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Antibiotic Efficacy and Interaction in Escherichia coli during Varying Nutrient Conditions

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Antibiotic Efficacy and Interaction in *Escherichia coli* during Varying Nutrient Conditions

A Thesis Presented by

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To the W. M. Keck Science Department

Of Scripps, Claremont McKenna, and Pitzer Colleges

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Thesis in Biology

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Abstract

Due to the recent rise in antibiotic resistant pathogens, and the difficulties surrounding the quest for new antibiotics, many researchers have started revisiting antibiotic interactions in hopes of finding new treatment options. The primary outcome of this project was to examine the efficacy of concomitant antibiotic use under varying nutrient conditions, to identify variations in antibiotic interactions. Antibiotic interactions were studied, utilizing *E. coli* as a model bacterial system, grown in four different media types. *E. coli* cultures were treated with streptomycin, tobramycin, erythromycin, and amikacin individually and in a pairwise fashion at varying doses. We found that at least some antibiotic efficacies were dependent on the environmental nutrient conditions *E. coli* was grown in, as the antibiotics were not equally effective in all media types. *E. coli* grown in potato dextrose broth, in particular, showed extremely high tolerance to antibiotic inhibition. In addition, we observed several variations in antibiotic interactions, depending on the combination of antibiotics and environmental conditions utilized. It is predicted that differences in available nutrients is the primary cause of the observed discrepancies in antibiotic properties between media. The observation of changes in antibiotic efficacy under different environmental and nutrient conditions has serious implications for use of antibiotic combinations as drug treatments. Not all microenvironments within the human body have identical nutrient make-up. If the interactions antibiotics are reported to have in one environmental condition change under another, reckless prescription of combinations could lead to a serious adverse reaction. Thus, this is an important area for future *in vitro* and *in vivo* research.
Introduction

Antibiotic resistance has become a major issue in modern society. Contraction of life threatening, multi-drug resistant infections used to be limited to hospitals and, as such, relatively contained. However, recently both methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant tuberculosis (MDR-TB) have been found in the general community. Although these two strains, which exhibit the most complete resistance, are still relatively rare, resistance genes themselves are quite common. Many pathogens exhibit at least some level of resistance (Fernandes, 2006). Additionally, resistance genes are not specific to pathogens. One study, that surveyed a large collection of soil bacteria, revealed a large and unexpected reservoir of resistance factors to all known antibiotics (Cottarel & Wierzbowski, 2007). This discovery was concerning due to the ease with which horizontal gene transfer could lead to the sharing of these defense mechanisms (Davies & Davies, 2010). Some scientists fear humanity will soon be returning to the pre-antibiotic era, as the current antibiotics are rapidly becoming obsolete (Fernandes, 2006; Davies & Davies, 2010).

During the Golden Age of antibiotics, these chemical weapons were discovered in mass and medicine thought it had found its cure for bacterial infections. More than half of the major antibacterial drug classes, such as aminoglycosides, cephalosporins, glycopeptides, macrolides, tetracyclines, sulfonamides and penicillins were discovered at that time (Amato et al., 2014, Fernandes, 2006). With the exception of folic acid inhibitors, the discovery of new antibiotics has virtually come to a halt in recent history. There are several factors that have contributed to this stall. First of all, during the Golden Age, all of
the easily identified antibiotic compounds were discovered. As such, the discovery of completely new antibiotic compounds, with novel mechanisms, has become difficult and time consuming. Researchers instead turned to chemically modifying and improving the existing compounds. While this was highly effective for a time, new and inventive modifications are increasingly hard to come by. Additionally, it is extremely difficult to get antibiotics approved by regulatory agencies. These agencies have a low tolerance for adverse side effects in antibiotics. In contrast, anti-cancer medications often have severe adverse side, but are more easily approved. It has been speculated that this discrepancy is due to the fact that most bacterial infections are not life threatening and thus adverse side effects are not tolerated as opposed to those in cancer treatment where it is apparent the treatment is essential even if it is basically poison. Finally, large pharmaceutical companies lack the motivation to pursue antibiotic research. There is little economic incentive for this type of research. The research itself is expensive and lengthy. Additionally, the moment the drug is approved and begins being used, resistance starts to evolve, which will ultimately render the drug useless. When a new antibiotic is placed on the market, it is often saved as a “last defense” against the already highly resistant bacteria, on which nothing else will work. Doctors are told to use these precious drugs sparingly, and thus, there are not enough profits made before patents expire to recuperate the costs invested (Fernandes, 2006). As such, researchers have turned their attentions back towards antibiotics that are currently in circulation in hopes that better understanding, through improved technologies, could be illuminating.
Background

Physiology:

Under optimal conditions *Escherichia coli* cells can divide every 20 to 30 minutes. All *E. coli* needs in order to maintain this level of rapid growth are some basic macro- and micro-nutrients. Macronutrients are molecules that must be present in relatively high concentrations for sustained bacterial growth. These macronutrients include sources of carbon, nitrogen, phosphorus, hydrogen, oxygen and sulfur. These are the most basic building blocks necessary for synthesis of carbohydrates, lipids, nucleic acids, and proteins. Additionally several cations, such as K⁺, Mg²⁺, Ca²⁺, and Fe²⁺, are also considered macronutrients and act as intercellular and extracellular signals as well as cofactors for many enzymes. In contrast, only trace amounts of micronutrients, like cobalt, manganese, nickel, zinc, molybdenum, and copper, are necessary for sustained bacterial growth. These micronutrients often take on the role of enzyme cofactors. While this limited list of ingredients is all it takes to grow *E. coli*, many bacteria require many more nutrients provided for them and numerous species have adapted to their specific environment to such a degree that scientists have yet to discover how to grow them in a laboratory setting at all (Slonczewski & Foster, 2011). Because of the rapid cellular growth and ease of use, *E. coli* has been extensively studied as an experimental model for *in vitro* experiments regarding the efficacy of antibiotics and was chosen as our model system for this body of work.
Bacteria do not replicate at their fastest possible rate for their entire life cycle, not even the highly prolific *E. coli*, but rather have various life stages marked by different rates of population growth. The lag phase occurs when cells are first transferred from an old culture to a new one. Bacteria have complex sensory mechanisms, which register and process information from the environment such as, what nutrients are available, or what toxins are present. This information is used to regulate gene expression accordingly, and synthesize the components necessary to prepare for rapid growth. It would make sense then for the rapid growth phase to follow, as it does in exponential phase. Exponential phase is named for the exponential growth of the culture as each cell replicates at its maximum rate. It is also the phase at which individual cells are at their largest to accommodate all of the bulky machinery required for growth. As nutrients begin to become scarce, because they have been mostly used up, and waste products begin building up, the bacteria enter stationary phase. A fairly steady population density characterizes stationary phase. In order to maintain this steady stationary phase, many bacteria initiate protective genes to compensate for the increased risk of damage due to oxygen radicals and other metabolic byproducts, some going so far as to sporulate or encyst. Finally the cells enter death phase where cells begin to die from toxic byproduct buildup. Culture death, like culture growth, happens on a logarithmic regression (Slonczewski & Foster, 2011). For a visual representation of bacterial growth view Figure 1. Experiments looking at antibiotic interactions for the body of work in this thesis focused on the exponential phase for *E. coli*, to ensure inclusion of the time interval of maximal bacterial growth (Kacena, 1999).
Figure 1: Theoretical model of bacterial growth curve (Komorniczak, 2009).

