The Effects of Human Behavioral Changes due to the COVID-19 Pandemic on the Reservoir of Lytic *Escherichia coli* and *Staphylococcus aureus* Bacteriophage on Humans at a South Carolina University

Korinne M. Swanson, Owen R. Smith, and Paul E. Richardson*

Department of Chemistry, Coastal Carolina University, Conway, SC

Bacteriophages are viruses, whose unique ability to infect and lyse bacterial cells may provide valuable insight for evading the repercussions of a post-antibiotic era in medicine. This study isolated and characterized *Escherichia coli* and *Staphylococcus aureus* bacteriophage on students and faculty at Coastal Carolina University as a means to understand the viability of human bacteriophage reservoirs for bacteriophage therapy. From September 2021 to March 2022, nasal and postauricular swab samples and a behavioral survey were collected from ninety-three randomly selected participants. Additionally, sixteen participants contributed nasal and postauricular swab samples and a behavioral survey on a monthly basis in a longitudinal study. The purpose of this study was to establish insight into behavioral factors, namely face mask wearing, handwashing/ sanitizing, and perceived stress levels, that potentially contribute to the *E. coli* and *S. aureus* bacteriophage dynamics. During the current collection cycle, 2021-2022, there has been a reemergence of bacteriophage since their disappearance the previous collection year. Results indicate participant engagement in mask wearing or handwashing/ sanitizing does not affect coliphage presence, but participants with coliphage presence generally had lower perceived stress levels than those lacking coliphage. Data was limited and correlations could not be made between *S. aureus* bacteriophage presence and the aforementioned behavioral factors. However, limited data suggests face mask wearing may be correlated to a lack of *S. aureus* bacteriophage presence on a host.

Introduction

Bacteriophages, otherwise known as phages, are viruses whose hosts are bacterial cells. Some phages are lytic and therefore can induce bacterial cell lysis[1]. Phages accomplish their innate bactericidal ability through the injection of their viral genomic material into the bacterium followed by hijacking and utilizing the host cell machinery to synthesize new phage particles. The excretion of endolysin and holin proteins triggers the lysis of the bacterial cell and subsequent release of phage particles capable of infecting the same bacterial strain with high specificity[2]. Due to this mechanism, phage therapy has been a popular topic of research within European countries since the co-discovery in 1915 and 1917 by Fredrick W. Twort, a bacteriologist from England, and Felix d'Herelle, a French-Canadian microbiologist at the Institut Pasteur in Paris. In 1919 at the Hôpital des Enfants-Malades in Paris, d'Herelle introduced the possibility of phage therapeutics by using phage isolated from clinical research to treat severe hemorrhagic dysentery. d'Herelle's discovery was succeeded by the efforts of Richard Bruynoghe and Joseph Maisin to utilize phage therapy in the treatment of staphylococcal skin diseases[3]. Despite the positive outlooks for phage therapy, its research was marginalized following the discovery of antibiotics in 1940[4].

After their discovery, antibiotics became the standard for treating bacterial infections and for nearly 70 years antibiotics have significantly diminished the morbidity and mortality rates of bacterial infections. However, shortly after the discovery of antibiotics, antibiotic resistance was discovered. Since then, the number of antibiotic resistant bacterial infections has drastically increase due to improper and overuse of antibiotics. Literature suggests that in approximately 30% to 50% of antibiotic treated bacterial infections, the treatment indication, antibiotic of choice, or duration of therapy is incorrect, which can lead to the development of antibiotic resistance[5]. Bacteria's rapid mutation rate of 1 in 10^5 to 10^8, has also been a determinant in the rise of antibiotic resistant bacterial infections[6]. Therefore, the discovery and utilization of novel treatments against bacterial infections is pertinent to ensuring public health.

In response, bacteriophage therapy research has reemerged as a potential solution to antibiotic resistant bacterial infections. Treating antibiotic resistant infections with phage therapy has multifarious advantages. Due to the nature of the lytic life cycle, the bactericidal capabilities of phages are invaluable and prove more effective than bacteriostatic treatments[7]. The biochemical composition of phages allows for the prevention of toxic-product accumulation when phages are degraded by the immune system. Furthermore, automated dosing mechanisms are amongst the most specialized features associated with phage treatments. They mimic the bodies homeostatic defense mechanism better than any pharmaceutical, as they incorporate highly specific bacterial lysis and phage production at the site of infection rather than systemically[8]. The non-targeted, and even targeted capabilities of broad-spectrum antibiotics have the potential to cause dysbacteriosis, a disruption to the microbiota homeostasis which can cause chronic and degenerative diseases[9]. The employment of phages against bacterial infections, even when administered in conjunction of other antibacterial agents, maintain a relatively narrow activity spectrum, resulting in a lower potential for side effects associated with dysbiosis[9,10]. Other advantages of phage therapy include the high availability and accessibility of phages, the versatility of pharmaceutical formulations and methods of delivery, and the ability to actively penetrate biofilms, complex bacterial clusters bound by extracellular polymeric substances (EPS) which incur more resistance to antibiotics[7,11]. As there are a tremendous number of benefits for the application of phage therapy, it is crucial to find phage repositories which can be utilized in its administration.

