UNIVERSITÀ DEGLI STUDI DI TORINO

Department of Clinical and Biological Sciences

Doctoral School in Life and Health Sciences

PhD Program in "Complex System for Life Sciences"

PhD Thesis

Innovative 'green' & multifaceted antimicrobial approaches for food preservation

Coordinator: Prof. Enzo Medico

Tutor: Prof. Caterina Guiot

PhD Candidate: Zunaira Munir

XXXV Cycle

DEDICATED TO MY PARENTS

Acknowledgements

I am thankful to **Allah** Almighty for His blessings that gave me strength and patience to complete this thesis.

The last three years in my life have been an extraordinary experience and for this I have many people to thank. Firstly, I would like to express my gratitude to my wonderful supervisor, **Prof. Caterina Guiot.** I would like to express my gratitude to her for her suggestions during this work and supporting me throughout the lab work with her expertise. Her positive attitude and appreciation for my work encouraged me to move forward. I am highly grateful for her assistance, continuous support, valuable advice, corrections and perceptive comments regarding the thesis. Being an international PhD student she always treats me as a daughter to make me as stronger as a mother do for her real daughter. I am fortunate to have Prof. Caterina Guiot as my supervisor. I could not imagine having a better advisor and mentor for my research work.

It is a genuine please to express my deep sense of thanks to **Prof. Roberta Cavalli,** Professor, Department of Drug Science and Technology, University of Turin who provided me an opportunity to work in her group and for her invaluable guidance during the course of this thesis. Her timely advice, meticulous scrutiny, scholarly advice, and scientific approach have helped me to a very great extent to accomplish this task.

I am grateful to **Prof. Giuliana Banche and Prof. Narcisa Mandras** Professors, Department of Public Health and Pediatric Sciences, University of Turin for having welcomed me so well in their Department. They not only gave me the opportunity to explore and innovate in their laboratory and opened up new areas of interest, but also provided constant support, guidance and encouragement.

I would like to acknowledge to Coordinator **Prof. Enzo Medico** and **Prof. Michele Debortoli**, for ensuring smooth working conditions and encouraging atmosphere towards scientific work.

Deepest thanks to my seniors and project fellows, **Shoeb Anwar Mohammad Khawja, Lorenza Cavallo and Eleonora Ficiarà**. Without their moral and practical support, I could not have succeeded in completing this task. My profound gratitude goes out to **Monica Argenziano** and **Ilaria Stura**, who has consistently served as a best colleague.

I would also like to acknowledge my lab mates, Irfan Ansari, Chiara Molinar, Maria Tannous, Domitilla, Simonetta and Sara Comini for always being there for me and supporting me through both the good and bad moments during my Ph.D. days.

I want to thank all my amazing friends especially **Chiara Ferraris** (my only Italian friend), **Yasmeen Safari, Fehmida Usmani and Annila Munsaf**. I have no words for the friendship, affection and moral support that I received from my friends. I would also like to thanks to my flatmate **Manisha Padhi** for her care and loving nature.

My acknowledgment would never be complete without a special mention to my beloved **Prof. Giuseppe Saglio** and his wonderful wife **Lucia**. They consider me as a family member and care as parents. I believe that words are inadequate to express how I feel about them.

It is an honor for me to express my gratitude to my husband, **Dr. Nabeel Ashraf**, who has supported me through all of my difficulties and has been there for me through all of the ups and downs, the late nights and early mornings, and for keeping me sane over the last year. He gave me support and help, discussed ideas, and prevented several wrong turns

Last but most importantly, my family, my father **Muhammad Munir** (Late) and my mother **Mrs. Sajida Munir** for their constant love, who supported me at every step of my life. It is due to their prayers that I have completed my research work. A special thanks to my brothers and sister who are always there for me. I want to express my gratitude to my beloved brother **Muhammad Shehzad Ali** for his patient, supportive advice and unwavering support throughout my academic career.

Zunaira Munir

Declaration

I undersigned declare that, the content of this thesis is the result of my own original work during last three years. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification in this or any other university or other institute of learning..

Zunaira Munir

ABSTRACT

Antibiotic resistance is one of the major current health concerns and a stricter limitation of the use of antibiotics in agriculture and farming is one of the adopted countermeasures. "Green" food requirements indicated by the Next Generation UE platform are based exclusively on biocompatible and biodegradable components.

Such a new perspective is very challenging and more innovative 'paraphernalia' are required, including nanoformulations and physical stimuli as photoactivation (in both cases complexity is brought into play since nanoscale exhibits nonstandard resonant effects).

Curcumin is largely employed in medicine due to its various and multiple potentials, which includes anticancer, antioxidant and antibacterial activity, but it also has substantial drawbacks. For instance, curcumin has extremely poor solubility in water (around 11 ng/mL). This makes it challenging to apply it in a water-soluble system, because of instability in solutions, limited bioavailability, poor absorption, and quick excretion from the body. Moreover, curcumin is also easily degradable under light, heat and in the presence of oxygen. To overcome these issues, encapsulation of curcumin in complexes or into nanobubbles (NBs) would strongly enhance its stability. Furthermore, curcumin is a natural photosensitizer whose antimicrobial efficacy can be enhanced by photoactivation with light of proper wavelenght.

In the first step of my work the antimicrobial effectiveness of a solution of curcumin in N-methyl-2-pyrrolidone on 3 food-borne bacterial strains (i.e. *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*) was evaluated both in the absence and in the presence of photoactivation with blue LED light. Most of this activity was performed in the *Microbiology Unit, Department of Sciences of Public Health and Pediatrics, University of Torino*.

In the second part of the research the effectiveness of a blue light photoactivated complex of curcumin/β-cyclodextrin on berries (strawberries and blueberries, in particular) and the possible impact on their shelf life was investigated under many point of views, including microbiological assays, oxidation, curcumin deterioration, and volatile component assessments (related to the retention of the original organoleptic properties).

According to the findings of this study, curcumin effectively preserves berries by decrease in bacterial count, expressed as colony forming units/ml (CFU/ml), without dramatically changing their odor or antioxidant qualities. Therefore, the investigated technique shows a good potential for a simple and

environmentally friendly approach to increase the shelf life of berries, which is possibly of interest for the market.

Finally, I devoted my third year of activity to prepare, characterize, and investigate the impact of curcumin-NBs on the food-born bacterial strains at the *Dept of Drug Science and Technology*. The effects of NBs loaded with curcumin/chitosan and NBs loaded with curcumin/chitosan and oxygen were studied. Their antibacterial properties were measured through microbiological and biochemical assays of minimum inhibitory concentration minimum bactericidal concentration and Time kill kinetics. The bacterial membrane damage was investigated by the Lactate Dehydrogenase (LDH) Release Assay.

According to the results of this study, it was ascertained that NBs (particularly those made of chitosan/curcumin and containing oxygen) following LED irradiation results in a better prevention of the microorganism proliferation with respect to that in absence of oxygen or photodynamic activation.

Interestingly, the present study also showed that NBs (curcumin, chitosan, oxygen & LED) were able to severely damage Gram negative bacteria, i.e *E.coli*, that are harder to kill in comparison with Gram positive ones, due to the different characteristics of the bacterium's cell envelope.

Therefore, the outcomes of the proposed study results in an innovative and 'green' approach against pathogenic microorganisms. Further study is required on the application of NBs directly on perishable food, i.e berries, as well as against other bacterial strain and fungus. Moreover, in order to further enhance the broad action of curcumin encapsulated into the NBs, more investigations are required to convert this nanoformulation into an 'antibiotic green drug' of interest for the market.

So, these findings may be included into a more comprehensive approach to fight pathogenic microorganisms that cause food deterioration in order to protect human health and raise food quality globally.

The research is partially financed by the Italian Ministry for Economic Development (MISE) and the POC TOINPROVE/2020 project of the University of Torino

Table of Contents

List of Figures

3.2	Killing activity against *E.coli*	66
3.3	Killing activity against *S. aureus*	67
3.4	Killing activity against *E.faecalis*	68
4.1	Description of the experimental groups and treatments	77
4.2	**A)** The larger structure on the left was designed to illuminate blue LEDs on up to 0.25 kg of berries after spraying with curcumin; **B)** On the right, the smaller one for in vitro study or a few berries	80
4.3	PEN e-nose during an analysis of a group of strawberries (10 vials)	82
4.4	Phase-solubility diagrams of curcumin with βCD	84
4.5	Filter paper-based microbial growth on BHA (above) and SAB (below) plates: **1.** Control (washing water from blueberries in a cyclodextrine solution), **2.** Washing water from Blueberries treated with Curcumin/β-CD solution 3. Washing water from blueberries treated with Curcumin /β-CD solution- along with 6h blue LED light	86
4.6	Microbial growth on BHA plates using unfiltered washing water: 1. Control, 2. Curcumin + Cyclodextrin solution treated blueberries, 3. Blueberries treated with cyclodextrin and curcumin solution along with 6 h Led light irradiation	86
4.7A	Top: PCA analysis of strawberries from e-nose data. Each dot represents a different sample. Different colors represent different treatment groups. Bottom: hierarchical cluster based on factor scores.	89-90
4.7B	Top: PCA analysis of blueberries from e-nose data. Each dot represents a different sample. Different colors represent different treatment groups. Bottom: hierarchical cluster based on factor scores.	90-91
4.8	**A)** Radar graph about CI (blue area), CICU (red area) and CICUB (grey area) in respect of CTR (green line). Blueberries and strawberries were both examined. All of the points outside of the green line indicate a better behaviour with respect to CTR, whilst the points inside the curve indicate a worse behaviour **B)** Radar graph about CICUB (grey area) and CICUBm (orange area) in respect of CTR (green line). A better behaviour with respect to CTR is shown by all the points outside of the green line, whereas a worse behaviour was indicated by all points inside the line.	92
4.9	**A)** calibration plot; **B)** oxidative stress in the five standard berries groups (CTR, CICU1, CICU2, CICUB1 and CICUB2) compared with standard control (linoleic acid) and berries+blue LED. The treatment (grey bars) reduces the oxidative stress with respect to controls.	93-94

List of Tables

CHAPTER 1: INTRODUTION

1.1 Food-related Bacteria and their Characteristics

Nowadays, the possibility of preserving food in an optimal way for long periods of time is increasingly important. In fact, food items are susceptible to more rapid degradation and may cause significant foodborne diseases in the absence of treatments that assure their optimal storage and decontamination from microbes **[1]**. Food can become contaminated by pathogenic microorganisms, mainly bacteria, at a number of phases, including manufacture, processing, storage, and delivery of the final product. Some pathogenic bacteria can create virulence factors and toxic compounds, like toxins, during the replication process, which play a role in the pathogenesis of many diseases **[2]**.

An example of a bacterial species responsible for foodborne illnesses is *Staphylococcus aureus,* a Gram-positive, commensal, and opportunistic pathogenic microorganism that can cause a broad range of infections (**Figure 1.1a**). *S. aureus* is well known for its resistance to a variety of chemical and physical conditions. In fact, it thrives in environments with temperature ranges between 7°C and 48.5°C (optimal 30°-37°C), pH ranges between 4.2 and 9.3 (optimal 7 to 7.5), and sodium chloride (NaCl) concentrations as high as 15%. Additionally, it can easily tolerate dry and stressful environments, so it can remain on inanimate surfaces for long periods. All of these properties encourage the growth of the organism in a variety of food products, which can serve as sources of contamination, particularly when they are improperly handled. The foods most at risk of contamination are vegetables, poultry, dairy, salads, and bakery items like pastries and cream-filled sweets. *S. aureus* produces numerous staphylococcal enterotoxins (SEs): i.e enterotoxin A is the most common cause of staphylococcal food-borne disease (SFD) **[3]** and has been accountable for a number of SFD epidemics in Japan, France, and the UK **[4].** Generally, SFD has a rapid and sudden onset and occurs soon after consuming *S. aureus*-contaminated food (about 3-5 hours). This is because bacterial contamination at the optimum temperature results in the synthesis of one or even more toxins, which are responsible for several symptoms, such as nausea, vomiting, abdominal cramps in the presence or absence of diarrhea. Usually SFD is self-limiting and resolves within 24-48 hours; however, it can be a severe condition if it affects infants, the elderly or immunocompromised patients, leading to hospitalization. The recommended therapy does not include the use of antibiotics: in fact, it is a mainly supportive therapy and full recovery takes about 20 hours approximately **[3].**

Escherichia coli (**Figure 1.1b**) is a Gram-negative, commensal bacterium. Particularly the enterohemorrhagic subtype O157: H7, is another causative cause of foodborne disease. Despite the fact that the overall number of infections is fewer compared to other enteric pathogens like *Salmonella* spp. or *Campylobacter* spp., this bacterium has caused significant public health concerns, particularly

in North America, Europe, and other parts of the world. In the United States, Japan, and the United Kingdom, *E. coli* O157:H7 has repeatedly been isolated from sick individuals **[5],** and it has also recently been found in the gastrointestinal tract of cattle, the natural reservoir of this bacterium, in water and in some contaminated foods, such as beef products and fresh produce polluted with fecal material (unpasteurized milk, apple cider, fresh spinach, and radish sprouts). In addition, inter human and zoonotic transmission has not been ruled out, as cases of infection have been reported in children attending kindergartens **[5]** and in people who have visited places such as zoos **[6]**, dairy farms, and campgrounds within which livestock had previously grazed **[7]**. The majority of *E. coli* strains harmlessly invade the gastrointestinal tracts of both people and animals; but certain strains have developed virulence factors via plasmids, transposons, bacteriophages, and/or islands of pathogenicity, making them pathogenic. In fact, *E. coli* O157:H7 produces multiple virulence factors, includes pathogenicity island components, toxins, and F-like plasmid pO157 products. In addition, it resists the acidic pH of the stomach and this characteristic increases the possibility of the bacterium to colonize the intestine and cause infection **[5]**. Infection caused by *E. coli* O157:H7 usually occurs asymptomatically or with episodes of non-bloody diarrhea, but cases of death following infection have been reported. In fact, in most cases, the infection manifests with non-bloody diarrhea that resolves without complications; however sometimes the infection can progress and lead to serious complications, such as bloody diarrhea. In 5-10% of patients with such diarrhea, the disease can progress to life-threatening *sequelae*, such as Hemolytic Uremic Syndrome (HUS) **[8]** or thrombocytopenic purpura (TTP) **[5].** Since there is no known treatment for *E. coli* O157:H7 infection and the use of antibiotics may be prohibited, the most popular strategy is essentially supportive therapy to reduce symptoms, stop the progression of the illness, and avoid systemic problems **[5]**.

Another group of bacteria whose significance has increased dramatically over the past several years are enterococci, which are by nature extremely resilient and capable of surviving, even for extended periods of time, under a wide range of adverse conditions. Enterococci are typical commensals of the gastrointestinal tract and are crucial for food maturation, the creation of distinct flavors, such as in different cheeses, and the rotting of particular meats. Their presence may also serve as an indicator of microbial contamination of fecal origin, in fact these Gram-positive bacteria can also be found in food and are the causative agents of a number of nosocomial diseases. The most common species among them is *Enterococcus faecalis* **(Figure 1.1c),** a bacterium frequently linked to a number of clinical illnesses, such as bacterial infections of the urinary tract, bacteremia, neonatal infections, dental diseases, and wound infections **[9].** In addition, it has been found in some foods such as raw milk and in pigs **[10, 11**]. For this reason, a possible transmission to humans, via the zoonotic route, cannot be excluded **[9].** *E. faecalis* possesses a number of toxic factors involved in

the pathogenesis of these infections, such as enterococcal superficial protein, hyaluronidase, gelatinase, and aggregation substance. In addition, it produces biofilms, i.e. a matrix of polysaccharides, proteins, extracellular DNA, and bacteria **[12],** that serves as a defense against antimicrobials and the immune system of the host body. Thanks of it, bacteria may thrive in unfavorable environments and proliferate across the ecosystem **[13]** and transfer their genes to other species making them resistant to antibiotics [**9].** In fact, *E. faecalis* possesses multiple resistance to several antibiotics, such as, beta-lactams, glycopeptides, quinolones and macrolides. However, if the bacterium does not exhibit higher resistance to beta-lactams, aminoglycosides and glycopeptides, combination antibiotic therapy can be utilized to treat the infection. One can combine a beta-lactam antibiotic or a glycopeptide with an aminoglycoside to have a bactericidal effect, or proceed to administer the antibiotic for which the bacteremia has not developed resistance **[14]**.

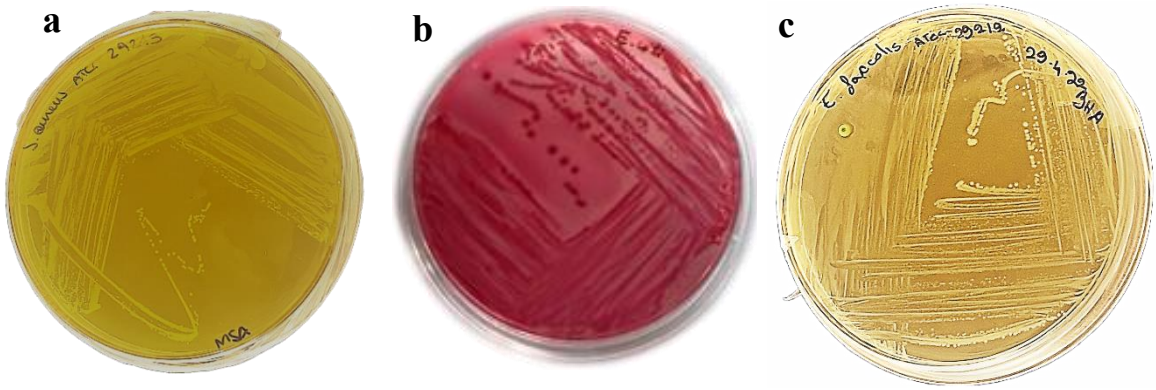


Figure 1.1: **a.** *Staphylococcus aureus* on Mannitol Salt Agar **b.** *Escherichia coli* on MacConkey Agar (MAC) and **c.** *Enterococcus faecalis* on Brain Heart Agar.

Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide **(Figure 1.2).** The outer membrane controls the entry and release of numerous substances, including ions, nutrients, and environmental pollutants, and it aids in osmoprotection and it acts as a permeability barrier. On the other hand, Gram-positive bacteria have a thicker layer of peptidoglycan and an inner plasma membrane, but lack an outer membrane that serves as protection **[15].** Because of this, Gram-negative bacteria are more resistant to antimicrobial agents than Gram-positive ones **[16].**

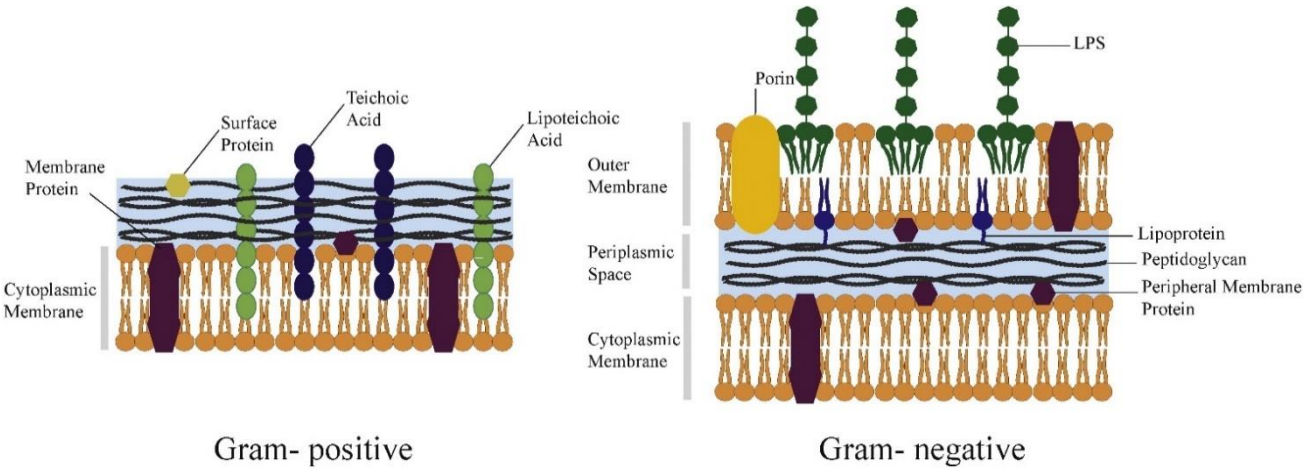


Figure 1.2: Structure of the envelope in Gram-positives and Gram-negatives **[17].**

The above- noted pathogenic bacteria are not the only ones that can contaminate food; countless other pathogens that cause diseases are also able to attack different food products. Scientific and technological research has recently started to demonstrate a developing interest in the creation of new antimicrobial materials and innovative techniques of food sanitization in order to avoid microbiological contamination, increase the shelf life of food items, and assure human safety.

1.2 Microbiological Analysis and Evaluation of Antibacterial Agents Effectiveness

We have performed microbiological analyses by administrating the antimicrobial compound and the photoactivation procedure on bacterial cultures and on foods, both by analysing the washing water or directly on a small amount of substance, in our case fruit (in-fructo).

Details on the bacterial cultures will be given in the following chapters.

A number of microbiological tests were performed to test the effectiveness of the antibacterial agents.

Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent or substance (expressed in µg/mL) which, under controlled *in vitro* conditions, completely prevents any visible growth of the microbial test strain **[19]**. The MIC experiments were carried out according to the CLSI protocol **[20].** MIC was used to evaluate the impact of different concentrations of an antimicrobial agent on inhibiting the growth of the microbial population.

Minimum Bactericidal Concentration

The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibacterial agent needed to induce a 3 log reduction in the size of the standard inoculum over a predetermined time of incubation, such as 24 or 48 hours. The MBC can be measured to identify the concentration that decreases the initial bacterial inoculum's survival by a specified reduction, such as ≥ 99.9%. The MBC is complementary to the MIC and it indicates the amount of bacterial killing: the MBC displays the lowest concentration of antimicrobial agent that leads in microbial mortality as opposed to the MIC test, which displays the lowest concentration of antimicrobial agent that significantly suppresses their growth **[21].**

Time Kill Kinetic

Time kill kinetic assays are performed to evaluate the *in vitro* reduction of a microbial population after exposure to an antimicrobial agent along time. Time kill is analyzed using the MIC values **[22]**.

Also the analyses on fruits, i.e. the Washing water analysis and the 'in-fructo' analysis, will be fully described in the following chapters.

1.3 New 'green' Food Sanitation Methods

Some of the traditional processes for food preservation, for example heating, freezing and drying, have been used by industries. Adopting heat treatment and congealing processes, however, comes with a variety of drawbacks. In fact, these treatments can act negatively on the organoleptic characteristics of the food, determining, for example, the alteration of the consistency, the shrinkage of the product, the loss of nutrients and organic properties. As a new and efficient strategy for food preservation, additives, coatings, and various polyphenolic plant extracts have recently utilized in chemical and microbiological treatments **[23].** Additionally, there are new preservation methods that are being developed or in used, such as, high pressure, electroporation, nanothermosonication, and addition of bacteriolytic enzymes, act on the inactivation of counting-mining microorganisms **[1]**. In any case it is essential to develop techniques that are unlikely to encounter or elicit resistance.

We will focus mainly on two innovative approaches:

- PhotoDynamic Inactivation
- Biocompatible nanosystems

- PhotoDynamic Inactivation

Photodynamic Inactivation (PDI) has shown to be a useful ally in a variety of fields. The mechanism of action is based on the locally or systemically activation of photosensitizers (PS) by light of proper wavelength in the presence of an oxygen source, eliciting the production of reactive oxygen species (ROS), which are responsible for the antimicrobial effects **[24].** Generally, a PS agent is introduced, allowed to propagate and then accumulate at the border or inside the treated cells **[25].** Following the accumulation phase, the PS agent exposed to a light of a certain wavelength while it is in its ground state (PS0) **(Figure 1.3).** After illumination, the PS agent undergoes into the singlet excited state (1PS*) by absorbing energy. The PS agent can either experience an intersystem crossover (ISC) into the excited triplet state (3PS*) when in the singlet excited state (1PS*), or it can fluoresce back to the ground state **[26].** The photosensitizer in the triplet excited state can provide an electron/ hydrogen atom or confer energy to molecular oxygen in its ground state (type I mechanism or type II mechanism, respectively). In the type I pathway, the PS interacts with the nearby tissutal biomolecules while in the excited triplet state it picks up an electron or a hydrogen atom and produces ROS, including hydroxyl ions (HO•), superoxide anion (O2 •), peroxide radicals (HO•), and hydrogen peroxide (H_2O_2) (Sun et al 2018). The subsequent set of oxidative reactions induced by ROS eventually lead to cellular toxicity (**Figure 1.3**). On the other hand, energy is transmitted directly from the triplet excited state of the photosensitizer to the oxygen molecule in pathway II producing a highly reactive and cytotoxic singlet oxygen ($^1O2$) **[27].** The cytotoxic singlet oxygen interacts with the surrounding biological molecules such lipids, nucleic acids and proteins of the cell membrane (or bacteria) to trigger cell death by apoptosis or necrosis **[27].**

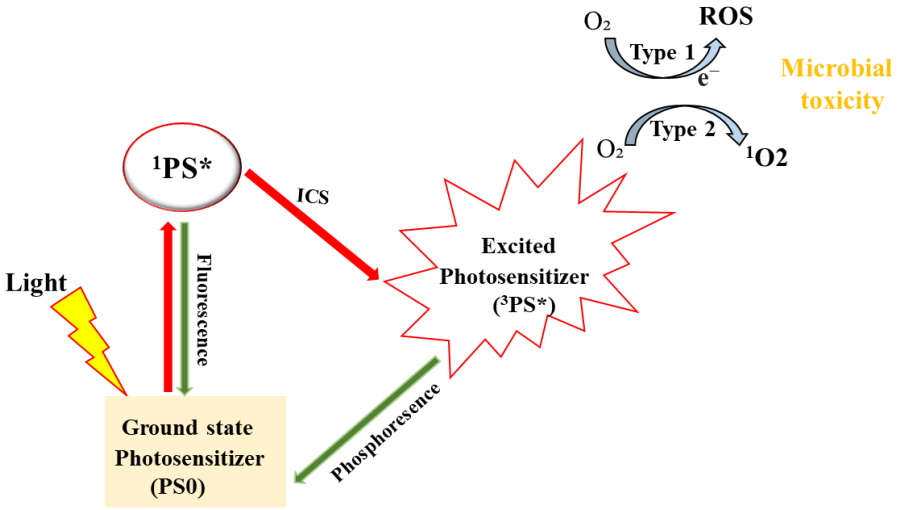


Figure 1.3: Jablonski diagram outlining PDT's method of action.

In recent years, researchers have placed particular interest on improving the antimicrobial activity of natural PS, such as curcumin or other natural substances by photoexcitation **[28].** PDI has been referred as a viable approach to combat food-borne microbes because of its advantages of safety, environmental protection, and low energy consumption **[29, 30].** The fundamental tenet of PDI is that specific light wavelengths activate photosensitizers, which then produce ROS which trigger a cytotoxic reaction that results in cell death **[31]**. For instance, targeting guanine nucleotides by ROS may result in DNA damage, according to **[32]**, and **[33]** concluding that cytoplasmic DNA and proteins were degraded by curcumin-mediated PDI. Furthermore, the oxidative burst of ROS alters the homeostasis of microbes, and the antioxidant enzyme system develops a crucial cellular defense network: primarily catalase, superoxide dismutase, and glutathione peroxidase **[34].** Several studies have shown that some harmful bacteria can be photodynamically inactivated using a light emitting diode (LED) light source **[35].** Since curcumin is a naturally occurring photosensitizer, it can be photoactivated by LEDs at a wavelength of 425 nm, which makes it suitable for decontamination of food surfaces. LEDs provide a number of benefits over conventional light sources, including being extremely energy-efficient, affordable, robust, and emitting little heat, which can be utilized to sterilize food surfaces. Additionally, when curcumin exposed to light of proper wavelength, it produces ROS including superoxide anion and hydroxyl radicals, among others, which inhibit the growth of bacteria **(Figure 1.4**). Therefore, the use of photosensitizers in conjunction with LED lighting would reasonably result in the deactivation of microbes on the surface of food. Additionally, using photoactivated curcumin would be a good choice because it is secure, prevalent in nature, and has no impact on the organoleptic qualities of food goods **[28].**

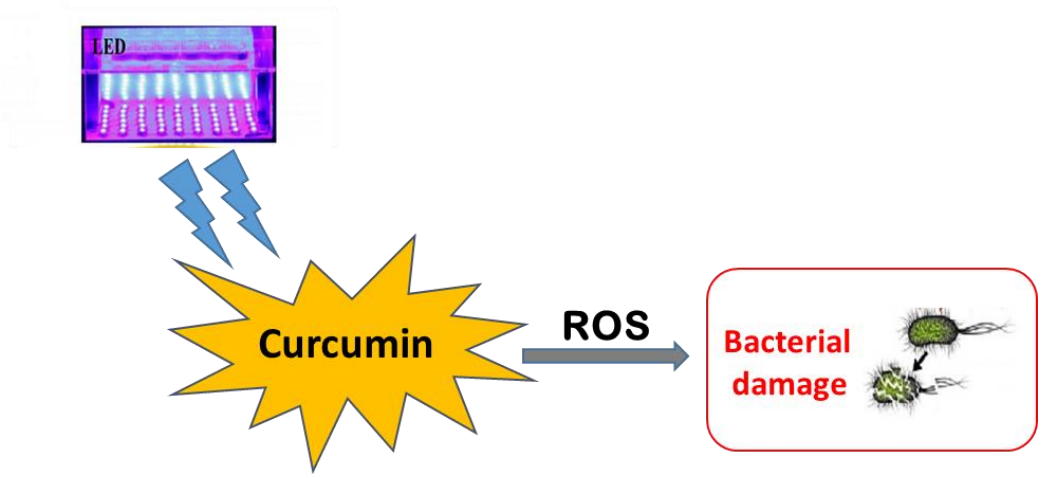


Figure 1.4: Curcumin as a photosensitizer with photodynamic therapy.

- **Biocompatible Nanosystems**

Nanotechnology, from the Greek term ‘nano’ that refers to "dwarf", is the technique of creating, manufacturing, and modifying particles with sizes generally less than 500 nanometer. The numerous tools developed by nanotechnology are used at the molecular level to identify and cure a wide range of diseases **[36, 37]**.

Nanotechnology has incredible potential for enhancing drugs effects by improving their pharmacokinetic profile, boosting solubility, bioavailability, delivering prolonged release, or even the targeted administration by the conjugation with specific ligands. There are many different kinds of nanomedicines being studied, including nanoemulsions, nanosponges, nanobubbles, nanosuspensions, and nanotubes. Over the last two decades, it developed into a well-known and successful formulation strategy in the pharmaceutical sector. It is expected to expand quickly through all phases of development, from initial detection to late-stage drug testing **[38].** We will mainly focus on NanoBubbles (NBs).

Nanobubbles (NBs), commonly referred to as ultrafine bubbles, are incredibly small in comparison to micron (MBs, around 1000 nm), and typical air bubbles (CBs, >10 um), exhibit a variety of distinct physical and physicochemical characteristics **[39].** Although showing some similarities with Surface nanoparticles bulk nanobubbles (NBs), and micro-pancakes , (the formation of surface NPs occurs at solid-liquid interfaces, micro-pancakes are quasi-two-dimensional gaseous domains, while bulk NBs are spherical and generally have diameters of 100–200 nm **[40, 41]),** they differ for the presence of a solid-gaseous interface mediated by a surfactant layer.

The idea of NBs was first proposed in 1954 as result of the expansion as gas nuclei developed upon cavitation **[42].** A research at McGill University in the start of the 1990s examined the function of cavitation and gas nucleation in flotation **[43].** Later in 2000, Lou et al. **[43]**, who acquired the very first image of NBs on the hydrophobic solid surface, revealed the morphology of NBs using atomic force microscopy (AFM). Liquids containing NBs have gained interest in recent years in industry and academics due to their unique characteristics as well as several uses, namely nanoscopic cleaning **[44]**, slip control in microfluidics **[45],** mineral flotation **[46-49]**, chemical industries **[50]**, and the treatment of wastewater **[51].** Additionally, several investigations on the NB's dimensions, shape, surface charge properties, stability, and dynamics have been conducted **[52-54].** The extraordinary stability of NBs, which can continue for many weeks or even months, has recently come to the attention of the scientific community **[55].**

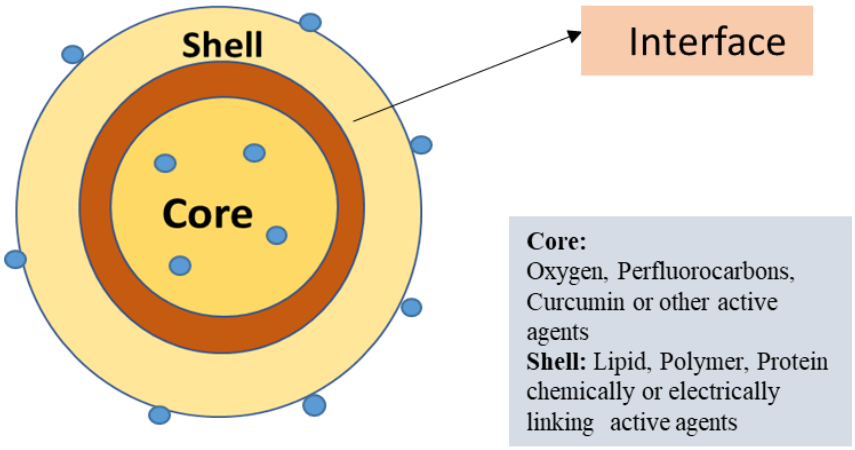


Figure 1.5: Nanobubbles structure

Figure 5 depicts the three components that make up an NB: the core, shell, and interface (surfactant layer). For the nanobubble's formulation, it is best to choose components that are safe, biocompatible, biodegradable, and regulatory agencies.

1.4 NBs Biodegradable Shell Components

Shell composition is a crucial aspect to take into account while formulating bubbles. Lipids, or polymers are frequently used to create the exterior (shell) of nanobubbles **[56].** Shell composition impacts on how gases are exchanged from the core to the exterior medium **[56].** Shell composition can significantly affect bubble half-life, in fact, nanobubble stability is influenced by shell thickness and elasticity **[57].** For example, extremely delicate shells can break with relatively small pressure changes, whereas highly stiff shells cannot oscillate (as often desired when used as contrast agents for sonography). Phospholipids are the building blocks of the most elastic shells; and the hydrocarbon tails of these lipids have a considerable effect on the properties of the shell. Increasing the amount of carbon atoms lowers surface tension and improves gas permeation resistance; however, prolonging the carbon chain's extension increases the viscosity of the shell that results in a stronger, echo-resistant bubble. Polymers are often used as alternative. The shell of polymer-based NBs is thicker (150-200 nm), has better acoustic characteristics, is more biocompatible, and has a longer half-life **[58].** Moreover, the polymeric shell may enable the incorporation of a greater quantity of drug **[59].**

For this reasons we selected polysaccharides as shell component. Polysaccharides are long, linear or branching chains of sugar units that are bonded together by glycosidic linkages. They are naturally quite abundant, are often relatively cheap to process **[60].** They can be of algal, plant, microbial or animal origin. Polysaccharides are thought to be promising biopolymers for achieving desired

increased functionality since they have several reactive groups, a wide range of molecular weight, and diverse chemical compositions.