**Environmental Stressors**

Along with, and in addition to, the growth phases, many environmental factors have considerable effects on bacterial growth. Temperature (Raghubeer & Matches, 1990) and pH (Gale & Epps, 1945; as reviewed by Conner, 1995) have been shown to play a significant role in both enzyme activity and overall cellular growth. Osmolarity (Record et al., 1998) and nutrient limitation (Slonczewski & Foster, 2011) lead to changes in cellular morphology and physiology, such as changes in membrane permeability and water influx and efflux, decreases in size, and the synthesis of distinct stress resistant enzymes. Each of these responses takes energy and resources away from cellular growth. Access to oxygen can affect which catabolic pathways bacteria can use, further affecting the rate and efficacy of catabolic activity (White, 2007). Our experiments utilized previously published and studied temperature and pH data (Yeh et al., 2006) and manipulated nutrition. Finally, destructive
chemicals, such as antibiotics synthesized by competing microbes or reactive oxidative species that are the by-product of metabolism, reap further havoc on bacterial growth. Reactive oxidative species damage DNA, while antibiotics are capable of disrupting cell wall synthesis, protein synthesis, DNA translation, and transcription, interfering with membrane permeability, and inhibiting essential enzymes (as reviewed by Conner, 1995, White, 2007).

These environmental factors activate several stress responses bacteria have evolved to cope with environmental stress, for example, the SOS, TAG, and SR responses. The SOS response is triggered by DNA damage or DNA replication inhibition. It induces the transcription of over 20 unlinked genes, including: DNA reparatory genes, genes that stall cell division, and translesion synthesis genes, that allow replication to skip damaged sections (White, 2007). The TAG response is activated by changes in cellular carbon fluxes and triggers the accumulation of the lipid triacylglycerol in preparation for a long period of inactivity (Sedwick, 2011; White, 2007). This long period of inactivity is characterized by little to no growth, immensely slowed metabolic rate and increased antibiotic resistance (Baek et al., 2015). The stringent response (SR) is induced by guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) and is a common bacterial response to carbon, amino acid, and iron starvation (White, 2007; Nguyen et al. 2011). This starvation response causes the cell to reallocate cellular resources and halts the synthesis of DNA, stable RNA, ribosomal proteins and membrane components in favor of producing factors crucial for stress resistance, glycolysis, and amino acid synthesis (Dalebroux & Swanson, 2012). The large changes in transcription profile caused by the stringent response, just as
those of the SOS, TAG, and indole response, lead to a significant increase in antibiotic and other stressor tolerance (Nguyen et al., 2011; Penesyan et al., 2015).

Metabolism

Every living organism has the ability to breakdown molecules it absorbs from its environment into smaller components. This process, called catabolism, produces the building blocks necessary to rebuild macromolecules and releases energy that can be harnessed for cellular use. Anabolism then consists of all of the consumption of that energy in order to rebuild macromolecules essential to the survival and growth of the organism. There are three major nutrient groups: carbohydrates, lipids, and proteins. Each of these nutrients is made up of different components and as such need different pathways for breakdown. Additionally, breakdown of the individual monomers themselves differs greatly. Thus, bacterial metabolism is a highly dynamic system of anabolic and catabolic pathways highly dependent on nutrients found in the environment.

Carbohydrate, and particularly sugar breakdown is perhaps the simplest and most studied catabolic pathway. When glucose enters the cell, it will be broken down by one of three different central metabolic pathways. Two of these pathways, glycolysis and the pentose phosphate pathway (PPP), can be found in both prokaryotic and eukaryotic cells, but the third, the Entner – Doudoroff pathway (ED) is isolated to prokaryotes. Each of these pathways will ultimately break the glucose down into a three-carbon modified sugar called pyruvate; however, their mechanisms differ slightly. Glycolysis produces two pyruvate, two ATP energy molecules, and 2 NADH electron carrier molecules. Intermediate products of
glycolysis are also important precursor metabolites for many other pathways including polysaccharides, pentose phosphates, aromatic amino acids, amino sugars, phospholipids, and the amino acids glycine, serine, and cysteine. PPP produces phosphoglyceraldehyde, CO$_2$ and the electron carrier NADPH. NADPH is an important source of electrons for biosynthesis and phosphoglyceraldehyde can either re-enter the glycolytic pathway to end up as pyruvate or be used as a precursor for nucleic acids. ED catalyzes the same overall reaction as glycolysis but produces only one ATP and one NADH plus one NADPH. While ED produces less cellular energy, it is more effective at breaking down some sugars and particularly aldonic acids than its counterparts. Some bacteria only use one of these pathways, while others use all three under varying circumstances. This allows the bacterium to have increased flexibility when responding to changes in environmental conditions (White, 2007).

