mJ/cm^2

For both bacteria the inactivation rate constant when exposed to UV-C LEDs that emit light at 280 nm was higher than when exposed to UV-C LEDs emitting light at 260 nm, indicating that 280 nm is more germicidal than 260 nm. This agrees with what was reported by Aoyagi et al. [51]. The authors compared two wavelengths, LEDs emitting light at 255 nm which is close to the wavelength used in this work, 260 nm, with LEDs emitting light at 280 nm. Their study suggested that LEDs emitting light at 280 nm are more fitting for water purification because of its external quantum efficiency, being higher than for LEDs emitting light at 255 nm.

3.1.1 Cell morphology analysis

The cell membranes and DNA of *S. enterica* Typhimurium and *L. monocytogenes* were stained with FM 4-64 (red) and DAPI (blue) to analyse possible morphological damages in the cells after exposure to UVs. Microscopy images (Figure 9 and 10) were then obtained for cells exposed to UV-C LEDs

that emit light at 260 and 280 nm, with the same UV fluence of 5.43 mJ/cm^2, as well as samples that were not exposed.

The difference in terms of brightness/intensity of the colour may be related with the overlap of cells, which ends up positioning some of them closer to the microscope lenses, resulting in a stronger fluorescence signal.

The dyes strained the cell membranes and DNA but it's impossible to conclude that DNA suffered damage by exposure to UV-C LEDs. Future assays should be conducted with a higher UV fluence.

