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Effects of Altering Physiologically Relevant Cholesterol Levels and Media Types on Porin Gene Expression in Klebsiella pneumoniae and the Resulting Impact on Antibiotic Resistance

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Effects of Altering Physiologically Relevant Cholesterol Levels and Media Types on Porin Gene Expression in *Klebsiella pneumoniae* and the Resulting Impact on Antibiotic Resistance

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THESIS/DISSERTATION CERTIFICATE OF APPROVAL

The thesis of Megan Camden is approved by

Dr. Terri Ellis, Committee Chair	Date
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Dr. Candice Tahimic	Date

Dedication

To my mom, for always being my number one fan. To my stepdad for inspiring me to push past everything that could easily hold me back. To my dad for giving me encouragement from Heaven. To my grandparents, siblings, and friends for supporting me, even if that meant less time with you. To my husband, for being by my side in both good and bad. To my puppies, for being my unfailing emotional support. And to God for putting all these amazing people in my life, and for giving me opportunities I could only dream of.

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Abstract

Klebsiella pneumoniae is a Gram-negative, non-motile bacterium belonging to the *Enterobacteriaceae* family. *K. pneumoniae* is a rising threat in the clinical setting, as there has been a large increase in the presence of antibiotic resistant isolates. While much research is conducted on laboratory and clinical strains of bacteria, not much is known regarding the impact that human physiology can have on bacterial gene expression, and in response, to antibiotic susceptibility. The goal of this study is to determine if physiologically relevant cholesterol levels and media types impact porin gene expression and antibiotic resistance in *K. pneumoniae*. To accomplish this, quantitative-PCR and minimum inhibitory concentration assays were utilized. The results of this study indicate that physiologically relevant levels of cholesterol and media types can have several significant effects on porin gene expression and on antibiotic resistance. Firstly, laboratory strain *K. pneumoniae* was more impacted by changes in cholesterol than clinical strain *K. pneumoniae* regarding changes to antibiotic susceptibility; however, expression varied largely across all cholesterol concentrations and genes. Secondly, gene expression was downregulated for all six tested genes in the physiologic media compared to the laboratory media; also, the physiologic media often had significantly lower antibiotic susceptibility than the laboratory media. Lastly, it was found that surfactant can have significant effects on both gene expression and antibiotic susceptibility analyses. The results indicate that physiological conditions can impact gene expression and antibiotic susceptibility. Hence, careful consideration should be given to experiments designed to mimic physiological conditions.

Chapter One

Introduction

Introduction

Outer Membrane Structure Can Contribute to Increased Bacterial Resistance to Antibiotics

Gram-negative bacteria possess an inner and an outer membrane, as shown in **Figure 1**. These outer membrane components surround a thin-layer of peptidoglycan, which helps prevent the lysis of cells, due to the surrounding environment often being hypotonic as compared to the internal cellular environment [1]. Lipopolysaccharides (LPS) act to maintain a barrier, preventing hydrophobic molecules from crossing the outer membrane through passive diffusion [2]. LPS composes much of the outer-leaflet of the outer membrane. The phospholipids compose much of the inner leaflet of the outer membrane, forming a bilayer, which is an essential component for retaining structure of the outer membrane. This component of the membrane is also needed for fluidity, and selective permeability [3]. The outer membrane can also contain cholesterol, but it is not required (**Figure 1.1**). Bacteria are unable to synthesize cholesterol; Meaning, bacterial species that utilize cholesterol in the outer membrane must acquire cholesterol from secondary sources [4]. These secondary sources are often the surrounding environment and, if applicable, host cells [5]. Cholesterol can change the structure, stability, and fluidity of the outer membrane [6]. Outer membrane proteins, which includes porins, are necessary for maintaining membrane structure, defense against outside threats, and the acquisition of nutrients and other vital components [7]. Outer membrane proteins are positioned such that the proteins transverse the outer membrane or through both the outer- and inner membrane.

Porins are transmembrane beta-barrel proteins which allow for the passive transport of small hydrophilic molecules through the outer membrane of Gram-negative bacteria, and into the periplasmic space [8]. Beta-lactam antibiotics are among the molecules able to transverse the

membrane through porin structures. However, bacteria with mutations in porin genes can show differential porin expression, with some bacteria downregulating or upregulating the genes, and others not possessing certain porins at all [9]. This allows for less of the antibiotic to cross the membrane and enter the bacterial periplasm.

Porin genes are of particular importance when evaluating antibiotic resistance in *K. pneumoniae* due to the frequent loss of porins in antibiotic-resistant strains [10,11]. The loss of porins can be beneficial due to most porins being the method through which antibiotics transverse the outer membrane and enter the periplasmic space [8]. The porin genes of highest importance, with regards to the development of antibiotic resistance are *ompF* and *ompC* in *Escherichia coli*, or the homologs of these genes *ompK35* and *ompK36* in *Klebsiella pneumoniae,* respectively [10,12].

OmpK35 and OmpK36 have 35kDa and 36kDa molecular weights, respectively, and are non-specific. One study found that OmpK35 and OmpK36 possess larger and more permeable channels than their OmpF and OmpC homologs. OmpK35 specifically was more permeable to lipophilic and large compounds, when compared to OmpK36, OmpF, and OmpC [10]. Expression of one porin over the other has been shown to provide differential survival rates depending on the conditions of exposure [13,14] . Ultimately, there can be a cost to porin-loss, which is often lower nutrient acquisition [15]. However, porin-loss can reduce the ability of applicable antibiotics from entering the periplasm.

OmpK26 is a porin that is similar in structure to OmpK35 and OmpK36 but is 26kDa in size. This porin is not utilized for the passage of antibiotics and is only utilized for oligogalacturonate passage. This porin is often not active under normal conditions but is often seen expressed in strains that are deficient in OmpK35 and/or OmpK36 [16].

OmpA is a beta-barrel porin that has many functions, including immune evasion and outer membrane stabilization through anchoring of the outer membrane to the peptidoglycan layer [17–19]. OmpA has been highly conserved among members of the *Enterobacteriaceae* family. This porin is 40kDa in size [20]. OmpA is important for helping the bacteria evade the immune system, which has been shown in both *in vitro* and *in vivo* models [19].

Lpp is a murein lipoprotein, and is important to the capsule retention, with deletion mutants showing reductions in resistance to complement, with a complete loss of the ability to grow in serum [21]. This porin is approximately 6kDa in size [22]. Lpp is important in cell envelope stability, with Lpp mutants also showing an increase in the periplasmic protein leakage, as well as the increased production of outer membrane vesicles [23].

LamB is porin that is utilized for the uptake of maltose and maltodextrins but is permeable to carbapenem antibiotics [24,25] . This porin is approximately 46kDa in size [26]. One study showed that LamB, when combined with the loss of OmpK35 and/or OmpK36, increased resistance to various classes of antibiotics. Specifically, the isolate expressing neither OmpK35 nor OmpK36 showed 2-fold expression of *lamB* [27].

The various effects of the alternate porins (OmpK26, OmpA, Lpp, and LamB) allows for evaluation of the impacts of different environmental conditions, such as changes in cholesterol concentration, on gene expression relative to changes in the essential porins (OmpK35 and OmpK36) in *K. pneumoniae* cells. Changes in porins have also been shown to impact other virulence factors, such as capsule and lipopolysaccharide, as well as survival within macrophages [28].

Porin-loss in *Klebsiella pneumoniae* has dramatic impacts on the effectiveness of antibiotics, specifically beta-lactam antibiotics [29–31] . Beta-lactam antibiotics act by binding penicillin-binding proteins, which are enzymes needed to complete peptidoglycan cross-linking [32]. Due to the peptidoglycan layer in Gram-negative bacteria being protected by the outer membrane, a loss of porins necessary for antibiotic transport can impact the effectiveness of beta-lactam antibiotics.

Porin-loss in bacteria is often seen clinically to be accompanied by another resistance mechanism. In *Klebsiella pneumoniae,* one such mechanism is the production of beta-lactamases, which are enzymes that hydrolyze the beta-lactam ring, making the antibiotic ineffective [29–32]. Bacteria previously exposed to beta-lactam antibiotics also can become producers of extended-spectrum beta-lactamases (ESBLs), which extend the ineffectiveness to more beta-lactamase antibiotics, even those newly developed [33]. The genes responsible for this are often located on plasmids and can horizontally transfer between members of the *Enterobacteriaceae* family [34]. Infections containing one or more of the mechanisms above have become common in hospital and clinical environments around the world [34,35].

The loss of porins results in a decrease in the ability of phagocytes to recognize and phagocytize the *Klebsiella pneumoniae* bacteria. The loss of one or more porin structures also leads to the development of other virulence factors, which could explain an increase in antibiotic resistance as well. There was a noted decrease in the production of capsule and an increase in the production of lipopolysaccharide. The loss of one or more porins also decreased oxidative burst by macrophages [36]. This is significant, as the findings reflect a differential change in survival between the porin-containing, and the porin-loss bacterial strains.

Pathology of *Klebsiella pneumoniae*

Understanding the background and pathology of *Klebsiella pneumoniae* is essential for understanding how physiological cholesterol levels and media types could impact this species with relation to porin expression and antibiotic susceptibility.

Klebsiella pneumoniae is a Gram-negative, non-motile bacteria, belonging to the family *Enterobacteriaceae*, which is of importance because it has shown an increase in antibiotic resistance in the last decade[37]. *Klebsiella* species are blamed for 8% out of all endemic hospital acquired infections in the United States and Europe [38]. Hospital-acquired infections (HAIs) infect nearly 2 million people annually in the United States, killing approximately 100,000 [39]. One major component that is significant to the pathogenicity of *Klebsiella pneumoniae* is its outer membrane.

Klebsiella pneumoniae is known for causing conditions such as urinary tract infections, liver abscesses, pneumonias, meningitis, and bacteremia. *Klebsiella pneumoniae* belongs to a group of pathogens, known as the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species). These pathogens are significant in their ability to exhibit multidrug resistance, cause high mortality risks, and have increased incidence of occurrence [40].

Klebsiella pneumoniae possesses the ability to utilize many different methods to increase virulence and pathogenicity. These factors can include becoming hypermucoviscous, forming biofilms, producing beta-lactamases or other enzymes, utilizing drug-efflux pumps, adjusting capsule and lipopolysaccharide composition and presence, producing siderophores and fimbriae,

as well as adjusting gene expression of other important factors such as porin gene expression [37,41].

As mentioned, *Klebsiella pneumoniae* can survive in numerous locations within the human body. Several of these locations could provide a potential source of cholesterol for Gram-negative bacteria. The average adult 20 years of age and older has a range of 125-200 mg/dL of cholesterol within the blood [42]. Due to the ability of *Klebsiella pneumoniae* to utilize cholesterol, it is possible for this species to utilize the cholesterol within human blood to alter the outer membrane structure. It is also possible that plasma derived from human blood, or synthetic media designed to mimic human plasma could have effects on outer membrane structure and on antibiotic susceptibility.

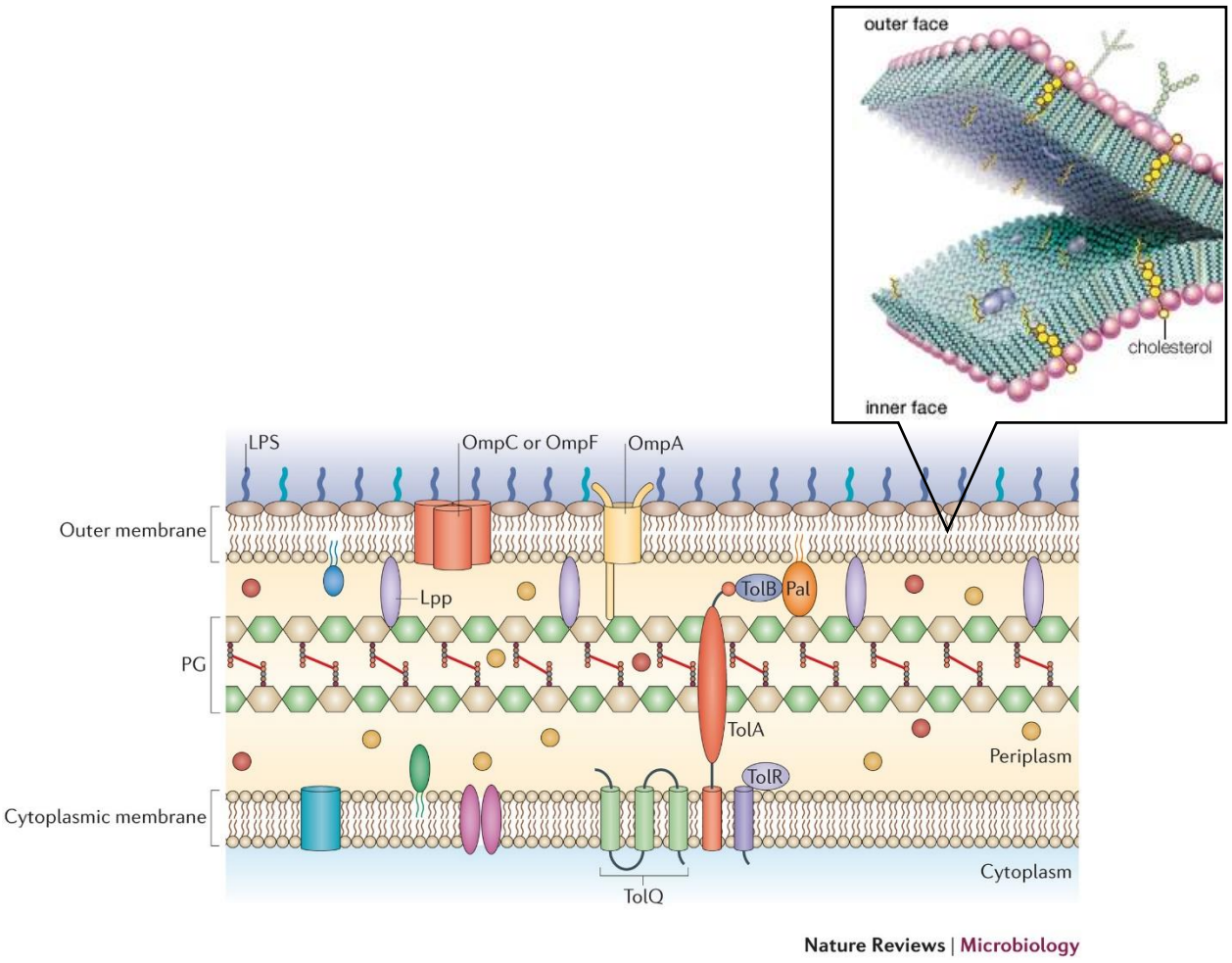


Figure 1.1. Outer Membrane Structure of Gram-Negative Bacteria [43]. Top Image Displays Zoomed-In and Separated Membrane Containing Cholesterol [44]. Image Shows Porin Structures OmpF, OmpC, and OmpA. The porin genes of highest importance, with regards to the development of antibiotic resistance are *ompF* and *ompC* in Escherichia coli, or the homologs of these genes *ompK35* and *ompK36* in *Klebsiella pneumoniae*, respectively [10,12] .

Chapter Two

Effects of Altering Physiologically Relevant Cholesterol Levels on Porin Gene Expression in *Klebsiella pneumoniae* and the Resulting Impact on Cephalothin Antibiotic Resistance

Introduction

Cholesterol May Impact Expression of Outer Membrane Genes and Antibiotic Susceptibility

The Gram-negative outer membrane can contain cholesterol, but cholesterol is not required. Bacteria are unable to synthesize cholesterol [4]. Bacteria that acquire cholesterol from host cells most often do so through direct contact between the bacterium and a mammalian host cell, or through outer membrane vesicles [45].

Cholesterol can change structure, stability, and fluidity of the outer membrane [6]. Often, bacterial membranes do not possess cholesterol at all, however one study in Gram-negative bacteria showed an absence of cholesterol made it easier for antimicrobial peptides (AMPs) to disrupt the membrane. Cholesterol increases membrane stiffness and cohesion, which may make the bending of the membrane utilized by many AMPs difficult [46]. In *Borrelia burgdorferi*, a Gram-negative spirochete, the presence of cholesterol and cholesterol glycolipids was significant to the formation of lipid rafts [47,48].

It was found that *Klebsiella pneumoniae,* in the presence of cholesterol on membrane-associated lipid rafts, increased macrophage-mediated phagocytosis, as well as decreased expression of *ompA* (10-fold decrease), *ompK35* (10-fold decrease), and *ompK36* (27-fold decrease) [49]. Similar results were found in *Escherichia coli*, by evaluating nine outer membrane proteins with beta-barrel structures [50]. The presence of cholesterol was concentration dependent, with protein-folding efficiency decreasing as concentration is increased [50,51].

Thesis Aims

Due to the prevalence of healthcare-associated infections, as well as the concerning increase in antibiotic resistant bacteria, evaluating the effects of cholesterol on porin gene expression and subsequent antibiotic susceptibility would fill a gap in this area. It is expected that as the concentration of cholesterol increases, porin gene expression will decrease due to the rigidity that cholesterol molecules can create within membranes [46]. If this occurs, then the overall membrane permeability should decrease, which should decrease antibiotic susceptibility. Also, assessing the impact that porin gene expression has on other outer membrane proteins in the presence of physiologically relevant levels of cholesterol would provide insight into the way *Klebsiella pneumoniae* responds to cholesterol levels through alterations in its outer membrane.

To evaluate how *Klebsiella pneumoniae* responds to physiologically relevant cholesterol levels *in vitro*, one laboratory strain and four clonally-related clinical isolates were individually exposed to one of five different cholesterol concentrations ranging from 25 mg/dL to 125 mg/dL. These concentrations were chosen due to their relevance to concentrations within human blood, the highest value being the bottom of the average cholesterol range in average adult human blood [42]. Levels below 15 mg/dL for low-density lipoprotein cholesterol (LDL-C) have been recorded in patients treated with alirocumab, which is in a class of medications called proprotein convertase subtilisin kexin type 9 (PCSK9) inhibitor monoclonal antibodies [52]. Following exposure to cholesterol overnight, gene expression and antibiotic susceptibility will be evaluated and compared.

It was hypothesized that as the levels of cholesterol increase, porin expression in *Klebsiella pneumoniae* would decrease and antibiotic susceptibility would decrease.

Materials and Methods

Bacterial Strains and Plasmids

Klebsiella pneumoniae clinical isolates were provided by Dr. Sebastian Alberti (University of the Balearic Islands, Palma de Mallorca, Spain) and were isolated from a single patient during a clinical outbreak in Spain [53]. All clinical isolates are clonally related. Isolate CSUB10R (10R) expresses neither OmpK35 nor Ompk36. CSUB10S (10S) expresses only OmpK36. The pSHA16K plasmid, carrying the *ompK35* gene was transformed into both clinical strains to create CSUB10R+pSHA16K and CSUB10S+pSHA16K. CSUB10R+pSHA16K and CSUB10S+pSHA16K were grown with 50 ug/mL of kanamycin (Fisher BioReagents, Pittsburgh, PA) for maintenance of the plasmid. These clinical isolates are extended spectrum β-lactamase (ESBL) positive [9,53].

Klebsiella pneumoniae laboratory strain ATCC 43816 was used for comparison (American Type Culture Collection). This strain is ESBL negative and contains no known resistance plasmids. Clinical and laboratory strain gene expression for *ompK35* and *ompK36* is shown in **Table 2.1**.

Growth Conditions

To mimic physiological cholesterol conditions, a water-soluble cholesterol mixture (Sigma-Aldrich, St. Louis, MO) was used. To allow for solubility of the cholesterol, the cholesterol was pre-mixed with the surfactant methyl-β-cyclodextrin (Sigma-Aldrich). The ratio of cholesterol to methyl-β-cyclodextrin is batch dependent, therefore controls were adjusted as needed. To create each culture, the appropriate concentration of water-soluble cholesterol was determined (25, 50, 75, 100, or 125 mg/dL) and added to Luria-Bertani (LB) broth (Difco,

Franklin Lakes, NJ). For later normalization, control LB broth was created with the amount of methyl-β-cyclodextrin equivalent to that in each water-soluble cholesterol culture. LB broth alone was used as an untreated control. All cultures were grown at 37℃ with 200 rpm shaking, overnight. This process was completed with the four clinical strains and the laboratory strain. CSUB10R+pSHA16K and CSUB10S+pSHA16K were grown additionally with 50 ug/mL of kanamycin (Fisher BioReagents) for maintenance of the plasmid.

Minimum Inhibitory Concentration (MIC) Assay

Overnight cultures were diluted to an optical density (OD) of 0.6 in LB broth with the same concentration of cholesterol as the overnight culture and added to a 96-well microtiter plate containing a 2-fold dilution series of cephalothin antibiotic (Thermofisher, Waltham, MA). LB only wells were used as positive control wells. Respective methyl-β-cyclodextrin control 2-fold dilution series of cephalothin antibiotic were ran as comparisons to the cholesterol series. Plates were incubated for 24 hours, then the absorbance was measured using a Biotek Gen5 Plate Reader at 630 nm (OD_{630}). The MIC50, MIC90, and MIC Breakpoint values were determined following Clinical and Laboratory Standards Institute (CLSI) guidelines [54,55]. Dilution plate counts were used to confirm the MIC50 values. If MIC50 was not reached, no MIC50 dilution plates were completed.

