ISSN: 2634 - 8861

Journal of Infectious Diseases & Case Reports

SCIENTIFIC Research and Community

Research Article

Open Access

Ethanol Induces a Persister Cell Phenotype in E. Coli and S. Epidermidis

Matthew Wagner[1], Kaden Bentley[1], Corey Philpot[1], Cole Farnsworth[1], Mark Stoll[1] and Victor M Jimenez Jr[1,2*]

[1]Department of Biomedical Sciences, Noorda College of Osteopathic Medicine 2162 S 180 E, Provo UT 84606, USA

[2]Department of Pharmacy, Roseman University of Health Sciences, 10920 S River Front Pkwy, South Jordan, Utah 84095, USA

ABSTRACT

Persister cells are a subpopulation of bacteria capable of surviving high doses of antibiotics and various stressors, contributing to chronic infections and antibiotic resistance. Ethanol exposure is known to induce significant morphological and physiological changes, such as alterations in fatty acid composition, ion leakage, and increased membrane fluidity, which may promote biofilm formation. In the current study, Escherichia coli and Staphylococcus epidermidis were cultured in LB broth supplemented with varying concentrations of ethanol (0 – 10%). Concurrently, growth curves were recorded, and membrane potential was assessed using flow cytometry to determine the impact of ethanol stress on cellular membrane alterations. Following growth, cells were centrifuged to remove ethanol and resuspended in fresh LB broth for an additional 18-hour static incubation to promote biofilm formation. Biofilms were subsequently fixed, stained, washed, and solubilized in acetic acid for quantification via OD530 measurements. The results demonstrated that bacteria maintain growth under high ethanol stress while exhibiting a significant reduction in membrane potential, consistent with the induction of a dormant persister phenotype. Moreover, ethanol exposure markedly enhanced biofilm formation, particularly in S. epidermidis, with robust biofilm production observed even at ethanol concentrations as high as 10%. In conclusion, these findings provide a comprehensive and quantitative analysis of bacterial responses to ethanol-induced stress, linking decreased membrane potential and enhanced biofilm formation to persister cell development. This work deepens our understanding of the mechanisms underlying bacterial persistence and may inform the design of novel therapeutic strategies to combat chronic infections and curb the emergence of antibiotic resistance.

***Corresponding author**

Victor M Jimenez Jr, Department of Biomedical Sciences, Noorda College of Osteopathic Medicine 2162 S 180 E, Provo UT 84606, USA.

Received: March 28, 2025; **Accepted:** April 07, 2025; **Published:** April 12, 2025

Keywords: Persister Cells, Alcohol, Ethanol, Escherichia Coli, Staphylococcus Epidermidis, Biofilm

Introduction and Discussion

In the late 1920s Alexander Fleming discovered the uses of penicillin. Since then, the continual discoveries of antibiotics have been the most important discoveries of medical science [1]. Despite the notable success in the field of infectious diseases, the Centers for Disease Control (CDC) states that more than 23,000 Americans die annually because of antibiotic-resistant bacteria, with the rate of morbidity and mortality rising globably [2]. Many pathogens colonize their host, evade the immune system and persist over time due to antibiotic resistance and tolerance. Although several bacterial survival mechanisms are well described, the formation of phenotypic divergent persister cells is not well understood [3]. A persister cell is an individual bacterium that survives exposure to a toxin that kills much of the existing population [4]. Likewise, the term persistence is used to describe a small subpopulation of bacterial cells that are transiently tolerant to a lethal activity of toxins [5]. Bacteria can evade modern medical treatments by many factors relating to the patient and the drug-specific evasion [5]. However, to better understand the development of persister cells, it is important to distinguish between resistance and tolerance. Antibiotic resistance indicates a resistance or inhibition from antibiotics and antibiotic tolerance denotes an ability to survive the duration of an antibiotic treatment. While antibiotic tolerance describes slow killing of the bacterial population, persistence refers to a small fraction of the population that is tolerant. In recent years, evidence has become increasingly supportive of persister cells being involved in the relapse of infection. For instance, uropathic *E. coli* (UPEC) was sequenced from a set of patients that were treated with antibiotics for urinary tract infections (UTI). It was found that the persister cells were genetically identical to the original strain indicating antibiotic tolerance and persistence [6]. Similarly, these persistent phenotypes have been shown with other microbes such as *Salmonella and S. pyogenes* [7,8]. The molecular mechanisms underlying persister cell formation and maintenance under stress cues are complex and not yet fully understood.

