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ORIGINAL RESEARCH

Monitoring the Antibacterial Activity of the Green Synthesized ZnO Nanoparticles on the Negative and Positive Gram Bacteria Mimicking Oral Environment by Using a Quartz Tuning Fork (QTF) Micromechanical Sensor

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Introduction: Green-synthesized nanoparticles show promise as anti-biofilm and antibacterial agents in medical applications, including dental implants and oral devices. However, conventional antibacterial testing methods are laborious and lack sensitivity. Quartz tuning fork (QTF)-based biosensors offer a compelling alternative due to their high sensitivity, compact size, and cost-effectiveness. This study evaluates a QTF biosensor for quantifying the antibacterial activity of green-synthesized ZnO nanoparticles against negative and positive Gram bacteria.

Methods: The antibacterial activity of ZnO nanoparticles was tested in a simulated oral environment against *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) using a QTF biosensor. Changes in resonance frequency and quality factor were measured to assess bacterial growth inhibition. Experiments were conducted with varying ZnO concentrations (eg, 1 mm) to correlate sensor responses with antibacterial effects.

Results: The QTF biosensor detected significant antibacterial activity as resonance frequency decreased by 5.69 ± 3.81 hz (*S. aureus*) and 30.57 ± 4.01 hz (*E. coli*) in 1 mm ZnO. Quality factor declined by 31.75 ± 7.55 for *E. coli* but remained stable for *S. aureus*. Higher bacterial concentrations (lower ZnO doses) increased damping effects, reducing the quality factor. *S. aureus* exhibited greater sensitivity to ZnO nanoparticles than *E. coli*.

Discussion: The QTF biosensor successfully quantified the antibacterial effects of green-synthesized ZnO nanoparticles, demonstrating its potential as a rapid, sensitive alternative to traditional methods. The differential responses of *S. aureus* and *E. coli* suggest species-specific interactions with ZnO, warranting further study. This approach could streamline the development of biocompatible, antibacterial medical materials.

Keywords: ZnO nanoparticles, QTF, antibacterial, positive and negative gram bacteria

Introduction

Metal oxide nanoparticles have potential applications in sensors, medical delivery, medical imaging, and antibacterial and anticancer applications.[1–10] The antibacterial properties of ZnO nanoparticles have been used in various dental applications such as ortho wires, resto/endo materials, and oral bite guard materials.[11] Green-synthesized ZnO nanoparticles (NPs) function as antibacterial materials and exhibit high growth inhibition.[12] ZnO NPs have been reported to reduce the growth of several harmful bacteria.[13] The size and shape of ZnO nanoparticles have a major influence on their antibacterial activity. It has been demonstrated that the antibacterial activity of ZnO is inversely proportional to its

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nanoparticle size.[14] The antibacterial activity of ZnO nanoparticles has been attributed to a variety of mechanisms such as the creation of reactive oxygen species (ROS),[15–18] direct contact between ZnO nanoparticles and cell walls,[19–21] and the release of Zn^{2+}.[15,22] ROS is produced upon exposure to light. In contrast, alternative investigations have revealed that ZnO exhibits antibacterial activity, even in the dark.[23–26] The release of Zn^{2+} in a solution containing bacteria and ZnO nanoparticles was hypothesized to be one of the ZnO antibacterial mechanisms through the inhibition of active transport, disruption of enzyme systems, and effects on amino acid metabolism.[15,22,27] Nevertheless, the contribution of Zn^{2+} to the antibacterial effect of ZnO nanoparticles is low, owing to the small amount of soluble Zn released from ZnO in water.[28,29] Conversely, it has been claimed that ROS are formed in the dark by capturing electrons that react with dissolved oxygen via oxygen vacancies associated with defects to produce ROS.[24] In the dark, ROS are formed as hydrogen peroxide, superoxide radicals, or both without the production of hydroxyl radicals.[24,25] The ROS produced act as antibacterial agents.[24–26,30,31] In contrast, the direct contact of ZnO nanoparticles with bacterial walls results in breakdown of the bacterial cell structure.[32] There are still unanswered questions regarding the chemical mechanism by which nanoparticles cross the outer membrane and cell wall before causing damage to bacteria. It has been discovered that gram-negative bacteria exhibit greater vulnerability to the nanoparticles than gram-positive bacteria.[33–35] This variation in susceptibility was assumed to be caused by variations in cell wall composition. One theory argues that gram-negative bacteria are more prone to cell wall breakdown when they come into contact with nanoparticles because of their thin peptidoglycan covering. Gram-negative bacteria also have an outer membrane consisting of lipopolysaccharides, which carry a negative charge. The presence of positively charged nanoparticles in the outer membrane can result in their accumulation, causing damage to bacterial cells.[4,35] Furthermore, the size and structure of nanoparticles have a significant impact on the antibacterial activity of ZnO.[32,36] Smaller ZnO nanoparticles have been demonstrated to be more bactericidal than larger ones, possibly because smaller particles are more easily absorbed and transfected across the cell membrane. Furthermore, the larger surface-area-to-volume ratio of smaller particles may result in the formation of more reactive oxygen species.[37] ZnO nanoparticles are promising antimicrobial coating materials for biomedical applications because of their antibacterial activity under dark conditions, biocompatibility, and non-toxicity to humans.[38–41] ZnO nanoparticles can be used as antibacterial coatings for oral bite guards, as illustrated in Figure 1.

