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Manipulation of the seagrass-associated microbiome reduces disease severity

Olivia J. Graham[1] | **Emily M. Adamczyk**[2] | **Siobhan Schenk**[3] | **Phoebe Dawkins**[1] | **Samantha Burke**[1] | **Emily Chei**[1] | **Kaitlyn Cisz**[1] | **Sukanya Dayal**[1] | **Jack Elstner**[1] | **Arjun Lev Pillai Hausner**[1] | **Taylor Hughes**[1] | **Omisha Manglani**[1] | **Miles McDonald**[1] | **Chloe Mikles**[1] | **Anna Poslednik**[1] | **Audrey Vinton**[1] | **Laura Wegener Parfrey**[2,3] | **C. Drew Harvell**[1]

[1]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York, USA

[2]Department of Zoology and Biodiversity Research Centre, Unceded xʷməθkʷəy̓əm (Musqueam) Territory, University of British Columbia, Vancouver, British Columbia, Canada

[3]Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada

Correspondence
Olivia J. Graham, Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA.
Email: ojg5@cornell.edu

Present addresses
Phoebe Dawkins, Department of Ecology and Evolutionary Biology, University of California, Irvine, California, USA; Samantha Burke, Evolution and Ecology Research Centre, School of Biological, Earth, and Environmental Sciences, The University of New South Wales, Sydney, New South Wales, Australia; Emily Chei, School of Biological Sciences, The Swire Institute of Marine Science, The University of Hong Kong, Pokfulam, Hong Kong; Kaitlyn Cisz and Anna Poslednik, Virginia Institute of Marine Science, William and Mary, Gloucester Point, Virginia, USA; Sukanya Dayal, Department of Biology and Marine Biology, University of North Carolina, Wilmington, North Carolina, USA; Jack Elstner, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California, USA; Taylor Hughes, School of Marine and Environmental Affairs, University of Washington, Seattle, Washington, USA; and Chloe Mikles, Department of Biology, Stanford University, Palo Alto, California, USA.

Abstract

Host-associated microbes influence host health and function and can be a first line of defence against infections. While research increasingly shows that terrestrial plant microbiomes contribute to bacterial, fungal, and oomycete disease resistance, no comparable experimental work has investigated marine plant microbiomes or more diverse disease agents. We test the hypothesis that the eelgrass (*Zostera marina*) leaf microbiome increases resistance to seagrass wasting disease. From field eelgrass with paired diseased and asymptomatic tissue, *16S rRNA* gene amplicon sequencing revealed that bacterial composition and richness varied markedly between diseased and asymptomatic tissue in one of the two years. This suggests that the influence of disease on eelgrass microbial communities may vary with environmental conditions. We next experimentally reduced the eelgrass microbiome with antibiotics and bleach, then inoculated plants with *Labyrinthula zosterae*, the causative agent of wasting disease. We detected significantly higher disease severity in eelgrass with a native microbiome than an experimentally reduced microbiome. Our results over multiple experiments do not support a protective role of the eelgrass microbiome against *L. zosterae*. Further studies of these marine host–microbe–pathogen relationships may continue to show new relationships between plant microbiomes and diseases.

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INTRODUCTION

Plant-associated microbes can improve host growth, health, and resilience (Ford & King, 2016; Trivedi et al., 2020; Turner et al., 2013). Microbiomes also serve as a first line of defence against pathogens, opportunistic bacteria, fouling, and grazing (Inaba et al., 2017; O'Connor et al., 2022; Trivedi et al., 2020). Even on land, the role of plant microbiomes in pathogen defence has been tested against only some types of pathogens, such as bacteria, oomycetes, and fungi (Bais et al., 2004; Berg & Koskella, 2018; Carisse et al., 2003; Chen et al., 2018), though, few manipulative experiments test the role of plant-associated bacteria in defence against diverse disease agents. While some microbiomes of terrestrial plants are protective, others can facilitate infection (Armstrong et al., 2000; Hayes et al., 2010; Stevens et al., 2021; Tovaglieri et al., 2019), providing metabolites that can fuel pathogen growth (Li et al., 2019). Marine plant pathogens can significantly compromise host health and alter ecosystem functions, particularly when they infect foundation species like eelgrass (*Zostera marina*), a temperate seagrass species. *Labyrinthula zosterae* (Lz) is a protist and the causative agent of seagrass wasting disease, which historically ravaged seagrass ecosystems throughout the Atlantic, thereby decimating animal populations, and fisheries, and dramatically reshaping coastal environments (Rasmussen, 1977). Given its close association with warming temperatures and recent eelgrass declines, seagrass wasting disease is considered a major ecological threat (Aoki et al., 2022; Groner et al., 2016, 2021). There is a clear need to understand the interactions between Lz and the eelgrass microbiome. Though the eelgrass microbiome is hypothesized to broadly support host health (Fahimipour et al., 2017) by enhancing growth, fixing nitrogen, and helping cope with stressors like elevated sulfide (Celdrán et al., 2012; Küsel et al., 2006; Mohr et al., 2021), there are no manipulative experiments that examine the role of the eelgrass microbiome in pathogen defence (Beatty et al., 2022; Tarquinio et al., 2019).

A growing number of studies have characterized the eelgrass microbiome (Adamczyk et al., 2022; Ettinger et al., 2017; Ettinger & Eisen, 2020; Sanders-Smith et al., 2020; Segovia et al., 2021; Ugarelli et al., 2017). We use the term 'microbiome' to refer to phyllosphere bacteria and archaea living on the surface of eelgrass leaves, though we acknowledge this excludes many members of the eelgrass microbiome, including eukaryotes and viruses, as well as communities in the endosphere and rhizosphere (reviewed in Tarquinio et al., 2019). Eelgrass leaves are selective surfaces (Kurilenko et al., 2007) with bacterial communities that are distinct from surrounding environments (Adamczyk et al., 2022; Sanders-Smith et al., 2020). These studies provide a foundation for examining the ecological roles of the eelgrass microbiome. For example, eelgrass-associated bacteria produce algicidal compounds (Inaba et al., 2017) and can positively influence host or epiphyte growth (Celdrán et al., 2012). While characterizing the eelgrass microbiome is the first step, there is a need for manipulative experiments to discern the functional response of these microbes to pathogens or environmental factors. Already, a protective microbiome against bacterial, oomycete, and fungal pathogens has been shown for terrestrial plants including tomato (Berg & Koskella, 2018), cucumber (Carisse et al., 2003), *Arabidopsis* (Bais et al., 2004), and wheat (Chen et al., 2018). However, the role of the eelgrass microbiome in pathogen defence remains untested, and there is a need to characterize the eelgrass microbiome across disease states.

Seagrass wasting disease is among the mounting global threats to the sustainability of seagrass meadows and their valuable ecosystem services (Orth et al., 2006; Rasmussen, 1977). Lz infects eelgrass and other seagrasses, consumes chloroplasts (Muehlstein, 1992), kills plant cells, and impairs photosynthesis (Ralph & Short, 2002). Notably, wasting disease caused worldwide eelgrass die-offs in the 1930s and 1940s (Muehlstein, 1992; Renn, 1936), resulting in cascading effects on nearshore ecosystems and fisheries. Lz infection also impairs eelgrass growth and rhizome sugar reserves in situ (Graham et al., 2021), likely undermining the sustained persistence of eelgrass meadows. Understanding eelgrass-Lz-microbiome interactions is especially important, given the mounting stressors of Lz and climate change.

Environmental conditions and Lz independently and synergistically impact eelgrass (Muehlstein, 1992; Renn, 1936; Strydom et al., 2020). With accelerated ocean warming and more frequent, intense heat waves

(Oliver et al., 2018), rising temperature is the most prominent global change factor impacting seagrass ecosystems (Short et al., 2016; Smale et al., 2019). Warming temperatures likely influence wasting disease dynamics, since Lz is temperature-sensitive (Dawkins et al., 2018). Recently, high wasting disease prevalence in natural meadows was associated with warm water anomalies and significant meadow declines (Aoki et al., 2022, 2023; Groner et al., 2021). Understanding the role of the eelgrass microbiome in Lz defence is an important first step in clarifying these complex interactions and is broadly relevant to eelgrass conservation and preservation.

Here, we examine the stability and composition of the eelgrass microbiome in different disease states and the role of the eelgrass microbiome in Lz defence. Specifically, we tested two hypotheses: (i) Diseased and asymptomatic eelgrass tissue in the field has different bacterial communities, and (ii) disease will be less severe in eelgrass with a native microbiome compared to eelgrass with a reduced microbiome. We used *16S rRNA* gene amplicon sequencing of diseased and asymptomatic eelgrass samples from a natural meadow and experimental samples and conducted two controlled inoculation experiments paired with measurements of wasting disease. This is the first project to pair measurements of wasting disease impacts on eelgrass microbiome composition in the field with experimental manipulations of the eelgrass microbiome to test for the impact of Lz infection (Figure 1).

