*Review*

# Exploring the Variability in Antibacterial Testing of Resin Dental Composites among Investigators: A Narrative Review

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**Abstract:** Caries is a common dental problem brought on by factors like excessive sugar consumption, poor oral hygiene, and the presence of microorganisms in the mouth. This dental pathology is treated with a variety of filling materials, including tooth-colored direct resin dental composite (RDC), glass ionomer cement (GIC), and dental amalgam (also known as silver filling). RDC is the most preferred filling material in dental clinics due to its excellent esthetics and minimal tooth preparation, making it the need of the modern era. However, antimicrobial agents were added to this material in order to enhance its ability to prevent secondary caries. The antibacterial activity of RDC has been tested using a variety of methods, but testing protocols have been found to vary. Thusly, the point of this article is to examine the disparity in the strategy involved by specialists for testing the antibacterial properties of RDCs.

**Keywords:** resin dental composites; antibacterial testing; agar diffusion; colony-forming unit; incubation time; sterilization

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## 1. Introduction

Caries remains one of the world's biggest dental problems [1]. The common etiological factors of dental caries include the presence of microorganisms in the oral cavity, poor oral hygiene, and a high intake of sugar. This dental problem is progressed by the acid levels produced during the fermentation of sugary food in the oral cavity [2].

This irreversible microbial pathology shows the initial clinical sign with the appearance of a white spot lesion on the decayed tooth, which then leads to cavity formation by the demineralization of the inorganic component followed by the destruction of the organic components of the tooth structures [3]. If the cavity is left untreated, it consequently leads to the involvement of dental pulp and eventually may result in infection, pain, an abscess, and sometimes tooth loss. To counter this problem, different restorative dental materials have been used in clinical practice, for instance, dental amalgam, resin-based composites, and glass ionomer cements (GICs), among which composites are the most widely recommended materials due to their realistic tooth appearance and sufficient durability.

In today's modern era, the demand for esthetics and the call for minimum-intervention dentistry has made resin-based dental composites (RDCs) the most desirable materials for dental restorations because of their remarkable esthetic properties and least compromise of the sound tooth structure during pre-filling tooth preparation. Irrespective of the brand, RDCs have shown an acceptable survival rate in direct posterior restorations, about 73% in 33 years of clinical service, along with a minimal annual failure rate of only 1.1% of restoration out of 353 required repairs, and the damage in 2.5% was beyond repair [4]. This failure is accredited to multiple reasons which include material fracture, endodontic lesions, and, most importantly, secondary caries, which account for about 54.1% of all failures [5]. Secondary caries has been evidently proven to be a leading cause of the long-term failure of resin-based dental composites [6]. Thus, the prevention of secondary caries is necessary to improve the durability of the RDC restoration.

From Figure 1, it can be elucidated that secondary caries surmounts all other reasons for the failure of RDCs [7]. Secondary caries, also referred to as recurrent caries, are a multifactorial, biofilm-associated dental pathology occurring in previously restored teeth. They are strongly related to the substrate and its interaction with the complex environment of the oral cavity. The tooth surfaces as well as the dental restorations are not immaculate and are lined with a thin, acellular, organic film that is formed after being exposed to saliva; this organic film is called the salivary pellicle. It provides a site of attachment to various microbes, forming complex polymicrobial colonies that mature to form biofilms [8]. Different surfaces aid the formation of various microbial colonies, i.e., studies suggest that biofilms adherent to GICs have fewer genotypic variations as compared to those associated with amalgam and RDCs, apparently resultant of their cariostatic properties and fluoride release. The metabolites of the biofilms lead to the demineralization of dental hard tissues, and for the RDCs, the esterase from the bacterial activity results in degradation of the restoration. The degradation products, i.e., residual monomers and photo initiators, in turn alter gene expressions in the biofilm to favor microbial growth, and the vicious cycle continues, ultimately leading to the failure of the dental restorations [8].