The primary structural components of plants and animals' exoskeletons are naturally occurring polysaccharides for example carrageenan, chitosan, cellulose, chitin, etc. Natural polysaccharides including chitosan, starch, cellulose, cyclodextrin, and dextran have all been thoroughly investigated and altered with a range of reactants. Chitin and chitosan are increasingly getting more attention because of their fundamental physicochemical and biological characteristics. Chitosan and chitin have a number of noteworthy properties, including non-toxicity, ability to form films and fibers, the capacity to bind metal ions, the ability to coagulate solutes or suspensions, and a variety of bioactivities. As a result, chitosan and chitin are recognized as bio-functional polymers with increased prospective in a variety of disciplines. They are noteworthy not only as abundant resources but also as an unique type of polymer **[61].**

A polysaccharide known as chitosan is formed when the chitin in crab shells is deacetylated in an alkaline environment. Its deacetylation levels range from 40 to 98 %. Due to its ability to bind to lipids, it finds extensive usage in the pharmaceutical industry, biological applications, and as a diet adjuvant **[62-64]**. In 1859, Rouget figured out the process by which chitin partial N-deacetylation produces the chemical chitosan (**Figure 1.6**) **[65].** It has a helix-like structure comprised of hydrogen bonds that join D-glucosamine with N-acetyl-D-glucosamine.

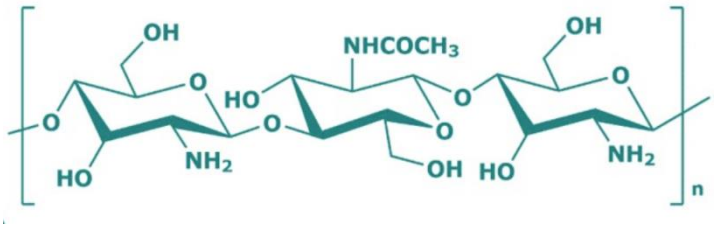


Figure 1.6: Chitosan chemical structure **[65].**

NBs Interface

The phase between the shell and the core is known as the interface. In this area, amphiphilic surfactants and co-surfactants form an interfacial monolayer that lowers interfacial tension, controls the size of nanobubbles, and enhances system stability. Stability rises as nanobubble diameter

decreases. NBs are stable at ambient temperature, but when heated to physiological body temperatures, they may aggregate to form microbubbles.

For the formation of stable NBs, Laplace pressure is one of the parameters that must be considered (Equation i). Laplace pressure is the term used to describe the pressure difference between the interior and exterior of a bubble **[56],** where r is the bubble's diameter and θ is the interfacial tension. Inverse relationship exists between NB size and Laplace pressure.

$$\Delta P = P\ inside - P\ outside = 2\theta/r \quad \textbf{Equation (i)}$$

The interfacial tension is decreased by the addition of surfactants and co-surfactants. The inclusion of the polymer shell can prevent NB coalescence **[58]**.

NBs Core

A hydrophobic gas or vaporizable compounds, such as sulphur hexafluoride and perfluorocarbons (PFCs) , is used to create the core **[66].**

The stability of the NBs is influenced by the kind of PFCs in the core **[67].** PFCs have different transitional characteristic from the liquid to gas phase (many of them, as perfluoropropane or perfluorobutane are gaseous at ambient temperatures, while perfluropentane and decafluoropentane are liquids) and this term refers as acoustic droplet vaporization (ADV). The boiling temperatures of each PFC measure the amount of energy requirement for this phase-shift from a liquid to a gaseous form **[68]**. Notably, oxygen can be loaded in the core thanks to its huge solubility in PFC.

The NBs developed in this dissertation for the delivery of curcumin, being manufactured using decafluoropentane, which is liquid at room temperature, should be referred as nanodroplets (NDs), but we generalized the term NBs for sake of simplicity.

1.5 Applications of NBs Technology to Food

Nanotechnology has recently emerged as a crucial tool for many applications, including effective solutions for extending the shelf life of food. When nanoscale particles (100-500 nm) are utilized, materials acquire distinctive and enhanced properties, such as the ability of releasing their cargo in a controlled and prolonged way. This approach enables the use of hydrophilic and lipophilic chemicals with antibacterial and antioxidant characteristics in the food industry. The shelf-life of a variety of products, including fruits, vegetables, dairy products, and other food items, can be extended by

releasing these substances during storage times. Using edible coatings, a thin layer that is applied to the food's surface to prolong its shelf life, is the best technique to maintain a food's characteristics, functioning, and organoleptic features. These coatings can be applied quickly and easily using rubbing, dipping, or spraying methods. Additionally, they are made using natural polymers that are cheap, accessible, and free of toxins, such as essential oils and other natural extracts **[69].**

In the last few years, these applications have often been used as vectors to deliver natural substances with antimicrobial and antioxidant activity **[70],** including essential oils, vitamins, as well as other plant products including polyphenols **[69].** Polysaccharide or protein-based substances called "nanocomposites" contain nanosystems (such as nanoemulsions and polymeric nanoparticles). The production of nanocomposites has made it possible to deliver active ingredients through edible coverings. Additionally, biodegradable synthetic polymers and liquid or solid lipids can be used to create nanosheets at room temperature, which maintain the appearance of the nanosystem while extending the food's shelf life **[69].**

References

1. Yoon J.-H.Lee S.-Y.,"*Comparison of the effectiveness of decontaminating strategies for fresh fruits and vegetables and related limitations",*Critical reviews in food science and nutrition, 2018. **58**(18): 3189-3208.
2. Martinović T., Andjelković U., Gajdošik M.Š., Rešetar D.Josić D.,"*Foodborne pathogens and their toxins",*Journal of proteomics, 2016. **147**: 226-235.
3. Kadariya J., Smith T.C.Thapaliya D.,"*Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health",*BioMed research international, 2014. **2014**.
4. Argudín M.Á., Mendoza M.C.Rodicio M.R.,"*Food poisoning and Staphylococcus aureus enterotoxins",*Toxins, 2010. **2**(7): 1751-1773.
5. Lim J.-Y., Yoon J.-W.Hovde C.J.,"*A brief overview of Escherichia coli O157: H7 and its plasmid O157",*Journal of microbiology and biotechnology, 2010. **20**(1): 5-14.
6. Heuvelink A., Van Heerwaarden C., Zwartkruis-Nahuis J., Van Oosterom R., Edink K., Van Duynhoven Y.De Boer E.,"*Escherichia coli O157 infection associated with a petting zoo",*Epidemiology & Infection, 2002. **129**(2): 295-302.
7. Jiang X., Morgan J.Doyle M.P.,"*Fate of Escherichia coli O157: H7 in manure-amended soil",*Applied and environmental microbiology, 2002. **68**(5): 2605-2609.
8. Banatvala N., Griffin P.M., Greene K.D., Barrett T.J., Bibb W.F., Green J.H.Wells J.G.,"*The United States national prospective hemolytic uremic syndrome study: microbiologic, serologic, clinical, and epidemiologic findings",*The Journal of infectious diseases, 2001. **183**(7): 1063-1070.
9. Anderson A.C., Jonas D., Huber I., Karygianni L., Wölber J., Hellwig E., Arweiler N., Vach K., Wittmer A.Al-Ahmad A.,"*Enterococcus faecalis from food, clinical specimens, and oral sites: prevalence of virulence factors in association with biofilm formation",*Frontiers in microbiology, 2016. **6**: 1534.
10. Gelsomino R., Vancanneyt M., Cogan T., Condon S.Swings J.,"*Source of enterococci in a farmhouse raw-milk cheese",*Applied and environmental microbiology, 2002. **68**(7): 3560-3565.
11. Larsen J., Schønheyder H.C., Singh K.V., Lester C.H., Olsen S.S., Porsbo L.J., Garcia-Migura L., Jensen L.B., Bisgaard M.Murray B.E.,"*Porcine and human community reservoirs of Enterococcus faecalis, Denmark",*Emerging infectious diseases, 2011. **17**(12): 2395.
12. Tolker-Nielsen T.,"*Biofilm development",*Microbial Biofilms, 2015: 51-66.

13. Del Pozo J.L.,"*Biofilm-related disease"*,Expert review of anti-infective therapy, 2018. **16**(1): 51-65.
14. Raza T., Ullah S.R., Mehmood K.Andleeb S.,"*Vancomycin resistant Enterococci: A brief review"*,J Pak Med Assoc, 2018. **68**(5): 768-772.
15. Rajagopal M.Walker S.,"*Envelope structures of Gram-positive bacteria"*,Protein and sugar export and assembly in Gram-positive bacteria, 2017: 1-44.
16. Shlar I., Droby S., Choudhary R.Rodov V.,"*The mode of antimicrobial action of curcumin depends on the delivery system: Monolithic nanoparticles vs. supramolecular inclusion complex"*,RSC advances, 2017. **7**(67): 42559-42569.
17. Silhavy T.J., Kahne D.Walker S.,"*The bacterial cell envelope"*,Cold Spring Harbor perspectives in biology, 2010. **2**(5): a000414.
18. Fan L.Song J.,"*Microbial quality assessment methods for fresh-cut fruits and vegetables"*,Stewart Postharvest Rev, 2008. **4**(3): 1-9.
19. Phillips I., Acar J., Bergan T., Degener J., Baquero F., Forsgren A., Schito G.Wiedemann B.,"*Methods for the determination of susceptibility of bacteria to antimicrobial agents. Terminology, EUCAST Definitive Document"*,Clin. Microbiol. Infect, 1998. **4**: 291-296.
20. Wikler M.A.,"*Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard"*,Clsi (Nccls), 2006. **26**: M7-A7.
21. Andrews J.M.,"*Determination of minimum inhibitory concentrations"*,Journal of antimicrobial Chemotherapy, 2001. **48**(suppl_1): 5-16.
22. Hacek D.M., Dressel D.C.Peterson L.R.,"*Highly reproducible bactericidal activity test results by using a modified National Committee for Clinical Laboratory Standards broth macrodilution technique"*,Journal of Clinical Microbiology, 1999. **37**(6): 1881-1884.
23. Sridhar A., Ponnuchamy M., Kumar P.S.Kapoor A.,"*Food preservation techniques and nanotechnology for increased shelf life of fruits, vegetables, beverages and spices: a review"*,Environmental Chemistry Letters, 2021. **19**: 1715-1735.
24. Kübler A.C.,"*Photodynamic therapy"*,Medical Laser Application, 2005. **20**(1): 37-45.
25. Sun J., Kormakov S., Liu Y., Huang Y., Wu D.Yang Z.,"*Recent progress in metal-based nanoparticles mediated photodynamic therapy"*,Molecules, 2018. **23**(7): 1704.
26. dos Santos A.l.F., De Almeida D.R.Q., Terra L.F., Baptista M.c.S.Labriola L.,"*Photodynamic therapy in cancer treatment-an update review"*,Journal of cancer metastasis and treatment, 2019. **5**: 25.
27. Mroz P., Yaroslavsky A., Kharkwal G.B.Hamblin M.R.,"*Cell death pathways in photodynamic therapy of cancer"*,Cancers, 2011. **3**(2): 2516-2539.

28. Aurum F.S.Nguyen L.T.,"*Efficacy of photoactivated curcumin to decontaminate food surfaces under blue light emitting diode",*Journal of Food Process Engineering, 2019. **42**(3): e12988.
29. Gonzales J.C., Brancini G.T., Rodrigues G.B., Silva-Junior G.J., Bachmann L., Wainwright M.Braga G.U.,"*Photodynamic inactivation of conidia of the fungus Colletotrichum abscissum on Citrus sinensis plants with methylene blue under solar radiation",*Journal of Photochemistry and Photobiology B: Biology, 2017. **176**: 54-61.
30. Majiya H.Galstyan A.,"*Dye extract of calyces of Hibiscus sabdariffa has photodynamic antibacterial activity: A prospect for sunlight-driven fresh produce sanitation",*Food science & nutrition, 2020. **8**(7): 3200-3211.
31. Abrahamse H.Hamblin M.R.,"*New photosensitizers for photodynamic therapy",*Biochemical journal, 2016. **473**(4): 347-364.
32. Kim M.-J.Yuk H.-G.,"*Antibacterial mechanism of 405-nanometer light-emitting diode against Salmonella at refrigeration temperature",*Applied and environmental microbiology, 2017. **83**(5): e02582-02516.
33. Huang J., Chen B., Li H., Zeng Q.-H., Wang J.J., Liu H., Pan Y.Zhao Y.,"*Enhanced antibacterial and antibiofilm functions of the curcumin-mediated photodynamic inactivation against Listeria monocytogenes",*Food Control, 2020. **108**: 106886.
34. Montllor-Albalate C., Colin A.E., Chandrasekharan B., Bolaji N., Andersen J.L., Outten F.W.Reddi A.R.,"*Extra-mitochondrial Cu/Zn superoxide dismutase (Sod1) is dispensable for protection against oxidative stress but mediates peroxide signaling in Saccharomyces cerevisiae",*Redox biology, 2019. **21**: 101064.
35. Endarko E., Maclean M., Timoshkin I.V., MacGregor S.J.Anderson J.G.,"*High-intensity 405 nm light inactivation of Listeria monocytogenes",*Photochemistry and photobiology, 2012. **88**(5): 1280-1286.
36. El-Sayed I.H., Huang X.El-Sayed M.A.,"*Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer",*Nano letters, 2005. **5**(5): 829-834.
37. Thrall J.H.,"*Nanotechnology and medicine",*Radiology, 2004. **230**(2): 315-318.
38. Cornier J., Owen A., Kwade A.Van de Voorde M., *Pharmaceutical Nanotechnology, 2 Volumes: Innovation and Production*2017: John Wiley & Sons.
39. Nirmalkar N., Pacek A.Barigou M.,"*On the existence and stability of bulk nanobubbles",*Langmuir, 2018. **34**(37): 10964-10973.

40. Weijs J.H., Seddon J.R.Lohse D.,"*Diffusive shielding stabilizes bulk nanobubble clusters",*ChemPhysChem, 2012. **13**(8): 2197-2204.
41. Fox F.E.Herzfeld K.F.,"*Gas bubbles with organic skin as cavitation nuclei",*The journal of the Acoustical Society of America, 1954. **26**(6): 984-989.
42. Zhou Z., Xu Z.Finch J.,"*On the role of cavitation in particle collection during flotation-a critical review",*Minerals Engineering, 1994. **7**(9): 1073-1084.
43. Lou S.-T., Ouyang Z.-Q., Zhang Y., Li X.-J., Hu J., Li M.-Q.Yang F.-J.,"*Nanobubbles on solid surface imaged by atomic force microscopy",*Journal of Vacuum Science & Technology B: Microelectronics and Nanometer Structures Processing, Measurement, and Phenomena, 2000. **18**(5): 2573-2575.
44. Eklund F.Swenson J.,"*Stable air nanobubbles in water: the importance of organic contaminants",*Langmuir, 2018. **34**(37): 11003-11009.
45. Li D., Jing D., Pan Y., Bhushan B.Zhao X.,"*Study of the relationship between boundary slip and nanobubbles on a smooth hydrophobic surface",*Langmuir, 2016. **32**(43): 11287-11294.
46. Maoming F., Daniel T., Honaker R.Zhenfu L.,"*Nanobubble generation and its applications in froth flotation (part II): fundamental study and theoretical analysis",*Mining Science and Technology (China), 2010. **20**(2): 159-177.
47. Nazari S., Shafaei S., Shahbazi B.Chelgani S.C.,"*Study relationships between flotation variables and recovery of coarse particles in the absence and presence of nanobubble",*Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2018. **559**: 284-288.
48. Zhou W., Niu J., Xiao W.Ou L.,"*Adsorption of bulk nanobubbles on the chemically surface-modified muscovite minerals",*Ultrasonics Sonochemistry, 2019. **51**: 31-39.
49. Zhou W., Liu K., Wang L., Zhou B., Niu J.Ou L.,"*The role of bulk micro-nanobubbles in reagent desorption and potential implication in flotation separation of highly hydrophobized minerals",*Ultrasonics Sonochemistry, 2020. **64**: 104996.
50. Lu X.-m., Yuan B., Zhang X.-r., Yang K.Ma Y.-q.,"*Molecular modeling of transmembrane delivery of paclitaxel by shock waves with nanobubbles",*Applied Physics Letters, 2017. **110**(2): 023701.
51. Temesgen T., Bui T.T., Han M., Kim T.-i.Park H.,"*Micro and nanobubble technologies as a new horizon for water-treatment techniques: A review",*Advances in colloid and interface science, 2017. **246**: 40-51.

52. Cho S.-H., Kim J.-Y., Chun J.-H.Kim J.-D.,"*Ultrasonic formation of nanobubbles and their zeta-potentials in aqueous electrolyte and surfactant solutions",*Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2005. **269**(1-3): 28-34.

53. Najafi A.S., Drelich J., Yeung A., Xu Z.Masliyah J.,"*A novel method of measuring electrophoretic mobility of gas bubbles",*Journal of colloid and interface science, 2007. **308**(2): 344-350.

54. Bhondayi C.,"*Flotation froth phase bubble size measurement",*Mineral Processing and Extractive Metallurgy Review, 2022. **43**(2): 251-273.

55. Michailidi E.D., Bomis G., Varoutoglou A., Efthimiadou E.K., Mitropoulos A.C.Favvas E.P., *Fundamentals and applications of nanobubbles,* in *Interface science and technology*2019, Elsevier. p. 69-99.

56. Cavalli R., Soster M.Argenziano M.,"*Nanobubbles: a promising efficienft tool for therapeutic delivery",*Therapeutic delivery, 2016. **7**(2): 117-138.

57. Delalande A., Postema M., Mignet N., Midoux P.Pichon C.,"*Ultrasound and microbubble-assisted gene delivery: recent advances and ongoing challenges",*Therapeutic delivery, 2012. **3**(10): 1199-1215.

58. Khan M.S., Hwang J., Lee K., Choi Y., Kim K., Koo H.-J., Hong J.W.Choi J.,"*Oxygen-carrying micro/nanobubbles: Composition, synthesis techniques and potential prospects in photo-triggered theranostics",*Molecules, 2018. **23**(9): 2210.

59. Xiong X., Zhao F., Shi M., Yang H.Liu Y.,"*Polymeric microbubbles for ultrasonic molecular imaging and targeted therapeutics",*Journal of Biomaterials Science, Polymer Edition, 2011. **22**(4-6): 417-428.

60. Liu Z., Jiao Y., Wang Y., Zhou C.Zhang Z.,"*Polysaccharides-based nanoparticles as drug delivery systems",*Advanced drug delivery reviews, 2008. **60**(15): 1650-1662.

61. Kurita K.,"*Chitin and chitosan: functional biopolymers from marine crustaceans",*Marine biotechnology, 2006. **8**: 203-226.

62. Kumar P., Choonara Y.E., du Toit L.C., Modi G., Naidoo D.Pillay V.,"*Novel high-viscosity polyacrylamidated chitosan for neural tissue engineering: fabrication of anisotropic neurodurable scaffold via molecular disposition of persulfate-mediated polymer slicing and complexation",*International journal of molecular sciences, 2012. **13**(11): 13966-13984.

63. Dodane V.Vilivalam V.D.,"*Pharmaceutical applications of chitosan",*Pharmaceutical Science & Technology Today, 1998. **1**(6): 246-253.

64. Chandy T.Sharma C.P.,"*Chitosan-as a biomaterial",*Biomaterials, artificial cells and artificial organs, 1990. **18**(1): 1-24.

65. Rouget C.,"*Des substances amylacées dans les tissus des animaux, spécialement des Articulés (chitine)"*,Comp. Rend, 1859. **48**: 792-795.

66. Sayadi L.R., Banyard D.A., Ziegler M.E., Obagi Z., Prussak J., Klopfer M.J., Evans G.R.Widgerow A.D.,"*Topical oxygen therapy & micro/nanobubbles: a new modality for tissue oxygen delivery"*,International wound journal, 2018. **15**(3): 363-374.

67. Sheeran P.S.Dayton P.A.,"*Phase-change contrast agents for imaging and therapy"*,Current pharmaceutical design, 2012. **18**(15): 2152-2165.

68. Phillips L.C., Puett C., Sheeran P.S., Dayton P.A., Wilson Miller G.Matsunaga T.O.,"*Phase-shift perfluorocarbon agents enhance high intensity focused ultrasound thermal delivery with reduced near-field heating"*,The journal of the Acoustical Society of America, 2013. **134**(2): 1473-1482.

69. Zambrano-Zaragoza M.L., González-Reza R., Mendoza-Muñoz N., Miranda-Linares V., Bernal-Couoh T.F., Mendoza-Elvira S.Quintanar-Guerrero D.,"*Nanosystems in edible coatings: A novel strategy for food preservation"*,International journal of molecular sciences, 2018. **19**(3): 705.

70. Maizura M., Fazilah A., Norziah M.Karim A.,"*Antibacterial activity and mechanical properties of partially hydrolyzed sago starch–alginate edible film containing lemongrass oil"*,Journal of Food Science, 2007. **72**(6): C324-C330.

CHAPTER 2: CURCUMIN AS A 'GREEN' PHOTOSENSITIZER ANTIMICROBIAL COMPOUND

Extended report from:

2022 Exploitation of the Antibacterial Properties of Photoactivated Curcumin as 'Green' Tool for Food Preservation [*N. Mandras and E. Ficiarà are the corresponding authors]. DOI: 10.3390/ijms23052600. In INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES vol. 23 (5) *Munir Z.; Banche G.; Cavallo L.; Mandras N.; Roana J.; Pertusio R.; Ficiara E.; Cavalli R.; Guiot C.*

2.1 Role of Curcumin

Turmeric/curcumin has been utilized as spice for centuries, especially in India and other Asian nations. Additionally, it is widely used to treat pathological conditions, particularly inflammatory ones **[1].** The rhizomes of the plant *Curcuma longa* L. are the source of a polyphenolic compound known as curcumin (C21H20O6; 1, 7-bis-4-hydroxy-3-methoxyphenyl-1, 6-heptadiene-3, 5-dione, molecular weight = 368, 38 g/M) (**Figure 2.1**).

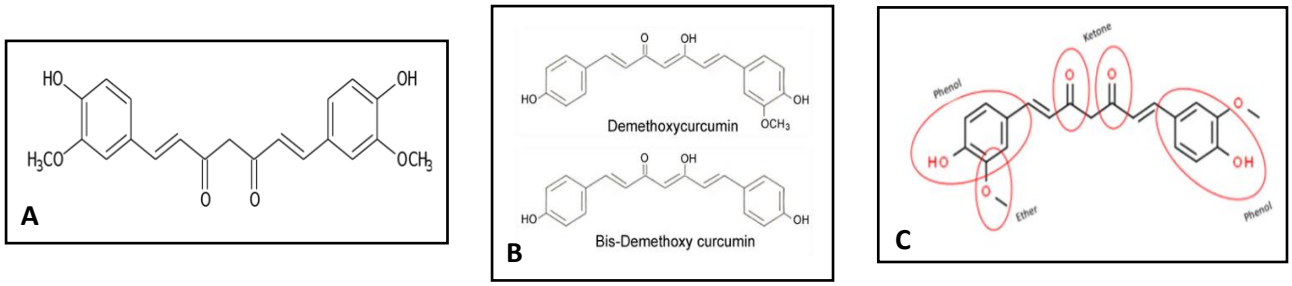


Figure 2.1: Curcumin's structural composition (**A**); demethoxycurcumin & bis-demethoxycurcumin (**B**); functional groups of the curcumin (**C**) **[2].**

Curcumin has a seven-carbon aliphatic chain connecting two highly polar aromatic rings, as well as two, β-unsaturated carbonyl groups (β-diketone) in its chemical structure. Curcumin acquires its hydrophobic characteristics from this chain, which also has the tautomeric conformations ketone and enol. These two forms are present because the β-diketone molecule facilitates the intramolecular transfer of hydrogen atoms. Due to the strong intramolecular hydrogen bonding, this form is more stable than the ketone form both in solution and in the solid phase.

The tautomeric ketone form is more prevalent in cell membranes and acidic/neutral aqueous solutions, while the enolic form is more prevalent in alkaline conditions. Some of the factors that affect the proportional contribution of tautomeric forms in solution include temperature, aromatic ring substitution and solvent polarity **[3]**. However, certain cutting-edge nuclear magnetic resonance (NMR) experiments have revealed that the enolic form of curcumin predominates in the majority of organic solvents **[4]**. Furthermore, it is not completely ruled out that the two forms could re-equilibrate in certain circumstances (such as an acidic environment). In nonpolar, aprotic solvents, the transformation of curcumin to the enolic form is mediated via intramolecular hydrogen transfer (i.e. deuterated chloroform), while curcumin is converted from its enolic form to its ketonic form in protic solvents (such as methanol) via the breakage of an intramolecular hydrogen bond with an intermolecular hydrogen bond **[3].** This step is important because inter- and intra-hydrogen bonding

may be the reason of curcumin's poor solubility in aqueous solutions **[5].** Usually, the numerous biological functions of curcumin are determined by the presence of different functional groups in the curcumin molecule, such like carbon-carbon double bonds, β-dictyl groups and phenyl rings with different methoxyl and hydroxyl substituents. Curcumin, for example, has a strong antioxidant activity and has the ability to neutralize a wide range of reactive species, such as nitrogen dioxide radicals, superoxide anion and ROS **[6]**. By neutralizing these chemicals, biomacromolecule damage can be avoided. On the other hand, curcumin exhibits both anti- and pro-oxidant action; in fact, when exposed to light, it can generate cytotoxic ROS **[3]**. Curcumin's oxidation mechanism and antioxidant power are governed by the number and orientation of its hydroxyl groups inside the aromatic ring, which also makes the enolic form of the compound more oxidizable than the ketone form.

Curcumin has a number of biological properties, including the potential to heal wounds and exhibit antibacterial and antiproliferative properties against certain cancer cells **[7]**. It interacts with a variety of targets implicated in the inflammatory response, including interleukins and tumor necrosis factor alpha **[1]**. For example, in the case of *Helicobacter pylori* infection, curcumin inhibits NF-κB activation, IL-8 release, and cellular shedding, leading to a reduction in gastric tissue inflammation **[8].** It also has *in vitro* antibacterial properties against a variety of pathogens **[1].**

2.2 Antibacterial Properties: Mechanisms of Action

The curcumin antibacterial properties are especially effective against pathogenic microorganisms. In order to prove its antibacterial action, several investigations have been carried out; for instance, in one study, turmeric oil was examined, and its antibacterial activity against *Bacillus subtilis*, *B. coagulans*, *B. cereus*, *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa* was confirmed **[9].** Additionally, curcumin has been proven to suppress methicillin-resistant *S. aureus* (MRSA) strains **[10]**. Curcumin's antibacterial properties originate from its capacity to prevent bacterial cell development **[7]** and by acting as an anti-biofilm. This was proven by a study performed on plants (*Arabidopsis thaliana)* and animal models (*Caenorhabditis elegans*) contaminated with *Ps. aeruginosa*, where the capability of curcumin to suppress the expression of genes essential for the initial generation of biofilms was demonstrated **[11]**.

Curcumin's main mode of action is by interference with the functions of Filamenting temperature sensitive mutant Z (FtsZ), a protein essential for bacterial cell division of the eukaryotic tubulin-containing cytoskeletal protein. Curcumin actually has the ability to stop the assembly of FtsZ and the Z-loop, which are required for bacterial cytokinesis and as a result inhibits the bacterial growth **[7].**

Curcumin sensitivity to the bacterium depends on the structure of the bacterial cell membrane **(see figure 2 in Introduction)**: consequently, compared to Gram-negative bacteria, Gram-positive bacteria exhibit higher curcumin sensitivity **[12].**

Numerous investigations have been conducted to determine the antibacterial effectiveness of curcumin against various bacterial strains. For instance, researches on MRSA were performed to verify curcumin's antibacterial effectiveness against this Gram-positive bacterium showing that curcumin can adhere to the cell wall and challenge the integrity of the bacteria. Actually, it harms cell membranes; in fact, transmission electron microscopy (TEM) revealed that after 8 hours of curcumin treatment, the cytoplasmic membrane was destroyed, leading to cell lysis **[13].** Similar findings from another investigation on *B. subtilis* were published. Following curcumin administration, it was discovered that several proteins, mostly involved in bacterial cell division, cell wall production, fatty acid synthesis, and central metabolism were damaged. In fact, fluorescence microscopy revealed considerable changes in the morphology of bacterial cells **[14]**. In order to assess the antibacterial activity of curcumin on Gram-negative bacteria, a recent investigation was carried out on a strain of *E. coli*. An apoptotic-like baptismal response was observed at high curcumin doses. In fact, at these doses, curcumin really led to DNA fragmentation, membrane depolymerization, ROS accumulation, altered membrane potential, and cell death **[15].** In order to stop bacterial development, curcumin can either target the bacterial cell wall, membrane, protein, DNA, and other cellular components or it can prevent bacterial growth by disrupting the quorum sensing (QS) mechanism **(Figure 2.2)**.

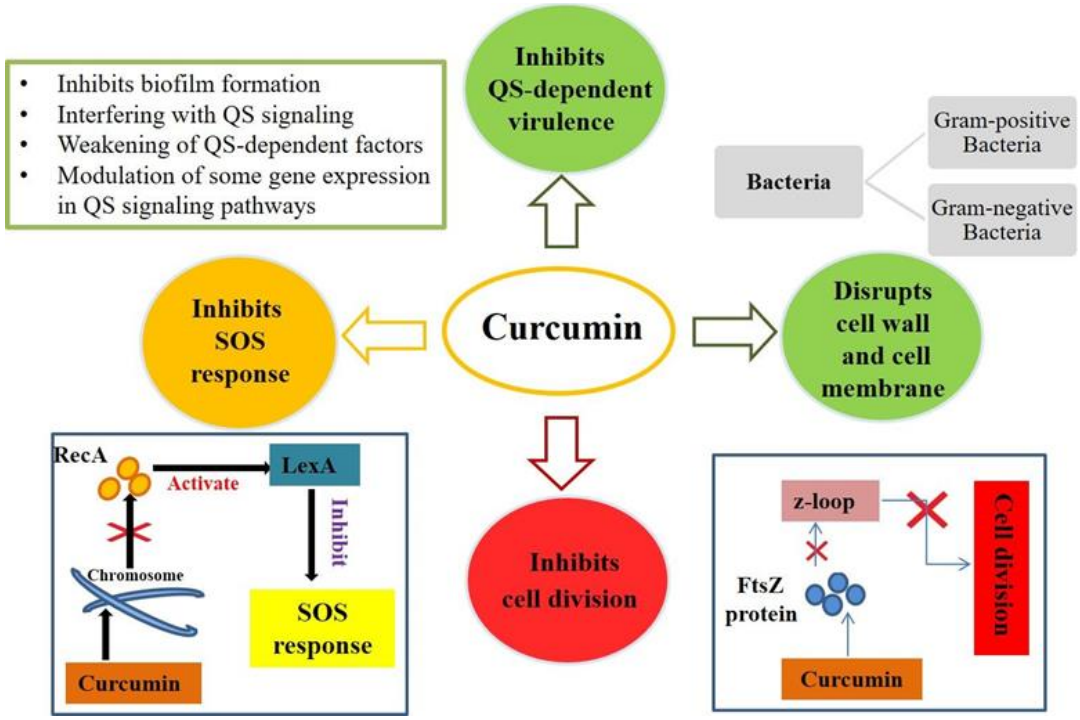


Figure 2.2: The various mechanisms used by curcumin to prevent bacterial growth: by specifically targeting the cell wall, protein, DNA, and other cellular elements. Additionally, it has the ability to disable the quorum sensing (QS) mechanism **[16].**

Quorum sensing (QS), a communication method among microbial cells, uses chemical signals (self-inducers) that are produced and perceived to assess cell density and species complexity in a population. QS enable bacteria to act as a cohesive community by regulating collective activity **[17].** Several ways exist for how curcumin suppresses QS activity (**Figure 2.3**). It can reduce QS-dependent components including exopolysaccharide production in bacteria like *E. coli*, *P. aeruginosa*, and *S. aureus*. Curcumin has also been demonstrated to reduce a number of phenotypes linked to QS suppression, such as motility and clustering, two of the most detrimental factors in the formation of biofilms in various bacterial strains. Through the QS system, curcumin can lessen the traits associated with virulence. The virulence factors can change in response to the signaling molecules of the QS system **[18]**. Additionally, curcumin prevents the formation of biofilms by interfering with the bacterial QS system. This is accomplished without harming the microorganisms or removing the developed biofilm. Clusters of microbial tissues that are covered in extracellular bacterial macromolecules are known as biofilms. The QS system regulates dynamic processes such as bacterial adhesion, biofilm maturation, and development in order to create biofilms **[19].**

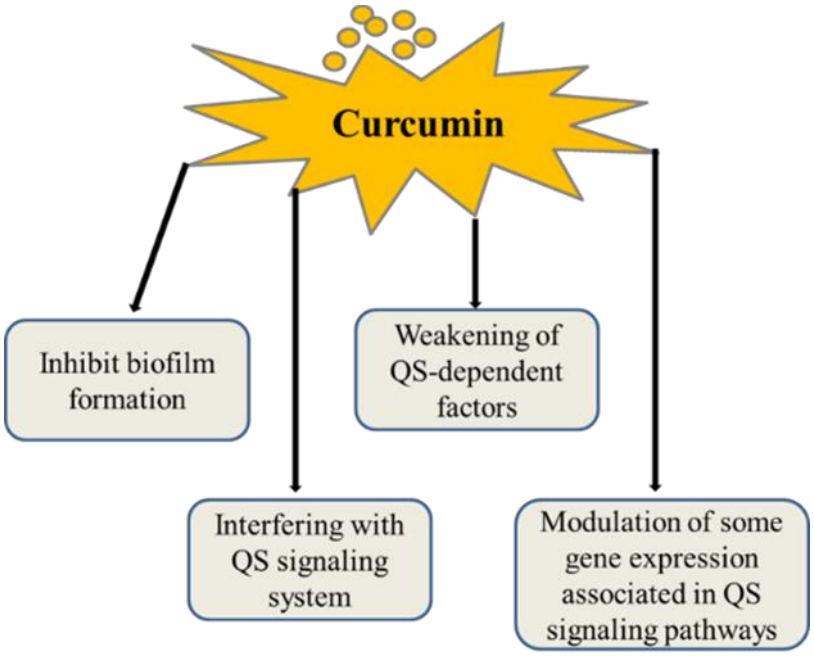


Figure 2.3: Curcumin reduces QS function in a number of different ways.