RNA Isolation, cDNA Synthesis, and qPCR

RNA was isolated from all cultures in mid-logarithmic phase. Samples were treated with RNA Protect (Qiagen, Germantown, MD) and proteinase K (New England Biolabs), then RNA was isolated using the RNeasy Minikit (Qiagen) following the instructions of the manufacturer. First-strand synthesis was completed using 1 µg of total RNA and the Protoscript II reverse

transcriptase kit (New England Biolabs, Ipswich, MA). Quantitative polymerase chain reaction (qPCR) was performed on the Eppendorf Mastercycler Realplex 2, using Luna Universal qPCR Master Mix (New England Biolabs). Data was analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Genes evaluated were *ompK35*, *ompK36*, *ompK26*, *ompA*, *lpp*, and *lamB*, then compared to the *gapA* gene for normalization. Primers are listed in **Table 2.2**.

Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. This was done, as opposed to evaluation of transcription levels in untreated cultures, after comparison of the impact of methyl-β-cyclodextrin to untreated cultures was determined to have a significant impact on expression All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. This process was completed for each cholesterol condition in *K. pneumoniae* ATCC 43816, and the clinical strains listed in **Table 2.1**.

Statistical Analysis

Experiments were completed with an $n \geq 4$. Significance was determined using a one-way ANOVA, followed by Tukey's post-hoc test. These analyses were completed using XLSTAT software. Significance was determined at $p < 0.05$.

Results

Surfactant Can Impact Gene Expression

Prior to evaluating the impacts of cholesterol on expression for each gene of interest, it needed to be determined if the surfactant used to solubilize the cholesterol, methyl-β-cyclodextrin, would impact expression. The results of this experiment show a significant

difference ($p < 0.001$) in expression of both *ompK35* and *ompK36* between the untreated *K. pneumoniae* ATCC 43816 cultures and the methyl-β-cyclodextrin 125 mg/dL cholesterol control cultures (**Figure 2.1**). All MBC treatments resulted in downregulation of expression of both genes. Therefore, all results were normalized to methyl-β-cyclodextrin controls as opposed to untreated controls.

Laboratory Strain MIC50 Values were Similar Between Treatments, While Breakpoint Values Indicated Differences

To evaluate the impact of cholesterol on antibiotic susceptibility, MIC assays were completed. The antibiotic utilized was cephalothin, a first-generation cephalosporin and beta-lactam antibiotic [57]. The mechanism of action of cephalothin is to inhibit cell wall synthesis. This occurs when the beta-lactam ring binds the penicillin-binding proteins, which are important for peptidoglycan cross-linking in the process of cell wall synthesis [58].

The average MIC50 values were similar between untreated *K. pneumoniae* ATCC 43816 and all MBC and cholesterol treatments. All treatments, including untreated, cholesterol treated, and MBC treated, had average MIC50 values of between 8 µg/mL and 12 µg/mL (data not shown). The similarity between these values indicates that neither cholesterol nor MBC impacts resistance at the tested levels.

Dilution plate counts were used to confirm MIC50 values found for all treatments as well as the untreated *K. pneumoniae* ATCC 43816 cultures. This was done due to the turbidity of the cultures as a result of the added cholesterol impacting the ability to rely solely on OD values. The plate counts found statistically similar values for all cholesterol, MBC, and untreated wells. The range (lowest to highest) was 9.06×10^5 CFUs/mL for the untreated culture and 9.31×10^5

CFUs/mL for the 125 mg/dL cholesterol culture (data not shown). Ultimately, a similar number of colony-forming units were able to grow within all tested MBC and cholesterol treatments when compared to the untreated control. This indicates that none of the treatments significantly changed the ability of the bacteria to grow at the MIC50 concentration.

The average MIC breakpoints for all MBC and cholesterol treatments were elevated above untreated controls, and most indicated a similar response (**Figure 2.2**). Breakpoints for all treatments except the untreated *K. pneumoniae* control were over the 32 μg/mL threshold for resistance per CLSI guidelines [54]. The increases being at similar treatment values indicated that MBC likely impacted the ability of the bacteria to be resistant to the cephalothin, and that the resistance seen here is not likely as a result of the cholesterol exposure. At the lowest dose for both the MBC control and the cholesterol, the MBC likely had a smaller impact on bacterial resistance. However, once the dose reached between the 50 mg/dL and the 75 mg/dL treatments, the impact of the MBC elevated to the maximum effect seen on resistance within this experiment. After that jump, the breakpoints remain similar between all treatments, meaning this was the range where the MBC reached its full effect, remaining there at all higher doses. The resistance values are similar between MBC and cholesterol, likely attributed to the MBC, not the cholesterol exposure.

Clinical Strains All Produced Similar Results Between Treatments for MIC50 and Breakpoint Values

To evaluate the impact of cholesterol on antibiotic susceptibility, MIC assays were completed. Cephalothin antibiotic was utilized. The average MIC50 values were similar between each clinical strain, but dissimilar to the untreated *K. pneumoniae* ATCC 43816 culture. For the clinical strains CSUB10R, CSUB10S, CSUB10R+pSHApSHA16K, and CSUB10S+pSHA16K

MIC50 values were not able to be determined, as the bacteria grew robustly at all concentrations of antibiotic for all treated and untreated cultures. The presence of the ESBLs in the four clinical strains contributes to the high resistance to cephalothin. MIC breakpoint values for the clinical strains were not reached for any of the cholesterol, MBC, or untreated clinical strain cultures for MIC assays in cephalothin and were therefore reported as >500 μg/mL.

Gene Expression Indicated Large Differences Between Cholesterol and MBC Treatments in the Laboratory Strain

To evaluate gene expression, six genes of interest, *ompK35*, *ompK36*, *ompK26*, *ompA*, *lpp*, and *lamB* were compared to *gapA*, which acted as the house-keeping gene. The average expression for both *ompK35* (**Figure 2.3**) and *ompK36* (**Figure 2.4**) showed trends of generally increased average expression for all treatments, with the only exception being a 1.5-fold decrease in average expression for the 25 mg/dL cholesterol treatment in *ompK35*. The trend in *ompK35* indicates that the lowest dose of cholesterol leads to the downregulation of this porin gene, but between the 25 mg/dL and the 50 mg/dL treatment of cholesterol, the cholesterol exposure produces a switch to upregulation within this gene (**Figure 2.3**). As the concentration of cholesterol increases, only the upregulation of *ompK35* only increases. This indicates that there is a positive effect of cholesterol on the regulation of *ompK35* once a certain threshold is reached somewhere between 25 mg/dL and 50 mg/dL of cholesterol. The trend for *ompK36* indicates that exposure to cholesterol causes upregulation of this gene at all concentrations, though there is not a notable stepwise pattern across doses (**Figure 2.4**). The largest increase was seen in the highest dose of cholesterol, with an over 4-fold increase. Aside from the 50 mg/dL treatment, the general trend indicates that as cholesterol dose increases, *ompK36* expression increases.

The average expression of *ompK26* (**Figure 2.5**) and *ompA* (**Figure 2.6**) showed a trend of generally decreased expression for all treatments, with the only exception being a 2-fold increase in average expression for the 125 mg/dL cholesterol treatment in *ompK26*. The trend for *ompK26* indicates that exposure to cholesterol causes statistically significant downregulation of this gene at the four lowest concentrations of cholesterol, but upregulation at the highest dose (**Figure 2.5**). Cholesterol exposure has a negative effect on this gene, but that effect switches to positive between 100 mg/dL and 125 mg/dL. There is not a notable stepwise pattern across doses. The trend for *ompA* indicates that exposure to cholesterol causes similar downregulation of this gene at all concentrations (**Figure 2.6**). This implies that cholesterol exposure leads to downregulation in *ompA*, but changing dose does not significantly impact the scale of the downregulation.

The average expression for *lpp* (**Figure 2.7**) and *lamB* (**Figure 2.8**) showed no consistent trends. In *lpp* both the 25 and 100 mg/dL treatments showed similar decreases in average expression, while both 50 and 75 mg/dL treatments showed similar modest increases in average expression, (**Figure 2.7**). There is not a remarkable trend regarding dose of cholesterol and regulation of *lpp*. There is a threshold effect in *lamB* regulation by cholesterol dose exposure (**Figure 2.8**). The lowest three doses of cholesterol show large downregulation within the gene, which then switches to upregulation for the highest two doses.

Clinical Strain Gene Expression Indicated Large Differences Between Treatments and Genes for Each Strain

The aim of this experiment was to assess if changes in the evaluated porin genes occurred in response to different concentrations of cholesterol and to see if there was any impact on genes within and across treatments for clinical strains with known resistance mechanisms and

differential porin gene expression. As with the laboratory strain, the cholesterol treatments for the clinical strains were compared to the respective MBC control treatment, not to an untreated culture because of surfactant on gene expression.

The average expression in *ompK35* showed a trend of increased average expression for CSUB10R+pSHA16K, except for a 2-fold decrease in average expression at 25 mg/dL of cholesterol (**Figure 2.9**). This trend was also seen in CSUB10S+pSHA16K, however there was an exception in both 25 and 50 mg/dL treatments, which both showed a 1-fold decrease in average expression.

The average expression in *ompK36* showed no trend between treatments for CSUB10S or CSUB10S+pSHA16K (**Figure 2.10**). CSUB10S showed increased expression with 50, 100, and 125 mg/dL of cholesterol, and decreased expression at 25 and 75 mg/dL. CSUB10S+pSHA16K showed the opposite pattern, with an exception at 125 mg/dL of cholesterol, which also produced an increase in expression. This indicates that at 25 mg/dL and 75 mg/dL of cholesterol, the absence of OmpK35 in strain CSUB10S led to downregulation of *ompK36*, while the presence of OmpK35 in strain CSUB10S+pSHA16K led to upregulation of *ompK36*. This pattern is then completely reversed at the 50 mg/dL and 100 mg/dL treatments.

The average expression in *ompK26* showed no trend within any of the clinical isolates or across the treatments (**Figure 2.11**). One notable point is at 75 mg/dL of cholesterol in strain CSUB10R. This point showed a nearly 6.5-fold decrease in average expression (**Figure 2.11**). While there is no trend across isolates or across treatments, it is worth noting that at the 25 mg/dL dose, the two strains lacking OmpK35 were upregulated, while the two strains possessing OmpK35 were downregulated. A similar trend is seen at the 75 mg/dL dose, where strains lacking in OmpK36 were downregulated and strains possessing OmpK36 were upregulated. At

the 50 mg/dL treatment, the only strain that was downregulated was CSUB10R+pSHA16K, which possesses OmpK35, but not OmpK36. At the 100 mg/dL treatment, the only strain not downregulated was the CSUB10R, which is lacking both OmpK35 and OmpK36. Interestingly, this pattern is reversed at 125 mg/dL of cholesterol.

The average expression in *ompA* showed no trend within isolates CSUB10R, CSUB10R+pSHA16K, or CSUB10S+pSHA16K. CSUB10S showed a general increase in average expression across treatments (**Figure 2.12**). While there is no trend across isolates or across treatments, it is worth noting that at 25 mg/dL of cholesterol only CSUB10S, which lacks OmpK35 and has OmpK36 is upregulated. At 50 mg/dL, only strain CSUB10S+pSHA16K, which possesses both porins is downregulated, while at 75 mg/dL only strain CSUB10R, which lacks both porins is downregulated. At the 100 mg/dL treatment, all treatments are upregulated, despite OmpK35 or OmpK36 being present or absent. At the 125 mg/dL treatment, both CSUB10R and CSUB10R+pSHA16K, which lack OmpK36 are downregulated for *ompA* expression, and CSUB10S and CSUB10S+pSHA16K, which both possess OmpK36 are upregulated for *ompA* expression.

The average expression in *lpp* showed a general decrease in average expression for all isolates across most treatments (**Figure 2.13**). The only exceptions to this pattern were for CSUB10S and CSUB10S+pSHA16K in the 25 mg/dL treatment, CSUB10R+pSHA16K and CSUB10S+pSHA16K in the 75 mg/dL treatment, and CSUB10S in the 100 mg/dL treatment. While the general trend is downregulation, which was seen for all strains using 50 mg/dL and 125 mg/dL of cholesterol, the exceptions are notable. At the 25 mg/dL dose, the two downregulated strains, CSUB10R and CSUB10R+pSHA16K, both lack OmpK36, while the two upregulated strains, CSUB10S and CSUB10S+pSHA16K, both possess OmpK36. At the 75

mg/dL dose, the two downregulated strains, CSUB10R and CSUB10S, both lack OmpK35, while the two upregulated strains, CSUB10R+pSHA16K and CSUB10S+pSHA16K, both possess OmpK35. And at the 100 mg/dL dose, only the strain CSUB10R+pSHA16K, which has OmpK35, but lacks OmpK36 was upregulated.

The average expression in *lamB* showed no trend within isolates CSUB10S, CSUB10R+pSHA16K, or CSUB10S+pSHA16K (**Figure 2.14**). CSUB10R showed a general decrease in average *lamB* expression across treatments. While there is no trend across isolates or across treatments, it is worth noting that at 25 mg/dL of cholesterol, the two strains lacking OmpK36, CSUB10R and CSUB10R+pSHA16K, were downregulated for *lamB* expression, and the two strains possessing OmpK36, CSUB10S and CSUB10S+pSHA16K were upregulated for *lamB* expression. At 50 mg/dL of cholesterol, only strain CSUB10S, which lacks OmpK35 but possesses OmpK36 is upregulated. At both 75 mg/dL and 100 mg/dL of cholesterol, the two strains that were downregulated for *lamB* expression are either missing both OmpK35 and OmpK36 (CSUB10R) or possess both porins (CSUB10S+pSHA16K). The two strains that were upregulated for *lamB* expression either have OmpK35 and lack OmpK36 (CSUB10S) or lack OmpK35 and have OmpK36 (CSUB10R+pSHA16K). At 125 mg/dL of cholesterol, the two strains that lack OmpK35, CSUB10R and CSUB10S, were downregulated for *lamB* expression, and the two strains that have OmpK35, CSUB10R+pSHA16K and CSUB10S+pSHA16K, were upregulated for *lamB* expression.

A heatmap showing all treatments and clinical strains is included in **Figure 2.15**. There are no distinct trends across the isolates or the treatments. However, it is notable that many of the largest downregulations of gene expression observed occurred in strain CSUB10R. The overall

largest downregulation occurred for *lamB* at 125 mg/dL of cholesterol, and the largest overall upregulation occurred for *lamB* in CSUB10R+pSHA16K at 125 mg/dL of cholesterol.

Discussion

The aim of this study was to evaluate the effects of cholesterol, within a physiological range of 25 mg/dL to 125 mg/dL, on *K. pneumoniae* porin gene expression and on subsequent antibiotic susceptibility. It was expected that as the concentration of cholesterol increased, porin gene expression and antibiotic susceptibility would decrease in *K. pneumoniae*. However, it was found that in addition to cholesterol impacting gene expression and antibiotic susceptibility the surfactant, methyl-β-cyclodextrin, also impacted both gene expression and antibiotic susceptibility. MIC50 values were similar between MBC and cholesterol treatments within the laboratory strain, *K. pneumoniae* ATCC 43816, while breakpoints differed. For the MBC and cholesterol treated *K. pneumoniae* clinical strains, MIC50 and breakpoint values were similar between each strain (CSUB10R, CSUB10S, CSUB10R+pSHA16K, CSUB10S+pSHA16K). And ultimately, gene expression for both the laboratory strain and clinical strains indicated large differences between cholesterol and MBC treatments across most of the tested genes.

The impact of surfactant was evident in both gene expression and antibiotic susceptibility assays. However, due to the hydrophobicity of cholesterol, a surfactant, methyl-β-cyclodextrin, was pre-mixed, before purchase, with the cholesterol and quantified to allow the cholesterol to be water-soluble. The surfactant, therefore, was not chosen for this study specifically, but was accounted for as described in the Materials and Methods section. This mixture was designed for cell and tissue cultures and can impact cells and tissues in numerous ways. One study found that nerve growth factor-differentiated PC12 cells and immortalized Schwann cells were not

impacted by a low percentage of MBC at 0.12%, however, at higher percentages (such as 0.25%) there was an increase in cell death and apoptosis [59]. Another study found that MBC was able to inhibit differentiation of myoblasts and block cholesterol-dependent apoptosis [60]. A third study found that MBC was able to potentiate a benzyl isothiocyanate-induced anti-cancer pathway for human colorectal cancer [61]. The varied effects of MBC appear to occur in numerous alternate cell and tissue types, in addition to the effects seen within this study.

When gene expression was evaluated for both *ompK35* and *ompK36*, MBC exposure produced significantly different effects from the untreated cultures of *K. pneumoniae* ATCC 43816. This was also clearly demonstrated in the MIC assay for breakpoint values. The MBC breakpoint values were more like those of the cholesterol treatments than to those of the untreated controls. The data indicated MBC reached the maximum effect between 50 mg/dL and 75 mg/dL for the respective cholesterol treatments. This impact on both gene expression and antibiotic susceptibility due to the surfactant posed an issue for adequately evaluating antibiotic susceptibility and gene expression. To compare antibiotic susceptibility values, MBC respective values to each cholesterol treatment were utilized. For gene expression, cholesterol values were calibrated to MBC, as opposed to untreated cultures.

A similar experiment was conducted in LB using cholesterol and a surfactant, tyloxapol [49]. The treatments were LB with no supplement, LB with 0.05% tyloxapol, and 0.05% tyloxapol with 50 μM cholesterol (supplementation with antibiotics was also completed where required). When evaluating the impact of the tyloxapol on *ompA*, *ompk35*, and *ompK36* expression, the gene expression of the tyloxapol was more like the untreated than the cholesterol and tyloxapol treated cultures [49]. The impact of surfactant on expression is different than what was seen within this study. This could indicate that at a low total percentage (0.05%) the surfactant may have less

of an impact on expression, or that the specific surfactant of tyloxapol may have less of an impact on expression than the methyl-β-cyclodextrin utilized within our study.

The chemistry and chemical structures of tyloxapol and methyl-β-cyclodextrin are also different (**Figure 2.16)**. Tyloxapol is a much smaller compound, with a molecular weight of approximately 298.4 g/mol, while methyl-β-cyclodextrin has a molecular weight of approximately 1320 g/mol [62,63]. Tyloxapol is a nonionic polymer, which forms spherical micelles that are 7 nm in size. Often tyloxapol is utilized to form micelles that act as "supramolecular carriers" to transport molecules (most commonly pharmaceutical drugs connected via non-covalent binding), and in this case, cholesterol [64]. Methyl-β-cyclodextrin is a heptasaccharide, meaning it has 7 glucopyranose units. The shape of this molecule has been described as "donut-" shaped, with the inner circle being highly hydrophobic and electron-rich. This is the location where the cholesterol molecule binds [63]. The size difference and the differences in interaction between both molecules, cholesterol, and the bacterial cells could possibly be a contributing factor for the differences observed between the present study and the study by Ares *et al.*[49] .

The outer membrane of bacterial species that utilize cholesterol are often regulated to protect membrane-fluidity; *K. pnuemoniae* is one of these species. Too much cholesterol incorporated into the membrane can make the outer membrane rigid. Membrane rigidity can be detrimental, as high rigidity affects the process of outer membrane protein folding, including for proteins such as porins. Lipids allow for elasticity within the outer membrane for folding to occur. As the ratio of lipids to other membrane components (proteins, cholesterol, etc.) decreases, the rigidity increases substantially [65]. This results in a decrease in outer membrane proteins, including porins, and in response, a decrease in transport through porins. This is significant, as

porins are the structures through which the antibiotics travel to enter the periplasmic space. This is why it was expected that as cholesterol increased, antibiotic susceptibility should decrease.

MIC50 values, which were utilized due to their higher sensitivity to environmental changes, were ultimately unchanged due to cholesterol or MBC presence compared to the untreated in the laboratory strain, *K. pneumoniae* ATCC 43816. Additionally, in the four clinical *K. pneumoniae* strains this measure showed similar results, with all clinical strains growing too robust to identify a specific MIC50 value or range for any of the cholesterol or MBC treatments. MIC breakpoint measurements showed more variation within the laboratory strain, *K. pneumoniae* ATCC 43816, however, due to the impact of the MBC, these results cannot be attributed to the cholesterol alone. The breakpoint results for the clinical strain were all similar, indicating that in already resistant clinical strains, MBC and/or cholesterol treatments may not impact resistance. The similarities within the laboratory strain and across the clinical strains for MIC50 and breakpoint measures indicate that part of the tested hypothesis regarding the impact of cholesterol on antibiotic susceptibility could potentially be supported, however, the large impact of the MBC makes it impossible to conclusively support or reject this part of our hypothesis.

The impact of cyclodextrins as carriers for antibiotics could indicate a possible reason for the effect of the surfactant on antibiotic susceptibility seen in this study. Regarding the cyclodextrin family of molecules, some studies indicate that the presence of cyclodextrin complexes increased the degradation rate of some antibiotics, with other studies indicating that the presence of cyclodextrin enhanced solubility and antibacterial activity [66,67]. One study found that forming a complex between meropenem and a β-cyclodextrin increased antibacterial activity in several Gram-positive and Gram-negative strains, however no significance was found between

meropenem only and the meropenem complex in the tested *K. pneumoniae* ATCC 31488 strain [68]. Methyl-β-cyclodextrin has also been shown to deplete cholesterol from the membrane of host cells, which then decreased engulfment of *K. pnuemoniae* ATCC 43186R (ATCC 43816 isolated from mice) by the MH-S mouse cell line [69]. The ultimate impact of the cyclodextrin family on antibiotic susceptibility is largely varied. Therefore, the presence of methyl-β-cyclodextrin, a member of the cyclodextrin family, could possibly account for the unexplainable decrease in antibiotic susceptibility.