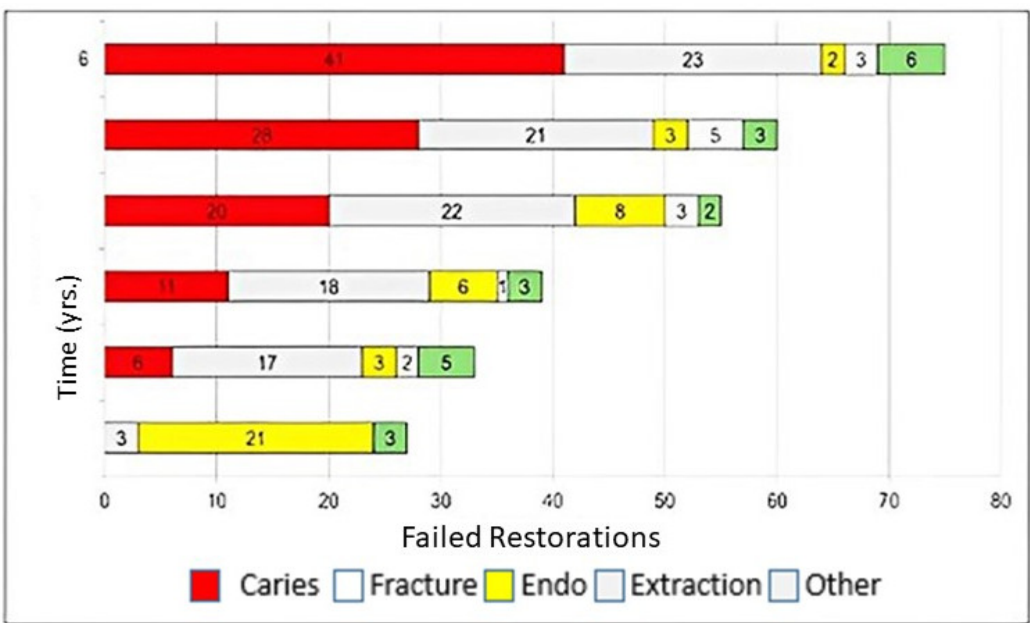


**Figure 1.** Six-year analysis of failed restorations and failure types [7].

To improve the durability by reducing the chance of failure due to secondary caries, RDCs need to possess antibacterial potential so that the restoration can withstand the damage done by the cariogenic bacteria of the oral cavity.

To achieve this objective, many researchers have attempted to deliver an RDC with inherent antibacterial potential that may last longer [9,10]. The three main strategies

employed by the material scientist include the release of the antimicrobial agent from the restoration. This approach enables an abundant availability of antimicrobial agents at the repair site of restoration; however, the antimicrobial effect is short-lived and the leeching out of agents from the RDCs adversely affects the physicomechanical properties of the restoration. The next strategy utilizes the property of antibacterial agents to damage the bacterial cell wall when it comes in direct physical contact with the restoration. This scheme enables the RDCs to maintain their physical and mechanical properties but does not provide sufficient antimicrobial effects. The third approach makes use of more than one antibacterial agent which acts synergistically to provide more effective antibacterial action [11].

This highlights that developing RDCs with antibacterial potential is of paramount importance to ensure their durability and effectiveness. One crucial aspect of the incorporation of antibacterial agents into RDCs is the evaluation of their antibacterial properties. Given their application within the oral cavity, where the physical environment, as well as the bacterial colonization, is dynamic and complex, understanding how these materials interact with oral microorganisms is critical.

The assessment of the antibacterial properties of RDCs necessitates a complex interplay of factors, including the choice of the bacterial strains used and the design of the experimental and assessment protocols employed. This multifaceted nature of antibacterial testing for RDCs has directed researchers worldwide to employ far-ranging and extensive antibacterial testing methodologies. While these efforts have remarkably added to our understanding of the performance of RDCs, they have also unveiled striking discrepancies in testing methodologies.

There has been a myriad of research conducted to develop a clinically sound antibacterial RDC; however, there is a lack of consensus among the researchers on this subject which needs to be addressed.

The aim of this paper is to provide a comprehensive overview of the testing methodologies used by researchers to evaluate the antibacterial properties of resin dental composites. We will go through the pre-testing protocols and highlight various methodologies for antibacterial testing, shedding light on major disparities that exist in this field of research. This review aims to enhance our understanding of the issues involved with the antibacterial assessment of RDCs by providing a critical examination of various testing methodologies and their differences. Through a review of these aspects of the antibacterial testing of RDC, we aim to provide a comprehensive perspective on the current state of antibacterial testing for resin dental composites, with a particular emphasis on the inconsistencies and discrepancies observed across studies.

We aspire that this paper will serve as an exceptional resource for researchers, clinicians, and dental materials scientists, directing them toward a better selection of testing protocols in the quest to develop RDCs that not only restore esthetics and function but also prevent secondary caries by inhibiting bacterial colonization.

The PubMed and Science Direct databases were screened for studies from 2013 to 2023. Article selection and data extraction were performed by using the keywords “antibacterial testing” and “resin-based dental composites”. The languages was restricted to English. The articles related to the antibacterial testing of RDCs for restorative purposes were included. All articles based on adhesive resins or resin-based cements were excluded. About eight hundred articles were initially identified from which thirty articles were reviewed for their full text. Only original research articles were included, while all the systematic reviews and meta-analyses were excluded as shown in Figure 2. The fundamental methods for determining the number of bacterial populations are the plate count method or spectrophotometry (turbidimetric) method [12].