2.3 Curcumin as Natural Photosensitizer

Recently, research has focused on employing photoexcitation to boost curcumin's antibacterial activity **[3].** Curcumin is photodegradable (light-sensitive) in aqueous solution and self-degrades in dark **[20]**; however, via illumination (400-500 nm wavelength), natural photosensitizers, such as curcumin, produce ROS, and hydroxyl radicals, able to kill bacterial cells **[21]**.

The ability of curcumin to absorb light is partly a result of its chemical components and is mostly attributable to the carbon chain's alternating single and double bonds. Curcumin is capable of absorbing a wide range of light, including visible light and light with a wavelength of around 430 nm, i.e. in the range of blue light. However, when curcumin transforms into its ketone form, it absorbs the UV radiation (approximately 389 nm) **[3].**

Less is known about the light intensity and the time of irradiation required for effective antimicrobial photoinhibition.

The **Figure 2.4** shows as such light produced by LED (LES Flex Strips LEDYDEL IP64, Turin, Italy) with a wavelength between 465 and 470 nm arranged in order to obtain a uniform illumination of the targets.

Figure 2.4: Irradiation chambers using LED (LES Flex Strips LEDYDEL IP64, Turin, Italy).

In our irradiation chambers the light flow per unit surface of 700 ± 25 lx, measured with the luxmeter mod. LMT B500 s/n 0180233 (photocell mod. P30SC0, s/n 287032) produced by LMT Lichtmesstechnik GmbH.

Curcumin exhibits antioxidant properties. The most often used lipid peroxidation product for antioxidant assays to evaluate the antioxidant activity of substances in lipid peroxidation systems is the malondialdehyde (MDA) test. MDA is produced when polyunsaturated fatty acids undergo oxidative degradation in acidic environments. MDA then reacts with thiobarbituric acid (TBA) to form the MDA-TBA adduct **[22].** The MDA-TBA assay gained popularity in lipid peroxidation studies and is still frequently used to evaluate the antioxidant properties of various natural substances In the current study, photoactivation was also utilized to enhance the antibacterial activities of nanostructures containing curcumin and chitosan shells.

Since the antimicrobial activity elicited by photoexcitation is based on oxidation processes, some caution is required when the treatment targets fruits which are often preferred because of their natural antioxidant properties, like blueberries, raspberries and strawberries, etc.

In conclusion, curcumin now appears to be of tremendous interest to the food industry due to its accessibility, affordability, natural chlorination, antimicrobial, and flavorful characteristics. Additionally, due to its property of being a natural photo-sensitizer, it might be utilized to disinfect the surface of food while combating a variety of pathogens. Because of this, it is regarded as a secure and "green" alternative compared to typical antibacterial drugs manufactured by pharmaceutical companies **[3]**.

2.4 Low Curcumin Solubility in Water

Curcumin is largely employed in medicine due to its several potentials, which include anticancer, antioxidant and antibacterial activity but it also has substantial drawbacks, for instance, poor solubility in water (around 11 ng/mL) **[23, 24],** instability in solutions, limited bioavailability, poor absorption, and quick excretion from the body. This makes it challenging to apply curcumin in water-soluble systems.

Different approaches are possible:

2.5 Use of Organic Solvents

Since curcumin has poor water solubility, there is a need for an organic solvent for its dissociation. The polar aprotic solvent N-methyl-2-pyrrolidone (NMP), which has been demonstrated to increase the solubility and permeability of a variety of compounds, also reported as a co-solvent and a complexing agent in other applications **[25-28]** was selected. Additionally, the choosing of a solvent should also consider its biological characteristics. In the pharmaceutical industry, N-methyl-2-pyrrolidone is used in the formulation for drugs by both oral and transdermal delivery route **[29, 30]**. In rats, NMP is absorbed rapidly after inhalation, oral, and dermal administration, distributed throughout the organism, and eliminated mainly by hydroxylation to polar compounds, which are excreted via urine. About 80% of the administered dose is excreted as NMP and NMP metabolites within 24 hours (from Wikipedia). Additionally, the utilization of N-methyl-2-pyrrolidone as a solvent in the production of curcuminoids was already addressed by our research team **[31].**

This highly soluble curcumin in NMP solution was added to the *in-vitro* cultures in both the microbiological investigations on the effect of 'bulk' curcumin (explained in this chapter 2) and of the curcumin-loaded nanobubbles (described in chapter 4).

2.6 Encapsulation/ inclusion complexation

Since curcumin is easily degradable under light, heat and in the presence of oxygen **[32]** the choice of a mean able to increase its solubility which could at the same time overcome these issues, is encapsulation in derivatives of cyclodextrins (CD) i.e β cyclodextrin.

With the use of cyclodextrins, several drugs have been rendered more soluble **[33]** and more than 30 pharmaceutical preparations based on cyclodextrin are available on the market **[34].** Cyclodextrins are therefore intriguing options for enhancing curcumin solubility.

The cyclic oligosaccharides known as cyclodextrins are made up of (α-1,4)-linked -D-glucopyranose units. They comprise a hydrophilic external surface and a lipophilic hollow in the center **(Figure 2.5).** The naturally occurring α-, β- and γ-cyclodextrin are composed of glucopyranose units, that are divided into six, seven, and eight glucopyranose units, respectively **[35].**

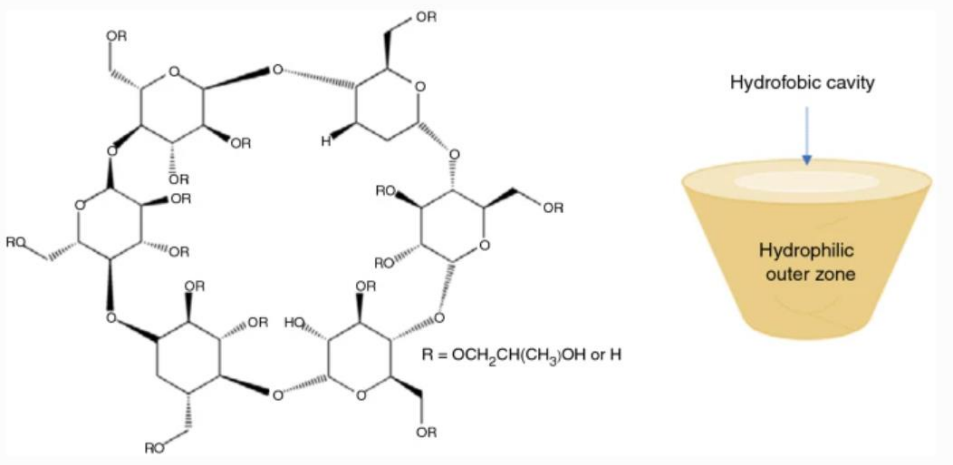


Figure 2.5: The general composition of cyclodextrin **[36].**

An inclusion complex is a type of supramolecular system that is created when one component (the host molecule) effectively encapsulates another. In the host's cavity, the enclosed substance (guest molecule) is present without materially altering the host's framework structure **(Figure 2.6)**.

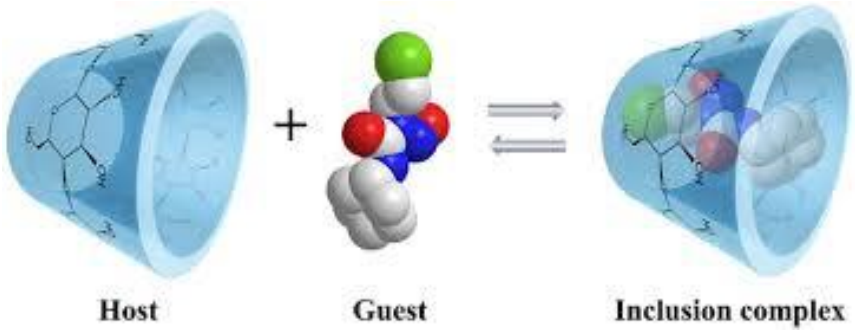


Figure 2.6: The concept of inclusion Complex [37].

Despite being completely soluble in water, the CD molecule's interior is generally non-polar and produces a hydrophobic microenvironment. CDs are therefore hollow, cup-shaped structures having an interior hydrophobic chamber and an exterior hydrophilic surface **(Figure 2.5) [36]**. They can

bind insoluble substances inside of their hydrophobic core, enhancing solubility and, as a result, improving chemical and enzymatic stability **[38].** The cavity size enables β-CD to create the proper inclusion complexes with molecules that include aromatic rings **[39]**, like curcumin **[40] (Figure 2.7).** Cyclodextrins enhance aqueous solubility of compounds i.e it shows about 100-fold increase in the solubility of curcumin.

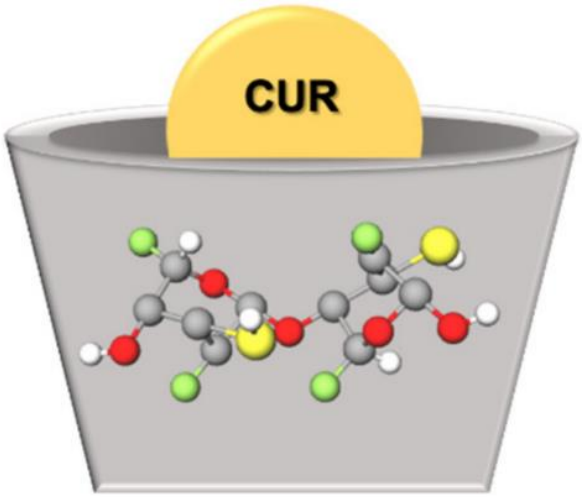


Figure 2.7: Schematic representation of curcumin in CD **[40].**

Phase solubility studies are widely used to determine the possibility for complexation to enhance substrate aqueous solubility. It provides a clear picture of the formed complex by the calculation of a number of essential features, such as the stability constant (k) and the complexation efficiency (CE) as well as the complexation ratio. Additionally, it enables quick determination of the solubilizing effectiveness of the cyclodextrin and the optimal molar ratio between the cyclodextrin polymer and the substrate that under investigation. As a result, it is crucial in assessing the use of cyclodextrin in pharmaceutical formulations **[41].**

The Higuchi and Connors method can be used to conduct phase solubility investigations **[42].** Curcumin was added in excess to a number of aqueous solutions that contained an increasing concentration of β -cyclodextrin (0 to 70 mM). Mixtures were constantly shaken at 500 rpm in the dark at room temperature for 72 hours. The resultant suspensions were then centrifuged for 25 minutes at 6000 rpm to separate the supernatant, which contained the curcumin. The supernatant's curcumin concentration was determined using a spectrophotometer at wavelength of 425 nm, referring to a previously obtained calibration plot. The apparent stability constants (Ks) of curcumin-βCD complexes were determined by the phase-solubility diagrams (where the acqueos β –

cyclodextrin concentration is reported in the abscissa and the curcumin solubility in the ordinate) using the equation:

$$Ks = \frac{slope}{S°\,(1 - slope)}$$

Where the slope of the phase solubility diagram's linear plot, S°, represents the intrinsic solubility of curcumin in the absence of cyclodextrin polymer. The following equation was used to compute the complexation efficiency, which is equivalent to the complex that was formed:

$$CE = \frac{slope}{(1 - slope)}$$

And finally the following equation **[43]** was used to compute the molar ratio of curcumin to CD:

$$\text{Curcumin: } \beta\text{CD} = 1 + \frac{\text{slope}}{(1 - \text{slope})}$$

2.7 Possibility of Curcumin Nanoformulations

Oxygen-loaded Nanocarriers/Nanodroplets manufactured with natural polymers i.e chitosan have great impact on the enhancement of drug bioavailability and exhibit robust antimicrobial effects. Based on these promising results we purposed this study to produce and characterize polymeric nanobubbles effectively loaded with curcumin and verifying their antimicrobial effect against bacterial strains. Nanobubbles with a perfluoropentane core and a biopolymer shell have already been described in the literature to release curcuminoids (Curc) and encapsulate curcumin in various quantities depending on the dose required **[31].**

These NBs are created by covering nanobubble core with a polysaccharide i.e chitosan whose chemical modifications is facilitated by the presence of reactive amino groups and hydroxyl groups. Therefore, a lot of work has been done recently to functionalize chitosan to increase both its solubility and effectiveness **[44-47]**. Numerous studies have demonstrated the feasibility of structural modification techniques for producing more desired chitosan derivatives, including carboxylation **[48]**, alkylation **[49, 50]** and physiologically active molecule conjugation **[51, 52].** The majority of the fundamental components of chitosan conjugates include chitosan transporters, conjugated bonds, and active molecules (drugs, small molecule, natural substances, proteins, nucleic acids, etc.). Depending on this conjugation approach, it was discovered that active substances such as ferulic acid **[53]**, tannic acid **[54]**, catechin **[55]**, curcumin **[56]**, eurycomanone **[57],** and streptomycin **[58]** successfully link with chitosan.

There have been several research papers on polysaccharide conjugates in the last 10 years, as well as some interesting conclusive publications **[59, 60],** but with regard to antibacterial chitosan conjugates. Chitosan conjugates, which are employed in antimicrobials, did not receive significant attention until 2010. Furthermore, the coupling of natural compounds like polyphenols such as curcumin **[61]**, organic acids **[62],** lysozyme **[63],** or commercial antimicrobials products like sulfadiazine and gibberellin **[64]** was shown to be a common coupling technique for chitosan conjugates. Due to the variety of coupled functional molecules, wide ranges of coupling techniques have been employed. The Schiff Base coupling was used as coupling method in this study, although these covalent techniques for chitosan conjugates and their potential uses in antibacterial characteristics have not been thoroughly evaluated and reviewed yet. As a result, our effort will concentrate a lot on the conjugation method of chitosan conjugates with curcumin, which are then used as a shell component in nanobubbles for antimicrobial applications.

Among the many possible chitosan conjugates, there has recently been a lot of interest in imine-linked chitosan conjugates. It is simple for the main amino group in the chitosan skeleton to condense with an acyl molecule (aldehyde, ketone), forming a Schiff base (**Figure 2.8**) **[65-70]**. During the reaction, no harmful chemicals are added. Due to its high selectivity and environmental friendliness, the reaction has several benefits. The successful coupling of curcumin **[56],** inulin **[71],** and caffeic acid [72] with chitosan to produce the appropriate conjugates has already been demonstrated in literature.

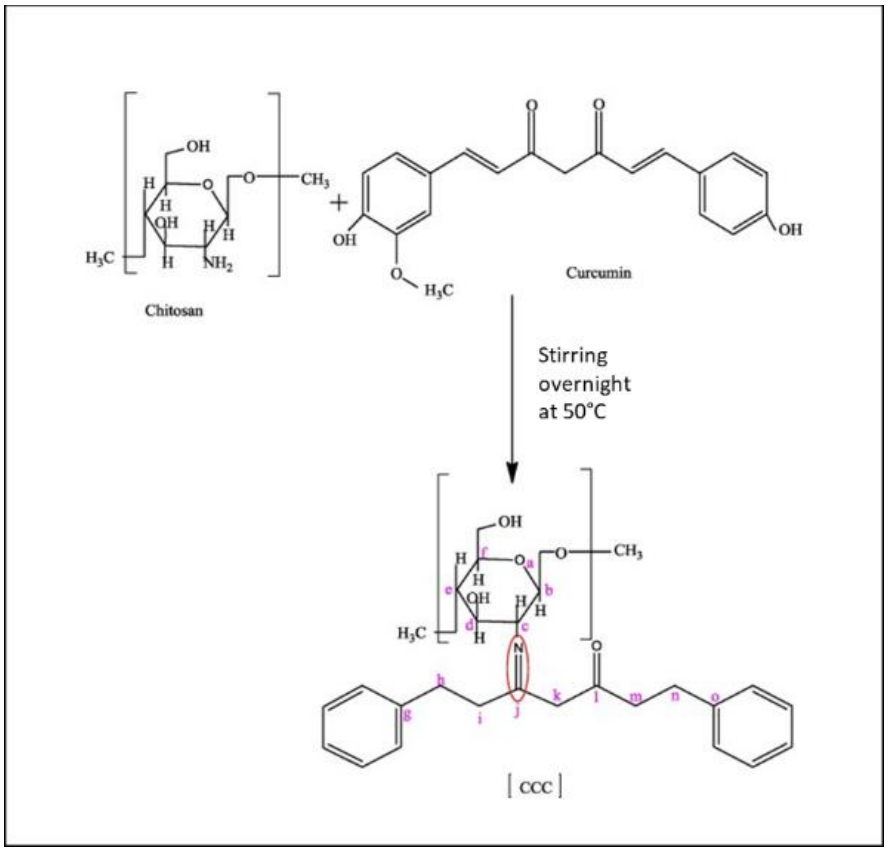


Figure 2.8: Curcumin-Chitosan Conjugation **[65].**

2.8 Possible Adverse Curcumin Organoleptic Properties

Organoleptic analysis can be performed either based on qualitative and quantitative basis.

1) Qualitative: This analysis is based on the judgement of trained or untrained people. Many characteristics may be evaluated such as: O: Olfaction, V: Sight, G: Taste, T: Touch

2) Quantitative: This analysis is based on the **electronic nose technique** that is described below. The management of aromatic properties in food production is crucial for determining the product's suitability. Furthermore, before a product is ready onto the market, it can also be part of the product design to meet customer satisfaction. Food's aromatic attributes are influenced by a variety of chemicals that also give it flavor and distinguishing characteristics. A crucial continuing effort in the creation of new products is accurately defining and quantifying optimal flavor and its attributes. This difficult task has generally been handled by so-called odor experts. However, the distinctive assessments of these specialists always include subjective aspects of personal opinions. The electric-nose can be used instead to avoid this subjectivity. The idea of an electronic nose (e-nose) ingerating chemical sensors and pattern recognition was initially described by Persaud and Dodd in 1982 **[73].**

An electronic nose is a machine that utilizes a sensor array composed of typically generic (non-specific) sensors that have been treated with various odor-sensitive chemical or biological substance to identify and differentiate between complex odors **[74]**. So, the e-nose system consists of non-selective sensors which interact with volatile molecules (gases or liquids), generating a physical or chemical alteration that transmits a signal to a computer, that subsequently categorizes the material based on a calibration and mentoring process that results in pattern recognition **(Figure 2.9).** The e-nose allows for measurements that are objective, repeatable, dependable, and economical. E-nose analysis is quick, easy, and possible in real time. It is intended to test, identify, and specify volatile compounds at extremely low concentrations (parts per billion).

The possibility of e-nose development for new product creation in the food sector was validated by recent research. For instance, Ortega et al. carried out an e-nose investigation to comprehend the crucial elements for Internet connectivity operations in the olive oil industry **[75]**. Other methods have emphasized on examining the fundamental ingredient, or water, that was utilized to make the supplied food. As a result, it has been feasible to roughly categorize the quality of this key raw resource **[76, 77].** Other research have primarily focused on categorization methods to identify if the food is fresh or contaminated, such as chicken or fish **[78-81].** Some other studies have attempted to categorize various liquids, like alcohol, and sesame oil, with positive outcomes **[82-84]**. Additionally, the researchers of **[85-88]** have categorized additional goods as black tea, green coffee, and several kinds of rice.

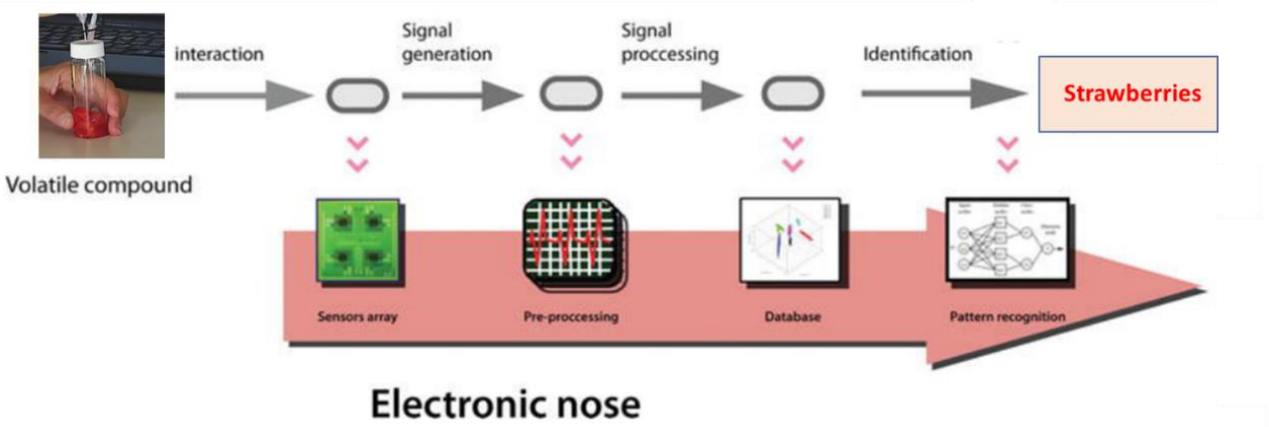


Figure 2.9: Electronic nose system **[89].**

There are two common methods used to analyze volatile ingredients in food: quality sensory panel assessment and conventional gas chromatography with mass spectrometry. These two methods, however, require a lot of time, effort, and money. They also have a minimal ability to create new products. On the contrary, the e-nose could be a good replacement for existing methods.

The current work utilized the Portable Electronic Nose (PEN3) System, which is based on a metal-oxide gas sensor array, to assess volatile substances. Ten distinct metal oxide single thick film sensors were employed for this study. The metal-oxide sensor's conductance changes as a result of gas molecule adsorption. This alteration in conductivity can be used to determine the quantity of volatile organic compounds adsorbed.

References

1. Tyagi P., Singh M., Kumari H., Kumari A.Mukhopadhyay K.,"*Bactericidal activity of curcumin I is associated with damaging of bacterial membrane",*PLoS One, 2015. **10**(3): e0121313.
2. Wolosewicz K., Podgorska K., Rutkowska E.Lazny R.,"*Synthesis of dicarbonyl curcumin analogues containing the tropane scaffold",*European Journal of Organic Chemistry, 2019. **2019**(29): 4662-4674.
3. Seidi Damyeh M., Mereddy R., Netzel M.E.Sultanbawa Y.,"*An insight into curcumin-based photosensitization as a promising and green food preservation technology",*Comprehensive Reviews in Food Science and Food Safety, 2020. **19**(4): 1727-1759.
4. Payton F., Sandusky P.Alworth W.L.,"*NMR study of the solution structure of curcumin",*Journal of natural products, 2007. **70**(2): 143-146.
5. Heger M., van Golen R.F., Broekgaarden M.Michel M.C.,"*The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer",*Pharmacological reviews, 2014. **66**(1): 222-307.
6. Menon V.P.Sudheer A.R.,"*Antioxidant and anti-inflammatory properties of curcumin",*The molecular targets and therapeutic uses of curcumin in health and disease, 2007: 105-125.
7. Rai D., Singh J.K., Roy N.Panda D.,"*Curcumin inhibits FtsZ assembly: an attractive mechanism for its antibacterial activity",*Biochemical journal, 2008. **410**(1): 147-155.
8. Zorofchian Moghadamtousi S., Abdul Kadir H., Hassandarvish P., Tajik H., Abubakar S.Zandi K.,"*A review on antibacterial, antiviral, and antifungal activity of curcumin",*BioMed research international, 2014. **2014**.
9. Negi P., Jayaprakasha G., Jagan Mohan Rao L.Sakariah K.,"*Antibacterial activity of turmeric oil: a byproduct from curcumin manufacture",*Journal of agricultural and food chemistry, 1999. **47**(10): 4297-4300.
10. Mun S.-H., Joung D.-K., Kim Y.-S., Kang O.-H., Kim S.-B., Seo Y.-S., Kim Y.-C., Lee D.-S., Shin D.-W.Kweon K.-T.,"*Synergistic antibacterial effect of curcumin against methicillin-resistant Staphylococcus aureus",*Phytomedicine, 2013. **20**(8-9): 714-718.
11. Rudrappa T.Bais H.P.,"*Curcumin, a known phenolic from Curcuma longa, attenuates the virulence of Pseudomonas aeruginosa PAO1 in whole plant and animal pathogenicity models",*Journal of agricultural and food chemistry, 2008. **56**(6): 1955-1962.

12. Shlar I., Droby S., Choudhary R.Rodov V.,"*The mode of antimicrobial action of curcumin depends on the delivery system: Monolithic nanoparticles vs. supramolecular inclusion complex",*RSC advances, 2017. **7**(67): 42559-42569.
13. Mun S.-H., Kim S.-B., Kong R., Choi J.-G., Kim Y.-C., Shin D.-W., Kang O.-H.Kwon D.-Y.,"*Curcumin reverse methicillin resistance in Staphylococcus aureus",*Molecules, 2014. **19**(11): 18283-18295.
14. Reddy P.J., Sinha S., Ray S., Sathe G.J., Chatterjee A., Prasad T.K., Dhali S., Srikanth R., Panda D.Srivastava S.,"*Comprehensive analysis of temporal alterations in cellular proteome of Bacillus subtilis under curcumin treatment",*PLoS One, 2015. **10**(4): e0120620.
15. Yun D.G.Lee D.G.,"*Antibacterial activity of curcumin via apoptosis-like response in Escherichia coli",*Applied microbiology and biotechnology, 2016. **100**: 5505-5514.
16. Munir Z., Banche G., Cavallo L., Mandras N., Roana J., Pertusio R., Ficiarà E., Cavalli R.Guiot C.,"*Exploitation of the antibacterial properties of photoactivated curcumin as 'green' tool for food preservation",*International journal of molecular sciences, 2022. **23**(5): 2600.
17. Hawver L.A., Jung S.A.Ng W.-L.,"*Specificity and complexity in bacterial quorum-sensing systems",*FEMS microbiology reviews, 2016. **40**(5): 738-752.
18. Zheng D., Huang C., Huang H., Zhao Y., Khan M.R.U., Zhao H.Huang L.,"*Antibacterial mechanism of curcumin: A review",*Chemistry & Biodiversity, 2020. **17**(8): e2000171.
19. De Kievit T.R., Gillis R., Marx S., Brown C.Iglewski B.H.,"*Quorum-sensing genes in Pseudomonas aeruginosa biofilms: their role and expression patterns",*Applied and environmental microbiology, 2001. **67**(4): 1865-1873.
20. Mondal S., Ghosh S.Moulik S.P.,"*Stability of curcumin in different solvent and solution media: UV–visible and steady-state fluorescence spectral study",*Journal of Photochemistry and Photobiology B: Biology, 2016. **158**: 212-218.
21. Aurum F.S.Nguyen L.T.,"*Efficacy of photoactivated curcumin to decontaminate food surfaces under blue light emitting diode",*Journal of Food Process Engineering, 2019. **42**(3): e12988.
22. Yen G.-C., Chang Y.-C.Su S.-W.,"*Antioxidant activity and active compounds of rice koji fermented with Aspergillus candidus",*Food chemistry, 2003. **83**(1): 49-54.
23. Hewlings S.J.Kalman D.S.,"*Curcumin: A review of its effects on human health",*Foods, 2017. **6**(10): 92.
24. Yu H., Shi K., Liu D.Huang Q.,"*Development of a food-grade organogel with high bioaccessibility and loading of curcuminoids",*Food chemistry, 2012. **131**(1): 48-54.

25. Benhabbour S.R., Kovarova M., Jones C., Copeland D.J., Shrivastava R., Swanson M.D., Sykes C., Ho P.T., Cottrell M.L.Sridharan A.,"*Ultra-long-acting tunable biodegradable and removable controlled release implants for drug delivery",*Nature communications, 2019. **10**(1): 4324.
26. Kohn-Polster C., Bhatnagar D., Woloszyn D.J., Richtmyer M., Starke A., Springwald A.H., Franz S., Schulz-Siegmund M., Kaplan H.M.Kohn J.,"*Dual-component gelatinous peptide/reactive oligomer formulations as conduit material and luminal filler for peripheral nerve regeneration",*International journal of molecular sciences, 2017. **18**(5): 1104.
27. Lee P.J., Langer R.Shastri V.P.,"*Role of n-methyl pyrrolidone in the enhancement of aqueous phase transdermal transport",*Journal of pharmaceutical sciences, 2005. **94**(4): 912-917.
28. Uch A.S., Hesse U.Dressman J.B.,"*Use of 1-methyl-pyrrolidone as a solubilizing agent for determining the uptake of poorly soluble drugs",*Pharmaceutical research, 1999. **16**(6): 968.
29. Jouyban A., Fakhree M.A.A.Shayanfar A.,"*Review of pharmaceutical applications of N-methyl-2-pyrrolidone",*Journal of Pharmacy & Pharmaceutical Sciences, 2010. **13**(4): 524-535.
30. Strickley R.G.,"*Solubilizing excipients in oral and injectable formulations",*Pharmaceutical research, 2004. **21**: 201-230.
31. Bessone F., Argenziano M., Grillo G., Ferrara B., Pizzimenti S., Barrera G., Cravotto G., Guiot C., Stura I.Cavalli R.,"*Low-dose curcuminoid-loaded in dextran nanobubbles can prevent metastatic spreading in prostate cancer cells",*Nanotechnology, 2019. **30**(21): 214004.
32. Dei Cas M.Ghidoni R.,"*Dietary curcumin: correlation between bioavailability and health potential",*Nutrients, 2019. **11**(9): 2147.
33. Loftsson T.Brewster M.E.,"*Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization",*Journal of pharmaceutical sciences, 1996. **85**(10): 1017-1025.
34. Tiwari G., Tiwari R.Rai A.K.,"*Cyclodextrins in delivery systems: Applications",*Journal of Pharmacy and Bioallied Sciences, 2010. **2**(2): 72-79.
35. Del Valle E.M.,"*Cyclodextrins and their uses: a review",*Process biochemistry, 2004. **39**(9): 1033-1046.
36. D'Aria F., Pagano B.Giancola C.,"*Thermodynamic properties of hydroxypropyl-β-cyclodextrin/guest interaction: a survey of recent studies",*Journal of Thermal Analysis and Calorimetry, 2022. **147**(8): 4889-4897.

37. Tian B., Xiao D., Hei T., Ping R., Hua S.Liu J.,"*The application and prospects of cyclodextrin inclusion complexes and polymers in the food industry: A review",*Polymer International, 2020. **69**(7): 597-603.

38. Kurita T.Makino Y.,"*Novel curcumin oral delivery systems",*Anticancer research, 2013. **33**(7): 2807-2821.

39. Davis M.E.Brewster M.E.,"*Cyclodextrin-based pharmaceutics: past, present and future",*Nature reviews Drug discovery, 2004. **3**(12): 1023-1035.

40. Smith A.J., Oertle J.Prato D.,"*Multiple actions of curcumin including anticancer, anti-inflammatory, antimicrobial and enhancement via cyclodextrin",*Journal of cancer therapy, 2015. **6**(03): 257.

41. Loftsson T., Hreinsdóttir D.Másson M.,"*The complexation efficiency",*Journal of Inclusion Phenomena and Macrocyclic Chemistry, 2007. **57**: 545-552.

42. Higuchi T.,"*Phase-solubility techniques",*Adv. Anal. Chem. Instr., 1965. **4**: 117-212.

43. Kfoury M., Landy D., Ruellan S., Auezova L., Greige-Gerges H.Fourmentin S.,"*Determination of formation constants and structural characterization of cyclodextrin inclusion complexes with two phenolic isomers: carvacrol and thymol",*Beilstein journal of organic chemistry, 2016. **12**(1): 29-42.

44. El Knidri H., Belaabed R., Addaou A., Laajeb A.Lahsini A.,"*Extraction, chemical modification and characterization of chitin and chitosan",*International journal of biological macromolecules, 2018. **120**: 1181-1189.

45. Butola B.,"*Recent advances in chitosan polysaccharide and its derivatives in antimicrobial modification of textile materials",*International journal of biological macromolecules, 2019. **121**: 905-912.

46. Sajid M.A., Shahzad S.A., Hussain F., Skene W., Khan Z.A.Yar M.,"*Synthetic modifications of chitin and chitosan as multipurpose biopolymers: A review",*Synthetic Communications, 2018. **48**(15): 1893-1908.

47. Mourya V.Inamdar N.N.,"*Chitosan-modifications and applications: Opportunities galore",*Reactive and Functional polymers, 2008. **68**(6): 1013-1051.

48. Rekha M.Sharma C.P.,"*Simultaneous effect of thiolation and carboxylation of chitosan particles towards mucoadhesive oral insulin delivery applications: an in vitro and in vivo evaluation",*Journal of biomedical nanotechnology, 2015. **11**(1): 165-176.

49. Popescu V., Muresan A., Popescu G., Balan M.Dobromir M.,"*Ethyl chitosan synthesis and quantification of the effects acquired after grafting it on a cotton fabric, using ANOVA statistical analysis",*Carbohydrate polymers, 2016. **138**: 94-105.

50. Mati-Baouche N., Delattre C., De Baynast H., Grédiac M., Mathias J.-D., Ursu A.V., Desbrières J.Michaud P.,"*Alkyl-chitosan-based adhesive: Water resistance improvement",*Molecules, 2019. **24**(10): 1987.

51. Mu Y., Wu G., Su C., Dong Y., Zhang K., Li J., Sun X., Li Y., Chen X.Feng C.,"*pH-sensitive amphiphilic chitosan-quercetin conjugate for intracellular delivery of doxorubicin enhancement",*Carbohydrate polymers, 2019. **223**: 115072.

52. Ding J., Xu Z., Qi B., Cui S., Wang T., Jiang L., Zhang Y.Sui X.,"*Fabrication and characterization of soybean oil bodies encapsulated in maltodextrin and chitosan-EGCG conjugates: An in vitro digestibility study",*Food Hydrocolloids, 2019. **94**: 519-527.

53. Wang D., Mao L., Dai L., Yuan F.Gao Y.,"*Characterization of chitosan-ferulic acid conjugates and their application in the design of β-carotene bilayer emulsions with propylene glycol alginate",*Food Hydrocolloids, 2018. **80**: 281-291.

54. Jing Y., Diao Y.Yu X.,"*Free radical-mediated conjugation of chitosan with tannic acid: Characterization and antioxidant capacity",*Reactive and Functional polymers, 2019. **135**: 16-22.

55. Cho Y.-S., Lee D.-S., Kim Y.-M., Ahn C.-B., Kim D.-H., Jung W.-K.Je J.-Y.,"*Protection of hepatic cell damage and antimicrobial evaluation of chitosan-catechin conjugate",*Journal of the Korean Society for Applied Biological Chemistry, 2013. **56**: 701-707.

56. Saranya T., Rajan V., Biswas R., Jayakumar R.Sathianarayanan S.,"*Synthesis, characterisation and biomedical applications of curcumin conjugated chitosan microspheres",*International journal of biological macromolecules, 2018. **110**: 227-233.

57. Bhat I.A., Nazir M.I., Ahmad I., Pathakota G.-B., Chanu T., Goswami M., Sundaray J.Sharma R.,"*Fabrication and characterization of chitosan conjugated eurycomanone nanoparticles: In vivo evaluation of the biodistribution and toxicity in fish",*International journal of biological macromolecules, 2018. **112**: 1093-1103.

