

## ORIGINAL ARTICLE

# Development and evaluation of a formulation of probiont *Phaeobacter inhibens* S4 for the management of vibriosis in bivalve hatcheries

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## Abstract

Larval eastern oysters (*Crassostrea virginica*) grown in shellfish hatcheries are susceptible to bacterial diseases, particularly vibriosis. Probiotics are microbes that confer health benefits to the host and have been identified as promising tools to manage diseases in aquaculture. The marine bacterium *Phaeobacter inhibens* S4 (S4) protects larval eastern oysters against challenge with the bacterial pathogen *Vibrio coralliilyticus* RE22 (RE22). A concentrated liquid formulation of probiont S4 that maintained high cell viability after long-term storage was developed for commercial use in shellfish hatcheries. The safety and efficacy of the formulation were tested in six different trials in two hatcheries. The S4 formulation was added to *C. virginica* larvae culture tanks daily at 10<sup>4</sup> colony forming units (CFU)/mL from Day 1 post fertilisation until Day 6, 12 or 14, depending on the trial. Treatment of larvae in the hatchery with the S4 formulation did not significantly affect the survival and growth of the larvae. Formulated probiont S4 treatment in the hatchery led to a significant increase in relative percent survival (RPS) when larvae were subsequently challenged with the pathogen RE22 (10<sup>5</sup> CFU/mL) for 24 h in a laboratory challenge as compared to probiotic-untreated RE22-challenged larvae (RPS increase of 46%–74%,  $p < 0.05$ ). These results suggest that this novel S4

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formulation is a safe, easy-to-use and effective tool in preventing larval losses due to vibriosis in hatcheries.

#### KEYWORDS

bivalve, *Crassostrea virginica*, formulation, hatchery, larvae, probiotic, vibriosis

## 1 | INTRODUCTION

The eastern oyster, *Crassostrea virginica*, is a bivalve species with significant ecological and economic importance to the Gulf of Mexico and Atlantic coastal communities of North America (Azra et al., 2021; Grabowski et al., 2012). Oyster production through aquaculture in the United States totaled 219 million dollars (USD) in 2018 (NOAA Fisheries, 2019). Hatchery production of oyster seed is crucial for ensuring a constant and sufficient supply of juveniles to support the oyster industry. However, changes in environmental conditions and disease outbreaks are limiting factors for the growth of aquaculture production (Stentford et al., 2012). Vibriosis, a disease caused by pathogenic bacteria in the genus *Vibrio*, has been an issue of particular concern in bivalve hatcheries. Various strains of *Vibrio* spp. that are pathogenic to oyster larvae lead to a rapid and high rate of larval mortality in hatcheries, resulting in substantial economic loss to the oyster industry (Dubert et al., 2017; Elston, 1993; Elston et al., 2008; Richards et al., 2015). Techniques for managing disease outbreaks in hatcheries include the use of water treatment systems (e.g., filtration, ultraviolet light and pasteurisation) and labour-intensive biosecurity measures (e.g., cleaning of equipment) to avoid the introduction and spread of pathogens (Dubert et al., 2017). Antibiotic usage in bivalve shellfish hatcheries is discouraged because of the potential development of resistance by bacteria and negative impacts on healthy oyster microbiota (Lokmer et al., 2016; Prado et al., 2010). Despite significant efforts to treat the water supply, pathogenic vibrios are still detected in shellfish hatcheries and may cause shellfish mortality in opportunistic conditions (Dubert et al., 2017).

The use of probiotics has emerged as a potential tool to reduce mortalities in the rearing of aquatic organisms and manage disease outbreaks in aquaculture (Cruz et al., 2012; Newaj-Fyzul et al., 2014; Verschuere et al., 2000; Yeh et al., 2020). Probiotics are defined as live, non-pathogenic microorganisms that, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2006). In aquaculture, probiotics are administered as either a food supplement or as an additive to the water (Cha et al., 2013; Gioacchini et al., 2010; Hai, 2015; Zhou et al., 2009). Candidate probiotics for use in invertebrate aquaculture include a variety of gram-negative and gram-positive bacteria, yeast and unicellular algae. Depending on the probiotic species used, these health benefits are derived from a variety of complementary mechanisms including improvement of water quality, enhancement of host nutrition through the production of supplemental digestive enzymes, competition with pathogenic bacteria, production of antimicrobial compounds, host immunomodulation and

modulation of microbial community structure to promote health (Cruz et al., 2012; Kesarcodi-Watson et al., 2008; Macey & Coyne, 2005; Modak & Gomez-Chiari, 2020; Nandi et al., 2018; Nayak, 2010).

The marine bacterium *Phaeobacter inhibens* S4 (S4) is a gram-negative alpha-Proteobacterium in the *Rhodobacteraceae*. Several *Phaeobacter* spp. and other members of the *Roseobacter* group exhibit inhibitory activity against a wide variety of marine pathogens such as *Vibrio coralliilyticus* RE22, *V. anguillarum*, *V. tubiashii* and *Alliroseovarius crassostreae* (Belas et al., 2009; D'Alvise et al., 2012; Grotkjær et al., 2016; Karim et al., 2013; Sonnenschein et al., 2021) and have been shown to effectively colonize surfaces by forming dense biofilms (Zhao et al., 2016). Previous studies have also demonstrated the probiotic ability of probiont S4 to prevent larval eastern oyster mortality against bacterial infection in laboratory and hatchery experiments (Karim et al., 2013; Sohn et al., 2016a). Mechanisms of S4 protection include biofilm formation, secretion of the antibiotic tropodithietic acid, quorum quenching by which S4 represses gene expression of virulence factors in the shellfish pathogen *V. coralliilyticus* RE22 and host immune modulation (Modak & Gomez-Chiari, 2020; Zhao et al., 2016, 2019).