EXPERIMENTAL PROCEDURES

Field sample collection for microbiome composition analyses: 2018 and 2020

Eelgrass leaves from a natural meadow at Fourth of July Beach, San Juan Island, Washington, USA (48°28.120′ N, 123°0.190′ W) were sampled to determine in situ microbial communities by *16S rRNA* gene amplicon sequencing. This site was selected given its robust eelgrass meadow and recent, high levels of wasting disease (Aoki et al., 2022; Groner et al., 2021). Third-youngest eelgrass leaves with visible wasting disease lesions and asymptomatic tissue were haphazardly collected from the intertidal eelgrass meadow in April 2018 ($n = 9$ leaves) and June 2020 ($n = 10$ leaves). Leaves were collected along the same, shallow depth gradient (+1 m mean lower low water) in the upper middle intertidal; they were stored in individual bags with a small amount of seawater and promptly returned on ice to the lab for processing. Collection and processing were performed wearing gloves and using a sterile technique (sterilizing blades and forceps with 2% HCl followed by 70% ethanol and flame). To compare microbial communities on diseased and asymptomatic eelgrass tissue, paired samples of lesion and asymptomatic eelgrass tissue were swabbed using a cotton-tip applicator ($n = 18$ in 2018, $n = 20$ in 2020; Figure 2A; Puritan, USA). In 2018, diseased samples were collected by swabbing a dark lesion on an

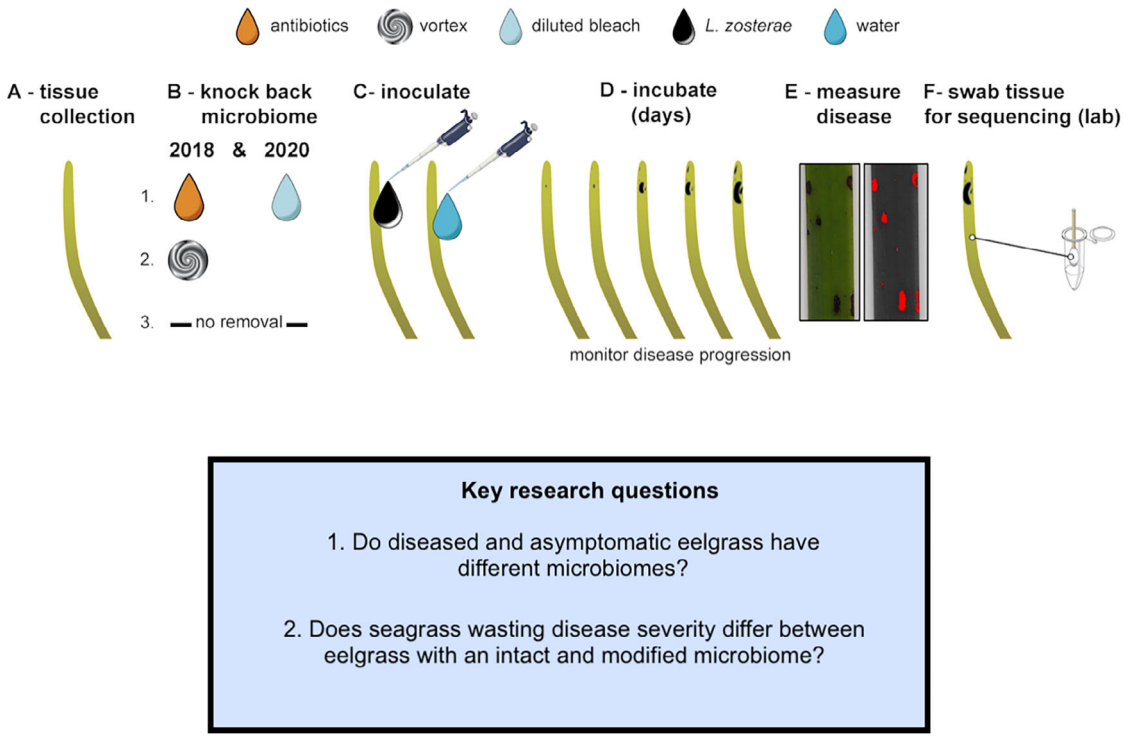

FIGURE 1 Eelgrass microbiome project overview and key research questions. (A–F) General experimental setup, including swabbing eelgrass for microbial sequencing. Illustration by Sylvia Heredia.

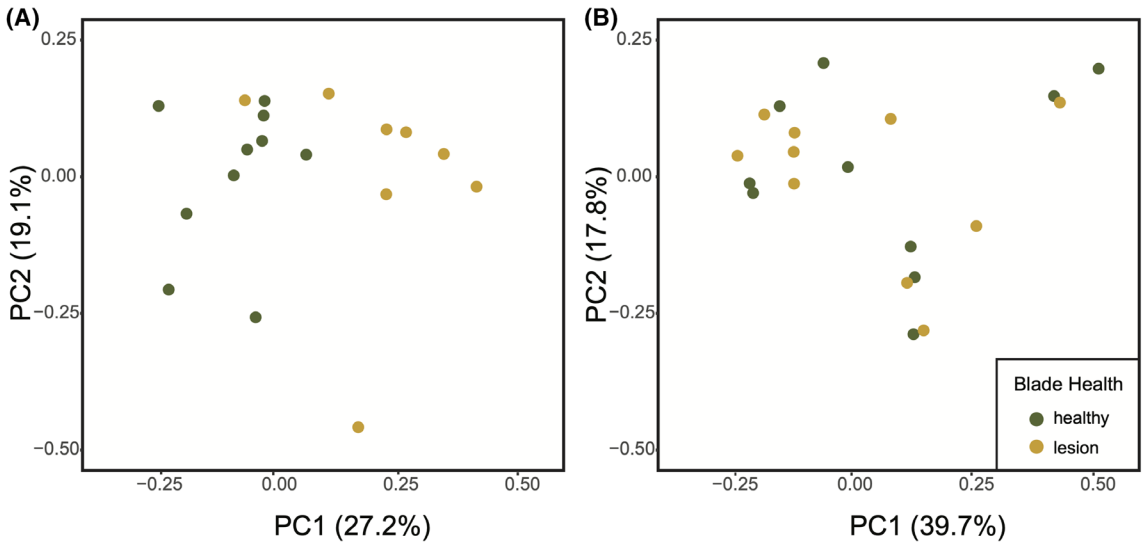

FIGURE 2 Microbial community composition differed between diseased and asymptomatic eelgrass in (A) spring 2018, but not in (B) summer 2020 (PERMANOVA; $p < 0.001$ in 2018; $p > 0.05$ in 2020). PERMANOVA and PCoA plots were based on Bray–Curtis dissimilarity indices.

eelgrass leaf for 10 s; asymptomatic samples were collected from the same leaf by swabbing an asymptomatic, 4 cm-long section of tissue 2 cm above the sheath for 10 s. In 2020, diseased samples were collected in the same manner, and asymptomatic samples were collected by swabbing an adjacent, asymptomatic section of tissue for 10 s. Applicator tips were snapped off into 1.5 mL cryotubes that were frozen at −80°C until DNA extraction.

In the summer of 2020, pathogen load in diseased and asymptomatic eelgrass was quantified via quantitative PCR (qPCR) to (i) confirm Lz presence in lesioned tissue and (ii) assess relationships between pathogen load and microbial richness. The same eelgrass leaves that were collected for sequencing ($n = 10$) were used for qPCR analyses to quantify the Lz load in diseased and asymptomatic tissue. After swabbing each leaf with a cotton-tip applicator, a section of asymptomatic and diseased tissue ($n = 20$ total) was excised from each leaf and preserved in 70% molecular-grade ethanol using sterile technique and stored in a sterile 1.5 mL screw cap microcentrifuge tube (Thermo Fisher Scientific, USA) until subsequent DNA extractions, consistent with established protocols (Aoki et al., 2022; Groner et al., 2018).

2018 laboratory antibiotics experiment

In addition to the field microbiome sampling, we conducted two full factorial lab experiments. For the first experiment (hereafter referred to as the 'antibiotics' experiment), intertidal eelgrass shoots were collected from Fourth of July Beach in April 2018 (Figure 1). Shoots were collected 1–2 m apart via snorkelling using gloved hands and were broken off above the rhizomes to minimize cross-contamination from the rhizosphere (Crump & Koch, 2008; Ettinger et al., 2017; Fahimipour et al., 2017). Because the eelgrass microbiome can vary with water depth (Bengtsson et al., 2017), eelgrass shoots were collected in the upper middle intertidal (+1 m mean lower low water). All shoots were stored in sealed plastic bags with seawater and placed on ice for transportation to the lab.

In the lab, eelgrass leaves were processed using a sterile technique, gloved hands, and on foil sheets; the latter two were sprayed with 10% bleach and 70% ethanol beforehand to create sterile work surfaces. Because the eelgrass microbiome can vary with the age of the leaf (Sanders-Smith et al., 2020), the third-youngest leaf from each shoot was selected for consistency. All other leaves on each shoot were discarded. Epiphytes were removed by gently scraping eelgrass leaves with sterile, plastic rulers, running gloved hands along each leaf, then carefully swishing leaves in a sterile basin with sterile, filtered seawater (FSW) to rinse for several minutes until the water was no longer cloudy with epiphytes. FSW was obtained by filtering seawater through a 1 μm filter bag (Knight Corporation, USA), sterilized at 120°C for 20 min on a liquid cycle (SX-500 Autoclave, Tomy, USA), and cooled to room temperature before use.

Using sterile technique, each eelgrass leaf was cut into six, 3 cm pieces, one for each of the six treatments ($n = 144$ pieces). Eelgrass pieces were stored in Petri dishes with low salinity, 10 parts per thousand FSW overnight at approximately 20°C to allow cut ends to heal and to flush any potential surface Lz from the

surface tissues, as low salinities are associated with reduced Lz infection in the field (Burdick et al., 1993) and lab (Muehlstein et al., 1988).

