

Gravitational and Space Research

Validation of Gentamicin and Streptomycin Stability for On-Orbit Microbial Monitoring

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Abstract

*The Genomic Enumeration of Antibiotic Resistance in Space (GEARS) flight project seeks to characterize the frequency and genomic identity of antibiotic-resistant organisms on the ISS and expand in-space sequencing-based diagnostic capabilities. This project selects for antibiotic-resistant microbes on space station surfaces using agar plates containing antibiotics, which may require storing the plates for several months. However, little published data is available on the longevity of antibiotics in agar plates. Here, we describe the process for selecting and validating antibiotic stability for GEARS. A literature review was conducted on several antibiotics for their intrinsic and acquired resistance in *Enterococcus* and *Staphylococcus* and stability in solution and in agar. Gentamicin and streptomycin were selected for a long-term study tracking changes in the bactericidal ability of the antibiotics in agar plates. After 6.5 months of storage at 4 °C, we found that both antibiotic types remained stable throughout the test period, although they both exhibited evidence of slight degradation. These findings indicated that either antibiotic would be sufficient for the GEARS study based on stability under cold storage conditions. Furthermore, this study generated a methodology for the study of and information on the long-term stability of antibiotics in agar, which can be useful for microbiology applications beyond spaceflight studies.*

Keywords

microbiology • antibiotic resistance • microbial monitoring

Introduction

Antibiotic resistance has existed for at least tens of thousands of years (D'Costa et al., 2011; Perron et al., 2015; Perry et al., 2016), and potentially millions of years or more (Barlow and Hall, 2002; Bhullar et al., 2012; Torres et al., 2025), and has emerged as a major health risk over the past century. Following the discovery of penicillin in 1928, antibiotics were rapidly and widely harnessed for human and veterinary medicine, livestock, agriculture, and biotechnology. This has revolutionized modern medicine and fundamentally impacted livelihood on Earth, but has also driven the extensive proliferation of antibiotic resistance. Today, antimicrobial resistance, which includes antibiotic resistance, is identified by the World Health Organization, Centers for Disease Control and Prevention, and the World Medical Association as one of the most prevalent contemporary public health issues (CDC, 2024; WHO, 2019, 2023; WMA, 2022).

Antibiotic-resistant microbes are widespread across outdoor and built environments (Bengtsson-Palme et al., 2017; Larsson and Flach, 2022; Mahnert et al., 2019), and areas that are enclosed and frequently cleaned can have higher resistance prevalence (Mora et al., 2016). Culture-based methods are commonplace in medicine, agriculture, livestock, and wastewater monitoring, enabling rapid, high-

throughput screening for antibiotic-resistant microbes (Caruso, 2018; Haulisah et al., 2021; Kuper et al., 2009; Lemonnier et al., 2023; Manaia et al., 2018; McLain et al., 2016; Reinthaler et al., 2003; Smaill, 2000; Tomida et al., 2013).

Although agar plates containing antibiotics can be used for such methods, little data exist on the long-term stability of antibiotics in agar (over weeks, months, or years). Lyophilized antibiotics are generally stable for months to years, but degrade quickly once reconstituted in water. This is generally not an issue for laboratory settings, as media can be prepared and inoculated with antibiotics as needed. However, certain situations (i.e., remote medicine, spaceflight) require longer-term storage of antibiotics in agar plates if real-time preparation of plates is not feasible, and specific data on the stability of various antibiotics is critical for planning culture-based screening in remote environments.

Over the past few decades of crewed space exploration, the effects of spaceflight conditions on microbes and their potential implications for crew health have become increasingly apparent. In space, microbes can demonstrate increased growth, virulence, pathogenicity, and biofilm formation, as well as changes in metabolism (Huang et al., 2018; Kacena et al., 1999; Kim et al., 2013; Landry et al., 2020; Ott et al., 2020; Rosenzweig et

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al., 2010; Sharma and Curtis, 2022; Vaishampayan and Grohmann, 2019; Wilson et al., 2007). The unique conditions of spaceflight can likewise exacerbate antibiotic resistance in microbes. This has been characterized in simulated-microgravity experiments on the ground and in culture-based experiments in space (Aunins et al., 2018; Moatti et al., 1986; Tirumalai et al., 2019; Tixador et al., 1985; Urbaniak et al., 2022)

Spaceflight can suppress astronaut immune systems (Sonnenfeld, 1998; Stratis et al., 2023), which increases the risks imposed by these microbial responses to spaceflight. Microbial monitoring is routinely conducted to survey the genomic and functional composition of crewed spacecraft (Yamaguchi et al., 2014). Such monitoring aboard the International Space Station (ISS) has indicated that antibiotic-resistant microbes are present on surfaces proximal to crew members, and may pose risks to astronaut health (Bryan et al., 2021; Urbaniak et al., 2022). Bryan et al. specifically investigated *Enterococcus faecalis* isolates recovered from the ISS, showing that antibiotic-resistant *E. faecalis* exists on-station with potential pathogenicity, and indicating the need to further assess the abundance of antibiotic-resistant microbes on-station.

Genomic Enumeration of Antibiotic Resistance in Space (GEARS) is a NASA-funded spaceflight mission aimed at characterizing the frequency and genomic identity of antibiotic-resistant microbes on the ISS. This study focuses on enterococci due to their status as the second-leading cause of hospital-acquired bacteremia (Dubin and Pamer, 2017; Hidron et al., 2008; Wisplinghoff et al., 2004) and their presence aboard the ISS (Bryan et al., 2021). This project will use contact slides filled with antibiotic-spiked agar to screen for antibiotic-resistant microbes on internal surfaces of the ISS and will leverage in-space nanopore sequencing instrumentation to identify microbes on orbit. The GEARS study is one of three complementary investigations aimed at better understanding the adaptation and antibiotic resistance of enterococci in the spaceflight environment. The other two investigations, Enterococcus Growth Advantage Investigation via Tn-seq (EnteroGAIT) and Adaptation and Evolution of Resilient Enterococcus in Space (AERES), are concurrently underway.

To prepare for the GEARS study, it was necessary to first determine which antibiotics would be most useful to generally screen for *Enterococcus*, as the main genus of interest for this project, as well as *Staphylococcus*, another genus with high propensity for antibiotic resistance and human pathogenicity. Next, it was necessary to determine the “shelf life” of these antibiotics once reconstituted in agar. For GEARS, it was important to determine how long antibiotic stability could be maintained to plan mission logistics and prevent significant variability due to loss of antibiotic potency. This manuscript

describes the process of antibiotic selection and the validation of antibiotic stability and reports results on the stability of gentamicin and streptomycin over a 6.5-month period.

