IDENTIFICATION AND CHARACTERIZATION OF *ENTEROCOCCUS SPP.* IN LOCAL SURFACE WATERS

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ABSTRACT

In order to identify and characterize *Enterococcus spp.*, we isolated bacteria from surface water samples taken from four sites in Morris County, NJ. We then performed various biochemical tests to identify the isolates by species, as well as determine their antibiotic resistance and virulence. Through the use of bacterial count data, this study indicated that none of the four local water sources were suitable for bathing or drinking water, according to federal safe water standards. Naturally found in animal digestive tracts, *Enterococcus spp.* is often utilized as fecal indicators in surface waters. In addition, we concluded that many common antibiotics, such as penicillin, are no longer effective in combating *Enterococcus spp.* Due to the recent increase in the number of nosocomial pathogens, many studies have shifted their focus to two of the more reoccurring and pathogenic species - *Enterococcus faecalis* and *Enterococcus faecium*. These two *Enterococcus* species are known for their resilience and high level of antimicrobial resistance. *E. faecium* was isolated from all four of the study sites; however, *E. faecalis* was only present at Loantaka Brook and Drew Pond. These two particular species of *Enterococcus* are responsible for a variety of diseases, including bacteriemia, endocarditis, urinary tract infections, and wound infections [1].

INTRODUCTION

Enterococci are gram-positive cocci that are usually arranged in pairs, singly, or in short chains [2].In 1984 a new genus of prokaryotic cells, *Enterococcus spp.*, was defined. The enterococci were split from the existing genus *Streptococcus* based on nucleic acid analysis and basic physiological differences. Currently there are sixteen species within the genus. On the basis of microscopic morphology, enterococcal isolates cannot be separated from strains of streptococcus [1]. Enterococci can be isolated from soil, food, water, animals, and plants. The prevalence of different enterococcal species is influenced by several factors including type of host, age, and diet. Since enterococci are generally found in the intestinal tract of warm-blooded animals, enterococci can be used as fecal indicator organism for water quality analysis.

They have cell walls with the group D glycerol teichoic acid, which is an example of a group-specific antigen and is identified as the streptococcal group D antigen [1]. The genus consists of facultative anaerobic organisms; this means that they prefer the use of oxygen, but can survive without it. Most strains of enterococci are homofermentative, meaning that lactic acid is the end product of glucose fermentation.

There are several ways to isolate *Enterococcus spp.* from other bacteria using selective techniques. One way is by incubating the sample in a nutrient broth containing 6.5% NaCl concentration. Enterococci are one of the few bacterial species that can grow in the presence of high salt. A second method of selection is growing the cultures in a broth containing esculin. All enterococci can hydrolyze esculin. The enterococci breakdown esculin into two parts, one of the parts reacts with iron from the solution to form a dark brown/black complex [1].

The two species of *Enterococcus* of medical significance to humans are *Enterococcus faecalis* ("pertaining to feces") and *Enterococcus faecium* ("of feces") [2]. Both organisms are commonly isolated from the human gastrointestinal tract. These two strains of enterococci are considered nosocomial pathogens, causing up to 10% of all hospital-acquired infections. These infections are spread through ingestion of contaminated water or food. It is important to monitor enterococci levels in water sources that humans drink from (such as lakes, ponds, and rivers) to ensure that humans will not come in contact with contaminated water [2].

Enterococci generally have a limited potential for causing disease, since they do not naturally produce any known toxins. What makes them pathogenic is their ability to obtain and express virulence factors. Some of the more serious virulence factors expressed by enterococci are hemolysins, cytolysins, gelatinase production, and biofilm formation. Typically enterococci cause urinary tract infections, wound infections, bacteremia, and endocarditis [1]. *E. faecalis* is now the leading cause of bacterial endocarditis. One of the main concerns that the medical community is facing with *Enterococcus spp.* is their ability to acquire resistance to antibiotics. Many strains of enterococci are resistant to different antibiotics. This means that it is much more difficult to treat a person who comes down with an infection that originates from the bacteria.

Antibiotics are antimicrobial compounds that terminate or impede the proliferation of bacteria and are derived from certain microorganisms. Antibiotics are used to treat bacterial infections; they are ineffective against viruses. Antibiotics exhibit selective toxicity, meaning that they are capable of targeting bacteria without harming the host cells [3]. If an antibiotic is highly selective, it can disrupt enzymes or the arrangement that is idiosyncratic to a certain bacterium. If its selective toxicity is low, the antibiotic inhibits the same process in the bacterium and in the host cell [4]. As a result, the lack of selectivity may prove to be deleterious to the host cell [5]. Broad spectrum antibiotics can be used to treat a variety of diverse species of bacteria; other antibiotics may be combined to treat specific bacterial infections. The efficacy of a treatment depends on many aspects, such as the location of the infection, the availability of antibiotic to the site of infection, and the susceptibility of the bacteria to the antibiotic [6].

Antibiotics are typically classified by their modes of action. For example, the antibiotic tetracycline inhibits protein synthesis by binding to ribosomes (internal cellular structures that create protein). Tetracycline hinders the assembly of the components required to create new bacterial cells by inhibiting protein synthesis [1]. The antibiotic penicillin, in the class of β-lactams, was once a reliable, effective remedy against an often fatal bacterium, *Staphylococcus aureus*. *Staphylococcus aureus* is a common cause of skin infections and was effectively treated with penicillin in the 1940s and 1950s. However, due to growing antibiotic resistance, penicillin has become ineffective against many bacteria. *Staphylococcus aureus* produced an enzyme capable of breaking down penicillin, rendering it useless against many gram-positive bacterial

infections [7]. One method of resisting penicillin is the production of β-lactamase, an enzyme that is capable of breaking down a β-lactam ring. B-lactam rings are four-atom rings that are a crucial component of the molecule's antibiotic properties. This breaks the β-lactam ring of penicillin, rendering it ineffective. Inhibiting the formation of peptidoglycan, essential for cell wall structural integrity, cross links in the bacterial cell wall, β-lactam antibiotics are bactericidal and bind to the enzyme that links the peptidoglycan molecules which weakens the cell wall of the bacterium [8].

