INTERACTION OF *CHROMOBACTERIUM* SPECIES WITH *BATRACHOCHYTRIUM DENDROBATIDIS* AT DIFFERENT TEMPERATURES

By

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ABSTRACT

Chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) has been identified as one of the major culprits in amphibian population declines worldwide. This fungus grows upon and colonizes the keratin layer of the amphibian skin. Amphibian skin also harbors a rich diversity of bacteria species, and some of these bacteria have been found to inhibit *Bd* growth and colonization. *Janthinobacterium lividum,* a violacein-producing bacteria species is one of the most studied anti-*Bd* bacteria, but there are *Chromobacterium* species that also produce similar metabolites but have received less attention regarding their efficacy against *Bd*. Violacein produced by *J. lividum* has been reported to be antifungal, but the effect of temperature on the efficacy of this bacterial metabolite has not been examined. Also, the anti-*Bd* properties of *Chromobacterium* spp. has not been tested.

In this study, we isolated and identified four *Chromobacterium* spp. from the skin of cricket frogs (*Acris gryllus*) in ponds around Tuskegee, AL. We grew the different species (*C. amazonesis*, *C. substugae, C.* etc) in a tryptone broth culture for three days and collected purple pigmented, cell-free supernate of the cultures (which contained violacein, confirmed by mass spectrometry). Using a 96 well plate, each identified bacterial species supernate as well as the supernate from *J. lividum* (YO8846) was tested against three strains of *Bd* (JEL 423, JEL 310 and JEL 197), that have been documented to be highly pathogenic to frogs at a range of environmentally relevant temperatures (12, 16, 22 and 24°C).

Each well absorbance was recorded every other day for 27 days, a inhibition score calculated, o analyze the degree of inhibition of each bacterial ribotype (the 4 *Chromobacterium* and *J. lividum*) for each *Bd* strain. We found that temperature had a significant effect on the strength of *Bd* inhibition by all bacteria species, with significantly more inhibition observed at the lower tested temperatures. Also, all tested species of *Chromobacterium* significantly inhibited *Bd* growth more than *J. lividum* at almost all tested temperatures.

We also tested the effect of temperature on proliferation of the isolated *Chromobacterium spp.* and characterized the potency of violacein produced from the different bacterial isolates We found the tested *Chromobacterium spp*. grew faster the J. *lividum* at all tested temperatures and produced violacein at all these temperatures, though in varying quantity. In contrast, *J. lividum* did not produce violacein at 12℃ and 30℃. We also found that all *Chrombacterium* to have significantly higher inhibitory ability than *J. lividum* at equivalent dilution factors, only becoming equivalent to *J. lividum* at the 1:1000 fold dilution. v. Overall, this study shows that these set of violacein-producing bacteria are abundant in majority of sampled frogs and could provide a vital protection against *Bd* infection.

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INTRODUCTION

Worldwide amphibian population declines have been widely associated with mortality due to chytridiomycosis, a condition caused by a lethal fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) from the phylum Chytridiomycota (Berger et al., 1998; Longcore et al., 1999; Lips et al., 2005;). These declines were observed in areas with diverse amphibian fauna (i.e. Australia, the Caribbean and Central America; Berger et al., 1998; Lips 1999; Bosch 2001). However, while some amphibian populations exposed to chytrid fungus experienced declines, others exhibited little to no impact from this type of exposure (Lips et al., 2005; Peterson et al., 2007; Briggs et al., 2010; Davidson et al., 2019). This observation implies differences in virulence of chytrid strains or some protective response in certain amphibian hosts. Additionally, the relative impact of chytrid infection may also be aggravated by other factors like natural habitat destruction, land fragmentation, urbanization, and climate change (Collins and Storfer 2003; Pounds et al., 2006). The chytrid fungus is widespread, inhabiting wetlands, soils and water bodies, and is a common parasite of plants, algae and insects (Kilburn et al., 2011) and its widespread presence and infection potential present a continual challenge to amphibian health. *B. dendrobatidis* (*Bd*) attacks amphibians by colonizing their skin, invading the keratinized layer of the epidermis, and using the keratin and other skin components as a nutrient source (Longcore et al., 1999; Fellers et al., 2001; Berger et al., 2005). *Bd* zoospores attach to the epidermal layer, release their cellular material through a germ-tube into the epidermis, where they germinate, disrupting skin integrity and physiology (Longcore et al., 1999).The frog skin is an important site of homeostatic activities, such as maintaining osmotic balance and electrolyte transport

(Boutilier et al., 1992; Boutilier et al., 1997). The later stages of *Bd* infection results in hyperkeratosis, causing disrupted respiration and impaired osmoregulation which can prove fatal (Peterson et al., 2007; Rosenblum et al., 2010; Campbell et al., 2011; Voyles et al., 2011). The amphibian skin is also the first line of immune defense against chytrid infection, possessing glands that produce antimicrobial peptides (AMPs) providing some defense against *Bd* infection (Apponyi et al., 2004; Clarke, 2007; Rollins-Smith et al., 2011; Voyles et al., 2011). AMPs, even combined with adaptive immunity, are not totally effective against *Bd* infection due to variation in their concentration and degradation by proteases secreted by symbiotic bacteria or the frog itself (Pask et al., 2012). Another defense to *Bd* infection is increasing skin-sloughing as a way of getting rid of the thickening skin layer but this is metabolically costly (Berger et al., 2005; Voyles et al., 2011, Peterson et al., 2013).

Several studies have shown the importance of amphibian skin microbiomes in ameliorating the level of *Bd* infection, with bacteria being the most studied group of microorganisms (Becker et al., 2009; Harris et al., 2009; Lauer et al., 2007; Muletz-Wolz et al., 2017a; Wiggins et al., 2011). These symbiotic bacteria produce secondary metabolites as well as volatile organic compounds and bacteriocins (Bletz et al., 2013) that are inhibitory to *Bd*; the best studied examples of these metabolites are violacein produced *by Janthinobacterium lividum* (Becker et al., 2009) and 2,4-diacetylphloroglucinol (DAPG) produced by *Pseudomonas fluorescens* (Lam et al., 2011; Woodhams et al., 2016).

B. dendrobatidis zoospores have been documented to avoid antifungal bacterial metabolites on amphibian skin, as well as *in vitro* by swimming away from them (Lam et al., 2011; Moss et al., 2008). One of the most studied of these bacterial metabolites is violacein which is produced by diverse species of gram-negative bacteria like *J. lividum* (Brucker et al., 2008; Harris

et al., 2009), *Chromobacterium sp.* (Ahmad et al., 2012; Rettori, 1998), as well as *Duganella sp.*(Aranda et al., 2011), *Massillia sp.* (Hitosi et al., 2011), *Collimonas* (Hakvåg et al., 2009). These bacteria species have been isolated from an array of environment such as soils (Choi et al. 2015; Aranda et al. 2011; Hitosi et al. 2011), plant surfaces (Blackburn et al., 2017), and water (Young et al., 2008; Menezes et al., 2012; Soby et al., 2013).

Violacein is a bis-indole derivative produced through a metabolically regulated mechanism called quorum sensing (McClean et al., 1997; Rettori and Durán 1998; Duran and Menck 2001). The pigmented metabolite gives the producers their characteristic purple or violet color. The most studied of these violacein producers is *J. lividum* as it relates to chytrid fungus (Brucker et al., 2008; Becker et al., 2009; Harris et al., 2009; Muletz et al., 2012) and *C. violaceum* in relation to tumor cells, parasites, bacteria and dye production (Duran et al., 2016; Duran and Menck 2001; Inniss and Mayfield 1979; Rettori and Durán 1998). Other *Chromobacterium* spp. have also been described but less explored for the properties of their violacein; these include *Chromobacterium vacinni* (Soby et al., 2013), *Chromobacterium amazonense* (Menezes et al., 2015), *Chromobacterium sphagnii* (Blackburn et al., 2017) and *Chromobacterium subtsugae* (Martin et al., 2007). Although there were few reports of *C. violaceum* being an opportunistic pathogen (Liu et al., 2012; Ponte and Jenkins, 1992; Yang and Li, 2011), there have been many studies showing the economic importance of violacein, which focus on their antifungal (Becker et al., 2009; Harris et al., 2009), antimicrobial (Cazoto et al., 2011; Rajalakshmi et al., 2011), antiparasitic (Lopes et al., 2009) and antitumor properties. Violacein is also used commercially as an additive in body care products and dyes (Duran et al., 2016).

Since violacein-producing bacteria are part of the bacterial symbiotic community on the skin of amphibians, several studies have documented their anti- *Bd* property (Brucker et al., 2008a,

Brucker et al.,2008b). However, the focus of the anti-*Bd* research has been on *J. lividum*, which has been tested *in vitro* and *in vivo* as a treatment option (Becker et al., 2009; Harris et al., 2009; Wiggins et al., 2011; Woodhams et al., 2016) as well as for bioaugmentation options. Amphibians exposed to *J. lividum* had a reduction in infection by *Bd*. These trials have experienced a differing rate of success; it would be useful to consider other violacein-producing species as well as the influence of an abiotic factor like temperature on the effectiveness of the inhibition. A few hundred of amphibian bacterial symbionts have been pinpointed to inhibit *B. dendrobatidis* (Harris et al., 2009; Kueneman et al., 2014; Muletz-Wolz et al., 2017, a,b) but there is still more yet to be tested as well as their relationship with temperature, especially in the genera *Chromobacterium*, which has not been previously described as part of the frog skin microbiota. *Chromobacterium* sp. produces violacein and have not been described for their *Bd*-inhibitory properties. There has, however, been documentation that recently described *Chromobacterium* spp. isolated from aquatic environments are toxic to some lepidopteran species (Soby et al., 2013; Blackburn et al., 2017;). Since only *J. lividum* has been used in anti- *Bd* experiments, it would be useful to determine the relative efficacy of other violacein-producing bacterial species.