This study aims to identify potential correlations between the presence of coliphage and *S. aureus* phage with human behaviors to determine the viability of humans serving as a bacteriophage repository for phage therapy. A treatment which could be utilized against antibiotic resistant strains of *S. aureus* and *E. coli*. *E. coli* is a common bacterium typically associated with the gut microbiota[12]. However, it is also the third most prevalent bacterial species to be isolated from skin and soft tissue infections subsequent to *S. aureus* and *Pseudomonas aeruginosa*. It is the causal agent of neonatal omphalitis, cellulitis localized to lower or upper limbs, necrotizing fasciitis, surgical site infection, infection to burn injuries, among other infections. The antibiotic susceptibility of *E. coli* has had a recent dramatic decline and many strains no longer respond to treatment with ampicillin, tetracycline, and fluoroquinolones[13]. *S. aureus* is another common bacterium typically found on the skin microbiome of 30% of individuals in the United States. While it does not always cause infection, it is the leading causative agent of skin and soft tissues infections[13]. Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a common antibiotic resistant strain that poses a threat to the public health of the United States[14]. MRSA is typically acquired by patients who are already hospitalized, athletes, men who have sex with men, military personnel, prison inmates, and children who attend daycare[14,15]. It can lead to mild to serious infection and can cause skin/soft tissue infections, osteomyelitis, sepsis, endocarditis, toxic shock syndrome, and necrotizing pneumonia even in healthy individuals[16]. Many antibiotics such as methicillin, linezolid, vancomycin, teicoplanin, and daptomycin have become increasingly ineffective against MRSA, making its treatment ever so elusive[17]. In 1999-2005, MRSA cases rose to endemic and even epidemic levels as

there was a 50% increase in hospitalizations due to MRSA[14]. This prompted a public health initiative to decrease the number of hospitalizations due to MRSA, which worked. In the past eleven years there has been a 74% decrease in MRSA cases at hospitals in the United States[18]. Despite the decrease in MRSA hospitalizations, the dilemma of antibiotic resistant infections will never completely resolve without innovation due to the nature of the antibiotic resistance acquisition. Analogously, other antibiotic resistant bacterial strains will not cease to exist. Therefore, it is pertinent to further develop and understand alternate treatments for bacterial infections such as bacteriophage therapy.

Bacteriophages are abundant and ubiquitous nucleic acid-based entities vital to maintaining the biological homeostasis of earth and likewise humans[19]. Hence humans can potentially serve as a reservoir for bacteriophage to be utilized in bacteriophage therapy. To further understand the potential of human bacteriophage repositories, one study collected 225 samples from students and faculty at Coastal Carolina University to determine the *S. aureus* and *E. coli* phage population. It was determined that 42.9% and 36.8% of samples were lytic to *S. aureus* and *E. coli* respectively[20]. L. Pieterse, et al. conducted a pilot study which monitored bacteriophage presence on 16 individuals monthly, aiming to quantify fluctuations in coliphage and *S. aureus* phage presence over an academic year. During that time *S. aureus* and *E. coli* phage presence fluctuated monthly, with temporal competition potentially causing an inverse relationship between *S. aureus* and *E. coli* bacteriophage presence[20]. In a follow-up study, bacteriophage monitoring from 2020-2021 amid the COVID-19 pandemic demonstrated a 100% and 94.8% decrease in the *S. aureus* and *E. coli* bacteriophage population, respectively[21].

This study randomly selected 93 participants from Coastal Carolina University to provide postauricular (directly behind the ear) and intranasal swab samples. Additionally, participants completed and returned a behavioral survey which was used to determine behavioral factors that potentially influence coliphage and *S. aureus* phage population dynamics. To further cultivate viral samples, sterile filtration and amplification of samples was utilized. Microbial testing was used to confirm phage presence in a sample while molecular testing was used to determine presence of specified phage types. In tandem with the aforementioned study, a long-term study was conducted to better understand factors, namely perceived stress, which influence bacteriophage population dynamics. The purpose of this study was to further investigate behavioral factors which could influence the coliphage and *S. aureus* phage repository on humans. This led to the following questions: 1. Is there a correlation between the regularity of facemask usage and coliphage/ *S. aureus* phage presence on a participant? 2. Is there a correlation between the consistency of handwashing/ sanitizing and coliphage/ *S. aureus* phage presence on a participant? 3. Is there a correlation between perceived stress levels and coliphage/ *S. aureus* phage presence on a participant? 4. Does perceived stress level affects fluctuations in coliphage/ *S. aureus* phage populations during an academic year?

In 2017, L. Pieterse, et al. conducted a pilot study, in addition to typical phage monitoring at Coastal Carolina University. The pilot study monitored bacteriophage presence on 16 individuals monthly, aiming to quantify fluctuations in coliphage and *S. aureus* phage presence over an academic year. During that time *S. aureus* and *E. coli* phage presence fluctuated monthly, with temporal competition potentially causing an inverse relationship between *S. aureus* and *E. coli* bacteriophage presence[20]. In addition to the sporadic study, the long-term study was continued to better understand factors which influence bacteriophage population dynamics. In this study, 16 participants provided monthly swab samples from September 2021 to March 2022. The purpose of this study was to understand the effects of fluctuations in perceived stress on the bacteriophage population over an academic year.

Methods and Materials

Study Design

Two studies, a sporadic study and a long-term study, were conducted concurrently during the 2021-2022 academic year. In the sporadic study, ninety-three samples were collected randomly from students and faculty at Coastal Carolina University. Participants in the sporadic study provided a nasal and postauricular swab sample and completed a behavioral survey once during annual collection. Conversely in the long-term study, 16 participants provided a nasal and postauricular swab sample and completed a behavioral survey on a monthly basis throughout the academic year, excluding the month of December.

During collection, participants self-collected postauricular and intranasal swab samples to maintain COVID-19 safety protocols. Verbal cues were given to the participants to ensure proper technique was used. Directly after collection, the end of the swab was broken into a 2 mL microcentrifuge tube (Carolina Biological Supply Company) containing 1000 µL of phosphate buffered saline (PBS) (PBS Tablets, Calbiochem). Additionally, participants completed a behavioral survey which inquired about perceived stress levels and engagement in mask wearing, handwashing, and hand sanitizing, as shown in Figure 1. The behavioral survey was anonymous and completed through Google Forms to maintain COVID-19 safety protocols.

Sterile Sample Filtration

Once samples were obtained, the swab incubated in the PBS solution for 30 minutes before filtration. After incubation, the PBS from the sample was placed in a syringe with a 0.45- micron PTFE membrane filter (VWR International) attached to a 10 mL Luer-Lok Tip syringe (BD) and filtered into a new microcentrifuge tube.

