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Determining the Role of Propionate and SigB on Benzalkonium Chloride Resistance in *Listeria monocytogenes*

Honors Thesis

Emma Schaefer

Department: Biology

Advisor: Yvonne Sun, Ph.D.

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Abstract

Listeria monocytogenes (*L. monocytogenes)* is a deadly food borne pathogen that causes listeriosis infection in humans with a high mortality rate. *L. monocytogenes* can form biofilms in food processing environments and become resistant to antimicrobial agents such as benzalkonium chloride (BC) and other quaternary ammonium chlorides (QACs). BC is used for cleaning and disinfection of food processing areas and is known to disrupt cell membranes of *L. monocytogenes*, causing cytosol leakage and the degradation of proteins and nucleic acids. Previous research shows that QAC resistance is associated with the upregulation of certain efflux pump genes (*mdrL*, *brcABC*, *qacH*, and *emrE*). Moreover, biofilm formation can also contribute to QAC resistance and subsequent persistence of *L. monocytogenes* in the environment. In addition, propionate is a commonly used food additive for flavoring and spoilage prevention that can potentially regulate *L. monocytogenes* biofilm formation. Biofilm formation and the expression of efflux pumps can both be regulated by the stress response sigma factor SigB in *L. monocytogenes*. However, it is not clear whether propionate affects this regulatory pathway. Therefore, my Honors Thesis research aims to investigate whether propionate can be used to increase *L. monocytogenes* susceptibility to BC and to determine the role of transcription factors, such as SigB, in conferring BC resistance. Results indicate that BC decreases planktonic growth in the presence of propionate in aerobic conditions, but not anaerobic conditions. Additionally, the growth of the Δ*sigB* mutant is significantly reduced by BC under anaerobic but not aerobic conditions. These results highlight that SigB as well as the presence or absence of oxygen all play critical roles in regulating *L. monocytogenes* susceptibility to BC. Therefore, environmental conditions and genetic composition of *L. monocytogenes* can both contribute to the efficacy of our antimicrobial efforts in the food processing industry.

Acknowledgements

I would like to thank the University of Dayton Biology Department and especially Dr. Yvonne Sun for her support, encouragement, and mentorship throughout the research and writing of this Honors Thesis. Thank you to the other undergraduate student researchers in the lab for your advice, collaboration, and company. Finally, thank you to the Dean's Summer Fellowship for funding a portion of this Honors Thesis research.

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Introduction

Listeria monocytogenes as a Pathogen

Listeria monocytogenes (*L. monocytogenes)* is a deadly foodborne pathogen that causes listeriosis infection in humans with a high mortality rate, including severe side effects such as meningitis and sepsis.[1] The CDC estimates around 1,600 people are infected with listeriosis each year and 260 are fatal cases.[2] The most at risk groups for listeriosis infection include pregnant women, the elderly, and the immunocompromised.[3] In the 1990s, *L. monocytogenes* infections were mostly linked to meat processing facilities, but more recently, outbreaks have been traced back to dairy and produce processing environments.[2] Immunocompromised and pregnant individuals are at greatest risk for experiencing the severe effects of listeriosis. It is believed that the principal route of infection of *L. monocytogenes* is through consumption of contaminated food.

L. monocytogenes is classified as a Gram-positive, rod-shaped, facultative anaerobe.[4] *L. monocytogenes* is a non-spore-forming bacterium that does not contain a capsule and is motile through the use of flagella.[5] The foodborne pathogen is present in rural environments and is transported to food processing facilities through the contamination of organic matter.[4] Previous *L. monocytogenes* outbreaks have been associated with raw fruits and vegetables, unpasteurized dairy products, and deli meats.[6] Its persistence in food processing facilities through biofilm formation is thought to contribute to outbreaks and listeriosis infection.

Listeria monocytogenes Persistence in the Environment

The FDA has set industry guidelines for food processing facilities to decrease *L. monocytogenes* persistence and listeriosis outbreak. The FDA reported that foods with a pH of less than 4.4 and a water activity of less than 0.92 typically do not support *L. monocytogenes* growth because the bacteria do not survive well in these conditions.[3] Some foods naturally have these characteristics, while other food products are treated with antimicrobials and kept in temperature controlled environments, which works in combination with the intrinsic properties of the food to control bacteria growth.[3] Currently in food processing facilities, the FDA recommends including the following steps in a written sanitation procedure: dry clean, pre-rinse, soap and scour, post-rinse, prepare for inspection, pre-op inspection, sanitize and assemble.[3]

Even with strict sanitation protocols, *L. monocytogenes* can persist in food processing environments due to its ability to survive in harsh conditions and colonize in areas that are difficult to properly sanitize. *L. monocytogenes* has been shown to survive at a large pH range and in environments with high salt concentrations and refrigeration temperatures.[7] Cross-contamination is another concern in food processing facilities that can contribute to bacteria persistence and disease outbreak. Even though high heat has been shown to inactivate *L. monocytogenes*, such as during the pasteurization process of dairy products, postprocessing contamination can occur.[7] This postprocessing contamination risk can be attributed to how cleanable the equipment is and the presence or absence of hygienic zoning in the facility.[8]

Benzalkonium chloride (BC), a quaternary ammonium chloride (QAC), is used for cleaning and disinfection of food processing areas. Most benzalkonium chlorides are

on the commercial market as a compound mixture with varying lengths of alkyl chain, with C_{12} and C_{18} derivatives eliciting the strongest biocidal effects.[9] The known mechanism of action of QACs involves disruption of cell membranes causing cytosol leakage and the degradation of proteins and nucleic acids.[10] BC-resistant strains of *L. monocytogenes* have been found at food processing sites and in cases of human listeriosis infection. There is also evidence that BC resistance can have cross-protective effects on other antimicrobials, thereby raising concerns over the use of BC in the health of the general public.[10]

Initial research investigating QAC resistance mechanisms showed that QAC-resistant *L. monocytogenes* appeared to have structural modifications in the peptidoglycan cell wall. Moreover, QAC resistance is associated with the upregulation of certain efflux pump genes (*mdrL, brcABC, qacH,* and *emrE*). With these genes capable of being transferred horizontally, the potential for BC resistance to spread in a *L. monocytogenes* biofilm is high. Therefore, it is important to figure out how to combat *L. monocytogenes* resistance to BC to ensure safe food processing environments and reduce the risk of listeriosis outbreak.

BC exposure has also been shown to increase antibiotic resistance in other bacterial species besides *L. monocytogenes.* Kim et. al reported that resistance to tetracycline and ciprofloxacin in *Pseudomonas aeruginosa,* a Gram-negative opportunistic bacterium, increased after being exposed to BC for three years.[11] Additionally, *Escherichia coli* upon BC exposure has been shown to form persisters, mutant variants better at survival, which contribute to the evolution of BC tolerance within the bacterial population.[12] This further supports the claim that bacteria treated with

BC could possess cross-protective effects for other antimicrobials and develop cross-resistant mechanisms for antibiotics—making dangerous pathogens more difficult to eliminate.