Owing to the growing interest in the properties of new antimicrobial products, it is essential to develop cost-effective, real-time, sensitive, and quantitative antimicrobial testing methods. There are several antimicrobial testing methods, such as disc diffusion, thin layer Chromatography, Dilution, adenosine triphosphate (ATP) bioluminescence assays, and flow cytometry.[40] Diffusion techniques such as the agar disk diffusion method are qualitative tests. However, they have notable limitations, including issues with precision, reproducibility, and interpretation, as well as difficulties in quantifying the amount of diffused antimicrobial agent in agar medium. Additionally, thin-layer chromatography (TLC)

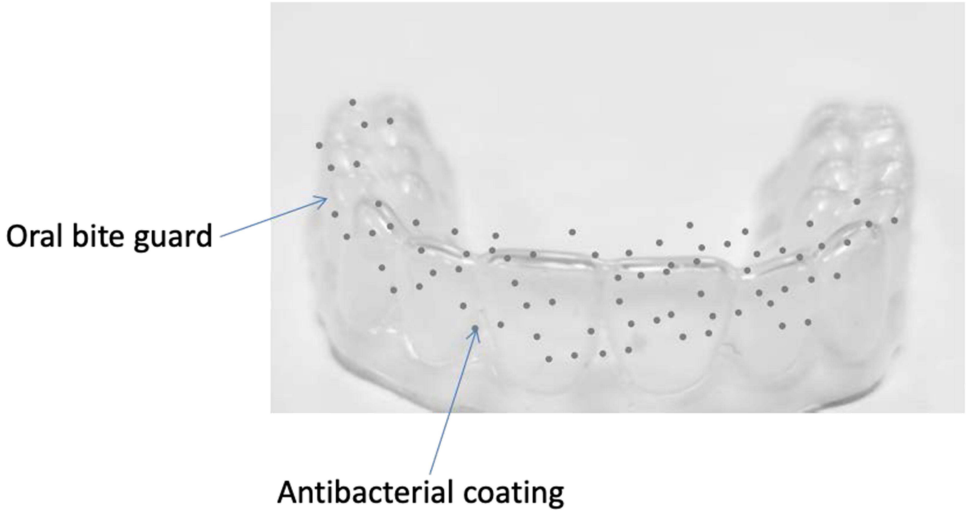

Figure 1 Schematic diagram for a coated oral bite guard.

bioautography methods are time-consuming and labor-intensive. Dilution methods are used to determine the minimum inhibitory concentration (MIC) of an antimicrobial agent. Similarly, ATP bioluminescence assays and flow cytometry are labor-intensive techniques. An MEMS-based microchannel cantilever was utilized to monitor nanomechanical fluctuations and bacterial bacteriophage interactions.[42] Quartz tuning fork (QTF) micromechanical sensors are a potential technology for biosensing owing to their low cost, high sensitivity, simplicity, and rapid detection. The QTF-based micromechanical sensor is a sensitive and precise technique that operates on the principle of vibrating two prongs of a quartz crystal; it vibrates at its resonant frequency when a voltage is applied to the electrodes on the quartz. The principle of the QTF-based sensor depends on the shifting of its mechanical resonance frequency, which is used as an indication of the physical, chemical, or biological interactions with high precession and sensitivity. The QTF surface can be functionalized by host molecules that can selectively bind to target molecules, resulting in mass loading and shifting the QTF resonance frequency to lower values.[43–45] The QTF resonance frequency increases as the number of target molecules attracted to the probe increases. In addition, the resonance frequency and quality factor of a vibrating QTF can also be changed if it is immersed in different media with different physical properties.[46] QTF can be used to monitor and diagnose diseases caused by cytomegalovirus (CMV) and germ-like bacteria.[45,47] This work introduced a new antibacterial test method based on a QTF-system biosensor to monitor the antibacterial activity of nanoparticles under controlled conditions and at low volumes on the microliter scale. The QTF is coast-effective, highly sensitive, and works at microscale liquid volumes. Therefore, it can be used to detect low concentrations of bacteria and nanoparticle antibacterial activity in both liquid and wet media. This study aimed to investigate the ability of a QTF-based biosensor to detect the antibacterial activity of green-synthesized ZnO nanoparticles using *Rosmarinus officinalis* L. extract in the oral environment of Gram-positive and Gram-negative bacteria, which can be used as antibacterial oral bite guards. The green synthesis method of metal oxide nanoparticles is a cost-effective, eco-friendly solvent, and yields productivity on a large scale.[48] In addition, green-synthesized nanoparticles are more biocompatible and soluble in water.[49] The using of *Rosmarinus officinalis* extract was used because it contains many phenolic compounds that reduce and stabilize nanoparticles.

