

Investigating the Inhibitory Potential of Solfeggio Frequencies on Quorum Sensing-Dependent Biofilm Formation of *Enterococcus faecalis*

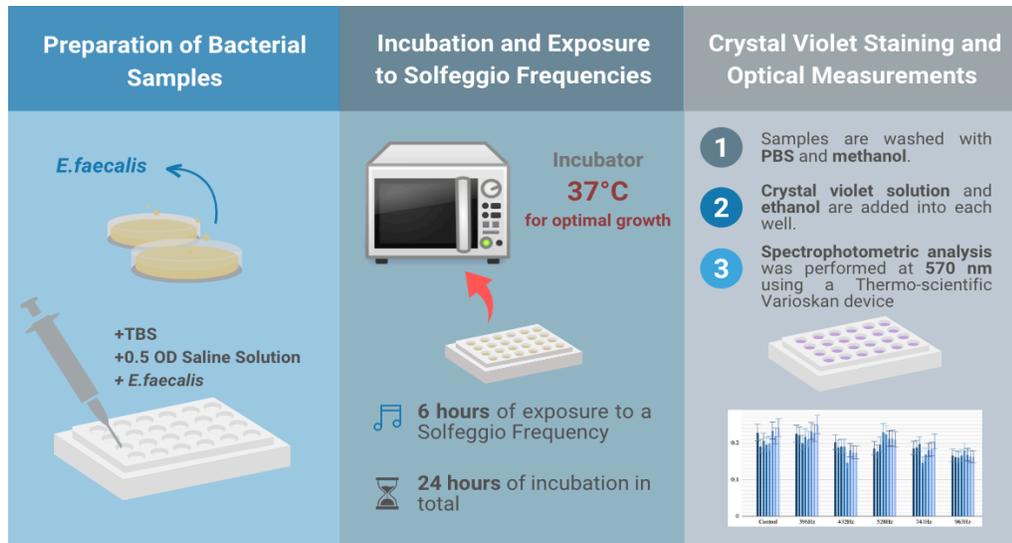
Alara Urunga
American Collegiate Institute

Abstract

Quorum sensing (QS), a complex biochemical communication mechanism utilized by bacteria, plays a pivotal role in orchestrating the transcription and expression of genes involved in crucial pathogenic activities such as biofilm formation and virulence. Biofilms are microbial communities encased in a self-produced matrix, which helps bacteria adhere to surfaces and resist immune defenses. This study aims to investigate the QS ability of *Enterococcus faecalis*, a gram-positive bacterium predominantly found in the gastrointestinal tracts of humans, by exploring the intricate interplay between the exposure of bacterial samples to selected solfeggio frequencies (396 Hz, 432 Hz, 528 Hz, 741 Hz, and 963 Hz) and the resulting differences in biofilm thickness. The biofilm formation (BF) in each sample has been evaluated through the crystal violet staining method along with spectrophotometric analysis. The results obtained from this research revealed noteworthy variations in the patterns of biofilm formation, intriguingly influenced by the different solfeggio frequencies applied. Specifically, the groups subjected to the distinctive frequencies of 432 Hz, 741 Hz, and 963 Hz demonstrated a notable reduction in biofilm thickness, suggesting a discernible attenuation in the quorum sensing of the *Enterococcus faecalis* strain. These findings provide experimental evidence that specific solfeggio frequencies (SF) possess the capacity to modulate quorum sensing, thus presenting a highly promising avenue for manipulating bacterial behavior and offering potential therapeutic interventions to combat the formidable challenges posed by biofilm-related infections.

Key Words: Quorum sensing, Enterococcus faecalis, Biofilm formation, Crystal Violet Staining, Solfeggio frequencies

Graphical Abstract



Impact Statement

This research seeks to elucidate the inhibitory effect of solfeggio frequencies (432 Hz, 528 Hz, 741 Hz and 963 Hz) on the quorum sensing (QS) mechanism of *E. faecalis*. Quorum Sensing, a vital mechanism for bacterial communication, plays a crucial role in biofilm formation and pathogenicity. The proposed investigation holds substantial significance in the context of combating biofilm-related infections, advancing our understanding of microbial behaviors and replacing antibiotics that pose a global health threat. Biofilms are microbial communities that exhibit increased resistance to antibiotics and host immune responses, posing a persistent challenge towards public health. Therefore, exploring novel strategies to disrupt QS holds immense promise in mitigating these issues, hence, this study offers a unique approach by examining the effect of specific sound frequencies on QS. In addition, it has the potential to offer new avenues for therapeutic interventions and inspire further research on solfeggio frequencies, which are specific frequencies that have physiological and psychological impacts on living organisms such as influencing brainwave patterns and heart rate. However, their effectiveness in medical fields is still being investigated. Accordingly, the outcomes of this study may open up new perspectives toward the use of solfeggio frequencies in medicine.

Data Summary

All data associated with optical density values and statistical tests in this paper is obtained directly from the scientific experiments conducted by the author at the Pharmaceutical Microbiology Department of Ege University. Additional websites are utilized for the purposes of generating sound frequencies, obtaining data and plotting graphs for the results. In addition, the method is adapted from the article with the DOI 10.1111/j.1600-0463.2007.apm_630.x: "Quantification of biofilm in

microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci”, which has also been cited in the bibliography section. Sounds (396 Hz, 432 Hz, 528 Hz, 741 Hz and 963 Hz) are generated from “Zenmix”: <https://zenmix.io/solfeggio-frequencies-generator>, and checked for accuracy of the specific frequencies. For the creation of the sine graphs, the website “Desmos” is utilized: <https://www.desmos.com/>. In addition, data is illustrated through Google Spreadsheets. For the calculations and visualizations of statistical analysis, Statistics Kingdom ANOVA Calculator has been used: www.statskingdom.com.

1. Introduction

Bacteria were thought to be independent, self-sufficient entities. These unicellular organisms were believed to lack the sophistication needed to form multicellular groupings, as demonstrated by plants and animals [1]. However, studies regarding the communal behaviors and pathogenicity of bacteria revealed that bacteria communicate with each other in the same medium through a process called “quorum sensing” (QS). This study aims to investigate the impact of solfeggio frequencies on the quorum sensing mechanism of *Enterococcus faecalis* through biofilm evaluation. To facilitate the reader’s understanding, the introduction part of the article is divided into five subtopics: Quorum Sensing, Quorum Sensing Inhibition, Biofilm Formation, *Enterococcus faecalis*, and Solfeggio Frequencies.