Viral Amplification

Samples were amplified using *E. coli* B, *E. coli* K12, and *S. aureus* cultures (Carolina Biological Supply Company). Approximately 3.0 mL of LB Miller Broth (Sigma-Aldrich) and 200 µL *E. coli* B culture was combined in a 15 mL culture tube (Thermo Fisher). It was then incubated at 37 °C for 30 minutes in a shaking incubater. Following the 30 minutes incubation period, 100 µL of the filtered sample were added to the 15 mL culture tube. The sample incubated in a shaking incubator at 37°C overnight. Viral amplification was repeated for each sample with *E. coli* K12 and *S. aureus*.

Plaque Assays

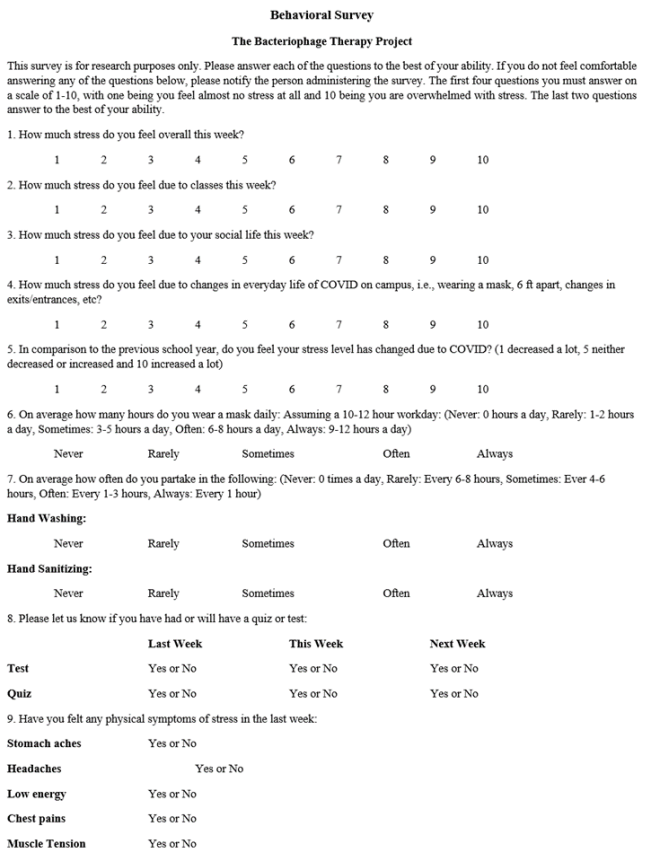
Plaque assays were prepared by adding 100 µL of *E. coli* B liquid culture to a sterile LB Agar plate. Sterile spreading techniques were used to establish a bacterial lawn on the plate. Spread plates were incubated for 20 minutes at 37°C. Five microliter (5 µL) of amplified sample was added to three of four areas of the plate. Five microliters (5 µL) of *E. coli* B culture was added to the fourth area of the plate which was designated for a negative control. Lids were removed from the plates as they dried in the incubator for 10 minutes at 37°C. Lids were replaced, plates were flipped upside down and remained in the incubator overnight. This was repeated using *E. coli* K12 and *S. aureus* as the bacterial hosts, and their respective amplified samples. Plaque assays were analyzed the following day to determine presence of lytic activity. Results were classified as 0 (no lytic activity), 1 (minimal lytic activity), 2 (some lytic activity), 3 (lytic region encompassed area sample was placed), 4 (lytic region slightly extended beyond where sample was placed), 5 (lytic region greatly surpassed area sample was placed), or non-determinant (sample contaminants obstructed visualization of potential lytic zones).

DNA Extraction

Samples identified to have positive (1-5) or non-determinant plaque assay results were subjected to molecular analysis. One hundred microliters (100 µL) of amplified sample were placed into a 2.0 mL microcentrifuge tube and centrifuged at 2,500 rpm for 5 minutes. The supernatant was transferred to a new microcentrifuge tube. Five microliters (5 µL) of proteinase K (Thermo Fisher) was added and the sample incubated at room temperature on a shaking incubator for 60 minutes. Samples were then placed in a heat block for five minutes at 95 °C.

Polymerase Chain Reaction (PCR)

Following DNA extraction, polymerase chain reaction (PCR) was



Behavioral Survey

The Bacteriophage Therapy Project

This survey is for research purposes only. Please answer each of the questions to the best of your ability. If you do not feel comfortable answering any of the questions below, please notify the person administering the survey. The first four questions you must answer on a scale of 1-10, with one being you feel almost no stress at all and 10 being you are overwhelmed with stress. The last two questions answer to the best of your ability.

1. How much stress do you feel overall this week?

1 2 3 4 5 6 7 8 9 10

2. How much stress do you feel due to classes this week?

1 2 3 4 5 6 7 8 9 10

3. How much stress do you feel due to your social life this week?

1 2 3 4 5 6 7 8 9 10

4. How much stress do you feel due to changes in everyday life of COVID on campus, i.e., wearing a mask, 6 ft apart, changes in exits/entrances, etc?

1 2 3 4 5 6 7 8 9 10

5. In comparison to the previous school year, do you feel your stress level has changed due to COVID? (1 decreased a lot, 5 neither decreased or increased and 10 increased a lot)

1 2 3 4 5 6 7 8 9 10

6. On average how many hours do you wear a mask daily: Assuming a 10-12 hour workday: (Never: 0 hours a day, Rarely: 1-2 hours a day, Sometimes: 3-5 hours a day, Often: 6-8 hours a day, Always: 9-12 hours a day)

Never Rarely Sometimes Often Always

7. On average how often do you partake in the following: (Never: 0 times a day, Rarely: Every 6-8 hours, Sometimes: Ever 4-6 hours, Often: Every 1-3 hours, Always: Every 1 hour)

Hand Washing:

Never Rarely Sometimes Often Always

Hand Sanitizing:

Never Rarely Sometimes Often Always

8. Please let us know if you have had or will have a quiz or test:

	Last Week	This Week	Next Week
Test	Yes or No	Yes or No	Yes or No
Quiz	Yes or No	Yes or No	Yes or No