Experimental Procedure

Materials

Green synthesized ZnO nanoparticles using *Rosmarinus officinalis* L. extract and healthy wild *Rosmarinus officinalis* L. (KSU Herbarium No: 9012) leaves were collected from the Abha region in southern Saudi Arabia. Zinc nitrate hexahydrate and sodium hydroxide were obtained from BHD® (Avantor, Inc., Radnor, PV, U.S). All chemicals were used without further purification. Bacterial growth media (Nutrient Broth from Scharluau microbiology), *Escherichia coli* bacteria code (ATCC25922)10^2 CFU/mL, *Staphylococcus aureus* bacteria code (ATCC29213)10^2 CFU/mL, and QTF sensors.

Green Synthesis Nanoparticles Preparation for Antibacterial Test

Rosmarinus Officinalis Extract Preparation

Leaf extract was prepared following the method described by Saad Algarni et al (2024).[50] Fresh leaves were washed, shadow-dried for 2–3 weeks, and then ground. The solution extract was prepared by dissolving 10 g of leaf powder in 100 mL deionized water and heating at 60 °C for 15 min, then filtering and storing at 4 °C.

Synthesis of ZnO NPs Using *Rosmarinus* Leaf Extract

The green-synthesized ZnO nanoparticles (NPs) were prepared according to the method published by T. Saad Algarniet al (2024).[50] Zinc nitrate hexahydrate (5 g) was dissolved in 30 mL of aqueous leaf extract and 18 mL of 2M NaOH was added to achieve a pH of 5.2. The mixture was heated to 80 °C and stirred for 3 h. Subsequently, the precipitate material was separated, washed multiple times with a distilled water-ethanol (3:1) solution, and then dried at 80 °C overnight. Finally, the product material was calcined at 500 °C for 3 h to obtain the ZnO NPs. Figure 2 shows the microstructural Scanning electron microscopy (SEM) image and Energy dispersive x-ray spectroscopy (EDS) of green-synthesized ZnO, the particle size in the range of 53–67 nm.

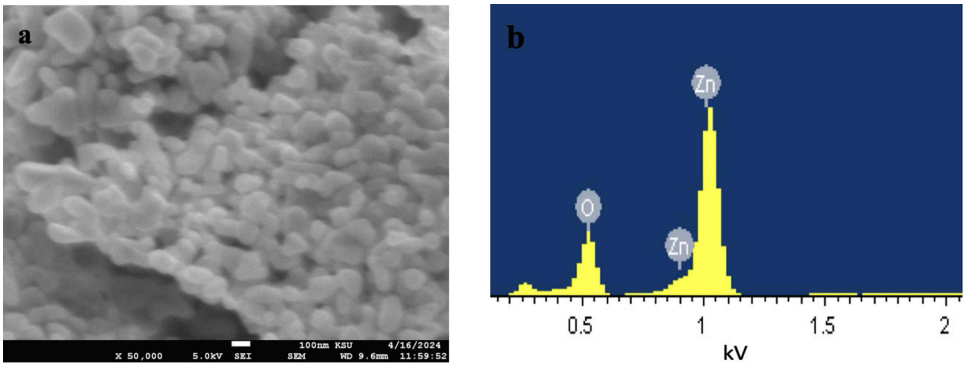

Figure 2 (**a**) SEM image (**b**) EDS spectrum of the green synthesized ZnO nanoparticle.

Bacteria Growth and Antibacterial Test

The antibacterial activity of the green-synthesized ZnO NPs against gram-positive and gram-negative bacteria was assessed. *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were used as gram-positive and gram-negative bacteria, respectively. The ZnO NPs were suspended in sterilized deionized (DI) water using ultrasonic vibrations at a frequency of 45 kHz for 1 h and then added to the growth media (Nutrient Broth, PH of 7.4) at different concentrations (1, 0.5, 0.25, and 0.125 mm). Bacteria with a starting concentration of 10^2 CFU/mL were grown individually in growth media with different concentrations of ZnO nanoparticles (1, 0.5, 0.25, 0.125, and 0 mm). All the bacterial samples were incubated in the dark at 37 °C with continuous shaking for 8 h.