Pyruvate produced from these central catalytic pathways can go down several paths from here. In the presence of oxygen, pyruvate will go through further oxidation in the Citric Acid Cycle, where more electron transport molecules will be produced. If energy is plentiful, pyruvate would be tagged for storage and undergo gluconeogenesis. If energy is needed, and oxygen is present, pyruvate will go through further oxidation in the Citric Acid Cycle, where more electron transport molecules will be produced. Electron transport molecules will carry their packages to the electron transport chain of oxidative phosphorylation to produce ATP. Finally, if no oxygen is present pyruvate will be fermented in order to regenerate oxidized electron carrying molecules NAD$^+$ and NADP$^-$ so that glucose breakdown can continue to occur (Slonczewski & Foster, 2011).
If the steps listed above were not already complicated enough, complex carbohydrates must undergo steps prior to the central catabolic pathways in order to break down polymers into monomers and proteins and lipids have their own complex catabolic pathways. The overall result is a highly regulated web of metabolic processes responsible for the breakdown of every nutrient absorbed and the building of every macromolecule essential for bacterial survival and growth (Slonczewski & Foster, 2011). Metabolism is an important consideration for this body of research, as it is a primary effector of various bacterial physiological traits. We varied our media, and nutrient availability, to determine what effect nutrient availability, and the resulting metabolic processes these trigger, has on bacterial tolerance to antibiotics.

*The Bacterial Ribosome*

![Bacterial Ribosome](image)

**Figure 2:** Bacterial Ribosome drawn by David Goodsell from PDB files 2wdk and 2wdl. Small (30S) subunit in front (green) and large (50S) subunit behind (blue) (Goodsell, 2010).
Protein synthesis occurs at the ribosome, which functions as the GTP-powered machinery that converts genetic material into polypeptide chains. Ribosomes are perhaps the most primitive specialized organelle and are conserved from prokaryotes to eukaryotes. They can be found free-floating in the cytosol or embedded in the endoplasmic reticulum of eukaryotic cells (Slonczewski & Foster, 2011). Single-stranded messenger RNA (mRNA) carries the genetic template for proteins to the ribosome, where tRNA molecules are used to translate the nucleotides into an amino acid sequence. tRNA molecules are L-shaped and mostly double stranded; however, the anticodon loop and the 3’ end, the functional sites, are single stranded. This allows the anticodon loops to associate with the mRNA threaded through the small subunit and the 3’ ends to interact with amino acids, peptides, and the large subunit. The ribosome has three tRNA binding sites in the peptidyl-transferase center (PTC): the aminoacyl- (A), the peptidyl- (P), and the exit- (E) sites. Each elongation cycle is made up of one decoding, one forming of the peptide bond, and one release of deacylated tRNA step. These steps follow a tRNA as it advances, along with the mRNA it is bound to, from the A to the P to the E site (as reviewed by Yonath, 2005).
**Figure 3:** Computer rendered representation of the bacterial ribosome activation complex with important features labeled. Yellow represents the 30S ribosomal subunit, green represents the 50S subunit. mRNA is represented by orange. A and P sites labeled purple and green respectively. Elongating polypeptide emerging through protein exit tunnel in 50S subunit. Image created by J. Lorsch from the work of Cate, J. H. et al.

The bacterial ribosome has RNA and protein elements. Long RNA chains make up approximately two thirds of the ribosome’s mass and wrap around many different protein components. All ribosomes have two subunits, a large subunit and a small subunit. In eukaryotic ribosomes these are the 60S and 40S subunits respectively. Bacterial ribosomes are slightly smaller and instead have 50S and 30S subunits. The 50S ribosomal subunit has a molecular weight of 1.45 MDa and contains two RNA chains plus 31 to 35 proteins. These two RNA chains, 23S and 5S, contain approximately 3000 nucleotides total. The protein exit
tunnel and PTC are important features of the large subunit. Two of the main differences essential for antibiotic binding selectivity between the 50S ribosomal subunit and the 60S ribosomal subunit consist of single nucleotide switches from adenine in prokaryotes to guanine in eukaryotes. The decreased hydrogen bonding capacity of guanine makes antibiotic binding unfavorable in eukaryotes. The 30S ribosomal subunit is composed of only one RNA chain and between 20 and 21 proteins. These proteins, plus the approximate 1500 nucleotides that make up the 16S RNA chain, give the small subunit a molecular weight of 0.85 MDa. The decoding center is the central feature of the small subunit, making it responsible for translation fidelity. The 50S and 30S subunits are held together in an active ribosomal complex by flexible components of both subunits, which form intersubunit bridges, as well as by tRNA molecules that associate with both subunits during protein synthesis (as reviewed by Yonath, 2005).

Each of the important features of the bacterial ribosome is a potential area of attack for antibiotics. These targets include: the decoding site, the PTC, the protein exit tunnel, and the mRNA threading and advancement machinery. The decoding center has two prominent features, the upper portion of helix H44 and helix H27. The upper region of H44 is responsible for almost all of the intersubunit contact in the assembled ribosome. If a compound other than the 50S subunit bound to H44, it would block assembly of a functional ribosome. H27, on the other hand, is a mobile component of the ribosome. It is referred to as the switch helix, because it undergoes conformational changes that play a role in tRNA and mRNA progression. The ribosomal catalytic site, containing the PTC, is similarly important for tRNA and mRNA progression. Both sideways and rotary motions are
necessary for translocation within the catalytic site, and as such, the delicate machinery can easily be disrupted. In addition, it is also the site of peptide-bond formation. If any substrate binding is inhibited, peptide synthesis would fail. The exit tunnel is located below the PTC and spans the entire large subunit. It used to be thought of as a stationary and passive aspect of the ribosome; however, recent data suggests it is a dynamic participant contributing to elongation discrimination and protein folding (as reviewed by Yonath, 2005).

Protein Synthesis Inhibitors

![Chemical structure of streptomycin, tobramycin, erythromycin, and amikacin.](image)

Figure 4: Chemical structure of A: streptomycin, B: tobramycin, C: erythromycin, and D: amikacin.

The antibiotics studied in this project included streptomycin, tobramycin, erythromycin, and amikacin (Figure 4). Previously published literature implicates inhibition of protein synthesis as a possible contributing factor to antibiotic tolerance (Nguyen et al.,
Bernier et al., 2015; Prax & Bertram, 2014; Cho et al, 2014; Amato et al., 2014). This was demonstrated by the tolerance induced by the lack of certain essential amino acids (Nguyen et al., 2011; Bernier et al., 2015), the tolerance displayed by auxotrophs (Bernier et al., 2015) and the tolerance associated with the persister phenotype, which is believed to be induced by protein synthesis inhibition (Penesyan et al. 2015; Prax & Bertram, 2014; Cho et al, 2014; Amato et al., 2014). Due to this overlap in protein synthesis inhibition, nutrient starvation, and tolerance, antibiotics targeting the ribosome and protein synthesis became the focus of this study. Specifically, streptomycin, tobramycin, and erythromycin were chosen for their commonality and amikacin for its relatively recent introduction to the market.