Materials and Methods

Antibiotic Selection

A literature review was conducted to identify antibiotics for further study, focusing on two main mechanisms of antibiotic impact related to cell wall biosynthesis (beta-lactams, carbapenems, penicillin-like antibiotics) and ribosome synthesis (aminoglycosides, lincomycin, macrolides, tetracyclines) and associated resistance mechanisms observed in *E. faecalis* (Arias and Murray, 2012). Data on intrinsic and acquired resistance in *Enterococcus* and *Staphylococcus*, and on long-term (several weeks) stability in solution and on agar plates at 4 °C, were aggregated from peer-reviewed literature, vendor information, and medical documentation. Antibiotic recommendations for use in the experimental portion of this study were made based on the presence of intrinsic resistance in *Enterococcus* and *Staphylococcus* and long-term antibiotic viability following reconstitution.

Contact Slide Preparation

Three concentrations (high, low, zero) for two antibiotic types (streptomycin, gentamicin) were used in this study. Antibiotic concentrations were determined via preliminary testing on how a model antibiotic-resistant strain, *Enterococcus faecalis* OG1RF (ATCC 47077), grew at various antibiotic concentrations. The “high” concentration was the minimum tested concentration needed to prevent any visible growth of *E. faecalis* OG1RF colonies, while the “low” concentration was the minimum tested concentration needed for *E. faecalis* OG1RF colonies to be visibly smaller than those growing on no-antibiotic TSA. The tested concentrations were 250 µg/mL (high) and 150 µg/mL (low) for gentamicin and 400 µg/mL (high) and 250 µg/mL (low) for streptomycin.

Tryptic soy agar (TSA) (Thermo Scientific R455002) powder was reconstituted in 18 MΩ water according to the manufacturer’s instructions, then autoclaved at 121 °C for 15 minutes. To ensure that antibiotics were added to agar at a consistent temperature, agar bottles were kept in a water bath at 50 °C after autoclaving, for at least 30 minutes and until time of use. Gentamicin (MP Biomedicals 02194530) and streptomycin (MP Biomedicals 0219454125) were reconstituted according to the manufacturer’s instructions and then filtered through a 0.2 µm filter. Antibiotic solutions were prepared fresh daily and stored at 4 °C until immediately before use. Aliquots of antibiotics were added to agar, then agar bottles were swirled gently to mix. Each HYCON contact

slide (Millipore 144024) was filled with 12 mL of agar, then left to cool and solidify in the biological safety cabinet for 30 minutes before being closed and transferred to the refrigerator. Contact slides were sealed using the provided film and stored at 4 °C in airtight and light-shielded conditions for the duration of the stability testing period of 6.5 months.

Antibiotic Stability Assay

Approximately once per month, a group of contact slides (high, low, and no antibiotic) was removed from storage, and their stability was assessed by inoculating with *E. faecalis* OG1RF. Fresh cultures were prepared and applied to the group of contact slides each month using the following workflow:

A wire loop was flamed and allowed to cool, then dipped into *E. faecalis* OG1RF stocks stored at -80 °C and streaked on three standard TSA plates. Plates were incubated for 24 hours at 37 °C. One isolated colony from each plate was used to inoculate a 100 mL flask of tryptic soy broth (TSB). The three flasks were then incubated for 24 hours at 37 °C while shaking at 200 rotations per minute (rpm).

Previous work determined an optimal dilution of 10^{-6} to yield an appropriate number of countable colonies for this organism and set of growth conditions. Accordingly, liquid cultures were diluted to 10^{-6} , then 50 μ L aliquots were plated on contact slides. For each time point, plating was done in triplicate for each group of high/low/zero antibiotic concentration slides (i.e., 3 plates at 250 μ g/mL gentamicin, 3 plates at 150 μ g/mL gentamicin, 3 plates at 400 μ g/mL streptomycin, 3 plates at 250 μ g/mL streptomycin, and 3 plates at zero antibiotic). Contact slides were incubated for 48 hours at 37 °C.

After 48 hours of incubation, contact slides were removed, colony-forming units (CFUs) were counted, and images of the contact slides were taken from a standardized distance at a resolution of 26.3 pixels/mm (0.038 mm/pixel). Following imaging, the group of contact slides for that sampling interval was discarded. Images were loaded into ImageJ (Schneider et al., 2012) and the scale was set using the middle line on each contact slide as a reference length, then the ImageJ ROI Manager Tool was used to measure colony diameter.

Statistical testing was performed to determine whether differences in colony counts within each sampling interval were significant. Single-factor ANOVA was performed on CFU counts from contact slides containing no antibiotic and low concentrations of gentamicin and streptomycin. No statistically significant differences were found, so no multiple-comparison tests were performed.

Statistical testing was also performed to determine whether colony diameters were significantly different between antibiotic types over the course of the experiment and within time points. A linear mixed-effects model was applied to the dataset using MATLAB's fitlme function. Timepoint, antibiotic type, and their interaction were set as fixed effects; replicate

number was set as a random effect; and colony diameter was set as the response variable. Post-hoc comparisons were performed for diameters from each antibiotic contact slide type against the no-antibiotic contact slide diameters. P-values were adjusted using a Holm-Bonferroni multiple testing correction ($\alpha = 0.05$).

Results

Target Antibiotic Selection

The literature review focused on 13 antibiotics from 7 classes (FIG.1.). These antibiotics were selected based on their impacts on cell wall biosynthesis (beta-lactams, carbapenems, penicillin-like antibiotics) and ribosome synthesis (aminoglycosides, lincomycin, macrolides, tetracyclines), which links them to key antibiotic resistance mechanisms in enterococci (Arias and Murray, 2012). Data on long-term stability in solution and in agar and prevalence of intrinsic and acquired resistance in *Enterococcus* and *Staphylococcus* were collected. These results are shown in Table 1.

Following this review, gentamicin and streptomycin were selected for further analysis. These antibiotics were among the higher-ranking options for stability in solution, and previous work had indicated that streptomycin is stable in agar for at least 30 days (Ryan et al., 1970). Furthermore, these specific antibiotics are relevant to the GEARS study – both *Enterococcus* and *Staphylococcus*, the main targets of this project, often display resistance to these antibiotics. Enterococci possess low-level intrinsic resistance to aminoglycosides overall (a category that includes both gentamicin and streptomycin), and can further acquire high-level resistance (Chow, 2000; Hollenbeck and Rice, 2012; Miller et al., 2014). Furthermore, staphylococci can develop resistance to both antibiotics (Cattoir, 2016; Domínguez et al., 2002; Jensen and Lyon, 2009; Vestergaard et al., 2016).