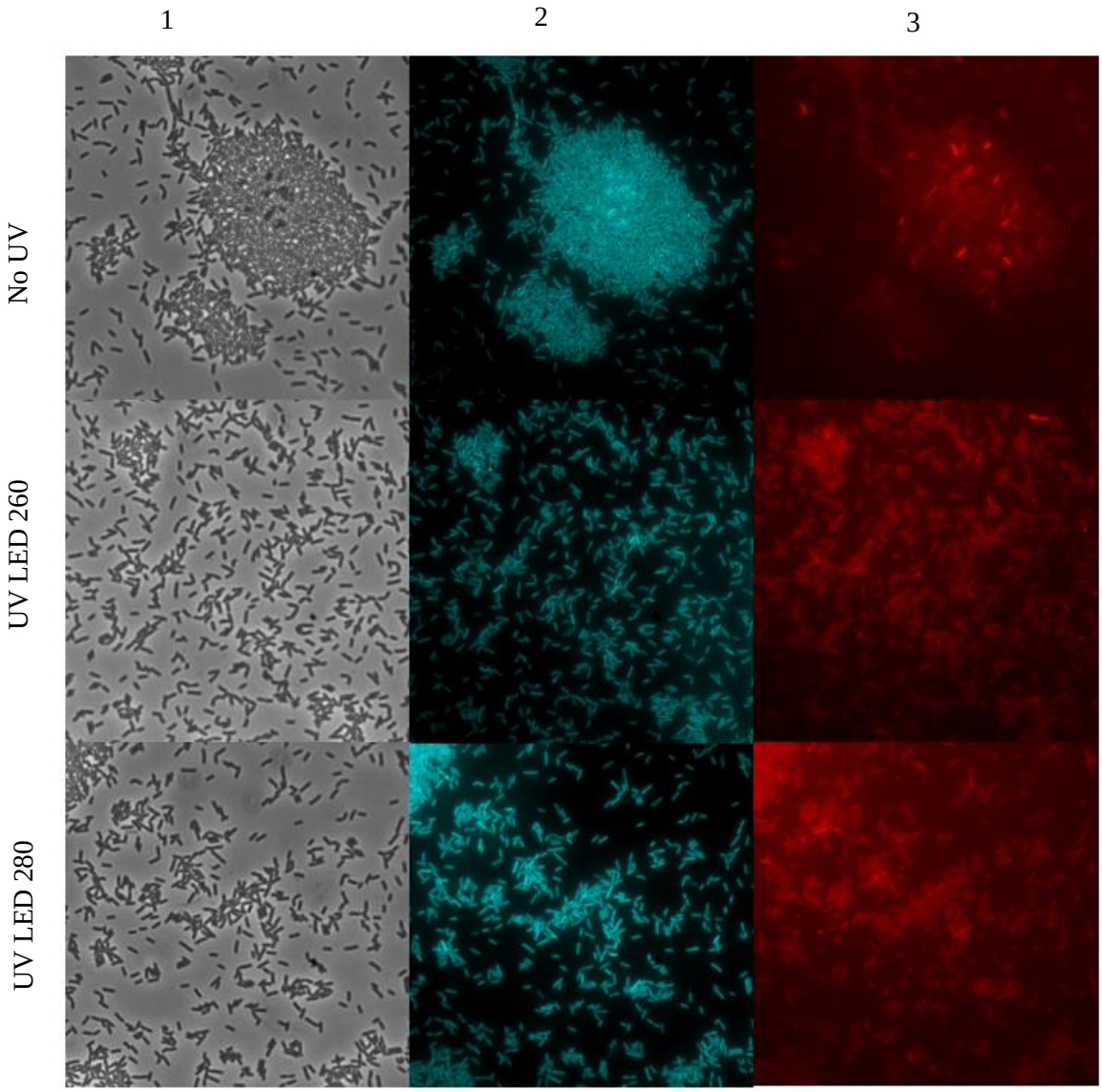


Figure 9 - Fluorescence microscopy analysis of *Listeria monocytogenes*:
1 - Microscopy images, employing phase contrast (depicted in grayscale), presents unfiltered images that offer a comprehensive perspective of the cellular architecture.
2 - Staining of *Listeria monocytogenes* DNA utilizing DAPI (rendered in blue).

3 - Employing the FM4-64 dye to stain the cellular membrane (depicted in red).