9. Have you felt any physical symptoms of stress in the last week:

Stomach aches Yes or No

Headaches Yes or No

Low energy Yes or No

Chest pains Yes or No

Muscle Tension Yes or No

Figure 1: The behavioral survey developed by Dr. Paul E. Richardson's lab. It was designed and used to determine a potential correlation between human behavior (namely face mask wearing, hand washing, hand sanitizing, and perceived stress levels) and *E. coli* and *S. aureus* phage presence.

conducted. Twenty-five microliters (25 µL) of Gotaq Green Master Mix (Promega Corporation), 21 µL template (DNA extraction), and 4 µL of the corresponding primer set were added to a 0.2 mL PCR tube (VWR International). PCR analysis was conducted in the BIORAD T100 Thermocycler. PCR analysis was conducted as follows: an initial 4-minute DNA unwinding step at 95°C, followed by 39 cycles of DNA denaturation (30 seconds at 94°C), primer annealing (1 minute at 55°C), and DNA extension (72°C for 2 minutes). After completion, PCR products were held at 4°C for short-term storage. Primer sets for coliphage comprised of CPA, CPB, and CPO; primer sets for *S. aureus* phage comprised of SPA and SPB as delineated by Table 1.

Gel Electrophoresis

PCR products were imaged by gel electrophoresis using 2% agarose (Agarose I, VWR) gels and 1x Tris-acetate EDTA (TAE) buffer. Five microliters (5µL) of ethidium bromide were used as a staining agent within the 2% agarose gel. Ten microliters (10 µL) of 1 kb DNA ladder (Promega Corporation) and 10 µL of PCR product were loaded into designated wells. Gel electrophoresis was conducted at 60 volts for 120 minutes before being imaged under UV light with the Molecular Imager ChemiDoc XRS+ Imaging System from BioRad Laboratories, Inc.

PCR Primers

Coliphage primers consisted of CPA, CPB, and CPO. Primer set CPA is comprised of conservative genomic sequences to identify coliphage types K1F, 933, Micro, T4, and Mu. Primer set CPB is comprised of conservative genomic sequences to characterize coliphage types Mu, N4, Jk, and Lambda. CPA and CPB primer sets were derived from a 2009 UNC Chapel Hill dissertation by Hee Suk Lee[22]. The CPO primers sets were derived from a study done by Cannon, et al[23]. and comprised of ORF23 and ORF43[20]. *S. aureus* primers used included

Table 1: Primer sets CPA, CPB, and CPO aided in the identification of coliphage. The dissertation of Hee Suk Lee aided in the derivation of CPA and CPB primer sets[21]. Primer set CPO was derived from L. Pieterse, et al.[20]. Primer sets SPA and SPB were used in the identification of *S. aureus* phage. Both primer sets originated from R. Pantůček, et al[22].

Primer Set	Target Family/ Organism	Gene Target	PCR Fragment Length (bp)	Primer Name	Primer Size (bp)	Primer Sequences (5' to 3')
CPA	*Podoviridae*	CKV1F, gp34	2110	K1FFor	16	TGGAAGCCCGTGAGAC
				K1FRev	18	GCAGCGTCAATCGCTCGG
	Podoviridae	933Wp09, *hkaG* gene	488	933For	18	GCAATACATCAAACGCCG
				933Rev	16	GCGAATGCCAGCGGCG
	Microviridae	Hypothetical protein	1039	MicroFor	25	GCTGCCGTCATTGCTTATTATGTTC
				MicroRev	25	GYTAYCGBMMCATYAAYTAHTCACG
	Myoviridae	Major head protein (gene 23)	704	T4setFor	20	GATATTTGTGGYGTTCAGCC
				T4setRev	24	GTCAAATACACCAGCTTTAGAACC
	Siphoviridae	cII protein	177	HKsetFor	20	CACAGCGAGAAATTGATCGC
				HKsetRev	19	CTAATCGGACTGATGTCTG
CPB	*Myoviridae*	Tail fiber gene (MUP49)	171	MusetFor	21	GAAAACGACTCAATCCTTGCC
				MusetRev	20	TCATCAGGTCTTTTGTTGTGG
	Podoviridae	Hypothetical protein	2285	N4For	20	GCACATGCAGAATAAGGTTG
				N4Rev	20	CCATTAGTAACACCATCTGC
	Siphoviridae	Tail fiber protein	878	JKsetFor	16	GYGAYCAGATGGTTCC
				JKsetRev	16	CAATRTCYTCYTARTTG
	Siphoviridae	B gene	307	LambdaFor	20	TGGGCGTACTTTATGGGGCG
				LambdaRev	20	CGGACCTGCTGGGCAAAAAT
CPO	Coliphage T2/ T4	ORF 23 (Major capsid protein)	405	ORF23For	20	TGGCGCAGTAACTCAGATTG
				ORF23Rev	20	GCACAGCTTCCATTTGTTT
	Coliphage T2/ T4	ORF 43 (DNA polymerase)	198	ORF43For	20	CCCTGCGCCTTTCATAATAA
				ORF43Rev	20	ATCGCAGGAACAGCTCCTAA
SPA	3A-like phages	Tail Fibers	744	3A-like For	20	TATCAGGCGAGAATTAAGGG
				3A-like Rev	23	CTTTGACATGACATCCGCTTGAC
	Twort-like phages	Major capsid protein	331	Twort-like For	20	TGGGCTTCATTCTACGGTGA
				Twort-like Rev	23	GTAATTTAATGAATCCACGAGAT
SPB	11-like phages	Hypothetical tail proteins	405	11-like For	22	ACTTATCCAGGTGGCGTTATTG
				11-like Rev	23	TGTATTTAATTTCGCCGTTAGTG
	77-like phages	Hypothetical tail proteins	155	77-like For	19	CGATGGACGGCTACACAGA
				77-like Rev	23	TTGTTCAGAAACTTCCCAACCTG