Antibiotic Stability Over 6.5-month Test Period

During each testing interval, colony-forming units (CFUs) were counted on each set of contact slides (high/low gentamicin and streptomycin, no antibiotic). No CFUs grew on the high-concentration contact slides for either antibiotic type throughout the testing period. Single-factor ANOVA was performed on CFU counts for contact slides containing no antibiotic and low concentrations of gentamicin and streptomycin. These tests found no statistically significant differences in colony counts among the no-antibiotic, low-gentamicin, and low-streptomycin plates at each time point. Since no statistically significant differences were found, no multiple correction testing was performed. FIG.2. displays these CFU counts over 6.5 months.

Table 1. Antibiotic literature review results. Antibiotics are organized alphabetically within their respective classes. Stability in solution and in agar at 4 °C is given, then resistance in *Enterococcus* and *Staphylococcus* is briefly described.

Class	Antibiotic	Stability in solution at 4°C	Stability in agar plates at 4°C	Enterococcus resistance		Staphylococcus resistance
				Low-level	High-level	
Aminoglycoside	Amikacin	60 days (WHO, 2014)	Unknown	Intrinsic (Chow, 2000)	Can be acquired (Chow, 2000)	Can be acquired (Gold et al., 2014)
	Gentamicin	4 weeks (Bastani et al., 2005)	Unknown	Intrinsic (Hollenbeck and Rice, 2012)	Can be acquired (Hollenbeck and Rice, 2012)	Intrinsic (Cattoir, 2016; Domínguez et al., 2002; Vestergaard et al., 2016)
	Kanamycin	12 months (US Biological Life Sciences, 2023)	30+ days (Ryan et al., 1970)	Intrinsic (Hollenbeck and Rice, 2012)	Unknown	Observed, unknown if intrinsic or acquired (Domínguez et al., 2002)
	Streptomycin	2 weeks (Millipore Sigma, 2021)	30+ days (Ryan et al., 1970)	Intrinsic (Hollenbeck and Rice, 2012)	Can be acquired (Chow, 2000)	Intrinsic (Jensen and Lyon, 2009)
	Tobramycin	96 hours (GlobalRPH, 2001)	Unknown	Intrinsic (Hollenbeck and Rice, 2012)	Unknown	Observed, unknown if intrinsic or acquired (Domínguez et al., 2002)
Beta-lactam	Cephalosporin	7-30 days (Xu et al., 2002)	Unknown	Intrinsic (Hollenbeck and Rice, 2012)	Intrinsic (Hollenbeck and Rice, 2012)	Intrinsic (Bruns and Keppeler, 1980)
Carbapenem (beta-lactam with hydrolase resistance)	Ertapenem	24 hours (GlobalRPH, 2008)	Unknown	Intrinsic (Werth, 2022)	Unknown	Can be acquired (Gesser et al., 2004; Ghosh and Banerjee, 2016)
Lincomycin	Clindamycin	32 days (GlobalRPH, 2007)	Unknown	Intrinsic (Hollenbeck and Rice, 2012)	Unknown	Can be acquired (Bastani et al., 2005)
Macrolide	Erythromycin	7 days (Al-Ramahi et al., 2015)	30+ days (Ryan et al., 1970)	Can be acquired (Kristich et al., 2014)	Can be acquired (Kristich et al., 2014)	Can be acquired (Miklasinska-Majdanik, 2021)
Penicillin-like beta-lactams	Amoxicillin	10 days (Al-Ramahi et al., 2015)	Unknown	Can be acquired (Conceição et al., 2012)	Can be acquired (Conceição et al., 2012)	Intrinsic (Bruns and Keppeler, 1980)
	Ampicillin	3 weeks (Sigma-Aldrich, 2016)	7 days (Ryan et al., 1970)	Can be acquired (Hollenbeck and Rice, 2012; Kristich et al., 2014)	Can be acquired (Hollenbeck and Rice, 2012; Kristich et al., 2014)	Intrinsic (Bruns and Keppeler, 1980)
	Penicillin	2 weeks (21)	7 days (Ryan et al., 1970)	Intrinsic (Hollenbeck and Rice, 2012)	Unknown	Intrinsic (Bruns and Keppeler, 1980; Lowy, 2003)
Tetracycline	Tetracycline	2 weeks (Barrick Lab, 2022)	30+ days (Ryan et al., 1970)	Can be acquired (Wilcks et al., 2005)	Can be acquired (Wilcks et al., 2005)	Can be acquired (Grossman, 2016)

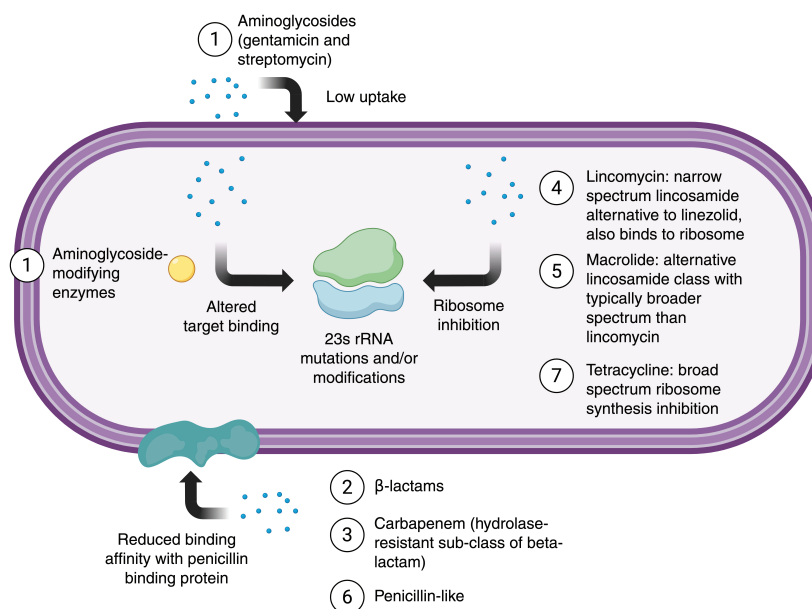


Figure 1. Mechanisms of resistance for antibiotics included in literature review. Antibiotics are numbered based on order in table.1. Adapted from Arias & Murray 2012.