Porin loss can not only affect cell membrane integrity and permeability, but it can also impact the expression of other outer membrane porins. Proteins that have been evaluated in respect to OmpK35 and OmpK36 are OmpK26, OmpA, Lpp, and LamB, as they have important functions for the outer membrane [28] .

When evaluating gene expression for the laboratory strain of *K. pneumoniae* ATCC 43816, both *lpp* and *lamB* indicated no consistent trends (at least 2 out of 5 treatments disagreeing with the remaining treatments). This lack of consistent trend appears throughout many of the genes tested with different concentrations of cholesterol in the four tested clinical strains as well (at least 2 out of 5 treatments disagreeing with the remaining treatments), including CSUB10R for *ompK26* and *ompA*, CSUB10S for *ompK36*, CSUB10R+pSHA16K for *ompA*, *lpp*, and *lamB*, and CSUB10S+pSHA16K for all six tested genes. This could indicate that the specific interactions between the presence or absence of OmpK35 and/or OmpK36 could lead to highly sensitive responses to cholesterol presence, and therefore regulation of the tested genes.

For the laboratory strain, *ompK35* and *ompK36* showed a generally increased trend of expression (at least 4 out of 5 treatments increased). The clinical strains with generally increased

expression (at least 4 out of 5 treatments increased) are CSUB10S for *ompK26*, *ompA*, and *lamB*, and CSUB10R+pSHA16K for *ompK35*. The generally increased expression for *ompK35* and *ompK36* in the laboratory strain contrast those found by Ares *et al.*, which reflected a 10-fold decrease for *ompK35* and a 27-fold decrease for *ompK36* [49]. The reason for this variation is unknown, however, the different surfactants utilized between studies could be a contributing factor when evaluating *ompK35* and *ompK36* expression. The generally increased trend in CSUB10S for *ompK26*, *ompA*, and *lamB* is significant, as CSUB10S lacks OmpK35. CSUB10S, which only possesses OmpK36, may potentially upregulate *ompK26*, *ompA*, and *lamB* due to the absence of OmpK35 when exposed to cholesterol. CSUB10R+pSHA16K upregulating *ompK35* is also significant, as this strain lacks OmpK36. Upregulating *ompK35* could potentially compensate for the lack of OmpK36 in the presence of cholesterol within this strain.

For the laboratory strain, *ompK26* and *ompA* show a generally decreased trend of expression (at least 4 out of 5 treatments decreased). The clinical strains with generally decreased expression (at least 4 out of 5 treatments decreased) are CSUB10R for *lpp* and *lamB*, CSUB10S for *lpp*, and CSUB10R+pSHA16K for *ompK26*. The generally decreased expression for *ompA* in the laboratory strain support those found by Ares *et al.*, however, the 10-fold decrease found in their study was larger than the 1-fold decrease found in the present study. Much like the results found in studies for *ompK35* and *ompK36*, it is possible that the differences in fold-changes could be a result of the differing surfactants as well. The generally decreased trend in *lpp* for both CSUB10R and CSUB10S and *lamB* for CSUB10R is significant, as both strains lack OmpK35. This could suggest that it is beneficial to downregulate *lpp* and/or *lamB* in the absence of OmpK35 and in the presence of cholesterol for these strains. For CSUB10R+pSHA16K, which lacks OmpK36 the generally decreased expression for *ompK26* could indicate that it is

beneficial to downregulate this gene in the absence of OmpK36 in the presence of cholesterol for this strain.

One interesting trend was observed when comparing the average expression of *ompK35* for CSUB10R+pSHA16K and CSUB10S+pSHA16K. The strain containing OmpK35, but lacking OmpK36 (CSUB10R+pSHA16K) had to transition to upregulation of *ompK35* at a lower dose than the strain containing both OmpK35 and OmpK36 (CSUB10S+pSHA16K). This indicates that OmpK35 may have a compensatory function in the absence of OmpK36 once a threshold is reached (between 25-50 mg/dL). The strain that possesses both porins did not need to begin upregulation of *ompK35* until between 50-75 mg/dL.

Conclusions

Research regarding cholesterol exposure and the resulting impact on gene expression and antibiotic resistance is lacking for both Gram-negative and Gram-positive bacteria. However, one related study evaluated the interaction between cholesterol crystals and bacterial adhesion to cardiac valves. The study found that the presence of cholesterol crystals significantly impacted bacterial adhesion [70]. Another related study found that the acquisition of cholesterol from hosts in bacterial liposomes was significant for resistance to AMPs [71]. The present study evaluated the impact of physiologically relevant cholesterol on porin gene expression and the resulting impact on antibiotic resistance. Ultimately, there does appear to be an effect of cholesterol on bacteria under certain conditions, making this an area that would benefit from more research.

This study found that porin gene expression can be highly sensitive when exposed to different environmental cholesterol conditions, regardless of whether the strain was a laboratory strain (lacking known resistance mechanisms) or a clinical strain (possessing numerous

resistance mechanisms). The changes between porin gene expression and overall membrane permeability occur primarily due to changes in the environment. The first way is due to changes in porin gene regulation as a result of the EnvZ/OmpR and CpxA-CpxR regulatory systems. The second way is due to differential survival of mutants expressing different porin genes or lacking the porins altogether.

OmpK35 and OmpK36 are regulated using several regulatory systems. The first major two-component regulatory system involved is the EnvZ/OmpR system. EnvZ responds to signals indicating a change in the extracellular environment, and OmpR regulates porin response. An example of this system under varying pH and osmolality can be seen in **Figure 2.17**. Here the expression of porins differed, with OmpF being the major porin at low osmolality and neutral pH, and OmpC being the major porin at high osmolality and acidic pH [72].

The second major two-component regulatory system that regulates porin expression is the CpxA-CpxR system. This system responds more specifically to conditions involving envelope stress. The activation of this system results in a decrease in *ompF* expression and increase in *ompC* expression [73]. Changing regulation of porin expression dependent on environmental conditions allows for differential survival and can ultimately lead to a change in virulence and pathogenicity [74].

In addition to alterations in porin expression as a result of external cellular conditions through the EnvZ/OmpR and CpxA-CpxR regulatory systems changing the outer membrane permeability, changes can occur as a result of differential survival. Differential survival as a result of porin gene expression can occur when the presence or absence of one of more porin genes allows a phenotype to survive the external cellular conditions. This pattern was observed with the clinical strains utilized for this study. CSUB10S, which was first isolated from the

patient, had only shown the absence of the OmpK35 porin. However, as conditions changed, the loss of OmpK36 occurred within the population, producing the CSUB10R isolate, which was isolated from the same patient later. These isolates were clonally related, showing that the under the conditions the population was exposed to, porin loss strains benefited over strains that possess OmpK35 and OmpK36 [75]. Ultimately, changes in environmental conditions can result in changes in porin expression or presence, and furthermore on the outer membrane permeability. This could imply that significant care should be placed on environmental conditions when porin gene expression is to be evaluated, especially if cholesterol is to be a component in the growth media. In addition, more research on other physiologically relevant conditions is needed. Also, more research on the impacts of these conditions on bacterial genes and antibiotic resistance should be completed.

This study also found that surfactant, specifically methyl-β-cyclodextrin, can largely impact both gene expression and antibiotic susceptibility assays, so testing surfactants against untreated controls is essential for avoiding unintended impacts. More research should also be completed regarding the impact of physiologically relevant cholesterol levels on porin gene expression and antibiotic susceptibility using other surfactants and/or antibiotics.

Ultimately, the hypothesis that as the levels of cholesterol increase, porin expression in *Klebsiella pneumoniae* should decrease and antibiotic susceptibility should decrease was neither completely supported nor rejected. The part of the hypothesis that was supported was that as cholesterol concentration increased, antibiotic susceptibility decreased. However, this was not a step-wise increase like was expected, but rather a jump in breakpoints from the untreated to 25 mg/dL, then from the 25 to 50 mg/dL of cholesterol. After this concentration the breakpoints remain the same for all concentrations. Due to the impact of the MBC, it is not possible to

attribute this only to the impact of changing cholesterol concentrations. The first part of the hypothesis that was rejected was that as there was a connection between increasing cholesterol concentration and antibiotic susceptibility in clinical strains (as MIC50 and breakpoint were similar between each clinical strain and each cholesterol concentration). The second part of the hypothesis that was rejected was that as cholesterol concentration increased, porin gene expression would decrease. This was not seen as a clear pattern within any of the tested strains or genes. There were decreases in expression seen, but there was no clear link between the decreases in expression and increasing cholesterol concentration.

The porin gene expression was largely inconsistent for most genes. The only trends (at least 4 out of 5 treatments agreeing) for increased gene expression were seen in *K. pneumoniae* ATCC 43816 for *ompK35* and *ompK36*, as well as CSUB10S for *ompK26*, *ompA*, and *lamB*, and CSUB10R+pSHA16K for *ompK35*. The only trends (at least 4 out of 5 treatments agreeing) for decreased gene expression were seen in *K. pneumoniae* ATCC 43816 for *ompK26* and *ompA*, as well as CSUB10R for *lpp* and *lamB*, CSUB10S for *lpp*, and CSUB10R+pSHA16K for *ompK26*. The inconsistency in gene expression could indicate a larger trend regarding sensitivity within porin genes to certain environmental conditions. Antibiotic susceptibility was largely consistent regardless of cholesterol treatment within strains, however, the large impact of methyl-β-cyclodextrin on antibiotic susceptibility made it difficult to attribute those results to the cholesterol only. This indicates that surfactant could potentially have a significant impact on antibiotic susceptibility assays, so the use of surfactant should be carefully considered when designing an experiment.

Tables and Figures

Table 2.1. Expression of Outer Membrane Porins OmpK35 and OmpK36

Porin Expression	CSUB10R	CSUB10S	CSUB10R+ pSHA16K	CSUB10S+ pSHA16K	ATCC 43816
Abbreviation	10R	10S	10R+16K	10S+16K	43816
ompK35	-	-	+	+	+
ompK36	-	+	-	+	+

Table 2.2. Primers Used for qPCR

Gene	**Forward Primer 5'- 3'**	**Reverse Primer 5'- 3'**
ompK35	TTCGACAACGCTATCGCACTGTCT	AGTACATGACGGCCGCATAGATGT
ompK36	CCGTCAACCAGACCGAAGAA	CAGGCCTGAAATTTGGCGAC
ompK26	GAACAACGCCCGGCAAGATGATGA	AGCTGCGGGCATAGACATAGTTCA
ompA	ACGTGCTCAGTCCGTTGTTGACTA	AGTAACCGGGTTGGATTCACCCAT
lpp	CGGTAATCCTGGGTTCTACTCT	TGCTCAGCTGGTCAACTTTAG
lamB	GCGGGTAAACGCTTCTATCA	GGTCAACGTTTTCCAGACCT
gapA	TTGACCTGACCGTTCGTCTGGAAA	AGCATCGAACACGGAAGTGCAAAC

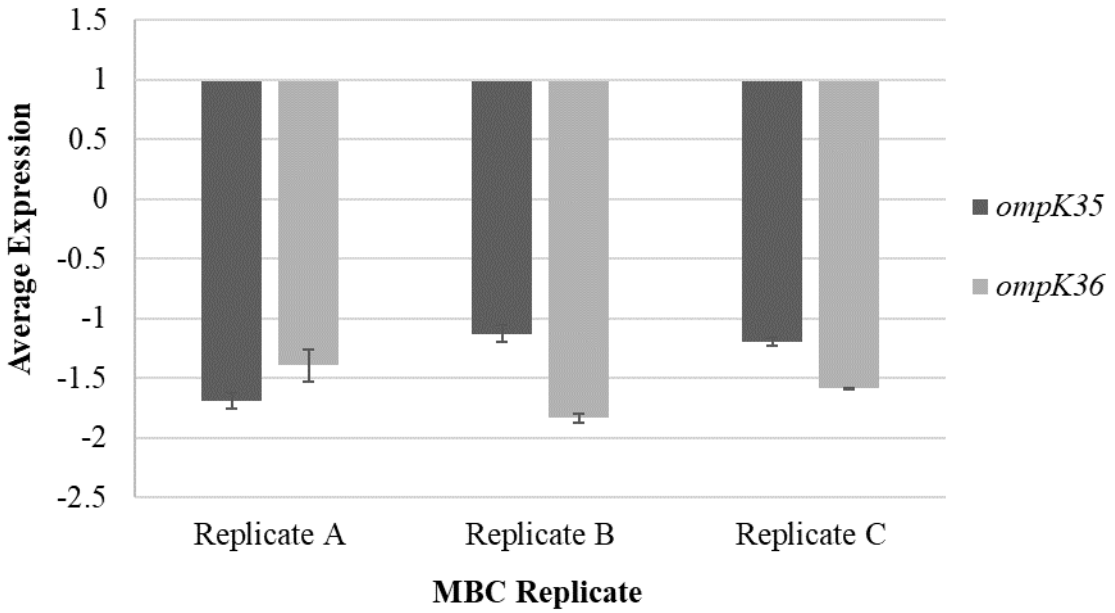


Figure 2.1. Average Expression Corrected of (MBC) 125 mg/dL Control Compared to Untreated ATCC 43816 Control in LB Broth (n=3 biological replicates). The results of this experiment show a significant difference ($p < 0.001$) in expression of both *ompK35* and *ompK36* between the untreated *K. pneumoniae* ATCC 43816 cultures and the methyl-β-cyclodextrin 125 mg/dL cholesterol control cultures. All MBC treatments resulted in downregulation of expression of both genes. Therefore, all results were normalized to methyl-β-cyclodextrin controls as opposed to untreated controls. ANOVA p-value is <0.001 for both *ompK35* and *ompK36*. Significance was determined at $p < 0.05$.

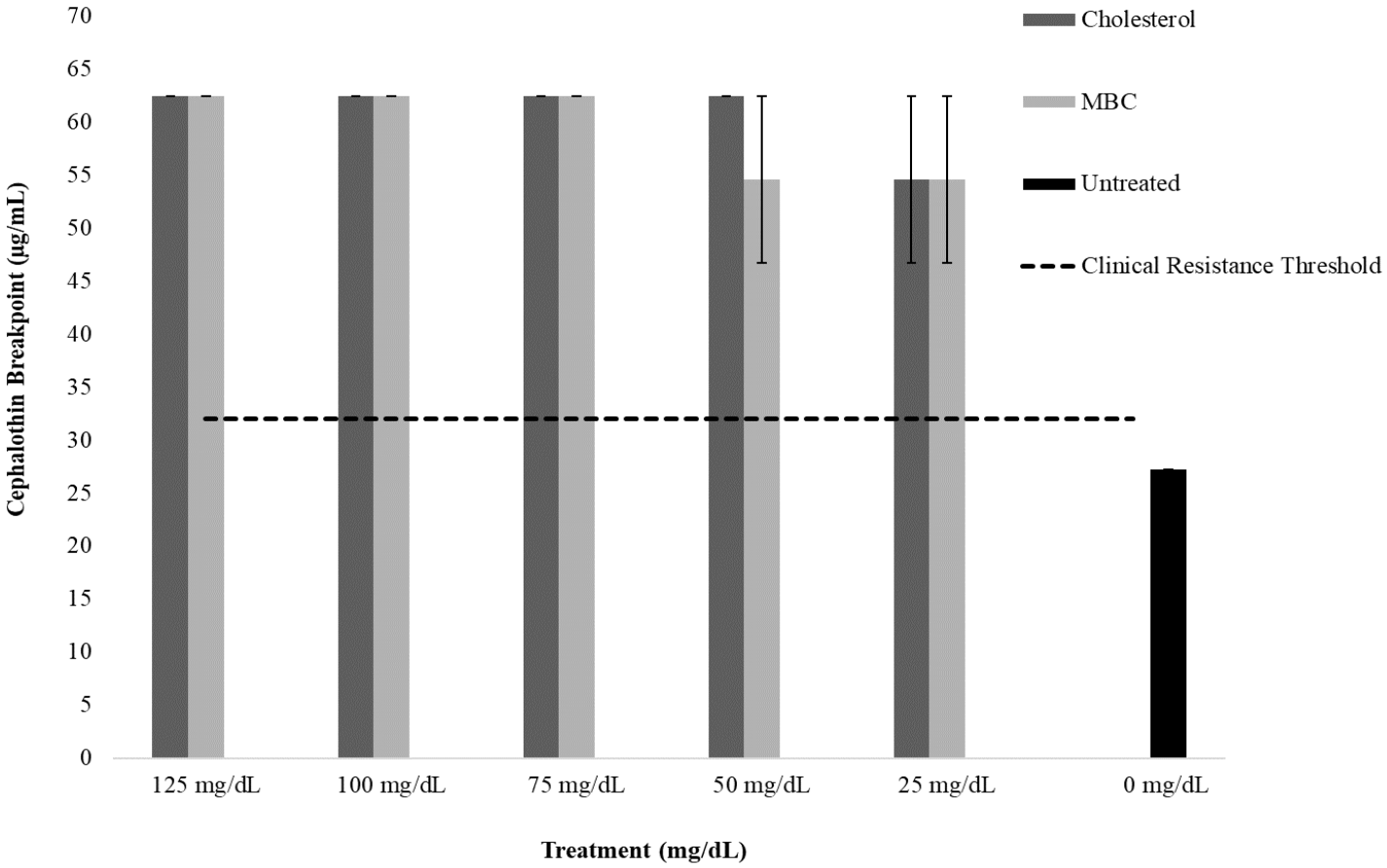
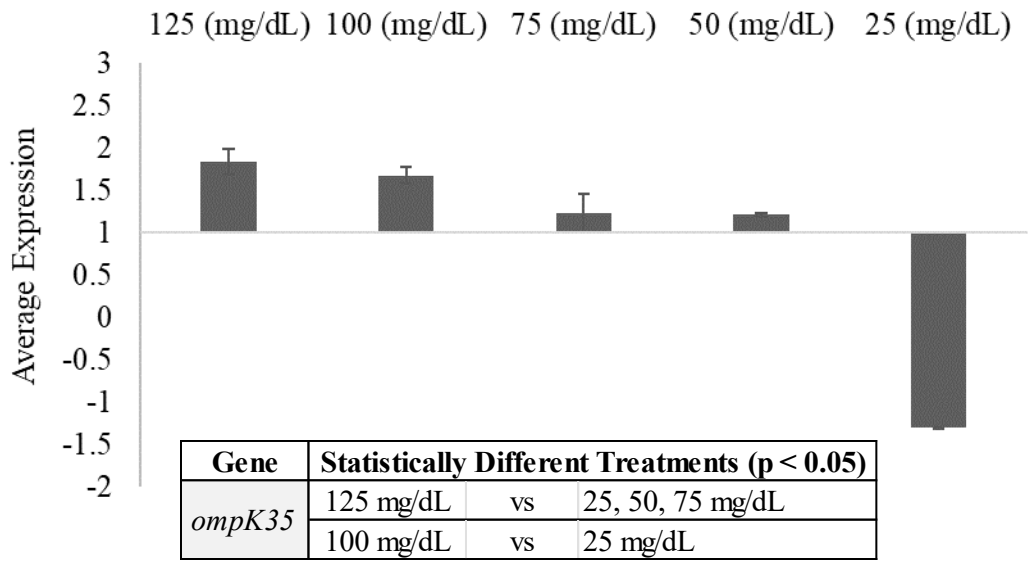
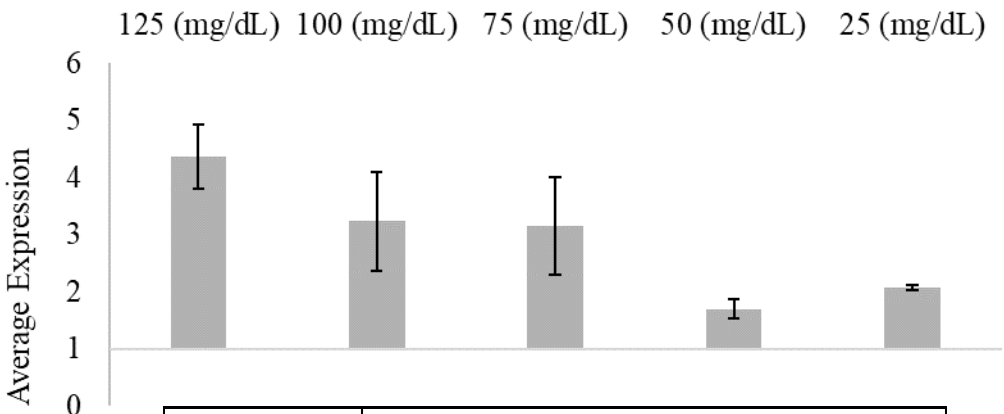


Figure 2.2. Strain ATCC 43816 Breakpoint Values for Cholesterol and MBC Control Treatments in Cephalothin Antibiotic (n=4). Overnight cultures were diluted to an OD of 0.6 in LB broth with the same concentration of cholesterol as the overnight culture and added to a 96-well microtiter plate containing a 2-fold dilution series of cephalothin antibiotic. LB only wells were used as positive control wells. Respective methyl-β-cyclodextrin control 2-fold dilution series of cephalothin antibiotic were ran as comparisons to the cholesterol series. Plates were incubated for 24 hours, then the absorbance was measured at 630 nm (OD_{630}). The MIC Breakpoint values were determined following CLSI guidelines [53,54]. Cholesterol and MBC can both impact antibiotic susceptibility for laboratory strain *K. pneumoniae*. Cholesterol and MBC treated bacteria both exceed the resistance threshold at all cholesterol treatments. The untreated control bacteria remain below the resistance threshold. Horizontal dashed black line indicates cephalothin antibiotic breakpoint threshold at 32 µg/mL [54].Error bars represent standard error.