Biofilm Formation

L. monocytogenes can form biofilms in food processing environments and become resistant to antimicrobial agents such as benzalkonium chloride and other quaternary ammonium chlorides (QACs).[4] *L. monocytogenes* formation of biofilms in the food processing environment poses risk for persistent contamination and increases the frequency of outbreaks. One study reported that 60% of foodborne outbreaks can be attributed to biofilm.[13] Blackman et al. demonstrated the ability of *L. monocytogenes* to form biofilms on both hydrophobic and hydrophilic surfaces in food processing facilities, specifically Teflon and stainless steel.[14] The same study also found that complex growth media, composed of hydrolyzed proteins and other organic polymers, increased *L. monocytogenes* biofilm formation on Teflon and stainless steel surfaces after 18 days compared to growth in chemically defined minimal media.[14]

L. monocytogenes resistance to QAC disinfectants also contributes to biofilm formation. Toro et al. reported *L. monocytogenes* exposed to sub-inhibitory concentrations of QAC disinfectants showed an increase in biofilm formation speed.[15] Additionally, the overexpression of biofilm formation genes was seen in *L. monocytogenes* strains resistant to QACs.[15] Another factor that contributes to *L. monocytogenes* biofilm formation is flagella driven motility. Studies have shown that flagella can help overcome repellant forces that could prevent attachment and also assist with initiating the attachment of bacteria cells to the surface.[13] A combination of internal

and external factors seem to work in combination to promote QAC resistance, enhance biofilm formation, and increase *L. monocytogenes* persistence in the environment.

SigB Stress Response

One additional factor that can influence *L. monocytogenes* biofilm formation is the stress response sigma factor SigB. SigB is the regulator of class II stress genes and is known to activate synthesis of proteins that control the response to stress environments, such as efflux pumps.[4] For example, one study showed that *L. monocytogenes* response to acidity was *sigB*-dependent, represented as decreased growth of a *sigB* deletion mutant compared to wildtype growth in acidic conditions.[16] Another study reported that mutations in RsbU, the upstream regulator of SigB, increased *L. monocytogenes* biofilm formation.[17] Because BC elicits stress in the environment of *L. monocytogenes*, it is important to investigate the role of SigB in conferring BC resistance.

The number and types of sigma factors vary and reflect the different environmental conditions and needs of the bacteria.[18] It is thought that the general stress response is conserved among Gram-positive bacteria, such as *L. monocytogenes*, and many Gram-positive strains contain the SigB transcription factor. However, this stress response regulation is not seen in all Gram-negative bacteria with many not possessing SigB, but instead expressing other sigma transcription factors.[18] Therefore, to fully understand *L. monocytogenes* BC resistance and biofilm formation, it is important to consider the role of the SigB transcription factor in different environmental conditions.

CodY Metabolic Regulation

Another notable factor that has implications in *L. monocytogenes* growth and persistence is the CodY global regulator. CodY is known to regulate many cellular processes, including regulating and responding to branch chain amino acid (BCAA) levels. In *L. monocytogenes*, CodY has been shown to suppress amino acid metabolism under high BCAA concentrations.[19] Furthermore, CodY has been shown to regulate *sigB*, which activates PrfA, the main virulence activator in *L.* monocytogenes.[19] This link suggests that during *L. monocytogenes* infection, CodY could promote *prfA* transcription by blocking *sigB* repression.[19] Additionally, high c-di-GMP levels coupled with suppression of *codY* gene expression and therefore *prfA* expression have been shown to decrease *L. monocytogenes* invasion into mammalian cells.[20] Studies suggest the role of CodY to be complex, and the transcriptional regulator could function as both an activator and repressor in both high and low BCAA levels. This complexity suggests that the role of CodY in conferring *L. monocytogenes* resistance to BC and biofilm formation should be studied under conditions that elicit a stress response.

Relevant Environmental Factors

It is important to consider relevant environmental factors contributing to *L. monocytogenes* resistance mechanisms, such as the presence or absence of oxygen. A study conducted by Tapia et al. examined biofilm formation on the surface at the air and liquid interface and demonstrated that *L. monocytogenes* biofilm formation is dependent on glycerol-induced aerotaxis towards the surface where there is more oxygen to couple with heterotrophic growth.[21] However, food-related surfaces can have reduced oxygen levels that may interfere with the aerotaxis process. Therefore, oxygen levels and

potentially other environmental signals may affect *L. monocytogenes* biofilm formation and resistance to BC.

Sodium propionate is a Food and Drug Administration approved and commonly used food additive for flavoring and spoilage prevention. Previous research has shown that propionate affects *L. monocytogenes* membrane composition as well as biofilm formation. Rinehart et al. found that exogenous propionate can reduce *L. monocytogenes* adherent growth in anaerobic conditions by altering its central carbon metabolism.[22] In contrast, in aerobic conditions, propionate resulted in increased *L. monocytogenes* adherent growth, suggesting that the presence or absence of oxygen can affect how propionate modulates biofilm formation in *L. monocytogenes*.[22] Furthermore, another study found that increased biofilm formation in the presence of salt was *sigB*-dependent.[16] It has also been shown that *L. monocytogenes* treated with propionate in anaerobic conditions had enhanced rates of intracellular infection of RAW264.7 macrophages and L-fibroblasts.[23] Currently, it is not clear whether propionate affects the regulation of efflux pump expressions. Similarly, how propionate affects *L. monocytogenes* BC resistance is unknown. More research is needed to examine how propionate and BC interact to establish how propionate plays a role in *L. monocytogenes* resistance to BC in aerobic and anaerobic conditions.

Research Goal

The overarching goal of my Honors Thesis aims at understanding how environmental factors influence *L. monocytogenes* persistence in the environment. **The main research objective is to investigate factors that influence *L. monocytogenes***

susceptibility to BC. I explored the following genetic and environmental factors: transcription regulators SigB and CodY, planktonic or biofilm growth conditions, propionate supplementation, and the presence or absence of oxygen. Our findings can help develop more effective strategies to eliminate *L. monocytogenes* contamination in food production pipelines.

Methods

Bacteria and Bacterial Culture Conditions

Listeria monocytogenes strain 10403s and isogenic mutants were used for this study. Aerobic growth of bacteria was performed with agitation (250 rpm). Anaerobic growth was performed inside an anaerobic chamber with 2-3% (v/v) H_2 in a N_2 atmosphere. Unless otherwise noted, bacterial culture media used was brain heart infusion (BHI) filter-sterilized to avoid heat treatments. Sodium propionate solution (1 M) in deionized water was prepared by filter-sterilization and was then aliquoted and stored in the freezer to avoid volatilization.