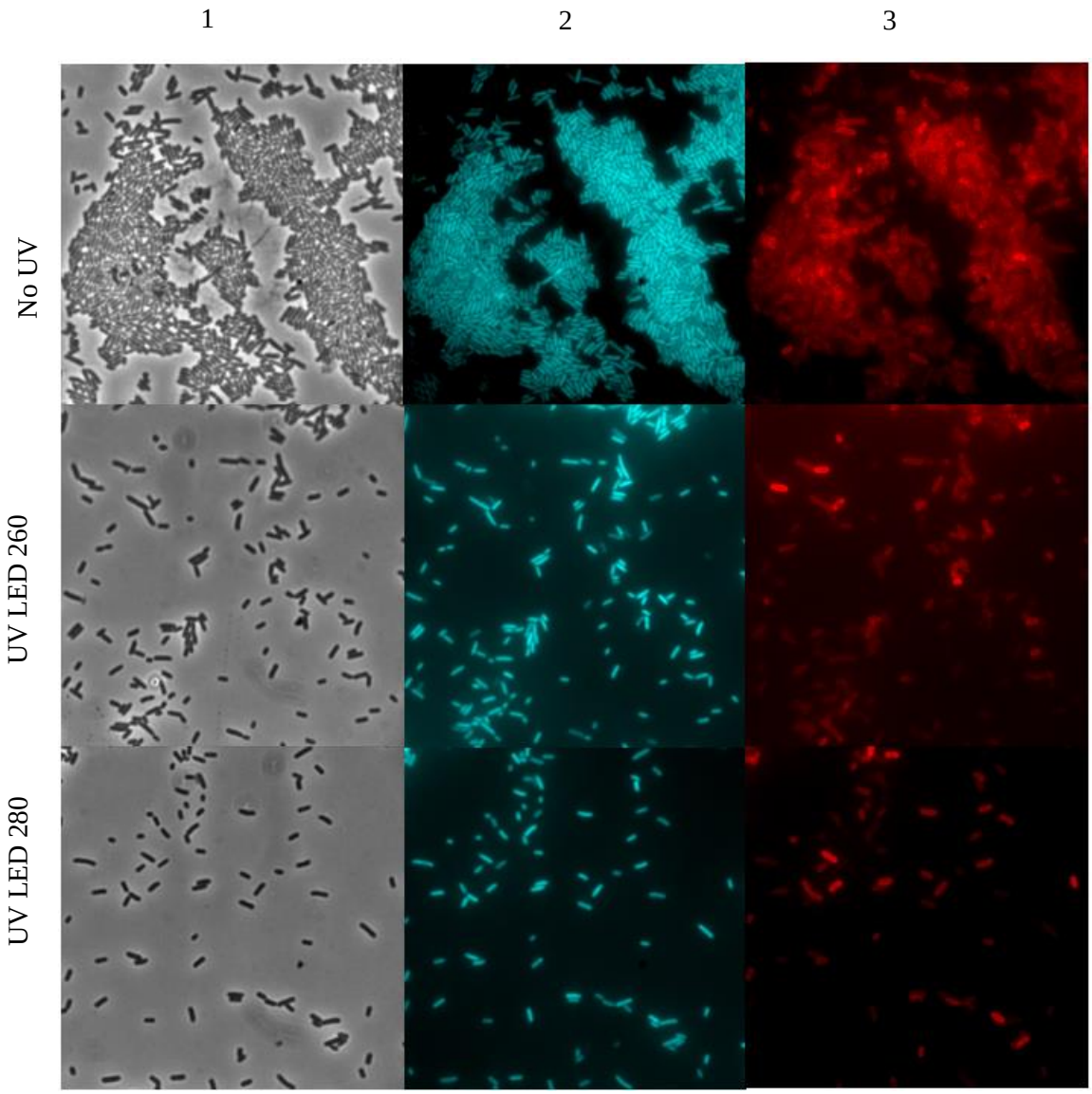
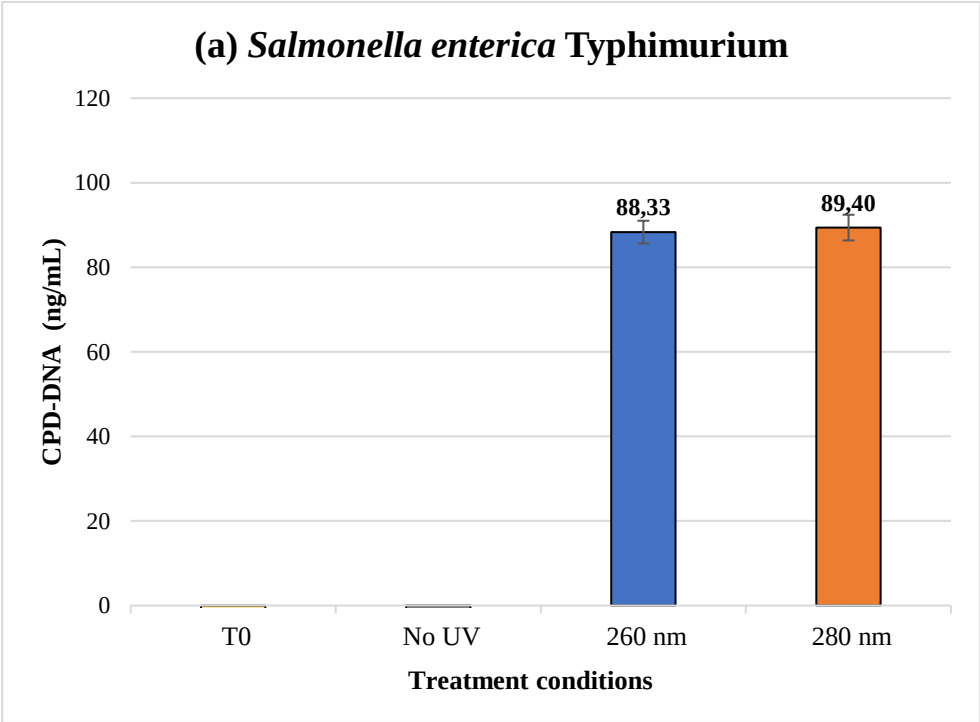


Figure 10 - Fluorescence microscopy analysis of *Salmonella enterica* Typhimurium:
1 - Microscopy images, employing phase contrast (depicted in grayscale), presents unfiltered images that offer a comprehensive perspective of the cellular architecture.
2 - Staining of *Salmonella enterica* Typhimurium DNA utilizing DAPI (rendered in blue).
3 - Employing the FM4-64 dye to stain the cellular membrane (depicted in red).