Erythromycin, which is usually administered to people allergic to penicillin, is a macrolide antibiotic. It inhibits bacteria growth by binding to the RNA of ribosomes, which impedes protein synthesis. Until recently, vancomycin was used successfully against *Staphylococcus aureus*. Vancomycin is a glycopeptide antibiotic that inhibits cell wall fusion and is utilized in the prophylaxis and treatment of an infection caused by a gram-positive bacterium [5]. It prevents the amalgamation of N-actylmuramic acid and N-acetylglucosamine-peptide subunits which inhibits the normal, healthy cell wall synthesis in gram-positive bacteria. However, increasing resistance has been documented and observed, indicating that vancomycin may inevitably be useless against *Staphylococcus aureus* as well as many other bacteria. For this reason, vancomycin is usually used as a "last resort" treatment in the case of the continuous failure of other antibiotics [1]. The Centers for Disease Control and Prevention (CDC), whose mission is to protect the health and safety of all Americans and provide essential human services, have created procedures to follow when treating a bacterium with vancomycin due to the escalating threat of vancomycin-resistant enterococci [9].

Other antibiotics utilized to test for enterococcal resistance were cephalothin, streptomycin, ciprofloxacin, gentamicin, and nitrofurantoin. Cephalothin is a first-generation cephalosporin antibiotic that impedes cell wall production. Streptomycin, an aminoglycoside, originates from the actinobacterium *Streptomyces griseus* and prevents bacterial proliferation by marring the cell membranes and inhibiting protein synthesis. The first antibiotic to be synthetically manufactured on a large scale was chloramphenicol, which is bacteriostatic, obstructs protein manufacture, and is derived from *Streptomyces venezuelae.* Ciprofloxacin (a bactericidal antibiotic) is a fluoroquinolone that is responsible for inhibiting bacterial DNA gyrase. Its mode of action relies on its ability to block bacterial DNA replication by binding to DNA gyrase, an enzyme that regulates the unwinding of a DNA helix into two strands. It is active against gram-positive and gram-negative bacteria. Gentamicin, an aminoglycoside, is commonly employed against gram-negative infections. It binds to a certain area on a bacterial ribosome, which leads to the misrepresentation of the bacterium's genetic code and prevents protein creation. Another antibiotic, nitrofurantoin, is bacteriocidal and is used clinically in treating cystitis. Nitrofurantoin inhibits protein synthesis mostly in the translation phase [1].

Antibiotic resistance is the potential of a microorganism to resist and endure the effects of an antibiotic. Like all other active living organisms, bacteria evolve and change over time in response to environmental obstacles. This allows the remaining bacteria to transfer their antibiotic resistant genes through plasmid exchange to other bacteria which have not been exposed to the antibiotic to develop resistance [3]. Another predicament arises when the surviving bacteria procreate, because their offspring will contain the antibiotic resistance gene. After many exposures and transfers a bacterium may become multi-resistant (a carrier of several

resistance genes). The bacteria react to an environmental pressure, antibiotic exposure, and form a genetic mutation in order to survive.

Nosocomial infections continue to escalate among patients in hospitals. Nosocomial is derived from the Greek word, *nosokomeion*, which means hospital. These infections often result from the rising number of antimicrobial-resistant gram-positive bacteria consequentially appearing forty-eight hours or more after a treatment from a hospital. The diffusion of the nosocomial infection requires a source of infectious bacteria, a vulnerable host, and a mode of transport for the microbes [10]. One of the most resistant bacteria is *Enterococcus*. *Enterococcus* is even resistant to the "last resort" drug vancomycin. Since *Enterococcus* emerged, the number of vancomycin-resistant microorganisms has multiplied radically due to the genetic coding *Enterococcus* contains [11]. This is important to because the spread of antibiotic-resistant bacteria strains have only just begun to disclose themselves, which may prove to be fatal to the human race.

However, antibiotic resistance cannot justify the virulence of *Enterococcus*. Virulence is the strength of its pathogenicity the ability to cause disease or illness to the host of a microorganism. Virulence factors are the products of expressed genes that enable a bacterial stain to invade and colonize a host. Virulent *Enterococcus* strains that express virulence pose an increased threat to our health. Bacteria can acquire virulence factors through mutation or the swapping of genes between pathogenic and non-pathogenic bacteria. *Enterococci* acquire the genes responsible for virulence either by mutation or plasmid swapping [12].

Mutations, or changes to the organism's genetic code, may allow the bacteria to express pathogenic characteristics. Recent findings have shown that these bacteria often carry the genes that code for virulence factors, but they are not expressed. Mutations can cause the expression of these genes, making the bacteria pathogenic [13]. These virulence factors can then be shared between bacteria through conjugation or some other form of gene swapping; spreading the pathogenic virulence factor throughout a population over a relatively short period of time.

Several different types of virulence factors act synergistically to enhance an organism's pathogenicity, enabling the bacteria to easily invade and colonize a host. Adhesion factors allow bacteria to stick themselves to the surface of host cells, while invasion factors allow the bacterial cell to produce proteins and enzymes that help the bacteria break through the cell membrane (or simply stimulate endocytosis) and access the host's cytoplasm [14].

For instance *E. faecalis* uses an Aggregation Substance (AS), a bacterial adhesin which protrudes from its cell wall as hair-like strands that allow the bacteria to cling to the walls of the digestive and biliary tract in humans. The AS can then be used as an aid for conjugation, allowing bacterial virulence factors to spread quickly between organisms [15].

Infection comes when the bacteria form biofilms – massive colonies of resistant, symbiotic bacteria. As biofilms are often composed of many different species of bacteria (each species may have its own purpose in the biofilm, making prokaryotic species behave in a similar matter to eukaryotic cells in a multicellular organism) they are inherently hard to kill, even with strong antibiotics [16].

We tested for the expression of genes that produce hemolysins, cytolysins, bacteriocins, and gelatinase – secreted substances that are harmful to the host. Hemolysins specifically target red blood cells causing β-hemolysis (complete destruction of the cell – the cell lyses causing cell death). Bacteria perform hemolysis to extract hemoglobin for use in the bacteria [17].

Cytolysins are any of a number of excreted proteins that cause host cell death by osmotic lysis. This is usually attained in a hypotonic solution as water diffuses into the cell. The cytolysin allows water to diffuse into the cell until the plasma membrane cannot hold against the high pressure and bursts, spilling the contents of the cell [18]. In this lab we also tested for the production of gelatinases – proteolytic compounds that hydrolyze gelatin and collagen, breaking them into smaller peptide segments [19].