Although probiont S4 demonstrated promising results for limiting *V. coralliilyticus* infections in bivalve aquaculture hatcheries, the probiont needs to be delivered daily to be effective (Sohn et al., 2016a, 2016b), and daily preparation of fresh cultures in the hatchery is impractical (personal communication with hatchery personnel). A standardised, stable, commercially produced formulation of probiont S4 would offer advantages such as ease and convenience in storage, handling and delivery at the hatchery. Commercially formulated probiotics mostly include dry products such as wettable powders, dusts, granules and liquid products such as cell suspensions in water, oils and emulsions (Cruz et al., 2012). Most commercial probiotics available in the market for aquaculture are formulated from a mixture of gram-positive bacteria that show high survival after freeze-drying. Examples include Prosol (*Bifidobacterium longum*, *Lactobacillus acidophilus*, *L. rhamnosus*, *L. salivarius* and *L. plantarum*), Engest Probiotics (*Bacillus subtilis*, *B. licheniformis* and *B. megaterium*) for shrimp (Gupta & Dhawan, 2011; Nisar et al., 2022) and Bioplus (*B. subtilis* and *B. licheniformis*) for rainbow trout (Bagheri et al., 2008). To the best of our knowledge, the only gram-negative bacteria commercially formulated is Eco-Pro (*Rhodospseudomonas palustris*), used for improving water quality in ponds (Hasan & Banerjee, 2020).

Based on previous research showing the efficacy and safety of using daily treatments of freshly grown cultures of *P. inhibens* S4 as a probiont in bivalve larval culture, both in laboratory and hatchery experiments

(Karim et al., 2013; Sohn et al., 2016a, 2016b), the present study developed a novel liquid formulation for the gram-negative bacterium S4, allowing for ease of routine application in the hatchery at a commercial scale. This novel liquid formulation was tested for its safety, efficacy, host protection, ease in handling and delivery in bivalve hatchery facilities. The results demonstrate that the formulation showed similar performance in the hatchery as previously reported for freshly cultured S4 (Sohn et al., 2016a, 2016b) and pre-treatment in the hatchery consistently protected eastern oyster larvae from experimental challenge with the bacterial pathogen *V. coralliilyticus* RE22.

## 2 | MATERIALS AND METHODS

### 2.1 | Bacterial strains

Bacterial strains *P. inhibens* S4Sm (probiotic, named S4 thereafter) and *V. coralliilyticus* RE22Sm (pathogen, RE22; both are streptomycin-resistant strains by spontaneous mutation) were maintained as stocks in 50% glycerol at  $-80^{\circ}\text{C}$  until use. Bacteria were cultured on yeast peptone with 3% sea salt (mYP30) media (5 g/L of peptone, 1 g/L of yeast extract, 30 g/L of ocean salt (Red Sea Salt) at  $27^{\circ}\text{C}$  with shaking at 175 rpm as described in Karim et al. (2013) unless otherwise indicated.

### 2.2 | Development of a liquid probiotic formulation for *P. inhibens* S4

Initial trials in the development of spray-dried or freeze-dried (lyophilised) formulations for *P. inhibens* S4Sm using mannitol or sucrose were not successful (Dao, 2015). Therefore, a liquid formulation was developed. Bacteria from glycerol stocks stored at  $-80^{\circ}\text{C}$  were streaked for isolation on a mYP30 agar plate and incubated at  $27^{\circ}\text{C}$  for 24–48 h. A single S4Sm colony was inoculated into Luria Broth with 3% sea salt (mLB30, pH 7) or mYP30 growth medium and incubated at  $27^{\circ}\text{C}$  with shaking for 48 h, until reaching stationary phase ( $\sim 10^9$  colony forming units [CFU]/mL). Four different formulation methods were tested for viability after storage for 6 weeks: (1) S4 mLB30 stationary broth undiluted cultures stored without shaking at  $4^{\circ}\text{C}$  (LB\_4); (2) S4 mLB30 stationary broth cultures diluted 1:1 with 3% filtered sterile artificial seawater (FSSW) and stored at  $4^{\circ}\text{C}$  (LB\_SW\_4) or (3)  $22^{\circ}\text{C}$  (LB\_SW\_22) and (4) S4 mYP30 stationary broth cultures diluted 1:1 with FSSW and stored at  $4^{\circ}\text{C}$  (YP\_SW\_4). The viability of the formulations was determined at 0, 2, 4 and 6 weeks in each of the storage conditions by spot plating serial dilutions in triplicate on mYP30 agar plates and counting CFU (CFU/mL) after 24–48 h of growth (Zhao et al., 2016). Culture and formulation of *P. inhibens* S4 were scaled up for commercial production by Kennebec River Biosciences using proprietary methods for large-scale bacterial production and the liquid formulation protocol reported here (i.e., dilution of stationary high titer S4 mYP30 cultures 1:1 in FSSW).

### 2.3 | Laboratory challenge of larvae treated with freshly cultured or formulated S4 probiotic

Laboratory challenge assays were conducted following protocols described by Karim et al. (2013). Briefly, eastern oyster larvae (7 days post fertilisation- [dpf],  $100\text{--}150\ \mu\text{m}$  in size) were obtained from the Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science (VIMS) hatchery. Oyster larvae (50–100 per well) were placed into 6-well plates (three replicates per treatment) with 5 mL of FSSW (28 Practical Salinity Units [PSU]). Larval oysters were fed with commercial algal paste (20,000 cells/mL; Reed Mariculture Inc.) prior to the addition of probiotics to enhance the ingestion of probiotics. Freshly cultured or formulated S4 was added to larvae in wells designated for each probiotic treatment at a concentration of  $10^4$  CFU/mL and incubated at room temperature with gentle shaking. After 24 h, the pathogen RE22 was added to each well at a final concentration of  $10^5$  CFU/mL. Control wells included unchallenged larvae (no S4 or RE22) and larvae incubated with S4 but without the pathogen. Each treatment was run in triplicate. Larval survival was determined 24 h after the pathogen was added using the neutral red technique (Gómez-León et al., 2008). Survival was calculated by using the formula: Survival (%) =  $100 \times (\text{number of live larvae} / \text{total number of larvae})$ . The relative percent survival (RPS) of probiotic pre-treated and RE22-challenged larvae, compared to the RE22-challenged control, was calculated using the formula: RPS (%) =  $[1 - (\% \text{ Mortality treatment} / \% \text{ Mortality control})] \times 100$  (Karim et al., 2013).