The current study aimed to evaluate whether ethanol, as an environmental stressor, induces the formation of phenotypically distinct persister cells in *Escherichia* coli and *Staphylococcus epidermidis*. First, both bacterial species were exposed to a range of ethanol concentrations and culture growth was monitored by measuring optical density (OD600). As shown in Figure 1, growth was observed in all ethanol-supplemented media in a dose-dependent manner, whereas no growth occurred in the presence of bleach, confirming that the ethanol treatments imposed a dose-specific stress without causing outright cell death. Recent

studies have highlighted the importance of specific signaling pathways and regulatory factors. For example, an alarmone is a small intracellular signaling molecule that can facilitate persister cell formation in *Escherichia coli* and other bacteria [9,10]. The global transcriptional regulator HipA has been implicated in the maintenance of persister cells in *Mycobacterium tuberculosis* [11,12]. Other factors that have been linked to persister cell formation and survival include stress response systems, such as the toxin-antitoxin molecules, and the bacterial cell envelope [13]. In addition to molecular mechanisms, bacterial metabolic modulation is critical for persistence in stressful environments. Persister cells are metabolically dormant which improves survival from most bacteriostatic antibiotic regimens [4,5,14]. The mechanism of metabolic dormancy is not fully understood; however, toxin-antitoxin (TA) molecules are linked to metabolism and persister cell formation [15]. Toxin-antitoxin (TA) molecules encode a toxin that can inhibit metabolic processes that decrease the proton motive force and ATP levels, thus improving survival in harsh environments. After the stressor is removed, an antitoxin is able to neutralize the activity of the toxin and the cell suspends dormancy and reinstates normal growth strategies [16]. Similar TA systems implicated in persister cell formation have been identified in *M. tuberculosis* and *Staphylococcus* aureus that inhibit a broad range of cellular processes, including translation, DNA metabolism, and the proton moving force [17-25].

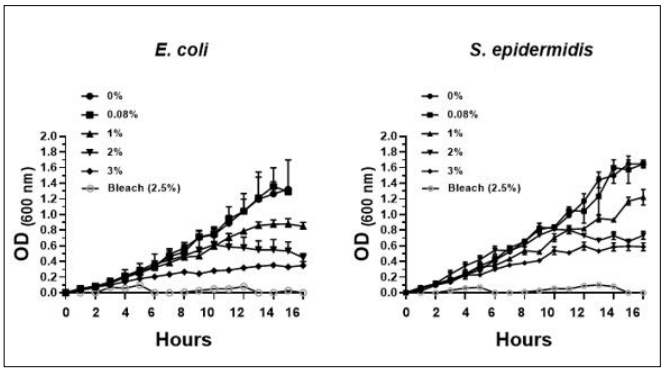

Figure 1: *E. coli* (left) and *S. epidermidis* (right) growth for 16 hours. Growth was measured at OD600 every hour in the same growth conditions and the media supplemented with various concentrations of ethanol (0.0%, 0.08%, 1%, 2%, 3%) or bleach (2.5%). Optimal growth was reached at 0% ethanol. Low ethanol concentrations (0.08%) were not different from control. A dose-dependent decrease in growth was observed in bacteria grown in media supplemented with >1% ethanol