1.1. Quorum Sensing

Quorum sensing is a process in which bacteria monitor their cell-population density by measuring the concentration of signal molecules called autoinducers. The individual organisms secrete the autoinducers into the extracellular environment when the population of QS bacteria expands. Therefore, there is a correlation between cell population density and the concentration of external autoinducer. Bacteria are able to detect one another by keeping track of the extracellular autoinducer concentration and adjust gene expression correspondingly [2].

Currently, three paradigmatic classifications are used to evaluate QS systems: Gram-negative bacteria have quorum-sensing systems of the LuxI/LuxR type, Gram-positive bacteria utilize oligopeptide/two-component-type QS circuits and LuxS (autoinducer-2) chemical signaling molecules are used in interspecies communication. In fact, the specific genes and signaling molecules that modulate QS differ among microorganisms. Through studies conducted from the past to the present, it has been discovered that bacterial communication (QS) enables bacteria to carry out several pathogenic activities such as biofilm formation, synthesis of virulence factors, conjugation, and bioluminescence along with swarming/motility, oxidative stress tolerance and pigment production [3]. In addition, QS pathways allow bacteria to adapt to changing conditions [4].

Consequently, the excessive use of antibiotics drives the evolution of antimicrobial resistance (AMR) in pathogenic bacteria, rendering treatments less effective and leading to persistent, hard-to-treat infections [5]. This poses a global health threat as resistant bacteria can spread rapidly, which limits treatment options and increases mortality rates particularly in hospital settings. Therefore, it is crucial to find new approaches to replace antibiotics for fighting bacterial infections or to develop supplementary treatments that can be used alongside antibiotics to reduce their overuse. For this reason, exploring novel strategies to disrupt quorum sensing holds immense promise in mitigating the consequences of AMR and controlling bacterial pathogenicity.

1.2. Quorum Sensing Inhibition

Diverse molecules and chemical signals have been synthesized for the purpose of inhibiting bacterial quorum sensing. It has been suggested that in order to be an effective quorum sensing inhibitor (QSI), a molecule must satisfy at least a few of the following requirements: it must be highly specific for a particular QS regulator, it must have no negative effects on the bacteria or the host, it must be chemically stable and resistant to degradation by different host metabolic systems, and it should preferably be longer than the native AHL. These qualities of a QSI make it less likely for bacteria to develop resistance to the drug(s), which typically exert selection pressure during the treatment of infections, and these substances are less likely to have an impact on the population of helpful bacteria present in the communities housing the host [6].

An example of a quorum sensing inhibitor is mBTL.

Meta-bromo-thiolactone (mBTL) is found to inhibit both the production of the virulence factor pyocyanin and BF. However, there has not been any in vivo study regarding its effect on humans yet [7]. Caffeine and vanilla extracts are also noted down for their ability to abate quorum sensing [8].

Moreover, it is being investigated whether physical stimuli are able to inhibit bacterial communication. Accordingly, scientists have figured that exposure to static magnetic field stimulated quorum sensing in luminescent *Vibrio* strains [9].

1.3. Biofilm Formation

Biofilms refer to structured microbial collectives anchored to surfaces, consisting of stationary cells (bacteria and/or fungi), ensconced within an extracellular matrix that includes polysaccharides, DNA, and assorted constituents [10]. Biofilms can develop on various surfaces, including medical devices, pipes, and natural environments, playing a significant role in various processes. BF contains about 80% water, bacteria/microorganisms and extracellular polymeric substances (EPS) [11]. In addition, EPS could make up 50% to 90% of the overall organic carbon content within biofilms [12].

The microorganisms that make up the biofilm initiate the activation of genes that induce the expression of stress-related genes. Consequently, these stress-related genes prompt a transition towards resistant phenotypes in response to specific alterations, such as variations in cell density, nutrient availability, temperature, pH, ultraviolet light, radiation and osmolarity [10]. Thus, the advantages of BF to microorganisms include protection against these environmental stresses. BF occurs in four stages: In the initial stage (Stage 1), bacterial cells undergo a reversible attachment process onto a surface. In Stage 2 of BF, an irreversible attachment of these bacterial cells to the substrate surface takes place. This attachment is facilitated by the expression of QS signaling molecules and the creation of extracellular polymeric material. Transitioning to Stage 3, a fully developed biofilm emerges, characterized by a three-dimensional structure wherein cells are densely packed into clusters. The gaps or channels between these clusters permit the movement of water and nutrients, as well as the removal of waste materials. Finally, at Stage 4, the biofilm experiences detachment and dispersion of individual cells. This detachment marks the initiation of a new cycle of BF [13].

In this study, the groups were exposed to SF for 6 hours specifically to allow the SF to affect the stages 1 and 2 of BF. Acknowledging that QS regulation takes place predominantly in stage 2, the limitation of exposure to 6 hours reinforces the interpretation of the experimental results and the effect of QS on BF.

1.4. *Enterococcus faecalis*

Enterococci, facultative anaerobic gram-positive bacteria known for their ability to adhere to a variety of surfaces and form biofilms, are attributed to causing significant nosocomial infections encompassing conditions such as urinary tract infections, bloodstream infections, and endocarditis [14]. Being one of the most widespread antibiotic-resistant pathogens for the past few decades, *E. faecalis* is responsible for bloodstream infections in 7% of European and 10% of US care units [15]. It can grow between 10 and 45 °C, with its optimal growth conditions being 35 and 37°C [16].

E. faecalis is commonly obtained from root canals in instances of unsuccessful endodontic procedures, and it is studied intensely in dentistry [17]. Moreover, its QS system is closely related to the BF and virulence [18]. Four primary QS regulators identified the QS of *E. faecalis* include cytolysin (CylR1, CylA), gene encoding gelatinase (GelE) associated with the Frs-QS system, and serine protease (SprE). Research indicates that cytolysin is also linked to pathogenicity in human infections [19, 20].

1.5. Solfeggio Frequencies

“Sound is a mechanical wave that propagates longitudinally through a physical medium (solid or fluid) and can be heard” [21]. Sound frequencies that fall within the range of 20 Hz - 20,000 Hz are audible by humans and

the intensity of sound is expressed in a logarithmic scale, corresponding to the term “decibel” (dB). Several sound waves have been utilized in previous studies to restrict the growth of pathogenic organisms. In a study conducted in India, scientists observed that the audible sound in the form of classical music was able to affect growth, metabolism, and antibiotic susceptibility of prokaryotic as well as eukaryotic microbes [22]. In another recent study conducted to evaluate the effect of solfeggio frequencies (SF), which are specific frequencies that are speculated to have physiological and psychological influences on organisms, it was found that SF reversed cognitive and endocrine deficits evoked by a 24-h light exposure in adult zebrafish [23]. In this paper, the frequencies 396 Hz, 432 Hz, 528 Hz, 741 Hz and 963 Hz, which are classified as SF, were applied on *E. faecalis* during incubation for BF comparison purposes.