SPA and SPB, which were derived from R. Pantůček, et al. Primer set SPA comprised of conservative genomic sequences to identify *S. aureus* phage types 3A and Twort, while SPB was comprised of conservative genetic sequences to identify *S. aureus* phage types 11 and 77[24]. Coliphage primer set CPA consisted of 0.2 µL HK Forward ("For"), 0.2 µL HK Reverse ("Rev"), 0.2 µL 933For, 0.2 µL 933Rev, 0.2 µL T4For, 0.2 µL T4Rev, 0.2 µL MicroFor, 0.2 µL MicroRev, 0.2 µL K1FFor, 0.2 µL K1FRev, along with 2.0 µL nuclease-free water (Promega Corporation)[22]. Coliphage primer set CPB consisted of 0.22 µL MuFor, 0.22 µL MuRev, 0.22 µL Lambda For, 0.22 µL Lambda Rev, 0.22 µL JKFor, 0.22 µL JKRev, 0.22 µL N4For, 0.22 µL N4Rev, along with 2.22 µL of nuclease-free water[22]. Coliphage primer set CPO consisted of 0.67 µL ORF23 For, 0.67 µL ORF23 Rev, 0.67 µL ORF43 For, 0.67 µL ORF43 Rev, and 1.33 µL of nuclease-free water[23]. *S. aureus* primer set SPA consisted of 0.5 µL 3A-Like For, 0.5 µL 3A-Like Reverse, 0.5 µL Twort Forward, 0.5 µL Twort Reverse, and 2.0 µL of nuclease-free water[24]. The *S. aureus* primer set SPB consisted of 0.5 µL 11-Like Forward, 0.5 µL 11-Like Reverse, 0.5 µL 77-Like Forward, 0.5 µL 77-Like Reverse, and 2.0 µL of nuclease-free water[24]. The above CPA, CPB, CPO, SPA, and SPB primers each totaled 4.0 µL and were used individually in the PCR reactions.

Statistical Analysis

Statistical analysis was conducted via RStudio. To statistically analyze samples from the sporadic study to survey data, Chi Square test, Fisher's Exact test, and T-test were used to determine potential correlations between behavioral factors namely as perceived stress, handwashing/ sanitizing, and facemask usage and coliphage or *S. aureus* phage presence. To analyze longitudinal data from the long-term study, the Pearson correlation test was used to determine possible correlation between fluctuations in perceived stress levels and changes in coliphage and S. *aureus* phage population amongst long-term participants.

Results

Sporadic Study: E.coli

During the 2021-22 collection year, 93 samples were collected and analyze to determine potential coliphage presence. Of the 93 samples,

25.8% formed lytic zones when subjected to plaque assays. None of the samples deemed positive or non-determinant via microbial tests were characterized via PCR using primer sets CPA, CPB, and CPO.

No significant differences in the extent participants reported wearing a face mask were determined between those who tested positively for coliphage and those who tested negatively for coliphage (p-value= 0.63; Figure 2a). Similarly, there were no significant changes in the number of positive coliphage results once Coastal Carolina University lifted the campus-wide mask mandate (p-value= 0.65; Figure 2b). Similarly, it was determined there was no correlation between participants' self-reported engagement in handwashing/ sanitizing and presence of coliphage on the participant (p-value=0.77 and 0.36 respectively; Figure 3). It was determined, with 95% confidence, that subjective stress levels were 0.065-2.56 points higher in participants who did not have coliphage present on them (p-value=0.0398; Figure 4).

Sporadic Study: S.aureus

In the 2021-22, 93 samples were collected and analyzed to determine potential *S. aureus* phage presence. Of the 93 samples, 4.30% formed lytic zones when tested via plaque assays. Positive and non-determinant samples were subjected to molecular testing and 5 were characterized via PCR using the SPA and SPB primer sets. All were identified as *S. aureus* phage type 77.

Due to a small sample of positive *S. aureus* plaque assay results, a correlation between the extent a participant self-reported wearing a face mask and presence of *S. aureus* phage on them could not be confidently determined. However, after Coastal Carolina University lifted the campus-wide mask mandate, requiring constant facemask usage, it was 18.22 times more likely that participants would test positively for *S. aureus* phage (p-value=0.0033; Figure 5). Again, the small sample of positive *S. aureus* bacteriophage results meant statistical analysis could not confidently determine if a participant's self-reported engagement in handwashing or hand sanitizing was correlated to *S. aureus* phage presence on the participant. It was found there was no correlation between the participant subjective stress level and *S. aureus* phage presence on the participant (p-value= 0.88; Figure 6).

Long-Term Study

Monthly fluctuations in the number of samples containing coliphage or *S. aureus* phage were prevalent throughout the study. There were 0, 4, 7, 1, 7, and 3 long-term samples which were lytic to *E. coli* in September, October, November, January, February, and March, respectively (Figure 7). There were no positive microbial tests for *S. aureus* phage, until the month of March, when there were two (Figure 7). The monthly average perceived stress levels were 7.0, 5.8, 6.9, 5.2, 5.8, and 6.6 for the months of September, October, November, January, February, and March, respectively. No linear correlation was determined between average stress level and coliphage or *S. aureus* phage presence (p-value= 0.93 and 0.64 respectively).