Given that growth persisted at all ethanol levels, further analyses were conducted using flow cytometry to determine whether the surviving cells exhibited characteristics of persister cells rather than nonspecific physiological adaptations. Persister cells are documented as a dormant subpopulation with reduced metabolic activity, low ATP levels, and diminished membrane potential, which contribute to their tolerance persister characteristics. *E. coli* expresses a membrane potential near (-)220mV in the early exponential phase, stabilizing near (-)140mV during late phase growth. Because the membrane potential of gram-negative bacterial is not primarily driven by the H+ gradient, the membrane potential of *S. epidermidis* alone was tested. Whereas *S. epidermidis* and other similar gram-positive bacteria have a membrane potential of smaller magnitude that is dominated by intracellular pH, and the H+ ion and the internal control DiOC2 moves proportionally to the H+ ion gradient. In the current study, 3% ethanol induced a moderate growth restriction which translated to a significant drop in membrane potential. By 4% ethanol, both growth and membrane potential were significantly affected. The membrane potential is affected by ethanol at concentrations as low as 0.5%. Furthermore, membrane potential of S. epidermidis was decreased by alcohol in a dose-dependent manner. Similarly, using JC1, researchers were able to induce persister cell by measuring fluorescently labeled membrane potential, where persister cells of different bacterial genus have a reduction of their membrane potential [24,26,27].

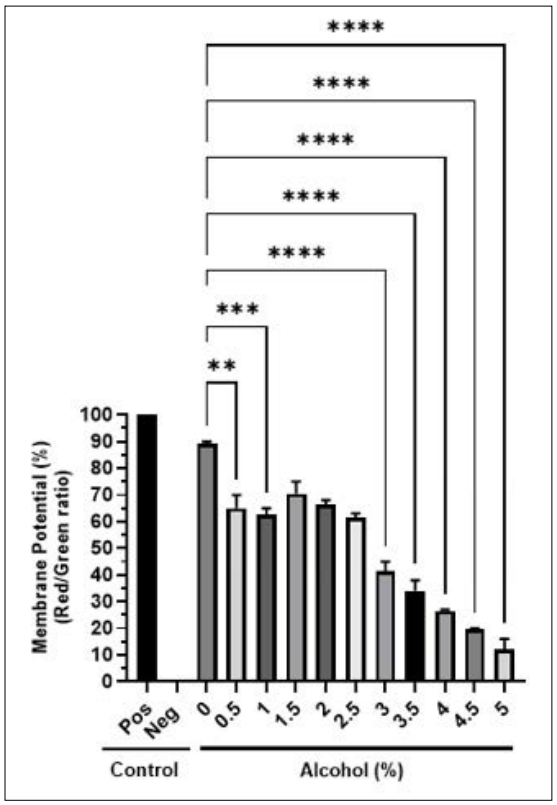

Figure 2: S. epidermidis membrane potential. Bacterial cells grown in media supplemented in various concentration of ethanol (0 – 5%) were fluorescently labeled and membrane potential measured via flow cytometry – red to greed ratio (%). Positive control (Pos) represents CCCP is a protonionophore which embeds itself into cellular membranes effectively depolarizing H+ membrane potentials. Negative control (Neg) represents cells not labeled. Bars represent two independent experiments and data are expressed as means ± SEM. Statistically significant results by one-way ANOVA indicated by asterisks **p<0.01, ***p < 0.001, ****p<0.0001

Bacterial stress responses can activate the formation of persister cells. These cells frequently participate in biofilm formation by contributing to the extracellular matrix, thereby enhancing community-level survival under hostile conditions. In the current study, biofilms were grown, fixed, stained, and solubilized, and their OD was measured (Figure 3). Both species exhibited increased biofilm formation between 3% and 5% ethanol, with biofilm production still evident at the highest ethanol concentrations tested. Biofilm offers distinct advantages, including enhanced protection from the host immune system and reduced susceptibility to antibiotics. These protective effects are primarily attributed to the dense and intricate structure of the biofilm matrix, which acts as a physical barrier against antimicrobial agents [28,29]. Moreover, biofilms facilitate intercellular communication through quorum