2. Methods

2.1. Choice of Method

For the detection and quantification of biofilm formation in *E. faecalis* when exposed to SF, crystal violet assay and spectrophotometric analysis were performed. Specifically, the crystal violet assay was selected for this experiment to measure the thickness of biofilms formed after exposure to SF. The biofilms' matrix, which is made up of proteins, polysaccharides, and extracellular DNA, is stained with crystal violet dye. Being compatible with a variety of bacterial strains, crystal violet assay is a standardized procedure that enables the measurement of biofilm development. Even though the method is qualitative, spectrophotometric analysis that was applied after the staining process to measure the optical density values (OD) of the dyed samples generated a quantitative measurement for the comparison and interpretation of the results. Accordingly, The alternative hypothesis was formed according to the notion that five SF (396 Hz, 432 Hz, 528 Hz, 741 Hz, 963 Hz) during a 6-hour incubation on *E. faecalis* would reduce biofilm formation by disrupting the QS mechanism of the bacteria. As a result, the OD values of samples were expected to be greater compared to the control group, indicating a higher extent of crystal violet staining bacterial biofilms in the wells.

2.2. Chemicals and Materials

The specific materials and amounts that were used in this research are 18 mL of Tryptic Soy Broth (TSB) with 2.5% glucose, *E. faecalis* strain, 10 mL Mueller Hinton Agar (MHA), 19 mL of 0.1% crystal violet solution, 60 mL of phosphate buffered saline (PBS) solution (pH = 7.2), 20 mL of methanol (CH₃OH), 15 mL of ethanol (C₂H₆O). Additionally, 1 P20 (2-20 µL) micropipette, 1 multichannel micropipette, 2 inoculation loops, approx. 300 micropipette tips, 3 glass tubes, 4 plastic petri dishes, 6 cell culture microplates (96 wells), 1 sound generator (Philips BT Speaker), 2 100 mL beakers, 1 microplate reader (Thermo-scientific Varioskan™), 2 incubators, 1 vortex, 2 bunsen burners, and 1 decibel meter.

2.3. Bacterial Strain

The *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper (ATCC® 29212™) preceptrol culture used in this research was originally isolated from a urine source and freeze-dried. It was stored at 2°C to 8°C aerobic atmosphere storage conditions.

2.4. Preparation of Bacterial Samples

Enterococcus faecalis was taken out of the bacterial culture and inoculated into nutrient-enriched Mueller Hinton Agar (MHA) inside of a plastic petri dish with an inoculation loop. The bacteria were incubated for 24 hours aerobically at 37°C for revival. After the incubation period, bacterial colonies were placed in a test tube gently in sterile conditions, *E. faecalis* suspension was made in saline (0.9%) solution and then vortexed. Using a McFarland device, the optical densities (OD) of the suspensions were adjusted to 0.5. Using a multichannel micropipette, 180 μ L of tryptic soy broth (TSB) containing 2.5% glucose was added to each 8 wells of a 96-well microplate to enable the formation of bacterial biofilms. 20 μ L of *E. faecalis* suspension was added to each well [24].

2.5. Exposure to SF

In order to prevent the samples from being impacted by different frequencies, experiments were carried out at different intervals. To quantify the impact of these factors on QS, the frequencies and sound intensity utilized in the experiment were maintained at a specified level (70-80 dB) that did not kill bacteria, and this variable was controlled through a tuning app (Soundcorset) that was run from a mobile device. Each step was repeated for the samples exposed to 396 Hz, 432 Hz, 528 Hz, 741 Hz, 963 Hz frequencies. The control groups were not exposed to any frequencies. Samples were incubated for a total of 24 hours at 37°C for maximum bacterial growth. The sound frequency generator used for this experiment (BT speaker) was set to the desired frequency and positioned 10 cm from the plates such that the sound intensity was between 70 and 80 dB. The BT speakers were turned off after six hours of exposure. Samples were taken out of the incubator after a total of 24 hours. Figure 1 demonstrates all the frequencies detected inside of the incubator by the application *Academio* while the bacteria were being exposed to the selected SF. Figure 2 depicts the mathematical illustrations of the sine waves of the sounds used. The amplitude of each graph indicates the sound intensity (dB), and periods show the energy level of the frequencies.

2.6. Staining the Biofilms

Once the culture medium was removed, 200 microliters of sterile PBS were injected into the wells to wash the biofilms. This process was repeated three times. The plates were turned over, and the wells were left to dry for five minutes. 200 μ L of methanol was added to each well of the microplate

for the fixation of biofilms. After 15 minutes, methanol was removed and the microplate was inverted again. 200 microliters of a 0.1% crystal violet solution were poured into the wells to begin the biofilm staining process. The wells were washed three times with 200 μ L of water, and the plates were dried. 150 μ L absolute ethanol was added to each well gently to solubilize the dye and the microtiter plate was covered with the lid to evade evaporation. After 15 minutes, spectrophotometric measurements were performed at 570 nm using a Thermo-scientific Varioskan device, and the optical density values for each experimental group were recorded for further analysis. The experimental steps were performed for each SF. The groups and number of repeats are listed in Table 1.