Zone of Inhibition

Overnight *L. monocytogenes* wildtype 10403s strain cultures were grown aerobically in 3 mL BHI for 16-18 hours. The following day, 12 BHI agar plates were labeled as aerobic or anaerobic and numbered 1-5 in a circle to indicate the different concentrations of BC. A 4 mg/mL BC stock solution (in sterile water) was diluted with sterile H_2O to make 1 µg/mL, 2 µg/mL, 4 µg/mL, and 6 µg/mL BC solutions. Using glass beads, 5µL of *L. monocytogenes* culture was spread on 6 BHI plates as control samples. A second set of 6 BHI agar plates were spread with 5µL of *L. monocytogenes* culture and 100 µL of a

sterile 1 M propionate solution. Then, 5 sterile filter disks were placed in a circle using tweezers to match up with the labeling on the plate lid. Sterile water or BC solutions at various concentrations (1, 2, 4, or 6 µg/mL) were then added to the filter discs (5 µL per disc). The 6 BHI plates labeled aerobic were incubated for 24 hours at 37°C. The 6 BHI plates labeled anaerobic were incubated in the anaerobic chamber at 37°C for 48 hours. After both plates were incubated, pictures of each plate were taken and downloaded into ImageJ software. ImageJ was used to measure each zone of inhibition diameter in pixels which were then converted to millimeters.

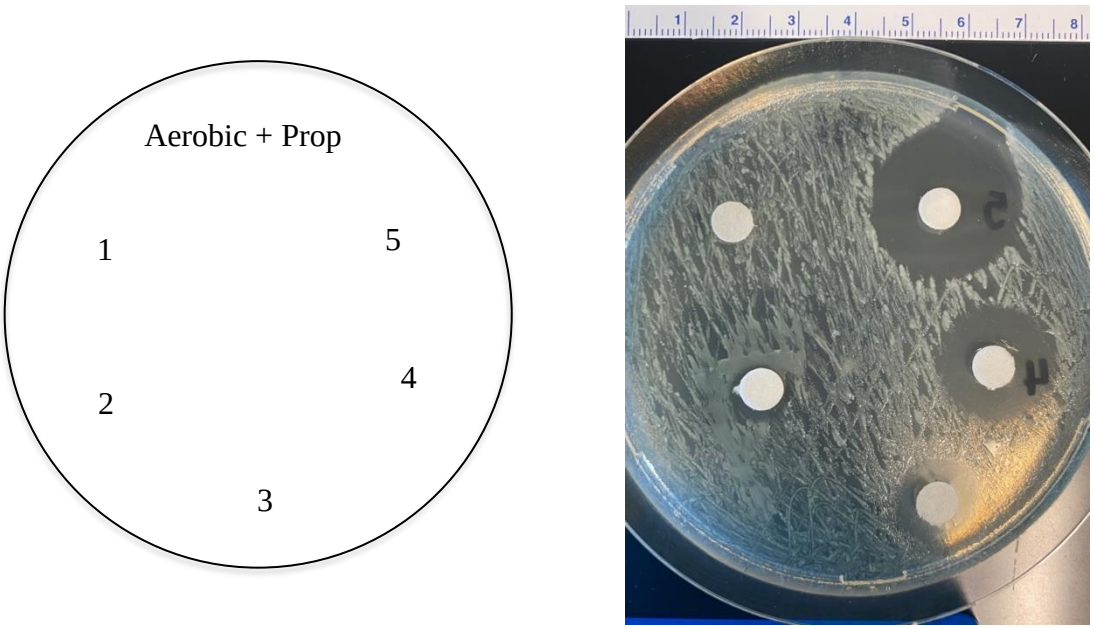


Figure 1. Labeled BHI plate schematic

Planktonic Growth in 96-well Plates

A microcentrifuge tube with 250 µL of BHI was inoculated with a *L. monocytogenes* wildtype 10403s colony and this was repeated with a Δ*sigB* mutant colony. Then, eight conical tubes were labeled and filled with 3 mL of BHI, 30 µL of either *L. monocytogenes* wildtype or Δ*sigB* mutant culture, and a specific BC concentration (0 µg/mL, 7.5 µg/mL, 75 µg/mL, or 150 µg/mL) made from a 4 mg/mL stock solution of BC. Propionate was first added to the 96-well plastic plates where 1.5 µL of the 1 M propionate stock solution was added to designated 15 mM propionate wells and 2.5 µL was added to designated 25 mM propionate wells. Next, the bacterial mixtures were aliquoted into the 96-well plates with 200µL per well and 4 replicate wells per condition. The aerobic 96-well plate was incubated for 24 hours at 37°C on a shaker plate and the anaerobic 96-well plate was incubated in the anaerobic chamber at 37°C for 24 hours. Following incubation, optical density of the planktonic growth was measured at 600 nm with a BHI blank.

Aerobic		0mM P	15mM P	25mM P
WT	0 BC 1 BC 10 BC 20 BC	x x x x		
ΔsigB	0 BC 1 BC 10 BC 20 BC			

Anaerobic		0mM P	15mM P	25mM P
WT	0 BC 1 BC 10 BC 20 BC	x x x x		
ΔsigB	0 BC 1 BC 10 BC 20 BC			

Figure 2. Schematics of 96-well plate set up for planktonic growth measurements

Quantification of Biofilm Growth in 96-well Plates

L. monocytogenes strain 10403s wildtype or the isogenic Δ*codY* mutant cultures were grown aerobically in 3 mL of BHI for 16-18 hours. The following day, cells from 2 mL of the wildtype culture were harvested by centrifugation at 10,000 rpm for 3 minutes. The supernatant was suctioned off and the pellet was resuspended in 2 mL of diluted (1/10 v/v) BHI (1/10 BHI). Then, 2 mL of cell suspension and 18 mL of diluted 1/10 BHI was added to a conical tube. Next, two 96-well plates were filled with 200 µL of distilled H_2O in the outer edge wells. Various combinations of BC, propionate, and NaCl were added into the inner wells along with 100 µL of wildtype cell suspensions in diluted 1/10 BHI, followed by brief gentle shaking to mix. The aerobic plate was incubated for 24 hours at 37°C on a shaker plate and the anaerobic plate was incubated in the anaerobic chamber at 37°C for 24 hours. Optical density of planktonic growth was measured at 600 nm after 24 hour incubation. Then, cultures were removed, and wells were washed five times with sterile water and dried for 30 minutes. To stain the adherent bacteria, crystal violet (3% w/v in water, 150 µL per well) was added to each well. After 45 min of staining, plates were washed five times with water and dried for 30 minutes. Ethanol (95%, 200 µL per well) was then added to each well to solubilize the stain. Absorbance of the dissolved crystal violet was read at 595 nm to represent the amount of biofilm growth.