sensing, enabling coordinated physiological responses among bacterial cells [30]. The findings in the current study suggest that persister cells actively contribute to biofilm development by participating in the formation of the extracellular matrix and promoting community interactions. The divergent persister cell phenotype ensures the survival of the biofilm, even when a majority of the bacterial population is eradicated [31]. Elucidating the underlying mechanisms governing persister cell formation and their role in biofilm growth is an area of intense research. However, recent studies have revealed various factors influencing persister cell formation, including genetic regulation, nutrient availability, stress responses, and the presence of quorum-sensing molecules [30,32]. Additionally, the emergence of persister cells can be influenced by stochastic processes, leading to phenotypic heterogeneity within bacterial populations [33,34]. Novel imaging techniques and single-cell analysis have provided valuable insights into the dynamics of persister cells within biofilms.

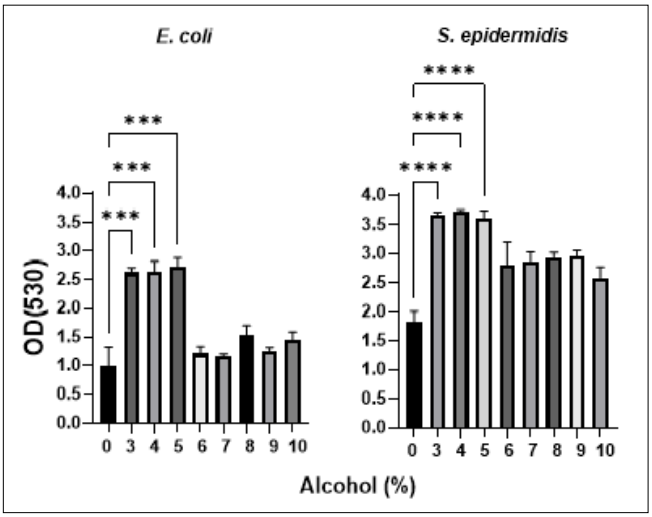

Figure 3: *E. coli* (left) and *S. epidermidis* (right) biofilm growth. Bacteria grown in media supplemented with various concentrations of ethanol (0-10%), followed by removal of ethanol and static biofilms grown. Biofilms were fixed, processed, and measured at OD530. Bars represent two independent experiments and data are expressed as means ± SEM. Statistically significant results by one-way ANOVA indicated by asterisks ***$p < 0.001$, ****$p<0.0001$

Overall, the objective of the current study was to establish an optimal set of conditions for testing ethanol-induced persister cells for future research. It is crucial to underscore the broader implications of ethanol-induced persister cell formation. In clinical settings such as hospitals and surgical centers, ethanol is a ubiquitous disinfectant used for surface sanitation and instrument sterilization. However, sublethal exposure—common in scenarios where ethanol rapidly evaporates or is diluted by organic matter—may inadvertently trigger stress responses in bacteria that favor the formation of persister cells. These dormant cells are notably tolerant to antibiotics and standard sterilization protocols, which poses a significant challenge for infection control and may contribute to the persistence and recurrence of nosocomial infections. This study not only provides insights into the molecular basis of ethanol-induced persistence in E. coli and S. epidermidis but also emphasizes the need for improved sterilization procedures that can effectively eliminate these resilient subpopulations.

Furthermore, ethanol-induced persister cell formation is critical for understanding how hazardous alcohol consumption may contribute to the emergence of more resilient, biofilm-forming, and antibiotic-tolerant persistent bacteria. Sustained high blood alcohol concentrations can chronically expose the normal flora—particularly in the gastrointestinal, respiratory, and oral cavities—to sublethal ethanol stress. Such exposure may drive normal commensal bacteria to adapt by forming persister cells, which are not only more tolerant to antibiotics but also display an enhanced capacity for biofilm formation and distinct physiological changes. Altered gut microbiota in chronic alcoholics has been linked to increased intestinal permeability, dysbiosis, and systemic inflammation, thereby contributing to a higher risk of infections and reduced effectiveness of conventional antimicrobial therapies. These ethanol-induced persister cells can persist undetected by the host immune system and may serve as reservoirs for recurrent infections or even transform into more virulent forms over time.