Discussion

From 2014-2018, the monitoring of *S. aureus* and *E. coli* phage population on students and faculty at Coastal Carolina University resulted in approximately 42.9% and 36.8% of samples demonstrated lytic activity to *S. aureus* and *E. coli* phage, respectively[20]. *S. aureus* colonization typically occurs as a persistent infection on 20% of the human population, transiently on 30% of the human population, and the rest of the population are noncarriers[25]. Typically, *S. aureus* colonizes around the nasal cavity and approximately 20% of the human population has nasal *S. aureus* colonization[25,26]. Assuming phage presence in all colonized bacteria, the percentage of *S. aureus* phage at Coastal Carolina University would indicate typical rates of *S. aureus* dermal colonization. Conversely, *E. coli* is not a common contributor to the skin microbiome, as *E. coli* presence on the skin induces keratocytes to secrete psoriasin, an antimicrobial peptide adept at eradicating *E.coli*[27]. Hence, the high percentage of coliphage found from swab samples may be indicative of a population with higher-than-normal rates of dermal *E. coli* colonization. However, there was a drastic change in the phage population amidst the COVID-19 pandemic. During 2020-21 school year, results from bacteriophage monitoring indicated a 100% decrease in the *S. aureus* phage population and a 94.8% decrease in the coliphage population[21]. From September 2021-March 2022, the most recent collection year, the phage population remerged as 4.30% of samples were lytic to *S. aureus* and 25.8% of samples were lytic to *E. coli* during microbial testing.

Merely 27.7% of samples lytic to *S. aureus* were characterized by primer set SPA. All of which were identified as 77-like bacteriophage. No samples were characterized using primer set SPB. Primer sets SPA and SPB derived from R. Pantůček, et al. were comprised of conserved genomic sequences specific to *S. aureus* phage types 3A, 11, 77, and Twort[24]. The small portion of lytic samples that were characterized through PCR could be due to the limited scope of the primer sets as only five of the eleven *S. aureus* phage serotypes, A, B, F, L, and D are represented[28]. Alternatively, a mere 250 staphylococcal phages have been identified and described in literature. Recombination and horizontal gene transfer have been shown to cause high diversity and mosaicism in the genetic makeup of staphylococcal phages that have been characterized[24]. Thus, it is likely that the somatic *S. aureus* phage repository is constituted of a diverse population that include phages that have yet to be characterized. Correspondingly, there was a lack of characterization of samples lytic to *E. coli* as none were characterized through PCR using primer sets CPA, CPB, nor CPO. Again, this could be attributed to the vast diversity of somatic coliphages and the inadequate scope of characterization these primers provide. Due to this, questions from the behavioral survey were analyzed against microbial data.

It was determined that the engagement in mask wearing, handwashing, or hand sanitizing had no effect on coliphage presence on a participant. Conversely, it was determined that participants with coliphage present were more likely to have a perceived stress level 0.065 -2.56 points higher than participants lacking coliphage presence (p-value=0.040). This refutes the theory that stress-induced immunosuppression would lead to higher rates of *E. coli* colonization amongst participants. General Adaptation Syndrome (GAS) describes the process humans goes through when introduced to a stressor over a long period of time. GAS consists of three stages; stage 1:Alarm (the "fight-or-flight response is triggered due to an increase in sympathetic-adrenal-medullary (SAM) and the hypothalamic-pituitary-adrenal (HPA) responses); Stage 2: Resistance (as the HPA response takes over, somatic processes work at their maximum capabilities and stressor removal triggers the body to revert to normalcy); Stage 3: Exhaustion (in the case of prolonged stress, somatic functions begin to deteriorate and even become ineffective. Sympathetic autonomic nervous system action reappears, and the adrenal cortex suffers damage. Thus, leading to parasympathetic action, which can include energy storage failure. At this point the immune system collapses and morbidity of stress-related disease increases)[29]. Strictly speaking, during the resistance phase somatic systems operate with increased efficiency to cope with the addition of a stressor. However, the resistance phase turns to the exhaustion phase after rigorous use damages somatic systems and causes problems such as immunosuppression. Literature suggests that the duration of the resistance stage cannot be quantified due to the variability and multiplicity of individual responses to stress[30]. It can be speculated that in response to the COVID-19 pandemic, there are individuals who are experiencing the resistance phase of GAS, thus causing them to have higher perceived stress levels without immunosuppression or an increased rate of *E. coli* colonization and subsequently phage population. Assuming participants move to the exhaustion stage, as time continues, the existing negative correlation between high stress levels and coliphage presence theoretically would shift to a positive correlation.

The long-term study resulted in no correlation between monthly average stress levels and coliphage presence (p=0.93). This could be due to a small sample size and an exceedingly small fluctuation in average monthly perceived stress with the highest average stress level being 7.00 in September 2021 and the lowest average stress level being 5.81 in October 2021. Despite the lack of correlation, this could also be attributed to the GAS. Theoretically those who are not affect by GAS should have healthy immune systems and low levels of *E. coli* colonization, coliphage presence, and perceived stress. Analogously,

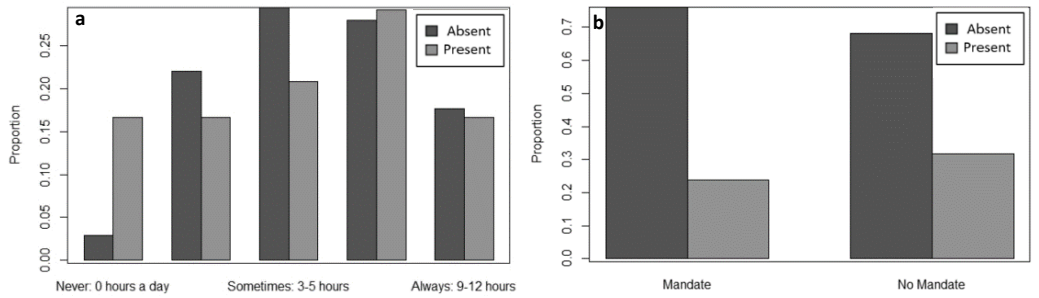


Figure 2a: Depicts the proportion of positive (1,2,3,4,5) and negative (0 and nondeterminate) *E. coli* plaque assays for each level of participant engagement in mask wearing. Never= 0 hours, Often= hours, Sometimes=3-5 hours, Often= hours, and Always = 9-12 hours. No correlation was determined between mask wearing and coliphage presence (Chi-squared analysis; p-value=0.63). **Figure 2b:** depicts proportion of positive (1,2,3,4,5) and negative (0 and nondeterminate) *E. coli* plaque assays before and after the mask mandate was lifted. No significant differences were noted after the mask mandate was lifted (Chi-squared analysis; p-value=0.65).