Additionally, temperature is an important environmental factor that influences *Bd* growth and proliferation and, thus, successful colonization of the amphibian skin (Berger et al., 2004; Forrest & Schlaepfer, 2011; Muletz-Wolz et al., 2017; Rollins-Smith et al., 2011; Woodhams and Alford, 2005). Woodhams and Alford (2005) suggested that *Bd* has an optimal growth temperature between 17-23°C, though they can grow at lower temperatures (Piotrowski et al., 2004; Rohr et al., 2008). Different bacterial species might have different temperature sensitivities, resulting in different growth rates (and, potentially, violacein production rates) than *J. lividum* and, thus, provide inhibitive ability against *Bd* over a greater range of environmental temperatures.

The effect of temperature on the interaction of anti-*Bd* bacterial metabolite(s) was tested *in vitro* using some species of *Pseudomonas, Stenotrophonas, Chryseobacterium,* and *Acinetobacter* against several *B. dendrobatidis* genotypes (Muletz-Wolz et al., 2017b). However, *Chromobacterium* spp., which are prolific producers of violacein, have not been found nor tested.

In this study, we tested the degree of *Bd* inhibition by four violacein producing *Chromobacterium* spp. ribotypes isolated from the skin of cricket frogs (*Acris gryllus*) in east-central Alabama (Tuskegee, AL). We compared the *Bd*-inhibitory activity of these ribotypes to the widely tested *J. lividum* across ecologically relevant temperatures. These sets of bioassays were done to establish the anti- *Bd* properties of violacein from *Chromobacterium* spp. at different temperatures as well as exploring their effectiveness against three virulent strains of *B. dendrobatidis*. We predicted that temperature would have an effect on the *Bd* inhibition ability of these bacterial ribotypes and that they would show differing inhibition profiles across exposure temperatures and among the chytrid strains. We also tested the effect of temperature on proliferation of the isolated *Chromobacterium* spp. ribotypes, determined whether the purple pigment the ribotypes produced had a mass spectography signature consistent with that of violacein and characterized the potency of violacein produced from the different bacterial isolates.

MATERIALS AND METHODS

Sampling

We hand captured (wearing powder-free nitrile gloves) southern cricket frogs (*Acris gryllus*), an abundant species, in ponds near Tuskegee, AL throughout the species' breeding season (April-September; N=146). Frogs were found either on the grassy edge of the pond or in the water and rinsed with distilled water before swabbing to remove transient bacteria. A sterile cotton-tipped swab was drawn along the webbing of the feet, dorsal and ventral surfaces of each frog (following the protocol described in Stevens et al., 2012) and streaks were made on sterile Tryptic Soy Agar (TSA) plates and sealed with parafilm. Frogs were released to the site of capture immediately after swabbing. We also recorded the air temperature and took water temperature at each time of sampling.

Culture processing

Streaked plates were sealed and returned to the Auburn campus. All plates were incubated at 25°C for 48 hours. Plates were observed for purple colored colonies and were picked and streaked on new TSA plates. Isolated colonies were purified by continuous streaking; cultures were then frozen at -80°C and prepared for DNA extraction. Each pure bacterial colony was grown overnight in Luria Bertani (LB) broth, and bacterial genomic DNA was extracted using a Bacterial DNA extraction kit (E.Z.N.A® Omega-Biotek). Isolated Bacterial DNA quality was checked on the Nano-drop (Thermofisher® Scientific).

Bacterial Identification

Obtained DNA were amplified by nested Polymerase Chain Reaction (PCR) with bacterial specific 16S rRNA gene primers 27F (5'- AGAGTTTGATCMTGGCTCAG-3'), 1492R (5'- TACCTTGTTACGACTT-3'), and 907R (5'- CCGTCAATTCMTTTGACTTT-3'). The 50µl reaction contained 0.5µl of each primer, 25µl PCR 2X master mix (Lucigen® Middleton, WI), 1µl (10ng/µl) of template DNA and 23µl of water. The thermocycler condition was: 94℃ for 2 min, then 30 cycles of 94℃ for 30 sec, 65℃ for 30 sec, 72℃ for 1min and a final extension at 72℃ for 10 mins. Amplified products were checked on 1% agarose stained with ethidium bromide. PCR products were cleaned (E.Z.N.A® Omega-Biotek) and amplified DNA was checked on Nano-drop to confirm the concentration of DNA. Sanger sequencing was done at ACGT Inc, Wheeling, IL. Using Geneious bioinformatics molecular sequence software, obtained consensus sequences were obtained by alignment of the two reverse and forward amplicons (1492R, 907R and 27F). Consensus sequences were submitted on the Ezbiocloud database (www.ezbiocloud.net/identify) to identify sequences with the highest similarity (Yoon et al., 2017). A phylogenetic tree was generated to observe how related the identified bacteria were to one another using MEGA software.

Chytrid Fungus

Three chytrid fungus cultures (JEL 423, JEL 310 and JEL 197) were obtained from the University of Maine, Orono. These strains of *Bd* were chosen because they have previously been tested with *J. lividum* and are highly virulent (Lauer et al., 2008; Becker et al., 2009; Harris et al., 2009) and they were isolated from sites in Panama (JEL 423 and JEL 310) and Washington, D.C (JEL 197). *J. lividum* (YO8846) culture was provided by Dr. Carly Multez-Wolz, National zoological park, Washington, D.C.

Bd-Challenge Assay

The *in vitro* challenge assay was performed following the protocol in Muletz-Wolz et al. (2017b) with the three *B. dendrobatidis* strains, four isolates of identified *Chromobacterium* ribotype and the *J. lividum* (YO8846) – isolated from skin of salamander, *Hemidactylum scutatum* from George Washington National forest, Virginia (Lauer et al., 2008; Harris et al., 2009). We used 96-well plates and incubated each plate at four ecologically relevant temperatures (12℃, 16℃, 22℃ and 24℃). These temperatures were chosen because they have been previously used in *J. lividum* and other *Bd*-inhibition assays and were within the range of water temperature observed during the sampling months.

Bacteria isolates were streaked on 1% tryptone agar plates to grow for 24 hours before inoculating into 40 ml of 1% tryptone broth. One colony from each culture were diluted in 1ml tryptone broth before adding 100 µl of culture was added to 30 ml fresh tryptone broth. Broth cultures were placed on a shaker (100 rpm) and incubated at room temperature for three days so that high densities of inhibitory metabolites would have been produced. To extract cell-free supernatants (CFSs), each bacteria culture was suspended in a sterile 15 ml falcon tubes and spun at 3000rpm (Beckman Allegra 6R) for 1 hour We withdrew the supernatant from the CFS using 5 ml syringes with an 18 gauge needle and filtered them using sterile disposable 0.2 µm pore syringe filters . This CFS was used to determine the inhibitory properties of violacein produced by each of the bacterial isolates against live *Bd* zoospores. Live bacterial cell cultures were not used to avoid competition for available nutrient by bacteria and *Bd* zoospores, so, inhibition will only be dependent on the activity of CFS.

Each strain of *Bd* has been passed at least three times on 1% tryptone agar plates before use for this assay. *Bd* strains were inoculated in 1% tryptone broth for ten days before growing them on

1% tryptone agar (7-10 plates for each one) for 7 days at 20°C. To harvest zoospores, plates were flooded with 1% tryptone broth and left to sit for 10 mins. Sterile disposable pasteur pipettes were used to withdraw broth, and adult cells (zoosporangia) were filtered out using a sterile coffee filter (placed in a funnel and covered with foil before autoclaving).

Experimental design

Figure 1 illustrates the treatment groups for the inhibition assay. *Bd* zoospores were counted using a disposable hemocytometer (Neubauer, Bulldog-Bio Inc.). As needed, zoospores suspension was diluted to obtain approximately 1 x 10^6 zoospores/ml. To each experimental well, 50µl of zoospores was added (50,000 zoospores/well). There were 5 treatment groups; (1) Negative control (NC), (2) Nutrient-depleted negative controls (NDNC), (3) Heat-killed blank (HK); killed at 60°C for 1 hour, (4) CFS Heat-killed, Bacteria Treated (HKBT) and, (5) Bacterial treatments (BT). Each set of plates were incubated at 12°C, 16°C, 22°C and 24°C for 27 days. Optical density at 492 nm (OD_{492}) of each well in all 96-well plates was measured using a microplate spectrophotometer. Plates were read every other day, with the first day being day 0.

Inhibition score calculation

To determine the inhibition score for each bacterial isolate, we subtracted the OD of heat-killed (HKBT & HK) from the corresponding experimental wells (BT & NDNC respectively) to correct for baseline zoospore OD. As described by Muletz-Wolz et al. (2017), corrected OD was transformed using the equation; $\log(OD_{corrected} \times (1- OD_{corrected}) + 1)$, to achieve normality of the values. The slopes of NDNC was taken to account for nutrient depletion. All calculations and statistical analysis were done using R version 3.2.1. Setting intercept at zero, we fit a linear regression to the values generated over time (change in optical density over time) for each well.

Inhibition score was then calculated by dividing the slope of each BT well by average slope of its corresponding NDNC wells on the same plate, where resulting fraction was subtracted from one (1- (BT slope/ NDNC slope). The primary condition for inhibition was that resulting values has to be higher than 0.

Dose-response assay

Ten-fold dilutions of extracted CFS from each bacterial species was made. *Batrachochytrium* zoospores were prepared as described above, and they were diluted to obtain about 2500 zoospores/ml. A 24-well plate assay was set up by adding 100µl of Bd zoospores and 100µl of CFS to each well in triplicates. A total of 15 plates were used for this assay. All plates were incubated at 16 ℃ for 10days. Resulting zoosporangia in each well were counted using an inverted microscope by counting the number sporangia in an ocular frame at 10X objective.