Bacteriocins are secreted compounds that target other local bacteria, preventing them from causing harm or taking nutrients form the infectious strain. *E. faecalis* is known to secrete AS-48, a type of bacteriocin, to kill all similar bacteria in the surrounding area, whether they are similar species or similar strains of the *same* species. The use of bacteriocins is crucial in situations where there are limited nutrients; killing off rival strains ensures that all nutrients in the region will go towards strengthening the infection [20].

MATERIALS AND METHODS

Culture Isolation

The *Enterococcus spp.* colonies were isolated from surface water samples taken from four sites in Morris County, NJ. The four sites from which samples were taken were Burnham Pond, Drew Pond, Loantaka Brook, and the Whippany River. Water samples were taken by submerging a sterile glass bottle into the surface of the water near the shoreline. One ml, 5 ml, 10 ml, and 25 ml samples were sterile filtered through a 0.45µl nitrocellulose filter. The enterococci, approximately one micrometer in diameter, were trapped on the surface of the filter. The filters were placed on mEI agar plates which were incubated at 44.5°C for 48 hours. All samples were run in duplicate. The mEI agar is highly selective for enterococcus growth. It contains cycloheximide to inhibit fungal growth and sodium azide to inhibit growth of gram-negative bacteria. mEI also contains esculin, which is hydrolyzed by enterococci with the production of a brown-black precipitate, as well as chromogen indoxyl-β-D-glucoside, from which enterococci produce an indigo blue complex. After incubation, the plates were examined for signs of enterococcal growth. The presence of indigo blue colonies indicated the presence of enterococci. We counted the number of these blue colonies on each of the plates to determine the concentration of *Enterococcus spp.* in the water sample.

Using sterile toothpicks, we picked 48 random colonies, differing in shape and size, and transferred them into microtitre trays containing 180 µl of 6.5% NaCl in nutrient broth. Each of the 48 wells on the tray contained a unique colony. The 6.5% NaCl broth serves as a method of further selecting the enterococci. After incubating the samples for four days at 37°C, we marked the lid of the tray above the wells that showed growth. From the wells that showed growth in NaCl, we took samples and transferred them into new microtitre wells filled with 180 µl of ENT

broth on the other side of the microtitre tray. Enterococci possess the ability to hydrolyze esculin, producing dark brownish black color. Esculin hydrolysis is a second method of selection for enterococci. After incubating the samples in ENT broth at 44.5°C for twenty four hours, we marked any wells that did not show esculin hydrolysis. Only isolates in wells demonstrating growth in 6.5% NaCl broth and esculin hydrolysis were included in the data set.

Speciation

Each enterococcal species ferments specific sugars. To identify the species in our study, our enterococcal samples were replica plated onto six different 1.0% sugar solutions in phenol red broth: arabinose, mannitol, methyl α-D-glucopyranoside (MGP), ribose, sorbose, and sorbitol. The Bokel replicator was dipped into ethanol and flamed between each stamping to ensure sterilization. The samples were then incubated at 37°C and checked at 24 and 48 hours. A change in color of the phenol red indicator from red to yellow in the sugar solutions indicated the presence of acid produced by the bacteria during sugar fermentation. We marked the samples that changed color after both 24 and 48 hours incubation to avoid false negatives due to alkaline reversion. When bacteria exhaust the sugar supply, they then use the proteins in the medium as an energy source. Proteins are composed of amino acids, which when hydrolyzed release ammonia, a base causing the phenol red pH indicator to revert to red. We recorded which wells (isolates) indicated fermentation (change in color) per sugar, then used a dichotomous key to identify the colonies. The dichotomous keys used were compiled using published biochemical fermentation data charts (Facklam, Sahm, and Teixeira). A dichotomous key is a method for determining the identity of different strains of *Enterococcus spp*. based on a series of paired choices of characteristics.

Antibiotic Resistance

The antibiotic susceptibility of each of the enterococcal isolates was tested by replica plating the isolates onto TSA (tryptic soy agar) supplemented with the following antibiotics: penicillin (10µg/ml), cephalothin (30µg/ml), tetracycline (30µg/ml), streptomycin (10µg/ml), gentamycin (10µg/ml), chloramphenicol (30µg/ml), ciprofloxacin (5µg/ml), vancomycin (30µg/ml), erythromycin (15µg/ml), and nitrofurantoin (300µg/ml). Isolates were also replica plated into two TSA plates that served as controls. All plates were incubated at 37˚C for 48 hours and checked for growth. If a particular isolate grew on an antibiotic plate, it indicated that the specific isolate was resistant to that antibiotic.

Virulence Factors

All *Enterococcus faecium* and *Enterococcus faecalis* colonies were identified and picked onto a nutrient agar plate. All isolates were identified by a grid pattern, which served as a reference. Because *E. faecalis* and *E. faecium* are considered nosocomial pathogenic species, we tested them for virulence factor expression. The virulence factors we evaluated were categorized as hemolysins, bacteriocins, or gelatinases. If a strain of bacteria tests positive for a bacteriocin, then it possesses the ability to kill off closely related species of bacteria. Hemolysins enable the bacteria to lyse red blood cells and utilize the iron in the hemoglobin for cellular respiration. Gelatinase provides bacteria the ability to hydrolyze collagen, which would allow the bacteria to

break through the connective tissues of the body and spread from one area to another. Samples from the initial grid reference plate were picked into a horse blood agar plate. The presence of hemolysins in our isolates was determined using horse blood because it very closely resembles human blood agar. If the agar under the isolate turned green, this was determined to be an alpha hemolysis. If the agar under the isolate showed clearing in the blood, this was determined to be beta hemolysis.

The samples from our initial grid of isolates were then picked onto bacteriocin test plates. The test plates were seeded with confluent lawns of the following species: *E. faecalis* ATCC 29212, *Streptococcus mitis PI 519, E. mundtii O, E. mundtii P,* or *E. mundtii R.* Bacteriocin expression was determined by examining the size of the clearing in the bacterial lawn that indicated a lysing of bacteria. Areas of no clearing ranked as 0 on our bacteriocin scale. Areas between 0 and 5 mm ranked +, areas between 5 and 10 mm ranked ++, and areas greater than 10 mm ranked +++. Finally, we picked the isolates onto a grid-mapped plate of gelatin agar to test for the production of gelatinase.