QTF Antibacterial Measurements Test

The sensing part of the QTF sensor was treated with UV-ozone for 15 min, followed by immersion in a Piranha H_2SO_4:H_2O_2 (3:1) solution for 5 min, rinsing with DI water three times, twice with absolute ethanol, and drying using high-purity dry nitrogen. To investigate the antibacterial activity of the ZnO nanoparticles, the QTF-based biosensor was immersed in 100 µL of bacteria grown in media containing a certain concentration of ZnO nanoparticles. The sensor response was measured as the resonance frequency shift and the quality factor of the QTF sensor. The resonance frequency was measured after immersing the QTF sensor in a bacterial sample, as illustrated in Figure 3. Measurements were performed after 5 min for each sample.

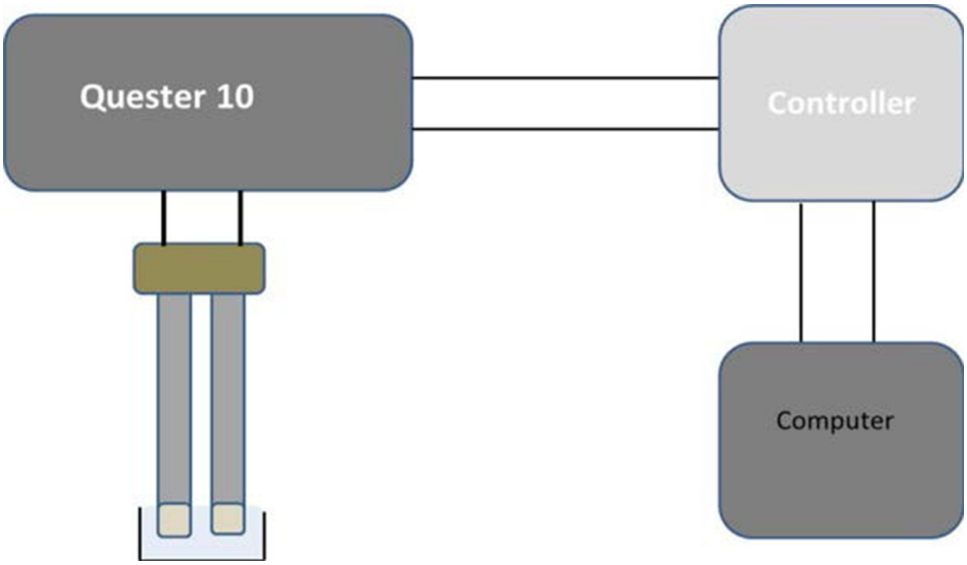

Figure 3 Schematic diagram for the QTF based antibacterial tests system.

The resonance frequency of the QTF samples was measured using a Quester-10 QTF measurement system from FOURIEN Company, Canada, which has been described in more detail in the literature.[46]

Results and Discussion

Results

The antibacterial activity of the green-synthesized ZnO nanoparticles was evaluated using the resonance frequency of the QTF-based biosensor. The variation in the resonance frequency of the QTF immersed in the bacterial solution with different contents of green-synthesized ZnO nanoparticles was measured as an indicator of the antibacterial activity of the nanoparticles. The resonance frequency and quality factor variations reflect changes in the bacterial concentration. Figure 4a shows the resonance frequency of the QTF immersed in *E. coli* grown in growth media containing different concentrations of ZnO nanoparticles (0, 0.125, 0.25, 0.5, and 1 mm). As the ZnO nanoparticles content in the bacterial solution increased, the resonance frequency of the QTF also increased. Conversely, a decrease in the ZnO nanoparticles content in the bacterial solution resulted in a decrease in the resonance frequency. The resonance frequency of the QTF sensor immersed in *E. coli* bacteria solution without ZnO nanoparticles incubated for 8 h decreased by 199.64±2.64 hz compared to that immersed in the growth media. On the other hand, the resonance frequency of the QTF sensor immersed in *E. coli* solution media with 1 mm ZnO nanoparticles incubated for 8 h decreased by 30.57±4.01 hz. The resonance frequency shift (Δf) was determined by subtracting the resonance frequency of the sensor immersed in the bacterial solution (f_B) from the resonance frequency of the QTF immersed in the growth medium (f_M) according to Equation (1):

$$\Delta f = f_B - f_M$$

Clearly, the resonance frequency shift increased as the concentration of ZnO nanoparticles decreased, as illustrated in Figure 4b. Furthermore, the quality factor increased as the concentration of ZnO nanoparticles increased, as shown in Figure 5a and b. The quality factor of the QTF sensor decreased as the concentration of the ZnO nanoparticles decreased.