After 12 h, the six pieces from each eelgrass leaf were randomly assigned to each of the six groups: (i) antibiotic rinse, (ii) vortex, and (iii) no microbiome disruption treatment for both Lz-and FSW-inoculated eelgrass. FSW-inoculated eelgrass served as lab contamination controls to ensure sound inoculation methods and that Lz-inoculations were effective at inducing wasting disease infection. Eelgrass with a native microbiome served as experimental controls since they were not exposed to a microbiome disruption treatment.

Two microbiome disruption treatments were used—dilute antibiotics (Penicillin–Streptomycin, VWR, USA) and vortexing—to determine which was more effective at manipulating the microbiome. For the antibiotic treatment, leaf pieces were stored in individual Petri dishes containing a solution of 1% v/v PenStrep (10,000 U/mL penicillin, 10 mg/mL streptomycin stock) diluted with FSW for 1 h. Specifically, PenStrep was used because it targets both Gram-negative and Gram-positive bacteria and is consistent with published methods for surface-sterilizing algae (Raghukumar et al., 1992). Following the antibiotic treatment, each eelgrass piece was transferred to a separate, sterile container with FSW to rinse any remaining antibiotic and reduce the possibility of antibiotics directly interacting with Lz, sterilizing forceps with HCl, ethanol, and flame sterilizing in between each. For the vortex treatment, two pieces from each leaf were placed in a 50 mL falcon tube filled with 10 mL FSW and vortexed on high for 30 s. Each piece was transferred to a separate, sterile container with FSW until inoculation. To minimize leaf cross-contamination, the FSW was changed in between each pair of leaf pieces. Both the PenStrep and vortex approaches were used to disrupt the plant microbiome in other studies (Ettinger & Eisen, 2020; Groner et al., 2018). Control eelgrass leaves were not exposed to microbiome disruption treatments, and were maintained in FSW until inoculation.

All eelgrass pieces were then inoculated with Lz in FSW or FSW alone. Eelgrass pieces were inoculated with Lz strain 0063FB, which was isolated from diseased eelgrass in False Bay, San Juan Island, WA, USA (48°28.964′ N, 123°4.452′ W). To inoculate leaf pieces, the centre of each piece was lightly nicked with a sterile scalpel, transferred to a sterile seawater agar petri dish, and inoculated with 15 μL of FSW or Lz inoculum (6.15×10^6 cells/mL), changing pipette tips in between each inoculation. To prevent Lz cells from clumping, we pulse vortexed the inoculum at medium speed for 5 s intervals approximately every 10 inoculations. Other seagrass wasting disease experiments have used a similar inoculation method (Dawkins et al., 2018; Groner et al., 2018). The identity of Lz cultures was confirmed via microscopic observations; all Lz cultures had characteristic features, as did lesions resulting from laboratory inoculations (Muehlstein et al., 1988). Following inoculations with Lz or FSW, all Petri dishes containing eelgrass were sealed with Parafilm (Heathrow Scientific, USA) and transferred to a 12.8°C cold room, where they sat under full-spectrum, aquatic plant LED lights (MarineLand, USA) set to a 14 h light: 10 h dark cycle for 6 h. After this initial pathogen exposure period, each petri dish was flooded with 2 mL FSW, resealed with Parafilm, and then returned to the 12.8°C cold room with LED lights for 4 days, at which point the experiment was terminated.

At the end of the experiment, four to six randomly chosen eelgrass pieces from each treatment were swabbed for *16S rRNA* gene sequencing, following the protocol outlined above ($n = 29$ samples). Working with a sterile technique and on foil trays sterilized with 10% bleach and 70% ethanol, a 1.5 cm-long section of a leaf piece was swabbed with a cotton-tip applicator for 10 s, after which the tip was snapped off and stored in a sterile 1.5 mL cryotube (Thermo Fisher Scientific, USA). This process was repeated for the remaining eelgrass pieces. All cryotubes were then transferred to a −80°C freezer.

Subsequently, all eelgrass pieces were scanned using a CanoScan LiDE 220 photocopy scanner (Canon, USA) for image analyses in ImageJ (Schneider et al., 2012) for key disease metrics: severity (proportion of lesioned leaf area relative to total leaf area), presence (presence/absence of lesions), prevalence (proportion of leaves with visible Lz lesions), and number of lesions on each leaf (Figure 1). All are important indicators of disease (Dawkins et al., 2018; Groner et al., 2014, 2016, 2018, 2021). To minimize bias, two researchers separately analysed each eelgrass image. If there was greater than a 5% difference in severity in measurements, a third person independently measured disease levels and averaged the two closest measurements for each leaf; all final severity measurements were within <2% difference from one another. ImageJ protocols are in the Supplementary Materials.

2020 laboratory bleach experiment

For the second laboratory experiment in March 2020 ('bleach' experiment), intertidal eelgrass shoots were collected from Fourth of July Beach, San Juan Island, WA, following the protocol outlined above for the 2018 experiment. In the lab, each plant was processed on a foil tray sterilized with 10% bleach and 70% ethanol, epiphytes were removed with plastic rulers and gloved hands, and all leaves except the third-youngest were gently removed at the top of the sheath, leaving the sheath and third-youngest leaf intact. Leaves were

stored in approximately 9L FSW in a 10.3°C cold room under MarineLand LED lights set to a 14 h light: 10 h dark cycle overnight. This full-salinity FSW soak replaced the low-salinity FSW soak used in 2018 to avoid further disruption of the eelgrass microbiome, as salinity can influence seagrass microbial taxa (Trevathan-Tackett et al., 2020; Vogel et al., 2020).

After 15 h, all leaves were visually assessed for lesion development that may have occurred overnight; any leaves that developed disease signs (i.e., black lesions) were discarded. The remaining leaves ($n = 113$) were separated into two bins: one for bleached eelgrass and one for untreated eelgrass. Here, dilute bleach was used to modify the microbiome—rather than antibiotics—to induce a larger microbiome disruption. For the bleach treatment, 56 leaves were placed in a basin with a dilute bleach solution (0.1% sodium hypochlorite and 0.001% Tween 20 [VWR, USA] in FSW). Leaves were gently submerged and swirled in the dilute bleach solution for 10 s before transferring to a new, sterile basin with reverse osmosis water to remove any trace bleach solution. They were swished in reverse osmosis water for 1 min and then transferred to a new basin with reverse osmosis water. This process was repeated for a total of three reverse osmosis water rinses to thoroughly remove any trace bleach and minimize potential negative interactions with Lz. Following these initial rinses, leaves were moved to another basin with FSW and mixed for 1 min. Leaves were stored in FSW at ambient temperature while the inoculum was prepared. Meanwhile, the remaining eelgrass leaves ($n = 57$) were left untreated in the original basin with FSW. To minimize eelgrass stress, the concentration and exposure period to sodium hypochlorite were more conservative than those used in other studies to modify seagrass and algae microbiomes (Ettinger & Eisen, 2020; Muehlstein, 1992; Newell & Fell, 1982; Raghukumar et al., 1992; Ravikumar et al., 2010).

All leaves were transferred to individual, sterile 370 mL WhirlPaks (Whirl-Pak, USA) and then inoculated with concentrated Lz in FSW or FSW alone. Lz-inoculated leaves ($n = 57$; bleached and untreated) were placed in individual WhirlPaks with 99 mL FSW and 1 mL of Lz inoculum. The inoculum was made with Lz strain 4JB-MVA, which was originally isolated from diseased eelgrass from Fourth of July Beach, San Juan Island, WA (Agnew et al., 2022); the final inoculum had a cell concentration of 1.2×10^6 cells/mL. The inoculum was pipetted directly into each WhirlPak, rather than nicking the surface of eelgrass leaves and pipetting it directly onto eelgrass tissue, to more accurately mimic natural infection mechanisms. Pipette tips were changed in between each inoculation. To prevent Lz cells from clumping, the inoculum was pulse vortexed for 5 s on medium speed after every 10 inoculations. Bleached and untreated control leaves ($n = 56$) were placed in individual WhirlPaks with 100 mL FSW.

Afterward, each WhirlPak was tightly sealed and gently agitated to distribute the inoculum and FSW. To confirm the inoculum contained live Lz cells, 20 μL of the stock inoculum was plated onto 5 serum seawater agar Petri dishes (Porter, 1990), which were incubated at 24°C and monitored for characteristic Lz growth. For negative controls, 20 μL of FSW were plated onto 5 serum seawater agar Petri dishes.

Once all eelgrass leaves were inoculated with Lz or FSW, WhirlPaks were moved to a 10.3°C cold room, where they were randomly arranged under MarineLand LED marine plant lights set to a 14 h light: 10 h dark cycle. Leaves were monitored once daily for disease progression. During each visit, each WhirlPak was gently agitated to circulate FSW and Lz. Forty-eight hours after the inoculations, each leaf was transferred to a new WhirlPak with 100 mL FSW, using a sterile technique and transferring all controls first to minimize the chances of cross-contamination from Lz-inoculated eelgrass.

The experiment was terminated 5 days after inoculation. Characteristic Lz cultures grew from the concentrated Lz inoculum plated on 5 serum seawater agar Petri dishes, thereby verifying the isolate was Lz. At the end of the experiment, six randomly chosen leaves from each of the four treatments were swabbed for final timepoint *16S rRNA* gene amplicon sequencing ($n = 24$ samples). Each leaf was processed with gloved hands, sterile technique, and on foil trays sterilized with 10% bleach and 70% ethanol. For each leaf, a 4 cm-long section of tissue located 2 cm above the sheath was swabbed for 10 s using a cotton applicator. Applicator tips were promptly stored in cryovials at −80°C until subsequent analyses. Finally, all eelgrass leaves were scanned using a Canon photocopy scanner to measure disease severity, presence, prevalence, and number of lesions in ImageJ.