DNA damage analysis

The CPDs quantitation gives an idea of the number of dimers formed that, when in high number in the DNA, will block DNA replication and inhibit cellular division. This is an indicator of DNA damage caused by UV radiation. Figure 11 shows the results obtained for the determination of CPDs concentration.

3.1.2



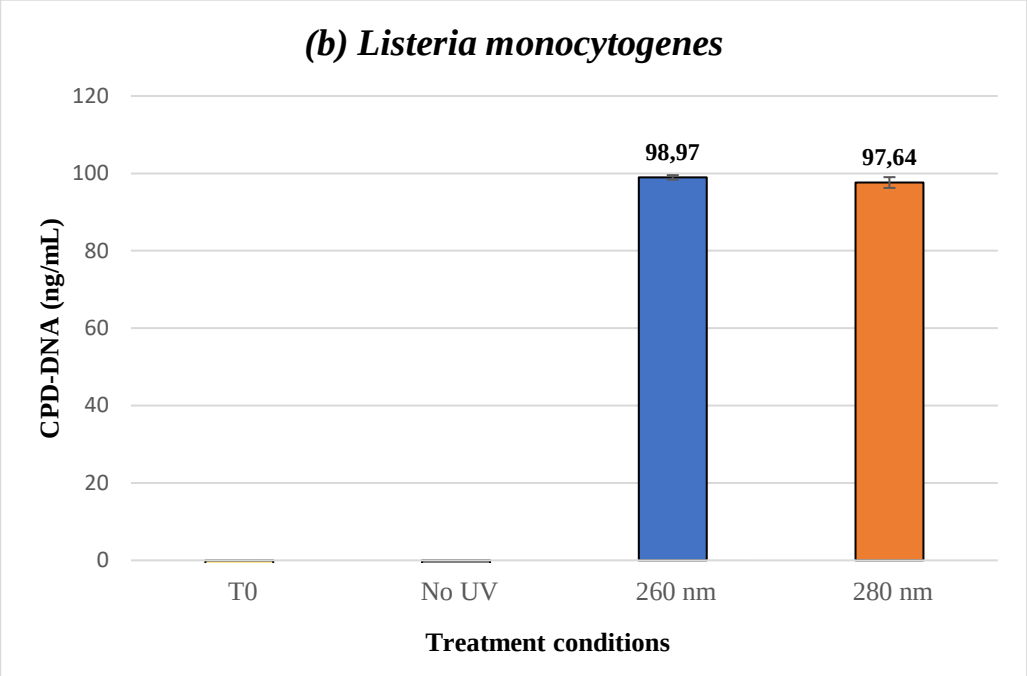


Figure 11 - The concentration of cyclobutane pyrimidine dimers formed after exposure to the same UV fluence (5.43 mJ/cm^2) using UV-C LEDs that emit light at 260 nm and 280 nm: (a) *Salmonella enterica* Typhimurium; (b) *Listeria monocytogenes.* The error bars correspond to the results obtained in duplicates and two independent inactivation experiments for *Salmonella enterica* Typhimurium, while for *Listeria monocytogenes* the error bars correspond to the results obtained in duplicates.

According to the obtained results it was observed that for samples that were not exposed (T0) and samples that were kept in the dark (no UV) during the inactivation experiments no formation of CPDs occurs, while for samples exposed to UV-C LEDs that emit light at 260 nm and 280 nm there was formation of CPDs. These results show that, for the target bacteria, both LEDs affect the DNA through the formation of CPDs. The higher effect of the LEDs that emit at 260 nm, due to the maximum absorbance wavelength of DNA, was not observed for the strains tested in this study. Further studies will be conducted to understand the reason behind the higher inactivation results obtained using the UV-C LEDs that emit light at 280 nm.

3.2 Microbial inactivation of *Salmonella enterica* Typhimurium on lettuce

Salmonellosis is the second most registered outbreak after campylobacteriosis in the European Union. Every year, around 91000 cases of salmonellosis are reported. Salmonellosis has a negative impact, according to EFSA, in the world economy, it is believed that more than 3 billion euros is spent in the fight against the pathogen. In 2021, European union accounted 60 050 cases of salmonellosis, increasing the cases by 14.3% in comparison with 2020 [14]. Therefore, in this study, *Salmonella* spp. was selected to study the effect of UV-C LEDs that emit at 260 nm and 280 nm on the inactivation of food (lettuce leaves).