The maximum quality factor was observed when the sensor was immersed in growth media without bacteria, while the minimum value for the sensor was observed when the sensor was immersed in bacteria incubated in media without ZnO nanoparticles. As the concentration of the ZnO nanoparticles increased, the quality factor increased.

On the other hand, to assess the antibacterial activity of ZnO nanoparticles on the positive gram bacteria by using the QTF biosensor, the resonance frequency and quality factor of the QTF immersed in the in *S. aureus* grown in media with different concentrations of the green synthesized ZnO nanoparticles was measured as a sensor response. The resonance frequency of the QTF sensor immersed in *S. aureus* with 1 mm ZnO nanoparticles was lower than that of the sensor immersed in the growth media by 5.69±3.81 hz. Moreover, as shown in Figure 6, the resonance frequency shift decreased

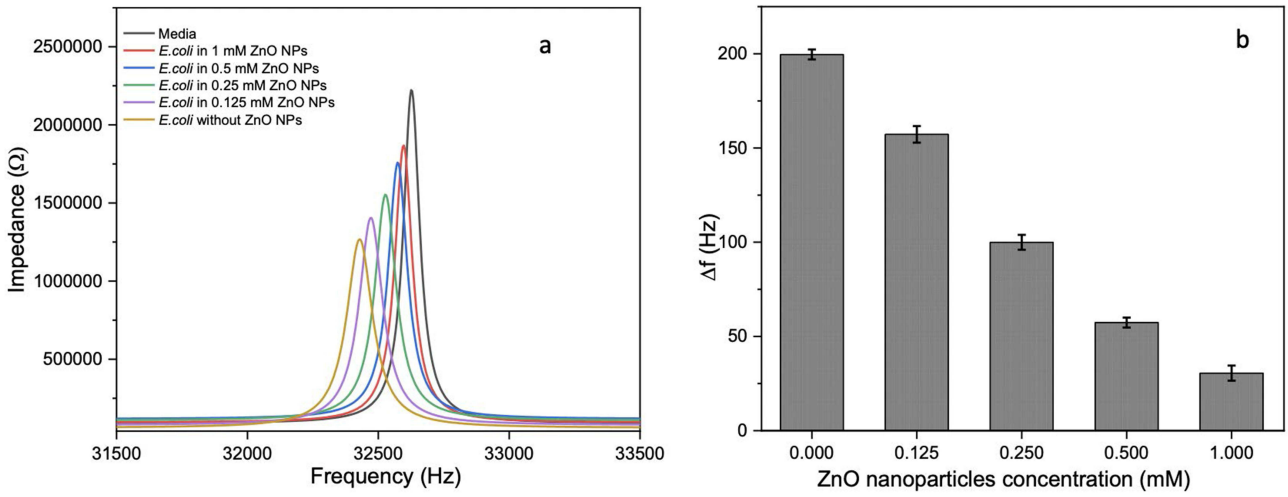

Figure 4 QTF (**a**) resonance frequency, (**b**) frequency shift after immersion in *E. Coli* bacteria growth in different ZnO nanoparticles content.

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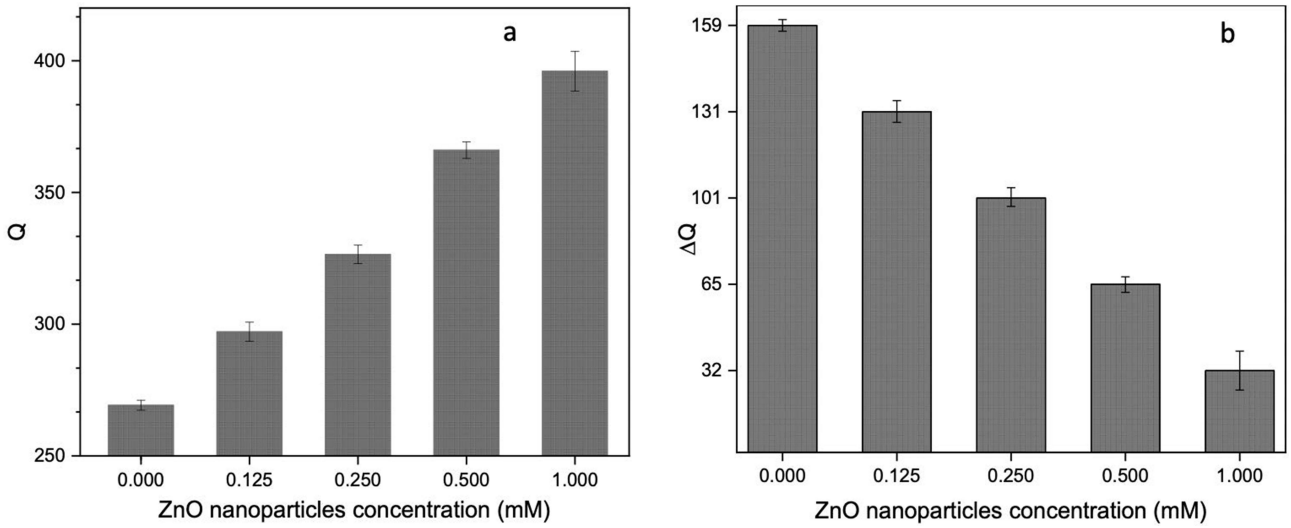