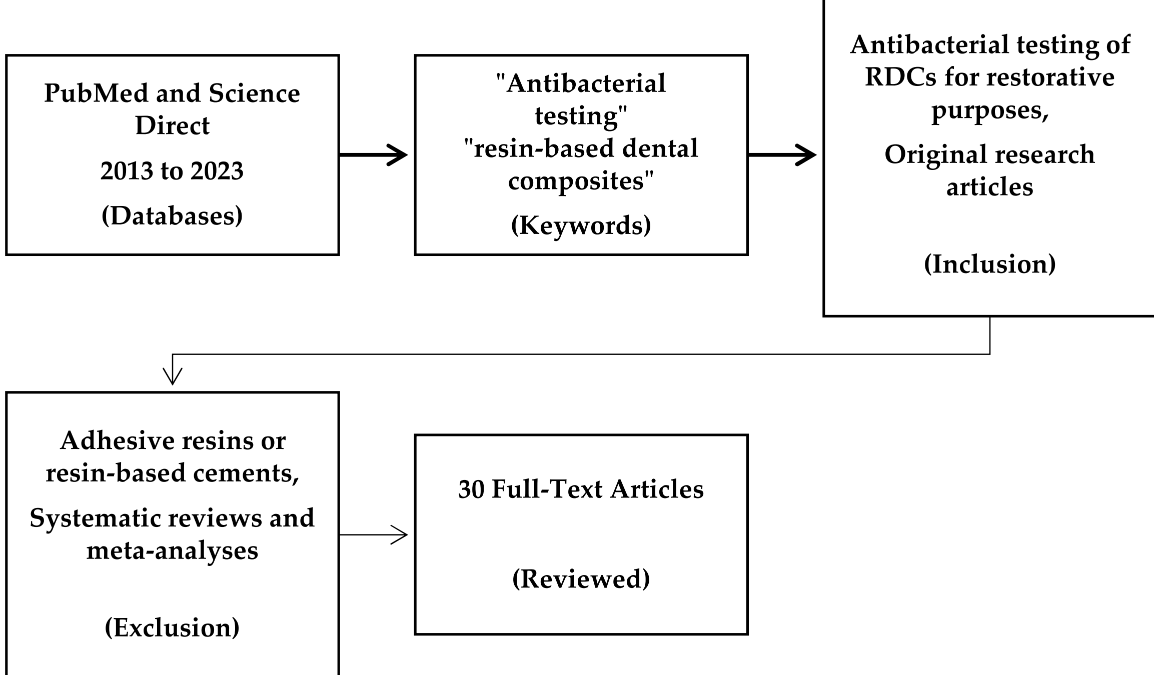


**Figure 2.** Flowchart of literature search strategy.

## 2. Methods for Testing the Antibacterial Properties of RDCs

### 2.1. Colony-Forming Units (CFU)

To evaluate the antibacterial efficacy of RDCs, the researchers primarily rely on assessing the viability of microorganisms. This is typically done by quantifying the colony-forming units (CFUs) of the bacterial cells. To do this, the researchers dilute the bacterial inoculum serially and plate it onto a nutrient-rich agar medium. The resulting colonies that form represent viable cells, and their number is used to estimate the concentration of viable cells in the original inoculum. By comparing the CFU counts of bacteria treated with RDCs to those of untreated bacteria, the researchers can determine the extent to which the RDCs have inhibited bacterial growth [13].

The CFU method is still the most commonly utilized approach for measuring cell growth, despite its limitations. It has both advantages and disadvantages and is best employed when the investigator does not need to analyze non-viable cells or cell debris. To ensure the accuracy of this experimental method, it is crucial to account for every viable cell that produces a colony, which requires appropriate dilution of the bacterial inoculum. When using the CFU method, there is a risk of overlooking cells that do not visibly form colonies on the plate. When applied to biofilms, it is necessary to ensure that the biofilm is fully dispersed before plating to avoid heterogeneity in the collected cell suspension. Such heterogeneity can result in an erroneous increase in the number of viable cells that initiate colony formation. In fact, certain biofilms, including oral biofilms like *S. mutans*, can be challenging to disperse into individual cells.

Obtaining a suspension of individual *S. mutans* cells can be challenging, which can result in a single colony forming from anywhere between one to a hundred cells. This can lead to inaccuracies in biofilm characterization since a colony may not accurately reflect the true composition of the biofilm. In situations where it is not feasible to obtain an ideal microbial cell suspension, the results obtained from characterization methods may be uncertain [14].

### 2.2. Spectrophotometry

The population density of microbial cultures was measured using spectrophotometers for over sixty years. This technique is based on turbidimetric analysis of the bacterial

growth, measured as the optical density (suspension turbidity) of bacterial cultures. It is employed to measure the light transmitted through the bacterial solution and gives an estimation of the optical density of the solution [15]. This transmitted light is recorded as absorbance or optical density. Spectrophotometric analysis is the indirect, qualitative, and less precise measure that approximates the total biomass of both live and dead cells, which can often be used alongside other testing methods. It is less arduous than the standard plate count but can only compute the measurements for a bacterial suspension of at least 107 cells. The increased bacterial growth will decrease the amount of light transmitted through the solution, and the increased bacterial biomass is indicated by increased turbidity in the bacterial solution (Figure 3). Despite being quick, optical density measurements have a narrow range of bacterial concentrations and are complicated by bacterial cell size variations, the formation of small to large clumps, and biofilm formation. A major drawback of OD measurements is the inability to directly measure cell numbers. This phenomenon is also related to the specific instrument configuration, as it is due to light scattering rather than absorption. Therefore, calibration protocols should be developed to compare results using reference substances, to relate OD measurements to cell numbers, and to compare measurements between instruments and experiments [16,17].