Violacein production at different temperatures

Each bacterium was grown in 1% tryptone broth for 24 hours before suspending 100 µl of each into a 15 ml tubes containing 5 ml of 1% tryptone. Tubes were incubated (on a shaker) at 12℃, 16℃, 20℃, 24℃ and 30℃ for 3 days. 200µl of each set of Bacterial culture was added to each well on a 96-well plate (four replicates). Optical density was read at 600 nm to obtain cell density, and each culture was visually observed for violacein production.

Statistical Analysis

All statistical analysis was adapted from Muletz-Wolz et al. (2017). For each bacterium, a linear mixed-effect model (*lme* function in the "*nlme*" package) was used to determine the slope of each treatment well groups and *Bd* strain. Inhibition score was the response variable and bacteria, *Bd* strain and temperature being the explanatory variable; plate was a random effect. Also, a two-way

ANOVA was done to determine the significance of each fixed effects. *Post-hoc* analysis (Tukey HSD) was done to see significant differences between temperature and individual *Batrachochytrium* strain (*TukeyHSD* function). We used a 3-way ANOVA to compare all across all bacteria species, *Bd*, and temperature. We then generated temperature versus *Batrachochytrium* interaction plots for each strain of bacteria. We also compared the inhibition score of each corresponding NC wells to that of the BT and NDNC wells to determine if any of the bacteria strains were promoting the growth of *Batrachochytrium*. Using the same random effects, we used a linear mixed-effects model to compare each temperature, type of well, and their interaction with the inhibition scores generated. For the dose-response, we used ANOVA analysis to determine the significance of each fixed effect on the number of sporangia in each well to bacterial CFS concentration for each of the *Bd* strains.

RESULTS

Bacteria Identification

Out of the 146 frogs swabbed, 71% had at least one bacteria type that produced violacein (Figure 2). We found isolated purple pigment producing colonies each time of sampling, though, these colonies were not equally distributed on every culture plate. Purple colored colonies were picked out based on the type of similar morphologies in the culture plate. 10 isolates were sequenced, and six of them had 99% similarity to *C. amazonense*, two had a 98% similarity to *C. substugae*, one had a 98% similarity to *C. vacinni*, and one was *C. sphagnii* (99%) (Table 1). *C. amazonense* was the most abundant from our culture collection. Four representative strains were selected from the sequenced collection, and *J. lividum* (YO8846) served as the control (i.e. previously characterized) violacein-producing strain. The strength of inhibition of violacein from each strain was compared across temperatures.

Bacteria-*Bd* Challenge Assay

All *Chromobacterium* spp., *as well as J. lividum* (Y08846), showed inhibition of the three *Bd* strains at each temperature of incubation (Figure 3). Significant differences were observed in the anti-*Bd* activity of *Chromobacterium* spp. compared to *J. lividum*, with temperature playing a significant role in the antifungal activity of bacterial treatments.

The percentage of inhibition was higher at lower temperatures (12℃ and 16℃) for all bacterial CFS used. *C. amazonense* (Figure 3a) inhibited all *Bd* strains with variation across temperatures

(F = 10.4, p = 0.002) and the extent of inhibition did not differ significantly among the *Bd* strains. *C. vacinni* (Figure 3b) was strongly inhibitory of all *Bd* with a non-significant temperatureinteraction (F= 3.95, p = >0.05) and was equally effective against the three *Bd* strains. The strength of inhibition of *C. substugae* (Figure 3c) showed no significant interaction among *B. dendrobatidis* used in this assay with a significant temperature variation (F = 18.1, p = 0.001). Inhibition scores for *C. sphagnii* varied across temperature (F = 6.1, p = 0.02) but *Bd* strains inhibition did not differ in their responses to temperature (Figure 3d). The reference bacteria strain, *J. lividum* (Y08846) (Figure 3e) was significantly less inhibitory of all Bd strains, with a significant difference (F = 4.9, p = 0.01) and variation across all temperatures of incubation (F = 8.45, p = 0.005).

Analysis of variance (3-way ANOVA) comparing the inhibition score of all bacteria strains showed a significant difference with *J. lividum* especially (F = 41.1, $p = 9.26 \times 10^{-10}$), and the temperature still had significant effect (F = 18, $p = 9.37 \times 10^{-13}$). Overall, 16℃ to 20℃ appeared to be the optimum temperature for *B. dendrobatidis* growth while 16℃ was observed to be the best inhibition temperature across all Bd strains (~ 90% above). *J. lividum* was inhibitory to JEL 423 only at 16℃ but was significantly lesser in inhibition of JEL 310 and JEL 197. Pairwise comparison, in this case, showed that inhibition score of JEL 423 to be different from the other two strains. While the other strains had a wider temperature range for the inhibition effectiveness. Also, linear-mixed effect model comparing control wells to treated wells showed that none of the bacterial CFS aided the growth of *Bd* (Figure 4a-d). Negative inhibition score indicates the quantity of growth in those control wells, where 16℃ showed the highest growth as well as being the most inhibitory temperature.

Dose-Response of Bacteria CFS

Undiluted CFS from each bacteria species showed inhibition across all *Bd* strains with a significant interaction among bacteria ($F = 26.7$, $p = <2 \times 10^{-16}$). and interaction among Bd strains ($p = <2 \times 10^{-16}$. JEL 423 showed significant difference in the inhibition across bacteria species ($F = 17.4$, $p = 1.56 \times 10^{-9}$) as well that of JEL 310 ($F = 24.6$, $p = 4.4 \times 10^{-12}$) and JEL 197 ($F = 14.7$, $p = 1.86 \times 10^{-8}$). All other higher fold dilutions were weakly inhibitory to chytrid (Figure 5a-f). The growth of zoosporangia in each well indicates that zoospores were able to survive the antifungal effects of the bacterial metabolites, and the lower concentration was less effective against chytrid. We also observed the presence of motile zoospores in the less concentrated wells; this suggests that slight presence of violacein and or the possible presence of other unidentified anti-*Bd* metabolites may be slowing down the development of these zoospores into adult cells.

Effects of Temperature on Bacterial Growth (Violacein Production)

The turbidity measured by optical density at 600nm, for each bacteria species at five temperatures (Figure 6) indicates the growth of each one at the represented temperatures. At 12℃, all *Chromobacterium* spp. had lighter turbidity but produced purple pigment, except for *J. lividum* (Y08846), which did grow minimally with slight production of violacein. Growth and production of violacein increased between 16℃ and 30℃, with the exception of *J. lividum,* that did not produce violacein at 30℃. We observed that 16℃, 20℃, and 24℃ appear to favor violacein production for each of the identified and tested bacteria species. Hence, the comparison between strains at each growth temperature shows significant differences among the bacteria; 16℃ ($p = 5.84 \times 10^{-10}$), 20℃ ($p = 2.2 \times 10^{-16}$) and, at 24℃ ($p = 2.2 \times 10^{-16}$). Mass-spectrometry analysis of supernatant extracts and a violacein standard indicated that the bacterial supernatants contain

violacein and other bacterial metabolites (Figure 7a-f) that could contribute to the observed anti-fungal activity.”

DISCUSSION

Sampling a common and abundant local amphibian species by swabbing revealed an abundance of *Chromobacterium* spp., which have not been previously reported as part of frog skin microbiota. To date, these *Chromobacterium* spp. have been recorded in soil (Lopes et al. 2009; Short et al. 2018) and water samples (Moss et al., 1978; Soby et al. 2013) as well as plant surfaces (Blackburn et al., .2017). Brucker et al. (2008) reported that bacteria found colonizing the amphibian skin are generally a division of the environmental microbiome in which they are resident. Our findings suggest that *Chromobacterium* spp. found in this locale is a subset of the frog's immediate environment.

We found four strains of violacein-producing bacteria that had not been previously described in this area of the United States. To date, the only violacein producing bacteria from the US have been reported in states of Virginia and California, have all been *J. lividum*. These identified *Chromobacterium* species of violacein-producing bacteria produced the same metabolite as the more widely tested *J. lividum.*

Since *J. lividum* has been the only reported violacein-producing bacteria from amphibian skin, it is the species that has been characterized for its anti-*Bd* properties (Brucker et al. 2008; Harris et al. 2009; Muletz et al. 2012). However, this study's *Bd* challenge assay comparing the four *Chromobacterium* spp. against *J. lividum* documented that the *Chromobacterium* spp. were significantly more inhibitory across all the tested incubation temperatures incubation when compared to this strain of *J. lividum*. Muletz-Wolz et al. (2017b) found that lower temperatures

enhanced the probiotic activity of anti-*Bd* metabolites produced by members of the bacterial community on the skin of amphibians, although, violacein-producing bacteria were not tested; we also observed higher inhibitions by the four tested strains at 12℃ and 16℃.

Chytridiomycosis is reported to be more lethal at lower temperatures (~20℃) (Longo et al., 2010; Stevenson et al., 2013); water temperatures ranged from 17℃ - 29℃ during the sampling period which corresponded to the breeding season; thus, we tested effectiveness at lower and higher temperatures. This study suggests that the effect of infection will be dampened by the presence of violacein-producing bacteria. Although phenotypic characteristics of each strain of *B. dendrobatidis* may differ, thereby affecting the strength of inhibition (Piotrowski et al., 2004), we only observed differences in the inhibition *Bd* strains for *J. lividum* (YO8846). All four *Chromobacterium* spp. were equally inhibitory to the three tested strains of *Bd* at all temperatures. Though *J. lividum* (YO8846) was not as effective against JEL 310 and JEL 197 *Bd*, strains at 16℃, which is the optimum temperature for chytrid growth. Thus, this strain *J. lividum* (YO8846) is less effective against certain chytrids at the optimum temperature for this pathogen's growth. We have not yet sequenced the *Bd* present in our locale but we predict that the identified *Chromobacterium* spp. will be very effective against the local strains.