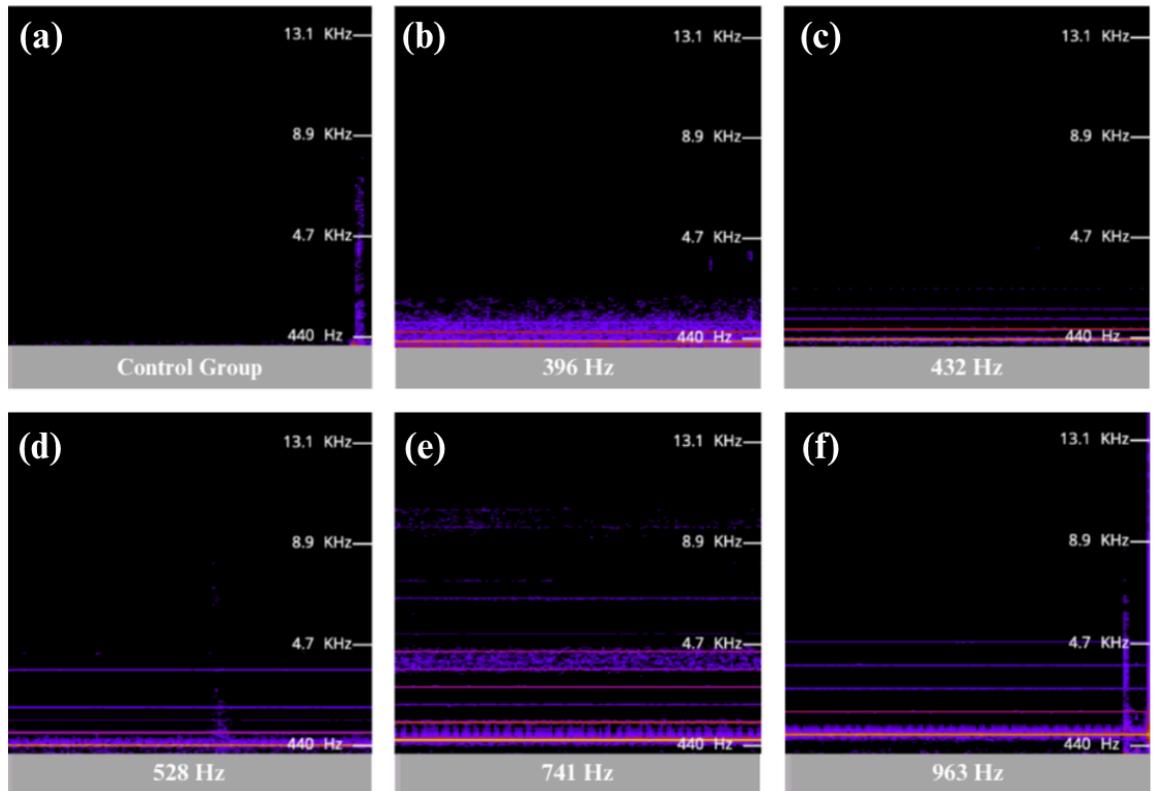


FIGURE 1. Detected Frequencies (Formed by <https://academo.org/>). Figures 1 (a) to (f) demonstrate the detected sound frequencies inside of the incubator as the samples were being exposed to the indicated solfeggio frequencies. External sounds were recorded in purple lines, and lines closer to red indicate the detected frequencies with the highest intensities (in decibel). This figure visually confirms the accurate exposure of the bacterial samples to the targeted solfeggio frequencies, ensuring the reliability of the experimental conditions for assessing their impact on biofilm formation.

2.7. Control Variables and Accuracy

There are several variables that were kept in the same levels or conditions.

These include temperature, sound intensity (dB) and the humidity of the environment. Temperature was kept stable by utilizing the incubator at a fixed value of 37°C for optimum growth conditions. Moreover, humidity was set at 95% in the incubator for all samples formed in the experiment. Sound intensity measured by dB was standardized in the range of 70-80 dB adjusting the distance between the BT speaker and the samples inside the incubator accordingly.

To ascertain whether *E. faecalis* was able to form biofilms, negative control groups were prepared without bacteria under the same procedure steps and conditions, and compared to the experimental groups. Each independent variable was repeated 8 times to increase the reliability of the data.

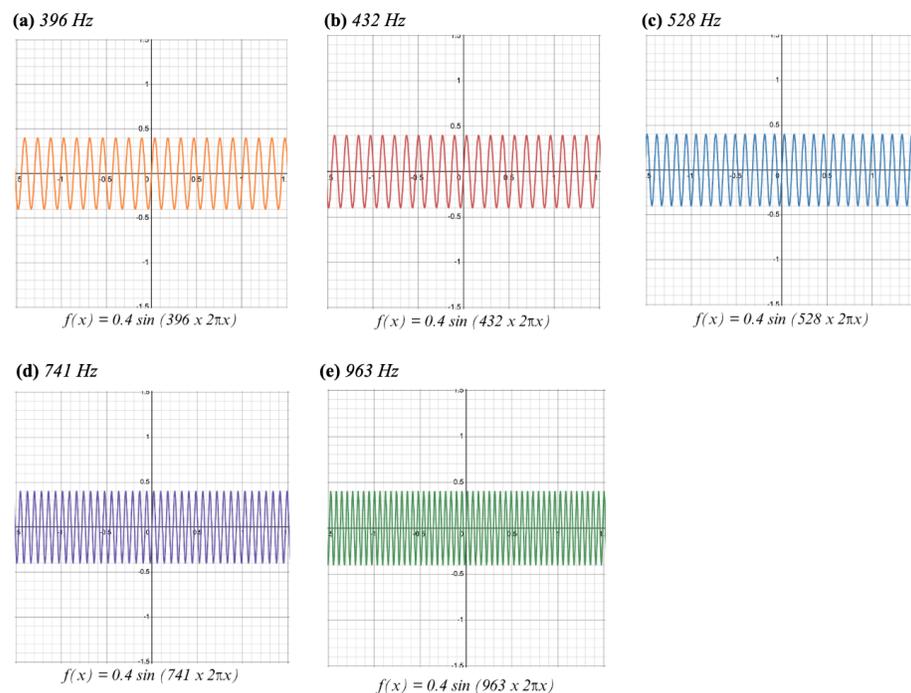


FIGURE 2. Mathematical Modeling of Solfeggio Frequencies (Created by <https://www.desmos.com/>) Graphs (a) to (e) illustrate the mathematical properties of the solfeggio frequencies used in this experiment (396 Hz, 432 Hz, 528 Hz, 741 Hz, 963 Hz), generated using the formula $I(t) = \sin(2\pi ft)$. This figure provides a visual reference for the wave properties of the frequencies to ensure a clear understanding of their potential effects on bacterial quorum sensing and biofilm formation.

Control Group	Wells in which <i>E. faecalis</i> samples were inoculated that are held without any outside disturbance (8)
Experimental Group 1	<i>E. faecalis</i> samples exposed to 396 Hz (8)
Experimental Group 2	<i>E. faecalis</i> samples exposed to 432Hz (8)
Experimental Group 3	<i>E. faecalis</i> samples exposed to 528 Hz (8)
Experimental Group 4	<i>E. faecalis</i> samples exposed to 741 Hz (8)
Experimental Group 5	<i>E. faecalis</i> samples exposed to 963 Hz (8)

TABLE 1. Control and Experimental Groups. Table 1 shows the frequencies each experimental group was exposed to.

3. Results

3.1. BF Analysis

The investigation into the impact of SF on QS-dependent BF in *Enterococcus faecalis* yielded varied results. The BF assay conducted using crystal violet staining and subsequent spectrophotometric analysis provided quantitative data for the evaluation of biofilm thickness through optical density measurements across the experimental groups consisting of five distinct SF. The eight samples of the control group, which consisted of *E. faecalis* samples exposed to standard growth conditions without any external frequency influence, exhibited a notable level of biofilm formation. This baseline measurement served as a reference point for the comparative analysis with the experimental groups subjected to SF.