Protein synthesis inhibitors have many modes of action including: interference with proper coding, tRNA binding at the decoding center or PTC, minimization of ribosomal mobility, and blockage of the protein exit tunnel. Streptomycin, tobramycin and amikacin are aminoglycoside antibiotics. These bactericidal antibiotics contain a cyclohexane ring and amino sugars (Slonczewski & Foster, 2011; as reviewed by Yonath, 2005). At high concentrations, they bind irreversibly to a single amino acid of protein S12 in the 30S-mRNA-tRNA initiation complex (KEGG drug, 2016) preventing binding of the 50S subunit (Slonczewski & Foster, 2011). This leads to a total shut down of translation and cellular growth. In addition, at low concentrations, streptomycin and amikacin bind to the four nucleotides of 16S rRNA that forms part of the decoding A site (Slonczewski & Foster, 2011; KEGG Drug, 2016). This causes translation to become “sloppy” with lots of mistranslated protein sequences. These mistranslated polypeptides are often toxic to the cell. Amino
Acid binding sites are unique to prokaryotes due to just two A to G differences in ribosomal DNA sequence between prokaryotes and eukaryotes (as reviewed by Yonath, 2005). Tobramycin has also been seen to destabilize the bacterial membrane (KEGG Drug).

Erythromycin on the other hand is a macrolide antibiotic and targets the 50S subunit. These antibiotics have large lactone rings of 12-22 carbon atoms. They bind to the protein L15 and 23S rRNA in the peptidyltransferase cavity which triggers release of peptidyl-tRNA from the P site (KEGG Drug, as reviewed by Yonath, 2005). This ultimately prevents peptide bond formation, protein synthesis, and cell growth or replication (Slonczewski & Foster, 2011; as reviewed by Yonath, 2005).

*Antibiotic Interactions*

There are three basic drug interactions: additive, synergistic, and antagonistic. In essence an additive interaction is no interaction. It occurs when the effects of two drugs used together is equally effective as the sum of the effectiveness of using both drugs alone. A synergistic interaction is when two drugs used together have a larger than additive effect. Finally, an antagonistic interaction is when the combination yields a smaller than additive effect (Yeh et al., 2006). There are two subclasses of antagonistic interactions: antagonistic buffering, when one drug masks the effect of the other, and antagonistic suppression, when one drug counteracts the effects of the other leading to a higher growth rate than at least one of them alone (Yeh et al., 2006; Yin et al., 2014). The current dominant hypothesis underlying synergy is that synergistic pairs tend to have targets that are neighbors in underlying biological networks, for example successive steps in the same pathway or
components in two separate pathways that eventually contribute to the same end-product (Yin et al. 2014; Gonzales et al. 2015; Black, 1962). The antibiotic combinations I explored have the following published interactions in Lysogeny Broth media: synergistic between streptomycin and tobramycin, streptomycin and amikacin, and tobramycin and amikacin; additive interactions between erythromycin and tobramycin as well as erythromycin and amikacin; and an antagonistic suppressive relationship between streptomycin and erythromycin (Yeh et al., 2006).

Due to the recent rise in antibiotic resistant pathogens (Fernandes, 2006; Davies & Davies, 2010) and the many issues surrounding the quest for new antibiotics (Fernandes, 2006), many scientists have started revisiting antibiotic interactions in hopes of finding new treatment options. Synergistic antibiotic combinations, in particular, have clinical value as they allow for a stronger inhibitory effect at lower dosages when compared with single drugs (Mitosch & Bollenbach, 2014). One study achieved great success treating highly antibiotic resistant and lethal MRSA with a triple drug combination containing: cabapenems, penicillins and β-lactamase inhibitors (Gonzales et al., 2015). Despite the success of such regimens, many are wary of their effects on resistance evolution. Several studies show a correlation between rate of resistance adaptation and the degree of synergism between individual antibiotics (Mitosch & Mollenback, 2014; Hegreness et al., 2008). Other studies are more optimistic and rely on the logic that inhibiting multiple targets, despite the interaction, might delay resistance evolution, as it is harder to accumulate mutations for multiple targets rapidly (Cottarel & Wierzbowski, 2007). Under the same logic, antagonistic interactions are currently being looked at as a promising mechanism to delay or even
reverse resistance evolution. Two studies, performed by Ocampo et al. and Yin et al. in 2014, found that antagonism between antibiotics seems to delay the emergence of resistance in a lab setting and one study, performed by Chait et al. in 2007, showed that it could even help reverse the resistance that currently exists by selecting for the non-resistant population. Further research in vitro and in vivo and delineation of optimal concomitant antibiotic use could become essential for clinical practice, particularly with the antibiotics studied here. These antibiotics are efficacious, all of these antibiotics are available, commonly utilized, and approved for treatment for bacterial infections. Thus, research in multiple antibiotic interactions may have a significant clinical impact, and may improve and optimize patient care for difficult-to-treat infections.

Our Study

Changes in antibiotic interaction could have large implications in the clinical setting. For example, if studies currently being run in LB provide clear indication of possibly promising treatment options, it would be important to know these interactions are consistent across all environments. The only way to better understand these interactions is to start changing factors. Any of the environmental factors that affect cellular metabolism and growth could be studied, but our study focuses on changes in nutrient availability by growing E. coli in various growth media.

Lysogeny broth, or LB, media is a traditionally and commonly used media in which to study E. coli. This is partially due to the fact that LB is a convenient media where E. coli grows consistently. Additionally, it is such a common E. coli growth media that it provides a
direct link for comparison purposes across literature. However, it is also a rather arbitrary choice of study medium, particularly when you consider much of this research is intended for use in the human body. LB media’s composition differs from that of human tissues, for example blood, where bacteria are likely to cause infection. It is understood that factors such as pH, nutrient availability, salt, etc. cause significant changes in bacterial metabolism and growth. These changes in turn can affect antibiotic efficacy. Thus we hypothesize interactions may also be affected by changes in environmental conditions.