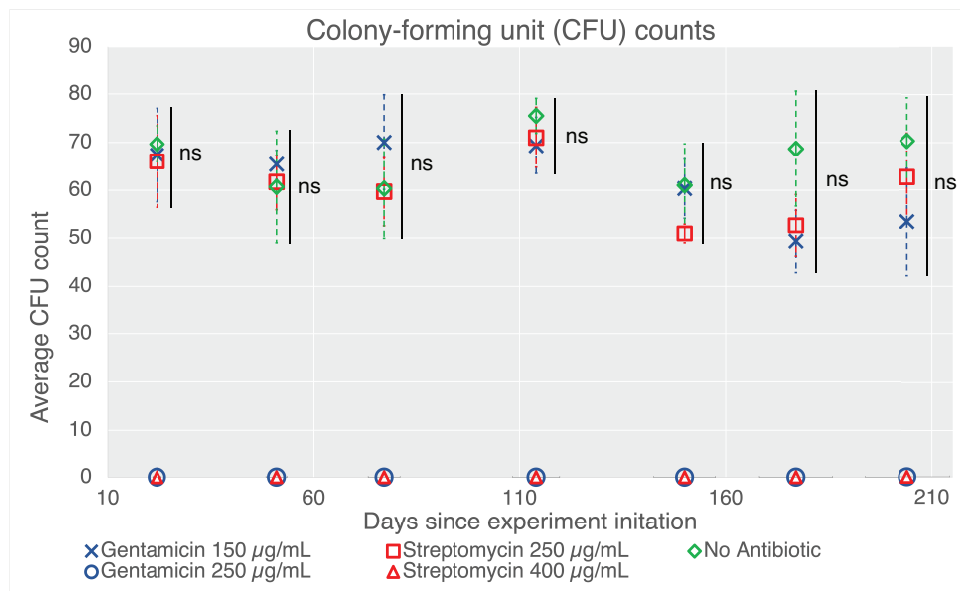


Figure 2. Average colony-forming unit (CFU) counts for contact slides containing no antibiotic, low-concentration and high-concentration gentamicin, and low-concentration and high-concentration streptomycin over 6.5 months. Colored bars indicate standard deviation. Asterisks denote level of statistical significance from single-factor ANOVA tests performed on CFU counts for the contact slides containing no antibiotic and low-concentration gentamicin and streptomycin (no significance: ns; $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****).

During preliminary testing, it was observed that colony diameter decreased with increasing antibiotic concentration. While a direct relationship between colony diameter and antibiotic concentration was not quantified, this observation suggests that colony diameter could also serve as an indicator of functional antibiotic concentration, suggesting its stability. Colony diameter was tracked throughout the study as an additional exploration of a potentially useful but currently unvalidated method to further inform antibiotic selection (see Discussion for further details). A linear mixed-effects model was applied to investigate the effects of timepoint, antibiotic type, and their interaction on colony diameter, while accounting for variability between replicates. All fixed effects were highly significant ($p < 0.05$). The model estimated the colony diameter on plates containing no antibiotic at hypothetical day 0 to be 2.77 mm (95% CI 2.72 to 2.82). Meanwhile, the estimated colony diameter at hypothetical day 0 was significantly smaller on plates containing gentamicin (-0.68 mm, 95% CI -0.75 to -0.60) and streptomycin (-0.77 mm, 95% CI -0.84 to -0.70). Throughout the experiment, colony size on plates with no antibiotics decreased with time (-0.0011 mm/day; 95% CI -0.0015 to -0.00068), which suggests an aging effect of agar plates. Meanwhile, colony diameters gradually increased on plates containing gentamicin (0.0032 mm/day, 95% CI 0.0025 to 0.0038) and streptomycin (0.0020 mm/day, 95% CI 0.0014 to 0.0026), which is consistent with antibiotic degradation over time. The standard deviation for replicates was zero, indicating no significant plate-to-plate differences and suggesting consistent growth conditions. Post-hoc testing

(FIG.3) revealed that at all timepoints, both antibiotic conditions had colony diameters that were significantly different than no antibiotic, with one exception: at the day 204 timepoint, the G150 condition had no significant difference in colony diameter. This suggests some degradation of the antibiotic by this timepoint, although longer-duration experimentation would have been necessary to further resolve this potential trend of decreased antibiotic efficacy.

Discussion

In this study, we present data on the long-term stability of gentamicin and streptomycin in tryptic soy agar, which was used to inform antibiotic selection and planning for the GEARS spaceflight mission. We discuss the criteria for selecting test antibiotics, show that both tested antibiotics remain stable over the 6.5-month test period, and present a novel method for using colony diameter to gain additional insight into antibiotic stability.

Based on the literature review, gentamicin and streptomycin were selected for further validation in the GEARS study due to their viability in solution and on agar plates and the prevalence of resistance to these drugs in *Enterococcus* and *Staphylococcus*. *Enterococcus* possess low-level intrinsic resistance to aminoglycosides (a class that includes both gentamicin and streptomycin), based on poor drug uptake and drug inactivation by *Enterococcus*-produced enzymes (Miller et al., 2014). *Staphylococcus* similarly exhibits

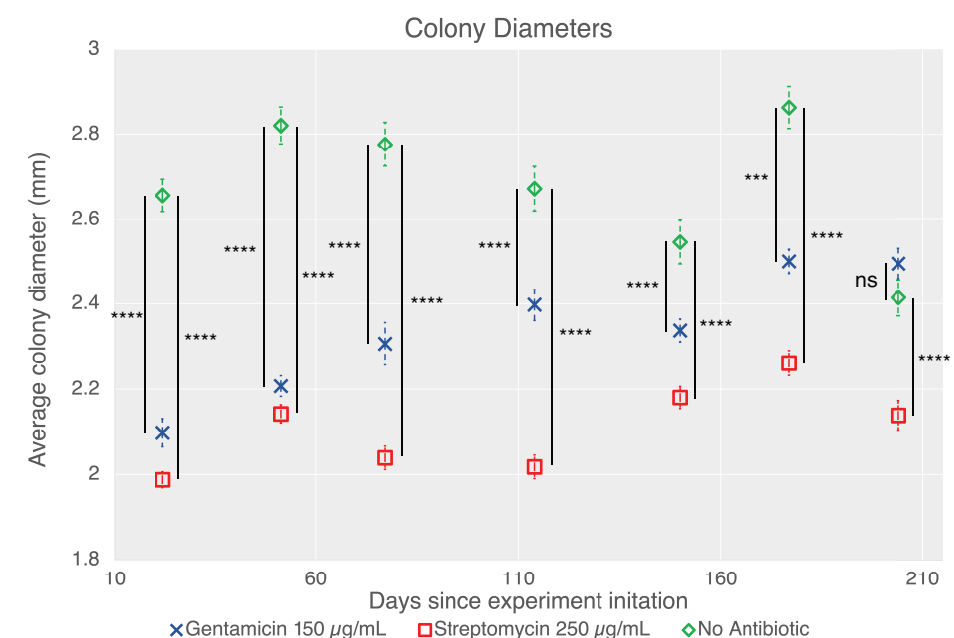


Figure 3. Average colony diameter on contact slides containing no antibiotic, low-concentration gentamicin, and low-concentration streptomycin over 6.5 months. Colored bars indicate standard error of the mean. Asterisks denote adjusted level of statistical significance from post-hoc comparisons (no significance: ns; $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***; $p < 0.0001$: ****).