The abundance of violacein-producing bacteria species symbiont on amphibian skin help dampen the rate of infection by *B. dendrobatidis* (Becker et al., 2009). Brucker et al. (2008b) demonstrated that metabolites produced by bacteria species on the skin of free-living salamanders occur in a concentration high enough to kill *B. dendrobatidis* strains. All bacteria species in this study were able to grow at temperatures ranging from 12℃ to 30℃ (Figure 6). However, it was observed that all four *Chromobacterium species* had significantly higher optical density than *J. lividum* especially, at temperatures of 16, 20, 24 and 30℃. Since 16-20℃ is the optimum

temperature range for chytrid growth and development, the greater densities observed for the *Chromobacterium sp.* at these temperatures indicates a greater production of violacein. Since this pigment is expressed in a cell-density dependent manner (Rettori and Durán 1998; Duran and Menck 2001; Durán et al., 2007)); thus, more protection would be expected under these conditions against chytrids when compared to *J. lividum*. This differences in growth and production of metabolites could be attributed to possible stressors like nutrient availability, environmental stressors like temperature, slower growth rate (Bibb 2005; Pantanella et al., 2007; De Carvalho and Fernandes 2010; Jančič et al., 2016) and or, the biosynthetic gene cluster (BGC) encoding violacein in each of these species are likely under a different transcriptional regulatory pathway (Cimermancic et al., 2014; Medema et al., 2015; Baral et al., 2018;). Choi et al (2015) review of violacein-producer clones suggested that mutations can occur in the gene cluster (*VioABCDE),* which could cause over-production of the metabolite. Production of purple pigment at lower density in the case of *Chromobacterium* spp. in this study, might require more exploration to understand the mechanism of action, as certain stressors might influence the BGC or the possibility of a violacein derivative that is not density dependent.

A study by Loudon et al. (2014) suggested that frog skin bacteria produce a higher concentration of antifungal metabolites when in competition with other bacteria in their community. Consequently, bacteria species tested are expected to produce high enough violacein as in the stock concentration from the dose-response assay. Even if the densities or concentration of violacein on the amphibian skin is not equivalent to that of the undiluted CFS used in the assay, the dose-response experiment indicated that the produced amount of violacein remains effective at a 1:100 dilution, with the *Chrombacterium sp*. being either more potent or concentrated than an exposure to supernatant obtained from an equivalently treated *J. lividum.* Overall,

Chromobacterium spp. appears to be a better biocontrol direction, compared to *J. lividum* (Y08846). Mass spectrometry peaks for each of the bacterial extracts show that they produce violacein based on comparison to a violacein standard (Figure 7a-f). Additional research would be required to determine if the observed anti-Bd activity observed in this study was due to violacein and or other secondary metabolites expressed by these bacterial isolates. We did not test for other compounds that might be present; like violacein derivative; deoxyviolacein (Choi et al., 2015), indole-3-carboxaldehyde (Brucker et al., 2008), although both compounds were described to be produced in lesser quantities compared to violacein. Besides the faster growth rate of *Chromobacterium* spp., several studies have shown their ability to produce hydrogen cyanide (HCN) (Michaels and Corpe 1965; Logan 1989; Michelsen and Stougaard 2012; Short et al., 2018). HCN is known to be lethal to mosquitoes and some fungal species (Michelsen & Stougaard 2012). Assessing HCN production could give a clearer view of the differences in anti-*Bd* properties of these bacteria species and determine if the greater inhibition is due to greater violacein production rate or if, in fact, other metabolites are being produced that have anti-fungal activity.

Bd has been identified in a variety of frog populations in the southeastern US (Peterson et al., 2007; Rothermel et al., 2008) but there have not been observed population declines due to its presence in these areas as in other localities across the world (Peterson et al., 2007) . These purple pigment-producing bacteria were found to be persistently abundant on the cricket frog's skin at each time of sampling, it is presumed that the abundance of this group of antifungal bacteria species is influencing their susceptibility to infection. These sets of bioassays suggest that metabolites produced by *Chromobacterium* spp. are produced at levels high enough across to be extremely effective against *Bd* and the antifungal factors produced are retained even when the bacteria cells are absent or lysed. It could be that the more varied and faster growing over a wider

temperature range, *Chromobacterium* species are affording frogs in more southern US populations a greater protection than what has been observed in more northern US populations in the anti-*Bd* property for each of the *Chromobacterium* spp. tested in this study

No	Identity	Top hit – strain	% similarity	Accession number
6	*Chromobacterium amazonense*	CBMAI3310	99.57	KF137653
1	*Chromobacterium vacinni*	MWU205	98.42	JZJL01000120
2	*Chromobacterium substugae*	PRAA4-1	98.49	JYKAO1000038
1	*Chromobacterium sphagnii*	IIBBL 14B-1	99.71	MKCT01000094

Table 1: Identified *Chromobacterium* spp. isolated from the skin of Cricket frogs