Figure 5 The QTF microsensor (**a**) quality factor, (**b**) quality factor shift after immersion in *E. Coli* bacteria with different ZnO nanoparticles content.

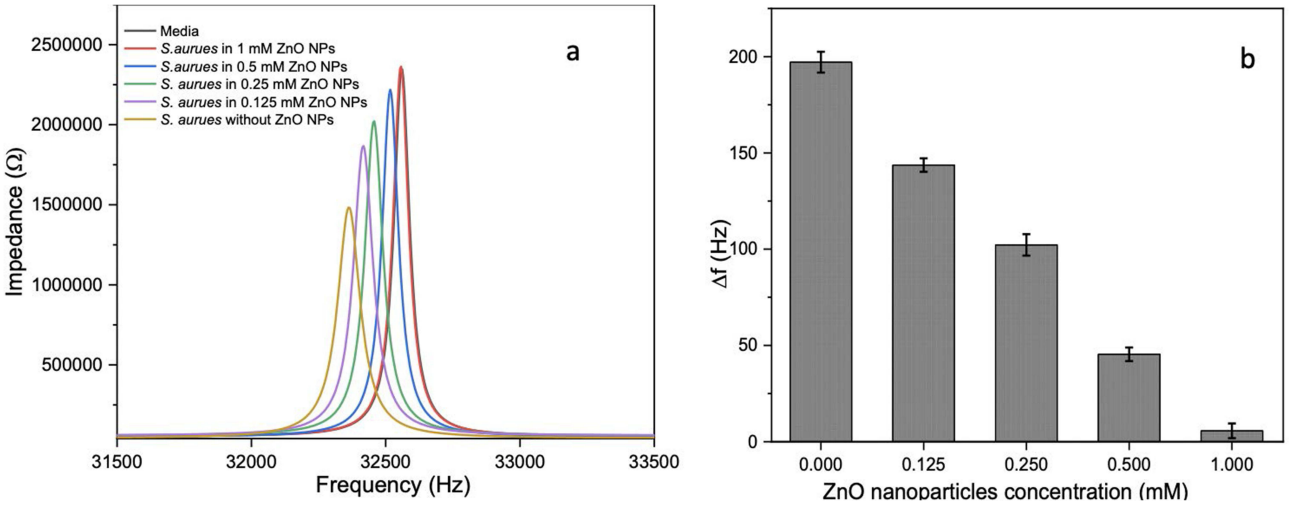

Figure 6 QTF (**a**) resonance frequency curves (**b**) frequency shift after immersion in *S. aureus* bacteria growth different ZnO nanoparticles content.

as the ZnO nanoparticle content increased. Figure 7 illustrates the quality factor and quality factor shift of the QTF sensor incubated in *S. aureus* with different ZnO nanoparticle concentrations. The quality factor of the sensor did not change when the sensor was incubated with *S. aureus* and 1 mm ZnO nanoparticles.

In comparison, the resonance frequency and quality factor of the sensor shifted more for the QTF immersed in *E. coli bacteria* than *S. aureus* at the same concentration of ZnO nanoparticles.

Discussion

The lower reduction in resonance frequency and quality factor values for the bacteria grown in higher ZnO nanoparticle concentrations indicates a decrease in the bacterial concentration in the solution. The resonance frequency and quality factor shift can be used as a response to bacterial concentration variations in growth media. The bacterial concentration decreased as the green-synthesized ZnO nanoparticle content increased due to the antibacterial effect, which killed the bacteria and inhibited their growth. Conversely, the resonance frequency decreased as the concentration of ZnO nanoparticles increased with an increase in the number of bacteria attached to the sensor surface. Bacteria have a high ability to attach to the silicon oxide surface. Therefore, as the concentration of bacteria increases, the resonance frequency decreases