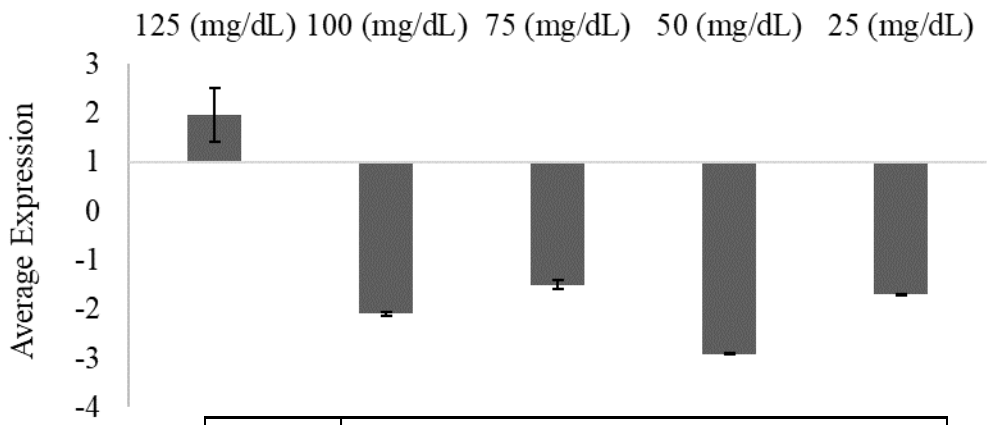
Gene	Statistically Different Treatments ($p < 0.05$)		
ompK35	125 mg/dL	vs	25, 50, 75 mg/dL
	100 mg/dL	vs	25 mg/dL

Figure 2.3. Average *ompK35* Expression Corrected for Cholesterol Treatments in Strain ATCC 43816 (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression showed trends of generally increased average expression for all treatments, with the only exception being a 1.5-fold decrease in average expression for the 25 mg/dL cholesterol treatment. As the concentration of cholesterol increases, only the upregulation of *ompK35* only increases. This indicates that there is a positive effect of cholesterol on the regulation of *ompK35* once a certain threshold is reached somewhere between 25 mg/dL and 50 mg/dL of cholesterol. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



Gene	Statistically Different Treatments (p < 0.05)		
ompK36	125 mg/dL	vs	25, 50 mg/dL

Figure 2.4. Average *ompK36* Expression Corrected for Cholesterol Treatments in Strain ATCC 43816 (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The trend for *ompK36* indicates that exposure to cholesterol causes upregulation of this gene at all concentrations, though there is not a notable stepwise pattern across doses. The largest increase was seen in the highest dose of cholesterol, with an over 4-fold increase. Aside from the 50 mg/dL treatment, the general trend indicates that as cholesterol dose increases, *ompK36* expression increases. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



Gene	Statistically Different Treatments (p < 0.05)		
ompK26	125 mg/dL	vs	25, 50, 75, 100 mg/dL

Figure 2.5. Average *ompK26* Expression Corrected for Cholesterol Treatments in Strain ATCC 43816 (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The trend for *ompK26* indicates that exposure to cholesterol causes statistically significant downregulation of this gene at the four lowest concentrations of cholesterol, but upregulation at the highest dose. Cholesterol exposure has a negative effect on this gene, but that effect switches to positive between 100 mg/dL and 125 mg/dL of cholesterol. There is not a notable stepwise pattern across doses. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.

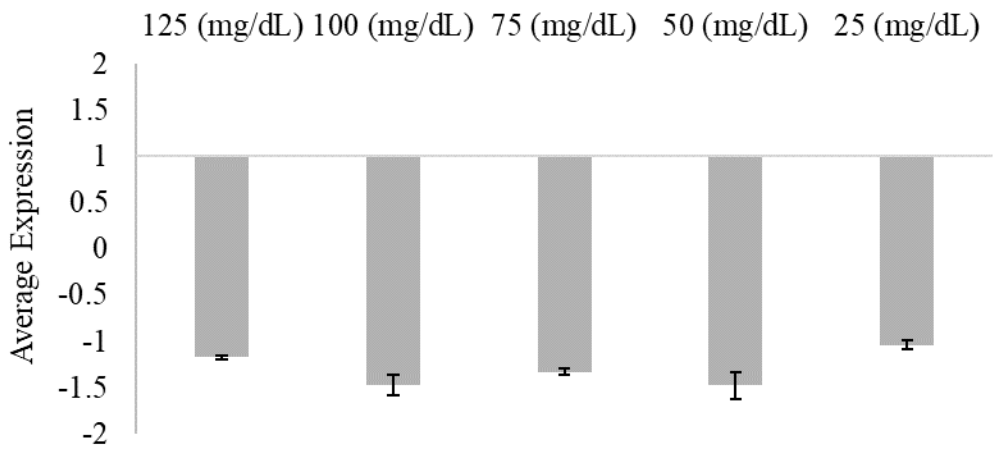
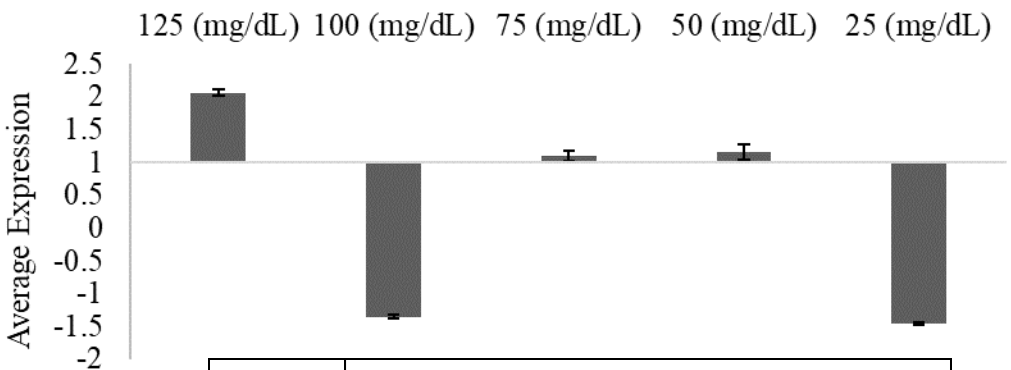
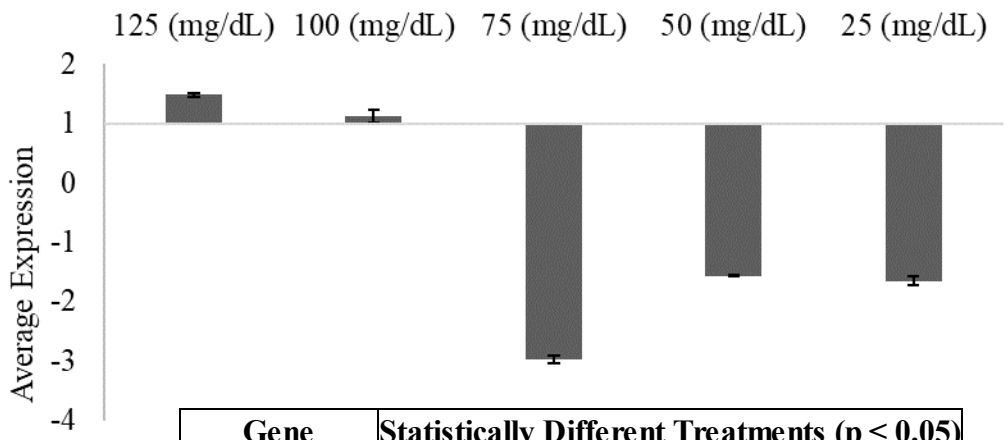


Figure 2.6. Average *ompA* Expression Corrected for Cholesterol Treatments in Strain ATCC 43816 (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The trend for *ompA* indicates that exposure to cholesterol causes similar downregulation of this gene at all concentrations. This implies that cholesterol exposure leads to downregulation in *ompA*, but changing dose does not significantly impact the scale of the downregulation. No treatments were determined to be statistically significant at $p < 0.05$. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



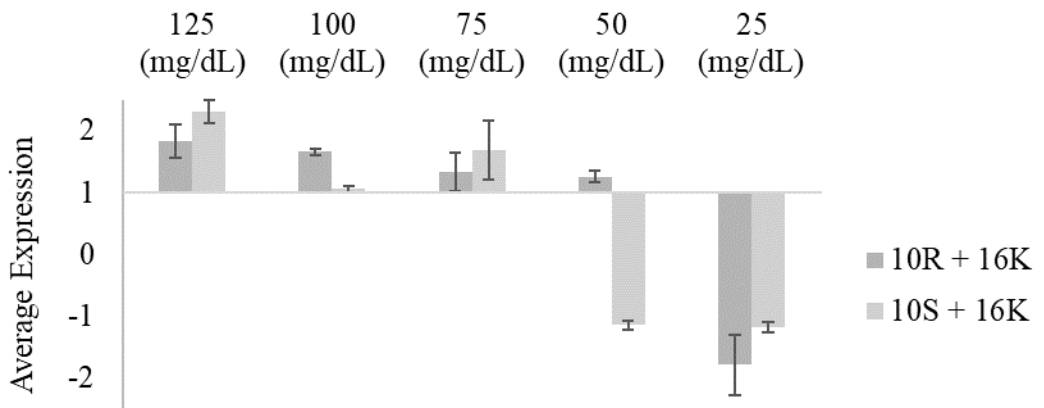
Gene	Statistically Different Treatments ($p < 0.05$)		
lpp	125 mg/dL	vs	25, 50, 75, 100 mg/dL
	100 mg/d	vs	50, 75 mg/dL
	75 mg/dL	vs	25 mg/dL
	50 mg/dL	vs	25 mg/dL

Figure 2.7. Average *lpp* Expression Corrected for Cholesterol Treatments in Strain ATCC 43816 (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression for *lpp* showed no consistent trends. In *lpp* both the 25 and 100 mg/dL treatments showed similar decreases in average expression, while both 50 and 75 mg/dL treatments showed similar modest increases in average expression. There is not a remarkable trend regarding dose of cholesterol and regulation of *lpp*. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



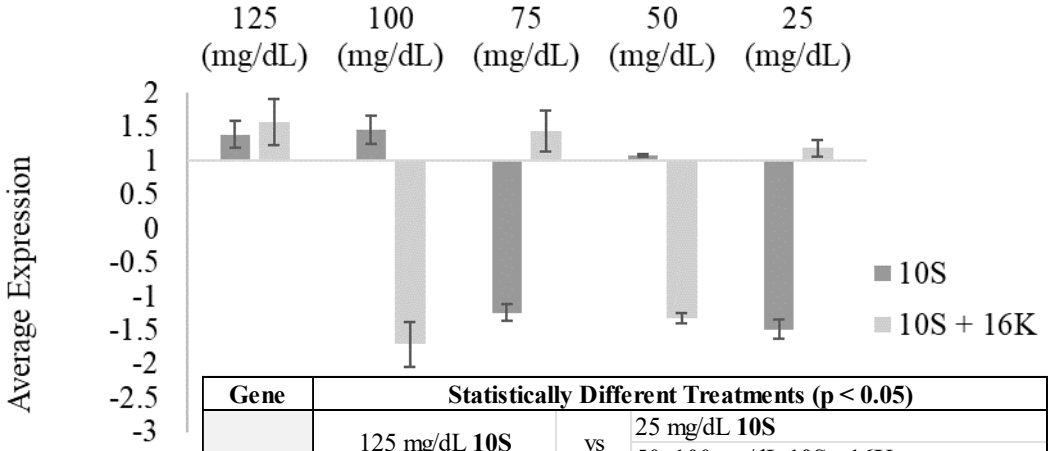
Gene	Statistically Different Treatments (p < 0.05)		
lamB	125 mg/dL	vs	25, 50, 75, 100 mg/dL
	100 mg/d	vs	25, 50, 75 mg/dL
	75 mg/dL	vs	50 mg/dL

Figure 2.8. Average *lamB* Expression Corrected for Cholesterol Treatments in Strain ATCC 43816 (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression for *lamB* showed no consistent trends. There is a threshold effect in *lamB* regulation by cholesterol dose exposure. The lowest three doses of cholesterol show large downregulation within the gene, which then switches to upregulation for the highest two doses. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



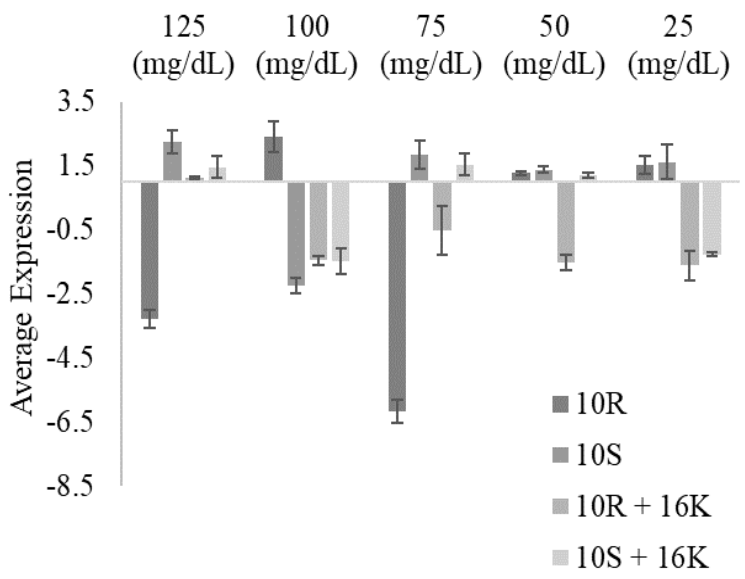
Gene	Statistically Different Treatments ($p < 0.05$)		
ompK35	125 mg/dL **10R +16K**	vs	25 mg/dL **10R +16K**
	125 mg/dL **10S +16K**	vs	25, 50 mg/dL **10R +16K**
			25, 50, 100 mg/dL **10S +16K**

Figure 2.9 Average *ompK35* Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression in *ompK35* showed a trend of increased average expression for CSUB10R+pSHA16K, with the exception of a 2-fold decrease in average expression at 25 mg/dL of cholesterol. This trend was also seen in CSUB10S+pSHA16K, however there was an exception in both 25 and 50 mg/dL treatments, which both showed a 1-fold decrease in average expression. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



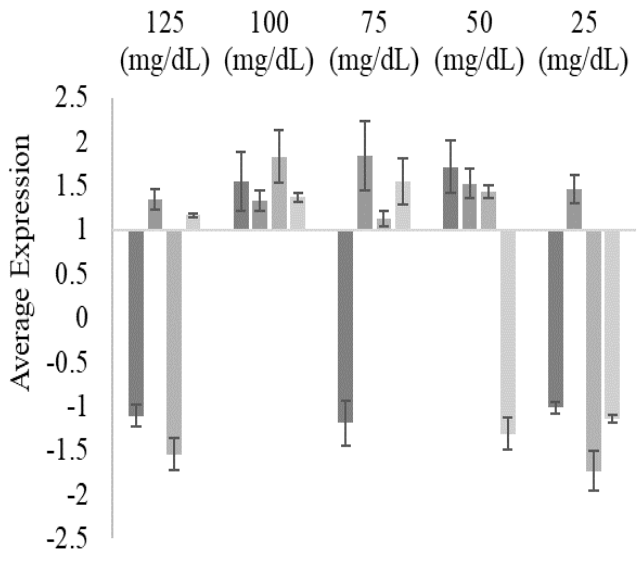
Gene	Statistically Different Treatments ($p < 0.05$)		
ompK36	125 mg/dL **10S**	vs	25 mg/dL **10S**
			50, 100 mg/dL **10S +16K**
	100 mg/dL **10S**	vs	100 mg/dL **10S +16K**
	125 mg/dL **10S +16K**	vs	25 mg/dL **10S**
			50, 100 mg/dL **10S +16K**
	75 mg/dL **10S +16K**	vs	100 mg/dL **10S +16K**

Figure 2.10. Average *ompK36* Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression in *ompK36* showed no trend between treatments for CSUB10S or CSUB10S+pSHA16K. CSUB10S showed increased expression with 50, 100, and 125 mg/dL of cholesterol, and decreased expression at 25 and 75 mg/dL. CSUB10S+pSHA16K showed the opposite pattern, with the exception at 125 mg/dL of cholesterol, which also produced an increase in expression. This indicates that at 25 mg/dL and 75 mg/dL of cholesterol, the absence of OmpK35 in strain CSUB10S led to downregulation of *ompK36*, while the presence of OmpK35 in strain CSUB10S+pSHA16K led to upregulation of *ompK36*. This pattern is then completely reversed at the 50 mg/dL and 100 mg/dL treatments. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



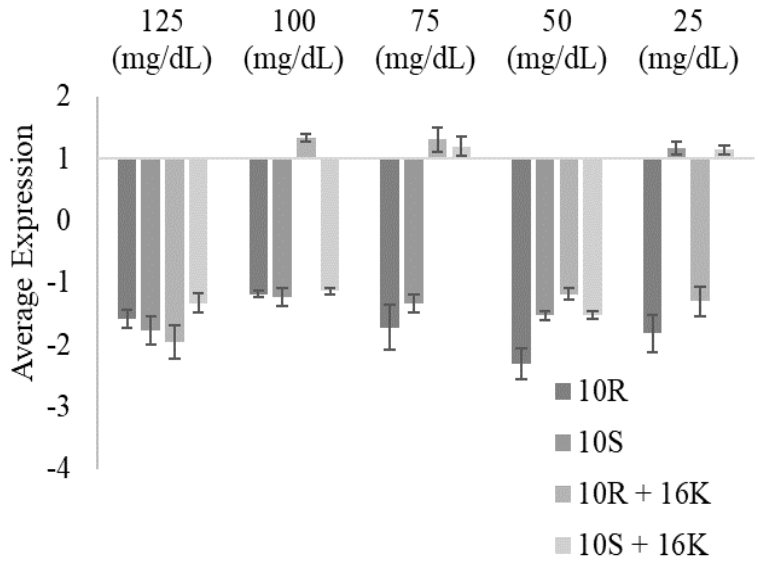
Gene	Statistically Different Treatments ($p < 0.05$)		
ompk26	100 mg/dL **10R**	vs	75, 125 mg/dL **10R**
			25, 50, 75, 100, 125 mg/dL **10R +16K**
			50, 100 mg/dL **10S**
			25, 50, 75, 100 mg/dL **10S +16K**
	50 mg/dL **10R**	vs	75, 125 mg/dL **10R**
	25 mg/dL **10R**	vs	75, 125 mg/dL **10R**
			100 mg/dL **10S**
	125 mg/dL **10S**	vs	75, 125 mg/dL **10R**
			25, 50, 75, 100, 125 mg/dL **10R +16K**
			50, 100 mg/dL **10S**
			25, 50, 75, 100 mg/dL **10S +16K**
	75 mg/dL **10S**	vs	75, 125 mg/dL **10R**
			25, 50, 100 mg/dL **10R +16K**
			100 mg/dL **10S**
			25, 100 mg/dL **10S +16K**
	50 mg/dL **10S**	vs	75 mg/dL **10R**
	25 mg/dL **10S**	vs	75, 125 mg/dL **10R**
			100 mg/dL **10S**
	125 mg/dL **10S +16K**	vs	75, 125 mg/dL **10R**

Figure 2.11. Average *ompK26* Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression in *ompK26* showed no trend within any of the clinical isolates or across the treatments. One notable point is at 75 mg/dL of cholesterol in strain CSUB10R. This point showed a nearly 6.5-fold decrease in average expression. While there is no trend across isolates or across treatments, it is worth noting that at the 25 mg/dL dose, the two strains lacking OmpK35 were upregulated, while the two strains possessing OmpK35 were downregulated. A similar trend is seen at the 75 mg/dL dose, where strains lacking in OmpK36 were downregulated and strains possessing OmpK36 were upregulated. At the 50 mg/dL treatment, the only strain that was downregulated was CSUB10R+pSHA16K, which possesses OmpK35, but not OmpK36. At the 100 mg/dL treatment, the only strain not downregulated was the CSUB10R, which is lacking both OmpK35 and OmpK36. Interestingly, this pattern is reversed at 125 mg/dL of cholesterol. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



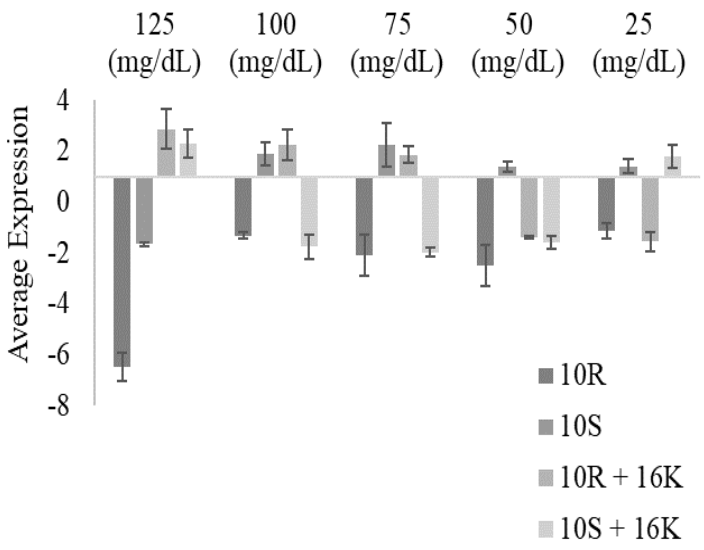
Gene	Statistically Different Treatments ($p < 0.05$)		
ompA	100 mg/dL **10R**	vs	25 mg/dL **10R +16K**
	50 mg/dL **10R**	vs	25, 75, 125 mg/dL **10R**
			25, 125 mg/dL **10R +16K**
			100 mg/dL **10S +16K**
	100 mg/dL **10R +16K**	vs	25, 75, 125 mg/dL **10R**
			25, 125 mg/dL **10R +16K**
			100, 125 mg/dL **10S +16K**
	75 mg/dL **10S**	vs	25, 75, 125 mg/dL **10R**
			25, 125 mg/dL **10R +16K**
			100, 125 mg/dL **10S +16K**
	50 mg/dL **10S**	vs	25 mg/dL **10R +16K**
	75 mg/dL **10S +16K**	vs	25 mg/dL **10R +16K**