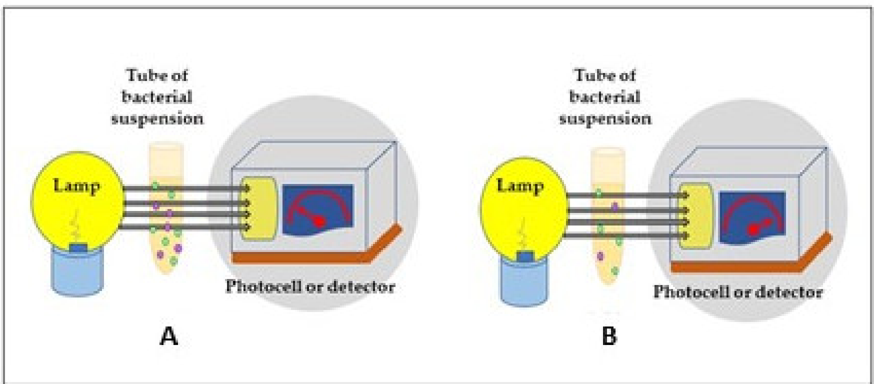


**Figure 3.** A schematic representation of the spectrophotometer. (**A**) Elucidates low biomass in the bacterial solution which can be interpreted by high value of optical density. (**B**) Elucidates increased biomass in the bacterial solution which can be interpreted by high value of optical density.

### *2.3. Agar Diffusion Test (ADT)*

Agar diffusion stands as one of the earliest techniques employed for regular laboratory assessment of antimicrobial susceptibility, with its inception dating back to the year 1940 [18]. In this method, test discs of RDC containing the antimicrobial substance are positioned on a nutrient-rich agar plate that has been inoculated with the bacteria under examination (as shown in Figure 4). Following an incubation period, the areas where bacterial growth has been restricted around the samples are assessed. Primarily, agar diffusion serves as a qualitative determinant of the microbial growth of RDCs modified by antibacterial agents. The foremost benefits of using the ADT include its simple procedure that does not necessitate any specialized equipment and it being the most economical among all susceptibility techniques. The hydrophobicity/hydrophilicity of the antimicrobial agent affects leaching and diffusion from the test material, which can lead to misleading results. Note that this test is only suitable for the diffusion of antimicrobial agents. Furthermore, the drawbacks of this method include it not being suitable for accurately testing all bacteria that are fastidious or slow-growing and being unsuitable for assessing the minimum inhibitory concentration (MIC) due to the inability to measure the precise quantity of antimicrobial agent that has diffused into the agar substrate [19–21]. This method is also affected by a variety of factors like pH, temperature, and evaporation that limit its use for precise diagnostic results [22].

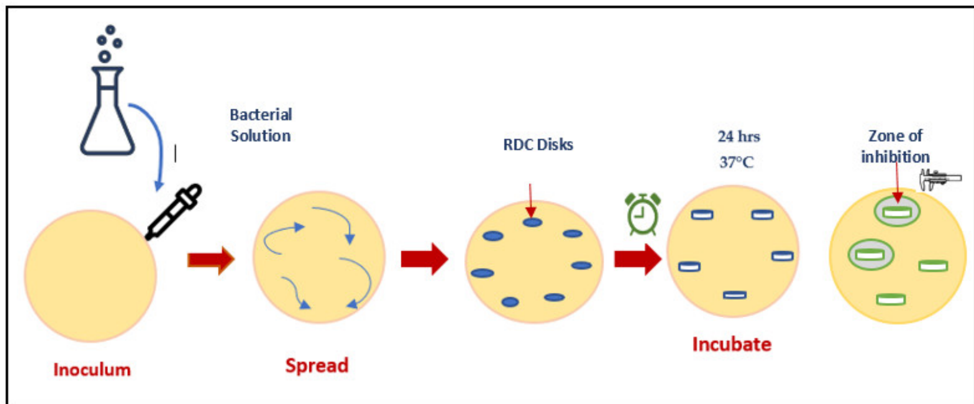


**Figure 4.** The steps of the agar diffusion test.

### 2.4. XTT/MTT

The MTT assay is a widely used method for evaluating cell proliferation, cytotoxicity, and metabolic activity. This assay relies on the reduction of a yellow tetrazolium salt to a violet formazan by cellular oxidoreductases. The resulting color change is used to determine the viability, proliferation, and metabolism of cells and has been applied to various types of cells including bacteria in antibacterial activity assays. The MTT assay is a valuable tool for researchers to assess the effects of compounds on cellular processes, and its versatility has contributed to its popularity in the scientific community [23].

During the MTT assay, metabolically active cells (such as those containing functional mitochondria and cytosolic enzymes) reduce the yellow MTT compound to a blue-purple formazan product (Figure 5). Since this product cannot permeate intact cell membranes, it accumulates within the bacterial cells. However, upon removal of the aqueous medium and solubilizing the cells in DMSO or isopropanol, the formazan product becomes soluble and can be measured spectrophotometrically. Since only viable cells have the ability to reduce MTT, the amount of reduced MTT formazan is proportional to the intensity of the color and correlates with the degree of cell viability. Overall, the MTT assay is a reliable tool for researchers to assess cell viability and metabolic activity [24].