Optical Density Measurements in Each Sample Following the 24-hour Incubation Period												
#	Control Group		396 Hz		432 Hz		528 Hz		741 Hz		963 Hz	
	E.f.	Neg. Control	E.f.	Neg. Control	E.f.	Neg. Control	E.f.	Neg. Control	E.f.	Neg. Control	E.f.	Neg. Control
1	0.228852	0.0865148	0.22617	0.0840759	0.202216	0.0835527	0.186034	0.0903696	0.186257	0.0960052	0.166398	0.102686
2	0.190975	0.0918221	0.22177	0.0813005	0.188644	0.076491	0.176816	0.0874831	0.187943	0.0862124	0.162248	0.0959753
3	0.206391	0.0836833	0.200431	0.0758618	0.191014	0.0786201	0.196455	0.0833782	0.197524	0.0792688	0.160806	0.091739
4	0.196075	0.0906942	0.217075	0.0820557	0.190928	0.078839	0.230503	0.0916562	0.147483	0.0840617	0.165841	0.0932546
5	0.198924	0.0922093	0.211016	0.0873923	0.147514	0.0917481	0.224656	0.0930004	0.168879	0.070923	0.181231	0.0992728
6	0.234031	0.094558	0.232956	0.088669	0.180534	0.0807718	0.212131	0.0879606	0.181093	0.089763	0.168231	0.104916
7	0.219128	0.0900806	0.227308	0.090887	0.174721	0.0789073	0.213328	0.096189	0.183669	0.0840923	0.16338	0.103372
8	0.24205	0.102539	0.250253	0.097242	0.174453	0.0908447	0.210385	0.087789	0.204012	0.0882102	0.162264	0.118179

TABLE 2. Optical Density Measurements. On the table above, raw data for all the experimental groups is shown. Negative control groups (NC) are composed of samples that were not containing *E. faecalis* biofilms. NC groups were formed to assess whether *E. faecalis* was able to form biofilms successfully or not. The values that are specified inside the boxes are in the optical density unit (ODU). It does not have any scientific unit, but can be expressed in the form of $-\text{Log}_{10}(1/T)$ (T:transmittance). Although these values are a result of a 24-hour incubation, it is significant to note that the

SF were only applied during the first 6 hours of incubation that roughly corresponds to the first and second stages of BF.

The results of the experimental groups were compared through one-way right-tailed ANOVA and Tukey's Honestly Significant Difference (HSD) test to determine whether the differences between the experimental groups with the control group were statistically significant. ANOVA has been utilized to compare the means of groups exposed to distinct SF to determine whether there are noteworthy differences in biofilm thickness among these groups. In addition, Tukey's test was performed to identify which SF were more effective in influencing BF, and to figure out the significant differences between control group and experimental groups.

Means, Standard Deviations and Coefficient of Variation			
Groups	Average	Standard Deviation	Coeff. of Variation (%)
Control	0.21455325	0.01915766155	8.929094081
396 Hz	0.223372375	0.01492333238	6.680921212
432 Hz	0.181253	0.01652141944	9.115115027
528 Hz	0.2062885	0.01851543176	8.97550361
741 Hz	0.1821075	0.01750944116	9.614892939
963 Hz	0.166299875	0.006527986376	3.925430717

TABLE 3. Descriptive Statistics Results. The analysis of the standard deviation of the groups are in the range 0.01-0.02, which indicates the quality and precision of the data as it appears to be closer to the 0, thus to the mean. All the coefficients of variation expressed in the table are less than 10%, which shows that the data sets have low variability, reinforcing consistency and reliability.

3.2. Statistical Analysis

3.2.1. ANOVA Analysis

Table 3 displays the means, standard deviations and coefficient of variation of the distinct groups. When the means of the experimental groups are divided into the mean of the control group, it can be deduced that the samples exposed to 396 Hz have an average OD value increased by approximately 4% compared to the control group. Mean of samples exposed to 432 Hz have a mean OD value decreased by 16%, the mean OD value of samples exposed to 528 Hz decreased by 4%, the mean OD value of samples exposed to 741 Hz decreased by 15%, and finally the average value of samples exposed to 963 Hz decreased by 23%. Statistical significance of these data are analyzed through the statistical tests indicated below.

Source	DF	Sum of Square	Mean Square	F Statistics	P-value
Groups (between groups)	5	0.01993	0.00399	15.3821	1.374 x 10 ⁻⁸
Error (within groups)	42	0.01088	0.00026		
Total	47	0.03081	0.00066		

TABLE 4. ANOVA Calculations. “DF” is an abbreviation for degrees of freedom. Sum of Square (SS) of groups represents the difference between the groups’ means and the total mean. SS of Error (within groups) indicate the variation within the groups. SS (total) represents differences from the overall mean. The greater the F value, the higher the probability that not all the means of the groups are equal/statistically insignificant. Conversely, the smallness of the P-value demonstrates the variance in between the groups’ data.

3.2.2. Tukey HSD Analysis

Table 4 shows the F statistics and P-value for the interpretation of the variance within the groups. Since p-value (1.374 x 10⁻⁸) is less than α , which is the significance level predetermined as 0.05, H₀ is rejected: Some of the groups' means are considered to be distinct. As a continuation of the ANOVA statistical test, HSD Tukey was performed. The Tukey test and graphical results are specified below in Table 5 and Figure 3.

	Difference	SE	Q	Lower CI	Upper CI	Critical Mean	p-value
Control-396 Hz	0.008819	0.005691	1.5496	-0.01521	0.03285	0.02403	0.8803
Control-432 Hz	0.0333	0.005691	5.8512	0.009273	0.05733	0.02403	0.002146
Control-528 Hz	0.008265	0.005691	1.4522	-0.01576	0.03229	0.02403	0.9062
Control-741 Hz	0.03245	0.005691	5.7009	0.008418	0.05647	0.02403	0.002941
Control-963 Hz	0.04825	0.005691	8.4786	0.02423	0.07228	0.02403	0.00005792

TABLE 5. Tukey HSD / Kramer Data. SE stands for Standard Error. CI stands for Critical Interval. The green values in the column “p-value” represent the experimental groups that were statistically significant compared to the control group.

