Novel anti-infectives against pathogenic bacteria

Neue Antiinfectiva gegen pathogene Bakterien

Doctoral thesis for a doctoral degree
at the
Graduate School of Life Sciences
Julius-Maximilians-Universität Würzburg

Section: Infection and Immunity

submitted by

Srikkanth Balasubramanian

from

Chennai, India

Würzburg, 2018

Submitted on: …………………………………………………………….…….

Members of the Thesis Committee:

Chairperson: Prof. Dr. Thomas Dandekar

Primary Supervisor: Dr. Tobias Ölschläger

Supervisor (Second): Prof. Dr. Ute Hentschel-Humeida

Supervisor (Third): Prof. Dr. Ulrike Holzgrabe

Supervisor (Fourth): Dr. Usama Ramadan Abdelmohsen

Date of Public Defence:

Date of Receipt of Certificates:

Affidavit

I hereby confirm that my thesis entitled "Novel anti-infectives against pathogenic bacteria" is the result of my own work. I did not receive any help or support from commercial consultants. All source and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,   _____

Srikkanth Balasubramanian

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Neue Antiinfectiva gegen pathogene Bakterien" eigenständig, d. h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

_____

Würzburg,   Srikkanth Balasubramanian

Dedication

This dissertation is dedicated to

My Parents

Acknowledgements

Table of contents

Summary

Marine sponge-associated actinomycetes are reservoirs of diverse natural products with novel biological activities. Their antibiotic potential has been well explored against a range of Gram positive and negative bacteria. However, not much is known about their anti-infective or anti-virulence potential against human pathogens. This Ph.D. project aimed to investigate the anti-infective (anti-Shiga toxin and anti-biofilm) potential of sponge-derived actinobacteria through identification and isolation of their bioactive metabolites produced and characterizing their mechanism of action by transcriptomics. This thesis is divided into three studies with the overall objective of exploring the anti-infective efficacy of actinomycetes-derived extracts and compound(s) that could possibly be used as future therapeutics.

The first study deals with investigation on the anti-Shiga toxin effects of sponge-associated actinomycetes. Diarrheal infections pose a huge burden in several developing and developed countries. Diarrheal outbreaks caused by Enterohemorrhagic *Escherichia coli* (EHEC) could lead to life-threatening complications like gastroenteritis and haemolytic uremic syndrome (HUS) if left untreated. Shiga toxin (Stx) produced by EHEC is a major virulence factor that negatively affects the human cells, leading them to death via apoptosis. Antibiotics are not prescribed against EHEC infections since they may enhance the risk of development of HUS by inducing the production and release of Stx from disintegrating bacteria and thereby, worsening the complications. Therefore, an effective drug that blocks the Stx production without affecting the growth needs to be urgently developed. In this study, the inhibitory effects of 194 extracts and several compounds originating from a collection of marine sponge-derived actinomycetes were evaluated against the Stx production in EHEC strain EDL933 with the aid of Ridascreen® Verotoxin ELISA assay kit. It was found that treatment with the extracts did not lead to significant reduction in Stx production. However, strepthonium A isolated from the culture of *Streptomyces* sp. SBT345 (previously cultivated from the Mediterranean sponge *Agelas oroides*) reduced the Stx production (at 80 µM concentration) in EHEC strain EDL933 without affecting the bacterial growth. The structure of strepthonium A was resolved by spectroscopic analyses including 1D and 2D-NMR, as well as ESI-HRMS and ESI-HRMS^2 experiments. This demonstrated the possible application of strepthonium A in restraining EHEC infections.

In the second study, the effect of marine sponge-associated actinomycetes on biofilm formation of staphylococci was assessed. Medical devices such as contact lenses, metallic implants, catheters, pacemakers etc. are ideal ecological niches for formation of bacterial biofilms, which thereby lead to device-related infections. Bacteria in biofilms are multiple fold more tolerant to the host immune responses and conventional antibiotics, and hence are hard-to-treat. Here, the anti-biofilm potential of an organic extract derived from liquid fermentation of *Streptomyces* sp. SBT343 (previously cultivated from the Mediterranean sponge *Petrosia ficiformis*) was reported. Results obtained *in vitro* demonstrated its anti-biofilm (against staphylococci) and non-toxic nature (against mouse macrophage (J774.1), fibroblast (NIH/3T3) and human corneal epithelial cell lines). Interestingly, SBT343 extract could inhibit staphylococcal biofilm formation on polystyrene, glass and contact lens surfaces without affecting the bacterial growth. High Resolution Fourier Transform Mass Spectrometry (HR-MS) analysis indicated the complexity and the chemical diversity of components present in the extract. Preliminary physio-chemical characterization unmasked the heat stable and non-proteinaceous nature of the active component(s) in the extract. Finally, fractionation experiments revealed that the biological activity was due to synergistic effects of multiple components present in the extract.

In the third study, anti-biofilm screening of 50 organic extracts generated from solid and liquid fermentation of 25 different previously characterized sponge-derived actinomycetes was carried out. This led to identification of the anti-biofilm organic extract derived from the solid culture of *Streptomyces* sp. SBT348 (previously cultivated from the Mediterranean sponge *Petrosia ficiformis*). Bioassay-guided fractionation was employed to identify the active fraction Fr 7 in the SBT348 crude extract. Further purification with semi-preparative HPLC led to isolation of the bioactive SKC1, SKC2, SKC3, SKC4 and SKC5 sub-fractions. The most active sub-fraction SKC3 was found to be a pure compound having BIC_{90} and MIC values of 3.95 µg/ml and 31.25 µg/ml against *S. epidermidis* RP62A. SKC3 had no apparent toxicity *in vitro* on cell lines and *in vivo* on the greater wax moth *Galleria melonella* larvae. SKC3 was stable to heat and enzymatic treatments indicating its non-proteinaceous nature. HR-MS analysis revealed the mass of SKC3 to be 1258.3 Da. Structure elucidation of SKC3 with the aid of 1D and 2D-NMR data is currently under investigation. Further, to obtain insights into the mode of action of SKC3 on *S. epidermidis* RP62A, RNA sequencing was done. Transcriptome data revealed that SKC3 was recognized by RP62A at 20 min and SKC3 negatively interfered with the central metabolism of staphylococci at 3 h. Taken

together, these findings suggest that SKC3 could be a lead structure for development of new anti-staphylococcal drugs.

Overall, the results obtained from this work underscore the anti-infective attributes of actinomycetes consortia associated with marine sponges, and their applications in natural product drug discovery programs.

Zusammenfassung

Meeresschwamm-assoziierte Actinomyceten stellen ein Reservoir für verschiedene natürliche Produkte mit neuartigen biologischen Aktivitäten dar. Ihr antibiotisches Potenzial gegenüber einer Reihe von Gram-negativen und -positiven Bakterien ist bereits intensiv erforscht worden. Wenig ist allerdings über ihre antiinfektive und antivirulente Wirksamkeit gegenüber menschlichen Pathogenen bekannt. Ziel dieser Doktorarbeit war es, die antiinfektiven Fähigkeiten (anti-Shiga-Toxin und anti-Biofilm) der aus Schwämmen isolierten Actinobakterien zu untersuchen. Hierfür wurden bioaktive Metabolite der Actinobakterien identifiziert und isoliert und abschließend wurde ihr Wirkmechanismus mit Hilfe einer Transkriptomanalyse charakterisiert. Diese Arbeit ist in drei Studien gegliedert, welche alle zum Ziel hatten die antiinfektive Wirksamkeit von aus Actinomyceten gewonnenen Extrakten und Komponente(n), welche möglicherweise als zukünftige Therapeutika dienen könnten, zu untersuchen.

Die erste Studie befasst sich mit den anti-Shiga-Toxin Effekten der Meeresschwamm-assoziierten Actinomyceten. Durchfallinfektionen stellen in vielen Entwicklungsländern aber auch in Industrieländern eine große Gefahr dar. Durchfallerkrankungen die durch enterohämorrhagische *Escherichia coli* (EHEC) hervorgerufen werden, können sich zu lebensbedrohlichen Komplikationen wie Gastroenteritis oder dem hämolytisch urenischen Syndrom (HUS) weiterentwickeln. Das von den EHEC Stämmen produzierte Shiga-Toxin (Stx) stellt hierbei den Haupt Virulenz Faktor dar, welcher die eukaryotische Proteinsynthese menschlicher Zellen negativ beeinflusst, was wiederum den Zelltod durch Apoptose zur Folge hat. Die Behandlung der EHEC-Patienten mit Antibiotika wird nicht empfohlen, da dies zu einem Anstieg von freigesetztem Stx der zersetzen Bakterien führen könnte, wodurch das Risiko für die Entwicklung des HUS ansteigt. Aus diesem Grund werden effektive Medikamente dringen benötigt, welche die Stx Produktion blockieren ohne das Wachstum der Bakterien zu beeinflussen. In dieser Studie wurden 194 Extrakte und einige isolierte Komponenten von aus Schwämmen gewonnenen Actinomyceten auf ihren negativen Einfluss auf die Stx Produktion des EHEC Stammes EDL933 mit der Hilfe des Ridascreen® Verotoxin ELISA Kits untersucht. Es konnte gezeigt werden, dass die Zugabe der Extrakte keinen signifikanten Einfluss auf die Stx Produktion hatte. Strepthonium A auf der anderen Seite, welches aus *Streptomyces* sp. SBT345 isoliert wurde (vom mediterranen Schwamm *Agelas oroides*) konnte die Stx Produktion von EDL933 bei einer Konzentration von 80 µM

reduzieren ohne das Wachstum des EHEC Stammes zu beeinflussen. Die Struktur von Strepthonium A wurde mittels spektroskopischer Analyse (1D- und 2D-NMR), sowie mittels ESI-HRMS und ESI-HRMS[2] Experimenten entschlüsselt. Basierend auf diesen Ergebnissen könnte Strepthonium A eine mögliche Alternative oder Zusatz in der Behandlung einer EHEC Infektion darstellen.

In der zweiten Studie wurde der Einfluss der Meeresschwamm-assoziierten Actinomyceten auf die Biofilmbildung von Staphylokokken bewertet. Medizinische Produkte wie Kontakt Linsen, metallische Implantate, Katheter, Herzschrittmacher, usw. stellen optimale ökologische Nischen für die Ausbildung von bakteriellen Biofilmen dar, wodurch Infektionen im Menschen hervorgerufen werden können. Bakterien in einem Biofilm sind deutlich toleranter gegenüber der Immunantwort ihres Wirtes sowie gegenüber konventionellen Antibiotika und sind daher schwer zu bekämpfen. In dieser Studie wurde das anti-Biofilm Potential eines organischen Extrakts der flüssigen Fermentation von *Streptomyces* sp. SBT343 (vom mediterranen Schwamm *Petrosia ficiformis*) ermittelt. *In vitro* Ergebnisse zeigten, dass das organische Extrakt anti-Biofilm (gegenüber *Staphylococci*) Fähigkeiten besitzt und nicht toxisch für Maus Makrophagen (J774.1), Fibroblasten (NIH/3T3) und humane korneale Epithelzellen ist. Zudem konnte gezeigt werden, dass das SBT343 Extrakt die Ausbildung eines Biofilms von Staphylokokken auf den Oberflächen von Polystyrol, Glass und Kontaktlinsen unterbinden konnte ohne das bakterielle Wachstum zu beeinflussen. Die hochauflösende Fouriertransformation-Massenspektrometrie (HR-MS) Analyse konnte die Komplexität sowie die chemische Vielfalt an Komponenten im Extrakt aufzeigen. Eine vorläufige, physio-chemische Charakterisierung deutet darauf hin, dass die aktive Komponente im Extrakt hitzestabil und nicht proteinartiger Natur ist. Abschließend konnte durch Fraktionierungsexperimente gezeigt werden, dass die biologische Aktivität auf synergistischen Effekten mehrerer Komponenten im Extrakt beruht.

In einer dritten Studie wurden 50 organische Extrakte, welche aus fester und flüssiger Fermentierung von 25 verschiedenen aus Meeresschwämmen isolierten Actinomyceten gewonnen wurden, auf anti-Biofilm-Aktivität untersucht. Hierbei wurde die anti-Biofilm Aktivität des organischen Extrakts der Festkultur von *Streptomyces* sp. SBT348 (vom mediterranen Schwamm *Petrosia ficiformis*) identifiziert. Eine Bioassay gestützte Fraktionierung führte zu der Identifikation der aktiven Fraktion Fr 7 im SBT348 Extrakt. Durch weitere Aufreinigung des Extrakts mit einer semipräparativen HPLC, konnten die bioaktiven Sub-Fraktionen SKC1, SKC2, SKC3, SKC4 und SKC5 isoliert werden. Die Sub-

Fraktion SKC3 hatte den stärksten anti-Biofilm Effekt und bestand aus einer reinen Verbindung mit BIC_{90} und MIC Werten von 3,95 μg/ml und 31,25 μg/ml gegen *S. epidermidis* RP62A. SKC3 zeigte weder erkennbare Toxizität gegenüber Zelllinien *in vitro* noch gegenüber den Larven der großen Wachsmotte *Galleria melonella in vivo*. SKC3 war Hitze- und Enzym-resistent, was auf eine nicht proteinartige Natur hindeutet. Eine HR-MS Analyse ergab, dass die Masse von SKC3 1258,3 Da beträgt. Die Strukturanalyse von SKC3 durch 1D und 2D-NMR ist zurzeit in Bearbeitung. Um weiteres Verständnis über den anti-Biofilm Wirkmechanismus von SKC3 auf *S. epidermidis* RP62A zu erlangen, wurde eine RNA Sequenzierungsanalyse durchgeführt. Die Transkriptomanalyse zeigte, dass SKC3 von RP62A nach einer 20-minütigen Inkubationszeit erkannt wird und dass SKC3 den zentralen Metabolismus des Staphylokokken Stammes nach 3 h negativ beeinflusst. Zusammengenommen deuten die Ergebnisse darauf hin, dass SKC3 als Leitstruktur für die Entwicklung neuer anti- Staphylokokken Medikamente dienen könnte.

Zusammenfassend heben die Ergebnisse dieser Arbeit die antiinfektiven Eigenschaften der Meeresschwamm-assoziierte Actinomyceten hervor und bieten eine Möglichkeit für die Nutzung dieser in Wirkstoffentwicklungsprogrammen.

1. General introduction

1.1. Infectious diseases and antibiotic resistance

Infectious diseases have continued to threaten the achievements of modern medicine for the past 70-80 years (Levy and Marshall, 2004). The mortality rates by infectious diseases (particularly of bacterial origin) account to one-fifth of the global deaths and is considered to be the major killer for children aged <5 years (WHO, 2009). The discovery of antibiotics was a major turn point in the management of bacterial infections which has led to substantial benefits on human and animal health. Antibiotics work against bacteria by targeting essential processes such as negative interference with cell wall/membrane synthesis/organization leading to bacterial cell death (bactericidal), or by blocking DNA/RNA/protein synthesis arresting the bacterial growth (bacteriostatic) (Coates et al., 2002; Aminov, 2010).

The discovery of penicillin by Alexander Fleming marked the onset of the "Golden age of antibiotics", the period between 1940 and 1960s (Brannon and Hadjifrangiskou, 2016). In this time-frame, plethora of new antibiotics were discovered by empirical approaches involving fermentation of soil microbes. However, their extensive over mining programs by the end of 1960s has brought an end to the initial era of antibiotic discovery (Lewis, 2012). By late 1970s-until now, the glory of traditional fermentation approaches has gradually diminished (Silver, 2011; Stallforth and Clardy, 2014; Silva et al., 2016) and currently there is a phase of void in the discovery of new antibiotics (**Figure 1**).
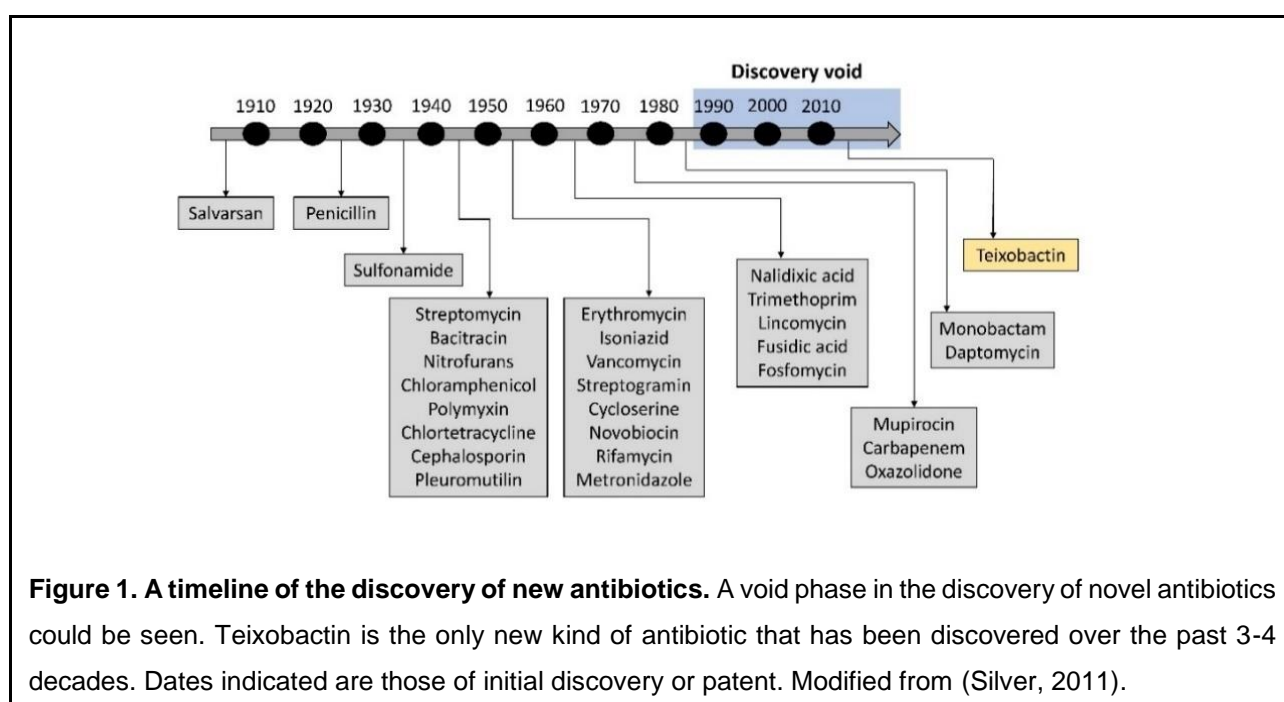
Figure 1. A timeline of the discovery of new antibiotics. A void phase in the discovery of novel antibiotics could be seen. Teixobactin is the only new kind of antibiotic that has been discovered over the past 3-4 decades. Dates indicated are those of initial discovery or patent. Modified from (Silver, 2011).

Lack of innovation and adequate investments by pharmaceutical venture capitalists (owing to the huge cost involved in the drug discovery process, uncertain life cycles of new drugs in the market and increasing stringent drug regulation processes) are some of the main reasons behind this sharp fall-off in the antibiotic drug discovery timeline (Hogberg et al., 2010; Gill et al., 2015). Over the last 40 years, only one new broad-spectrum antibiotic Teixobactin has been discovered so far (Ling et al., 2015). Teixobactin was discovered in a screen of 10,000 uncultured bacteria using the innovative iChip technology (iChip is a multichannel miniature device that can cultivate rare microbial cells directly in their source environments in a high-throughput manner). The discovery of Teixobactin highlights the potential of innovative approaches in fueling the existing dry antibiotic pipeline with the yet undiscovered drugs (Arias and Murray, 2015).

Alexander Fleming during his Nobel Prize lecture in 1945, clearly warned that the inappropriate usage of antibiotics could lead to development of resistance (Fleming, 1945). However, the medical community and public have failed to recognize this risk, and this has led to a global overuse and misuse of antibiotics. Consequently, bacterial strains have evolved to become insensitive and tolerant to existing antibiotics. The emergence of multidrug-resistant, extensively drug-resistant and pan drug-resistant bacterial strains have now posed fears of an expected post-antibiotic era in which many infections could become untreatable (Sousa et al., 2015; Hauser et al., 2016).

The inefficacy of conventional antibiotics against drug-resistant bacteria has become a global health and economic concern (Sommer, 2014; Fitchett, 2015; Tillotson, 2015). The magnitude of this problem on a global scale has been outlined in the WHO's Global Report on Surveillance (WHO, 2014). Estimates suggest that antimicrobial resistance could lead to about 25,000 deaths per year in the European Union (EU) and 23,000 deaths per year in the USA. The total economic cost is estimated to be around €1.5 billion per year in the EU and is as high as $20 billion per year in the USA (WHO, 2015; CDC, 2013; CDC, 2014). According to a report from the UK, the human cost of antibiotic resistance crisis is estimated to be around 300 million cumulative premature deaths by 2050, together with a global economy-related loss of $100 trillion (Neill, 2014; Arias and Murray, 2015). The need for expensive drugs for second line treatments, longer hospital stays, and prolonged sick leaves are some of the obvious reasons behind this economic burden (Coast and Smith, 2003).

Drug-resistant bacteria could persist, multiply, and produce virulence despite the presence of antibiotic drugs. Several resistance mechanisms aid bacterial tolerance to antibiotics. Like

antibiotics, the antibiotic resistance mechanisms are ancient and existed even before the antibiotics introduction into the market or their usage (Davies and Davies, 2010; D'Costa et al., 2011). Antibiotic resistance could be exogenous or endogenous (Silver, 2011). Endogenous resistance occurs endogenously in the bacterial pathogen by mutations and selection pressure. As an outcome of endogenous resistance, bacteria could possess the following properties:

- reduced target(s) affinity to drugs
- remodeling of the target(s)
- reduced drug influx and efflux
- upregulation of target(s)

Exogenous resistance occurs by horizontal gene transfer (HGT) mediated transmission of resistance to human bacterial pathogens from environmental organisms (such as antibiotic producers, non-human pathogens and commensals). As an outcome of exogenous resistance, bacteria could display the following properties leading to ineffectiveness of antibiotics:

- class specific efflux of drugs
- class specific modification or degradation of drugs
- target(s) protection or modification

From the existent data, it envisaged that resistance to antibiotics is almost inevitable and it could emerge soon after the introduction into the market (**Figure 2**). Thus, efforts aiming at discovery of new antibiotics and alternate approaches should continue to circumvent this inexorable rise of antibiotic resistance and inexistence of effective drugs in the market. In parallel to this, the following should be done:

- Rational dose regimens based on pharmacodynamic and pharmacokinetic profiles should be prescribed by the medical practitioners to avoid the antibiotics overuse (Cheng et al., 2016).
- Antibiotic prescriptions for treating diseases with non-microbial origin should be strictly avoided.
- Antibiotics must be carefully used in animal and agricultural context to avoid the spread of resistance via food chains and environments (Chang et al., 2015).
- Hygiene conditions should be improved to avoid the accumulation and spread of resistant bacteria in the environment (WHO, 2001; Hogberg et al., 2010).

- Coordinated networking of medical professionals, microbiologists, natural product chemists and pharmacologists together with investor pharmaceutical companies could drive the existing effective drugs towards clinical applications and thereby bolster the treatment regimens of patients experiencing drug-resistant infections.

Antibacterial introduced into the market

Chloramphenicol
Tetracycline
Streptomycin

Methicillin
Ampicillin

Erythromycin
Vancomycin

Linezolid
Daptomycin

Sulfonamides
Penicillin

1930　1940　1950　1960　1970　1980　1990　2000　2010

Sulfonamides
Penicillin

Methicillin

Ampicillin

Linezolid
Daptomycin

Tetracycline
Streptomycin
Chloramphenicol

Erythromycin
Vancomycin

First antibacterial resistance reported

Figure 2: Timeline depicting the development of resistance. From introduction of antibiotics into market to development of significant clinical resistance. Modified from (O'Connell et al., 2013).

An ideal target for development of new antibiotic drugs should possess the following properties:

- It should not be vulnerable to the development of rapid resistance.
- The structure of the target should be conserved among different bacterial species if broad-spectrum activity needs to be achieved.
- Its essentiality to the organism of the function should be there.
- It should not be structurally or functionally homologous with humans (to avoid toxic effects).

With these properties it is likely that the rate-limiting steps of conventional antibiotic discovery could be overcome, and the resistance phenomenon will be avoided (Silver, 2011). The inexorable rise of antibacterial resistance with the conventional drugs has led to a massive shift in the drug-discovery research paradigms. Development of resistance-

resistant drugs (that act against drug-resistant pathogens), anti-resistant drugs (that could augment the activity of existing antibiotics by circumventing the drug-resistance mechanisms; e.g. β-lactamase inhibitors, efflux pump inhibitors, membrane permeabilizers), host-directed therapies (that modulate the host immune systems and provoke infection clearance), alternate treatments (phage therapy, microbiota therapy, usage of probiotics and prebiotics) etc. are some of the blooming areas of research against drug-resistant pathogens (Gill et al., 2015).

1.2. Anti-virulence strategies

Bacteria encounter different challenges in the host environment such as pH changes, reduced oxygen levels, active immune response, secretions from the host (like mucus), existing host microbiota etc. To establish themselves in the host and cause a disease, they are equipped with an arsenal of components called virulence factors (Staskawicz et al., 2001). Examples of these factors include motility proteins, enzymes, toxins, secretion systems, adherence and colonization components (pili, curli and biofilms), cell-cell communication molecules (quorum sensing components) (Escaich, 2008). These factors are non-essential for bacterial growth, but are coordinately expressed during an infection in the host (Allen et al., 2014). Targeting the virulence or infectivity of the pathogen without directly affecting its survival (anti-virulence/anti-pathogenic/anti-infective approach) could combat the bacterial diseases. They are specifically aimed at disarming the pathogens of their virulence factors that lead to the disease without hampering the growth (Rampioni et al., 2014; Sousa et al., 2015; Silva et al., 2016). The subsequent neutralization or inhibition of virulence factors could block the pathogen progression to cause a disease and thereby, allowing the pathogen elimination through host immunity or antibiotic therapy (Then and Sahl, 2010; Allen et al., 2014; Johnson and Abramovitch, 2017). Anti-virulence therapy is an approach that is even older than antibiotic usage. In 1893, the German physiologist Emil von Behring treated diphtheria affected children with immune antiserum raised against diphtheria toxin.

There has been a considerable increase in the development of anti-virulence approaches over the past two decades (**Figure 3**). Currently, the United States Food and Drug Administration (US-FDA) approved anti-virulence therapies exist only for *Bacillus anthracis*, *Clostridium botulinum* and *C. difficile* infections. There are also several anti-virulence drugs in preclinical trials (Dickey et al., 2017).
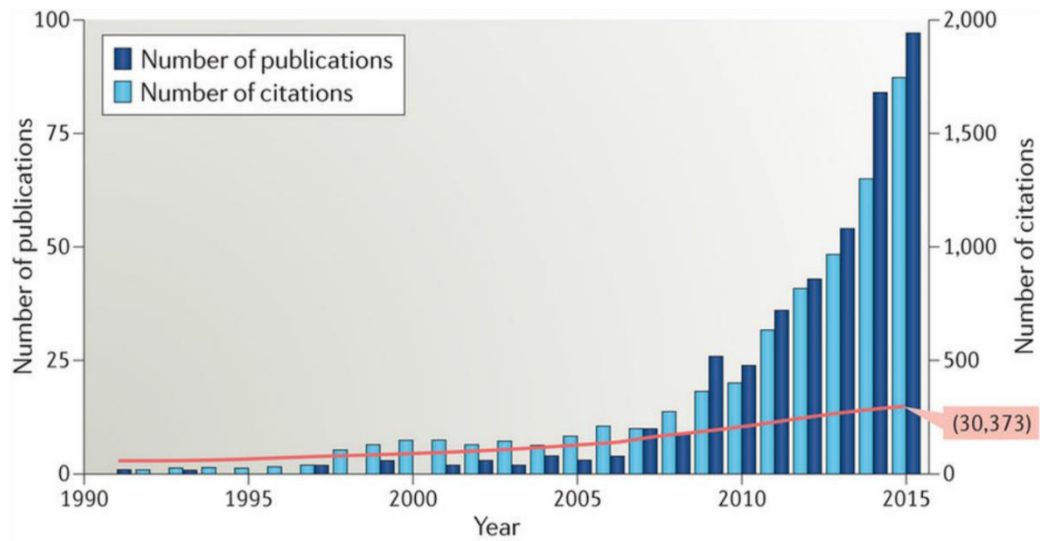
Figure 3: The upsurge of anti-virulence strategies. The increase in the number of anti-virulence publications and citations over time. The red base line indicates the number of antibiotic publications; indicated in the brackets. Adapted with permission from Nature Reviews Drug Discovery, Springer Nature (Dickey et al., 2017). © 2017.

Anti-virulence strategies have the following advantages over the conventional antibiotic therapies (Escaich, 2008; Johnson and Abramovitch, 2017):

1. They target specific virulence factors than the metabolism, and potentially reduce the selective evolutionary pressure for development of resistance.
2. They don't damage the host microbiota as they do not affect the bacterial growth.
3. They can be used as stand-alone medications or in combinations with existing antibiotics.
4. Anti-virulence compounds have limited off-target effects.

Even though they possess several advantages, these approaches also have the following limitations (Shakhnovich et al., 2007; Allen et al., 2014; Curtis et al., 2014; Johnson and Abramovitch, 2017):

1. They have a narrow range of spectrum and limited specificity against the pathogens. This limits their usage against polymicrobial infections.
2. Their targets should have constitutive than inducible expression for effective functioning.
3. Before being used in combination with antibiotics, potential drug-interactions, pharmacokinetic properties of the drugs in combination must be carefully studied.

4. The potential lifetime of anti-virulence drugs and their usage in the world of continuous bacterial evolution remains unclear.

5. The recovery of virulence during the anti-virulence drug treatment (anti-virulent drug-resistance) has also been reported in certain cases.

The vast knowledge acquired in the fields of bacterial pathogenesis and virulence factor identification (Freiberg and Brotz-Oesterhelt, 2005; Burrack and Higgins, 2007; Roemer et al., 2011; Anthouard and Dirita, 2015), and the potential of anti-virulence strategies serve as a ray of hope in the discovery of novel therapeutics against bacterial pathogens (Boucher et al., 2009; Brannon and Hadjifrangiskou, 2016; Hauser et al., 2016). Even though anti-virulence strategies are thought to reduce the development of bacterial resistance, efforts should be taken such that they are not accumulated in the environment as that of the antibiotics (Gill et al., 2015).

1.3. Enterohemorrhagic *Escherichia coli* and Shiga toxin

The human gastrointestinal tract (GI) is a complex environment consisting of a wide range of microorganisms, comprising the host microbiota (Pifer and Sperandio, 2014). It is estimated that the number of bacterial cells in the GI tract is 10 times higher than their numbers in the body, and more than 1000 different individual species could be present (Hooper and Gordon, 2001; Gill et al., 2006; Hugon et al., 2015). The complexity of adult GI microbiota is a result of hygiene, medication, diet and lifestyle over the years, starting from "absolutely zero microbe levels" in the fetal stage (Koenig et al., 2011). The association between the human host and GI microbiota is symbiotic, facilitating beneficial effects like shaping the immunity, physiology, behavior and nutrition to humans, and nutrient availability and exchange to the microbes (Gordon and Klaenhammer, 2011; Grenham et al., 2011; Thursby and Juge, 2017). Any disturbance to this symbiotic relationship leading to an imbalance between the host and microbiota (dysbiosis), could lead to augmentation of GI tract infections and diseases like inflammatory bowel disease (IBD) and autism (Grenham et al., 2011). Alteration of the GI microbiota and the resulting dysbiosis is often a consequence of antibiotics therapy or infections with enteric pathogens. Both these factors reduce the GI tract microbial diversity and shift the community composition leading to development of enteric diseases (Dethlefsen and Relman, 2011; Jandhyala et al., 2015).

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a Gram negative, food-borne enteric pathogen, that is often associated with bloody diarrhea (BD), hemorrhagic colitis,

hemolytic uremic syndrome (HUS) and death (Karmali et al., 1983; Jacob et al., 2013; Lee et al., 2014). More than 63,000 infections ascribed to food borne illnesses, caused by EHECs are recorded annually in the United States (Scallan et al., 2011). Further, the implications and severity of EHEC infections on global health and economy could be realized from their notable outbreaks over the years. The largest and the most recent 2011 EHEC outbreak in Germany led to 3842 illnesses and 53 deaths (RKI, 2011). Low infectious doses of 50-100 CFUs are enough for EHECs to establish in the host and cause a disease (Tilden et al., 1996; Pifer and Sperandio, 2014). Outbreaks have been associated with consumption of contaminated food such as ground beef, ready-to-eat salad, cheese salami, lettuces, salmon roe, radish sprouts, fenugreek seeds, apple cider and unpasteurized dairy products (Vojdani et al., 2008; McCollum et al., 2012; King et al., 2014; Marder et al., 2014). Person-person transmission of EHECs has also been found in nurseries, day-care centers and certain institutions (Pennington, 2010).

Shiga toxin (Stx) is the major virulence factor responsible for the pathogenesis of EHECs. With its initial discovery in the 1980s, Stx has emerged as one of the important toxins responsible for virulence in EHECs and other enteric pathogens (Konowalchuk et al., 1977; Stearns-Kurosawa et al., 2010). Production of Stx along with other virulence factors in the GI tract induces hemorrhagic colitis and its entry into the circulatory system could lead to the life-threatening complication of HUS (Griffin and Tauxe, 1991; Smith et al., 2014).

There are two kinds of Shiga toxin (Stx) produced by the EHECs, namely the Stx1 and Stx2. While, Stx1 is structurally similar to the Stx produced by *Shigella dysentriae*, Stx2 which shares 55% similarity (amino acid level) with Stx1, is more virulent and heat stable. EHEC strains possess several allelic variants of Stx1 (Stx1, Stx1c, Stx1d) and Stx2 (Stx2a, Stx2c, Stxc2, Stx2d, Stx2d$_{activable}$, Stx2e, Stx2f) with different immunological reactivity and pathogenic potentials (Tarr et al., 2005; Pacheco and Sperandio, 2012).

Stx is an A_1B_5 toxin which is encoded by a bacteriophage integrated in the EHEC chromosome (O'Brien and Holmes, 1987; Tyler et al., 2004). The activation of this Stx prophage in the EHEC chromosome, phage DNA replication and subsequent bacterial cell lysis leads to release of Stx and Stx phages (Schmidt, 2001). Consequently, the released Stx phages could convert the commensal *E. coli* to Stx-producing *E. coli* (STEC), leading to spread of infection and STEC strains (Gamage et al., 2004; Yue et al., 2012). Various factors that provoke the SOS response (like antibiotics) could induce the transcription of phage and *stx* genes via the activation mediated by the protein RecA (Los et al., 2011). Since antibiotics

enhance the Stx production *in vitro* and *in vivo* (Kimmitt et al., 2000;Zhang et al., 2000), antibiotic based chemotherapeutic measures are not recommended for treating EHEC infections (Tarr et al., 2005).

The mechanism of Stx is illustrated in **Figure 4**. Briefly after the release of Stx, the pentameric B subunit binds to glycosphingolipids on the eukaryotic cell surfaces and gets internalized via endocytosis. This endocytosis-mediated internalization of Stx leads to the activation of N-glycosidase activity of the A subunit (32 kDa) leading to the disruption of ribosomal protein elongation, blockade in protein synthesis and ultimately cell death by apoptosis (MacConnachie and Todd, 2004; Bauwens et al., 2011; Betz et al., 2012; Bauwens et al., 2013).

Figure 4. Mechanism of action of Stx. The first step (1) involves the binding of the B-subunit of Stx to globotriaosylceramide (Gb3) receptor (expressed by certain eukaryotic cells), the next step (2) involves the internalization of Stx via endocytosis and the subsequent retrograde transport (3) to trans-Golgi network (TGN) and endoplasmic reticulum (ER). Finally, in the ER, Stx inactivates ribosomes, blocks protein synthesis and leads to apoptotic cell death (4). Modified from (Pacheco and Sperandio, 2012).

Current management of EHEC outbreak (Braeye et al., 2014) typically involves the following steps:
1. Early detection of the infection.
2. Timely identification of the suspected food vehicle to avoid the spread of strains.
3. Subsequent control measures to curb the infection intensities.

The frequent diarrheal outbreaks, emergence of highly pathogenic EHEC strains (e.g. EHEC O104:H4) and the inexistence of effective anti-EHEC strategies have altogether necessitated the need for development of novel anti-Stx approaches in targeting EHEC

infections (Goldwater and Bettelheim, 2012). Further, it is envisaged that modulating the virulence through toxin-suppressing therapeutics could be promising for treating EHEC infections without affecting the host endogenous microbiome (Clatworthy et al., 2007).

The **Chapter 2** of this Ph.D. thesis provides an attempt taken towards identification of anti-Stx substances in nature.

1.4. Staphylococci and biofilms

Medical devices like (implants, central venous catheters, peritoneal dialysis catheters, prosthetic joints, pacemakers, heart valves etc.) and biomaterials (like contact lenses and conjunctival plugs) have greatly helped in improving the quality of human health (Vinh and Embil, 2005; Suter et al., 2011). However, in health care facilities the surfaces of these devices are often attacked by microorganisms. Bacteria from perioperative contaminations (originating from either the patient's own body, health care worker's body or health care environments) form strong communities called "biofilms" and lead to nosocomial and device-related infections (DRIs) (Percival et al., 2015; Aljabri et al., 2018). The observation of biofilms in human niches dates back to their identification on teeth by Antonie van Leeuwenhoek in the 17th century (Percival, 2011).

Biofilms are three-dimensional resistant networks of bacteria that are enmeshed in a self-produced matrix composed of polysaccharides, proteins, lipids, extracellular DNA, RNA and water (Costerton et al., 1999; Hall-Stoodley et al., 2004; Hoiby et al., 2011). Water channels are responsible for the flow of essential nutrients to and within the biofilm (Sutherland, 2001; Lu and Collins, 2007). The thickness of matrix is usually between 200-1000 nm (Sleytr, 1997). The viscoelastic nature of the matrix is responsible for the mechanical stability of biofilms to shear stresses (Shaw et al., 2004). Biofilms are formed on biotic or abiotic surfaces. Biofilms on medical devices could be caused by single class of bacteria (mono-species biofilm) or a mixture of different classes of bacteria (mixed biofilm) depending on the nature and the extent of contamination (Donlan, 2002).

The formation of biofilm confers resistance to bacteria against stressful conditions such as UV, lack of nutrients, presence of host-immune systems and antibiotics etc. (Hoiby et al., 2010; de la Fuente-Nunez et al., 2013). The presence of biofilm matrix itself, reduced growth rates, persister cell formations, efflux pumps, plasmid exchange, target mutations, antibiotic-deactivating enzymes etc. are some of the common factors contributing to the resistance

phenotype of bacteria in biofilms (Stewart and Costerton, 2001; Hall-Stoodley et al., 2004). Biofilm-driven DRIs are resilient to treatments and hence, are linked with increased morbidity and mortality rates, and corresponding increased economic losses in health-care settings (Barros et al., 2014; Kleinschmidt et al., 2015; Leary et al., 2017). The necessity of a second surgery for removal of infected medical devices (e.g. implants, pacemakers), extended second-line antibiotic usage, longer hospital stays are some of the obvious reasons connected to the increased health care losses with DRIs (Bryers, 2008; Otto, 2012).

Currently, biofilm-associated infections represent 80% of the nosocomial infections, and staphylococci are the leading etiological agents in this aspect (Bryers, 2008; Hoiby et al., 2010; Becker et al., 2014). Staphylococci are clustered Gram-positive cocci, that are non-motile and non-spore forming facultative anaerobic bacteria belonging to the phylum Firmicutes. Based on their ability to produce coagulase (the enzyme responsible for clotting of blood), they are classified as Coagulase negative (CoNS) and coagulase positive staphylococci (CoPS).

S. epidermidis (CoNS) and *S. aureus* (CoPS) are commensal bacteria residing on human skin and mucous membranes (Otto, 2008). Through formation of biofilms on medical devices, they could lead to complications like blood-stream infections, prosthetic joint infections, early-onset neonatal sepsis, endocardial and urinary tract infections (Barros et al., 2014; WHO, 2014; Widerstrom, 2016). Insufficient hand hygiene, inadequate disinfection and/or sterilization of medical devices and surfaces are presumed to be the possible reasons behind transmission of staphylococci to medical devices. An example of *in vitro* staphylococcal (*S. epidermidis* RP62A) biofilm on contact lens surface is shown in **Figure 5C**. The array of problems caused by staphylococcal biofilms and the emergence of methicillin and vancomycin resistant staphylococcal strains is far from resolved. It is predicted that the resistance problem is greater for CoNS than CoPS, however, subsequent therapeutic options are extremely limited in both cases (Becker et al., 2014).

Staphylococcal biofilm formation on medical devices is a complex and multifactorial phenomenon involving attachment, accumulation, maturation and detachment phases (**Figure 5A**). The different phases of biofilm development process are explained below:

1.4.1. Initial attachment and microcolony formation

The first step of biofilm life cycle involves the reversible attachment of staphylococcal cells to an abiotic surface. Various physical forces and non-specific interactions like van der

Waal's forces, electrostatic interactions etc. govern this step (Muszanska et al., 2012). Physiochemical characteristics of the surface like hydrophobicity, surface energy, chemical composition of material, temperature and roughness of the surface also contributes to the initial adherence of bacteria (Dunne, 2002). Bacteria tend to attach more likely to hydrophobic (non-polar) surfaces than hydrophilic (polar) surfaces (Pringle and Fletcher, 1983). Staphylococcal surface molecules like the protein autolysin (AtlE), serine-aspartate family protein (Sdr), accumulation associated protein (Aap), wall teichoic acids (WTAs) also govern the attachment of bacteria to biotic or abiotic surfaces (Otto, 2009). Once the attachment becomes stable, bacterial multiplication and division leads to formation of micro-colonies. The micro-colonies then coordinate with each other in multiple aspects, facilitating the exchange of substrate, exchange and excretion of metabolic products (Costerton et al., 1999).