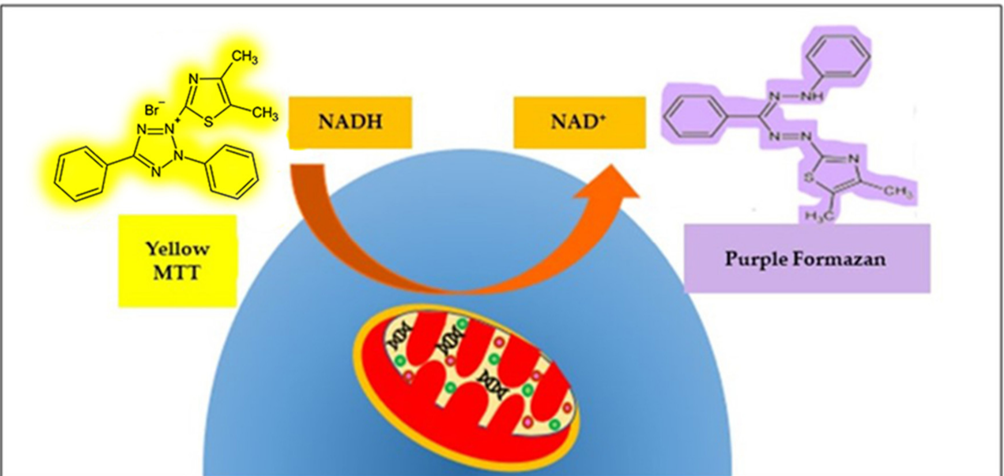


**Figure 5.** Structure of MTT and colored formazan product.

XTT is a soluble alternative to MTT that can be used to measure metabolic activity and vitality in cells. XTT is soluble in the majority of aqueous media, unlike MTT. This yellow salt is reduced to a colored formazan product by dehydrogenases in metabolically active cells, making it a valuable tool for researchers to evaluate cellular processes. The XTT assay has several advantages over the MTT assay, including its solubility and ease of use in various experimental conditions [25]. Another advantage of this assay is its suitability for

cells in suspension, making it a useful alternative to other assays that are not optimized for suspension cultures. The XTT assay works by the extracellular reduction of XTT, which is facilitated by NADH produced in the mitochondria through trans-plasma membrane electron transport and an electron mediator. This process results in the formation of a colored formazan product that can be measured spectrophotometrically to evaluate cellular metabolic activity and vitality. Compared to other assays, XTT's solubility in aqueous media and ability to work with cells in suspension make it a versatile and reliable tool for assessing cellular processes [26].

### *2.5. Lactic Acid Production*

Lactic acid is a strong determinant in the pathogenesis of tooth caries [27]. In 1857, Pasteur's discovery revealed that lactic acid is not an inherent constituent of milk; instead, it is a byproduct generated through the fermentation of microorganisms [28]. The fermentation of carbohydrates by the cariogenic microbes such as *Streptococcus mutans* [29] in the oral cavity results in the production of lactic acid which demineralizes the dental hard tissues and is a major cause of secondary caries. This suggests that an efficient antibacterial agent must impede the production of lactic acid by cariogenic bacteria. This can be evaluated by incubating the antibacterial test samples for 3 h in contact with the bacterial solution in 5% $CO_2$ and 37 °C to release lactic acid in buffer peptone water, followed by measuring the lactic acid concentration, using a microplate reader at an absorbance set at 340 nm. Lactic acid standards were used to generate a standard curve [30].

### *2.6. Confocal Laser Scanning Microscope*

Quantifying surface biofilm formation using conventional methods such as CFU is difficult and more prone to human error. When compared to conventional light microscopy, which employs high-resolution methods that allow 3D visualization of biofilm structures, confocal laser scanning microscopy (CLSM) offers significantly better image resolution and contrast.

The 3D biofilm may be optically divided Into horizontal and vertical sections using CLSM. To see dense biofilm samples in detail, image-processing methods are utilized for the quantitative investigation of biofilms. This method was also used to assess bacterial viability and biofilm availability on transparent and opaque surfaces [31]. Fluorescent dyes are applied to biofilms to distinguish between live and dead bacteria, allowing bacteria to be differentiated according to the permeability of the cytoplasmic membrane. CLSM can capture a series of image scans showing significant changes in bacterial cell viability over time, allowing for real-time visualization of microbial death. It consists of a mixture of two nucleic acid-binding dyes named Syto 9 and propidium iodide. Svto 9 stains all viable bacteria green, while propidium iodide stains membrane-damaged bacteria (non-viable bacteria) red, as shown in Figure 6 [32]. Disadvantages of confocal microscopy include high cost and a relatively narrow field of view. This technique uses a high-intensity laser beam that damages living tissue [33].

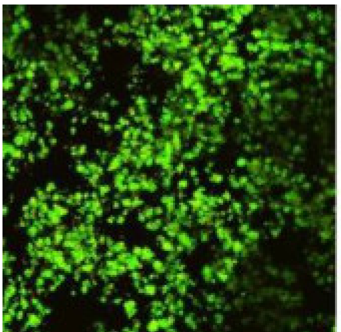
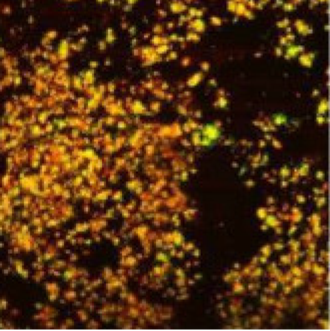

**Figure 6.** The confocal microscopy images of live/dead stained biofilms formed on composite. Live bacteria stain green, whereas dead bacteria are red.

## 3. Pre-Testing Protocols: Incubation Time and Sterilization

Material samples are usually disinfected or sterilized prior to antimicrobial testing to avoid contamination with the organisms. The conventional methods of sterilization in an autoclave subject the test material to high temperature, pressure, and humidity, which leads to degradation of the polymer matrix of RDCs.