Forty technical replicates were performed to study the variability of the initial concentrations in the spiked lettuce leaves prior to UV exposure, since we could not evaluate the same lettuce piece prior to irradiation. Forty leaves pieces with 12.6 cm^2 were therefore spiked with *S. enterica* Typhimurium and analyzed as detailed in section 2.2. The average concentration measured prior to UV exposure was 1.2×10^6 CFU/12.6 cm^2.

Ten lettuce leaves inoculated with *S. enterica* Typhimurium were exposed to 3 small UV-C LEDs that emit light at 260 nm, 280 nm and the combination of the wavelengths. Each side of the of the leaves was irradiated for 10 minutes. Figure 12 shows the inactivation results obtained with the UV-C LEDs that emit light at different wavelengths. The log reductions obtained were 1.8, 2.5 and 2.6 for 260 nm, 280 nm and their combination, respectively. The results presented in Figure 12 agree with the results obtained in PBS in terms of the combination of wavelengths achieving a similar result to using the LEDs that emit light at 280 nm.

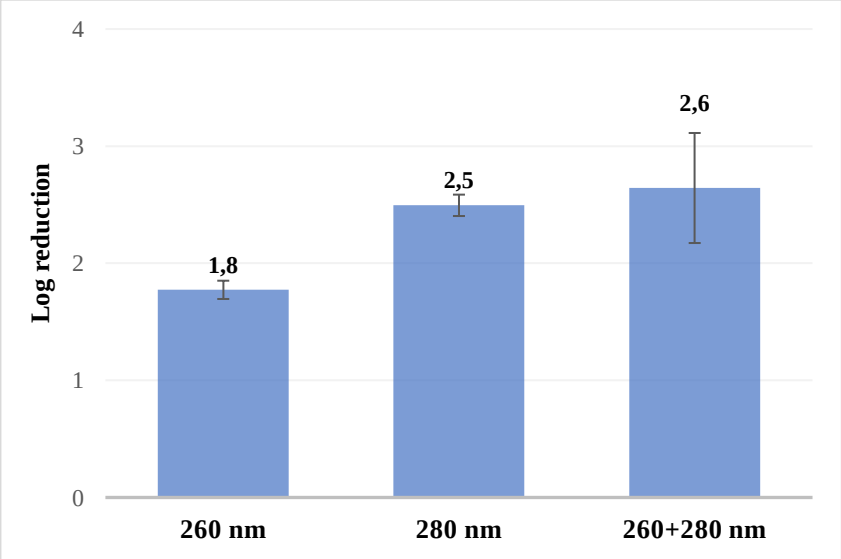


Figure 12 - Log reduction of Salmonella enterica Typhimurium spiked in lettuce leaves after 10 minutes exposure (on each side) to UV-C LEDs that emit light at 260 nm, 280 nm, and the combination of the two wavelengths. The error bars correspond to the results obtained for two independent experiments.

The pearl beam reactor was also set to emit light at 260 and 280 nm to each sample at a time that corresponds to the same UV fluence of 206.53 mJ/cm^2. This UV fluence corresponds to 10 minutes exposure using the LEDs that emit light at 260 nm and to 4 minutes and 7 seconds exposure using the UV-C LEDs that emit light at 280 nm. Each lettuce leaf before irradiation was placed in the middle of sterile lid petri dish over ice to keep the samples refrigerated, with 2 cm distance from the UV-C LEDs.

The results obtained in the inactivation assay conducted with the same UV fluence are shown in Figure 13, with log reductions of 1.85 for the LEDs that emit light at 260 nm and 2.45 for the LEDs that emits light at 280 nm. These results show that after exposing the lettuce pieces to the same UV fluence, the LEDs that emit light at 280 nm show a better inactivation efficiency than the LEDs that emit light at 260 nm. The inactivation results obtained in the lettuce leaf samples (Figure 13) agree with the inactivation results obtained in PBS (Figure 5 and Table 1).

