Can aquatic plant *Lemna Minor* facilitate the emergence of *Enterococci faecalis* superbugs?

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ABSTRACT

Bacterial antibiotic resistance genes are ubiquitous in aquatic environments, especially those subject to wastewater discharge. Antibiotic resistance is often transferred through bacterial conjugation; however, the role of aquatic macrophytes in facilitating genetic exchange has yet to be fully elucidated. Therefore, the effect of the common macrophyte *Lemna minor* on the conjugal transfer of vancomycin resistance genes in *Enterococci faecalis* was evaluated as a model for potential aquatic pathogen emergence. The resident microbial populations and thallus versus root *Enterococci* association were tested by ultrasonicating *L.minor* for increasing time intervals and plating isolate on Slanetz and Bartley agar. A vancomycin susceptible and resistant strain of *E.faecalis* were incubated with *L.minor,* ultrasonicated, and then plated on double selection plates to enumerate any transconjugants. The study found no resident *Enterococci* on the *L.minor*, and 90 seconds of ultrasonication yielded the best cell *E.faecalis* recovery post-incubation ($p<0.001$). *L.minor* thalli had higher *E.faecalis* association with an average of 97 more cells per mm^2 than on *L.minor* roots ($p<0.001$). The higher bacterial association on thalli is predicted to be attributed to differences in *E.faecalis* subpopulation surface charges. Both thalli and roots reduced the number of *E.faecalis* cells in suspension by 2-fold, therefore *L.minor* could mitigate bacterial contamination in wastewater. After co-incubation with *E.faecalis* strains, *L.minor* did not facilitate the conjugal transfer of resistance genes. Only transconjugants from controls grew on double selection plates suggesting *L.minor* does not pose a high risk for increasing the antibiotic resistance in aquatic environments. Conjugation may still occur in the presence of *L.minor*, but such transfer events are likely to be common and pose little threat in aquatic environments.

1. INTRODUCTION

Antibiotic resistance has been identified as an emerging threat to mankind in environmental (Berendonk *et al.* 2015), clinical (World Health Organization, 2014), and political (Carlet *et al.* 2014) contexts. The observed increase in antibiotic resistance is attributed to the acquisition of antibiotic resistance genes (ARG) which in turn has led to the emergence of multi-drug resistant pathogens (Berendonk *et al.* 2015; Lupo *et al.* 2012). The compounding effects of the increased use of antibiotics and their release into environmental reservoirs are suggested to have contributed to the increase in ARG detected in environmental bacteria (Cantas *et al.* 2013).

Antibiotic resistance acquired by opportunistic pathogens is a primary source of hospital infections, and with the current inadequate supply of new antibiotics, characterizing the spread of ARG in the environment is imperative to tackling antibiotic resistance (Berendonk *et al.* 2015). Bacterial contaminants can spread in the environment, along with their constituent ARG, and aquatic environments appear to have the capability of hosting antimicrobial resistant communities (Taylor *et al.* 2011). In such communities, non-pathogenic bacteria can act as gene reservoirs and progressively accumulate resistance genes (Baquero *et al.*,2008). Bodies of water associated with anthropogenic discharge, such as wastewater (Berendonk *et al.* 2015), may provide the proximity microbes require for horizontal gene transfer of ARG. Aquatic environments can provide an alternate set of selective pressures allowing antibacterial resistant microbes to thrive since antibacterial agents can be potentially active even after wastewater treatment (Baquero *et al.* 2008). While it is predictable that the release of antibiotics into aquatic systems through treatment plant effluents and other means alters selective pressures and prompts antibacterial resistance (Baquero *et al.* 2008; Bouki *et al.* 2013), the biotic and abiotic factors that contribute to the increase of ARG in environmental bacteria have yet to be fully elucidated.

Enterococcus faecalis is a gram-positive, facultatively anaerobic bacterium that has emerged as a multi-resistant hospital pathogen (Sava *et al.* 2010). *E.faecalis* can efficiently transfer vancomycin resistance through plasmid conjugation (Sterling *et al.* 2020), and *E.faecalis* is ubiquitous in aquatic environments (Byappanahalli *et al.* 2012). The transfer of plasmids and transposons, which can code for antibacterial resistance, is often accomplished through conjugation in aquatic environments (Yin and Stotzky, 1997). Resistance genes have been detected in a variety of freshwater systems (Lupo *et al.* 2012), including in both drinking water and wastewater biofilms (Schwartz *et al.* 2003). Since *E.faecalis* is spread through animal and human faeces, its presence in aquatic environments can be used as an environmental indicator of faecal contamination (Wade *et al.* 2006). In lake and seawater, *E.faecalis* is frequently found in a viable but non-culturable state (VBNC), suggesting conventional protocols for microbial quantification in aquatic systems may need revaluating for adequate detection (Ramamurthy *et al.* 2014). The gap in knowledge regarding horizontal gene transfer by *E.faecalis* in aquatic environments, particularly environments subject to wastewater discharge, leaves an unbalanced understanding of how current treatment plant effluents could influence disease reservoirs.

The ability for genetic exchange to concur in an aquatic context had been explored in recent years, and it has been shown that the transfer of vancomycin resistance genes between *E.faecalis* strains can be facilitated through filter-feeding zooplankton of the *Daphnia* genus (Olanrewaju *et al.* 2019) and through *Ephydatia fluviatilis* freshwater sponge colonies (Cartwright *et al.* 2020). The effect filter feeders have on ARG transfer shows that aquatic animals can potentially facilitate horizontal gene transfer in environments. Also, some algae can be associated with high numbers of enterococci (Byappanahalli *et al.* 2003; Whitman *et al.* 2003) and some species increased conjugal gene transfer rates between *E.faecalis* (Olanrewaju *et al.* 2019). Biofilms have been identified as ARG hotspots in aquatic environments (Abe *et al.* 2020), and cells in biofilms have higher ARG transfer efficiency than planktonic cells (Angles *et al.*, 1993). These findings suggest that other forms of aquatic life, such as biofilm supporting aquatic plants, could potentially facilitate horizontal gene transfer between *E.faecalis* strains.

Aquatic plants have been proposed as an attractive solution for bioremediation of wastewater and other forms of waste treatment (Muradov *et al.* 2014). *Lemna minor*, known as common duckweed, is an aquatic, free-floating macrophyte that is often used as a plant model in biochemical testing due to its sensitivity and cost-effectiveness (ISO 20079, 2005). *L.minor* is found in all European countries except Iceland and is tolerant of seasonal temperature variation (Tutin 2010). *L.minor* can grow in wastewater (Zirschky and Reed, 1988) and can be used to recover nutrients and pollutants, such as phenol, during wastewater treatment (Muradov *et al.* 2014; Younis *et al.* 2016). Duckweed wetlands have also been shown to reduce pollutants generated from dairy waste and take up nitrogen and phosphorus from wastewater systems (Adhikari *et al.* 2015; Cedergreen and Madsen 2002; Walsh *et al.* 2021). *L.minor* can produce starches from wastewater nutrients and likewise produce substantial biomass (Chen *et al.* 2018). Micronutrients important in livestock feed can also be accumulated from agricultural effluent in *L.minor* continuous circulation systems (Devlamynck *et al.* 2021). Thus, *L.minor*'s biotechnological qualities subject it to being a promising source of biofuel and animal feed (Bog *et al.* 2019; Cheng and Stomp 2009). Therefore, researching *L.minor* in wastewater treatment systems has commercial, scientific, and political value.