1.4.2. Accumulation

This phase is mediated in intercellular attachment and development of multicellular agglomerates leading to the development of three-dimensional biofilm structures. This step of biofilm formation could be either polysaccharide intercellular adhesin (PIA) (also known as poly-N-acetylglucosamine, PNAG) dependent or independent. Many staphylococcal strains encode a functional *icaADBC* operon responsible for PIA synthesis (detailed in **Figure 5B**). The products of the *ica* locus, IcaA and IcaD synthesize a chain of activated monomers of N-acetylglucosamine (GlcNAc) and the transmembrane protein IcaC, by its transporter function exports this chain. Cell-surface located enzyme IcaB then, partially de-acetylates this chain which induces positive charges in the otherwise neutral polymer PIA (Heilmann et al., 1996; Gerke et al., 1998; Vuong et al., 2004). The cationic nature of PIA is essential for its surface binding and multiple roles in biofilm formation. A variety of environmental stresses and multiple global virulence factors are known to influence the PIA synthesis process (Otto, 2008; 2009). Thus, synthesis of PIA is a crucial step in the life cycle of staphylococcal biofilms and has a major role in its pathophysiology *in vitro* and *in vivo* (Mehlin et al., 1999; Wang et al., 2007; Stevens et al., 2009).

PIA-independent biofilm formation is mainly mediated by proteins. A variety of surface proteins (like accumulation associated protein (Aap), biofilm-associated protein (Bap), SasG, SasC, protein A, fibronectin-binding proteins like FnBPA and FnBPB) and cell wall anchored proteins (CWA) (like clumping factors A and B, autolysins A and E, WTA, fibronectin binding protein SdrG/Fbe and lipoteichoic acids (LTA)) are known to assist this

mode of biofilm formation (Speziale et al., 2014). Under certain conditions, staphylococci can switch between these two modes of biofilm formation and still form a tough biofilm (Rohde et al., 2005; Hennig et al., 2007). Thus, it can be understood that both proteins and polysaccharides could contribute to the aggregation and accumulation of cells within a biofilm.

1.4.3. Structuring and maturation of biofilms

Structuring and maturation phase of biofilm formation is facilitated by a cell-cell signaling phenomenon (quorum sensing) mediated by accessory gene regulator (Agr) systems (Otto, 2012). At this stage, secretion of certain autoinducer peptides (cell signaling molecules) lead to multi-layered structuring of the biofilm. Interstitial voids are produced in the biofilm matrix which serve as a circulatory system for supply of essential nutrients to bacterial microcolonies and subsequent removal of their metabolic waste (Mack et al., 1996; Periasamy et al., 2012). Typically, biofilms resemble mushroom shaped structures where bacteria with low metabolic activity (due to oxygen and nutrient limitations) are embedded in the bottom. Few persister cells (that neither grow nor die but become tolerant to antibiotics) may also be present in a biofilm (Rani et al., 2007). Bacteria with high metabolic activity (rapidly dividing cells) are usually present at the surface of the biofilms. The upregulation of *agr*-related genes at these surfaces further leads to augmentation in dispersal of free bacteria from biofilms (Yarwood et al., 2004).

1.4.4. Detachment

In this stage, sessile bacteria get detached from biofilms and get transition to mobile forms in a natural pattern or under conditions of mechanical stress (Costerton et al., 1999). Dispersal of cells in a staphylococcal biofilm could be mediated by enzymatic degradation of matrix (like proteases, hydrolases, nucleases) or by disruption of non-covalent interactions through detergent-like substances (like phenol soluble modulins (PSMs)) (Otto, 2009; Kaplan et al., 2012). Once detached free bacteria get disseminated to a new site and continue the spread of an infection (Otto, 2008).

The wide range of health complications caused by staphylococcal biofilms with their resistant and recalcitrant nature, and the inexistence of effective anti-staphylococcal drug formulations, has urged the need for discovery of novel anti-biofilm-based therapeutics in staphylococcal disease management. The **Chapters 3 and 4** of this Ph.D. thesis provide an attempt in achieving this goal.

Figure 5: Staphylococcal biofilms. (A) Biofilm growth cycle depicting the different stages of biofilm formation (attachment, accumulation, maturation and dispersal or detachment). **(B)** The exopolysaccharide PIA synthesis mechanism. PIA is a deacetylated β 1-6 linked N-acetylglucosamine (GlcNAc) homopolymer synthesized by the products of *icaADBC* operon. The membrane located IcaA that has the N-acetlyglucosamine transferase activity works with IcaD and generates poly-GlcNAc chains, which is then transported by the IcaC membrane protein. After export, the surface-associated IcaB protein partially de-acetylates PIA by removing some of the N-acetyl groups, and this gives the PIA a cationic character necessary for attachment to hydrophobic surfaces. Expression of *icaADBC* operon is modulated by the repressor protein IcaR and several other regulatory proteins, environmental factors and nutrient limitation. Insertion elements like IS256 can also switch on/off the PIA expression. CW, cell wall; CM, cell membrane; PG, peptidoglycan; PSMs, phenol soluble modulins. Images A and B modified from (Otto, 2009; Richards and Melander, 2009). **(C)** Scanning electron micrograph of a 24 h staphylococcal biofilm on contact lens. Net like structures represent the biofilm matrix. Image was false-colored using the ImageJ program. Scale bar: 0.5 µm.

1.5. Bioactive potential of marine natural products (MNPs)

Natural products (NPs) are small chemical compounds (molecular weight<3000 Da) produced by living organisms. NPs are structurally complex and possess defined orientation in the space (Montaser and Luesch, 2011; Martins et al., 2014). They are mainly secondary metabolites, which are unessential for the growth and development of the producing organism. Chemical defense against predators, intra or inter-species communication, survival mechanisms are some of the ecological roles of these secondary metabolites in the producers. NPs are widely probed in drug discovery programs owing to their potential in interacting with diverse drug targets with greater efficiency and biochemical specificities (Martins et al., 2014). Estimates suggest that more than half of the present-day drugs were made using NPs or their derivatives (Fenical and Jensen, 2006; Newman and Cragg, 2007; Molinski et al., 2009; Subramani and Aalbersberg, 2013).

Depending on the origin, NPs could be terrestrial or marine. The frequent re-discovery and excessive overmining of terrestrial natural products has shifted the research focus towards MNPs which have chemical novelty and enormous pharmacological potential. The marine environment is a treasure trove for discovery of new compounds with antibacterial, antiviral, antiparasitic, antioxidant, anticancer and immunomodulatory activities (Villa and Gerwick, 2010; Zhou et al., 2013; Abdelmohsen et al., 2014). Particularly, their potential against drug-resistant bacterial, fungal, viral and parasitic infections has been increasingly studied in detail (Rahman et al., 2010; Eom et al., 2013; Abdelmohsen et al., 2017). Several MNPs have already entered phase 1, 2 and 3 clinical trials, and six MNP-based drugs have been approved for usage by the US-FDA and EU (Mayer et al., 2010; 2018). Extreme conditions in the oceans such as temperature differences, variations in light intensity, salinity, pH, pressure and the presence of certain chemicals are some of the reasons for the presence of diverse and novel antibiotic compounds in the marine environment (Lane, 2008; Rateb and Ebel, 2011; Abdelmohsen et al., 2017).

Marine invertebrates are the most bio-prospected organisms in MNPs research owing to their rich chemical and biological diversity. Bioprospecting efforts for discovery of new drugs majorly target two classes of marine invertebrates, namely, "marine sponges and cnidarians" (Leal et al., 2012; Leal et al., 2014). Particularly, marine sponges and their associated actinomycetes are ranked the highest for discovery of novel anti-infectives and presence of chemically diverse metabolites (Stamatios Perdicaris, 2013; Abdelmohsen et al., 2014).

Nutritional scarcity and chemical defense are often linked to the reasons for their production of MNPs (Montaser and Luesch, 2011).

1.5.1. Marine sponges and their microbial consortia

Sponges (phylum Porifera) are primitive filter feeders living on the benthic habitats and their evolutionary origin dates 700-800 million years back (Belarbi el et al., 2003; Thomas et al., 2010). Estimates suggest that there are more than 20,000 species of sponges on this earth and only around 8800 of these species are currently known (Hooper et al., 2013; Van Soest et al., 2018). Tropical reefs, polar latitudes, deep sea, fresh water lakes and rivers are the common habitats where marine sponges are found (Schmitt et al., 2012). Pictures of marine sponges involved in this study are shown in **Figure 6 (A, B)**. Through filter feeding, sponges absorb and pump out constant volumes of sea water through their bodies to retain food and remove waste particles. 1 kg of sponge has the potential to pump out 24,000 l of water per day (Taylor et al., 2007). Microbes including bacteria, unicellular algae, fungi and viruses, and certain nano- and pico-eukaryotes are commonly acquired by these sponges through filter feeding (Thacker and Freeman, 2012; Webster et al., 2012). The microbial content in marine sponges contribute to about 35% of the total sponge biomass and the microbial density in a sponge is 3-4 orders of magnitude greater than the surrounding sea water (Taylor et al., 2007). From an ecological perspective, it is presumed that microbes in sponges offer beneficial effects to them e.g. protection against predators via production of defense compounds, protection against environmental stresses, nutrient acquisition, stabilization of sponge skeletons, metabolic waste processing etc. (Lam, 2006; Abdelmohsen et al., 2014). In addition, microbial symbionts of marine sponges are benefited by constant nutrient supply as a consequence of filter feeding activities, as well as access to scarce elements like nitrogen (from the sponge metabolic end product ammonia) (Hentschel et al., 2012).

Figure 6: Photographs of Mediterranean marine sponges under investigation in this Ph.D. thesis. **(A)** *Agelas oroides*, **(B)** *Petrosia ficiformis* (underwater photography by Dr. Thanos Dailianis)

Various innovative cultivation-dependent (Abdelmohsen et al., 2010; Cheng et al., 2015) and -independent techniques (16S rRNA gene library construction, denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), amplicon tag sequencing, metagenomics, metaproteogenomics, single cell genomics etc.) (Schmitt et al., 2012; Simister et al., 2012; Jin et al., 2014; Rodriguez-Marconi et al., 2015) are now available to get useful insights to the microbial diversity associated with marine sponges. Both marine sponges and their associated microbiomes offer an interesting chemical and metabolic repertoire that could be used to produce biologically active compounds (Piel, 2006; Blunt et al., 2007). A wide range of marine sponge compounds possessing anti-diabetic, antioxidant, anti-inflammatory, antitumor, immunosuppressive, antimicrobial and antibiofilm activities have been reported (Blunt et al., 2007; Mehbub et al., 2014; Skropeta and Wei, 2014). However, the daunting challenge associated with the large-scale production and marketability of these compounds is the cultivability of sponges in normal environments. The majority of sponges from benthic habitats do not survive in seawater aquaria due to their slow growth rates, seasonal influences, inability to adapt in the artificial sea environment, and infection with parasites (Belarbi el et al., 2003). Further, yields of compounds produced by aquaculture of sponges are invariably low and cost of maintenance is high. Strategies such as identification of pharmacophore linked with synthetic chemistry and metabolomics-based approaches could initiate the scale-up of drugs from these marine prototypes (Kersten and Dorrestein, 2009; da Silva et al., 2015; Kurita et al., 2015).

The evidence of production of bioactive compounds by the sponge microbiota and the sponge, has led the parentage of natural products from sponges a question of debate (Leal et al., 2014). Using the sponge microbiota for production of new compounds could be an alternate approach as it overcomes the above-mentioned bottlenecks and large-scale cultivation of these microbes is possible with the usage of bioreactors. The large fraction of uncultivable microbes in marine sponges represents a major draw-back in this strategy and this could be resolved with the application of metagenomics-based techniques for identification of biosynthetic gene clusters. This could in turn bolster the discovery of new MNPs from these uncultivable microbes (Brady et al., 2009; Donia et al., 2011; Wilson and Piel, 2013). Around 32 bacterial phyla and candidate phyla were described from marine sponges so far. The most common phyla associated with marine sponges include Actidobacteria, Actinobacteria, Chloroflexi, Cyanobacteria, Nitrospira, Bacteriodetes, Planctomycetes, Gemmatimonadetes, Spirochetes and Proteobacteria (α and γ) (Hentschel et al., 2012; Schmitt et al., 2012). **Figure 7** indicates the percentage distribution of compounds produced by sponge-associated microbes.

It could be seen that the phylum Actinobacteria among the bacterial sponge symbionts are prolific producers of secondary metabolites followed by the members of phylum Proteobacteria (Thomas et al., 2010).

Figure 7: Percentage distribution of compounds produced by: (A) bacterial and fungal sponge associates, **(B)** bacteria-phylum wise. Modified from (Thomas et al., 2010).
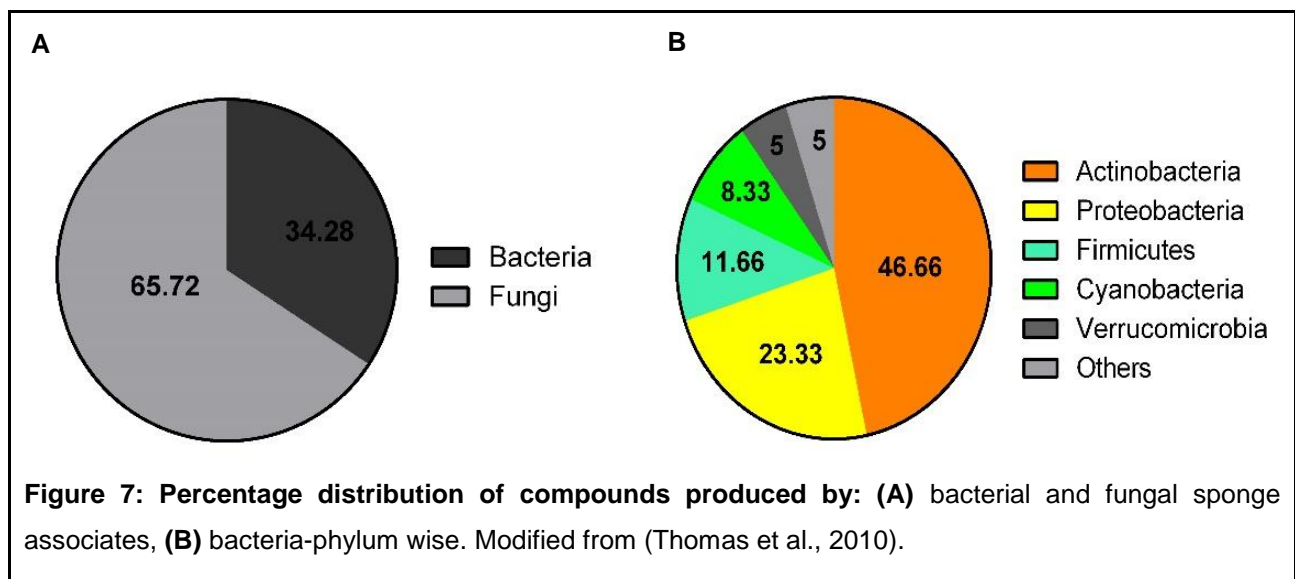
1.5.2. Marine sponge-derived actinomycetes

Actinomycetes are Gram positive bacteria with high GC contents (up to 70%) and diverse colony morphologies (Waksman, 1950; Korn-Wendisch, 1992). They exist in terrestrial and

marine environments and produce a broad spectrum of NPs with massive chemical diversity and a range of biological activities (Li and Vederas, 2009; Nett et al., 2009; Abdelmohsen et al., 2014). However, the frequent re-discovery of compounds from terrestrial actinomycetes and the rich metabolic diversity of marine actinomycetes has made the exploitation of actinomycetes from marine habitats a hotspot in NP-based drug discovery (Lam, 2006; Fischbach and Walsh, 2009; Subramani and Aalbersberg, 2012; 2013). A total of 10,400 actinomycete 16S rRNA gene sequences were so far described from marine origin (cultivated from sea water, marine sediments, invertebrates like soft corals, tunicates, fish and marine sponges) (Abdelmohsen et al., 2014). It is well known that the majority of the marine actinomycetes isolated from marine invertebrates comes from marine sponges (Zhang et al., 2006; Selvin, 2010). Abdelmohsen et al. (2014) extensively studied the diversity of marine sponge-derived actinomycetes. 60 different genera of marine sponge-derived actinomycetes were identified by a search in NCBI database (until August 2013). These genera are represented in **Figure 8**. Over half of the genera of actinomycetes isolated from sponges were of the suborder Micrococcineae (*Micrococcus*, *Microbacterium* and *Arthrobacter*). Members of Micrococcineae are fast-growing but produce only a few chemotypes (Lang et al., 2004). Many of the chemically rich *Streptomyces* were represented by hundreds of sequence entries. Several new and rare actinomycetes (like *Actinokineospora*, *Actinomadura*, *Knoellia*, *Nonomurea*, *Pseudonocardia*, *Saccharopolyspora*, *Saccharomonospora* and *Verrucosispora*) have also been reported from sponges, pointing their undiscovered potential in producing clinically relevant compounds.
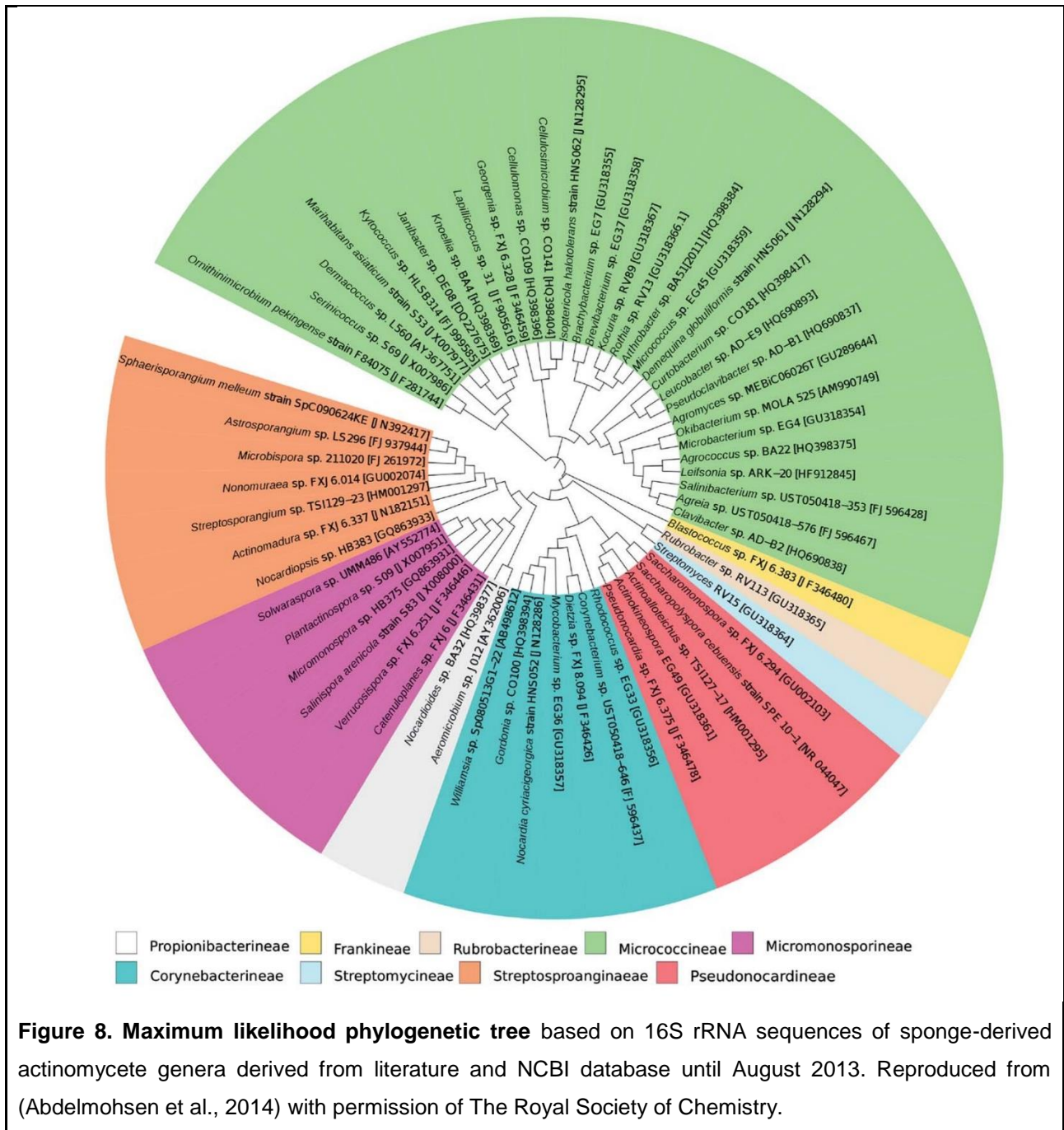
Figure 8. Maximum likelihood phylogenetic tree based on 16S rRNA sequences of sponge-derived actinomycete genera derived from literature and NCBI database until August 2013. Reproduced from (Abdelmohsen et al., 2014) with permission of The Royal Society of Chemistry.

There has been a considerable rise in the discovery of new actinomycetes and even genera from marine sponges (Kwon et al., 2006; Supong et al., 2013a; Supong et al., 2013b). The following modifications in the isolation protocols have been made to facilitate the recovery of new sponge-associated actinomycetes:

- Heat shock application which reduces the numbers of Gram negative bacteria from sea water (Takizawa et al., 1993)

- Pretreatment with ultraviolet (UV) radiation and high frequency waves to induce spore germination (Bredholdt et al., 2007)

- Supplementation of cultivation media with antibiotics to inhibit the growth of Gram negatives and fungi (Webster et al., 2001)

- Media formulation with low nutrients for recovery of oligotrophic bacteria (Olson et al., 2000)

- Addition of sponge extract to the cultivation media (Kampfer et al., 2014)

- Encapsulation of cells in gel microdroplets (Zengler et al., 2002)

- Diffusion chambers, microbial traps and isolation chips (Gavrish et al., 2008; Lewis et al., 2010; Pahlow et al., 2013)

Actinobacteria produce the major fraction of MNPs among the different microbial phyla in marine habitats, with antiprotozoal, antiviral, anticancer, antioxidant, anti-inflammatory and antibiotic activities against drug-resistant pathogens (Wei et al., 2011; Palomo et al., 2013; Abdelmohsen et al., 2014; Abdelmohsen et al., 2017). It is due to the production of unique chemotypes, these actinomycetes are regarded as economically and biotechnologically profitable prokaryotes (Lam, 2006; Subramani and Aalbersberg, 2013). NPs produced by sponge-derived actinomycetes include several classes of compounds like polyketides, alkaloids, fatty acids, peptides and terpenes. About 22% of total MNPs by marine actinomycetes were obtained from sponge-associated actinomycetes (**Figure 9A**). Further, the number of NPs from marine actinomycetes (reported over the years) is depicted in **Figure 9B**.

Figure 9. Distribution of MNPs. (A) Percentage distribution of NPs from marine actinomycetes in different environments, **(B)** NPs discovery rates from marine actinomycetes; data collected from MarinLit® 2013 and literature. Modified from (Abdelmohsen et al., 2014).
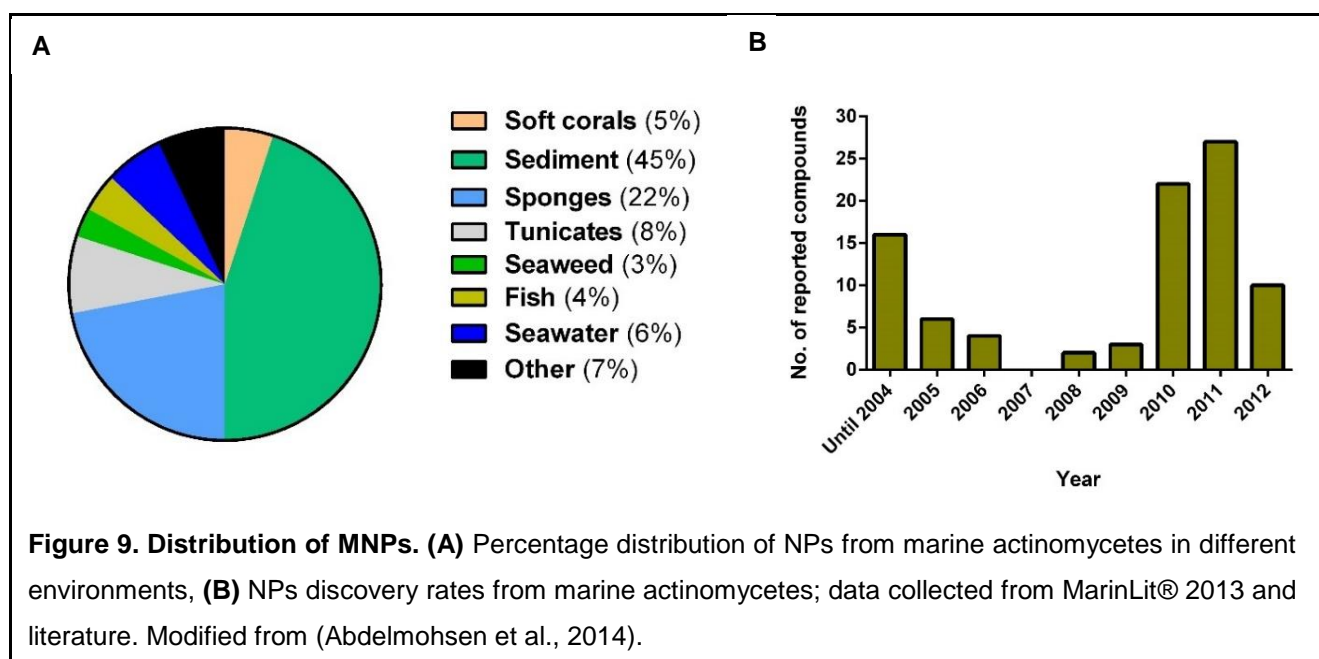
Few anti-infective compounds derived from these actinomycetes have been illustrated in **Figure 10.** From the diversity, abundance and the available genome mining data it is evident that there is still room for discovery of new anti-infectives from these talented phyla.
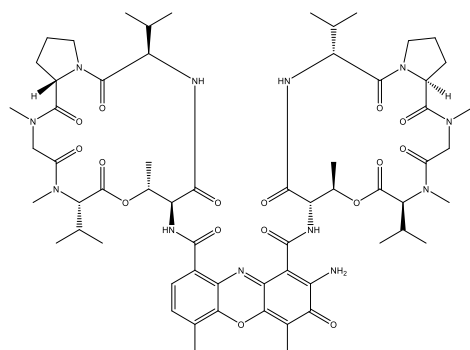
Actinomycin D (Lee et al., 2016)
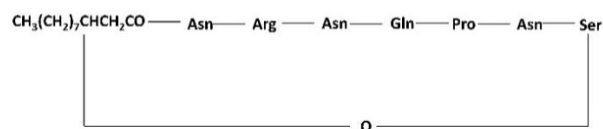
Coryxin (Dalili et al., 2015)
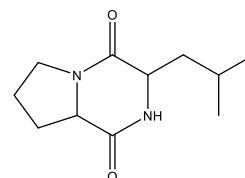
Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) (Rajivgandhi et al., 2018)
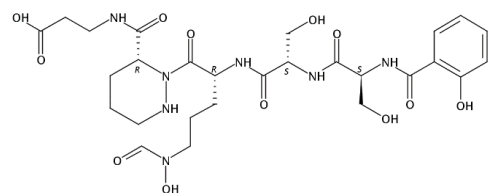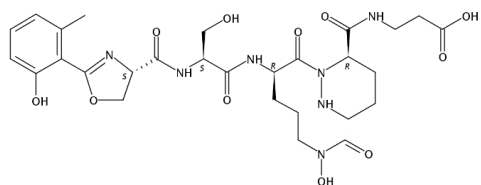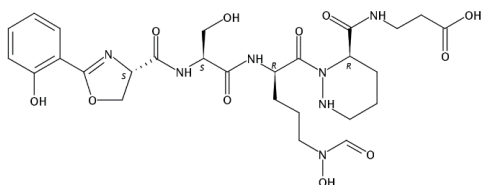
Cahuitamycins A, B, C (Park et al., 2016)
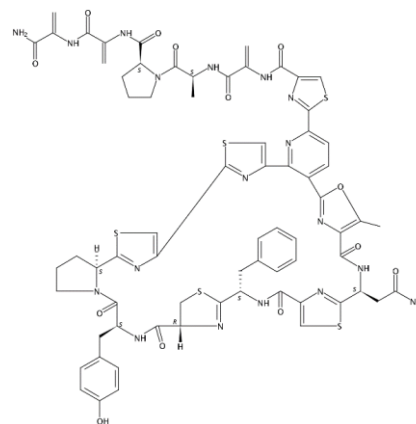
Kocurin (Palomo et al., 2013)
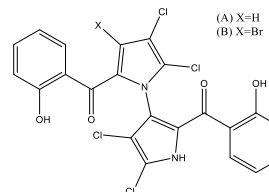
Marinopyrroles A and B (Hughes et al., 2008)

Figure 10. Diverse chemistry of anti-infective compounds from actinomycetes. Few examples from existing literature have been shown.

1.6. Scope of the study

The alarming levels of drug-resistant bacterial infections, impressive array of evolved bacterial protection mechanisms against drugs, as well as the current inexistence of effective therapeutics in the market, have urged the continuation in search of novel anti-infective agents. Marine sponge-associated actinomycetes have been increasingly mined for discovery of new antibiotics. The main goal of this Ph.D. thesis is to investigate the anti-infective or anti-virulence potential of marine sponge-associated actinomycetes against Shiga toxin production in EHEC and biofilm formation in staphylococci.

The first objective of the study (**Chapter 2**) was to evaluate the inhibitory effect of the compound strepthonium A isolated from *Streptomyces* sp. SBT345 (previously cultivated from the Mediterranean sponge *Agelas oroides*) in curtailing Stx production in EHEC strain EDL933. Structural elucidation as well as the biological activity has been reported.

The second objective of the study (**Chapter 3**) was to investigate the anti-biofilm effect of an organic extract obtained from liquid fermentation of *Streptomyces* sp. SBT343 (previously cultivated from the Mediterranean sponge *Petrosia ficiformis*) in restraining staphylococcal biofilm formation *in vitro*. The biofilm inhibitory effects of SBT343 extract were studied on polystyrene, glass and contact lens surfaces using crystal violet assay, scanning electron and confocal microscopies. Toxicity of SBT343 extract was evaluated *in vitro* (cell lines: mouse macrophage (J774.1), fibroblast (NIH/3T3), human corneal cells) and *in vivo* (greater wax moth *Galleria melonella* larvae). Physio-chemical characterization of the extract (heat and enzymatic treatments) was done to ascertain the nature of active component(s). Finally, fractionation experiments were done to isolate and identify the active component(s).

The third objective of the study (**Chapter 4**) was to investigate the anti-biofilm effect of an organic extract obtained from solid fermentation of *Streptomyces* sp. SBT348 (previously cultivated from the Mediterranean sponge *Petrosia ficiformis*) in blocking staphylococcal biofilm formation *in vitro*. Bioassay-guided fractionation and semi-preparative HPLC methods were employed to isolate and identify the active compound(s). Anti-biofilm and anti-staphylococcal effects of the most active compound SKC3 in the extract was extensively studied using *in vitro* assays. Finally, RNA sequencing was done to understand the mechanism of action of SKC3 on staphylococci.

The experimental **Chapters (2, 3 and 4)** are preceded by a general introduction (**Chapter 1**), followed by a general discussion (**Chapter 5**) on the anti-infective potential of actinobacteria from marine sponges, and conclusion and future perspectives (**Chapter 6**). Further, the materials and methods used in this Ph.D. thesis have been detailed in chapters 2, 3 and 4. For further information, readers are requested to refer to these chapters.

2. Inhibitory potential of strepthonium A against Shiga toxin production in enterohemorrhagic *Escherichia coli* (EHEC) strain EDL933

This article was published in the peer-reviewed journal Natural Product Research.

For documentation of individual contributions to this work and consent of all authors for second publication in this thesis please refer to the appendix.

Supplementary information to this article could be accessed online at: http://dx.doi.org/10.1080/14786419.2017.1297443

Taylor & Francis
Taylor & Francis Group

Check for updates

SHORT COMMUNICATION

Inhibitory potential of strepthonium A against Shiga toxin production in enterohemorrhagic *Escherichia coli* (EHEC) strain EDL933

Cheng Cheng[a‡], Srikkanth Balasubramanian[b‡], Agnes Fekete[c], Markus Krischke[c], Martin J. Mueller[c], Ute Hentschel[d], Tobias A. Oelschlaeger[b] and Usama Ramadan Abdelmohsen[a1]

[a]Department of Botany II, Julius-von-Sachs Institute for Biological Sciences, University of Würzburg, Würzburg, Germany; [b]Institute for Molecular Infection Biology, University of Würzburg, Würzburg, Germany; [c]Department of Pharmaceutical Biology, Julius-von-Sachs Institute for Biological Sciences, University of Würzburg, Würzburg, Germany; [d]GEOMAR Helmholtz Centre for Ocean Research, RD3 Marine Microbiology, and Christian-Albrechts University of Kiel, Kiel, Germany

ABSTRACT

The production of shiga toxin (Stx) is a critical step in the establishment and progress of enterohemorrhagic *Escherichia coli* (EHEC) infections. The possible release of Stx from dead and dying bacteria, and the risk of resistance development have restricted the usage of antibiotics against EHEC. The chlorinated quaternary ammonium compound, strepthonium A, was isolated from the culture of *Streptomyces* sp. SBT345 that was cultivated from the Mediterranean sponge *Agelas oroides*. The structure was elucidated and confirmed by spectroscopic analyses including 1D and 2D NMR, ESI-HRMS, as well as ESI-HRMS^2. Strepthonium A follows Lipinski's rule of five with respect to its molecular weight, CLogP values and the number of hydrogen acceptors and donors. Verotoxin ELISA assay demonstrated that Strepthonium A reduced the Stx production in EHEC strain EDL933 at 80 μM concentration without growth inhibition. This study demonstrates the potential of strepthonium A in restraining the production of Stx in EHEC infections.

Marine sponge: *Agelas oroides*   *Streptomyces* sp. SBT345   Strepthonium A

Strain isolation

Compound purification and structure elucidation

Inhibition of Shiga toxin production (Determination by Verotoxin ELISA)

Stx A
Stx B
Shiga toxin

Enterohemorrhagic *E. coli* strain EDL933
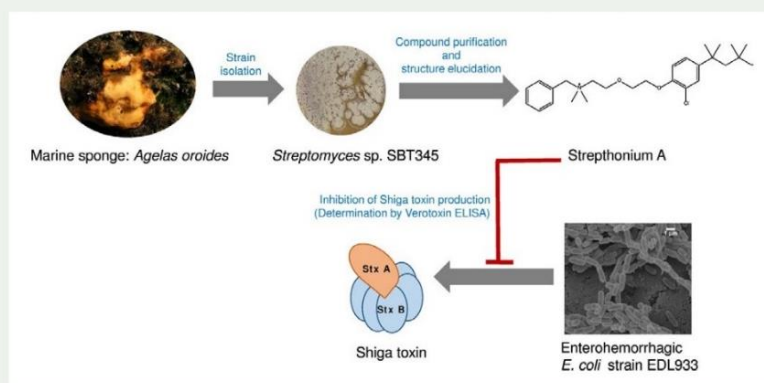
CONTACT Tobias A. Oelschlaeger ✉ t.oelschlaeger@uni-wuerzburg.de; Usama Ramadan Abdelmohsen ✉ usama.ramadan@uni-wuerzburg.de
[1]Permanent address: Department of Pharmacognosy, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt.
‡ These authors contributed equally to this work.

⊕ Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14786419.2017.1297443.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a foodborne pathogen that causes a wide spectrum of gastrointestinal illness, ranging from bloody diarrhoea (BD) to life-threatening complications such as haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Lee et al. 2014). The largest and the most recent 2011 EHEC outbreak in Germany led to 3842 cases and 53 deaths (Rund et al. 2013). Low infectious doses of 50–100 CFUs are sufficient for EHEC to cause disease in healthy individuals, highlighting the propensity of the infection (Pacheco & Sperandio 2012). Administration of antibiotics, antimotility agents, narcotics and non-steroidal anti-inflammatory drugs in EHEC infections is generally not recommended due to the effect on the induction of phage lytic cycle with release of Stx, but of course may be considered in individual cases (Rund et al. 2013).

The production of Shiga toxin (Verotoxin) by EHEC has been considered as the basis for their ability to cause the life-devastating haemorrhagic colitis, gastroenteritis and HUS. Modulating the toxin activity and/or production through the usage of synthetic toxin binders, toxin neutralisers, designer probiotics, monoclonal antibodies (MAbs), etc. is a possible strategy to ameliorate and/or prevent the transmission of EHEC infections for which effective treatments are elusive (Goldwater & Bettelheim 2012). Therefore, seeking for novel effective treatments with less expensive prescription is highly demanded.

The marine environment has offered a vast potential for the discovery of novel molecules with a broad range of biological activities (Blunt et al. 2015). Marine sponge-associated actinomycetes are one of the prolific producers of biologically active marine natural products. This could be seen from the growing discovery of novel bioactive compounds from them (Abdelmohsen et al. 2014). Herein, we report the structure elucidation and bioactivity of strepthonium A, which was purified from the culture of the marine sponge-associated actinomycete *Streptomyces* sp. SBT345 (Cheng et al. 2015).

2. Results and discussion

2.1. Structure elucidation

Strepthonium A was obtained as a white amorphous powder. The molecular formula was determined to be $C_{27}H_{41}ClNO_2^+$ from its HRESI-MS (m/z 446.2878 $[M]^+$ calculated for $C_{27}H_{41}ClNO_2^+$, 446.2826 with chlorine isotopic pattern, (Figure S1) indicating eight degrees of unsaturation. The ^1H NMR spectrum (Figure S2) exhibited eight aromatic protons in two different rings and six methylene multiplets. Other signals were attributed to seven methyl groups which were further confirmed by proton integrations and their carbon chemical shifts displayed in HSQC spectrum (Figure S3). The ^{13}C and DEPT135 spectra (Figure S4 and S5) displayed 27 carbon signal resonances including 7 methyls, 6 methylenes (3 oxygenated ones and 2 attached to nitrogen), 12 aromatic ones and 2 quaternary carbons. HMBC spectrum (Figure S6) indicated that both methyl groups were connected to the same hetero atom. In addition, these two methyl groups were also correlated with δ_C 64.6 and 70.4 which unveiled the presence of a quaternary amine in its structure. Analysis of COSY (Figure S7) and HMBC data indicated a structure of two different phenyl rings connected by a heteroaliphatic chain which was interrupted by a dimethylammonium group and two oxo-bridged oxygens. An aliphatic chain was assigned to C-15, in the phenoxy ring (Figure S8) based on HMBC correlations from the methyl groups (H-24 and H-25) and the methylene proton (H-19)
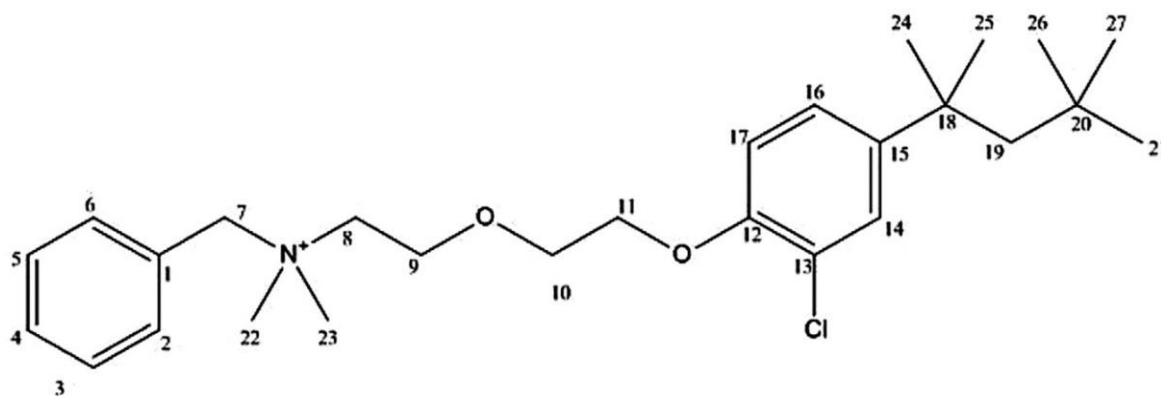
Figure 1. Structure of strepthonium A.

to C-15 (Figure S6). The remaining aromatic carbon at C-13 carried a chlorine. To confirm the structure elucidation, fragmentation pattern of strepthonium A was determined using product ion scan (Figure S9a) and a mechanistic pathway for the fragmentations was proposed (Figure S9b). We conclude the structure of this compound to be N-benzyl-2-(2-(2-chloro-4-(2,4,4-trimethylpentan-2-yl)phenoxy)ethoxy)-N,N-dimethylethan-1-aminium and proposed its new name as strepthonium A (Figure 1).

Strepthonium A is a chlorinated quaternary ammonium compound and is structurally similar to the synthesised non-chlorinated quaternary ammonium surfactant benzethonium which has been widely used as a bactericide and antiseptic in medicaments (Hikiba et al. 2005;). Strepthonium A also follows the Lipinsky rule of five in terms of low molecular weight less than 500 Da, a CLogP value of 4.908, three hydrogen acceptors and no hydrogen donors. However, strepthonium A is a permanent cation and therefore likely not membrane permeable. This could be an advantage, since; no systemic effects after oral uptake are expected. In a previous study, it was found that a group of structurally similar compounds to strepthonium A that were chlorinated at different positions in the aromatic rings were found to possess less toxic and irritating properties than the non-chlorinated quaternary ammonium compounds (Weiss et al. 1951). Therefore, we suspect a low toxicity of strepthonium A and the chlorination in the 2-position of the phenoxy group may contribute to the possible lower cytotoxicity. The striking structural similarity of strepthonium A to the synthetic benzethonium raises the possibility of strepthonium A to be a bio-transformation product. However, this is unlikely since benzethonium was neither present in the microbiological media nor in the production facility. Further experiments are needed to evaluate the cytotoxicity of strepthonium A. Due to the limited yield of the compound obtained from the isolation procedure, future experiments depend on the chemical synthesis of this compound.