Commonly used sterilization methods are ethylene oxide gas, ultraviolet (UV) radiations, gamma radiations, and ethanol. Chemical disinfectants, namely, glutaraldehyde and 70% ethanol, are generally used for disinfecting the testing material. However, these chemicals leave behind their remnants on the test specimens which may end up in compromised bacterial growth on the specimens [34].

### 3.1. 70% Ethanol

Immersing the test samples in 70% ethanol for 1 min has shown to be an effective method to disinfect the RDC samples, but immersion in aqueous solutions may cause the leaching of unreacted monomers and, at the same time, solubilize the resins from the organic matrix of the restorative material. RDCs are not inert materials; thus, they are highly prone to alterations when treated with such chemicals [35].

### 3.2. Ethylene Oxide

Heat-sensitive materials are sterilized using ethylene oxide (Eto). This process subjects the material to a temperature of about 40 to 55 °C, which may deteriorate the physiochemical properties of the RDC. A major drawback of ethylene oxide sterilization is the aeration time required for the removal of toxic and carcinogenic residues of Eto [35].

### 3.3. Gamma Radiation

Gamma radiation may kill or harm live cells, degrade materials by raising the temperature of polymers, and enhance the degree of conversion of dental resins [35].

### 3.4. UV Light

Another useful sterilization tool is UV light, which possesses a bactericidal effect and can sufficiently decontaminate the RDC test specimens. UV light of wavelengths between 200 and 280 nm is also used for the sterilization of heat-sensitive testing materials. This method also has the advantage of no chemical exposure to the RDC which may alter its inherent properties. Consequently, it is environmentally safe in comparison with the previously mentioned methods. However, RDCs, when subjected to UV light, are susceptible to possible changes in their chemical and physical properties due to the oxidation of the exposed surface, altering the hydrophobic properties and increasing the surface energy of the RDC test specimen. It can be concluded that sterilization with UV light for 1 min is impeccable among all other sterilization methods, resulting in the least deleterious effects, as inherent properties of the RDC remain intact [35].

### 3.5. Incubation Time/Biofilm Maturity

The literature depicts an evident variability in the incubation time/biofilm maturity. Greater incubation time results in highly mature biofilm which poses the test material to an increased bacterial challenge. Most researchers use a 24 h incubation time; however, biofilms that matured for up to several weeks have been employed in some studies. Different organisms require different incubation conditions. Bacterial biofilms are a major cause of pathogenic processes in the oral environment. Biofilms are functionally organized, layered collections of microorganisms (bacteria, algae, and fungi) attached to biological and abiotic surfaces. Biofilms in the oral cavity are highly complex and dynamic in nature and are influenced by the flow of saliva and oral hygiene method and are encouraged by the high humidity, moderate temperature, and quantity of nutrients in the oral environment. In the oral cavity, biofilm production is a step-wise process that involves four steps such as the

formation of acquired pellicle, colonization and coaggregation, and finally, establishment of a mature biofilm, as shown in Figure 7 [36].

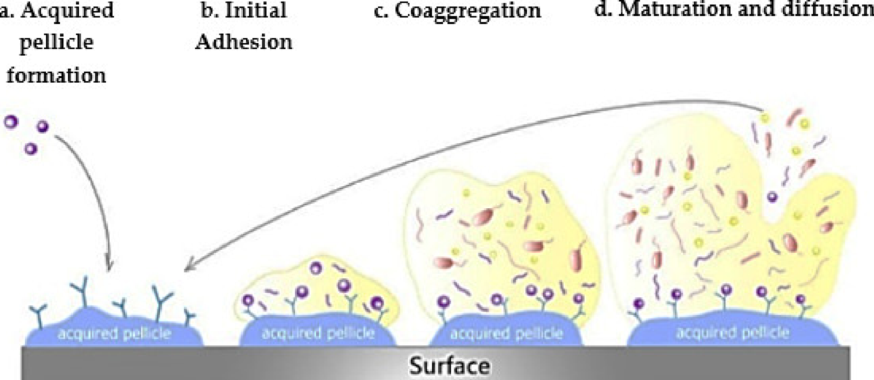


**Figure 7.** Formation of biofilm. (**a**) Formation of acquired pellicle (**b**) Adhesion (**c**) Coaggregation (**d**) Maturation of biofilm.

Brushing and the use of oral rinses modify and disrupt oral biofilms; thus, when choosing the incubation time and biofilm maturity level, researchers must consider the requirement of the restorative material and the clinical environment of the oral cavity [37]. There have been various studies over the years that consistently show resin dental composites to have increased thickness in the development of biofilm, for example, in the case where the percentage of *Streptococcus mutans* within the total CFU count in dental plaque was higher in resin composite restoration with an average mean value of 13.7. Research suggests that composite resins could potentially influence the microbial environment within dental plaque biofilms. This is based on findings indicating that resin composites have an inherent ability to stimulate bacterial growth. The heightened accumulation of biofilm on composite surfaces is the contributing factor to this phenomenon. Consequently, these composites might eventually be susceptible to cavity formation due to the escalated bacterial growth and subsequent plaque build-up [38].

Several studies have been conducted with the primary focus of developing an antibacterial RDC which has the ability to inhibit bacterial adherence, biofilm formation, and reduction in secondary caries.