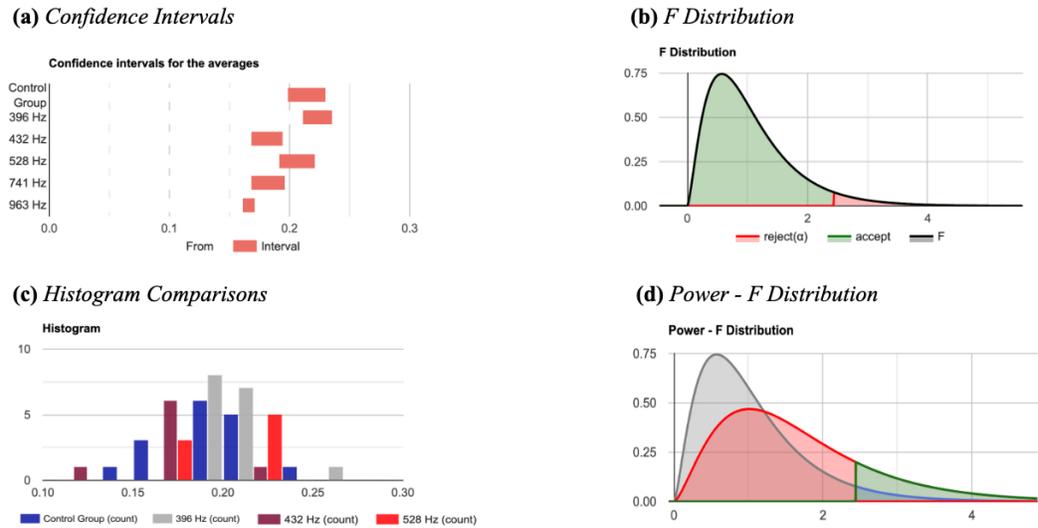


FIGURE 3. Visualization of the Data. (Analysis performed by <https://www.statskingdom.com/180Anova1way.html>). Graphs (a) to (d) demonstrate the statistical distributions of the data obtained in this study. Since $p\text{-value} < \alpha$, H_0 is rejected. In other words, the difference between the averages of some groups is big enough to be statistically significant. The observed effect size f is large (1.35). That indicates that the magnitude of the difference between the averages is large.

Comparison of the Effect of Solfeggio Frequencies on Biofilm Formation Optical Density Measurements

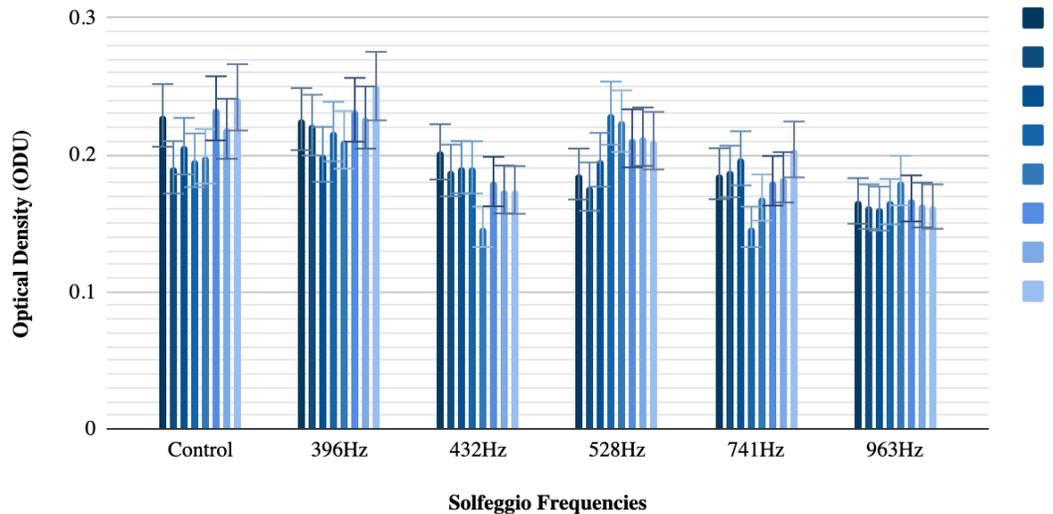


FIGURE 4. Graphical Comparison of the Effect of SF on BF

Comparison of Samples and Negative Control Groups

Ability of Biofilm Formation

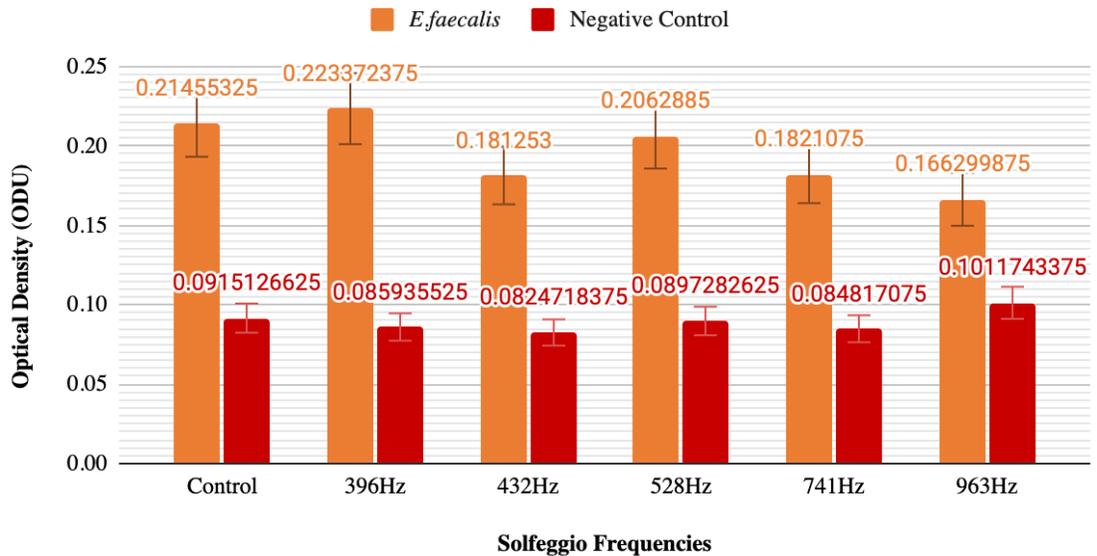


FIGURE 5. Graphical Comparison of Experimental Samples and Negative Control Groups

Overall, the results of the research are summarized in Figure 4 and Figure 5. Figure 4 visually demonstrates the OD values of all the samples containing *E. faecalis*. Coupled with ANOVA and Tukey statistical tests, it can be inferred that there is a statistically significant reduction in biofilm thickness in the samples exposed to 432, 741 and 963, where the most significant decline is seen at the samples exposed to 963 Hz. 396 Hz and 528 Hz, however, were found to have no statistically significant effect on the BF of *E. faecalis*. To accurately comment on BF of the bacteria, negative control groups were formed. Figure 5 displays the comparison of negative control samples that do not contain bacteria and samples that are exposed to SF. The fact that there is a statistical difference between samples and negative control values indicates the occurrence of BF in samples, thus, any differences in OD values can successfully be attributed to differences in biofilm thickness.