58. Mu H., Liu Q., Niu H., Sun Y.Duan J.,"*Gold nanoparticles make chitosan–streptomycin conjugates effective towards Gram-negative bacterial biofilm",*RSC advances, 2016. **6**(11): 8714-8721.

59. Basu A., Kunduru K.R., Abtew E.Domb A.J.,"*Polysaccharide-based conjugates for biomedical applications",*Bioconjugate chemistry, 2015. **26**(8): 1396-1412.

60. Hussain M., Abbas K., Jantan I.Bukhari S.,"*Polysaccharide-based materials in macromolecular prodrug design and development",*International Materials Reviews, 2017. **62**(2): 78-98.

61. Woo J.Y.Je J.Y.,"*Antioxidant and tyrosinase inhibitory activities of a novel chitosan–phloroglucinol conjugate",*International journal of food science & technology, 2013. **48**(6): 1172-1178.

62. Cong Y., Geng J., Wang H., Su J., Arif M., Dong Q., Chi Z.Liu C.,"*Ureido-modified carboxymethyl chitosan-graft-stearic acid polymeric nano-micelles as a targeted delivering carrier of clarithromycin for Helicobacter pylori: Preparation and in vitro evaluation",*International journal of biological macromolecules, 2019. **129**: 686-692.

63. Kim S., Cui Z.-K., Koo B., Zheng J., Aghaloo T.Lee M.,"*Chitosan–lysozyme conjugates for enzyme-triggered hydrogel degradation in tissue engineering applications",*ACS applied materials & interfaces, 2018. **10**(48): 41138-41145.

64. Dumitriu R.P., Profire L., Nita L.E., Dragostin O.M., Ghetu N., Pieptu D.Vasile C.,"*Sulfadiazine—Chitosan Conjugates and Their Polyelectrolyte Complexes with Hyaluronate Destined to the Management of Burn Wounds",*Materials, 2015. **8**(1): 317-338.

65. Antony R., Arun T.Manickam S.T.D.,"*A review on applications of chitosan-based Schiff bases",*International journal of biological macromolecules, 2019. **129**: 615-633.

66. Yin X., Chen J., Yuan W., Lin Q., Ji L.Liu F.,"*Preparation and antibacterial activity of Schiff bases from O-carboxymethyl chitosan and para-substituted benzaldehydes",*Polymer bulletin, 2012. **68**: 1215-1226.

67. Tamer T.M., Hassan M.A., Omer A.M., Baset W.M., Hassan M.E., El-Shafeey M.E.Eldin M.S.M.,"*Synthesis, characterization and antimicrobial evaluation of two aromatic chitosan Schiff base derivatives",*Process biochemistry, 2016. **51**(10): 1721-1730.

68. Liu W., Qin Y., Liu S., Xing R., Yu H., Chen X., Li K.Li P.,"*C-coordinated O-carboxymethyl chitosan metal complexes: Synthesis, characterization and antifungal efficacy",*International journal of biological macromolecules, 2018. **106**: 68-77.

69. Liu W., Qin Y., Liu S., Xing R., Yu H., Chen X., Li K.Li P.,"*Synthesis, characterization and antifungal efficacy of C-coordinated O-carboxymethyl chitosan Cu (II) complexes",*Carbohydrate polymers, 2017. **160**: 97-105.

70. Wei L., Tan W., Wang G., Li Q., Dong F.Guo Z.,"*The antioxidant and antifungal activity of chitosan derivatives bearing Schiff bases and quaternary ammonium salts",*Carbohydrate polymers, 2019. **226**: 115256.

71. Zhang G., Liu J., Li R., Jiao S., Feng C., Wang Z.A.Du Y.,"*Conjugation of inulin improves anti-biofilm activity of chitosan",*Marine drugs, 2018. **16**(5): 151.

72. Yang C., Han B., Zheng Y., Liu L., Li C., Sheng S., Zhang J., Wang J.Wu F.,"*The quality changes of postharvest mulberry fruit treated by chitosan-g-caffeic acid during cold storage",*Journal of Food Science, 2016. **81**(4): C881-C888.

73. Persaud K.Dodd G.,"*Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose",*Nature, 1982. **299**(5881): 352-355.

74. Gardner J., Hines E.Pang C.,"*Detection of vapours and odours from a multisensor array using pattern recognition: self-organising adaptive resonance techniques",*Measurement and control, 1996. **29**(6): 172-178.

75. Ortega J.B., Garcia J.G.Ortega J.G. *Precision of volatile compound analysis in extra virgin olive oil: The influence of MOS electronic nose acquisition factors*. in *2015 IEEE International Conference on Industrial Technology (ICIT)*. 2015. IEEE.

76. Adnan K., Yusuf N., Maamor H., Rashid F., Ismail S., Thriumani R., Zakaria A., Kamarudin L., Shakaff A.Jaafar M. *Water quality classification and monitoring using e-nose and e-tongue in aquaculture farming*. in *2014 2nd International Conference on Electronic Design (ICED)*. 2014. IEEE.

77. Carmona E.N., Sberveglieri V.Pulvirenti A. *Detection of microorganisms in water and different food matrix by Electronic Nose*. in *2013 Seventh International Conference on Sensing Technology (ICST)*. 2013. IEEE.

78. Chongthanaphisut P., Seesaard T.Kerdcharoen T. *Monitoring of microbial canned food spoilage and contamination based on e-nose for smart home*. in *2015 12th International Conference on Electrical Engineering/Electronics, Computer, Telecommunications and Information Technology (ECTI-CON)*. 2015. IEEE.

79. Sberveglieri G., Zambotti G., Falasconi M., Gobbi E.Sberveglieri V. *MOX-NW electronic nose for detection of food microbial contamination*. in *SENSORS, 2014 IEEE*. 2014. IEEE.

80. Timsorn K., Wongchoosuk C., Wattuya P., Promdaen S.Sittichat S. *Discrimination of chicken freshness using electronic nose combined with PCA and ANN*. in *2014 11th International Conference on Electrical Engineering/Electronics, Computer, Telecommunications and Information Technology (ECTI-CON)*. 2014. IEEE.

81. Guney S.Atasoy A. *Fish freshness assessment by using electronic nose*. in *2013 36th International Conference on Telecommunications and Signal Processing (TSP)*. 2013. IEEE.

82. Siadat M., Losson E., Ghasemi-Varnamkhasti M.Mohtasebi S.S. *Application of electronic nose to beer recognition using supervised artificial neural networks*. in *2014 International Conference on Control, Decision and Information Technologies (CoDIT)*. 2014. IEEE.

83. Siyang S., Seesaard T., Lorwongtragool P.Kerdcharoen T. *E-nose based on metallo-tetraphenylporphyrin/SWNT-COOH for alcohol detection*. in *2013 IEEE International Conference of Electron Devices and Solid-state Circuits*. 2013. IEEE.

84. Lihui M., Yongyang G., Hui S., Mingjun Q., Ting Z.Xiaohua H. *Rapid detection of sesame oil flavoring based on the gas sensor array*. in *2013 Fifth International Conference on Measuring Technology and Mechatronics Automation*. 2013. IEEE.

85. Bhattacharyya N., Bandyopadhyay R., Bhuyan M., Tudu B., Ghosh D.Jana A.,"*Electronic nose for black tea classification and correlation of measurements with "Tea Taster" marks"*,IEEE transactions on instrumentation and measurement, 2008. **57**(7): 1313-1321.

86. Tudu B., Metla A., Das B., Bhattacharyya N., Jana A., Ghosh D.Bandyopadhyay R.,"*Towards versatile electronic nose pattern classifier for black tea quality evaluation: An incremental fuzzy approach"*,IEEE transactions on instrumentation and measurement, 2009. **58**(9): 3069-3078.

87. Sberveglieri V., Fava P., Pulvirenti A., Concina I.Falasconi M. *New methods for the early detection of fungal contamination on green coffee beans by an Electronic Nose*. in *2012 Sixth International Conference on Sensing Technology (ICST)*. 2012. IEEE.

88. Yu H., Meng M.Yin Y. *The research on the application of electronic nose in discriminate the rice varieties*. in *Proceedings of the 2013 International Conference on Advanced Mechatronic Systems*. 2013. IEEE.

89. Santos J.P., Lozano J.Aleixandre M.,"*Electronic noses applications in beer technology"*,Brewing Technology, 2017. **177**.

CHAPTER 3: *In-VITRO* MICROBIOLOGICAL ASSAYS INVESTIGATING THE ANTIMICROBIAL PROPERTIES OF CURCUMIN DILUTED IN N-METHYL-2-PYRROLIDONE WITH AND WITHOUT LED PHOTOACTIVATION

Extended report from:

2022 Exploitation of the Antibacterial Properties of Photoactivated Curcumin as 'Green' Tool for Food Preservation [*N. Mandras and E. Ficiarà are the corresponding authors]. DOI: 10.3390/ijms23052600. In INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES vol. 23 (5) *Munir Z.; Banche G.; Cavallo L.; Mandras N.; Roana J.; Pertusio R.; Ficiara E.; Cavalli R.; Guiot C.*

2022 Antimicrobial activity of Curcumin and Curcumin/Chitosan loaded nanobubbles with photodynamic light. Poster Presentation "50° Congresso Nazionale società Italiana Di Microbiologia (SIM) 18-21 September 2022 in Napoli Italy

2021 Evaluation of Chitosan/Curcumin loaded Nanobubbles with and without Photoactivated light for Food Preservation. In Abstract Book
Zunaira Munir, Lorenza Cavallo, Francesca Menotti, Vivian Tullio, Sara Comini, Roberta Cavalli, Cat ...
Poster Presentation, Conference 'SIM (Societa Italiana Di Microbiologia) (16-17,20-21 September 2021)

2021 Application of Curcumin-loaded NBs with and without Photodynamic light (LED) for food preservation

Zunaira Munir, a Roberta Cavalli, c Giuliana Banche, b Narcisa Mandras, b Caterina Guiot

Nano Innovation Conference & amp; Exhibition' (21-24 September 2021)

2022 Antimicrobial activity of chitosan/curcumin loaded nanobubbles irradiated with photodynamic light for food preservation Munir Z., Cavallo L., Menotti F., Comini S., Cavalli R., Guiot C., Ficiarà E., Roana J., Mandras N., Banche G.

Poster Presentation International Conference on Nanomaterials Applied To Life Sciences NALS 2022, in Santander, Cantabria, Spain from the 27th to the 29th April 2022.

3.1 EXPERIMENTAL DESCRIPTION

3.1.1 MATERIAL AND METHOD

N-methyl-2-pyrrolidone and curcumin (Mw = 368.5) were acquired from Sigma-Adrich (Munich, Germany) and utilized as received). The following products were purchased from Oxoid SpA (Garbagnate Milanese, Italy): *Brain Heart Infusion* (BHI) *broth*, Trypticase Soy broth and agar (TSB, TSA).

The bacteria used in this study were from the American Type Culture Collection (ATCC) including Gram negative (*Escherichia coli* ATCC 25922), and Gran positive (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) strains. These strains were stored in the commercial Microbank Bacterial Preservation system (Pro-lab Diagnostics, Austin, Tex). Vials were inoculated according to the manufacturer's instructions and were kept in a −80°C freezer. LED system (Blue LED (LES Flex Strips LEDYDEL IP64, Turin, Italy) with wavelength from 465-470). All other chemicals, used and not listed above, were commercially available analytical grade product

3.1.2 Curcumin Solution Preparation

N-methyl-2-pyrrolidone and 0.9% saline solution were mixed in a 1:1 ratio to form a stock solution of 1 mg/ml of curcumin, which was then kept in the dark. The solution was then diluted in BHI according to the protocol to ascertain the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC).

3.1.3 Bacterial Inoculum

An inoculum was prepared for each bacterium from Microbank. Five tubes containing 5 ml of BHI broth were set up and scalar dilutions of 500 µl in 500 µl were made. Growth tests on TSA were performed in parallel. Subsequently, the tubes were kept at 37°C overnight. From the overnight culture, inocula were prepared in 0.9% sterile saline solution to reach 0.5 McFarland turbidity standard and diluted in BHI broth medium to final concentrations of 5×10^5 CFU/ml.

3.1.4 Set-Up of Multiwell Plates

For each bacterial strain, two 96-well microtiter plates were set up, one of which was a control plate without irradiation and the other subjected to 3 h of LED treatment. One hundred µl of BHI was placed in each well of the plate, except for the first well in which 200 µl of curcumin was put at 1 mg/ml of a concentration.

Then, serial dilutions were made, resulting in the following final concentrations of curcumin (mg /ml):

1	**0.50**	**0.25**	**0.125**	**0.06**	**0.03**	**0.015**	**0.0075**	**0.0037**	**0.0017**	**0.0009**

100µl of each bacterial suspension (inoculum in 10^5 CFU/ml) were then added to each well, resulting in a total volume of 200 µl per well. The final concentrations (mg /ml) obtained were as follows:

0.50	**0.25**	**0.125**	**0.06**	**0.03**	**0.015**	**0.0075**	**0.0037**	**0.0018**	**0.0009**	**0.00046**

In addition, three controls were set up: the medium control (200 µl BHI in one well), the bacterial fertility control (100 µl inoculum + 100 µl BHI) for each strain, and the curcumin solution control (100 µl curcumin 2 mg/ml + 100 µl BHI). One plate was subjected to PDI treatment by visible light at a wavelength of 425 nm (Light Emitting Diode, LED) for 3 hours, while the control plate without treatment was left under a fume hood covered with aluminium foil for the same time. A thermometer was associated to the container, on whose lid the LEDs were placed, to monitor the temperature during the application of PDI treatment **(Figure 3.1).**

Figure 3.1: Container containing the LEDs for photodynamic treatment.

At the end of 3 hours, the 96-well microtiter plates were kept in thermostate at 37°C for 24 hours. After 24 h, MIC reading was performed, indicating positivity in case of the presence of turbidity of the wells within the multiwells. MIC is established as the concentration corresponding to the first clear well without visible bacterial growth.

3.1.5 Minimun Bactericidal Concentration Determination

In order to establish the MBC, aliquots of 10 µl from all the wells which showed no visible bacterial growth were plated on BHA and incubated for 24 h at 37°C. The MBC corresponded to the first dilution in which the number of CFU was less than or equal to three **(Table 3.1).**

3.1.6 Time Kill Kinetics

Time-kill kinetics assay was analyzed using the MIC values, which evaluated before the experiment. . An overnight culture of bacteria was diluted in BHI broth. Test compound i.e curcumin solution was added to logatithmic-phase broth cultures of approximately 10^5 CFU/ml. Six well microtiter plates were prepared by using BHI medium, respective test compound at the concentration of 3xMIC and bacterial inoculum. After one hour of incubation into the hood, one plate was placed under irradiation of LED and other under dark for 3h. Then, the killing was evaluated at different time (T0, 1.5h, 6h, 20h, 24h, 48h and 72h). On the corresponding day, the bacterial colonies were counted and the time kill kinetics were plotted as CFU/mL against time. The mean percentage of survival compared to the non-treated (control) was used to determine bacterial survival. All experiments were performed in duplicate.

3.2 Statistical Analysis

Whenever data were assumed to be normally distributed, the results were depicted as the mean ± standard deviation (SD). The SD serves as a measure of the data's degree of dispersion from the mean. In case of progressively halving discrete values (e.g. MIC) the relative error is assumed to be 0.5Microsoft Office Excel was used for statistical analysis

3.3 RESULTS

3.3.1 Curcumin's antibacterial properties with and without LED irradiation

The broth microdilution method was used to determine the MIC of curcumin with and without LED irradiation against all bacterial strains. According to the study's findings, curcumin has a strong antibacterial impact on selected bacterial strains, with low MIC and MBC values under blue visible light (LED) (**Table 3.1**).

3.3.2 Minimum Inhibiting and Bactericidal Concentration

The results showed that *E. faecalis* was highly susceptible to irradiation, being the MIC following the curcumin administration equal to 0.125 mg/ml while after curcumin administration and under blue light (LED) for three hours, the MIC drastically decreased to 0.0037 mg/mL. Also *S. aureus* strain was sensitive to curcumin followed by LED exposure, exhibiting a MIC equal to 0.0075 mg/ml , compared to the value of 0.06 mg/ml when not exposed to photodynamic treatment. On the contrary, the MIC value (0.125 mg/ml) against *E. coli* was same with and without exposure to photodynamic treatment. **Table 3.1** displays the outcomes of MIC and MBC for all the tested strains of bacteria.

Bacterial strains	MIC without LED (mg/mL)	MIC with LED (mg/mL)	MBC without LED (mg/m)	MBC With LED (mg/mL)
E. faecalis	0.125 ± 0.063	0.0037 ± 0.002	>0.25	0.0037 ±0.002
S. aureus	0.06 ± 0.03	0.0075 ± 0.063	>0.25	0.0075 ± 0.004
E. coli	0.125 ± 0.063	0.125 ± 0.063	>0.25	>0.25

Table 3.1: MIC and MBC of curcumin without and with 3h photodynamic irradiation with LED.

The current study's findings demonstrated that curcumin with LED light exhibited a high level of bactericidal activity against *S. aureus* and *E. faecalis* strains, with MBC values of 0.0075 and 0.0037 mg/ml, respectively. While for *E. coli* the MBC is greater than 0.25 mg/ml with both conditions with and without LED light.

The photo-activated curcumin exhibits a significant antibacterial effect against Gram-positive bacteria but not Gram-negative bacteria, according to the findings of the MIC and MBC tests.

3.3.3 Time Kill Kinetics Assessment

Curcumin's antibacterial activity was tested against *S. aureus, E. faecalis,* and *E. coli* by performing killing assays. Curcumin was administered at a dose of 3MIC, and therefore the absolute concentration was different for the different strains and the different treatments. The results are shown in T**ables (3.2-4)** and F**igures (3.2-4).** Due to high MIC values, curcumin demonstrated a powerful killing capacity against *E. coli* **(Figure 3.2)** but, as expected, the effect of the LED irradiation was negligible. On the other hand, some killing potential was seen in *S. aureus* **(Figure 3.3)** and *E. faecalis* **(Figure 3.4)** that gradually decrease with time but with comparison to *E.coli* apparently less bacteria were killed due to the much lower doses of curcumin. In all cases, the result shows that at proper concentration and longer duration of time (72 h), the bacterial killing was effective.

***Escherichia coli* ATCC 25922**

The results about *E. coli* time kill kinetics show that 3XMIC curcumin concentration is effective at controlling bacterial growth for a longer time. *E. coli* is a Gram-negative bacterium, difficult to destroy with a low curcumin dosage, as comparison of other Gram-positive bacterial strains but it can be killed with a relatively high concentration of curcumin and is almost not affected by the exposure of LED light. Nevertheless, this study revealed that the curcumin at high concentration was rapidly bactericidal achieving complete elimination of *E. coli* **(Figure 3.2).**

Time (h)	Control (CFU/ml)	Curcumin 3XMIC without LED (CFU/ml)	Curcumin 3XMIC with LED (CFU/ml)
T0	4.8×10^6	2.2×10^3	3.1×10^1
1.5	1.4×10^7	1.0×10^1	1.0×10^1
6	3.3×10^9	1.0×10^1	1.0×10^1
20	$2.9x\ 10^9$	1.0×10^1	1.0×10^1
24	2.5×10^9	1.0×10^1	1.0×10^1
48	4.7×10^8	1.0×10^1	1.0×10^1
72	1.7×10^8	1.0×10^1	1.0×10^1

Table 3.2: Time kill kinetics of curcumin without (WI) and with (LED) PDI treatment against *E.coli.*

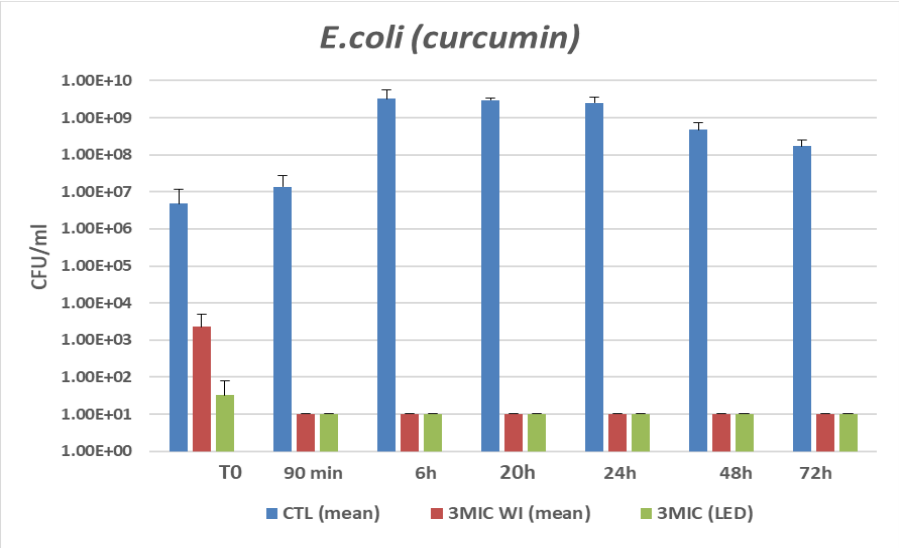


Figure 3.2: Killing activity against *E.coli*.

***Staphylococcus aureus* ATCC 29213**

According to the results, *S. aureus* growth was somewhat slowed down but not completely prevented by administering curcumin at low doses, corresponding to one half of the dose used for *E. coli* without LED irradiation and to about 6% of the case of LED irradiation **(see Table 3.1)**. Table 3 and Figure 3 indicate the decline in CFU/ml for bacteria treated with 3XMIC of curcumin after 20 hours of incubation without LED illumination. This is as a result of the higher MIC value. However, because of the extremely low curcumin concentration, the bacterial population in the 3XMIC of curcumin with LED-treated bacteria did not gradually decline.

Time (h)	Control (CFU/ml)	Curcumin 3XMIC without LED (CFU/ml)	Curcumin 3XMIC with LED (CFU/ml)
T0	8.3×10^5	1.8×10^3	2×10^3
1.5	6.7×10^6	1.8×10^3	3×10^3
6	3.6×10^8	1.8×10^3	3.5×10^3
20	$7.8x\ 10^8$	1.5×10^3	6×10^3
24	2.6×10^8	5.5×10^1	1.5×10^3
48	2.1×10^9	5.5×10^1	1.5×10^3
72	2×10^9	5.5×10^1	2.5×10^3

Table 3.3: Time kill kinetics of curcumin without (WI) and with (LED) PDI treatment against *S.aureus*.

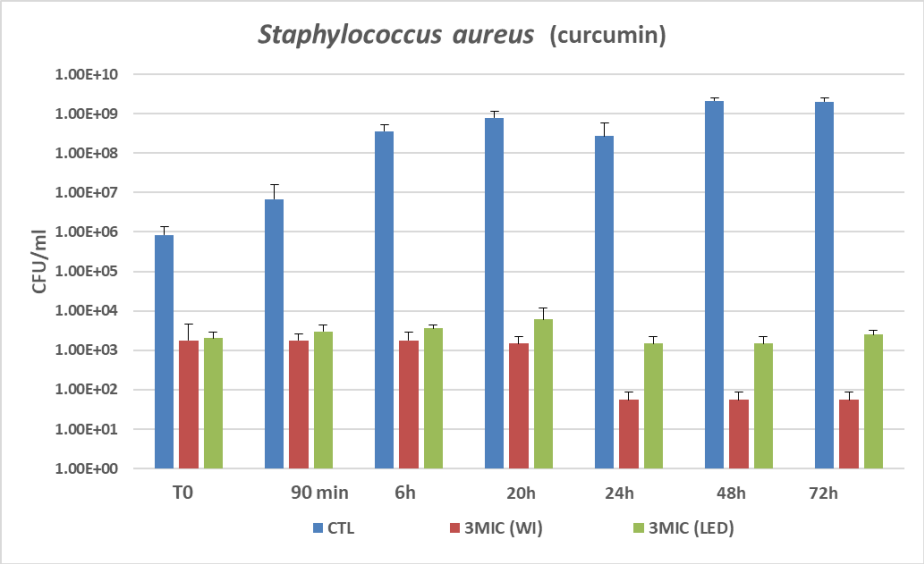


Figure 3.3: Killing activity against *S. aureus*.

***Enterococcus faecalis* (ATCC 29212)**

The results of *E. faecalis* were relatively similar to *S. aureus* since the MIC for both under light exposure was very low so, bacterial growth was not fully suppressed **(Figure 3.4**). Note that in this case, the curcumin dosage without LED irradiation is the same of that used for *E. coli* but in the case of LED irradiation was only 3% of the dosage (see Table 3.1) and is still effective in controlling the infection.

Time (h)	Control (CFU/ml)	Curcumin 3XMIC without LED (CFU/ml)	Curcumin 3XMIC with LED (CFU/ml)
T0	5.6×10^6	1.5×10^4	1.2×10^4
1.5	9.3×10^6	1.2×10^2	1.3×10^3
6	4.6×10^8	1.5×10^2	1.5×10^3
20	1.8×10^9	3.5×10^2	1.1×10^3
24	2.5×10^9	5.5×10^1	1.5×10^3
48	1×10^9	5.5×10^1	1.5×10^3
72	1.2×10^9	3.5×10^1	1.5×10^3

Table 3.4: Time kill kinetics of curcumin without (WI) and with (LED) PDI treatment against *E.faecalis*.

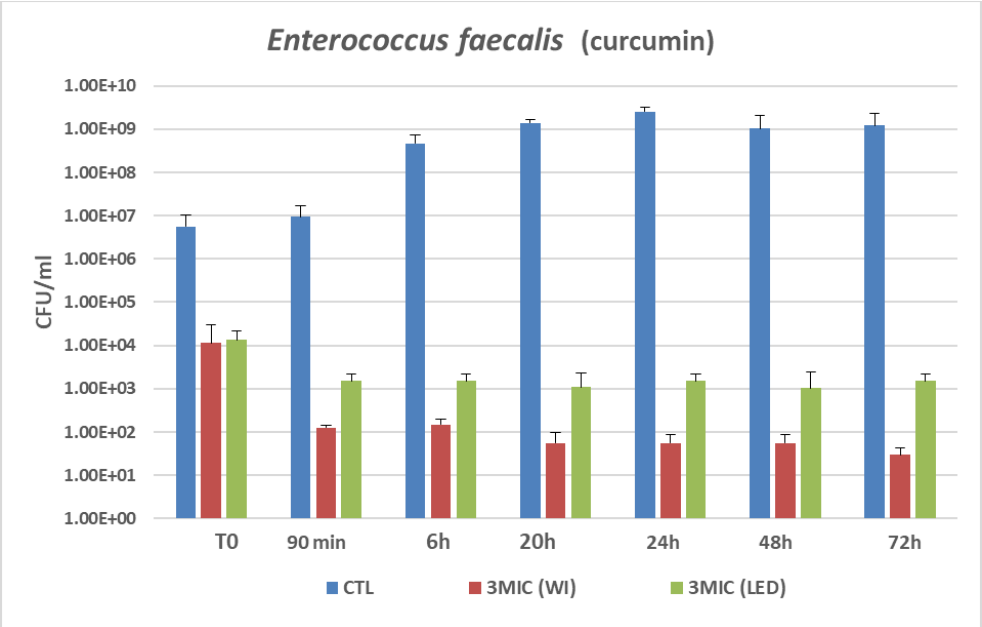


Figure 3.4: Killing activity against *E.faecalis*.

As a consequence, the overall findings demonstrated that the killing potential against bacterial strains depends on the concentration of curcumin, that here is expressed as multiple of the MIC, and that LED irradiation is effective al low doses for gram-positive bacteria but does not change the required inhibiting dose of curcumin for gram-negative bacteria.

3.4 DISCUSSION AND CONCLUSION

Curcumin administration followed by 3 hours of blue LED light irradiation affects Gram-positive bacteria more than Gram-negative bacteria because the bacterial cell membrane structures are different and impart different resistance to curcumin **[1].** In particular, Gram-negative bacteria have an inner cytoplasmic membrane surrounded by a thin layer of peptidoglycan and an outside lipopolysaccharide membrane. The outer membrane assists in osmoprotection, and also serves as a permeability barrier and regulates the in and out of a wide range of substances, including ions, nutrients, and environmental contaminants. On the other hand, Gram-positive bacteria, lack an external protective barrier **[2].** As a result, these bacteria were more easily destroyed because of the porous structure and simple penetration of photosensitizers. This explains why Gram-negative bacteria have higher antimicrobial agent resistance than Gram-positive bacteria **[1].** To manage this problem, we have developed a number of bio-conjugates such as nanobubbles containing curcumin, which are described in chapter 4.

Our results confirm what already observed in the literature. The antimicrobial activities of curcumin were initially reported by Schraufstatter et al **[3].** Numerous investigations on the broad-spectrum

inhibitory action of curcumin against a number of Gram-positive and Gram-negative bacteria have been conducted during the past seventy years. The concerned bacteria were *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, E. faecalis, S. epidermidis, Streptococcus pyogenes, Listeria innocua, S. aureus, Helicobacter pylori, Bacillus subtilis, E. coli, B. cereus* **[4-8]**. Curcumin has an antibacterial properties because it can make bacterial membranes more permeable **[9].**

Interestingly, curcumin also has strong antibacterial activity against Multidrug-Resistant (MDR) isolates including polymyxin-resistant *K.pneumoniae* and methicillin-resistant *S. aureus* (MRSA) **[6, 8, 10].** Curcumin's MIC values against clinical isolates of MRSA were found to be between 125 and 500 µg/mL in a recent study by Batista de Andrade et al **[11]**. Another investigation by Yasbolaghi Sharahi et al. found that the curcumin's MICs against *A. baumannii, Klebsiella pneumoniae* and *P. aeruginosa* MDR ranged from 128 to 512 µg/mL **[5].** Curcumin's MIC value was previously found to be 18.42-250 µg/ml against *S. aureus* **[12-15]**, 93.8 µg/ml-250 µg/ml against *E. coli* **[13, 14, 16],** and 62.5-293 µg/ml against *E. faecalis* **[14, 17]**. Importantly, multiple study groups observed considerable variations in the MICs of curcumin against various strains **[18]**. This might be because each research group used a different solvent (such water, DMSO, or ethanol), each of which had a different degree of solubility for curcumin **[18]**. These variations might also be influenced by the MIC test technique, the device's effect on the bacterial cell membrane, and the purity of the curcumin utilized in the research **[18].**

Due to its inherent photosensitizing properties, curcumin has the potential to have antimicrobial and antibacterial effects that can be strengthened by photodynamic activation. Recent suggestions for a variety of applications demonstrate that photodynamic activation can be a highly efficient sterilizing method. A method called (PDI) depends on the activation of non-thermal photophysical and photochemical processes, which depend upon light, photosensitizer, and the presence of oxygen **[19].** It functions on the theory that photosensitizers may be triggered at specific wavelengths to produce ROS with intense oxidation to inactivate abnormal cells and harmful microbes **[20].** Due to its multi-target nature, PDI has the benefit of not producing any harmful compounds, requiring just a light source for energy, and having a minimal risk of generating microbial resistance **[21].** Light-emitting diode (LEDs), lasers, and halogen lamps are common light sources. The wavelength of the light is an important consideration for PDI. LEDs offer the benefits of being inexpensive, having broader emission bands, being simple to utilize, and having more irradiation time flexibility.

According to preliminary findings from our team's research **[22],** curcumin exerts a significant antibacterial impact on a number of bacterial strains, with low MIC and MBC values. Additionally,

the effects of curcumin coupled with photodynamic activation using LED light for three hours on two strains of Gram-positive bacteria (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) and one strain of Gram-negative bacteria (*E. coli* ATCC 25922) were examined. And at quite low curcumin concentrations, the MIC was determined i.e 0.125, <0.0075, and <0.0037 mg/mL against *E. coli*, *S. aureus*, and *E. faecalis*, respectively. **(Table 1) [23].** According to these findings, the MIC of antibacterial activity against Gram-positive bacteria drastically altered, sometimes by two orders of magnitude, when curcumin was coupled with photodynamic therapy using LED light. Note that the minimum amount of curcumin needed to kill *S. aureus* (MIC equal to 20.3 μM with LED irradiation findings of this study) is significantly less than the amounts used to kill apples (80 μM) and beef, poultry, and pork meat (40 μM) reported by Corrêa et al. **[24]**.

Overall, the research stated above demonstrates that the efficiency of PDI is substantially influenced by the bacterial strain, Gram-negative strains being significantly less inhibited. Regrettably, quite few researchers have looked into why the antibacterial action of curcumin appears to vary depending on the bacterial strain. By generating membrane permeabilization, curcumin affects both Gram-positive and Gram-negative bacteria in a comparable way, as demonstrated by Tyagi et al. **[25].** LED irradiation alone produced minimal antibacterial effects, according to Aurum et al. **[26],** whereas photoactivated curcumin had substantial bactericidal effects also on *E. coli*. Our investigation showed a small effectiveness in *E. coli*, and this finding may be explained by the various bacteria inocula, curcumin formulations, and experimental setups we employed. These changes are conceivable because Gram-positive and Gram-negative bacteria have differing cell wall compositions. Gram-positive bacteria can more easily being destroyed because of their porous nature and the ease with which photosensitizers can penetrate. Gram-negative bacteria, on the other hand, are more difficult to be eradicated because of their outer membrane, which progressively suppresses curcumin's penetration effect. Similar findings have been reported by various authors, and it has been hypothesized that variations in results are due to differing membrane structures **[27-29].** Curcumin's method of biological action is dependent on the characteristics of the delivery mechanism, according to conclusive evidence from Shlar et al. [1]. However, the outer membrane of Gram-negative bacteria shields them from curcumin diffusion and photosensitization unless nanovectors are used, which are predicted to enhance membrane crossing **[30, 31].**

References

1. Shlar I., Droby S., Choudhary R.Rodov V.,"*The mode of antimicrobial action of curcumin depends on the delivery system: Monolithic nanoparticles vs. supramolecular inclusion complex",*RSC advances, 2017. **7**(67): 42559-42569.
2. Rajagopal M.Walker S.,"*Envelope structures of Gram-positive bacteria",*Protein and sugar export and assembly in Gram-positive bacteria, 2017: 1-44.
3. Schraufstätter E.Bernt H.,"*Antibacterial action of curcumin and related compounds",*Nature, 1949. **164**(4167): 456-457.
4. Liu M., Lu Y., Gao P., Xie X., Li D., Yu D.Yu M.,"*Effect of curcumin on laying performance, egg quality, endocrine hormones, and immune activity in heat-stressed hens",*Poultry Science, 2020. **99**(4): 2196-2202.
5. Sharahi J.Y., Ahovan Z.A., Maleki D.T., Rad Z.R., Rad Z.R., Goudarzi M., Shariati A., Bostanghadiri N., Abbasi E.Hashemi A.,"*In vitro antibacterial activity of curcumin-meropenem combination against extensively drug-resistant (XDR) bacteria isolated from burn wound infections",*Avicenna journal of phytomedicine, 2020. **10**(1): 3.
6. Taghavifar S., Afroughi F.Saadati Keyvan M.,"*Curcumin nanoparticles improved diabetic wounds infected with methicillin-resistant Staphylococcus aureus sensitized with HAMLET",*The international journal of lower extremity wounds, 2022. **21**(2): 141-153.
7. Morão L.G., Polaquini C.R., Kopacz M., Torrezan G.S., Ayusso G.M., Dilarri G., Cavalca L.B., Zielińska A., Scheffers D.J.Regasini L.O.,"*A simplified curcumin targets the membrane of Bacillus subtilis",*Microbiologyopen, 2019. **8**(4): e00683.
8. Kaur A., Sharma P.Capalash N.,"*Curcumin alleviates persistence of Acinetobacter baumannii against colistin",*Scientific reports, 2018. **8**(1): 11029.
9. De R., Kundu P., Swarnakar S., Ramamurthy T., Chowdhury A., Nair G.B.Mukhopadhyay A.K.,"*Antimicrobial activity of curcumin against Helicobacter pylori isolates from India and during infections in mice",*Antimicrobial agents and chemotherapy, 2009. **53**(4): 1592-1597.
10. Sundaramoorthy N.S., Sivasubramanian A.Nagarajan S.,"*Simultaneous inhibition of MarR by salicylate and efflux pumps by curcumin sensitizes colistin resistant clinical isolates of Enterobacteriaceae",*Microbial pathogenesis, 2020. **148**: 104445.
11. de Andrade Neto J.B., de Farias Cabral V.P., Nogueira L.F.B., da Silva C.R., Sa L.G.d.A.V., da Silva A.R., da Silva W.M.B., Silva J., Marinho E.S.Cavalcanti B.C.,"*Anti-MRSA activity of curcumin in planktonic cells and biofilms and determination of possible action mechanisms",*Microbial pathogenesis, 2021. **155**: 104892.

