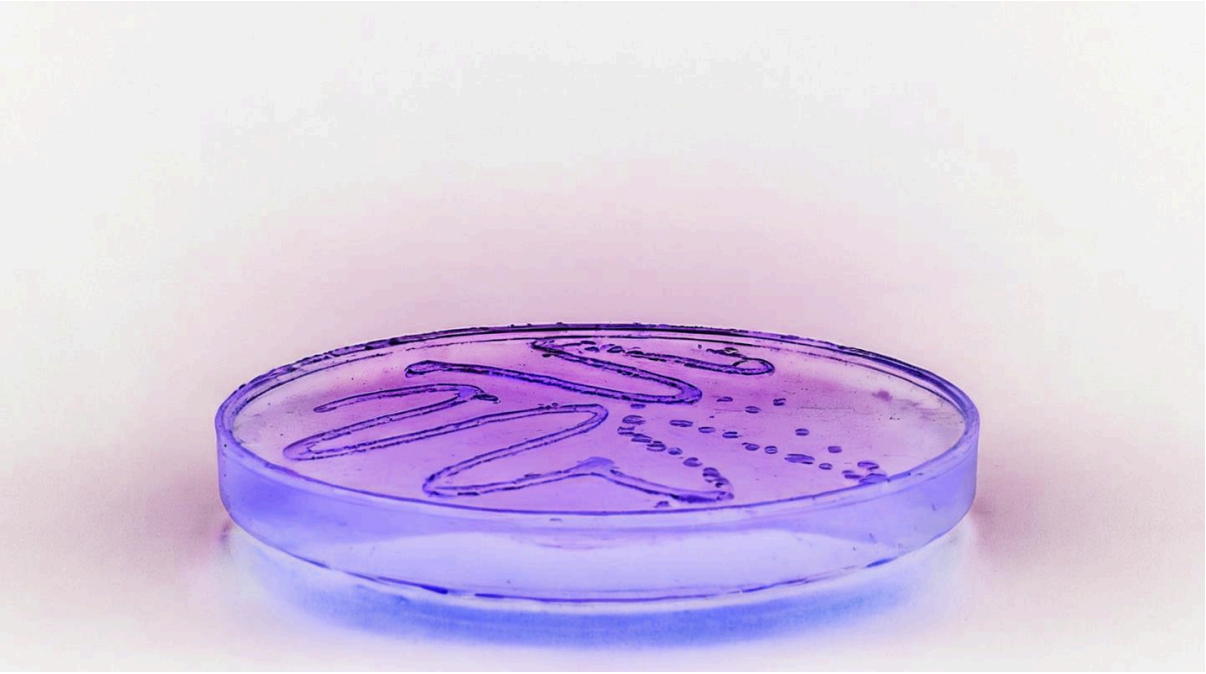
Photodynamic inactivation of *Escherichia coli*

Influencing virulence with blue light

Hannah Davidsson



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Photodynamic inactivation of *Escherichia coli*. Influencing virulence with blue light

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Abstract

The issue of sickness and death as a result of foodborne illness is a health concern spread worldwide. This indicates the need for effective antimicrobial technologies within the food industry. Photodynamic inactivation (PDI) is a technology which utilizes the overproduction of reactive oxygen species (ROS) within pathogens by activating endogenous photosensitizers, where the oxidative stress generated by ROS in turn leads to cell death. This paper has investigated the effect of blue light PDI on *Escherichia coli* O157:H7 *gfp*+ where the change in growth as well as changes of gene expressions have been explored. The results indicated that a wavelength of 420 nm generated the highest reduction of colony forming units per ml (CFU/ml). In addition, longer exposure to blue light also resulted in a higher reduction of CFU/ml. Refrigerating the samples further reduced the CFU/ml. On average, a percentage reduction of 91.9-95.5% of CFU/ml was observed in the treated samples. The findings of this research emphasizes the possible benefits of the implementation of blue light PDI in the food industry. However, due to the novelty of this technology, more research is required to ensure consumer completely safe food products.

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1. Introduction

Having access to safe and nutritious food is key for obtaining a healthy life (World Health Organisation, 2022). Yet, foodborne illness still remains worldwide where infections can be related to the intake of contaminated food or water. Annually, there are 600 million cases of illness globally related to foodborne pathogens and among the affected, 420 000 cases annually result in death (Hadi et al., 2020). The majority of cases and deaths are related to diarrheal diseases where major bacterial contributors are *Salmonella enterica*, *Campylobacter* spp. and *Escherichia coli*. Contamination can be related to unsafe storage solutions as well as polluted water, air and land, however, the incessant development of the food chain brings new aspects of concern for outbreaks of diseases (World Health Organisation, 2024). Over recent years, the production of food has grown to be more complex, resulting in an increase in contamination points (Langsrud et al., 2003). Today, the production volumes are larger, operations are more mechanical, there is a higher level of processed foods, and the time and distance between producers and consumers have increased. Pathogens can linger in food processing environments and food products and contamination can occur at any point during the production and distribution. The viability of pathogens together with the more complex production systems lead to an extensive need for efficient sanitary practices within the food industry, to avoid outbreaks of illness.

Blue light is an emerging technology for decontamination of food and surfaces (Lena et al., 2023). It uses the mechanism where blue light excites endogenous photosensitizers which result in the overproduction of reactive oxygen species (ROS), eventually leading to cell death of the pathogen. A process also called photodynamic inactivation (PDI). In this paper, PDI of *Escherichia coli* O157:H7 *gfp*+ has been studied by analyzing the change in growth as well as changes of gene expressions of virulence genes concerning attachment, when the bacteria was exposed to blue light.

2. Background

2.1 *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacteria (Lim, 2010). While most *E. coli* strains are harmless to humans, a few strains have developed pathogenic properties. Enterohemorrhagic *E. coli* (EHEC) serotype O157:H7 is the major source of food poisoning outbreaks among the different strains of *E. coli* (Nguyen & Sperandio, 2012; Folkhälsomyndigheten, 2020, Hadi et al., 2020). Symptoms of infection include bloody diarrhea and abdominal cramps but infection may also proceed to hemolytic uremic syndrome (HUS), which can lead to life-threatening complications (Nguyen & Sperandio, 2012). Since treating infections with antibiotics have shown to result in a higher production and release of Shiga toxins by the bacterial cells, which in turn increase the risk of HUS development, antibiotics are not used as a cure (Mühlen et al., 2020). Currently there are no other treatments available for EHEC infections (Nguyen & Sperandio, 2012).

Cattle are natural reservoirs of EHEC and colonization in adult ruminants is asymptomatic (Nguyen & Sperandio, 2012). Livestock transmit the bacteria through their feces which can lead to contaminated agricultural grounds as well as contaminated runoff water associated with the area (Xicohtencatl-Cortes et al., 2009). EHEC that has been transmitted to water can remain infectious for over 6 months making irrigation water from contaminated sources a major reservoir for *E. coli* O157:H7 (Hadi et al., 2020). Other sources of contamination include compost, insects and wildlife animals. The use of contaminated tools for harvest as well as equipment for transportation, processing equipment and human contamination throughout the process are further examples of contamination points in the industry.

Examples of foods where EHEC is a major contributor to foodborne illness are meat, dairy products, seafood and fruits and vegetables (Hadi et al., 2020).