In the above-mentioned studies, researchers employed various antimicrobial susceptibility tests (Table 1), with the colony-forming unit method being the most favored choice, and the second most used test was the agar diffusion test.

**Table 1.** Summary of study results: addressing variability in antibacterial property testing of resin dental composites.

| No. | Year | Pre-Testing Measures | Species Used | Performed Tests | References |
|---|---|---|---|---|---|
| **1** | 2013 | Incubation = 12 h<br>Sterilization = UV radiation | *Escherichia coli*, *Staphylococcus aureus*, Lactobacillus | Spectrophotometry<br>Agar Disk Diffusion | [39] |
| **2** | 2021 | Incubation = 24 h<br>Sterilization = UV radiation | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [40] |
| **3** | 2020 | Incubation = 36 h<br>Sterilization = ethanol | *Streptococcus mutans* | Colony-Forming Unit (CFU) LIVE/DEAD Assay-Confocal Laser Scanning Microscope | [41] |
| **4** | 2022 | Incubation = 24 h<br>Sterilization = 75% alcohol | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [42] |
| **5** | 2022 | Incubation = 24 h<br>Sterilization = UV irradiation | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [43] |

**Table 1.** *Cont.*

| No. | Year | Pre-Testing Measures | Species Used | Performed Tests | References |
|---|---|---|---|---|---|
| **6** | 2022 | Incubation = 24 h<br>Sterilization = ultraviolet light | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [44] |
| **7** | 2022 | Incubation = 24, 48, 72, 96, and 120 h<br>Sterilization = none | *Streptococcus mutans* | Colony-Forming Unit (CFU) Live/Dead Assay Fluorescent Microscope | [45] |
| **8** | 2017 | Incubation = 12 h of incubation for S. aureus and 72 h of incubation for *S. mutans* Sterilization = none | *Streptococcus mutans*<br>*Staphylococcus aureus* | Agar Diffusion Test | [46] |
| **9** | 2019 | Incubation = 48 h<br>Sterilization = none | *Porphyromonas gingivalis*, *Streptococcus mutans*, and *Staphylococcus aureus* for agar dilution method and *Streptococcus mutans* for the biofilm test | Agar Dilution Method<br>Biofilm Formation | [47] |
| **10** | 2022 | Incubation = 24 h<br>Sterilization = ultraviolet (UV) | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [48] |
| **11** | 2022 | Incubation = 24 h<br>Sterilization = ultraviolet (UV) irradiation | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [49] |
| **12** | 2021 | Incubation = 48 h<br>Sterilization = none | *Streptococcus mutans* and *L. acidophilus* | Biofilm Colony-Forming Unit (CFU) | [50] |
| **13** | 2021 | Incubation = 24 h<br>Sterilization = ultraviolet light for 2 h | *Streptococcus mutans* | Colony-Forming Units (CFU)<br>Metabolic Activity Test (CCK-8) | [51] |
| **14** | 2021 | Incubation = 48 h<br>Sterilization = ethylene oxide for 24 h | Dental Plaque Microcosm Biofilm | Colony-Forming Units (CFU),<br>Lactic Acid, And Metabolic Activity | [52] |
| **15** | 2022 | Incubation = 48 h<br>Sterilization = none | *Escherichia coli*<br>*Pseudomonas aeruginosa*<br>*E. faecalis* | Colony-Forming Unit (CFU) | [53] |
| **16** | 2020 | Incubation = 24 h<br>Sterilization = 70% ethanol solution | *Streptococcus mutans* | Colony-Forming Unit (CFU) | [54] |
| **17** | 2019 | Incubation = 48 h<br>Sterilization = ethanol | *Streptococcus mutans*,<br>*Staphylococcus aureus*,<br>*Pseudomonas aeruginosa* | Live/Dead Assay<br>Fluorescent Microscope | [55] |
| **18** | 2020 | Incubation = 24 h<br>Sterilization = ethylene oxide gas | *Streptococcus mutans* | Disk Diffusion Assay | [56] |
| **19** | 2022 | Incubation = 24 h<br>Sterilization = ultraviolet rays for 1 h | *Streptococcus mutans* | Colony-Forming Units (CFUs) | [57] |
| **20** | 2018 | Incubation = fresh samples, 1 week, 2 weeks<br>Sterilization = ultraviolet light<br>Saliva treatment | *Streptococcus mutans* | Colony-Forming Units (CFUs) | [58] |
| **21** | 2013 | Incubation = 24 h<br>Sterilization = none | *Streptococcus mutans* | Agar Diffusion Test<br>Colony-Forming Units (Cfus) | [59] |
| **22** | 2017 | Incubation = 96 h<br>Sterilization = UV | *Streptococcus mutans* biofilm | Colony-Forming Unit (CFU), | [60] |
| **23** | 2022 | Incubation = 24 h<br>Sterilization = none | *Streptococcus mutans* | Crystal Violet Biofilm Assay<br>LIVE/DEAD Assay,<br>Confocal Laser Scanning Microscope | [50] |