low-level intrinsic resistance to gentamicin (Cattoir, 2016; Vestergaard et al., 2016) and exhibits widespread emergent low-level resistance to streptomycin (Jensen and Lyon, 2009). Additionally, streptomycin has been shown to be viable in agar plates for at least 30 days (Ryan et al., 1970), and gentamicin and streptomycin are viable in solution for 4 weeks (Bastani et al., 2005) and 2 weeks (Millipore Sigma, 2021), respectively. We evaluated whether antibiotics remained stable on agar plates under cold storage conditions for up to 6.5 months. This timeframe was selected to support a GEARs mission lasting up to 4 months, with margins for time between slide preparation and launch, as well as potential delays on the ground or on-orbit. The “shelf life” would limit the time between preparation and usage, and since GEARs plates are being returned to Earth, would imply limits on the time between launch and landing to avoid overgrowth of plates. These two antibiotics underwent additional stability testing to inform GEARs flight planning.

Perhaps the most prominent general finding from the literature review was the deep lack of data on the stability of antibiotics in agar. This is not surprising: conventional laboratory operations allow for rapid preparation of antibiotics for use in agar or solution, and general best practices for antibiotic resistance research dictate using freshly-prepared antibiotics. However, it is useful to have information on the long-term stability of antibiotics in agar for applications where the tools of a standard microbiology lab may not be accessible

(e.g., spaceflight, remote medicine, livestock monitoring). By validating the long-term stability of our antibiotics of interest for the GEARs study, we collected data that may be useful in other situations where antibiotic resistance monitoring is needed but the continuous production of antibiotic-containing consumables is not feasible. Culture-based microbial monitoring is critical to medical, agricultural, livestock, and wastewater systems. Data on antibiotic stability after reconstitution in agar plates can extend the shelf life of plates needed for these applications and enable microbial monitoring in remote or resource-limited settings. Furthermore, this data can inform the longevity of antibiotics polluting environmental systems, which is a major driver of the rapid proliferation of antibiotic resistance seen today (Larsson and Flach, 2022). In addition, these findings may have implications for the natural use of antibiotics by microbes; extended stability of antibiotics could support ecological functions such as niche maintenance, defense, intercellular signaling, or inter-species interactions (Davies, 2006; Martínez, 2008; Sengupta et al., 2013).

Throughout the 6.5-month testing period, there were no significant differences between CFU counts on low-antibiotic and no-antibiotic contact slides, although the colonies on the low-antibiotic contact slides were visibly smaller than those of the no-antibiotic contact slides. Additionally, no colonies ever grew on the high-antibiotic contact slides. This indicates that the chosen test concentrations were well-suited for

growing the test model organism on antibiotic concentrations that partially and fully attenuated growth, and that antibiotics remained stable enough to have these effects throughout the test period.

Antibiotic decay was estimated to be no greater than 40% for gentamicin and 37.5% for streptomycin as described below. No colonies were observed on the high-concentration antibiotic contact slides (250 µg/mL for gentamicin and 400 µg/mL for streptomycin) throughout 6.5 months of storage, while colonies were observed on the low-concentration antibiotic contact slides (150 µg/mL for gentamicin and 250 µg/mL for streptomycin) at the initiation of the experiment, when the contact slides were freshly made. This implies that the high-concentration antibiotic contact slides did not decay down to an equivalent functional concentration of the low-concentration antibiotic contact slides during the testing period. Thus, the remaining functional antibiotic doses must be greater than 150 µg/mL / 250 µg/mL or 60% of the original for gentamicin (<40% decay), and 250 µg/mL / 400 µg/mL or 62.5% for streptomycin (<37.5% decay).

Based on these findings, it was determined that either antibiotic option would be suitable for the GEARS study when targeting 30-45 day duration Commercial Resupply Services (CRS) missions. Ultimately, gentamicin was selected due to satisfactory stability and anecdotal observations of streptomycin interfering with downstream DNA extraction and sequencing, although at tested doses higher than considered for culturing.

In this experiment, we used colony diameter as an additional proxy metric for antibiotic efficacy. This was based on an observation in preliminary testing that colony diameter decreased with increasing antibiotic concentration. This observation can be extended to suggest that, as an antibiotic decays, its reduced functional concentration will be reflected in larger colony diameters than on a plate with fresh antibiotics. Thus, we decided to record colony diameter to explore how it changed as antibiotics decayed over the test period. It is important to note that this is not a validated method for directly measuring antibiotic efficacy and is intended as a potentially useful metric that enriches our conclusions and may merit further exploration. Further testing would be necessary to determine whether there is a quantifiable relationship between antibiotic concentration/efficacy and colony diameter, and to explore how this relationship may translate into clinical applications. These results imply that for longer duration CRS missions, up to six months, some degradation may be present, but does not exceed 40%, and could be lower.

The GEARS study is currently underway, with the first launch and operations occurring in March and April 2024, the second in December 2024, and the third in June 2026. This mission may reveal additional information about the utility of antibiotics under the unique conditions of spaceflight (e.g., launch,

radiation, and microgravity). As space missions increase in duration, distance from Earth, and crew size, it will be critical to continually update our understanding of the microbes that coexist with the crew, which can have drastic implications for astronaut health.

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Author Disclosure Statement

No competing financial interests exist.