4. Discussion

This study aimed to explore the potential of solfeggio frequencies as modulators of quorum sensing-mediated biofilm formation in *Enterococcus faecalis*. The findings provide evidence that three specific frequencies (432 Hz, 741 Hz, and 963 Hz) significantly reduced BF, as observed through decreased OD values in spectrophotometric analysis. These results highlight the potential of SF as a novel, non-invasive approach to modulating bacterial behavior and combating biofilm-related infections. Given the increasing threat of antibiotic-resistant biofilms, alternative

methods of bacterial control are urgently needed. Thus, understanding how sound frequencies influence QS could open new avenues for antimicrobial strategies, particularly in addressing antibiotic-resistant biofilms. This section will further elaborate on the significance of these findings, contextualize them within existing literature, and propose directions for future research.

4.1. Prior Research

Although there is limited research in the effectiveness of SF on model organisms and their QS abilities, several experiments have been conducted utilizing a range of sound frequencies on microorganisms. Researchers from Liverpool John Moores University discovered that the exposure of *Pseudomonas aeruginosa* to vibration at 100, 800 and 1600 Hz for 48 hours caused a significant increase in BF, with the most significant growth seen at 800 Hz [25]. Another study focusing on marine ecosystems and the effect of ultrasound on microorganisms revealed that application of ultrasounds in membrane bioreactor for treating wastewater could be utilized as a potential reductive agent for biofouling formation [26]. Therefore, the diversity of the results provided by sound frequencies indicate the necessity of further research to evaluate the effects of specific frequencies that have not been applied on organisms in previous research since it could potentially inhibit QS.

4.2. Additional Considerations

A substantial mismatch among the results and the hypothesis was that the impact of frequencies is not necessarily linked to their energy levels (period lengths of the sound waves). Consequently, there is no consistency among groups that follow a decreasing or increasing trendline respectively as 396 Hz, 432 Hz, 528 Hz, 741 Hz and 963 Hz. This supports the notion that the effects of frequencies arranged according to their energy levels may exhibit inconsistency among each other, and specific frequencies could have varied effects on the QS mechanisms of microorganisms. These findings reinforce the complexity of bacterial communication systems and highlight the need for further research to identify the precise mechanisms underlying these effects.

4.3. Further Discussions

These findings shed light on the intricate dynamics of quorum sensing (QS), biofilm formation (BF), and their susceptibility to solfeggio frequencies (SF). However, further investigation is essential to unravel the molecular mechanisms underlying these effects, particularly in determining whether SF directly interferes with QS signal molecules, membrane dynamics, or gene regulation. Additionally, the potential therapeutic implications of these findings warrant careful exploration to assess their clinical and translational significance.

To further elucidate the precise effects of SF on *E. faecalis* and

QS-dependent BF, more advanced laboratory techniques should be employed. Methods such as genome sequencing, high-performance liquid chromatography (HPLC), bioluminescent assays, piezoelectric sensors, and fluorescence microscopy could provide deeper insights into the biochemical and genetic changes induced by SF [27]. While the crystal violet staining assay is effective in quantifying BF, it does not reveal the underlying molecular mechanisms at play. It is also important to acknowledge that this study focused specifically on *E. faecalis*, and the observed effects of SF may not be consistent across different bacterial species. Investigating a broader range of pathogens will be crucial in determining whether this phenomenon is species-specific or a more universal principle.

By demonstrating that certain SF can reduce BF, this study lays the foundation for future research into non-traditional antimicrobial approaches. These findings suggest that sound frequencies could serve as a novel, non-invasive strategy for disrupting QS, offering potential applications in combating biofilm-related infections."

Conflicts of Interest

The author declares that there are no conflicts of interest.

References

1. Greenberg EP. Bacterial communication: Tiny teamwork. *Nature* [Internet]. 2003 Jul [cited 2023 Jul 18];424(6945):134–4. Available from: <https://www.nature.com/articles/424134a>
2. Federle MJ, Bassler BL. Interspecies communication in bacteria. *Journal of Clinical Investigation* [Internet]. 2003 Nov 1 [cited 2023 Jul 24];112(9):1291–9. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC228483/>
3. ÖNEM E. Ulusal Tez Merkezi | Anasayfa [Internet]. tez.yok.gov.tr. [Süleyman Demirel Üniversitesi]; 2013 [cited 2023 May 21]. Available from: <https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=1GJZ95Jd b636UOBAAGgMNw&no=IHY55ec2njZ2LC2Ef1-X-A>
4. Habboush Y, Guzman N. Antibiotic Resistance [Internet]. PubMed. Treasure Island (FL): StatPearls Publishing; 2023 [cited 2023 Aug 6]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK513277/>
5. Bhardwaj AK, Vinothkumar K, Rajpara N. Bacterial quorum sensing inhibitors: attractive alternatives for control of infectious pathogens showing multiple drug resistance. *Recent Patents on Anti-Infective Drug Discovery* [Internet]. 2013 Apr 1 [cited 2023 Jul 30];8(1):68–83. Available from: [https://pubmed.ncbi.nlm.nih.gov/23394143/#:~:text=Quorum%20sensing%20\(QS\)%20is%20a](https://pubmed.ncbi.nlm.nih.gov/23394143/#:~:text=Quorum%20sensing%20(QS)%20is%20a)
6. Kalia VC. Quorum sensing inhibitors: An overview.