While *L.minor* has proven to be productive in wastewater treatment systems, it has also been used to study human microbial pathogenesis. Biofilms were demonstrated to grow on this macrophyte in vitro (Zhang *et al.* 2016; Zhang *et al.* 2010); thus, *L.minor* could be a potential host for Enterococci growth in wastewater systems. Given that *L.minor* is often present in

wastewaters populated with *E.faecalis* and microbes have been shown to associate with *L.minor,* there is potential for these aquatic macrophytes to facilitate horizontal gene transfer between *E.faecalis* strains. Enterococci have been identified on several aquatic macrophytes and there is strong scientific evidence supporting plant-associated enterococci (Byappanahalli *et al.* 2012). Yet, there have been few studies exploring conjugal gene transfer rates in environments that are host to both antibacterial resistant enterococci and aquatic macrophytes. Exploring the relationship between *L.minor* and *E.faecalis* could provide insight into potential mechanisms giving rise to aquatic bacterial pathogens and the suitability of *L.minor* in wastewater systems.

This study aims to determine if *Lemna minor* can facilitate conjugative gene transfer of antibacterial resistance genes between vancomycin-resistant and vancomycin susceptible *Enterococci faecalis* strains. It is hypothesized that coculturing vancomycin-resistant and vancomycin susceptible *E.faecalis* with *L.minor* will increase conjugation between the bacteria strains as seen by the growth of *E.faecalis* colonies on vancomycin and rifamycin double selection plates. The objectives of the research project are to determine if *L.minor* is host to resident enterococci, and if so, if it can be sterilized; to determine if *Enterococci* cells can be recovered from *L.minor* thalli and roots, to assess and compare the impact of different vortexing and sonication methods on *E.faecalis* recovery from *L.minor*; to determine the doubling time of *E.faecalis* to maximize potential conjugation; to analyze the number and surface area relationship of transconjugants isolated after culturing vancomycin-resistant and vancomycin-susceptible *E.faecalis* strains with *L.minor*.

2. METHODS AND MATERIALS

2.1 Test Organisms

Lemna minor were collected from the Ulster University Pond (Figure 1). Taxonomic descriptions in Bog *et al.* (2020) assisted in *L.minor* identification. *Enterococcus faecalis* strains used were MF06036^{Van} (donor) and MW01105^{Rif} (recipient) as described in Olanrewaju *et al.* (2019). Before a conjugation experiment was executed, preliminary experiments characterized the association of *E.faecalis* to *L.minor* as described herein. The *L.minor* plants selected for testing were composed of one thallus per root unless otherwise stated.

2.2 Lemna minor resident enterococci and sterilization

To ensure that *L.minor* were not already resident to enterococci*, L.minor* were sterilized in a 0.5% Troclosene sodium solution made from Haz-Tab tablets (Guest Medical) for 4 minutes, rinsed with phosphate-buffered saline solution (PBS) twice, and transferred into Hoagland's No.2 basal salt medium (Sigma Aldrich). Surviving plants were used to re-inoculate sterile cultures of *L.minor.* Additionally, *L.minor* was placed directly on Slanetz and Bartley (SB) agar plates and incubated first at 35°C for four hours and then 44°C for 44 hours to determine if resident enterococci were present (Ewald and Eie, 1992; Slanetz and Bartley, 1957). Dark red colonies on SB plates were counted as presumptive enterococci.

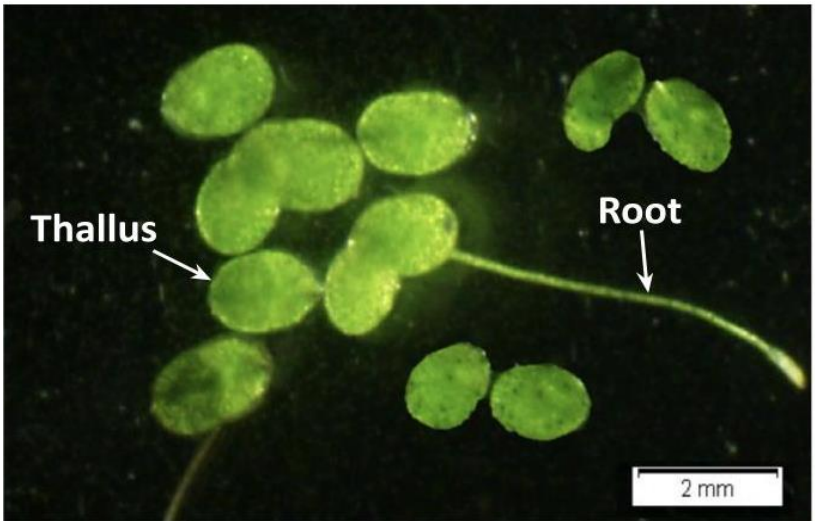

Figure 1. The thalli and roots of *Lemna minor* collected from the Ulster University Pond.

2.3 Lemna minor surface area

The average surface area of *L.minor* thalli and roots was calculated by imaging sixty-five plants using an OLYMPUS SZX16 microscope (OLYMPUS Co, Japan) and taking plant area measurements in OLYMPUS cellSense imaging software (Figure 3). The average *L.minor* root diameter (0.176 mm) was informed by the values reported by 4/29/2022 4:33:00 PM and used to calculate the roots' cylindrical surface area. Thallus surface area is described only as the side of the plant in contact with the medium.

2.4 Recovery of E.faecalis from Lemna minor

To determine if *E. faecalis* could be recovered from biofilms grown on *L.minor*, sterilized *L.minor* with a total of 9 thalli per OECD (2006) guidelines, was placed into twelve 60mm Petri dishes. Then, 9 mL of PBS was added to each dish per the procedure modified from Mateos-Cárdenas *et al.* (2019). The twelve dishes, each with nine thalli, were inoculated with 1 mL of *E. faecalis* overnight that had been diluted 1:10 in tryptic soy broth (TSB) so that the bacterial concentration

was comparable to Olanrewaju *et al.* (2019). The cultures were incubated for 24 hours at 30°C. Post incubation, *L.minor* were washed twice in 1 mL of PBS and resuspended in 1 mL of sterile PBS. As a control, three *L.minor* samples were vortexed for 30 seconds. Next, three samples of 9 thalli and 9 roots were ultrasonicated for 15 seconds, vortexed for 30 seconds, and then ultrasonicated again for 15 seconds in modification of the more intense procedure by He *et al.* (2012). Following the same procedure, three samples were ultrasonicated for 30 seconds before and after vortexing, and the last three *L.minor* samples were ultrasonicated for 45 seconds before and after vortexing (Figure 2). From each sample, 100 µL of solution was plated onto SB agar plates and incubated at 44°C for 48 hours to confirm plated cells were enterococci**.** The colony forming units (CFU) on the SB agar plates were counted and the statistics were summarized in Table 1. The 45 second sonication method had the most promising cell recovery and was used for the following conjugation experiments (Figure 4).