RESULTS

After comparing the total abundances of enterococci in the four water sources, we found that the Whippany River had the highest concentration of *Enterococcus spp.* with 3150 colonies per 100 mL, which was more than double the number of colonies than any of the other water samples. Loantaka Brook had the second highest concentration of enterococci with 1100 colonies per 100 mL, Drew Pond contained 880 colonies per 100 mL, and finally Burnham Pond consisted of the lowest concentration of enterococci with only 240 colonies per 100 mL (Figure 1).

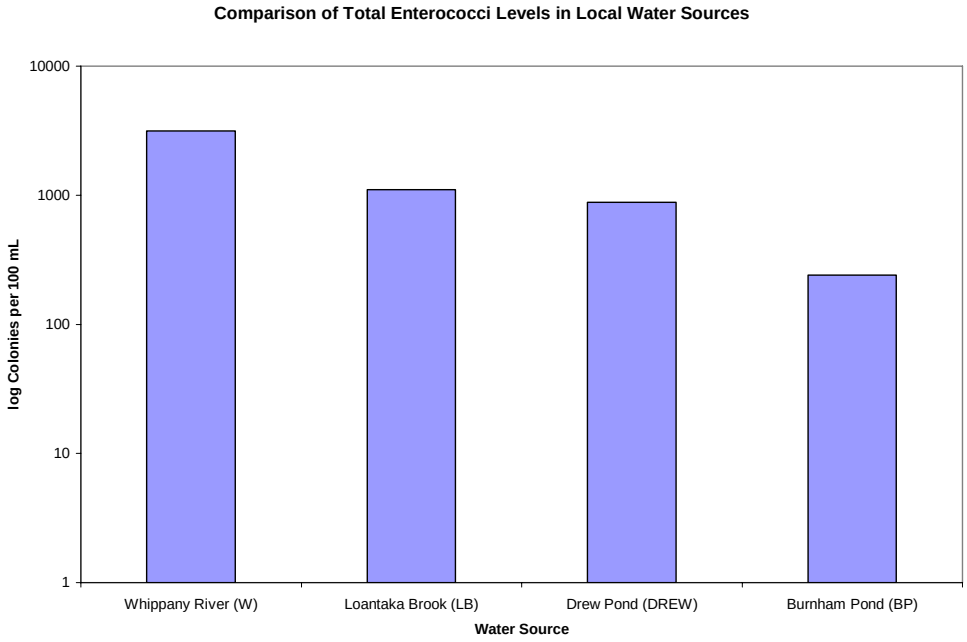

Fig. 1: Comparison of Total *Enterococci* Levels in Local Water Sources

After isolating the enterococci, we determined the relative abundances of various *Enterococcus spp.* in the water samples (Figure 2). Whippany River, which contained the highest overall concentration of enterococci, had relatively equivalent concentrations of each of the six *Enterococcus spp.* with the notable exception of *E. faecalis*, none of which were found in the river sample. Loantaka Brook, which had the second highest concentration of enterococci contained a high concentration of *E. casseliflavus*, with an overwhelming 35% of all the enterococci found being part of that species. *E. faecium* and *E. durans/hirae* also had a strong presence in the pond, and *E. mundtii* also had a moderately large presence. However, it was interesting to note that hardly any colonies of *E. faecalis* and *E. gallinarium* were observed in the brook sample. The pond at Drew University contained an extremely high concentration of *E. faecium,* as well as large amounts of *E. durans* and *E. casseliflavus.* Again, however, there were only paltry amounts of *E. faecalis.* Even more interestingly, there was a complete absence of organisms from the species *E. mundtii* and *E. gallinarium.* Both of these species had a moderate concentration in all the other local water supplies, so it was noteworthy that there were absolutely none to be found in Drew Pond. Finally, in the samples from Burnham Pond, we found numerous organisms of the species *E. casseliflavus, E. durans*, and *E. hirae*. Absent from the pond, however, were colonies of the more pathogenic species – *E. faecium* and *E. faecalis.*

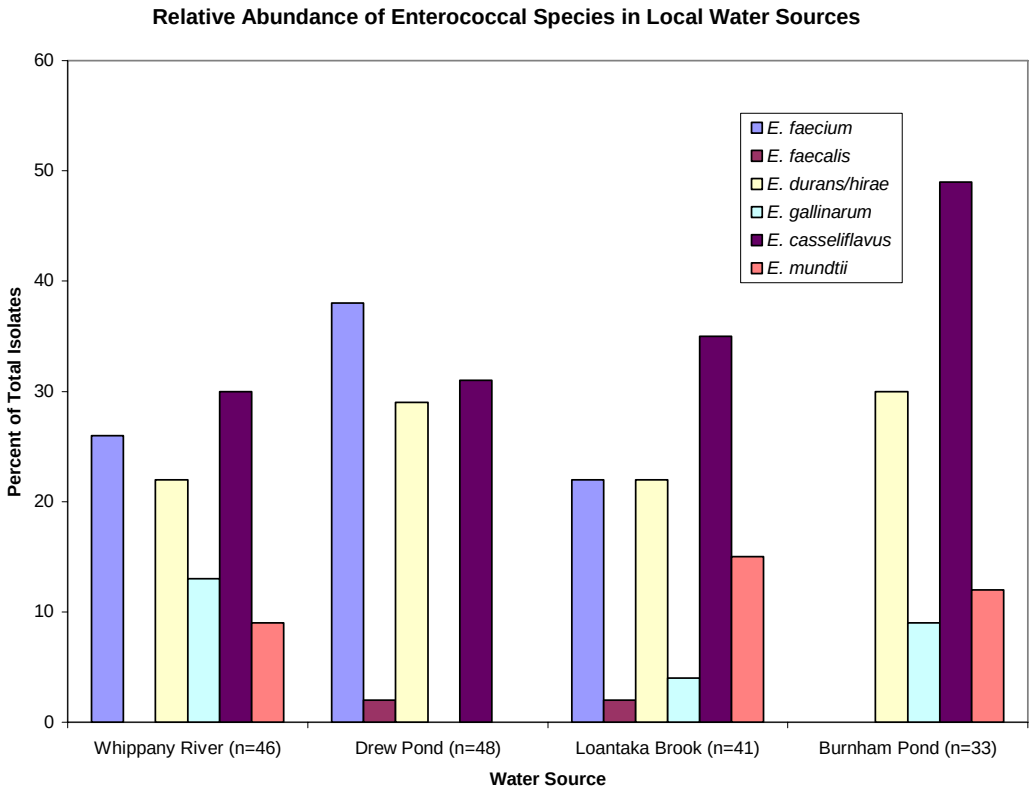

Fig. 2: Relative Abundance of Enterococcal Species in Local Water Sources

Because of the importance of antibiotic resistance in combating bacterial pathogens, we then tested the resistance of the bacterial isolates to various commonly used antibiotics in the field of medicine today. (Table 1) We were particularly interested in the antibiotic resistance of *E. faecium* and *E. faecalis*, the two *Enterococcus* strains that are pathogenic to humans (Figure 3).

