

Toxicity of Bioactive and Probiotic Marine Bacteria and Their Secondary Metabolites in *Artemia* sp. and *Caenorhabditis elegans* as Eukaryotic Model Organisms

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We have previously reported that some strains belonging to the marine *Actinobacteria* class, the *Pseudoalteromonas* genus, the *Roseobacter* clade, and the *Photobacteriaceae* and *Vibrionaceae* families produce both antibacterial and antivirulence compounds, and these organisms are interesting from an applied point of view as fish probiotics or as a source of pharmaceutical compounds. The application of either organisms or compounds requires that they do not cause any side effects, such as toxicity in eukaryotic organisms. The purpose of this study was to determine whether these bacteria or their compounds have any toxic side effects in the eukaryotic organisms *Artemia* sp. and *Caenorhabditis elegans*. *Arthrobacter davidanieli* WX-11, *Pseudoalteromonas luteoviolacea* S4060, *P. piscicida* S2049, *P. rubra* S2471, *Photobacterium halotolerans* S2753, and *Vibrio coralliilyticus* S2052 were lethal to either or both model eukaryotes. The toxicity of *P. luteoviolacea* S4060 could be related to the production of the antibacterial compound pentabromopseudilin, while the adverse effect observed in the presence of *P. halotolerans* S2753 and *V. coralliilyticus* S2052 could not be explained by the production of holomycin nor andrimid, the respective antibiotic compounds in these organisms. In contrast, the tropodithietic acid (TDA)-producing bacteria *Phaeobacter inhibens* DSM17395 and *Ruegeria mobilis* F1926 and TDA itself had no adverse effect on the target organisms. These results reaffirm TDA-producing *Roseobacter* bacteria as a promising group to be used as probiotics in aquaculture, whereas *Actinobacteria*, *Pseudoalteromonas*, *Photobacteriaceae*, and *Vibrionaceae* should be used with caution.

Treatment of microbial diseases is necessary in humans, animals, and plants and, in the past several decades, it has become increasingly challenging due to the rapid development of microbial resistance to antibiotics and other antimicrobials. This leads to extra health care costs and losses in productivity, and the discovery of novel antimicrobial compounds is essential not only to ensure human health but also societal economy (1).

The marine environment represents a promising source of new bioactive compounds (2), and bacteria and fungi associated with marine macroorganisms, such as algae, invertebrates, or sponges, seem to be the most important producers (3, 4). The spectrum of activity of these biological active compounds ranges from pathogenic bacteria, fungi, and viruses to tumor cells (4). During two marine research expeditions (Galathea 3 and LOMROG II), our group isolated bacterial species with antibacterial activities. These species predominantly belonged to the *Actinobacteria* class, the *Pseudoalteromonas* genus, *Roseobacter* clade, and the *Photobacteriaceae* and *Vibrionaceae* families (3, 5, 6). Due to their antagonistic activity, live cultures of bacteria from *Pseudoalteromonas*, *Phaeobacter*, *Ruegeria*, and *Vibrio* genera are not only of interest as sources of novel pharmaceuticals but also as probiotics in fish and shellfish aquaculture systems (7–9).

Although many of the secondary metabolites derived from marine bacteria seem to be promising leads as new antibiotics, little is known about their potential effect on eukaryotes. Some *Pseudoalteromonas*, *Phaeobacter*, and *Vibrio* strains associated with macroalgae have a negative effect on the colonization of the algal surfaces by other bacteria and the settlement of invertebrates, such as barnacles or polychaetes (10). This effect has been related to the production of secondary metabolites, which seem to give an ecological advantage to the producing bacteria both against other microorganisms but also against bacterivorous eukaryotic preda-

tors (10–12). Consequently, the application of the producing bacteria or the bioactive compounds might cause adverse effects on the organisms to be treated (e.g., humans, animals, or plants) or, in the case of aquaculture, negatively affect the other organisms involved in fish or shellfish culture such as algae or live prey (e.g., rotifers and *Artemia* sp.). Hence, the toxicity of live bacterial cultures and bioactive compounds on the target organisms should be tested and any adverse effect ruled out before they can be applied. A complete toxicology assessment is a significant task, and we therefore, as a preassessment, determined the effect of the marine bacteria and their bioactive products in two model eukaryotic systems: *Caenorhabditis elegans* and *Artemia* sp.

C. elegans is a bacterivorous nematode that has a short life cycle, is easy to cultivate, and is one of the most commonly used organisms in biological research and toxicology studies (12, 13). *C. elegans* has been developed as a model to assess virulence of many human, animal, and plant microbial pathogens (14) and the toxicity of different compounds (11). The effects observed in *C. elegans* are similar to those observed in mammalian models (15).