Aerobic	0 µg/mL BC	8 µg/mL BC
0 Prop	x x x x. x x x x x. x	
25 mM Prop		
25 mM NaCl		

Anaerobic	0 µg/mL BC	8 µg/mL BC
0 Prop	x x x x. x x x x x. x	
25 mM Prop		
25 mM NaCl		

Figure 3a. Schematics of 96-well plate set up for wildtype planktonic and biofilm growth measurements

Aerobic	Wildtype	Δ*codY* mutant
0 Prop	x x x x. x x x x x. x	
25 mM Prop		
25 mM NaCl		

Anaerobic	Wildtype	Δ*codY* mutant
0 Prop	x x x x. x x x x x. x	
25 mM Prop		
25 mM NaCl		

Figure 3b. Schematics of 96-well plate set up for wildtype and Δ*codY* mutant planktonic and biofilm growth measurements

Results

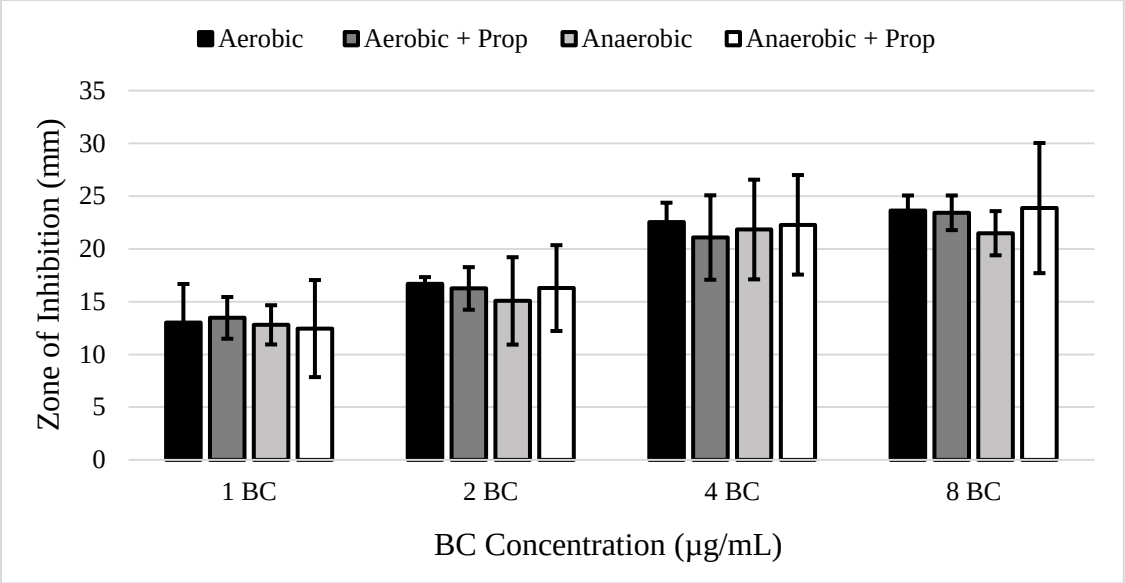
Zone of Inhibition

2 experiments

3 replicates each (6 total)

To determine *L. monocytogenes* susceptibility to BC, I first conducted zone of inhibition assays using wildtype strain 10403s. There was a trend of increasing concentrations of BC with increasing diameters of zone of inhibition; however, there was no significant difference in zone of inhibition diameter between *L. monocytogenes* grown with or without propionate or in aerobic or anaerobic conditions (Figure 4). These results suggest that BC is effective in inhibiting *L. monocytogenes* growth regardless of propionate and presence or absence of oxygen. To confirm this observation, additional 96-well plate assays were performed to determine the effects of BC on planktonic and adherent growth.

Figure 4 BC susceptibility assay against wildtype strain 10403s. BHI agar plates were spread with *L. monocytogenes* with or without propionate (25 mM). Filter discs containing BC (vol) at various concentrations were placed on BHI agar plates. The diameters of the zone of inhibition were measured after 24 hours at 37°C for aerobically incubated samples and 48 hours at 37°C for anaerobically incubated samples. Averages of 6 replicates over 2 independent experiments are plotted with error bars representing standard errors of the means.