We found that the *E. faecium* from the Whippany River was 100% resistant to cephalothin and streptomycin but 0% resistant to tetracycline and ciprofloxacin. Vancomycin resistance was a relatively low 8%. *E. faecium* from the Drew Pond was 100% resistant to penicillin, cephalothin, and streptomycin, and 0% resistant to tetracycline and ciprofloxacin, like those found in the Whippany River. Vancomycin resistance was a higher, but still relatively low 11%. *E. faecium* found in Loantaka Brook displayed 0% resistance to vancomycin and 100% resistance to penicillin. *E. faecalis* showed 100% resistance to penicillin, cephalothin, streptomycin, and gentamycin. They showed 0% resistance to the remaining six antibiotics including vancomycin. However, only two isolates of *E. faecalis* were identified, and thus, these results are not statistically relevant.

Table 1: Relative Antibiotic Resistance of *Enterococcus spp.* in Local Water Sources

Water Source	Enterococci Species	PEN	CEP	TET	STR	GEN	CHL	CIP	VAN	ERY	NIT
W	FM (n=12)	75%	100%	0%	100%	67%	0%	0%	8%	0%	0%
n=46	FS (n=0)	-	-	-	-	-	-	-	-	-	-
	DH (n=10)	60%	100%	0%	100%	60%	0%	0%	0%	0%	0%
	G (n=6)	50%	100%	0%	100%	83%	0%	0%	100%	0%	0%
	CAS (n=14)	64%	100%	7%	100%	29%	0%	0%	43%	0%	0%
	MUN (n=4)	75%	100%	0%	100%	75%	0%	0%	0%	0%	0%
	Total	65%	100%	2%	100%	57%	0%	0%	28%	0%	0%
BP	FM (n=0)	-	-	-	-	-	-	-	-	-	-
n=33	FS (n=0)	-	-	-	-	-	-	-	-	-	-
	DH (n=10)	50%	90%	0%	60%	50%	0%	0%	10%	0%	0%
	G (n=3)	100%	100%	33%	100%	100%	0%	0%	33%	0%	0%
	CAS (n=16)	100%	100%	6%	100%	31%	0%	6%	75%	0%	0%
	MUN (n=4)	100%	100%	0%	100%	75%	0%	0%	0%	0%	0%
	Total	85%	97%	6%	88%	48%	0%	3%	42%	0%	0%
DREW	FM (n=18)	100%	100%	0%	100%	100%	0%	0%	11%	0%	0%
n=48	FS (n=1)	100%	100%	0%	100%	100%	0%	0%	0%	0%	0%
	DH (n=14)	100%	100%	0%	100%	100%	0%	0%	7%	0%	0%
	G (n=0)	-	-	-	-	-	-	-	-	-	-
	CAS (n=15)	100%	100%	0%	100%	100%	0%	0%	33%	0%	0%
	MUN (n=0)	-	-	-	-	-	-	-	-	-	-
	Total	100%	100%	0%	100%	100%	0%	0%	17%	0%	0%
LB	FM (n=9)	100%	89%	22%	78%	78%	0%	11%	0%	0%	0%
n=41	FS (n=1)	100%	100%	0%	100%	100%	0%	0%	0%	0%	0%
	DH (n=9)	100%	100%	0%	100%	100%	0%	0%	0%	0%	0%
	G (n=2)	100%	100%	0%	100%	0%	0%	0%	50%	0%	0%
	CAS (n=14)	100%	100%	0%	100%	71%	0%	0%	79%	0%	0%
	MUN (n=6)	100%	100%	0%	100%	67%	0%	0%	0%	0%	0%
	Total	100%	98%	5%	95%	76%	0%	2%	29%	0%	0%

Key	FM = *E. faecium*	**Key**	W = Whippany River	**Key**	PEN = Penicillin
Enterococcus	FS = *E. faecalis*	Water	BP = Burnham Pond	Antibiotic	CEP = Cephalothin
spp.	DH = *E. durans/E. hirae*	Source	DREW = Drew Pond		TET = Tetracycline
	G = *E. gallinarum*		LB = Loontaka Brook		STR = Streptomycin
	CAS = *E. casseliflavus*				GEN = Gentamycin
	MUN = *E. mundtii*				CHL = Chloramphenicol
					CIP = Ciprofloxacin
					VAN = Vancomycin
					ERY = Erythromycin
					NIT = Nitrofurantoin