An important virulence trait for *E. coli* O157:H7 is the ability to produce attaching and effacing (A/E) lesions on the cells that line the inner surface of the small and large intestine (enterocytes) (Ogierman et al., 2000). In *E.coli* O157:H7, the outer membrane protein intimin is essential for attachment. When the pathogen attaches to the intestinal epithelium of the

host, brush border microvilli are effaced and A/E lesions can be formed (Deng et al., 2004). The capacity to produce A/E lesions on intestinal absorptive cells is encoded by the locus for enterocytes effacement (LEE), which is a pathogenicity island present in *E. coli* O157:H7. Pathogenicity islands are found in the genome of pathogenic strains of a bacteria and they encode for different virulence factors. LEE carries eae genes which encodes for the intimin required for the intimate attachment to epithelial cells (Ogierman et al., 2000).

2.2 Photodynamic inactivation

Blue light is currently being explored as an alternative to the commonly used UV irradiation, for inactivation of microorganisms within the food industry (Kim & Kang, 2021). In contrast to UV irradiation, which is germicidal due to its direct ability to disrupt cell metabolism and reproduction of the pathogen, blue light achieves bactericidal effects through photodynamic inactivation (Singh et al., 2021). Photodynamic inactivation is a process which generates oxidative stress by utilizing the overproduction of reactive oxygen species (ROS) (Hasenleitner & Plaetzer, 2019). Three components are required for photodynamic inactivation to occur. These are irradiation of visible light, photosensitizers and oxygen (Kim & Kang, 2021). ROS are released when intracellular photosensitizers are exposed to visible light. Radiation from the light source excites photosensitizer molecules which in the excited state either transfers the excess energy to molecular oxygen (type II process), or transfers an electron to a neighboring molecule (type I process) (Vinagreiro, 2020). The type II process generates singlet oxygen whereas the type I process leads to the formation of other ROS. The reactive oxygen species affect cellular structures by damaging proteins, lipids and nucleic acids which results in loss of cell viability (Fila et al., 2017). Singlet oxygen is a highly reactive form of molecular oxygen and it affects the cell by oxidizing proteins and lipids which results in cell destruction (Maisch et al., 2007). Singlet oxygen is considered to be the most important ROS involved in photooxidative damage (Alves et al., 2014).

2.3 Control of microorganisms in the food industry

Methods to remove the presence of bacteria and other undesired microorganisms are important elements within the food industry to reduce outbreaks of infection and foodborne illnesses (Sansebastiano et al., 2007). The rules and guidelines concerning food safety within

the food industry are extensive, and the subject is studied incessantly (Europaparlamentet, 2023). Disinfection, sterilization and sanitation are different strategies defined by the degree of eliminative effect. Sansebastiano et al (2007) defines disinfection, sterilization and sanitation as follows:

> Disinfection: elimination on a given substrate of pathogen microorganisms, viruses and bacteria, in a vegetative form through the use of chemical or physical means.
>
> Sterilization: elimination on a given substrate of all the microorganisms including bacterial spores through the use of chemical or physical means.
>
> Sanitation: reduction of the contaminating bacterial load present on a given substrate in order to reach safety levels for human health

Examples of methods for combating microorganisms are thermal sterilization technologies, high pressure processing and pulsed electric field (Sansebastiano et al., 2007). Examples of commonly used disinfectants are chlorine dioxide, iodine, hydrogen peroxide and peracetic acid. Today, thermal sterilization is the dominating sterilization method within the food industry (Li & Farid, 2016).

Reducing the amount or eliminating pathogens from foods does not only lead to a reduction in outbreaks of foodborne illness, but also to prolonged shelf life of the product (Li & Farid, 2016).

3. Aim and research questions

The aim of this study was to analyze changes concerning attachment and growth of *E. coli* O157:H7 *gfp*+ when the bacteria was exposed to blue light. Further was the aim to discuss photodynamic inactivation of *E. coli* O157:H7 *gfp*+ from a food-safety perspective. The following research questions were composed:

- How do different wavelengths of blue light impact the growth of *Escherichia coli* O157:H7 *gfp*+?
- How does the time of exposure influence the growth of *Escherichia coli* O157:H7 *gfp*+?
- How does the temperature after exposure to blue light affect the growth of *Escherichia coli* O157:H7 *gfp*+?
- Can blue light stimulate changes in gene expressions of virulence genes concerning attachment?
- How does blue light influence the virulence of *Escherichia coli* O157:H7 *gfp*+?

4. Material and method

The study was carried out by a light experiment followed by DNA and RNA extractions. The nucleic acids were then studied by Droplet Digital PCR. The data was analyzed by using Welch's unequal variances t-test and linear regression models.

4.1 Light experiment

Escherichia coli O157:H7 *gfp*+ (Swedish Institute for Communicable Disease Control, Solna Sweden. Registry no. E81186) was plated on LB agar with ampicillin and the plate was incubated at 37℃ for 24 hours. After the incubation 20 colonies were transferred to 20 individual tubes containing 6 ml LB broth supplemented with ampicillin. The tubes were then incubated at 37℃ for 24 hours.

After incubation, the tubes were centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and 10 ml of 0,85% NaCl was added to the tubes which were then vortexed. The tubes were then centrifuged at 5000 rpm for 10 min. The supernatant was discarded and 6 ml of LB broth was added to the tubes. The bacterial solution was then divided into four tubes and the optical density at 590 nm was measured. The sample was diluted until a value of 0,2 was observed. To create control samples, bacterial solution from each of the tubes was serial diluted (10^{-1}-10^{-8}) and drops of 20 µl were placed on LB agar supplemented with ampicillin and arabinose. The control plates were incubated at 37℃ for 24 hours and the CFU were counted.

Of the remaining bacterial solution, 2 ml was transferred into 6 wells of 8 microtitre plates. Two replicates of plates were put under the LED lamp (Heliospectra Dyna, Heliospectra AB, Gothenburg Sweden) for each time interval (2 minutes, 20 minutes, 3 hours and 4 hours). The intensity of the lamp was set to 200 µmol $m^{-2}s^{-1}$.

After light exposure, one of the replicate plates was stored in the fridge for 24 hours while samples from the remaining plates were collected. After 24 hours, the procedure was repeated for the refrigerated plates. 2 ml from three of the wells were transferred into tubes which were centrifuged at 14 000 rpm for 3 minutes. The supernatant was discarded and 750 µl of

DNA/RNA SHIELD solution (zymo Research) was mixed with the pellet. The tubes were then stored in the freezer at -20℃ to be used later for DNA and RNA extractions. 1 ml was collected from each of the remaining 3 wells and the bacterial solution was serial diluted (10^{-1}-10^{-8}) and drops of 20 µl was placed on LB agar supplemented with ampicillin and arabinose. The plates were incubated at 37℃ for 24 hours and the CFU were counted.

The process was repeated for each of the wavelengths tested (400 nm, 420 nm, 460 nm).

4.2 DNA and RNA extractions

DNA and RNA was extracted using the *ZymoBIOMICS DNA/RNA Miniprep Kit*, following the manufacturer's protocol. The DNA was stored at -20℃ and RNA was stored at -80℃.