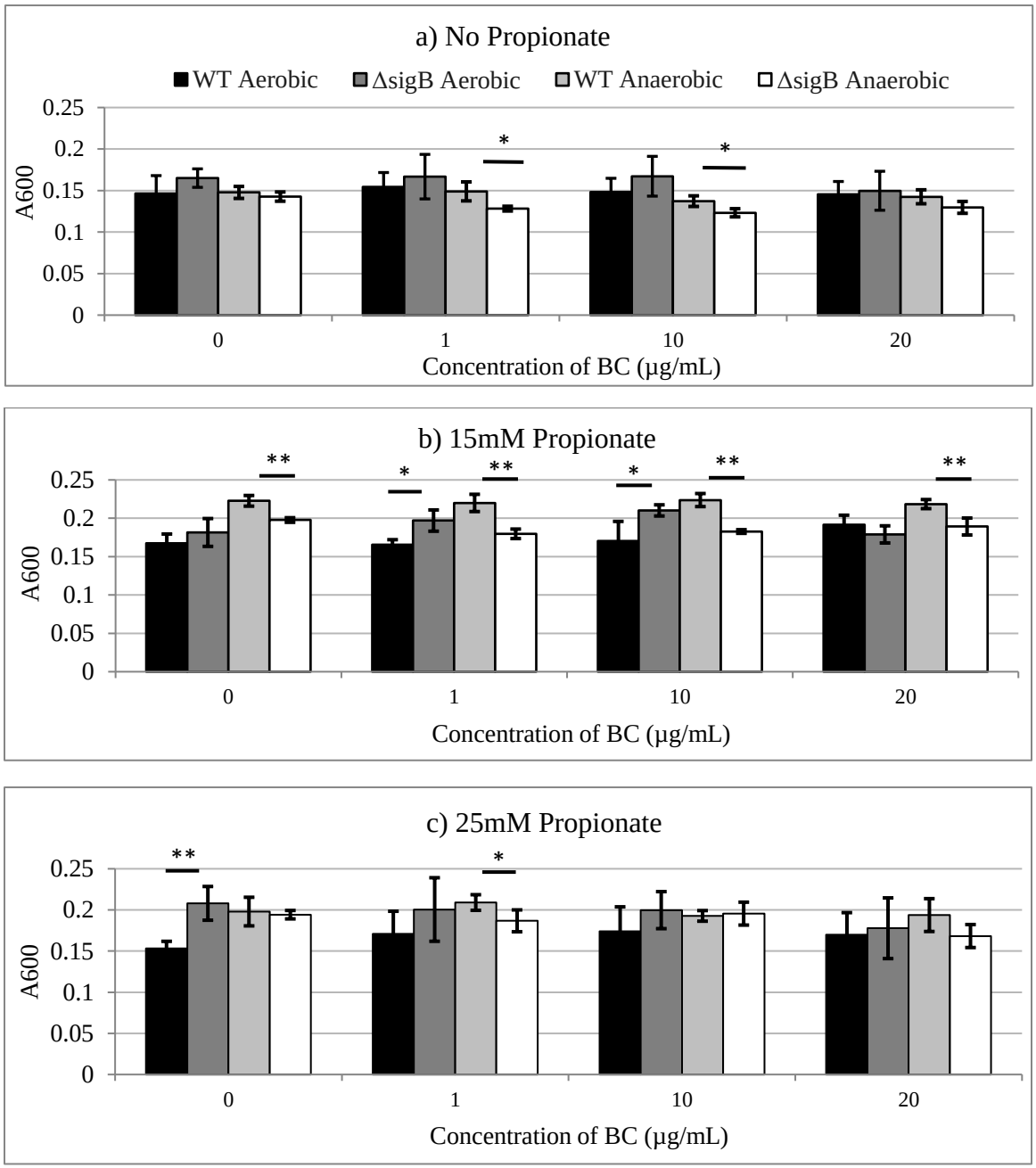
Planktonic Growth in 96-well Plates

1 experiment

4 replicates

To investigate the role of propionate, genetic factors, and BC on planktonic growth in aerobic and anaerobic conditions, a susceptibility assay was performed using both wildtype and Δ*sigB* mutant isogenic strains. In aerobic conditions, 25 mM propionate significantly decreased wildtype growth when no BC was added, however there was no significant difference between wildtype and Δ*sigB* mutant growth when BC was added. In contrast to wildtype, in anaerobic conditions, BC at 1µg/mL and 10 µg/mL significantly inhibited Δ*sigB* mutant growth in diluted BHI without propionate (Figure 5a). When 15 mM propionate was added, under anaerobic conditions, BC inhibited Δ*sigB* mutant growth at all concentrations, including the control (Figure 5b). This was also seen when 25 mM propionate was used, but only at 1µg/mL BC (Figure 5c). In contrast, under aerobic conditions, 15 mM propionate and BC at 1 µg/mL and 10 µg/mL significantly enhanced Δ*sigB* mutant growth (Figure 5b). These results indicate that under anaerobic conditions, SigB plays a role in BC resistance. However, in aerobic conditions, SigB and propionate could increase *L. monocytogenes* susceptibility to BC. Therefore, it is possible that *L. monocytogenes* adapted to propionate in anaerobic environments are more resistant against BC, but the presence of oxygen could increase susceptibility. Future research is needed to identify mechanisms by which oxygen exposure and propionate confer *L. monocytogenes* BC resistance, perhaps through the role of specific efflux pumps.

Figure 5 BC susceptibility assay in wildtype strain 10403s and an isogenic Δ*sigB* mutant strain with increasing propionate concentrations in aerobic and anaerobic conditions for planktonic growth. Wildtype or ΔsigB mutant strains were inoculated in microcentrifuge tubes filled with either 0 mM, 15 mM, or 25mM propionate and either 0 µg/mL, 1 µg/mL, 10 µg/mL, or 20 µg/mL BC. Optical density was measured after 24 hours at 37°C in aerobic or anaerobic conditions. Averages of 4 replicates from 1 experiment are plotted with error bars representing standard errors of the means. T tests were performed for each pair-wise comparison indicated by horizontal lines with asterisks representing samples with statistically significant differences. (* for 0.01<p<0.05; ** for 0.001<p<0.01)