### 2.4 | Hatchery trial set up

Hatchery experiments were conducted at the Aquaculture Genetics and Breeding Technology Center, VIMS hatchery and Mook Sea Farms (MOOK) hatchery. At VIMS, four independent trials (Trials 1–4) were run with 60 L flat-bottom larval rearing tanks used for each trial (Table 1). Tanks (minimum of three per treatment) were randomly assigned to the following treatments: no probiotics (control) or S4 formulation (probiotic treatment). Broodstock was spawned at the hatchery using standard techniques for each trial, and experiments were initiated by adding  $3.6 \times 10^5$  to  $6 \times 10^5$  larvae (6.2–10 larvae/mL) to each static tank on Days 1–2 post fertilisation. Larvae in each tank were fed with a hatchery-reared microalgal diet consisting of *Pavlova pingus*, *Chaetoceros negrocile* and *Tetraselmis chui*. Trials 5 and 6 were conducted at MOOK. In Trial 5,  $5.2 \times 10^7$  larvae (17.3 larvae/mL) were raised in each of two single 3000 L static tanks (one control, one treated with S4) from Days 1 to 8 post fertilisation, and then larvae from each tank were distributed into  $3 \times 200$  L flowthrough tanks from Days 9 to 12. In Trial 6 at MOOK, larvae were raised in 15 L buckets from Days 1 to 12. Larvae in each tank at MOOK were fed with a hatchery-reared microalgal diet consisting of *P. lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), *Tisochrysis lutea* (CCMP1324) and *C. muelleri* (CCMP1316). The probiotic formulation was added daily at a dose of  $10^4$  CFU/mL at the time of algal feeding from Day 1 (24 h post

**TABLE 1** Hatchery trials performed in this study

Trial	Hatchery	Tanks per treatment	Trial length (dpf)	Trial date	Data collected for each trial		
					Survival/growth	Culturable vibrios on TCBS	Pathogenic challenge (RE22)
1	VIMS	Control = 3; S4 = 6	12	June 2019	Yes	–	–
2	VIMS	Control = 3; S4 = 3	6	July 2019	Yes	Yes	–
3	VIMS	Control = 4; S4 = 4	14	May 2020	Yes	Yes	Yes
4	VIMS	Control = 4; S4 = 4	7	June 2020	Yes	Yes	Yes
5	MOOK	Control = 1; S4 = 1 (static, Days 1–8) Control = 3, S4 = 3 (flowthrough, Days 9–12)	12	January 2021	Yes	–	–
6	MOOK	Control = 4; S4 = 4	12	June 2021	Yes	Yes	Yes

Abbreviations: Control = no probiotic provided; dpf, days post fertilisation; MOOK, Mook Sea Farms; RE22 = *Vibrio coralliilyticus* RE22; S4, *Phaeobacter inhibens* S4 formulation added at  $10^4$  CFU/mL; TCBS, thiosulfate-citrate-bile salts-sucrose medium; VIMS, Virginia Institute of Marine Science.

fertilisation) until the termination of the trial. Daily dosage of the probiotic was based on a previous study (Karim et al., 2013) showing that the length of protection conferred to the eastern oyster larvae exposure to the probiotic is 24 h.

The trials ended at Day 12 or 14 (immediately prior to larval setting, Trials 1, 3, 5 and 6) or earlier if larval performance was low (6 days, Trial 2) or the hatchery needed the tanks for routine production (7 days, Trial 4). The proportions and species of microalgae diet fed to larvae in the hatchery differed with the age of the larvae. Larval tanks were drained down every other day for size grading of larvae, cleaning of tanks and for maintenance of water quality (Helm et al., 2004). Environmental data (temperature, pH and salinity) were also measured from tank water daily in all the trials (Table S1).

## 2.5 | Evaluation of the effect of S4 formulation on larval growth and survival during hatchery trials

Data for each trial were collected every 2 days during the trial period at the time of drain down. Larvae from each tank were collected on nylon mesh screens, rinsed and transferred to 150 L tanks filled with 100 L in Trial 5 and 400 mL beakers filled with filtered seawater to 200 mL in Trials 1, 2, 3, 4 and 6. At MOOK, after gentle stirring of the water to evenly distribute larvae, a micropipette was used to collect six 1000  $\mu$ L larval samples into 6-well plates and then immobilised with 70% isopropyl alcohol and counted using a dissecting microscope. Larval sizes were estimated based on the percentage of larvae retained on standard mesh sizes (325, 270, 230, 200, 170, 140, 120, 100, 80 and 75  $\mu$ m; Mook and VIMS) and by image analysis using ImageJ (VIMS only). For image analysis and health assessments, a micropipette was used to collect four 50  $\mu$ L larval samples. Each sample was placed on a gridded Sedgewick Rafter counting cell installed on the microscope stage. Larvae were initially observed under a 4x objective for motility, overall shape and gut coloration to make a health assessment and

were assigned a health rating from 1 (poor health) to 3 (good health; Table S1). Larvae were then temporarily immobilised with a 2:1 mixture of freshwater and 70% isopropyl alcohol. Larvae were counted under a microscope and percentage survival was calculated and recorded. Larval sizes were observed under 10x objective magnification and an ocular micrometre was used to measure the longest axis of each larval shell length. Larval specific growth rate (SGR) at the end of each trial was calculated from the larval sizes using the formula: SGR (Specific growth rate (% per day)) =  $((LnLt - LnLo)/t) \times 100$  where  $LnLt$  =  $\ln$  final shell length ( $\mu$ m),  $LnLo$  =  $\ln$  initial shell size ( $\mu$ m), and  $t$  = time (days) (Nimrat et al., 2011).

## 2.6 | Determination of levels of *Vibrio* spp. in hatchery larval samples

The total number of culturable *Vibrio* spp. was determined for trials 2, 3, 4 and 6 using a plate count method on thiosulfate-citrate-bile salts-sucrose medium (TCBS, Difco; Sohn et al., 2016a). Samples were collected from water in the rearing tank (10 mL) and larval oysters (~1000) during drain-down events in the hatchery. Oyster larvae were rinsed with FSSW, homogenised using a sterile pestle and suspended in 1 mL FSSW. Samples were serially diluted and 10  $\mu$ L of each dilution were spot-plated on TCBS agar plates in triplicate. The inoculated plates were incubated for 16–20 h at 28°C, and colonies were counted. Results were expressed as CFU/mL.

## 2.7 | Laboratory pathogen challenge of probiotic-treated larvae from hatchery

Since pathogens could not be introduced into the hatcheries, a subsample of about 1000 larvae from each tank was collected during drain-down events and shipped overnight on ice (kept cool, not frozen) to the laboratory at the University of Rhode Island. Oyster larvae

(~50–100 per well) were placed in 6-well plates and challenged with the pathogen RE22 at a final concentration of  $10^5$  CFU/mL following the methods described in the laboratory challenge section above. Controls included three wells of non-challenged larvae per tank and treatment.