4.3 Droplet digital polymerase chain reaction (ddPCR)

To determine the concentration of a specific gene in the original sample, a ddPCR was performed. The primers eaeA and eaeL were tested as well as absolute quantification of *E. coli*. The sample was prepared by mixing a mastermix of 10 µl QX200 EvaGreen Digital™ PCR Supermix, 0,5 µl forward primer, 0,5 µl reverse primer as well as 7 µl DNase/RNase free MilliQ-water per well. The 18 µl of mastermix was added to each well of a 96-well PCR plate together with 2 µl of the target nucleic acid from DNA and RNA extractions. The extracted RNA was converted to cDNA with iScript. The plate was then put in a droplet generator. Once the droplets were generated, PCR amplification was performed. After amplification, the samples were loaded into a droplet reader. QuantaSoft™ software was used to run the instrument and analyze the data.

4.4 Statistical analysis

To analyze the results, Welch’s unequal variances t-test was performed. This is because the different treatment groups do not necessarily have the same variance. The Welch’s t-test is defined using the formula:

$$t = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{s^2_{\overline{X}_1} - s^2_{\overline{X}_2}}}, \qquad df = \frac{\left(\frac{s_1^2}{N_1} - \frac{s_2^2}{N_2}\right)^2}{\frac{s_1^4}{N_1^2(N_1-1)} - \frac{s_2^4}{N_2^2(N_2-1)}},$$

$$s^2_{\overline{X}_i} = \frac{s_i^2}{N_i}, \qquad s_i^2 = \frac{1}{(N_i-1)^2} \sum_{k=1}^{N_i} (x_k - \overline{X}_i)^2,$$

where $\overline{X}_i$ is the mean of group *i* and N_i is its size. Using the test statistic t and the degrees of freedom df, the corresponding t-distribution threshold for a two-tailed t-test with $\alpha = 0.05$ can be calculated. If the absolute value of t is larger than the corresponding threshold, then the null hypothesis can be discarded, meaning that there is a significant difference between the means of the two treatment groups. The statistical analysis was done using Python and the statistical packages *statsmodels* and *SciPy*.

5. Results

5.1 Distribution of log(CFU/ml) over treatment time, wavelength and refrigeration

To analyze the change in culturability of *E. coli* O157:H7 *gfp*+ after light exposure, the number of colony forming units (CFU) were counted. To compare the effects between the treated and control samples, the log concentration of CFU/ml for each treatment group was investigated. A visual analysis indicated that the treated samples had a lower log concentration of CFU for all treatment groups (figure 1). Treatment groups refers to collections of samples that share a specific time, wavelength or refrigeration setting. For example, the treatment group *time=20 min* refers to all samples that were treated with an exposure time of 20 minutes, regardless of wavelength or refrigeration.

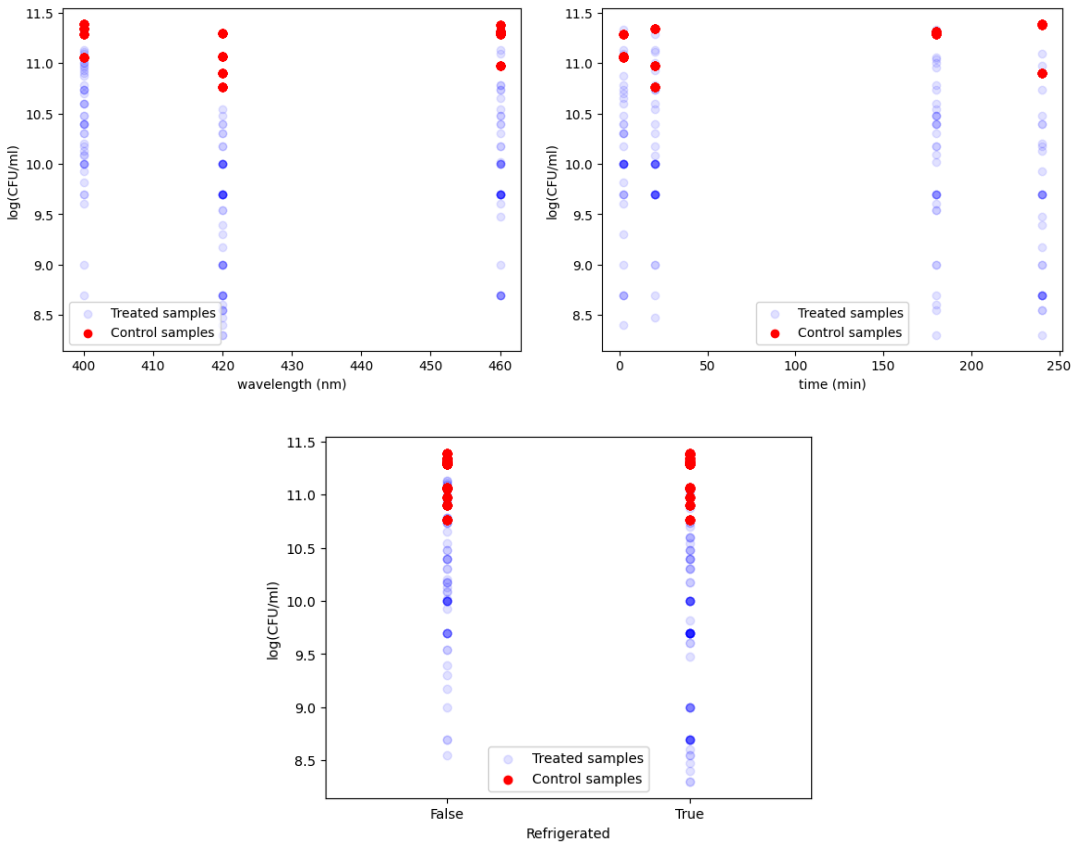

Figure 1: Distribution on log(CFU/ml) in relation to time (top-left), wavelength (top-right), and refrigeration (bottom).

5.2 Log-transformation of colony forming units

When working with bacterial concentrations it is common to use log-transformations due to the exponential growth inherent to bacteria. To verify that this transformation is sound the distribution of samples is compared to a normal distribution, since normally distributed data is necessary in order to apply many statistical methods. This comparison is done for both the linear CFU/ml and the log-transformed log(CFU/ml). For this, quantile-quantile (QQ) plots are used, which compare samples from two given distributions. If the points follow a straight line, the two distributions are similar. The log-transformed concentrations (figure 2, right) follow the line to a greater extent than the linear concentrations (figure 2, left). This shows that using log concentrations of CFU is reasonable for statistical purposes.

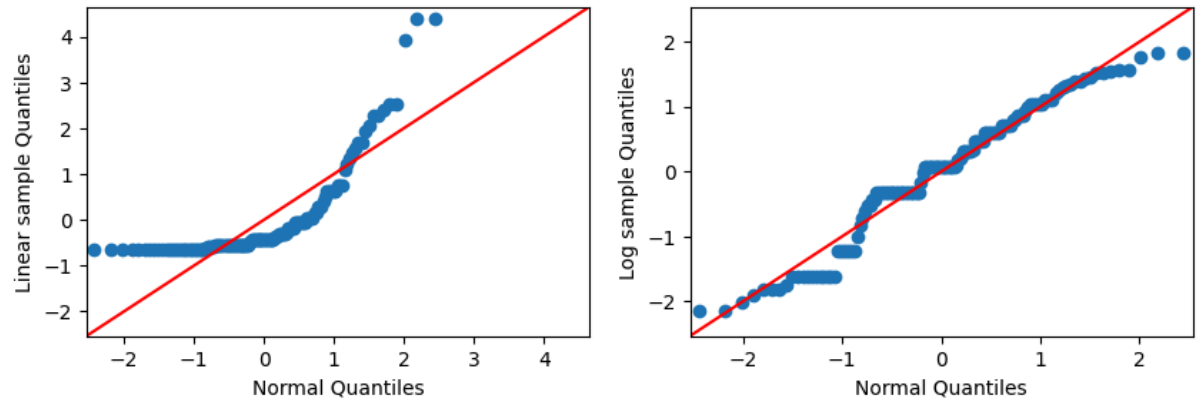

Figure 2: QQ-plots comparing a normal distribution to the untransformed linear concentration (left) and the log-transformed concentration (right). A straight line indicates that the distribution of data follows a normal distribution. The log-transformed concentration follows the line to a greater extent, showing that it is more normally distributed.