Quantification of Biofilm Growth in 96-well Plates

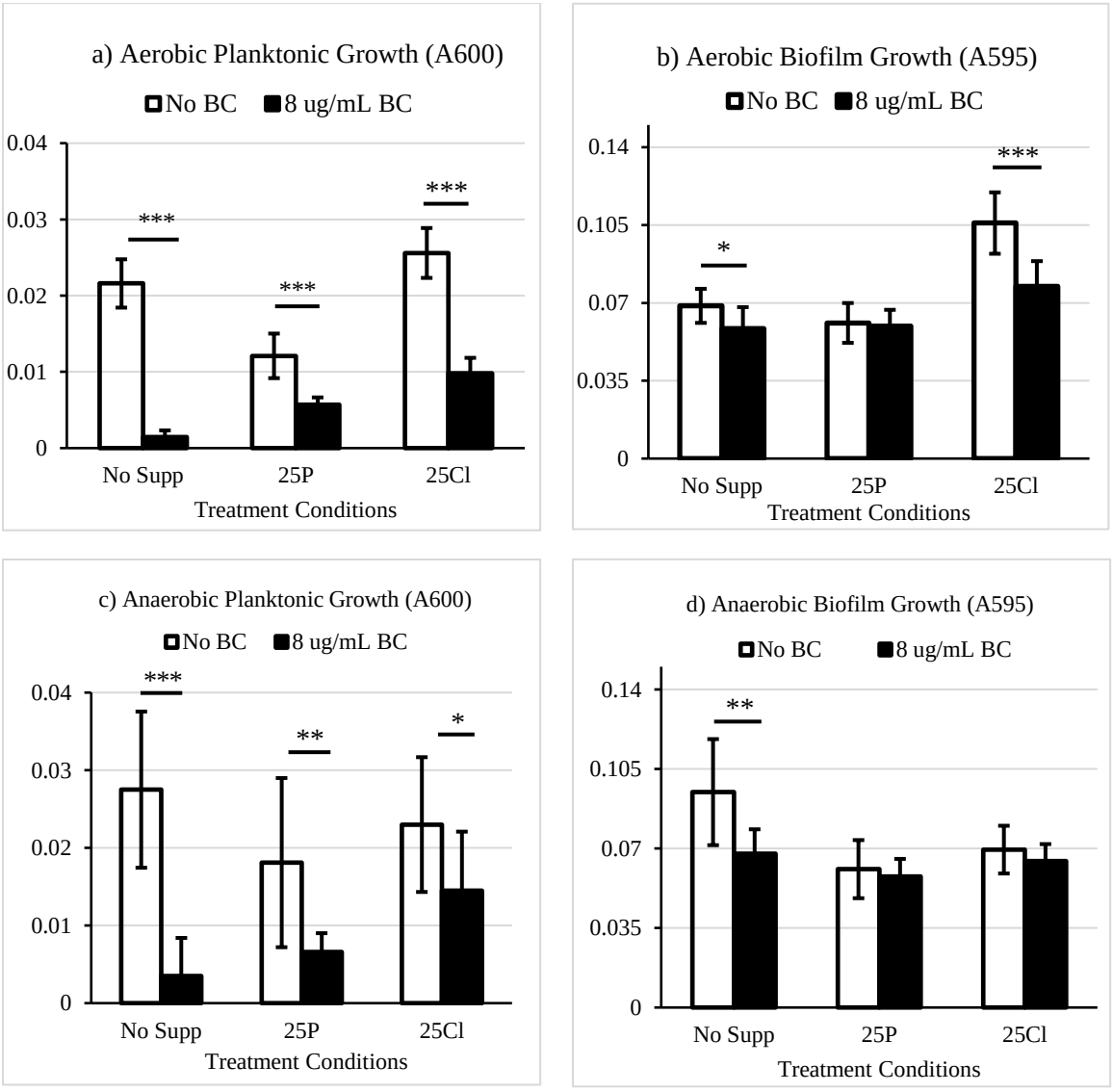
1 experiment

10 replicates

To assess the role of propionate and oxygen availability in conferring BC resistance, I performed biofilm assays in the wildtype strain and compared biofilm growth in *L. monocytogenes* treated with and without BC. Regardless of oxygen availability, BC at 8 ug/mL significantly inhibited planktonic growth in diluted BHI in 96-well plates (Figure 6a, 6c). In contrast, under aerobic conditions, BC inhibited biofilm growth in no treatment control and NaCl treatment samples but not in propionate treatment samples (Figure 6b). Under anaerobic, BC inhibited biofilm growth in no treatment but not in propionate or NaCl treatment samples (Figure 6d). These results indicate that at a concentration where BC is effective in inhibiting *L. monocytogenes* planktonic growth, it is not as effective against biofilm growth, regardless of the availability of oxygen. Moreover, while propionate exhibits little to no effects on the BC activity against planktonic *L. monocytogenes*, it compromises the efficacy of BC in inhibiting adherent *L. monocytogenes*. Therefore, it is likely that adherent *L. monocytogenes* adapted to propionate is more resistant against BC and will likely persist in environments where BC is the main choice of disinfectant. Future research can help elucidate the types of adaptations from propionate exposure that contribute to BC resistance.

Figure 6 BC susceptibility in planktonic or biofilm wildtype strain 10403s under aerobic or anaerobic conditions. An overnight aerobic culture of wildtype strain 10403s in BHI was used as the inoculum for these experiments. Experimental cultures were grown in 2 96-well plates with diluted BHI (1/10, v/v) with BC (0 µg/mL or 8 µg/mL BC), propionate (0 mM or 25 mM), or NaCl (0 mM or 25 mM). Optical density was used to measure planktonic growth after 48 hours at 37°C in aerobic or anaerobic conditions. After planktonic growth was measured, crystal violet staining was performed, and optical density was used to measure biofilm growth. Averages of 8 replicates from 1 experiment are plotted with error bars representing standard errors of the means. T tests were performed for each pair-wise comparison indicated by horizontal lines with asterisks representing samples with statistically significant differences.

(* for $0.01<p<0.05$; ** for $0.001<p<0.01$)



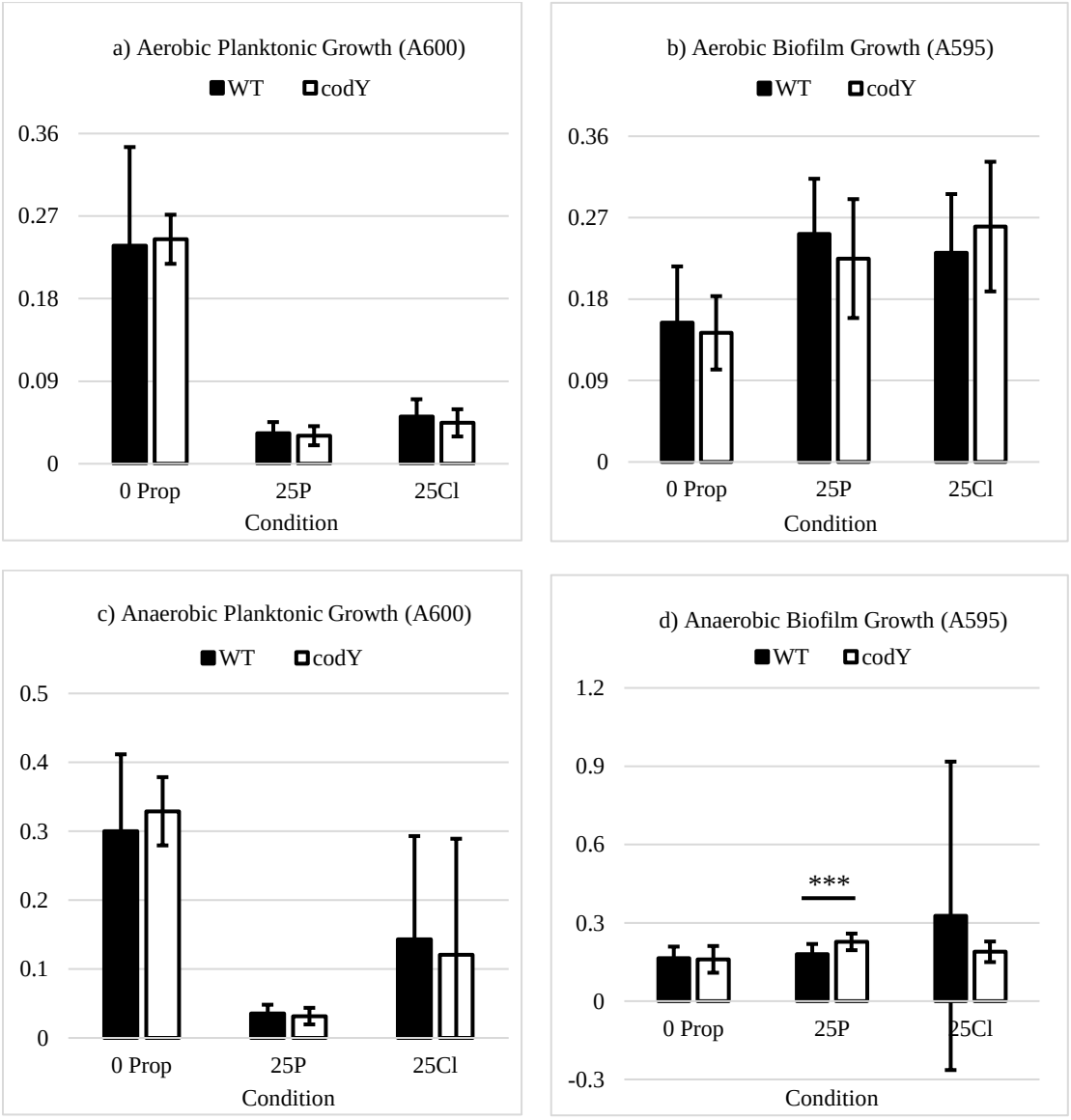
Role of CodY in Biofilm Growth

2 experiments

10 replicates each (20 total)

To investigate potential genetic factors associated with the propionate effects on BC susceptibility, I first performed biofilm assays and compared the biofilm formation between WT *L. monocytogenes* and the Δ*codY* mutant (Figure 7). Under all conditions tested, a significant difference between WT and the Δ*codY* mutant was only observed under anaerobic biofilm growth in the presence of propionate (Figure 7d). These results suggest that the Δ*codY* mutant had similar biofilm-forming ability as WT bacteria under most conditions except for during anaerobic propionate exposure. Therefore, CodY likely exerts a negative effect on *L. monocytogenes* biofilm formation in response to anaerobic propionate treatment. How CodY activities influence *L. monocytogenes* biofilm formation or response to propionate remains unclear.