## 2.8 | Statistical analysis

All statistical analyses were performed in the R statistical computing environment, version 4.0.2 (R Development Core Team, 2019). Data were checked for normality and homogeneity of variance prior to selection of the statistical method. The effect of formulation on S4 viability and on the effect of S4 treatment in the hatchery on concentration of culturable vibrios in the hatchery was evaluated using one-way Analysis of Variance (ANOVA). The effect of trial and probiotic treatment on growth (SGR) and percent in the hatchery, as well as the effect of probiotic treatment in the hatchery on larval survival after challenge with pathogen RE22, was analysed using generalised linear models (GLMs). A  $p$ -value  $\leq 0.05$  was considered to be statistically significant.

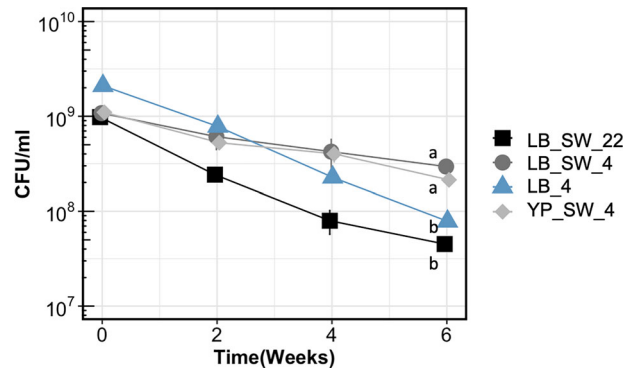
## 3 | RESULTS

### 3.1 | Viability of formulated S4 under various storage conditions

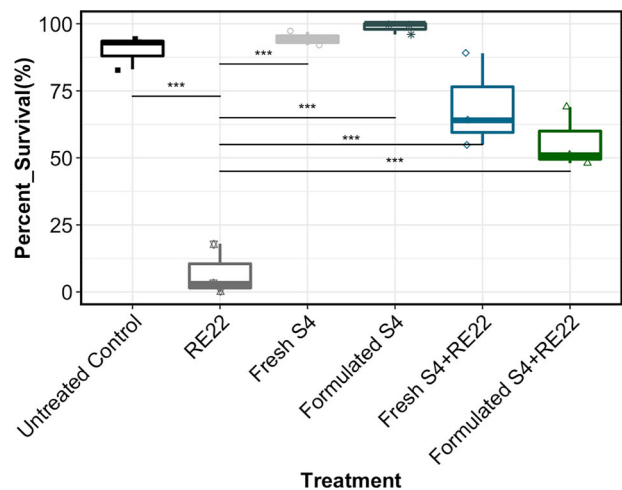
The viability of probiont S4 stored using different formulation methods (varying in storage media and temperature) was assessed biweekly during storage for 6 weeks. Formulation method had a significant effect on the viability of probiont S4 at the end of the 6 weeks (one-way ANOVA;  $p < 0.05$ ; Figure 1). The formulations in which dense cultures of S4 (grown in either mLB30 or mYP30) were diluted 1:1 in FSSW and then stored at 4°C (LB\_SW\_4 and YP\_SW\_4) showed significantly higher viability at the end of the 6 weeks (declines of 0.52 and 0.6 log) as compared to undiluted cultures stored at 4°C (LB\_4) or the diluted cultures stored at 22°C (LB\_SW\_22; declines of 1.32 and 1.34 log). Based on these results, the YP\_SW\_4 formulation was used in the hatchery trials.

### 3.2 | Effect of fresh and formulated S4 on the survival of eastern oyster larvae after pathogen challenge in the laboratory

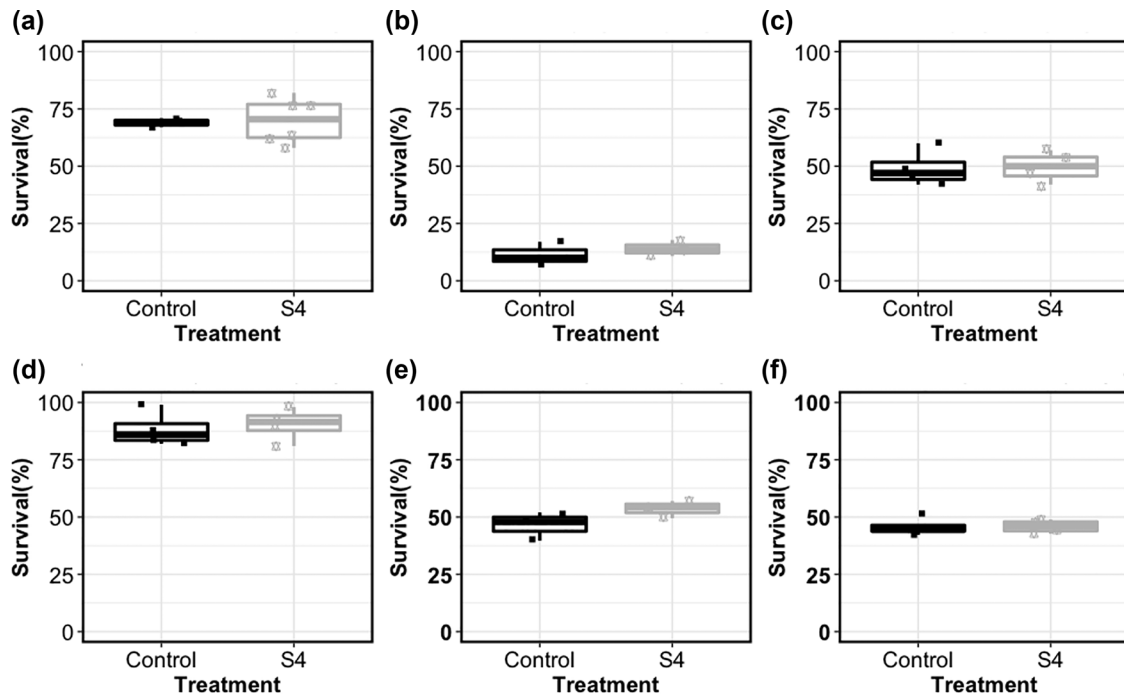
Pretreatment of larvae with freshly cultured S4 or formulated S4 (YP\_SW\_4) had no detrimental effect on larval survival over a 48-h period (Figure 2). Challenge of untreated control larvae with the pathogen RE22 led to significant larval mortality (90% decrease, GLM,  $p < 0.05$ ). Pretreatment with either the freshly prepared or the formulated S4 significantly increased larval survival following RE22 challenge as compared to non-treated controls (40%–60%;  $p < 0.05$ ).