5.3 Change in log concentration compared to the size of the original sample

In order to determine if the treatments have a similar effect regardless of if the starting concentration of CFU is high or low, a linear regression model was employed. By analyzing the slope of the linear model, the impact of starting CFU/ml can be evaluated. Below is a 95% confidence interval for this slope (table 1). Since the interval contains 0, the effect is not statistically significant. This is also indicated by the gentle incline of the fitted linear model

(figure 3). Thus, it cannot be stated that the treatment's relative effect on the log concentration of bacteria is affected by the amount of bacteria in the control sample.

Table 1: 95% confidence interval for bacterial reduction in relation to sample size. The interval stretches from negative to positive, meaning that the treatments have a similar effect regardless of starting CFU/ml.

	Lower Bound	**Upper Bound**
Intercept	-5.265275	8.981965
Slope	-0.913877	0.362183

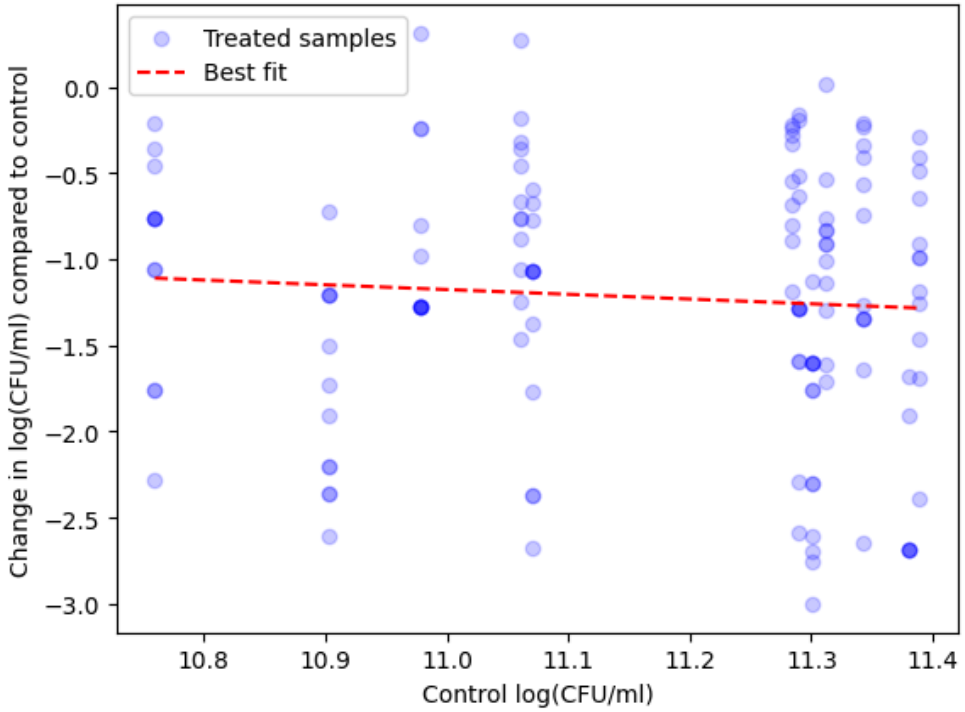

Figure 3: Difference in log(CFU/ml) between treated and control samples as a function of control sample log(CFU/ml).

5.4 Overall treatment effect

To demonstrate the overall treatment effect on the growth of *E. coli* O157:H7 *gfp*+, a 95% confidence interval demonstrating the average change in log(CFU/ml) compared to the control group was constructed (table 2), using all treated samples. The interval is completely below zero which indicates that overall, the treatments resulted in a lower concentration of colony forming units. This confidence interval was transformed into a percentage reduction in CFU/ml, yielding an average reduction of 94.0%. To investigate the effects of individual treatment parameters, similar confidence intervals were constructed for each treatment group (figure 4). All such intervals were completely below zero, indicating that all investigated treatments resulted in a significant reduction of CFU/ml.

Table 2: 95% Confidence Interval for change in log(CFU/ml) for all treated samples compared to control groups. The interval is completely below zero which means that the treatment has significantly reducing effects. The general percentage reduction can also be observed in the table.

	Lower Bound	Mean	Upper Bound
change in log(CFU/ml)	-1.350	-1.221	-1.092
% reduction in CFU/ml	91.9%	94.0%	95.5%

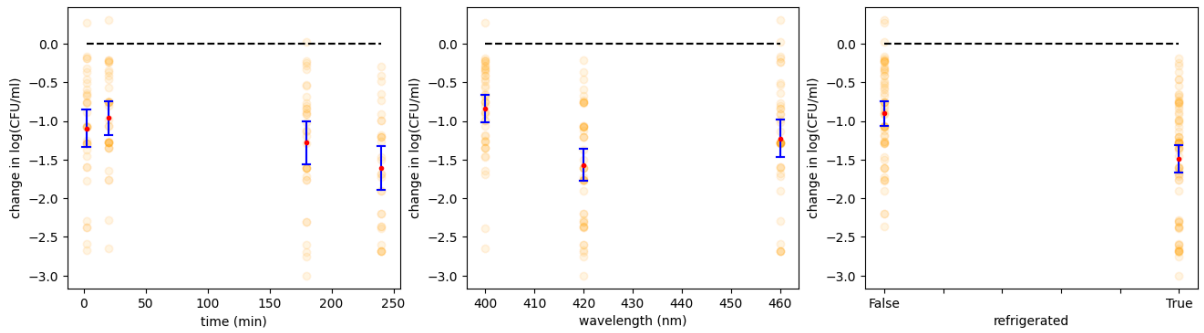

Figure 4: 95% confidence intervals for change in log(cfu/ml) compared to control group over treatment time, wavelength and refrigeration. All intervals are completely below zero which means that each treatment group has a significant reducing effect.

5.5 Comparison between treatments

Comparison using linear regression model

To compare the effect of different treatments a linear regression model has been employed. This model is trained to predict the change in log(cfu/ml) using time, wavelength and refrigeration as inputs, according to the equation

$$change = a + b * time + c * wavelength + d * is\_refrigerated,$$

where the parameters a, b, c and d are learned by the model using the collected data. The confidence intervals for the estimates of the parameters of the model were calculated (table 3). For time and refrigeration, the coefficient confidence interval is completely below zero. This means that in general, increasing treatment time or applying refrigeration to the sample further increases the effect of the treatment. This cannot be said for increasing the wavelength.