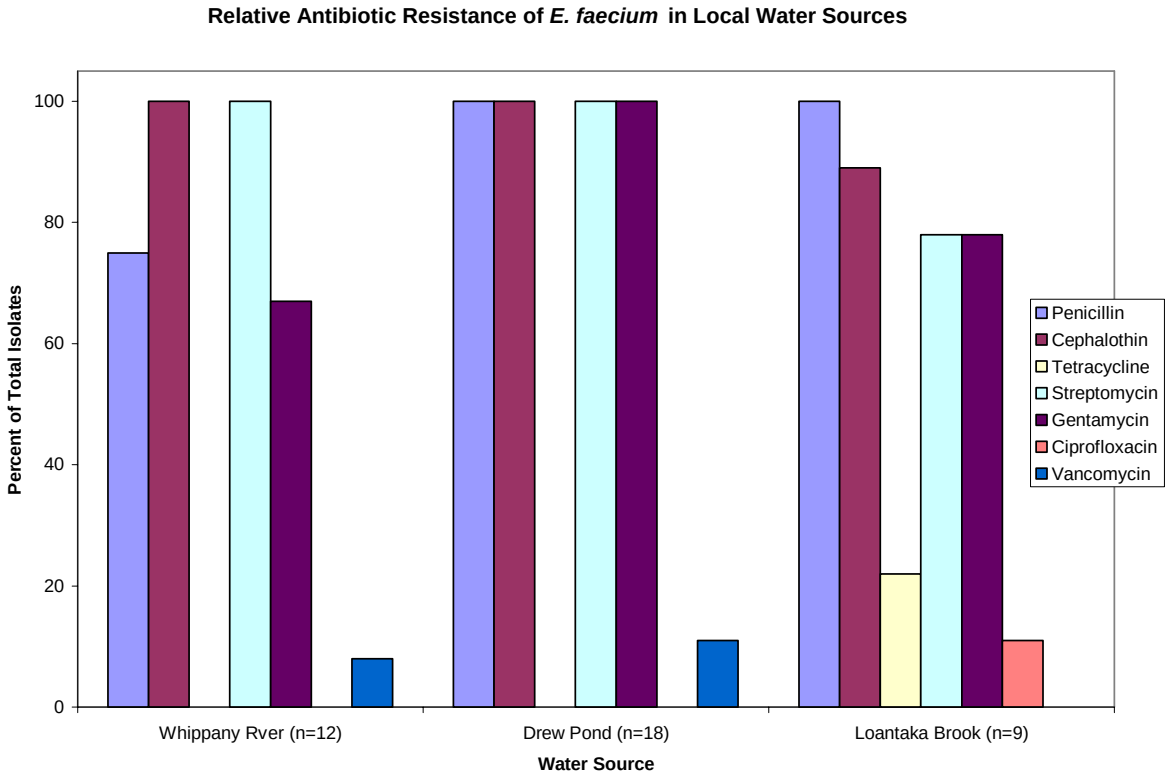

Fig. 3: Relative Antibiotic Resistance of *E. faecium* in Local Water Sources
Note: a) Burnham Pond contained no *E. faecium* isolates, and thus is not represented on this graph. b) All *Enterococcus spp.* displayed 0% resistance to chloramphenicol, erythromycin, nitrofurantomycin, and thus are also not represented on this graph.

Furthermore, we were interested in the total resistance of all the *Enterococcus spp.* to the various antibiotics (Figure 4). Enterococci from Whippany River were most (100%) resistant to cephalothin and streptomycin and least (0%) resistant to ciprofloxacin, a trend that was fairly consistent across water sources. Also noteworthy, 28% of enterococci from Whippany River were resistant to vancomycin. The largest percent of enterococci resistance to vancomycin occurred in Burnham Pond (48%), and the lowest percent resistance occurred in Drew Pond (17%). The greatest vancomycin resistance was demonstrated by *E. gallinarium* from Burnham Pond. In general, the enterococci displayed greatest resistance to penicillin, cephalothin, and streptomycin and least resistance to chloramphenicol, erythromycin, and nitrofurantomycin.

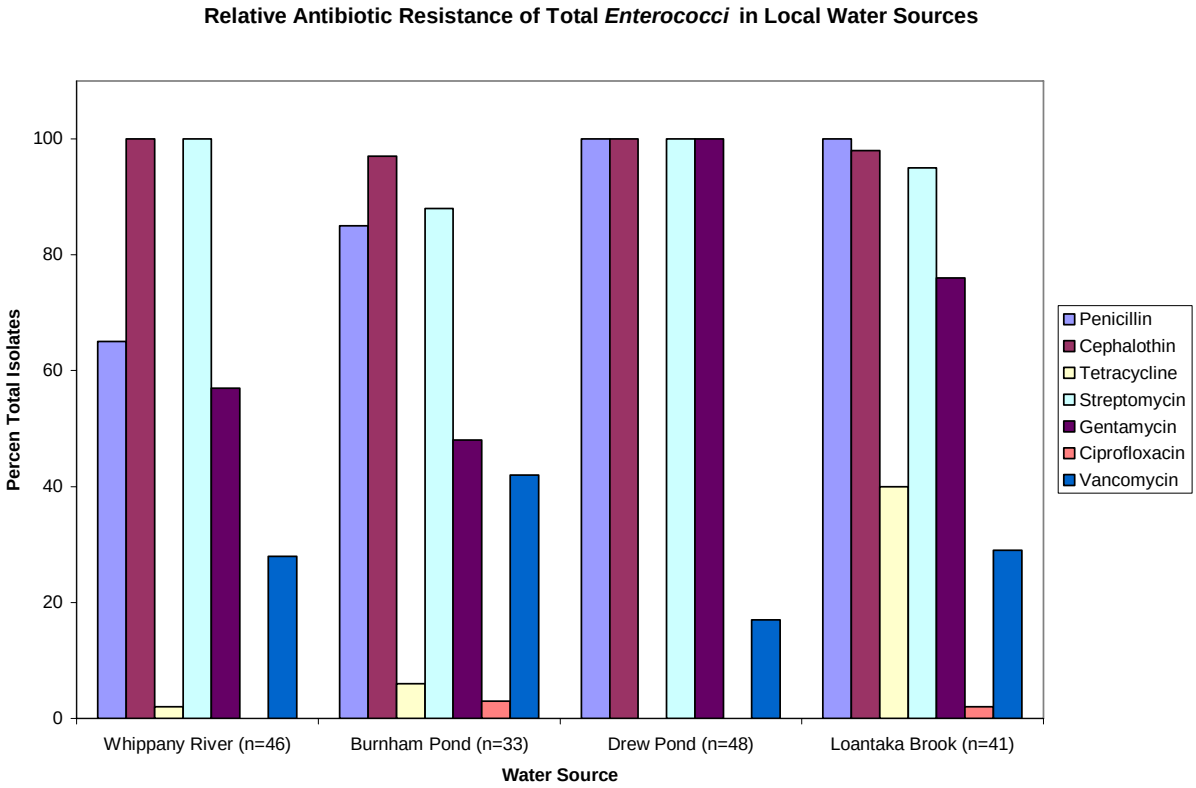

Fig. 4: Relative Antibiotic Resistance of Total Enterococci in Local Water Sources
Note: a) All *Enterococcus spp.* displayed 0% resistance to chloramphenicol, erythromycin, nitrofurantoin, and thus are also not represented on this graph.