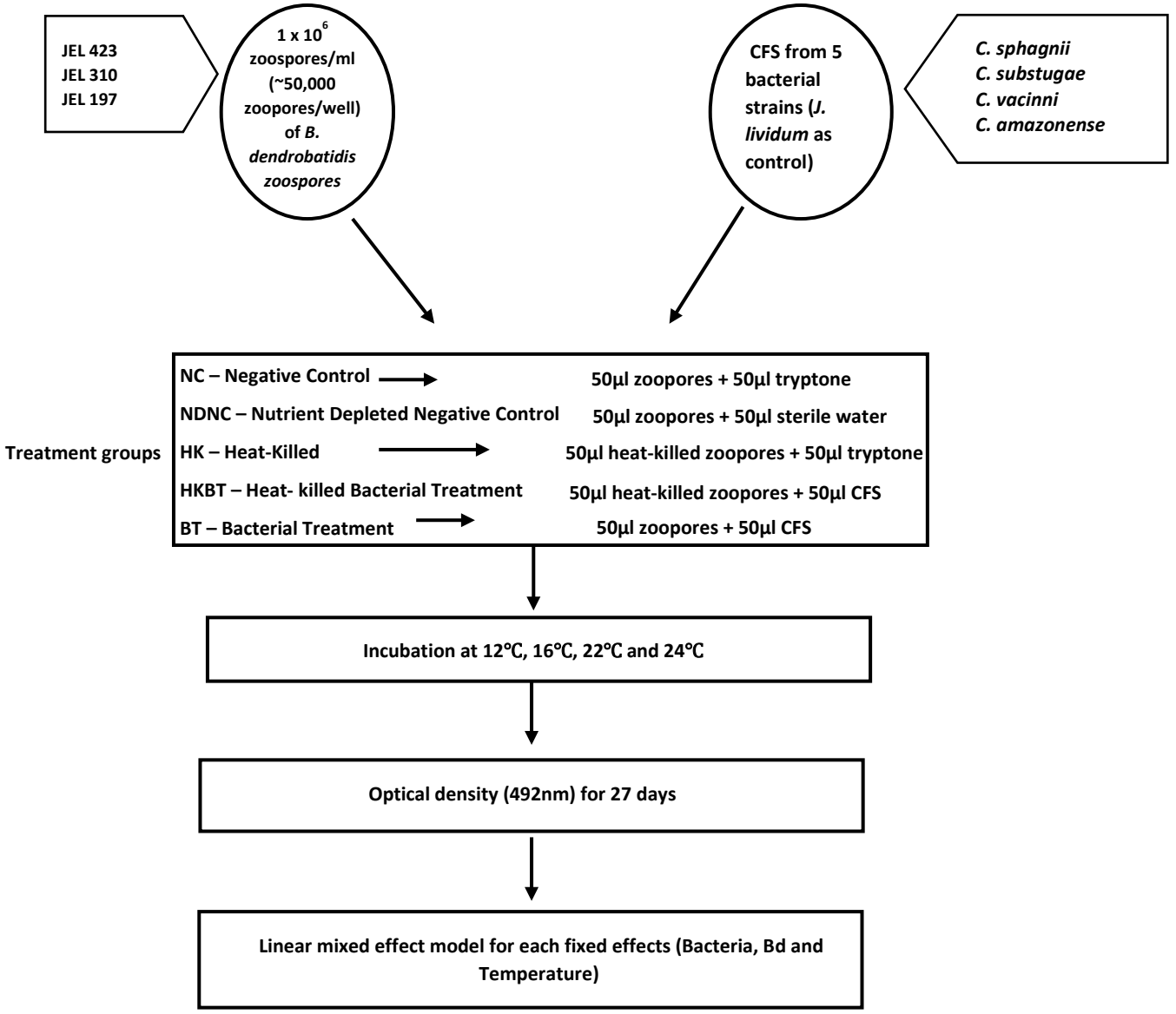

Figure 1: Flow chart of Bacteria-*Bd* challenge assay protocol

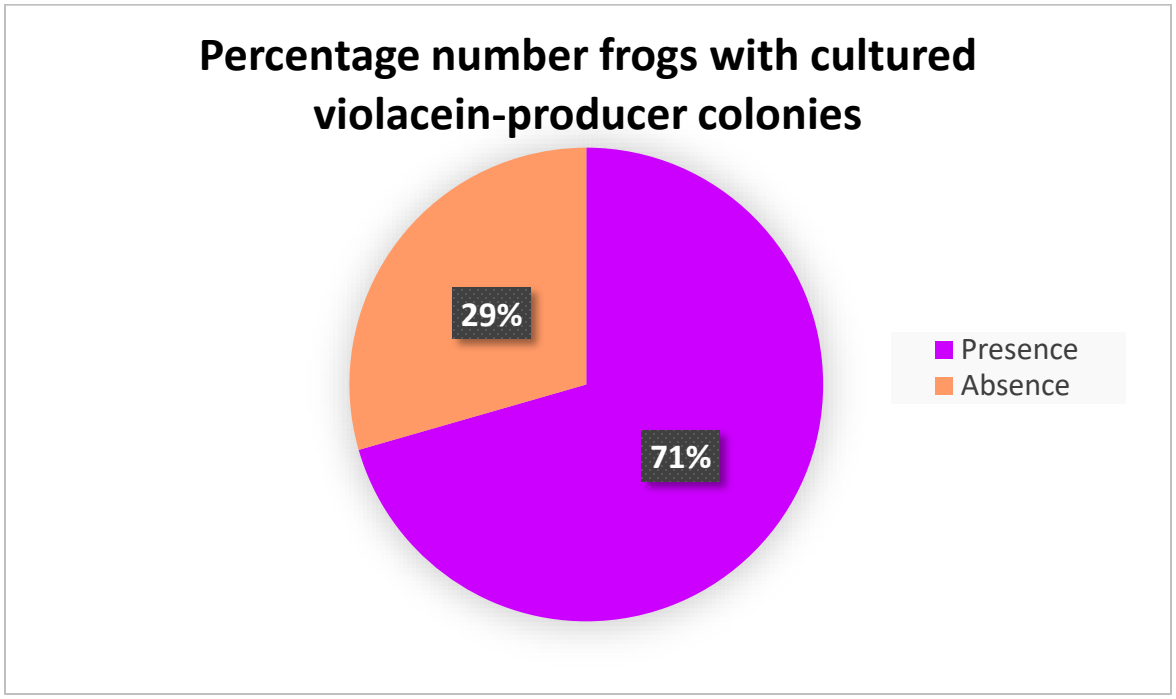

Figure 2: Percentage prevalence of Violacein-producing bacteria found on sampled cricket frogs (N=146)

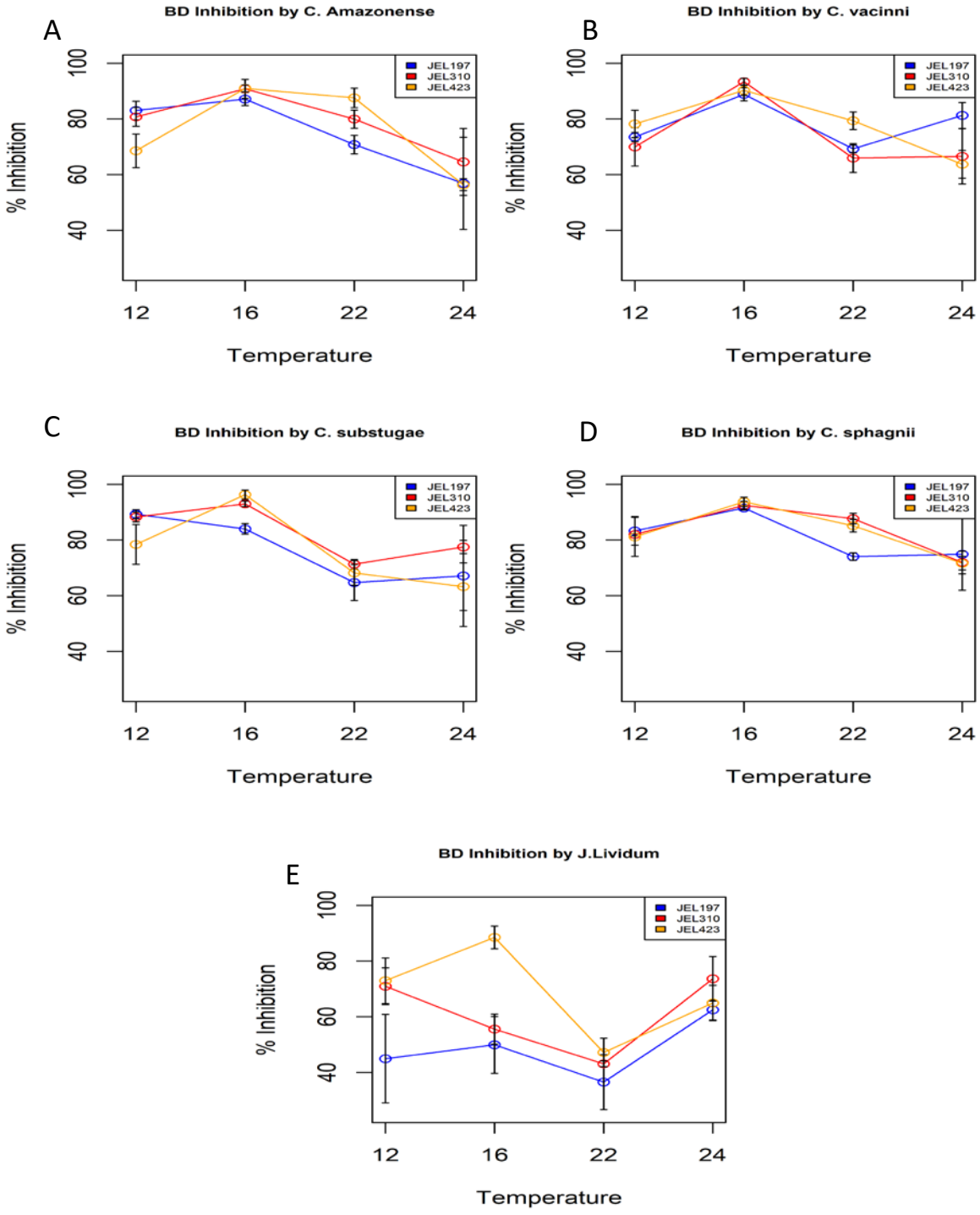

Figure 3: Interaction plots of each Bacteria with *Batrachochytrium dendrobatidis* and temperature. % Inhibition score (Mean ± Standard deviation of % inhibiton score). **(a)***Chromobacterium amazonense* **(b)** *Chromobacterium sphagnii* **(c)** *Chromobacterium vacinni* **(d)** *Chromobacterium substugae* **(e)** *Janthinobacterium lividum*

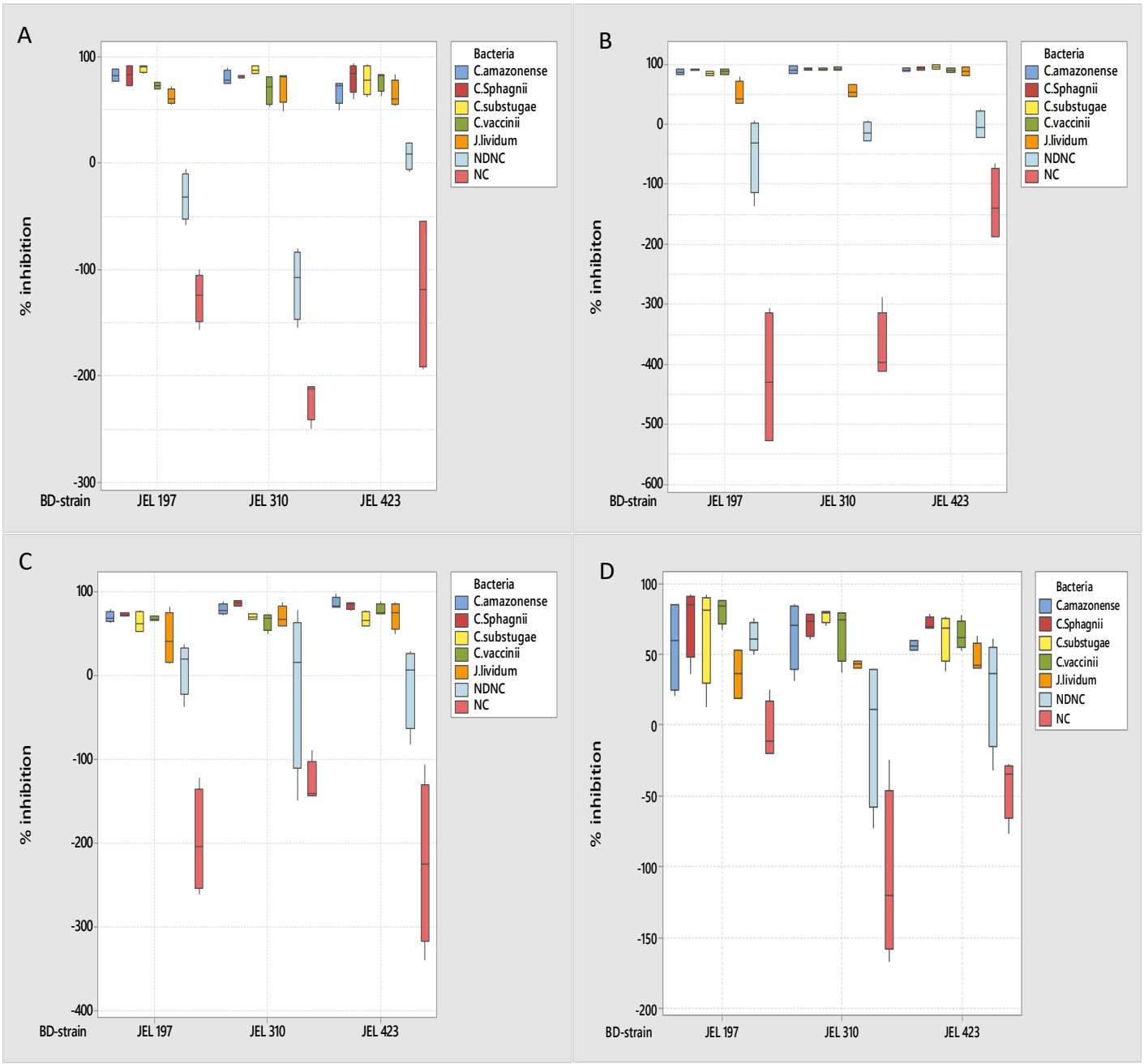

Figure 4: Comparison of % inhibition of each Bacteria treatment wells and control wells for each temperature (NDNC- Nutrient depleted negative control, NC- Negative control). **(a)** 12℃ **(b)** 16℃ **(c)** 22℃ **(d)** 24℃.