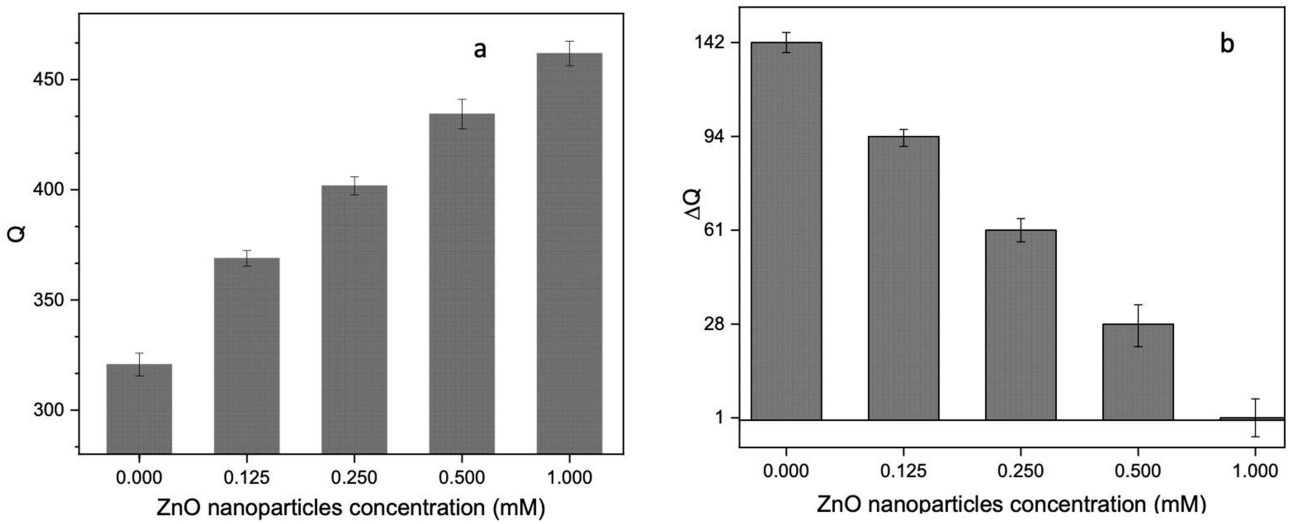

Figure 7 (**a**) quality factor (**b**) quality factor variation of the QTF microsensor immersed in *S. aureus* with different ZnO nanoparticle contents.

because of the increase in mass loading resulting from the attachment of bacteria to silicon oxide on the QTF surface.[45,51] The attachment of bacteria to the surface is related to several mechanisms, such as adhesion pili, Van der Waals forces, and hydrogen bonds, and zeta potential also plays a role in cell adhesion.[51–53] The decrease in the quality factor at lower ZnO concentrations and higher bacterial concentrations can be attributed to the increased viscosity of the solution caused by the growing bacterial population. This increased viscosity leads to a greater damping effect on the QTF. Additionally, the change in quality factor can serve as a useful indicator of bacterial concentration, as it reflects the interaction between the sensor and the bacteria in the growth solution. The observed differences in resonance frequency and quality factor shifts between *S. aureus* and *E. coli* can be attributed to the different antibacterial effects of *Rosmarinus officinalis-synthesized ZnO* nanoparticles on gram-positive and gram-negative bacteria. The lower resonance frequency shift in *S. aureus* suggests that the *Rosmarinus officinalis* synthesized ZnO nanoparticles have a more significant impact on *S. aureus* gram-positive bacteria than on *E. coli* gram-negative bacteria due to differences in cell wall structure or nanoparticle interactions. The green *Rosmarinus officinalis* synthesized ZnO nanoparticles exhibited antibacterial effects against both *Sauries* and *E. coli*. However, *S. aureus* exhibited a slightly higher susceptibility to the ZnO nanoparticles used in this study. The lower effect of ZnO nanoparticles on gram-negative bacteria is because they have a complex cell wall that consists of an outer membrane with lipopolysaccharides, a thin peptidoglycan layer, and inner lipid components; however, gram-positive bacteria possess simpler cell walls composed mainly of thick, compact peptidoglycan layers.[54] Table 1 shows some literature reports on the antibacterial effect of green-synthesized ZnO particles.

It has been reported that ZnO nanoparticles act as antibacterial materials to inhibit bacterial growth through the attachment of ZnO nanoparticles to the bacterial surface, resulting in an increase in Zn^{2+} in the bacterial cytoplasm.[23,58] In addition, it has been reported in the literature that ROS are produced in the dark; ROS such as hydrogen peroxide and superoxide radicals are generated, while hydroxyl radicals are not produced, which act as

Table 1 A Comparison Between Studies Using the Antibacterial Effect of Green-Synthesized ZnO Particles

ZnO type	Test Method	Bacteria Type	Main Results	Ref
Green synthesized by *Saccharomyces cerevisiae* Size 15.0 nm	Zone of inhibitory test (ZOI): agar-disc diffusion MIC: serial dilutions technique	Gram positive: *S. aureus* Gram positive: *E. coli*	It showed antibacterial for both types but *S. aureus* more susceptible than *E. coli* in both ZOI and MIC	[54]

(*Continued*)

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Table 1 (Continued).