Figure 7 Planktonic and biofilm assay in wildtype strain 10403s and an isogenic Δ*codY* mutant strain under aerobic or anaerobic conditions. An overnight aerobic culture of wildtype strain 10403s and an isogenic Δ*codY* mutant strain in BHI was used as an inoculum for these experiments. Experimental cultures were grown in 2 96-well plates with diluted BHI (1/10, v/v), propionate (0mM or 25mM), or NaCl (0mM or 25mM). Optical density was used to measure planktonic growth after 48 hours at 37°C in aerobic or anaerobic conditions. After planktonic growth was measured, crystal violet staining was performed, and optical density was used to measure biofilm growth. Averages of 8 replicates from 2 independent experiments are plotted with error bars representing standard errors of the means. T tests were performed for each pair-wise comparison indicated by horizontal lines with asterisks representing samples with statistically significant differences. (* for 0.01<p<0.05; ** for 0.001<p<0.01)



Discussion

Biofilm formation is a key attribute for many pathogens, including *L. monocytogenes*. In other human pathogens, such as *Vibrio cholerae* and *Pseudomonas aeruginosa*, biofilm formation also poses a significant threat to public health. First, bacterial biofilms can help pathogens escape phagocytosis by physically evading immune detection and destruction.[24] For example, the production of biofilm polysaccharides in *V. cholerae* is required for intestinal colonization.[25,26] Biofilm production by *P. aeruginosa* both protects the bacteria from immune destruction and induces hyperinflammation that can result in tissue damage.[27] In addition, biofilm formation promotes environmental persistence for these human pathogens as well. Biofilm formation contributes to *V. cholerae* persistence in marine aquatic environments in between seasonal outbreaks.[28] Additionally, *P. aeruginosa*, has developed persister cells in biofilm that can survive biofilm-targeting nanoparticle antimicrobial treatments.[29]

Challenges in removing biofilms can have detrimental consequences. Persistent biofilm formation by foodborne pathogens on food processing equipment adds to the threat of outbreaks. On medical devices, such as pacemakers and catheters, or various body surfaces, such as the skin or mucosal linings in the digestive tract, persistent biofilms can cause life-threatening infections.[24] More alarmingly, bacteria in biofilms often contain and share mechanisms for antibiotic and disinfectant resistance that further compromise our ability to eliminate these bacterial threats. Therefore, persistent biofilms can create challenges inside and outside of the human body.

Besides L. monocytogenes, previous research suggests that BC resistance in biofilm formations occurs in other human pathogens, such as *Staphylococcus aureus* and *Salmonella*. In these bacterial species, using BC above the minimum inhibitory concentrations (MIC) did not significantly inhibit planktonic or biofilm growth and using doses below the sub-MIC enhanced biofilm formation.[30] Another study suggested that BC is effective in decreasing biofilm formation in S. Typhimurium, *S. aureus,* and *L. monocytogenes* however, it was unable to fully remove biofilm.[31] The study pointed out that most disinfectant protocols are based on planktonic growth susceptibility and additional biofilm research needs to be integrated in protocols. Furthermore, research studying the effects of BC on dual species *L. monocytogenes-E. coli* suggests that complex biofilm formation across multiple species does not always imply greater resistance. Perhaps this is because there is competition for nutrients which depletes metabolic activity and resistance mechanisms.[32]

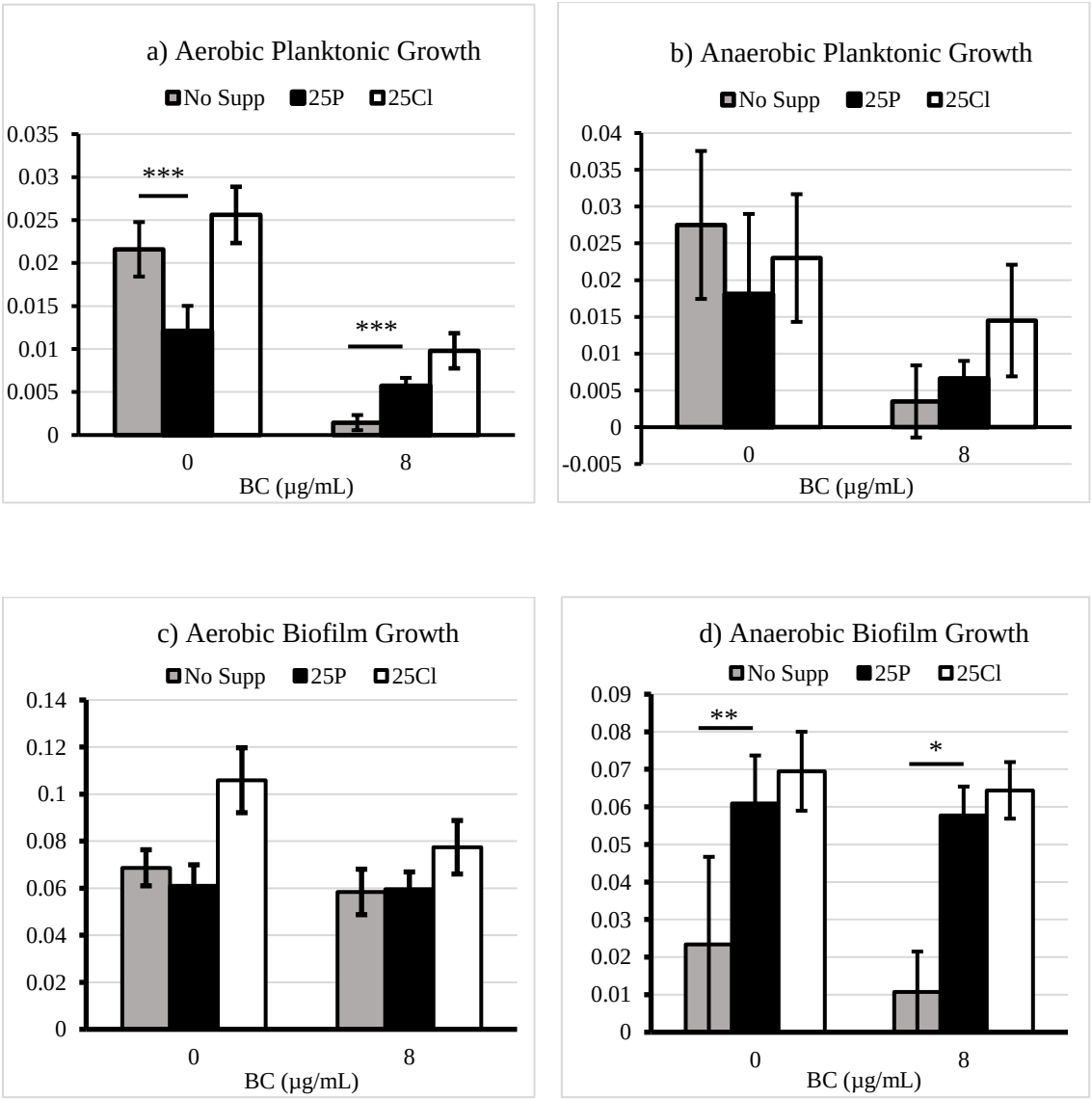
Several limitations of this research should be addressed in future studies to add to the body of research surrounding *L.* monocytogenes biofilm formation and improve current disinfectant protocols for food processing environments. This biofilm research was conducted in plastic, 96-well plates, which is not representative of the different surfaces *L. monocytogenes* can form biofilm on in food processing facilities. Future research should conduct BC-exposed biofilm assays on various surfaces, such as stainless steel and Teflon, which has been shown to support biofilm formation in *L. monocytogenes*.[14] Additionally, future research should explore biofilm formation in other *L. monocytogenes* strains. This study used the 10403s wildtype and isogenic mutant strains, however previous research shows that there are differences in biofilm formation across serotypes.[33] Furthermore,