**FIGURE 1** Formulation method had an effect on the viability of *Phaeobacter inhibens* S4. Bacterial cultures were stored for 6 weeks in four different conditions and sampled biweekly. Data expressed as mean  $\pm$  SD of colony forming units (CFU)/mL of S4 ( $n = 3$ ). S4 = *P. inhibens* S4; LB\_4: S4 Luria Broth with 3% sea salt (mLB30) undiluted broth culture stored at 4°C; LB\_SW\_4: S4 stored in diluted mLB30 broth (1:1 with filtered sterile seawater [FSSW]) at 4°C; LB\_SW\_22: S4 stored in diluted mLB30 (1:1 with FSSW) at 22°C; YP\_SW\_4: S4 stored in diluted yeast peptone with 3% sea salt (mYP30) broth (1:1 with FSSW) at 4°C. Different letters indicate statistically significant differences based on Tukey's pairwise comparisons (one-way ANOVA,  $p < 0.05$ ; for samples on Week 6).



**FIGURE 2** Treatment of larvae with S4 formulation in the laboratory led to increased larval survival to challenge with the pathogen RE22. Effect of pre-incubation of oyster larvae with *P. inhibens* S4 fresh culture (Fresh S4) or formulation (formulated S4, S4 stored in mYP30 diluted 1:1 with FSSW at 4°C) on survival after challenge with *Vibrio coralliilyticus* RE22 ( $n = 50$ –100 larvae per well, three wells per treatment). Survival was measured 24 h after RE22 challenge ( $10^5$  CFU/mL) and 48 h after addition of the probiotic S4 ( $10^4$  CFU/mL). Data are shown as box plots (median is shown by the line that divides the box into two parts; upper quartile—upper edge of the box—represents 75% value between the median and highest survival; lower quartile represents 25% value between the lowest and the median survival). \*\*\* indicates statistically significant differences between the treatments connected by the line (generalised linear model [GLM],  $p < 0.05$ ).



**FIGURE 3** Daily treatment of eastern oyster larvae in the hatchery with S4 formulation did not affect survival. Larval oysters were treated daily with S4 at a dose of  $10^4$  CFU/mL of water in the tank from Day 1 post fertilisation. Box plot (median, upper and lower quartile, see legend for Figure 2) represents the data for survival of larval oysters (in percent of total larvae stocked in tanks) at the end of each trial period (6–14 days post fertilisation,  $n = 3 - 6$  tanks per treatment, Table 1). (A) Trial 1, Virginia Institute of Marine Science (VIMS); (B) Trial 2, VIMS; (C) Trial 3, VIMS; (D) Trial 4, VIMS; (E) Trial 5, Mook Sea Farms (MOOK); (F) Trial 6, MOOK. Control: no probiotic provided; S4: *P. inhibens* S4 formulation.

Survival did not differ between larvae treated with fresh or formulated cultures of S4 and then challenged with RE22 (GLM;  $p > 0.05$ ; Figure 2).

### 3.3 | Effect of formulated probiotic treatment in the hatchery on the growth and survival of eastern oyster larvae

Based on the protection conferred by the formulation to the bacterial challenge in the laboratory trials, the formulation (YP\_SW\_4) was tested in hatchery conditions. Variability in larval growth and survival between trials within a hatchery and between hatcheries was observed, with one trial (Trial 2; VIMS, July 2019) showing lower survival (i.e., larval crash) for both control and probiotic-treated tanks (Figure 3b; GLM  $p < 0.05$ ; Supplementary Tables S3 and S4). Daily treatment of larvae with the formulation in the hatcheries did not have a significant impact on larval survival or growth (SGR) in any of the trials in the hatcheries in the absence of pathogen (GLM;  $p > 0.05$ ; Figures 3 and 4; Supplementary Tables S2 and S3).

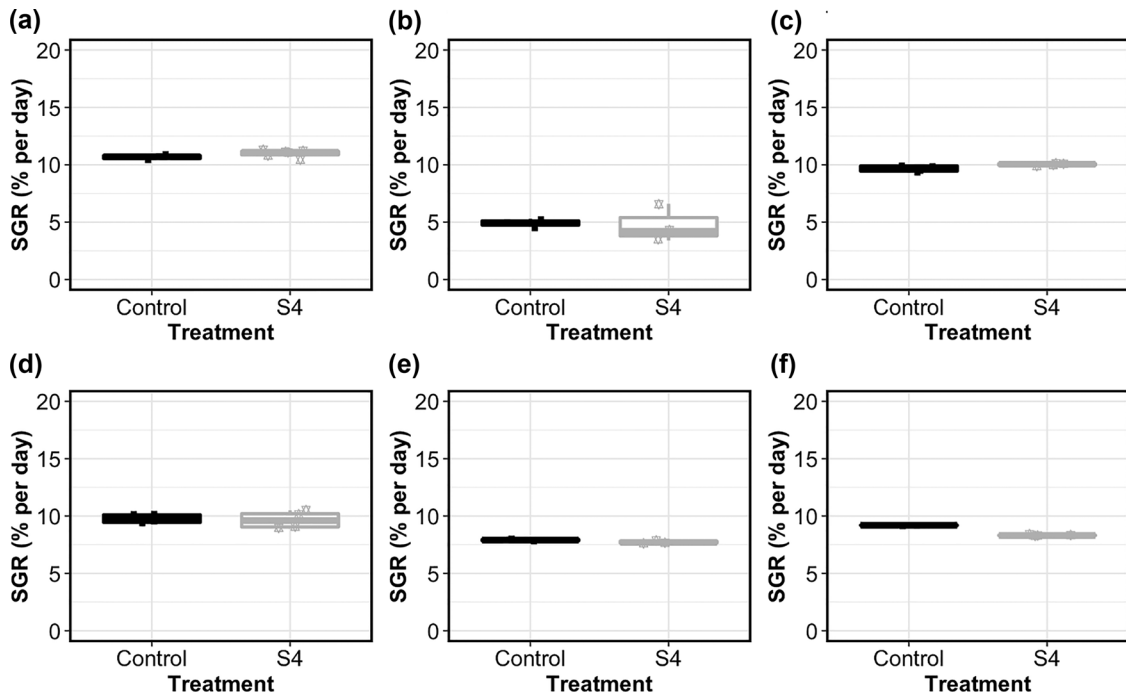
### 3.4 | Effect of probiotic treatment in the hatchery on the amount of total culturable *Vibrio* spp. in eastern oyster larvae