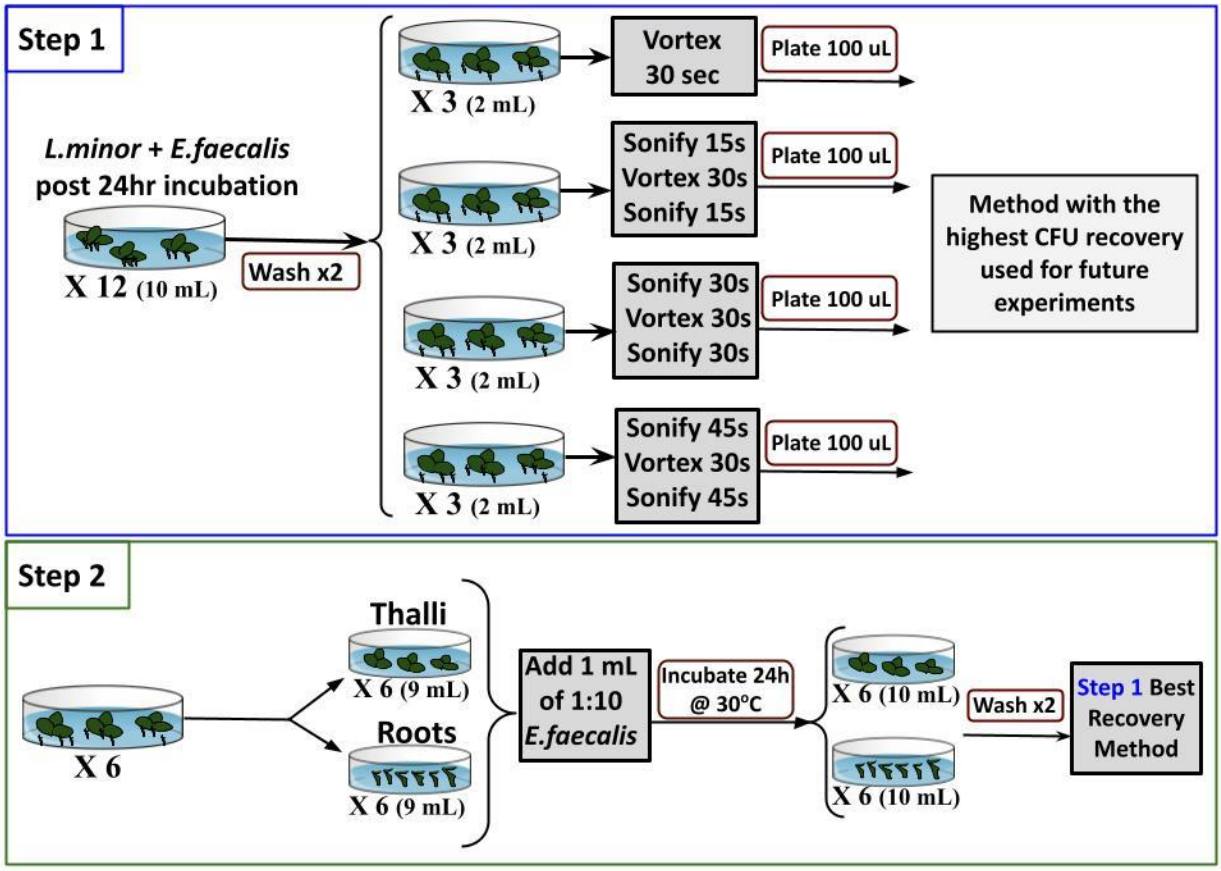

Figure 2. Schematic of the recovery of *E.faecalis* from *L.minor* through increasing ultrasonication times. Step one diagrams the four treatments for cell recovery where 30 seconds of vortexing serves as the control and ultrasonication times increases in a total of 30 seconds between each condition. The most effective method from Step 1 was then used to compare the *E.faecalis* CFU between *L.minor* thalli and roots.

After the recovery methods were evaluated, six replicates of 9 *L.minor* were placed into 9 mL of PBS. *L.minor* roots were separated from the thalli with sterile scissors and placed into six separate dishes. A 1 mL aliquot of 1:10 diluted MF06036^{Van} *E.faecalis* was added into each dish and samples were incubated at 30ºC for 24 hours. Afterward, *L.minor* roots and thalli were washed twice in sterile PBS, then 1 mL aliquots were ultrasonicated for 45 seconds, vortexed for 30 seconds, and ultrasonicated for an additional 45 seconds. From each suspension, 200 µL were incubated on SB agar at 44ºC for 48 hours. *E.faecalis* CFU recovered from thalli and roots were normalized according to plated volume and plant surface area (Figure 5).

2.5 Determination of E.faecalis doubling time

After identifying the most effective recovery method for *E.faecalis* and determining if *E.faecalis* attached to *L.minor* thalli and roots, the doubling time of the MF06036^{Van} strain was calculated to confirm the time needed to enter cells into their mid-exponential phase. An *E.faecalis* culture was diluted 1:10 in TSB and placed in a 37ºC water bath. The culture's optical density (OD) at 600 nm was measured using a Hach DR 2500 photometer every 15 minutes for four hours to establish the doubling time (dT) by using OD measurements from Figure 6 in Equation 1 which was rearranged from Wood *et al*. (2019).

$$\mathrm{dT} = t/(\frac{1}{ln2} * \ln\frac{OD_2}{OD_1}) \quad (1)$$

In Equation 1, *t* is the difference in time between OD_1 and OD_2 which represent the optical densities measured during the beginning (OD_1) and end (OD_2) of the log growth phase. The dT calculated was compared to the typical *E.faecalis* doubling time of 47- 56 minutes to check for irregular culture growth (Dean *et al.* 2020).

2.6 Conjugal transfer of antibiotic resistance

Using the methods modified from Conwell *et al.* (2017) and executed by Olanrewaju *et al.* (2019), *E.faecalis* cultures of donor and recipient strains were diluted 1:10 and incubated for 155 minutes to enter cells into their exponential growth phase for optimal conjugation (Kohler *et al.* 2019). Cells were mixed to a 1:14 donor to recipient ratio, and twenty-five 60mm dishes were filled with 9 mL of PBS. After *L.minor* were separated into their thalli and roots with sterile scissors, 9 thalli, 9 roots, 18 thalli, and 18 roots were respectively added into the dishes in replicates of five. No *L.minor* were added to the last 5 dishes and 1 mL of the bacterial

suspension was aliquoted into all 25 dishes. Dishes were incubated for 24 hours at 30°C. After incubation, the *L.minor* were washed two times in 2 mL of sterile deionized water and resuspended in 1 mL of PBS. *L.minor* thalli and roots were ultrasonicated for 45 seconds, vortexed for 30 seconds, and then ultrasonicated for an additional 45 seconds. A 500 µL aliquot of the sonicated bacterial suspension and a 500 µL aliquot of the original incubated medium were plated on TSA double selection plates prepared with 10 µg/mL vancomycin and 100 µg/mL rifampicin (Conwell *et al.* 2017). Equivalent aliquots were diluted 6-fold and spread on TSA plates. Plates were incubated at 37°C for 48 hours and any colonies counted on double selection plates were presumed transconjugants (TC). Colonies recovered from both the *L.minor* and the incubated cell suspension were summed in Table 2. Colonies counted on TSA plates were used to compare post incubation CFU concentrations (Figure 7). TC numbers were normalized to surface area and volume for each condition (Table 2).

The concentration of parent *E.faecalis* strains was determined by counting colonies after a 6-fold serial dilution. The transfer efficiency was calculated for the *E.faecalis* incubated with and without *L.minor* by enumerating the number of TC per donor and per recipient strain in Table 2 (Olanrewaju *et al.* 2019). The parent strain suspensions were plated on double selection plates and single selection plates to confirm they did not possess multidrug resistance.