Disease data processing and analyses

All data were processed and analysed in the software R (v. 1.4.1106; R Core Team, 2020); graphs were developed using the ggplot2 (v. 3.3.6), ggpubr (v. 0.4.0), and RColorBrewer (v. 1.1–3) R packages (Kassambara, 2020; Neuwirth, 2014; Wickham, 2016). In both the antibiotics and bleach experiments, Lz inoculation methods were effective. This is reflected by the lower disease severity among FSW-inoculated controls compared to Lz-inoculated eelgrass (Table S1). This indicated Lz infection did not result from contamination during inoculations. Severity analyses focused on Lz-inoculated eelgrass, instead of the FSW-inoculated controls, which were strictly lab contamination controls to monitor for potential lab contamination with Lz.

Different statistical approaches were required to assess whether disease severity in Lz-inoculated eelgrass differed between leaves with a native microbiome

compared to leaves treated with antibiotics in 2018, or compared to leaves treated with bleach in 2020. This is because in 2018 eelgrass leaves were cut and distributed across each treatment, whereas in 2020 entire eelgrass leaves were used in each treatment. In the 2018 experiment, a linear mixed effects model was constructed to assess whether disease severity in Lz-inoculated eelgrass differed between leaves with a native microbiome and those treated with antibiotics (lmer package, version 1.1-14; Bates et al., 2015); the vortex treatment was not effective in disrupting the microbiome (see below), and thus was not analysed here. Leaf identity was included as a random effect since a piece of each eelgrass leaf was replicated in each of the six treatments. Severity data met the assumptions for equal variances and normality, indicated via visual inspection of plots with fitted and residual values (Zuur et al., 2010). In the 2020 experiment, a Welch's *t*-test was then used to determine differences in severity between bleached and untreated eelgrass inoculated with Lz. Severity data from the 2020 bleach experiment were not normally distributed, as indicated by visually evaluating Q–Q plots. Logit-transformed severity data met the assumption for normality.

For the qPCR data analyses, the pathogen load of diseased and asymptomatic eelgrass collected from a natural meadow in 2020 did not have equal variances, so a Welch's *t*-test was used to compare the mean Lz load between diseased and asymptomatic eelgrass.

Molecular methods

In 2018, microbial DNA was extracted from swabs using the MoBio PowerSoil®-htp 96 well DNA extraction kit (USA) following the manufacturer's recommended protocol. In 2020, DNA was extracted from swabs using a DNeasy PowerSoil Pro extraction kit (Qiagen, USA) following the manufacturer's recommended protocol. Both years included negative controls (sterile swabs, $n = 9$ total). The *16S rRNA* gene was used to characterize bacteria and archaea; archaea were not detected and we refer to only bacteria henceforth. In 2018, DNA extracts were sent to the Integrated Microbiome Resource at Dalhousie University (Halifax, Nova Scotia, Canada) for PCR amplification with barcoded primers targeting the V4-V5 region (515f = GTGYCAGCMGCCGCGGTAA and 926r = CCGYCAATTYMTTTRAGTTT), library preparation, and sequenced with according to standard protocols (Comeau et al., 2017). In 2020, due to facility shutdowns related to the COVID-19 pandemic, PCR amplification and library preparation were performed in house at the University of British Columbia with barcoded primers targeting the V4 region based on Caporaso et al. (2012): 515f (5′-GTGYCAGCMG CCGCGGTAA-3′), tagged with a 12 bp Golay barcode to facilitate sample pooling, and 806r (5′-GGAC-TACNVGGGTWTCTAAT-3′). Each PCR contained 10 μL of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific™, USA), 1 μL of both 515f and 806r primers (final concentration of 10 μM each), 1 μL of 5 μM peptide nucleic acid (PNA) chloroplast blocking primer (PNA Bio Inc., USA; Lundberg et al., 2013), 1 μL of DNA (or 1 μL of molecular-grade water for negative controls), and 7 μL PCR grade water for a final volume of 20 μL. The following thermocycler settings were used to run 25 PCR cycles: denaturation at 98°C for 1 s, PNA annealing at 70°C for 5 s, primer annealing at 50°C for 5 s, and extension at 72°C for 24 s. Each run included an initial denaturation step at 98°C for 10 s and a final extension step of 72°C for 1 min. Gel electrophoresis was used to check for amplicon bands of the target length prior to quantification with the Qubit™ dsDNA HS assay kit (Thermo Scientific™, USA), and pooled at equal concentration. The final pool was purified using a QIAquick PCR Purification Kit (Qiagen, USA). Library quantitation and paired-end Illumina MiSeq sequencing (2 × 300 bp) were performed at the Hakai Institute (Quadra Island, British Columbia, Canada). PCR negative controls (molecular grade water, $n = 2$ total) were included in all sequencing runs.

For the qPCR analyses, published protocols were followed for Lz DNA extraction, creating standard Lz cell curves, and running qPCR analyses (Aoki et al., 2022; Groner et al., 2018, 2021). Briefly, Lz DNA was extracted from diseased and asymptomatic eelgrass samples using a DNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's instructions. Before each extraction, each eelgrass tissue sample was extracted from its microcentrifuge tube using a sterile technique, gently wiped dry on a KimWipe (Kimberly Clark, USA) to remove ethanol, weighed, transferred to a 2-mL XXTuff reinforced microvial (Biospec, USA) with a tungsten bead (Qiagen, USA), and frozen by dropping each vial into a thermos filled with liquid nitrogen. Samples were disrupted for 1 min with a Mini-Beadbeater-16 (BioSpec, USA). All qPCR reactions were run in 96-well microplates. Individual wells contained 20 μL reactions: 10 μL TaqMan Fast Universal PCR Master Mix (Applied Biosystems by Life Technologies, USA), 0.8 μL forward primer (400 nM, Laby_ITS_Taq_f: 5′-TTGAACGTAACATTCGACTTTCGT-3′), 0.8 μL reverse primer (400 nM, Laby_ITS_Taq_r: 5′-ACGCAT-GAAGCGGTCTTCTT-3′), 5.5 μL of molecular-grade water (VWR, USA), 0.4 μL of probe (100 nM, Laby_ITS_probe: FAM-5′-TGGACGAGTGTGTTTTG-3′-MGB-NFQ), 0.5 μL of BSA (20 mg/mL, New England BioLabs, USA), and 2 μL of DNA template (diluted 1:10). The probe was modified from (Bockelmann et al., 2013), consistent with previous Lz qPCR analyses (Aoki et al., 2022; Groner et al., 2018, 2021). Lz cells were used to make six cell standards, ranging from 0.1 to 10^4 cells. Six cell standards were run in

triplicate; samples and negative controls (wells with molecular-grade water, rather than DNA) were run in duplicate. Plates were run on a CFX Connect Real-Time PCR Detection System (BioRad, USA) with the following reaction conditions: 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

Microbiome data processing

Raw, demultiplexed sequencing reads were read into R for processing with the DADA2 pipeline (Callahan et al., 2016) with separate processing for the 2018 and 2020 data. Briefly, the quality of the reads was assessed with the plotQualityProfile function. Primers were then removed and reads were truncated based on their quality with the filterAndTrim function—275 bp (forwards reads) and 200 bp (reverse reads) for 2018 data and 250 bp (forward and reverse reads) for 2020 data; truncation decisions were based on the quality of reads, which varied between sequencing runs, as is standard protocol (Callahan et al., 2016). Then, paired reads were merged using the mergePairs function and a sequence table was constructed for 2018 and 2020 separately. Next, the two sequence tables were merged with the mergeSequenceTables function, which combines all unique ASV sequences into a single table. Sequences differed in length between the 2018 and 2020 datasets because they were generated with different primers; as a result, there are separate ASVs for each year. Finally, taxonomy was assigned to the amplicon sequence variants (ASV) with the DADA2 assignTaxonomy and addSpecies functions (RDP method within DADA2 (Callahan et al., 2016; Wang et al., 2007); trained on the SILVA database (v. 138.2; Quast et al., 2012) that was clustered 99% similarity formatted for DADA2 (McLaren, 2020)); a minimum bootstrap value of 50 was used.

After taxonomic assignment, non-target sequences (eukaryotes, chloroplasts, mitochondria, and taxa unassigned at the domain level) were removed. Samples with less than 10 total reads were removed. This included all 2020 negative controls ($n = 11$). For the 2018 sequencing run, all negative controls ($n = 4$) failed sequencing, indicating minimal contamination during the extraction and PCR steps. Then, ASVs found in two samples or less were removed; counts of two or fewer in the ASV table were converted to 0 to minimize the effect of barcode switching. All remaining samples had at least 1000 reads per sample (Table S2). In cases where taxonomic ranks were blank following taxonomy assignment, taxonomic information was propagated from higher ranks to enable genus-level comparisons (e.g., an ASV identified only to family Saprospiraceae would be listed as unassigned Saprospiraceae). Anaerobic taxa characteristic of the mammalian gut (e.g., Negativicutes, Clostridia) and skin (e.g., Propionibacteria) were detected in two samples from 2018 and suspected to be contaminants from the sequencing facility, as human skin and faecal and murine faecal samples were processed at similar times; they were removed.