Conclusion

The current study identified the ethanol range of 0.5% to 2.5% as the initiating concentration for an emerging persister cell phenotype, the range of 3%-5% ethanol as inducing a divergent persister cell phenotype, and concentrations above 5% ethanol as a near to full bactericidal level for most bacteria with tolerance levels increased for some bacteria that resulted in biofilm formation. Additionally, the differences seen between *E. coli* and *S. epidermidis* demonstrate that these levels are affected by the physiological processes unique to each Genus and species of bacteria, namely: tendency to grow biofilm, extracellular matrix structure, ions used to maintain the membrane potential, and likely many others [35-37]. Testing ethanol-induced persister cells is vital not only for advancing our understanding of bacterial stress responses and persistence mechanisms but also for informing the development of improved sanitation protocols in hospitals and surgical centers, as well as guiding both public health strategies aimed at reducing alcohol-related microbial dysbiosis and the development of targeted therapeutic approaches to manage infections in chronic alcohol users.

Limitations and Future Directions

This study has proven useful for developing a protocol for ethanol-induced persister cell formation and identifying concentrations of ethanol that will induce said phenotype. Future studies should explore different sets of gram-positive and gram-negative bacteria beyond *E. coli* and *S. epidermidis*. Furthermore, studies should include various antibiotics and mechanisms of action. Phenotypic changes to persister cells have been noted and studied, yet the lack of microscopic visualization techniques for biofilm and planktonic cells limits the scope of the study.

Materials and Methods

Bacterial Growth Conditions and Growth Curves

To determine the effects of ethyl alcohol (EtOH) on bacterial growth patterns under standard laboratory conditions, the concentration of ethanol was varied by supplementing Luria-Bertani (LB) broth media solution (LB Broth Base, Lennox Cat. 12,780,052) to various concentrations (0-10% v/v). To each test tube, sterile LB broth media was added to the corresponding percentage ethanol. Total volume was adjusted to 30 mL. *E. coli* (ATCC W1485) and *S. epidermidis* (ATCC NCTC 11047, R. Hugh 2466) were inoculated equally in separate sterile 50mL test tubes (Nunc Conical Sterile Polypropylene Centrifuge tubes, Cat. 339653) using a sterile loop (10 µL or one CFU). The tubes were then placed in a shaking incubator (MaxQ 6000 Incubated/ Refrigerated Shaker, Cat. SHKE6000) at 37°C and left for 24 hours. For bacterial growth curves, the optical density (OD 600)

measurements were taken in 1-hour increments in triplicate via spectrophotometer (Barnstead International Turner SP-830, SM110215). Each bacterial strain was measured hourly until the plateau phase was determined by optical density.

Membrane Potential Detection

E. coli and *S. epidermidis* grown in LB media supplemented with various concentrations of ethanol as previously described. Membrane potential was measured using the ThermoFisher Attune NxT Blue Red Laser Flow Cytometer and the ThermoFisher Invitrogen BacLight™ Bacterial Membrane Potential Kit (catalog: B34950). Manufacturer guidelines were followed with the exception that the kit called for 1mL of isolate at a concentration of 1x106 per mL to be measured with 10uL of dye. Instead 500uL of isolate was measured using 5uL of dye. Positive controls were made using CCCP. Cells were stained by DiOC2, and fluorescent intensity was measured. Green was collected on the FITC channel and red was collected on the PE channel. The primary readout was red and green intensity which was then compared ratiometrically to indicate the relative magnitude of the membrane potential compared to the positive control.