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TABLE 1 Bacterial strains and antibacterial compounds analyzed in this study

Group	Species	Strain	Bioactive compound	Reference(s)
Actinobacteria class	<i>Arthrobacter davidanieli</i>	WX-11	Arthrobacilin	6
<i>Pseudoalteromonas</i> genus	<i>Pseudoalteromonas luteoviolacea</i>	S4054	Indolmycin	3, 18
		S4047-1	Indolmycin	
		S4060	Pentabromopseudilin	
		S2607	Pentabromopseudilin	
		S2049	Unknown	
	<i>Pseudoalteromonas piscicida</i>	S2049	Unknown	
	<i>Pseudoalteromonas rubra</i>	S2471	Prodigiosin	
<i>Roseobacter</i> clade	<i>Phaeobacter inhibens</i> (formerly <i>Phaeobacter gallaeciensis</i>)	DSM17395	Tropodithietic acid	19
		<i>Ruegeria mobilis</i>	F1926	Tropodithietic acid
<i>Photobacteriaceae</i> family	<i>Photobacterium halotolerans</i>	S2753	Holomycin	3,5
<i>Vibrionaceae</i> family	<i>Vibrio coralliilyticus</i>	S2052	Andrimid	3, 5
	<i>Vibrio nigripulchritudo</i>	S2604	Nigribactin	3, 20

The crustacean *Artemia* spp. have also, in the recent years, gained importance as a model organism in ecotoxicology (16) since it adapts easily to changes in nutrients, salinity, temperature, and oxygen, is easy to culture, is resistant to manipulation, and has a short life cycle (17). Furthermore, *Artemia* spp. are used as live feed for cultured fish and shellfish in aquaculture, and this gives it additional value as a toxicity model in aquaculture systems, e.g., for testing probiotic cultures.

The purpose of the present study was to determine whether marine bacteria that produce antibiotic and/or antivirulence compounds had any potential toxic side effects in eukaryotic organisms. The toxicity of all live bacterial cultures was analyzed by using both *Artemia* sp. and *C. elegans* as eukaryotic models but, since the majority of the compounds were not commercially available and could be extracted only in small amounts, the toxicity of the pure compounds was only tested on *Artemia* sp.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains analyzed in the present study are listed in Table 1. *Vibrio coralliilyticus* DSM19607 (21) and *Pseudoalteromonas tunicate* D2 (22) were used as positive killing controls in *Artemia* sp. and *C. elegans* assays, respectively, and *Escherichia coli* OP50 (Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN) was used as a negative killing control in *C. elegans* assays. *Vibrio anguillarum* 90-11-287 (23) was used as the target strain in a bioassay for testing the antagonism of the extracted secondary metabolites (24).

All marine strains were grown in marine broth (MB; Difco 2216) or marine agar (MA; Difco 2216). *Escherichia coli* OP50 was grown in Luria-Bertani (LB) broth (Miller Difco) and LB agar (Miller Difco). In the toxicity assay using *C. elegans*, bacterial strains were grown on Väättänen nine salts solution (VNSS) (25). All marine strains were incubated at 25°C for 24 to 48 h and stagnant conditions, and *E. coli* OP50 was incubated at 37°C and 250 rpm for 24 h. The bacterial strains were preserved as frozen stocks prepared in skim milk freeze medium (26) and stored at -80°C.

Pure antibacterial compounds. Indolmycin and violacein were purchased from Bioaustralis (Australia) and Sigma (USA), respectively, and tropodithietic acid (TDA) was purchased from BioViotica (Germany). Andrimid and holomycin were purified as previously described (5), and pentabromopseudilin was extracted from a 3-day-old *P. luteoviolacea* S4060 culture grown in 6 liters of marine minimal medium (27) with 0.4% mannose and 0.3% Casamino Acids as a carbon source, followed by incubation at 25°C and 100 rpm. Briefly, 12 g of sterile Diaion HP-20 resin

(Supelco)/liter was added to the outgrown culture. After 24 h, the resin was filtered off, and the bacterial cells were pelleted. The resin was extracted with 1 liter of methanol, while the pellet was extracted with 1 liter of methanol (MeOH)-ethyl acetate (EtOAc) (30/60 [vol/vol], 110 rpm overnight). The extracts were dried on a rotatory evaporator, redissolved in MeOH, and pooled. Violacein was quantitatively removed from the extract by cation-exchange. The extract was adsorbed onto a 10-g Septra SCX (Phenomenex) and dry-loaded onto a 50-g SNAP column (Biotage) with 40 g of pure SCX resin at the bottom. An Isolera flash purification system (Biotage) was used to fractionate the extracts with a mixed pH and solvent gradient (35 ml/min). The fractionation started with 100% water with 1% formic acid (FA; pH 2, 5 min) increasing to 100% MeOH with 1% FA over 10 min (pH 2), switched directly to MeOH with 2% ammonium hydroxide (pH 11, 20 min), and finally washed with MeOH (pH 7, 5 min). Fractions were automatically collected using UV detection (200 and 400 nm). Liquid chromatography-ultraviolet/electrospray ionization mass spectrometry (LC-UV/EI-MS) operated in negative ionization mode was used to determine the fractions containing the pentabromopseudilin as previously described (28). In addition, a bioassay against *V. anguillarum* was performed as previously described (24) to validate the antibacterial activity of the fractions. Pentabromopseudilin containing fractions (eluting with pH 2) were pooled and further purified by normal-phase chromatography. The active fractions were dry-loaded onto 2 g of Isolute diol (Biotage) in a 25-g SNAP column with 20 g of pure resin in the base. Fractions were collected by using solvents ranging from heptane, dichloromethane (DCM), and EtOAc to pure MeOH (15 ml/min). Pentabromopseudilin was recovered from the fractions eluting with heptane to DCM, together with an unidentified analogue ($M_r = 496.7068$).