The taxonomy table, filtered sequence table (hereafter referred to as the ASV table), and metadata were merged into a phyloseq object ('phyloseq' version 1.34.0; McMurdie & Holmes, 2013). Coverage-based rarefaction was then conducted for alpha diversity analyses (Chao & Jost, 2012). Briefly, the filtered ASV tables were prepared for the package iNEXT (Hsieh et al., 2016) with the metagMisc::prepare_inext function with the singleton correction set to true. Next, the ASV tables were sampled 1000 times with the coverage set to 0.8 (Chao & Jost, 2012; Chiu & Chao, 2016) using the phyloseq_coverage_raref function; all 1000 ASV tables were then averaged to rebuild the rarefied ASV table.

Microbiome data analyses

Statistical analyses of microbiome data were conducted separately for each year because different primer sets were used. Microbiome data from field surveys and lab experiments were also analysed separately because microbial communities change in lab experiments (Pound et al., 2022), and this change was more pronounced in 2018 when the experimental design included the low salinity rinse.

The most relatively abundant taxa at the genus rank were plotted to visually assess differences in the dominant taxa across years for the field samples and across treatments for the 2018 and 2020 lab experiments. The 20 (field) or 15 (2018 lab and 2020 lab) most abundant were calculated separately for each of these three datasets and relative abundance taxa plots were generated in R. Ranked relative abundance was used to define the most abundant taxa. The taxonomy table was grouped according to the genus level and separated by field or lab experiment (year). Within these three data subsets (field, 2018 lab, 2020 lab), the relative abundance of each genus was calculated. Then the 15 genera with the greatest relative abundance within the groups were selected and plotted. In this dataset, this equated to abundances of 0.46% or greater. The taxa plots were made by plotting the relative abundance of these most abundant taxa within a single sample. All of the remaining taxa that were not the most abundant were grouped and plotted as 'other' such that the total relative abundance of each group was summed to 1.

To compare differences in microbial community composition, Bray–Curtis dissimilarity indices were calculated based on rarefied reads and visualized using principal coordinate analysis (PCoA) within the vegan package in R (v. 2.5-7; Oksanen et al., 2013). Permutational multivariate analysis of variance (PERMANOVA) was performed to test for significance using the adonis2

function in the vegan package (Anderson, 2008; McMurdie & Holmes, 2013), along with a pairwise post hoc test using the pairwise. adonis function (v. 0.0.1, Oksanen et al., 2013). The PERMANOVA assumption for equal variance was tested with the betadisper function (Oksanen et al., 2013) and was always met. PERMANOVAs for the field surveys included individual leaf identity (leafID) and leaf health (diseased or asymptomatic) as factors. PERMANOVAs for the lab experiments included the microbiome removal method (2018: antibiotics, vortex, no removal; 2020: bleach, no removal), inoculum (Lz or FSW), and interaction between the removal method and inoculum. Each PERMANOVA was reiterated 999 times.

Species richness was calculated using the specnumber function and analysed using analysis of variance (ANOVAs) and Tukey post hoc tests in R. Mean microbial richness for eelgrass in each treatment group—antibiotics, vortexed, bleach, and untreated—met assumptions for ANOVA, *t*-tests, and Pearson's correlation test. Paired *t*-tests were used to compare the microbial richness of diseased and asymptomatic tissue from a natural meadow. Finally, a Pearson's correlation test was used to determine correlations between severity and bacterial richness among experimental eelgrass, and separately between Lz load and bacterial richness on diseased tissue from a natural meadow. Throughout the analyses, a *p*-value lower than 0.05 was considered as the benchmark to reject the null hypothesis (i.e., no difference between groups).

RESULTS

Microbiome composition analyses

Microbiome composition analyses: Eelgrass from a natural meadow (2018 and 2020)

Microbial communities were significantly different between diseased and asymptomatic eelgrass tissue in spring 2018 (PERMANOVA, pseudo-$F_{1,17} = 3.99$, $R^2 = 0.18$, $p < 0.001$; Figure 2A; Table S3), but not in summer 2020 (Figure 2B; Table S3). The 20 most abundant taxa were fairly consistent between diseased and asymptomatic tissue and across the different sampling years (Figure S1). *Loktanella*, *Sulfitobacter*, and *Maribacter*—commonly associated with seagrasses—were among the most abundant taxa on diseased and asymptomatic eelgrass (Figure S1).

In 2018, disease status (i.e., leaf health) accounted for 18% of the total variance in eelgrass microbial community composition (PERMANOVA, pseudo-$F_{1,17} = 3.99$, $R^2 = 0.18$, $p < 0.001$; Table S3). In 2020, disease status did not account for a significant percentage of variance in the microbiome, though leaf identity did (PERMANOVA, pseudo-$F_{9,19} = 3.41$, $R^2 = 0.74$, $p < 0.001$; Table S3). However, differences in dispersion (variance) of microbial communities were not detected between diseased and asymptomatic eelgrass in either year (2018 field samples: $F_{1,16} = 0.0015$, $p = 0.977$; 2020 field samples: $F_{1,18} = 0.1906$, $p = 0.649$). The microbial richness of diseased eelgrass was significantly greater compared to asymptomatic eelgrass tissue in spring 2018, but richness did not differ between diseased and asymptomatic eelgrass in summer 2020 (paired *t*-test, $t(7) = -3.53$, $p = 0.0096$; Figure 3, Table S4).

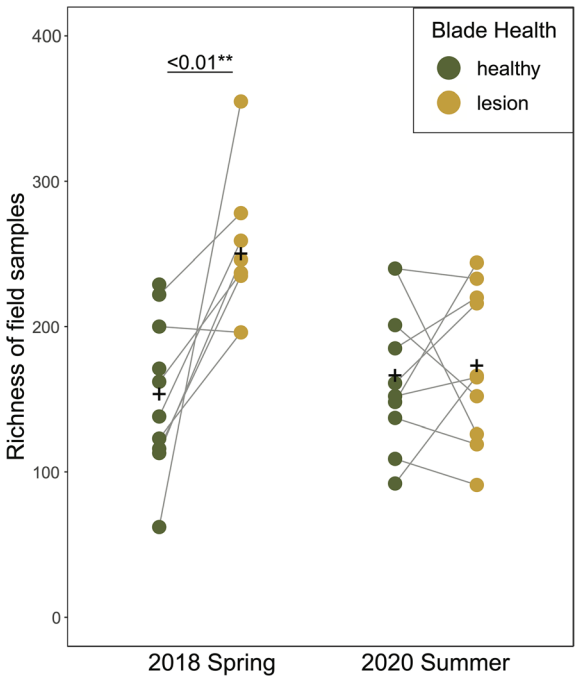

FIGURE 3 Microbial richness varied between diseased and asymptomatic eelgrass in spring 2018, but not in summer 2020 (paired *t*-test; $p = 0.0096$ for 2018; $p = 0.74$ for 2020).

The presence of Lz in eelgrass lesions collected from a natural meadow in 2020 was confirmed via qPCR. Diseased eelgrass had significantly higher pathogen loads compared to asymptomatic eelgrass (Welch's *t*-test, $t(34.6) = 4.98$, $d = 1.31$, $p < 0.0001$; Figure S2). The mean Lz load ($\log_{10}$ cells/mg) was 1.04 ± 0.94 in diseased eelgrass and 0.14 ± 0.24 in asymptomatic eelgrass (mean ± SD). Among eelgrass collected from a natural meadow in 2020, bacterial species richness did not correlate with mean Lz load, contrary to predictions (Pearson's correlation test, $p > 0.05$; Figure S3).

Microbiome composition analyses: Eelgrass from laboratory experiments (2018 and 2020)

Vortexing was not an effective method for microbiome disruption, reflected by the fact that microbial