**Table 1.** *Cont.*

| No. | Year | Pre-Testing Measures | Species Used | Performed Tests | References |
|---|---|---|---|---|---|
| **24** | 2021 | Incubation = 24 h<br>Sterilization = none | *Streptococcus Mutans*<br>*Lactobacillus acidophilus* | Colony-Forming Unit (CFU) | [61] |
| **25** | 2015 | Incubation = 24 h<br>Sterilization = none | *Streptococcus mutans*,<br>Total Streptococci,<br>Total microorganisms | Colony-Forming Unit (CFU) Counts | [62] |
| **26** | 2015 | Incubation = 24 h | *Streptococcus mutans* | Biofilm Inhibition Test | [63] |
| **27** | 2023 | Incubation = 24 h<br>Sterilization = 70° alcohol and UV light irradiation<br>Incubation = 24–72 h | *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus gordonii* | Bacterial Sensitivity Test by Agar Diffusion Technique, Monospecies Biofilm Inhibition Assay | [64] |
| **28** | 2020 | Incubation = 48 h<br>Incubation = 24 h | *Streptococcus mutans* | Disk Diffusion Test,<br>Direct Contact Test | [65] |
| **29** | 2021 | Incubation = 24 h<br>Sterilization = ethylene oxide | *Streptococcus mutans* | Agar Diffusion Test<br>Colony-Forming Unit (CFU) Counts | [66] |
| **30** | 2015 | Incubation = 48 h | *Enterococcus faecalis*,<br>*Actinomyces viscousus*,<br>*Streptococcus mutans*,<br>*Lactobacillus casei* | Direct Contact Test (DCT) | [67] |

The CFU test is the dominant technique for checking the viability of bacteria, offering direct insight into measures of bacterial reproduction. The agar diffusion test is an old reliable test but is dependent on the release of water-soluble agents from the RDC. Hence, further tests are also required for accurate results [68].

The second most significant source of variability in these experimental studies was the utilization of divergent incubation time periods (Table 1), making accurate comparisons challenging. The selection of the time period is heavily dependent on the specific bacteria tested for antibacterial susceptibility. Thus, there is an imperative need for standardization in defining incubation time parameters to enhance the reliability of antibacterial testing protocols.

Incubating RDC samples in bacterial solution for 24 h is the most common practice employed in the included studies. Incubating RDCs in bacterial solution mimics the oral environment and depicts how the material may interact with the oral microflora. Dental restorations are intended to remain in the mouth for an extended period of time; therefore, it is important to assess their antibacterial efficacy over time. Incubating samples for a prolonged time allows researchers to evaluate how well the RDC can prevent bacterial growth and biofilm formation during this extended exposure. Longer incubation periods may allow researchers to study the sustained antibacterial effects of RDCs. Some RDCs may initially inhibit bacterial growth but lose their effectiveness over time, while others might be able to maintain their antibacterial properties over an extended period. This information is essential for understanding how the material performs clinically.

The primary focus of the majority of these research studies was on *Streptococcus mutans*, a Gram-positive bacterium well-known for its role in causing tooth caries (Table 1). *S. mutans* has a variable incubation period, usually lasting between 24 h and 48 h, depending on the specific factors of the environment, such as the presence of aerobic or anaerobic settings. The necessity of accurately replicating oral conditions led to the selection of an incubation period of twenty-four hours. This decision was made in accordance with the fact that *S. mutans* grow in low-oxygen environments, particularly those that are nearer to the gingival surface, where caries tend to develop more frequently [69].

Another significant variability among researchers was found to be the sterilization protocols. The majority of studies employed the ultraviolet (UV) light sterilization method to eliminate contaminants before testing for antibacterial activity. In these research studies, the least used techniques for sterilization were ethanol and ethylene oxide. Notably, twelve research studies did not report the sterilization technique. UV provides fast and effective

bactericidal properties by damaging the DNA and RNA of microorganisms, including bacteria, viruses, and fungi. Together with this effective bactericidal property, the non-chemical approach is an added advantage since it does not involve the use of any harmful chemicals that may leave residues or byproducts and possibly interact with the RDC samples. It also exempts the generation of excessive heat, which is very crucial for RDCs, since it can massively determine the structure and properties of RDCs. However, the use of UV light must be done with extreme caution, ensuring the use of personal protective equipment (PPE) shielding from UV radiation, and proper safety precautions must be employed while using UV light [35].

From these studies, we have come to know that there are a wide variety of antibacterial tests available. With that, there is an even higher need for investigators to understand the strengths and weaknesses of a particular test so that they may select the most appropriate test for a particular study in order to avoid misleading results. So, there will be room for significant improvement in the standardization of the tests.

The sterilization method, incubation time, bacterial species, and relevant tests must be standardized to ensure the consistency and comparability of test results across different studies and laboratories. Researchers can use this standardization to make meaningful comparisons between different composite materials or antibacterial agents.

## 4. Conclusions

In order to lessen the failure rate and increase the durability of dental composite resins, antimicrobial agents have shown a significant improvement. However, to check the outcome of the antibacterial agents in RDCs, numerous antibacterial tests are performed.

To summarize this review, it is recommended to employ multiple antibacterial techniques when evaluating resin-based restorative materials. Quantitative tests that encompass the intricacy of naturally occurring polymicrobial biofilms and provide quantification of bacterial viability, such as counting colony-forming units (CFU), should be prioritized as a measurable outcome. It is advised to avoid relying solely on a test that measures only metabolic activity or membrane integrity (live/dead assay).

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