2.2. Influence of strepthonium A on growth and Shiga toxin production of EHEC O157:H7 strain EDL933

None of the various proposed anti-EHEC strategies, whether phytopharmaceuticals, designer probiotics, synthetic toxin binders or neutralisers or monoclonal antibodies have entered the market until now. As a part of EHEC drug discovery screening programme, we tested the effect of strepthonium A on inhibiting the Stx production in EHEC O157:H7 strain EDL933 by the Verotoxin ELISA assay. Results showed that incubation of EHEC O157:H7 strain EDL933
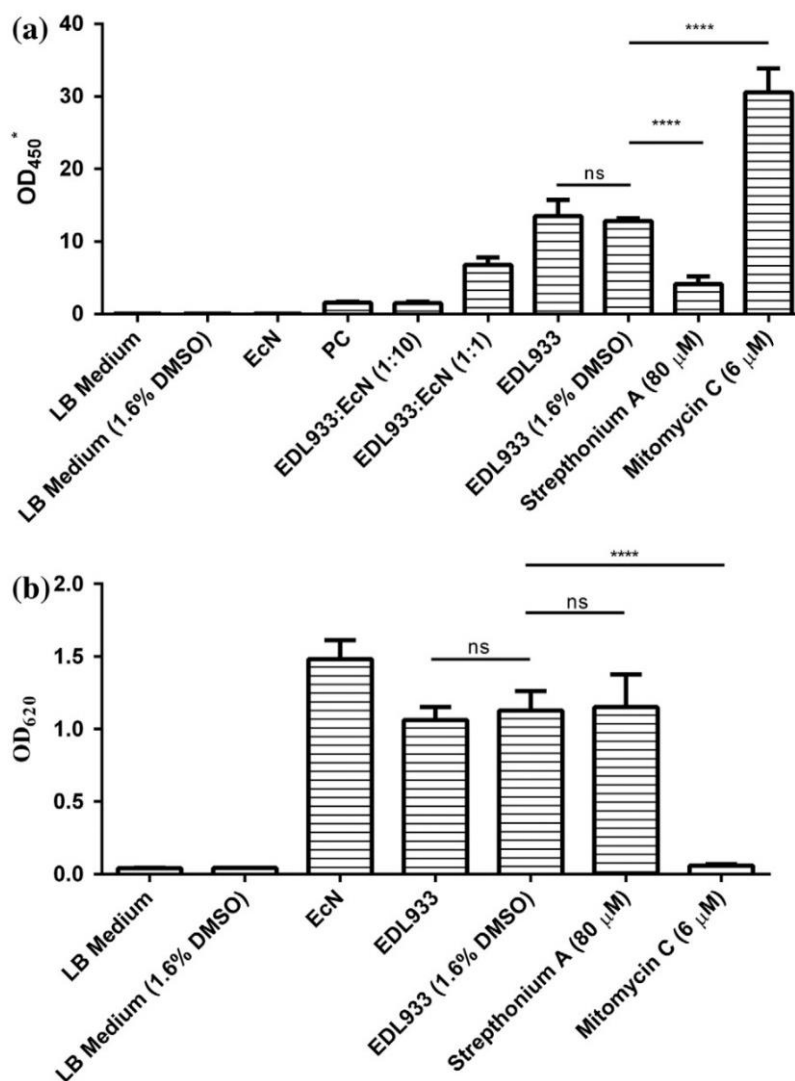
Figure 2. Strepthonium A inhibited the Stx production in EHEC O157:H7 without affecting the growth. (a) Effect on Stx production, (b) Effect on growth.

The graphs represent mean values ± SEM from three independent repetitions of the experiment done in quadruplicates. *Samples were diluted 1:10 before the Verotoxin ELISA was performed (ns: not significant, ****$p < 0.0001$). PC, positive control was provided with the Ridascreen ® Verotoxin ELISA.

with strepthonium A reduced the Stx levels by 68%. The positive control, the probiotic strain EcN, in co-culture with EDL933 at two different ratios (EDL933: EcN (1:1 and 1:10)) reduced the Stx levels by 50 and 89%, respectively. The antibiotic mitomycin C is known to trigger an SOS response activating the Stx expression (Pacheco & Sperandio 2012). Addition of mitomycin C (6 µM) enhanced the Stx production by 138.32% compared to the control (Figure 2(a)). Antibiotics are preferably not used to treat EHEC infections because of possible antibiotic resistance development and the risk of HUS development resulting from Stx release from dead/dying bacteria. Our findings indicate that the strepthonium A at the tested concentration has no growth inhibition effects on EHEC O157:H7 strain EDL933 and the antibiotic mitomycin C (used here as a positive control) severely impaired growth (Figure 2(b)). Thus, strepthonium A was shown to down-regulate Stx production in EHEC O157:H7 strain EDL933 without inhibiting its growth.

3. Conclusion

In summary, our results show that the identified compound strepthonium A exhibits anti-Stx potential and is able to inhibit Stx production without influencing bacterial growth. The study highlights the potential of marine actinomycetes in providing new natural products with novel bioactivities.

Authors Contributions

CC and URA (isolation and structure elucidation of strepthonium, manuscript preparation), SB and TAO (bioactivity testing and analysis of results, manuscript preparation), AF and MK (mass spectrometry analysis of strepthonium, manuscript preparation), UH, MJM, TAO and URA, (manuscript preparation and revision). All authors read and approved the final manuscript.

Acknowledgements

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

References

Abdelmohsen UR, Bayer K, Hentschel U. 2014. Diversity, abundance and natural products of marine sponge-associated actinomycetes. Nat Prod Rep. 31(3):381–399.
Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR. 2015. Marine natural products. Nat Prod Rep. 32:116–211.
Cheng C, MacIntyre L, Abdelmohsen UR, Horn H, Polymenakou P, Edrada-Ebel R, Hentschel U. 2015. Biodiversity, anti-trypanosomal activity screening, and metabolomics profiling of actinomycetes isolated from Mediterranean sponges. PLoS ONE. doi:10.1371/journal.pone.0138528.
Goldwater PN, Bettelheim KA. 2012. Treatment of enterohemorrhagic *Escherichia coli* (EHEC) infection and hemolytic uremic syndrome (HUS). BMC Med. 10:775.
Hikiba H, Watanabe E, Barrett JC, Tsutsui T. 2005. Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells. J Pharmacol Sci. 97:146–152.
Lee JH, Kim YG, Ryu SY, Cho MH, Lee J. 2014. Ginkgolic acids and Ginkgo biloba extract inhibit *Escherichia coli* O157:H7 and *Staphylococcus aureus* biofilm formation. Int J Food Microbiol. 174:47–55.
Pacheco AR, Sperandio V. 2012. Shiga toxin in enterohemorrhagic *E. coli*: regulation and novel anti-virulence strategies. Front Cell Infect Microbiol. 2:81.

Rund SA, Rohde H, Sonnenborn U, Oelschlaeger TA. 2013. Antagonistic effects of probiotic *Escherichia coli* Nissle 1917 on EHEC strains of serotype O104:H4 and O157:H7. Int J Med Microbiol. 303(1):1–8.

Weiss P, Cordasco MG, Carman W, Reiner L. 1951. The effect of ring chlorination on the antibacterial action of (aryloxyethoxyethyl) benzyldimethylammonium chlorides. J Am Pharm Assoc (1912–1977). 40:267–272.

3. Marine sponge-derived *Streptomyces* sp. SBT343 extract inhibits staphylococcal biofilm formation

This article was published in the peer-reviewed journal Frontiers in Microbiology, Section: Antimicrobials, Resistance and Chemotherapy.

For documentation of individual contributions to this work and consent of all authors for second publication in this thesis please refer to the appendix.

Supplementary information to this article could be accessed online at: https://www.frontiersin.org/article/10.3389/fmicb.2017.00236/full#supplementary-material

Marine Sponge-Derived *Streptomyces* sp. SBT343 Extract Inhibits Staphylococcal Biofilm Formation

Srikkanth Balasubramanian[1], Eman M. Othman[2,3], Daniel Kampik[4], Helga Stopper[2], Ute Hentschel[5], Wilma Ziebuhr[1], Tobias A. Oelschlaeger[1]* and Usama R. Abdelmohsen[6,7]*

[1] Institute for Molecular Infection Biology, University of Würzburg, Würzburg, Germany, [2] Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany, [3] Department of Analytical Chemistry, Faculty of Pharmacy, Minia University, Minia, Egypt, [4] Department of Ophthalmology, University Hospital Würzburg, Würzburg, Germany, [5] GEOMAR Helmholtz Centre for Ocean Research, RD3 Marine Microbiology, and Christian-Albrechts University of Kiel, Kiel, Germany, [6] Department of Botany II, Julius-von-Sachs Institute for Biological Sciences, University of Würzburg, Würzburg, Germany, [7] Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia, Egypt

Staphylococcus epidermidis and *Staphylococcus aureus* are opportunistic pathogens that cause nosocomial and chronic biofilm-associated infections. Indwelling medical devices and contact lenses are ideal ecological niches for formation of staphylococcal biofilms. Bacteria within biofilms are known to display reduced susceptibilities to antimicrobials and are protected from the host immune system. High rates of acquired antibiotic resistances in staphylococci and other biofilm-forming bacteria further hamper treatment options and highlight the need for new anti-biofilm strategies. Here, we aimed to evaluate the potential of marine sponge-derived actinomycetes in inhibiting biofilm formation of several strains of *S. epidermidis*, *S. aureus*, and *Pseudomonas aeruginosa*. Results from *in vitro* biofilm-formation assays, as well as scanning electron and confocal microscopy, revealed that an organic extract derived from the marine sponge-associated bacterium *Streptomyces* sp. SBT343 significantly inhibited staphylococcal biofilm formation on polystyrene, glass and contact lens surfaces, without affecting bacterial growth. The extract also displayed similar antagonistic effects towards the biofilm formation of other *S. epidermidis* and *S. aureus* strains tested but had no inhibitory effects towards *Pseudomonas* biofilms. Interestingly the extract, at lower effective concentrations, did not exhibit cytotoxic effects on mouse fibroblast, macrophage and human corneal epithelial cell lines. Chemical analysis by High Resolution Fourier Transform Mass Spectrometry (HRMS) of the *Streptomyces* sp. SBT343 extract proportion revealed its chemical richness and complexity. Preliminary physico-chemical characterization of the extract highlighted the heat-stable and non-proteinaceous nature of the active component(s). The combined data suggest that the *Streptomyces* sp. SBT343 extract selectively inhibits staphylococcal biofilm formation

33

without interfering with bacterial cell viability. Due to absence of cell toxicity, the extract might represent a good starting material to develop a future remedy to block staphylococcal biofilm formation on contact lenses and thereby to prevent intractable contact lens-mediated ocular infections.

INTRODUCTION

Ocular devices such as intraocular lenses, posterior contact lenses, conjunctival plugs and orbital implants have aided in restoring and improving human vision. However, contamination of these devices with bacterial biofilms can lead to device-related ocular infections such as endophthalmitis, crystalline keratopathy, corneal ulceration, keratitis, lacrimal system, and periorbital infections (Bispo et al., 2015; Cho et al., 2015). The National Institute of Health (NIH) estimates that biofilms contribute to about 75% of the human microbial infections. Biofilms are surface-associated sessile microbial communities that are enmeshed in a self-produced extracellular matrix composed of polysaccharides, proteins, lipids and DNA (Flemming and Wingender, 2010; Oja et al., 2015). Compared to the free-living planktonic counterparts, bacteria in biofilms are 1000-fold more resistant to conventional antibiotic therapies and host immune responses (Donlan, 2001; Otto, 2009; Burmolle et al., 2010). The highly persistent and detrimental nature of biofilm-associated infections and rapid emergence of multidrug resistant strains (Barros et al., 2014; Sakimura et al., 2015) has imposed a major burden on health-care and medical settings. The current inexistence of effective biofilm-based therapeutics (Bjarnsholt et al., 2013) has necessitated the need for development of novel antibiofilm strategies for prophylaxis and/or treatment of the multitude of biofilm-associated ocular infections.

Staphylococci, particularly *S. epidermidis* and *S. aureus* are the most common causative agents of device-related infections. Infections caused by other staphylococci are far less frequent (Otto, 2009). *S. epidermidis* and *S. aureus* are commensal Gram positive bacteria found on human skin and nares, causing a wide range of indwelling medical device-related infections (Rogers et al., 2009; Uribe-Alvarez et al., 2016). The biofilm-based lifestyle of *S. epidermidis* and *S. aureus* on medical devices is a hallmark of the sub-acute and chronic recalcitrant infections caused by them (Reiter et al., 2014). Biofilm formation in *S. epidermidis* and *S. aureus* is facilitated predominantly by the synthesis of the homopolymer polysaccharide intercellular adhesion (PIA) by the enzymes coded by the *ica* locus. PIA-independent biofilm formation, mediated by surface proteins such as biofilm associated protein (Bap/Bhp) and accumulation associated protein (Aap), eDNA release, autolysins and cell sortase-anchored proteins have also been reported in several staphylococcal strains (Otto, 2009; Archer et al., 2011; McCarthy et al., 2015).

Marine actinomycetes represent an untapped reservoir of a broad range of biologically active compounds of pharmaceutical importance (Subramani and Aalbersberg, 2012; Zotchev, 2012;

Lee et al., 2014; Azman et al., 2015). Particularly, the marine sponge-associated actinomycetes are well documented for their intrinsic chemical repertoire (Abdelmohsen et al., 2014a; Santos-Gandelman et al., 2014; Reimer et al., 2015). Novel secondary metabolites with discrete biological activities have been reported from sponge-associated actinomycetes (Abdelmohsen et al., 2014a; Grkovic et al., 2014; Cheng et al., 2015). These include antimicrobial (Hentschel et al., 2001; Eltamany et al., 2014), antiparasitic (Dashti et al., 2014; Viegelmann et al., 2014), immunomodulatory (Tabares et al., 2011), antichlamydial (Reimer et al., 2015), antioxidant (Abdelmohsen et al., 2012; Grkovic et al., 2014), anticancer (Vicente et al., 2015) and anti-biofilm (Oja et al., 2015; Park et al., 2016) activities. The extreme and dynamic conditions offered by the oceans (differences in temperature, pH, pressure, light intensities etc.) are the potential reasons often linked to the production of secondary metabolites by marine actinomycetes (Abdelmohsen et al., 2017). The frequent rediscovery of bioactive compounds and redundancy of sample strains from terrestrial environment has further made the marine actinomycetes as hotspots for discovery of new compounds (Dalisay et al., 2013). Among actinomycetes, the genus *Streptomyces* are considered to be the most prolific producers of secondary metabolites for medical, agriculture and veterinary usage (Tan et al., 2016). Over two-thirds of natural products isolated to date are from *Streptomyces* which indicates their huge biosynthetic potential and chemistry profiles. The rich genetic and metabolic diversity, and the ability to catabolize a wide range of compounds, has made the genus *Streptomyces* to be probed for discovery of novel compounds that could be translated to clinical applications (Hassan et al., 2016; Ser et al., 2016; Yang and Sun, 2016; Perez et al., 2016; Zhao et al., 2016).

In our continuing effort for discovery of anti-biofilm agents, we employed a crystal violet-based screening method to identify anti-biofilm activity of organic extracts generated from marine sponge-derived actinomycetes. The biofilm forming reference strain *S. epidermidis* RP62A was employed as a model for screening. Herein we report the inhibitory effects of an organic extract from marine sponge-associated *Streptomyces* sp. SBT343 against the biofilm formation of *S. epidermidis* RP62A on polystyrene, glass and contact lens surfaces. The potential anti-biofilm activity was tested on two other strains of *S. epidermidis*, four different *S. aureus* strains and two different *P. aeruginosa* strains. The results obtained highlighted that the extract exhibited potent anti-biofilm effects on all the staphylococcal strains tested but did not exert any effect on the *Pseudomonas* strains. Preliminary evaluations on the physico-chemical characterization of active component(s) in the extract suggested their heat stable and non-proteinaceous nature.

34

MATERIALS AND METHODS

Pathogenic Strains and Growth Conditions

Bacterial strains used in the study are listed in **Table 1**. All strains for the biofilm study were propagated in Tryptic Soy Broth (TSB; Becton Dickinson) (17.0 g/l pancreatic digest of casein, 3.0 g/l papaic digest of soybean meal, 5.0 g/l sodium chloride, 2.5 g/l dipotassium hydrogen phosphate, 2.5 g/l glucose) and incubated at 37°C.

Fermentation Conditions and Extract Preperation

Streptomyces sp. SBT343 was cultivated from the Mediterranean sponge *Petrosia ficiformis* that was collected offshore Pollonia, Milos, Greece (N36.76612°; E24.51530°) in May 2013 (Cheng et al., 2015). Briefly, 1% (v/v) inoculum of a well grown culture of *Streptomyces* sp. SBT343 was inoculated in 150 mL modified ISP2 medium (2.5 g/l malt extract, 1.0 g/l yeast extract, D-mannitol 25 mM, in artificial sea water) in a 250 mL conical flask and the strain was subjected to batch fermentation (incubation at 30°C with shaking at 150 rpm) for 10 days. After harvesting, the filtrate of the fermented culture was extracted twice with 250 mL ethyl acetate for each time. The extract was generated by evaporating the solvent in a rotational evaporator (Heidolph Laborota, 4001, Büchi Vacuum Controller V-805). The modified ISP2 broth medium control ensured the purity of the fermentation and was also extracted separately in a similar manner and this served as the medium control for the bioactivity testing. Extracts were dissolved in DMSO (final concentration 3.75% on the cells) and used for anti-biofilm assays.

Quantification of Biofilm Formation

Quantification of biofilm formation was performed according to Weisser et al. (Weisser et al., 2010). Briefly, overnight culture of bacterial strains were diluted with TSB medium to OD_{600} of 0.05, and incubated statically in a 96-well flat bottom polystyrene plate (Greiner bio-one, GmbH, Germany) with or without the varying concentrations of the *Streptomyces* sp. SBT343 extract (0, 31.25, 62.5, 125, and 250 µg/ml) at 37°C (for *S. epidermidis*

and *P. aeruginosa*) or 30°C (for *S. aureus*) for 24 h. Extract from the modified ISP2 medium control (250 µg/ml) served as the control for the experiment. For biofilm quantification, planktonic bacteria were discarded, and the wells were rinsed carefully with sterile 1X phosphate buffered saline (PBS) twice, and the adherent cells in the plate were heat-fixed at 65°C for 1 h. This was followed by staining with 0.3% crystal violet for 5 min. The stained biofilm was washed with sterile water thrice. Plates were dried in an inverted position and OD_{492} readings were measured to compare the extent of biofilm inhibition in the extract treated sets vs. the modified ISP2 medium control treated set. The biofilm-negative *S. epidermidis* (ATCC12228) and *S. carnosus* TM300 served as the negative controls in the experiment.

Effect of the Extract on Existing Biofilms

To study the effect of the *Streptomyces* sp. SBT343 extract on the existing biofilm, biofilms were established by incubating the bacteria (inoculation OD_{600} 0.05) in TSB medium in a 96-well flat bottom polystyrene plate at 37°C or 30°C for 24 h. Planktonic bacteria were discarded by inverting the plate on paper stacks. This was followed by addition of fresh TSB medium with varying concentration of the *Streptomyces* sp. SBT343 extract and the plate was again incubated statically at 37°C or 30°C for 24 h. The effect of the extract in eradication of preformed biofilm by the extract was measured by the crystal violet assay as explained above. Extract from the modified ISP2 medium control (250 µg/ml) and sodium metaperiodate (40 mM) served as the negative and positive controls in the experiment.

Growth Measurements

The effect of *Streptomyces* sp. SBT343 extract on the growth of staphylococcal strains was evaluated (Nithya et al., 2010) by growth curve analysis. The growth curves were determined up to 24 h. The extract at 50% biofilm inhibitiory concentration (BIC_{50}) and the highest tested concentration (125 µg/ml) (non-toxic to cell lines), was added to a conical flask containing bacteria at an OD_{600} of 0.1. The flasks were incubated 37°C under shaking conditions at 200 rpm. TSB medium without the bacteria served as negative control. Growth medium with the modified ISP2 medium extract (125 µg/ml) and bacteria served as the

TABLE 1 | Strains used in this study.

Strain	Description	Reference and/or source
S. epidermidis ATCC 12228	Non-infection associated strain	ATCC collection
S. carnosus TM300	Meat starter culture	Rosenstein et al., 2009
S. epidermidis RP62A	Reference strain isolated from intra-vascular catheter associated sepsis	ATCC collection
S. epidermidis O-47	Clinical isolate from septic arthritis	Heilmann et al., 1996
S. epidermidis 1457	Clinical isolate from a patient with infected central venous catheter	Mack et al., 1992
S. aureus SH1000	MSSA; *rsbU* derivative of 8325-4 *rsbU*+	Horsburgh et al., 2002
S. aureus RN4220	Restriction-deficient transformation recipient; originally derived from NCTC 8325-4	Kreiswirth et al., 1983
S. aureus Newman	MSSA isolate from osteomyelitis patient	Lipinski et al., 1967
S. aureus USA300	CA-MRSA isolate from a wrist abscess	McDougal et al., 2003
P. aeruginosa PAO1	Clinical isolate from wound	Dr. Vinay Pawar, Braunschweig, Germany
P. aeruginosa PA14	Clinical isolate from burn wound	Dr. Vinay Pawar, Braunschweig, Germany

35

control. The reading was observed continuously up to 24 h at 2 h intervals. The experiment was carried out with three independent cultures.

Investigation on the Appearance of Biofilm-Switches Upon Extract Treatment

In order to study the appearance of biofilm-switches [PIA-dependent to PIA-independent (protein-mediated) biofilms] in the presence of the extract, the residual biofilms after the extract treatments were challenged with metaperiodate (40 mM NaIO$_4$) for PIA-dependent and proteinase K (1 mg/ml in 100 mM Tris-Cl) for PIA-independent (protein-mediated) modes of biofilm formation with the procedure described elsewhere (Wang et al., 2004).

Scanning Electron Microscopy

In order to evaluate the effect of the extract on the biofilm formation on soft contact lens (Proclear®, CooperVision® Lensbest, Germany), round pieces (diameter of 6 mm) were punched out of each contact lens using a sterile puncher. Each piece was washed twice with sterile 1XPBS and placed in a 24 well plate (Greiner bio-one, GmbH, Germany) containing 1 mL of *S. epidermidis* RP62A (OD$_{600}$ 0.05) in TSB with the extract (at concentrations of 62.5, 125, and 250 μg/ml). For testing the effect of the extract on the biofilm formation on glass surface, sterile glass cover slips (diameter of 12 mm) were placed in a 24 well plate containing 1 mL *S. epidermidis* RP62A (OD$_{600}$ 0.05) in TSB with the extract (at concentrations of 62.5, 125, and 250 μg/ml). In each case, bacteria treated with extract (250 μg/ml) from modified ISP2 medium served as the control, while wells with the TSB and the contact lens or cover slips alone served as sterile controls, respectively. Plates containing the contact lens and cover slips sets were then incubated statically at 37°C for 24 h. Samples were then washed carefully with sterile 1XPBS twice, and fixed overnight with 6.25% gluteraldehyde (in 50 mM phosphate buffer pH 7.4). After overnight fixation, samples were washed 5 times with Sörenson buffer (100 mM KH$_2$PO$_4$ and 100 mM Na$_2$HPO$_4$) and transferred to the electron microscopy unit, where they were dehydrated and then coated with gold by a low vacuum sputter coating, and scanned by scanning electron microscopy.

Confocal Microscopy

Samples on the contact lens and the cover slips were prepared and treated as above. After overnight incubation in 24 well plates, samples were subjected to a rapid epifluoroscence staining method employing the bacterial viability kit Live/Dead Bac Light, Invitrogen Ltd., Paisley, UK. The kit employs Syto 9 green and propidium iodide red fluorescent nucleic acid stains for distinguishing live and dead cells, respectively. The dye was prepared and added to the samples according to the manufacturer's specifications. Samples with the dye were incubated in the dark for 15 minutes and then assessed by a confocal microscope. The final step comprised of acquiring

photographs of the samples with a Leica Microystems (Leica TCS SP5, Leica Microsystems, Germany) at excitation levels of 488 nm for Syto 9 and 543 nm for propidium iodide. Images were acquired at 1.5 μm intervals. Further, images representing two dimensional [compressed z series (x–y sides) and compressed (x–z) views] and three dimensional views of the biofilm were acquired with IMARIS v 8.1.2. The thickness of the biofilms was also calculated with IMARIS v 8.1.2.

Cytotoxicity Studies

Human Epithelial Corneal Cells (HCEC) were cultivated in DMEM/Ham's F12 (Invitrogen Life Technologies, USA), supplemented with 5% (v/v) FBS, 1% (w/v) L-glutamine, 0.4% (w/v) antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin), insulin (5 mg/l) and EGF (10 μg/l) (PAA Laboratories GmbH, Austria). To assess the cytotoxicity on HCEC, vitality assay was performed with slight modifications (Schmitt et al., 2002). Briefly, vitality staining was performed with different concentrations of SBT343 extract for 24 h. 3.5 × 10^5 cells were seeded in 6-well plates for 24 h in a control medium. After treatment, cells were collected, and 70 μl of the cell suspension was stained with 30 μl staining solution [Gel RedBiotrend (Köln, Germany) and fluorescein diacetate (Kreiswirth et al., 1983)]. Twenty microliter of this mixture was applied to the slide, and the fractions of green and red cells in a total of 200 cells were counted at a 500-fold magnification with a fluorescence microscope. Macrophage (J774.1) and mouse fibroblast (NIH/3T3) cell lines were cultured in RPMI 1640 (1X)+Glutamax^{TM}-I and DMEM (1X)+Glutamax^{TM}-I (Life Technologies^{TM},USA) supplemented with 10%FCS, without antibiotics. Cytotoxicity on J774.1 and NIH/3T3 was assesssed employing alamar blue assay (Huber and Koella, 1993). 1 × 10^5 cells/ml were seeded in 96-well plates containing the extract at different concentrations (ranging from 31.25 to 500 μg/ml) and were incubated for 24 h at 37°C with 5% CO$_2$. After incubation, 20 μL alamar blue (Thermofischer scientific, USA) was added to each well and the plates were further incubated for 24 h at 37°C with 5% CO$_2$. Finally, the OD$_{550}$ values of the plates were measured and normalized with OD$_{630}$ values. The extent of cytotoxicity was determined by comparing the extract treated sets with the control. The final DMSO concentration on the cells was 1%.

Physico-chemical Characterization of Anti-biofilm Component(s)

To understand the nature of the active component(s), the extract was subjected to thermal and enzymatic (proteinase K and trypsin) treatments. Briefly, the extract was subjected to heat treatments at 50, 75, and 100°C for 1 h and cooled on ice. For the enzymatic treatment, proteinase K or trypsin (at a final concentration of 1 mg/ml) was added to the extract (at a final concentration of 0.125 mg/ml) and the reactions were incubated for 1 h at 37°C. As controls, extracts were incubated for 1 h at 37°C without the enzymes, a treatment that did not impair the anti-biofilm effect. For each of the above tests, the

36

biofilm inhibitory effects of treated and untreated extracts were compared using the microtitre plate assay (for biofilm formation) against all the staphylococcal strains tested. Each data point is composed of three independent experiments performed in quadruplicate.

In parallel, the activity of proteinase K and trypsin (1 mg/ml each) in the presence of DMSO were independently assessed employing the quantitative azocasein assay (Hasegawa et al., 2008).

Quantitative RT-PCR

After complementation of *S. epidermidis* RP62A with SBT343 extract at 62.5 (BIC_{50}) and 250 μg/ml for 24 h at 37°C, total RNA was isolated from planktonic bacteria and those in biofilm employing Trizol reagent (Invitrogen, Paisley, UK) and FastPrep® disrupter (Thermo Savant, Qbiogene, Inc., Cedex, France). Firstly, 1 ml of cells (from planktonic and biofilm states; the biofilm was gently removed from 24 well plate using a sterile scraper and re-suspended in 1 ml of fresh TSB medium) were centrifuged at 13000 rpm for 10 min at 4°C and pellets were re-suspended in 1 ml Trizol reagent. This suspension was briefly homogenized in a Lysing Matrix E tube (MP Biomedicals Germany, GmbH, Eschwege, Germany) in the FastPrep® cell disrupter and subjected to chloroform-based RNA extraction method. Purity and concentration of the extracted total RNA was spectrophotometrically assessed using a NanoDrop ND-1000 (peqLab Biotechnologie, GmbH). The A_{260}/A_{280} values of (range 1.8–2.0) indicated the purity of the RNA samples and the mean RNA yield obtained was 182.63 μg/ml. All the RNA samples were digested with DNase I (Thermo Scientific). Briefly, 1 μg of RNA was digested with 1 μl of DNase by incubation at 37°C for 30 min. This was followed by addition of 1.5 μl of DNase stop solution (50 mM EDTA) and incubation at 70°C for 10 min. After quality assessment of RNA after DNase treatment, about 50 ng/μl of RNA was used as template for qPCR experiment.

cDNA synthesis and qPCR amplification was performed simultaneously by employing the power SYBR® Green RNA-to-C_T^{TM} 1-step kit (Applied Biosystems, GmbH, Germany). Primers used in the study were designed according to the literature (Reiter et al., 2014), and were commercially produced (Eurofins MWG Synthesis GmbH, Germany). These primers were chosen based on their thermodynamic and sequence parameters. The reaction mixture for qPCR contained 1 μl of RNA template, 5 μl of power SYBR® Green RTPCR mix, 1 μl each of forward and reverse primers (10 μM), 0.08 μl of reverse transcriptase provided by the manufacturer and 1.92 μl of RNase free water. qPCR was performed using the Bio-Rad C1000 Touch^{TM} thermal cycler with the following cycle parameters: holding stage of 48°C for 30 min and 95° C for 10 min, followed by 50 cycles of 95°C for 15 s and then 55°C for 1 min, with a final melting curve determination. Experiment was peformed with three technical and biological replicates each. A difference of ≥7 Ct (cycle threshold) between the cDNA sample and no-template PCR control was considered negligible for relative quantification analysis. Finally, the relative expression of the target genes (*IcaA* and *IcaR*) in the presence of SBT343 extract

in relation to the modified ISP2 extract treated control was determined using the comparison with the expression level of the reference gene, DHFR (the expression of DHFR gene stayed constant in the conditions tested). The significance of the relative quantification was assessed by Student's *t*-test (GraphPad Prism® version 6.01).

Statistical Analysis

Experiments were repeated at least three times in quadruplicates and the data were expressed as mean ± standard error mean. The Student's *t*-test was used and $p < 0.05$ was considered as statistically significant. GraphPad Prism® version 6.01 was used for statistical analysis of the experimental data.

LC-MS Analysis

Analytical grade reagents, Methanol (MeOH), dichloromethane (DCM), acetonitrile (MeCN), and formic acid were purchased (Fisher Scientific, Hemel Hempstead, UK). In-house HPLC grade water was used from a direct Q-3 water purification system (Millipore, Watford, UK). Samples and medium control samples were prepared at a concentration of 1 mg/mL in 80:20 MeOH: DCM with a solvent blank. Experiments were performed with an Exactive mass spectrometer with an electrospray ionization source attached to an Accela 600 HPLC pump with Accela autosampler and UV/Vis detector (Thermo Scientific, Bremen, Germany). The mass accuracy was set to less than 3.0 ppm. The Orbitrap mass analyzer can limit the mass error within ±3.0 ppm. The instrument was calibrated to maintain a mass accuracy of ±1.0 ppm by applying the lock mass function. The instrument was externally calibrated per the manufacturer's instructions before the run and was internally calibrated during the run using lock masses. Mass spectrometry was carried out over a mass range of 100–2000 *m/z* in positive and negative ionization modes with spray voltage of 4.5 kV and capillary temperature at 270°C. Ten microliter were injected from each vial, at a flow rate of 300 μL/min. The column used was an ACE5 C18 column (5 μm × 75 mm × 3 mm) (Hichrom Limited, Reading, UK). A binary gradient method was utilized. The two solvents were A (water and 0.1% formic acid) and B (MeCN and 0.1% formic acid). The gradient was carried out for 10 min and the program followed; at zero minutes A = 90% and B = 10%, at 30 min A = 0% and B = 100% at 36 min A = 90% and B = 10% until end at 45 min. The UV absorption wavelength was set at 254 nm, the sample tray temperature was maintained at 4°C and the column maintained at 20°C. The samples were run sequentially, with solvent and media blanks analyzed first. LC-MS data was acquired using Xcalibur version 2.2 (Thermo Scientific, Bremen, Germany). LC-MS chromatograms were subsequently obtained using MassLynx v 4.10. This was followed by dereplication strategy. Since, the modified ISP2 medium is a complex mixture of constituents and could interfere with the identification of secondary metabolites in the SBT343 extract, a medium blank was analyzed together with the bacterial extract and obtained features were regarded as interference and subtracted for detection of true bacterial secondary metabolites.

37

FIGURE 1 | (A) Dose dependent inhibition of *Streptomyces* sp. SBT343 extract on biofilm formation of *S. epidermidis* RP62A on polystyrene flat-bottom 96-well plates. *S. epidermidis* RP62A treated with the extract from modified ISP2 medium (250 μg/ml) served as the appropriate control in the experiment. **(B)** Growth curve of *S. epidermidis* RP62A in the presence of BIC_{50} (62.5 μg/ml) and highest tested concentration (125 μg/ml) of the SBT343 extract. **(C)** Investigation of the *Streptomyces* sp. SBT343 for induction of PIA-independent biofilm formation in *S. epidermidis* RP62A. Control and the residual biofilms after the extract treatment (250 μg/ml) were subjected to two parallel treatments (i) with metaperiodate ($NaIO_{4-T}$), which is suitable to dissolve the PIA-matrix proportion of the biofilm, and (ii) with proteinase K (proteinase K_{-T}) which will reduce proteins engaged in the biofilm matrix. Biofilm measurements were done shortly after the respective treatments, and the total biofilm levels were compared with the untreated control and residual biofilm sets. The biofilm-negative *S. epidermidis* ATCC12228 served as the negative control. *S. epidermidis* RP62A treated with the extract from modified ISP2 medium (250 μg/ml) served as the appropriate control. Graphs represent mean values ± SEM from three independent repetitions of the experiment done in quadruplicate. ns, not significant, ****$p < 0.0001$.

Finally, the m/z values were searched for possible hits in the MarinLit® database (Abdelmohsen et al., 2014b; Macintyre et al., 2014).

RESULTS

Anti-biofilm Effect of the *Streptomyces* sp. SBT343 Organic Extract

Our continuing effort for discovery of anti-biofilm agents from marine sponge-derived actinomycetes against the model isolate *S. epidermidis* RP62A, led to identification of the anti-biofilm *Streptomyces* sp. SBT343 extract. The presence of the extract at 31.25 μg/ml during the bacterial growth caused a significant ($p < 0.0001$) reduction in the biofilm formation after 24 h of growth. At 62.5 μg/ml about 50% of the biofilm formation was inhibited and this was designated as the Biofilm Inhibitory Concentration (BIC_{50}). The anti-biofilm activity of the extract was dose-dependent, leading to 71.35% inhibition of biofilm formation at the maximum concentration (250 μg/ml) tested (**Figure 1A**). Even after the addition of extract at BIC_{50} and highest tested concentration, the growth of *S. epidermidis* RP62A was at the same level as that of the control. These results confirm that the biofilm inhibition by the extract is not due to growth effect (**Figure 1B**). SBT343 extract showed no effect on detaching/dispersing the bacteria from preformed biofilm at any of the tested concentrations (data not shown).

As the strain *S. epidermidis* RP62A is known as a strong PIA matrix biofilm producer, SBT343-mediated biofilm inhibition strongly suggests interference with PIA-mediated biofilm formation. Since, staphylococci are known to switch from PIA-dependent to PIA-independent biofilm formation under different conditions; the presence of these spontaneous switches in the presence of the extract was assessed. The ineffectiveness of proteinase K treatment on residual biofilms highlight that there is no spontaneous switch from PIA-dependent to PIA-independent (protein-mediated) biofilm formation in the presence of SBT343 extract (**Figure 1C**).

SEM Analysis

The biofilm inhibition potential of the SBT343 extract was studied on glass and contact lens surfaces using microscopic techniques. Electron microscopy of biofilm formation on glass cover slips and contact lenses in the presence of 62.5, 125, and 250 μg/ml SBT343 extract confirmed the results obtained by the *in vitro* crystal violet assay (**Figure 2**). In the control glass cover slips, mushroom shaped, three-dimensional biofilm was observed, whereas, multi-layered biofilm was observed on control contact lenses. In the glass cover slips and contact lenses incubated with the SBT 343 extract (62.5, 125, and 250 μg/ml), a dose-dependent reduction in the biofilm was clearly seen (**Figure 2A**). The reduction of biofilm with the extract was even pronounced in the contact lens model suggesting its possible application in contact lens solution and storage systems. Surfaces were distinctly seen between sporadic microcolonies in the glass cover slip and the contact lens incubated with higher concentrations of the extract. Representative SEM images at higher magnification indicated that the extract did not affect the morphology of the staphylococcal cells. Further, the presence of fibrous, net-like structures in the biofilm matrix was greatly reduced in both the glass cover slips and contact lenses incubated with SBT343 extract (**Figure 2B**). These findings suggest that the extract possibly works by altering the biofilm matrix composition or interferes with the production of extracellular matrix.

FIGURE 2 | Representative SEM images of staphylococcal biofilm on (A) Glass cover slip and contact lens at X1500 magnification. **(B)** Glass cover slip and contact lens at X10000 magnification. The white arrows indicate the presence of fibrous, net like structures which are typical features of PIA-dependent biofilms and the black arrows indicate the absence of the same. *S. epidermidis* RP62A treated with the extract from modified ISP2 medium (250 μg/ml) served as the appropriate control in the microscopy experiments.

FIGURE 3 | Confocal laser scanning microscopy analyses of staphylococcal biofilm in the presence of the SBT343 extract, visualized by fluorescence vital dye. Images were acquired by Leica TCS SP5 with a 20X objective lens (scale bar 30 μm). **(A)** Biofilm images on the glass cover slip. **(B)** Biofilm images on contact lens. In **(A,B)**, compressed z series (top), where multiple x–y planes from top to bottom of the biofilm are combined and the smaller image (bottom) represents the compressed x–z (side) of the biofilm. The thickness of the biofilms in control was 28.7 μm, whereas after extract treatment it was only 15.1 μm (62.5 μg/ml) and 4.53 μm (125 μg/ml) on the glass cover slips. Similarly, the thickness of the biofilms in the extract treated contact lens samples was only 13.6 μm (62.5 μg/ml) and 7.55 μm (125 μg/ml).

Confocal Microscopy Analysis

For the confocal microscopy studies, effect of the SBT343 extract on the biofilm formation was also examined with two systems, first in the cover slips, where the control sample showed compact and condensed biofilm, while the treatment with the extract attenuated the formation of the biofilm in a dose dependent matter. The same observations were obtained in the contact lens as a second system for examination. The ability of the extract to inhibit the biofilm formation was stronger in the coverslips experiments in comparison to the contact lens, but in both systems the inhibition was significant in comparison to the control (**Figure 3**). Noteworthy, very few non-viable bacteria (stained red) were spotted in the experiment both in the treated and the untreated sets. This further confirms that the extract does not interfere with bacterial cell viability. As a control, cells in biofilm were exposed to 75% ethanol for 5 mins and stained

39

with propidium iodide and SYTO green. In this case a large population of dead cells were spotted in the microscope (data not shown). Images representing compressed x–y and x–z (side) views (**Figure 3**) indicated that the number of the bacteria in the biofilm and the total biofilm thickness in the extract treated cover slips and contact lens sets were greatly reduced in comparison with the compact and condensed biofilm control. These images further corroborated the results that the reduction in fluorescence in the presence of the extract, was primarily due to the repression of biofilm formation, and has no negative effects on bacterial cell viability.

Anti-biofilm Effect of SBT343 Extract on Other Pathogens

The anti-biofilm activity of the SBT343 extract was investigated with other biofilm-forming *S. epidermidis*, *S. aureus*, and *P. aeruginosa* strains (**Table 2**). SBT343 extract (125 µg/ml) significantly reduced the biofilm formation of all the *S. epidermidis*, MSSA and MRSA strains tested, while the biofilm formation of *P. aeruginosa* was unaffected (**Figure 4A**). A dose dependent reduction in biofilm formation of the staphylococcal strains was observed upon extract treatment (**Figure 4B**), while the growth of the strains were unaltered in the presence of the extract (**Figure 4C**). Further, the extract had no effects on the preformed biofilms of all the strains tested at the highest concentration tested (data not shown). This indicates selectivity of the extract in inhibiting staphylococcal biofilms.

Cytotoxicity Analysis

We further investigated the cytotoxicity profile of the SBT343 extract against mouse fibroblast (NIH/3T3), macrophage (J774.1) and human corneal epithelial cells (HCEC). Results from vitality test and alamar blue assay demonstrated that cells did not suffer from significant toxicity after 24 h with effective concentrations of the extract (in the range of 31.25–125 µg/ml). The highest concentration 500 µg/ml displayed moderate to high cytotoxic effects on the cell lines tested (**Table 3**).

TABLE 2 | Biofilm formation of the investigated bacterial strains employing crystal violet assay.

Strain	Biofilm (OD 492 nm)
S. epidermidis ATCC 12228	0.124 ± 0.010
S. carnosus TM300	0.204 ± 0.013
S. epidermidis RP62A	2.861 ± .0.143
S. epidermidis O-47	1.387 ± 0.044
S. epidermidis 1457	2.165 ± 0.069
S. aureus SH1000	0.994 ± 0.112
S. aureus RN4220	1.177 ± 0.092
S. aureus Newman	0.691 ± 0.149
S. aureus USA300	0.659 ± 0.031
P. aeruginosa PAO1	1.337 ± 0.101
P. aeruginosa PA14	1.331 ± 0.067

Each data point is composed of three independent experiments performed in quadruplicate. Standard errors are reported.