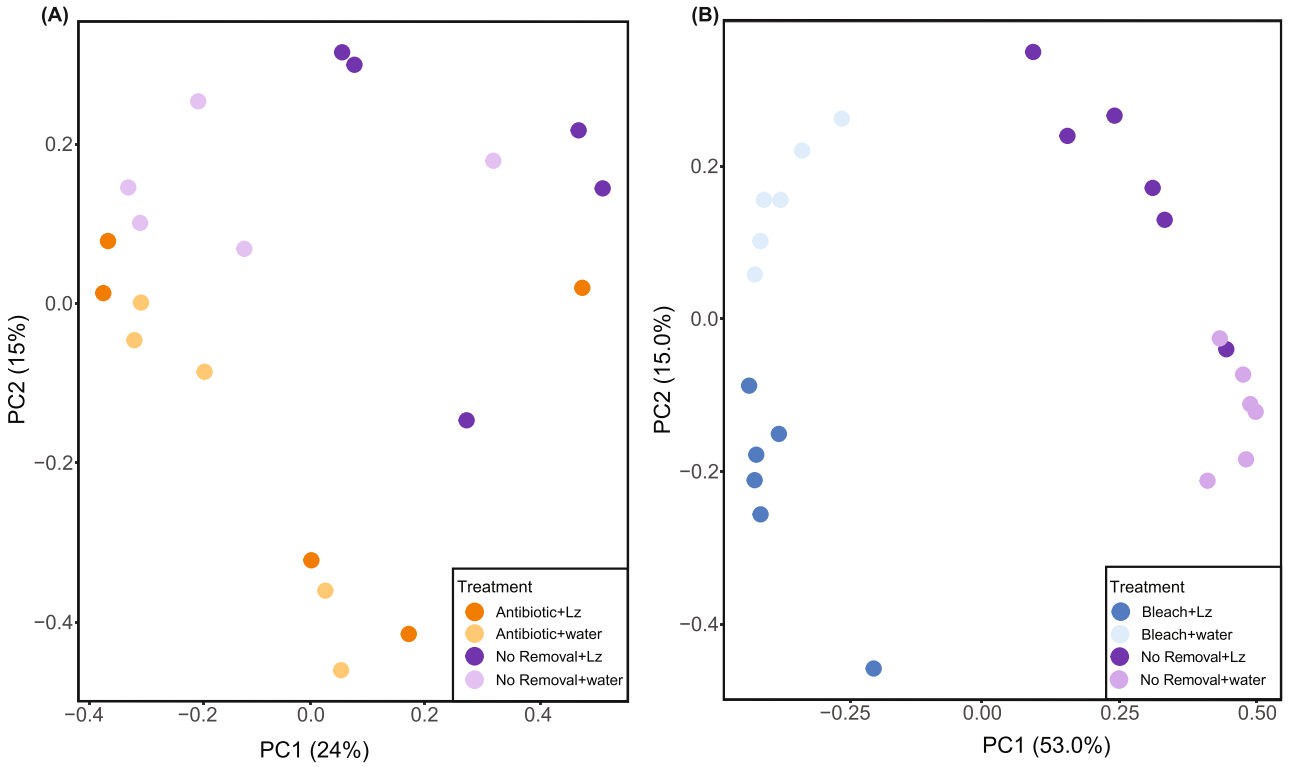

FIGURE 4 Eelgrass-associated microbiota varied significantly by microbiome disruption treatment and inoculum. PCoA plots based on Bray–Curtis dissimilarity indices reflect differences in microbial communities from experiments in (A) 2018 and (B) 2020, sampled at the final time point ($p < 0.014$ in 2018; $p < 0.001$ in 2020).

community composition was not significantly different between untreated eelgrass and vortexed eelgrass in the 2018 experiment (pairwise adonis post hoc test, pseudo-$F_{1,28} = 0.47$, $R^2 = 0.027$, $p = 1.00$; Table S5). Thus, data from the vortex treatment were not included in subsequent disease or microbiome composition analyses.

Microbial community composition strongly differed among eelgrass with and without a native microbiome (Figure 4, Table S5). In 2018, the 15 most abundant bacteria present at the final time point mostly belonged to Flavobacteriales and opportunistic Alteromonadales (Figure S4A; Adamczyk et al., 2022). In 2020, some bacterial taxa previously identified as key constituents of the eelgrass microbiome in other studies (Adamczyk et al., 2022; Beatty et al., 2022; Sanders-Smith et al., 2020; Trevathan-Tackett et al., 2020)—Flavobacteriaceae and *Methylotenera*—and *Glaciecola* were abundant on untreated eelgrass but disappeared in many bleached eelgrass samples (Figure S4B). The two experiments shared some taxa (Figure S4). Among the 15 most abundant bacterial taxa, seven were found in both 2018 and 2020 experiments. *Pseudoalteromonas* and *Marinomonas* had the highest relative abundance in the 2018 and 2020 experimental eelgrass, respectively (Figure S4).

Microbiome disruption treatment accounted for 11% of the total variance in eelgrass microbial community composition in the 2018 experiment (PERMANOVA, pseudo-$F_{1,19} = 2.21$, $R^2 = 0.11$, $p = 0.019$) and 50% of the variance in the 2020 experiment (PERMANOVA, pseudo-$F_{1,23} = 32.78$, $R^2 = 0.50$, $p = 0.001$; Figure 4, Table S5). Inoculum (Lz versus water controls) did not influence eelgrass-associated microbial composition in the 2018 experiment (PERMANOVA pseudo-$F_{1,19} = 1.55$, $R^2 = 0.075$, $p = 0.092$; Table S5), but did in the 2020 experiment (PERMANOVA pseudo-$F_{1,23} = 5.19$, $R^2 = 0.078$, $p = 0.005$; Table S5). Similarly, the interactive effect of the microbiome disruption method and inoculum did not influence the eelgrass microbial communities in the 2018 experiment (PERMANOVA, pseudo-$F_{1,19} = 0.89$, $R^2 = 0.043$, $p = 0.56$; Table S5), though it did in the 2020 experiment (PERMANOVA, pseudo-$F_{1,23} = 8.19$, $R^2 = 0.12$, $p = 0.002$).

Differences in the dispersion of microbial communities were not detected between eelgrass exposed to different microbiome disruption treatments (2018 experiment: $F_{1,18} = 3.51$, $p = 0.079$; 2020 experiment: $F_{1,22} = 1.02$, $p = 0.347$). Likewise, inoculum (FSW or Lz) did not influence microbial dispersion (2018 experiment: $F_{1,18} = 0.35$, $p = 0.57$; 2020 experiment: $F_{1,22} = 0.92$, $p = 0.35$). Bleach significantly reduced bacterial richness in the 2020 experiment (ANOVA, $F_{1,20} = 37.42$, $p < 0.001$), though antibiotics did not in the 2018 experiment (ANOVA, $F_{1,16} = 0.39$, $p = 0.54$; Figure 5; Table S6). Among Lz-inoculated plants in both experiments, microbial richness, and disease

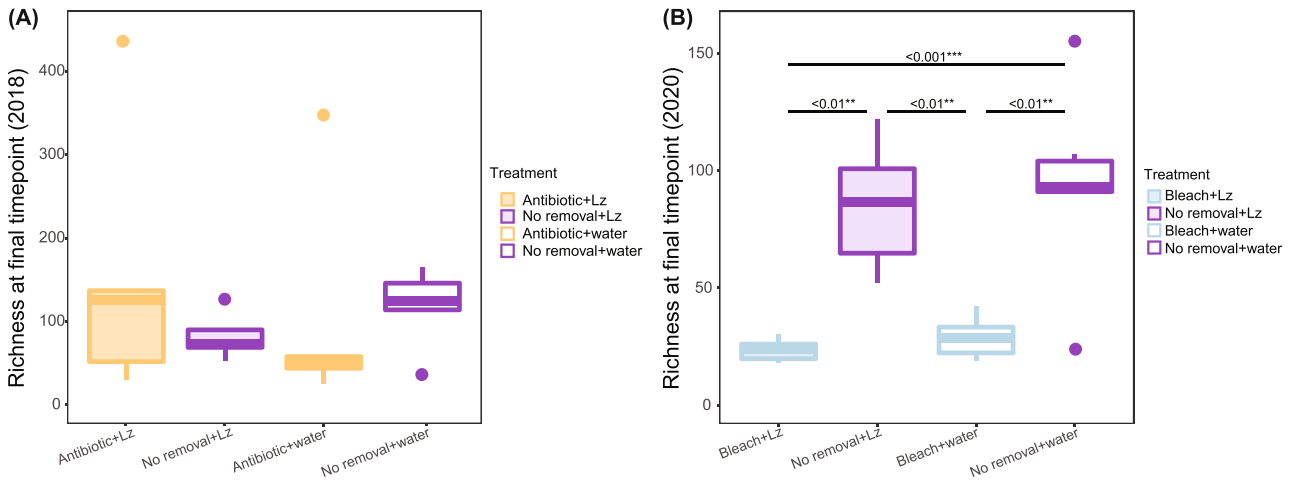

FIGURE 5 The eelgrass-associated coverage-based rarefied microbial richness from experiments in (A) 2018 ($n = 4$–5 leaf pieces/treatment) and (B) 2020 ($n = 6$ leaves/treatment). Richness varied significantly by microbiome disruption treatment in 2020, but not in 2018. Note the different y-axis scales. Asterisks indicate significant differences ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***).

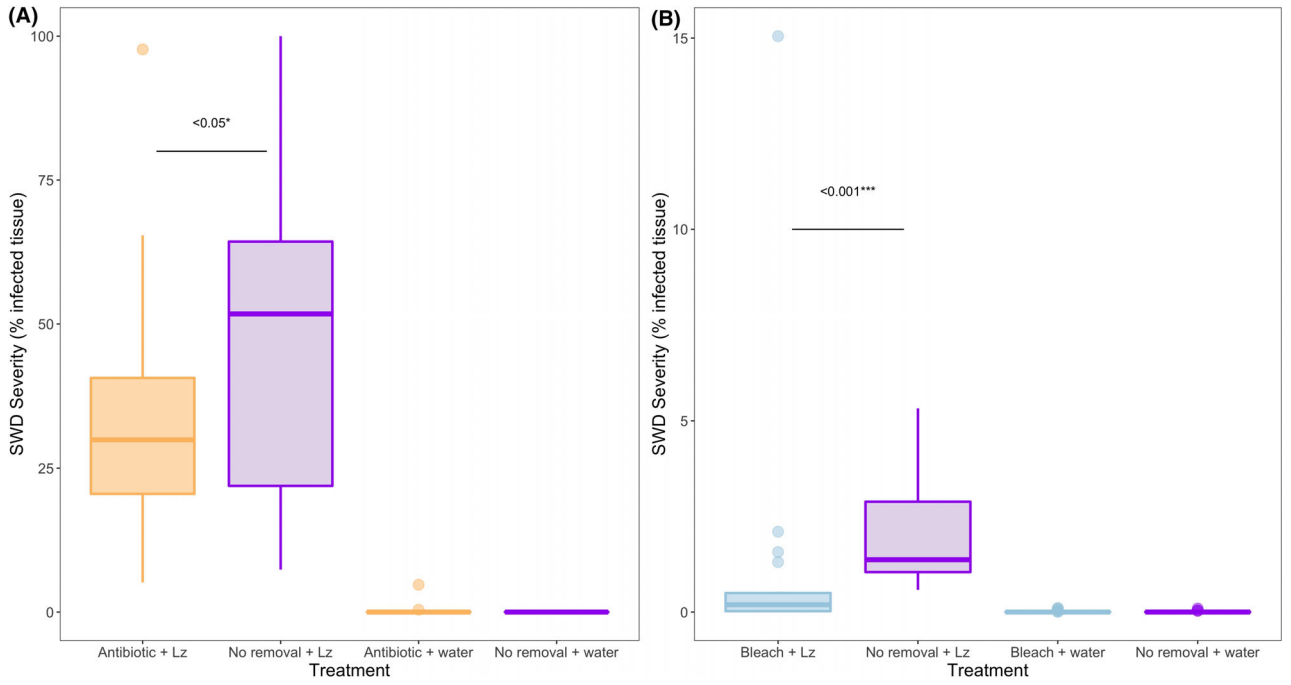

FIGURE 6 Mean disease severity was consistently greater among eelgrass with native microbiomes compared to those reduced with antibiotics or bleach in the (A) 2018 ($n = 24$ leaf pieces/treatment) and (B) 2020 experiments ($n = 28$–29 leaves/treatment). Note the different y-axis scale.

severity were not correlated (Pearson's correlation test, $p > 0.05$; Figure S5A,B).