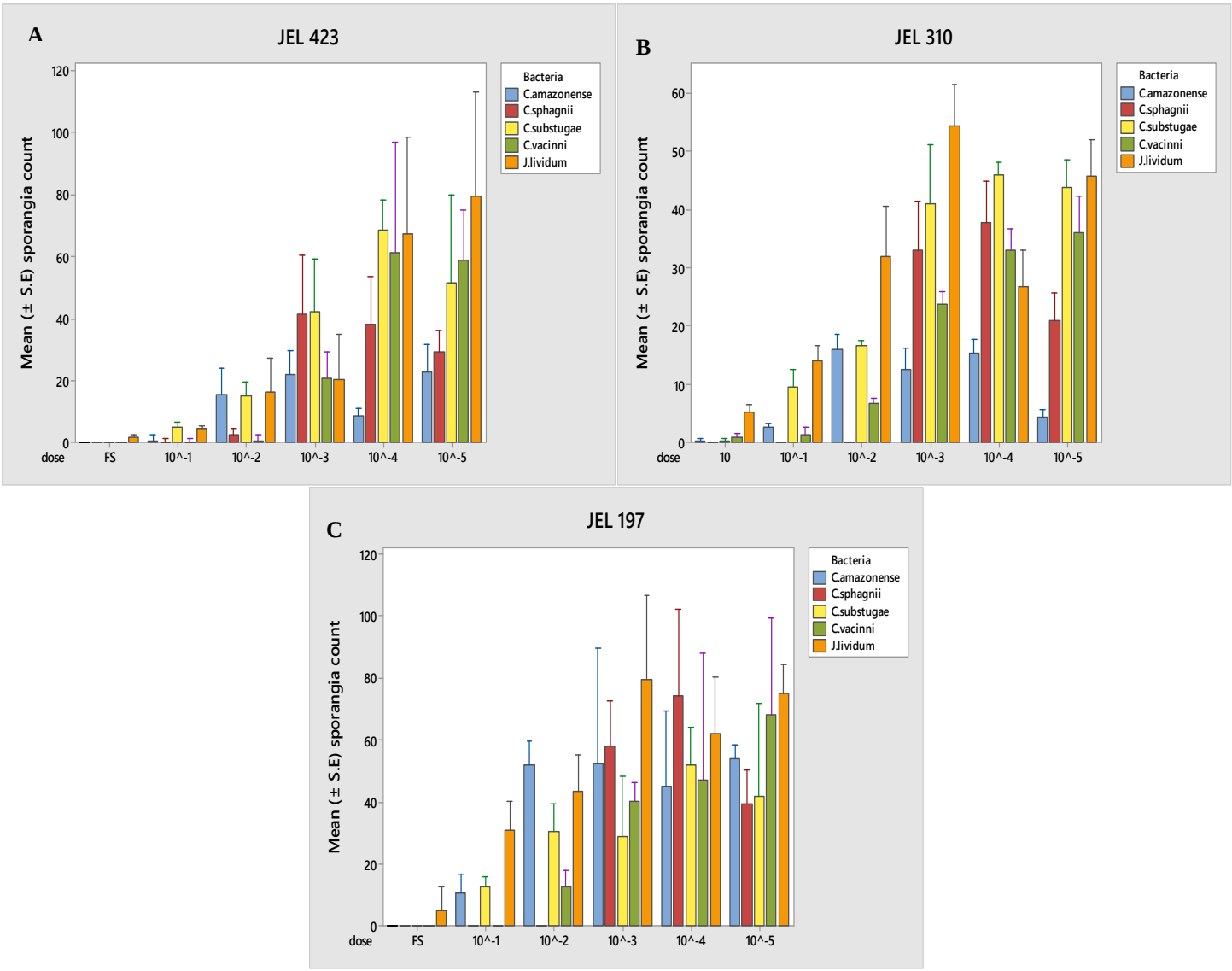

Figure 5: Dose-response comparing the mean sporangia count of *Batrachochytrium* zoosporangia and Bacteria CFS concentration; FS- Undiluted cell-free supernatant. **(a)** JEL 423**(b)** JEL 310 **(c)** JEL 197

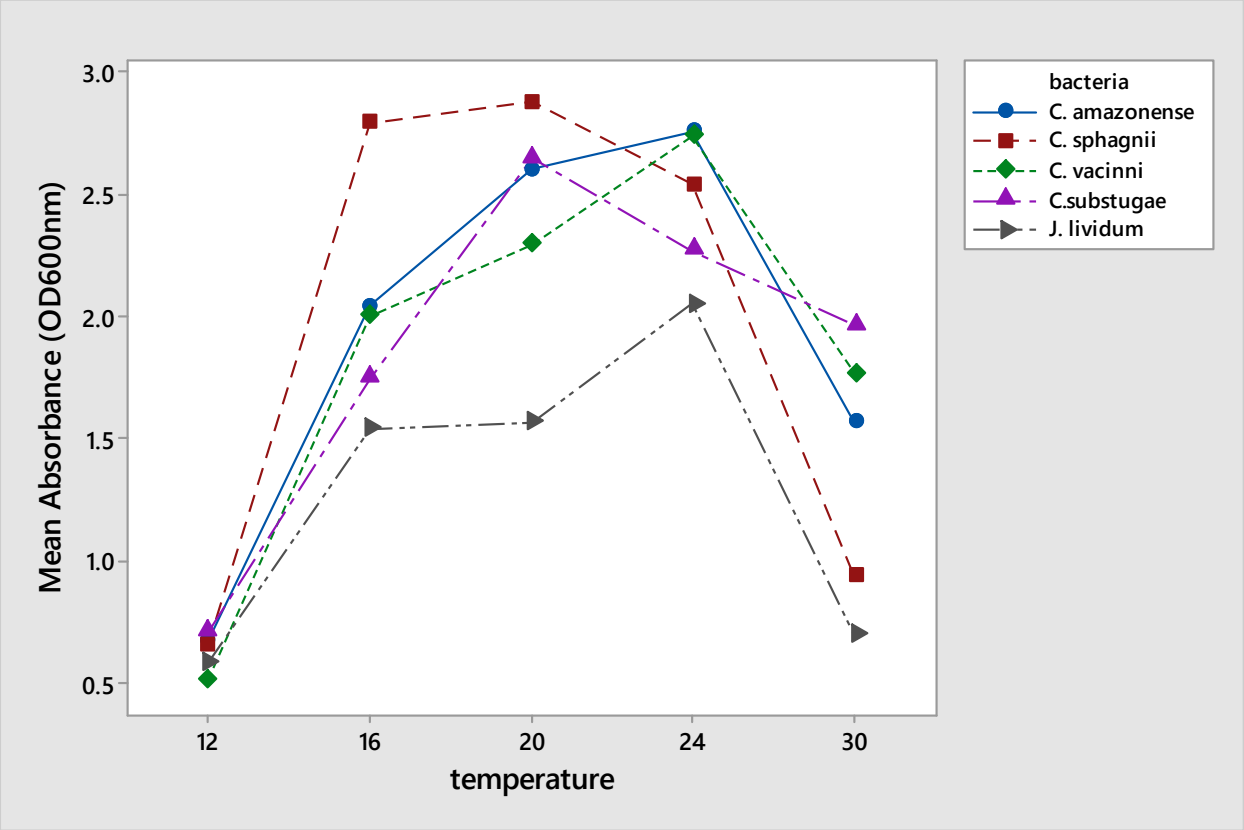

Figure 6: Growth and Violacein production of the five bacterial species across temperatures