Physico-chemical Characterization of Anti-biofilm Component(s)

Preliminary physical and chemical characterization of the biofilm inhibiting component(s) in the extract was assessed by subjecting the extract to physical (heat) and chemical (proteinase K or trypsin) treatments. Both the physical and chemical treatments, did not reduce the activity of the extract against all the staphylococcal strains (**Figure 5**). However, a slight increase in the activity was observed upon heat treatment, in *S. epidermidis* RP62A and 1457, and *S. aureus* SH1000 and RN4220 strains. Similarly, a slight increase in the anti-biofilm activity was observed upon enzyme treatment in *S. aureus* SH1000 and RN4220 strains (**Figure 5**). This suggests that the active component(s) in the extract is thermo-stable and non-proteinaceous in nature, and the extract contains compound(s) with anti-biofilm activity that could work similarly on different staphylococcal strains. DMSO at the tested concentrations did not influence proteinase K and trypsin activities (Supplementary Figure 1).

LC-MS Analysis

To identify the putative bioactive secondary metabolites in the SBT343 extract, the LC-UV/MS signature of the extract generated by high resolution Fourier Transform mass spectrometry was compared with the MarinLit database (a database for marine natural products). The dereplication of the chemical profile of SBT343 extract by comparison of HRMS data with MarinLit, led to identification of several known and unknown metabolites which were previously isolated from the genus *Streptomyces*. Both the positive and negative modes of electrospray ionization spectral data were used for dereplication purposes. The total ion chromatogram of the SBT343 extract showing the distribution of known and unknown compounds is depicted in **Figure 6A**. Further, the known compounds detected from the dereplication strategy are mentioned in **Table 4** and their structures are depicted in **Figure 6B**.

DISCUSSION

Biomaterials used in clinical and medical settings are ideal niches for formation of microbial biofilms (Shanmughapriya et al., 2012). Even though a number of natural and synthetic anti-biofilm agents have already been discovered (Richards and Melander, 2009; Gupta et al., 2016; Miquel et al., 2016; Tran et al., 2016), none of them has entered the market, owing to obstacles in translational research and lack of interest by pharmaceutical and biomedical companies (Romero and Kolter, 2011). Hence, there is a large unmet need for development of anti-biofilm formulations to tackle the problem of biofilms.

Various culture dependent and independent techniques have revealed the bacterial biofilm diversity in contact lens-related corneal diseases (Hall and Jones, 2010; Wiley et al., 2012). Staphylococci, particularly *S. epidermidis*, are frequent contaminants of a range of ocular devices. It was estimated by Hou et al. (2012) that many ophthalmic isolates of *S. epidermidis* could form biofilms *in vitro*. Strong biofilm formation by

FIGURE 4 | Anti-biofilm effect of SBT343 extract on other staphylococcal strains. (A) Photographs of wells of 96-well plate showing biofilms in the absence and presence of SBT343 extract after crystal violet staining. Control (modified ISP2 medium extract: 125 μg/ml) and SBT343 extract treated (125 μg/ml). **(B)** Dose dependent inhibitory effect of *Streptomyces* sp. SBT343 extract on biofilm formation of other staphylococcal strains on polystyrene flat-bottom 96-well plates. Staphylococcal strains treated with the extract from modified ISP2 medium (250 μg/ml) served as the appropriate control in the experiment; **(C)** Growth curves of staphylococcal strains in the presence of BIC_{50} and highest tested concentration (125 μg/ml) of the SBT343 extract. Graphs represent mean values ± SEM from three independent repetitions of the experiment done in quadruplicate. ns: not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

S. epidermidis has been observed on various intraocular and contact lens (IOLs) materials (Okajima et al., 2006). Bacteria in the biofilms of contact lenses are frequently resistant to antimicrobials in the soft contact lens care products (Szczotka-Flynn et al., 2009). This has sharpened the need to develop new anti-biofilm-based contact lens products for combating ocular infections. The anti-biofilm potential of natural product based preparations, extracts, and compounds have increasingly been reported in *in vitro* biomaterial-based models (Pacheco da Rosa et al., 2013; Nair et al., 2016). Several researchers have tried to assess the anti-biofilm aspects of marine bacteria (Nithya et al., 2011; Gowrishankar et al., 2012; Papa et al., 2015; Wu et al., 2016), particularly, marine actinomycetes (Oja et al., 2015; Saleem et al., 2015; Park et al., 2016).

Leshem et al. (2011) and Cho et al. (2015) have previously reported the inhibitory effect of natural product based extract and several compounds on staphylococcal biofilm formation on contact lens and contact lens cases. We have shown that the organic extract from marine *Streptomyces* sp. SBT343 exhibits similar anti-biofilm effects at much lower concentrations (62.5–250 μg/ml). The extract had no adverse effects on the contact lens material at the highest concentrations tested (data not shown). At the same time, the lowest effective concentration of the extract did not show apparent cytotoxic effects on the three different cell lines tested which indicates the possibility of using the extract for the human subjects. The selective anti-biofilm effect of the SBT343 extract against different staphylococcal strains without interference with the bacterial cell growth suggests the less possible appearance of resistant mutants with the usage of this extract. However, further experiments are needed to prove this hypothesis. The basis of employing TSB medium as the growth medium in the study is to provide optimal growth conditions at which the effects of SBT343 extract could be determined in short periods.

Under certain conditions, *S. epidermidis* is known to switch between the PIA-dependent and independent modes of biofilm lifestyle (Rohde et al., 2005; Hennig et al., 2007). Our findings

TABLE 3 | Cytotoxic evaluation for SBT343 extract.

Cell line	% reduction in cell viability		
	500 μg/ml	**250 μg/ml**	**31.25–125 μg/ml**
HCEC	90.75 ± 1.23****	20.66 ± 5.10*	NC
NIH/3T3	21.56 ± 2.43****	NC	NC
J774.1	33.83 ± 2.27****	NC	NC

Each data point is composed of three independent experiments performed in quadruplicate. Standard errors are reported. Differences in mean were compared to untreated control and considered statistically significant when p (*p < 0.05, ****p < 0.0001) as per Student's t-test. NC, not cytotoxic.

41

FIGURE 5 | Physico-chemical characterization of anti-biofilm component(s). (A) Influence of heat treatment on anti-biofilm activity. UT, untreated; HT, heat treated. **(B)** Influence of enzyme treatment on anti-biofilm activity. UT, untreated; PKT, proteinase K treated; TT, trypsin treated. ns, not significant; ****$p < 0.0001$.
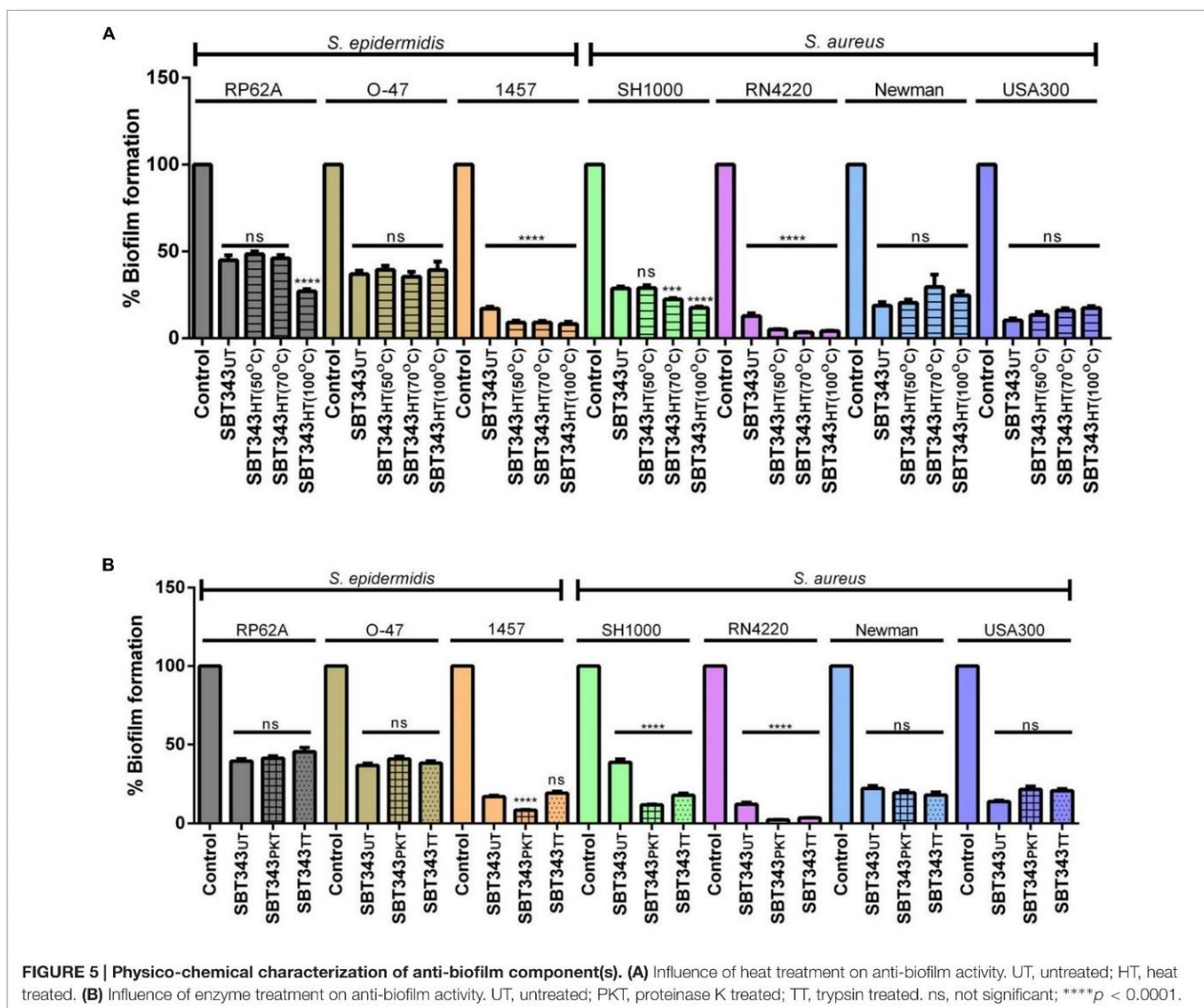
suggest that there are no switches in the biofilm lifestyle of the organism in the presence of the extract (**Figure 1C**). For a better understanding of the mechanism of biofilm inhibition by the extract, the relative mRNA expression of *icaA* and *icaR* in *S. epidermidis* RP62A (after 12 and 24 h) was determined in the planktonic and the biofilm cells in the presence of the extract. However, no significant changes in the expression levels of *icaA* and *icaR* were noted in the extract treated planktonic and biofilm cells (data not shown). These results indicate that the extract possibly works by an alternate mechanism and a global gene expression analysis would assist in deciphering the exact mode of action of the extract. The universal anti-biofilm activity against different staphylococcal strains with no effects on preformed biofilm and low cytotoxicity of this extract suggests its potential usage in contact lenses storage cases to prevent contact lens-associated ocular infections. Further studies are necessary to determine the anti-biofilm effect of SBT343 extract on different contact lens and storage cases materials.

The preliminary physical and chemical characterization of the anti-biofilm component(s) in the extract indicates that the active component(s) is of thermo-stable and non-proteinaceous nature and could act similarly on different staphylococcal strains tested. Dereplication strategies are often used in the natural products-based research for rapid identification of secondary metabolites in the crude bacterial extracts (Abdelmohsen et al., 2014b; Cheng et al., 2015). Currently, several analytical methods and tools are available for dereplication of metabolites in complex mixtures. Comparison of the HRMS data at positive and negative modes with the MarinLit database resulted in identification of several putative compounds in the *Streptomyces* sp. SBT343 extract. It is clear from the chromatogram that several peaks were not identified by comparison to the database, including some of the major components that showed strong peak intensity and good resolution. The high number of unidentified compounds highlights the chemical potential of this strain as a source of new natural products. Most of the compounds

42

FIGURE 6 | (A) Total ion chromatogram of crude extract of *Streptomyces* sp. SBT343 in both positive and negative modes, annotated to indicate metabolites identified in **Table 4**. Annotation and dereplication was done by eliminating the metabolites from the extract of modified ISP2 medium. Those annotated with an asterisk * are sodium ion adducts. **(B)** Structures of the putatively identified and dereplicated compounds.

identified in the extract were previously isolated from marine *Streptomyces*. An extensive literature search on the biological activities of these compounds revealed that none of them have been tested/reported to have anti-biofilm effects, while several of the putative compounds identified are known to possess anti-fungal, anti-cancer, anti-mycobacterial and anti-malarial effects.

TABLE 4 | Putatively identified and dereplicated compounds from the high-resolution mass spectral data sets of the crude ethyl acetate extract of *Streptomyces* sp. SBT343 using MarinLit® database with a precision of ±0.1–1.0.

Peak ID	ESI Mode	m/z*	Rt (min)	Hits (m/z**)
1	P	1095.624	5.85	Azalomycin (1095.682)
2	P	325.197	3.44	Streptcytosine B (325.109)
3	P	307.187	3.83	Streptcytosine C (307.153)
4	P	350.098	5.03	Daryamide A (350.184)
5	N	510.265	4.85	Azamerone (510.168)
				Antimycin B1 (510.221)
6	N	272.154	3.38	Usabamycin A (272.152)
7	N	659.355	5.15	Actinoramide D (660.738)

*P, positive ESI mode; N, negative ESI mode; Rt, retention time; * indicates the neutral m/z values of peaks found in the crude ethyl acetate extract of Streptomyces sp. SBT343; ** indicates the m/z values of the corresponding hits identified with the MarinLit® database.*

This further highlights the novelty in discovery of compound(s) with specific anti-biofilm effects from the SBT343 extract. Up-scaling of the fermentation process and consequent bio-assay guided fractionation would help in isolation and identification of active compound(s) in the extract. In conclusion, our results show that the chemically rich *Streptomyces* sp. SBT343 extract has the potential to prevent the staphylococcal biofilm formation on polystyrene, glass, and contact lens surface without exhibiting toxic effects on bacterial and mammalian cells. Future characterization of lead compounds in this extract may yield novel anti-biofilm compound(s) of pharmaceutical interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: UA, TÖ, UH, WZ. Performed the experiments: SB, EO. Analyzed the data: SB, EO, TÖ, UA. Manuscript preperation: SB, EO, DK, UA, HS, UH, WZ, TÖ. Manuscript revision: SB, EO, HS, UA, UH, WZ, TÖ. All authors read and approved the final manuscript.

FUNDING

43

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00236/full#supplementary-material

REFERENCES

Abdelmohsen, U. R., Balasubramanian, S., Oelschlaeger, T. A., Grkovic, T., Pham, N. B., Quinn, R. J., et al. (2017). Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. *Lancet Infect. Dis.* 17, e30–e41. doi: 10.1016/S1473-3099(16)30323-1

Abdelmohsen, U. R., Bayer, K., and Hentschel, U. (2014a). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat. Prod. Rep.* 31, 381–399. doi: 10.1039/c3np70111e

Abdelmohsen, U. R., Cheng, C., Viegelmann, C., Zhang, T., Grkovic, T., Ahmed, S., et al. (2014b). Dereplication strategies for targeted isolation of new antitrypanosomal actinosporins A and B from a marine sponge associated-*Actinokineospora* sp. EG49. *Mar. Drugs* 12, 1220–1244. doi: 10.3390/md12031220

Abdelmohsen, U. R., Szesny, M., Othman, E. M., Schirmeister, T., Grond, S., Stopper, H., et al. (2012). Antioxidant and anti-protease activities of diazepinomicin from the sponge-associated *Micromonospora* strain RV115. *Mar. Drugs* 10, 2208–2221. doi: 10.3390/md10102208

Archer, N. K., Mazaitis, M. J., Costerton, J. W., Leid, J. G., Powers, M. E., and Shirtliff, M. E. (2011). *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. *Virulence* 2, 445–459. doi: 10.4161/viru.2.5.17724

Azman, A. S., Othman, I., Velu, S. S., Chan, K. G., and Lee, L. H. (2015). Mangrove rare actinobacteria: taxonomy, natural compound, and discovery of bioactivity. *Front. Microbiol.* 6:856. doi: 10.3389/fmicb.2015.00856

Barros, M., Branquinho, R., Grosso, F., Peixe, L., and Novais, C. (2014). Linezolid-resistant *Staphylococcus epidermidis*, Portugal, 2012. *Emerg. Infect. Dis.* 20, 903–905. doi: 10.3201/eid2005.130783

Bispo, P. J., Haas, W., and Gilmore, M. S. (2015). Biofilms in infections of the eye. *Pathogens* 4, 111–136. doi: 10.3390/pathogens4010111

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M., and Hoiby, N. (2013). Applying insights from biofilm biology to drug development – can a new approach be developed? *Nat. Rev. Drug Discov.* 12, 791–808. doi: 10.1038/nrd4000

Burmolle, M., Thomsen, T. R., Fazli, M., Dige, I., Christensen, L., Homoe, P., et al. (2010). Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. *FEMS Immunol. Med. Microbiol.* 59, 324–336. doi: 10.1111/j.1574-695X.2010.00714.x

Cheng, C., Macintyre, L., Abdelmohsen, U. R., Horn, H., Polymenakou, P. N., Edrada-Ebel, R., et al. (2015). Biodiversity, anti-trypanosomal activity screening, and metabolomic profiling of actinomycetes isolated from mediterranean sponges. *PLoS ONE* 10:e0138528. doi: 10.1371/journal.pone.0138528

Cho, P., Shi, G. S., and Boost, M. (2015). Inhibitory effects of 2,2'-dipyridyl and 1,2,3,4,6-penta-O-galloyl-b-D-glucopyranose on biofilm formation in contact lens cases. *Invest. Ophthalmol. Vis. Sci.* 56, 7053–7057. doi: 10.1167/iovs.15-17723

Dalisay, D. S., Williams, D. E., Wang, X. L., Centko, R., Chen, J., and Andersen, R. J. (2013). Marine sediment-derived *Streptomyces* bacteria from British Columbia, Canada are a promising microbiota resource for the discovery of antimicrobial natural products. *PLoS ONE* 8:e77078. doi: 10.1371/journal.pone.0077078

Dashti, Y., Grkovic, T., Abdelmohsen, U. R., Hentschel, U., and Quinn, R. J. (2014). Production of induced secondary metabolites by a co-culture of sponge-associated actinomycetes, *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163. *Mar. Drugs* 12, 3046–3059. doi: 10.3390/md12053046

Donlan, R. M. (2001). Biofilms and device-associated infections. *Emerg. Infect. Dis.* 7, 277–281. doi: 10.3201/eid0702.010226

Eltamany, E. E., Abdelmohsen, U. R., Ibrahim, A. K., Hassanean, H. A., Hentschel, U., and Ahmed, S. A. (2014). New antibacterial xanthone from the marine sponge-derived *Micrococcus* sp. EG45. *Bioorg. Med. Chem. Lett.* 24, 4939–4942. doi: 10.1016/j.bmcl.2014.09.040

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Gowrishankar, S., Duncun Mosioma, N., and Karutha Pandian, S. (2012). Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and -susceptible *Staphylococcus aureus* biofilms. *Evid. Based Complement. Alternat. Med.* 2012:862374. doi: 10.1155/2012/862374

Grkovic, T., Abdelmohsen, U. R., Othman, E. M., Stopper, H., Edrada-Ebel, R., Hentschel, U., et al. (2014). Two new antioxidant actinosporin analogues from the calcium alginate beads culture of sponge-associated *Actinokineospora* sp. strain EG49. *Bioorg. Med. Chem. Lett.* 24, 5089–5092. doi: 10.1016/j.bmcl.2014.08.068

Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S., and Tribedi, P. (2016). Biofilm, pathogenesis and prevention–a journey to break the wall: a review. *Arch. Microbiol.* 198, 1–15. doi: 10.1007/s00203-015-1148-6

Hall, B. J., and Jones, L. (2010). Contact lens cases: the missing link in contact lens safety? *Eye Contact Lens* 36, 101–105. doi: 10.1097/ICL.0b013e3181d05555

Hasegawa, H., Lind, E. J., Boin, M. A., and Hase, C. C. (2008). The extracellular metalloprotease of *Vibrio tubiashii* is a major virulence factor for pacific oyster (*Crassostrea gigas*) larvae. *Appl. Environ. Microbiol.* 74, 4101–4110. doi: 10.1128/AEM.00061-08

Hassan, R., Shaaban, M. I., Abdel Bar, F. M., El-Mahdy, A. M., and Shokralla, S. (2016). Quorum sensing inhibiting activity of *Streptomyces coelicoflavus* isolated from soil. *Front. Microbiol.* 7:659. doi: 10.3389/fmicb.2016.00659

Heilmann, C., Gerke, C., Perdreau-Remington, F., and Gotz, F. (1996). Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect. Immun.* 64, 277–282. doi: 10.1016/j.ijmm.2006.12.001

Hennig, S., Nyunt Wai, S., and Ziebuhr, W. (2007). Spontaneous switch to PIA-independent biofilm formation in an ica-positive *Staphylococcus epidermidis* isolate. *Int. J. Med. Microbiol.* 297, 117–122.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., and Hacker, J. (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol. Ecol.* 35, 305–312. doi: 10.1111/j.1574-6941.2001.tb00816.x

Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K., and Foster, S. J. (2002). SigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. *J. Bacteriol.* 184, 5457–5467. doi: 10.1128/JB.184.19.5457-5467.2002

44

Hou, W., Sun, X., Wang, Z., and Zhang, Y. (2012). Biofilm-forming capacity of *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* from ocular infections. *Invest. Ophthalmol. Vis. Sci.* 53, 5624–5631. doi: 10.1167/iovs.11-9114

Huber, W., and Koella, J. C. (1993). A comparison of three methods of estimating EC50 in studies of drug resistance of malaria parasites. *Acta Trop.* 55, 257–261. doi: 10.1016/0001-706X(93)90083-N

Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'reilly, M., Schlievert, P. M., Bergdoll, M. S., et al. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 305, 709–712. doi: 10.1038/305709a0

Lee, L. H., Zainal, N., Azman, A. S., Eng, S. K., Goh, B. H., Yin, W. F., et al. (2014). Diversity and antimicrobial activities of actinobacteria isolated from tropical mangrove sediments in Malaysia. *Sci. World J.* 2014:698178. doi: 10.1155/2014/698178

Leshem, R., Maharshak, I., Ben Jacob, E., Ofek, I., and Kremer, I. (2011). The effect of nondialyzable material (NDM) cranberry extract on formation of contact lens biofilm by *Staphylococcus epidermidis*. *Invest. Ophthalmol. Vis. Sci.* 52, 4929–4934. doi: 10.1167/iovs.10-5335

Lipinski, B., Hawiger, J., and Jeljaszewicz, J. (1967). Staphylococcal clumping with soluble fibrin monomer complexes. *J. Exp. Med.* 126, 979–988. doi: 10.1084/jem.126.5.979

Macintyre, L., Zhang, T., Viegelmann, C., Martinez, I. J., Cheng, C., Dowdells, C., et al. (2014). Metabolomic tools for secondary metabolite discovery from marine microbial symbionts. *Mar. Drugs* 12, 3416–3448. doi: 10.3390/md12063416

Mack, D., Siemssen, N., and Laufs, R. (1992). Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect. Immun.* 60, 2048–2057.

McCarthy, H., Rudkin, J. K., Black, N. S., Gallagher, L., O'neill, E., and O'gara, J. P. (2015). Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*. *Front. Cell Infect. Microbiol.* 5:1. doi: 10.3389/fcimb.2015.00001

McDougal, L. K., Steward, C. D., Killgore, G. E., Chaitram, J. M., Mcallister, S. K., and Tenover, F. C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J. Clin. Microbiol.* 41, 5113–5120. doi: 10.1128/JCM.41.11.5113-5120.2003

Miquel, S., Lagrafeuille, R., Souweine, B., and Forestier, C. (2016). Anti-biofilm activity as a health issue. *Front. Microbiol.* 7:592. doi: 10.3389/fmicb.2016.00592

Nair, S. V., Baranwal, G., Chatterjee, M., Sachu, A., Vasudevan, A. K., Bose, C., et al. (2016). Antimicrobial activity of plumbagin, a naturally occurring naphthoquinone from *Plumbago rosea*, against *Staphylococcus aureus* and *Candida albicans*. *Int. J. Med. Microbiol.* 306, 237–248. doi: 10.1016/j.ijmm.2016.05.004

Nithya, C., Begum, M. F., and Pandian, S. K. (2010). Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. *Appl. Microbiol. Biotechnol.* 88, 341–358. doi: 10.1007/s00253-010-2777-y

Nithya, C., Devi, M. G., and Karutha Pandian, S. (2011). A novel compound from the marine bacterium *Bacillus pumilus* S6-15 inhibits biofilm formation in gram-positive and gram-negative species. *Biofouling* 27, 519–528. doi: 10.1080/08927014.2011.586127

Oja, T., San Martin Galindo, P., Taguchi, T., Manner, S., Vuorela, P. M., Ichinose, K., et al. (2015). Effective antibiofilm polyketides against *Staphylococcus aureus* from the pyranonaphthoquinone biosynthetic pathways of *Streptomyces* Species. *Antimicrob. Agents Chemother.* 59, 6046–6052. doi: 10.1128/AAC.00991-15

Okajima, Y., Kobayakawa, S., Tsuji, A., and Tochikubo, T. (2006). Biofilm formation by *Staphylococcus epidermidis* on intraocular lens material. *Invest. Ophthalmol. Vis. Sci.* 47, 2971–2975. doi: 10.1167/iovs.05-1172

Otto, M. (2009). *Staphylococcus epidermidis*–the 'accidental' pathogen. *Nat. Rev. Microbiol.* 7, 555–567. doi: 10.1038/nrmicro2182

Pacheco da Rosa, J., Korenblum, E., Franco-Cirigliano, M. N., Abreu, F., Lins, U., Soares, R. M., et al. (2013). *Streptomyces lunalinharesii* strain 235 shows the potential to inhibit bacteria involved in biocorrosion processes. *Biomed. Res. Int.* 2013:309769. doi: 10.1155/2013/309769

Papa, R., Selan, L., Parrilli, E., Tilotta, M., Sannino, F., Feller, G., et al. (2015). Anti-biofilm activities from marine cold adapted bacteria against *Staphylococci* and *Pseudomonas aeruginosa*. *Front. Microbiol.* 6:1333. doi: 10.3389/fmicb.2015.01333

Park, S. R., Tripathi, A., Wu, J., Schultz, P. J., Yim, I., Mcquade, T. J., et al. (2016). Discovery of cahuitamycins as biofilm inhibitors derived from a convergent biosynthetic pathway. *Nat. Commun.* 7:10710. doi: 10.1038/ncomms10710

Perez, M., Schleissner, C., Fernandez, R., Rodriguez, P., Reyes, F., Zuniga, P., et al. (2016). PM100117 and PM100118, new antitumor macrolides produced by a marine *Streptomyces caniferus* GUA-06-05-006A. *J. Antibiot.* 69, 388–394. doi: 10.1038/ja.2015.121

Reimer, A., Blohm, A., Quack, T., Grevelding, C. G., Kozjak-Pavlovic, V., Rudel, T., et al. (2015). Inhibitory activities of the marine streptomycete-derived compound SF2446A2 against *Chlamydia trachomatis* and *Schistosoma mansoni*. *J. Antibiot.* 68, 674–679. doi: 10.1038/ja.2015.54

Reiter, K. C., Sant'anna, F. H., and D'azevedo, P. A. (2014). Upregulation of icaA, atlE and aap genes by linezolid but not vancomycin in *Staphylococcus epidermidis* RP62A biofilms. *Int. J. Antimicrob. Agents* 43, 248–253. doi: 10.1016/j.ijantimicag.2013.12.003

Richards, J. J., and Melander, C. (2009). Controlling bacterial biofilms. *Chembiochem* 10, 2287–2294. doi: 10.1002/cbic.200900317

Rogers, K. L., Fey, P. D., and Rupp, M. E. (2009). Coagulase-negative staphylococcal infections. *Infect. Dis. Clin. North Am.* 23, 73–98. doi: 10.1016/j.idc.2008.10.001

Rohde, H., Burdelski, C., Bartscht, K., Hussain, M., Buck, F., Horstkotte, M. A., et al. (2005). Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol. Microbiol.* 55, 1883–1895. doi: 10.1111/j.1365-2958.2005.04515.x

Romero, D., and Kolter, R. (2011). Will biofilm disassembly agents make it to market? *Trends Microbiol.* 19, 304–306. doi: 10.1016/j.tim.2011.03.003

Rosenstein, R., Nerz, C., Biswas, L., Resch, A., Raddatz, G., Schuster, S. C., et al. (2009). Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. *Appl. Environ. Microbiol.* 75, 811–822. doi: 10.1128/AEM.01982-08

Sakimura, T., Kajiyama, S., Adachi, S., Chiba, K., Yonekura, A., Tomita, M., et al. (2015). Biofilm-forming *Staphylococcus epidermidis* expressing vancomycin resistance early after adhesion to a metal surface. *Biomed Res. Int.* 2015:943056. doi: 10.1155/2015/943056

Saleem, H. G., Aftab, U., Sajid, I., Abbas, Z., and Sabri, A. N. (2015). Effect of crude extracts of selected actinomycetes on biofilm formation of *A. schindleri*, *M. aci*, and *B. cereus*. *J. Basic Microbiol.* 55, 645–651. doi: 10.1002/jobm.201400358

Santos-Gandelman, J. F., Giambiagi-Demarval, M., Oelemann, W. M., and Laport, M. S. (2014). Biotechnological potential of sponge-associated bacteria. *Curr. Pharm. Biotechnol.* 15, 143–155. doi: 10.2174/1389201015666140711115033

Schmitt, E., Lehmann, L., Metzler, M., and Stopper, H. (2002). Hormonal and genotoxic activity of resveratrol. *Toxicol. Lett.* 136, 133–142. doi: 10.1016/S0378-4274(02)00290-4

Ser, H. L., Tan, L. T., Palanisamy, U. D., Abd Malek, S. N., Yin, W. F., Chan, K. G., et al. (2016). *Streptomyces antioxidans* sp. nov., a novel mangrove soil actinobacterium with antioxidative and neuroprotective potentials. *Front. Microbiol.* 7:899. doi: 10.3389/fmicb.2016.00899

Shanmughapriya, S., Francis, A. L., Kavitha, S., and Natarajaseenivasan, K. (2012). In vitro actinomycete biofilm development and inhibition by the polyene antibiotic, nystatin, on IUD copper surfaces. *Biofouling* 28, 929–935. doi: 10.1080/08927014.2012.717616

Subramani, R., and Aalbersberg, W. (2012). Marine actinomycetes: an ongoing source of novel bioactive metabolites. *Microbiol. Res.* 167, 571–580. doi: 10.1016/j.micres.2012.06.005

Szczotka-Flynn, L. B., Imamura, Y., Chandra, J., Yu, C., Mukherjee, P. K., Pearlman, E., et al. (2009). Increased resistance of contact lens-related bacterial biofilms to antimicrobial activity of soft contact lens care solutions. *Cornea* 28, 918–926. doi: 10.1097/ICO.0b013e3181a81835

Tabares, P., Pimentel-Elardo, S. M., Schirmeister, T., Hunig, T., and Hentschel, U. (2011). Anti-protease and immunomodulatory activities of bacteria associated with Caribbean sponges. *Mar. Biotechnol.* 13, 883–892. doi: 10.1007/s10126-010-9349-0

45

Tan, L. T., Chan, K. G., Lee, L. H., and Goh, B. H. (2016). *Streptomyces* bacteria as potential probiotics in aquaculture. *Front. Microbiol.* 7:79. doi: 10.3389/fmicb.2016.00079

Tran, P. L., Huynh, E., Pham, P., Lacky, B., Jarvis, C., Mosley, T., et al. (2016). Organoselenium polymer inhibits biofilm formation in polypropylene contact lens case material. *Eye Contact Lens* doi: 10.1097/ICL.0000000000000239 [Epub ahead of print].

Uribe-Alvarez, C., Chiquete-Felix, N., Contreras-Zentella, M., Guerrero-Castillo, S., Pena, A., and Uribe-Carvajal, S. (2016). *Staphylococcus epidermidis*: metabolic adaptation and biofilm formation in response to different oxygen concentrations. *Pathog. Dis.* 74:ftv111. doi: 10.1093/femspd/ftv111

Vicente, J., Stewart, A. K., Van Wagoner, R. M., Elliott, E., Bourdelais, A. J., and Wright, J. L. (2015). Monacyclinones, new angucyclinone metabolites isolated from *Streptomyces* sp. M7_15 associated with the Puerto Rican sponge *Scopalina ruetzleri*. *Mar. Drugs* 13, 4682–4700. doi: 10.3390/md13084682

Viegelmann, C., Parker, J., Ooi, T., Clements, C., Abbott, G., Young, L., et al. (2014). Isolation and identification of antitrypanosomal and antimycobacterial active steroids from the sponge *Haliclona simulans*. *Mar. Drugs* 12, 2937–2952. doi: 10.3390/md12052937

Wang, X., Preston, J. F. III, and Romeo, T. (2004). The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J. Bacteriol.* 186, 2724–2734. doi: 10.1128/JB.186.9.2724-2734.2004

Weisser, M., Schoenfelder, S. M., Orasch, C., Arber, C., Gratwohl, A., Frei, R., et al. (2010). Hypervariability of biofilm formation and oxacillin resistance in a *Staphylococcus epidermidis* strain causing persistent severe infection in an immunocompromised patient. *J. Clin. Microbiol.* 48, 2407–2412. doi: 10.1128/JCM.00492-10

Wiley, L., Bridge, D. R., Wiley, L. A., Odom, J. V., Elliott, T., and Olson, J. C. (2012). Bacterial biofilm diversity in contact lens-related disease: emerging role of *Achromobacter*, *Stenotrophomonas*, and *Delftia*. *Invest. Ophthalmol. Vis. Sci.* 53, 3896–3905. doi: 10.1167/iovs.11-8762

Wu, S., Liu, G., Jin, W., Xiu, P., and Sun, C. (2016). Antibiofilm and anti-infection of a marine bacterial exopolysaccharide against *Pseudomonas aeruginosa*. *Front. Microbiol.* 7:102. doi: 10.3389/fmicb.2016.00102

Yang, N., and Sun, C. (2016). The inhibition and resistance mechanisms of actinonin, isolated from marine *Streptomyces* sp. NHF165, against *Vibrio anguillarum*. *Front. Microbiol.* 7:1467. doi: 10.3389/fmicb.2016.01467

Zhao, F., Qin, Y. H., Zheng, X., Zhao, H. W., Chai, D. Y., Li, W., et al. (2016). Biogeography and adaptive evolution of *Streptomyces* strains from saline environments. *Sci. Rep.* 6:32718. doi: 10.1038/srep32718

Zotchev, S. B. (2012). Marine actinomycetes as an emerging resource for the drug development pipelines. *J. Biotechnol.* 158, 168–175. doi: 10.1016/j.jbiotec.2011.06.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LC and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

46

4. A new bioactive compound from the marine sponge-derived *Streptomyces* sp. SBT348 inhibits staphylococcal growth and biofilm formation

Authors

Srikkanth Balasubramanian[1], Joseph Skaf[2], Ulrike Holzgrabe[2], Richa Bharti[3], Konrad U. Förstner[3], Wilma Ziebuhr[1], Ute Hentschel[4], Usama Ramadan Abdelmohsen[5*], Tobias A. Oelschlaeger[1, *]

Affiliations

[1]Institute for Molecular Infection Biology, University of Würzburg, Josef-Schneider-Str. 2/D15, 97080 Würzburg, Germany

[2]Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

[3]Core Unit Systems Medicine, University of Würzburg, 97080 Würzburg, Germany

[4]GEOMAR Helmholtz Centre for Ocean Research, RD3 Marine Microbiology, and Christian-Albrechts University of Kiel, Düsternbrooker Weg 20, D-24105 Kiel, Germany

[5]Department of Pharmacognosy, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt

* Corresponding authors

t.oelschlaeger@uni-wuerzburg.de; Tel.: +49-931-31-82150 (TAO)

usama.ramadan@mu.edu.eg; Tel: +20-1111595772 (URA)

This article was submitted to the peer-reviewed journal Frontiers in Microbiology, Section: Antimicrobials, Resistance and Chemotherapy and is currently under review.

For documentation of individual contributions to this work and consent of all authors for second publication in this thesis please refer to the appendix.

Supplementary tables to this article are provided in a CD attached to this PhD thesis.

Abstract

Staphylococcus epidermidis, the common inhabitant of human skin and mucosal surfaces has emerged as an important pathogen in patients carrying surgical implants and medical devices. Entering the body via surgical sites and colonizing the medical devices through formation of multi-layered biofilms leads to refractory and persistent device-related infections (DRIs). Staphylococci organized in biofilms are more tolerant to antibiotics and immune responses, and thus are difficult-to-treat. The consequent morbidity and mortality, and economic losses in health care systems have strongly necessitated the need for development of new anti-bacterial and anti-biofilm-based therapeutics. In this study, we describe the biological activity of a marine sponge-derived *Streptomyces* sp. SBT348 extract in restraining staphylococcal growth and biofilm formation on polystyrene, glass, medically relevant titan metal and silicone surfaces. A bioassay-guided fractionation was performed to isolate the active compound (SKC3) from the crude SBT348 extract. Our results demonstrated that SKC3 effectively inhibits the growth (MIC: 31.25 µg/ml) and biofilm formation (sub-MIC range: 1.95-<31.25 µg/ml) of *S. epidermidis* RP62A *in vitro*. Chemical characterization of SKC3 by heat and enzyme treatments, and Mass Spectrometry (HRMS) revealed its heat-stable and non-proteinaceous nature, and high molecular weight (1258.3 Da). Cytotoxicity profiling of SKC3 *in vitro* on mouse fibroblast (NIH/3T3) and macrophage (J774.1) cell lines, and *in vivo* on the greater wax moth larvae *Galleria melonella* revealed its non-toxic nature at the effective dose. Transcriptome analysis of SKC3 treated-*S. epidermidis* RP62A has further unmasked its negative effect on central metabolism such as carbon flux as well as, amino acid, lipid and energy metabolism. Taken together, these findings suggest a potential of SKC3 as a putative drug to prevent staphylococcal DRIs.

Keywords: Marine sponges, *Streptomyces*, Staphylococci, Device-related infections, Bioassay guided-fractionation, Transcriptome

1. Introduction

Surgical implants and medical devices have greatly assisted in improving the survival and recovery of patients from physical ailments (Vinh and Embil, 2005). However, they also are ideal niches for colonization and biofilm formation by microorganisms from patient's own skin, healthcare workers' skin or hospitalized settings (Percival et al., 2015). Biofilms are networks of microorganisms that are entrapped in a self-produced gluey matrix made up of polysaccharides, proteins, lipids and eDNA (Otto, 2009; Flemming and Wingender, 2010). Microbes in biofilms exhibit 10-1000-fold increased resistance to antibiotics and host immune systems; and a number of mechanisms are supposed to contribute to this phenomenon such as the presence of biofilm matrix itself, slow growth rate and persister cell formation, efflux pumps, plasmid exchange, target mutations and antibiotic-modifying enzymes etc. (Stewart and Costerton, 2001; Hall-Stoodley et al., 2004; Percival et al., 2011; Rajput et al., 2018). Current treatment of biofilm based DRIs involves complete removal of the infected implant or device by a surgical procedure followed by prolonged antibiotic treatments (Otto, 2012). Biofilm based infections thus, lead to increased patient morbidity and mortality, and increased health care costs (Shida et al., 2013; Kleinschmidt et al., 2015; Leary et al., 2017).

The majority of the DRIs reported till date are a consequence of biofilm formation by coagulase negative (*e.g. S. epidermidis*) and positive (*e.g. S. aureus*) staphylococci (Mack et al., 2007; Becker et al., 2014; Windolf et al., 2014). Predominantly, *S. epidermidis* an inhabitant of skin and mucosa is the leading cause of nosocomial and DRIs (Otto, 2009; Franca et al., 2012; Namvar et al., 2014; Sabate Bresco et al., 2017). The development of complications like catheter-related blood stream infections, prosthetic joint infections, early-onset neonatal sepsis etc. and the rapid emergence of drug-resistant staphylococcal strains in hospital and community settings has challenged the effectiveness of current therapeutic regimes (Barros et al., 2014; WHO, 2014; Sakimura et al., 2015; Widerstrom, 2016). Therefore, it is imperative to develop novel antibacterial and antibiofilm based therapeutics for management of the hard-to-treat staphylococcal infections (Bjarnsholt et al., 2013).

Marine bioprospecting has gained much attention in the recent years owing to its massive chemical and biological diversity (Mayer et al., 2010; Gerwick and Moore, 2012; Martins et al., 2014; Thompson et al., 2017). A variety of bioprospecting techniques (including cultivation-dependent to independent approaches) have been described so far towards harnessing the bioactive potential of the marine realm (Abdelmohsen et al., 2015; Kodzius

and Gojobori, 2015; Indraningrat et al., 2016). Particularly, marine sponges and their associated actinomycetes are abundant reserves of novel natural products with distinct biological activities of pharmaceutical importance (Thibane et al., 2010; Abdelmohsen et al., 2014a; Abdelmohsen et al., 2015; Abdelmohsen et al., 2017). A wide spectrum of anti-staphylococcal compounds and extracts possessing antibacterial and/or antibiofilm activities have been reported from marine sponges and microbes (Rahman et al., 2010; Stowe et al., 2011; Beau et al., 2012; Palomo et al., 2013; Gomes et al., 2014; Balasubramanian et al., 2017).