2.8 Statistical analysis

The normality of each data set was evaluated using a Shapiro-Wilk test. An ANOVA test evaluated the differences in mean cell counts between the four enterococci recovery methods**.** The difference in cell counts between thalli and roots was adjusted to surface area and evaluated using the student's t-test**.** Variation in mean CFU/mm^2 between thalli and roots was calculated and used in Equation 2 determine how many replicates would be needed to see a significant difference and to inform the number of replicates for attempted conjugation experiments. Equation 2 was described by Van Emden (2008, pp.310-313).

$$\textit{Number of replicates} = (t^2 \times 2 \times V)/ D^2 \qquad (2)$$

In Equation 2, t is the test statistic for the experiment at the 0.05 alpha level, V is the overall variance, and D is the difference between sample means. The CFU/mL between treatments was analyzed with an ANOVA, and the differences in TC/mL was evaluated using a Kruskal–

Wallis test. Post Hoc tests were used when appropriate. The effect size for the difference between mean TC was enumerated using *Cohen's d* coefficient as described in Lakens (2013).

$$\textit{Cohen's d} = \frac{(\overline{X}\,1 - \overline{X}\,2)}{\text{Pooled SD}} \quad (3)$$

In Equation 3, the numerator is the difference between the control and treatment sample means and the denominator is the pooled standard deviation. The *Cohen's d* coefficient is used to interpret the magnitude of the difference between sample means. *IBM SPSS Statistics v. 27* was used for statistical analysis and *Microsoft Excel v. 16.58* was used to graph results.

3. RESULTS

3.1 L.minor Sterilization and Morphology

The *L.minor* used in the study were host to a variety of bacteria and fungi; yet they were not resident to enterococci as no colonies grew on SB agar. No colonies grew on TSA when the *L.minor* were sterilized in 0.5% Troclosene sodium solution, and sterilized plants maintained their pigmentation after being cultivated in Hoagland's media for one week. *E.faecalis* was recoverable and viable from sterilized *L.minor*, although improved colony growth was recorded for *E.faecalis* kept at 35^{o}C during the first four hours of their incubation period.

The average surface area (n = 65) of a *L.minor* thallus was 2.24 mm^2 and the average surface area of a root was 3.50 mm^2 (Figure 3). Thallus and root surface areas were normally distributed (M = 5284.7 mm^2, SD = 174.4, W(64) = 0.976, p = 0.224 for roots; M = 5325 mm^2, SD = 336.2, W(64) = 0.976, p = 0.236 for thalli). Therefore, average surface areas were used for cell abundance calculations (Figure 5). Root and thallus average surface areas had a mean difference of 40.3 mm^2.

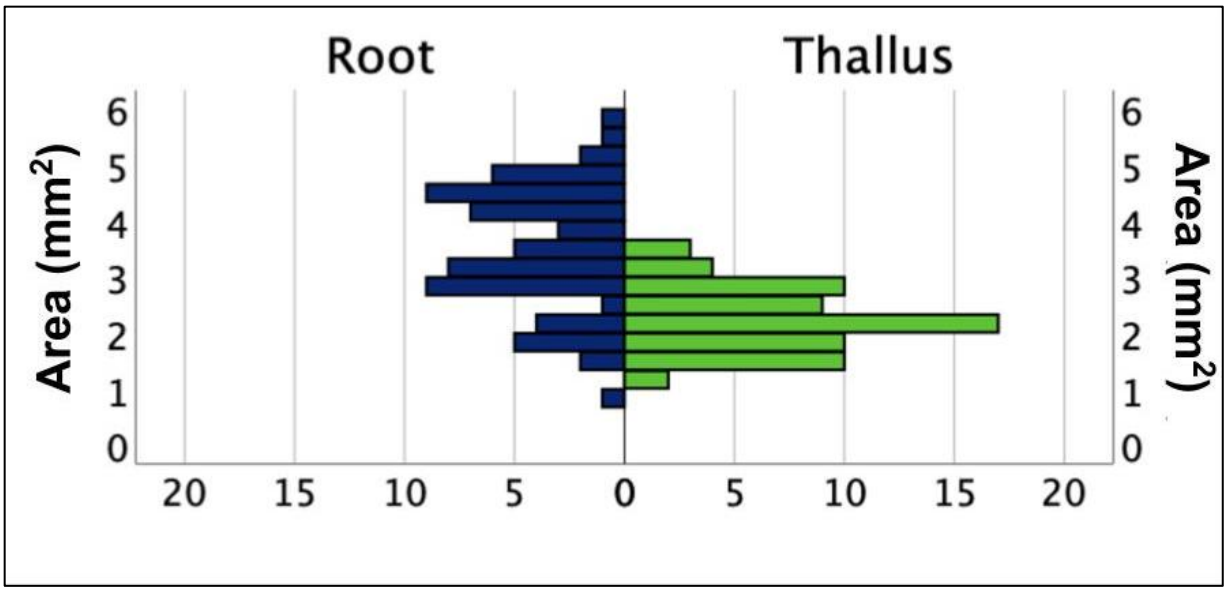

Figure 3. The distribution of surface area for 65 separated *L.minor* roots and thalli in mm^2 calculated in OLYMPUS cellSense imaging software. Thallus surface area represents only the surface area in contact with water.

3.2 Recovery of Enterococci from L.minor

When *L.minor* incubated with *E.faecalis* were ultrasonicated and vortexed, CFU/mL yield increased with ultrasonication time and 30 seconds of vortexing had the lowest mean cell recovery of 2,370 CFU/mL. A 90 second ultrasonication time had the highest mean cell recovery of 8,990 CFU/mL (Table 1 & Figure 4). Overall, ultrasonication time had a significant effect on cell recovery, $F(3,16) = 68.3$, $p<0.001$. Post hoc comparisons indicated CFU/mL from 60s (M = 5080, SD = 9 33.8) and 90s (M = 8994, SD = 1056) ultrasonication were significantly higher than CFU/mL recovered from vortexing (M = 2374, SD = 266.3) both with $p < 0.001$. Additionally, the CFU/mL from 90s sonication was significantly higher than CFU/mL recovered from 30s sonication (M = 3552, SD = 613.8), $p = 0.008$. A significant difference was only observed if the difference in sonication times were greater than 30 seconds.