Table 3: 95% confidence intervals for the linear regression coefficients. The confidence interval for the coefficients is completely below zero for time and refrigeration, but not wavelength.

Parameter	**Lower Bound**	**Upper Bound**
Intercept (a)	-0.904497	3.109831
Time (b)	-0.003125	-0.000862
Wavelength (c)	-0.008969	0.000417
Refrigerated (d)	-0.776502	-0.318759

5.6 Comparison using pairwise statistical t-test

To compare the treatments in more detail, t-tests were performed between treatments grouped by a specific time, wavelength or refrigeration. Since the variance is not necessarily the same between groups, an *unequal variance t-test* (Welch's t-test) was used and the p-values for the comparison between treatment groups were calculated (figure 5). The null hypothesis is as follows:

H_0: *no difference in treatment effect*

H_1: *difference in treatment effect*

If the p-value is below 0.05, the difference between treatments is considered to be statistically significant. This confirms the results from the linear regression, that longer exposure time and added refrigeration leads to a higher reduction in CFU concentration. This is however not statistically significant between all time exposures, as it could only be said that 4 hours resulted in a larger reduction compared to 2 and 20 minutes. The results also show that 420 nm is the optimal wavelength, as it results in a significantly higher reduction in CFU concentration compared to 400 and 460 nm.

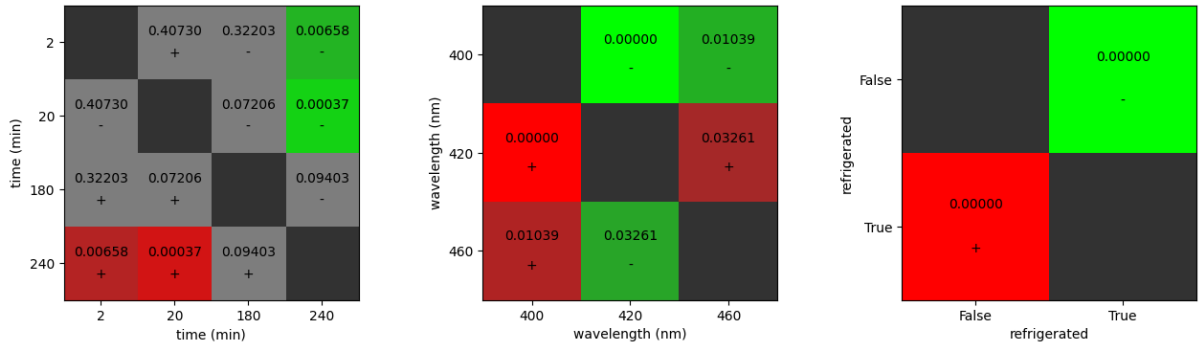

Figure 5: P-values of t-test between treatment groups, comparing x-axis to y-axis. Statistically significant differences (p-value < 0,05) are highlighted. The sign (+/-) indicates whether the data suggests that the treatment from the x-axis has reduced log(CFU/ml) compared to the y-axis (-) or not (+).

5.7 Result of ddPCR

To analyze the changes of the genome, a ddPCR was performed. The virulence genes eaeA and eaeL, related to attachment, were investigated by examining cDNA. An absolute quantification of *E. coli* O157:H7 *gfp*+ was further performed to estimate the amount of *E. coli* O157:H7 *gfp*+ in the sample. The absolute quantification of *E. coli* O157:H7 *gfp*+ was also performed with cDNA. Concentration refers to the concentration of positive drops after droplet reading. A positive drop means that the desired gene expression was found in the drop.

5.7.1 eaeA

The gene expressions of eaeA were examined and the distribution of log concentration of positive drops for each treatment group was analyzed. To further examine the mean concentration as well as the distribution within each treatment group, 95% confidence intervals were constructed (figure 6). To determine if one specific treatment had greater impact on the final concentration compared to another, a pairwise t-test with unequal variance was performed. The only treatment which had a statistically significant reduction was when the bacterial solution was refrigerated after light exposure. As for the other treatments, even though an increase or decrease in concentration was observed, the p-value is above 0,05 which means that the changes are not statistically significant (figure 7).

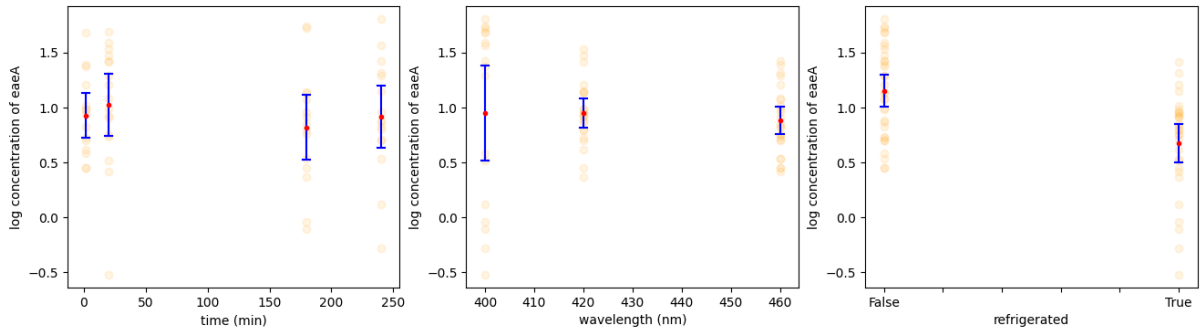

Figure 6: Confidence interval for log concentration of eaeA over treatment time, wavelength and refrigeration. Sample points are marked in yellow and the mean value with red.

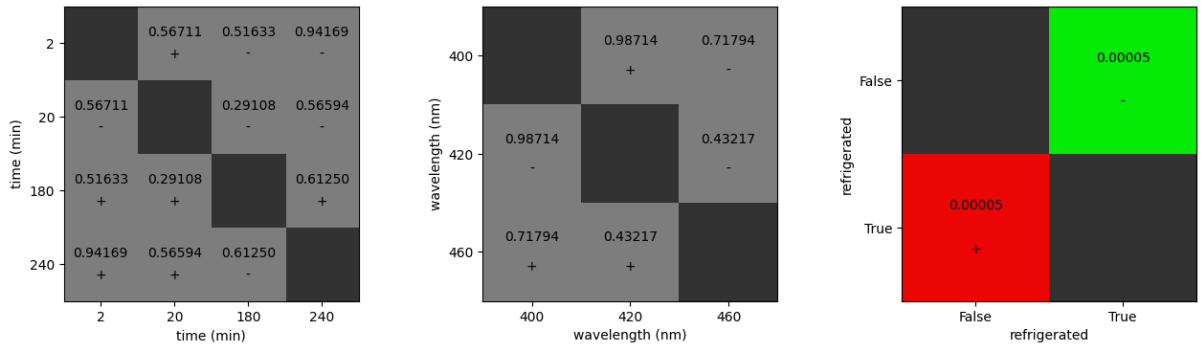

Figure 7: P-values of t-test between treatment groups for eaeA, comparing x-axis to y-axis. Statistically significant differences (p-value < 0,05) are highlighted. The sign (+/-) indicates whether the data suggests that the treatment from the x-axis has reduced eaeA concentration compared to the y-axis (-) or not (+).