A

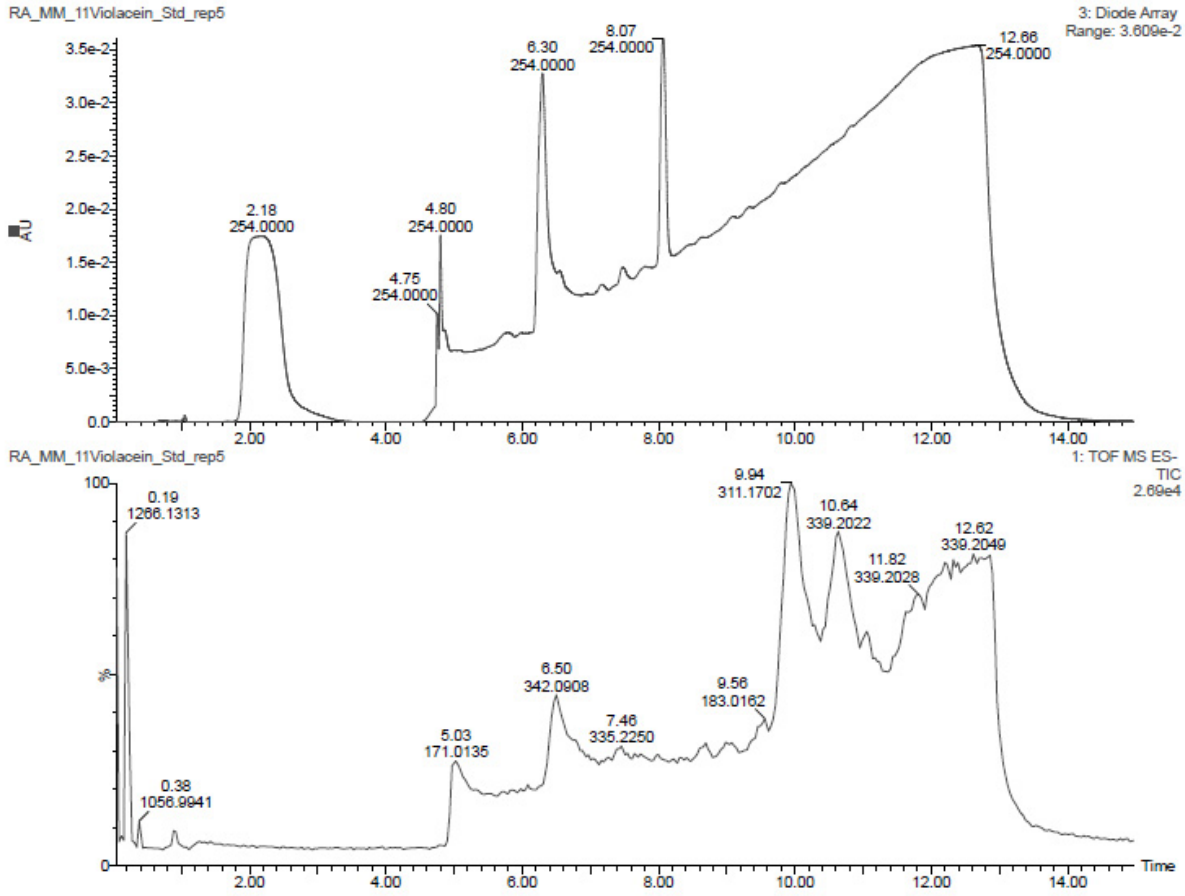

Figure 7a: Mass spectrometry chromatograph for a Violacein standard (Adipogen life sciences®)