12. Sivasothy Y., Sulaiman S.F., Ooi K.L., Ibrahim H.Awang K.,"*Antioxidant and antibacterial activities of flavonoids and curcuminoids from Zingiber spectabile Griff",* Food Control, 2013. **30**(2): 714-720.
13. Tajbakhsh S., Mohammadi K., Deilami I., Zandi K., Fouladvand M., Ramedani E.Asayesh G.,"*Antibacterial activity of indium curcumin and indium diacetylcurcumin",* African Journal of Biotechnology, 2008. **7**(21).
14. Sadeghi-Ghadi Z., Behjou N., Ebrahimnejad P., Mahkam M., Goli H.R., Lam M.Nokhodchi A.,"*Improving antibacterial efficiency of curcumin in magnetic polymeric nanocomposites",* Journal of Pharmaceutical Innovation, 2022: 1-16.
15. Wang Y., Lu Z., Wu H.Lv F.,"*Study on the antibiotic activity of microcapsule curcumin against foodborne pathogens",* International journal of food microbiology, 2009. **136**(1): 71-74.
16. Gunes H., Gulen D., Mutlu R., Gumus A., Tas T.Topkaya A.E.,"*Antibacterial effects of curcumin: an in vitro minimum inhibitory concentration study",* Toxicology and industrial health, 2016. **32**(2): 246-250.
17. Yadav S., Singh A.K., Agrahari A.K., Sharma K., Singh A.S., Gupta M.K., Tiwari V.K.Prakash P.,"*Making of water soluble curcumin to potentiate conventional antimicrobials by inducing apoptosis-like phenomena among drug-resistant bacteria",* Scientific reports, 2020. **10**(1): 14204.
18. Teow S.-Y., Liew K., Ali S.A., Khoo A.S.-B.Peh S.-C.,"*Antibacterial action of curcumin against Staphylococcus aureus: a brief review",* Journal of tropical medicine, 2016. **2016**.
19. Penha C.B., Bonin E., da Silva A.F., Hioka N., Zanqueta É.B., Nakamura T.U., de Abreu Filho B.A., Campanerut-Sá P.A.Z.Mikcha J.M.G.,"*Photodynamic inactivation of foodborne and food spoilage bacteria by curcumin",* LWT-Food Science and Technology, 2017. **76**: 198-202.
20. Jiang Y., Liang Y., Zhang H., Zhang W.Tu S.,"*Preparation and biocompatibility of grafted functional β-cyclodextrin copolymers from the surface of PET films",* Materials Science and Engineering: C, 2014. **41**: 1-7.
21. Costa L., Tomé J.P., Neves M.G., Tomé A.C., Cavaleiro J.A., Faustino M.A., Cunha Â., Gomes N.C.Almeida A.,"*Evaluation of resistance development and viability recovery by a non-enveloped virus after repeated cycles of aPDT",* Antiviral Research, 2011. **91**(3): 278-282.

22. Munir Z., Cavallo L., Menotti F., Tullio V.C., Comini S., Cavalli R., Guiot C., Cuffini A., Banche G.Mandras N. *Evaluation of Chitosan/Curcumin loaded Nanobubbles with and without Photoactivated light for Food Preservation*. in *Abstract Book*. 2021. SIM.
23. Munir Z., Banche G., Cavallo L., Mandras N., Roana J., Pertusio R., Ficiarà E., Cavalli R.Guiot C.,"*Exploitation of the antibacterial properties of photoactivated curcumin as 'green' tool for food preservation",* International journal of molecular sciences, 2022. **23**(5): 2600.
24. Correa T.Q., Blanco K.C., Garcia E.B., Perez S.M.L., Chianfrone D.J., Morais V.S.Bagnato V.S.,"*Effects of ultraviolet light and curcumin-mediated photodynamic inactivation on microbiological food safety: A study in meat and fruit",* Photodiagnosis and Photodynamic Therapy, 2020. **30**: 101678.
25. Tyagi P., Singh M., Kumari H., Kumari A.Mukhopadhyay K.,"*Bactericidal activity of curcumin I is associated with damaging of bacterial membrane",* PLoS One, 2015. **10**(3): e0121313.
26. Aurum F.S.Nguyen L.T.,"*Efficacy of photoactivated curcumin to decontaminate food surfaces under blue light emitting diode",* Journal of Food Process Engineering, 2019. **42**(3): e12988.
27. Dahl T.A., McGowan W.M., Shand M.A.Srinivasan V.S.,"*Photokilling of bacteria by the natural dye curcumin",* Archives of microbiology, 1989. **151**: 183-185.
28. de Oliveira E.F., Tosati J.V., Tikekar R.V., Monteiro A.R.Nitin N.,"*Antimicrobial activity of curcumin in combination with light against Escherichia coli O157: H7 and Listeria innocua: Applications for fresh produce sanitation",* Postharvest Biology and Technology, 2018. **137**: 86-94.
29. Maisch T., Szeimies R.-M., Jori G.Abels C.,"*Antibacterial photodynamic therapy in dermatology",* Photochemical & Photobiological Sciences, 2004. **3**(10): 907-917.
30. Agel M.R., Baghdan E., Pinnapireddy S.R., Lehmann J., Schaefer J.Bakowsky U.,"*Curcumin loaded nanoparticles as efficient photoactive formulations against gram-positive and gram-negative bacteria",* Colloids and Surfaces B: Biointerfaces, 2019. **178**: 460-468.
31. Ryu V., Ruiz-Ramirez S., Chuesiang P., McLandsborough L.A., McClements D.J.Corradini M.G.,"*Use of micellar delivery systems to enhance curcumin's stability and microbial photoinactivation capacity",* Foods, 2021. **10**(8): 1777.

CHAPTER 4: EVALUATION OF THE EFFECTIVENESS OF PHOTOACTIVATED CURCUMIN ON BERRIES SAMPLES

Extended Report From:

2022 Exploitation of the Antibacterial Properties of Photoactivated Curcumin as 'Green' Tool for Food Preservation [*N. Mandras and E. Ficiarà are the corresponding authors]. DOI: 10.3390/ijms23052600. In INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES vol. 23 (5) *Munir Z.; Banche G.; Cavallo L.; Mandras N.; Roana J.; Pertusio R.; Ficiara E.; Cavalli R.; Guiot C.*

2022 "BLUE STRAWBERRIES": ROLE OF CURCUMIN AND β-CYCLODEXTRIN WITH AND WITHOUT PHOTOACTIVATION FOR FRUIT PRESERVATION

Munir Z, Cavalli R, Torri L, Cavallo L, Mandras N, Banche G, Guiot C, Stura I

Poster Presentation V International Summer School on "Advances in cyclodextrin chemistry and applications" in Ascea (Italy) 4 – 7 September 2022

Nanoinnovation: https://www.nanoinnovation2022.eu/home/poster/STURA_Ilaria.pdf

Also SIF communication: https://2022.congresso.sif.it/talk/221

4.1 PURPOSE OF WORK

The study aims at evaluating the effectiveness of a treatment based on a natural antibacterial photo-activable compound, e.g. curcumin, in order to increase the fresh fruit's shelf life, especially the easily perishable berries (strawberries, raspberries, blueberries, etc.), preserving their organoleptic and antioxidant properties.

Due to the administration of curcumin to foods, for both safety and organoleptic reasons, we investigated the use of β-cyclodextrin as solubilization medium, assessing its effectiveness in term of curcumin concentration in the berries and its impact on the fruit taste.

4.2 MATERIAL AND METHOD

4.2.1 Materials and Chemicals

Unless otherwise noted, Sigma-Aldrich (St. Louis, MO, USA) was the source of the components used. β-cyclodextrin was a gift of Roquette (Roquette Freres, Lestrem, France). Curcumin from Curcuma longa was purchased from Sigma Aldrich. For microbiological studies, Liofilchem ® provides Tripticase Soy Agar (TSA) plates.

The used instrument were:

- Spectrophotometer: Duo Beckman
- Centrifuge: Rotofix 32A Hettich Centrifugen
- Lux meter: mod. LMT B500 s/n 0180233 (photocell mod. P30SC0, s/n 287032), LMT Lichtmesstechnik GmbH
- Ultrasonic bath: Bandelin Sonorex Digitec; Bandelin Electronic GmbH & Co. KG, Berlin, Germany

4.2.2 The Experiment's Setting

For the investigation, blueberries and strawberries were utilized in four separate tests that were each conducted independently. All the berries were purchased in a nearby market in the summer of 2022. They have not been frozen or put through any other kind of preservation process. The series of four tests are listed below.

In the **preliminary experiment** (E0), three experimental conditions were set up:

i. Blueberries treated with a solution of cyclodextrin as a control

ii. Blueberries treated with solution of curcumin and β-cyclodextrin (1mg/ml) without being irradiated.

iii. Blueberries treated with curcumin and cyclodextrin solution (1mg/ml) and exposed to LED light for 6 hours each day for three days.

The three single-layer berries trays were prepared according to the above experimental setup and treatment placed in a refrigerator at temperature (4°C). Three to four berries were randomly selected from each tray every day at the same time, and transferred to tubes containing sterile water, and shaken for a short period of time.

Full Experiment

In the **first** experiment (E1), 2 kg of **strawberries** were divided into four experimental groups:

i. berries without treatment (CTR);

ii. berries sprayed with β cyclodextrin solution (CI);

iii. berries sprayed with a solution of 0.5 mg/ml curcumin in β cyclodextrin (CICU); and

iv. berries sprayed with a solution of 0.5 mg/ml curcumin in β cyclodextrin and exposed to blue light for 3h (CICUB) after 1 h of incubation.

In the **second experiment** (E2), **blueberries** were divided into 4 groups:

1. berries without treatment (CTR);

2. berries sprayed with a solution of 1 mg/ml curcumin in β -cyclodextrin (CICU);

3. berries sprayed with a solution of 1 mg/ml curcumin in β -cyclodextrin and exposed to blue light for 3h (CICUB) after 1 h of incubation;

4. berries prepared as CICUB and illuminated for 3h every day for three days (CICUBm).

In the **third one** (E3), **blueberries** were divided into five groups:

1. berries without treatment (CTR);
2. berries sprayed with β -cyclodextrin solution (CI);
3. berries sprayed with a solution of 1 mg/ml curcumin in β -cyclodextrin (CICU);
4. berries sprayed with a solution of 1 mg/ml curcumin in β -cyclodextrin and ex-posed to blue light for 3h (CICUB) after 1 h of incubation;
5. berries prepared as CICUB and illuminated for 3h every day for three days (CICUBm).

After treatment, all groups were stored at refrigeration temperature (4°C).

All experiments can be regarded as Closed Systems as no nutrients are added to the system and no metabolic waste products are removed. When nutrients are lacking, growth stops and metabolic waste products accumulate in the medium. In order to prevent this Death Phase, visual and microbiological controls were performed in all four groups at the conclusion of treatment (T0), as well as at one, two,

and three days later (T24, T48, and T72). A longer period would also result in the fruits becoming bad.

Qualitative analysis was performed for the preliminary experiment (E0).

In contrast, for **quantitative** study volatile compound analysis was carried out after four days for the first (E1) and third (E3) tests, but not for the second (E2) test due to management issues. Additionally, fruit samples treated with 3h of LED irradiation (CICUB1 and CICUB2, respectively) and sprayed with two different concentrations of curcumin (CICU1 with 0.5 mg/ml and CICU2 with 1 mg/ml) were examined for oxidation and quantitative detection of curcumin content.

In **Figure 4.1** the experimental procedure is sketched.

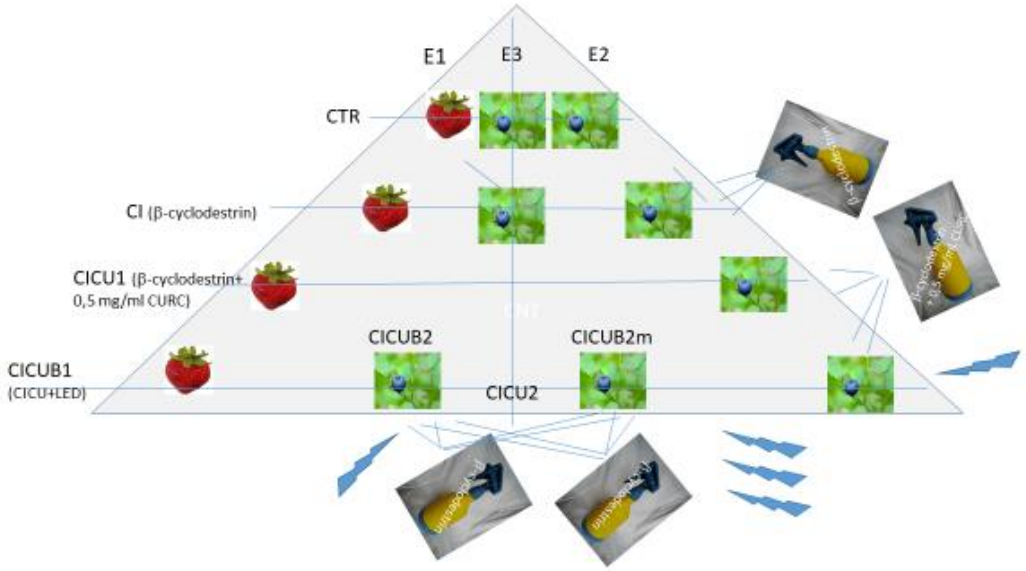


Figure 4.1: Description of the experimental groups and treatments.

4.2.3 Phase solubility study of the β-cyclodextrin/curcumin inclusion complex

Phase solubility studies were carried out using the method developed by Higuchi and Connors **[1].** An excess amount of Curcumin was added to a series of aqueous so-lutions containing an increased concentration of β-cyclodextrin (0 to 70mM) **[2, 3].** Mixtures were continuously shaken at 28 Relative centrifugal force (RCF) for 72h at room temperature in the dark. The resultant dispersions were then centrifuged for 25 minutes at 4025 RCF and the curcumin containing supernatant was separated and its curcumin concentration was determined using a spectrophotometer at wavelength of 425nm. The curcumin concentration was estimated on the basis of a previously obtained calibration plot. The

apparent stability constant (Ks) of curcumin–βCD, complexes were calculated from phase-solubility diagrams according to the following equation:

$$Ks = \frac{Slope}{S°\,(1 - slope)} \qquad (1)$$

Where the slope is obtained from the linear part of the phase solubility diagram, and S° is curcumin's intrinsic solubility in the absence of cyclodextrin polymer.

4.2.4 Preparation of Curcumin /β-Cyclodextrin inclusion Complex 1:1

A 1:1 molar ratio of curcumin and β-cyclodextrin was used to produce the inclusion complexes by adding 10mg of curcumin to 10mL of a water solution containing β-cyclodextrin at the concentration of 3 mg/mL. At room temperature and in the dark, the mixture was stirred continuously for 48 hours. The resultant dispersion was centrifuged for 10 minutes at 1370 RCF. Finally, the complex-containing supernatant was collected for characterization and further studies. For both blueberries and strawberries, this inclusion complex was employed.

4.2.5 Preparation of the Curcumin-Complex Solution

The curcumin β-cyclodextrin complex as freeze-dried powder was weighed and dissolved in water under magnetic stirring to prepare two aqueous solutions of 0.5 and 1 mg/ml of curcumin respectively.

4.2.6 Curcumin Concentration in Fruits after Teatment

The concentration of curcumin on berries was assessed in three distinct situations to see whether the sprayed curcumin/βCD complex adheres to the fruit peel efficiently and is not destroyed by LED light. Just after the spraying the berries were eventually kept at ambient temperature for 1 h and then treated for three hours using LED blue light (with or without waiting for 1 h between spraying and light). This procedure was carried out on both types of berries and at two different curcumin concentrations (0.5 and 1 mg/ml).

For curcumin quantitation, the following process was used: Approximately 15 berries were subjected under three conditions i.e

(a) Curcumin-βCD (0.5 mg/ml & 1mg/ml) spray,

(b) Curcumin-βCD (0.5 mg/ml & 1mg/ml) spray, LED 3h

(c) Curcumin-βCD (0.5mg/ml & 1mg/ml) spray, 1 h rest, LED 3h

5g of berries from each group were homogenized in distilled water using an Ultraturrax homogenizer and the chloroform extraction procedure was performed to extract curcumin. For that, 4 ml of chloroform and 2 ml of homogenized berries were combined, vortexed for 1 minute, and then cooled in an ice bath. This process created two layers: an aqueous one containing berries and curcumin and other with chloroform. The extraction was performed twice or three times more (until reaching a white color) after separating the chloroform layer. At the end of the extraction, a nitrogen pump was used to evaporate the chloroform and then–1 ml of methanol was added to the dried curcumin, and the absorbance of curcumin solution was determined using a UV spectrophotometer at 425 nm. A calibration curve was also determined by comparing standard curcumin concentration and UV Spectrophotometer lectures. After quantifying the absorbance of each sample the calibration equation was used to calculate the concentration of curcumin that is enlisted in **Table 4.3.**

4.2.7 Photoactivation

Blue LED (LES Flex Strips LEDYDEL IP64, Turin, Italy) with a wavelength between 465 and 470 nm was used to photoactivate the curcumin. Berries underwent a 3-hour radiation treatment by each side in one of the two available irradiation chambers. The first one, shown in **Figure 4.2A**, was used to irradiate quantities of berries that weighed up to 0.25 kg and were placed on a grill at a half-height. Ten led strips (each with 27 leds) were fitted on the base, and ten more were positioned on top, for a total light output of 693 ±83 lx per unit surface. The experiment was carried out in a chemical hood with laminar flow to avoid heating.

For the laboratory essays, such as the oxidation test, on tiny fruit samples (2-3 berries maximum), the second smaller irradiation chamber (Figure 4.2B) was used. A Petri dish can be placed inside on the plastic supports; three led strips (6 led each) on top, three on bottom and one on the edges of the box generate a light flow per unit area of 700 ± 25 lx, which is similar to the other. The luxmeter mod. LMT B500 s/n 0180233 (photocell mod. P30SC0, s/n 287032) developed by LMT Lichtmesstechnik GmbH was used to measure the brightness.

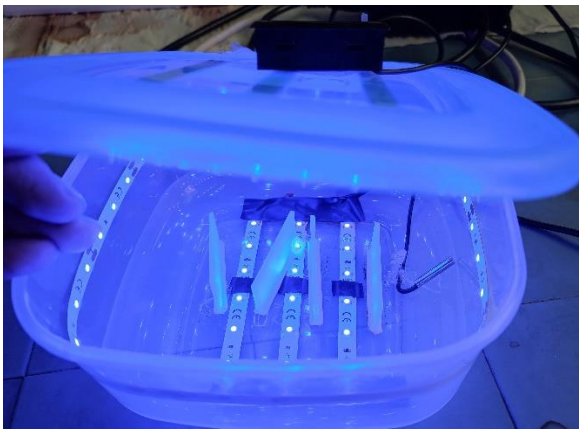


Figure 4.2: **A)** The larger structure on the left was designed to illuminate blue LEDs on up to 0.25 kg of berries after spraying with curcumin; **B)** On the right, the smaller one for in vitro study or a few berries.

4.2.8 Microbiological Analyses

After the photoactivation treatments, the plate counting technique **[4]** was used to quantify the amount of microorganisms on both the fruit samplesand the washing water.

In the **preliminary experiment (E0**) according to the experimental protocol, the washing water from the treated berries was filtered using a vacuum filter. To evaluate the presence of bacteria and fungi, the filter paper was then plated on BHA and SAB plates respectively. To enable for microbial colonies development, all agar plates were incubated for 24-48 hours at 37 °C.

For experiment E1, E2 and E3; initially, 100 µl of washing water was plated onto the Trypticase Soy Agar (TSA) plates, after washing the strawberries in 10 ml of sterile water. The bacterial counts on these samples was labelled as "Berry" at different times in the Result section.

The samples of blueberries and strawberries (5g each) were taken out of the water, homogenized in 5 ml of sterile water, progressively diluted, and plated onto TSA.

Additionally, blueberries were put in tubes containing 0.9% NaCl saline solution and subjected to sonication (35 kHz; Bandelin Sonorex Digitec; Bandelin Electronic GmbH & Co. KG, Berlin, Germany) for 10 min to detach bacteria without affecting their viability in order to measure fruits-adherent bacteria on berries **[5].** After sonication, the fruits were taken out, and the tubes underwent a 10-minute centrifugation at 1789 RCF. The resulting pellets were resuspended in saline solution, and serially diluted. Each dilution was inoculated on TSA. The bacterial counts on these samples are labelled as "Peel" at different times in the Result section.

Following 24-48 hours of optimum microbial growth on all agar plates at 37°C, the total numbers of viable colonies on each plate were counted.

4.2.9 Qualitative Organoleptic Analysis

In order to assess the berries' organoleptic qualities for E0 (sight, smell, touch, and taste), berries were analyzed after being removed from the washing water. Volunteers A, B, C, and D were specifically instructed to eat and evaluate the berries's appearance using their senses of taste, smell, touch, and sight. They were then asked to give a score of 0 for an altered appearance and 1 for a normal appearance.

4.2.10 E-nose Organoleptic Analysis

Volatile compound analysis was carried out for E1, E2, and E3 by using a commercially available portable electronic nose (Win Muster Airsens Analytic Inc., Schwerim, Germany, **Figure 4.3**). This nose is fitted with 10 metal oxide semiconductors (MOS) that have various chemical compositions (see Table 1) and thickness to provide selectivity towards different classes of volatile compounds as reported by Torri **[6].** In particular, the sensors are W1C (Aromatic compounds), W5S (Broad range sensitivi-ty, nitrogen oxides), W3C (Ammonia), W6S (Hydrogen, breath gases), W5C (Alkanes, aromatic compounds), W1S (Methane, environment), W1W (Sulphur compounds), W2S (Alcohols), W2W (Sulphur aromatic compounds), W3S (high concentrations >100 ppm, methane).

Name of the sensor	Sensitive substances	Reference
W1C	Aromatic compounds	Toluene, 10 ppm
W5S	Broad range sensitivity, nitrogen oxides	NO_2, 1 ppm
W3C	Ammonia	Propane, 1 ppm
W6S	Hydrogen, breath gases	H_2, 100 ppb
W5C	Alkanes, aromatic compounds, less polar compounds	Propane, 1 ppm
W1S	Methane (environment)	CH_3, 100 ppm
W1W	Sulfur compounds, terpenes, limonene, pyrazine	H_2S, 1 ppm
W2S	Alcohols, a broad range of aromatic compounds	CO, 100 ppm
W2W	Sulfur aromatic compounds	H_2S, 1 ppm
W3S	Reacts on high concentrations >100 ppm, methane	CH_3, $10CH_3$, 100 ppm

Table 4.1 Sensors' description of the used e-nose **[6].**

Figure 4.3: PEN e-nose during an analysis of a group of strawberries (10 vials).

Each batch of berries was split into ten samples, of 10.0± 0.1 g, and placed in conventional clear glass vials (40 ml), which were hermetically sealed with a PTFE/silicone septum and a screw cover.
The vials underwent a one-hour equilibration period at room temperature (26 °C) and analyzed at the same temperature in standardized conditions. At a rate of 400 mL/min for 30 s, the device probe vacuumed the volatile components from the vial headspace through the sensor array. Before injecting the next sample, the system was purged for 70 s at a flowrate of 600 mL/min with filtered air, allowing the instrument to re-establish a baseline.
The Principal Component Analysis (PCA) of the data and hierarchical cluster on the first three factors were performed using the XLSTAT statistical software package, version 2020.5 (Addinsoft, Paris, France).

4.2.11 Oxidation Stress Analysis

Malondialdehyde test

Malondialdehyde (MDA) is the most used oxidative stress test for assessing the antioxidant activity of substances in lipid peroxidation systems. MDA is produced when polyunsaturated fatty acids undergo oxidative degradation in acidic environments. It then reacts with thiobarbituric acid (TBA) to form the MDA-TBA adduct **[7].**

CTR, CTR + 3h LED, CICU1, CICU2, CICUB1 and CICUB2 groups were tested. A control (only with Linolenic acid) was prepared following the same procedure without a test sample. The tested samples consisting in 100 µl of homogenized berries were added to 0.1mL of distilled water, 0.2mL of 4% sodium dodecyl sulfate (SDS), 1.5 mL of Phosphoric acid and 100µl of 0.1% linoleic acid. The

mixture was accomplished by adding 1 ml of TBA solution, and it was then heated for 45 minutes at 100°C. After cooling on an ice bath, 4ml of 1-butanol was added to generate two layers from which the supernatant (containing the TBA-MDA adduct) was separated and were analysed by UV-spectrophotometer (Duo Beckman) at 535nm.

For the MDA standard curve the same process was used, with the exception that instead of adding 100 µl of the sample solution, the standard solution (1,1,3,3 tetra methoxy-propane) was added.

DPPH Free Radical Scavenging Activity

Curcumin's antioxidant activity was investigated when it was sprayed on blueberries and strawberries by testing its ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH). Curcumin's DPPH free radical scavenging capacity was also assessed three hours after exposure to blue light. A lipophilic free radical known as DPPH exhibits purple coloration in solution and UV absorption at a wavelength of 517 nm. When DPPH undergoes a redox reaction with an antioxidant, it turns yellow with a decrease in absorbance at 517 nm.

To perform the DPPH free radical scavenging assay, 100 µL of each sample (i.e. homogenized berries sprayed with curcumin) were added to a DPPH methanolic solution (900 µL, 0.1 mM) and incubated at room temperature for 30 min in the dark. Absorbance was measured at 517 nm against a blank using a spectrophotometer. All experiments were performed in triplicate. The percentage of antioxidant activity was calculated according to the following formula:

DPPH scavenging (%) = [(A DPPH - A sample)/A DPPH] X 100.

4.3 Statistical analysis

The results of repeated microbiological experiments (in triplicate) were reported as averages with standard deviations. Differences between observation times were studied with ANOVA, while comparisons between curcumin concentrations and between oxidative tests were evaluated by t-test. The p values were reported, considering a type I error of 5%. Calculations were done using SAS® Statistic Software v 9.4.

4.4 RESULTS

4.4.1 Phase Solubility Study

The solubility characteristic of curcumin increases in proportion to the amount of β-cyclodextrin. The curcumin/βCD inclusion complex's A_L type of solubility phase profile, as described by **[1],** shows a linear rise in solubility as a function of β-cyclodextrin concentration **(Figure 4.4).** The resulting value of Ks = 834.4 M-1 assessed that curcumin was satisfactorily solubilized.

The slope of the resulting initial linear part of the solubility phase diagram was used to compute the relevant parameters.

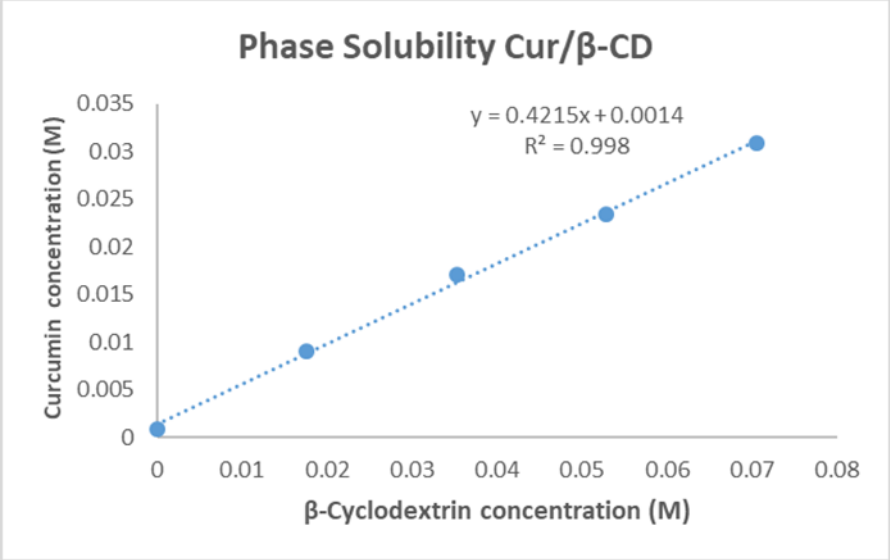


Figure 4.4: Phase-solubility diagrams of curcumin with βCD.

	Equation	R^2	K_s (M^{-1})	CE	Ratio	Type of curve
Curcumin / βCD (72h)	y = 0.4215x + 0.0014	0.998	834.4	0.59	1:1	A_L

Table 4.2: The linear part of the phase solubility diagram was used as the basis for determining the complexation constant (Kc), complexation efficiency (CE), and regression line equation.

4.4.2 Curcumin Concentration in Fruits after Treatment

The concentration of curcumin on berries was assessed in three distinct situations to determine if the spraying was effectively adheres to the fruit peel and is not degraded by LED light: immediately following the spraying and three hours after irradiation (with or without waiting 1 h between spraying

and lighting). This procedure was carried out for all berry varieties and curcumin concentrations in the spray solution (0.5 or 1 mg/ml).

Table 4.3 presents the findings in µg of curcumin per g of berries. P values describe a comparison between fruits of irradiated and non-irradiated, with or without the rest of 1 h. It is important to note that the final concentration is solely dependent on the fruit's surface area. Actually, the solution sprayed only covers the berry's surface, not its whole volume. A ratio of 0.5 between is expected assuming that the mean surface of blueberries is around half that of strawberries.

The higher amount of curcumin was observed when berries were exposed to LED radiation 1 hour after being sprayed, most likely as result of a better curcumin penetration across the peel. The difference between the two concentrations of strawberries and the 0.5 mg/ml concentration of blueberries is statistically significant, while the difference in 1 mg/ml concentration on blueberries is not statistically significant. This is most likely caused by a maximum carrying capacity that is achieved in the case of blueberries. The amount of curcumin observed per gram of fruit obviously increased when the curcumin concentration in the sprayed liquid was doubled.

	Blueberries				**Strawberries**			
	Spray 0.5 mg/ml	**p-value**	**Spray 1 mg/ml**	**p-value**	**Spray 0.5 mg/ml**	**p-value**	**Spray 1 mg/ml**	**p-value**
Curcumin+β-cyclodextrin spray	0.69 ± 0.01 µg/g		0.9 ± 0.3 µg/g		0.35 ± 0.01 µg/g		0.39 ± 0.01 µg/g	
Curcumin+β-cyclodextrin spray, blue light with LED 3h	0.6 ± 0.2 µg/g	0.29	1.16 ± 0.05 µg/g	0.14	0.33 ± 0.01 µg/g	0.03	0.47 ± 0.03 µg/g	0.004
Curcumin+β-cyclodextrin spray, 1 h rest, blue light with LED 3h	1.00 ± 0.05 µg/g	<0.0001	1.09 ± 0.08 µg/g	0.22	0.49 ± 0.02 µg/g	<0.0001	0.82 ± 0.02 µg/g	<0.0001

Table 4.3: Concentration of curcumin on berries, evaluated at different times.

4.4.3 Preliminary Experiment (E0):

Antibacterial effectiveness of Curcumin and LED on Blueberries

The results showed that both bacterial and fungal growth were clearly inhibited in the third experimental condition that comprised treating blueberries with a curcumin+β cyclodextrin solution and exposing them to LED light for 6 hours **(Figures 4.5-6).**

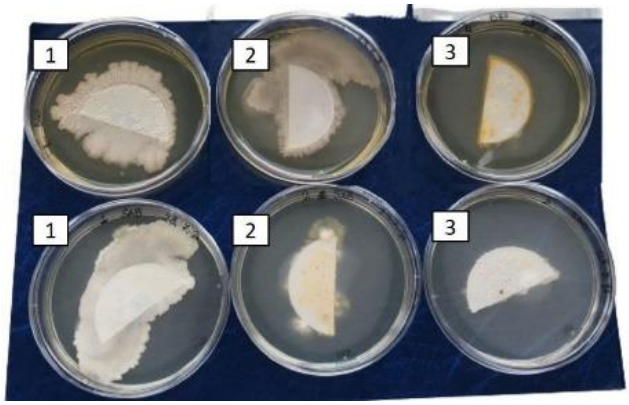


Figure 4.5: Filter paper-based microbial growth on BHA (above) and SAB (below) plates: **1.** Control (washing water from blueberries in a cyclodextrine solution), **2.** Washing water from Blueberries treated with Curcumin/β-CD solution 3. Washing water from blueberries treated with Curcumin /β-CD solution- along with 6h blue LED light.

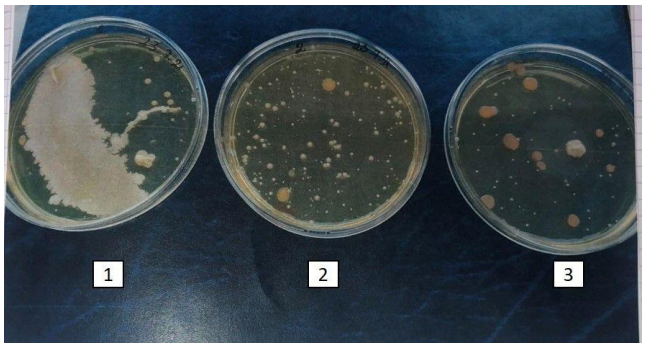


Figure 4.6: Microbial growth on BHA plates using unfiltered washing water: 1. Control, 2. Curcumin+Cyclodextrin solution treated blueberries, 3. Blueberries treated with cyclodextrin and curcumin solution along with 6 h Led light irradiation.