References

- Al-Ramahi RJ, Zaid AAN, Anabousi H (2015) Problems associated with reconstitution, administration, and storage of antibiotic suspensions for pediatrics: A cross-sectional study in Nablus city, Palestine. *BMC Research Notes*, 8, 760. <https://doi.org/10.1186/s13104-015-1746-z>
- Arias CA, Murray BE (2012) The rise of the Enterococcus: Beyond vancomycin resistance. *Nature Reviews Microbiology*, 10(4), 266–278. <https://doi.org/10.1038/nrmicro2761>
- Aunins TR, Erickson KE, Prasad N, Levy SE, Jones A, Shrestha S, Mastracchio R, Stodieck L, Klaus D, Zea L, Chatterjee A (2018) Spaceflight modifies *Escherichia coli* gene expression in response to antibiotic exposure and reveals role of oxidative stress response. *Frontiers in Microbiology*, 9. <https://doi.org/10.3389/fmicb.2018.00310>
- Barlow M, Hall BG (2002) Phylogenetic analysis shows that the OXA b-lactamase genes have been on plasmids for millions of years. *Journal of Molecular Evolution*, 55(3), 314–321. <https://doi.org/10.1007/s00239-002-2328-y>
- Barrick Lab (2022) *Barrick Lab—Antibiotic concentrations and stock solutions*. <https://barricklab.org/twiki/bin/view/Lab/ProtocolsAntibioticStockSolutions>
- Bastani B, Amin K, Herr A (2005) Prolonged stability of stored vancomycin, gentamicin, and heparin for use in the antibiotic-lock technique. *ASAIO Journal (American Society for Artificial Internal Organs: 1992)*, 51(6), 761–763. <https://doi.org/10.1097/01.mat.0000183656.75618.09>

- Bengtsson-Palme J, Larsson DGJ, Kristiansson E (2017) Using metagenomics to investigate human and environmental resistomes. *Journal of Antimicrobial Chemotherapy*, 72(10), 2690–2703. <https://doi.org/10.1093/jac/dkx199>
- Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD (2012) Antibiotic Resistance Is Prevalent in an Isolated Cave Microbiome. *PLoS ONE*, 7(4). <https://doi.org/10.1371/journal.pone.0034953>
- Bruns W, Keppeler H (1980) Mechanism of intrinsic penicillin-resistance in *Staphylococcus aureus*. Binding of penicillin G to the cytoplasmic membrane of resistant staphylococci. *Arzneimittel-Forschung*, 30(9), 1469–1475.
- Bryan NC, Lebreton F, Gilmore M, Ruvkun G, Zuber MT, Carr CE (2021) Genomic and functional characterization of *Enterococcus faecalis* isolates recovered from the International Space Station and their potential for pathogenicity. *Frontiers in Microbiology*, 11. <https://doi.org/10.3389/fmicb.2020.515319>
- Caruso G (2018) Antibiotic resistance in *Escherichia coli* from farm livestock and related analytical methods: A review. *Journal of AOAC INTERNATIONAL*, 101(4), 916–922. <https://doi.org/10.5740/jaoacint.17-0445>
- Cattoir V (2016) Mechanisms of antibiotic resistance. In J. J. Ferretti, D. L. Stevens, and V. A. Fischetti (Eds.), *Streptococcus pyogenes: Basic Biology to Clinical Manifestations*. University of Oklahoma Health Sciences Center. <http://www.ncbi.nlm.nih.gov/books/NBK333414/>
- CDC (2024) 2019 Antibiotic Resistance Threats Report. Antimicrobial Resistance. <https://www.cdc.gov/antimicrobial-resistance/data-research/threats/index.html>
- Chow JW (2000) Aminoglycoside resistance in enterococci. *Clinical Infectious Diseases*, 31(2), 586–589. <https://doi.org/10.1086/313949>
- Conceição N, de Oliveira CDC HB, da Silva LEP, de Souza LRC, de Oliveira AG (2012) Ampicillin susceptibility can predict in vitro susceptibility of penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis* isolates to amoxicillin but not to imipenem and piperacillin. *Journal of Clinical Microbiology*, 50(11), 3729–3731. <https://doi.org/10.1128/JCM.01246-12>
- Davies, J (2006) Are antibiotics naturally antibiotics? *Journal of Industrial Microbiology and Biotechnology*, 33(7), 496–499. <https://doi.org/10.1007/s10295-006-0112-5>
- D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD (2011) Antibiotic resistance is ancient. *Nature*, 477(7365), 457–461. <https://doi.org/10.1038/nature10388>
- Domínguez E, Zarazaga M, Torres C (2002) Antibiotic resistance in *Staphylococcus* isolates obtained from fecal samples of healthy children. *Journal of Clinical Microbiology*, 40(7), 2638–2641. <https://doi.org/10.1128/JCM.40.7.2638-2641.2002>
- Dubin K, Pamer EG (2017) Enterococci and their interactions with the intestinal microbiome. *Microbiology Spectrum*, 5(6), 10.1128/microbiolspec.bad-0014–2016. <https://doi.org/10.1128/microbiolspec.bad-0014-2016>
- Gesser RM, McCarroll KA, Woods GL (2004) Efficacy of ertapenem against methicillin-susceptible *Staphylococcus aureus* in complicated skin/skin structure infections: Results of a double-blind clinical trial versus piperacillin-tazobactam. *International Journal of Antimicrobial Agents*, 23(3), 235–239. <https://doi.org/10.1016/j.ijantimicag.2003.07.013>
- Ghosh S, Banerjee M (2016) Methicillin resistance and inducible clindamycin resistance in *Staphylococcus aureus*. *The Indian Journal of Medical Research*, 143(3), 362–364. <https://doi.org/10.4103/0971-5916.182628>
- GlobalRPH. (2001). *Tobramycin*. <https://globalrph.com/dilution/tobramycin/>
- GlobalRPH. (2007). *Clindamycin*. <https://globalrph.com/dilution/clindamycin/>
- GlobalRPH. (2008). *Ertapenem*. GlobalRPH. <https://globalrph.com/dilution/ertapenem-invanz/>
- Gold RM, Cohen ND, Lawhon SD (2014) Amikacin resistance in *Staphylococcus pseudintermedius* isolated from dogs. *Journal of Clinical Microbiology*, 52(10), 3641–3646. <https://doi.org/10.1128/JCM.01253-14>
- Grossman TH (2016) Tetracycline antibiotics and resistance. *Cold Spring Harbor Perspectives in Medicine*, 6(4), a025387. <https://doi.org/10.1101/cshperspect.a025387>
- Haulisah NA, Hassan L, Bejo SK, Jajere SM, Ahmad NI (2021) High levels of antibiotic resistance in isolates from diseased livestock. *Frontiers in Veterinary Science*, 8. <https://doi.org/10.3389/fvets.2021.652351>
- Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK, Facilities NHSNT, PNHSN (2008) Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control and Hospital Epidemiology*, 29(11), 996–1011. <https://doi.org/10.1086/591861>
- Hollenbeck BL, Rice LB (2012) Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*, 3(5), 421–569. <https://doi.org/10.4161/viru.21282>
- Huang B, Li DG, Huang Y, Liu CT (2018) Effects of spaceflight and simulated microgravity on microbial growth and secondary metabolism. *Military Medical Research*, 5. <https://doi.org/10.1186/s40779-018-0162-9>
- Jensen SO, Lyon BR (2009) Genetics of antimicrobial resistance in *Staphylococcus aureus*. *Future Microbiology*, 4(5), 565–582. <https://doi.org/10.2217/fmb.09.30>
- Kacena MA, Merrell GA, Manfredi B, Smith EE, Klaus DM, Todd P (1999) Bacterial growth in space flight: Logistic growth curve parameters for *Escherichia coli* and *Bacillus subtilis*. *Applied Microbiology and Biotechnology*, 51(2), 229–234. <https://doi.org/10.1007/s002530051386>
- Kim W, Tengra FK, Young Z, Shong J, Marchand N, Chan HK, Pangule RC, Parra M, Dordick JS, Plawsky JL, Collins CH (2013) Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa*. *PLOS ONE*, 8(4), e62437. <https://doi.org/10.1371/journal.pone.0062437>