5.7.2 eaeL

The gene expressions of eaeL were examined and the distribution of log concentration of positive drops for each treatment group was analyzed. To further examine the mean concentration as well as the distribution within each treatment group, 95% confidence intervals were constructed (figure 8). To determine if one specific treatment had greater impact on the final concentration compared to another, a pairwise t-test with unequal variance was performed. 460 nm had a statistical significantly higher concentration compared to both 400 nm and 420 nm. A lower concentration was also observed among the refrigerated samples, compared to the non-refrigerated samples, and this difference was statistically significant (figure 9).

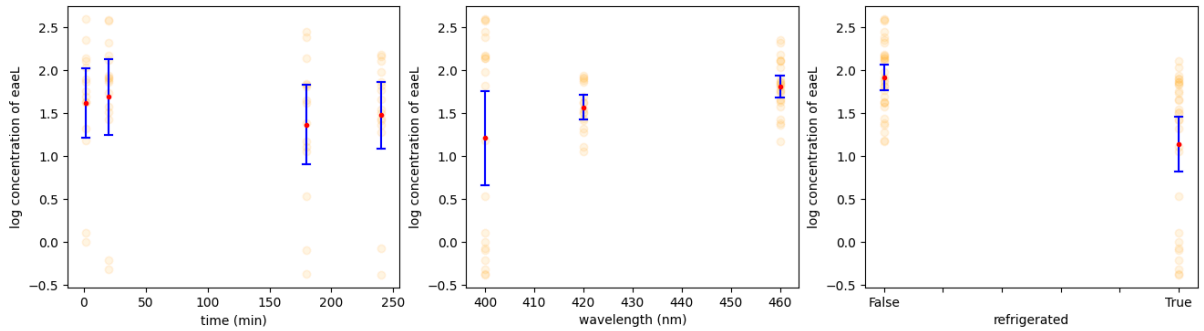

Figure 8: Confidence interval for log concentration of eaeL over treatment time, wavelength and refrigeration. Sample points are marked in yellow and the mean value with red.

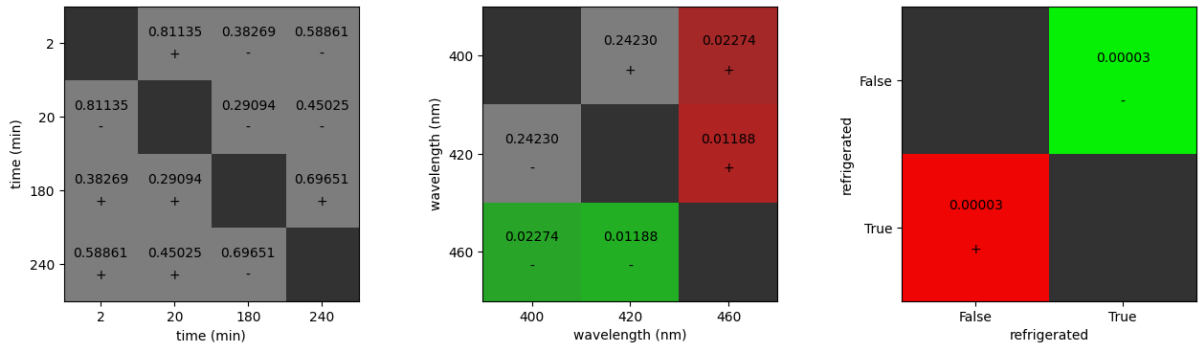

Figure 9: P-values of t-test between treatment groups for eaeL, comparing x-axis to y-axis. Statistically significant differences (p-value < 0,05) are highlighted. The sign (+/-) indicates whether the data suggests that the treatment from the x-axis has reduced eaeL concentration compared to the y-axis (-) or not (+).

5.7.3 Absolute quantification of *E. coli* O157:H7 gfp+

The amount of *E. coli* O157:H7 *gfp*+ was analyzed by absolute quantification and the distribution of log concentration of positive drops for each treatment group was noted. To further examine the mean concentration as well as the distribution within each treatment group, 95% confidence intervals were constructed (figure 10). To determine if one specific treatment had greater impact on the final concentration compared to another, a pairwise t-test with unequal variance was performed. Both 420 nm and 460 nm resulted in higher concentrations of positive drops compared to 400 nm. There was however no statistical significant difference between 420 and 460 nm. A lower concentration was also observed among the refrigerated samples, compared to the non-refrigerated samples, and this difference was statistically significant (figure 11).

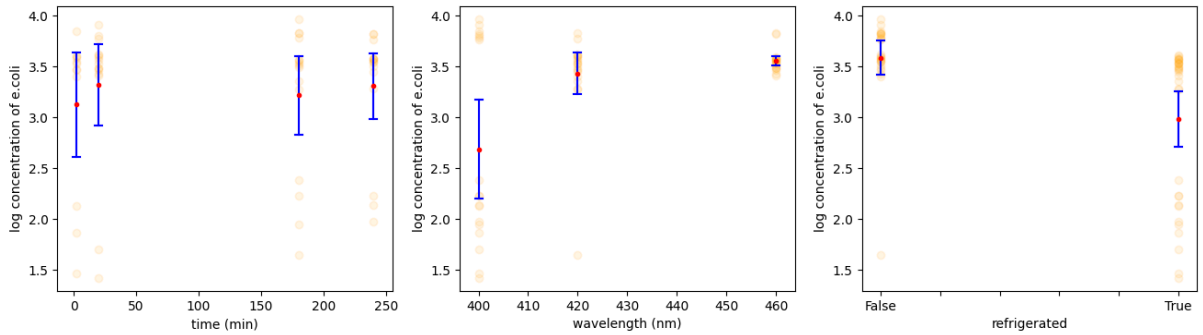

Figure 10: Confidence interval for log concentration of E. coli O157: H7 gfp+ over treatment time, wavelength and refrigeration. Sample points are marked in yellow and the mean value with red.

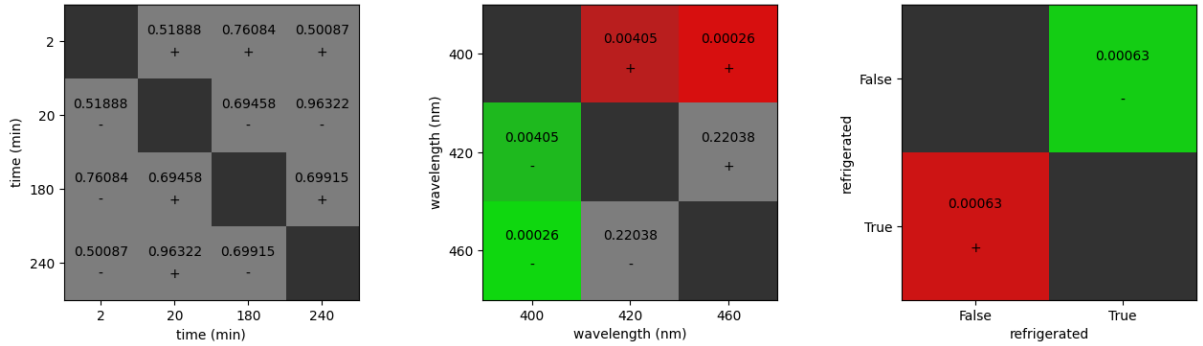

Figure 11: P-values of t-test between treatment groups, comparing x-axis to y-axis. Statistically significant differences (p-value < 0,05) are highlighted. The sign (+/-) indicates whether the data suggests that the treatment from the x-axis has reduced E. coli O157:H7 gfp+ concentration compared to the y-axis (-) or not (+).