The last step in the data collection was the determination of virulence among the *E. faecium* and *E. faecalis* isolates. We ran three tests for virulence, one to test the presence of gelatinase, for which the results were inconclusive and no data was gathered, one to test the presence of hemolysin, for which all the isolates displayed alpha hemolysin, which also resulted in inconclusive and generally irrelevant data. The third group of tests was the most productive of the three. This series of five tests assessed the presence of bacteriocins and the ability of the enterococci to lyse other types of bacteria (Table 2). The majority (33%) of the enterococci from the Whippany River sample lysed all five of the bacteria, indicating a greater virulence capacity among enterococci from this water source. On the other hand, in the Drew Pond and Loantaka Brook the majority of bacteria (36% and 44% respectively) lysed none of the bacteria, indicating a relatively lower capacity for virulence.

In both Drew Pond and Loantaka Brook, the *E. faecalis* isolates demonstrated an ability to lyse all five bacterial strains, indicated strong virulence capabilities.

Table 2: Virulence Factors of *E. faecium* and *E. faecalis* in Local Water Sources

		Number of Bacterial Strains Lysed					
Water Source	Enterococci Species	5	4	3	2	1	0
W	FM (n=12)	4	2	2	2	1	1
	FS (n=0)	0	0	0	0	0	0
BP	FM (n=0)	0	0	0	0	0	0
	FS (n=0)	0	0	0	0	0	0
DREW	FM (n=14)	2	2	3	2	0	5
	FS (n=1)	1	0	0	0	0	0
LB	FM (n=9)	1	4	0	0	0	4
	FS (n=1)	1	0	0	0	0	0

Additionally, we identified the virulence patterns of the enterococcal isolates in order to identify similarities and differences among the virulence capacities of various *E. faecium* and *E. faecalis* isolates (Table 3).

Table 3: Virulence Factors of *E. faecium and E. faecalis* in Local Water Sources

		Bacteriocin				
Water Source	Enterococci Species/Sample	EF	SM	O	P	R
W	*FM*/A1					
	FM/B5		+		+	+
	FM/B6		+		+	+
	FM/E1	+	+	+	+	+
	FM/E2		+	+	+	+
	FM/E3	+	+	+		+
	FM/E5		+		+	
	FM/F1	+	+	+	+	+
	FM/F3		+			+
	FM/F5	+				
	FM/G2	+	+	+	+	+
	FM/H5	+	+	+	+	+
BP	No *FM/FS* present.					
DREW	*FM*/A1					
	FM/A4				+	+
	FM/A5		+		+	+
	FM/A6					
	FM/B5					
	FM/B6		+	+		+
	FM/C1		+	+	+	+
	FM/D1					
	FM/D6	+	+	+	+	+
	FM/F1		+	+	+	+
	FM/F3	+	+	+	+	+
	FM/F5		+		+	+
	FM/G2					
	FS/D2	+	+	+	+	+

LB	*FM*/A4		+	+	+	+
	FM/C5	+	+	+	+	+
	FM/D5					
	FM/E2	+		+	+	+
	FM/E5		+	+	+	+
	FM/E6					
	FM/G1	+		+	+	+
	FM/G4					
	FM/H6					
	FS/D1	+	+	+	+	+
Key	FM = *E. faecium*					
Enterococcus	FS = *E. faecalis*					
spp.						
Key	W = Whippany River					
Water	BP = Burnham Pond					
Source	DREW = Drew Pond					
	LB = Loantaka Brook					
Key	EF = *Enterococcus faecalis* ATCC 29212					
Bacteriocin	SM = *Streptococcus mitis* PI 519					
	O = *Enterococcus mundtii* O					
	P = *Enterococcus mundtii* P					
	R = *Enterococcus mundtii* R					

DISCUSSION

General Discussion

This survey provides a comparison of *Enterococcus spp.* presence in local water supplies in Morris County, NJ, as well as the antimicrobial resistance and virulence of these gram-positive bacteria.

The study of *Enterococcus spp.* had many aims, including the identification of various *Enterococcus* species and their relative numbers in local water sources; additionally, it was conducted to assess the "safeness" of the potentially nosocomial pathogens in fresh water environments. Discrepancies in the results could possibly have been caused by various experimental errors. In order to make the survey feasible, a relatively small sample size was implemented; as a result, proper statistical analysis could not be obtained. To produce conclusive data, the team would have had to perform the experiments with several samples from various depths and locations in order to acquire accurate representations of the water sources; however, the team only sampled from the edges of the sources, and as a result, the spectrum of bacteria presence may have been skewed.

Bacterial Presence

We were able to draw several important conclusions after analyzing our data. The count of bacteria in the Whippany River was extremely high (3150 isolates of *Enterococcus* per 100 mL) when compared to the concentration of colonies in the other water supplies. Drew Pond and Loantaka Brook had similar concentrations of *Enterococcus* in them (880 isolates in every 100 mL sample in Drew Pond and 1100 isolates in every 100 mL sample from Loantaka Brook), but

these were much lower than that found in the Whippany River. Burnham Pond, on the other hand, had a dramatically lower amount of *Enterococcus* (240 isolates per 100 mL sample). However, even the cleanest of these water sources contains levels of *Enterococcus* that are far too high to be safe for drinking or bathing. The federally-allowed maximum level of *Enterococcus* that can be found in water to be used for drinking is 7 isolates per 100 mL sample and for water to be used for bathing the maximum is 100 per 100 mL sample. Consequently, none of the four water sources we tested are even close to being safe for either drinking or bathing.