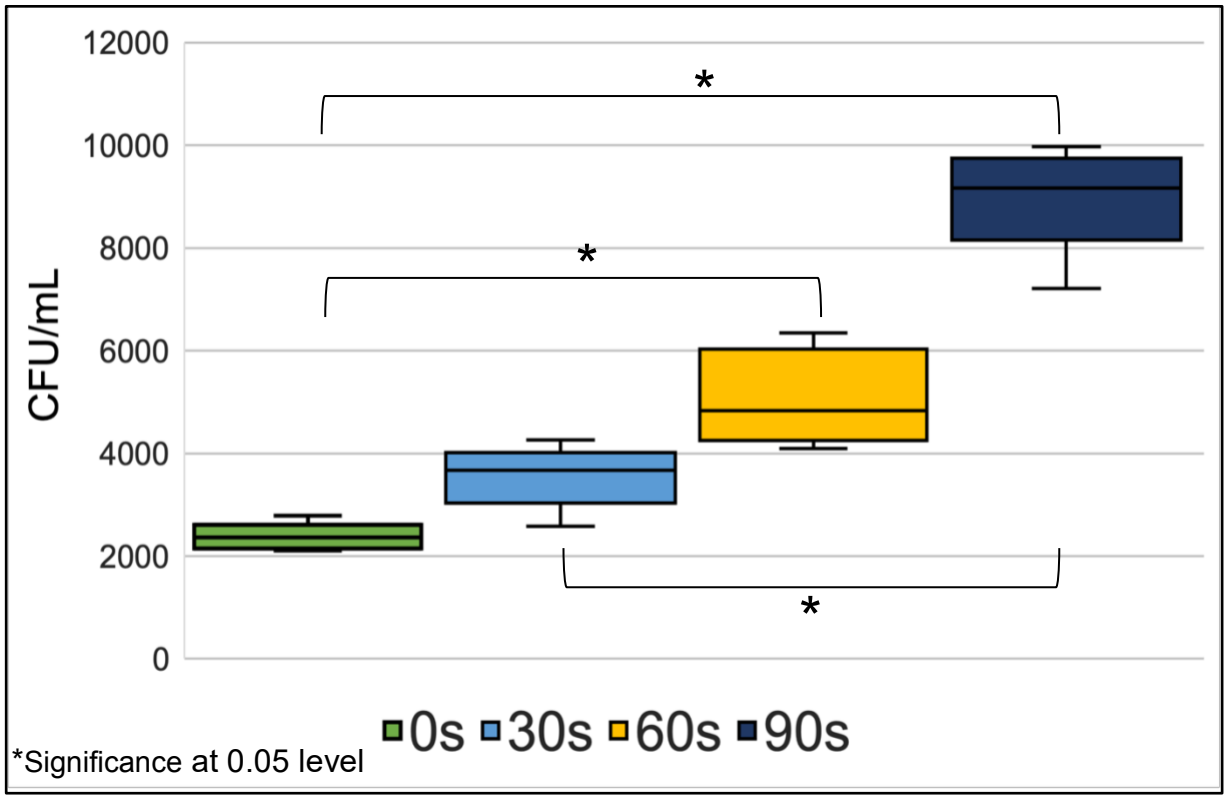

Figure 4. *E.faecalis* in CFU/mL recovered from *L.minor* after ultrasonication and vortexing three replicates of 9 *L.minor* for increasing intervals. Sonication times in the figure are the total time *L.minor* were exposed to ultrasonication and are the composite of two rounds of ultrasonication with an intermediate 30s vortexing step. The 0s control was vortexed for 30 seconds. Significance was evaluated using a one-way ANOVA at the 0.05 significance level.

Table 1. The mean (n = 3), SEM, and 95% confidence intervals for E.*faecalis* CFU recovered from *L.minor* after 24 hours of incubation by means ultrasonication for a total of 30 seconds, 60 seconds, and 90 seconds. Each sonication interval was divided in half and interrupted with 30 seconds of vortexing. Vortexing samples for 30 seconds acted as the experimental control.

Recovery Method	Mean ($\bar{x}$)	SEM	95% CI for the Mean (μ)
Vortex (30 sec)	2.37×10^3	119	(2.14×10^3, 2.61×10^3)
Ultrasonicated (30 sec)	3.55×10^3	274	(3.01×10^3, 4.09×10^3)
Ultrasonicated (60 sec)	5.08×10^3	417	(4.26×10^3, 5.90×10^3)
Ultrasonicated (90 sec)	8.99×10^3	472	(8.07×10^3, 9.92×10^3)

SEM= Standard Error of the Mean; CI= Confidence Interval

3.3 Comparison of Enterococci Attachment Between Thalli and Roots

The average concentration of *E.faecalis* recovered from *L.minor* thalli was 5,325 CFU/mL and from roots was 5,285 CFU/mL. The CFU counts were adjusted to *L.minor* surface area to evaluate if thalli or roots had a higher *E.faecalis* abundance (Figure 5). The CFU/mm^2 recovered from roots and thalli were normally distributed; $W(6) = 0.942$, $p = 0.679$ for roots; and $W(6) = 0.909$, $p = 0.429$ for thalli. However, the CFU/mm^2 was significantly higher for thalli (M = 264, SD =16.4) when compared to roots (M = 167, SD = 5.5); $t(10) = -13.4$, $p < 0.001$. While there was less variance in thallus surface area, the standard deviation of CFU/mm^2 for thalli was three times the standard deviation for roots. Thalli had an average of 97 CFU/mm^2 more than roots, since CFU counts were adjusted to the roots being an average of 40.3 mm^2 larger.

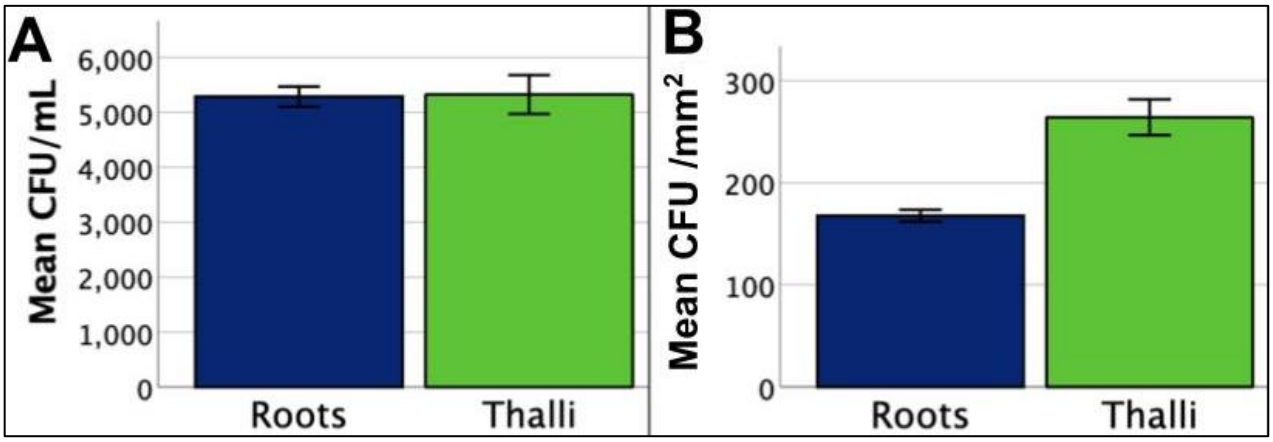

Figure 5. (A) The average *E.faecalis* CFU/mL recovered from six replicates of 9 *L.minor* roots and thalli after 90 seconds of ultrasonication, (B) and recovered *E.faecalis* CFU adjusted to average surface area of 9 *L.minor* roots, 31.50 mm^2; and 9 *L.minor* thalli, 20.16 mm^2. Only the thallus side in contact with media was measured for surface area. Error bars show +/- 2 standard deviations.

Using equation 2, the number of replicates needed to see a significant difference between root and thalli CFU/mm^2 was calculated to be 4.6 with the calculated V (119.9), t (-13.4), and D (97) values. Therefore, five replicates were used in the conjugation experiments.

3.4 Enterococci Doubling Time

The OD readings from the MF06036^{Van} *E.faecalis* culture were steady during the first 80 minutes in the water bath, after which OD began to increase (Figure 6). The culture's OD steadily increased between 80 minutes and 240 minutes until its OD stabilized at 0.332. Using Equation

1, the dT was calculated to be 36.7 min where $OD_1 = 0.048$, $OD_2 = 0.263$, and $t = 90$. The maximum absorbance (0.324 nm) was reached at 250 minutes when the culture entered the stationary phase. A 155-minute incubation entered the $MF06036^{Van}$ strain into the mid-exponential phase.