ZnO type	Test Method	Bacteria Type	Main Results	Ref
Green synthesized by using garlic extract Particle size 14 nm	Agar diffusion method for ZOI Microdilution method for MIC	*S. aureus* exhibited the highest antibacterial activity followed by *E. coli, P. aeruginosa*, *L. monocytogenes*, *S. typhimurium*, and B. subtilis For MIC the *S. aureus* it showed the highest sensitivity with the lowest MIC	*S. aureus* *S. typhimurium* *E. coli* *L. monocytogenes B. subtilis* *P. aeruginosa*	[55]
Synthesized by using basil extract Particle size 25 nm	Agar diffusion method for ZOI Microdilution method for MIC	Antiracial was the highest for *S. aureus, E. coli*, *P. aeruginosa*, *L. monocytogenes*, *B. subtilis, S. typhimurium* respectively. For MIC B. subtilis, and *P. aeruginosa* exhibited the lowest MIC.	*S. aureus* *S. typhimurium* *E. coli* *L. monocytogenes* *B. subtilis* *P. aeruginosa*	[55]
Green synthesized by using Rosemary extract Size (54 nm)	Agar diffusion method for ZOI Microdilution method for MIC	Antibacterial activity was the highest for *S. aureus, E. coli, P. aeruginosa*, *L. monocytogenes*, *S. typhimurium*, *B.* subtilis respectively *S. aureus* exhibited the lowest MIC.	*S. aureus*, *S. typhimurium* *E. coli.* *L. monocytogenes* *B. subtilis* *P. aeruginosa*	[55]
Green synthesize *Cassia fistula* Particle diameter 68.1 nm, *Melia azedarach* L. Particle diameter 3.62 nm	Disc diffusion method	*E. coli* showed a higher Zone of inhibition than *S. aureus* for the ZnO nanoparticles synthesized by *Melia azedarach* L. *more efficient against S. aureus*	*S. aureus* and *E. coli*	[56]
Green synthesis of zinc oxide nanoparticles using *Pisonia Alba* P. leaf 48 nm	Disc diffusion method	*S. aureus exhibited a higher susceptibility than K. pneumophila*	*S. aureus* and *K. pneumophila*	[57]
Green synthesized ZnO nanoparticles Rosemary extract	QTF	*S. aureus exhibited a* higher susceptibility than *E. coli*	*S. aureus and E. coli*	Current study

antibacterial agents.[24,25] The smaller particles can penetrate into the cell membrane and produce ROS inside the cell resulting in more effect, as well as the penetrated nanoparticles causes mechanical damage to the cell membrane resulting in the leaking of bacterial cell cytoplasm.[59,60] In addition, the QTF sensor exhibited a slightly greater decrease in both resonance frequency and quality factor when immersed in *E. coli* without ZnO nanoparticles, indicating that *E. coli* may exert a stronger mechanical effect owing to its larger cell size compared to *S. aureus*. These findings suggest that the mechanical properties of the bacterial cells, combined with the antibacterial activity of ZnO nanoparticles, influence the response of the sensor.

Conclusion

The QTF system successfully detected the effect of the green synthesized ZnO nanoparticles on both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria in an oral-mimicking environment through the changes in resonance frequency and quality factor as a function of ZnO concentration. Therefore, the QTF micromechanical biosensor can work as a low coast, real-time, label-free, and quantitative a sensing platform to assess the antibacterial activity. The green-synthesized ZnO nanoparticle exhibited good antibacterial activity against both *E. coli* and *S. aureus*. As the concentration of ZnO nanoparticles in the growth solution increased, the resonance frequency and quality factor increased, indicating enhanced antibacterial activity and reduced bacterial presence. Notably, the QTF system revealed a stronger antibacterial effect of the green synthesized by *Rosmarinus officinalis* ZnO nanoparticles on *S. aureus* than *E. coli*, demonstrating its potential for comparative microbial studies. The work exhibited the ability of the QTF micromechanical sensor to convert nanoscale biological interactions into measurable mechanical signals in a short time, eliminating the need for dyes, biomarkers, and lengthy incubation steps required in the conventional methods. In conclusion, the QTF sensor can work as a powerful alternative technology for label-free, real-time, and miniaturized antimicrobial effects suitable for both research and potential clinical applications.

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Disclosure

The authors report no conflicts of interest in this work.

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