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# Comparison between methods for detecting bacterial concentration for the determination of bacterial radioresistance value.

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## 1. Introduction

The value of radioresistance can be called the D10 value, measured in kiloGray (kGy), and it is the dose required to inactivate 90% of the population at the initial moment. This value is determined by a simple linear regression. The kinetics of microbial death is directly related to the doses of applied irradiation. The radioresistance of a microorganism is associated with many factors, such as temperature, habitat, atmosphere, types of cells (Gram-positive or Gram-negative), and physiological age of the cell [1].

Currently recommended by the International Organization for Standardization (ISO) [2] is that the sterilization dose should be adjusted for each type of product, depending on its microbial load [3]. The chosen sterilization doses must be in accordance with the initial microbial load, the level of sterility (SAL), the relative combination of various microorganisms present in the product with their different D10 values, and the radiosensitivity of the microorganisms present. It is observed that the fraction of microbial survival is inversely proportional to the absorbed dose [4].

Microorganisms can be destroyed by physical or chemical agents, and this destruction follows an exponential law. The finite probability of survival can be calculated based on the number of species existing in the product, the lethality of the sterilization process, and in some cases, taking into account the environment in which the microorganisms live. Thus, the sterility of a particular item among a population of sterilized products cannot be absolutely assured. For the sterilization and inactivation process to follow logarithmic reduction criteria, it is necessary to ensure a low initial microbial load. For a microorganism to be considered dead, it must no longer proliferate in culture media considered rich, in which its proliferation can be observed under normal conditions [5].

To determine the radioresistance value of the bacterium, it is necessary to have the bacterial concentration value before irradiation, as well as the value of the concentration after irradiation, with the care not to allow duplication during sample preparation for data reading. One of the commonly used microbiological tests is the microbial enumeration test, which is based on the growth of CFU (Colony Forming Units) on Petri dishes or filter membranes. The number of colonies from an enumeration of a specific dilution aliquot provides the microorganism count per unit of sample [5]. This technique takes a minimum of 24 hours for results. Two methods can be used to estimate the number of microorganisms in a particular sample: direct or indirect. The most commonly used direct method is through particle counters, which are equipment based on optical and electronic deviations, counting chambers such as the Neubauer Chamber, and stained smears for visualization under a microscope. Direct methods are faster as there is no need to wait for colony growth, thus being a rapid method. Serial dilutions enable defining the volume containing the recommended cell density; this dilution needs to be made from the stock solution. Through plate counting, it is possible to determine the microbial density for each dilution [5;6].

## 2. Methodology

E. coli ATCC strains were used for the study. They were inoculated in TSB broth (Tryptic Soy Broth), which is a rich and non-selective culture medium to obtain a large and healthy bacterial population. After inoculation, the tubes were incubated in a bacteriological incubator at 37°C for 24 hours. The next

day, the optical density of the culture medium was measured using a spectrophotometer to determine the initial concentration. We irradiated 11 cm diameter plates containing 0.5 mL of saline solution and bacteria at the known concentration with increasing irradiation doses according to what is already established in the literature, from 0.5 kGy to 3 kGy (in intervals of 0.5 kGy).

2.1 Plate Count
After irradiation, we performed serial dilution according to the estimated bacterial concentration so that colony counting on plates would be possible. I inoculated 3 Müller Hinton agar plates with the highest dilution and the previous one of each irradiation dose, as well as the negative control for irradiation. The plates were incubated at 37°C for 24 hours and then read. After reading, the values can be plotted on a Cartesian plane.With the plotted graph, we will be able to observe the decay rate in the bacterial population present, making it possible to establish the D10 value of this bacterium through simple linear regression.

2.2 Microdilution
In a 96-well plate, I added 180 µL of TSB medium and 20 µL of sample at the known concentration in the first wells corresponding to dilution 0, and serial dilution was performed for the other wells up to dilution -3. This plate was incubated at 37°C for 24 hours with agitation. Then it was read by a plate spectrophotometer. With the data, it was possible to plot the data on a Cartesian plane for the determination of the bacterium's radioresistance value.

2.3 InCell Analyzer
In a 96-well plate, 270 µL of DPBS (Phosphate Buffered Saline Solution) and 30 µL of sample were added. We added Calcein solution (1 µM concentration) and Propidium Iodide (1.5 mM concentration) so that live bacteria were stained green and dead bacteria orange. This plate is placed in the InCell Analyzer equipment, where it will be incubated for 12 hours and photographed microscopically every 30 minutes; data analysis was done in Fiji software. With this data, it was possible to plot the data and compare the analysis methods.