6. Discussion

This paper has investigated the impact of blue light on *Escherichia coli* O157:H7 *gfp*+. Both changes in bacterial growth and changes of gene expressions have been explored.

The results indicated that the blue light treatment did affect the growth of *E. coli* O157:H7 *gfp*+, and that both the frequency of the wavelength, length of exposure as well as refrigeration were important factors in influencing the final number of observed CFU/ml. As seen in figure 4, all treatment groups showed a significantly reduced number of colony forming units per ml (CFU/ml), compared to the control group. However, after closer observations, differences in efficacy between the treatment groups could be discovered.

Looking at the growth in relation to wavelength, the linear regression model (table 3) did not show that an increase in wavelength would mean further reductions of CFU/ml. This can be stated since the confidence interval for the wavelength coefficient included 0. However, the results of the pairwise statistical t-test showed that there was a significant difference in efficiency between all wavelengths (figure 5). The optimal wavelength was found to be 420 nm, as it resulted in a significantly higher reduction compared to both 400 nm and 460 nm light. Additionally, 460 nm showed a greater effect in reducing CFU/ml compared to 400 nm. The fact that 420 nm was superior to 400 and 460 nm explains why the linear regression model didn't show a significant coefficient for the wavelength, as the relation between wavelength and log(CFU/ml) reduction was not linear. Comparing the results with other studies, Hadi et al (2020) argue that the optimal spectrum of blue light for the inactivation of *E. coli* O157:H7 is between 400-450 nm. However, another study by Jung & Yoon (2023) claims that the optimal spectrum of blue light for inactivation of *E. coli* O157:H7 was between 415-425 nm. The study by Jung and Yoon is more recent which could mean that their suggested spectrum is more relevant. In comparison, the results of this paper shows that the mean reduction of log(CFU/ml) was significantly higher for 420 nm, which is in line with these previous findings. The different inactivation rates observed by different wavelengths can be explained by the mechanism of the photosensitizer, since different endogenous photosensitizer molecules absorb light at different wavelengths (Lena et al., 2023). This will result in more or less production of reactive oxygen species (ROS) depending on which, and the number of, photosensitizer molecules are present in the targeted organism. Therefore,

further studies on different photosensitizers are also needed to fully understand the effect of blue light on microbes of different species.

When observing the change in growth in relation to time, all time periods resulted in a reduction of CFU/ml (figure 4). This can also be observed in the linear regression model which indicated that an increase in time would lead to further reductions of CFU/ml (table 3). This can be stated since the confidence interval for the time coefficient is fully negative. However, when further studying the difference between treatment groups using the pairwise statistical t-test (figure 5), a statistically significant effect could be observed between the 4 hour exposure, compared to the 2 and 20 minute exposures, where 4 hours was found lead to a higher reduction in log(CFU/ml). There was, however, no discernable difference between any other exposure times. If PDI should be implemented in the food industry, 4 hours of exposure is perhaps not reasonable. However, even though the difference between 2 minutes and 4 hours was statistically significant, it was not large (figure 4). The mean reduction of log(CFU/ml) was -1.097 and -1.608 for 2 minutes and 4 hours respectively, corresponding to a percentage reduction in CFU/ml of 92.0% and 97.5% respectively. This leaves the theoretical option of treating the bacteria for 2 minutes instead of 4 hours, with the end result of a lower, but still substantial, reduction. An alternative solution to decrease exposure time is to add exogenous photosensitizers to the suspension (Cossu et al., 2021; Lena et al., 2023). By adding photosensitizers to the sample the inactivation time decreases as more ROS can be generated, hence accelerating cell death of the targeted microorganism (Lena et al., 2023). However, added photosensitizers must first be established as safe for consumption as well as confirmed not to affect the quality of the food product if this was to be implemented within the food industry.

Looking at the bacterial growth in relation to refrigeration, both treatment groups resulted in a significant reduction of bacteria (figure 4). However, the negative coefficient to the refrigeration variable indicates that refrigerating the sample after light exposure resulted in a greater reduction of CFU/ml (table 3). This result is in line with the pairwise statistical t-test which showed that refrigerating the sample resulted in a significantly greater reduction of bacteria, compared to the non-refrigerated samples. This could be due to the changes of the cell membrane when microorganisms experience colder temperatures. To ensure membrane function, microorganisms adjust the composition of membrane lipids when exposed to colder temperatures (Lena et al., 2023). This is done to increase the fluidity of the cell membrane by

increasing the amount of unsaturated fatty acids in the membrane. These fatty acids are more sensitive to oxidation by ROS, which in turn results in further damage to the cell membrane. Due to this change of the membrane when exposed to colder temperatures, it would be interesting to study if a colder temperature during the light exposure would further impact the final reduction.

Exploring the changes in gene expressions, the ddPCR of eaeA showed no statistically significant difference between treatments, except that refrigeration resulted in a clear decrease in the presence of eaeA (figure 6). Whether this is due to an effect of the light treatment or just the refrigeration itself is not certain. A similar result was found in the ddPCR of eaeL, showing a decrease when refrigerating the sample. However, for this gene, an increase in gene presence was observed when using a wavelength of 460 nm. When the analysis of the samples was performed by ddPCR, the samples containing DNA did not give a result. This means that there is no control group to compare with and that the concentration of drops can only be compared with the result of the ddPCR with cDNA.Thus, we cannot be sure if the increase of gene presence from 460 nm is due to random variation or some property of the treatment. Further, since a majority of the treatment groups generated similar levels of positive drops, and there is no reference point due to the absence of results from ddPCR of DNA, we cannot establish if the light treatment affected the gene presence.

When analyzing the absolute quantification of *E. coli* O157:H7 *gfp*+, a difference in drop concentration in relation to wavelength could be observed. A higher concentration of bacteria was observed after the light treatment for wavelengths 420 and 460 nm, compared to 400 nm (figure 14). However, this contradicts the result observing the number of CFU where those higher wavelengths resulted in a greater reduction of colony forming units (figure 5). A possible explanation could be the formation of viable but non-culturable (VBNC) bacteria. The VBNC state is a condition in which bacteria still are metabolically active, but lacks the ability to grow on conventional media (Pienaar et al., 2016). Many bacterial strains can enter a VBNC state as a response to environmental stress, as an adaptation strategy for long-term survival. When bacteria was put under the lamp, the environmental stress could have resulted in the formation of VBNC bacteria. This would explain why fewer CFU were observed while the ddPCR indicated a higher concentration of bacteria for those wavelengths. As VBNC bacteria don't grow on conventional media they can be difficult to detect by conventional methods within the food industry, which can lead to an underestimation of viable cells

(Pienaar et al., 2016). Since VBNC *E. coli* has the ability to return to a culturable state, this underestimation can be of public health concern.