- Kristich CJ, Rice LB, Arias CA (2014) Enterococcal infection—treatment and antibiotic resistance.. In M. S. Gilmore, D. B. Clewell, Y. Ike, and N. Shankar (Eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary. <http://www.ncbi.nlm.nih.gov/books/NBK190420/>
- Kuper KM, Boles DM, Mohr JF, Wanger A (2009) Antimicrobial susceptibility testing: a primer for clinicians. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 29(11), 1326–1343. <https://doi.org/10.1592/phco.29.11.1326>
- Landry KS, Morey JM, Bharat B, Haney NM, Panesar SS (2020) Biofilms—impacts on human health and its relevance to space travel. *Microorganisms*, 8(7), Article 7. <https://doi.org/10.3390/microorganisms8070998>
- Larsson DGJ, Flach CF (2022) Antibiotic resistance in the environment. *Nature Reviews Microbiology*, 20(5), 257–269. <https://doi.org/10.1038/s41579-021-00649-x>
- Lemonnier D, Machuel M, Obin O, Outurquin G, Adjidé C, Mullié C (2023) Trends in antibiotic-resistant bacteria isolated from screening clinical samples in a tertiary care hospital over the 2018–2022 period. *Antibiotics*, 12(8), Article 8. <https://doi.org/10.3390/antibiotics12081314>
- Lowy, FD (2003) Antimicrobial resistance: The example of *Staphylococcus aureus*. *Journal of Clinical Investigation*, 111(9), 1265–1273. <https://doi.org/10.1172/JCI200318535>
- Mahnert A, Moissl-Eichinger C, Zojer M, Bogumil D, Mizrahi I, Rattei T, Martinez JL, Berg G (2019) Man-made microbial resistances in built environments. *Nature Communications*, 10(1), 968. <https://doi.org/10.1038/s41467-019-08864-0>
- Manaia CM, Rocha J, Scaccia N, Marano R, Radu E, Biancullo F, Cerqueira F, Fortunato G, Iakovides IC, Zammit I, Kampouris I, Vaz-Moreira I, Nunes OC (2018) Antibiotic resistance in wastewater treatment plants: Tackling the black box. *Environment International*, 115, 312–324. <https://doi.org/10.1016/j.envint.2018.03.044>
- Martínez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science*, 321(5887), 365–367. <https://doi.org/10.1126/science.1159483>
- McLain JE, Cytryn E, Durso LM, Young S (2016) Culture-based methods for detection of antibiotic resistance in agroecosystems: advantages, challenges, and gaps in knowledge. *Journal of Environmental Quality*, 45(2), 432–440. <https://doi.org/10.2134/jeq2015.06.0317>
- Miklasińska-Majdanik M (2021) Mechanisms of resistance to macrolide antibiotics among *Staphylococcus aureus*. *Antibiotics*, 10(11), 1406. <https://doi.org/10.3390/antibiotics10111406>
- Miller WR, Munita JM, Arias CA (2014) Mechanisms of antibiotic resistance in enterococci. *Expert Review of Anti-Infective Therapy*, 12(10), 1221–1236. <https://doi.org/10.1586/14787210.2014.956092>
- Millipore Sigma (2021) *Penicillin/Streptomycin, 100X, Tissue Culture Grade—Calbiochem*. https://www.emdmillipore.com/US/en/product/Penicillin-Streptomycin-100X-Tissue-Culture-Grade-Calbiochem,EMD_BIO-516106
- Moatti N, Lapchine L, Gasset G, Richoilley G, Templier J, Tixador R (1986) Preliminary results of the “Antibio” experiment. *Naturwissenschaften*, 73(7), 413–414. <https://doi.org/10.1007/BF00367282>
- Mora M, Mahnert A, Koskinen K, Pausan MR, Oberauner-Wappis L, Krause R, Perras AK, Gorkiewicz G, Berg G, Moissl-Eichinger C (2016) Microorganisms in confined habitats: microbial monitoring and control of intensive care units, operating rooms, cleanrooms and the International Space Station. *Frontiers in Microbiology*, 7, 1573. <https://doi.org/10.3389/fmicb.2016.01573>
- Ott CM, Crabbé A, Wilson JW, Barrila J, Castro-Wallace SL, Nickerson CA (2020) Microbial stress: spaceflight-induced alterations in microbial virulence and infectious disease risks for the crew. In A. Choukèr (Ed.), *Stress Challenges and Immunity in Space: From Mechanisms to Monitoring and Preventive Strategies* (pp. 327–355). Springer International Publishing. https://doi.org/10.1007/978-3-030-16996-1_18
- Perron GG, Whyte L, Turnbaugh PJ, Goordial J, Hanage WP, Dantas G, Desai MM (2015) Functional characterization of bacteria isolated from ancient Arctic soil exposes diverse resistance mechanisms to modern antibiotics. *PLoS ONE*, 10(3), e0069533. <https://doi.org/10.1371/journal.pone.0069533>
- Perry J, Waglechner N, Wright G (2016) The prehistory of antibiotic resistance. *Cold Spring Harbor Perspectives in Medicine*, 6(6), a025197. <https://doi.org/10.1101/cshperspect.a025197>
- Reinthal FF, Posch J, Feierl G, Wüst G, Haas D, Ruckebauer G, Mascher F, Marth E (2003) Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research*, 37(8), 1685–1690. [https://doi.org/10.1016/S0043-1354\(02\)00569-9](https://doi.org/10.1016/S0043-1354(02)00569-9)
- Rosenzweig JA, Abogunde O, Thomas K, Lawal A, Nguyen YU, Sodipe A, Jejelowo O (2010) Spaceflight and modeled microgravity effects on microbial growth and virulence. *Applied Microbiology and Biotechnology*, 85(4), 885–891. <https://doi.org/10.1007/s00253-009-2237-8>
- Ryan KJ, Needham GM, Dunsmoor CL, Sherris JC (1970) Stability of antibiotics and chemotherapeutics in agar plates. *Applied Microbiology*, 20(3), 447–451.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671–675. <https://doi.org/10.1038/nmeth.2089>
- Sengupta S, Chattopadhyay MK, Grossart HP (2013) The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology*, 4, 47. <https://doi.org/10.3389/fmicb.2013.00047>
- Sharma G, Curtis PD (2022) The impacts of microgravity on bacterial metabolism. *Life*, 12(6), Article 6. <https://doi.org/10.3390/life12060774>
- Sigma-Aldrich (2016) *Product Information—Ampicillin sodium salt*. <https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/349/565/a0166pis.pdf>
- Smaill F (2000) Antibiotic susceptibility and resistance testing: an overview. *Canadian Journal of Gastroenterology and Hepatology*, 14(10), 382415. <https://doi.org/10.1155/2000/382415>