Qualitative Analysis (Organoleptic)

Table 4.4 shows the outcomes of the three samples' organoleptic quality analysis. Sample 1 that was not rinsed with curcumin, has not changed taste smell and odour. Sample 2 that was rinsed with curcumin solution showed that its organoleptic quality remained unaltered. The organoleptic

characteristics of the last sample 3 remained unchanged after being rinsed with curcumin and exposed to blue LED light for 6 hours.

	1	2	3
Subject	**V/O/T/G**	**V/O/T/G**	**V/O/T/G**
A	1/1/1/1	1/1/1/1	1/1/1/1
B	1/1/1/1	1/1/1/1	1/1/1/1
C	1/1/1/1	1/1/1/1	1/1/1/1
D	1/1/1/1	1/1/1/1	1/1/1/1

Table 4.4: Three samples' organoleptic characteristics: 1. Control Blueberries (without any treatment); 2. Blueberries washed with curcumin; 3. Blueberries washed with curcumin and exposed to blue LED light for 6 hours; O: Olfaction, V: Sight, G: Taste, T: Touch; 0 indicates a change in the characteristic, 1 indicates no change in the characteristic.

These findings were based on the assessment of four volunteers in order to understand the differences between berries in the control group and treated, however there is a high chance that human errors or low sensitivity contributed to the findings. According to the reports, human nose possesses 400 smell receptors and is capable of detecting at least one trillion smells **[8].** The human nose can rank smells, but since people's opinions might be biased, it cannot for instance detect harmful gases. The human nose also has limits on what gases it can detect. Because of these limitations, the human nose cannot be used as a universal tool for all smell-related categorization and discrimination. So, that's the reason why e-nose technique was used for the E1, E2, and E3 experiments.

4.4.4 Full experiment

Microbial analysis on Blueberries and Strawberries

Microbiological studies were classified into "Berry" and "Peel" types. "Berry" refers to the examination of the smoothed berry, and "Peel" to the water in which the entire berry was washed. The test revealed that there were less bacteria in the CICU, CICUB, and CICUBm groups, particularly in the E3 experiment, which takes into account the greater curcumin content **(see Table 4.5).** In this instance, the variations between the groups and the times are statistically significant (p-values for washing water and smoothed fruit, respectively, are 0.003 and 0.01). However, the differences in E1 and E2 experiments, with the lowest curcumin concentration, were not statistically significant

(p>0.05). Curcumin at a concentration of 0.5 mg/ml probably was not enough to kill bacteria for an extended period of time.

Experiment	Group	Washing water (peel)					Smoothed berry (5 g)				
		0	24	48	72	p-value	0	24	48	72	p-value
Strawberries (1 mg/ml)	CTR	4.4	3.3	3.5	4.6	0.66	2.3	2.8	2.9	2.6	0.66
	CI	4.6	2.7	3.9	5.1		1.9	2.8	2.1	3.0	
	CICU	4.2	4.0	4.2	4.0		3.0	2.0	3.2	2.2	
	CICUB	4.1	3.6	4.1	3.4		2.2	2.5	2.7	2.0	
Blueberries (1 mg/ml)	CTR	5.4	5.3	6.9	6.0	0.94	1.5	3.1	3.3	5.1	0.60
	CICU1	4.7	3.5	3.3	4.6		2.9	2.1	1.6	1.6	
	CICUB1	5.8	4.7	7.0	4.8		2.1	4.1	2.8	1.6	
	CICUBm1	2.9	3.2	4.4	4.8		1.4	1.6	2.8	2.9	
Blueberries (2 mg/ml)	CTR	3.3	3.7	5.8	3.9	0.003	2.1	2.0	3.1	3.1	0.01
	CI	5.0	5.4	4.1	4.9		3.0	3.0	2.9	2.9	
	CICU2	4.2	4.3	5.6	3.8		2.5	2.4	3.2	2.6	
	CICUB2	3.2	3.9	3.7	3.4		2.3	2.7	2.4	2.5	
	CICUBm2	3.3	3.4	4.7	3.6		2.4	2.7	3.0	2.5	

Table 4.5: Results of the three experiments. Values are in logarithms of the number of bacterial colonies at T0 and after 24, 48, and 72 h, for both peel and smoothed berries.

Odour Analysis

Concerning the odor analysis, the first two main components of PCA showed 75.38% and 84.87% of the variance of data respectively in strawberries and blueberries' cases (**Figure 4.7A-B** shows the biplots in output from PCA analysis on top).

The two principal components are F1 (sensors: W1C, W3C, W6S, W1W, W2W, W3S) and F2 (sensors: W5S, W5C, W1S, W2S). These two components can be given a practical meaning, as done by **[9]** in a related situation. In reality, methane, ammonia, and breath gases have a significant influence on F1, which signifies the 'degradation' of berries. The 'fruitiness' is indicated by F2 because it identifies esters, or the berries' natural odor. The radar graphs use these definitions **(Figure 4.8).**

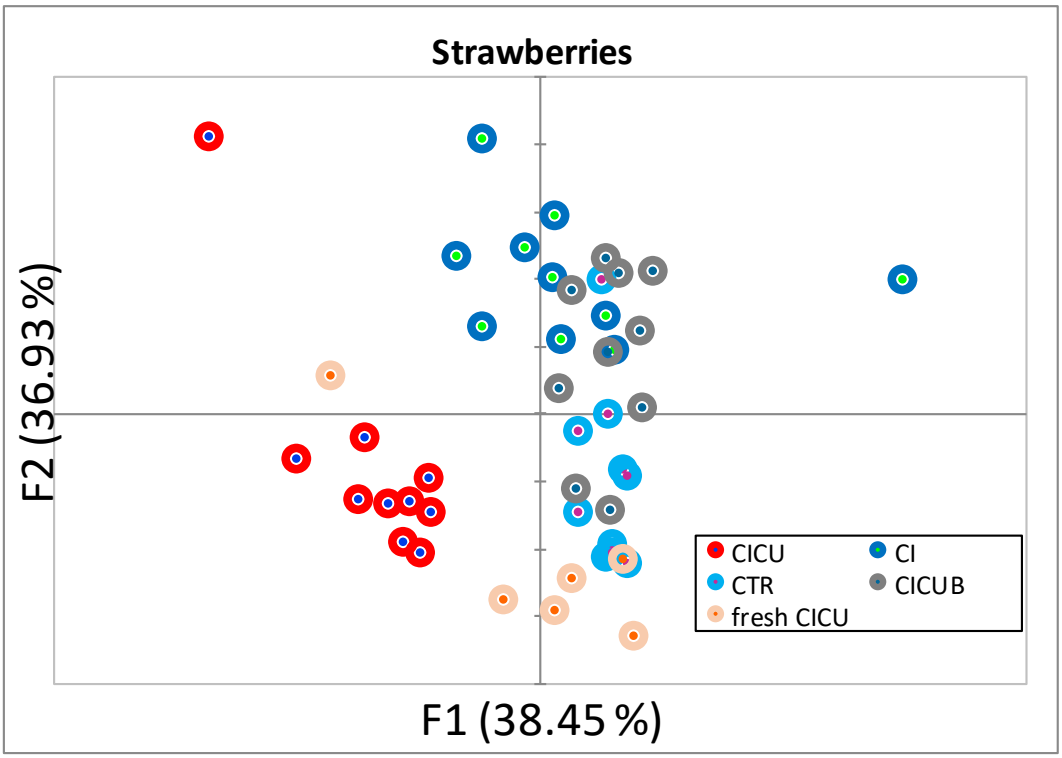
In particular, CTR berries (light blue circles in top **Figures 4.7A-B**) are not distinguishable by CICUB (grey circles) ones, in both experiments, showing an overlap of odors of the two groups. CI (blue circles) changes in the F1 component, probably because of the deodorant effect of β-cyclodextrin.

The CICU group (red circles) is distinguishable from CTR in strawberries but not in blueberries. The CICUBm group has a strong F2 component, showing an alteration of the odor. Considering the hierarchical clusters (bottom Figures 7A-B), calculated on factor scores, an overlap of groups is seen in the strawberries' case. Indeed, two major clusters are found: the first one contains CTR, CICU, and fresh CICU (in prevalence), but also some CICUB samples; the second one CI and CICUB (in prevalence), but also CTR and CICU samples. In the blueberries' case, CICUBm creates a very strong cluster, while the other groups are in another one. No significant changes in odor are therefore assessed in the two cases, except for the CICUBm group. Multiple irradiations probably significantly degrade the berries.

In conclusion, CI (blue area) does not exhibit any antimicrobial effect, but it is interesting to note the cluster created by the CI (blue) group enhancing the 'fruitiness' of the samples is a natural deodorizing product that can mask the volatile compound of berries. The addition of curcumin (CICU, red area), although impacting the odor characteristics, improves the microbial resistance especially at 72 h both in the fruits and in the washing water.

LED blue light irradiation (CICUB, grey area) further enhances the antimicrobial effect at all times. The repetition of LED irradiation on different days (CICUBm, yellow area) does not radically modify the microbiological effectiveness but only worsens the fruit's pleasantness.

7A.



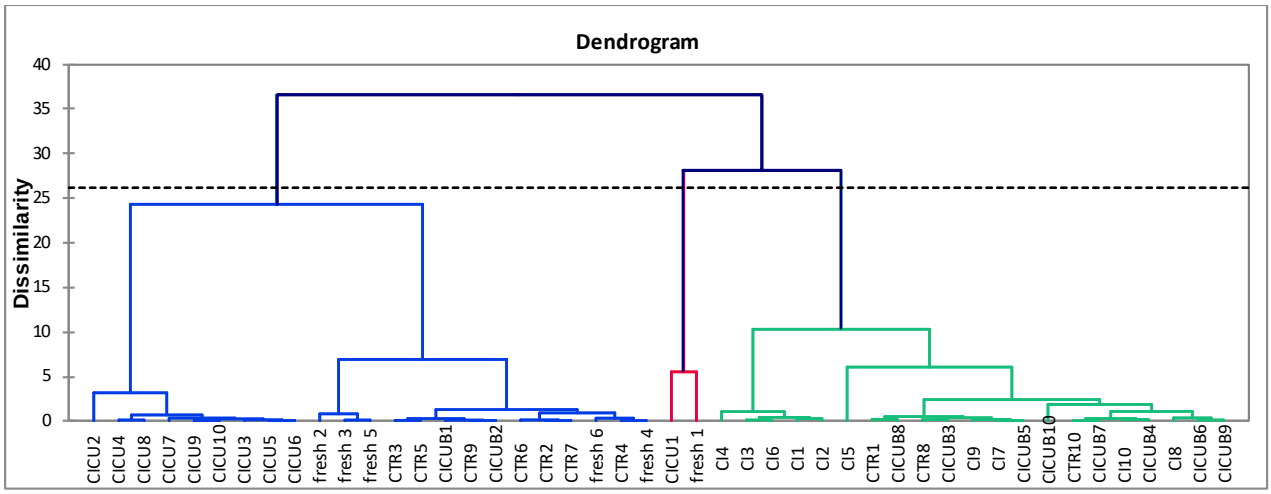
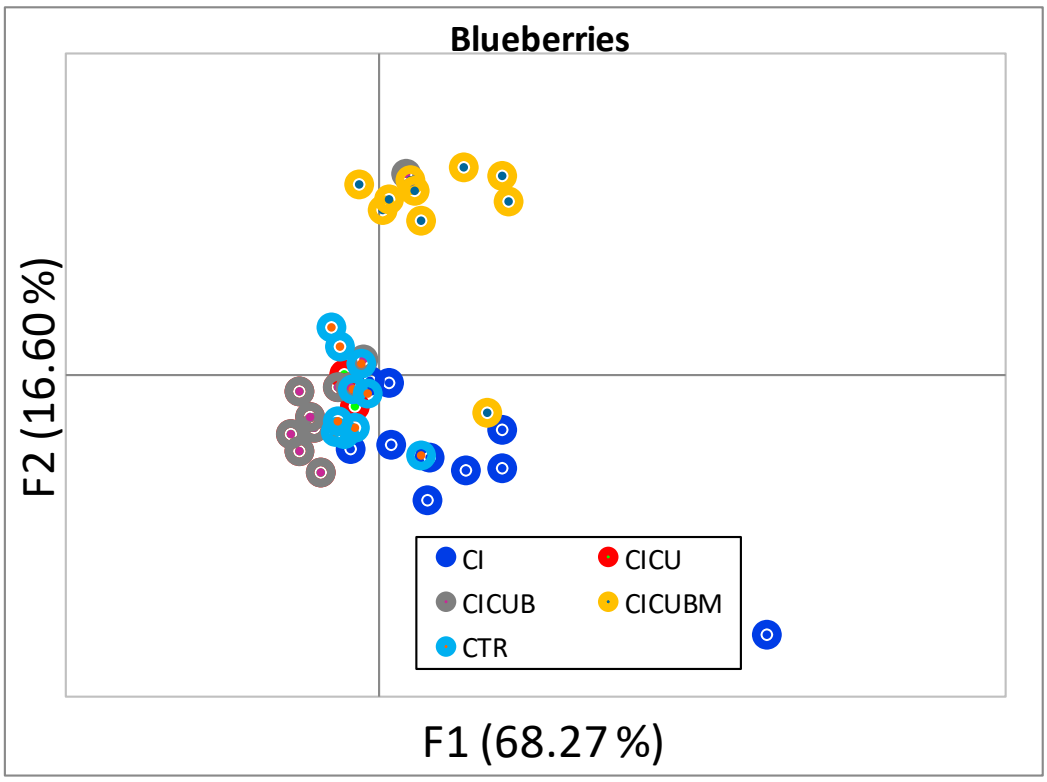


Figure 4.7A. Top: PCA analysis of strawberries from e-nose data. Each dot represents a different sample. Different colors represent different treatment groups. Bottom: hierarchical cluster based on factor scores.

7B.



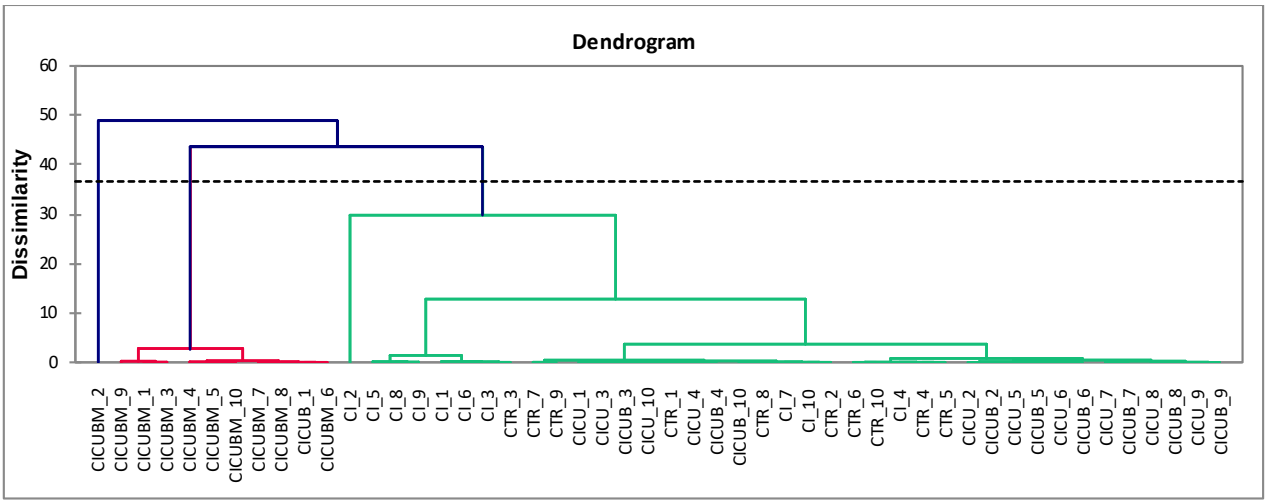


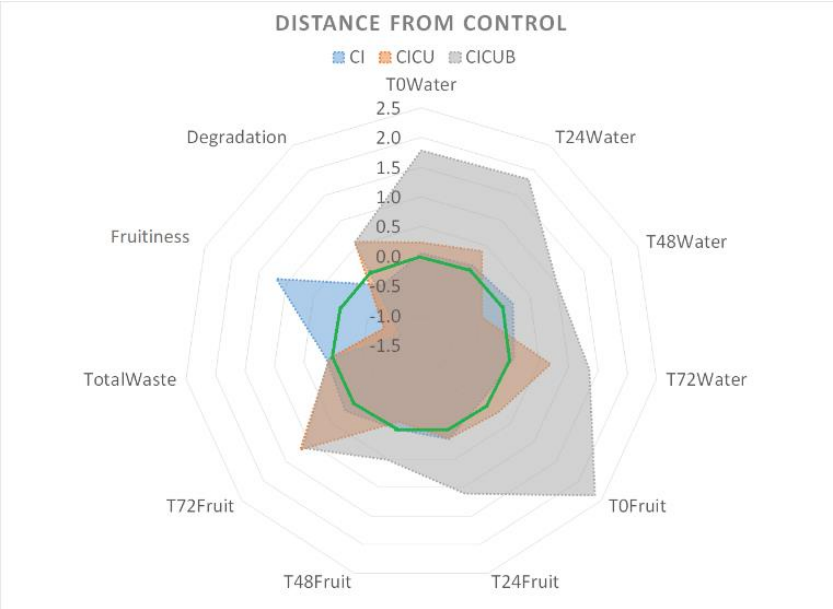
Figure 4.7B. Top: PCA analysis of blueberries from e-nose data. Each dot represents a different sample. Different colors represent different treatment groups. Bottom: hierarchical cluster based on factor scores.

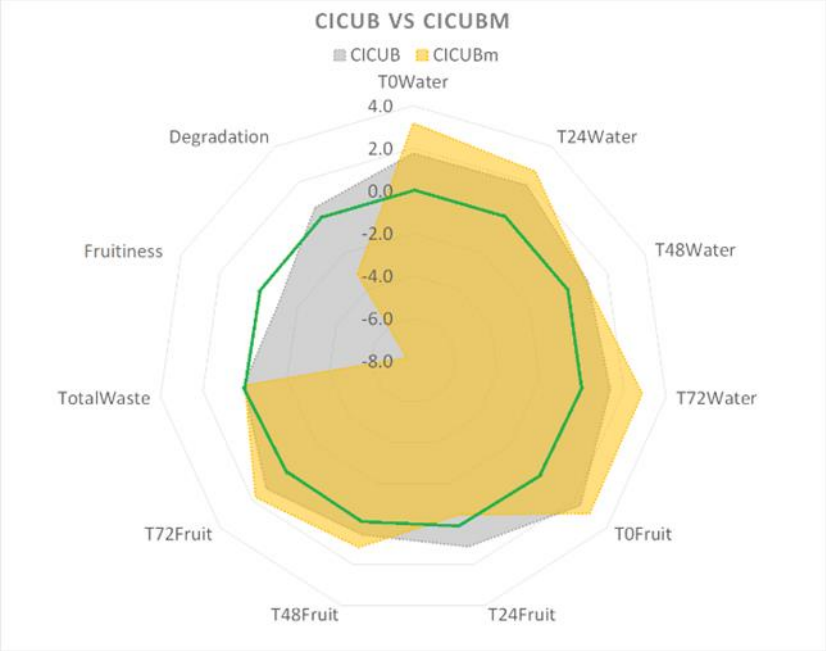
Global Gain Analysis

Figure 8 summarizes the outcomes of the three studies using radar graphs, while related images are displayed above (**Figure 4.8A & B**). In order to compare the three tests, which differ from one another in terms of methodologies and berries, radar charts were employed. Radar graphs are actually created by normalizing the data in comparison to the control, which allows data from many tests to be comparable, as is discussed in more detail in the following paragraphs.

To summarize the results, radar graphs in Figure 8A-B are produced by normalizing data concerning the CTR group (green line). The area contained within the green line assesses to what extent the treatment is less performant than no treatment, while the area that extends outside the green line quantifies how the treatment is more efficient than the control.

8A.





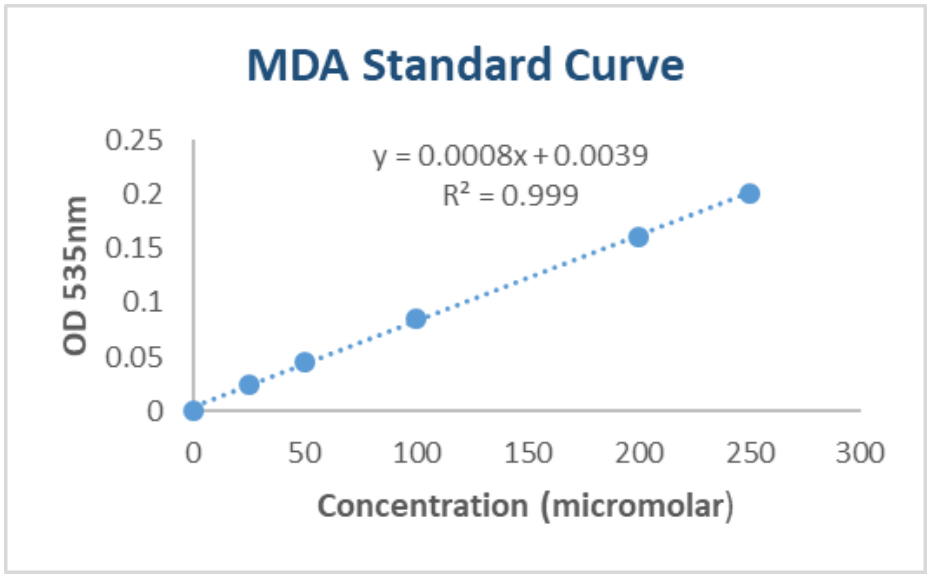
8B.

Figure 4.8: A) Radar graph about CI (blue area), CICU (red area) and CICUB (grey area) in respect of CTR (green line). Blueberries and strawberries were both examined. All of the points outside of the green line indicate a better behaviour with respect to CTR, whilst the points inside the curve indicate a worse behaviour **B)** Radar graph about CICUB (grey area) and CICUBm (orange area) in respect of CTR (green line). A better behaviour with respect to CTR is shown by all the points outside of the green line, whereas a worse behaviour was indicated by all points inside the line.

4.4.5 Oxidative Stress Evaluation

In MDA test less oxidative stress was detected in CICU1, CICU2, CICUB1 and CICUB2 (57 and 32 PPM, respectively) compared to CTR (220 PPM) and irradiated CTR with blue light (132 PPM) **(Figure 4.9B).** DPPH test partially confirms this result, showing a better antioxidant activity in CICUB groups, while a worse one in CICU groups **(see Table 4.6).** Indeed, the curcumin may potentiate the antioxidative properties of the berries. Moreover, although LED irradiation is responsible for some oxidative stress as expected, the curcumin (at both concentrations) counteracts such effect by keeping the oxidative stress lower than the control values.

9A.



9B.

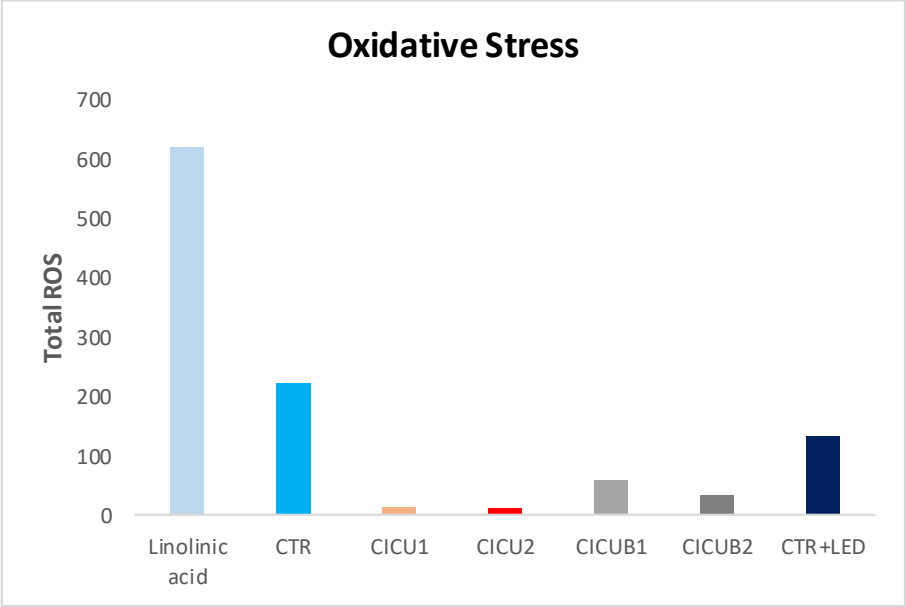


Figure 4.9: A) calibration plot; **B)** oxidative stress in the five standard berries groups (CTR, CICU1, CICU2, CICUB1 and CICUB2) compared with standard control (linoleic acid) and berries+blue LED. The treatment (grey bars) reduces the oxidative stress with respect to controls.

DPPH Scavenging (%)	**Strawberries (%)**	**Blueberries (%)**	**Only curcumin (%)**
CTR	78 ± 3	76 ± 3	50 ± 2
CTR + blue LED	78 ± 2	79 ± 1	89 ± 2
CICU1 (0.5 mg/ml)	71 ± 1	77 ± 2	
CICUB1 (0.5 mg/ml)	83 ± 3	76 ± 3	
CICU2 (1 mg/ml)	76 ± 2	73 ± 2	
CICUB2 (1 mg/ml)	84 ± 1	83 ± 3	

Table 4.6: DPPH scavenging (%) of treated/not treated blue- and strawberries.

4.5 DISCUSSION AND CONCLUSION

The results from this preliminary investigation confirm that curcumin is effective for berries preservation, reducing microbial degradation without significantly affecting the odour and antioxidant properties.

After showing that curcumin was satisfactorily solubilized, with a complexation Ks = 834.4 M-1 **(Table 4.2),** and that curcumin was effectively delivered to the berries, some further considerations can be drawn from the global gain analysis.

Microscopic investigation of the CICU, CICUB, and CICUBm groups demonstrated a decreased amount of bacterial colonies, specifically in the E2 and E3 tests, which employed blueberries (**Table 4.5**). Strawberries typically respond less effectively to the treatment because of their surface's greater porosity and irregularity. The assessment of curcumin concentration after treatment is also interesting, showing that it remains on the berry's surfaces performing its antibacterial effect for a long time. A larger concentration in the solution or a slower release, e.g. by curcumin incorporation in more long-lasting complexes, will keep on controlling the bacterial concentration on fruits possibly prolonging berries' shelf life.

In terms of the odour analysis, the first two major PCA components : F1 (named ‘degradation’) and F2 (named ‘fruitiness’) explain most of the data variances 75.38% and 84.87%, respectively, see **Figure 4.7**.).

The radar graphs in **Figure 4.8 A-B** show that CI (blue area) has no antimicrobial effects, but enhances the "fruitiness" of the samples acting as a natural deodorant, which hides the volatile chemical of berries. Despite having an effect on the organoleptic qualities, the addition of curcumin (CICU, red area) increases microbial resistance, particularly at 72 hours, in both the fruits and the washing water.

The antibacterial action is constantly enhanced by the use of LED blue light (CICUB, grey region). Repeated LED irradiation on several days (CICUBm, yellow region) does not significantly alter the microbiological efficacy; instead, it merely makes the fruit less appealing. For all groups, the amounts of berries discarded throughout the experiments were comparable.

The proposed treatments are safe, effective and the organoleptic and antioxidant properties of the berries are retained.

In conclusion, also in the case of berries, we confirm the consolidated lettering and the results already present in the literature. Curcumin is used and recognized worldwide for its wide range of potential health benefits in many different ways. For example, curcumin has been used as a colorant in China, a beverage additive in Korea, an anti-inflammatory in Pakistan, an antiseptic in Malaysia, and as a

preservative and colouring agent in cheese, chips, and butter in the United States. Additionally, curcumin can also be found in a variety of products, such as capsules, energy drinks, ointments, soaps, and tablets **[10].** The US Food and Drug Administration (FDA) has designated curcuminoids as "Generally Recognized As Safe" (GRAS) **[10].** Because of its several potential advantages, curcumin was employed in this study as a dose for red fruits. It is known that curcumin may have both antibacterial qualities and displays photodynamic properties which would increase the effectiveness of its antimicrobial activities **[11].**

According to the early findings of this study, curcumin effectively preserves berries by minimizing microbial decay without dramatically changing the odour or antioxidant qualities.

The antibacterial impact is further enhanced by photodynamic activation in a targeted manner. We can therefore extend to berries, which are highly expensive and sensitive fruits, a method that has been effective on other fruits, such as dates, mango, litchis and kumquat **[12-16],** vegetables as tomatoes and lettuce **[17]** and fresh-cut fruits **[18-20]**. After three hours of exposure to LED blue light, the β -cyclodextrin/curcumin inclusion complex demonstrated its ability to stick to the fruit peel and function as an efficient photosensitizer to induce PDI.

The organoleptic qualities of the fruits handled by this method are not harmful. The presence of the β-cyclodextrin/curcumin inclusion complex lowers the amount of ROS, protecting the inherent antioxidant qualities of berries provided by anthocyanins, even though the LED light, as predicted, created considerable oxidative products when applied to untreated berries **[21].**

Repeating the LED illumination every day for three times results in a further development of the antibacterial actions, but the berry's odour qualities drastically diminish. Additionally, it could be challenging to establish multi-illuminations throughout the supply chain. In fact, berries should be packed or at least crammed when being transported and stored in marketplaces, while their whole surface should be exposed to blue light for the treatment to be successful.

It's also noteworthy to measure the curcumin concentration after treatment since it demonstrates how long curcumin stays on berries to exert its antibacterial effects. In order to effectively manage the amount of microbes on fruits and extend the shelf life of berries, the solution's concentration should be higher or its release should be slower.

The solution's ingredients are all organic, risk-free, and have no known side effects **[22].** Additionally, the developed complex is a solution and does not contain nanodimensional ingredients, which the European Food Safety Authority (EFSA) restricts in some circumstances since they may pose a harm to human health **[23].**

Further investigations, are ongoing. We aim to specifically shorten the illumination period to make the treatment practical for companies. In fact, the current production process cannot support a three-hour product halt in order to photoactivate the curcumin. In order to be effective, the treatment period must be decreased to less than an hour. We are investigating a number of options, such as photoactivating curcumin while spraying it or using other nanostructures to increase the curcumin concentration and omit the illumination step. Additionally, more research about ~~use of~~ nanocarriers able to slowly release curcumin is planned.

Finally, despite a number of limitations, such as the brief observation period and the small number of samples, our experiments have demonstrated that the treatment based on the inclusion of β-cyclodextrin and curcumin and LED blue light irradiation is a promising way to prolong the shelf-life of berries without changing their aroma.

References

1. Higuchi T.,"*Phase-solubility techniques",*Adv. Anal. Chem. Instr., 1965. **4**: 117-212.
2. Argenziano M., Haimhoffer A., Bastiancich C., Jicsinszky L., Caldera F., Trotta F., Scutera S., Alotto D., Fumagalli M.Musso T.,"*In vitro enhanced skin permeation and retention of imiquimod loaded in β-cyclodextrin nanosponge hydrogel",*Pharmaceutics, 2019. **11**(3): 138.
3. Yang Z., Argenziano M., Salamone P., Pirro E., Sprio A.E., Di Scipio F., Carere M.E., Quaglino E., Cavallo F.Cavalli R.,"*Preclinical pharmacokinetics comparison between resveratrol 2-hydroxypropyl-β-cyclodextrin complex and resveratrol suspension after oral administration",*Journal of Inclusion Phenomena and Macrocyclic Chemistry, 2016. **86**: 263-271.
4. Fan L.Song J.,"*Microbial quality assessment methods for fresh-cut fruits and vegetables",*Stewart Postharvest Rev, 2008. **4**(3): 1-9.
5. Comini S., Scutera S., Sparti R., Banche G., Coppola B., Bertea C.M., Bianco G., Gatti N., Cuffini A.M.Palmero P.,"*Combination of Poly (ε-Caprolactone) Biomaterials and Essential Oils to Achieve Anti-Bacterial and Osteo-Proliferative Properties for 3D-Scaffolds in Regenerative Medicine",*Pharmaceutics, 2022. **14**(9): 1873.
6. Torri L., Rinaldi M.Chiavaro E.,"*Electronic nose evaluation of volatile emission of Chinese teas: from leaves to infusions",*International journal of food science & technology, 2014. **49**(5): 1315-1323.
7. Yen G.-C., Chang Y.-C.Su S.-W.,"*Antioxidant activity and active compounds of rice koji fermented with Aspergillus candidus",*Food chemistry, 2003. **83**(1): 49-54.
8. Bushdid C., Magnasco M.O., Vosshall L.B.Keller A.,"*Humans can discriminate more than 1 trillion olfactory stimuli",*Science, 2014. **343**(6177): 1370-1372.
9. Aghilinategh N., Dalvand M.J.Anvar A.,"*Detection of ripeness grades of berries using an electronic nose",*Food science & nutrition, 2020. **8**(9): 4919-4928.
10. Gupta S.C., Patchva S.Aggarwal B.B.,"*Therapeutic roles of curcumin: lessons learned from clinical trials",*The AAPS journal, 2013. **15**: 195-218.
11. Neelakantan P., Cheng C.Q., Ravichandran V., Mao T., Sriraman P., Sridharan S., Subbarao C., Sharma S.Kishen A.,"*Photoactivation of curcumin and sodium hypochlorite to enhance antibiofilm efficacy in root canal dentin",*Photodiagnosis and Photodynamic Therapy, 2015. **12**(1): 108-114.
12. Sarwar S., Netzel G., Netzel M.E., Mereddy R., Phan A.D.T., Hong H.T., Cozzolino D.Sultanbawa Y.,"*Impact of curcumin-mediated photosensitization on fungal growth,*

*physicochemical properties and nutritional composition in Australian grown strawberry",*Food Analytical Methods, 2021. **14**: 465-472.