There are many factors which point to the possibility of implementing PDI for sanitation within the food industry. Today, there are a wide range of sanitation methods currently used in food production to combat microbes, all with different inconveniences. While thermal processing is commonly used within the food industry, it may lead to loss of nutrients as well as undesired changes of structure and taste of the final product (Li & Farid, 2016). Alternatives to thermal processing are high-pressure processing (HPP) and pulsed electric field (PEF). While these leave the produce more intact, both HPP and PEF are more costly and less energy efficient than thermal processing. Another light-based technology which has germicidal abilities is Ultraviolet (UV) irradiation (Singh et al., 2021). UV, and especially UVC light, has the ability to damage the DNA of pathogens and UVC light is currently used to free dairy products, liquid foods (juice, soft drinks etc.) and meat products from undesired microorganisms. UV irradiation has also been used to disinfect wastewater, water, air and surfaces. However, while light on the UV spectrum shows great germicidal effect, it has a significant disadvantage as it can cause severe damage to the employers working with the food during sanitation. Constant exposure to UV radiation increases the risk of developing different types of skin cancer, face erythema as well as complications to the skin, eyes, nails and hair of the workers (Hadi et al., 2020). Other sanitation methods include the use of disinfectant chemicals (Sansebastiano et al., 2007). While the most commonly used disinfectants often prove to be effective in eliminating microorganisms, they bring other disadvantages (Wirtanen & Salo, 2003). Some of the highly used chemicals for disinfection include chlorine dioxide, hydrogen peroxide, iodine and peracetic acid and they all bring complications. Among other things, chlorine dioxide for example generates toxic by-products, hydrogen peroxide requires high concentrations to be effective, iodine alters the taste and peracetic acid is corrosive. Disinfectant chemicals also bring the issue of development of resistance among bacteria (Wirtanen & Salo, 2003). All are factors which may affect either the consumer, workers in the facility or the environment.

In comparison, photodynamic inactivation is a more energy-efficient sanitation method and there is also a lower cost of operation, compared to thermal processing, HPP and PEF (Cossu et al., 2021). As for changes in nutrient content, texture and color that are related to other current sanitation methods, these factors have not been extensively studied due to the novelty

of the technology. However, Bhavya et al (2021) showed in a recent study on pineapple that blue light did not have a significant effect on color or levels of antioxidants. To be certain of structural changes of the food product, more research is required. Comparing blue light to UV, blue light has a clear benefit concerning the workers environment. As previously stated, exposure to UV has a profound impact on human health, since exposure to blue light is less harmful to mammalian cells, it can bring a more safe work environment for the employees (Hadi et al., 2020). Another issue of conventional methods is the question of resistance. Throughout the evolutionary history of pathogenic bacteria, resistance to antimicrobial agents has been a reality (Varela et al., 2021). Today, antimicrobial resistance is a cause of public health concern on a global scale. Since the ROS generated as a consequence of irradiation attacks cellular structures at random, the mode of action is unspecific (Hasenleitner & Plaetzer, 2019). As a result, the development of resistance to PDI is considered unlikely. This is another aspect which promotes the implementation of blue light photodynamic inactivation in the food industry.

However, PDI also has its limits. To achieve bactericidal effects, the bacteria needs to be directly exposed to the light (Kim et al., 2017). If parts of the sample are in shadow, it would in turn impact the final reduction. This speaks to the potential difference in bactericidal effects that can be related to different types of products where the light cannot be evenly distributed. The treated samples in the experiment showed a reduction of CFU/ml after exposure to blue light, the exposed bacteria was however contained in a clear liquid. Should the product not be in a clear liquid form but instead for example a vegetable or meat with a rough surface, parts of the surface may be shadowed. This would mean that not all bacteria are exposed to the light, hence affecting the final reduction. Due to the novelty of PDI in relation to the food industry, few studies have been carried out where different surfaces have been studied (Lena et al., 2023). More studies are hence needed to ensure similar levels of bacterial reductions on surfaces, as in clear liquids.

Another issue is the growth of bacteria within a product. If a contaminated food product has been cut or damaged along the production line, bacteria may have entered the product through the wounds (Tournas, 2005). This indicates bacterial growth within the product. Today, there is a lack of studies regarding the penetration capacity of blue light on different surfaces (Lena et al., 2023). For comparison, UV light has a penetration capacity of a few millimeters, depending on the optical characteristics of the food (Hinds et al., 2019).

However, if there is bacterial growth within a non-liquid food product, it is unlikely that the light would eliminate all bacteria, since the size of a majority of solid foods exceeds a few millimeters in depth.

7. Conclusion

Photodynamic inactivation using blue light proved to impact the growth of *E. coli* O157:H7 *gfp+* to great extent, which speaks for its potential for future implementation in the food industry. However, the possible presence of viable but non-culturable bacteria as a result of blue light exposure, emphasizes the opposite conclusion. With the great amount of both advantages and disadvantages related to the method, as well as the current lack of extensive studies on the subject, it is clear that more research is required to fully understand how this technology affects the microorganisms, the quality of the food as well as the safety of the work environment.

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Popular Science Summary

Livsmedelsindustrin idag är mycket komplex, särskilt om man jämför med hur den såg ut ett par decennier tillbaka. I takt med växande innovationer har industrin bland annat blivit mer maskinell, utbudet av processad mat ökar och idag transporteras livsmedel över hela världen för att nå slutkonsumenten. De här förändringarna medför en ökad andel mellanhänder som hanterar vår mat, vilket då också leder till en ökad spridning av bakterier och andra mikroorganismer som kan göra oss sjuka. En av de bakterier som orsakar mest sjukdomsfall i världen är Shiga-toxin producerande *Escherichia coli* som vid smitta, i värsta fall, kan leda till döden. Därför letar man nu ständigt efter nya tekniker som kan avlägsna *E. coli*-bakterier, och andra mikroorganismer, från maten vi äter.

En teknologi som nyligen uppmärksammats inom livsmedelsindustrin är användandet av blått ljus för att döda mikroorganismer. När en mikroorganism träffas av blått ljus händer något som kallas för fotodynamisk inaktivering. Det betyder att det inuti mikroorganismen bildas en överproduktion av reaktiva syreradikaler. Dessa är mycket skadliga för olika organismer och kan då leda till att mikroorganismen slutligen dör. När vi belyste *E. coli*-bakterier med blått ljus kunde vi se en genomsnittlig reduktion av bakterier på 94%! Vi kunde även se att olika våglängder av blått ljus påverkar hur mycket bakterier som dör. Spektrat för blått ljus är mellan ca 400-480 nanometer och man har sett att 420 nanometer har funkat bättre jämfört med 400 och 460 nanometer. Andra faktorer som kan påverka hur mycket bakterier som dör är hur länge bakterierna exponeras för ljuset, samt temperaturen efter exponeringen. Skulle blått ljus användas inom livsmedelsindustrin kan man därför själv bestämma vilken våglängd, tid och temperatur som mikroorganismerna utsätts för, för att anpassa efter just den typ av produktion som pågår i fabriken.

Men varför ska vi använda blått ljus när det redan finns andra metoder för att döda mikroorganismer? Till att börja med är behandling med blått ljus mycket mer energieffektivt jämfört med andra metoder som används idag. Behandlar man men blått ljus undviker man också de farliga kemikalier som ofta används inom industrin, vilket är bra för både arbetare i fabriken och miljön. Dock finns det fortfarande osäkerheter inom teknologin eftersom man nyligen börjat upptäcka dess potential inom livsmedelsindustrin. Därför är det viktigt att fortsätta forska på ämnet så vi kan vara helt säkra på att maten vi äter fri från oönskade mikroorganismer.

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