Daily treatment of larval tanks with the probiotic formulation did not significantly decrease the total number of culturable vibrios (as

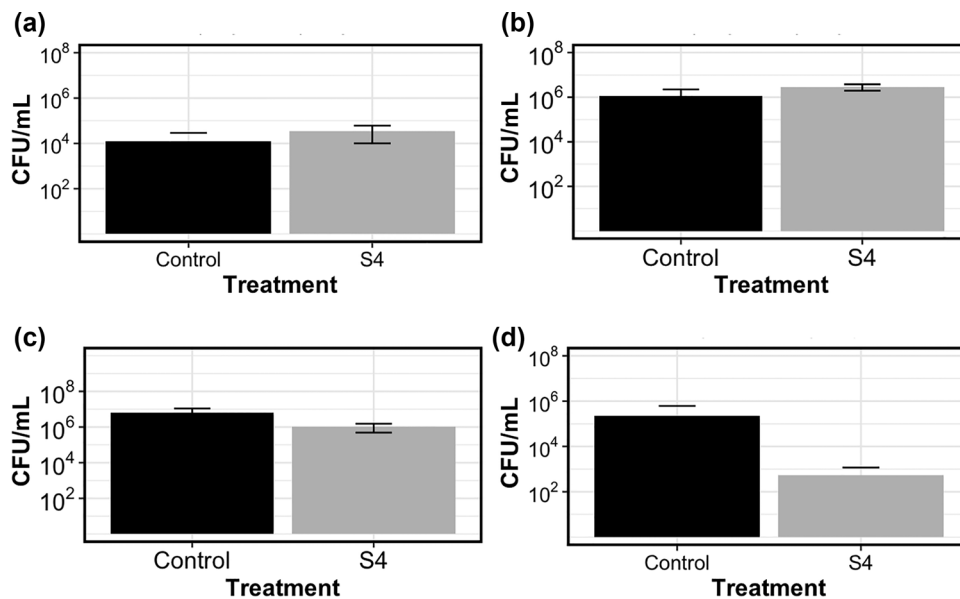
detected by culture in TCBS media) in the oyster larvae, compared to control-treated tanks in any of the hatchery trials ( $p > 0.05$ , one-way ANOVA for each hatchery trial; Figure 5). Variability in *Vibrio* counts between tanks within treatments and trials was observed (from  $10^3$  to  $10^6$  CFU/mL), likely due to handling issues and seasonal and regional differences. Culturable vibrios in water samples were below the level of detection in all trials and both hatcheries (less than  $10^2$  CFU/mL, data not shown).

### 3.5 | Effect of probiotic formulation treatment in the hatchery on the survival of eastern oyster larvae to challenge with the pathogen *V. coralliilyticus* RE22

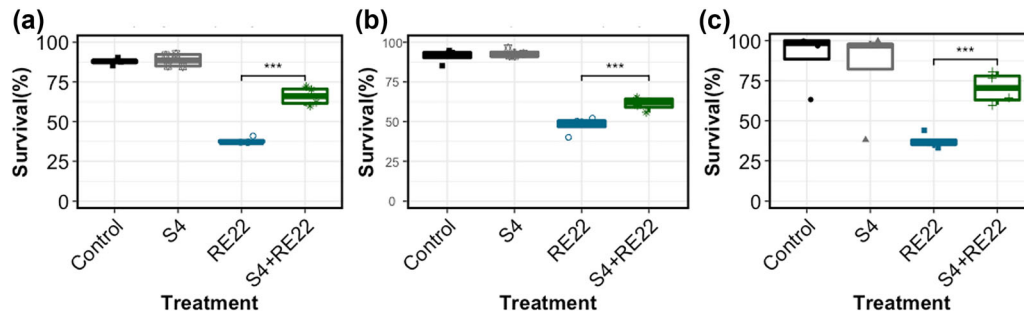
Exposure of eastern oyster larvae to the S4 probiotic formulation in the hatchery significantly increased larval survival during subsequent challenge with the bacterial pathogen *V. coralliilyticus* RE22 in the laboratory as opposed to treatment with RE22 alone (Trials 3, 4 and 6; GLM;  $p < 0.05$ , Figure 6; Supplementary Table S4). Bacterial challenge assays for Trials 1, 2 and 5 were not performed because they were designed to confirm the safety of the probiotic formulation in each of the hatcheries before conducting further studies. In the laboratory assays, survival ranged between 72% and 92% for unchallenged larvae collected from both control and probiotic-treated tanks. For larvae challenged with the pathogen, survival in the larvae collected from the control tanks ranged from 37% to 41%, while survival of larvae from the probiotic-treated



**FIGURE 4** Daily treatment of eastern oyster larvae in the hatchery with S4 formulation did not affect growth. Larval oysters were treated daily with S4 at a dose of  $10^4$  CFU/mL of water in the tank from Day 1 post fertilisation. Box plot (median, upper and lower quartile, see legend for Figure 2) represents the data for the specific growth rate (SGR) of larval oysters at the end of each trial period (6–14 days post fertilisation,  $n = 3–6$  tanks per treatment, Table 1). (A) Trial 1, VIMS; (B) Trial 2, VIMS; (C) Trial 3, VIMS; (D) Trial 4, VIMS; (E) Trial 5, MOOK; (F) Trial 6, MOOK. Control: no probiotic provided; S4: *P. inhibens* S4 formulation.