B

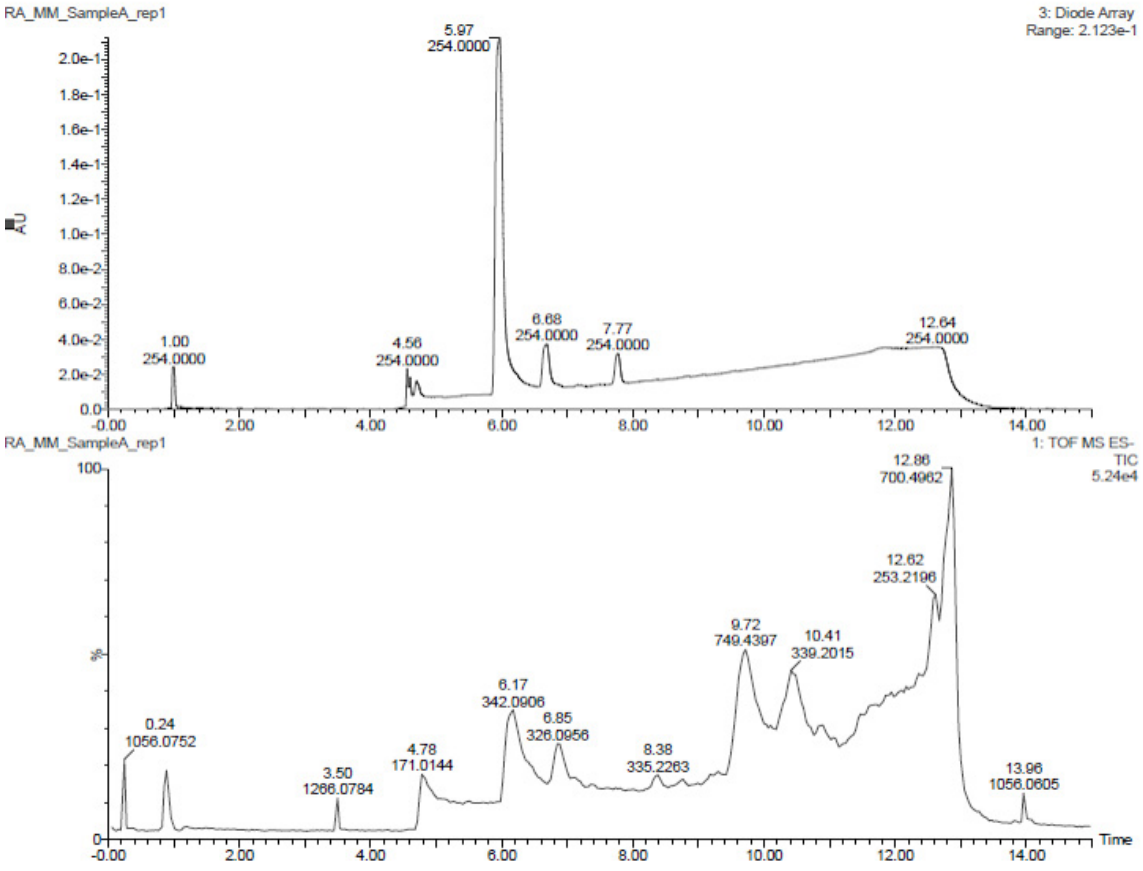

Figure 7b: Mass spectrometry chromatograph for Violacein of *C. amazonense*

C

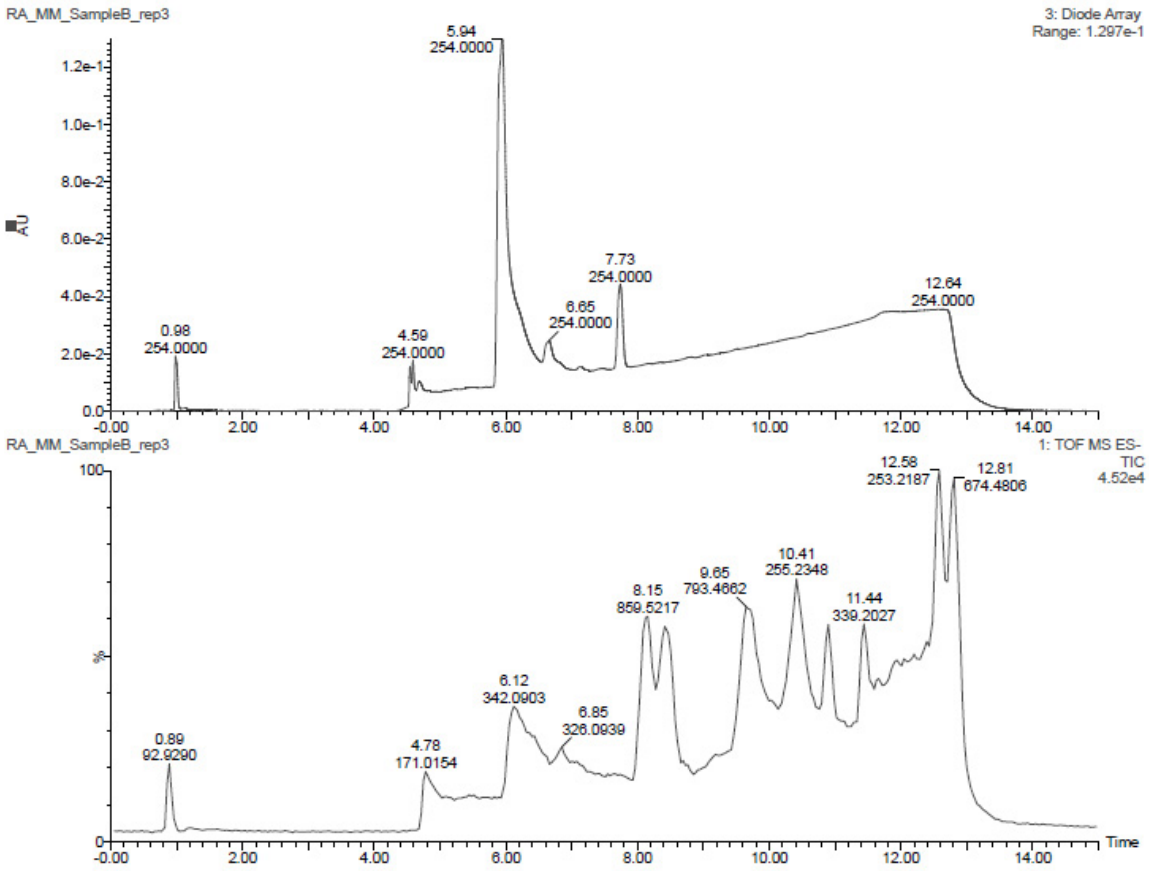

Figure 7c: Mass spectrometry chromatograph for Violacein from *C. sphagnii*

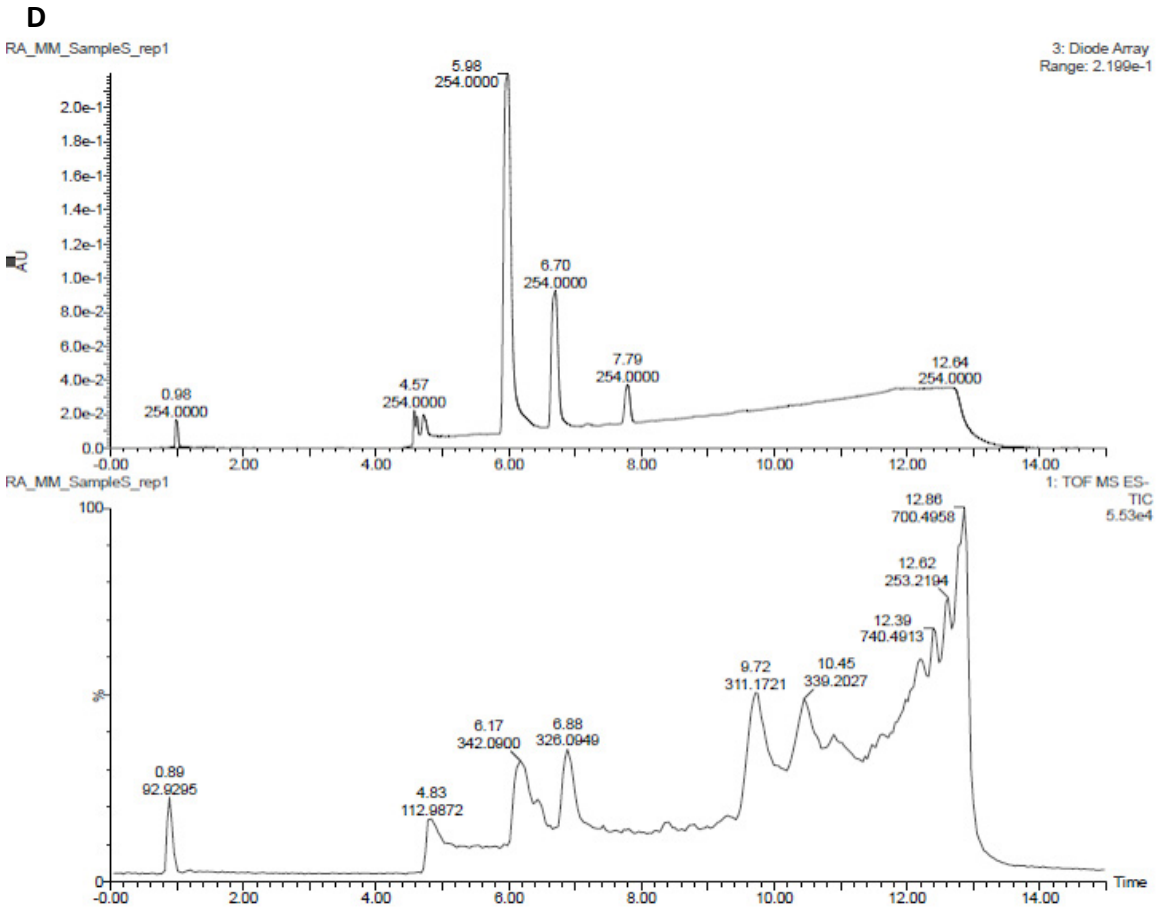

Figure 7d: Mass spectrometry chromatograph for Violacein from *C. substugae*

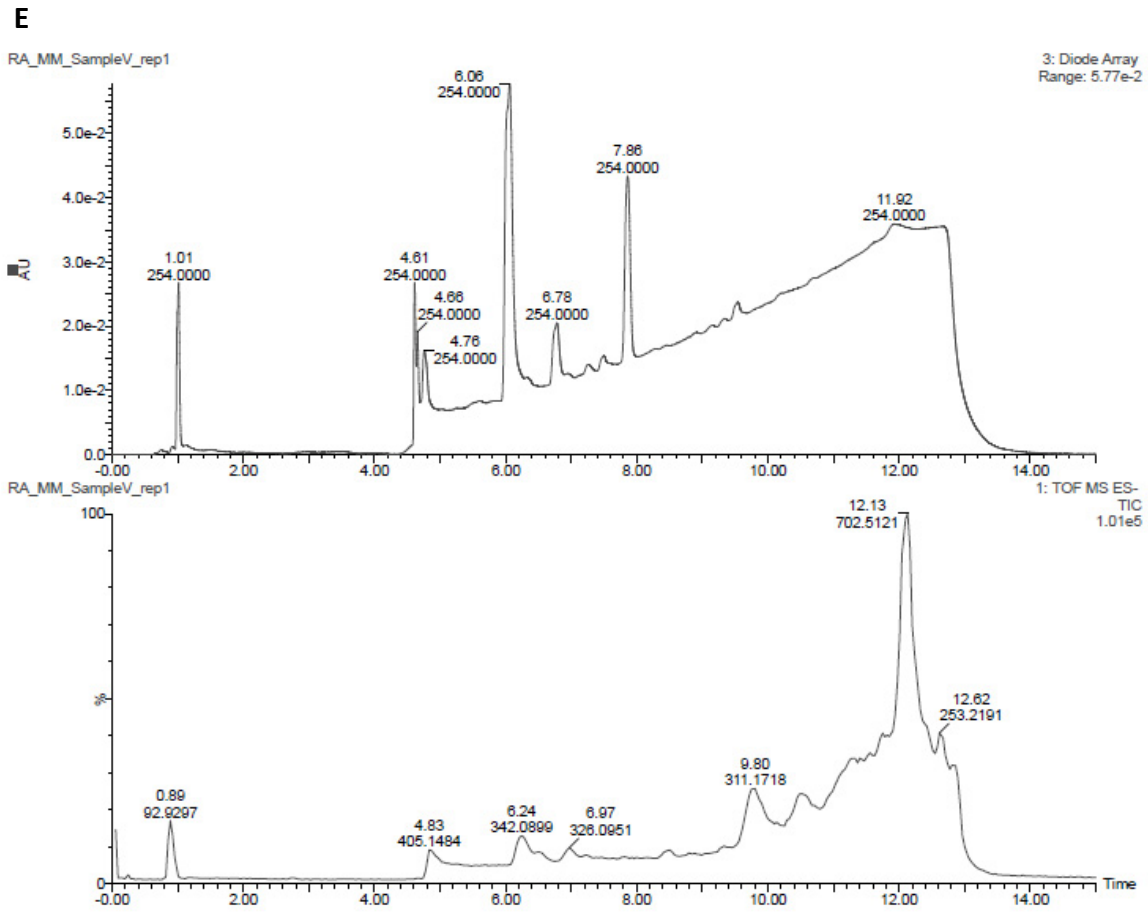

Figure 7e: Mass spectrometry chromatograph for a Violacein from *C. vacinni*

F

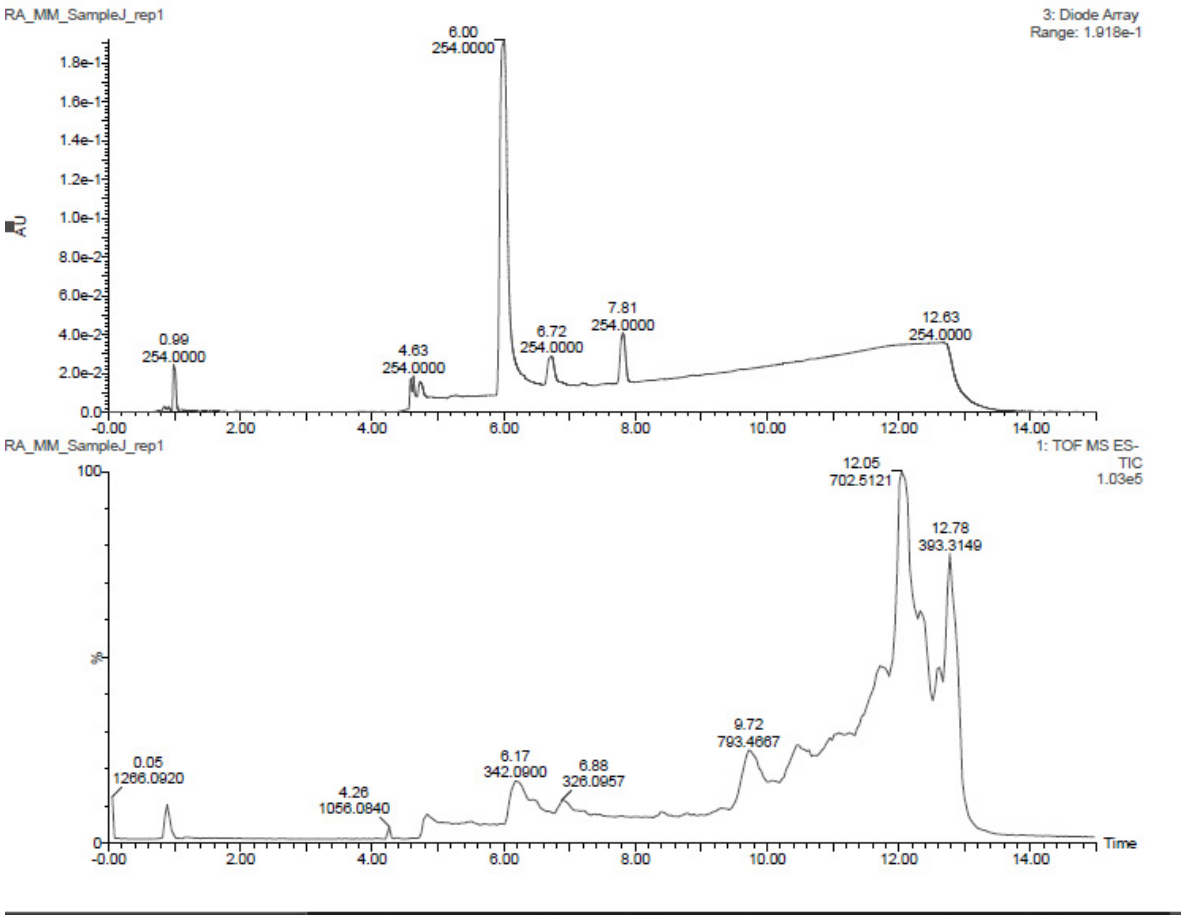

Figure 7f: Mass spectrometry chromatograph for Violacein from *J. lividum*

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