Media

Lysogeny broth (LB) has traditionally been used to study *E. coli* due to its convenience and thus *E. coli* growth in this media is well understood (Kasina et al., 1999). LB is a complex media made up of yeast cell lysate, peptone, and salt. Yeast extract (YE) and potato dextrose (PD) are also common media for microbial growth and study. YE is composed solely of the lysate of yeast cells and PD is made up of sliced unwashed and unpeeled potatoes combined with dextrose sugar. LB and YE contain all of the components of normal cells, including: lipids, proteins, amino acids, nucleic acids, and some carbohydrates. LB has further protein content in the form of added peptone. These media provide fairly diverse nutrient options; however, they tend to lack simple sugars. In contrast, PD is composed of only starches and sugars. This protein deficient environment is considerably different than those provided by YE and LB. While neither LB nor PD represent human tissues to a more accurate extent than LB does, they do provide differing nutrient environments in which to observe antibiotic efficacies and interactions.
Materials and Methods

*Media preparation and storage:* Lysogeny broth (LB), yeast extract (YE), and potato dextrose broth (PD) were prepared from Fisher Scientific-brand dehydrated powder. LB powder came in premeasured capsules that were dissolved in 1 L milliQ water. 23 g YE powder and 10 g NaCl, or 24 g PD powder and 10 g NaCl were dissolved in 1 L milliQ water. Media was sterilized and stored at room temperature in sealed bottles. LB and PD broth were used alone; additionally a combination of 90% lysogeny and 10% potato dextrose broth was utilized.

**Table 1:** Composition of various media.

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<td>LB</td>
<td>10 g peptone 140, 5 g yeast extract, 10 g NaCl</td>
</tr>
<tr>
<td>YE</td>
<td>Extraction of yeast cell contents</td>
</tr>
<tr>
<td>PD</td>
<td>Sliced unwashed and unpeeled potatoes and dextrose sugar</td>
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*Antibiotic preparation and storage:* Antibiotics were prepared in a 10 mg/mL concentration; 0.1 g streptomycin, amikacin, and tobramycin were each dissolved in 10 mL milliQ water. 0.1 g erythromycin was dissolved in 10 mL ethanol. Streptomycin, amikacin, and erythromycin were stored away from light at -37 degrees Celsius. Tobramycin was stored away from light at -4 degrees Celsius. New antibiotics prepared monthly to minimize freeze/thaw efficacy issues.
**Cell culture preparation and storage:** *E. coli* cell line, *Escherichia coli* K-12 mg1655, was stored in – 80°C freezer in 15% glycerol solution. When needed, single aliquot was removed and thawed. 20 μL thawed aliquot in 2 mL LB broth was incubated for 4 to 6 hours at 37°C, non-shaking. Culture was then diluted to $10^{-4}$ concentration to inoculate 96 well plate conditions.

**Basic assay structure:** Every assay included: negative control – media only, positive control – media plus 25 μL of $10^{-4}$ dilution *E. coli* culture, and testing conditions. Testing conditions were prepared by combining 25 μL of $10^{-4}$ dilution *E. coli* culture, between 5 and 100 μL antibiotics and medium of choice to bring the total volume to 1 mL. 100 μL was pipetted into 4 wells of a *Brand* 96 well plate for each condition. 96 well plates were sealed with parafilm, wrapped in tin foil, and placed in 37°C incubator shaking at 300 rpm for 18 hours.

**Determining single-drug concentrations:** A range of concentrations were tested for each drug. A concentration was chosen that reduced growth rate by a significant but nonlethal amount (approximately 10-40% inhibition).

**Drug/media interaction assay:** Streptomycin, tobramycin, erythromycin and amikacin were assessed individually and in a pairwise fashion at varying doses. The *E. coli* dilution remained constant throughout all experiments. These experiments were assessed across various media.
**pH assay:** 40 μL of each positive control condition (media + *E. coli*) pipetted onto Fisher Scientific pH strips prior to 96 well plate inoculation and color change was noted and recorded. After 18 hours and plate reading, 40 μL of each positive control condition pipetted from first well onto pH strips. Again color change was noted and recorded.

**Reading plates:** 96 well plates were read after 18 hours of incubation in 37°C incubator shaking at 300 rpm. Plates’ population density was measured with VERSA max microplate reader produced by Molecular Devices to determine amount of bacteria present in each 100-μl culture. Optical density readings were taken at wavelength 600 after shaking once and testing conditions were compared to positive and negative controls.

**Statistics:** Replicates from each plate were averaged into one data point per condition per plate. Negative control (media only) optical density was subtracted from positive control and each testing condition with coinciding media. Growth in testing conditions was then calculated as percent growth of the positive control’s uninhibited growth. GraphPad Prism4 statistics and graphing software was used for statistical analysis and figure generation. Specifically repeated measures ANOVA was used to assess the significance of differences in percent inhibition for each antibiotic individually in the three different preparations of broth studied at different concentrations of antibiotic, the expected additive combination value, and actually experimental combination value. Tukey’s multiple comparisons test was then used to determine the significance of specific interactions therein. *P* values less than 0.05 were considered significant.
Results

In order to begin pair-wise drug interaction trials, appropriate antibiotic concentrations for each drug had to be determined in each media. Experimental single drug concentrations were chosen that decreased *E. coli* cell growth by a significant but nonlethal amount, approximately 10-40% inhibition (Fig 5, 6, 7). This range was crucial in order to differentiate between synergistic and additive effects. If the predicted additive effect was already greater than 100% inhibition, it would be impossible to distinguish synergy. These drug concentrations (Table 2) were then utilized in the experiments measuring the relationship between multiple antibiotic exposures, which were the primary outcome measure for this research. Streptomycin and amikacin concentrations for LB media were received from colleague Donahey, G., from unpublished data.

![Figure 5: Mean ± SE for percent inhibition for various antibiotic concentrations in LB media (N = 3).](image-url)
Figure 6: Mean ± SE for percent inhibition for various antibiotic concentrations in PD media (N = 3).

Figure 7: Mean ± SE for percent inhibition for various antibiotic concentrations in YE media (N = 3).
Table 2: Antibiotic concentrations used in antibiotic interaction assays.