Biofilm Formation and Detection

E. coli and *S. epidermidis* grown in LB media supplemented with various concentrations of ethanol as previously described. Following ethanol treatment, bacteria were centrifuged at 500G using an Allegra C – 30R Centrifuge, supernatant removed, and bacterial pellet resuspended in ethanol-free LB broth. 100 µl of each bacterium was added to a 24 well plate (Thermo Scientific Nunc Non-Treated Multi-dishes) in a 1 to 10 dilution with LB growth media. Plates were placed in a static incubator at 37◦C overnight. Following biofilm formation, each well was fixed by removing the excess solution and adding 98.5% methanol for 30 minutes. The methanol was removed, and the plate was allowed to air dry for 15 minutes. To stain the biofilm, 0.1% safranin was added and incubated for 30 minutes. Excess solution was removed then 2 rinses of phosphate buffer were performed with a 10-minute air dry between each rinse to remove any nonadherent bacteria. The biofilm was solubilized by adding 30% acetic acid and incubated for 30 minutes. Plates were then read using a BioTek synergy 2 plate reader at OD530

Author Contributions

Matthew Wagner: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. Kaden Bentley: Methodology, Investigation, Writing – review & editing. Corey Philpot: Methodology, Investigation, Writing – review & editing. Cole Farnsworth: Methodology, Investigation, Writing – review & editing. Mart Stoll: Methodology, Investigation, Supervision, Writing – review & editing. Victor M. Jimenez Jr: Writing – review & editing, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding Statement

The following study was supported by The Noorda College of Osteopathic Medicine under Award Number [NRDA2023IA-01].

Acknowledgments

Authors have no financial interests, or conflicts of interest to disclose. A special thank you to Roseman University and Noorda-COM for funding this study and providing laboratory facilities and non-material support.

References

1. Adedeji WA (2016) The Treasure Called Antibiotics. Ann Ib Postgrad Med 14: 56-57.
2. Antimicrobial Resistance Collaborators (2022) Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 399: 629-655.
3. Van den Bergh B. Fauvart M, Michiels J (2017) Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 41: 219-251.
4. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, et al. (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17: 441-448.
5. Fisher RA, Gollan B, Helaine S (2017) Persistent bacterial infections and persister cells. Nat Rev Microbiol 15: 453-464.
6. Russo TA, Stapleton A, Wenderoth S, Hooton TM, Stamm WE (1995) Chromosomal restriction fragment length polymorphism analysis of Escherichia coli strains causing recurrent urinary tract infections in young women. J Infect Dis 172: 440-445.
7. Okoro CK, Kingsley RA, Quail MA, Kankwatira AM, Feasey NA, et al. (2012) High-resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive nontyphoidal Salmonella typhimurium disease. Clin Infect Dis 54: 955-963.
8. Bingen E, Denamur E, Lambert-Zechovsky N, Braimi N, el Lakany M, et al. (1992) DNA restriction fragment length polymorphism differentiates recurrence from relapse in treatment failures of Streptococcus pyogenes pharyngitis. J Med Microbiol 37: 162-164.
9. Korch SB, Hill TM (2006) Ectopic overexpression of wild-type and mutant hipA genes in Escherichia coli: effects on macromolecular synthesis and persister formation. J Bacteriol 188: 3826-3836.
10. Amato SM, Brynildsen MP (2014) Nutrient transitions are a source of persisters in Escherichia coli biofilms. PLoS One 9: e93110.
11. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230: 13-18.
12. Dahl JL, Kraus CN, Boshoff HI, Doan B, Foley K, et al. (2003) The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of Mycobacterium tuberculosis in mice. Proc Natl Acad Sci USA 100: 10026-10031.
13. Dörr T, Lewis K, Vulić M (2009) SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet 5: e1000760.
14. Gollan B, Grabe G, Michaux C, Helaine S (2019) Bacterial Persisters and Infection: Past, Present, and Progressing. Annu Rev Microbiol 73: 359-385.
15. Lewis K (2010) Persister cells. Annu Rev Microbiol 64: 357-372.
16. Gerdes K, Christensen SK, Løbner-Olesen A (2005) Prokaryotic toxin-antitoxin stress response loci. Nat Rev Microbiol 3: 371-382.
17. Slayden RA, Dawson CC, Cummings JE (2018) Toxin-antitoxin systems and regulatory mechanisms in Mycobacterium tuberculosis. Pathog Dis 76.
18. Ma D, Mandell JB, Donegan NP, Cheung AL, Ma W, et al. (2019) The Toxin-Antitoxin MazEF Drives Staphylococcus aureus Biofilm Formation, Antibiotic Tolerance, and Chronic Infection. mBio 10: 01658-19
19. Germain E, Castro-Roa D, Zenkin N, Gerdes K (2013) Molecular mechanism of bacterial persistence by HipA. Mol Cell 52: 248-254.