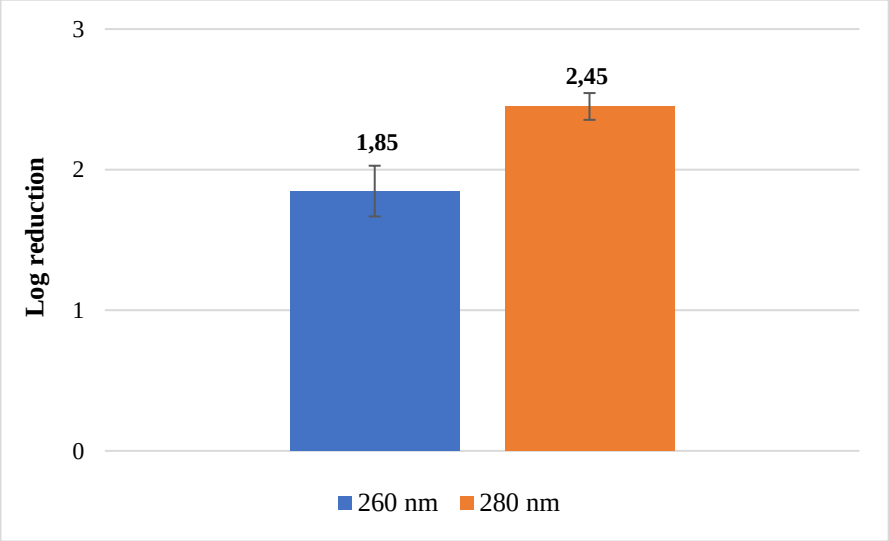


Figure 13: Log reduction of *Salmonella enterica* Typhimurium spiked into lettuce leaves exposed to the same UV fluence (206.53 mJ/cm^2) on both sides. The error bars correspond to the results obtained in three replicates.

Bohrerova and Lee [8] also stated that the use of UV fluence at 150, 450, 900 mJ/cm^2 lead to a range of log reduction from 1.96 to 2.52 for *Salmonella enterica* Typhimurium when similar experiments were conducted.

4

CONCLUSION

The obtained results show that:

- Three single small diodes that emit light at 260nm and 280nm were extremely effective for the inactivation of bacteria associated with foodborne diseases. With a small UV fluence of 4 mJ/cm^2, a log reduction higher than 5.5 was achieved for the target bacteria spiked in phosphate buffer solutions.
- UV-C LEDs that emit light at 280 nm were more effective at achieving inactivation of *S. enterica* Typhimurium and *L.monocytogenes* compared to the UV-C LEDs that emit light at 260 nm. Using a combination of wavelengths was not notoriously beneficial compared to using the UV-C LEDs that emit light at 280nm.
- *Listeria monocytogenes* was found to be more sensitive to irradiation than *S. enterica* Typhimurium.
- Higher UV fluences should be tested to observe morphological damage using fluorescent dyes.
- The concentration of cyclobutane pyrimidine dimers formed in the DNA, when exposed to the same UV dose of 5 mJ/cm^2, using the two wavelengths tested (260 nm and 280 nm) were similar for both bacteria tested.
- In inactivation assays conducted using spiked lettuce leaves exposed for 10 minutes to three single small LEDs that emit light at 280 nm, 2 log reduction was obtained.
- In inactivation assays conducted using spiked lettuce exposed to the same UV fluence of 206.53 mJ/cm^2 for both LEDs emitting light at 260 and 280 nm, a log reduction of 1,85 and 2,45 was obtained, respectively. With these results, 280 nm showed to be the most effective in inactivating *Salmonella.*

- This disinfection system could be a promising alternative to the traditional UV mercury lamps and be applied to guarantee the quality and safety of food products.