<table>
<thead>
<tr>
<th>Media</th>
<th>Antibiotic</th>
<th>Concentration [μg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Streptomycin</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Tobramycin</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td>0.6</td>
</tr>
<tr>
<td>PD</td>
<td>Streptomycin</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Tobramycin</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td>6</td>
</tr>
<tr>
<td>YE</td>
<td>Streptomycin</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Tobramycin</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*E. coli* was grown in 96 well plates for 18 hours to determine the efficacy and interactions between streptomycin, tobramycin, erythromycin, and amikacin. Interactions were assessed in LB, YE, and PD media with the concentrations displayed in Table 2. In LB, the experimental combination value differed from the expected additive value for the combinations of streptomycin and tobramycin, streptomycin and amikacin, and tobramycin and amikacin (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3). Each of these combinations showed synergistic interactions, where combination treatment inhibited almost twice as much as would be expected if the effects were additive. The combinations of erythromycin with each of the other three antibiotics provided experimental values were less than the additive in each case; however, these effects were not significant (Figure 8).
**Figure 8:** Mean ± SE for percent inhibition of antibiotics individually, and in pairwise combinations, in LB media. The additive bar is what would be anticipated to occur, if the two antibiotics studied worked via an additive interaction, and is a calculated measure. The experimental bar on the far right is the experimental value obtained when the two antibiotics were placed together in media. Green bar represents a synergistic interaction and purple an additive interaction. S stands for streptomycin, T for tobramycin, E for erythromycin, and A for amikacin (N=3 plates with 4 wells each).
In YE, the combinations of amikacin with both streptomycin and tobramycin had significantly greater experimental inhibition than would be expected if the effects were additive (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.001, N = 3), showing synergistic interactions. In addition, the combination of amikacin and erythromycin had significantly less experimental inhibition than would be expected if the effects were additive (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3), exhibiting an antagonistic interaction. This antagonistic interaction can be further classified as antagonistic buffering as the experimental combination value does not differ significantly from either antibiotic alone (Tukey’s Multiple Comparison’s Test, df = 3,2, p > 0.05, N = 3). The combinations of streptomycin and tobramycin as well as erythromycin with both streptomycin and tobramycin provided experimental values were less than the additive in each case; however, these effects were not significant (Figure 9).
Figure 9: Mean ± SE for percent inhibition of antibiotics individually, and in pairwise combinations, in YE. The experimental bar on the far right is the experimental value obtained when the two antibiotics were placed together in media. Green bar represents a synergistic interaction, purple an additive interaction, and red an antagonistic interaction. S stands for streptomycin, T for tobramycin, E for erythromycin, and A for amikacin (N=3 plates with 4 wells each).
The concentration of antibiotics used in PD testing was 10 times that used in LB conditions. Although these concentrations achieved the desired inhibition during the previously performed antibiotic concentration determination assays, these results were not seen in the antibiotic interaction assay. In contrast, *E. coli* grown in PD showed little to no inhibition due to any of the antibiotics. There were no significant differences between the experimental combination inhibition and the expected additive inhibition in PD (Repeated Measures ANOVA, $df = 3,2$, $p > 0.05$, $N = 3$). As such, all interactions were additive (Figure 10).
**Figure 10:** Mean ± SE for percent inhibition of antibiotics individually, and in pairwise combinations, in PD. The experimental bar on the far right is the experimental value obtained when the two antibiotics were placed together in media. Green bar represents a synergistic interaction, purple an additive interaction, and red an antagonistic interaction. S stands for streptomycin, T for tobramycin, E for erythromycin, and A for amikacin (N=3 plates with 4 wells each).
Antibiotics were not equally effective in all media types (Figure 11). *E. coli* grown in PD media, in particular, showed extremely high tolerance to antibiotic inhibition.

**Figure 11:** Antibiotic concentrations used in antibiotic interaction assays. Orange bars represent concentrations for LB media, yellow bars are for PD media, and pink bars are for YE media.

It was noted that the overall uninhibited growth in LB and YE seemed to be greater than that in PD. To determine whether the increased tolerance seen in PD media was the result of this overall deceleration in cellular growth or to the nutritional contents of PD media, LB was diluted with PD at intervals of 10%. If the antibiotic tolerance was based
solely on the decreased growth rate in PD media, we would expect high levels of tolerance to coincide with a dilution that exhibited significant reduction in growth rate. However, if this tolerance was instead dependent on other factors having to do with the composition of PD media, we could see signs of tolerance at any dilution.

When 100% PD was compared to 100% LB in this follow-up study, the overall uninhibited growth was, in fact, significantly less than that in LB media (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.0001, N = 3). Uninhibited growth differed significantly between 100% LB media at every dilution (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3, Figure 12 A). Antibiotic concentrations used in this assay were four times those used in the 100% LB assay. Inhibition due to streptomycin was less than that at 100% LB for each dilution; however, the decrease was not significant until 50% LB/50% PD media (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3). Inhibition due to streptomycin and tobramycin was less than that at 100% LB for each dilution; however, the decreases were not significant until 50% LB/50% PD media (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3). Inhibition due to erythromycin was significantly less than that at 100% LB for every dilution (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.001, N = 3). Inhibition due to amikacin was less than that at 100% LB for each dilution; however, the decreases were only significant for 60% LB/40% PD, 40% LB/60% PD, and 20% LB/80% PD media (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3, Figure 12 B). Although the first signs of significant tolerance did not appear to coincide with a major decrease in uninhibited growth, which would point towards media components being the larger contributing factor, these data were not satisfactorily conclusive.
Figure 12: A: Mean ± SE for uninhibited growth in positive controls for dilutions of LB with PD. B: Mean ± SE for percent inhibition under testing conditions with each antibiotic for dilutions of LB with PD (N=3 plates with 4 wells each).
Starting and final pH was noted to track changes made throughout incubation to get an increased idea of *E. coli*'s active metabolic processes therein. Many metabolic pathways have specific byproducts that are excreted into the environment and effect pH, such as, the catabolism of proteins, which releases basic byproducts that increase pH (Burstain et al., 2003)). Starting pH for LB, YE, and 90% LB/10% PD media was relatively similar and almost neutral, at approximately pH 6. It was discovered that the starting pH of PD media, approximately 4, was more acidic. Final pH increased for LB, YE, and 90% LB/10% PD media, but remained constant for PD media, as well as, remaining constant, or even decreasing, for all other dilutions. This suggests protein breakdown was occurring in the LB, YE, and 90% LB/10% PD but not in PD or any other dilutions. For specifics see Table 3.