Effect of live bacterial cultures on *Artemia* nauplii. The *Artemia* cysts (INVE Aquaculture, Salt Lake City, UT) were disinfected and decapsulated by sodium hypochlorite (NaOCl) treatment (29). In brief, 0.1-g portions of *Artemia* cysts were treated with 1.5 ml of 0.5% NaOCl (vol/vol) and vortexed for 3 min. The cysts were filtered on a 10- μ m-pore-size NY10 filter (Millipore) and washed three times with 25 ml of sterile deionized water. The cysts were transferred to a sterile 50-ml Falcon tube by washing the filter in 20 ml of sterile 3% (wt/vol) Sigma sea salts (SSS; Sigma-Aldrich), and the cysts hatched after being exposed to light for 24 h while shaking at 50 to 60 rpm and 25°C. Then, 20-ml portions of disinfected *Artemia* nauplius solution were transferred to 250-ml bottles containing 78 ml of 3% SSS.

Bacterial cultures were grown for 2 days in MB, and the concentration was estimated by plating 10-fold dilutions of the cultures on MA plates that were incubated at 25°C for 48 h. All bacterial cultures were added to the *Artemia* cysts at a final concentration of $\sim 10^7$ CFU/ml. The *Artemia*

cultures exposed to the bacteria were incubated at 25°C and 90 to 100 rpm. MB was used as a negative control, and *V. coralliilyticus* DSM19607 was used as a positive control for infection (21). Live nauplii were counted daily in a home-made methacrylate counting chamber using a stereomicroscope (SZ40; Olympus). For each time point, three samples were counted after 0, 4, 24, and 48 h. The bacterial density was determined by plating of 10-fold serial dilutions on MA plates. Two independent biological replicates were carried out.

Effect of live bacterial cultures on *C. elegans*. *C. elegans* strain Bristol N2 (wild type) was maintained on nematode growth medium (NGM) plates seeded with *E. coli* OP50 as a food source (30), followed by incubation at room temperature (22°C). The synchronization protocol for *C. elegans* was adapted from Strange et al. (31). In brief, adult nematodes from NGM agar plates were washed with sterile deionized water and transferred to a 50-ml sterile tube. The tube was stored on ice to allow the nematodes to settle down. Settled nematodes were transferred to a fresh tube containing 15 ml of sterile deionized water and washed two times more or until the supernatant was clear of bacteria. Then, the nematodes were lysed by incubation with 3 ml of 10 to 15% NaOCl and 2 ml of 2.5 M NaOH for 5 min. The lysis reaction was stopped by adding 10 ml of egg-buffer (47.2 ml of 2.5 M NaCl, 16 ml of 3 M KCl, 2 ml of 1 M MgCl₂, 2 ml of 1 M CaCl₂, 20 ml of 0.5 HEPES, and 930 ml of deionized water) and centrifugation at 275 × *g* for 3 min. The pellet was redissolved in 10 ml of fresh egg-buffer and again centrifuged. This step was repeated twice to remove all NaOCl and NaOH. Washed eggs were transferred to NGM plates without *E. coli* OP50 and allowed to hatch overnight. The next day, 500 μl of *E. coli* OP50 overnight culture was added, and the nematodes were incubated for 2 to 3 days at room temperature (22°C) to achieve the L4 stage.

Portions (50 μl) of a 2-day-old bacterial culture grown in MB were spread on VNSS agar, and the plates were incubated at 25°C for 2 days. *Pseudoalteromonas tunicata* D2 was used as a positive killing control (22). The negative control was *E. coli* OP50 from a 24-h LB liquid culture. Synchronized L4 stage nematodes were purified from *E. coli* OP50 as described, and approximately 30 to 40 synchronized and washed nematodes were transferred to each plate after pregrowth of the bacterial cultures. The plates were incubated at 25°C. The number of living nematodes was counted under a stereomicroscope (SZ61; Olympus) every day over a period of 4 days (96 h). The assay was carried out in duplicate.