A preliminary anti-biofilm screening (against the model isolate *S. epidermidis* RP62A) with 50 different organic extracts obtained from solid and liquid batch fermentations of 25 different marine sponge-derived actinomycetes led to the identification of the bioactive extract from *Streptomyces* sp. SBT348. Marine sponge-derived *Streptomyces* sp. SBT348 is a Gram-positive bacterium that was previously shown to possess distinct metabolomic and rich chemistry profiles with strong biological activities (Cheng et al., 2015; Cheng et al., 2017). In this study, bioassay-guided fractionation was performed to unravel the active component(s) in the SBT348 extract. The most active compound SKC3 was evaluated further for growth and biofilm inhibition on various *S. epidermidis*, *S. aureus* and *P. aeruginosa* strains. Results obtained highlighted the specific anti-biofilm nature of SKC3 with high potency and non-toxic nature. Chemical analysis revealed the heat-stable, non-proteinaceous and high-molecular weight of SKC3 (1258.3 Da). Finally, data from transcriptome analysis revealed the regulation of expression of several genes related to carbon, amino-acid, proteins, lipids, nucleotide and energy metabolism suggesting the possible interference of SKC3 with global metabolism of staphylococci.

2. Materials and methods

2.1. Instrumentation

Flash chromatography was done on an Interchim Puri-Flash 430 instrument (ultra performance flash purification) connected to an Interchim flash ELSD (Montlucon, France). Semi-preparative HPLC of the active fraction was perfomed with Agilent 1100 series (Waldbronn, Germany) using Gemini-NX5u-C18-110A column (250x10 mm, Phenomenex, USA) and detection at 254 nm. The following gradient was applied solvent A: water and solvent B: acetonitrile. Separation method: solvent B 20% for 4 min, 40% for 11 min, 40%

to 50% in 5 min, 50% to 90% in 1 min and again to 20% in 4 min; maximum pressure of 400 bar and a flow rate of 4 ml/min.

Analytical HPLC to assess the purity of the compound was done with the same HPLC system but with Gemini-NX5u-C18-110A column (250x4.60 mm, Phenomenex, USA) Separation method: solvent B 5% at 0 min, 5% to 100% for 25 min, 100% for 1 min, 100% to 50% in 2 min and again to 5% in 2 min; maximum pressure of 400 bar and a flow rate of 1 ml/min.

Fourier transform-infra-red-spectroscopy (FT-IR) of SKC3 were conducted using Jasco FT/IR-6100 spectrometer with an ATR unit (Groß-Umstadt, Germany) at room temperature. MS measurements were performed using Electron Spray Ionization (ESI) in a micrOTOF-QIII mass spectrometer (Bruker Daltonics, Billerica, Massachusetts, USA) coupled to an Agilent 1100 HPLC system. ESI was operated in positive mode with a capillary voltage of 4.5 kV. Nitrogen at 200 ºC and a flow rate of 7 l/min was used as the desolvation gas. Mass spectral data was obtained over a range of 50-3500 m/z.

Scanning electron microscopy (SEM) was done with JEOLJSM-7500F (Japan) with field emission gun system.

2.2. Bacterial strains and culture conditions

Bacterial strains used in the work are mentioned in **Table 1**. *Streptomyces* sp. SBT348 was grown in ISP2 medium (4 g/l yeast extract, 10 g/l malt extract, 4 g/l glucose in artificial sea water) at 30 ºC. All other strains in the study were cultured in Tryptic Soy Broth (TSB; Becton Dickinson) (17.0 g/l pancreatic digest of casein, 3.0 g/l peptic digest of soybean meal, 5.0 g/l sodium chloride, 2.5 g/l dipotassium hydrogen phosphate, 2.5 g/l glucose) and incubated at 37 ºC.

Table 1: Strains used in this study.

Strain	Origin	Relevant characteristics	Reference and/or source
Streptomyces sp. SBT348	Marine sponge-derived actinomycetes strain[#]	Filamentous and sporulating	(Cheng et al., 2015)
S. epidermidis RP62A	Reference strain isolated from intra-vascular catheter associated sepsis	+++	ATCC collection

S. epidermidis O-47	Clinical isolate from septic arthritis	++	(Heilmann et al., 1996)
S. epidermidis 1457	Clinical isolate from a patient with infected central venous catheter	+++	(Mack et al., 1992)
S. epidermidis ATCC 12228	Non-infection associated strain	---	ATCC collection
S. carnosus TM300	Meat starter culture	---	(Rosenstein et al., 2009)
S. aureus Newman	MSSA isolate from osteomyelitis patient	+	(Lipinski et al., 1967)
S. aureus USA Lac*	CA-MRSA isolate from a wrist abscess	+	(McDougal et al., 2003)
S. aureus RF122	Bovine mastitis isolate	-	(Fitzgerald et al., 2001)
S. aureus Mu50	Human MRSA isolate from surgical wound infections, vancomycin-resistant	-	(Kuroda et al., 2001)
S. aureus Col	Human MRSA isolate	-	(Dyke et al., 1966)
P. aeruginosa PAO1	Clinical isolate from wound	+++	Dr. Vinay Pawar, Braunschweig, Germany
P. aeruginosa PA14	Clinical isolate from burn wound	+++	Dr. Vinay Pawar, Braunschweig, Germany

Isolated from Mediterranean sponge *Petrosia ficiformis* that was collected offshore Pollonia, Milos, Greece (N36.76612º; E24.51530º), May 2013 (GeneBank accession No. KP238417). +++ strong biofilm former, ++ moderate biofilm former; + weak biofilm former, - no detectable biofilms under conditions tested, --- biofilm negative phenotype. Biofilm formation was assessed in TSB medium employing the standard crystal violet biofilm formation assay.

2.3. Large scale fermentation and extract preparation

1000 ISP2 agar plates (prepared with artificial sea water) were inoculated with a week-old liquid culture of *Streptomyces* sp. SBT348 respectively and were incubated at 30 ⁰C for 10 d (batch fermentation). Agar with bacterial biomass was cut into small pieces and transferred into 1 l of ethyl acetate. The solutions were subjected to shaking at 175 rpm in a shaker overnight. Subsequently, the macerations were filtered, and the filtrates were evaporated in vacuo to obtain the dried SBT348 organic extract. Agar plates without the actinomycetes were extracted in a similar manner and this was the medium control for the bioactivity testing. Extracts were dissolved in DMSO (final concentration 3.75% v/v) and used for in vitro assays. Additionally, scanning electron microscopy (SEM) was done for the *Streptomyces* sp. SBT348 10 d culture on the ISP2 agar plate. The SEM protocol has been described below.

2.4. Bioassay guided-fractionation for isolation for active component(s)

1.2 g of the dried extract obtained was subjected to fractionation using a flash chromatography with a cyclohexane/ethyl acetate/methanol gradient eluent yielding 10 major fractions. After biological evaluation of each major fraction *in vitro*, against the biofilm formation of *S. epidermidis* RP62A, the active fraction Fr 7 was found. Fr 7 was sub-fractionated by semi-preparative HPLC and this yielded 7 sub-fractions (including the bioactive SKC1, SKC2, SKC3, SKC4 and SKC5). The bioactive fraction was further purified on HPLC to yield the bioactive compound SKC3. Pure compound SKC3 was dissolved in DMSO (final concentration 3.75% on cells) or stored dry in amber colored vials at -80 ⁰C to ensure stability.

2.5. Characterisation of the active compound SKC3

2.5.1. Stability of compound to heat and enzyme treatments

SKC3 at the respective effective concentrations was subjected to heat (100 ⁰C for 1 h; followed by cooling on ice) and enzymatic (proteinase K and trypsin; final concentration of 1 mg/ml, 37 ⁰C for 1 h) treatments. As controls, DMSO (final concentration of 3.75%) was subjected to similar heat and enzymatic treatments. For each of the treatments, the growth and biofilm inhibitory effects of treated and untreated SKC3 was assessed using the microtiter 96 well plate assay against *S. epidermidis* RP62A. Each data point is composed of three independent cultures performed in duplicates.

2.6. Biofilm assay and MIC determination

Biofilm assay was perfomed as previously described (Balasubramanian et al., 2017). Bacterial strains (OD_{600} ~ 0.05 in TSB) were incubated in the presence of SBT348 extract or SKC3 at different concentrations at 37 ºC (for *S. epidermidis* and *P. aeruginosa*) or 30 ºC (for *S. aureus*) for 24 h. Experimental controls included bacteria treated with ISP2 medium extract or DMSO and TSB without bacteria. MIC was determined against the various pathogenic bacterial strains in this microbroth dilution assay according to CLSI protocols. OD_{630} values were used to determine the MICs. MIC was determined as the concentration of the test substance where the lowest OD_{630} values were recorded with no visible bacterial growth. After OD_{630} measurement, the planktonic bacteria were discarded by rinsing with sterile 1xPBS (sterile) and biofilm cells were heat fixed at 65 ºC for 1 h. Plates were then stained with 0.3% crystal violet for 5 min, washed thrice with sterile double-distilled water and air-dried briefly. Finally, OD_{492} measurements determined the extent of biofilm inhibition in test wells in comparison with control. *S. epidermidis* (ATCC12228) and *S. carnosus* TM300 were the biofilm negative strains used in the experiment.

For studying the effect on existing or pre-formed biofilms, biofilms were established shortly before the experiment with the above protocol. Formed biofilms were then treated with fresh TSB (control) or the test substance at their respective final concentrations and incubated further at 37 ºC or 30 ºC for 24 h. The extent of biofilm eradication was assessed with the crystal violet assay. $NaIO_4$ that digests the biofilm matrix (polysaccharides) was used as the positive control in the experiment.

2.7. Growth curve studies

The antagonistic effect of SBT348 extract and SKC3 on the growth of *S. epidermidis* RP62A was determined by growth curve measurements (Nithya et al., 2010). Briefly, SBT348 extract or SKC3 (MIC and $MBIC_{90}$) were added to tubes containing bacteria (initial OD_{600} of 0.1). Tubes were incubated at 37 ºC at 200 rpm. Bacterial growth was monitored for every 2 h up to 24 h by optical density and CFU measurements (every 4 h). TSB medium devoid of the bacteria was used as the negative control while medium extract or DMSO treated bacteria served as the appropriate controls in the experiment. Three independent cultures were used in this experiment to ensure reproducibility of results.

2.8. Antibiofilm effect on different surfaces

The antibiofilm effect of SBT348 extract and the compound SKC3 was studied at their respective BICs on different surfaces; glass cover slips (diameter of 12 mm), medically relevant titan metal plates (diameter of 1.5 cm; University clinic for dental, oral and jaw diseases, Würzburg, Germany), and silicone tubes (length 1 cm and 0.2 cm diameter; Biotronik, Berlin, Germany). Briefly, 1 ml of *S. epidermidis* RP62A (OD_{600} of 0.05), was transferred to 24 well plates (Greiner bio-one, GmbH, Germany) containing the surfaces of interest with the test substances. Control wells containing the medium extract and DMSO were maintained in parallel. Sterile controls containing the surfaces with TSB alone were included to ensure absence of contamination. All the plates were incubated at 37 ºC for 24 h under static conditions. Samples were then subjected to washing with sterile PBS (2X) and subjected to SEM studies.

For SEM, samples were fixed overnight with gluteraldehyde (6.25%) and washed with Sörenson buffer (100 mM KH_2PO4 and 100 mM Na_2HPO4). After dehydration with a series of steps with ethanol, samples were finally coated with gold by low vacuum sputter coating, and scanned in the electron microscopy unit, University of Würzburg.

2.9. Cytotoxicity profiling

2.9.1. *In vitro* on cell lines

Cytotoxicity of the purified compound SKC3 was assessed on macrophage (J774.1) and mouse fibroblast (NIH/3T3) cell lines using alamar blue assay (Huber and Koella, 1993). RPMI 1640 (1X) + Glutamax™-1 and DMEM (1X) + Glutamax™-1 (Life Technologies™, USA), supplemented with 10% FCS without antibiotics, were used for culturing J774.1 and NIH/3T3 cell lines, respectively. 10^5 cells/ml were seeded on 96 well plates containing SBT348 extract (62.5-500 µg/ml) or SKC3 (3.95-500 µg/ml) and the plates were incubated at 37 ºC with 5% CO_2 for 24 h. 20 µl of alamar blue (Thermofischer scientific, USA) was added to each well and the plates were incubated for a further period of 24 h at 37 ºC with 5% CO_2. Finally, the OD_{550} values of the plates were measured and normalized to OD_{630} values. The extent of cytotoxicity was measured by comparison of extract/SKC3 treated sets with the control. MeOH (toxic to the cells) was used as the positive control in the experiment. DMSO at a final concentration of 1% was used as the control.

2.9.2. *In vivo* on *G. melonella* larvae

G. melonella larvae (at their final stage) were purchased from Mouse Live Bait (Balk, The Netherlands). *In vivo* toxicity of SBT348 extract and SKC3 was assessed in *G. melonella* using the method described previously (Gibreel and Upton, 2013; Skaf et al., 2017). Healthy larvae (clear in color without the presence of any spots or pigmentation) were used in the experiment. SBT348 extract and SKC3 at their respective test concentrations were prepared in endotoxin-free PBS (Merck, Germany) (vehicle control) and were injected in the last left pro-leg of the larvae with sterile insulin pens (BD Micro-Fine™ + Demi). A total of 10 larvae were included per group. Negative controls included the group that underwent no injection and injection with vehicle control only, while positive control included the group injected with pure MeOH (Roth, Germany). Larval groups were incubated at 37 °C in petri dishes (devoid of light). Larval survival rates were recorded every 24 h up to 120 h. Larvae that were pigmented and did not respond to touch were scored dead and vice versa. Experiments were repeated three independent times to ensure the reproducibility of results.

2.10. RNA extraction, DNase treatment and RNA quality determination

S. epidermidis RP62A (OD_{600} of 1.0) was treated with SKC3 (62.5 µg/ml) and was statically incubated in a 6-well plate at 37 °C for 20 min and 3 h. Treatment with DMSO (final concentration of 3.75% v/v on the cells) served as the appropriate control in the experiment. RNAprotect bacteria reagent (Qiagen, Germany) was added at the respective time points for protection and stabilization of RNA. Subsequently, RNA isolation was done according to the customized protocol described by Franca et al., 2012. Three independent biological replicates each from a pool of three independent wells were performed in order to reduce the variability. Isolated RNA samples were subjected to treatment with Turbo DNA-free™ kit (Invitrogen, USA) following manufacturer's instructions and acid phenol: chloroform: isoamylalcohol (125:24:1) (Ambion, USA). Finally, pure RNA samples obtained, were precipitated with ethanol and checked for DNA contamination by PCR for the *icaA* gene (**Figure S1**).

Concentration and purity of the total RNA was evaluated spectrophotometrically using NanoDrop 2000 PEQLAB GmbH (Erlangen, Germany). The ratios A_{260}/A_{280} (mean values of all the samples was 1.97) and A_{260}/A_{280} (mean values of all the samples was 2.59) were used as indicators of protein and phenol/polysachharide contamination. Total RNA quality was also assessed with an Agilent 2100 Bioanalyzer (Agilent, CA). RNA integrity numbers of all samples were ~8.0 or more.

2.11. Ribosomal RNA depletion, library preparation and sequencing

Extracted RNA was depleted of ribosomal RNA using the Ribo-Zero rRNA Removal Kit for bacteria (Illumina) according to the manual. Depleted RNA was fragmented for 3 min at 94 °C using the NEBNext Magnesium RNA Fragmentation Module. The RNA ends were repaired with two consecutive T4 PNK incubations (-/+ ATP) and an RppH treatment. Library preparation was performed according to the NEBNext Multiplex Small RNA Library Preparation Guide for Illumina. All adapters and primers were diluted 1:4 and 15 and 16 cycles of PCR were used, respectively. No size selection was performed at the end of the protocol. 12 libraries were pooled and sequenced on a NextSeq 500 with a read length of 75 nt.

2.12. Analysis of deep-sequencing data

The quality of raw reads (Phred scores, amount of duplicates and adapter) were assessed using FastQC version-0.11.31 (Andrews, 2010). In order to assure a high sequence quality, the Illumina reads in FASTQ format were trimmed with a cut-off phred score of 20 by cutadapt version-1.15 (Martin, May 2011) that also was used to remove the sequencing adapter sequences. The following steps were performed using the subcommand "create", "align" and "coverage" of the tool READemption version 0.4.3 (Forstner et al., 2014) with default parameters. Reads with a length below 15nt where removed and the remaining reads were mapped to the reference genome sequences (NCBI accession no. NC_002976.3 (31 January 2014)) using segemehl (Hoffmann et al., 2009). Coverage plots in wiggle format representing the number of aligned reads per nucleotide were generated based on the aligned reads and visualized in the Integrated Genome Browser (Freese et al., 2016). Each graph was normalized to the total number of reads that could be aligned from the respective library. To restore the original data range and prevent rounding of small error to zero by genome browsers, each graph was then multiplied by the minimum number of mapped reads calculated over all libraries. The differentially expressed genes were identified using DESeq2 version 1.16.1 (Love et al., 2014). In all cases, only genes with maximum Benjamini-Hochberg corrected p-value (p_{adj}) of 0.05, were classified as significantly differentially expressed. The data were represented as MA plots using R.

Differentially expressed genes (cutoff of p adjusted ≤0.05 and $\log_2 FC = \pm 2$) was used to perform Gene enrichment using the R package clusterProfiler version 3.4.4 (Yu G. et al., 2012). Using enrichKEGG function enrichment in KEGG pathways was analyzed. Only the

pathways with Benjamini–Hochberg FDR threshold ≤0.05 defined as significantly enrichment terms.

The RNA-Seq data presented in this work has been deposited at the NCBI Gene Expression Omnibus (Edgar et al., 2002) and can be accessed through GEO series accession number GSE109983 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109983). Samples treated with SKC3 has been referred to as C3 in the submitted files.

2.13. Statistical analysis

All the experiments were performed three independent times with technical replicates. Data are expressed as mean±SEM. For all the comparisons, Student's t-test was used. For comparing different Kaplan-Meier survival curves from *in vivo G. melonella* experiments, Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test was used. p value<0.05 was considered as statistically significant. GraphPad Prism® version 6.01 was used for statistical analysis of experimental data.

3. Results

3.1. Antibiofilm potential of *Streptomyces* sp. SBT348

The antibiofilm potential of *Streptomyces* sp. SBT348 was identified with a preliminary anti-biofilm screening of different actinomycetes organic extracts against the strong biofilm forming *S. epidermidis* RP62A. *Streptomyces* sp. SBT348 was characterized by its wrinkled, rough, dry and light-yellow mycelia on ISP2 agar medium (t=10 d). SEM analysis revealed the filamentous nature of *Streptomyces* sp. SBT348. Branched networks with the presence of extracellular polymeric substance-like materials were identified in the scanning electron micrograph (**Figure 1A**). The ethyl acetate SBT348 extract significantly reduced the biofilm formation (at 24 h) in *S. epidermidis* RP62A (p<0.0001). Extract at a concentration of 62.5 µg/ml reduced the biofilm formation by ~90% and this was designated as the BIC_{90} (90% biofilm inhibition concentration). Notably, there were no significant differences in the effect beyond this concentration (**Figure 1B**). SBT348 extract at BIC_{90} or $2xBIC_{90}$ did not further alter the growth pattern of *S. epidermidis* RP62A (compared to extract from ISP2 medium control) (**Figure 1C**). SBT348 extract had no antagonistic effects on pre-formed *S. epidermidis* RP62A biofilms at any of the tested concentrations (15.62-500 µg/ml; data not shown). Cytotoxicity profiling of the extract *in vitro* on NIH/3T3 and J774.1 cell lines (**Table 3**), and *in vivo* on *G. melonella* larvae demonstrated the non-toxic nature of the extract

(**Figure 1D**). Further, no changes in the activity of the extract was observed after heat and enzymatic (proteinase K and trypsin) treatments (data not shown). This highlighted the presence of heat-stable and non-proteinaceous active proportion(s) in the extract.
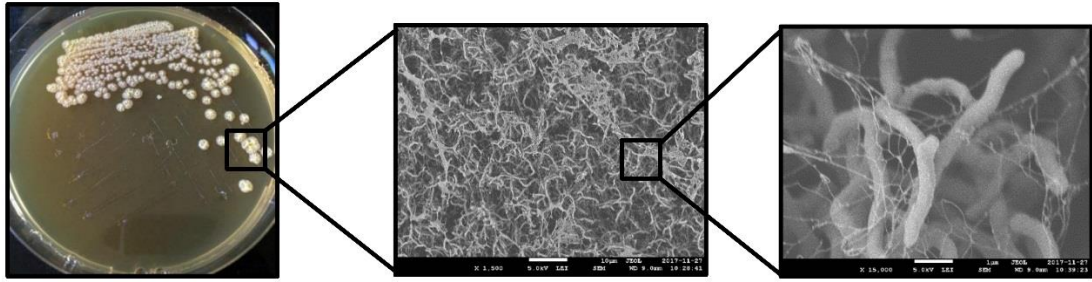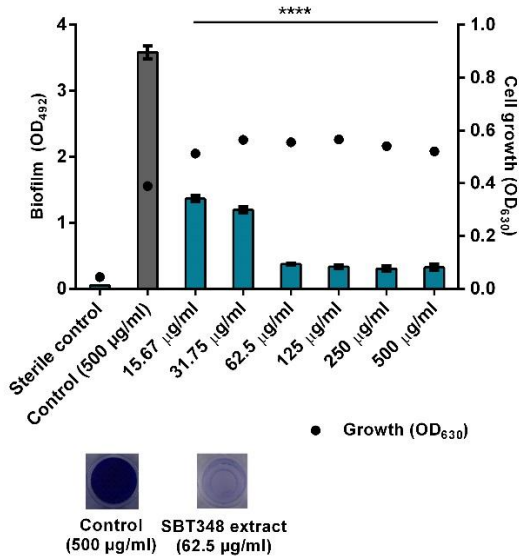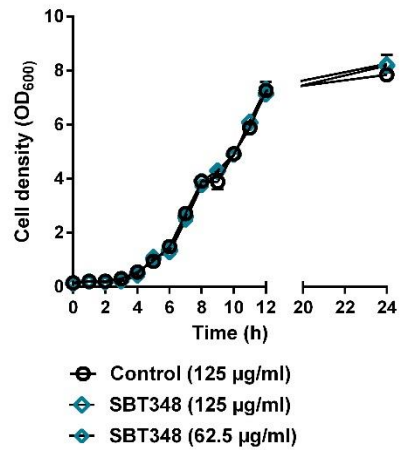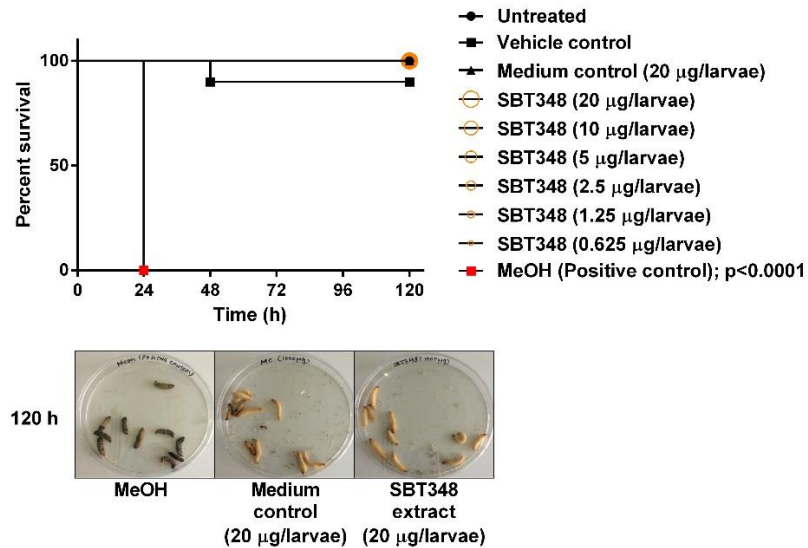
Figure 1. (A) Colony morphology and scanning electron micrograph (at X1500 and X15000 magnification) of *Streptomyces* sp. SBT348 after 10 d batch fermentation on ISP2 agar plate at 30 ºC. SEM indicates the presence of extracellular polymeric substance-like materials in *Streptomyces* sp. SBT348. Scale bar: 10 µm and 1 µm. **(B)** Dose-dependent inhibition of biofilm formation of *S. epidermidis* RP62A by ethyl acetate extract of *Streptomyces* sp. SBT348. **(C)** Influence of SBT348 extract on the growth of *S. epidermidis* RP62A

at BIC_{90} (62.5 µg/ml) and $2 \times BIC_{90}$ (125 µg/ml). (**D**) Kaplan-Meier survival curve of *G. melonella* larvae treated with $16 \times BIC_{90}$-$0.5 \times BIC_{90}$ of SBT348 extract (20 – 0.625 µg/larvae). MeOH (positive control) killed 100% of larval population at 24 h. Treatment with medium control or SBT348 extract (at the tested concentrations) did not lead to death of the larvae (100% survival).

Control in the experiments (**B-D**) consisted of ethyl acetate extract (at the respective highest concentration) from sterile ISP2 medium which was used as growth medium for *Streptomyces* sp. SBT348. Graphs represent the mean±SEM from three independent repetitions of experiment done with multiple technical replicates. ns, not significant; **** $p < 0.0001$.

3.2. Bioassay-guided fractionation and characterization of the active compound

The bioassay-guided fractionation approach followed to identify the active component SKC3 (MIC of 31.25 µg/ml and BIC_{90} of 3.95 µg/ml) in the extract that is shown in **Figure 2A**. SKC3 was further investigated in detail in the study. The pure compound SKC3 (**Figure S2**) was obtained as yellow crystalline solid and was soluble in polar solvents like water, DMSO and MeOH. Results obtained from LC and MS analysis revealed that SKC3 had a purity of 100% and a mass of approximately 1258.3 Da (**Figure 2B**). This mass was also found in the crude LC-MS chromatogram of SBT348 extract (data not shown). FT-IR spectra of SKC3 revealed some significant bands at 2936, 3326 and 1660 cm^{-1}, representing the presence of -C-H-stretches, -OH and C=O groups (**Figure 2C**). Mass search with 1258. 3 Da in databases like MarinLit® and Chemspider® did not yield any possible hits. Further, heat and enzymatic treatments did not significantly alter the biological activity of SKC3 (**Figure S3**). This was in line with the results obtained from the stability studies of the extract. The absence of relevant hits with the existing mass and spectral data indicated that SKC3 is likely to be a new compound. The structure elucidation of SKC3 is currently under investigation.

A

Petrosia ficiformis → *Streptomyces* sp. SBT348 ⊣ *S. epidermidis* RP62A

SBT348 extract
Large scale solid fermentation
(n=1000 plates)
Extraction solvent: EtOAc

← Flash chromatography

Fr 0 | Fr 1 | Fr 2 | Fr 3 | Fr 4 | Fr 5 | Fr 6 | Fr 7 | Fr 8 | Fr 9

Semi-preparative HPLC →

SF 1 | SF 2 | SKC 1 | SKC 2 | SKC 3 | SKC 4 | SKC 5

Inactive | Moderately active (BIC$_{50}$>125µg/ml) | Highly active (BIC$_{50}$<31.25µg/ml)

B

mV

10.001   Detector A 254nm

Intens. x10⁴
689.15687
922.50935
1096.55895
1281.32074
2017_3679_OEL_C3.d: +MS, 2.5-2.5min #147-148

C

Figure 2. (A) Bioassay-guided fractionation scheme employed for isolation of active compound SKC3 in the SBT348 extract. Crystal violet biofilm assay (on 96-well plate against S. epidermidis RP62A) was done in each step for identification of active anti-biofilm fraction (Fr), sub-fraction (SF), and pure compound (SKC3). **(B)** LC-MS chromatogram and ESI-MS spectra of SKC3 confirming the presence of single peak and mass of SKC3 (1258.3309 Da). ## Sodium ion adduct. **(C)** FT-IR spectra of SKC3 representing the presence of strong absorption troughs at 1660 cm^{-1} (*), 2936 cm^{-1}, 3326 cm^{-1} (**) indicating the presence of -OH, -CH stretch and C=O groups. # background signal from the instrument.

3.3. Antagonistic activities of SKC3 against staphylococci

SKC3 displayed an MIC of 31.25 µg/ml on *S. epidermidis* RP62A and the sub-MIC concentrations (1.95-<31.25 µg/ml) effectively inhibited the biofilm formation in the crystal violet biofilm assay (**Figure 3A**). BIC$_{90}$ value of SKC3 was 3.95 µg/ml. Interference of SKC3 (MIC) with the growth of *S. epidermidis* RP62A was further confirmed with the growth curve analysis (**Figure 3B**). Thus, presence of SKC3 at MIC, effectively inhibited bacterial growth (approximately 100-fold reduction in CFUs/ml; data not shown) while SKC3 at BIC$_{90}$ had no significant influence. Further, SKC3 (at the highest tested concentration: 500 µg/ml) had no clearing effect on existing biofilms of *S. epidermidis* RP62A (**Figure 3C**). Complete biofilm dispersal by NaIO$_4$ (40 mM) was used a positive control in this experiment. SKC3 was also effective in inhibiting the growth and biofilm formation of other strains used in the study (**Table 2**). Noteworthy, SKC3 was more effective against MSSA, MRSA and VRSA strains

used in the study, but was ineffective against the tested Gram negative *P. aeruginosa* strains.

Table 2: **Effect of SKC3 on other staphylococcal strains used in the study**

Strain	MIC	BIC$_{>75}$
S. epidermidis RP62A	>31.25 µg/ml	3.95 µg/ml
S. epidermidis O-47	>31.25 µg/ml	7.81 µg/ml
S. epidermidis 1457	>31.25 µg/ml	15.62 µg/ml
S. aureus Newman	>31.25 µg/ml	7.81 µg/ml
S. aureus USA300 Lac*	>15.62 µg/ml	3.95 µg/ml
S. aureus RF122	>31.25 µg/ml	ND
S. aureus Col	>15.62 µg/ml	ND
S. aureus Mu50	>15.62 µg/ml	ND
P. aeruginosa PAO1	-	-
P. aeruginosa PA14	-	-

MIC, minimum inhibitory concentration; BIC$_{>75}$ >75% biofilm inhibitory concentration; -, inactive; ND, not determined.
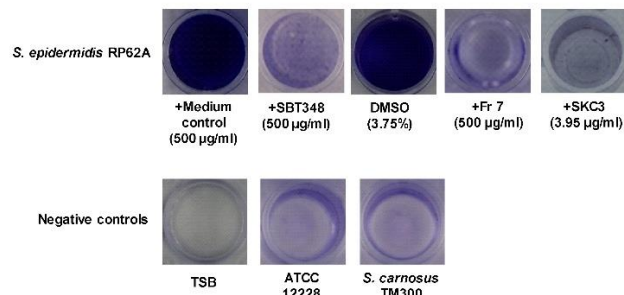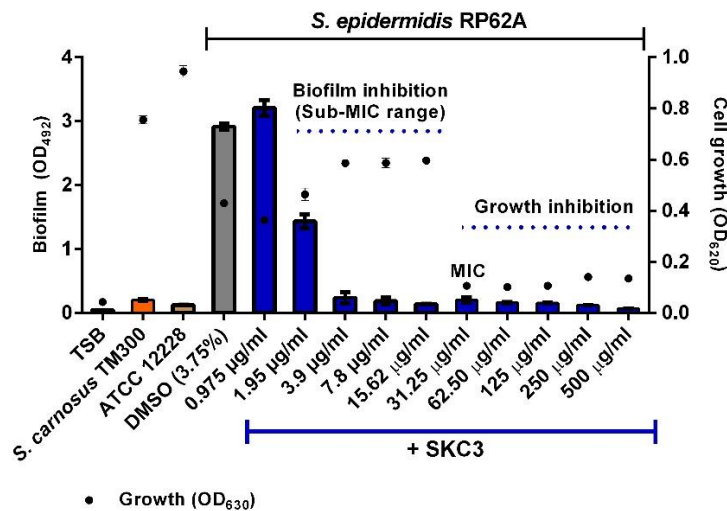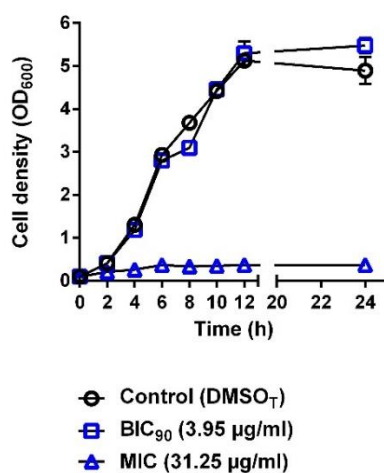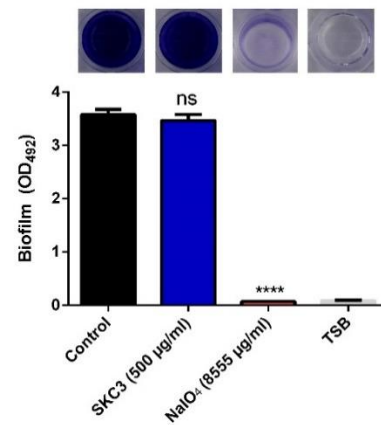
Figure 3. (A) Inhibition of growth (MIC: 31.25 µg/ml) and biofilm formation (sub-MIC range: <31.25 µg/ml) of *S. epidermidis* RP62A. Controls consisted of biofilm negative strains *S. epidermidis* ATCC 12228, *S. carnosus* TM300, DMSO treated *S. epidermidis* RP62A and sterile TSB without bacteria. **(B)** Effect of SKC3 on growth (OD_{600}) at MIC (31.25 µg/ml) and BIC_{90} (3.95 µg/ml) respectively. DMSO treated *S. epidermidis* RP62A was the appropriate control in the growth curve analysis. **(C)** Influence of SKC3 on pre-formed (existing) biofilms of *S. epidermidis* RP62A. $NaIO_4$ that digests the polysaccharide biofilms was used as the positive control. All the experiments were repeated at least three times in multiple technical replicates. ns, not significant; ****p<0.0001.

3.4. SEM analysis

Investigation of the antibiofilm efficacy of SKC3 at sub-MICs were further evaluated with SEM of *S. epidermidis* RP62A biofilms grown on glass, titan metal and silicone tube surfaces. From the scanning electron micrographs, clear differences in appearance were observed in the three sterile surfaces under study. In the control sets of the surfaces (treated with DMSO; 3.75%), three-dimensional dense biofilm structures were observed. Treatment with SKC3 (BIC_{90} and $2xBIC_{90}$) significantly reduced the biofilm formation on these surfaces and this further confirmed the results obtained from crystal violet biofilm assay (**Figure 4**). Particularly, the three-dimensional networks were absent, and the surfaces were clearly seen (between sporadic microcolonies or single cells) in the SKC3-treated sets. A closer look on the SEM images at higher magnification revealed no alterations in the cell morphology of staphylococci. These findings further point towards the antibiofilm potential of the isolated compound SKC3.
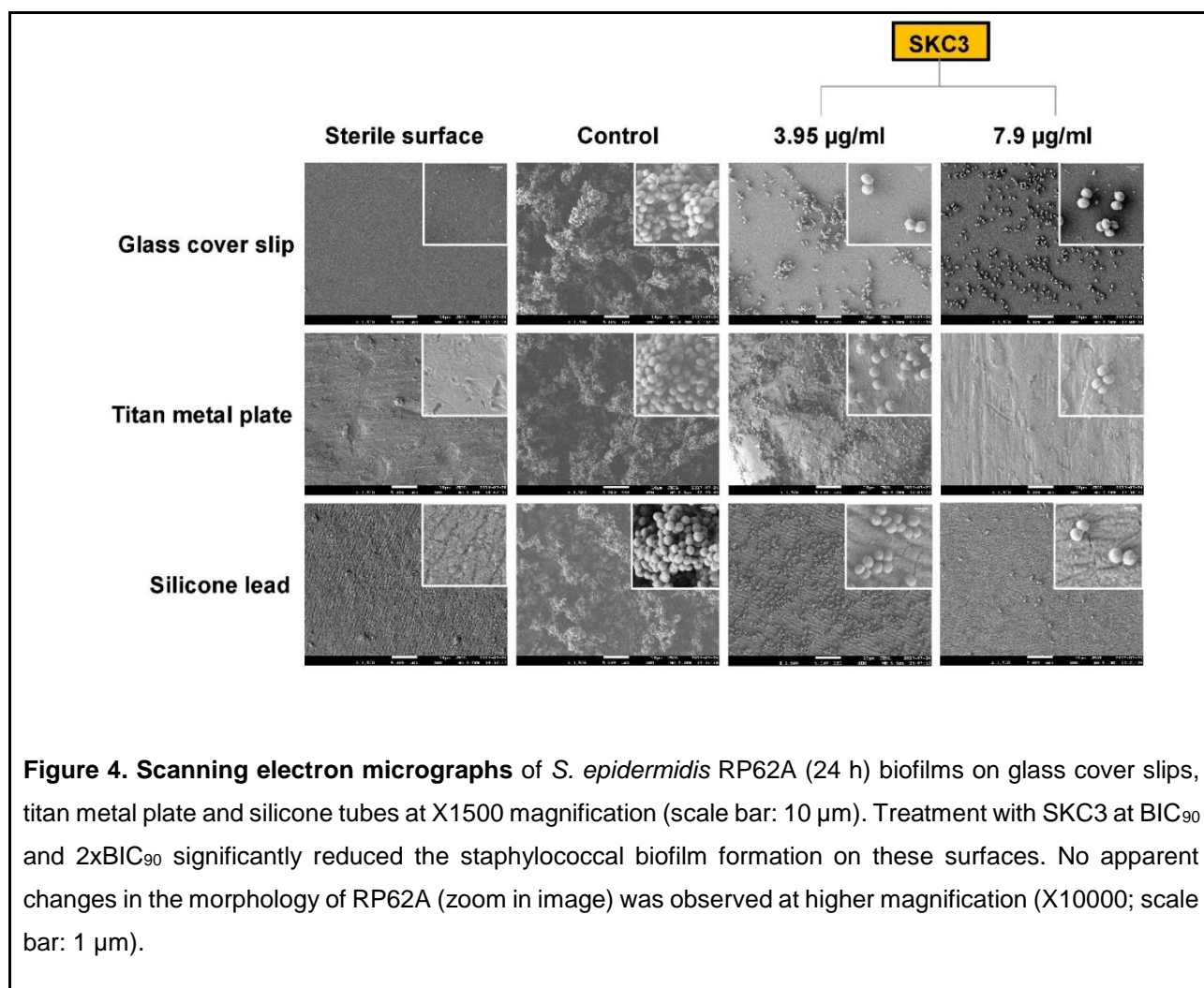
Figure 4. Scanning electron micrographs of *S. epidermidis* RP62A (24 h) biofilms on glass cover slips, titan metal plate and silicone tubes at X1500 magnification (scale bar: 10 µm). Treatment with SKC3 at BIC_{90} and $2xBIC_{90}$ significantly reduced the staphylococcal biofilm formation on these surfaces. No apparent changes in the morphology of RP62A (zoom in image) was observed at higher magnification (X10000; scale bar: 1 µm).

3.5. *In vitro* and *in vivo* toxicity of SKC3

In vitro toxicity assessment of SKC3 was done on mouse macrophage (J774.1) and fibroblast cell lines (NIH/3T3) using the alamar blue assay. Results from the cytotoxicity analysis demonstrated the non-toxic nature of SKC3 at effective concentrations (**Table 3**). Toxicity of SKC3 was additionally assessed *in vivo* in the greater wax moth larvae, *G. melonella*. In recent years, *G. melonella* larvae have emerged as an interesting model system for evaluating the toxicity and efficacy of novel compounds and for studying various microbial infections (Gibreel and Upton, 2013; Aparecida Procopio Gomes et al., 2016; Skaf et al., 2017). The ease of handling, low maintenance costs, absence of ethical concerns, survival at human physiological temperatures are some of the advantages of using *G. melonella* larvae for pre-screening of toxicity (Tsai et al., 2016). Survival rates of larvae treated with SKC3 (BIC_{90}-200XBIC_{90}) are shown in **Figure 5**. None of the tested concentrations lead to death of the larvae, whereas, the positive control MeOH lead to 90% reduction in the larval survival rates. Thus, SKC3 was completely non-toxic to the larvae at the tested concentration.

Table 3: *In vitro* cytotoxicity of SKC3 on cell lines

Cell line	% reduction in cell viability			
	500 µg/ml	250 µg/ml	125 µg/ml	3.9-125 µg/ml
NIH/3T3	41.07±1.37[****]	NC	NC	NC
J774.1	62.91±.83[****]	63.90±1.84[****]	32.78±7.00[***]	NC

Each data point is comprised of three independent trials done in quadruplicate. Mean±SEM are reported. Differences in the mean were compared to the control and considered statistically significant when p (***p<0.001, ****p<0.0001) calculated by Student's *t*-test. SBT348 extract (62.5-500 µg/ml) exhibited no significant toxicity on both the cell lines tested. Positive control, MeOH reduced the cell viability of NIH/3T3 by 66.73±0.59[****] and J774.1 by 72.10±2.16[****]. NC-no cytotoxicity.
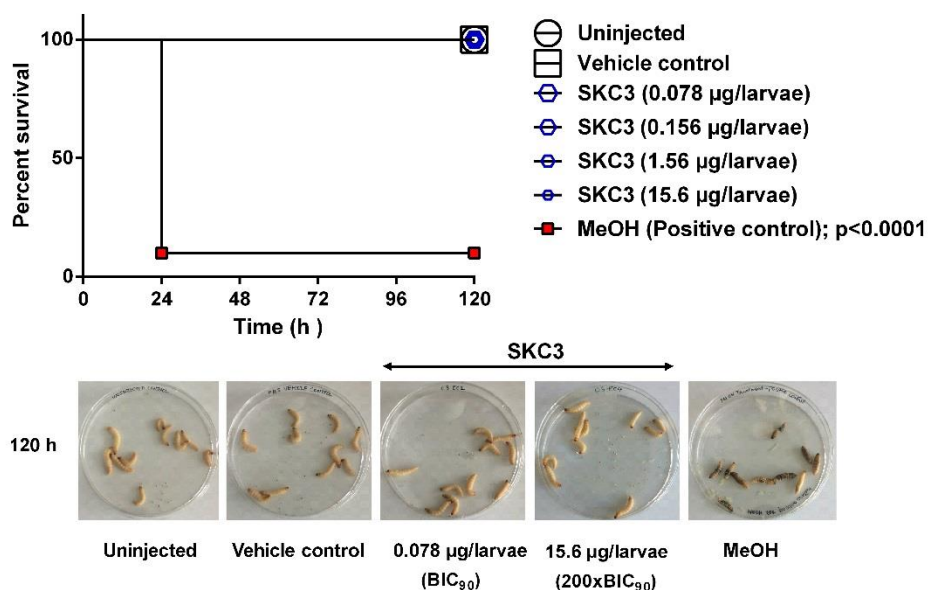
Figure 5. *In vivo* **toxicity evaluation of SKC3 on** *G. melonella* **larvae.** No death was observed in the larval groups treated with vehicle control (1xPBS; endotoxin-free) and SKC3 (at all the tested concentrations; BIC_{90}-200XBIC_{90}). MeOH treatment lead to 90% death of the larvae.