- Sonnenfeld G (1998) Immune responses in space flight. *International Journal of Sports Medicine*, 19, S195–S204. <https://doi.org/10.1055/s-2007-971992>
- Stratis D, Trudel G, Rocheleau L, Pelchat M, Laneuville O (2023) The transcriptome response of astronaut leukocytes to long missions aboard the International Space Station reveals immune modulation. *Frontiers in Immunology*, 14. <https://doi.org/10.3389/fimmu.2023.1171103>
- Tirumalai MR, Karouia F, Tran Q, Stepanov VG, Bruce RJ, Ott CM, Pierson DL, Fox GE (2019) Evaluation of acquired antibiotic resistance in *Escherichia coli* exposed to long-term low-shear modeled microgravity and background antibiotic exposure. *mBio*, 10(1), 10.1128/mbio.02637-18. <https://doi.org/10.1128/mbio.02637-18>
- Tixador R, Richoille G, Gasset G, Templier J, Bes JC, Moatti N, Lapchine L (1985) Study of minimal inhibitory concentration of antibiotics on bacteria cultivated in vitro in space (Cytos 2 experiment). *Aviation, Space, and Environmental Medicine*, 56(8), 748–751.
- Tomida J, Oumi A, Okamoto T, Morita Y, Okayama A, Misawa N, Hayashi T, Akaike T, Kawamura Y (2013) Comparative evaluation of agar dilution and broth microdilution methods for antibiotic susceptibility testing of *Helicobacter cinaedi*. *Microbiology and Immunology*, 57(5), 353–358. <https://doi.org/10.1111/1348-0421.12044>
- Torres MDT, Wan F, de la Fuente-Nunez C (2025) Deep learning reveals antibiotics in the archaeal proteome. *Nature Microbiology*, 1–15. <https://doi.org/10.1038/s41564-025-02061-0>
- Urbaniak C, Morrison MD, Thissen JB, Karouia F, Smith DJ, Mehta S, Jaing C, Venkateswaran K (2022) Microbial Tracking-2, a metagenomics analysis of bacteria and fungi onboard the International Space Station. *Microbiome*, 10(1), 100. <https://doi.org/10.1186/s40168-022-01293-0>
- US Biological Life Sciences (2023) *Kanamycin Sulfate 50mg/ml Solution CAS 25389-94-0*. <https://www.usbio.net/biochemicals/K0015/Kanamycin-Sulfate-50mgml-Solution>
- Vaishampayan A, Grohmann E (2019) Multi-resistant biofilm-forming pathogens on the International Space Station. *Journal of Biosciences*, 44(5), 125. <https://doi.org/10.1007/s12038-019-9929-8>
- Vestergaard M, Leng B, Haaber J, Bojer MS, Vegge CS, Ingmer H (2016) Genome-wide identification of antimicrobial intrinsic resistance determinants in *Staphylococcus aureus*. *Frontiers in Microbiology*, 7, 2018. <https://doi.org/10.3389/fmicb.2016.02018>
- Werth BJ (2022) *Carbapenems*. Merck Manual Professional Edition. <https://www.merckmanuals.com/professional/infectious-diseases/bacteria-and-antibacterial-medications/carbapenems>
- WHO (2014) Anti-TB drug information sheets. In *Companion Handbook to the WHO Guidelines for the Programmatic Management of Drug-Resistant Tuberculosis*. World Health Organization. <https://www.ncbi.nlm.nih.gov/books/NBK247415/>
- WHO (2019) *Ten health issues WHO will tackle this year*. <https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019>
- WHO (2023) *Antimicrobial resistance*. <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>
- Wilks A, Andersen SR, Licht TR (2005) Characterization of transferable tetracycline resistance genes in *Enterococcus faecalis* isolated from raw food. *FEMS Microbiology Letters*, 243(1), 15–19. <https://doi.org/10.1016/j.femsle.2004.11.028>
- Wilson JW, Ott CM, zu Bentrup KH, Ramamurthy R, Quick L, Porwollik S, Cheng P, McClelland M, Tsapralis G, Radabaugh T, Hunt A, Fernandez D, Richter E, Shah M, Kilcoyne M, Joshi L, Nelman-Gonzalez M, Hing S, Parra M, ... Nickerson CA (2007) Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proceedings of the National Academy of Sciences*, 104(41), 16299–16304. <https://doi.org/10.1073/pnas.0707155104>
- Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases*, 39(3), 309–317. <https://doi.org/10.1086/421946>
- WMA (2022) *WMA - The World Medical Association-WMA Statement on Antimicrobial Resistance*. <https://www.wma.net/policies-post/wma-statement-on-resistance-to-antimicrobial-drugs/>
- Xu QA, Trissel LA, Saenz CA, Ingram DS, Williams KY (2002) Stability of three cephalosporin antibiotics in AutoDose Infusion System bags. *Journal of the American Pharmaceutical Association (Washington, D.C.: 1996)*, 42(3), 428–431. <https://doi.org/10.1331/108658002763316851>
- Yamaguchi N, Roberts M, Castro S, Oubre C, Makimura K, Leys N, Grohmann E, Sugita T, Ichijo T, Nasu M (2014) Microbial monitoring of crewed habitats in space—current status and future perspectives. *Microbes and Environments*, 29(3), 250–260. <https://doi.org/10.1264/jsme2.ME14031>