**FIGURE 5** Daily treatment of eastern oyster larvae in the hatchery with S4 formulation did not affect total culturable vibrio levels in larvae. *Vibrio* levels (CFU/mL in samples containing around 1,000 homogenized larvae  $\pm$  SD) in oyster larval samples collected from larval tanks at the hatchery were measured at the end of each trial by spot plating dilutions of homogenized larvae on thiosulfate-citrate-bile salts-sucrose medium agar plates. (A) Trial 2, VIMS; (B) Trial 3, VIMS; (C) Trial 4, VIMS and (E) Trial 6, MOOK. Control = no probiotic provided; S4 = *P. inhibens* S4 formulation ( $p > 0.5$ , one way ANOVA for each trial on log-transformed data).



**FIGURE 6** Daily treatment of eastern oyster larvae in the hatchery with S4 formulation significantly increased larval survival to pathogen RE22 challenge. Larvae from each of the hatchery tanks were transported to the laboratory at the end of each trial and exposed to a 24-h challenge with RE22 (RE22: larvae from control tanks challenged with RE22; S4+RE22: S4-treated larvae challenged with RE22) or no pathogen challenge (control: larvae from control tanks, not challenged; S4: larvae from S4-treated tanks, not challenged). (A) Trial 3, VIMS; (B) Trial 4, VIMS and (C) Trial 6, MOOK. S4 = *P. inhibens* S4 formulation; RE22 = *V. coralliilyticus* RE22. \*\*\* indicates statistical significance between the treatments connected by the bracket ( $n = 50$ –100 larvae per well, three wells per treatment; GLM for each trial,  $p < 0.05$ ).

tanks ranged between 60% and 76% (RPS increase of 46% to 74%; Table S5).

## 4 | DISCUSSION

A novel liquid formulation was developed for the commercial delivery of the gram-negative probiont S4 in oyster hatcheries. The product was found to be stable and maintained viability at  $10^8$  CFU/mL or higher for a period of 6 weeks when stored at 4°C in an airtight container in the dark. The performance of the commercially prepared S4 formulation tested here compared favorably to the freshly cultured S4 (as reported in Sohn et al., 2016a, 2016b) in all laboratory experiments and hatchery trials, having no negative effect on larval growth or survival in the hatchery. As also shown previously for freshly cultured S4 (Karim et al., 2013; Sohn et al., 2016a), larvae treated with the probiotic formulation in the hatchery showed improved survival when experimentally challenged with the pathogen *V. coralliilyticus* RE22. This study demonstrates that the formulation is safe and effective for use in eastern oyster (and probably other bivalve spp.; Sohn et al., 2016b) hatcheries to prevent larval vibriosis.

The applicability of several *P. inhibens* strains as probiotics for marine aquaculture has been assessed in several studies (Belas et al., 2009; D'Alvise et al., 2012; Grotkjær et al., 2016; Karim et al., 2013; Sonnenschein et al., 2021). However, until now, no suitable formulations of *P. inhibens* strains have been described for use in production hatcheries. As a gram-negative, non-spore forming bacterium, S4 did not survive spray drying procedures commonly used to formulate gram-positive bacteria such as *Bacillus* spp. Our novel approach to the formulation of this bacterium takes advantage of prior knowledge in the mechanisms allowing planktonic marine bacteria to survive in the oligotrophic conditions sometimes observed in coastal and oceanic waters (Holmquist et al., 1993; Nelson et al., 1997). The novelty of the formulated S4 for applications in commercial aquaculture is that it is easily delivered as a live, actively metabolising bacteria in a liquid

medium. Bacteria in the formulation remain highly viable over a period of at least 6 weeks when stored at 4°C. This formulation method differs from other commonly used techniques, such as freeze or spray drying, that put bacteria in a state of dormancy.

An effective probiotic formulation should not be harmful to the cultured larvae in the hatchery or negatively impact production. The probiotic formulation did not cause any detrimental effects to the larvae in six trials performed at two different hatcheries, confirming its safety to the larvae at the provided dose. The present study showed that there was no difference between the effect of daily treatment with fresh S4 (as reported in Sohn et al., 2016a, 2016b) and the formulated S4 on eastern oyster larvae; i.e., both were safe and had no negative impact on larval growth or survival in the hatchery.

Importantly, this study showed that daily exposure to the probiotic formulation in the hatcheries significantly improved the survival of larval oysters when challenged with the pathogen RE22. This confirms results from the previous laboratory and hatchery challenge assays utilising freshly cultured S4 administered prophylactically to the oyster larvae (Karim et al., 2013; Sohn et al., 2016a). Some members of the *Roseobacter* group, including several *Phaeobacter* spp., have been shown to display a wide range of inhibitory activity against aquaculture pathogens, especially against members of the genus *Vibrio*, which are responsible for larval mortalities in aquaculture (Kesarcodi-Watson et al., 2012; Planas et al., 2006; Porsby et al., 2008; Prado et al., 2010; Prol et al., 2009). Despite showing a protective effect in oyster larvae against experimental challenge with the pathogen *V. coralliilyticus* RE22, daily treatment of larvae in the hatchery with the S4 formulation did not significantly decrease the level of culturable vibrios in the larvae in any of the hatchery trials. These results are consistent with previous studies with other *P. inhibens* strains, showing that probiotic treatment does not impact the abundance of culturable vibrios in larvae (Grotkjær et al., 2016; Porsby et al., 2008; Sohn et al., 2016a), and suggesting that the effects of *Phaeobacter* spp. may be species-specific. Previously, microbiome analysis performed in a different study showed that probiont *B. pumilus* RI0695 treatment in the hatchery

leads to an increase in *Vibrio* diversity, without affecting total levels of culturable vibrios, and a shift in the composition of the *Vibrio* community to non-pathogenic species, indicating a subtle beneficial effect on larval microbial communities (Stevick et al., 2019). Probiotic S4 may have direct and/or indirect effects on bacterial community diversity and composition in the hatchery due to its previously reported antibiotic, quorum quenching and immunomodulatory actions (Modak & Gomez-Chiari, 2020; Zhao et al., 2016, 2019). Additional research is warranted to determine the effect of S4 on the larval microbiome, including effects on *Vibrio* spp.