Each of the water sources had a different breakdown of *Enterococcus spp.*. In the Whippany River, the relative abundances of *Enterococcus faecium*, *Enterococcus durans,* and *Enterococcus casseliflavus* were in the same range and were all moderately large. Drew Pond had a high relative abundance of *Enterococcus faecium*, with 38% of the *Enterococcus* found in the pond belonging to that species. Loantaka Brook, on the other hand, had a high concentration of *Enterococcus casseliflavus*, with 35% of the *Enterococcus* found belonging to that species. It was interesting to see, however, that the top three species found in both Drew Pond and Loantaka Brook were *Enterococcus faecium, Enterococcus durans,* and *Enterococcus casseliflavus.* Both ponds had at least moderate amounts of each of these species; each of the three species had at least 22% of the colonies found in both ponds. Additionally, there was very little *Enterococcus faecalis* found in both ponds, with no more than 4% of the isolates. However, there were slight differences between even these two parallel water supplies. Most noticeable were the relatively high presence of *Enterococcus mundtii* in Loantaka Brook and the complete absence of it from the Drew Pond. Such numbers indicate the parallels between these two water supplies, yet still remind us of the uniqueness of each pond because of the different environments. In Burnham Pond, interestingly, there were no *Enterococcus faecalis* or *Enterococcus faecium* found. However, there was a high abundance of *Enterococcus durans* and especially *Enterococcus casseliflavus*. It was interesting to note the low presence of *Enterococcus faecalis*. *Enterococcus faecalis and Enterococcus faecium* are common nosocomial pathogen, which means they are common causes of secondary infections in hospitals and are the only species of *Enterococcus* regarded as potential pathogens.

Antimicrobial Resistance

In the last two decades, antimicrobial resistance in gram-positive bacteria has developed into a major problem in clinical settings. Due to an increased number of immunosuppressed patients as well as ineffective infection control measures, rates of infections caused by nosocomial *E. faecalis* and *E. faecium* have increased tremendously [21].

Of the eleven antibiotics tested, three (penicillin, cephalothin, streptomycin) were virtually ineffective in curbing *Enterococcus spp.* growth. In Loantaka Brook samples, the isolated *Enterococcus spp.* displayed 100% resistance to penicillin, 98% resistance to cephalothin, and 95% resistance to streptomycin. Similarly, in Burnham Pond, the samples demonstrated 85% resistance to penicillin, 97% resistance to cephalothin, and 88% resistance to streptomycin. All three of these are first-generation antibiotics; penicillin was first utilized in 1929 [22] and streptomycin in 1943 [21]. On the other end of the spectrum, three other antibiotics (chloramphenicol, erythromycin, nitrofurantoin), demonstrated 100% effectiveness against the *Enterococcus spp.* tested. Chloramphenicol is extremely toxic, and as a result, highly

restricted and utilized in only very controlled environments; nitrofurantoin is a highly specific antibiotic used mostly in urinary tract infections. As a result, bacteria have not been exposed to these antibiotics enough to develop a significant resistance to them.

Generally, antimicrobial resistance trends were fairly consistent between the four different water sources (Table 1). Surprisingly, the *Enterococcus spp.* isolated from the Drew Pond sample displayed 100% resistance to four antibiotics – penicillin, cephalothin, streptomycin, and gentamycin, an occurrence not observed at any other water source, which may imply higher organismal clonality at this site.

For most antibiotics, the resistance patterns were similar for the *E. faecalis* and *E. faecium* isolates, with one exception. The only species of *Enterococcus* that seemed to be affected by vancomycin was *E. faecium*. At the Whippany River, 8% of *E. faecium* colonies were resistant to the antibiotic; a similar trend was seen in the Drew Pond samples, where 11% of *E. faecium* colonies were resistant to vancomycin. The Surveillance Network Database – USA reported that in patients with nosocomial bloodstream infections in 1995 and 1997, 94.5% of *E. faecalis* were susceptible to vancomycin and ampicillin as opposed to only 25.4% of *E. faecium* [11]. Our results supported these findings, demonstrating a 100% *E. faecalis* antibiotic susceptibility rate.

Virulence Factors

Enterococcus faecalis and *E. faecium* are considered potentially pathogenic because of their lack of susceptibility to antibacterial substances and because they express specific virulence factors. The virulence factors that we evaluated determined the ability of *Enterococcus* to invade the body (gelatinase), the ability to acquire iron in the body (hemolysin), and the ability to kill other similar bacteria (cytolysins).

In the study seven different tests for virulence were used: gelatinase, hemolysins, and five different cytolysins. The bacterial isolates were identical in regards to the gelatinase and hemolysin tests and thus these tests were not a help in identification or differentiation. This means that there were no isolates positive for β-hemolysis, which shows complete destruction of blood cells, and no gelatinase production, showing the bacteria's inability to invade different parts of the human body because of the collagen boundaries (collagen has a similar structure to gelatin).

The indicator strains that we used to evaluate bacteriocin production were *Enterococcus faecalis* ATCC 29212, *Streptococcus mitis* PI 519, as well as *Enterococcus mundtii* O, P, and R. The most effective *Enterococcus* isolates for bacteriocin production were the two *E. faecalis* strains. Both isolates lysed 100% of all bacteria that we tested. This is a signal that the overall virulence of *E. faecalis* is very high. The percentages of *E. faecium* that produced bacteriocins were 20% for five strains lysed, 23% for four lysed strains, 14% produced bacteriocins against three strains, 11% produced bacteriocins against two strains, 3% produced bacteriocins against one strain, and 29% could not lyse any bacteria at all. Since 100% of the *E. faecalis* could produce all five bacteriocins and *E. faecium* could not (some could not lyse any at all), *E. faecalis* is likely the more potentially virulent strain.

Conclusion

The federal clean water standards allow for a maximum of 7 isolates of *Enterococcus spp.* per 100 mL sample for drinking water and a maximum of 100 isolates of *Enterococcus spp.* per 100 mL sample for bathing water. Our lowest enterococcal counts were well above this 100 isolate maximum; therefore, none of the four local surface water sources are safe for drinking and/or bathing. We also concluded that penicillin, cephalothin, and streptomycin are virtually ineffective in combating *Enterococcus spp*. growth. In addition, we found that *E. faecalis* and *E. faecium,* recognized nosocomial pathogens, were present in our water samples. These species *displayed* similar resistance patterns, with the exception of vancomycin resistance. In terms of virulence, *E. faecalis* demonstrated the highest level of bacteriolysin activity. The Whippany River sample harbored the highest percentages of virulent bacteria.

ACKNOWLEDGEMENTS

This project would not have achieved its present level of success without the hard work and dedication of several individuals. Thanks to Dr. June Middleton for her guidance and perseverance. Thanks to Alex Kohl for constantly providing us with sustenance. The team would also like to thank Jeremy Tang for showing his zest and stealing our hearts.

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