**Table 3.** Starting and final pH data for all media types used in this experiment.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Starting pH</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% LB</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>YE</td>
<td>6.5</td>
<td>8</td>
</tr>
<tr>
<td>100% PD</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>90% LB/10% PD</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>80% LB/20% PD</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>70% LB/30% PD</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>60% LB/40% PD</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>50% LB/50% PD</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>40% LB/60% PD</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>30% LB/70% PD</td>
<td>4.5</td>
<td>4</td>
</tr>
<tr>
<td>20% LB/80% PD</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>10% LB/90% PD</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
In order to further parse out the effects of media nutrient conditions, overall growth rate, and pH, drug interaction assays were run using 90% LB/10% PD. 90% LB/10% PD media most closely resembled LB, our control media. Although 90% LB/10% PD had significantly less growth than 100% LB, its growth was much more similar to that of 100% LB than to that of 100% PD. In addition, its starting and final pH’s were identical to those of 100% LB. The antibiotic concentrations used in this antibiotic interaction assay were twice the antibiotic concentrations used in LB tests. The experimental combination value differed from the expected additive value for the combinations of amikacin with each other antibiotic (Tukey’s Multiple Comparison’s Test, df = 3,2, p < 0.05, N = 3). Each of these combinations showed synergistic interactions where combination treatment inhibited up to about five times as much as would be expected if the effects were additive. While the combination of streptomycin and tobramycin produced experimental inhibition that was greater than expected if the effects were additive, this effect was not significant. The combinations of streptomycin and erythromycin and tobramycin and erythromycin produced experimental values that were less than the additive in both cases, but these effects were again not statistically significant (Figure 13).
**Figure 13:** Mean ± SE for percent inhibition of antibiotics individually, and in pairwise combinations, in 90% LB and 10% PD media. The additive bar is what would be anticipated to occur, if the two antibiotics studied worked via an additive interaction, and is a calculated measure. The experimental bar on the far right is the experimental value obtained when the two antibiotics were placed together in media. Green bar represents a synergistic interaction, purple an additive interaction, and red an antagonistic interaction. S stands for streptomycin, T for tobramycin, E for erythromycin, and A for amikacin (N=3 plates with 4 wells each).
Some of the interactions between tested pairs of antibiotics differed with media and others remained constant (Table 4).

**Table 4:** Antibiotic interactions for all tested pairs in each type of media. S stands for streptomycin, T for tobramycin, E for erythromycin, and A for amikacin. Syn stands for synergistic, Add for additive, and Ant for antagonistic.

<table>
<thead>
<tr>
<th>Antibiotic combination</th>
<th>LB</th>
<th>YE</th>
<th>PD</th>
<th>90% LB/10% PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>S+T</td>
<td>Syn</td>
<td>Add</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>S+E</td>
<td>Add</td>
<td>Add</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>S+A</td>
<td>Syn</td>
<td>Syn</td>
<td>Add</td>
<td>Syn</td>
</tr>
<tr>
<td>T+E</td>
<td>Add</td>
<td>Add</td>
<td>Add</td>
<td>Add</td>
</tr>
<tr>
<td>T+A</td>
<td>Syn</td>
<td>Syn</td>
<td>Add</td>
<td>Syn</td>
</tr>
<tr>
<td>E+A</td>
<td>Add</td>
<td>Ant</td>
<td>Add</td>
<td>Syn</td>
</tr>
</tbody>
</table>

**Discussion**

We found that at least some antibiotic efficacies and interactions appear to be dependent on the environmental nutrient conditions *E. coli* is grown in (Figure 11 & Table 4). The differences in antibiotic efficacies are most apparent when comparing LB and PD media (Figure 11). The antibiotic concentrations used in PD were 10 times those used in LB, and even with such high concentrations inhibition was minimal and inconsistent (Figures 5 & 10). When studying the YE results tolerance appears more varied between the antibiotics tested (Figure 11). Tolerance was relatively high for amikacin (four times that of LB) and tobramycin (six times that of LB), but relatively low for streptomycin (twice that of LB) and
erythromycin (the same as LB). In addition, LB, YE and PD each showed varied antibiotic interactions for at least one drug interaction (Table 4).

There could be several causes for the observed discrepancies between media. The first is the apparent slowed uninhibited growth rate in PD when compared to LB or YE (Figure 12). The antibiotics studied here bind to the ribosome and inhibit protein synthesis. As such, it would make sense for E. coli exhibiting slower growth rates to show less inhibition, as slower growth rates are indicative of less protein synthesis. This leaves our antibiotics with fewer protein synthesizing ribosome targets to inhibit (Amato et al, 2015). This seems like an unlikely primary cause; however, because E. coli grown in YE and LB respectively had very similar uninhibited growth but expressed different antibiotic tolerances and different antibiotic interactions. In addition, as noted previously, during the serial dilutions experiment (Figure 12), increases in tolerance did not correspond to a large decrease in uninhibited growth. This suggests that something other than growth rate is affecting tolerance. Finally, 90% LB/10% PD had similar uninhibited growth to LB, approximately 80% growth compared to LB’s, and more than three times the uninhibited growth of PD (Figure 12). While the interactions in this hybrid media more closely resemble those in LB than PD, the interaction between streptomycin and tobramycin differed (Table 4) and E. coli grown in this media showed increased antibiotic tolerance, particularly for erythromycin and amikacin (Figure 12).