Effect of pure compounds on *Artemia* nauplii. Since several of the pure antibacterial compounds were only available in very limited amounts, the *Artemia* assay was scaled down to 96-well plates (Nunc, catalog no. 167008). Then, 10 to 15 disinfected *Artemia* nauplii per 100 μl of 3% SSS was transferred to each well, and the pure compounds were added to achieve a final volume of 200 μl in each well. The MIC of each compound against *S. aureus* was estimated based on literature searches (see Table S1 in the supplemental material) and 2× MIC, 1× MIC, 0.5× MIC, and 0.05× MIC were tested in the *Artemia* assay. TDA was tested at 1× MIC, 0.5× MIC, 0.25× MIC, and 0.025× MIC. All compounds were dissolved in acetonitrile except TDA, which was dissolved in dimethyl sulfoxide (DMSO). Solvent controls were included in every test using the appropriate concentrations. Live nauplii were counted at 0, 4, 24, and 48 h under an inverted microscope (IX51; Olympus). At each time point, two counts were made, and the assay was carried out in two independent trials.

Statistical analysis. A one-way analysis of variance and Tukey test (level of significance = 0.05) were used to determine the significance of the effect of bacterial cultures and pure compounds on the survival of *Artemia* nauplii and *C. elegans*.

RESULTS

Effect of live bacterial cultures on *Artemia* nauplii. The positive control *V. coralliilyticus* DSM19607 reduced survival of *Artemia* nauplii by 43 and 96% after 24 and 48 h in coculture, respectively. Similarly, the survival of the nauplii was significantly affected ($P < 0.05$) by *P. rubra* S2471, *P. piscicida* S2049, *P. halotolerans* S2753,

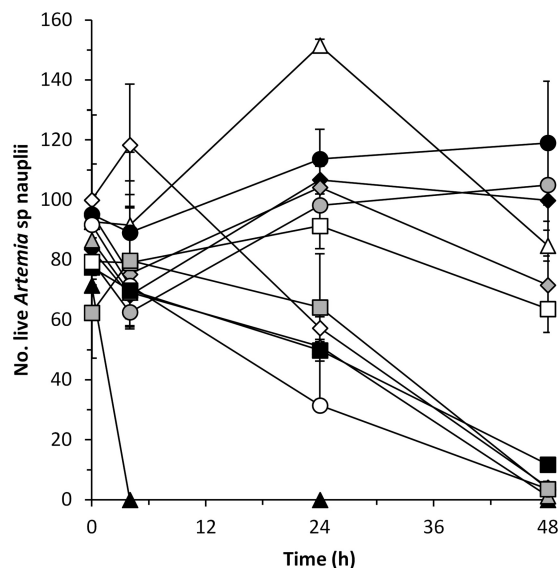


FIG 1 Influence of *A. davidanieli* WX-11 (△), *P. luteoviolacea* S4054 (gray diamond), *P. luteoviolacea* S4060 (▲), *P. rubra* S2471 (gray triangle), *P. piscicida* S2049 (○), *P. inhibens* DSM17395 (gray circle), *R. mobilis* F1926 (●), *V. coralliilyticus* S2052 (■), *V. nigripulchritudo* S2604 (□), and *P. halotolerans* S2753 (■) on the survival of *Artemia* nauplii. MB (◆) was used as a killing negative control, and *V. coralliilyticus* DSM19607 (◇) was used as a killing positive control. The results are means and standard deviations of two independent assays.

and *V. coralliilyticus* S2052, which reduced the viability of nauplii by 35 to 65% after 24 h and by 85 to 99% after 48 h in coculture. *P. luteoviolacea* S4060 killed all nauplii after 4 h of incubation. The presence of *A. davidanieli* WX-11, *P. inhibens* DSM 17395, *P. luteoviolacea* S4054, *R. mobilis* F1926, and *V. nigripulchritudo* S2604 had no effect on nauplius survival, which was not different ($P > 0.05$) from that in the MB control (Fig. 1).

P. luteoviolacea S4054 produces violacein and indolmycin, whereas *P. luteoviolacea* S4060 produces violacein and pentabromopseudilin (18). To check whether the different effect observed by these two strains in *Artemia* nauplii could be related to their different chemical profiles, we also analyzed the effect of *P. luteoviolacea* strains S4047-1 and S2607, which produce, respectively, indolmycin and pentabromopseudilin, together with violacein (18). The two strains producing pentabromopseudilin killed all of the *Artemia* nauplii after 4 h of incubation, whereas those producing indolmycin had no effect on nauplius survival and did not differ from the MB control ($P > 0.05$) (Fig. 2).