3.6. Transcriptome analyses of SKC3-treated *S. epidermidis* RP62A

Total RNA sequencing was done for *S. epidermidis* RP62A treated with SKC3 (62.5 µg/ml) at 20 min and 3 h points. Global transcriptome analysis with the obtained RNA sequencing results revealed the existence of several differentially expressed genes upon SKC3 treatment. The differentially expressed genes were identified by setting the threshold of Log_2foldchange±2.0 with an adjusted p value of <0.05 for statistical significance. From the MA plots (**Figure 6: A, B**), it is evident that higher number of genes were differentially expressed (upon SKC3 treatment) at 3 h than 20 min. This was additionally confirmed in the PCA plot and a well-distributed grouping of the different biological replicates were observed (**Figure S4**). According to the set threshold, a total of 31 genes representing 1.1% of the transcriptome were significantly altered in response to SKC3 at 20 min and a total of 509 genes representing 19.5% of the transcriptome were significantly altered in response to SKC3 at 3 h. Among these genes, 29 genes were upregulated, and 2 genes were downregulated at 20 min (**Table S1**), whereas, 265 genes were upregulated, and 244 genes were downregulated at 3 h (**Table S2**). After data filtering and searches in PubMed and UniProtKB, the differentially expressed genes at the two-time points were manually sorted in several categories based on their biological functions of the products they encode.

Majority of the differentially regulated genes in the entire data set consisted of hypothetical proteins. Transcriptome analysis revealed that at 20 min, several of the differentially expressed genes were attributed to signal transduction mechanism, transporters, transcription and antibiotic response-related functions (**Figure 6C**). This suggests that *S. epidermidis* RP62A responds to SKC3 by signal transduction mechanisms and by expressing several transcription, transporters and antibiotic-stress related genes. Transcriptome analysis further revealed that at 3h, several of the metabolic processes (pertaining to carbon, amino acid, protein, lipid, nucleotide and energy metabolism) and transport processes were strongly affected (**Figure 6D**). Functional enrichment analysis also yielded similar antagonistic effects of SKC3 on metabolism (**Figure S5**). A list of all differentially regulated metabolism-related genes and virulence genes upon SKC3 treatment at 3 h are further detailed in **Table 4**. Overall, the results from transcriptome analysis suggest that SKC3 possibly works by interference with the overall metabolism of staphylococci.

Figure 6. MA plots representing the significant gene expression changes (red dots; padj value<0.05) in SKC3 (62.5 µg/ml) treated-*S. epidermidis* RP62A at **(A)** 20 min and **(B)** 3 h time points in comparison with
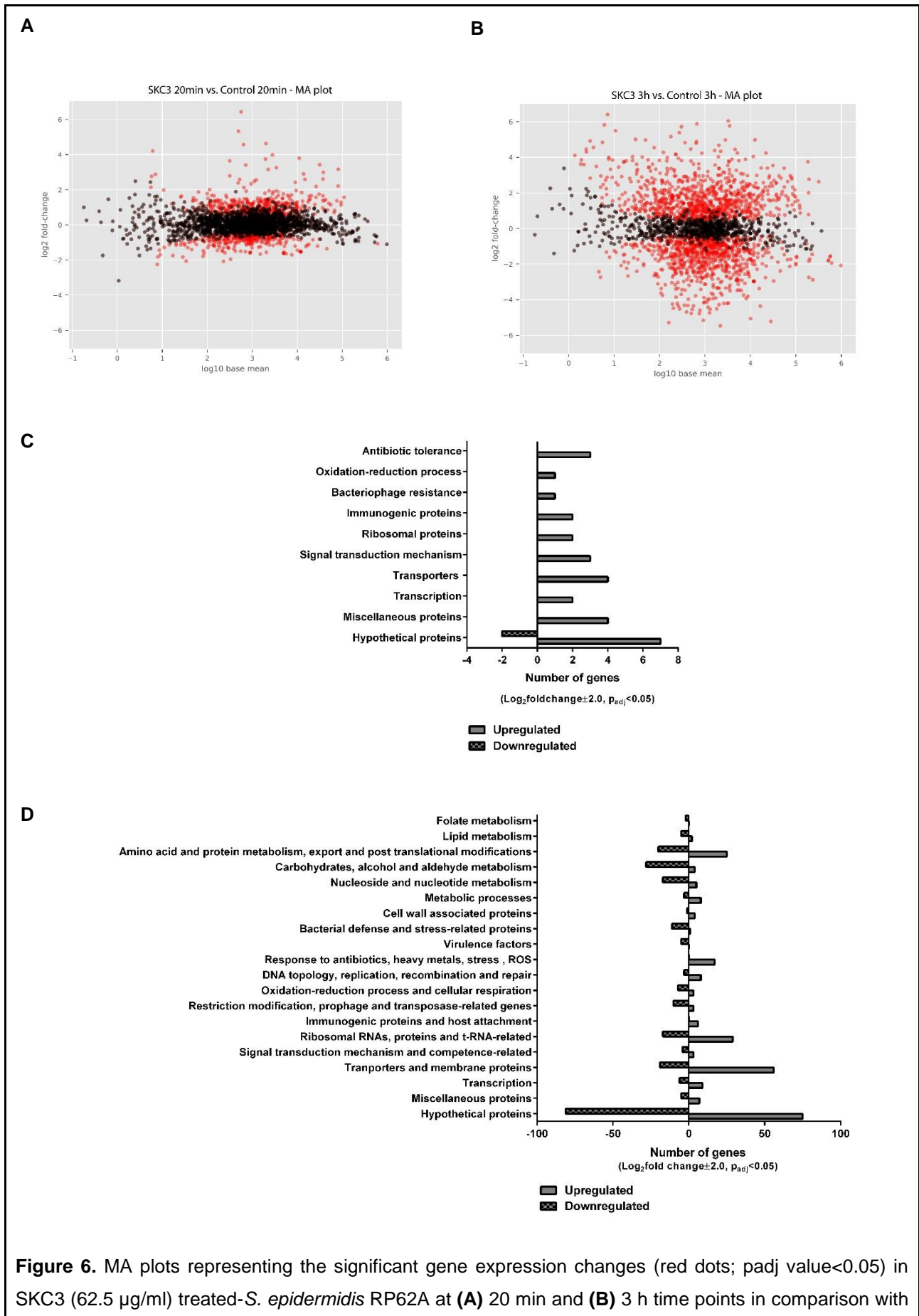
control (DMSO treated-*S. epidermidis* RP62A). Black dots represent the insignificant gene expression changes (padj value>0.05). Histogram of differentially expressed genes in the presence of SKC3 (62.5 µg/ml) at 20 min **(C)** and 3 h **(D)**. Results are summarized based on biological process. X-axis indicates the number of differentially expressed genes in a category. Positive and negative axes represent the numbers of up- and downregulated genes respectively. Absolute value of Log$_2$fold change±2 and padj value<0.05 was used as the threshold to screen the differentially expressed genes. UniProtKB was used to search for the biological function of differentially expressed genes.

Table 4: List of metabolism-related genes affected in response to SKC3 at 3 h. Only genes with a Log$_2$foldchange±2.0 and a padj value<0.05 were included.

Metabolic process and genes	Function	Log$_2$fold change	p$_{adj}$ value
Carbon metabolism			
glmU	UDP-N-acetylglucosamine pyrophosphorylase	-2.1757	3.88E-44
SERP0257	alcohol dehydrogenase zinc-containing	-2.9328	7.33E-24
fruK	1-phosphofructokinase	-2.0069	7.77E-11
hprK	HPr kinase/phosphatase	-2.3083	3.43E-37
pgk	phosphoglycerate kinase	-2.0399	3.02E-17
tpiA	triosephosphate isomerase	-2.2812	2.56E-15
pgi	glucose-6-phosphate isomerase	-3.4822	1.38E-49
pdhA	pyruvate dehydrogenase complex E1 component alpha subunit	-3.1290	1.06E-26
pdhB	pyruvate dehydrogenase complex E1 component beta subunit	-3.3469	7.02E-40
pdhC	pyruvate dehydrogenase complex E2 component dihydrolipoamide acetyltransferase	-3.5036	1.37E-27
pdhD	pyruvate dehydrogenase complex E3 component lipoamide dehydrogenase	-2.4225	1.55E-16
pyc	pyruvate	-2.7047	3.23E-51
trxA	thioredoxin	-2.3877	4.66E-11

tkt	transketolase	-2.3499	1.62E-104
SERP0974	acylphosphatase	-3.7088	5.81E-45
malA	alpha-glucosidase	-2.4798	3.93E-27
gnd	6-phosphogluconate dehydrogenase decarboxylating	-2.3314	5.38E-91
pfkA	6-phosphofructokinase	-2.5866	9.06E-34
SERP1290	PTS system IIBC components	2.6442	1.92E-32
tal	transaldolase	-2.1920	4.46E-19
sceD	sceD protein	4.8221	7.26E-69
lacR	lactose phosphotransferase system repressor	2.0861	7.09E-16
sdhA	L-serine dehydratase iron-sulfur-dependent alpha subunit	-2.1059	3.48E-44
SERP2112	alcohol dehydrogenase zinc-containing	-2.1470	2.46E-14
SERP2114	PTS system IIABC components	-3.3405	5.78E-34
budA	alpha-acetolactate decarboxylase	-2.4764	2.38E-37
budB	acetolactate synthase catabolic	-3.4875	2.65E-55
ldh	L-lactate dehydrogenase	-3.3363	1.49E-48
SERP2345	dihydroxyacetone kinase family protein	-2.0835	3.63E-14
gldA	glycerol dehydrogenase	-2.4748	1.96E-33
SERP2354	tributyrin esterase EstA putative	2.9073	1.37E-14
pflB	formate acetyltransferase	-3.6275	2.36E-49
Amino acid and protein metabolism			
SERP0033	cyclase putative	2.1197	1.70E-08
cysK	cysteine synthase	-2.5176	1.47E-38
cysE	serine acetyltransferase	-3.4603	1.64E-52
cysS	cysteinyl-tRNA synthetase	-2.7266	4.95E-54
ilvE	branched-chain amino acid aminotransferase	-2.5610	6.43E-11
SERP0349	deoxyribodipyrimidine photolyase putative	2.1987	2.22E-18

prfB	peptide chain release factor 2	-2.5844	5.22E-23
lgt	prolipoprotein diacylglyceryl transferase	-2.1091	7.12E-33
gcvH	glycine cleavage system H protein	-2.4191	2.29E-23
def	peptide deformylase	-2.1348	2.13E-24
def-2	polypeptide deformylase	2.1031	4.64E-59
fmt	methionyl-tRNA formyltransferase	2.1719	7.90E-22
glnR	glutamine synthetase repressor	-2.7030	7.01E-27
trpD	anthranilate phosphoribosyltransferase	4.0312	1.23E-14
trpC	indole-3-glycerol phosphate synthase	2.2689	1.50E-08
trpB	tryptophan synthase beta subunit	2.0434	6.37E-06
argB	acetylglutamate kinase	2.3225	6.68E-10
glyS	glycyl-tRNA synthetase	-2.9083	1.29E-22
SERP1176	peptidase U32 family	2.3811	1.83E-24
SERP1177	peptidase U32 family	2.6006	3.16E-30
infC	translation initiation factor IF-3	2.3684	4.36E-17
ald	alanine dehydrogenase	-2.7547	2.67E-09
SERP1292	serine protease HtrA putative	-2.7124	1.23E-30
SERP1310	dipeptidase family protein	-3.0286	8.51E-63
SERP1376	protein export protein PrsA putative	-2.6829	7.19E-41
SERP1549	death-on-curing family protein	2.3238	1.91E-56
leuB	3-isopropylmalate dehydrogenase	2.6290	7.14E-09
glyA	serine hydroxymethyltransferase	-2.3637	4.36E-33
secY	preprotein translocase SecY subunit	2.5474	1.38E-20
SERP2034	amino acid permease family protein	3.3899	3.46E-17
SERP2043	peptidase M42 family	-3.4218	1.54E-47
cysJ	sulfite reductase (NADPH) flavoprotein alpha-component	-2.1931	2.60E-23
cysH	phosophoadenylyl-sulfate reductase	-2.5134	3.48E-28
arcA	arginine deiminase	-2.3875	1.56E-39
sepA	extracellular elastase precursor	2.3264	4.49E-17

SERP2272	peptide methionine sulfoxide reductase putative	2.6559	5.30E-18
SERP2276	secA family protein	2.2325	1.96E-31
hisH	amidotransferase HisH	2.5488	6.82E-14
hisB	imidazoleglycerol-phosphate dehydratase	3.4466	1.62E-17
hisD	histidinol dehydrogenase	2.6795	3.23E-18
hisG	ATP phosphoribosyltransferase	2.8203	5.58E-10
SERP2338	peptide synthetase	2.4122	9.72E-67
SERP2364	peptidase M20/M25/M40 family	2.6489	1.23E-16
SERP2375	diaminopimelate epimerase family protein	2.6285	3.49E-18
serS	seryl-tRNA synthetase	-2.7533	3.76E-36
Lipid metabolism			
SERP0309	lipase/esterase putative	2.2785	1.41E-11
fabH	3-oxoacyl-(acyl-carrier-protein) synthase III	-2.1260	4.25E-10
plsX	fatty acid/phospholipid synthesis protein PlsX	-2.1079	1.80E-60
acpP	acyl carrier protein	-2.8822	7.31E-15
SERP1001	DegV family protein	-2.0926	2.95E-41
SERP2337	4-phosphopantetheinyl transferase family protein	2.9365	5.06E-42
SERP2523	glycerophosphoryl diester phosphodiesterase UgpQ putative	-2.4642	8.93E-19
Nucleotide and energy metabolism			
prsA	ribose-phosphate pyrophosphokinase	-2.2578	1.40E-57
SERP0371	exsD protein	6.0454	4.40E-29
SERP0372	6-pyruvoyl tetrahydrobiopterin synthase putative	5.8808	9.47E-31
SERP0373	exsB protein	5.7653	9.79E-77
folD	methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	-2.5692	3.02E-88

	phosphoribosylaminoimidazole carboxylase catalytic subunit	-4.1495	5.44E-70
purE			
purK	phosphoribosylaminoimidazole carboxylase ATPase subunit	-3.9826	4.21E-85
purC	phosphoribosylaminoimidazole-succinocarboxamide synthase	-4.4226	2.61E-60
purS	phosphoribosylformylglycinamidine synthase PurS protein	-4.5392	7.95E-95
purQ	phosphoribosylformylglycinamidine synthase I	-4.5973	2.90E-78
purL	phosphoribosylformylglycinamidine synthase II	-4.2042	6.09E-107
purF	amidophosphoribosyltransferase	-4.2181	3.25E-136
purM	phosphoribosylformylglycinamidine cyclo-ligase	-4.3477	2.30E-69
purN	phosphoribosylglycinamide formyltransferase	-3.8846	2.36E-80
purH	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	-3.7319	6.33E-113
purD	phosphoribosylamine--glycine ligase	-2.6846	2.00E-23
purB	adenylosuccinate lyase	-2.9759	1.15E-62
cdd	cytidine deaminase	-2.0209	1.32E-49
thiI	thiamine biosynthesis protein ThiI	2.6751	1.97E-48
fhs	formate--tetrahydrofolate ligase	-3.4566	2.24E-39
upp	uracil phosphoribosyltransferase	-2.2580	1.20E-35
adk	adenylate kinase	2.5524	1.03E-10
SERP1865	inosine-uridine preferring nucleoside hydrolase family protei	-2.0061	2.04E-09
rbsK	ribokinase	-2.2333	3.77E-29
Metabolic processes			
SERP0250	acetyltransferase GNAT family	-2.4617	3.97E-40
SERP0461	glyoxalase family protein	2.8462	1.43E-08

SERP0556	fumarylacetoacetate hydrolase family protein	-2.1019	4.39E-07
SERP0561	hydrolase haloacid dehalogenase-like family	-2.2982	1.85E-41
SERP1178	O-methyltransferase family protein	2.3694	4.74E-47
SERP1280	aminotransferase class V	2.7479	3.78E-43
SERP1918	amidohydrolase family protein	2.1722	5.93E-10
SERP1996	acetyltransferase GNAT family	2.0172	4.71E-05
SERP2054	glycosyl transferase group 1 family protein	2.5257	1.06E-23
SERP2299	N-acetyltransferase family protein	2.6212	2.47E-16
SERP2547	YjeF-related protein	3.2688	4.13E-16

4. Discussion

The increased use of implanted medical devices, the subsequent risk of biofilm formation on these devices and the emergence of drug-resistant strains has altogether imposed a heavy burden on patient and health care systems (Becker et al., 2014; WHO, 2014; Casillo et al., 2017). About 5027 anti-biofilm agents against Gram positive and negative bacteria, and fungi have been reported between 1988-2017 (Rajput et al., 2018). However, up to our knowledge none of them have been successfully translated to the market for clinical and medical applications. Our research aimed at harnessing the potential of marine sponge-derived actinomycetes for discovery of novel antibacterial and anti-biofilm compounds (Abdelmohsen et al., 2014a; Abdelmohsen et al., 2014b; Dashti et al., 2014). Actinomycetes from marine sponges represent an untapped reservoir of a wide range of unforeseen biological compounds (Xi et al., 2012; Abdelmohsen et al., 2015; Sun et al., 2015). Previous results have demonstrated the antibiofilm efficacy of an organic extract from *Streptomyces* sp. SBT343 isolated from marine sponge *Petrosia ficiformis* (Balasubramanian et al., 2017). In this paper, we describe the anti-staphylococcal activity of another strain *Streptomyces* sp. SBT348 isolated from the same sponge. We applied a bioassay-guided fractionation strategy to identify, isolate and purify the active compound responsible for this activity.

Streptomyces sp. SBT348 is a filamentous Gram-positive bacterium that was previously shown to possess distinct metabolomic and rich chemistry profiles with strong biological activities (Cheng et al., 2015; Cheng et al., 2017). SEM of the 10 d old *Streptomyces* sp. SBT348 culture used for extraction and isolation of the bioactive SKC3 indicated the presence of biofilm-like networks (**Figure 1A**). This extends the possibility of SKC3 to be a

compound produced in the biofilm networks that is antagonistic to other bacteria. However, more experiments are needed to confirm the same.

Leary et al., proposed a combination of autoclave and chlorhexidine treatment for complete removal of biofilms from orthopedic materials (Leary et al., 2017). Alternative, coating-based strategies have been proposed to prevent this phenomenon (Windolf et al., 2014). The isolated compound SKC3 effectively inhibited the growth and biofilm formation of different staphylococcal strains (**Figure 3**, **Table 2**). Further, the inefficacy of SKC3 against dispersing pre-formed biofilms highlights its usage in prevention of staphylococcal infections. This is advantageous, since, targeting the disassembly could lead to increased inflammatory response and severity of a disease (Franca et al., 2016). SKC3 was also shown to inhibit the staphylococcal biofilm formation on different medically relevant surfaces (glass, titan metal and silicone tubes). The non-toxic nature of SKC3 *in vitro* (cell lines) and *in vivo* (*G. melonella* larvae) explains its applicability as antimicrobial and antibiofilm agents on medical devices. As a step forward, the potential of SKC3 to protect *G. melonella* from *S. aureus* USA300 Lac* was also assessed in an independent experiment. Results obtained indicated that SKC3 could not protect the larvae from staphylococcal infection (data not shown). The exact reason behind this failure remains unclear. However, further investigations are needed to evaluate the toxicity and *in vivo* antimicrobial efficacy of SKC3 on higher *in vivo* model systems to support its usage. The huge mass (1258.3 Da), stability towards heat and enzymatic treatments, and the absence of relevant hits in several databases point towards a complex structure of SKC3. Thus, SKC3 is expected to be a new compound and further NMR spectrometric investigations to elucidate its complete structure are currently in progress.

Transcriptomics have been increasingly used for understanding the responses of staphylococci to antimicrobial agents and for obtaining insights into the antimicrobial mode of action (Sianglum et al., 2012; Wang et al., 2018). In our study, an overall view of the state of SKC3 treated *S. epidermidis* RP62A was achieved by RNA sequencing and transcriptome analysis (at 20 min and 3 h post treatment). Transcriptome data from early time point (20 min), indicated that genes encoding a two-component system (sensor histidine kinase and response regulator), several proteins involved in transport of macromolecules, such as ATP-binding cassette (ABC) transporters, quaternary ammonium compound efflux pumps (SugE) were significantly upregulated. ABC transporters are often involved in multi-drug resistance by serving as efflux pumps for transport of anti-infectives (Lage, 2003). SugE, a drug efflux pump belonging to the small multi drug resistance family (SMR) was shown to be involved

in resistance to a narrow range of quaternary ammonium compounds in *Escherichia coli* (Chung and Saier, 2002). However, these ABC transporters and *sugE* regulated by SKC3 have not been documented to be involved in resistance to antimicrobial compounds in *S. epidermidis* till date. Further studies are needed to understand the exact roles of these transporters and efflux pump in this organism. Thus, it could be presumed that at 20 min *S. epidermidis* RP62A recognizes SKC3 by a yet unknown two-component system and reacts by expressing a variety of transporters.

Transcriptome data from the late time point (3 h), indicated that genes encoding for hypothetical proteins were the most differentially regulated (representing 30.64% of the total differentially expressed genes at 3 h). Major fraction of the known differentially expressed genes at 3 h included the genes encoding proteins involved in global metabolism (representing 23.37% of the total differentially expressed genes), and transporters and membrane proteins (representing 14.73% of the total differentially expressed genes). In addition, bacterial stress and defense related proteins were strongly downregulated indicating the sensitivity of bacterial cells at this time point. Like the 20 min transcriptome data, several ABC transporters, ion transporters, drug transporters and efflux pumps were influenced in the presence of SKC3 at 3 h. These are speculated to be the typical responses of *S. epidermidis* to toxic agents (Putman et al., 2000; Cecil et al., 2011). However, the specific effects of SKC3 on metabolism are much stronger. Interference with metabolism involved differential regulation of genes involved in carbon metabolism (down regulation of genes related to processes of glycolysis, gluconeogenesis, pentose phosphate pathway, glycerol, fructose and lactose metabolism), lipid metabolism (repression of genes related to fatty acid biosynthesis and phospholipid metabolism), nucleotide and energy metabolism (repression of several genes related to purine biosynthetic process from *de novo* and salvage pathways), and amino acid, and protein metabolism (repression in biosynthesis of cysteine, isoleucine, leucine, valine, glycine, glutamine and lipoproteins; repression of alanine and arginine catabolism; up regulation in biosynthesis of tryptophan, arginine and histidine). Cecil et al. previously reported similar negative influence of the isoquinoline compounds IQ-143 and IQ-238 on purine and carbon metabolism in staphylococci (Cecil et al., 2011; Cecil et al., 2015). In another study, Sianglum et al. had demonstrated the influence of the phytochemical compound rhodomyrtone in altering metabolism in methicillin-resistant *S. aureus* (Sianglum et al., 2012). Our results thus, demonstrate the striking similarity of SKC3 in altering metabolism of staphylococci with the existing literature.

Interestingly, there was no direct remarkable influence of SKC3 on the transcription of *ica* locus (3 h) encoding the polysaccharide intercellular adhesin (PIA) responsible for biofilm formation in *S. epidermidis*. However, virulence factors like the phenol soluble modulins α and β, (proinflammatory cytolysins) involved in biofilm structuring and detachment processes, (Otto, 2009; Fey and Olson, 2010)) and hemolysin (putative) were down-regulated. The purine biosynthesis regulator *purR* and the iron sequestration system (*sitA*, *sitB*, *sitC* and *sirR*) were also found to be significantly down regulated. PurR is reported to positively regulate *ica* expression via an indirect mechanism (Mack et al., 2007; O'Gara, 2007). These results suggest that SKC3 could inhibit biofilm formation by repressing the expression of *purR*. *S. epidermidis* RP62A is also known to largely depend on the availability of iron to form biofilms (Massonet et al., 2006; Oliveira et al., 2017) and the down-regulation of iron acquisition system could be yet another explanation for biofilm inhibitory effect. Further investigations are required to get more insights into molecular mechanisms of biofilm inhibition by SKC3.

In conclusion, the anti-biofilm compound SKC3 was isolated from the chemically diverse strain *Streptomyces* sp. SBT348 with the aid of bioassay guided-fractionation. SKC3 exhibited antagonistic effects against growth and biofilm formation (at concentrations less than MICs) of several staphylococcal strains tested without exhibiting apparent *in vitro* and *in vivo* toxicity. Transcriptome analysis revealed the interference of SKC3 with several metabolic processes (carbon, protein, lipid, nucleotide and energy metabolism) of staphylococci. However, further experimental data is needed to elucidate the exact anti-staphylococcal mode of action of SKC3.

5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Author Contributions

Conceived and designed the experiments: TAO, URA, UHo, UHe, KUF, WZ. Performed the experiments: SB, JS, RB. Analyzed the data: SB, JS, RB, TAO. Manuscript preparation: SB, JS, RB, UHo, KUF, UHe, WZ, URA, TAO. Manuscript revision: SB, TAO, URA, UHo, UHe, KUF, WZ. All authors read and approved the final manuscript.

7. Funding

8. Acknowledgments

9. References

Abdelmohsen, U.R., Balasubramanian, S., Oelschlaeger, T.A., Grkovic, T., Pham, N.B., Quinn, R.J., and Hentschel, U. (2017). Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. *Lancet Infect Dis* 17**,** e30-e41.

Abdelmohsen, U.R., Bayer, K., and Hentschel, U. (2014a). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat Prod Rep* 31**,** 381-399.

Abdelmohsen, U.R., Grkovic, T., Balasubramanian, S., Kamel, M.S., Quinn, R.J., and Hentschel, U. (2015). Elicitation of secondary metabolism in actinomycetes. *Biotechnol Adv* 33, 798-811.

Abdelmohsen, U.R., Yang, C., Horn, H., Hajjar, D., Ravasi, T., and Hentschel, U. (2014b). Actinomycetes from Red Sea sponges: sources for chemical and phylogenetic diversity. *Mar Drugs* 12, 2771-2789.

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.

Aparecida Procopio Gomes, L., Alves Figueiredo, L.M., Luiza Do Rosario Palma, A., Correa Geraldo, B.M., Isler Castro, K.C., Ruano De Oliveira Fugisaki, L., Jorge, A.O.C., De Oliveira, L.D., and Junqueira, J.C. (2016). *Punica granatum L.* (Pomegranate) extract*: In vivo* study of antimicrobial activity against *Porphyromonas gingivalis* in *Galleria mellonella* model. *Scientific World Journal* 2016, 8626987.

Balasubramanian, S., Othman, E.M., Kampik, D., Stopper, H., Hentschel, U., Ziebuhr, W., Oelschlaeger, T.A., and Abdelmohsen, U.R. (2017). Marine sponge-derived *Streptomyces* sp. SBT343 extract inhibits staphylococcal biofilm formation. *Front Microbiol* 8, 236.

Barros, M., Branquinho, R., Grosso, F., Peixe, L., and Novais, C. (2014). Linezolid-resistant *Staphylococcus epidermidis*, Portugal, 2012. *Emerg Infect Dis* 20, 903-905.

Beau, J., Mahid, N., Burda, W.N., Harrington, L., Shaw, L.N., Mutka, T., Kyle, D.E., Barisic, B., Van Olphen, A., and Baker, B.J. (2012). Epigenetic tailoring for the production of anti-infective cytosporones from the marine fungus *Leucostoma persoonii*. *Mar Drugs* 10, 762-774.

Becker, K., Heilmann, C., and Peters, G. (2014). Coagulase-negative staphylococci. *Clin Microbiol Rev* 27, 870-926.

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M., and Hoiby, N. (2013). Applying insights from biofilm biology to drug development - can a new approach be developed? *Nat Rev Drug Discov* 12, 791-808.

Casillo, A., Papa, R., Ricciardelli, A., Sannino, F., Ziaco, M., Tilotta, M., Selan, L., Marino, G., Corsaro, M.M., Tutino, M.L., Artini, M., and Parrilli, E. (2017). Anti-biofilm activity of a long-chain fatty aldehyde from antarctic *Pseudoalteromonas haloplanktis* TAC125 against *Staphylococcus epidermidis* biofilm. *Front Cell Infect Microbiol* 7, 46.

Cecil, A., Ohlsen, K., Menzel, T., Francois, P., Schrenzel, J., Fischer, A., Dorries, K., Selle, M., Lalk, M., Hantzschmann, J., Dittrich, M., Liang, C., Bernhardt, J., Olschlager, T.A., Bringmann, G., Bruhn, H., Unger, M., Ponte-Sucre, A., Lehmann, L., and Dandekar, T.

(2015). Modelling antibiotic and cytotoxic isoquinoline effects in *Staphylococcus aureus*, *Staphylococcus epidermidis* and mammalian cells. *Int J Med Microbiol* 305**,** 96-109.

Cecil, A., Rikanovic, C., Ohlsen, K., Liang, C., Bernhardt, J., Oelschlaeger, T.A., Gulder, T., Bringmann, G., Holzgrabe, U., Unger, M., and Dandekar, T. (2011). Modeling antibiotic and cytotoxic effects of the dimeric isoquinoline IQ-143 on metabolism and its regulation in *Staphylococcus aureus*, *Staphylococcus epidermidis* and human cells. *Genome Biol* 12**,** R24.

Cheng, C., Macintyre, L., Abdelmohsen, U.R., Horn, H., Polymenakou, P.N., Edrada-Ebel, R., and Hentschel, U. (2015). Biodiversity, anti-trypanosomal activity screening, and metabolomic profiling of actinomycetes isolated from mediterranean sponges. *PLoS One* 10**,** e0138528.

Cheng, C., Othman, E.M., Stopper, H., Edrada-Ebel, R., Hentschel, U., and Abdelmohsen, U.R. (2017). Isolation of Petrocidin A, a new cytotoxic cyclic dipeptide from the marine sponge-derived bacterium *Streptomyces* sp. SBT348. *Mar Drugs* 15.

Chung, Y.J., and Saier, M.H., Jr. (2002). Overexpression of the *Escherichia coli sugE* gene confers resistance to a narrow range of quaternary ammonium compounds. *J Bacteriol* 184**,** 2543-2545.

Dashti, Y., Grkovic, T., Abdelmohsen, U.R., Hentschel, U., and Quinn, R.J. (2014). Production of induced secondary metabolites by a co-culture of sponge-associated actinomycetes, *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163. *Mar Drugs* 12**,** 3046-3059.

Dyke, K.G., Jevons, M.P., and Parker, M.T. (1966). Penicillinase production and instrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* 1, 835-838.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30**,** 207-210.

Fey, P.D., and Olson, M.E. (2010). Current concepts in biofilm formation of *Staphylococcus epidermidis*. *Future Microbiol* 5**,** 917-933.

Fitzgerald, J.R., Monday, S.R., Foster, T.J., Bohach, G.A., Hartigan, P.J., Meaney, W.J., and Smyth, C.J. (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J Bacteriol* 183, 63-70.

Flemming, H.C., and Wingender, J. (2010). The biofilm matrix. *Nat Rev Microbiol* 8**,** 623-633.

Forstner, K.U., Vogel, J., and Sharma, C.M. (2014). READemption-a tool for the computational analysis of deep-sequencing-based transcriptome data. *Bioinformatics* 30**,** 3421-3423.

Franca, A., Freitas, A.I., Henriques, A.F., and Cerca, N. (2012). Optimizing a qPCR gene expression quantification assay for *Staphylococcus epidermidis* biofilms: a comparison between commercial kits and a customized protocol. *PLoS One* 7**,** e37480.

Franca, A., Perez-Cabezas, B., Correia, A., Pier, G.B., Cerca, N., and Vilanova, M. (2016). *Staphylococcus epidermidis* biofilm-released cells induce a prompt and more marked *in vivo* inflammatory-type response than planktonic or biofilm cells. *Front Microbiol* 7**,** 1530.

Freese, N.H., Norris, D.C., and Loraine, A.E. (2016). Integrated genome browser: visual analytics platform for genomics. *Bioinformatics* 32**,** 2089-2095.

Gerwick, W.H., and Moore, B.S. (2012). Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. *Chem Biol* 19**,** 85-98.

Gibreel, T.M., and Upton, M. (2013). Synthetic epidermicin NI01 can protect *Galleria mellonella* larvae from infection with *Staphylococcus aureus*. *J Antimicrob Chemother* 68**,** 2269-2273.

Gomes, N.M., Bessa, L.J., Buttachon, S., Costa, P.M., Buaruang, J., Dethoup, T., Silva, A.M., and Kijjoa, A. (2014). Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the soil fungi *N. fischeri* and *N. siamensis*. *Mar Drugs* 12**,** 822-839.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2**,** 95-108.

Hoffmann, S., Otto, C., Kurtz, S., Sharma, C.M., Khaitovich, P., Vogel, J., Stadler, P.F., and Hackermuller, J. (2009). Fast mapping of short sequences with mismatches, insertions and deletions using index structures. *PLoS Comput Biol* 5**,** e1000502.

Huber, W., and Koella, J.C. (1993). A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. *Acta Trop* 55**,** 257-261.

Indraningrat, A.A., Smidt, H., and Sipkema, D. (2016). Bioprospecting sponge-associated microbes for antimicrobial compounds. *Mar Drugs* 14.

Kleinschmidt, S., Huygens, F., Faoagali, J., Rathnayake, I.U., and Hafner, L.M. (2015). *Staphylococcus epidermidis* as a cause of bacteremia. *Future Microbiol* 10**,** 1859-1879.

Kodzius, R., and Gojobori, T. (2015). Marine metagenomics as a source for bioprospecting. *Mar Genomics* 24 Pt 1**,** 21-30.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., and Hiramatsu, K. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357, 1225-40.

Lage, H. (2003). ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int J Antimicrob Agents* 22**,** 188-199.

Leary, J.T., Werger, M.M., Broach, W.H., Shaw, L.N., Santoni, B.G., Bernasek, T.L., and Lyons, S.T. (2017). Complete eradication of biofilm from orthopedic materials. *J Arthroplasty* 32**,** 2513-2518.

Lipinski, B., Hawiger, J., and Jeljaszewicz, J. (1967). Staphylococcal clumping with soluble fibrin monomer complexes. *J Exp Med* 126**,** 979-988.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15**,** 550.

Mack, D., Davies, A.P., Harris, L.G., Rohde, H., Horstkotte, M.A., and Knobloch, J.K. (2007). Microbial interactions in *Staphylococcus epidermidis* biofilms. *Anal Bioanal Chem* 387**,** 399-408.

Martin, M. (May 2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal* 17**,** 10-12.

Martins, A., Vieira, H., Gaspar, H., and Santos, S. (2014). Marketed marine natural products in the pharmaceutical and cosmeceutical industries: tips for success. *Mar Drugs* 12**,** 1066-1101.

Massonet, C., Pintens, V., Merckx, R., Anne, J., Lammertyn, E., and Van Eldere, J. (2006). Effect of iron on the expression of *sirR* and *sitABC* in biofilm-associated *Staphylococcus epidermidis*. *BMC Microbiol* 6**,** 103.

Mayer, A.M., Glaser, K.B., Cuevas, C., Jacobs, R.S., Kem, W., Little, R.D., Mcintosh, J.M., Newman, D.J., Potts, B.C., and Shuster, D.E. (2010). The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol Sci* 31**,** 255-265.

Mcdougal, L.K., Steward, C.D., Killgore, G.E., Chaitram, J.M., Mcallister, S.K., and Tenover, F.C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant

Staphylococcus aureus isolates from the United States: establishing a national database. *J Clin Microbiol* 41, 5113-5120.

Namvar, A.E., Bastarahang, S., Abbasi, N., Ghehi, G.S., Farhadbakhtiarian, S., Arezi, P., Hosseini, M., Baravati, S.Z., Jokar, Z., and Chermahin, S.G. (2014). Clinical characteristics of *Staphylococcus epidermidis*: a systematic review. *GMS Hyg Infect Control* 9, 23.

Nithya, C., Begum, M.F., and Pandian, S.K. (2010). Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. *Appl Microbiol Biotechnol* 88, 341-358.

O'gara, J.P. (2007). *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiol Lett* 270, 179-188.

Oliveira, F., Franca, A., and Cerca, N. (2017). *Staphylococcus epidermidis* is largely dependent on iron availability to form biofilms. *Int J Med Microbiol* 307, 552-563.

Otto, M. (2009). *Staphylococcus epidermidis*--the 'accidental' pathogen. *Nat Rev Microbiol* 7, 555-567.

Otto, M. (2012). Molecular basis of *Staphylococcus epidermidis* infections. *Semin Immunopathol* 34, 201-214.

Palomo, S., Gonzalez, I., De La Cruz, M., Martin, J., Tormo, J.R., Anderson, M., Hill, R.T., Vicente, F., Reyes, F., and Genilloud, O. (2013). Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Mar Drugs* 11, 1071-1086.

Percival, S.L., Hill, K.E., Malic, S., Thomas, D.W., and Williams, D.W. (2011). Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. *Wound Repair Regen* 19, 1-9.

Percival, S.L., Suleman, L., Vuotto, C., and Donelli, G. (2015). Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. *J Med Microbiol* 64, 323-334.

Putman, M., Van Veen, H.W., and Konings, W.N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 64, 672-693.

Rahman, H., Austin, B., Mitchell, W.J., Morris, P.C., Jamieson, D.J., Adams, D.R., Spragg, A.M., and Schweizer, M. (2010). Novel anti-infective compounds from marine bacteria. *Mar Drugs* 8, 498-518.

Rajput, A., Thakur, A., Sharma, S., and Kumar, M. (2018). aBiofilm: a resource of anti-biofilm agents and their potential implications in targeting antibiotic drug resistance. *Nucleic Acids Res* 46**,** D894-D900.

Rosenstein, R., Nerz, C., Biswas, L., Resch, A., Raddatz, G., Schuster, S.C., and Gotz, F. (2009). Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. *Appl Environ Microbiol* 75**,** 811-822.

Sabate Bresco, M., Harris, L.G., Thompson, K., Stanic, B., Morgenstern, M., O'mahony, L., Richards, R.G., and Moriarty, T.F. (2017). Pathogenic mechanisms and host interactions in *Staphylococcus epidermidis* device-related infection. *Front Microbiol* 8**,** 1401.

Sakimura, T., Kajiyama, S., Adachi, S., Chiba, K., Yonekura, A., Tomita, M., Koseki, H., Miyamoto, T., Tsurumoto, T., and Osaki, M. (2015). Biofilm-forming *Staphylococcus epidermidis* expressing vancomycin resistance early after adhesion to a metal surface. *Biomed Res Int* 2015**,** 943056.

Shida, T., Koseki, H., Yoda, I., Horiuchi, H., Sakoda, H., and Osaki, M. (2013). Adherence ability of *Staphylococcus epidermidis* on prosthetic biomaterials: an *in vitro* study. *Int J Nanomedicine* 8**,** 3955-3961.

Sianglum, W., Srimanote, P., Taylor, P.W., Rosado, H., and Voravuthikunchai, S.P. (2012). Transcriptome analysis of responses to rhodomyrtone in methicillin-resistant *Staphylococcus aureus*. *PLoS One* 7**,** e45744.

Skaf, J., Hamarsheh, O., Berninger, M., Balasubramanian, S., Oelschlaeger, T.A., and Holzgrabe, U. (2018). Improving anti-trypanosomal activity of alkamides isolated from *Achillea fragrantissima*. *Fitoterapia* 125, 191-198.

Stewart, P.S., and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* 358**,** 135-138.

Stowe, S.D., Richards, J.J., Tucker, A.T., Thompson, R., Melander, C., and Cavanagh, J. (2011). Anti-biofilm compounds derived from marine sponges. *Mar Drugs* 9**,** 2010-2035.

Sun, W., Zhang, F., He, L., Karthik, L., and Li, Z. (2015). Actinomycetes from the South China Sea sponges: isolation, diversity, and potential for aromatic polyketides discovery. *Front Microbiol* 6**,** 1048.

Thibane, V.S., Kock, J.L., Ells, R., Van Wyk, P.W., and Pohl, C.H. (2010). Effect of marine polyunsaturated fatty acids on biofilm formation of *Candida albicans* and *Candida dubliniensis*. *Mar Drugs* 8**,** 2597-2604.

Thompson, C.C., Kruger, R.H., and Thompson, F.L. (2017). Unlocking marine biotechnology in the developing world. *Trends Biotechnol* 35**,** 1119-1121.

Tsai, C.J., Loh, J.M., and Proft, T. (2016). *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7**,** 214-229.

Vinh, D.C., and Embil, J.M. (2005). Device-related infections: a review. *J Long Term Eff Med Implants* 15**,** 467-488.

Wang, J., Nong, X.H., Amin, M., and Qi, S.H. (2018). Hygrocin C from marine-derived *Streptomyces* sp. SCSGAA 0027 inhibits biofilm formation in *Bacillus amyloliquefaciens* SCSGAB0082 isolated from south China sea gorgonian. *Appl Microbiol Biotechnol* 102**,** 1417-1427.

WHO (2014). Antimicrobial resistance: global report on surveillance 2014.