## 3. Results and Discussion

After using the three methods described for determining bacterial concentration (plate count, microdilution, and InCell Analyzer), we can observe the following results:

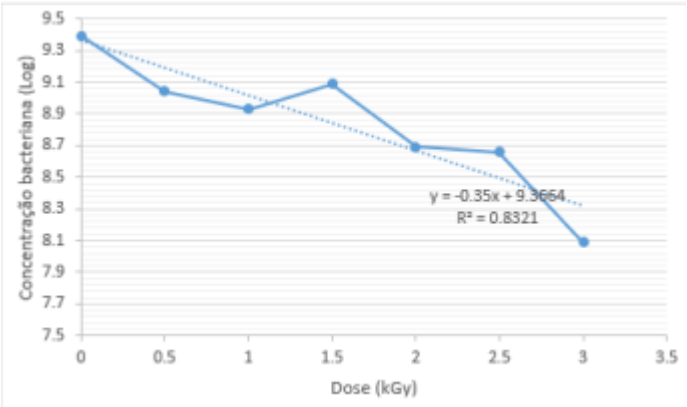


Figure 1. Bacterial concentration (CFU.log) of different absorbed radiation doses inoculated on Müller Hinton agar plates after 24 hours of incubation.

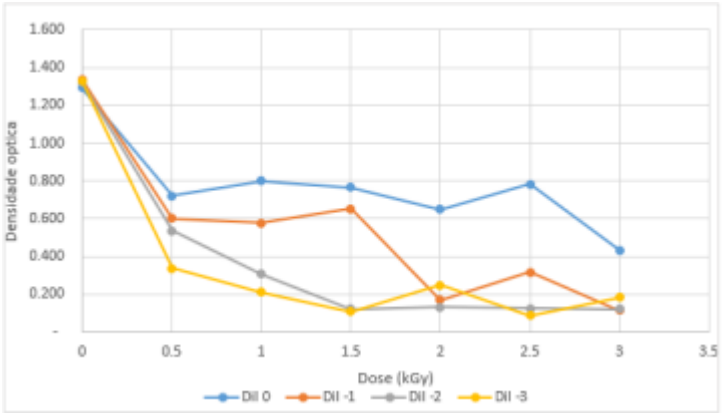


Figure 2. Optical density reading of the 96-well plate produced by the microdilution method.

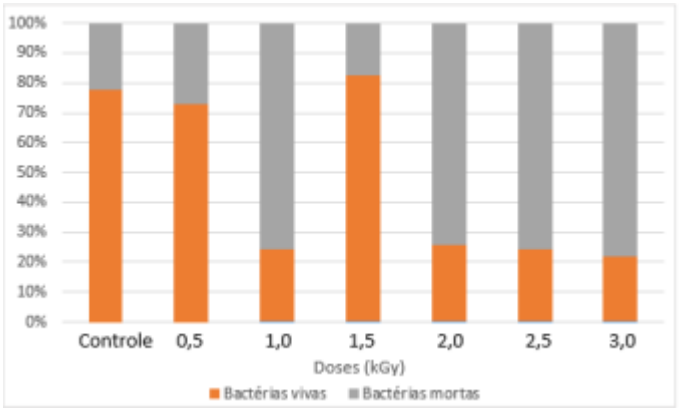


Figure 3. Reading of the 96-well plate by the InCell. Analyzer equipment and data processing using Fiji software.

The most commonly used method for counting and determining the concentration of colonyforming units is agar plate counting. However, when compared to the other two methods, it is the slowest, taking more than 24 hours for results. Another obstacle is the necessary dilutions for countable plates. The microdilution method is sensitive up to dilution -1, as we can see in the graph, the lines referring to the control and dilution -1 behave very similarly, whereas the smaller dilutions do not.

When the results of the three methods are compared, we can observe that they are similar, where we can note that at a radiation dose of 1.5 kGy, there is an increase in the bacterial population.

## 4. Conclusions

The methods for determining bacterial concentration after irradiation differ in time and the amount of inputs used. The fastest and most sensitive method among those analyzed in this study is the use of the InCell Analyzer equipment, but obtaining the equipment and dyes, as well as training for its use, increases the cost of this method. The traditional method of inoculation and reading on plates is the most known and used, as it is a low-cost method, but it takes at least 24 hours to obtain results.

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