13. Huang G., Huang L., Geng C., Lan T., Huang X., Xu S., Shen Y.Bian H.,"*Green and multifunctional chitosan-based conformal coating as a controlled release platform for fruit preservation",*International journal of biological macromolecules, 2022. **219**: 767-778.
14. Huang L., Yong K.W., Fernando W.C., Carpinelli de Jesus M., De Voss J.J., Sultanbawa Y.Fletcher M.T.,"*The inactivation by curcumin-mediated photosensitization of Botrytis cinerea spores isolated from strawberry fruits",*Toxins, 2021. **13**(3): 196.
15. Li M., Wang M., Hu S., Sun J., Zhu M., Ni Y.Wang J.,"*Advanced Coatings with Antioxidant and Antibacterial Activity for Kumquat Preservation",*Foods, 2022. **11**(15): 2363.
16. Al-Asmari F., Mereddy R.Sultanbawa Y.,"*The effect of photosensitization mediated by curcumin on storage life of fresh date (Phoenix dactylifera L.) fruit",*Food Control, 2018. **93**: 305-309.
17. de Oliveira E.F., Tikekar R.Nitin N.,"*Combination of aerosolized curcumin and UV-A light for the inactivation of bacteria on fresh produce surfaces",*Food Research International, 2018. **114**: 133-139.
18. Chai Z., Zhang F., Liu B., Chen X.Meng X.,"*Antibacterial mechanism and preservation effect of curcumin-based photodynamic extends the shelf life of fresh-cut pears",*LWT, 2021. **142**: 110941.
19. Zou Y., Yu Y., Cheng L., Li L., Zou B., Wu J., Zhou W., Li J.Xu Y.,"*Effects of curcumin-based photodynamic treatment on quality attributes of fresh-cut pineapple",*LWT, 2021. **141**: 110902.
20. Lin Y., Hu J., Li S., Hamzah S.S., Jiang H., Zhou A., Zeng S.Lin S.,"*Curcumin-based photodynamic sterilization for preservation of fresh-cut Hami melon",*Molecules, 2019. **24**(13): 2374.
21. Badria F.A.Blumenberg M., *Phenolic Compounds: Chemistry, Synthesis, Diversity, Non-Conventional Industrial, Pharmaceutical and Therapeutic Applications* 2022: BoD–Books on Demand.
22. Chainani-Wu N.,"*Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa)",*The Journal of Alternative & Complementary Medicine, 2003. **9**(1): 161-168.
23. https://ec.europa.eu/health/scientific_committees/opinions_layman/en/nanotechnologies/#6

CHAPTER 5: *IN VITRO* CHARACTERIZATION OF CURCUMIN-LOADED NANOBUBBLES AND EVALUATION OF MICROBIOLOGICAL ASSAYS WITH AND WITHOUT PHOTODYNAMIC BLUE LED LIGHT ACTIVATION

5.1 PURPOSE OF WORK

The objective of this study was the production and characterization of chitosan-shelled nanoformulations (i.e nanobubbles) loaded with curcumin to evaluate their antimicrobial properties against Gram positive and Gramnegative strains. Additionally, since curcumin is a naturally occurring photosensitizer, the potential synergistic effect with visible light (Light Emitting Diode) photodynamic activation was assessed on the chosen bacterial strains.

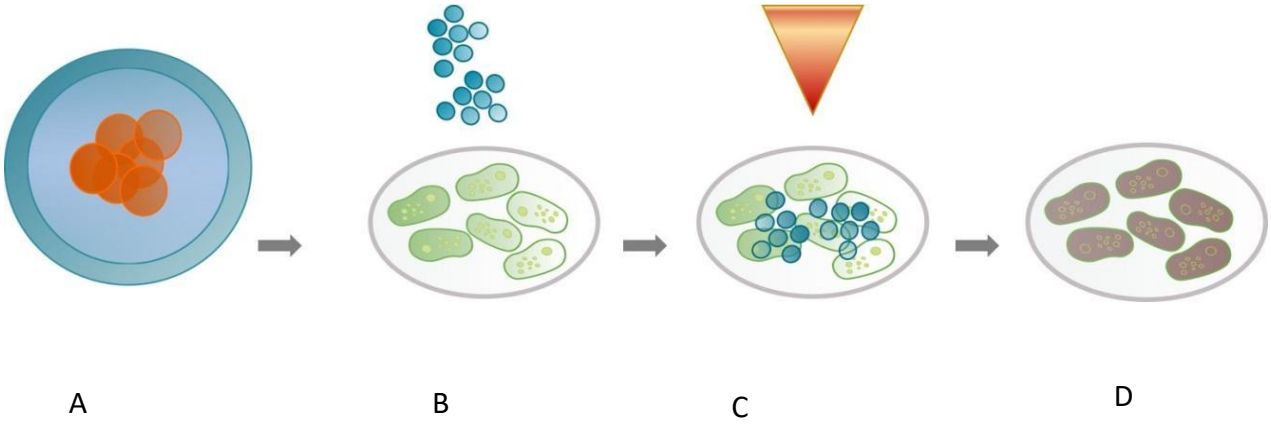


Figure 5.1: The planned procedure is depicted graphically: nanostructures containing Curcumin (A) interaction with pathogenic microbes (B), Curcumin-NB photo activation using LED light (C) increases the system's ability to be toxic to microorganisms (D).

5.2 MATERIALS AND INSTRUMENTS

5.2.1 Chemicals

Decaperfluoropentane and ethanol purchased from Sigma-Aldrich (St. Louis, MO, USA). Cargill kindly provided Epikuron 200® Palmitic acid was from Fluka (Buchs, CH). Curcumin (Mw =368.4), Chitosan, 1 methyl-2-pyrrolidinone, Oxoid SpA (Garbagnate Milanese, Italy) provided the Trypticase Soy Broth and Agar (TSB, TSA) and Sabouraud dextrose (SAB) broth and agar. The LDH release assay was carried out using the LDH cytotoxicity test kit (Cayman Chemical # 601170). Assay Buffer (Dissolve the Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of purified water. At room temperature, this buffer is stable for around a year). All additional chemicals that utilized but not listed above were analytical-grade and commercially accessible.

5.2.2 Bacterial Culture

The study was conducted on three different bacterial strains:

i. *Staphylococcus aureus* ATCC 29213 (Gram positive)
ii. *Enterococcus faecalis* ATCC 29212 (Gram positive)
iii. *Escherichia coli* ATCC 25922 (Gram negative)

The selected strains were seeded on Mannitol Salt Agar (MSA), Brain Hearth Infusion Agar (BHA), and MacConkey Agar (MAC), respectively and incubated at 37°C for 24h. Young colonies (18−24 h) were picked up to approximately 3−4 McFarland standard and inoculated into cryovials containing both cryopreservative fluid and porous beads to allow microorganisms to adhere. After inoculation, cryovials were kept at -80 °C for extended storage (Micro-bank, PRO-LAB Diagnostic, Canada).

5.2.3 INSTRUMENTATIONS

Dynamic Light Scattering

The average size and polydispersity index of the nanoformulations were ascertained by employing photon correlation spectroscopy (PCS) and dynamic light scattering (DLS) with a 90 Plus particle sizer (Brookhaven Instruments Corporation, USA) that outfitted with Mas option particle sizing software. The investigations were carried out at 25°C and a fixed scattering angle of 90°. The samples were properly dilute before each analysis. In the same equipment, an additional electrode was used to measure the zeta potential.

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) study, utilizing an HPLC system made up of a Shimadzu system equipped with a UV/Vis detector, was used to determine the quantitative amount of curcumin and the release of curcumin from nanobubbles. The procedure was specifically developed for curcumin. Details are given in **Table 5.1.**

Instrument	**Shimadzu system with UV/VIS detector**
Column	Agilent RP-C 18 (250 mm × 4.6 mm)
Moblie Phase	Acetonitrile and water (70:30 v/v)
Flow rate	1ml/min
Wavelength	425 nm
LC time	15 minutes
Retention time	13.06

Table 5.1: Basic information about HPLC.

LED System

Blue light emitted diode (LED) (LES Flex Strips LEDYDEL IP64, Turin, Italy) of wavelength 425 nm– 470 nm was used in this study **(Figure 5. 2).**

Figure 5.2: Blue led (LES Flex Strips LEDYDEL IP64, Turin, Italy).

5.3 METHODS AND PROCEDURES

5.3.1 Curcumin Nanobubbles Formulation Preparation

1. Preparation of Chitosan Nanobubbles Loaded with Curcumin in the Core

In order to produce the nanoformulations, ethanol was used to dissolve Epikuron 200® (Cargill) (3% w/w) and palmitic acid (0.5% w/w), which was used as a co-surfactant. An appropriate amount of curcumin solution was added in the mixture of Epikuron 200® and palmitic acid. Following that, 210 µl of decaperfluoropentane was gradually added. Using an Ultra-Turrax® high strength homogenizer, the entire mixture was homogenized for 2 minutes after adding the suitable amount of distilled water, resulting in the formation of a nanoemulsion. At 37°C, the nanoemulsion was saturated for 15 minutes. The nanobubbles' polymeric shell was then formed by dropwise addition of an aqueous solution of chitosan (2.7% w/w) in the nanoemulsion.

2. Method for Preparing Curcumin Conjugated Chitosan

By using the imine formation (Schiff base reaction) approach, curcumin conjugated chitosan (CCC) was formed **[1-5].** In order to prepare the conjugated complex, chitosan (2.7% w/w) was dissolved in acetic acid buffer pH 4.5 under stirring at 500 rpm. The chitosan mixture was then mixed with 0.1% curcumin solution that had been dissolved in 5 ml of ethanol. The reaction mixture was placed under continuous stirring for 30 min at 700 rpm and, after setting the temperature controller to 50°C, was maintained in continuous stirring and heat (60°C) overnight (**Figure 5.3**). The reaction mixture was cool down at room temperature.

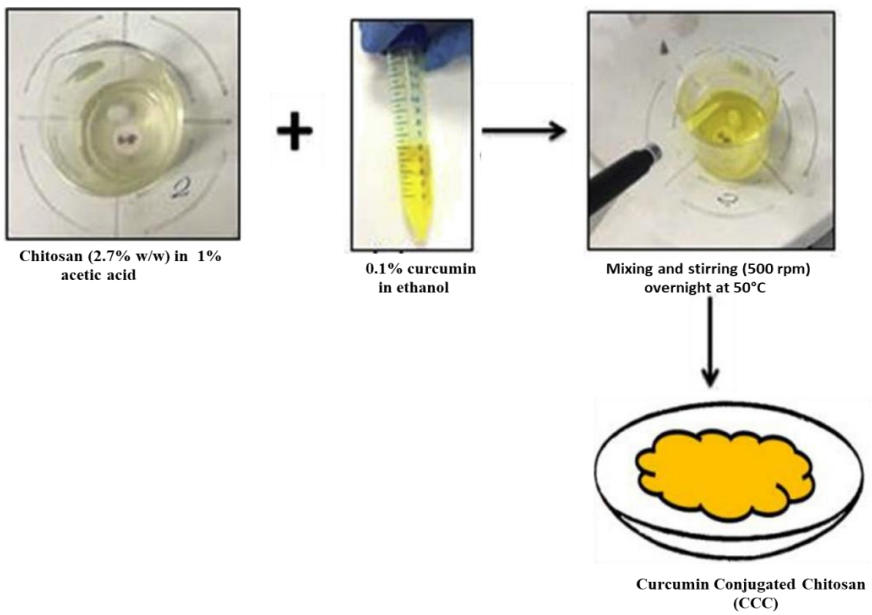


Figure 5.3: Curcumin Conjugated Chitosan preparation.

5.3.2 Nanobubbles Types

Table 5.2 lists the different types of NBs produced and tested in our Lab. Most of them were loaded with curcumin, with a concentration, which typically ranges from 10 µg/ml to 749 µg/ml, as well as with oxygen. Based on their MIC against bacteria we selected the most effective ones for additional studies.

Types of NB		**Curcumin in**		**Oxygen**
		core	Shell	
1	**Chitosan shell NB (blank)**	No	No	No
2	**Curcumin loaded chitosan shell NBs**	Yes	No	No
3	**Curcumin- conjugated-chitosan shell NBs**	No	Yes	No
4	**Curcumin loaded curcumin conjugated chitosan shell NBs**	Yes	Yes	No
5	**Curcumin oxygen loaded chitosan shell NBs**	Yes	No	Yes
6	**Curcumin oxygen loaded curcumin conjugated chitosan shell NBs**	Yes	Yes	Yes

Table 5.2: Detailed of the various NBs formulations that were investigated.

5.3 3 Physico-chemical Characterization of the Nanobubbles

In order to analyze the physicochemical properties the zeta potential and the particle size of the nanoformulations were taken into consideration. Dynamic light scattering spectroscopy (DLS) was used to assess the diameter, polydispersity index (PDI), and zeta potential of the NBs at ambient temperature. After dilution with ultrapure water (1:20 v/v), the analyses were conducted at a fixed angle of 90° and a temperature of 25 °C. The zeta potential was evaluated using an electric field of 15 V/m. The PDI indicates the size distribution within a population of nanobubbles.

5.3.4 Morphological analysis

The morphology of each NBs formulation was analyzed using fluorescence microscopy, taking advantage from the natural fluorescence properties of the curcumin, which emits light peaked around 543 nm **[6]**.

5.3.5 Encapsulation Efficiency (EE)

One hundred µl of curcumin-loaded NBs were put into a volumetric flask and were diluted using ethanol. The samples were ultrasonically disrupted for 15 minutes, and then 1 ml of the sonicated samples was centrifuged at 15000 rpm for 15 minutes and analyzed by spectrophotometer and HPLC. Encapsulation efficiency was calculated by subtracting the amount of free curcumin from the initial added amount, by using the equation below (Eq. 1)

$$\% \text{ EE} = (\text{Total drug} - \text{Free drug}) / (\text{Total drug}) \times 100 \qquad \textbf{(Eq. 1)}$$

5.3.6 Loading Capacity (LC)

In order to measure the loading capacity, freeze-dried NB samples were used. Around 5 mg of freeze-dried sample was weighed, and mixed in 1 ml of ethanol/methanol. After 15 minutes of sonication, samples were centrifuged for 5 minutes at 15000 rpm. A spectrophotometer was used to examine the absorbance at 425 nm of the sample's supernatant, and Equation 2 (Eq. 2) was used to determine the loading capacity. The loading capacity was also checked by HPLC using the extraction method.

$$\text{LC} = (\text{Total drug} - \text{Free drug}))/ (\text{NB weight}) \times 100 \qquad \textbf{(Eq. 2)}$$

5.3.7 Determination of Curcumin Concentration

The concentration of curcumin was calculated based on standard calibration curves. For producing the calibration curve an appropriate amount standard curcumin was weighed and mixed with methanol in a volumetric flask, to create a stock standard solution. The mobile phase was then used to dilute this solution, resulting in a series of standard solutions up to 50 μg/mL that were then injected into the HPLC system. Linear calibration curves for curcumin were obtained with regression coefficients of 0.999.

The chloroform extraction procedure was used to determine the curcumin concentration in the nanobubbles. This was done by combining 3 ml of the appropriate NBs with 3 ml of chloroform, vortexing the mixture for 1 minute, and then cooled in an ice bath. This procedure produced two layers: one aqueous with curcumin and the other with chloroform. After removing the chloroform layer, the extraction was repeated two or three more times (until the color turns white). Following the completion of the extraction, the chloroform was evaporated using a nitrogen pump. After removing the chloroform from the mixture, the dried curcumin was combined with 1 ml of methanol, and the HPLC was used to determine the pack area of the curcumin solution in the mobile phase. Following the determination of each sample's peak area, the calibration equation was applied to determine the concentration of curcumin indicated in **Table 5.5.**

5.3.8 Physical Stability

The physical stability of NBs kept at 4 °C was assessed for up to eight weeks by repeating the measurements of size, PDI, and zeta potential by dynamic light scattering.

5.3.9 *In Vitro* Curcumin Release Study

A multi-compartment rotating cell was used to test the in vitro curcumin release from curcumin-loaded NBs. Curcumin-loaded NBs were put inside the donor chambers. A semi-permeable cellulose membrane (cut-off 14 KDa) was used to separate the receiving and the donor chambers. Beta-cyclodextrin (5% w/v) was used as the receiving phase for all formulations. Moreover, throughout the 48-hour experiment, 1 mL of sample was extracted from the receiving phase at predefined intervals. After each extraction, the receiving phase was refilled with 1ml of cyclodextrin solution. HPLC (Shimadzu, UV/VIS detector SPD-20AV.) was used to monitor the curcumin release.

5.3.10 Antioxidant Activity Evaluation

Thiobarbituric acid Test

Antioxidant activity was evaluated by thiobarbituric acid assay (TBA). For the present study blank NB, Oxygen loaded NB, Curcumin loaded NB and Oxygen-curcumin loaded NB were tested. According to the procedure each test tube was filled with 0.1 ml of distilled water, 0.2 ml of sodium dodecyl sulfate (SDS) solution, 1.5 ml of phosphoric acid, and 100 ml of liposomes. Then 100 µl of the sample (NBs) was added to the appropriate test tube. The mixture was heated for 45 minutes at 100°C after adding 1 ml of the TBA solution (thiobarbituric acid). Before adding 4 ml of 1-butanol, samples were cooled down on an ice bath. After the butanol was added, two layers formed, from which the supernatant layer (TBA-MDA adducts) was separated. Using a UV spectrophotometer, the samples (supernatant layer) were examined at λ 535 nm.

5.3.11 Microbiological Assay

The MIC and MBC of NBs with and without exposure to 3h LED photodynamic activation were determined using the microdilution method according to the CLSI guideline (CLSI, 2007) in 96-well multiwell plates. For MIC an overnight culture of each bacterial strain was prepared. The inoculum was prepared to a turbidity of 0.5 McFarland standard (5 x 10^8 UFC/ml) that was further suspended in BHI media to get the concentration of bacteria cells of 10^5 UFC/mL ŧ further used in microdilution plates with diluted test compounds (i.e curcumin, NBs). In two microtiter 96-well plates an appropriate concentration of test compound was added in the first well that was further serially diluted. So, each well of microtiter plate contains different concentrations of each respective test compound. After, one plate was put under dark and the other one under LED system for three hours and then incubated for 24h at 37°C. A 10 µl of inoculum was taken from each well showing no visual growth after incubation; spotted onto BHA and incubated at 37°C for 24h. MBC was considered to be the lowest concentration of nanobubbles that completely inhibited bacterial growth.

5.3.12 Time Kill Kinetics

The antimicrobial nanobubbles bactericidal or bacteriostatic activity against a bacterial strain was evaluated using the Time-kill Kinetics test **[7]**. For time kill kinetics assays a bacterial inoculum of 10^5 cells/mL for each bacterial strain was prepared from overnight culture. Six well microtiters were prepared by using BHI media, each respective test compound at the concentration of 3MIC and bacterial inoculum. After one hour of incubation into the hood one plate was placed under LED irradiation and the other one under dark. Then, time kill kinetic was evaluated at different time (T0, 6h, 20h, 24h, 48h, and 72h).

5.3.13 Lactate Dehydrogenase Release Assay (Membrane Damage)

Lactate dehydrogenase (LDH) is a stable enzyme immediately released into the medium of the cell culture when the plasma membrane is ruptured **[8].** The LDH test, also known as the LDH release assay, is a cytotoxicity/cell death assay that assesses the severity of plasma membrane damage.

To detect LDH leakage into cell culture medium, a tetrazolium salt is utilized in this assay. It consists on two-step reactions. LDH catalyzes the reduction of NAD+ to nicotinamide adenine dinucleotide NADH and H+ by oxidizing lactate to pyruvate in the first step. The second step uses newly formed NADH and H+ in the presence of an electron acceptor to catalyze the reduction of a tetrazolium salt (INT) to a brightly coloured formazan that absorbs considerably at 490-520 nm **[9].** The amount of formazan produced directly corresponds with the amount of LDH released as a marker of cytotoxicity.

To perform this assay, a bacterial inoculum of 10^5 cells/mL for each bacterial strain was prepared from an overnight culture. Two microtiter plates (twenty-four-wells) were prepared using BHI media, each test compound (NBs, Curcumin) at a concentration of 3MIC, and the bacterial inoculum. After an hour of incubation under a hood, one plate was set up to be exposed to LED light for three hours, while another plate was set up to be placed into dark at room temperature. After the three hours, the cytotoxicity protocol was followed. All samples were centrifuged for 10 minutes at 13000 rpm along with samples for spontaneous release and maximum release, obtained by mixing 200 µl of bacterial suspension with 20 µl of assay buffer and 150µl of bacterial suspension with 45µl of Triton X-100 (3%), respectively. Supernatant (100 µl) was transferred to a new 96-well assay plate, and 100 µl LDH Reaction Solution was then added to each well. The plate was incubated at 37°C for 30 minutes while being gently shaken on an orbital shaker. After 30 minutes the optical density (O.D) was measured on a microplate reader at 490 nm. LDH was evaluated at T0, after 4h and at 24 h from incubation. Each experiment's findings were represented as "% cytotoxicity," which is a proportion of the overall quantity of LDH that was present in the target cells (Eq. 4).

% Cytotoxicity = **[**(Experimental Value) - (Spontaneous Release) **/** (Maximum Release) - (Spontaneous Release)**]** x 100 **(Eq. 4)**

5.4 Statistical Analysis

Results were shown as the mean ± standard deviation whenever data were presumed to be normally distributed (SD). For statistical analysis, Microsoft Office Excel was employed.

5.5 RESULTS

5.5.1 Physicochemical Characterization of NBs Formulations

All nanobubbles' physicochemical characteristics, such as average diameter, polydispersity, and zeta-potential, assessed using DLS are shown in **Table 5.3**. The average size was 521 nm. With the addition of oxygen, size diameter was slightly increased.

The chitosan on the NB surface was the reason of the positive value of the zeta potential. In comparison to the blank, the zeta potential reduced after the addition of the drug (curcumin), but the values were still high enough to prevent NB aggregation.

Additionally, the pH of all nanobubbles, which ranged from 4.8 to 5.4, was also assessed. Numerous NB types were prepared (with different concentration of curcumin) and characterized; however, only those containing a larger amount of curcumin were chosen for microbiological testing.

Formulation	**Average Diameter (nm)**	**Polydispersity Index**	**Zeta Potential (mV)**
Chitosan shell NBs (Blank)	438± 5	0.14 ± 0.06	+35 ± 1
Curcumin loaded chitosan shell NBs	472 ± 3	0.18 ± 0.01	+29 ± 2
Curcumin-conjugated-chitosan shell NBs	502 ± 6	0.19 ± 0.01	+30 ± 1
Curcumin loaded & curcumin-conjugated-chitosan shell NBs	511 ± 4	0.28 ± 0.01	+30 ± 2
Curcumin oxygen loaded chitosan shell NBs	519 ± 3	0.32 ± 0.01	+31 ± 1

Curcumin loaded & curcumin-conjugated-chitosan shell oxygen NBs	521 ± 3	0.39 ± 0.02	+31 ± 1

Table 5.3: The physicochemical characteristics of different formulations of NBs. The results were presented as mean ± SD.

5.5.2 Morphological Studies

All NBs formulations were examined for morphology using fluorescence microscopy. As shown in **Figure 5.4**, the chitosan-shelled NBs with curcumin conjugated to them exhibited a spherical morphology with well defined shells.

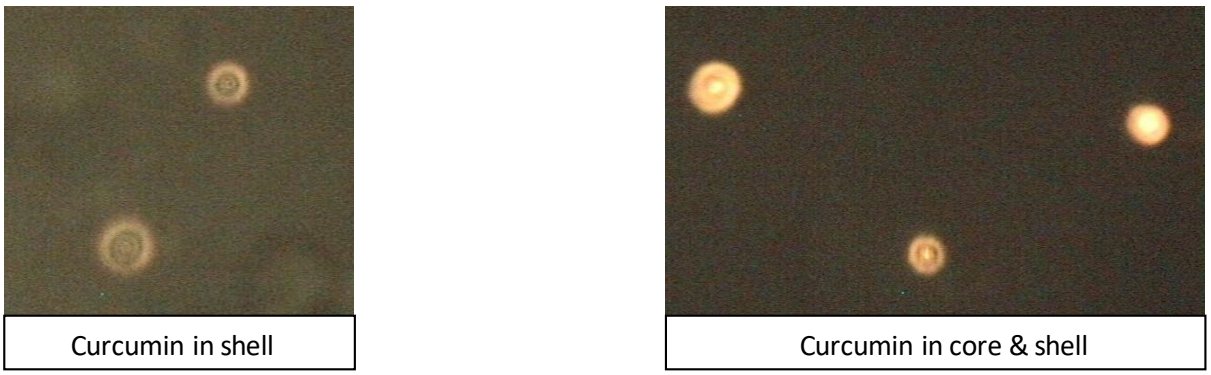


Figure 5.4: Morphological representation by fluorescence microscopy shows curcumin conjugated chitosan shelled NBs with (right) and without (left) curcumin in the core.

5.5.3 Encapsulation Efficiency and Loading Capacity

Equestions (2) and (3) were used to compute loading capacity (LC) and encapsulation efficiency (EE), and the results are shown in **Table 5.4.** For all formulation LC values < 10%. The range of EE for all formulations was between 80% and 88%. EE and LC slightly increased when curcumin was present also in the shell. In comparison to Curcumin-NBs, Curcumin-Oxygen-NBs show a moderate increase in both EE and LC. Despite the presence of oxygen, curcumin was encapsulated effectively in the chitosan shell and beyond the surfactant interface layer. Therefore, the loading capacity and encapsulation effectiveness of all formulations were unaffected by oxygen.

Formulation	Encapsulation Efficiency (%)	Loading Capacity (%)
Curcumin loaded chitosan shell NBs	84	1.5
Curcumin-conjugated-chitosan shell NBs	85.3	3.8
Curcumin loaded & curcumin-conjugated-chitosan shell NBs	87.7	4.3
Curcumin oxygen loaded chitosan shell NBs	88.1	5.1
Curcumin loaded & curcumin-conjugated-chitosan shell oxygen NBs	89.2	5.3

Table 5.4: Encapsulation efficiency and loading capacity of NBs. Mean of the percentages (n=3) are presented.

5.5.4 HPLC Analysis of Curcumin Concentration in Nanobubbles

HPLC was used to measure the curcumin concentration in all of the prepared NBs to see if the theoretical and actual concentrations were comparable. The chloroform extraction-derived curcumin solution was diluted in mobile phase before being injected into HPLC. The peak areas were determined by HPLC, and the equation for the calibration curve was applied to calculate the concentration of curcumin. The results were presented in **Table 5.5.**

Type of Nanobubbles	Theoretical Conc. of Curc.in NBs (µg/ml)	Conc. of Curc.in NBs by HPLC (µg/ml)
Curcumin loaded chitosan shell NBs	341 ± 25	335 ± 30
Curcumin- conjugated-chitosan shell NBs	405 ± 40	400 ± 42
Curcumin loaded & curcumin- conjugated-chitosan shell NBs	759 ± 55	743 ± 44
Curcumin oxygen loaded chitosan shell NBs	607 ± 47	595 ± 32
Curcumin loaded & curcumin- conjugated-chitosan shell oxygen NBs	759 ± 38	743 ± 49

Table 5.5: Curcumin concentration in all of the prepared NBs by HPLC.

Since according to both Table 4 and Table 5 the content in curcumin is maximal for the Curcumin loaded & curcumin- conjugated-chitosan shell NBs (Curc-CS-NBs) and the Curcumin loaded & curcumin- conjugated-chitosan shell oxygen NBs (Curc-Oxy-CS-NBs) only for these two samples the results from further investigations will be reported.

5.5.5 Nanobubbles Stability

The average diameter of all formulations was increased after an eight-week testing period, as shown in **figure 5.5**. Diameter size increase ranged from 9.2% to 12.19%. Although the change in size is statistically significant, however the NBs diameter kept always below 600 nm after 8 weeks. Zeta potential, pH, and polydispersity indexes were quite close to those shown in the **Table 5.3**.

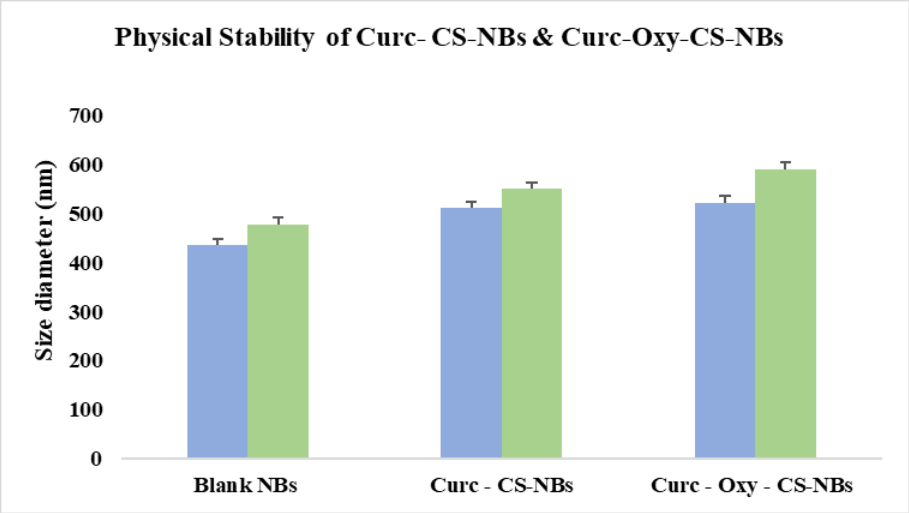
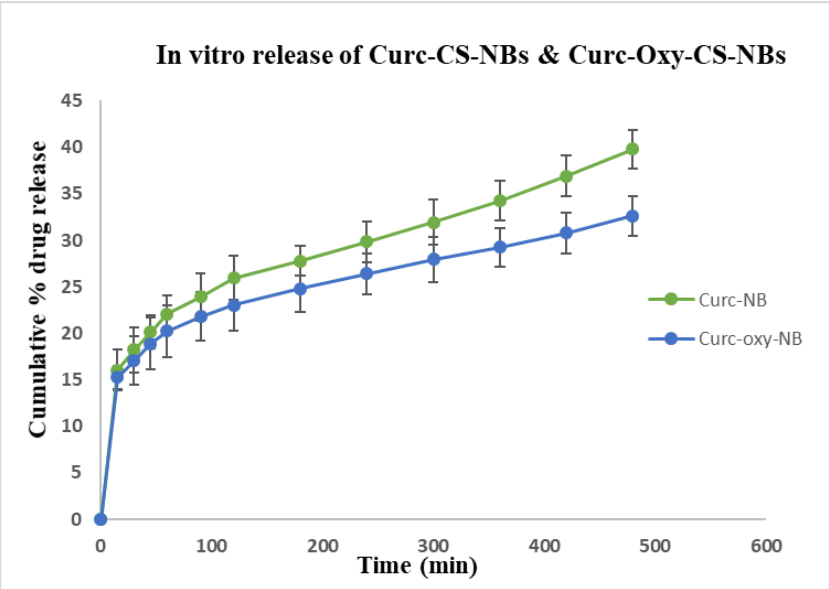


Figure 5.5: Physical stability of blank NBs, Curc-CS-NBs & Curc-Oxy-CS-NBs. Results are presented as mean ± SD (n=3). Both NBs were stored at 4°C and analysed for 8 weeks. Diameter size increase of Curc- CS-NBs & Curc-Oxy-CS-NBs after 8 weeks of study. Blue: Week 0. Green: Week 8.

5.5.6 *In Vitro* Release of Curcumin Study

In vitro release studies was conducted only on the selected NBs , i.e. Curc-CS-NBs and Curc-Oxy-CS-NBs. The release study was carried out for 48 hours, as reported in **Figure 5.6**. The percentage of curcumin released over 'short time' (Fig 5.7A) and 'long time (Fig 5.7B)' periods for both formulations was assessed. The findings that after 48 hours, the drug released from Curc-CS-NB and Curc-Oxy-CS-NB were 69.14% and 66.98% respectively (**Figure** 5.**6B)** showed that the effects were prolonged (i.e sustained release).

A.



B.

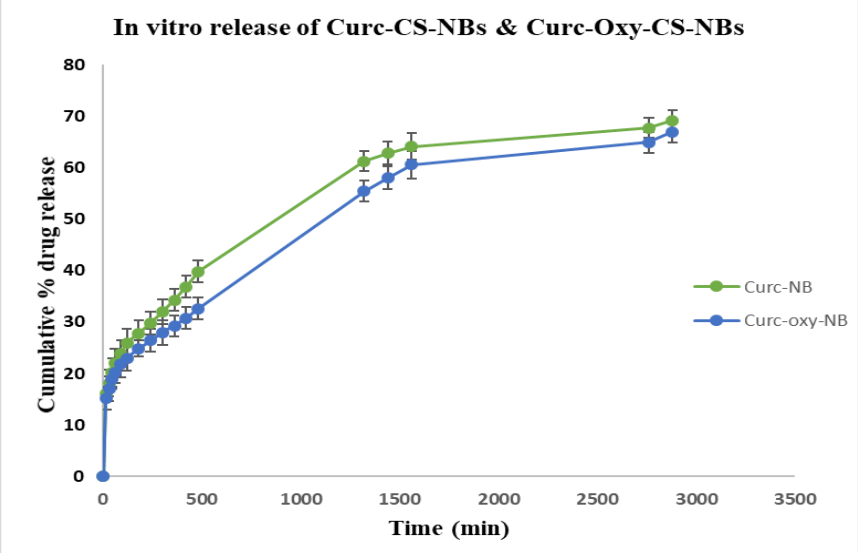


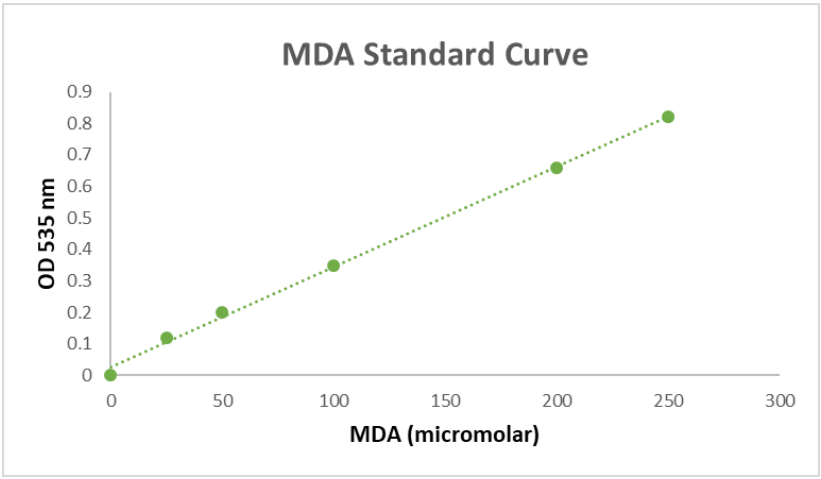
Figure 5.6: *In Vitro* release studies of the NBs. Results are presented as mean ± standard deviation (n=3). Green line: Curc-CS-NBs and blue line: Curc-Oxy-CSNBs. **A)**. in the 'Short time study' (up to eight hours), the release of curcumin was monitored to assess the burst impact. **B).** In the 'long time study' up to 48 hours, the curcumin release was monitored to check whether it was sustained over time.

5.5.7 Antioxidant Activity

Thiobarbituric Acid Assay

The results report how much MDA was produced by the various formulations, estimated by a calibration curve shown in Fig. 7A, and it is apparent that NBs loaded with curcumin produced significantly less MDA ($P < 0.001$) than the blank NBs **Figure 5.7B**. A reduced MDA production may suggest that curcumin~~'s~~ inhibits oxidation or enhances scavenging during the oxidation process.

A.



B.