Disease analysis: Laboratory experiments (2018 and 2020)

Severity is an integrated measure that combines the initial risk of infection with the realized progression of a lesion and is thus the most reliable, single metric of disease impact. Contrary to our second hypothesis, which states that eelgrass with a native microbiome has lower disease severity compared to eelgrass with a reduced microbiome, Lz-inoculated eelgrass with a native microbiome consistently had higher disease severity compared to eelgrass with a reduced microbiome—either when altered by antibiotics or dilute bleach (Figure 6, Table S1). Among Lz-inoculated eelgrass in the antibiotics experiment, mean severity was significantly lower among eelgrass treated with

antibiotics compared to untreated eelgrass (lmer, $t(23) = 2.30$, $p = 0.03$; Table S7, Figure 6A). In the bleach experiment, eelgrass with a native microbiome again had significantly greater disease severity compared to bleach-treated eelgrass (Welch's *t*-test, $t(29.7) = -5.49$, $d = -1.46$, $p < 0.0001$; Figure 6B). The prevalence and number of lesions results are in the Supplementary Materials.

DISCUSSION

Plant-associated bacteria can contribute to disease resistance by secreting antimicrobial compounds or competing for nutrients or space, among other mechanisms (Trivedi et al., 2020; Turner et al., 2013; Vannier et al., 2019). Even on land, the role of plant microbiomes in pathogen defence has been tested against only some types of pathogens, such as bacteria, oomycetes, and fungi (Bais et al., 2004; Berg & Koskella, 2018; Carisse et al., 2003; Chen et al., 2018). Compared to terrestrial systems, interactions between marine microbes and their hosts are even more poorly understood and little is known about how plant-associated microbiomes contribute to disease processes in the ocean. However, recent work suggests that a huge diversity of marine host-associated microbiomes not only influence host health and fitness but also significantly shape ecosystem structure and function (reviewed in Wilkins et al., 2019). Host–microbiome interactions—especially how microbiomes influence disease trajectories from diverse agents—are increasingly important in light of mounting anthropogenic and environmental stressors (Egan & Gardiner, 2016; Mera & Bourne, 2018).

Here, we explored the role of the eelgrass leaf microbiome in defence against the protist *L. zosterae*. Field sampling showed that the presence of disease lesions affected the microbiome in spring 2018, but not in summer 2020, suggesting a changing interaction between microbiome and pathogen and raising the question of how *L. zosterae* interacts with the host microbiome. Based on studies of terrestrial plants and bacterial, oomycete, and fungal pathogens, we expected a defensive role of the eelgrass microbiome against *L. zosterae*, but two experiments demonstrated that plants with a native microbiome were more severely infected than plants with a reduced microbiome. Further, even when experiments were repeated using different microbiome disruption treatments, eelgrass with a native microbiome had higher disease levels than eelgrass treated with antibiotics or bleach. These laboratory experiments indicate the powerful approach of coupled pathogen inoculations and microbiome disruption methods and indicate that changes in the eelgrass microbiome can influence disease outcomes.

Microbiome composition analyses

Microbiome composition analyses: Eelgrass from a natural meadow (2018 and 2020)

Diseased eelgrass had significantly different community structure and higher richness compared to asymptomatic tissue in spring 2018, but not in summer 2020. Seasonal stressors could account for shifts in the eelgrass microbiome and observed differences in microbial communities. The Anna Karenina Principle suggests that stressors—like disease or environmental conditions—have stochastic effects on the composition of host-associated microbial communities (Zaneveld et al., 2017). The summer 2020 collections likely coincided with warmer temperatures and higher natural disease levels compared to the spring 2018 collections, given that high levels of seagrass wasting disease are associated with warmer summer temperatures (Aoki et al., 2022). The combination of seasonally warmer temperatures and higher disease could have altered the eelgrass microbiome on both diseased and adjacent, asymptomatic tissue in the summer. This could account for the similar microbial communities and richness observed between asymptomatic and diseased tissue in the summer of 2020, compared to the different microbial communities and richness in the spring of 2018. These results align with recent summertime analyses also showing the similar microbial richness of diseased and adjacent, asymptomatic eelgrass along the Northeast Pacific (Beatty et al., 2022). The same study also found that eelgrass bacterial communities varied with local sea surface temperatures (Beatty et al., 2022). Microbial communities also differed between diseased and asymptomatic *Zostera muelleri* tissue (Hurtado-McCormick et al., 2021). Our spring 2018 sampling showing a distinct microbiome of diseased tissue shows the need for more detailed time series studies of seagrass microbiomes to investigate when and how the disease may disrupt native microbiomes.

These temporal differences could also be partially attributed to the location swabbed on each leaf and potential bacterial colonization time. In spring 2018, asymptomatic tissue was swabbed at the base of leaves (i.e., newer growth), while in summer 2020, asymptomatic tissue adjacent to lesions was swabbed. Differences in these outcomes again emphasize the value of both wider microbiome sampling along leaf surfaces to investigate the spatial extent over which lesions may influence microbiomes as well as longer, seasonal time series. Variability in microbial community structure may also reflect differences in seasonality and other physical and biological factors that influence the eelgrass microbiome. For example, the composition of the eelgrass leaf microbiome is variable at the meadow scale and may be due to disease prevalence,

temperature, and geography (Beatty et al., 2022). Other studies find variation in the eelgrass microbiome associated with geography (Adamczyk et al., 2022; Bengtsson et al., 2017; Sanders-Smith et al., 2020) and epiphytic algae (O'Connor et al., 2022).

Our qPCR results confirming Lz presence and load in lesioned eelgrass tissue are consistent with previously published work (Aoki et al., 2022; Groner et al., 2018, 2021). The most abundant bacteria identified on diseased and asymptomatic tissue were consistent with those in other eelgrass phyllosphere studies. For example, *Methylotenera*, *Granulosicoccus*, and Rhodobacterales are core members of the eelgrass microbiome (Adamczyk et al., 2022; Sanders-Smith et al., 2020) and are characteristic of eelgrass leaves (Beatty et al., 2022; Crump et al., 2018; Ettinger et al., 2017). The lack of a 'core' microbiome specific to asymptomatic and diseased eelgrass suggests the environment (Segovia et al., 2021)—rather than disease state—is a more significant driver of microbial community composition. Given the close and complex associations between these bacteria and their plant hosts, future work could incorporate metagenomics approaches to clarify the functional roles of specific bacteria.

Microbiome composition analyses: Eelgrass from laboratory experiments (2018 and 2020)

Dilute bleach effectively changed the eelgrass microbiome. This treatment in the 2020 experiment was associated with shifts in community composition and reduced bacterial richness. Antibiotics caused a less pronounced shift in community composition in the 2018 experiment but no significant change in richness. Vortexing was not effective in manipulating the eelgrass microbiome. In our studies, bleach was the most effective method for reducing the eelgrass microbiome. Though treatments were bigger drivers of microbial composition than inoculum (Lz or FSW), inoculum accounted for significant variation in eelgrass microbial composition in 2020, but not in 2018. Future experiments investigating whether this is a result of Lz eating or inhibiting particular bacteria may shed light on the mechanism by which the native microbiome facilitates Lz disease severity. Tracking infection and the eelgrass microbiome over time could provide further insights into how Lz influences the eelgrass microbiome.

The dramatic differences in the most abundant bacterial taxa between treated and untreated plants in 2020 reflect the efficacy of the bleach treatment in disrupting the eelgrass microbiome (Figure S4). Untreated eelgrass in 2020 had a greater number of most abundant taxa compared to bleached eelgrass and may be more representative of the native eelgrass microbiome. In 2020, untreated eelgrass harboured many taxa characteristic of seagrasses (e.g., *Methylotenera* and *Sulfitobacter*) in addition to Alteromonadales. *Marinomonas* and *Pseudoaltermonas* were the most abundant genera in bleached eelgrass at the end of the 2020 experiment, suggesting they are especially resilient to disturbance (Espinosa et al., 2010; Ettinger & Eisen, 2020). In the 2018 experiment, all leaves were incubated in low salinity (10 ppt) FSW prior to experimental treatments to remove naturally occurring Lz; this may have altered the microbiome. Eelgrass-associated bacterial communities in the 2018 experiment were dominated by *Loktanella* and Flavobacteriaceae, taxa associated with seaweed disease and known to degrade seagrass and seaweed polysaccharides (Zhang et al., 2020). This community makes sense given the disruption to the microbiome associated with the low salinity incubation in 2018.