Widerstrom, M. (2016). Significance of *Staphylococcus epidermidis* in health care-associated infections, from contaminant to clinically relevant pathogen: this is a wake-up call! *J Clin Microbiol* 54**,** 1679-1681.

Windolf, C.D., Logters, T., Scholz, M., Windolf, J., and Flohe, S. (2014). Lysostaphin-coated titan-implants preventing localized osteitis by *Staphylococcus aureus* in a mouse model. *PLoS One* 9**,** e115940.

Xi, L., Ruan, J., and Huang, Y. (2012). Diversity and biosynthetic potential of culturable actinomycetes associated with marine sponges in the China seas. *Int J Mol Sci* 13**,** 5917-5932.

Yu, G., Wang, L.G., Han, Y., and He, Q.Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16, 284-287.

Supplementary figures to chapter 4
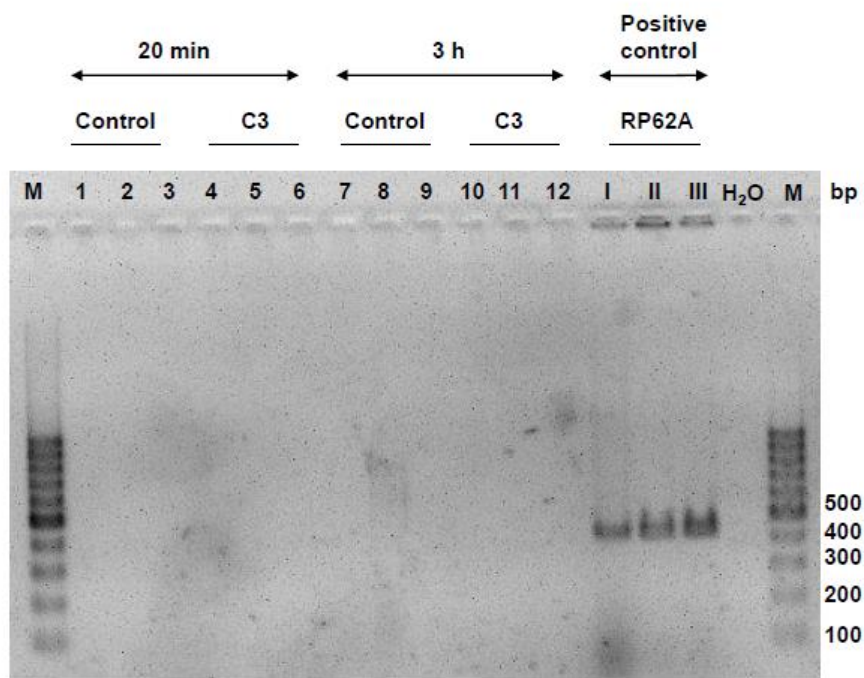
Figure S1. *icaA* PCR (quality control) for verification of the absence of DNA contamination before RNA sequencing. M, molecular ladder (100 bp). *icaA* amplicon at 414 bp seen in the three independent biological replicates (I, II, III) used for the transcriptome experiment. Primer sequence (5'-3') used for PCR:

icaA forward: GTCATTGATGACGATGCGCC

icaA reverse: AAGTACTTCATGCCCGCCTT
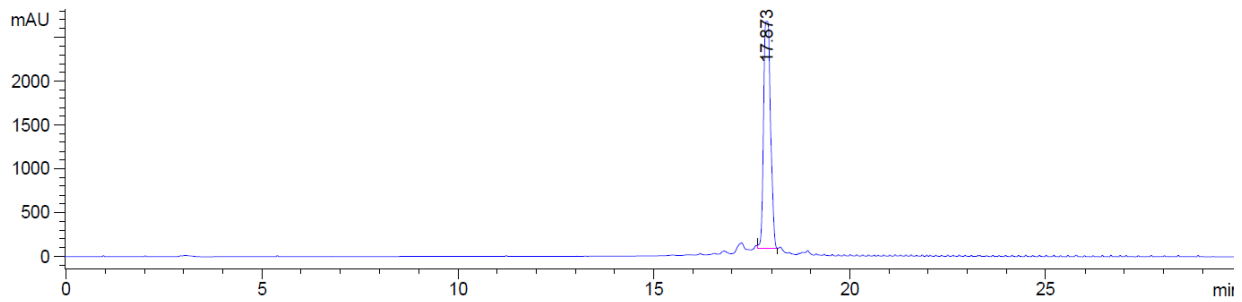
Figure S2. HPLC chromatogram of SKC3 at wavelength of 250 nm (retention time ~17.8 min) confirming its purity. SKC3 was dissolved in pure HPLC-grade MeOH and had a concentration of 500 μg/ml.
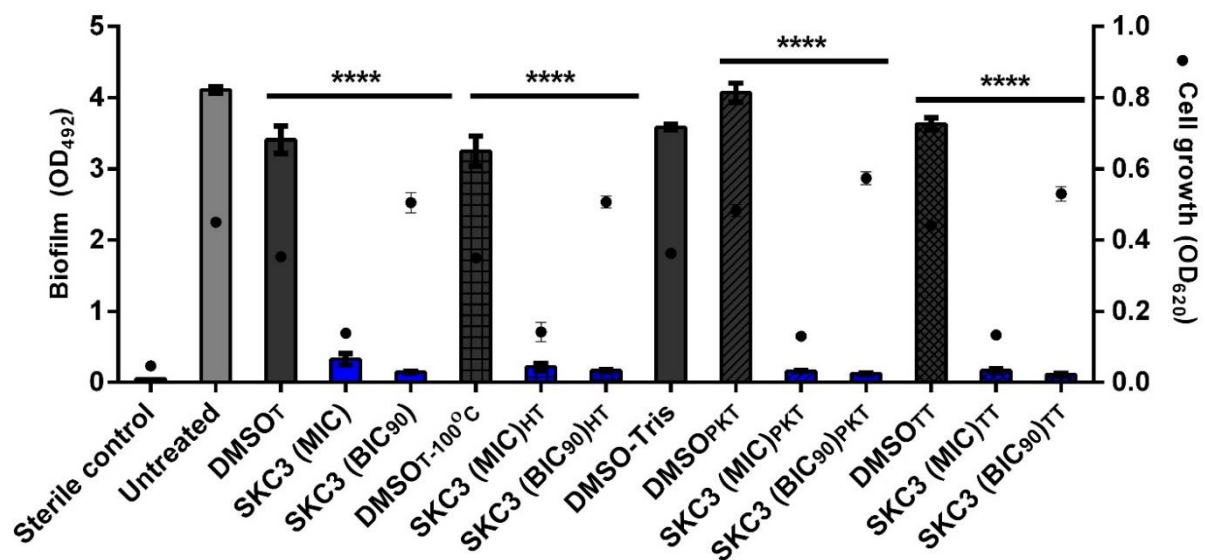
Figure S3. Effect of heat and enzymatic treatments on biological activity of SKC3 (physio-chemical characterization). HT, heat treatment; PKT, proteinase K treatment; TT, trypsin treatment (MIC: 31.25 μg/ml; BIC_{90}, 3.95 μg/ml). $DMSO_T$, DMSO treated (3.75% on cells), Sterile control, TSB with DMSO without *S. epidermidis* RP62A.
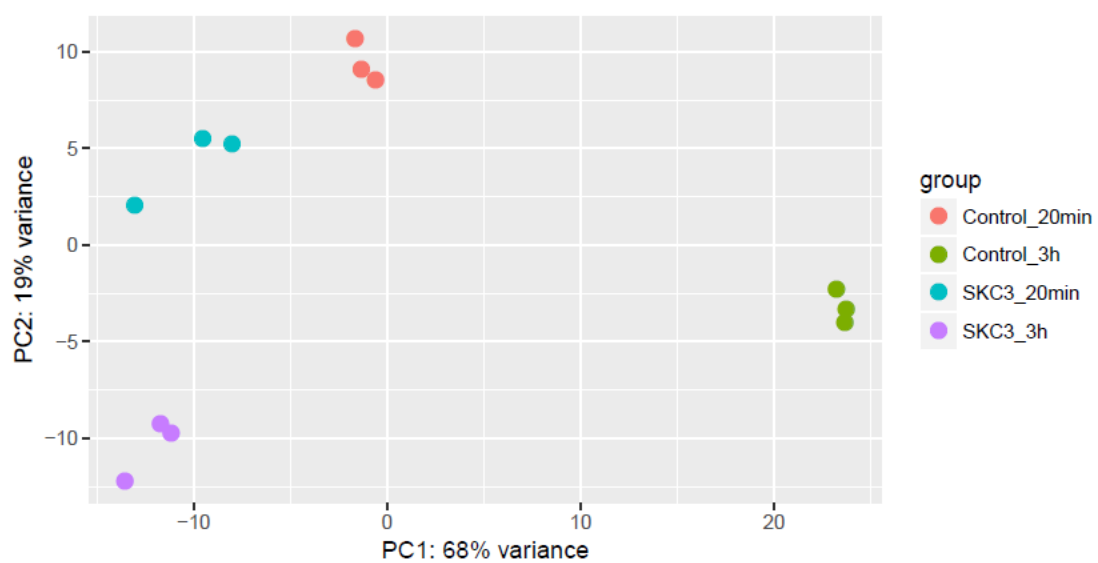
Figure S4. PCA plot representing the well-defined grouping of different biological replicates from RNA sequencing data. This plot also indicates the difference in level of gene expression changes between the control and SKC3 treated samples at 20 min and 3 h.
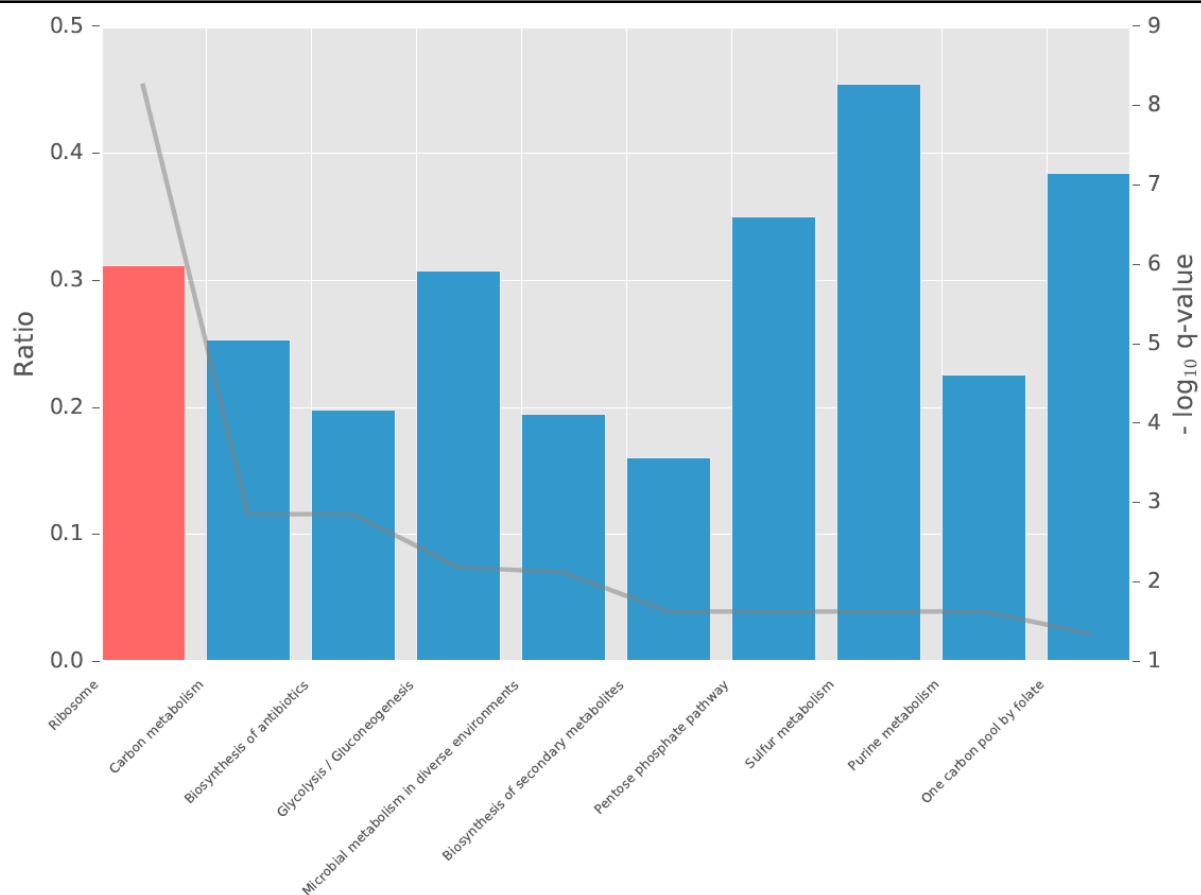
Figure S5. Function enrichment analysis (Filter: Log$_2$foldchange ± 2.0, p$_{adj}$ value: 0.05) of SKC3 treated *S. epidermidis* RP62A (3 h) revealed down-regulation of several pathways involved in carbon, sulfur and purine metabolism. Column bars reflect the ratio on the left. This ratio denotes the number of selected genes in a pathway per total number of genes in the pathway. The line in the graph reflects the -log$_{10}$ q-value, which is the p-value from the hypergeometric test adjusted for multiple testing. Red, up-regulation; blue, down-regulation.

5. General discussion

5.1. A retrospective of the bioactive potential of sponge-associated actinomycetes

Resistance against different classes of antibiotics and cross resistance to antibiotics of the same class are the major concerns in management of hospital and community-acquired infections. Today, even last resort antibiotics such as colistin, vancomycin and polymyxin B are facing the problems of bacterial resistance (Silver, 2011; Dickey et al., 2017). Big pharma companies have displayed reduced interests on antibiotic drug discovery initiatives owing to several reasons such as the absence of discovery of novel chemical scaffolds, frequent re-discovery of known compounds and limited financial return on investment (Bush and Page, 2017). Alternatively, modifications of existing chemical scaffolds have provided a temporary solution for treatment of resistant bacterial infections (Fischbach and Walsh, 2009; Brotz-Oesterhelt and Sass, 2010). These semi-synthetic analogues of existing antibiotics are known to display improved spectrum of activity, pharmacokinetic properties and act efficiently against resistant pathogens. Few derivatives of the existing antibiotics like vancomycin and teicoplanin (telavancin, oritavancin, and dalvabancin) have been approved for usage in treatment of complicated skin and skin structure infections (CSSSI), acute bacterial skin and skin structure infections (ABSSSI) caused by Gram positive bacteria (Leadbetter et al., 2004; Bouza and Burillo, 2010; Guskey and Tsuji, 2010). Their structures are shown in **Figure 1**.

Even though these compounds have been introduced into the clinic, the pipeline in the early stages of antibiotic discovery remains dry. Thus, discovery of novel anti-infectives with new modes of action is essential to address the urgent clinical needs. The marine realm has proven to be an attractive environment in this regard to find new compounds against infectious diseases (Abdelmohsen et al., 2017). Specifically, marine sponges and their microbial diversity have gained significant attention towards discovery of novel anti-infectives (Bessa et al., 2016; Wright et al., 2017; Buttachon et al., 2018).

Figure 1: Structures of existing antibiotics and their derivatives in the market. Modified from (Genilloud, 2017).

The *Streptomyces* strains used in this Ph.D. thesis were previously cultured from the Mediterranean marine sponges *Petrosia ficiformis* and *Agelas oroides.* Several biological activities have been reported from these sponges (Sauleau et al., 2017; Bayari et al., 2018). These include anticancer, antibacterial and antiparasitic activities (Ferretti et al., 2007; Ines et al., 2007; Tasdemir et al., 2007; Dyson et al., 2014). However, the main limitation of using the sponges for the large-scale production of compounds is the high sensitivity and resistance of these sponges to growth outside the oceanic environments. Further, the sponge-associated microbes are speculated to be the true producers of secondary metabolites in marine sponges (Bringmann et al., 2004; Lopez-Gresa et al., 2009; Thomas et al., 2010; Pagliara and Caroppo, 2011). The chemically diverse phylum Actinobacteria are known to be abundantly present in sponge niches (Abdelmohsen et al., 2014). Therefore, using the actinomycetes diversity of sponges is an ideal way for scaling up the production of bioactive compounds. From an ecological standpoint, using actinomycetes living on sponges for discovery of new compounds is an emerging strategy, since they are presumed to provide a line of protection to these sponges from predators through production of secondary metabolites.

Actinomycetes have contributed significantly to the discovery of new antibiotics. It is known that the majority of present day antibiotics like tetracyclines, rifamycins, aminoglycosides, macrolides, glycopeptides and β-lactams originate from actinomycetes (Genilloud, 2017). However, the isolation and characterization of many compounds is limited by the challenge in isolating and culturing their producer actinomycetes *in vitro* (Rappe and Giovannoni, 2003). Development has been made in cultivating the "uncultivable" actinomycetes and discovery of new antibiotic scaffolds by employing diffusion chamber technologies like iChip (Kaeberlein et al., 2002; Gavrish et al., 2008; Nichols et al., 2010; Versluis et al., 2017). A list of antibiotics derived from marine actinomycetes that have been cultured with new isolation methods is presented in **Table 1**.

Table 1: Antibiotic discovery from actinomycetes (cultivated from new isolation methods). Modified from (Genilloud, 2017). MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci.

Producer	Isolation scheme	Antibiotic	Antibiotic spectrum	Reference
Nocardia sp.	Diffusion chamber	Neocitreamicins I, II	Gram positive	(Peoples et al., 2008)
Streptosporangium sp.	Diffusion chamber	Novo 3	MRSA/VRE	(A. Peoples, 2012)
Amycolatopsis sp.	Diffusion chamber	Novo 4	MRSA/VRE	(A. Peoples, 2011)
Lentzea kentukyensis	iChip technology	Lassomycin	*Mycobacterium tuberculosis*	(Gavrish et al., 2014)

The *Streptomyces* strains SBT343, 345 and 348 used in this Ph.D. thesis were previously cultivated from the Mediterranean sponges using cultivation-dependent approaches. The sporulating strain *Streptomyces* sp. SBT343 was isolated on basic actinobacterial medium, M1, whereas, the filamentous and sporulating *Streptomyces* sp. SBT345 and SBT348 were isolated on ISP2_F medium (ISP2 agar covered by a layer of soft agar on top after inoculation) (Cheng et al., 2015).

The closest relative to these strains as revealed by BLAST searches is shown in **Table 2**.

Table 2: Isolation and identification of actinomycetes isolates used in the study. Adapted from (Cheng, 2016).

Streptomyces sp.	Sponge source	Sequence length	Closest relative by BLAST	Sequence identity (%)
SBT345	*A. oroides*	1398	*Streptomyces* sp. 56E35	99.86
SBT343	*P. ficiformis*	1448	*S. flavogriseus* P.S.461	99.65
SBT348	*P. ficiformis*	1152	*S. atroolivaceous* strain Nt1-5	99.91

Several compounds with biological activities have been reported from *Streptomyces* sp. SBT345 and SBT348. No biological activity has been reported so far from *Streptomyces* sp. SBT343. Morphology of the strains and the structures of compounds previously reported from the three strains in this study is shown in **Figure 2**.

Figure 2: **(A) Colony morphologies** of the sponge-derived *Streptomyces* strains in the study in ISP2 medium (prepared with artificial sea water). **(B) Structures of bioactive compounds** isolated from *Streptomyces* sp. SBT345 and SBT348, data derived from existing literature. No compound has been isolated so far from *Streptomyces* sp. SBT343 (Cheng, 2016b; a; Cheng et al., 2017).
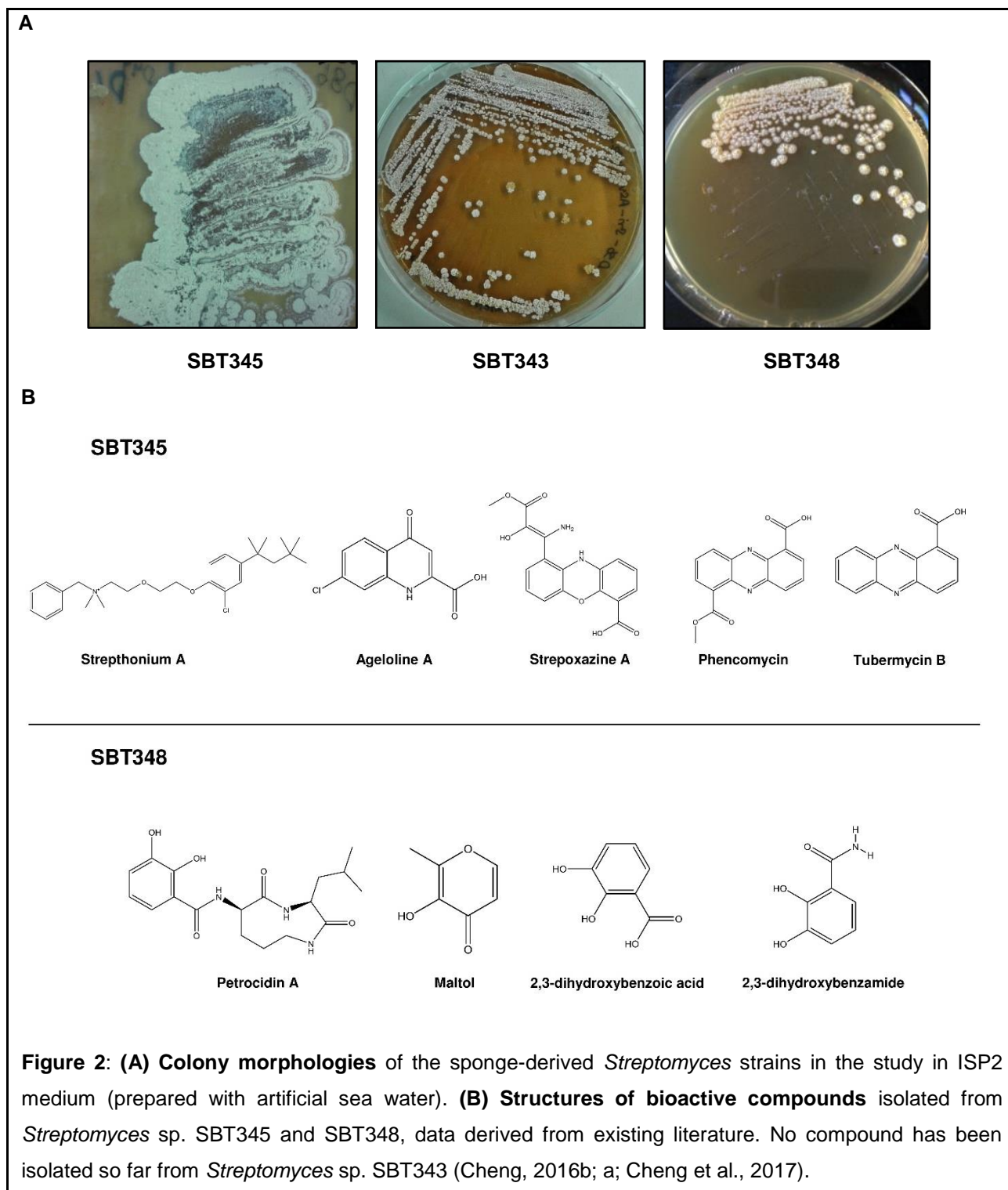
The existing knowledge in the abundance and diversity of bioactive actinomycetes in marine sponges and their increasingly available genomes have unmasked their enormous unprecedent abilities in production of novel secondary metabolites for pharmaceutical applications (Harjes et al., 2014; Horn et al., 2015; Dashti et al., 2017). However, they possess a large fraction of silenced biosynthetic gene clusters (encoding secondary

metabolites) that are not transcribed under conventional laboratory conditions (Seyedsayamdost, 2014; Abdelmohsen et al., 2015). Predictions from mathematical models have revealed thousands of undiscovered antibiotics from actinomycete genomes (Caboche, 2014; Cimermancic et al., 2014). Hence, it is postulated that a lot of biosynthetic genes could remain cryptic in the *Streptomyces* strains used in this Ph.D. thesis. Genome sequencing of these strains should be done to understand their real secondary metabolism potential. Bioinformatic algorithms such as antiSMASH (Blin et al., 2013), MIBiG (Medema et al., 2015) and NaPDoS (Ziemert et al., 2012) could help in identification of silenced gene clusters. The induction in expression of the silent genes in these strains could be then achieved by suitable elicitation strategies (e.g. co-cultivation with other microbes, addition of microbial lysates, microbial cell components, chemical elicitors) or by heterologous expression of the identified genes in a suitable host (Challis, 2008; Harvey et al., 2015). Thus, it is posited that with these genome targeted approaches the actual chemical diversity of these *Streptomyces* strains could be harnessed (Genilloud, 2017). A list of antibiotics discovered already with the genome-targeted approaches are enlisted in **Table 3**.

Table 3: Antibiotics discovered from the genome-based approaches from actinomycetes. Modified from (Genilloud, 2017).

Genome	Antibiotic	Antibiotic spectrum	Reference
Streptomyces mauvecolor	Piperidamycins	Gram positive	(Hosaka et al., 2009)
Streptomyces aureus	Phosacetamycin	Gram positive/negative	(Evans et al., 2013)
Streptomyces monomycini	Argolaphos A/B	Gram positive/negative	(Ju et al., 2015)
Streptomyces durhamensis	Valinophos	Gram positive/negative	(Ju et al., 2015)
Sphaerisporangium sp.	Sphaerimicin A	Gram positive	(Winn et al., 2010)
Nocardiopsis sp.	TP-1161	Gram positive	(Engelhardt et al., 2010)
Saccharomonospora sp.	Taromycin A	Gram positive	(Yamanaka et al., 2014)
Salinispora sp. *S.afghaniensis*	Thiolactomycin and analogs	Gram positive	(Tang et al., 2015)

5.2. Anti-Stx approaches: state-of-the-art

EHECs have become a major problem in subjects of human health. Several notable outbreaks have been recorded with EHEC infections (RKI, 2011; McCollum et al., 2012).

Current regimes for the treatment of EHEC infections include management of symptoms like bleeding, anaemia, fluid or electrolytes imbalance, central nervous system effects and renal failure (Pacheco and Sperandio, 2012; McGannon et al., 2010). Antimotility agents, narcotics, non-steroidal anti-inflammatory drugs and antibiotics could aggravate the neurological complications and HUS progression by EHEC infections and thus are not recommended (Pacheco and Sperandio, 2012). Antibiotics are known to induce the phage lytic cycle and thereby lead to expression and dissemination of Stx from the dying bacteria. They are believed to worsen the outcome of EHEC infections and their endorsement for treatment of EHEC infections remains debatable (Zhang et al., 2000; McGannon et al., 2010). Reports suggest that antibiotics that target the DNA synthesis like quinolones, Mitomycins, Amplicillin and Ciprofloxacin could increase the Stx production, whereas, antibiotics that target the protein synthesis like Azithromycin, Doxycycline, Fosfomycin and Gentamycin inhibits Stx production (Wong et al., 2000; Los et al., 2010; McGannon et al., 2010). Certain antibiotics like Ceftriaxone and Rifampin are documented to have no effects on Stx production (Zhang et al., 2000). Clearly, it is still premature to use antibiotics for treatment of EHEC infections. There are several other strategies that seem promising in the management of EHEC infections. They are discussed below. However, it is interesting to note that none of these strategies have entered the market so far and data with regards to their human efficacy is still lacking.

5.2.1. Quorum sensing inhibitors

Quorum sensing (QS) is defined as a process of bacterial communication. Pathogenic bacteria like EHEC, sense their cell density and communicate with one another using chemical molecules called autoinducers (Bassler, 1999; Hughes et al., 2009). QS controls the expression of bacterial virulence factors during an infection. QS inhibitors could interfere with Stx production and EHEC virulence. Anti-EHEC for development of QS inhibitors typically involves high-throughput screening of chemical libraries. LED209 is a small molecule that was shown to down-regulate the virulence and Stx production in EHEC via binding to the cognate receptor QseC. LED209 exhibited limited toxicity against bacterial and human cells. Specifically, LED209 was able to supress the Stx production, motility and A/E (attaching and effacing) lesions of EHECs *in vitro* (Rasko et al., 2008).

5.2.2. Pyocins

Bacteriocins like pyocins (produced by *P. aeruginosa*) bind to bacterial cell surface, forms pores on cell membrane, and thereby leads to membrane depolarization and cell death

(Scholl et al., 2009). Ritchie, et al. (2011) demonstrated that an engineered pyocin specific against *E. coli* O157 ameliorated the diseased condition caused by EHECs *in vivo* (Ritchie et al., 2011). LPS degradation was the reason ascribed to the mode of action of pyocins on EHECs.

5.2.3. Vaccines and immunotherapy

Vaccines are the most powerful tools in prophylaxis of infectious diseases. Vaccine-resistant strains have never emerged so far. This is thought to be due to the presence of multiple immunogenic epitopes on a vaccine (Abdelmohsen et al., 2017). With the fields of reverse vaccinology, novel adjuvants and omics technologies, it is now possible to develop vaccines against every pathogen (Rappuoli et al., 2011). Efforts in raising antibodies against the Stx have been undertaken in the previous years. Anti-Stx antibodies are postulated to prevent the pathogenesis of EHECs *in vivo* by neutralizing Stx (Yamagami et al., 2001; Bitzan et al., 2009; Lopez et al., 2010; Dickey et al., 2017). For example, the chimeric antibody against Stx2, Shigamab (developed by Bellus Health), neutralizes the toxin just after its production (Dickey et al., 2017).

5.2.4. Toxin binding inhibitors

These involve inhibitors that prevent the binding of Stx to its Gb3 receptor on human cells. Gb3 ligand mimics possessing the trisaccharide moieties could subsequently neutralize the toxin *in vitro* and *in vivo* (Kitov et al., 2000). Examples of these mimics include, SUPERTWIG, glycan encapsulated gold nanoparticles, Synsorb Pk etc. (Takeda et al., 1999; Nishikawa et al., 2005; Kale et al., 2008). However, most of these inhibitors are still at their infancy and immense translational research is needed for their clinical usage.

5.2.5. Probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Cordonnier et al., 2017). *E. coli* Nissle 1917, lactic acid bacteria (lactobacilli, enterococci, bifidobacteria) and yeasts (*Saccharomyces* genus) represent the most common probiotics clinically used (Rund et al., 2013; Stanford et al., 2014; McFarland, 2015; Mohsin et al., 2015; Varankovich et al., 2015). Probiotics are known to act on pathogenic bacteria via direct antagonism, immunomodulation or competitive exclusion mechanisms (Preidis et al., 2011). Probiotics like *Bifidobacterium* sp., *Clostridium butyricum*, *E. coli* Nissle 1917, *Lactobacilli* strains and *Saccharomyces cerevisiae* are reported to exhibit anti-EHEC effects (antagonism against growth, *stx*

expression, adhesion and colonization) (Cordonnier et al., 2017). An in-depth knowledge on several factors (like dosage and duration of course of probiotics, mode of administration, risk of possible adverse metabolic effects due to manipulation of existent microbiota and transfer of resistance from probiotics to the pathogenic strains in the GI tract) is needed before the marketing and usage of probiotics in treatment of EHEC infections (Cordonnier et al., 2017).

5.2.6. Anti-Stx NPs

NPs have always been valuable in treatment of bacterial infections. Nature is endowed with chemically diverse substances that can be used for clinical applications. Few terrestrial and plant based-NPs (phytochemicals) have shown promising effects against EHECs (Bommarius et al., 2013; Friedman and Rasooly, 2013; Lee et al., 2013; Noel et al., 2013). These NPs act against EHECs by negatively regulating the growth, Stx production, QS processes and inhibiting the cytotoxic effect of the produced Stx on human cells. Examples of anti-EHEC NPs include cinnamon oil, indole-3-acetaldehyde, curcumin, phenethyl isothiocyanate etc. (Sood et al., 2001; Moon et al., 2006; Lee et al., 2012; Nowicki et al., 2014; Sheng et al., 2016). Up to our knowledge, the potential of marine realm against EHEC infections has been left untouched so far. Thus, it is an exciting strategy to explore the anti-EHEC potential of MNPs.

In this Ph.D. thesis, the anti-Stx effect of strepthonium A isolated from marine sponge-derived *Streptomyces* sp. SBT345 has been reported. Previous metabolomic analysis revealed that *Streptomyces* sp. SBT345 is a chemically distinct strain (Cheng, 2016). The compounds isolated from this strain (in the previous years) is shown in **Figure 2B**. The chlorinated quinolone compound ageloline A isolated from liquid fermentation of *Streptomyces* sp. SBT345 exhibited anti-oxidant and anti-chlamydial effects (Cheng, 2016b). In another study, solid fermentation of *Streptomyces* sp. SBT345 resulted in production of the cytotoxic phenoxazin analogue streptoxaxine A along with the known phenoxazin antibiotics, phencomycin and tubermycin B (Cheng, 2016a). Overall, results indicate the rich chemical and biological potential of *Streptomyces* sp. SBT345. Further, genome mining and elicitation experiments with *Streptomyces* sp. SBT345 should be performed to shed lights on the yet undetected compounds produced by this strain.

Strepthonium A is a quaternary ammonium compound and shares structural similarity to the non-chlorinated surfactant benzethonium. The latter has been widely used as a bactericide and is predominantly present as an ingredient in deodorants, cosmetics, mouth washes and

medications (Hikiba et al., 2005; Oztekin and Erim, 2005; Sugimoto et al., 2008; Oyanagi et al., 2012). By contrast with benzethonium, strepthonium A (at 80 µM) displayed no effects on the growth of EHEC strain EDL933 rather selectively inhibited the Stx production. Thus, less pressure for development of resistance to strepthonium A is expected. Lipinski's rule of five is used for the analysis of drug-like physiochemical properties. It is an algorithm consisting of four rules (based on molecular weight, log P, hydrogen bond donor and hydrogen bond acceptor) that determines the drug-like nature of a candidate. It could be observed that strepthonium A follows the Lipinski's rule of five (molecular weight<500 Da, CLogP value:4.908, three hydrogen bond acceptors and no hydrogen bond donors) and is therefore drug-like. With the chlorination in the 2- position of the phenoxy group in strepthonium A, lower cytotoxic effects are expected. Further, its cationic nature could restrict its membrane permeability and the consequent systemic effects after the oral uptake. The absence of benzethonium in the production facilities and microbiological media points that the isolated compound is not a bio-transformation product. However, feeding experiments are necessary to confirm the biosynthetic origin of strepthonium A. Further, the compound needs to be chemically synthesized and induction of resistance (multi-passage experiments of EHECs with increasing concentrations of strepthonium A), cytotoxicity profiling (*in vitro* and *in vivo*), and detailed *in vitro* studies are certainly needed to prove its medicinal application in curtailing EHEC infections.

5.3. Anti-biofilm approaches: state-of-the-art

Bacteria typically race for surfaces, establish biofilms and lead to recalcitrant and resistant infections (Gristina et al., 1988; Busscher et al., 2012). The formation of biofilms on different surfaces (biotic or abiotic) is a massive concern in medical, marine and food industrial settings. Consequently, several antibiofilm strategies have been developed. These strategies fall into the four categories (**Figure 3**) aiming at prevention, weakening, disruption or killing of bacterial biofilms (Bjarnsholt et al., 2013; van Tilburg Bernardes et al., 2015; Gupta et al., 2016).

Figure 3: Existing anti-biofilm strategies aiming at preventing/weakening/disrupting/killing for management of biofilm infections. Active and slow metabolism cells represent the susceptibility of bacterial cells to antibiotic treatment. It is expected that a combination of the four strategies could lead to management of bacterial biofilms. Modified from (Bjarnsholt et al., 2013).

These strategies based on the stage of biofilm targeted are summarized below:

5.3.1. Prevention

Pre-conditioning of a surface by methods like treatment with chemicals, imprint special 3D patterns or surface hydrophobicity etc. are some of the technologies that inhibit the bacterial attachment to different surfaces (de Carvalho and da Fonseca, 2007; Pogodin et al., 2013; Kim et al., 2015b). Antibiotic coatings, pre-treating the surfaces with UV radiations and addition of antibodies or enzymes have proven to prevent the formation of bacterial biofilms on different surfaces (Frederiksen et al., 2006; Berra et al., 2008; Bak et al., 2011; O'Grady et al., 2011).

5.3.2. Weakening

Certain anti-biofilm approaches involve weakening the bacteria by disarming them. This helps to improve the activity of existing antibiotics on biofilm or even the host immune responses. Virulence factors, QS, sRNAs, iron metabolism are usually targeted. Antibodies, compounds, QS inhibitors, sRNAs, siderophore-metal or siderophore-antibiotic conjugates have been documented to weaken the bacteria in biofilms (Bjarnsholt et al., 2013). However,

many of these approaches are species or even strain specific which limits their clinical applicability.

5.3.3. Disruption

Breaking the bacterial networks by dispersing agents is another interesting approach. Typically, a disruption process could release bacterial cells from biofilms and render them susceptible to host immune clearance and antibiotic treatments. This strategy typically requires an antibiotic treatment in combination to clear the released cells. Disruption of biofilm could be performed by mechanical scrubbing, treatment with ultrasonic waves, enzymes (DNases, glycoside hydrolases, alginate lyases) or compounds acting on the matrix (e.g. cis-2-decanoic acid and nitric oxide) (Frederiksen et al., 2006; Kaplan, 2009; Young et al., 2010; Bjarnsholt et al., 2013; Fleming and Rumbaugh, 2017). However, the lack of *in vivo* data with these methods and the risk of induction of inflammatory response in the host by biofilm-released cells have warranted their usage (Franca et al., 2016).

5.3.4. Killing

Mono-therapy with single effective compounds like Gallium nitrate or combinatorial therapy with antibiotics (meropenem, colistin, azithromycin) that act under conditions of oxygen limitations and low metabolic activity, and phage therapy appear to be promising strategies in effectively killing bacterial cells in a biofilm (Hughes et al., 1998; Hill et al., 2005; Kohler et al., 2007; Pamp et al., 2008).

With the aid of "aBiofilm" platform, it is now possible to get access to all the anti-biofilm agents discovered till date (Rajput et al., 2018). This platform comprises 5027 anti-biofilm agents (1720 unique) reported between 1988-2017. The list includes chemical compounds, peptides, phages, secondary metabolites, extracts, nanoparticles and antibodies targeting over 140 organisms including bacteria and fungi. Particularly, with the predictor function of this database it is possible to identify the anti-biofilm potential of an unknown substance with an accuracy of ~80%. Further, the stages of biofilm targeted and chemical diversity of different compounds could be understood with this platform. However, this platform lacks prediction of strain specific anti-biofilm activity. Nevertheless, this platform could be used for determining the anti-biofilm effects of known or unknown compounds and aid in discovery of new anti-biofilm substances.

However, none of the agents described above have made it to the market. This is due to the lack of translational science to upscale the laboratory findings to industrial settings. The

enormous cost involved, lack of sufficient investments by the pharmaceutical sectors, the question of returns from these investments, vagueness in regulatory steps for approval are some of the other reasons tagged to the inexistence of biofilm control agents in the market. Glimmers of hope could still be seen from the growing discovery of new anti-biofilm agents applicable in medical areas of research (Romero and Kolter, 2011).

NPs are rich in antagonizing the biofilm processes in pathogenic bacteria. Various phytochemicals and MNPs were previously shown to possess anti-biofilm properties (Chusri et al., 2012; Nair et al., 2016; Silva et al., 2016; Algburi et al., 2017; Wang et al., 2017). Secondary metabolites produced by actinomycetes have been increasingly tested for anti-biofilm applications (Lee et al., 2012; Park et al., 2012; Park et al., 2016).

In this Ph.D. thesis, the anti-biofilm potential of *Streptomyces* sp. SBT343 and SBT348 is reported. Both these strains were previously isolated from the Mediterranean sponge *Petrosia ficiformis* (Cheng et al., 2015). SEM analysis indicated their similarity in morphology to other *Streptomyces* sp. reported in the literature (**Figure 4**). No biological activities have been reported until now from *Streptomyces* sp. SBT343. *Streptomyces* sp. SBT348 is a candidate actinomycetes strain that had been previously prioritized in a strain collection comprising 64 different Mediterranean sponge-associated actinomycetes based on its distinct HPLC, LC-HRMS and NMR profiles (Cheng et al., 2015; Cheng, 2016). Metabolomics, de-replication tools and PCA analysis had confirmed the chemical uniqueness of this strain. Extracts from both solid and liquid fermentation of SBT348 revealed chemical richness and displayed anti-oxidant, anti-microbial and anti-trypanosomal activities (Cheng, 2016). Four different compounds including the new cytotoxic cyclic dipeptide petrocidin A and the known 2,3-dihydroxybenzamide, 2,3-dihydroxybenzoic acid and maltol have been reported from the solid fermentation of *Streptomyces* sp. SBT348 (Cheng et al., 2017). Thus, these strains have a huge chemical potential in them which could be used for discovery of new metabolites through genome mining and elicitation platforms.