Effect of live bacterial cultures on *C. elegans*. After 24 h of incubation, the positive control *P. tunicata* D2 and *P. luteoviolacea* S4060 killed all of the nematodes. The presence of *P. rubra* S2471 and *P. piscicida* S2049 reduced *C. elegans* survival by 29 and 85%, respectively, after 24 h and killed all nematodes after 72 h in coculture (Fig. 3). *A. davidanieli* WX-11, *P. luteoviolacea* S4054, and *P. halotolerans* S2753 reduced *C. elegans* numbers by 92 to 100% after 72 h of incubation (Fig. 3). The survival of *C. elegans* was also reduced in the presence of *V. coralliilyticus* S2052, but it was only significantly different ($P < 0.05$) from the *E. coli* OP50 negative control after 72 h of incubation. No negative effect was observed in the presence of *P. inhibens* DSM17395, *R. mobilis* F1926, or *V. nigripulchritudo* S2604, where nematode survival was not different ($P > 0.05$) from the *E. coli* OP50 negative control (Fig. 3).

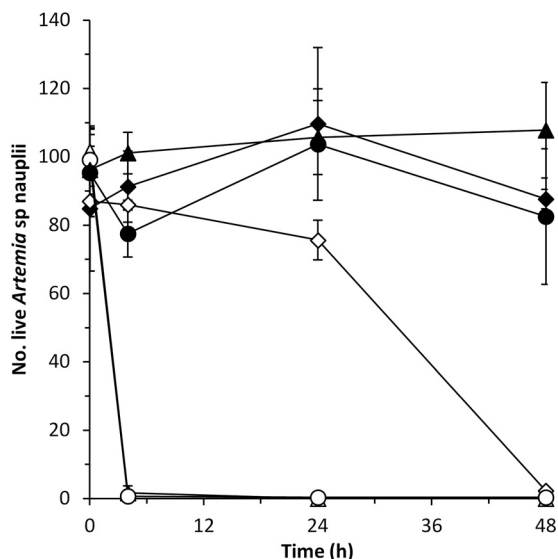


FIG 2 *Artemia* survival when cocultured with *P. luteoviolacea* S4060 (Δ) and S2607 (\circ), which produce violacein and pentabromopseudilin, and with S4054 (\blacktriangle) and S4047-1 (\bullet), which produce violacein and indolmycin. MB (\blacklozenge) was used as a killing negative control, and *V. coralliilyticus* DSM19607 (\diamond) was used as a killing positive control. The results are means and standard deviations of two independent assays.

Effect of pure compounds on *Artemia* nauplii. Target-guided purification of pentabromopseudilin resulted in a semipure fraction with two closely eluting compounds. One peak ($M_r = 547.6143$) was identified as pentabromopseudilin. The other peak ($M_r = 496.7068$) is potentially a new analogue due to a similar isotope pattern and it did not match any compound in AntiBase

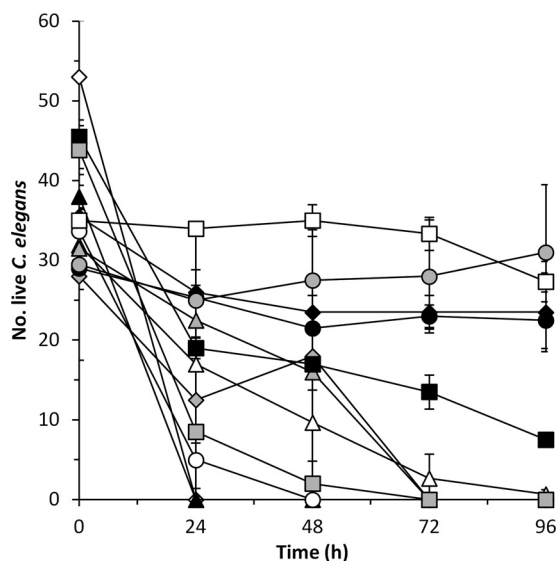


FIG 3 Influence of *A. davidanieli* WX-11 (Δ), *P. luteoviolacea* S4054 (gray diamond), *P. luteoviolacea* S4060 (\blacktriangle), *P. rubra* S2471 (gray triangle), *P. piscicida* S2049 (\circ), *P. inhibens* DSM17395 (gray circle), *R. mobilis* F1926 (\bullet), *V. coralliilyticus* S2052 (\blacksquare), *V. nigripulchritudo* S2604 (\square), and *P. halotolerans* S2753 (\blacksquare) on the survival of *C. elegans*. *E. coli* OP50 grown in VNSS medium (\blacklozenge) was used as a killing negative control, and *P. tunicata* D2 (\diamond) was used as a killing positive control. The results are means and standard deviations of two independent assays.

TABLE 2 Effect of acetonitrile and DMSO on the survival of *Artemia* nauplii^a

Solvent	Concn (%)	Mean survival (%) \pm SD	
		4 h	24 h
Acetonitrile	4	0 \pm 0	0 \pm 0
	2.5	88 \pm 8	100 \pm 0
	1	83 \pm 8	83 \pm 8
	0.625	84 \pm 19	81 \pm 13
	0.5	100 \pm 0	92 \pm 15
	0.25	96 \pm 8	79 \pm 8
	0.1	84 \pm 16	76 \pm 16
DMSO	7.5	0 \pm 0	0 \pm 0
	3.75	65 \pm 26	17 \pm 2
	1.88	100 \pm 0	91 \pm 0
	0.21	75 \pm 10	80 \pm 0
Control	3	100 \pm 0	100 \pm 0

^a The results are means of two independent experiments. *Artemia* nauplii were cultured in 3% Sigma Sea Salts.