- Biotechnology Advances [Internet]. 2013 Mar [cited 2023 Jun 19];31(2):224–45. Available from:
<https://pubmed.ncbi.nlm.nih.gov/23142623/>
7. O’Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF, Bassler BL. A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proceedings of the National Academy of Sciences* [Internet]. 2013 Oct 18 [cited 2023 Aug 5];110(44):17981–6. Available from:
<https://www.pnas.org/doi/10.1073/pnas.1316981110>
 8. Norizan S, Yin WF, Chan KG. Caffeine as a Potential Quorum Sensing Inhibitor. *Sensors* [Internet]. 2013 Apr 18 [cited 2023 Aug 1];13(4):5117–29. Available from:
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3673129/>
 9. Talà A, Side D, Buccolieri G. Exposure to Static Magnetic Field Stimulates Quorum Sensing Circuit in Luminescent *Vibrio* Strains of the *Harveyi* Clade. *National Library of Medicine* [Internet]. 2014 Jun 24 [cited 2023 Aug 2]; Available from:
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4069165/>
 10. Oli AK, Javaregowda PK, Jain A, Kelmani CR. Mechanism Involved in Biofilm Formation of *Enterococcus faecalis*. *www.intechopen.com* [Internet]. 2022 Apr 29 [cited 2023 Aug 9]; Focus on Bacterial Biofilms. Available from:
<https://www.intechopen.com/chapters/81571>
 11. Kamimura R, Kanematsu H, Ogawa A, Kogo T, Miura H, Kawai R, et al. Quantitative Analyses of Biofilm by Using Crystal Violet Staining and Optical Reflection. *Materials* [Internet]. 2022 Sep 28 [cited 2023 Aug 9];15(19):6727. Available from:
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9571847/>
 12. Donlan RM. Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases* [Internet]. 2002 Sep [cited 2023 Aug 9];8(9):881–90. Available from:
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2732559/>
 13. Arunasri K, Mohan SV. Chapter 2.3 - Biofilms: Microbial Life on the Electrode Surface. Mohan SV, Varjani S, Pandey A, editors. *ScienceDirect* [Internet]. 2019 Jan 1 [cited 2023 Aug 9];295–313. Available from:
<https://www.sciencedirect.com/science/article/abs/pii/B978044464052900011X>
 14. Hashem YA, Amin HM, Essam TM, Yassin AS, Aziz RK. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. *Scientific Reports* [Internet]. 2017 Jul 18 [cited 2023 Aug 9];7(1). Available from:
<https://www.nature.com/articles/s41598-017-05901-0>
 15. Franziska Woitschach, Kloss M, Karsten Schlodder, Borck A, Grabow N, Reisinger EC, et al. Bacterial Adhesion and Biofilm Formation of *Enterococcus faecalis* on Zwitterionic

- Methylmethacrylat and Polysulfones. *Frontiers in Cellular and Infection Microbiology* [Internet]. 2022 May 16 [cited 2023 Aug 9];12. Available from:
<https://www.frontiersin.org/articles/10.3389/fcimb.2022.868338/full>
16. Arias C, Murray B. Enterococcus - an overview | ScienceDirect Topics. In: *wwwsciencedirectcom* [Internet]. Science Direct; 2015 [cited 2023 Aug 9]. Available from:
<https://www.sciencedirect.com/topics/medicine-and-dentistry/enterococcus>
 17. Guerreiro-Tanomaru JM, de Faria-Júnior NB, Duarte MAH, Ordinola-Zapata R, Graeff MSZ, Tanomaru-Filho M. Comparative Analysis of Enterococcus faecalis Biofilm Formation on Different Substrates. *Journal of Endodontics* [Internet]. 2013 Mar [cited 2023 Aug 9];39(3):346–50. Available from:
<https://www.sciencedirect.com/science/article/abs/pii/S0099239912011594>
 18. Li Y, Pan J, Wu D, Tian Y, Zhang J, Fang J. Regulation of Enterococcus faecalis Biofilm Formation and Quorum Sensing Related Virulence Factors with Ultra-low Dose Reactive Species Produced by Plasma Activated Water. *Plasma Chemistry and Plasma Processing* [Internet]. 2018 Oct 29 [cited 2023 Aug 9];39(1):35–49. Available from:
<https://link.springer.com/article/10.1007/s11090-018-9930-2>
 19. Haas W, Shepard BD, Gilmore MS. Two-component regulator of Enterococcus faecalis cytolysin responds to quorum-sensing autoinduction. *Nature* [Internet]. 2002 Jan [cited 2020 Nov 11];415(6867):84–7. Available from:
<https://pubmed.ncbi.nlm.nih.gov/11780122/>
 20. Pinkston KL, Gao P, Diaz-Garcia D, Sillanpää J, Nallapareddy SR, Murray BE, et al. The Fsr Quorum-Sensing System of Enterococcus faecalis Modulates Surface Display of the Collagen-Binding MSCRAMM Ace through Regulation of gelE. *Journal of Bacteriology* [Internet]. 2011 Sep [cited 2023 Aug 9];193(17):4317–25. Available from:
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3165527/#:~:text=The%20Fsr%20quorum%2Dsensing%20system%20of%20E>
 21. Piasek A. Sound [Internet]. McGraw Hill's AccessScience. 2020 [cited 2023 Aug 9]. Available from:
<https://www.accessscience.com/content/article/a637200>
 22. Sarvaiya N, Kothari V. Effect of audible sound in form of music on microbial growth and production of certain important metabolites. *Microbiology* [Internet]. 2015 Mar [cited 2023 Aug 7];84(2):227–35. Available from:
<https://link.springer.com/article/10.1134/s0026261715020125>
 23. dos Santos AC, de Abreu MS, de Mello GP, Costella V, do Amaral

- NR, Zanella A, et al. Solfeggio-frequency music exposure reverses cognitive and endocrine deficits evoked by a 24-h light exposure in adult zebrafish. *Behavioural Brain Research* [Internet]. 2023 Jul 26 [cited 2023 Aug 5];450:114461. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S016643282300179>
24. STEPANOVIĆ S, VUKOVIĆ D, HOLA V, BONAVENTURA GD, DJUKIĆ S, ĆIRKOVIĆ I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* [Internet]. 2007 Aug [cited 2023 Jul 28];115(8):891–9. Available from: <https://pubmed.ncbi.nlm.nih.gov/17696944/>
25. Murphy MF, Edwards T, Hobbs G, Shepherd J, Bezombes F. Acoustic vibration can enhance bacterial biofilm formation. *Journal of Bioscience and Bioengineering* [Internet]. 2016 Dec [cited 2023 Jul 25];122(6):765–70. Available from: <https://pubmed.ncbi.nlm.nih.gov/27338651/>
26. Rodríguez-Calvo A, Gonzalez-Lopez J, Ruiz LM, Gómez-Nieto MÁ, Muñoz-Palazon B. Effect of ultrasonic frequency on the bacterial community structure during biofouling formation in microfiltration membrane bioreactors for wastewater treatment. *International Biodeterioration & Biodegradation* [Internet]. 2020 Nov [cited 2023 May 16];155:105102. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0964830520310337>
27. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. *The Brazilian Journal of Infectious Diseases: An Official Publication of the Brazilian Society of Infectious Diseases* [Internet]. 2011 Jul 1 [cited 2023 Jul 6];15(4):305–11. Available from: <https://pubmed.ncbi.nlm.nih.gov/21860999/>