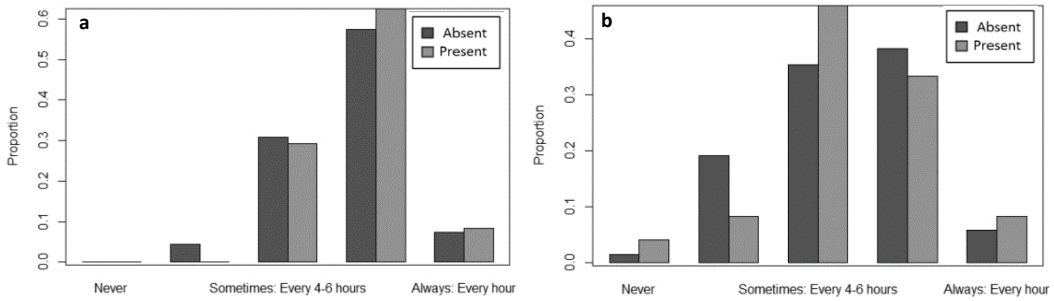


Figure 3a: Depicts the proportion of positive (1,2,3,4,5) and negative (0 and nondeterminate) *E. coli* plaque assays for each level of participant engagement in handwashing. Never, Often= every hour, Sometimes=every 4-6 hours, Often=every hours, and Always = every hour. No correlation was determined between mask wearing and coliphage presence (Chi-squared analysis; p-value=0.77). **Figure 3b:** Depicts the proportion of (1,2,3,4,5) and negative (0 and nondeterminate) *E. coli* plaque assays for each level of participant engagement in handwashing. Never, Often= every hour, Sometimes=every 4-6 hours, Often=every hours, and Always = every hour. No correlation was determined between mask wearing and coliphage presence (Chi-squared analysis; p-value=0.36).

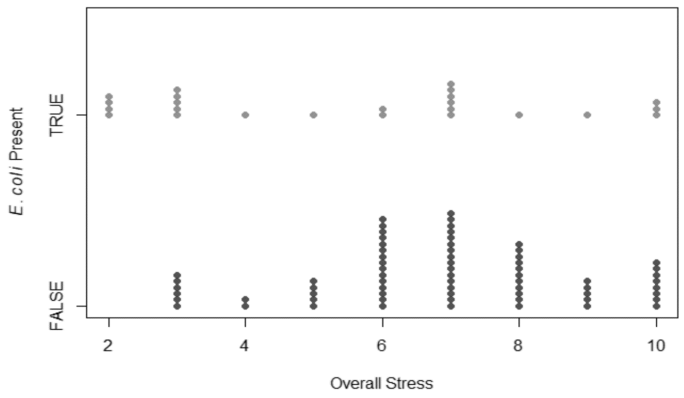


Figure 4: Depicts the stratification of positive (1,2,3,4,5) and negative (0 and nondeterminate) *E. coli* plaque assays based on participant perceived stress. It was determined with 95% confidence that participants with a negative *E. coli* plaque assay would have a perceived stress level 0.065 to 2.56 points higher than those who had positives ones (T-test; p-value= 0.040).

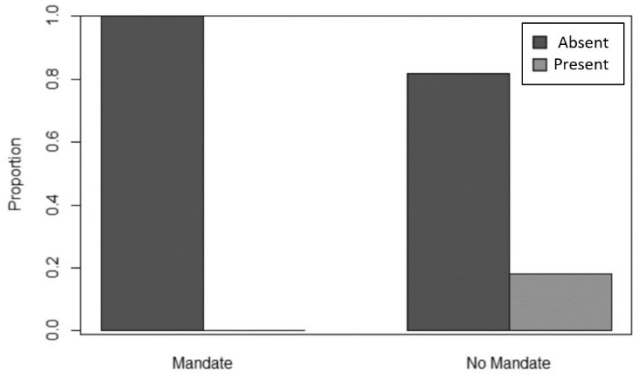


Figure 5: depicts proportion of positive (1,2,3,4,5) and negative (0 and nondeterminate) *S. aureus* plaque assays before and after the mask mandate was lifted. It was determined participants were 18.22 times more likely to have positive *S. aureus* plaque assays after the mask mandate was lifted (Fisher's Exact; p-value= 0.0033)

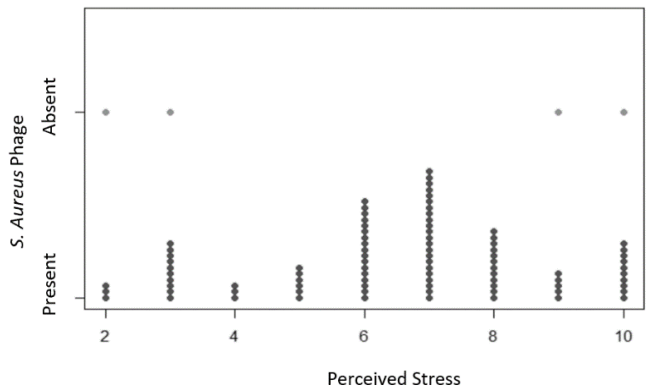


Figure 6: Depicts the stratification of positive (1,2,3,4,5) and negative (0 and nondeterminate) *S. aureus* plaque assays based on participant perceived stress. No correlation was determined between perceived stress levels and positive *S. aureus* plaque assays (T-test; p-value= 0.88).