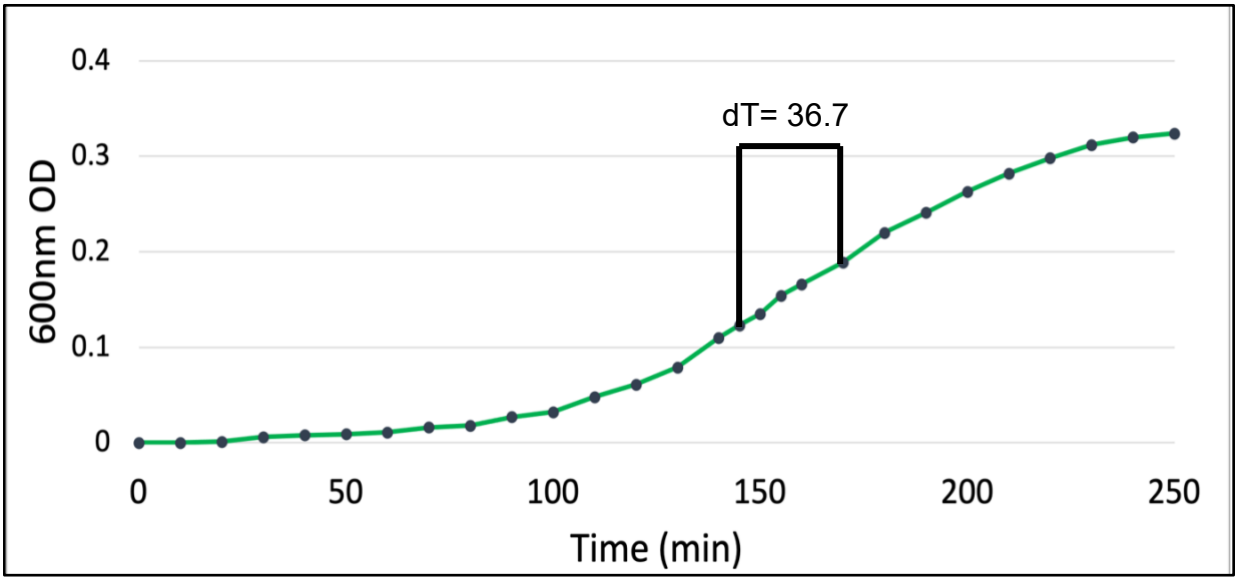

Figure 6. Growth curve of vancomycin resistant ($MF06036^{Van}$) *E.faecalis* derived from a 1:10 diluted *E.faecalis* culture suspended in TSB and kept in a 37°C water bath with 600 nm photometer optical density (OD) measurements taken every 15 minutes over 4 hours. The calculated doubling time in minutes (dT) is included.

3.5 Conjugal Transfer of Antibiotic Resistance

The $MW01105^{Rif}$ parent stain initially possessed vancomycin and rifampin multidrug resistance prior to conjugation. Therefore, only the CFU/mL between treatments was compared during the first round of experiments (Figure 7). The inoculum donor cell count was 6.58×10^8 cells/mL, and the recipient count was 3.48×10^8 cells/mL. When the bacterial suspension incubated with *L.minor* was diluted and enumerated on TSA, the control with no *L.minor* had double the CFU. There was a significant difference between the control CFU/mL counts and CFU/mL counts from the media incubated with *L.minor* thalli and roots; $F(4, 20) = 57.2$, $p < 0.001$. Post hoc comparisons indicated CFU/mL from the control ($M = 5.33 \times 10^5$, $SD = 6.2 \times 10^5$) was significantly higher than medium incubated with 9 thalli ($M = 1.95 \times 10^5$, $SD = 2.0 \times 10^5$); 18 thalli ($M = 1.87 \times 10^5$, $SD = 3.1 \times 10^5$); 9 roots ($M = 2.20 \times 10^5$, $SD = 4.6 \times 10^5$); and 18 roots ($M = 2.51 \times 10^5$, $SD = 5.7 \times 10^5$) all with $p < 0.0001$. However, media incubated with thalli and roots were not significantly different from one another, $F(3,16) = 2.48$, $p = 0.10$.

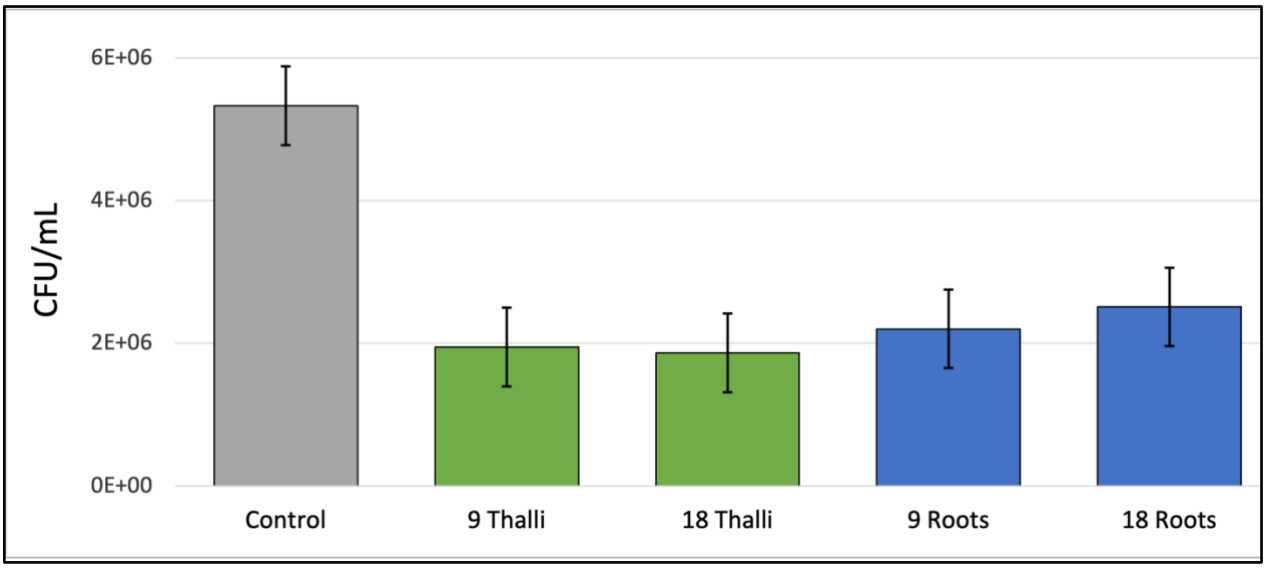

Figure 7. Average (n = 5) *E.faecalis* CFU recovered from 9 and 18 *L.minor* thalli and roots. *E.faecalis* was incubated with *L.minor* samples in a 10 mL bacterial suspension for 24 hours at 30°C. The incubated medium was diluted 6-fold before being plated on TSA. CFU were summed and adjusted to CFU/mL for controls and treatments. Error bars show +/- 2 standard deviations.

A new culture of the MW01105^{Rif} strain was tested for multidrug resistance and was susceptible to vancomycin while remaining resistant to rifampin. Therefore, a second conjugation experiment was run using equivalent procedures as the first experiment. After 24 hours of incubation, only the controls possessed TC growth (Table 2). There was an average of 4 TC/mL in controls (SD = 1.58) and an average of 0 TC/mL in all treatments (SD = 0). A Kruskal–Wallis test followed by a post hoc test showed a significant difference between the control and all treatment groups ($H(3) = 18.13$, $p = 0.001$). The mean rank scores and significance test results are enumerated in Table 3. Using equation 3, the effect size (d) between the treatment and control groups was 0.84 where $\overline{X}1 = 4$, $\overline{X}2 = 0$, and SD Pooled = 2.37. A d greater than 0.80 is considered large. TC abundance was unable to be calculated.

Table 2. The mean (n = 5) surface area of *L.minor* thalli and roots, transconjugant number, *E.faecalis* transconjugant abundance, and transfer efficiency of vancomycin resistance between the vancomycin resistant donor and rifampin resistant recipient. Total TC counts were determined by summing the CFU from both the media and the plant isolate. Cells were incubated with *L.minor* at 30°C for 24 hours and *E.faecalis* was recovered from separated *L.minor* thalli and roots.