Daily probiotic treatment did not significantly increase the growth or survival of larvae in any of the hatchery trials, which spanned different environmental conditions (Table S2) and hatchery protocols. Since larval survival was high in most of the trials, and levels of culturable *Vibrio* cells in larvae were low (i.e., there were no vibriosis outbreaks detected at the hatcheries), it was not expected that we would be able to detect a major effect on survival. However, S4 treatment was not able to prevent the larval crash observed in Trial 2, performed in July 2019 at VIMS, despite the consistent effect of S4 treatment protecting larvae against challenge with the bacterial pathogen RE22, and the fact that some *Phaeobacter* spp. strains consistently show the ability to inhibit bacterial pathogens (Kesarcodi-Watson et al., 2012; Planas et al., 2006; Porsby et al., 2008; Prado et al., 2010; Prol et al., 2009). The lack of protection by S4 to larval losses in this trial may be due to the inability of S4 treatment to protect larvae against crashes due to causes other than vibriosis. Some eastern oyster hatcheries in the Atlantic coast of the United States try to avoid spawning in July and August since larval performance is known to be low at this time of the year due to decreased water quality (personal communications from hatchery managers) and other potential causes such as adverse environmental (e.g., toxins from harmful algal blooms and water acidification) or physiological (e.g., poor conditioning of broodstock) conditions or other pathogens (Ashton et al., 2020; Gray et al., 2022).

Also, we observed that S4 treatment did not increase the growth of the larvae in any of the hatchery trials as reported in two other oyster species, Pacific and Kumamoto oysters (*C. gigas* and *C. sikamea*; Madison et al., 2022). Probiotic benefits are highly dependent on species and strain (Cruz et al., 2012; Kesarcodi-Watson et al., 2008; Macey & Coyne, 2005; Modak & Gomez-Chiari, 2020; Nandi et al., 2018; Nayak, 2010). Differences in probiotic efficacy between our study and Madison et al. (2022) could be due to the fact that we provided only one probiotic as opposed to a cocktail of several probiotics, differences in bacterial species or strain and their mechanisms of action, differences in host species and/or differences in other factors in the experimental design like culture and environmental conditions. For example, as it relates to effects on growth, other probiotics have been shown to provide direct nutritional benefits to the host through increased digestion through the release of digestive enzymes or as a direct nutritional source (Bagheri et al., 2008; Campa-Córdova et al., 2009; Freckelton et al., 2017; Hamdan et al., 2016; Macey & Coyne, 2005; Tan et al., 2016). Previous studies have also shown that probiotic treatment can increase the settlement (transition from planktonic larvae to sessile juveniles) success of oysters in the hatchery (Madison et al., 2022).

Unfortunately, due to logistic constraints derived from working in production conditions at hatcheries in different locations, we were not able to test the effect of *P. inhibens* S4 on eastern oyster settlement. Further research is required to understand the effect of S4 on settlement success, considering that the effect of bacteria on settlement is also species- and strain-specific (Freckelton et al., 2017; Tan et al., 2016).

As seen in previous hatchery experiments with the fresh S4 culture (Sohn et al., 2016a), levels of variability in all the parameters that were measured in the hatchery (growth, survival and culturable vibrios) were seen between tanks within treatment, between trials within a hatchery and between hatcheries. Variability in larval performance between tanks within treatments in a trial could be due to handling and husbandry activities in the hatcheries. The frequent handling of each tank during the drain down needed to sort the larvae and maintain water quality likely led to the introduction of slightly different bacterial communities in each tank (Arfken et al., 2021; Asmani et al., 2016; Stevick et al., 2019). Variability in the growth and survival of the larvae between hatcheries could be due to differences in location, culture systems, water filtration methods, feeding methods, spawning events, genetic variations in broodstock and environmental conditions, to mention a few. Despite the variability in environmental conditions and performance between trials and hatcheries, there was consistency in the safety and ability to protect the larvae against RE22 pathogenic bacteria challenge when the probiotic S4 formulation was applied in the hatcheries, suggesting that S4 provides a benefit by protecting larvae against the effect of *V. coralliilyticus* RE22 infection.

## 5 | CONCLUSION

This research provides evidence on the effectiveness of a newly developed approach to the formulation of marine gram-negative bacteria for use as probiotics in the eastern oyster larviculture in aquaculture. This formulation approach may be useful for developing formulations of other probiotics, especially gram-negative bacteria for use in marine aquaculture. The S4 formulation was shown to be safe, easy to handle and stable to use in the hatchery environment, and it may help manage the impact of vibriosis when used prophylactically in oyster hatcheries, although it may not offer protection against other largely uncharacterised causes of larval mortality. Future research should focus on identifying the effect of S4 formulation on the microbial community of larvae, water and rearing tanks in the hatchery and combining the use of S4 with other candidate probiotics or management methods to provide additional benefits to the larvae and/or prevent other causes of larval mortality.

### AUTHOR CONTRIBUTIONS

**Evelyn Takyi:** Data curation; formal analysis; investigation; methodology; visualisation; writing—original draft; writing—review and editing. **Jason LaPorte:** Formal analysis; investigation; methodology. **Sae-bom Sohn:** Formal analysis; investigation; methodology. **Rebecca J. Stevick:** Data curation; formal analysis; investigation; methodology;

supervision; validation; visualisation. **Erin M. Witkop:** Investigation; methodology. **Lauren S. Gregg:** Data curation; investigation; methodology. **Amanda B Chesler:** Data curation; investigation; methodology. **Jessica A Small:** Data curation; investigation; methodology. **Meredith M. White:** Data curation; investigation; methodology. **Cem Giray:** Methodology. **David C. Rowley:** Conceptualisation; funding acquisition; supervision; writing—review and editing. **David R. Nelson:** Conceptualisation; Funding acquisition; Resources; Supervision; Writing – review & editing. **Marta Gomez-Chiari:** Conceptualisation; funding acquisition; project administration; resources; supervision; visualisation; writing—review and editing.

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## CONFLICT OF INTEREST STATEMENT

Cem Giray used to work at Kennebec River Biosciences, a company licensed by the University of Rhode Island to commercialise the formulation of S4. His role in the research was to oversee the development of the commercial upscale of S4 production and provide the probiotic for the studies. He was not involved in the hatchery trials, the data collection or the analysis. Employees at each of the hatcheries were responsible for hatchery data collection and analysis.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

## ETHICS STATEMENT

The research involved samples collected in shellfish hatcheries as part of their routine production practices; larval oysters (invertebrates) were immediately preserved in ethanol or flash frozen, which serve as an anesthetic. The research was performed using all current ethical guidelines.

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## PEER REVIEW

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## SUPPORTING INFORMATION

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