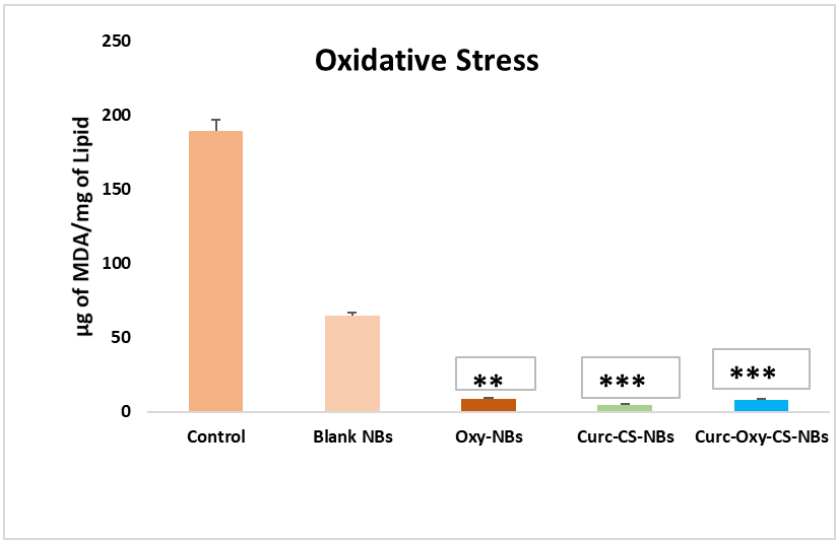


Figure 5.7: **A.** MDA standard curve. **B**. Curc-CS-NBs and Curc-Oxy-CS-NBs significantly reduce lipid peroxidation. Statistical significance ***indicates $P < 0.001$, *** indicates $P < 0.0001$.

5.5.8 Microbiological Study

MIC and MBC

Based on their content in curcumin, only Curc-CS-NBs and Curc-Oxy-CS-NBs were investigated for their antibacterial activity. MIC and MBC were evaluated both with and without photodynamic activation by LED light. Following photoactivation the tested NBs showed to be more effective against all the bacterial strains, especially for *E. faecalis* and *S. aureus* (**Table 5.6**).

Nanoformulation	Bacterial strain	MIC µg/ml		MBC µg/ml	
		No LED	LED	No LED	LED
Curc-CS-NBs	*E. coli*	46 ±10	46 ±10	93 ± 15	46 ±10
	S. aureus	93 ± 15	46 ±10	93 ± 15	46 ±10
	E. faecalis	46 ±10	23 ±7	93 ± 15	46 ±10
Curc-Oxy-CS NBs	*E. coli*	93 ± 15	46 ±10	93 ± 15	46 ±10
	S. aureus	46 ±10	12 ±5	93 ± 15	12 ±5
	E. faecalis	93 ± 15	46 ±10	93 ± 15	46 ±10

Table 5.6: MIC and MBC of Curc-CS-NBs and Curc-Oxy-CS-NBs tested with or without photodynamic treatment on three different bacterial strains.

The MIC range of *E. coli* was 46.4 μg/ml and 92.8 μg/ml in the absence of photodynamic activation for Curc-CS-NBs and Curc-Oxy-CS-NBs. The MIC, however, was 46.4 μg/ml when both types of NBs were exposed to LED light.

S. aureus showed MICs for Curc-CS-NBs and Curc-Oxy-CS-NBs of 92.8 μg/ml and 46.4 μg/ml, respectively, while not receiving photodynamic activation. The MIC for Curc-CS-NBs was 46.4μ g/ml, while the MIC for Curc-Oxy-CS-NBs reduced to 11.6 μg/ml, which is a significantly lower concentration.

E. faecalis's MIC values of 46.4 μg/ml and 92.8 μg/ml without photodynamic activation; reduced to 23.2 μg/ml and 46.4 μg/ml, respectively for Cur-CS-NBs and Curc-Oxy-CS-NBs, following LED exposure.

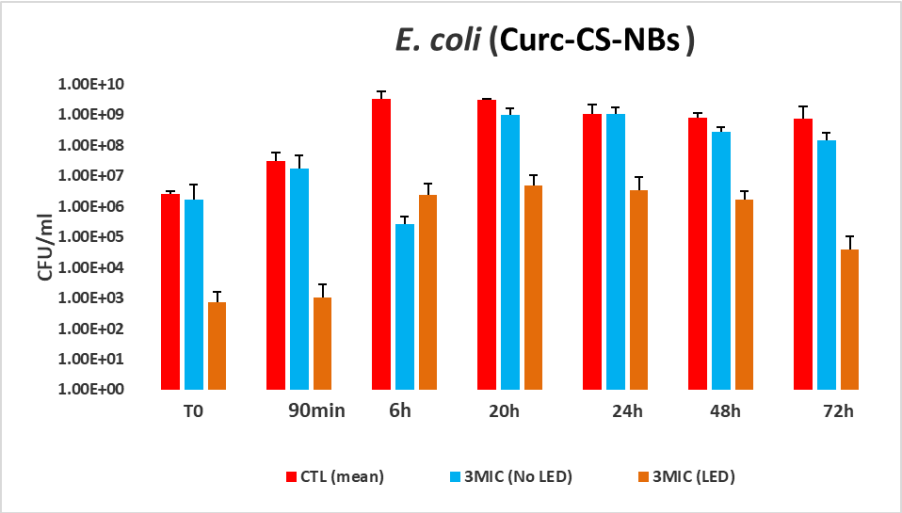
5.5.9 Time kill kinetics

The antibacterial activity of Curc-NBs and Curc-Oxy-CS-NBs against the bacterial strains was examined at the concentrations of 3XMIC of curcumin either after 3h under dark or under LED irradiations at different time points (T0, 90min, 6h, 20h, 24h, 48h and 72h). The percentage of survival was calculated after comparing the NBs-treated bacterial samples with the control samples (untreated). Stronger killing potential was shown for NBs with oxygen and LED irritation against all bacterial strains. Meanwhile along with LED irradiation the killing potential also depends on the MIC value (i.e the higher the MIC value the higher will be the killing activity).

Escherichia coli ATCC 25922

According to the results from the *E. coli* time kill kinetics, a number of parameters, such as the MIC value, are crucial for effectively controlling the spread of the bacterium over several days. But interestingly, our findings demonstrated that Curc-Oxy-CS-NBs at the low MIC concentration (46.4 g/ml) after 3 hours of LED irradiation were capable of killing bacteria for days as shown in **Figure 5.8B** green colour. This MIC value was three times lower than the MIC concentration of curcumin alone (**see the chapter 2**). Thus, this study showed that the curcumin loaded NBs when combined with oxygen and LED light can completely eradicate even gram-negative bacteria like ***E. coli***. The antibacterial effect was not so evident when evaluating the MIC and the MBC because those were almost 'instantaneous' measures, and the curcumin content was only minimally released by the NBs. On the contrary, in the time killing test, the antibacterial effect is enhanced by the longer sustained release of the curcumin (and the oxygen as well) to be activated by the light.

A.



B.

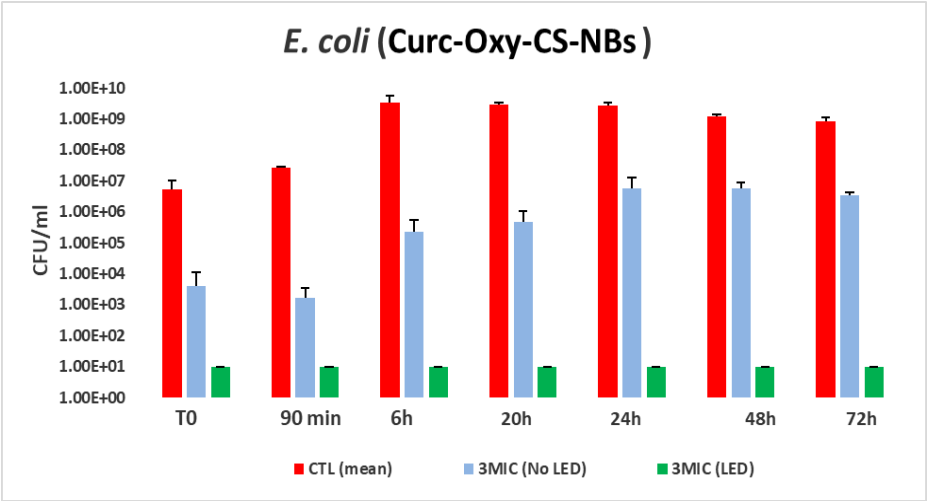
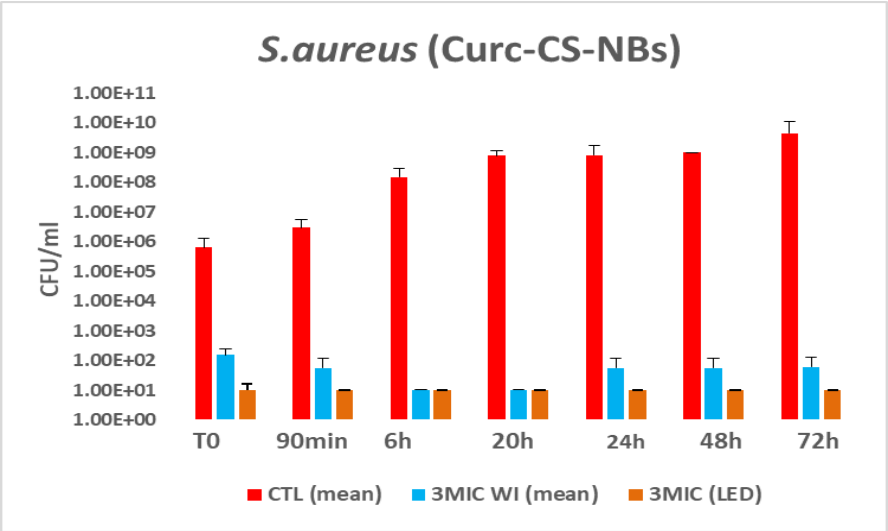


Figure 5.8: Graphical representation of killing activity against *E.coli*. A) Curc-CS-NBs with and without LED light. B) Curc-Oxy-CS-NBs with and without LED illumination.

***Sthaphylococcus aureus* ATCC 29213**

Generally, the results showed that both types of curcumin-loaded NBs (without and with oxygen) are very effective in inhibiting the growth of *S.aureus*. The MIC value of 11.6µg/ml for Curc-Oxy-CS-NBs upon photoactivation can damage the bacterial growth for days. The results also show without LED irradiation a MIC value of 92.8µg/ml was required to kill this bacterium since, as shown in **Figure 5.9B**, half concentration is not enough for controlling the bacterial growth for 3 days.

A.



B.

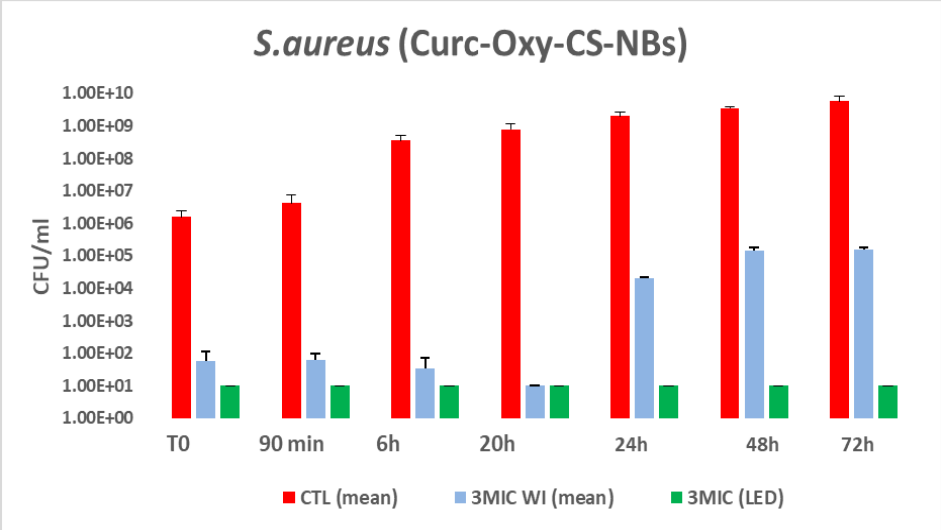
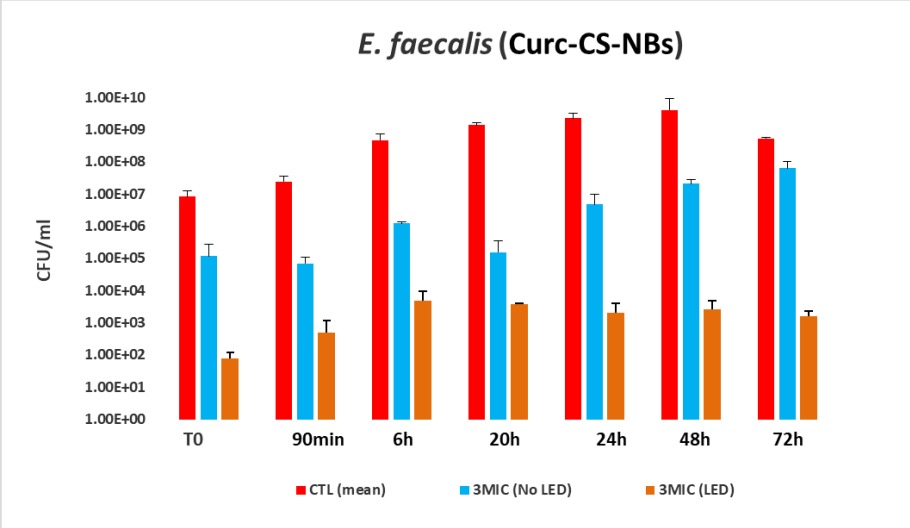


Figure 5.9: Graphical representation of killing activity against *S. aureus*. **A)** Curc-CS-NBs with and without LED light. **B)** Curc-Oxy-CS-NBs with and without LED illumination.

Enterococcus faecali ATCC 29212

The result with *E. faecali* showed again a stronger killing potential of Curc-Oxy-CS-NBs at the MIC value of 46.4µg/ml (**Figure 5.10B**). According to these findings, curcumin, oxygen, and LED were all essential for inhibiting bacterial growth. The NBs were unable to kill bacteria for days if even only one of these individual parameters was lacking.

A.



B.

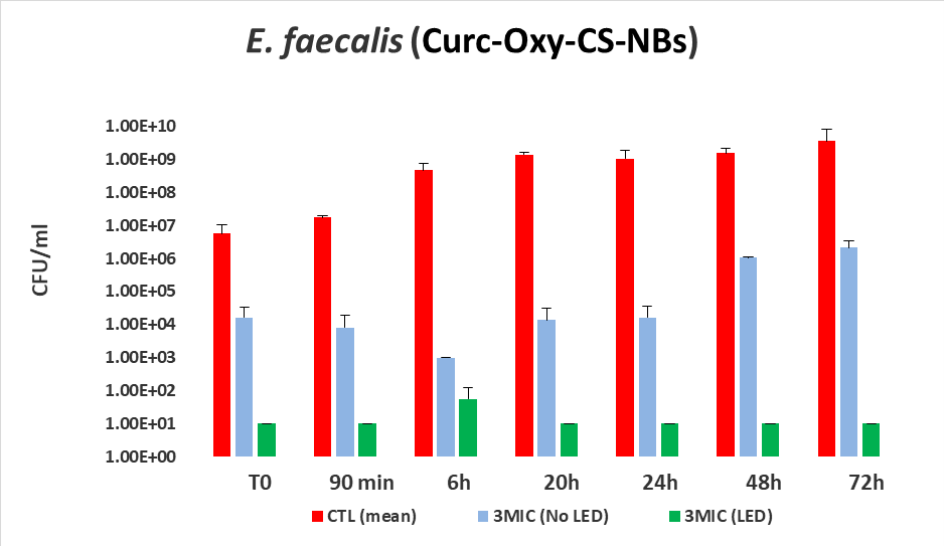
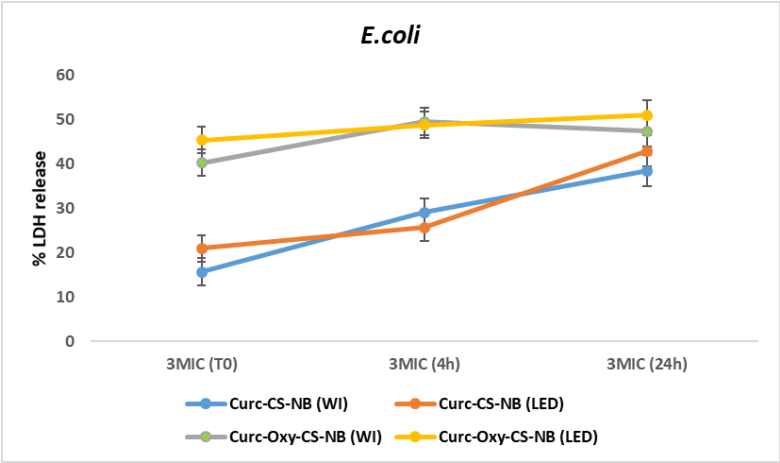


Figure 5.10: Killing effect against *E. faecalis*.

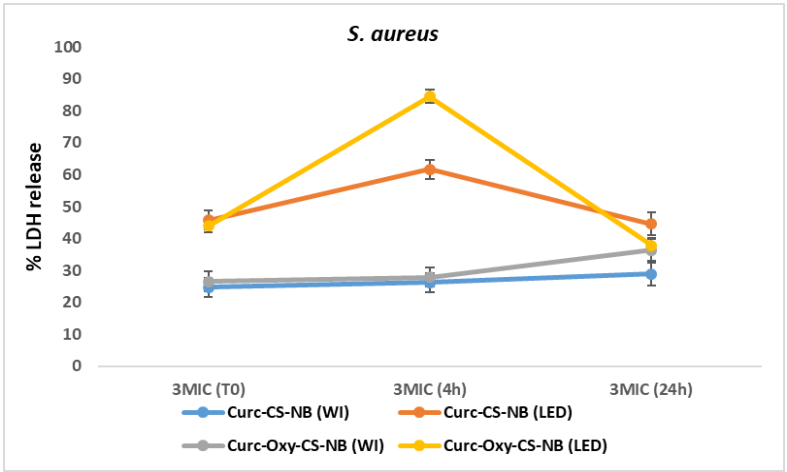
5.5.10 Lactate Dehydrogenase Activity

In addition to evaluating the membrane's integrity, the lactate dehydrogenase (LDH) activity was investigated to confirm the time kill studies. The results showed that Cur-Oxy-CS-NBs under LED irradiation significantly damaged the integrity of bacterial membranes, resulting in the release of the cytosolic enzyme LDH. The results were shown in **Figure 5.11 A, B and C**. The percentage of LDH released for Curc-CS-NBs and Curc-Oxy-CS-NBs ranged from 6% to 49% under dark conditions, on the contrary the percentage of LDH released for Curc-NBs and Curc-Oxy-CS-NBs ranged from 10% to 85% under light conditions. The timing of the damage was different for the 3 investigated bacterial strains, but in all cases the presence of oxygen and the LED irradiations, responsible for the formation of ROS, explain the effect on the bacterial membranes and the resulting antimicrobial action.

A.



B.



C.

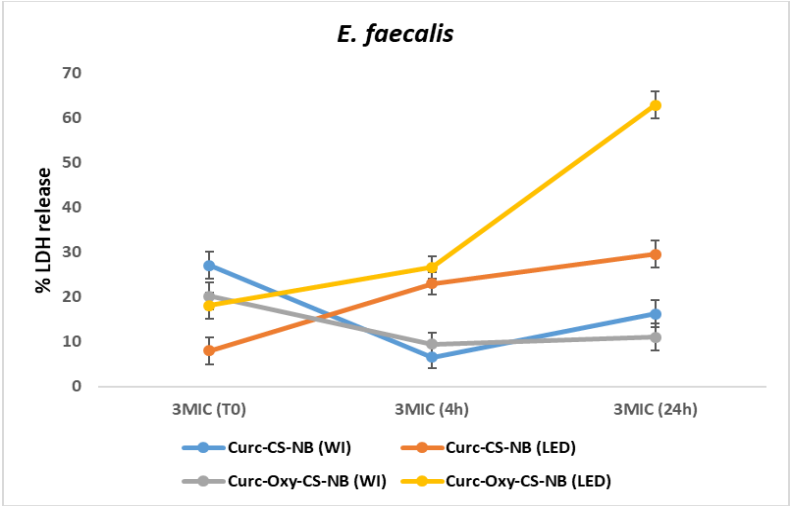


Figure 5.11: LDH release activity of Curc-CS-NBs and Curc-oxy-CS-NBs under LED and dark condition against the tested bacterial strains. **A)** LDH for *E.coli*, **B)** LDH for *S.aureus*, **C)** LDH for *E.faecalis*.

5.6 DISCUSSION AND CONCLUSION

This study is focused on the proposal of a new approach to the dramatic problem of controlling the bacterial infections by means of natural agents overcoming antibiotic resistance.

The innovative steps investigated here are two: first of all, curcumin is administrated via nanovectors of average diameter around 500 nm.

The technological problems have been successfully solved, and curcumin loading inside the core and also on the NBs shell allows the encapsulation of a satisfactory amount of curcumin, as proved also by direct measures by HPLC.

Besides solving the problems related to unsolubility and degradation of the curcumin, nanoformulations are also expected to improve the adhesion to the bacterial membranes (due to the well known mucoadhesive properties of the chitosan) and, above all, to locally release the curcumin in a continuous and sustained way.

After showing a satisfactory stability and the retention, or even an enhancement, of the antioxidant properties of the curcumin, the antibacterial ability of two nanoformulations, namely the Curc-CS-NBs and Curc-Oxy-CS-NBs, were tested.

By comparing their MIC values with those reported in Chapter 2 **[10]**, we note that that they are comparable for the *S. Aureus*, which was already very responsive to curcumin, while the MIC of the NBs is much lower that of the 'bulk' curcumin for *E. faecalis* and *E. Coli*.

These results suggest that a better penetration of the active principle is obtained. To appreciate the effect of the slower release a comparison between the Time kill kinetics is needed and it is possible since both for 'bulk' and nanoencapsulated curcumin the dosage was expressed as 3XMIC.

As far as the *S. aureus* is concerned, both the Curc-CS-NBs and Curc-Oxy-CS-NBs were very effective at all times, enhancing the already very good results obtained by administering 'bulk' curcumin.

For *E. faecalis* and *E. coli*, on the contrary, possibly due to the fact that their MIC, and therefore the overall administrated curcumin dose, was lower with respect to that of the 'bulk' curcumin, the control of the bacterial growth along 72 hours is worse.

The second important innovative step of this study is the curcumin photoactivation by means of the exposition to blue LED light for 3 hours. Since it is well known that most of the bactericidal effects are due to the production of ROS, we expect that the Curc-Oxy-CS-NBs should exalt the toxicity of the treatment, as confirmed by the observed increase of the bacterial membrane leakage showed by the LDH test.

Looking at the microbiological data, the MIC for the Curc-CS-NBs and Curc-Oxy-CS-NBs following 3h of LED illumination were larger than those estimated for the 'bulk' curcumin in the two gram-positive strains, while they were significantly lower for *E. coli*.

The results for *E. faecalis* and *S. aureus* are not surprising, since the MIC value is evaluated just three hour after the administration of the NBs, and therefore the content of curcumin already released and photoactivable is expected to be low.

The results obtained by the Time kill test, which show a much better bactericidal action, especially by Curc-Oxy-CS-NBs, along 72 hours for all the strains and, in spite of the lower curcumin dosage, expecially for *E. coli*, are in good agreement with our expectations related to the LDH release and the ROS production.

Our results confirm the previous studies described in the literature. Poor absorption and rapid degradation of curcumin has been an issue because of its poor solubility in water, instability at physiological pH and bioavailability. Different studies have shown that curcumin can be encapsulated in biocompatible nanosystems (such as polymeric nanoparticles and nanoemulsions), which have

several advantages: such as antimicrobial properties, are able to increase the bioavailability and solubility of curcumin **[11, 12].** Another study demonstrated that encapsulated curcumin can be used as carriers to transport natural substances with antimicrobial and antioxidant activity **[13, 14].**

Researchers already proved that curcumin produces a powerful photodynamic response to light by showing that neither curcumin alone, nor curcumin in the absence of light, could result in significant bacterial growth decreases. To measure the antibacterial photodynamic effects of curcumin against *Staphylococcus saprophyticus subsp. bovis* and *E. coli* DH5 alpha, Agel et al. **[11]** developed curcumin-loaded nanoparticles and curcumin-loaded nanoparticles with chitosan. The results of in vitro investigations were validated by the enhanced adhesion of the chitosan-modified nanoparticles to bacteria and the structural damage caused by photodynamic activation. Furthermore, the survival rate of *S. saprophyticus* was reduced to less than 0.0001% (>6.2 log10 decrease) when curcumin-loaded nanoparticles were used in conjunction with LED irradiation. When combined with light and nanoparticles containing curcumin and chitosan, the effectiveness was higher, with a survival rate of 0.0000045%. Contrarily, when applied to *E. coli*, the photoactivated curcumin-loaded nanoparticles displayed a survival rate of 0.13% (CFU only 2.9 log10) and an improvement in antibacterial efficacy with the curcumin-loaded and chitosan nanoparticles with LED, with a reduction in CFU of up to 5.9 log10 (0.00013% survival) **[11].**

In conclusion, the objective of this work was to optimize, synthesize, and assess the bactericidal activity of chitosan shelled, curcumin, and oxygen-loaded NBs. Curc-CS-NBs & Curc-Oxy-CS-NBs designed and manufactured for the present study, have shown interesting physicochemical characteristic and strong antimicrobial properties, further enhanced by the photodynamic activation by visible light (Light Emitting Diode, LED). It was shown that a proper combination of Curc-Oxy-CS-NBs and photodynamic treatment results in greater inactivation of the microorganism, compared with the condition of no oxygen and LED exposure.

Interestingly, the present study showed that the Curc-Oxy-CS-NBs (chitosan-shelled and curcumin, and oxygen loaded) when exposed to blue LED light were also able to damage gram negative bacteria i.e *E. coli* that are hard to kill due to their different characteristics of the bacterium's cell envelope, whose outer membrane contain lipopolysaccharide that serves as a permeability barrier, controls the entry and exit of different substances, conferring greater resistance against antimicrobic **[15-17].** Conversely, the outer membrane of Gram-negative bacteria shields them from curcumin diffusion and photosensitization when curcumin is supplied by nanovectors (which are predicted to enhance membrane crossing) **[11, 15, 18].**

Therefore, the results of the proposed study will be useful in the defense against pathogenic microorganisms affecting for instance the skin or other outer mucosae, since the current study demonstrated that very common and diffuse bacterial strains can be successfully eradicated when exposed to photoactivated curcumin and oxygen-loaded NBs. Additionally, further study is also encouraged on the application of nanobubbles directly on food, either against bacteria and fungi. Future researchs may aim to improve even further the experimental conditions by adding less curcumin to the NBs while maintaining their effectiveness and subjecting the system to a briefer photodynamic activation period still producing an effective antibacterial activity. These findings may be included into a more comprehensive approach to combat pathogenic microorganisms that cause illnesses in order to protect human health and raise food quality globally.

References

1. Antony R., Arun T.Manickam S.T.D.,"*A review on applications of chitosan-based Schiff bases",*International journal of biological macromolecules, 2019. **129**: 615-633.
2. Yin X., Chen J., Yuan W., Lin Q., Ji L.Liu F.,"*Preparation and antibacterial activity of Schiff bases from O-carboxymethyl chitosan and para-substituted benzaldehydes",*Polymer bulletin, 2012. **68**: 1215-1226.
3. Tamer T.M., Hassan M.A., Omer A.M., Baset W.M., Hassan M.E., El-Shafeey M.E.Eldin M.S.M.,"*Synthesis, characterization and antimicrobial evaluation of two aromatic chitosan Schiff base derivatives",*Process biochemistry, 2016. **51**(10): 1721-1730.
4. Saranya T., Rajan V., Biswas R., Jayakumar R.Sathianarayanan S.,"*Synthesis, characterisation and biomedical applications of curcumin conjugated chitosan microspheres",*International journal of biological macromolecules, 2018. **110**: 227-233.
5. Wei L., Tan W., Wang G., Li Q., Dong F.Guo Z.,"*The antioxidant and antifungal activity of chitosan derivatives bearing Schiff bases and quaternary ammonium salts",*Carbohydrate polymers, 2019. **226**: 115256.
6. Nguyen H.N., Ha P.T., Sao Nguyen A., Nguyen D.T., Do H.D., Thi Q.N.Thi M.N.H.,"*Curcumin as fluorescent probe for directly monitoring in vitro uptake of curcumin combined paclitaxel loaded PLA-TPGS nanoparticles",*Advances in Natural Sciences: Nanoscience and Nanotechnology, 2016. **7**(2): 025001.
7. Wayne P.,"*Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing: 20th informational supplement",*CLSI document M100-S20, 2010.
8. Burd J.Usategui-Gomez M.,"*A colorimetric assay for serum lactate dehydrogenase",*Clinica Chimica Acta, 1973. **46**(3): 223-227.
9. Korzeniewski C.Callewaert D.M.,"*An enzyme-release assay for natural cytotoxicity",*Journal of immunological methods, 1983. **64**(3): 313-320.
10. Munir Z., Banche G., Cavallo L., Mandras N., Roana J., Pertusio R., Ficiarà E., Cavalli R.Guiot C.,"*Exploitation of the antibacterial properties of photoactivated curcumin as 'green' tool for food preservation",*International journal of molecular sciences, 2022. **23**(5): 2600.

11. Agel M.R., Baghdan E., Pinnapireddy S.R., Lehmann J., Schaefer J.Bakowsky U.,"*Curcumin loaded nanoparticles as efficient photoactive formulations against gram-positive and gram-negative bacteria",*Colloids and Surfaces B: Biointerfaces, 2019. **178**: 460-468.
12. Bessone F., Argenziano M., Grillo G., Ferrara B., Pizzimenti S., Barrera G., Cravotto G., Guiot C., Stura I.Cavalli R.,"*Low-dose curcuminoid-loaded in dextran nanobubbles can prevent metastatic spreading in prostate cancer cells",*Nanotechnology, 2019. **30**(21): 214004.
13. Maizura M., Fazilah A., Norziah M.Karim A.,"*Antibacterial activity and mechanical properties of partially hydrolyzed sago starch–alginate edible film containing lemongrass oil",*Journal of Food Science, 2007. **72**(6): C324-C330.
14. Seidi Damyeh M., Mereddy R., Netzel M.E.Sultanbawa Y.,"*An insight into curcumin-based photosensitization as a promising and green food preservation technology",*Comprehensive Reviews in Food Science and Food Safety, 2020. **19**(4): 1727-1759.
15. Shlar I., Droby S., Choudhary R.Rodov V.,"*The mode of antimicrobial action of curcumin depends on the delivery system: Monolithic nanoparticles vs. supramolecular inclusion complex",*RSC advances, 2017. **7**(67): 42559-42569.
16. Rajagopal M.Walker S.,"*Envelope structures of Gram-positive bacteria",*Protein and sugar export and assembly in Gram-positive bacteria, 2017: 1-44.
17. Silhavy T.J., Kahne D.Walker S.,"*The bacterial cell envelope",*Cold Spring Harbor perspectives in biology, 2010. **2**(5): a000414.
18. Ryu V., Ruiz-Ramirez S., Chuesiang P., McLandsborough L.A., McClements D.J.Corradini M.G.,"*Use of micellar delivery systems to enhance curcumin's stability and microbial photoinactivation capacity",*Foods, 2021. **10**(8): 1777.

CONCLUSION

Bacterial infections are a serious threat for human health since the development of antibiotic resistant strains. As a consequence, new interest towards natural, green and sustainable approaches arose in the scientific community.

Although based on natural composites, such studies are not so naïve as they can seem. On the contrary, they make use of very sophisticated techniques, such as the bacterial Photo Dynamic Inactivation (PDI), able to activate natural photosensitizers, like curcumin, to produce lethal Reactive Oxygen Species (ROS) following visible light emission. Also the manufacture of nanovectors which encapsulate the antibacterial substances for a sustained, long time release in the tissues is a modern effective strategy to enhance the antimicrobial fight.

In the present dissertation three different investigations are described, aiming at proposing the application of curcumin, undergoing PDI and nanoformulations treatment, for preserving from bacterial infections, and therefore prolonging the shelf-life, of very delicate fruits such as berries, with a very little impact on their organoleptic and antioxidant properties.

- The first part of the study quantified the antibacterial properties of the curcumin solubilized in N-methyl-2-pyrrolidone, with or without PDI treatment, on both Gram positive and Gram-negative bacterial strains, showing that the Minimum Inhibitory Concentration (MIC) and the Minimum bactericidal Concentration (MBC) were very low with respect to other antibacterial substances and allowed a sustained Kill Kinetics for days.

- The second study was focused on the goal of extending the shelf life of berries. Different curcumin concentrations and irradiation approaches were used in three experiments. In specifically, the effects of 0.5 and 1 mg/ml of curcumin in β -cyclodextrin and single or multiple blue light irradiations were investigated. Due to the perishability of the berries and the closed system employed for microbiological studies, the main limitation on this preliminary study was the short observation period of 3 days. In spite of this, the treatment was successful and proved to be a viable preservation technique. Numerous tests on the solution's stability, adhesiveness, antioxidant, and preservation of organoleptic properties were conducted in order to guarantee the efficacy of the therapy. The treatment's short- and long-term effects on consumers' health was not investigated, although the safety of all

substances employed has been thoroughly established and approved by the European Food Safety Authority (EFSA).

- Following these encouraging findings, more research efforts have been done on novel coating carriers including nano-crystals and nano-bubbles, which ought to be able to release curcumin over a longer period. To sum up, the aim of the third part of the study was to optimize, synthesize, and evaluate the bactericidal effectiveness of chitosan shelled and oxygen-loaded NBs containing curcumin. Curc-CS-NBs and Curc-Oxy-CS-NBs were developed and produced in the current work, which displayed intriguing physicochemical characteristics as well as potent antibacterial capabilities. These features were further increased by photodynamic activation by visible light (Light Emitting Diode, LED). It was demonstrated that a proper combination of Curc-Oxy-CS-NBs and PDI treatment results in greater inactivation of the microorganism, even also capable of damaging gram negative bacteria, such as E. coli, that are difficult to kill due to their different characteristics of the bacterium's cell envelope.

Thus, we hope that the proposed study's findings will be helpful in the fight against pathogenic microorganisms that affect, for example, the skin or other outer mucosae, as the current study showed that very prevalent and diffuse bacterial strains can be successfully eliminated when exposed to photoactivated curcumin and oxygen-loaded NBs. Further research is also encouraged on the use of nanobubbles directly on food to fight fungi and bacteria. Future studies may further enhance the antimicrobial effects by reducing the amount of curcumin in the NBs while retaining their efficiency and exposing the system to photodynamic activation for a shorter amount of time while still creating an effective antibacterial activity. These results may be included into a more thorough strategy to fight pathogenic microorganisms that cause disease in order to safeguard human health and improve food quality worldwide.