Sample	*L.minor* Surface Area (count: mm^2)	Donor Count (CFU/mL)	Recipient Count (CFU/mL)	TC number (CFU/mL) MI + LI (T)	Transfer Efficiency (T:D)	Transfer Efficiency (T:R)	Transconjugant Abundance (CFU/mm2)
Control	0 : 0	8.00×10^8	7.40×10^8	4+0 (4)	5.0×10^{-9}	5.4×10^{-9}	-
Thalli	9 : 20.2	8.00×10^8	7.40×10^8	0+0 (0)	0	0	0
	18 : 40.3	8.00×10^8	7.40×10^8	0+0 (0)	0	0	0
Roots	9 : 31.5	8.00×10^8	8.00×10^8	0+0 (0)	0	0	0
	18 : 63.0	8.00×10^8	8.00×10^8	0+0 (0)	0	0	0

MI = media isolate; LI = *L.minor* isolate; T = total mean transconjugants; D = donor; R = Recipient

Table 3. Kruskal-Wallis test of significance results run in *SPSS* between average (n = 5) TC/mL counts from controls, and TC/mL counts from treatments where TC were recovered from *L.minor* thalli and roots after ultrasonicated samples were incubated at 37°C on rifampin and vancomycin double selection plates. Significance was determined at the 0.05 level.

Sample	n	Mean (TC/mL)	SD	Mean Rank	H-Value	P-Value	Effect Size (*d*)
Control	5	4	3.35	21	14.05	0.001	0.84
9 Thalli	5	0	0	11			
18 Thalli	5	0	0	11			
9 Roots	5	0	0	11			
18 Roots	5	0	0	11			

SD = Standard deviation

4. DISCUSSION

In the present study, it was hypothesized vancomycin resistance gene transfer between *E.faecalis* could be facilitated by the common aquatic macrophyte *L.minor,* observed through an increase in conjugation efficiency. Initial experimentation was necessary to characterize *L.minor*'s resident microbial population to determine if *E.faecalis* would associate with *L.minor*. *L.minor* has been shown to host 53 bacterial taxonomic groups at the phylum level and 479 groups at the family level. Yet, the *Firmicutes* phylum, which includes *Enterococcus spp*., was not among the resident bacteria recovered (Iwashita *et al.* 2020). In alignment with the findings of Iwashita *et al.* (2020), the *L.minor* from the Ulster University Pond did not host enterococci.

This finding suggests the stream restoration project for the Ballysally Blagh, adjacent to the Ulster University Pond, has mitigated the agricultural impact of animal feces on the pond's water quality (Smith 1999). However, even if there were numerous enterococci in the Ballysally Blagh, as historically found in Ulster University projects, limited fecal bacteria may leach into the pond producing undetectable contamination (Aislabie *et al.* 2001; Brennan *et al.* 2010). Current farming practices across Ireland, such as slurry spreading, introduce a higher risk of surface water microbial contamination in the form of untreated wastewater runoff (Nag *et al.* 2021; Sidhu and Toze, 2009). The use of Combine Sewer Overflows across the United Kingdom poses additional bacterial contamination threats (McGinnis *et al.* 2018). Yet, *L.minor's* resident microbial population suggests current practices near the sampling site were not promoting the spread of pathogenic organisms beyond the Ballysally Blagh. If pathogenic enterococci were present, they were below the level of detection. The *L.minor* samples populated TSA plates with bacterial and fungal growth. Therefore, sterilization was needed before experimentation. A 4-minute 0.5% troclosene sodium solution exposure sterilized plants but was shorter than the 7-minute exposure recommended by OECD for growth inhibition testing (2006) and used by Xue *et al.* (2018).

To confirm that *E.faecalis* could associate with *L.minor*, sterile *L.minor* were cultured with *E.faecalis* and ultrasonicated for increasing time intervals. Ultrasonication is superior to scaping methods for bacterial isolation on hard surfaces (Bjerkan *et al.* 2009) and macrophyte tissues (He *et al.* 2012; Kirzhner *et al.* 2009) because of its higher success in disrupting embedded bacterial biofilms. There was a significant increase in cell yield with ultrasonication time (Table 1 & Figure 4), and since *L.minor* were washed before ultrasonication, it was presumed recovered cells had attached to *L.minor* rather than being planktonic cells from the original inoculum. If CFU counts were adjusted to account for the 30 second vortexing step by subtracting the mean cell recovery from the control, then only be a significant difference existed between the 30 second and the 90 second ultrasonication times (p = 0.008; Figure 4). A 1.5-minute ultrasonication time falls within the recommended range of 1 - 5 minutes. Therefore, the 30 second and 60 second ultrasonication results agree with past studies suggesting samples need to be ultrasonicated more than one minute for adequate biofilm disruption (Kobayashi *et al.* 2007). Further cell yield may be possible since there was no observed decline in CFU recovery with increased ultrasonication time. The 90 second total ultrasonication time was still deemed sufficient for cell recovery, as times nearing 5 minutes can cause microbial inactivation and skew bacterial viability results (Kirzhner *et al.* 2009). The 90 seconds of ultrasonication did not

have a detrimental effect on *L.minor* plant tissues; although long ultrasonication exposures can cause plant damage (Zolghadrnasab *et al.* 2021).

The doubling time of MF06036^{Van} *E.faecalis* was calculated since conjugation is most likely to occur during the mid-exponential growth phase (Kohler *et al.* 2019). The MF06036^{Van} strain was incubated in a 37ºC water bath with samples taken every 15 minutes, during which time the culture flask was briefly removed from the water bath. As seen in Figure 6, the culture remained in its lag phase for 80 minutes which is typical for bacterial cultures (Bertrand 2019). The culture reached its mid-exponential phase after 155 minutes unlike in Olanrewaju *et al.* (2019), where an *E.faecalis* entered mid-exponential growth after 90 minutes in a 37ºC water bath. Removing the flask for sampling could have delayed the exponential growth phase, and antibiotic resistant microbes can have a longer lag duration to account for the additional time needed to replicate ARG material (Bertrand 2019; Li *et al.* 2016). The calculated doubling time for the MF06036^{Van} strain was 36.7 minutes which fell below the double times of 47 minutes and 56 minutes reported in Dean *et al.* (2020). However, Theophel *et al.* (2014) described how antibiotic resistant *Enterococcus* cultures with longer lag phases proceeded to divide faster than antibiotic susceptible controls. Thus, the doubling time did not suggest irregular cell growth.

E.faecalis CFU recovered from *L.minor* thalli and roots were compared since plant microenvironments can introduce a set of preferential microscale environments for epiphytic bacterial and fungal communities (Hurtado-McCormick *et al.* 2019). *L.minor* thalli had significantly higher CFU/mm^2 (Figure 5), but previous research suggests *L.minor* roots are resident to more microbes as a whole (Iwashita et al. 2020). It was expected that roots would have a higher *E.faecalis* association as the rhizosphere of aquatic plants is associated with intimate microbial relationships. Likewise, the rhizosphere and root systems of marsh plants have been shown to increase the metabolic activity in gram-positive microbes (Kroer *et al*. 1998). While *L.minor* lives on the water surface, completely submerged macrophytes have been shown to possess diverse microbial populations that are host-specific and seasonally variant (He *et al.* 2012; He *et al.* 2020; Korlević *et al.* 2021; Matsuzawa *et al.* 2010). *L.minor* thalli could offer *E.faecalis* a preferential microhabitat since certain groups of bacteria are linked to specific purposes within plant relationships. *E.faecalis* strains are often composed of two subpopulations with different surface charges where the majority subpopulation is more negatively charged (Cowan *et al.* 1992; van Merode *et al.* 2006). Therefore, the MF06036^{Van} *E.faecalis* culture could have been composed of two subpopulations with one population preferentially associating to the

thalli of *L.minor* due to subtle differences in surface charge. Higher levels of ammonium (NH_4^+) uptake in *L.minor* thalli is accompanied by proton expulsion which causes extracellular environments to be slightly acidified by H^+ ions (Cedergreen and Madsen, 2002; Escobar *et al.* 2006). This positive environment could attract the *E.faecalis* subpopulation with a stronger negative charge leading to the observed increase in CFU recovered from *L.minor* thalli. However, the *L.minor* studied in Cedergreen and Madsen (2002) differ from the one thallus and one root surface areas used in the present study (Figure 3). Therefore, the significant difference in ammonium uptake reported is not as extreme since uptake varied by area and existing nitrogen status.