Figure 2.12. Average *ompA* Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression in *ompA* showed no trend within isolates CSUB10R, CSUB10R+pSHA16K, or CSUB10S+pSHA16K. CSUB10S showed a general increase in average expression across treatments. While there is no trend across isolates or across treatments, it is worth noting that at 25 mg/dL of cholesterol only CSUB10S, which lacks OmpK35 and has OmpK36 is upregulated. At 50 mg/dL, only strain CSUB10S+pSHA16K, which possesses both porins is downregulated, while at 75 mg/dL only strain CSUB10R, which lacks both porins is downregulated. At the 100 mg/dL treatment, all treatments are upregulated, despite OmpK35 or OmpK36 being present or absent. At the 125 mg/dL treatment, both CSUB10R and CSUB10R+pSHA16K, which lack OmpK36 are downregulated for *ompA* expression, and CSUB10S and CSUB10S+pSHA16K, which both possess OmpK36 are upregulated for *ompA* expression. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



Gene	Statistically Different Treatments ($p < 0.05$)		
lpp	100 mg/dL **10R +16K**	vs	25, 50, 75, 100, 125 mg/dL **10R**
			25, 50, 125 mg/dL **10R +16K**
			50, 75, 100, 125 mg/dL **10S**
			50, 125 mg/dL **10S +16K**
	75 mg/dL **10R +16K**	vs	25, 50, 75, 125 mg/dL **10R**
			25, 125 mg/dL **10R +16K**
			50, 75, 100, 125 mg/dL **10S**
			50, 125 mg/dL **10S +16K**
	25 mg/dL **10S**	vs	25, 50, 75, 125 mg/dL **10R**
			125 mg/dL **10R +16K**
			50, 125 mg/dL **10S**
			50 mg/dL **10S +16K**
	75 mg/dL **10S +16K**	vs	25, 50, 75, 125 mg/dL **10R**
			125 mg/dL **10R +16K**
			50, 125 mg/dL **10S**
			50 mg/dL **10S +16K**
	25 mg/dL **10S +16K**	vs	25, 50, 75, 125 mg/dL **10R**
			125 mg/dL **10R +16K**
			50, 125 mg/dL **10S**
			50 mg/dL **10S +16K**

Figure 2.13. Average *lpp* Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression in *lpp* showed a general decrease in average expression for all isolates across most treatments. The only exceptions to this pattern were for CSUB10S and CSUB10S+pSHA16K in the 25 mg/dL treatment, CSUB10R+pSHA16K and CSUB10S+pSHA16K in the 75 mg/dL treatment, and CSUB10S in the 100 mg/dL treatment. While the general trend is downregulation, which was seen for all strains using 50 mg/dL and 125 mg/dL of cholesterol, the exceptions are notable. At the 25 mg/dL dose, the two downregulated strains, CSUB10R and CSUB10R+pSHA16K, both lack OmpK36, while the two upregulated strains, CSUB10S and CSUB10S+pSHA16K, both possess OmpK36. At the 75 mg/dL dose, the two downregulated strains, CSUB10R and CSUB10S, both lack OmpK35, while the two upregulated strains, CSUB10R+pSHA16K and CSUB10S+pSHA16K, both possess OmpK35. And at the 100 mg/dL dose, only the strain CSUB10R+pSHA16K, which has OmpK35, but lacks OmpK36 was upregulated. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.



Gene	Statistically Different Treatments ($p < 0.05$)		
lamB	125 mg/dL **10R +16K**	vs	25, 50, 75, 100, 125 mg/dL **10R**
			25, 50 mg/dL **10R +16K**
			25, 50, 125 mg/dL **10S**
			25, 75, 100 mg/dL **10S +16K**
	100 mg/dL **10R +16K**	vs	25, 50, 75, 100, 125 mg/dL **10R**
			25, 50 mg/dL **10R +16K**
			125 mg/dL **10S**
			50, 75, 100 mg/dL **10S +16K**
	75 mg/dL **10R +16K**	vs	50, 75, 125 mg/dL **10R**
			75 mg/dL **10S +16K**
	100 mg/dL **10S**	vs	50, 75, 125 mg/dL **10R**
			75 mg/dL **10S +16K**
	75 mg/dL **10S**	vs	25, 50, 75, 100, 125 mg/dL **10R**
			25, 50 mg/dL **10R +16K**
			125 mg/dL **10S**
			50, 75, 100 mg/dL **10S +16K**
	125 mg/dL **10S +16K**	vs	25, 50, 75, 100, 125 mg/dL **10R**
			25, 50 mg/dL **10R +16K**
			125 mg/dL **10S**
			50, 75, 100 mg/dL **10S +16K**
	25 mg/dL **10S +16K**	vs	75, 125 mg/dL **10R**

Figure 2.14. Average *lamB* Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). RNA was isolated from all cultures in mid-logarithmic phase. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Each cholesterol concentration was also evaluated relative to its respective methyl-β-cyclodextrin control. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression in *lamB* showed no trend within isolates CSUB10S, CSUB10R+pSHA16K, or CSUB10S+pSHA16K. CSUB10R showed a general decrease in average *lamB* expression across treatments. While there is no trend across isolates or across treatments, it is worth noting that at 25 mg/dL of cholesterol, the two strains lacking OmpK36, CSUB10R and CSUB10R+pSHA16K, were downregulated for *lamB* expression, and the two strains possessing OmpK36, CSUB10S and CSUB10S+pSHA16K were upregulated for *lamB* expression. At 50 mg/dL of cholesterol, only strain CSUB10S, which lacks OmpK35 but possesses OmpK36 is upregulated. At both 75 mg/dL and 100 mg/dL of cholesterol, the two strains that were downregulated for *lamB* expression are either missing both OmpK35 and OmpK36 (CSUB10R) or possess both porins (CSUB10S+pSHA16K). The two strains that were upregulated for *lamB* expression either have OmpK35 and lack OmpK36 (CSUB10S) or lack OmpK35 and have OmpK36 (CSUB10R+pSHA16K). At 125 mg/dL of cholesterol, the two strains that lack OmpK35, CSUB10R and CSUB10S, were downregulated for *lamB* expression, and the two strains that have OmpK35, CSUB10R+pSHA16K and CSUB10S+pSHA16K, were upregulated for *lamB* expression. Table within figure indicates gene being evaluated, treatments showing statistically significant differences, and p-value. Statistics were determined using Tukey's Post-Hoc test. Error bars represent standard error.

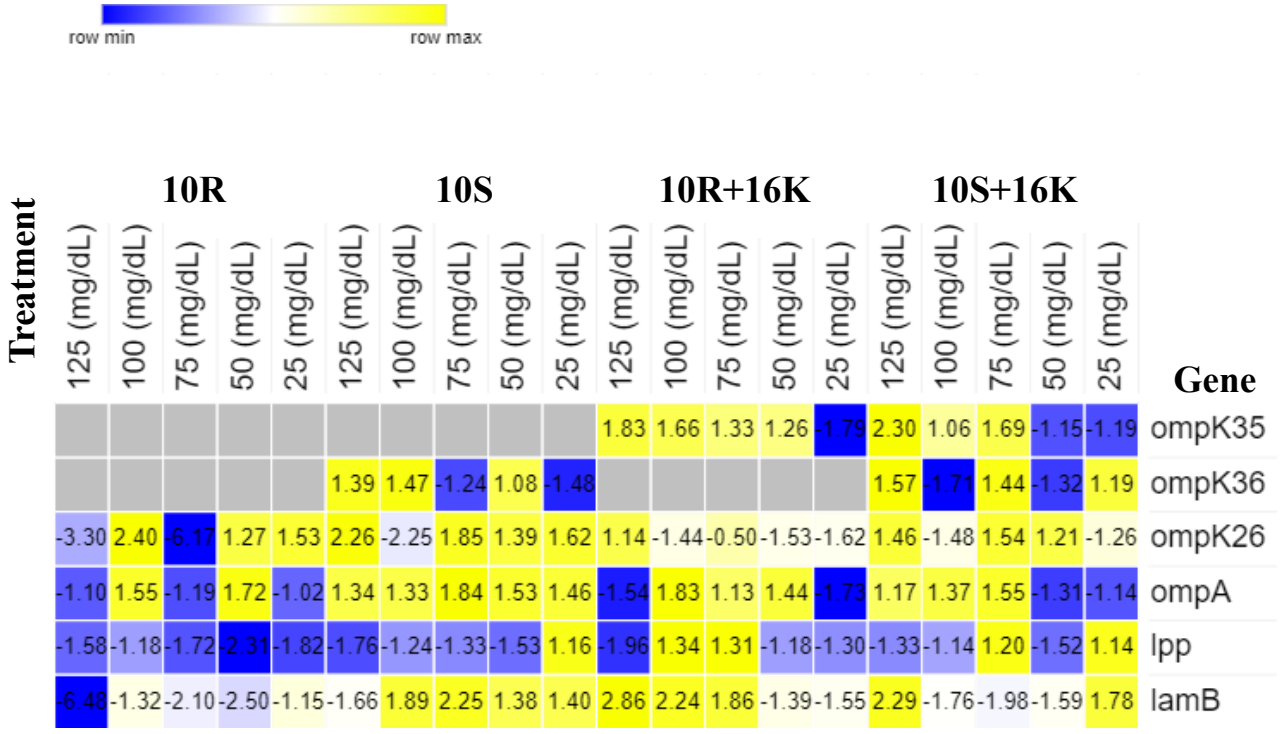


Figure 2.15. Heat Map Representing Average Expression Corrected for Cholesterol Treatments in Clinical Strains (n=4). Heat Map was Created Using Morpheus Software from the Broad Institute [76]. There are no distinct trends across the isolates or the treatments. However, it is notable that many of the largest downregulations of gene expression observed occurred in strain CSUB10R. The overall largest downregulation occurred for *lamB* at 125 mg/dL of cholesterol, and the largest overall upregulation occurred for *lamB* in CSUB10R+pSHA16K at 125 mg/dL of cholesterol.

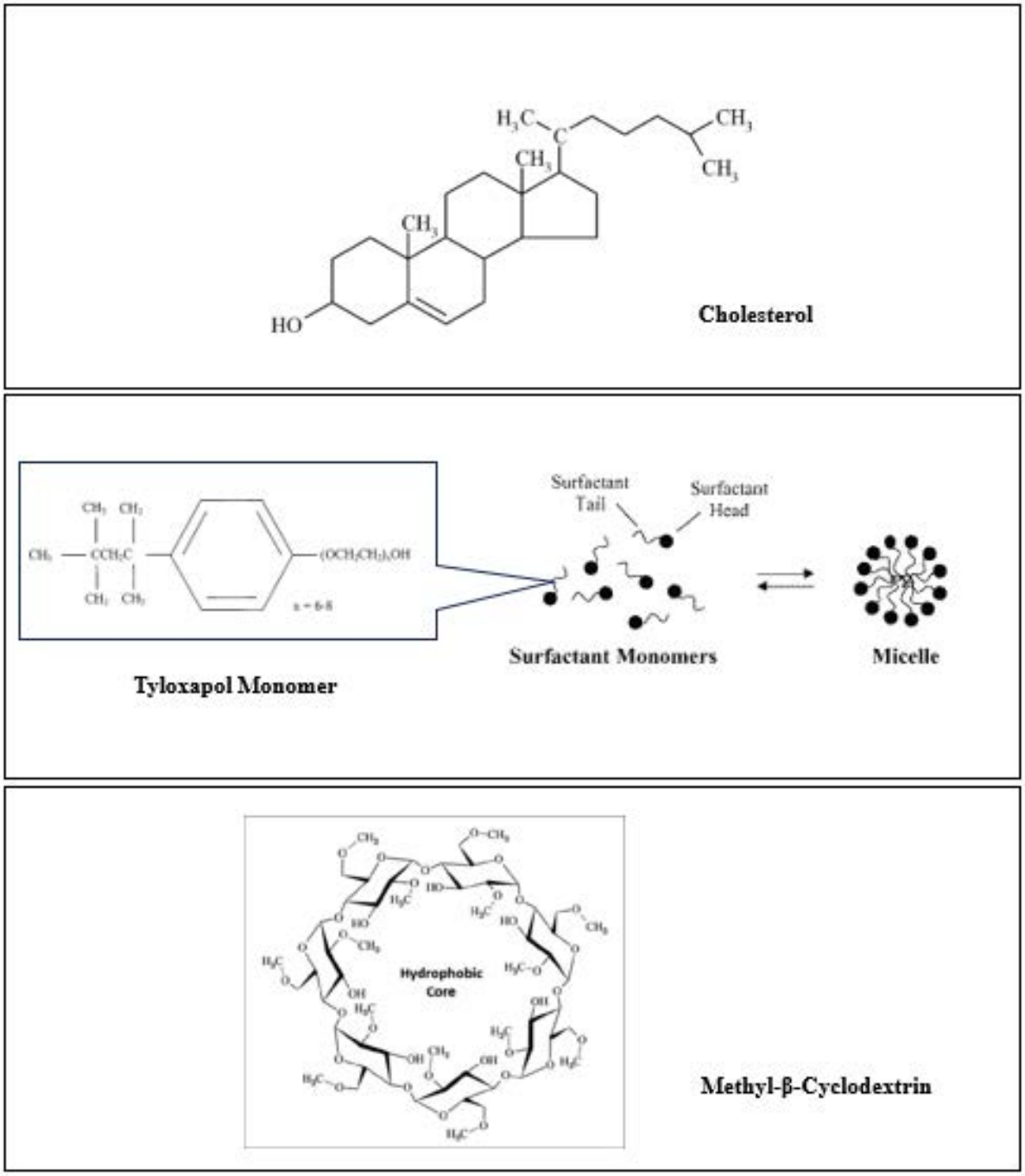


Figure 2.16. The Structures of Cholesterol (Top), a Tyloxapol Monomer and Micelle (Middle), and Methyl-β-Cyclodextrin (Bottom) [77–80]. The chemistry and chemical structures of tyloxapol and methyl-β-cyclodextrin are different, with tyloxapol being smaller and less complex than MBC [62,63]. Tyloxapol can also form micelle structures [64]. MBC has an inner circle that is highly hydrophobic and electron-rich [63].

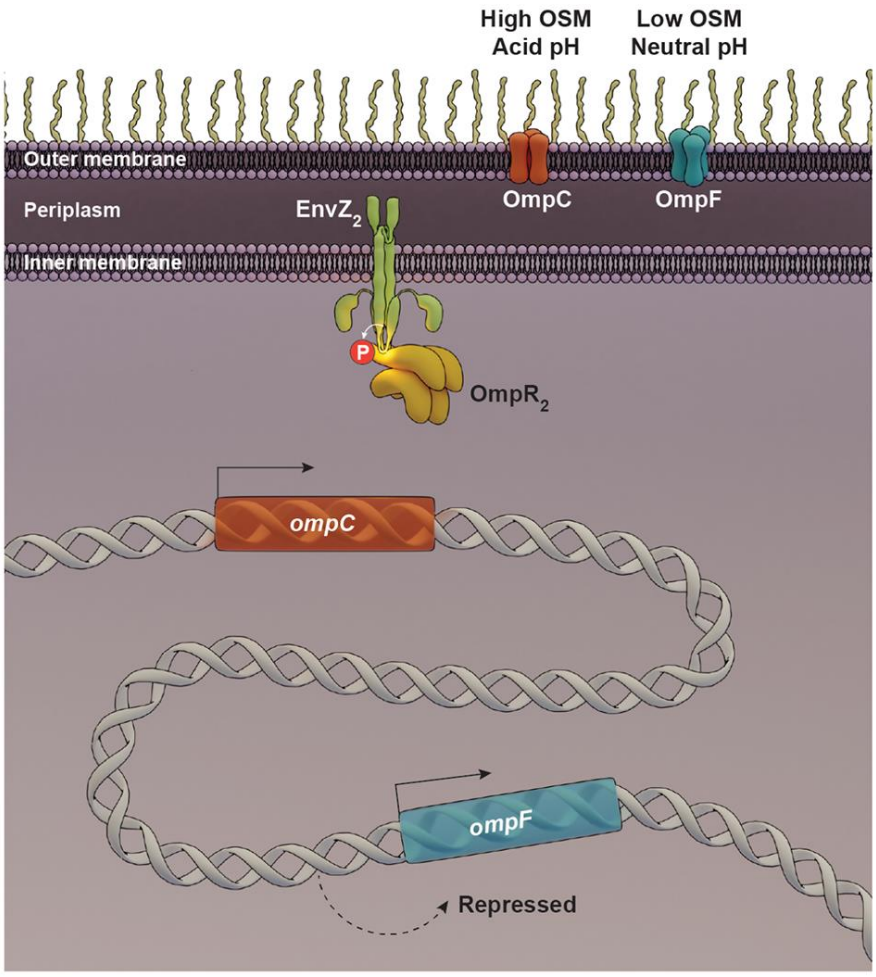


Figure 2.17. EnvZ/OmpR Regulatory System [72]**.** OmpK35 and OmpK36 are regulated using several regulatory systems. The first major two-component regulatory system involved is the EnvZ/OmpR system. EnvZ responds to signals indicating a change in the extracellular environment, and OmpR regulates porin response. An example of this system under varying pH and osmolality can be seen here [72].

Chapter Three

Effects of Physiological Media vs Laboratory Media on Porin Gene Expression in *Klebsiella pneumoniae* and the Resulting Impact on Antibiotic Resistance

Introduction

There are two main regulatory systems that are involved in porin regulation, the EnvZ/OmpR and the CpxA-CpxR systems [72,73]. The EnvZ/OmpR system is largely influenced by the extracellular environment, meaning that variations in media type could potentially alter porin expression. This system is largely impacted through changes in pH and osmolarity. The CpxA-CpxR system can also change porin expression as a result of changing external environmental conditions, particularly with respect to envelop stress. Activation of this system has been linked to changes in *ompF* and *ompC* [73].

Klebsiella pneumoniae can survive in numerous locations within the human body. Each of these locations has different conditions, specifically regarding pH or osmolarity. This could indicate that growing bacteria in a media type that is different from the location of infection could provide antibiotic susceptibility values dissimilar from the true susceptibility profile as well as differing gene expression.

Traditionally, many bacterial cultures and assays are created in/completed in media types that are complex and nutrient rich. This is to promote fast and dense bacterial growth. One common laboratory media used for this purpose is Luria-Bertani/Lysogeny Broth (LB). This media is created from tryptone, yeast extract, NaCl, and distilled water [81]. The exact contents can vary slightly (largely due to the yeast extract component), which is why this is not a defined media. The impacts of this variability on antibiotic susceptibility assays are not known. Nor is it known how close the antibiotic susceptibility values obtained in a complex and undefined media are to the true susceptibility values of the bacteria at the area of infection within a host.

Klebsiella pneumoniae can grow in human blood. Hence, evaluating bacterial growth in media like human blood could provide insight into how a physiological media compares to a

laboratory/traditional media. One synthetic media type like human blood is Human Plasma-Like Media (HPLM) [82].

HPLM was designed specifically to mimic adult human plasma. Therefore, HPLM was formulated with polar metabolite and salt ion concentrations comparable to human plasma. Originally HPLM was utilized to evaluate cellular metabolism, but due to its similarity to human plasma and its defined properties, it could potentially serve as a useful way to evaluate bacterial gene expression and antibiotic susceptibility [82]. Comparing results in HPLM to isolated human plasma, both heat-inactivated and native (not heat-inactivated) would also serve as a useful tool to evaluate these factors, as well as comparing different media types/sources.

Thesis Aims

Due to the prevalence of healthcare-associated infections, as well as the concerning increase in antibiotic resistant bacteria, evaluating the effects of physiologic media vs traditional media on porin gene expression and further on antibiotic susceptibility would provide information in an area where information is deficient. Also, assessing the impact that porin gene expression has on other outer membrane proteins for *Klebsiella pneumoniae grown* in different media types would provide insight into the way this species responds through alterations in its outer membrane.