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APPENDIX

Table A1: Concentration of the DNA isolated from the different bacterial cell suspension samples and the corresponding absorption ratios

Sample ID	DNA concentration (ng/µl)	Ratio (260/280)	Ratio (260/230)
S. enterica Typhimurium T0 not irradiated	162.83	1.91	2.21
S. enterica Typhimurium C0 not irradiated	176.00	1.91	2.18
S. enterica Typhimurium 260 nm	152.37	1.94	2.31
S. enterica Typhimurium 280 nm	148.45	1.92	2.20
S. enterica Typhimurium T0 not irradiated	145.52	1.92	2.22
S. enterica Typhimurium C0 not irradiated	144.88	1.91	2.21
S. enterica Typhimurium 260 nm	131.16	1.94	2.20
S. enterica Typhimurium 280 nm	144.34	1.89	2.18
L.monocytogenes T0 not irradiated	13.66	1.95	1.64
L.monocytogenes C0 not irradiated	77.64	1.88	2.25
L.monocytogenes 260 nm	87.03	1.89	2.28
L.monocytogenes 280 nm	69.37	1.92	2.22

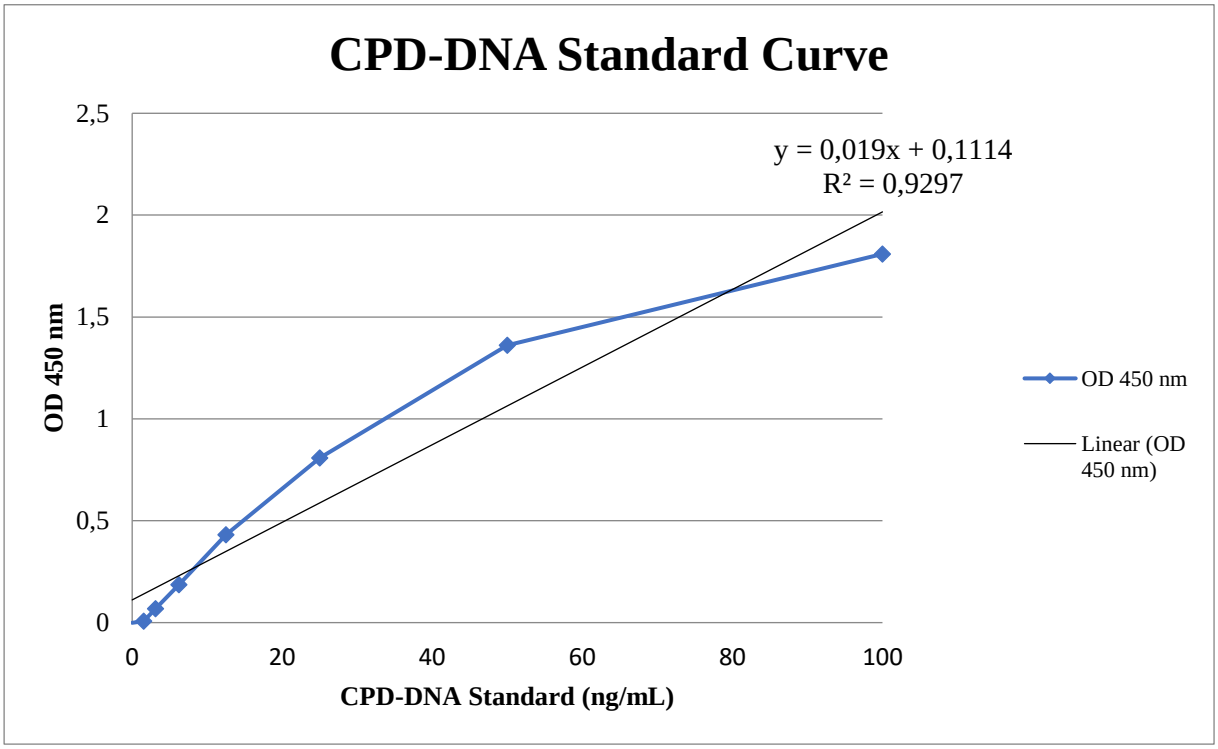

Figure A1 - CPD-DNA standard curve used to obtain the CPD concentration of the analyzed samples. The error bars represent the results obtained for the duplicates performed.

Concentration of CPD-DNA in [B units / V units] in the test samples was calculated as:

$$\text{CPD – DNA concentration} = \frac{B}{V} \times D$$

Where:

B - amount of CPD-DNA in the sample well calculated from standard curve in µL/mL

V - sample volume added in the sample wells in µL

D - sample dilution factor if sample is diluted to fit within the standard curve range (prior to reaction well set up).