2010 (32) or any known analogue. This semipure fraction of pentabromopseudilin was used and named the pentabromopseudilin mix.

Acetonitrile and DMSO were used as solvents for preparation of solutions of the different compounds. Acetonitrile killed almost all *Artemia* nauplii at concentrations between 25 and 4%, which are equivalent to that in the solutions with 2 \times MIC and 1 \times MIC of andrimid, holomycin, indolmycin, pentabromopseudilin mix, and violacein (Table 2). A similar effect was observed for DMSO at the concentrations present in TDA solutions with 1 \times MIC (7.5%) and 0.5 \times MIC (3.75%) values (Table 2). Therefore, we analyzed the effect of the different compounds based on 0.25 \times MIC and 0.025 \times MIC for TDA and 0.5 \times MIC and 0.05 \times MIC for the other compounds. The pentabromopseudilin mix killed all nauplii at a final concentration of 10 μ M (0.5 \times MIC) but did not have any lethal effect at 1 μ M (0.05 \times MIC) (Fig. 4) and was not different ($P > 0.05$) from the MB control. Andrimid, holomycin, indolmycin, TDA, and violacein did not have any significant effect on nauplius survival in the tested concentrations compared to the MB control ($P > 0.05$).

DISCUSSION

Marine microorganisms can produce potent bioactive compounds and, although some of these bioactive substances have been known for decades, little is known about their adverse effect on eukaryotic organisms, and only a few have been recently approved (4). In the present study, *Artemia* sp. and *C. elegans* were used as eukaryotic models to provide a first assessment of potential toxicity of bacterial bioactive compounds and the producing bacteria themselves. We have shown that some antibiotic-producing marine bacteria belonging to the *Actinobacteria* class, the *Pseudoalteromonas* genus, and the *Photobacteriaceae* and *Vibrionaceae* families have a lethal effect on one or both target organisms and hence should be used with great caution. In contrast, other bacteria, e.g., from the *Roseobacter* clade, had no adverse effect.

The actinobacterium *A. davidanieli* WX-11 had no effect on *Artemia* sp. but killed almost all nematodes after 72 h of incubation. Arthrobacilins A, B, and C have been detected in WX-11 (6)