To evaluate how *Klebsiella pneumoniae* responds in traditional media vs physiologic media, one laboratory strain and four clonally-related clinical isolates were individually exposed to either LB broth, HPLM, or isolated human plasma (heat-inactivated or not heat-inactivated). Following exposure to the chosen media type, gene expression and antibiotic susceptibility was evaluated and compared. Four different antibiotics were utilized for antibiotic susceptibility

testing due to the differing mechanisms of action. Cephalothin, a cephalosporin antibiotic, and imipenem, a carbapenem antibiotic both classified as beta-lactam antibiotics utilize a mechanism of action which aims to inhibit cell wall synthesis. This is done when the beta-lactam ring binds the penicillin-binding proteins needed for peptidoglycan cross-linking in the process of cell wall synthesis [58]. Cephalothin and imipenem are being compared due to cephalothin being a first-generation cephalosporin and imipenem being a carbapenem. Carbapenem antibiotics should show significantly lower MIC values, due to being designed to survive mechanisms which cephalothin antibiotic could not, particularly resisting hydrolysis to beta-lactamases or inhibiting them altogether. Carbapenem antibiotics are also known for their high potency within both Gram-negative and Gram-positive bacteria [83]. Kanamycin, an aminoglycoside antibiotic, and tetracycline, a tetracycline antibiotic, both utilized a mechanism of action which aims to inhibit protein synthesis. Kanamycin and tetracycline both interact with the 16S rRNA of the 30S ribosomal subunit. Kanamycin interacts at the A site, altering its conformation to prevent mRNA from being properly translated [58,84]. Tetracycline interacts with conserved sequences to prevent binding of tRNA to the A site [58].

It was hypothesized that growth of *Klebsiella pneumoniae,* as well as porin gene expression and antibiotic susceptibility in HPLM and human plasma would be different than in LB broth. As mentioned, porin regulation is highly impacted by pH and osmolarity. Human plasma has a pH of approximately 7.4, while LB broth has a pH of approximately 6.8-7.2 [81,85,86]. Changes of osmolarity can also occur when bacteria are grown in different media conditions [87]. Ultimately, changes in these conditions as well as other potential conditions changes between a laboratory media and a physiologic media should alter changes in porin gene

expression, which could alter expression of other outer membrane structures and therefore potentially change antibiotic susceptibility.

Materials and Methods

Bacterial Strains and Plasmids

Klebsiella pneumoniae clinical isolates were provided by Dr. Sebastian Alberti (University of the Balearic Islands, Palma de Mallorca, Spain) and were isolated from a single patient during a clinical outbreak in Spain [53]. All clinical isolates are clonally related. Isolate CSUB10R (10R) expresses neither OmpK35 nor Ompk36. CSUB10S (10S) expresses only OmpK36. The pSHA16K plasmid, carrying the *ompK35* gene was transformed into both clinical strains to create CSUB10R+pSHA16K and CSUB10S+pSHA16K. CSUB10R+pSHA16K and CSUB10S+pSHA16K were grown with 50 ug/mL of kanamycin (Fisher BioReagents) for maintenance of the plasmid. These clinical isolates are extended spectrum β-lactamase (ESBL) positive [9,53].

Klebsiella pneumoniae laboratory strain ATCC 43816 (American Type Culture Collection) was used for comparison. This strain is ESBL negative and contains no known resistance plasmids. Clinical and laboratory strain gene expression for *ompK35* and *ompK36* is shown in **Table 3.1**.

Descriptions of Media

Luria-Bertani (LB) broth (Difco) is used as a complex, nutrient rich medium that allows for rapid and high-yield growth. The formulation of LB broth is 10 g tryptone, 5 g yeast extract, and 10 g of sodium chloride combined in 1 L of deionized water [81]. Human Plasma-Like Media

(HPLM) (Thermofisher) is used as a synthetic, defined media meant to resemble the metabolic profile of human plasma. This media is formulated with over 60 polar metabolites and salt concentrations like human plasma [82]. The human plasma was Pooled Human Plasma Apheresis Derived Na Heparin (Innovative Research). This plasma was collected via apheresis from donors at an FDA-approved collection center. The plasma was tested and found negative for the following viral markers: HBsAg, HCV, HIV-1, HIV-2, HIV-1Ag or HIV-1 NAT, ALT, West Nile Virus NAT, Zika NAT and syphilis. The anticoagulant added is Na Heparin [88]. The plasma was thawed, aliquoted into 20 mL conical centrifuge tubes and centrifuged at 4,000 rpm for five minutes to remove the anticoagulants. Half of the plasma, referred to as native plasma, was then refrozen for later use. The remaining half of the plasma was heat inactivated. To heat inactivate the plasma, the plasma was heated at 56℃ for 30 minutes with occasional mixing, allowed to reach room temperature, then refrozen for later use.

Growth Curve Determinations

To evaluate differences in growth rates and patterns, *K. pneumoniae* 43816 was grown in either LB broth or HPLM overnight at 37℃ and 200 rpm. A 1:1000 dilution of overnight culture was made in fresh media of the same type and grown at 37℃ and 200 rpm. The optical density (OD) was measured at 600 nm every 30 minutes for 7 hours.

Dilution plate counts were used to determine the growth curve in human plasma. *K. pneumoniae* 43816 was grown in human plasma (Innovative Research) that was either heat inactivated or not heat inactivated. The bacteria were incubated overnight at 37℃ and 200 rpm shaking. A 1:1000 dilution of overnight culture was made in fresh media of the same type (heat-inactivated or Native) and grown at 37℃ and 200 rpm. Samples were taken every hour for eight hours for dilution plate counts on LB agar.

Minimum Inhibitory Concentration (MIC) Assays

LB vs HPLM

To evaluate the differences in antibiotic resistance, *K. pneumoniae* 43816 was grown in either LB broth or HPLM overnight at 37℃ and 200 rpm shaking. Overnight cultures were diluted to an optical density (OD) of 0.6 in their respective broth and added to a 96-well microtiter plate containing a 2-fold dilution series of cephalothin (Thermofisher), tetracycline (Sigma), kanamycin (Fisher BioReagents), or imipenem (Thermofisher) antibiotic. LB or HPLM only wells were used as positive controls. Plates were incubated for 24 hours, then the absorbance at 630 nm (OD_{630}) was measured using a Biotek Gen5 Plate Reader. The MIC50, MIC90, and MIC Breakpoint values were determined following Clinical and Laboratory Standards Institute (CLSI) guidelines [54,55]. This process was repeated using cephalothin and imipenem with the four clinical strains. Dilution plate counts were used to confirm the MIC values to determine bacteriostatic vs bactericidal effects.

Average CFUs were calculated from the plating of all the MIC50 wells. This was only completed for *K. pneumoniae* 43816 grown in LB or HPLM with cephalothin. This was to determine bacteriostatic vs bactericidal effects.

Altering Media Type

To evaluate the extent of the effect of media type on MIC values *K. pneumoniae* 43816 was grown in either LB or HPLM overnight at 37℃ and 200 rpm. Overnight cultures of bacteria were then centrifuged, all residual media removed, and the bacteria resuspended in the opposite media type. Optical density was used to ensure a similar number of bacteria in both media types for resuspension. The cultures were diluted until reaching an optical density of 0.6 at 600 nm.

The bacterial resuspensions were added to a 96-well microtiter plate containing a 2-fold dilution series of cephalothin in the new growth media. LB or HPLM only wells were used as positive controls. Plates were incubated for 24 hours, then the absorbance at 630 nm (OD_{630}) was measured using a Biotek Gen5 Plate Reader. The MIC50, MIC90, and MIC Breakpoint values were determined following Clinical and Laboratory Standards Institute (CLSI) guidelines [54,55]. Dilution plate counts were used to confirm the MIC values. **Figure 3.1** illustrates the procedure for the alteration of media types experiment.

Heat-Inactivated vs Native Human Plasma

To evaluate the changes in antibiotic resistance in human plasma, *K. pneumoniae* 43816 was grown in either heat-inactivated or native plasma. Overnight cultures were diluted to 10^3 CFUs/mL (determined using growth curve data). The cells were then added to a 96-well microtiter plate containing a 2-fold dilution series of cephalothin. Heat-inactivated or native plasma only wells were used as positive control wells, respectively. Plates were incubated for 24 hours, then the absorbance at 630 nm (OD_{630}) was measured using a Biotek Gen5 Plate Reader. The MIC50, MIC90, and MIC Breakpoint values were determined following Clinical and Laboratory Standards Institute (CLSI) guidelines [54,55]. Dilution plate counts were used to confirm the MIC values.

RNA Isolation, cDNA Synthesis, and qPCR

RNA was isolated from either LB or HPLM *K. pneumoniae* 43816 cultures in mid-logarithmic phase. Samples were treated with RNA Protect (Qiagen) and proteinase K (New England Biolabs), then RNA was isolated by treatment with TRI Reagent (Ambion), then using the Direct-zol RNA Miniprep kit (Zymo Research), following the instructions of the

manufacturer. Treatment with TRI Reagent was done using three volumes of TRI Reagent to one volume of sample. First-strand synthesis was completed using 1 µg of total RNA and the Protoscript II reverse transcriptase kit (New England Biolabs), following the instructions of the manufacturer. Quantitative polymerase chain reaction (qPCR) was performed on the Eppendorf Mastercycler Realplex 2, using Luna Universal qPCR Master Mix (New England Biolabs). Data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Genes evaluated were *ompK35*, *ompK36*, *ompK26*, *ompA*, *lpp*, and *lamB*, then compared to the *gapA* gene for normalization. Primers are listed in **Table 3.2**. Average expression levels for HPLM were evaluated relative to LB. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale.

Statistical Analysis

Experiments were completed with an $n \geq 3$. Significance was determined using a one-way ANOVA, followed by Tukey's post-hoc test. These analyses were completed using XLSTAT software. Significance was determined at $p < 0.05$.

Results

Growth Curves Indicate Differences between Media Types

LB Allowed Bacteria to Grow to Higher Densities than HPLM

To evaluate if growth rate and cell density were different between *K. pneumoniae* ATCC 43816 in LB broth and HPLM media, a growth curve plotting optical density at 600 nm over 7.5 hours was completed. The growth curve indicated similar growth within the first 1.5 hours (**Figure 3.2**). After this time point, LB grown *K. pneumoniae* showed a much steeper logarithmic

phase compared to HPLM grown *K. pneumoniae*. Ultimately, *K. pneumoniae* from both media types began to reach stationary phase at approximately 4.5 hours. The O.D. reached by *K. pneumoniae* grown in LB was closer to 2.1, whereas the *K. pneumoniae* grown in HPLM was closer to 1.4, meaning the HPLM grown *K. pneumoniae* grew to a lower cell density. The results of this experiment, particularly that the bacteria grown in HPLM did not grow as densely as bacteria grown in LB, were used to ensure that all experiments began with the same density of bacteria. This meant that a higher volume of HPLM grown bacteria was necessary for MIC assays and RNA isolation experiments to reach the same starting density of bacteria.

Heat-Inactivation of Plasma Allowed Bacteria to Grow to Higher Densities Compared to Bacteria Grown in Native Plasma

The goal of this experiment was to evaluate if *K. pneumoniae* ATCC 43816 grown in heat-inactivated plasma vs native plasma demonstrated different growth patterns or cell densities. To evaluate this, samples were taken from a growth curve culture made in each media type, every hour. The samples were diluted, plated, and after 24 hours the number of CFUs were counted, and CFUs/mL were determined. This method of evaluating growth (CFUs/mL as opposed to OD over time) was completed due to the turbidity of the plasma. Sample OD measurements taken prior to this experiment showed large variability for multiple readings of the same sample, and therefore OD was not an accurate measure of cell density. The resulting growth curve indicated similar growth within the first hour between both media types (**Figure 3.3**). After this time point, heat-inactivated plasma grown *K. pneumoniae* showed higher CFUs/mL during both the logarithmic and stationary phases. *K. pneumoniae* grown in heat-inactivated plasma reached 4.2×10^6 CFUs/mL, whereas *K. pneumoniae* grown in native plasma only reached 2.0×10^6 CFUs/mL.

Minimum Inhibitory Concentration is Impacted by Growth Media

To complete MIC assays, several different antibiotics were selected due to their differing mechanisms of action. The four tested antibiotics, cephalothin, imipenem, kanamycin, and tetracycline, tested both the inhibition of cell wall synthesis and the inhibition of protein synthesis. Testing antibiotics with differing mechanisms of action allows for comparisons between strains with and without beta-lactamases and between media types, and ultimately the impacts on resistance to antibiotics that work in different ways.

Laboratory Strain MIC50 and Breakpoint Values Varied Largely Between Media Types

To evaluate if MIC50 and breakpoint values were similar between different media types, a laboratory strain not known to possess resistance mechanisms, *K. pneumoniae* ATCC 43816 was grown in selected media types and resistance was evaluated with an MIC assay. MIC50 values were selected due these values showing the greatest sensitivity to changes, and antibiotic susceptibility as a result. The resulting MIC50 and breakpoint values varied largely depending on the antibiotic and the growth medium.

The first antibiotic tested in the MIC assay was cephalothin. This antibiotic was compared for *K. pneumoniae* ATCC 43816 grown in either LB, HPLM, heat-inactivated plasma, or native plasma. For cephalothin antibiotic, MIC50 values were the highest for HPLM, followed by LB, then both types of plasma. The largest change for cephalothin, however, was seen in the MIC breakpoint values, with the smallest seen in LB, and the largest seen in HPLM (**Table 3.3)**. The breakpoint value range of HPLM for cephalothin, 250-500 µg/mL, is the highest reported within this area of the study and is significantly above the ≥ 32 µg/mL resistance determination for cephalothin antibiotic [54]. Of all the tested media, only bacteria grown in HPLM were at or

above the cephalothin resistance threshold. Comparing HPLM to LB, it was seen that the synthetic media meant to mimic human plasma produced much higher breakpoint values. This result was also seen when comparing HPLM to both plasma types. Ultimately, this result shows that even well-defined media such as HPLM can produce MIC results significantly different from traditional laboratory media, and even the plasma it was designed to mimic.

To confirm the MIC50 values, evaluate bactericidal vs bacteriostatic effects and measure the respective CFUs for comparison between media types, dilution plate counts were completed. The plate count data showed significant differences in CFUs between each treatment and LB (**Table 3.4**). LB had the highest MIC50 plate count averages, followed by HPLM, native plasma, and lastly heat-inactivated plasma. All the treatments indicated a bacteriostatic effect, not a bactericidal effect since growth still occurred, but was shifted from what was expected.

To compare MIC50 and breakpoint values for different antibiotics in addition to cephalothin, *K. pneumoniae* ATCC 43816 was grown in either LB or HPLM. As seen in **Table 3.3**, tetracycline and kanamycin MIC50 values were consistently larger in HPLM than in LB, while imipenem MIC50 values were the same between media types. Regarding breakpoint values, all HPLM breakpoints were substantially larger than those found in LB. This indicates that bacteria grown in a defined and physiological media were able to grow in much higher antibiotic concentrations than the laboratory media.

Clinical Strain MIC50 and Breakpoint Values Varied Largely Between Media Types

To evaluate if MIC50 and breakpoint values were similar between different media types within strains knowing to possess resistance mechanisms, the following experiment was completed. MIC50 values were selected due to these values showing the greatest sensitivity to

changes, and antibiotic susceptibility as a result. The four tested clinical strains can be found in **Table 3.1**. The two antibiotics evaluated, cephalothin and imipenem, both act as cell wall synthesis inhibitors and are beta-lactam antibiotics [58]. As mentioned previously, cephalothin is a first-generation cephalosporin and imipenem is a carbapenem. Carbapenem antibiotics should show significantly lower MIC values, due to being designed to survive mechanisms which cephalothin antibiotic could not. This can include resisting hydrolysis to beta-lactamases or inhibiting them altogether [83]. However, in strains that produce ESBLs (as the four tested clinical strains do), and particularly carbapenemases, the efficacy of carbapenems can decrease, requiring higher concentrations of antibiotic to reach the breakpoint [89].

For cephalothin antibiotic, MIC50 and breakpoint values were the same for all four clinical strains and for both LB and HPLM (**Table 3.5**). The MIC50 was not reached, and the breakpoint was determined to be >1000 µg/mL for cephalothin. Due to the resistance threshold being ≥ 32 µg/mL for cephalothin, all strains in both media were considered clinically resistant [54].

For imipenem antibiotic, MIC50 values were largely similar between strains and media types, with CSUB10S+pSHA16K being the highest at 16 µg/mL (**Table 3.5**). This value was found in HPLM, but the other MIC50 values for the remaining strains were the same between media types. The breakpoint values were also noticeably similar between all strains in both media types, again with CSUB10S+pSHA16K being the highest at 31 µg/mL. This value was also found in the HPLM media. All breakpoint values were considered resistant according to the CLSI guidelines, which places the resistance threshold at ≥ 4 µg/mL for imipenem [55]. Ultimately, the MIC50 and breakpoint values were not significantly different between media types for imipenem.

Alternating Media Types Increased MIC50 and Breakpoint Values Compared to Not Alternating Media

The goal of this experiment was to evaluate if growing bacteria overnight in one media type, then transferring these bacteria to the alternate media type for MIC assays, would alter resistance values. This is seen clinically when bacteria are isolated from a patient where it lives in one set of conditions, then grown in another media type with differing conditions for MIC assays/resistance determinations. The alternation pattern used for this study is described in **Figure 3.1**.

As seen in **Table 3.6**, the *K. pneumoniae* ATCC 43816, grown overnight in HPLM and resuspended in LB, MIC50 for cephalothin was between 4 and 31 µg/mL. For *K. pneumoniae* ATCC 43816, grown overnight in LB and resuspended in HPLM, the MIC50 for cephalothin was between 16 and 31 µg/mL. The *K. pneumoniae* grown initially in LB, then resuspended in HPLM for the MIC assay showed a higher range for the MIC50 value. The breakpoint for both treatments is the same at >1000 µg/mL, which is above the resistance threshold for cephalothin, which is ≥ 32 µg/mL for cephalothin [54]. These results indicate that bacteria originally grown in LB then resuspended in HPLM for the MIC assay were able to survive at higher densities at higher concentrations of antibiotics, which resulted in a higher MIC50 range. The higher densities were seen when plating bacteria from all antibiotics and all concentrations to confirm if growth was occurring. At all concentrations, growth occurred, but was visually denser at higher concentrations for the LB resuspended in HPLM than for the HPLM resuspended in LB.

K. pneumoniae Grown in HPLM Showed Decreased Gene Expression Compared to K. *pneumoniae* Grown in LB

To evaluate gene expression, six genes of interest, *ompK35*, *ompK36*, *ompK26*, *ompA*, *lpp*, and *lamB* were compared to *gapA*, which acted as the house-keeping gene. Bacteria grown in HPLM were also compared against LB using the $2^{-\Delta\Delta CT}$ method [56]. Porins OmpK35 and OmpK36 were evaluated due to their non-specificity, which is often utilized to transport antibiotics into the periplasmic space, as well as their noted loss in many clinical strains. Due to their non-specificity, these porins are essential to the transport of many molecules, so the loss of OmpK35 and/or OmpK36 can have negative effects on the bacteria. This can include a loss of nutrient transport and an inability to exchange toxic metabolites [12]. Therefore, other porins or structures often compensate for the loss of OmpK35 and/or OmpK36. Porins OmpK26 and LamB were evaluated due to their noted compensation in porin function for strains deficient in OmpK35 and/or OmpK36. Porin OmpA and lipoprotein Lpp were evaluated due to their importance in envelope stability and outer membrane integrity.

The average expression for *K. pneumoniae* ATCC 43816 grown in HPLM showed a trend of decreased expression for all tested genes, when compared to growth in LB (**Figure 3.4**). Genes *ompK35*, *ompA*, and *lpp* showed similarly decreased expression, slightly below 3-fold, The *lamB* gene showed a decrease in expression just over 3-fold. The *ompK36* gene showed a decrease in expression nearly 7.5-fold. The largest change was seen in *ompK26* with a decrease in expression of approximately 9-fold.

Discussion

The aim of this study was to evaluate the effects of physiologically relevant media types on bacterial growth, porin gene expression, and on antibiotic susceptibility. It was hypothesized that growth of *Klebsiella pneumoniae*, as well as porin gene expression and antibiotic susceptibility in HPLM and human plasma would be different than in LB broth. It was found that growth between media types was different as bacteria grown in LB media grew to higher densities than when grown in HPLM, and bacteria grown in heat-inactivated plasma grew to higher densities than when grown native plasma. Heat-inactivated and native plasma were utilized in this study to allow for comparisons between a media designed to mimic human plasma and actual human plasma. MIC50 and breakpoint results for the laboratory strain *K. pneumoniae* ATCC 43816 indicate that bacteria grown in LB, heat-inactivated plasma, and native plasma were more susceptible to cephalothin, tetracycline, kanamycin, and imipenem antibiotics than bacteria grown in HPLM. In addition, bacteria grown in plasma and HPLM results in different MIC50 plate counts than bacteria grown in LB. The MIC50 and breakpoint values for the four tested clinical strains (CSUB10R, CSUB10S, CSUB10R+pSHA16K, CSUB10S+pSHA16K) in cephalothin and imipenem were largely similar within antibiotic however, between bacteria grown in LB and bacteria grown in HPLM. The MIC50, and particularly breakpoint values for cephalothin were greatly impacted when media types were altered between overnight to MIC resuspension. There were also significant differences in gene expression between bacteria grown in LB and bacteria grown in HPLM, as all genes for bacteria grown in HPLM were downregulated compared to bacteria grown in LB.