The second possible contributing factor is the differences in pH observed between the media types. The most drastic of which is PD’s relatively acidic pH when compared to
the relatively neutral pHs of LB and YE (Table 3). It is understood that pH plays a significant role in both enzyme activity and overall cellular growth (Gale & Epps, 1945; Conner & Toltrola, 1995). Thus, these discrepancies in starting pH could be a major contributing factor for the observed variations. In addition, the final pH of LB and YE was greater than that of the starting pH. As this is an indicator of protein catabolism, it reveals this metabolic process could be active in E. coli grown in LB and YE, while the lack of pH change in PD suggests protein catabolism is not occurring therein. This is an attractive option as a contributing factor but is probably not the soul contributor. 90% LB/10% PD had identical starting and final pHs to LB but still showed signs of increased tolerance (Figure 12) this indicates that at least some of this change is due to something other than pH. It does appear that the synergistic interactions between amikacin and both streptomycin and tobramycin were restored in 90% LB/10% PD as opposed to PD alone; however, the synergy between streptomycin and tobramycin was not restored and a novel synergy between erythromycin and amikacin was observed (Table 4). In addition, YE also had a very similar starting and final pH to LB and showed similar increases in pH throughout incubation (Table 4), suggesting a comparable amount of protein catabolism. However, it too showed antibiotic interactions (Table 4) and efficacies (Figure 11) that varied from those in LB. Thus, it appears neither starting pH, nor the presence of active protein break down is solely responsible for the observed variations between media types.

The remaining difference between the media, that could be culpable, is differences in the nutritional content of these media. Although YE and LB are extremely similar media, containing yeast cell lysate i.e., lipids, proteins, amino acids, nucleic acids, carbohydrates
etc., LB has added protein in the form of peptone. In contrast, PD is made up almost entirely of sugars and starches. Thus, differences between these media can be looked at in the form of deficiencies or richness. LB and YE are rich in protein and many other nutrients, including: lipids and nucleic acids, but deficient in sugars, while PD is rich in sugars but deficient in just about every other nutrient. It is possible that either the presence or the absence of metabolites could be responsible for the variation in antibiotic efficacies and interactions.

Sugar and nitrogen starvation are both serious environmental stressors for \textit{E. coli}. Reactions to these different starvation conditions trigger the activation of different genes, leading to vastly different transcription panels and highly nuanced responses to these different, but similar, threats (Hua et al. 2004). In addition to changes in overall gene transcription, very specific stress response pathways are activated by starvation. Some of these pathways include the triacylglycerol (TAG) and stringent (SR) responses (White, 2007). The TAG response is activated by changes in cellular carbon fluxes (Sedwick, 2011, White, 2007). It initiates the preparation for and entry into a state of cellular inactivity (Baek et al., 2015). In this state, little to no growth occurs and cells suppress metabolic activity. The SR response is a common bacterial response to carbon, amino acid, and iron starvation (White, 2007, Nguyen et al., 2011). In contrast to the reduced activity triggered by the TAG response, the SR response leads to the reallocation of cellular resources to increase protein synthesis of factors crucial for stress resistance, glycolysis, and amino acid synthesis at the expense of the production of DNA, stable RNA, ribosomal proteins, and membrane components (Dalebroux & Swanson, 2012). Previous studies suggest that nitrogen
starvation, in particular, can lead to increased tolerance to many harmful environmental factors, including antibiotics. This is achieved by the slowing of protein synthesis, because if no amino-acid building blocks are present, the cell cannot synthesize proteins (Nguyen et al., 2011; Bernier et al., 2015; Penesyan et al., 2015; Prax & Bertram, 2014; Cho et al., 2014; Amato et al., 2014).

The metabolism of sugars and starches varies immensely from the metabolism of proteins and amino acids (White, 2007; Slonczewski & Foster, 2011). These differences in metabolism could be responsible for the variations between PD and both LB and YE observed in this study. The differences between LB and YE were more surprising due to their relative similarity in nutritional content; however, metabolic processes are exceedingly complicated and it is possible that *E. coli* grown in YE have vastly different metabolic profiles than those grown in LB. If this were the case, underlying metabolism could be a prominent contributor to the observed changes in antibiotic efficacy and interaction. In addition, bacteria have extensive networks in place that sense their environments. These complicated sensory mechanisms activate pathways that produce changes in bacterial gene regulation, proteome, metabolism, physiology and behavior (Szurmant & Ordal, 2004).

Because differences in antibiotic interactions and efficacies exist between 90% LB/10% PD, it is possible that something in the PD media, most likely sugar, is acting as a signaling molecule. With such a small dilution between 100% LB and 90% LB, it would seem unlikely that the media is missing much of what is present in LB. Additionally, pH values indicate that protein catabolism is occurring in both 90% LB/10% PD and 100% LB making protein starvation an unlikely explanation for the variations between the two; however, even a
small addition of sugar could cause significant changes if it were a signaling molecule. If this turned out to be the case, it is unclear why the detection of sugar would lead to changes that result in increased antibiotic tolerance, as it would be expected that sugar would simply be used as a nutritional source and not an indicator of stressful conditions.

Although discrepancies in antibiotic efficacies and interactions were noted in this study and conjectures were made regarding their causes, more research would need to be performed to truly understand the mechanisms underlying these changes. In order to continue parsing out the differences between the media studied here, I would start with PD media and try to run similar experiments while controlling pH. If results were the same as seen here, further work could be done looking into the effects of adding proteins into this media. It would also be interesting to dilute YE with PD in order to see if this produced similar results to the dilutions of LB with PD. However, the use of fully defined minimal media would allow for the best tracking of specific changes in nutrition. Once a specific metabolite is identified as either a trigger for tolerance or changed interaction, *E. coli* mutants, deficient in certain metabolic processes, could be used to further understand the effects of underlying active metabolic pathways. In addition, YE and PD media were explored in this study and while they were useful in identifying media’s effect on antibiotic efficacy and interactions, they do not reflect human tissues any more accurately than LB. In order to get the most clinically relevant data, experiments should be run in media that best approximates human tissues. The easiest of these might achieve might be blood as it is already liquid and could easily be adapted as a growth medium. However, because *E. coli* most often causes infections of the urinary and digestive tracts, it might be worthwhile to
create media with the same components as are found in these locations. These next steps are crucial for the increased understanding of antibiotics and their interactions and for responsible patient care.

These findings could have serious implications for use of drug combinations as drug treatments. Not all microenvironments within the human body have identical nutrient make-up. If the interactions antibiotics are reported to have in one environmental condition change under another, reckless prescription of combinations could lead to a serious adverse reaction. Further research is necessary to more fully understand antibiotic interactions and antibiotics in general. This study only slightly scratched the surface by manipulating nutritional content. Every other environmental factor that triggers bacterial stress responses, effects growth, or changes bacterial metabolism could have an effect on antibiotic efficacy and interactions that are currently taken for granted.

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