varying core genetic features and molecular markers across genotypes in *Listeria* are associated with stress tolerance traits and the ability to form biofilm under sub-inhibitory BC concentrations.[33] The more we know, the more effective we can be at protecting vulnerable populations from dangerous pathogens.

Supplemental Figures

Supplemental Figure #1

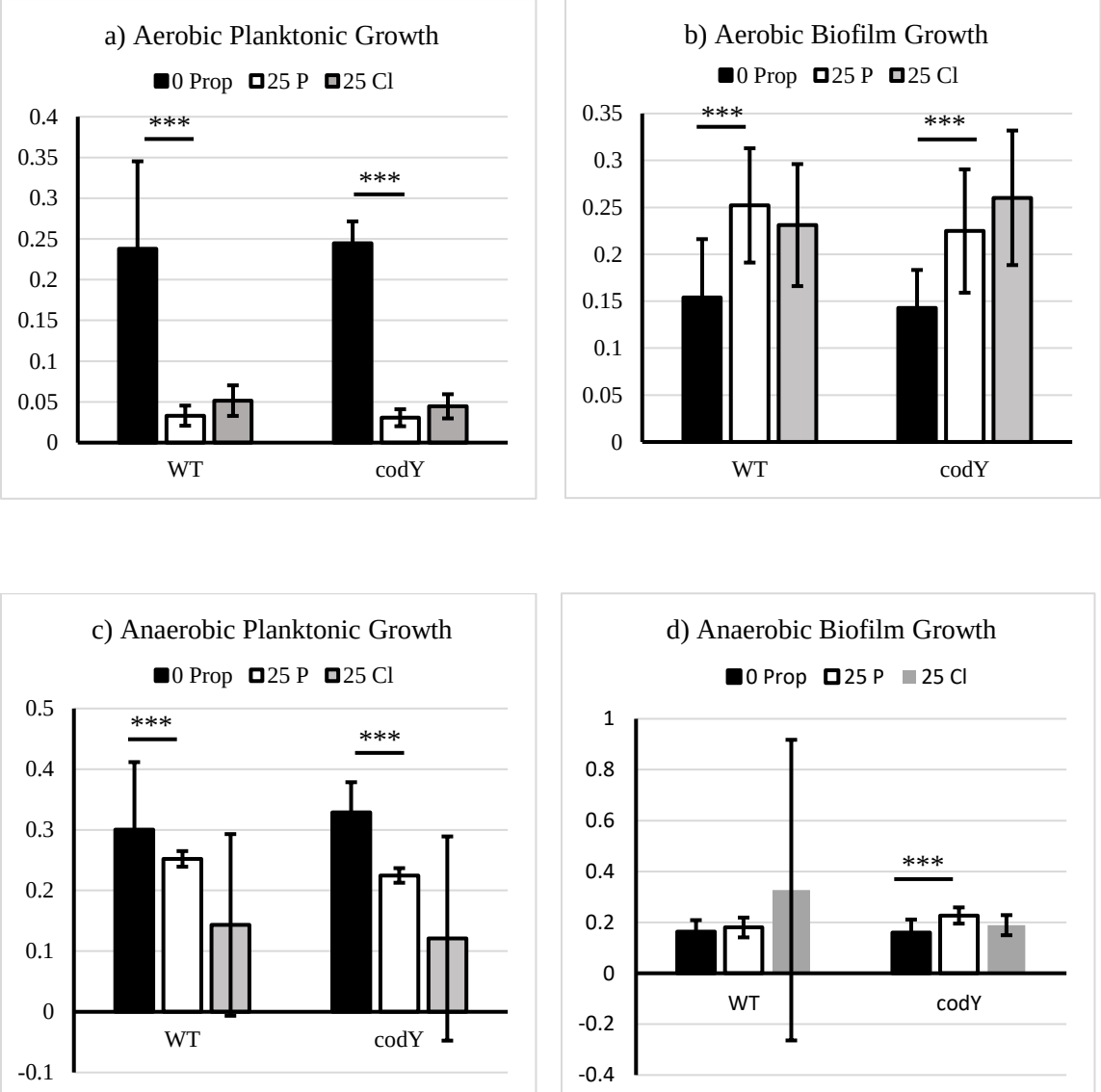
These are the same data from Figure 3.



Addition of 25mM propionate significantly reduced WT planktonic growth in conditions without BC but enhanced planktonic growth in conditions with 8 µg/mL BC grown aerobically (Supplemental Figure 1a). However, addition of propionate did not significantly affect anaerobic planktonic growth, regardless of the presence of absence of BC (Supplemental Figure 1b). Addition of 25mM propionate significantly enhanced WT biofilm growth in anaerobic, but not aerobic, conditions regardless of the presence or absence of BC (Supplemental Figure 1c, 1d).

Supplemental Figure #2

These are the same data from Figure 4.



Addition of 25mM propionate significantly decreased WT and Δ*codY* mutant planktonic growth in aerobic and anaerobic conditions (Supplemental Figure 2a, 2c). However, 25mM propionate significantly enhanced WT and Δ*codY* mutant biofilm growth in aerobic conditions (Supplemental Figure 2b). Addition of 25mM propionate enhanced Δ*codY* mutant biofilm growth in anaerobic conditions (Supplemental Figure 2d).

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