One potential reason for the variation in growth between LB and HPLM is that LB is a rich media designed with high density growth in mind. Comparing this to HPLM, which was

designed to be more representative of actual human plasma, the composition of media types is significantly different. LB broth is created using tryptone, yeast extract, NaCl, and distilled water [81], and due to the contents varying slightly, this media is not considered a defined media. The tryptone provides amino acids needed for protein synthesis, as well as serving as a source of carbon for the bacteria [90]. The yeast extract, often derived from a form of baker's yeast, serves to provide water-soluble vitamins, amino acids, peptides, and carbohydrates. This component is essential to the high rate of growth and cell density seen in LB grown bacteria [91,92]. The NaCl is primarily to aid in transport and providing an osmotic balance [93]. HPLM, which contains over 60 polar metabolites, including amino acids, vitamins, inorganic salts, reducing agents, and water-soluble acids, with a goal of mimicking human plasma closely [94]. The compounds utilized for HPLM serve primarily for mammalian cell culture, so the necessary agents for growth are included. For tissue culture this media can be supplemented with fetal bovine serum (FBS), but since our goal was to evaluate the media as it was, no FBS was added [95].

When growth was evaluated for *K. pneumoniae* ATCC 43816 in LB broth and HPLM similarities were present for the 1.5 hours, as OD between both media types followed a similar pattern, after 1.5 hours LB showed a steeper logarithmic growth phase. At 4.5 hours both media types began to reach stationary phase. The stationary phase for bacteria grown in LB, however, was at a higher OD than bacteria grown in HPLM. This indicates that HPLM grown bacteria grew to a lower cell density than LB grown bacteria. These results indicated that not only would ensuring a similar starting density be essential for experiments conducted comparing bacteria between LB and HPLM, but also that a potential reason may exist for why growth in HPLM was not able to reach as high of a cell density as growth in LB.

The human plasma utilized, which was either heat-inactivated or native, is Pooled Human Plasma Apheresis Derived Na Heparin. As described, half of the plasma remained in the native state and half was heat-inactivated.

When growth was evaluated for *K. pneumoniae* ATCC 43816 in heat-inactivated plasma and native plasma, similarity was seen for growth occurring within the first hour, after which growth was higher in heat-inactivated plasma. The bacteria grown in heat-inactivated plasma was able to grow to 4.2 x 10^6 CFUs/mL, whereas *K. pneumoniae* grown in native plasma only reached 2.0 x 10^6 CFUs/mL. This largely shows the effect of protein denaturation on growth for *K. pneumoniae* in human plasma. Some of proteins that are denatured are complement proteins, which are utilized by the innate immune system to clear bacteria quickly [96]. This potentially explains why the heat-inactivated plasma bacteria grew to higher cell densities, less complement should be present to kill the bacteria, allowing more to survive.

The Impact of Media Type, and of Altering Media Type, on Antibiotic Susceptibility

To compare antibiotics with differing mechanisms of action, four antibiotics were utilized: cephalothin, imipenem, kanamycin, and tetracycline. Cephalothin, a cephalosporin antibiotic, and imipenem, a carbapenem antibiotic both classified as beta-lactam antibiotics utilize a mechanism of action which aims to inhibit cell wall synthesis. This is done when the beta-lactam ring binds the penicillin-binding proteins needed for peptidoglycan cross-linking in the process of cell wall synthesis [58]. Cephalothin and imipenem were compared due to cephalothin being a first-generation cephalosporin and imipenem being a carbapenem. Carbapenem antibiotics should show significantly lower MIC values, due to being designed to survive mechanisms which cephalothin antibiotic could not, particularly resisting hydrolysis to

beta-lactamases or inhibiting them altogether. Carbapenem antibiotics are also known for their high potency within both Gram-negative and Gram-positive bacteria [83].

Kanamycin, an aminoglycoside antibiotic, and tetracycline, a tetracycline antibiotic, both utilized a mechanism of action which aims to inhibit protein synthesis. Kanamycin and tetracycline both interact with the 16S rRNA of the 30S ribosomal subunit. Kanamycin interacts at the A site, altering its conformation to prevent mRNA from being properly translated [58,84]. Tetracycline interacts with conserved sequences to prevent binding of tRNA to the A site [58]. These four antibiotics, therefore, test both the inhibition of cell wall synthesis and the inhibition of protein synthesis. Testing antibiotics with differing mechanisms of action allows for comparisons between strains with and without beta-lactamases and between media types, and ultimately the impacts on resistance to antibiotics that work in different ways.

The MIC50 and breakpoint results for the laboratory strain *K. pneumoniae* ATCC 43816 indicate that bacteria grown in LB, heat-inactivated plasma, and native plasma were more susceptible to cephalothin, tetracycline, kanamycin, and imipenem than bacteria grown in HPLM. In addition, MIC50 plate counts performed for bacteria tested in cephalothin indicated bacteria grown in LB was different than bacteria grown in both plasma types and HPLM. The increase in MIC50 and breakpoints for bacteria grown in HPLM for most antibiotics compared to the other tested media types indicates that not only does the HPLM media type, in some currently unknown way, allow the bacteria to survive to higher concentrations, but that the mechanism of action of the tested antibiotics is largely extraneous, as the MIC50 and breakpoints increased in nearly all antibiotics for HPLM grown bacteria.

One difference regarding antibiotic susceptibility was seen when comparing the MIC50 values between bacteria grown in LB and bacteria grown in HPLM for imipenem. The MIC50

values were the same, while the breakpoint values were much higher in HPLM. This indicates that in imipenem, which is a newer class of beta-lactam antibiotics, the sensitivity of the bacteria to the antibiotics was not largely influenced by media type. This is significant, as MIC50 values for cephalothin, which is a much older class of beta-lactam antibiotics, found the sensitivity of the bacteria to the antibiotics was largely influenced by media type. While the exact reason for this is not known, one explanation for this could include the size of imipenem vs cephalothin. Imipenem is a much smaller molecule, with a molecular weight of approximately 299.4 g/mol, whereas cephalothin is a larger molecule, with a molecular weight of approximately 396.4 g/mol. According to PubChem, cephalothin is also considered a more complex molecule [57,97]. This information suggests that perhaps due to imipenem being smaller and less complex of a molecule that is has an easier time travelling through the membrane and reaching the antibiotic target. This could explain why imipenem performs similarly at low concentrations between media types; changes hinder the ability of imipenem to transverse the membrane and interact with the antibiotic target much less due to it being a smaller and less complex molecule than cephalothin.

The MIC50 plate counts for bacteria tested in cephalothin indicated that bacteria grown in LB was different than bacteria grown in both plasma types and HPLM. This is significant, as it indicates that a bacteriostatic effect was occurring, not a bactericidal effect. Bacteriostatic means the bacteria is alive and in stationary phase, while bactericidal means the bacteria is killed. This indicates that under differing media types, antibiotics may have differing effects regarding if they act as bacteriostatic or bactericidal.

The MIC50 and breakpoint values for the four clinical strains (CSUB10R, CSUB10S, CSUB10R+pSHA16K, CSUB10S+pSHA16K) for cephalothin and imipenem were largely similar between LB and HPLM. The results of this are understandable, as the four clinical strains

possess known resistance genes. The MIC50 and breakpoint values remaining similar between media types indicates that strains possessing known resistance mechanisms may be less impacted by media type. The similarities between MIC50 and breakpoint values regardless of if the strain possessed, or did not possess, OmpK35 and/or OmpK36 indicates that media type did not alter the antibiotic susceptibility differently in the strains with different porin presence/absence.

To evaluate if growing bacteria overnight in one media type and then transferring them to the alternate media type for MIC assays would alter resistance values, the media type alteration experiment was completed. This alteration between media types is seen clinically when bacteria is isolated from a patient where it lives in one set of conditions, then grown in another media type with differing conditions for MIC assays/resistance determinations.

The MIC50, and particularly breakpoint values for *K. pneumoniae* ATCC 43816 tested using cephalothin, were greatly impacted when media types were altered between overnight to MIC resuspension. The MIC50 values were slightly higher in the bacteria grown in LB overnight to HPLM resuspension vs the bacteria grown in HPLM overnight to the LB resuspension. These MIC50 values are also higher than the bacteria grown in LB and bacteria grown in HPLM MIC50 values obtained when the MIC assays were performed in the same media as the overnight culture. The increase in MIC50 values not only indicates that the bacteria exposed to both media are able to survive at higher antibiotic concentrations, but also that the order of the exposure potentially matters. This is highly significant, as bacteria isolated from the human body are often tested in laboratory media for susceptibility determinations. If changes to antibiotic susceptibility can occur for bacteria between the human body and the laboratory media, the results of antibiotic susceptibility assays could be an inaccurate reflection of the true antibiotic susceptibility values.

The breakpoint values were the same for both combinations, with breakpoints >1000 µg/mL. This is significantly higher than the LB and the HPLM breakpoint values obtained when the MIC assays were performed in the same media as the overnight culture. This increase reflects the conclusion from the MIC50 data, that the bacteria exposed to both media can survive at higher antibiotic concentrations. However, order of exposure does not appear to have as large of an impact.

The reason for the increases in MIC50 and breakpoint values when the bacteria are exposed to both media types could imply that bacteria respond differently when switched between media types. One study evaluated the effects of pH modification on bacterial growth in *E. coli* and *K. oxytoca*, as well as the impact on antimicrobial activity. To test this *E. coli* ATCC 25922 and *K. oxytoca* ATCC 700324 were grown overnight culture on a Mueller-Hinton agar plate enriched with spores of *B, subtilis* ATCC 6633. The bacteria were then individually added to sterilized, pooled urine adjusted to have a pH of 5.0, 6.0, 7.0, or 8.0, or Mueller-Hinton broth, at an inoculum of approximately 5×10^5 CFU/mL. Bacteria were counted periodically over a 24-hour incubation time. Antibiotic susceptibility tests found that urine acidification decreased the susceptibility of both *E. coli* and *K. oxytoca* to all tested fluoroquinolone antibiotics [98]. Fluroquinolones work by inhibiting DNA gyrase and topoisomerase IV [99]. The pH 5 urine increased MIC 35- to 45-fold as compared to Mueller-Hinton broth, while pH 7 and 8 performed similarly to Mueller-Hinton broth. The reduction of antimicrobial activity was found to be reversible once urine incubated bacteria were returned to their original broth. When [C^{14}]-ciprofloxacin was introduced to the bacteria, following incubation, the impact of pH on ciprofloxacin uptake was evaluated. The results found that the mean uptake of [C^{14}]-ciprofloxacin was the lowest at pH 5 and increased stepwise as pH increased. The pH 7 and pH 8

samples had a ratio like that found in the Mueller-Hinton broth. The results largely indicate that pH can have a highly significant impact on MIC results [98].

When comparing the present study with the study on urine acidification, media type can have a highly significant impact on MIC results. Based on the results of their study, pH could be a contributing factor to the results seen here. Human plasma has a pH of approximately 7.4, while LB broth has a pH of approximately 6.8-7.2 [81,85,86]. While these pH values are similar, LB has a much more variable pH value. This could potentially cause similar results to what was seen in the other study [98]. One potential way to evaluate this would be to check the pH of media sources used within the present study and compare the pH to the MIC results. Another study evaluated the impact of urine vs cation-adjusted Mueller-Hinton broth on meropenem MIC results for *K. pneumoniae* (wild-type, clinical, and metallo-beta-lactamase-/*K. pneumoniae*-carbapenemase-harboring strains) and found metallo-beta-lactamase-mediated resistance was attenuated in urine [100]. The results of the present study and the two previous studies show how small changes in environmental conditions can cause large changes in antibiotic susceptibility. A few changes to the present study that could help determine why the changes occurred that we found would be to test alternate factors such as pH or incubation times between media types.

The adaptability of bacteria to different environments is one of the reasons bacteria are so often successful as conditions change. Bacteria can often undergo a process where they evaluate and respond to their environment known as quorum sensing. This can help bacteria to collectively sense the surrounding environment and respond as a population. Quorum sensing allows bacteria to respond quickly to environmental changes [101].

The Impact of Media Type on Porin Gene Expression

When evaluating gene expression between bacteria grown in LB and bacteria grown in HPLM for *K. pneumoniae* ATCC 43186, it was found that all genes for bacteria grown in HPLM were downregulated compared to bacteria grown in LB. The smallest downregulation occurred in *ompA*, and the largest downregulation occurred in *ompK26*. The downregulation of all genes evaluated is significant, as this indicates that the six tested genes could be highly sensitive to environmental changes. Also, for bacteria grown under the conditions of the physiologic media, the downregulation of all six genes occurred when compared to bacteria grown in LB. This is significant, as porins are important for the transport of nutrients across the outer membrane. If all the tested porin genes are downregulated, the bacteria could either be finding alternate porins to utilize, slowing down metabolic processes, or be potentially nutrient deprived. This impact on porin gene expression suggests that media type could be a highly influencing factor to evaluating gene expression. Furthermore, this suggests that media type should be carefully considered when designing studies, especially those meant to evaluate gene expression under physiologic conditions.

One study evaluated the impact of culture medium on porin gene expression and susceptibility of *Escherichia coli* to piperacillin-tazobactam [102]. To evaluate the impact of media type, specifically the change in osmolarity between media types, *E. coli* clinical isolates were grown in one of four media types, including: Mueller-Hinton (MH) medium, GN1 medium (utilized by many automated systems for the *Enterobacteriaceae* family, nutrient broth, and nutrient broth supplemented with 20% sorbitol. The nutrient broth (NB) and nutrient broth supplemented with 20% sorbitol (NBS) were utilized as controls. Clinical isolates were then organized into groups based on if the isolates were susceptible or resistant in MH media/susceptible or resistant in GN1 media. The results indicated that strains that were

susceptible in one media type could either remain susceptible in the other media type or become resistant. This same pattern was seen in strains that were shown resistant in one media type, the strain could either remain resistant in the alternate media type or become susceptible. This indicates that changing media type can potentially alter antibiotic susceptibility [102].

The present study additionally found that changing media could potentially impact susceptibility. This was seen when bacteria were grown overnight in one media type then resuspended in another. The resistance susceptibility to cephalothin decreased significantly for both combinations of overnight to resuspension, even when compared to the decrease in susceptibility seen for bacteria grown in HPLM for both overnight and resuspension (which was already significantly higher than bacteria grown in LB for both overnight and resuspension). This indicates that taking bacteria from one media type (clinically this is the human body) and resuspending the bacteria in another media type for susceptibility evaluations (often this is completed in a laboratory media) could potentially impact the resulting susceptibility analysis negatively.

Further evaluation by Pinet *et al.*, regarding the reason for the change in susceptibility found large changes in porin gene expression between media type. Immunodetection by specific antibodies and quantification of gene expression was assessed. Immunodetection of porins was completed by evaluation with antisera against OmpF and OmpC. OmpF is the *E. coli* homolog of OmpK35 and OmpC is the *E. coli* homolog of OmpK36. Ultimately, OmpC was over-expressed in GN1 and NBS media, while OmpF was over-expressed in MH and NB media. Quantification of gene expression using porin-beta-Galactosidase gene fusion found that porin expression, particularly expression of OmpF, was favored in MH, GN1, and NB media. Further modification

of media osmolarity resulted in changes to porin expression. High osmolarity resulted in a 2- to 3-fold increase in piperacillin-tazobactam MIC for all but two strains [102].

The results of the study by Pinet *et al.* are reflective of the results found within this study. Specifically, that porin gene expression can vary between media types and those changes can potentially impact antibiotic susceptibility. One difference between studies was seen when susceptibility was evaluated for clinical strains with known susceptibility values. The clinical strains remained resistant to cephalothin for both media types consistently. This is different from the results from the previous study which found that a strain could either remain resistant in the alternate media type or become susceptible. In the present study all the resistant strains remained resistant, with none becoming susceptible in any of the tested conditions.

Other studies have found differences in gene expression and changing culture media type as well. One study investigated the gene expression of *L. helveticus* in either milk or MRS broth. The results obtained from annotated gene probes found 42 genes that were upregulated in the milk and found numerous additional genes using titled microarrays [103]. Another study looked at gene expression for *P. aeruginosa* in a synthetic growth medium called modified artificial-sputum medium, which was designed to mimic lung sputum from patients with cystic fibrosis. The results found that fifty genes were differentially expressed in the artificial media compared to LB. Also, that the media was highly suitable to evaluating *P. aeruginosa* gene expression in cystic fibrosis [104].

Conclusions

The aim of this study was to evaluate the effects of physiologically relevant media types on growth, porin gene expression, and on antibiotic susceptibility. It was hypothesized that

growth of *Klebsiella pneumoniae*, as well as porin gene expression and antibiotic susceptibility in HPLM and human plasma would be different than in LB broth. The hypothesis was neither completely supported nor rejected. The first part of the hypothesis that was supported was that bacterial growth would be different between LB, plasma (heat-inactivated and native), and HPLM. The results indicated that LB was able to grow to much higher cell densities than HPLM, consistent with the higher nutrient content of LB.

The second part of the hypothesis that was supported was that porin gene expression would be different between LB and HPLM. The results indicated that all six tested genes were downregulated in bacteria grown in HPLM compared to bacteria grown in LB. This result is understandable as HPLM grown bacteria grew less robustly and densely when compared to LB grown bacteria. This potentially suggests less nutrients needed for bacterial growth are present in HPLM, so porin gene expression is downregulated, as less porins are needed for nutrient transport.

The third part of the hypothesis that was not fully supported was that media type would impact antibiotic susceptibility. The results that supported this did indicate that HPLM was significantly less susceptible to antibiotics compared to LB, heat-inactivated plasma, and native plasma for the laboratory strain, *K. pneumoniae* ATCC 43816. This is highly significant, as it indicates that bacteria grown in HPLM show different antibiotic susceptibility than laboratory media and actual human plasma. Therefore, testing bacterial antibiotic susceptibility in HPLM may not be reflective of true susceptibility values. Additionally, bacteria grown in both plasma types were found to be less susceptible than bacteria grown in LB. This indicates that utilizing a laboratory media for susceptibility determinations may not result in true susceptibility values. When applying this clinically, this could indicate that isolating bacteria from the human body,

then testing the bacteria in a laboratory media to determine susceptibility, may not be accurate for determining the amount of antibiotic or specific antibiotic needed for treatment.

The third part of the hypothesis, that media type would impact antibiotic susceptibility, was also not fully supported. The results that rejected this indicated that there was no significant difference between LB and HPLM when compared within four clinical strains. As the four clinical strains possess numerous known resistance mechanisms, whereas the laboratory strain does not, this result is understandable. Altering media types did indicate differences between bacteria grown overnight in one media type and resuspended in another. The results showed that the highest antibiotic concentrations required for cephalothin breakpoints occurred when media types were altered between overnight and resuspension for MIC assays.

While research is currently being completed to evaluate how gene expression differs between different types of culture media, more studies are needed. Also, more studies are needed to compare the changes in gene expression with changes in antibiotic susceptibility. Lastly, more experimentation is needed to compare both the gene expression and antibiotic susceptibility changes because of differing culture media between laboratory and clinical strains. Ultimately, more research is needed to decide which media is the most accurate for clinical determination of MICs, as our results indicate that different media types can produce different antibiotic susceptibility results when compared to each other and to conditions found within the human body.

Ultimately, the hypothesis that growth of *Klebsiella pneumoniae*, as well as porin gene expression and antibiotic susceptibility for bacteria grown in HPLM and human plasma would be different than for bacteria grown in LB broth, was largely supported (as explained above). The results observed indicate that careful consideration should be given for experiments evaluating

gene expression and antibiotic susceptibility, as large differences were observed. Also, thought should be given into how these differences could potentially reflect larger differences in a clinical setting.

Tables and Figures

Table 3.1. Expression of Outer Membrane Porins OmpK35 and OmpK36

Porin Expression	CSUB10R	CSUB10S	CSUB10R+ pSHA16K	CSUB10S+ pSHA16K	ATCC 43816
Abbreviation	10R	10S	10R+16K	10S+16K	43816
ompK35	-	-	+	+	+
ompK36	-	+	-	+	+

Table 3.2. Primers Used for qPCR

Gene	**Forward Primer 5'- 3'**	**Reverse Primer 5'- 3'**
ompK35	TTCGACAACGCTATCGCACTGTCT	AGTACATGACGGCCGCATAGATGT
ompK36	CCGTCAACCAGACCGAAGAA	CAGGCCTGAAATTTGGCGAC
ompK26	GAACAACGCCCGGCAAGATGATGA	AGCTGCGGGCATAGACATAGTTCA
ompA	ACGTGCTCAGTCCGTTGTTGACTA	AGTAACCGGGTTGGATTCACCCAT
lpp	CGGTAATCCTGGGTTCTACTCT	TGCTCAGCTGGTCAACTTTAG
lamB	GCGGGTAAACGCTTCTATCA	GGTCAACGTTTTCCAGACCT
gapA	TTGACCTGACCGTTCGTCTGGAAA	AGCATCGAACACGGAAGTGCAAAC

Table 3.3. MIC50 and Breakpoint Values for *Klebsiella pneumoniae* ATCC 43816 in LB, HPLM, Heat-Inactivated Plasma, and Native Plasma. Cells with An Asterisk (*) are Above the Breakpoint for the Tested Antibiotic (Cephalothin is ≥ 32 µg/mL, Tetracycline is ≥ 16 µg/mL, Kanamycin is ≥ 64 µg/mL, and Imipenem is ≥ 4 µg/mL).