We compared lab results to expectations of disease-induced shifts in the microbiomes of other marine organisms (Zaneveld et al., 2017). We anticipated that more severe Lz infections would be associated with altered bacterial richness. However, there was no association between disease severity and bacterial richness among eelgrass leaves in the lab experiment. Another expectation would be that increased disease severity could be associated with increased bacterial dispersion (beta diversity) of eelgrass in different microbiome disruption treatments or from a natural meadow. We did not find associations between disease and bacterial community dispersion in eelgrass from the lab experiment or a natural meadow.

While eelgrass microbial communities likely differ between plants from field and lab conditions, we noted similarities in major bacterial constituents. For example, the most abundant eelgrass bacteria from the experiments and field were shared taxa, including Altermonadales (*Glaciecola*), which is easily culturable; Rhodobacteriales (*Loktanella* and *Sulfitobacter*), which are characteristic of the eelgrass microbiome (Adamczyk et al., 2022; Beatty et al., 2022; Crump et al., 2018; Ettinger et al., 2017; Sanders-Smith et al., 2020); and the generalist Flavobacteriales (*Flavobacteriacea*), which is common on marine surfaces (Sanders-Smith et al., 2020).

Disease analysis: Laboratory experiments (2018 and 2020)

We experimentally altered the eelgrass microbiome to determine its role in pathogen defence. Disease severity was significantly higher in eelgrass with a native microbiome than in eelgrass treated with either antibiotics or bleach. The similarity in response to antibiotics and dilute bleach treatments, and our rigorous attention to the rinses following microbiome reductions as explained in the methods, allows us to discount the

likelihood that the treatments could have directly inhibited Lz infection, rather than indirectly by altering the microbiome. The finding in both iterations of this experiment that plants with a native microbiome are more susceptible to disease than those with a reduced microbiome is counter to our second hypothesis that the eelgrass microbiome facilitates Lz resistance and to previous work indicating the beneficial role of plant microbiomes in pathogen defence (Berendsen et al., 2018; Berg, 2009; Berg & Koskella, 2018; Ford & King, 2016; Pieterse et al., 2014; Trivedi et al., 2020). The increased susceptibility of the plants with a native microbiome compared to a reduced microbiome suggests unexpected ways that Lz interacts with the eelgrass microbiome.

Although the process by which the eelgrass microbiome might facilitate infection remains to be determined, we propose a potential explanation. Certain members of the native eelgrass microbiome may facilitate infection. Lz is also a known saprophyte and could consume eelgrass-associated bacteria as a food source, an idea previously suggested (Armstrong et al., 2000; Pokorny, 1967; Porter, 1972, 1990). When grown in axenic cultures, *Labyrinthula* sp. consume diatoms, bacteria, and yeast (Amon, 1968; Jepps, 1931; Perkins, 1973; Porter, 1972; Sakata & Fujisawa, 1996; Watson, 1957; Young, 1943), which they lyse with ectoplasmic networks. In lab trials, *Labyrinthula* sp. cultured with bacteria grew faster than non-bacteria controls (Armstrong et al., 2000). Microbiome–pathogen work in other systems also suggests that microbial metabolites can provide food for pathogens (Tovaglieri et al., 2019). Our results also align with recent work that suggests some commensal bacteria on eelgrass may facilitate wasting disease by degrading plant cellulose or overrunning the plant immune response (Beatty et al., 2022). More broadly, these results indicate Lz could play a key role in marine microbial food webs if it uses eelgrass-associated bacteria as a food source in natural meadows (Porter, 1990; Wahid et al., 2007). From a broader ecological perspective, this could mean that under certain environmental conditions (e.g., different salinity, temperature, nutrient inputs, pH), eelgrass could have a different microbiome that would then be more resistant to Lz infection, compared to eelgrass with a stable, core microbiome that could potentially serve as a food source for Lz. For example, we would normally expect that environmental stressors like extreme temperatures or high nutrient inputs would compromise eelgrass health. In the context of microbiome-Lz interactions, though, atypical environmental conditions may favour eelgrass because such conditions may disrupt the microbiome, thereby altering a potential food source for the opportunistic Lz. Of course, it is challenging to make generalized statements about expectations for these tripartite interactions across the range of environmental conditions in which eelgrass persists. Furthermore, these results raise new questions about how microbiome interactions might change with different types of pathogens or changing environmental conditions and are a reminder that we should be cautious of generalized expectations that microbiomes are beneficial or effective defences against different pathogens.

Broader impacts and next steps

Bacteria perform essential functions for plants and animals. While most studies show microbiome-mediated pathogen defence (Berg et al., 2017; Berg & Koskella, 2018; Carisse et al., 2003; Ford & King, 2016), host-associated microbes can also facilitate infection (Stevens et al., 2021). Indeed, microbiomes vary greatly over long and short timescales in response to selection from the host (Foster et al., 2017), physical conditions, and competition with other microbes, including episodic, new invaders like Lz. Given the complexity of interactions between hosts, microbiomes, and pathogens, experimental studies are needed to decouple mechanisms by which the host microbiome may support or hinder infection. This is especially true in light of climate change, which can impact all components of disease dynamics (Harvell et al., 2002) and microbial assemblages and functions (Hutchins et al., 2019).

Our experiments indicate that eelgrass-associated bacteria may facilitate infection by the protist pathogen Lz. This highlights the complexity of plant–microbe–pathogen interactions and underscores the need to understand the functional role of eelgrass microbial communities in disease resistance, especially in the face of mounting anthropogenic stressors: habitat loss, stress and collapse from climate change, and increased frequency of seagrass wasting disease outbreaks (Aoki et al., 2022, 2023; Burge et al., 2013; Cullen-Unsworth & Unsworth, 2018; Dunic et al., 2021; Groner et al., 2021; Serrano et al., 2021; Short et al., 2016; Strydom et al., 2020; Sullivan et al., 2018). Consequently, determining which factors support a healthy microbiome and how host microbiomes interact with diverse disease agents—from viruses to bacteria and protists—is crucial for the sustainability of seagrasses and other coastal foundation species (Trevathan-Tackett et al., 2019; Wilkins et al., 2019), given their essential ecosystem roles and promising natural solutions against climate change (Serrano et al., 2019). Specifically, future studies could combine metagenomics and metabolomics approaches to identify defensive functions and metabolic properties of phyllosphere bacteria (Bass et al., 2019). Understanding how to culture or recruit beneficial microbes involved in host defence could be a valuable biocontrol tool for managing and sustaining eelgrass meadows

facing disease and other environmental stressors (Tarquinio et al., 2019; Trevathan-Tackett et al., 2019). Microbial-based solutions have been proposed for disease management in other marine systems, including fish aquaculture and corals (Bentzon-Tilia et al., 2016; Rosado et al., 2019) and are used for agriculture (Berg, 2009; Berg et al., 2017; Busby et al., 2017; Erlacher et al., 2014) and amphibians (Woodhams et al., 2016). There is an urgent need for innovative research that spans disciplines and approaches to sustain global seagrass meadows (Tarquinio et al., 2019; Trevathan-Tackett et al., 2019). For example, coupling microbial omics-based tools and remote sensing approaches may allow for accurate predictions of climate-driven microbial shifts and disease outbreaks, which could have ecosystem-wide impacts (Beatty et al., 2021). Fundamental research identifying beneficial and antagonistic microbes—and their functions and interactions with pathogens—are crucial before these applications can be fully realized for the eelgrass-Lz and other marine systems (Bentzon-Tilia et al., 2016).

AUTHOR CONTRIBUTIONS

Olivia J. Graham: Conceptualization (lead); data curation (equal); formal analysis (supporting); funding acquisition (equal); investigation (lead); methodology (lead); project administration (lead); resources (equal); supervision (lead); visualization (equal); writing – original draft (lead); writing – review and editing (lead). **Emily Adamczyk:** Conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (supporting); writing – original draft (equal); writing – review and editing (equal). **Siobhan Schenk:** Data curation (equal); formal analysis (lead); methodology (equal); software (equal); visualization (equal); writing – review and editing (equal). **Phoebe Dawkins:** Investigation (supporting); supervision (supporting). **Samantha Burke:** Investigation (supporting). **Emily Chei:** Investigation (supporting). **Kaitlyn Cisz:** Investigation (supporting). **Sukanya Dayal:** Investigation (supporting). **Jack Elstner:** Investigation (supporting). **Arjun Lev Pillai Hausner:** Investigation (supporting); supervision (supporting). **Taylor Hughes:** Investigation (supporting). **Omisha Manglani:** Investigation (supporting). **Miles McDonald:** Investigation (supporting). **Chloe Mikles:** Investigation (supporting). **Anna Poslednik:** Investigation (supporting). **Audrey Vinton:** Investigation (supporting). **Laura Wegener Parfrey:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); investigation (supporting); methodology (equal); resources (equal); software (lead); visualization (supporting); writing – review and editing (equal). **C. Drew Harvell:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); methodology (equal); resources (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available in online repositories. Sequences and MiMarks-compliant metadata are available via the European Nucleotide Archive accession number PRJEB51437: https://www.ebi.ac.uk/ena/browser/view/PRJEB51437. Disease data (severity, prevalence, number of lesions, qPCR) and R scripts are available via Cornell University's eCommons: https://doi.org/10.7298/qhwv-eq20.

ORCID

Olivia J. Graham https://orcid.org/0000-0003-3834-264X

Siobhan Schenk https://orcid.org/0000-0003-0572-9303

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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