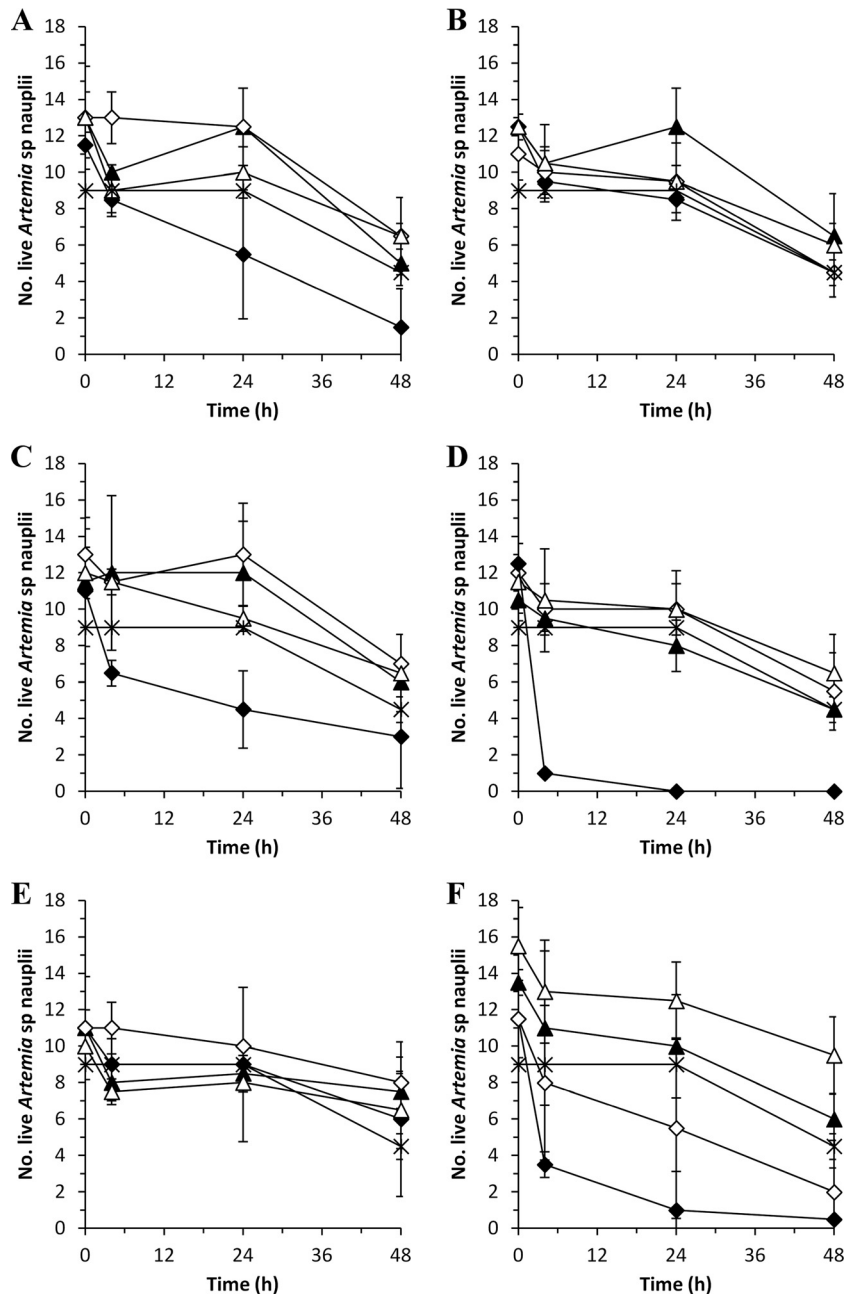


FIG 4 Survival of *Artemia* nauplii in the presence of andrimid (A), holomycin (B), indolmycin (C), pentabromopseudilin mix (D), TDA (E), and violacein (F) at the concentrations needed for 0.5× MIC (◆) and 0.05× MIC (▲), or at 0.25× MIC (◇) and 0.025× MIC (△) in the case of TDA, against *S. aureus*. MB (*) was used as a negative control. The effect of acetonitrile and DMSO concentrations used for the preparation of 0.5× MIC or 0.25× MIC (◇) and 0.05× MIC or 0.025× MIC (△) solutions is also represented. The results are means and standard deviations of two independent assays.

and, although they have antibacterial activity, it has been suggested that the antibacterial activity of WX-11 could also be related to other compounds, since arthrobaicilins were also detected in extracts without antibacterial activity (6). Arthrobaicilins have a cytotoxic effect against human cancer cells (33) and the closer relation of *C. elegans* to humans than to *Artemia* sp. could explain their different sensitivities to WX-11. The potential mode of action of arthrobaicilin is not known and, hence, the specific mechanism of toxicity is difficult to explain. *A. davidanieli* is used as a live vaccine for disease control in salmonids, although the effect is

probably due to the stimulation of the immune system rather than to antibacterial activity (34).

The *P. luteoviolacea* strains differed in their effect on survival of *Artemia* nauplii, and this could be explained by the different chemical profiles in the strains. Strains S4054 and S4047-1 produce violacein and indolmycin (18) and had no effect on *Artemia* nauplii, whereas strains S2607 and S4060 produce violacein and pentabromopseudilin (18) and killed all nauplii after 4 h in coculture. These results already indicated that violacein was not the compound responsible of the toxicity observed, and this was con-

firmed by testing the pure compounds. Violacein and indolmycin did not have any significant effect on the survival of *Artemia* nauplii, whereas the pentabromopseudilin mix killed all nauplii after 24 h of incubation. Both nauplii and nematodes were rapidly paralyzed in the presence of the pentabromopseudilin-producing strains, and this also indicates that pentabromopseudilin was the main toxic compound. Indeed, it has been demonstrated that pentabromopseudilin inhibits the myosin ATPase and motor activity in several myosins (35). At present, no toxic effect of indolmycin on eukaryotic organisms has been reported. In the present study, strain S4054 that produces violacein and indolmycin did not affect *Artemia* nauplii but had a negative effect on *C. elegans*. This could be explained by the different culture methods used, VNSS plates for the nematodes and 3% SSS in the *Artemia* assay, which could have led to variations in the production of the compounds. In addition, *Artemia* spp. have been shown to be less sensitive in ecotoxicology tests than other organisms, such as algae, insects, and other crustaceans (18), and having an exoskeleton might also prevent the uptake of toxins.

P. piscicida and *P. rubra* killed both *Artemia* sp. and *C. elegans*. A lethal effect of *P. piscicida* on eukaryotic organisms, such as algae and crustaceans, has previously been reported (36). *P. piscicida* S2049 produces several bromoalterochromides (37), which have inhibitory properties against *Bacillus subtilis* (38) and a toxic effect on eukaryotic organisms, such as sea urchins (37). The production of bromoalterochromides by S2049 could explain the toxic effect we observed in the tested eukaryotic organisms. *P. rubra* strains produce the pigment prodigiosin (18), which can antagonize bacteria (39) and have a negative effect on eukaryotic organisms, including algae (40), and parasites (41) corresponding to the effects observed in the present study.