The conjugation experiments were run twice, and during the first experiment, too many CFU to count appeared on double selection plates. The MW01105^{Rif} strain also grew on vancomycin controls. Therefore, the surrounding plant media was serially diluted on TSA plates to enumerate the CFU in each culture (Figure 7). There were significantly less CFU/mL in the cell suspensions incubated with *L.minor* thalli and roots, albeit all cell suspensions remained above 1E6 cells/mL. *L.minor* has been shown to possess antimicrobial properties (Gulcin *et al.* 2010), and the reduction in CFU supports past research suggesting floating macrophytes could reduce bacteria in aquatic systems (El-Din and Abdel-Aziz, 2018; Gulcin *et al.* 2010; Sooknah and Wilkie, 2004). The slight antibacterial effect observed in the experiment could be attributed to phenolic compounds or flavonoids found in *Lemna spp.* (González-Renteria *et al.* 2020). However, the degree of antibacterial activity *Lemna spp*. possess varies greatly and there is no direct evidence it demonstrates significant antagonism on *E.faecalis* (Al-Snafi, 2019). Although, even slight microbial reduction could have positive implications for the use of *L.minor* in systems subject to wastewater discharge.

While the first conjugation experiment suggested the MW01105^{Rif} strain was multidrug resistant, a new culture proved to be susceptible to both antibiotics. No TC were present in samples with *L.minor* after the second conjugation experiment. Yet, the controls with no *L.minor* had an average of 4 TC/mL. There was a significant difference between the control and all treatment groups (Table 3). The effect size was large at 0.84 and strongly suggests that *L.minor* does not facilitate conjugation of vancomycin resistance between *E.faecalis* strains. The control transfer efficiency between both the donor and recipient strains were more than 10 times less than the values reported in Olanrewaju *et al.* (2019) after *Daphnia* filtration (Table 2). Controls from Olanrewaju *et al.* (2019) yielded no TC despite *E.faecalis* inoculations from both studies being

equivalently diluted and cultured. The sporadic nature of the antibiotic resistance observed in this study supports suggestions made by Larsson and Flach (2022) stating that antibiotic resistance transfer in nature is common, and transfer events may have limited consequences if the ARG is directly transferred. The MW01105^{Rif} gaining multidrug resistance while stored in the laboratory, but not acquiring resistance in experiment treatments, additionally demonstrated how transmission events can be influenced by several poorly understood drivers. The presence of TC in controls suggests that *L.minor* changed the selective pressures (Berendonk *et al.* 2015) or reduced cell metabolic rates (Kroer *et al.* 1998) which then interfered with pheromone-induced conjugation. These findings reject the alternative hypothesis stating that *L.minor* facilitates conjugal gene transfer between *E.faecalis*. Therefore, the association between *L.minor* and *E.faecalis* does not function as a suitable model for the emergence of aquatic pathogens.

E.faecalis cells do not have a method of mobility outside the biofilm state, and close contact or even direct contact is needed for conjugation to occur (Cook *et al.* 2011). Recipient *E.faecalis* cells secrete pheromones to attract plasmid donors of the same species, and a low level of pheromone secreting recipients greatly reduces conjugation *in vitro* (Conwell *et al.* 2017; Hirt *et al.* 2018). Therefore, the chance of conjugation in a cell culture is highly dependent on *E.faecalis* pheromone secretion and the likelihood of recipient cells coming into close contact with donor cells. This introduced a limitation on the study, as the available surface area of *L.minor* thalli and roots could have been too little for cells to stick to the plants within the required conjugation vicinity. *L.minor* surface area may have also exceeded the optimal amount of surface area causing abundant cells to stick but trapping donors and recipients into stationary locations which reduced conjugation. Unlike the Daphnids in Olanrewaju *et al.* (2019), *L.minor* do not possess a filtering capacity to promote the active movement of cells, so donors and recipients have increased opportunities for interaction and pheromone-induced conjugation. Therefore, it would be advantageous in future experiments to incubate *E.faecalis* with a wide range of *L.minor* surface areas to see if intraspecies conjugation in the presence of *L.minor* operates under a conjugation efficiency bell curve.

Additionally, assumptions from previous Ulster University studies were made due to the restricted project timeline and supplies access. Two rounds of the conjugation experiments were executed and yielded contrasting results. Thus, further studies are needed to determine why conjugation may have been unfavorable in the provided conditions. The ratio of donor to

recipient *E.faecalis* was assumed to be 1:14 from a past study within the same Ulster University department where filter-feeding freshwater sponges were incubated with *E.faecalis* (Cartwright *et al.* 2020). Other studies have used donor and recipient ratios ranging from 1:1 (Price *et al.* 2019) to 1:9 (Conwell *et al.* 2017; Cook *et al.* 2011; Olanrewaju *et al.* 2019). The 1:14 assumption might not be best suited for aquatic, macrophyte facilitated conjugation. Additionally, colonies on double selection plates were presumed to be TC and PCR was unavailable to confirm the presence of *vanA* resistance gene. Therefore, the ability for colonies to grow on double selection plates cannot be attributed to the *vanA* gene with absolute certainty. Running PCR from TC colonies in future experiments would fortify results and confirm *vanA* gene transfer.

5. CONCLUSION

The aim of the report was met by incubating *L.minor* with vancomycin resistant and susceptible *E.faecalis* and determining that conjugation of antibiotic resistance was not facilitated by *L.minor*. *E.faecalis* cells were adequately recovered from *L.minor,* and significantly more *E.faecalis* CFU/mm^2 associated on *L.minor* thalli when compared to *L.minor* roots. This association could be attributed to the difference in plant surface changes and the presence of heterogeneous enterococci subpopulations. Additionally, *L.minor* significantly reduced the number of CFU/mL in its surrounding media which serves as evidence for bacterial attachment to *L.minor*. If enterococci attach to *L.minor* and are subsequently removed from the planktonic cell population, *L.minor* could function as a low-cost bacterial remediation tool in wastewater systems. Both *L.minor* roots and thalli were not found to support the conjugation of vancomycin resistance between *E.faecalis* strains, and the hypothesis stating *L.minor* would facilitate the transfer of ARG was rejected. Further studies are needed to elucidate the factors contributing to the inhibition of pheromone-induced conjugation in the presence of *L.minor* for both industrial and ecological purposes. Discerning the optimal plant surface area and donor to recipient ratio for potential conjugation could provide environmental managers key insight into the number of bacteria in waste effluent that could promote ARG transfer. The lack of transconjugants in treatments does not rule out the possibility of conjugation occurring in the presence of *L.minor* but suggests *L.minor* does not pose a serious ARG increase threat. With *L.minor's* value in water treatment and biomass production, the experimental results provided a promising outlook on the continued use of *L.minor* in bacterially contaminated environments.

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