LB (n≥8)		
Antibiotic	**MIC50 (µg/mL)**	**Breakpoint (µg/mL)**
Cephalothin	1 - 4	4 - 8
Tetracycline	0.5 - 4	2 - 8
Kanamycin	4	8 - 16
Imipenem	0.5	0.5 - 2

HPLM (n≥8)		
Antibiotic	**MIC50 (µg/mL)**	**Breakpoint (µg/mL)**
Cephalothin	4 - 16	250 - 500*
Tetracycline	4 - 8	62.5 - 125*
Kanamycin	1 - 16	62.5
Imipenem	0.5	8 - 16*

Heat-Inactivated Plasma (n=3)		
Antibiotic	**MIC50 (µg/mL)**	**Breakpoint (µg/mL)**
Cephalothin	1	16

Native Plasma (n=3)		
Antibiotic	**MIC50 (µg/mL)**	**Breakpoint (µg/mL)**
Cephalothin	1	16

Table 3. 4. Plate Counts of MIC50 for LB, HPLM, Heat-Inactivated, and Native Plasma for *Klebsiella pneumoniae* ATCC 43816 Following MIC Protocol to Determine Bacteriostatic vs Bactericidal Effects in Cephalothin. Cells with An Asterisk (*) are Statistically Different from LB ($p < 0.0001$). Determined by Tukey's HSD.

MIC50 Plate Count Averages (CFUs) (n=3)			
LB	**HPLM**	**Heat-Inactivated Plasma**	**Native Plasma**
8.85×10^{5}	1.09×10^{4} *	4.87×10^{3} *	5.23×10^{3} *

Table 3.5. Comparison of MIC values for Cephalothin and Imipenem in LB vs HPLM for Clinical Strains. Cells with An Asterisk (*) are Above the Breakpoint for the Tested Antibiotic (Cephalothin is ≥ 32 µg/mL and Imipenem is ≥ 4 µg/mL).

Cephalothin (n=4)					
Media	**Strain**	***ompK35***	***ompK36***	**MIC50 µg/mL Average**	**Breakpoint µg/mL Average**
LB	All clinical strains	N/A	N/A	Not Reached	>1000*
HPLM	All clinical strains	N/A	N/A	Not Reached	>1000*
Imipenem (n=4)					
Media	**Strain**	***ompK35*** **Expression**	***ompK36*** **Expression**	**MIC50 µg/mL Average**	**Breakpoint µg/mL Average**
LB	CSUB10R	-	-	8	16*
	CSUB10R + pSHA16K	+	-	8	16*
	CSUB10S	-	+	8	16*
	CSUB10S + pSHA16K	+	+	8	16 - 31*
HPLM	CSUB10R	-	-	8	16*
	CSUB10R + pSHA16K	+	-	8	16 - 31*
	CSUB10S	-	+	8	16*
	CSUB10S + pSHA16K	+	+	16	31*

Table 3.6. MIC50 and Breakpoint Values for *Klebsiella pneumoniae* ATCC 43816 in Cephalothin: Altered Media Type. Cells with An Asterisk (*) are Above the Breakpoint for the Tested Antibiotic (Cephalothin is ≥ 32 µg/mL)

Treatment (n=12)	MIC50 µg/mL Average	Breakpoint µg/mL Average
HPLM Overnight to LB Resuspension	4 - 31	>1000*
LB Overnight to HPLM Resuspension	16 - 31	>1000*

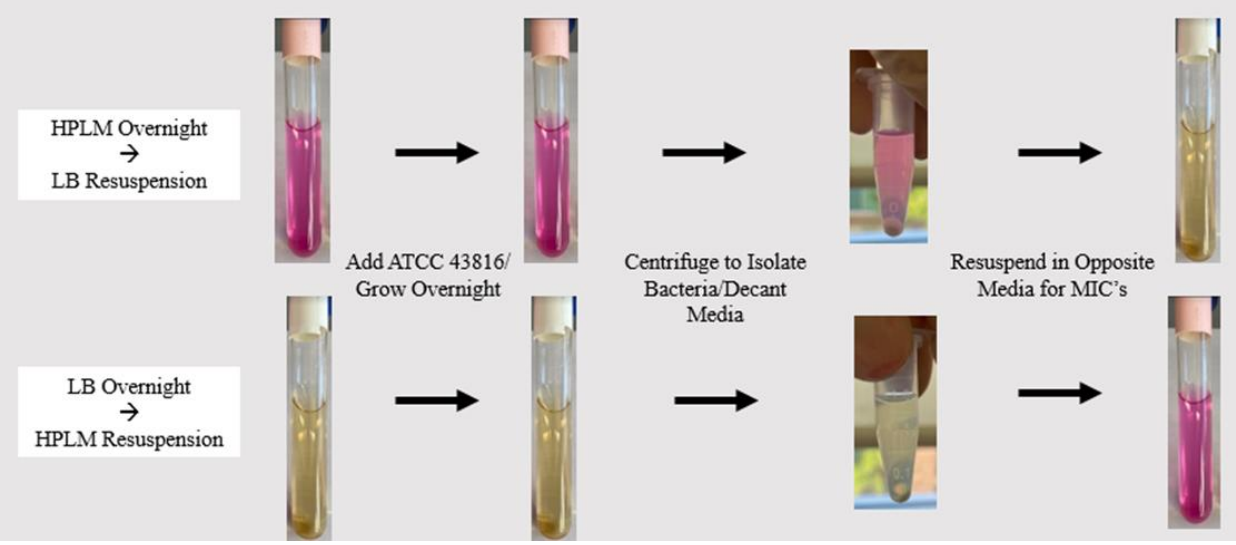


Figure 3.1. Procedure for Creating Overnight Cultures and MIC Resuspensions to Evaluate Altering Media Type. To evaluate the extent of the effect of media type on MIC values *K. pneumoniae* 43816 was grown in either LB or HPLM overnight at 37℃ and 200 rpm. Overnight cultures of bacteria were then centrifuged, all residual media removed, and the bacteria resuspended in the opposite media type. Optical density was used to ensure a similar number of bacteria in both media types for resuspension. The cultures were diluted until reaching an optical density of 0.6 at 600 nm. The bacterial resuspensions were added to a 96-well microtiter plate containing a 2-fold dilution series of cephalothin in the new growth media. LB or HPLM only wells were used as positive controls. Plates were incubated for 24 hours, then the absorbance at 630 nm (OD_{630}) was measured using a Biotek Gen5 Plate Reader. The MIC50, MIC90, and MIC Breakpoint values were determined following Clinical and Laboratory Standards Institute (CLSI) guidelines [54,55]. Dilution plate counts were used to confirm the MIC values.

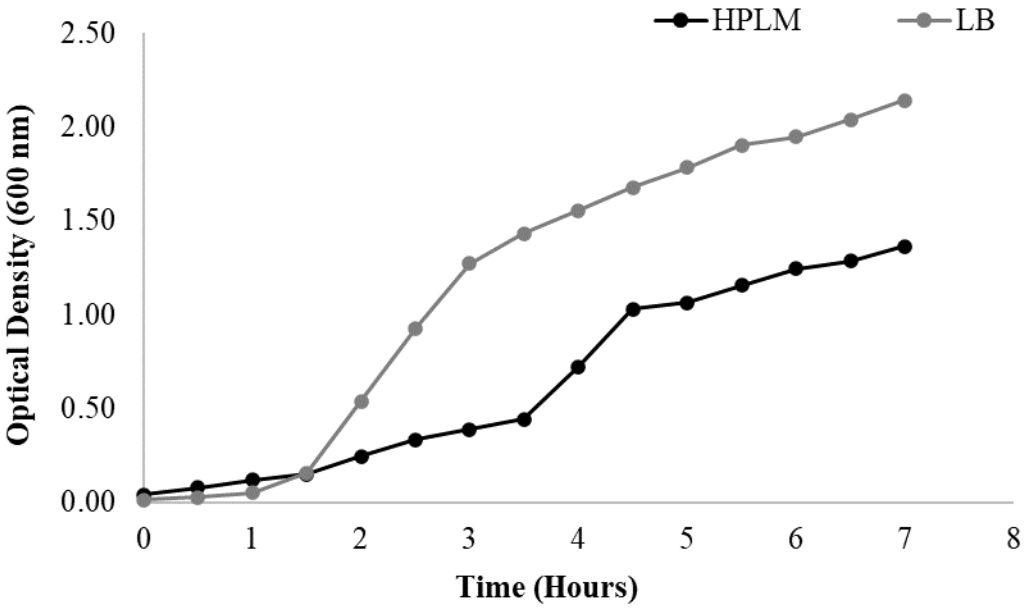


Figure 3.2. Growth Curve Comparing LB and HPLM for *Klebsiella pneumoniae* ATCC 43816. To evaluate differences in growth rates and patterns, K. pneumoniae 43816 was grown in either LB broth or HPLM overnight at 37℃ and 200 rpm. A 1:1000 dilution of overnight culture was made in fresh media of the same type and grown at 37℃ and 200 rpm. The optical density (OD) was measured at 600 nm every 30 minutes for 7 hours. The growth curve indicated similar growth within the first 1.5 hours. After this time point, LB grown *K. pneumoniae* showed a much steeper logarithmic phase compared to HPLM grown *K. pneumoniae*. Ultimately, *K. pneumoniae* from both media types began to reach stationary phase at approximately 4.5 hours. The O.D. reached by *K. pneumoniae* grown in LB was closer to 2.1, whereas the *K. pneumoniae* grown in HPLM was closer to 1.4, meaning the HPLM grown *K. pneumoniae* grew to a lower cell density.

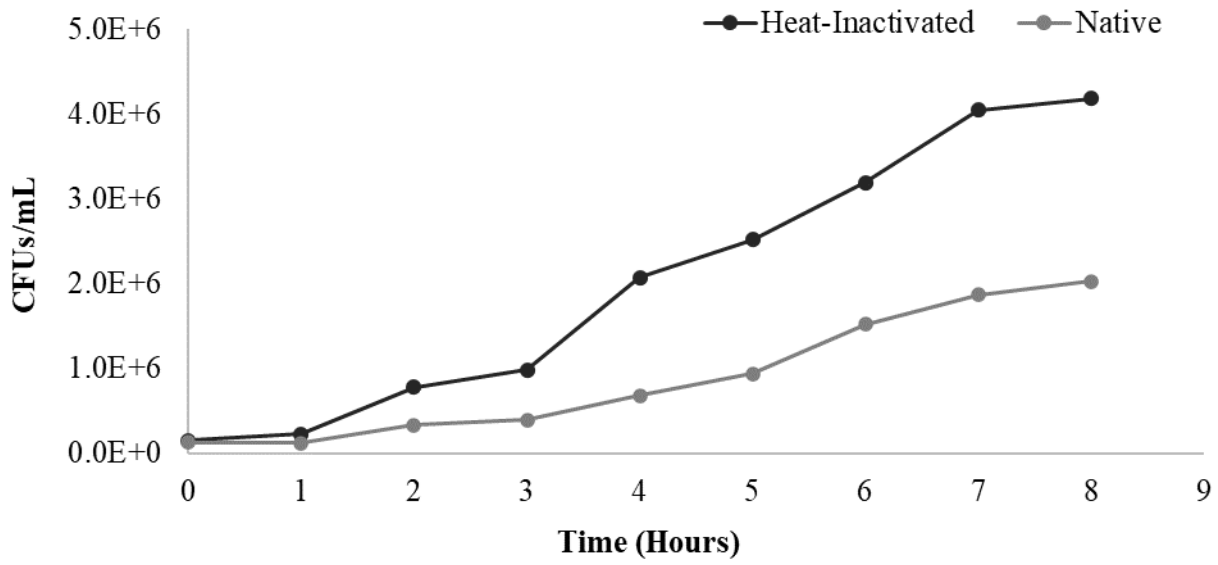


Figure 3.3. Growth Curve Comparing Heat-Inactivated and Native Human Plasma for *Klebsiella pneumoniae* ATCC 43816. Dilution plate counts were used to determine the growth curve in human plasma. *K. pneumoniae* 43816 was grown in human plasma that was either heat inactivated or not heat inactivated. The bacteria were incubated overnight at 37℃ and 200 rpm shaking. A 1:1000 dilution of overnight culture was made in fresh media of the same type (heat-inactivated or Native) and grown at 37℃ and 200 rpm. Samples were taken every hour for eight hours for dilution plate counts on LB agar. The growth curve indicated similar growth within the first hour between both media types. After this time point, heat-inactivated plasma grown *K. pneumoniae* showed higher CFUs/mL during both the logarithmic and stationary phases. *K. pneumoniae* grown in heat-inactivated plasma reached 4.2 x 10^6 CFUs/mL, whereas K. pneumoniae grown in native plasma only reached 2.0 x 10^6 CFUs/mL.

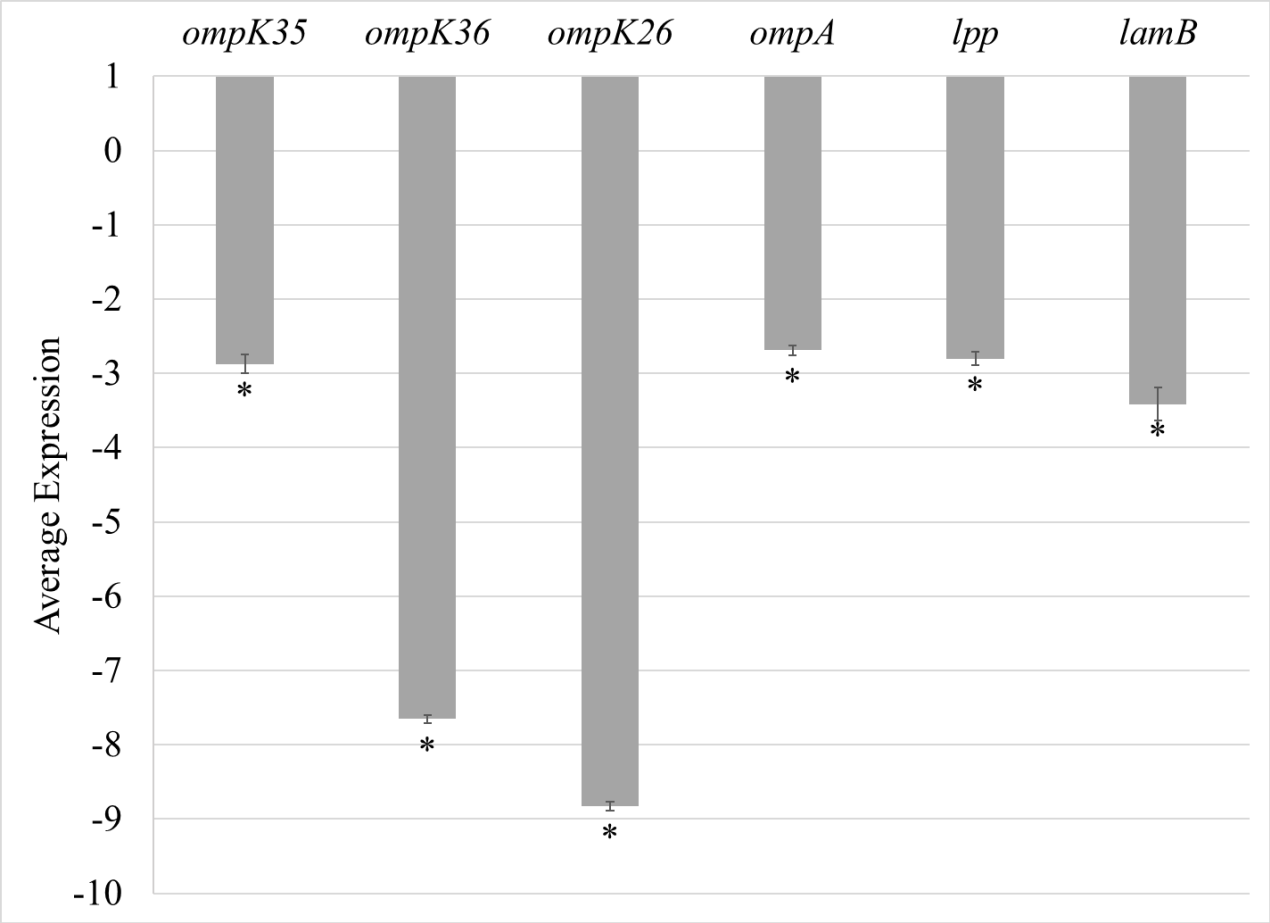


Figure 3.4. Average Expression Corrected for ATCC 43816 Grown in HPLM Compared to ATCC 43816 Grown in LB (n=3). RNA was isolated from either LB or HPLM *K. pneumoniae* 43816 cultures in mid-logarithmic phase. Samples were treated with RNA Protect and proteinase K. RNA was isolated by treatment with TRI Reagent, then using the Direct-zol RNA Miniprep kit. First-strand synthesis was completed using the isolated RNA. qPCR was performed and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [56]. Gene expression was compared to the *gapA* gene for normalization. Average expression levels for HPLM were evaluated relative to LB. All decimal values below 1.0 were corrected by dividing -1 by the decimal value (-1/decimal). This allowed for visualization of the decimals as proportional negatives on a fold-scale. The average expression for *K. pneumoniae* ATCC 43816 grown in HPLM showed a trend of decreased expression for all tested genes, when compared to growth in LB. Error Bars Represent Standard Error. p-values of <0.0001 (indicated by *) were Determined Using One-Way ANOVAs for Each Gene.

Conclusion

The major aim of this study was to determine if physiologically relevant cholesterol levels and media types impact porin gene expression and antibiotic resistance in *K. pneumoniae*. The aim of the first part of the study was to evaluate how *K. pneumoniae* responded to physiologically relevant cholesterol levels *in vitro*; one laboratory strain and four clonally related clinical isolates were individually exposed to different cholesterol levels. It was hypothesized that as the levels of cholesterol increase, porin expression in *K. pneumoniae* would decrease and antibiotic susceptibility would decrease. The aim of the second part of the study was to evaluate the effects of physiologic media vs traditional media on porin gene expression and further on antibiotic susceptibility. To evaluate how *K. pneumoniae* responds in traditional media vs physiologic media, one laboratory strain and four clonally related clinical isolates were individually exposed to either Lysogeny broth, Human Plasma-Like Media, or isolated human plasma (heat-inactivated or not heat-inactivated). The Lysogeny broth acted as the laboratory media, HPLM acted as the synthetic, physiologic media (which was designed/formulated to mimic human plasma), and the human plasma acted as the actual physiologic media with or without protein denaturation. It was hypothesized that growth of *K. pneumoniae*, as well as porin gene expression and antibiotic susceptibility in HPLM and human plasma would be different than in LB broth.

The first part of this study focused on evaluating the effects of cholesterol, within a physiological range on *K. pneumoniae* porin gene expression and further on antibiotic susceptibility. It was expected that as the concentration of cholesterol increased, porin gene expression and antibiotic susceptibility would decrease in *K. pneumoniae*. However, it was found that in addition to cholesterol impacting gene expression and antibiotic susceptibility the

surfactant, methyl-β-cyclodextrin, also impacted both gene expression and antibiotic susceptibility. MIC50 values were similar between MBC and cholesterol treatments within the laboratory strain, *K. pneumoniae* ATCC 43816, while breakpoints differed. For the MBC and cholesterol treated *K. pneumoniae* clinical strains, MIC50 and breakpoint values were similar between each strain (CSUB10R, CSUB10S, CSUB10R+pSHA16K, CSUB10S+pSHA16K). Gene expression seen for both the laboratory strain and clinical strains indicated large differences between cholesterol and MBC treatments across most of the tested genes.

The second part of this study focused on evaluating the effects of physiologically relevant media types on growth, porin gene expression, and on antibiotic susceptibility. It was hypothesized that growth of *K. pneumoniae*, as well as porin gene expression and antibiotic susceptibility in HPLM and human plasma would be different than in LB broth. It was found that growth between media types was different as bacteria grown in LB media grew to higher densities than when grown in HPLM, and bacteria grown in heat-inactivated plasma grew to higher densities than when grown native plasma. Heat-inactivated and native plasma were utilized in this study to allow for comparisons between a media designed to mimic human plasma and actual human plasma. MIC50 and breakpoint results for the laboratory strain *K. pneumoniae* ATCC 43816 indicate that bacteria grown in LB, heat-inactivated plasma, and native plasma were more susceptible to cephalothin, tetracycline, kanamycin, and imipenem antibiotics than bacteria grown in HPLM. In addition, bacteria grown in plasma and HPLM results in different MIC50 plate counts than bacteria grown in LB. The MIC50 and breakpoint values for the four tested clinical strains (CSUB10R, CSUB10S, CSUB10R+pSHA16K, CSUB10S+pSHA16K) in cephalothin and imipenem were largely similar within antibiotic however, between bacteria grown in LB and bacteria grown in HPLM. The MIC50, and

particularly breakpoint values for cephalothin were greatly impacted when media types were altered between overnight to MIC resuspension. There were also significant differences in gene expression between bacteria grown in LB and bacteria grown in HPLM, as all genes for bacteria grown in HPLM were downregulated compared to bacteria grown in LB.

In conclusion, this study evaluated the effects of altering physiologically relevant cholesterol levels and media types on porin gene expression in *K. pneumoniae* and the resulting impact on antibiotic resistance. The results of this study indicate that physiologically relevant levels of cholesterol and media types can have several significant effects on porin gene expression and on antibiotic resistance. Laboratory strain *K. pneumoniae* was more impacted by changes in cholesterol than clinical strains of *K. pneumoniae* regarding changes to antibiotic susceptibility; however, gene expression varied largely in all strains for genes and cholesterol concentration. Gene expression was downregulated for all six tested genes in the physiologic media compared to the laboratory media; also, the physiologic media often had significantly lower antibiotic susceptibility than the laboratory media. Finally, it was found that surfactant can have significant effects on both gene expression and antibiotic susceptibility analyses. Ultimately, the results indicate that physiological conditions can impact gene expression and antibiotic susceptibility, so careful consideration should be given to experiments designed to mimic physiological conditions.

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