V. corallilyticus S2052 had a lethal effect on *Artemia* sp. after 24 h in coculture, and this is in agreement with previous studies (56). It also affected the survival of *C. elegans*; however, the effects were not seen until after 96 h of incubation. Strain S2052 produces the antibiotic andrimid (5); however, andrimid in itself did not adversely affect *Artemia* nauplii. Andrimid targets the multisubunit acetyl coenzyme A carboxylase, which catalyzes the first step in fatty acid biosynthesis and is essential for bacterial growth (42). Eukaryotes carry out these reactions by a large multifunctional protein (43), and it is therefore not surprising that andrimid did not significantly affect *Artemia* sp. and *C. elegans* survival. S2052 is able to colonize and use the chitin from the exoskeleton of *Artemia* sp. as a nutrient (56), and, since respiration in *Artemia* spp. occurs through the surfaces of the legs, where the exoskeleton is thinner, it is possible that S2052 colonization interferes with *Artemia* oxygen uptake, compromising the survival of the nauplii and explaining the differences observed between *Artemia* sp. and *C. elegans*.

P. halotolerans S2753 killed both *Artemia* sp. and *C. elegans*. Biofilms of *P. halotolerans* can have an inhibitory effect in the settlement of the barnacle *Amphibalanus improvisus* in macroalgae, although the mechanism involved in such inhibition is not known (10). Similarly to andrimid, holomycin, which is the main antibacterial compound produced by S2753, did not affect *Artemia* survival. The activity of holomycin is related to the inhibition of RNA polymerase and, since prokaryotic and eukaryotic RNA polymerases are different, it was expected that holomycin did not affect the survival of *Artemia* sp. Similarly, Oliva et al. (44) showed that the yeasts *Saccharomyces cerevisiae* and *Candida kefyr* are not affected by holomycin. *P. halotolerans* produces two dep-

sipeptides called solonomamide A and B, which have a chemical structure similar to unnarmicins (45). Unnarmicins have been identified in a *Photobacterium* strain (46) and inhibit fungal ATP binding cassette transporters (ABC), which are involved in drug resistance (47). *C. elegans* possess several ABC transporters involved in drug resistance (48), and solonomamides could thus potentially inhibit one or several of these proteins, making the nematodes sensitive to holomycin or other secondary metabolites produced by S2753. Similarly to *V. corallilyticus*, *P. halotolerans* is able to use chitin (49) and form biofilms (10), and it is therefore possible that the toxic effect observed in *Artemia* sp. was caused by degradation of the exoskeleton in the nauplii.

V. nigripulchritudo S2604, *P. inhibens* DSMZ19395, and *R. mobilis* F1926 had no effect on the survival of *Artemia* sp. and *C. elegans*. The antibacterial activity of *V. nigripulchritudo* is linked to the production of the siderophore nigribactin (20). Siderophores are iron-chelating compounds, which have been related to virulence in bacterial pathogens. In contrast to our results, the link between siderophores and virulence in several human pathogens has been demonstrated by using *C. elegans* as a model organism (50). Similarly, several strains of *V. nigripulchritudo* have been identified as the cause of high mortalities in shrimp farms (51); however, different strains of *V. nigripulchritudo* have different virulence patterns, and it is not known what determines the pathogenicity in this species (51). Therefore, it is possible that S2604 is a nonpathogenic strain or that the culture conditions used in the present study were not appropriate for virulence expression.

P. inhibens DSMZ17395 and *R. mobilis* F1926 are innocuous for fish larvae (7), algae, and live prey (52). Their antibacterial activity is caused by production of TDA, which is a broad-spectrum antibiotic able to inhibit fish and shellfish pathogens (53) and is the main compound responsible for antagonism in laboratory broth cultures (54), in live feed, and in fish larval systems (52).

Pseudoalteromonas, *Roseobacter*, and *Vibrionaceae* bacteria have been suggested as probiotics in aquaculture (7–9). However, our results suggest that several *Pseudoalteromonas* and *Vibrionaceae* strains may not be suitable, since they can be toxic to *Artemia* sp. or potentially toxic (e.g., *V. nigripulchritudo*). Nevertheless, their secondary metabolites provide them with the capability to regulate bacterial virulence expression (20) and kill bacteria (18), algae causing harmful algal blooms (HAB) (40), parasites (41), or tumor cells (55). These bacteria could therefore have value, e.g., as control agents of biofouling and HAB in the marine environment, as producers of cancer treatment compounds, or as antibacterial or antiparasitic agents. In contrast, the lack of toxicity of the live cultures of the TDA-producing *Roseobacter* species reaffirms their suitability as probiotics in aquaculture. Furthermore, bacterial resistance against TDA is hard to develop (53), and this, together with the innocuousness of pure TDA in the tested eukaryotic organisms, also raises the interesting possibility of applications in the control of human infections.

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