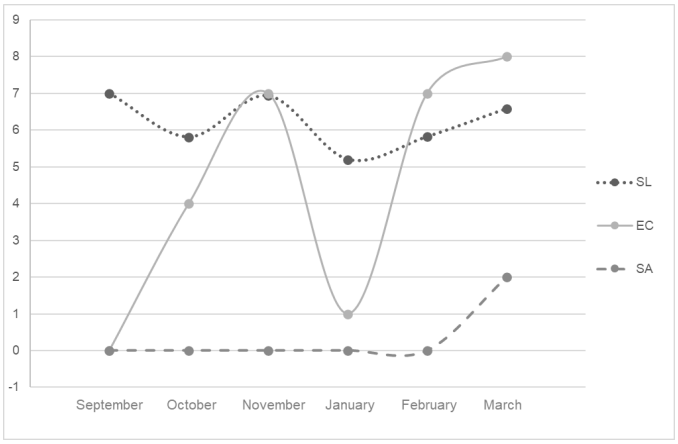


Figure 7: Summary of monthly positive (1,2,3,4,5) *E. coli* (EC) and *S. aureus* (SA) plaque assays and monthly average perceived stress levels (SL) throughout the 2021-2022 school year. No correlation was found between average monthly perceived stress level and the number of monthly positive E. coli/ S. aureus plaque assays (Person Correlation; p-value= 0.93 and 0.64 respectively). No data was collected in December 2021.

individuals in the resistance phase of GAS would have an active immune system causing low levels of *E. coli* colonization and subsequently coliphage but have higher perceived stress levels. Therefore, the limited data from the long-term study similarly presents evidence that perceived stress levels alone are not correlated to coliphage presence or absence, but rather coliphage presence could be attributed to those in the final stage of GAS, the exhaustion phage.

The diminutive sample size of positive *S. aureus* plaque assays (4.30%) led to an inability to determine if a correlation existed between participant engagement in mask wearing, hand washing, and hand sanitizing and *S. aureus* phage presence. However, the limited data collection did suggest possible correlation between face mask usage and *S. aureus* phage presence, as positive S. aureus plaque assays were 18.22 times more likely after the mask mandate cessation (p-value=0.0033). The introduction of persistent face mask usage amidst the COVID-19 pandemic has caused a dysbiosis in the facial microbiome. After a mere four hours of face mask usage, a trend towards altered beta-diversity occurs through increased temperature, increased moisture, and introduction of bacterial and yeast species typically not present in the facial microbiome from the mouth[31]. One study found that facemasks worn for 4 hours contained high bacterial loads and caused a 42% and 27% dissimilarity in the microbial composition of the skin and anterior nares of participants. The most common bacterial species isolated from facemasks were *Bacillus*, *Staphylococcus* (predominantly *Staphylococcus epidermis*), and *Acinetobacter*. *Streptococcus* was also prevalent on facemasks[31]. Evidence suggests highly temporal relationships between *S. aureus* and other bacterial strains, namely other strains of *Staphylococcus*, *Streptococcus*, and *Acinetobacter* cause inhibition to *S. aureus* growth[32]. Consequently, improper and prolonged facemask usage could result in the introduction of a high load of bacterial species which inhibit the growth of *S. aureus* and subsequently decreasing its prevalence in the human facial microbiome. In conjunction with the *S. aureus* population, the *S. aureus* phage population would also diminish.

Prior data suggested a potential temporal competition between *E. coli* and *S. aureus,* which leads to the inverse relationship between coliphage and *S. aureus* phage presence[20]. During this collection cycle, evidence shows a possible temporal relationship between the two bacterial strains. However, it was not the only factor causing fluctuations in coliphage presence. Positive *S. aureus* plaque assays only occurred in March. In March there were two samples which had positive *S. aureus* plaque assays and three samples which had positive *E. coli* plaque assays. In other months, the number of positive *E. coli* plaque assays varied from none to seven, thus demonstrating fluctuations independent of fluctuations in monthly positive *S. aureus* plaque assays. This is not to say that a temporal relationship does not exist between *E. coli* and *S. aureus*. *S. aureus* has been shown to produce phenol-soluble modulins (PSMs), namely a δ-toxin which exhibits bactericidal activity. However, further research has shown that the bactericidal activity is generally limited to a specific bacterial target. *E. coli* has also not been demonstrated to inhibit *S. aureus* growth[25]. While a temporal relationship between *E. coli* and *S. aureus* is possible, it is not the main factor influencing fluctuations in coliphage and *S. aureus* phage population dynamics.

Furthermore, both the sporadic and long-term studies presented crucial insight into behavioral factors that seemingly affect coliphage and *S. aureus* phage presence. However, fluctuations in the coliphage and *S. Aureus* phage population cannot be fully understood at this point. This study was indicative of continual drastic fluctuations in the coliphage and *S. aureus* phage population, which may be caused by external factors such as the human response to stress, face mask usage, and many other factors yet to be explored, such as UV exposure, prevalence of antimicrobial peptides on skin, and interactions with environmental phages.

Conclusion

Prior data has shown that the coliphage and *S. aureus* phage reservoir on humans at Coastal Carolina University fluctuates greatly throughout the year and in response to global social change. It was speculated that changes in human behavior contributed to this fluctuation. The current study did not demonstrate that mask wearing, handwashing, or hand sanitizing were correlated with coliphage presence nor absence on a participant. There was a correlation between higher stress levels and an absence of coliphage on a participant, which may be explained by GAS. In the midst of a global pandemic, many people are experiencing prolonged stress and hence GAS. Immunosuppression does not occur until the final exhaustion stage of GAS, therefore those in the resistance stage have somatic systems operating with high efficiency, thus potentially deterring *E. Coli* colonization. There was not enough data to determine if a relationship between *S. aureus* phage and mask usage, hand washing, hand sanitizing, or perceived stress existed. However, after the removing the mask mandate participants were 18.22 times more likely to have positive *S. aureus* plaque assays. This may be evidence that facemask usage causes a lack of *S. aureus* phage presence, due to the introduction of bacterial strains which inhibit *S. aureus* colonization. While GAS and mask-wearing explain changes in phage population dynamics post-pandemic, the causes of fluctuations in coliphage and *S. aureus* phage pre-pandemic are still uncertain. As many factors influencing phage population dynamics on humans remain unknown, it is imperative that the reliability and fluctuations of the phage repository on humans is further understood before its employment in the fight against antibiotic resistant infections.

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Notes and References

**Corresponding author email: prichar@coastal.edu*

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