20. Pedersen K, Zavialov AV, Pavlov MY, Elf J, Gerdes K, et al. (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell 112: 131-140.
21. Castro-Roa D, Garcia-Pino A, De Gieter S, van Nuland NAJ, Loris R, et al. (2013) The Fic protein Doc uses an inverted substrate to phosphorylate and inactivate EF-Tu. Nat Chem Biol 9: 811-817.
22. Bernard P, Kézdy KE, Van Melderen L, Steyaert J, Wyns L, et al. (1993) The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. J Mol Biol 234: 534-541.
23. Harms A, Stanger FV, Scheu PD, de Jong IG, Goepfert A, et al. (2015) Adenylylation of Gyrase and Topo IV by FicT Toxins Disrupts Bacterial DNA Topology. Cell Rep 12: 1497-1507.
24. Verstraeten N, Knapen WJ, Kint CI, Liebens V, Van den Bergh B, et al. (2015) Obg and Membrane Depolarization Are Part of a Microbial Bet-Hedging Strategy that Leads to Antibiotic Tolerance. Mol Cell 59: 9-21.
25. Dörr T, Vulić M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol 8: e1000317.
26. 26. Wang T, El Meouche I, Dunlop MJ (2017) Bacterial persistence induced by salicylate via reactive oxygen species. Sci Rep 7: 43839.
27. Roy S, Bahar AA, Gu H, Nangia S, Sauer K, et al. (2021) Persister control by leveraging dormancy associated reduction of antibiotic efflux. PLoS Pathog 17: e1010144.
28. Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358: 135-138.
29. Zhang Y (2014) Persisters, persistent infections and the Yin-Yang model. Emerg Microbes Infect 3: e3.
30. PredaVG, Săndulescu O (2019) Communication is the key: biofilms, quorum sensing, formation and prevention. Discoveries (Craiova) 7: e100.
31. Spoering AL, Lewis K (2001) Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol 183: 6746-6751.
32. de la Fuente-Núñez C, Reffuveille F, Fernández L, Hancock RE (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr Opin Microbiol 16: 580-589.
33. Manuse S, Shan Y, Canas-Duarte SJ, Bakshi S, Sun WS, et al. (2021) Bacterial persisters are a stochastically formed subpopulation of low-energy cells. PLoS Biol 19: e3001194.
34. Fox EP, Cowley ES, Nobile CJ, Hartooni N, Newman DK, et al. (2014) Anaerobic bacteria grow within Candida albicans biofilms and induce biofilm formation in suspension cultures. Curr Biol 24: 2411-2416.
35. Redelman CV, Madapolam C, Anderson GG (2012) Alcohol treatment enhances Staphylococcus aureus biofilm development. FEMS Immunology & Medical Microbiology 66: 411-418.
36. Luther MK, Bilida S, Mermel LA, LaPlante KL (2015) Ethanol and Isopropyl Alcohol Exposure Increases Biofilm Formation in Staphylococcus aureus and Staphylococcus epidermidis. Infect Dis Ther 4: 219-226.
37. Vani S, Vadakkan K, Mani B (2023) A narrative review on bacterial biofilm: its formation, clinical aspects and inhibition strategies. Future Journal of Pharmaceutical Sciences 9: 50.