Figure 4: SEM analysis of *Streptomyces* sp. SBT343 (A, B) and SBT348 (C, D). Magnifications of X1500 and X10000 are represented. Scale bar: 10 and 1 µm.
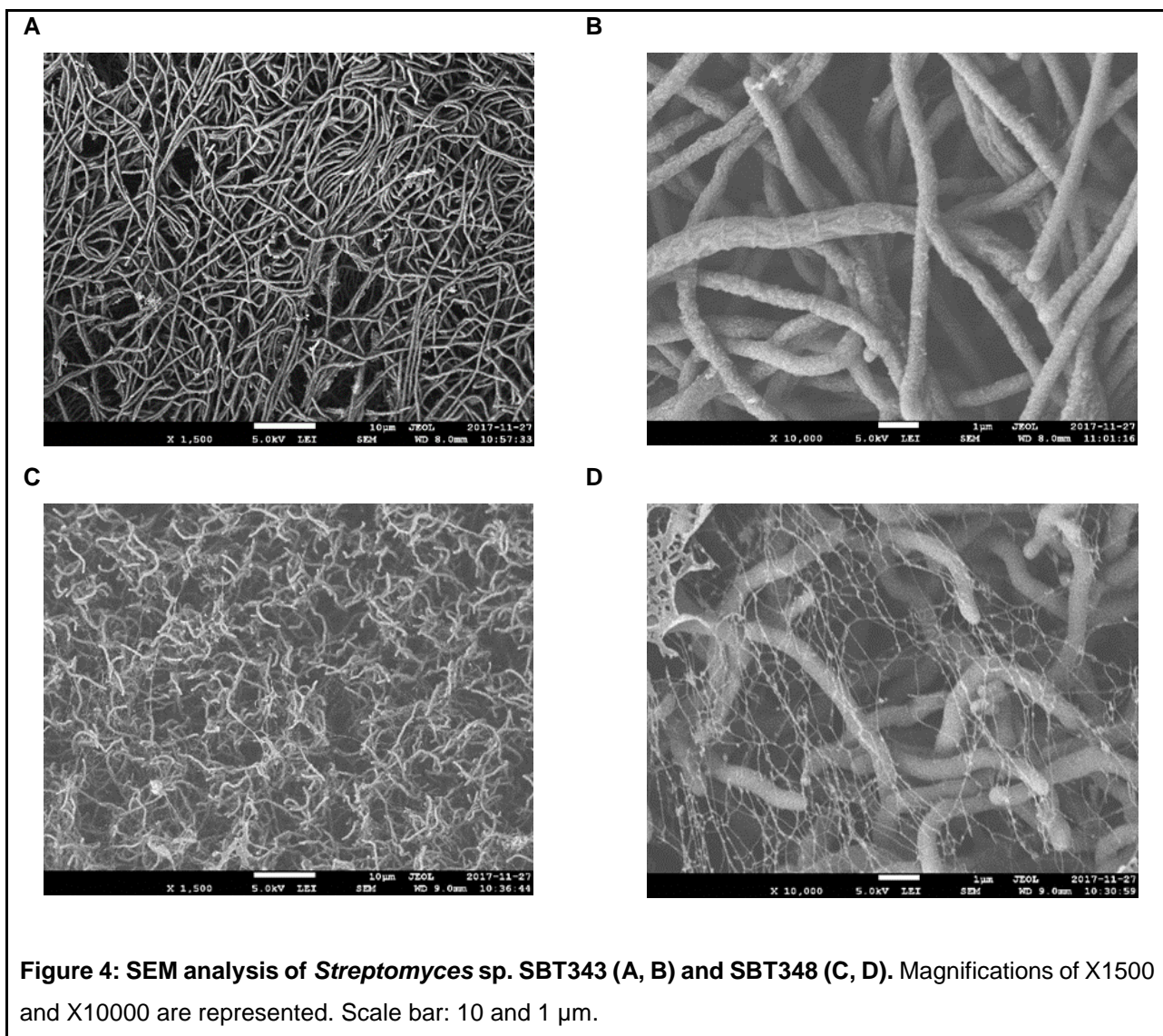
Up-scaling of the fermentation conditions and bioassay-guided fractionation was employed in both the studies with *Streptomyces* sp. SBT343 and SBT348 extracts (**Chapters 3 and 4**) to identify the active component(s) in the extract proportions. Batch fermentation was employed in large scale under liquid and solid fermentation conditions (50 l for SBT343; 1000 plates for SBT348). This was followed by extraction and fractionation experiments. Bioassay-guided isolation is conventionally used in NP drug discovery programs and essentially involves activity testing at every step in the fractionation procedure. Loss of activity during fractionation, limited isolation of trace compounds and repeated isolation of known compounds are some of the limitations of this strategy (Inui et al., 2012; Weller, 2012). However, with the aid of metabolomic tools, de-replication strategies and combining them with fractionation procedures it is now possible to identify different compounds in a mixture and linking them to their structural data.

A loss of activity was seen after bioassay-guided fractionation of the anti-biofilm SBT343 extract. However, a combination of the different fractions obtained (in equal volume ratios) and testing against biofilm inhibition revealed that the activity was due to the synergism of different compounds in the extract and was not an effect of a single compound (**Figure 5**). Hence, detailed investigations into these fractions of SBT343 extract were discontinued.

Figure 5: Bioassay-guided fractionation of SBT343 extract. None of the nine fractions generated from fractionation of SBT343 extract (n-hexane, F2, F5, F7, F8, F9, F11, F13, F14) resulted in anti-biofilm effects against biofilm formation (OD_{492}) of *S. epidermidis* RP62A, while the SBT343 extracts before and after fractionation procedures were still active. Further, combination of the nine fractions (F_{comb}) in equal volumes and testing (0.250 mg/ml) restored the activity. This indicates that the activity in SBT343 extract was due to synergism of various components. TSB, tryptic soy broth; $DMSO_T$, DMSO treatment (3.75%); $MC_{T(old)}$, medium control before fractionation; $SBT343_{(old)}$, extract before fractionation; $MC_{T(new)}$, remaining medium control after fractionation; $SBT343_{(new)}$, remaining extract after fractionation; ****p,0.0001 (as determined by Student's t-test).

In contrast to bioassay-guided fractionation of SBT343, results with SBT348 demonstrated the excellent applicability of this technique in successful isolation of active compound(s) (**Chapter 4, Figure 2A**). These observations indicate the complexity and resistance of different compound(s) (present in NP-based-extracts) to isolation procedures. Interestingly, fractionation of SBT348 extract resulted in identification of several anti-biofilm fractions (SKC1, SKC2, SKC3, SKC4, and SKC5). Of the five active fractions, only SKC3 displayed high purity (identified with analytical HPLC; **Chapter 4**, **Figure S2**) and potent anti-biofilm activities (BIC_{90}: 3.95 µg/ml; MIC: 31.25 µg/ml against *S. epidermidis* RP62A). Hence, SKC3

was investigated in detail for its mechanism of action, cytotoxicity profiling and broad-spectrum anti-staphylococcal effects.

The absence of impurities or contaminants in SKC3 was analysed with diffusion ordered spectroscopy (DOSY). DOSY is an NMR technique employed in NP chemistry research to identify the purity of component and presence of different components in a mixture. Diffusion parameters (diffusion coefficients) are different for different components in a mixture, and this forms the basis of DOSY analysis. DOSY spectra of SKC3 revealed that SKC3 is pure and devoid of contaminating impurities. Further, all the accompanying NMR spectra of SKC3 (provided in the CD attached to this thesis) indicates the high complexity and unusual structure of SKC3. Structural elucidation of SKC3 with the obtained NMR and HR-MS data is currently under investigation (in collaboration with Prof. Dr. Rolf Müller, Helmholtz Institute of Pharmaceutical Research, Saarland, Germany).

Transcriptomics has been increasingly used for studying the mechanism of action of anti-biofilm and antibiotic compounds (Pietiainen et al., 2009; Riordan et al., 2011; Sianglum et al., 2012; Kim et al., 2015a). It provides an overall view on the gene expression of various processes in bacteria, thereby the metabolic and physiological states. Selection of appropriate time points are essential for transcriptome experiments. Kinetics of biofilm formation of *S. epidermidis* RP62A (with a starting OD_{600} of 0.05) was assessed in the presence of SKC3 ($2xBIC_{90}$: 7.8 µg/ml) (**Figure 6A**). Results obtained indicated that the biofilm formation was inhibited between 3-4 h. However, to increase the RNA yield together with minimal usage of compound, the initial culture OD_{600} was increased to 1.0. Subsequently, it was found that the biofilm formation was inhibited between 1-3 h (**Figure 6B**). Thus, RNA was isolated at 20 min and 3 h post-treatment with SKC3 (62.5 µg/ml). In parallel, CFU determinations were done at time points of 0 h, 20 min and 3 h to eliminate the effect of SKC3 on growth at this concentration. It was observed that treatment with SKC3 did not significantly affect the CFUs of *S. epidermidis* (Log CFUs/ml remained at values around 8-8.5). The isolated RNA was subjected to sequencing and transcriptome analysis.

Figure 6: Biofilm kinetics of *S. epidermidis* RP62A in the presence of SKC3. (A) Kinetics with initial OD_{600} of 0.05. It could be seen that in the presence of SKC3, biofilm formation was inhibited between 3-4 h. **(B)** Kinetics with initial OD_{600} of 1.0. It could be seen that increasing the initial OD_{600} to 1.0, subsequently increased the effective concentrations. Further, a clear inhibition of biofilm formation was seen when SKC3 was used at 31.25 or 62.5 µg/ml at time points between 1-3 h. Thus, time points of 20 min (early) and 3 h (late) were chosen for RNA sequencing and transcriptome analysis.

In both the experiments above, SKC3 was added at t (0 h) and control consisted of treatment with DMSO (3.75%) at t (0 h). UT, untreated *S. epidermidis* RP62A; MIC, minimum inhibitory concentration (determined with initial OD_{600} of 0.05).

Evidences from transcriptome data has outlined that SKC3 possibly works by overall negative regulation on central metabolism of staphylococci. Transcriptome data of SKC3 treated staphylococci at 20 min time point revealed upregulation of transcripts of members of certain two-component systems (involving membrane bound histidine kinase and corresponding response regulator) indicating the possible recognition of SKC3 by staphylococci. A few genes encoding efflux proteins and proteins documented to confer antibiotic resistance in other bacteria were also upregulated at this time point. Additional data on the exact role of these efflux systems and resistance proteins in *S. epidermidis* RP62A is necessary to draw conclusions on the same. Nevertheless, experiments involving induction of staphylococcal resistance to SKC3 needs to be performed to better understand the life cycle of SKC3 in medicinal utilization aspects. Transcripts encoding several key proteins and enzymes involved in carbon (glycolysis, gluconeogenesis, TCA cycle), amino acids, proteins, lipids, energy (synthesis of purine, folate, pyrimidines) metabolism and export were significantly down-regulated at 3 h staphylococcal samples treated with SKC3. Further, the absence of direct influence of SKC3 on genes related to biofilm formation indicates that

biofilm inhibitory effects of SKC3 may be due to secondary effects (such as down-regulation of *purR* and iron-sequestration genes).

Cytotoxicity to mammalian systems is an important criterion regarding applicability of NPs to medications. MTT assay and Alamar blue assay are two commonly used assays in determining the cytotoxicity of compounds *in vitro* on cell lines. In this study, alamar blue assay demonstrated the non-toxic nature of the anti-biofilm SBT343 and SBT348 extracts and the compound SKC3 on the mouse macrophage J774.1 and fibroblast NIH/3T3 cell lines. In addition to the *in vitro* cytotoxicity data, *in vivo* toxicity information is essential to extrapolate the results to human systems before pre-clinical applications. Simple invertebrate animal model systems like *Artemia salina* (brine shrimp), *Caenorhabditis elegans* (round worm), *Danio rerio* (zebra-fish), *Drosophila melanogaster* (fruit fly) and *G. melonella* (greater wax moth larvae) have been increasingly employed to determine the toxic effects of NPs and compounds prior to mammalian models (Freires et al., 2017). This pre-screening step has the advantage in eliminating the concerned failure in mammalian model experiments and reducing the number of animals in pre-clinical screens. However, a single compound could elicit differential toxicity on different animal models and thus, the extrapolation of results to human applications should be carefully defined. In this thesis work, *G. melonella* has been used to study the toxicity of the antibiofilm extracts and compound SKC3 (**Figure 7A; Chapter 4, Figure 5)**. All the tested substances in the study showed no toxic effects on the larval system.

G. melonella is a very good *in vivo* model for studying the virulence of different staphylococci and other pathogenic bacteria (Junqueira, 2012). The advantages of *G. melonella* over the other *in vivo* model systems in toxicity analysis is explained in **Chapter 4**. *S. aureus* USA Lac* was the most virulent strain among the staphylococcal strains used in the study. It resulted in a completed death of all the larvae at 72 h pointing its associated pathogenicity (**Figure 7B**).

Figure 7: (A) *In vivo* toxicity of SBT343 extract on *G. melonella* larvae. Larvae were administered with different amounts of SBT343 extract (equivalent to their effective anti-biofilm concentrations). Medium control included the extract generated from modified ISP2 medium in which *Streptomyces* sp. SBT343 was grown. Toxicity was observed only in larval populations treated with SBT343 extract (250 µg or higher).

(B) Virulence potential of different *S. aureus* strains in the study. Larvae were administered with different strains (inoculation OD_{600} of 0.1) or with endotoxin-free PBS vehicle (vehicle control). Methicillin-resistant *S. aureus* Mu50, Col strains did not affect the larval survival significantly. However, *S. aureus* USA300 Lac* (MRSA), RF122 and Newman (MSSA) strains significantly reduced the survival rates. It could be observed that *S. aureus* USA300 Lac* strain was the most pathogenic strain and lead to 100% death of larvae (72 h), followed by *S. aureus* RF122 and Newman strains. Interestingly, *S. epidermidis* RP62A showed no effects on larval survival (data not shown). This could be due to the absence of virulence factors (other than biofilms) in this commensal bacterium. Positive control MeOH (Roth GmbH, Germany) killed all the larvae 1 h post injection.

Log-rank-test (Mantel-Cox) was used to determine the statistical significance ($p < 0.05$, statistically significant) of different survival curves. Each group consisted of 10 larvae and three independent experiments were performed to evaluate the statistical significance.

The ability of SKC3 to protect *G. melonella* from *S. aureus* USA Lac* was also independently assessed. Results indicated that administration of SKC3 (at effective or several fold higher concentrations) together with *S. aureus* USA Lac* could not increase the survival rates of larvae, in comparison to the infected group (**Figure 8**). The cause behind this failure is unknown and needs further *in vivo* evaluation of SKC3 on other model systems.

Figure 8: *In vivo* anti-staphylococcal activity of SKC3. Larvae were administered with cocktails of *S. aureus* USA300 Lac* (inoculation OD_{600} of 0.1) with endotoxin-free PBS vehicle (control) or SKC3 (MIC, 2xMIC, 200xMIC) to evaluate the *in vivo* antibiotic effects of SKC3. Negative controls included larvae that received no injection, PBS vehicle only, C3 only (200XMIC) or *S. aureus* USA300 Lac* only. Pure MeOH (Roth GmbH, Germany) that killed the larvae was the positive control in the experiment. Log-rank-test (Mantel-Cox) was used to determine the statistical significance ($p<0.05$, statistically significant) of different survival curves. No significant difference was observed between the groups that received the cocktail control and cocktail with SKC3. This reveals that SKC3 was ineffective in protecting the larvae from staphylococcal infection. Each group consisted of 10 larvae and three independent experiments were performed to evaluate the statistical significance. MIC, 15.62 µg/ml; ns, not significant.

Taken together, the findings reveal that both the *Streptomyces* spp. (SBT343 and SBT348) contain a reservoir of anti-biofilm compounds that are heat-stable and non-proteinaceous in nature. Further, their broad-spectrum effects on different staphylococcal strains, potency in

eliminating the biofilm formation on different surfaces (polystyrene, glass, silicone, contact lens and titan metal) and lack of toxicity suggest their possible applications in medications or antibiofilm coatings on clinically relevant materials. This could mollify the complications caused by DRIs.

6. Conclusion and future perspectives

Overall, this Ph.D. study highlights the potential of actinomycetes in producing new anti-infective substances against pathogenic bacteria like staphylococci and EHECs. The specific antagonistic activities of extracts and/or compounds derived from sponge-associated *Streptomyces* against biofilm formation and Shiga toxin production underpins their application in management of these bacterial infections.

As a step forward, genomes of the three *Streptomyces* sp. (SBT343, SBT345 and SBT348) should be sequenced. This will assist in the identification of biosynthetic gene clusters and the subsequent discovery of metabolic pathways responsible for synthesis of the anti-infective compound(s). The compounds could then be synthesized in greater amounts either by heterologous expression of the identified biosynthetic genes in a suitable host, or by semi-synthetic approaches. Through an in-depth understanding into the biosynthesis regulation, strain engineering and optimisation of fermentation conditions the yield of the compound production could be enhanced.

Elicitation experiments involving co-culture with pathogenic bacteria or addition of elicitors such as microbial cell components, lysates, inorganic compounds etc. could be done to activate the production of new compound(s) from these metabolically rich *Streptomyces* strains.

Further, omics-based approaches like genomics, transcriptomics and metabolomics will help in identification of self-resistance mechanism(s) in these strains and the generated data could help in understanding the mechanism of action of these compound(s). Another approach involving induction of resistance experiments through passaging of bacterial cells in the presence of increasing concentration of these compound(s) could help in assessment of the development of drug resistance in pathogenic bacteria.

An integration of all the above approaches could lead to the discovery of yet undiscovered drugs from these biotechnologically profitable actinomycetes.

7. Bibliography (introduction and discussion)

Abdelmohsen, U.R., Balasubramanian, S., Oelschlaeger, T.A., Grkovic, T., Pham, N.B., Quinn, R.J., and Hentschel, U. (2017). Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. *Lancet Infect Dis* 17, e30-e41.

Abdelmohsen, U.R., Bayer, K., and Hentschel, U. (2014). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat Prod Rep* 31, 381-399.

Abdelmohsen, U.R., Grkovic, T., Balasubramanian, S., Kamel, M.S., Quinn, R.J., and Hentschel, U. (2015). Elicitation of secondary metabolism in actinomycetes. *Biotechnol Adv* 33, 798-811.

Abdelmohsen, U.R., Pimentel-Elardo, S.M., Hanora, A., Radwan, M., Abou-El-Ela, S.H., Ahmed, S., and Hentschel, U. (2010). Isolation, phylogenetic analysis and anti-infective activity screening of marine sponge-associated actinomycetes. *Mar Drugs* 8, 399-412.

Algburi, A., Comito, N., Kashtanov, D., Dicks, L.M., and Chikindas, M.L. (2017). Control of biofilm formation: antibiotics and beyond. *Appl Environ Microbiol* 83.

Aljabri, K., Garlitski, A., Weinstock, J., and Madias, C. (2018). Management of device infections. *Card Electrophysiol Clin* 10, 153-162.

Allen, R.C., Popat, R., Diggle, S.P., and Brown, S.P. (2014). Targeting virulence: can we make evolution-proof drugs? *Nat Rev Microbiol* 12, 300-308.

Aminov, R.I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol* 1, 134.

Anthouard, R., and Dirita, V.J. (2015). Chemical biology applied to the study of bacterial pathogens. *Infect Immun* 83, 456-469.

Arias, C.A., and Murray, B.E. (2015). A new antibiotic and the evolution of resistance. *N Engl J Med* 372, 1168-1170.

Bak, J., Begovic, T., Bjarnsholt, T., and Nielsen, A. (2011). A UVC device for intra-luminal disinfection of catheters: *in vitro* tests on soft polymer tubes contaminated with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. *Photochem Photobiol* 87, 1123-1128.

Barros, M., Branquinho, R., Grosso, F., Peixe, L., and Novais, C. (2014). Linezolid-resistant *Staphylococcus epidermidis*, Portugal, 2012. *Emerg Infect Dis* 20, 903-905.

Bassler, B.L. (1999). How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Opin Microbiol* 2, 582-587.

Bauwens, A., Betz, J., Meisen, I., Kemper, B., Karch, H., and Muthing, J. (2013). Facing glycosphingolipid-Shiga toxin interaction: dire straits for endothelial cells of the human vasculature. *Cell Mol Life Sci* 70, 425-457.

Bauwens, A., Bielaszewska, M., Kemper, B., Langehanenberg, P., Von Bally, G., Reichelt, R., Mulac, D., Humpf, H.U., Friedrich, A.W., Kim, K.S., Karch, H., and Muthing, J. (2011). Differential cytotoxic actions of Shiga toxin 1 and Shiga toxin 2 on microvascular and macrovascular endothelial cells. *Thromb Haemost* 105, 515-528.

Bayari, S.H., Sen, E.H., Ide, S., and Topaloglu, B. (2018). Structural studies on Demospongiae sponges from Gokceada island in the northern Aegean sea. *Spectrochim Acta A Mol Biomol Spectrosc* 192, 368-377.

Becker, K., Heilmann, C., and Peters, G. (2014). Coagulase-negative staphylococci. *Clin Microbiol Rev* 27, 870-926.

Belarbi El, H., Contreras Gomez, A., Chisti, Y., Garcia Camacho, F., and Molina Grima, E. (2003). Producing drugs from marine sponges. *Biotechnol Adv* 21, 585-598.

Berra, L., Kolobow, T., Laquerriere, P., Pitts, B., Bramati, S., Pohlmann, J., Marelli, C., Panzeri, M., Brambillasca, P., Villa, F., Baccarelli, A., Bouthors, S., Stelfox, H.T., Bigatello, L.M., Moss, J., and Pesenti, A. (2008). Internally coated endotracheal tubes with silver sulfadiazine in polyurethane to prevent bacterial colonization: a clinical trial. *Intensive Care Med* 34, 1030-1037.

Bessa, L.J., Buttachon, S., Dethoup, T., Martins, R., Vasconcelos, V., Kijjoa, A., and Martins Da Costa, P. (2016). Neofiscalin A and fiscalin C are potential novel indole alkaloid alternatives for the treatment of multidrug-resistant Gram-positive bacterial infections. *FEMS Microbiol Lett* 363.

Betz, J., Bauwens, A., Kunsmann, L., Bielaszewska, M., Mormann, M., Humpf, H.U., Karch, H., Friedrich, A.W., and Muthing, J. (2012). Uncommon membrane distribution of Shiga toxin glycosphingolipid receptors in toxin-sensitive human glomerular microvascular endothelial cells. *Biol Chem* 393, 133-147.

Bitzan, M., Poole, R., Mehran, M., Sicard, E., Brockus, C., Thuning-Roberson, C., and Riviere, M. (2009). Safety and pharmacokinetics of chimeric anti-Shiga toxin 1 and anti-Shiga toxin 2 monoclonal antibodies in healthy volunteers. *Antimicrob Agents Chemother* 53, 3081-3087.

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M., and Hoiby, N. (2013). Applying insights from biofilm biology to drug development - can a new approach be developed? *Nat Rev Drug Discov* 12, 791-808.

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., and Weber, T. (2013). antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Res* 41, W204-212.

Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H., Northcote, P.T., and Prinsep, M.R. (2007). Marine natural products. *Nat Prod Rep* 24, 31-86.

Bommarius, B., Anyanful, A., Izrayelit, Y., Bhatt, S., Cartwright, E., Wang, W., Swimm, A.I., Benian, G.M., Schroeder, F.C., and Kalman, D. (2013). A family of indoles regulate virulence and Shiga toxin production in pathogenic *Escherichia coli*. *PLoS One* 8, e54456.

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B., and Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* 48, 1-12.

Bouza, E., and Burillo, A. (2010). Oritavancin: a novel lipoglycopeptide active against Gram-positive pathogens including multiresistant strains. *Int J Antimicrob Agents* 36, 401-407.

Brady, S.F., Simmons, L., Kim, J.H., and Schmidt, E.W. (2009). Metagenomic approaches to natural products from free-living and symbiotic organisms. *Nat Prod Rep* 26, 1488-1503.

Braeye, T., Denayer, S., De Rauw, K., Forier, A., Verluyten, J., Fourie, L., Dierick, K., Botteldoorn, N., Quoilin, S., Cosse, P., Noyen, J., and Pierard, D. (2014). Lessons learned from a textbook outbreak: EHEC-O157:H7 infections associated with the consumption of raw meat products, June 2012, Limburg, Belgium. *Arch Public Health* 72, 44.

Brannon, J.R., and Hadjifrangiskou, M. (2016). The arsenal of pathogens and antivirulence therapeutic strategies for disarming them. *Drug Des Devel Ther* 10, 1795-1806.

Bredholdt, H., Galatenko, O.A., Engelhardt, K., Fjaervik, E., Terekhova, L.P., and Zotchev, S.B. (2007). Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. *Environ Microbiol* 9, 2756-2764.

Bringmann, G., Lang, G., Steffens, S., and Schaumann, K. (2004). Petrosifungins A and B, novel cyclodepsipeptides from a sponge-derived strain of *Penicillium brevicompactum*. *J Nat Prod* 67, 311-315.

Brotz-Oesterhelt, H., and Sass, P. (2010). Postgenomic strategies in antibacterial drug discovery. *Future Microbiol* 5, 1553-1579.

Bryers, J.D. (2008). Medical biofilms. *Biotechnol Bioeng* 100, 1-18.

Burrack, L.S., and Higgins, D.E. (2007). Genomic approaches to understanding bacterial virulence. *Curr Opin Microbiol* 10, 4-9.

Bush, K., and Page, M.G.P. (2017). What we may expect from novel antibacterial agents in the pipeline with respect to resistance and pharmacodynamic principles. *J Pharmacokinet Pharmacodyn* 44, 113-132.

Busscher, H.J., Van Der Mei, H.C., Subbiahdoss, G., Jutte, P.C., Van Den Dungen, J.J., Zaat, S.A., Schultz, M.J., and Grainger, D.W. (2012). Biomaterial-associated infection: locating the finish line in the race for the surface. *Sci Transl Med* 4, 153rv110.

Buttachon, S., Ramos, A.A., Inacio, A., Dethoup, T., Gales, L., Lee, M., Costa, P.M., Silva, A.M.S., Sekeroglu, N., Rocha, E., Pinto, M.M.M., Pereira, J.A., and Kijjoa, A. (2018). Bis-indolyl benzenoids, hydroxypyrrolidine derivatives and other constituents from cultures of the marine sponge-associated fungus *Aspergillus candidus* KUFA0062. *Mar Drugs* 16.

Caboche, S. (2014). Biosynthesis: bioinformatics bolster a renaissance. *Nat Chem Biol* 10, 798-800.

Center for Disease Control and Prevention (CDC) (2013). Antibiotic resistance threats in the United States. http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf (accessed Aug 26, 2016).

Center for Disease Control and Prevention (CDC) (2014). Transatlantic taskforce on antimicrobial resistance: progress report.http://www.cdc.gov/drugresistance/pdf/tatfar-progress_report_2014.pdf (accessed Aug 26, 2016).

Challis, G.L. (2008). Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology* 154, 1555-1569.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., and Hanage, W.P. (2015). Antibiotics in agriculture and the risk to human health: how worried should we be? *Evol Appl* 8, 240-247.

Cheng, C. (2016). Metabolomics and dereplication-based isolation of novel bioactive natural products from marine sponge-associated actinomycetes. Ph.D. dissertation, University of Würzburg, Germany.

Cheng, C., Macintyre, L., Abdelmohsen, U.R., Horn, H., Polymenakou, P.N., Edrada-Ebel, R., and Hentschel, U. (2015). Biodiversity, anti-trypanosomal activity screening, and metabolomic profiling of actinomycetes isolated from Mediterranean sponges. *PLoS One* 10, e0138528.

Cheng, C., Othman, E.M., Stopper, H., Edrada-Ebel, R., Hentschel, U., and Abdelmohsen, U.R. (2017). Isolation of Petrocidin A, a new cytotoxic cyclic dipeptide from the marine sponge-derived bacterium *Streptomyces* sp. SBT348. *Mar Drugs* 15.

Cheng, C., Othman, E.M., Fekete, A., Krischke, M., Stopper, H., Ebel, R.E., Mueller, M.J., Hentschel, U., and Abdelmohsen, U.R. (2016a). Strepoxazine A, a new cytotoxic phenoxazin from the marine sponge-derived bacterium *Streptomyces* sp. SBT345. *Tetrahedron Letters* 57, 4196-4199.

Cheng, C., Othman, E.M., Reimer, A., Grüne, M., Pavlovic, V.K., Stopper, H., Hentschel, U., and Abdelmohsen, U.R. (2016b). Ageloline A, new antioxidant and antichlamydial quinolone from the marine sponge-derived bacterium *Streptomyces* sp. SBT345. *Tetrahedron Letters*.

Cheng, G., Dai, M., Ahmed, S., Hao, H., Wang, X., and Yuan, Z. (2016). Antimicrobial drugs in fighting against antimicrobial resistance. *Front Microbiol* 7, 470.

Chusri, S., Sompetch, K., Mukdee, S., Jansrisewangwong, S., Srichai, T., Maneenoon, K., Limsuwan, S., and Voravuthikunchai, S.P. (2012). Inhibition of *Staphylococcus epidermidis* biofilm formation by traditional Thai herbal recipes used for wound treatment. *Evid Based Complement Alternat Med* 2012, 159797.

Cimermancic, P., Medema, M.H., Claesen, J., Kurita, K., Wieland Brown, L.C., Mavrommatis, K., Pati, A., Godfrey, P.A., Koehrsen, M., Clardy, J., Birren, B.W., Takano, E., Sali, A., Linington, R.G., and Fischbach, M.A. (2014). Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters. *Cell* 158, 412-421.

Clatworthy, A.E., Pierson, E., and Hung, D.T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* 3, 541-548.

Coast, J., and Smith, R.D. (2003). Antimicrobial resistance: cost and containment. *Expert Rev Anti Infect Ther* 1, 241-251.

Coates, A., Hu, Y., Bax, R., and Page, C. (2002). The future challenges facing the development of new antimicrobial drugs. *Nat Rev Drug Discov* 1, 895-910.

Cordonnier, C., Thevenot, J., Etienne-Mesmin, L., Alric, M., Livrelli, V., and Blanquet-Diot, S. (2017). Probiotic and enterohemorrhagic *Escherichia coli*: An effective strategy against a deadly enemy? *Crit Rev Microbiol* 43, 116-132.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318-1322.

Curtis, M.M., Russell, R., Moreira, C.G., Adebesin, A.M., Wang, C., Williams, N.S., Taussig, R., Stewart, D., Zimmern, P., Lu, B., Prasad, R.N., Zhu, C., Rasko, D.A., Huntley, J.F., Falck, J.R., and Sperandio, V. (2014). QseC inhibitors as an antivirulence approach for Gram-negative pathogens. *MBio* 5, e02165.

D'costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., and Wright, G.D. (2011). Antibiotic resistance is ancient. *Nature* 477, 457-461.

Da Silva, R.R., Dorrestein, P.C., and Quinn, R.A. (2015). Illuminating the dark matter in metabolomics. *Proc Natl Acad Sci U S A* 112, 12549-12550.

Dalili, D., Amini, M., Faramarzi, M.A., Fazeli, M.R., Khoshayand, M.R., and Samadi, N. (2015). Isolation and structural characterization of Coryxin, a novel cyclic lipopeptide from *Corynebacterium xerosis* NS5 having emulsifying and anti-biofilm activity. *Colloids Surf B Biointerfaces* 135, 425-432.

Dashti, Y., Grkovic, T., Abdelmohsen, U.R., Hentschel, U., and Quinn, R.J. (2017). Actinomycete metabolome induction/suppression with N-acetylglucosamine. *J Nat Prod* 80, 828-836.

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74, 417-433.

De Carvalho, C.C., and Da Fonseca, M.M. (2007). Preventing biofilm formation: promoting cell separation with terpenes. *FEMS Microbiol Ecol* 61, 406-413.

De La Fuente-Nunez, C., Reffuveille, F., Fernandez, L., and Hancock, R.E. (2013). Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* 16, 580-589.

Dethlefsen, L., and Relman, D.A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci U S A* 108 Suppl 1, 4554-4561.

Dickey, S.W., Cheung, G.Y.C., and Otto, M. (2017). Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. *Nat Rev Drug Discov* 16, 457-471.

Donia, M.S., Ruffner, D.E., Cao, S., and Schmidt, E.W. (2011). Accessing the hidden majority of marine natural products through metagenomics. *Chembiochem* 12, 1230-1236.

Donlan, R.M. (2002). Biofilms: microbial life on surfaces. *Emerg Infect Dis* 8, 881-890.

Dunne, W.M., Jr. (2002). Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev* 15, 155-166.

Dyson, L., Wright, A.D., Young, K.A., Sakoff, J.A., and Mccluskey, A. (2014). Synthesis and anticancer activity of focused compound libraries from the natural product lead, oroidin. *Bioorg Med Chem* 22, 1690-1699.

Engelhardt, K., Degnes, K.F., and Zotchev, S.B. (2010). Isolation and characterization of the gene cluster for biosynthesis of the thiopeptide antibiotic TP-1161. *Appl Environ Microbiol* 76, 7093-7101.

Eom, S.H., Kim, Y.M., and Kim, S.K. (2013). Marine bacteria: potential sources for compounds to overcome antibiotic resistance. *Appl Microbiol Biotechnol* 97, 4763-4773.

Escaich, S. (2008). Antivirulence as a new antibacterial approach for chemotherapy. *Curr Opin Chem Biol* 12, 400-408.

Evans, B.S., Zhao, C., Gao, J., Evans, C.M., Ju, K.S., Doroghazi, J.R., Van Der Donk, W.A., Kelleher, N.L., and Metcalf, W.W. (2013). Discovery of the antibiotic phosacetamycin via a new mass spectrometry-based method for phosphonic acid detection. *ACS Chem Biol* 8, 908-913.

Fenical, W., and Jensen, P.R. (2006). Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat Chem Biol* 2, 666-673.

Ferretti, C., Marengo, B., De Ciucis, C., Nitti, M., Pronzato, M.A., Marinari, U.M., Pronzato, R., Manconi, R., and Domenicotti, C. (2007). Effects of *Agelas oroides* and *Petrosia ficiformis* crude extracts on human neuroblastoma cell survival. *Int J Oncol* 30, 161-169.

Fischbach, M.A., and Walsh, C.T. (2009). Antibiotics for emerging pathogens. *Science* 325, 1089-1093.

Fitchett, J.R. (2015). Antibiotics, copayments, and antimicrobial resistance: investment matters. *Lancet Infect Dis* 15, 1125-1127.

Fleming, A. (1945). Nobel lecture 1945.

Fleming, D., and Rumbaugh, K.P. (2017). Approaches to dispersing medical biofilms. *Microorganisms* 5.

Franca, A., Perez-Cabezas, B., Correia, A., Pier, G.B., Cerca, N., and Vilanova, M. (2016). *Staphylococcus epidermidis* biofilm-released cells induce a prompt and more marked *in vivo* inflammatory-type response than planktonic or biofilm cells. *Front Microbiol* 7, 1530.

Frederiksen, B., Pressler, T., Hansen, A., Koch, C., and Hoiby, N. (2006). Effect of aerosolized rhDNase (Pulmozyme) on pulmonary colonization in patients with cystic fibrosis. *Acta Paediatr* 95, 1070-1074.

Freiberg, C., and Brotz-Oesterhelt, H. (2005). Functional genomics in antibacterial drug discovery. *Drug Discov Today* 10, 927-935.

Freires, I.A., Sardi, J.C., De Castro, R.D., and Rosalen, P.L. (2017). Alternative animal and non-animal models for drug discovery and development: bonus or burden? *Pharm Res* 34, 681-686.

Friedman, M., and Rasooly, R. (2013). Review of the inhibition of biological activities of food-related selected toxins by natural compounds. *Toxins (Basel)* 5, 743-775.

Gamage, S.D., Patton, A.K., Hanson, J.F., and Weiss, A.A. (2004). Diversity and host range of Shiga toxin-encoding phage. *Infect Immun* 72, 7131-7139.

Gavrish, E., Bollmann, A., Epstein, S., and Lewis, K. (2008). A trap for *in situ* cultivation of filamentous actinobacteria. *J Microbiol Methods* 72, 257-262.

Gavrish, E., Sit, C.S., Cao, S., Kandror, O., Spoering, A., Peoples, A., Ling, L., Fetterman, A., Hughes, D., Bissell, A., Torrey, H., Akopian, T., Mueller, A., Epstein, S., Goldberg, A., Clardy, J., and Lewis, K. (2014). Lassomycin, a ribosomally synthesized cyclic peptide, kills *Mycobacterium tuberculosis* by targeting the ATP-dependent protease ClpC1P1P2. *Chem Biol* 21, 509-518.

Genilloud, O. (2017). Actinomycetes: still a source of novel antibiotics. *Nat Prod Rep* 34, 1203-1232.

Gerke, C., Kraft, A., Sussmuth, R., Schweitzer, O., and Gotz, F. (1998). Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J Biol Chem* 273, 18586-18593.

Gill, E.E., Franco, O.L., and Hancock, R.E. (2015). Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens. *Chem Biol Drug Des* 85, 56-78.

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M., and Nelson, K.E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science* 312, 1355-1359.

Goldwater, P.N., and Bettelheim, K.A. (2012). Treatment of enterohemorrhagic *Escherichia coli* (EHEC) infection and hemolytic uremic syndrome (HUS). *BMC Med* 10, 12.

Gordon, J.I., and Klaenhammer, T.R. (2011). A rendezvous with our microbes. *Proc Natl Acad Sci U S A* 108 Suppl 1, 4513-4515.

Grenham, S., Clarke, G., Cryan, J.F., and Dinan, T.G. (2011). Brain-gut-microbe communication in health and disease. *Front Physiol* 2, 94.

Griffin, P.M., and Tauxe, R.V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* 13, 60-98.

Gristina, A.G., Naylor, P., and Myrvik, Q. (1988). Infections from biomaterials and implants: a race for the surface. *Med Prog Technol* 14, 205-224.

Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S., and Tribedi, P. (2016). Biofilm, pathogenesis and prevention-a journey to break the wall: a review. *Arch Microbiol* 198, 1-15.

Guskey, M.T., and Tsuji, B.T. (2010). A comparative review of the lipoglycopeptides: oritavancin, dalbavancin, and telavancin. *Pharmacotherapy* 30, 80-94.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2, 95-108.

Harjes, J., Ryu, T., Abdelmohsen, U.R., Moitinho-Silva, L., Horn, H., Ravasi, T., and Hentschel, U. (2014). Draft genome sequence of the antitrypanosomally active sponge-associated bacterium *Actinokineospora* sp. Strain EG49. *Genome Announc* 2.

Harvey, A.L., Edrada-Ebel, R., and Quinn, R.J. (2015). The re-emergence of natural products for drug discovery in the genomics era. *Nat Rev Drug Discov* 14, 111-129.

Hauser, A.R., Mecsas, J., and Moir, D.T. (2016). Beyond antibiotics: new therapeutic approaches for bacterial infections. *Clin Infect Dis* 63, 89-95.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., and Gotz, F. (1996). Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* 20, 1083-1091.

Hennig, S., Nyunt Wai, S., and Ziebuhr, W. (2007). Spontaneous switch to PIA-independent biofilm formation in an *ica*-positive *Staphylococcus epidermidis* isolate. *Int J Med Microbiol* 297, 117-122.

Hentschel, U., Piel, J., Degnan, S.M., and Taylor, M.W. (2012). Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol* 10, 641-654.

Hikiba, H., Watanabe, E., Barrett, J.C., and Tsutsui, T. (2005). Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells. *J Pharmacol Sci* 97, 146-152.

Hill, D., Rose, B., Pajkos, A., Robinson, M., Bye, P., Bell, S., Elkins, M., Thompson, B., Macleod, C., Aaron, S.D., and Harbour, C. (2005). Antibiotic susceptabilities of

Pseudomonas aeruginosa isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions. *J Clin Microbiol* 43, 5085-5090.

Hogberg, L.D., Heddini, A., and Cars, O. (2010). The global need for effective antibiotics: challenges and recent advances. *Trends Pharmacol Sci* 31, 509-515.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35, 322-332.

Hoiby, N., Ciofu, O., Johansen, H.K., Song, Z.J., Moser, C., Jensen, P.O., Molin, S., Givskov, M., Tolker-Nielsen, T., and Bjarnsholt, T. (2011). The clinical impact of bacterial biofilms. *Int J Oral Sci* 3, 55-65.

Hooper, J.N., Hall, K.A., Ekins, M., Erpenbeck, D., Worheide, G., and Jolley-Rogers, G. (2013). Managing and sharing the escalating number of sponge "unknowns": The SpongeMaps project. *Integr Comp Biol* 53, 473-481.

Hooper, L.V., and Gordon, J.I. (2001). Commensal host-bacterial relationships in the gut. *Science* 292, 1115-1118.

Horn, H., Cheng, C., Edrada-Ebel, R., Hentschel, U., and Abdelmohsen, U.R. (2015). Draft genome sequences of three chemically rich actinomycetes isolated from Mediterranean sponges. *Mar Genomics* 24 Pt 3, 285-287.

Hosaka, T., Ohnishi-Kameyama, M., Muramatsu, H., Murakami, K., Tsurumi, Y., Kodani, S., Yoshida, M., Fujie, A., and Ochi, K. (2009). Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. *Nat Biotechnol* 27, 462-464.

Hughes, C.C., Prieto-Davo, A., Jensen, P.R., and Fenical, W. (2008). The marinopyrroles, antibiotics of an unprecedented structure class from a marine *Streptomyces* sp. *Org Lett* 10, 629-631.

Hughes, D.T., Clarke, M.B., Yamamoto, K., Rasko, D.A., and Sperandio, V. (2009). The QseC adrenergic signaling cascade in Enterohemorrhagic *Escherichia coli* (EHEC). *PLoS Pathog* 5, e1000553.

Hughes, K.A., Sutherland, I.W., and Jones, M.V. (1998). Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* 144 (Pt 11), 3039-3047.

Hugon, P., Dufour, J.C., Colson, P., Fournier, P.E., Sallah, K., and Raoult, D. (2015). A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect Dis* 15, 1211-1219.

Ines, T., Amina, B., Khaled, S., and Kamel, G. (2007). Screening of antimicrobial activity of marine sponge extracts collected from Tunisian coast. *Proc West Pharmacol Soc* 50, 152-155.

Inui, T., Wang, Y., Pro, S.M., Franzblau, S.G., and Pauli, G.F. (2012). Unbiased evaluation of bioactive secondary metabolites in complex matrices. *Fitoterapia* 83, 1218-1225.

Jacob, M.E., Foster, D.M., Rogers, A.T., Balcomb, C.C., Shi, X., and Nagaraja, T.G. (2013). Evidence of non-O157 Shiga toxin-producing *Escherichia coli* in the feces of meat goats at a U.S. slaughter plant. *J Food Prot* 76, 1626-1629.

Jandhyala, S.M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., and Nageshwar Reddy, D. (2015). Role of the normal gut microbiota. *World J Gastroenterol* 21, 8787-8803.

Jin, L., Liu, F., Sun, W., Zhang, F., Karuppiah, V., and Li, Z. (2014). *Pezizomycotina* dominates the fungal communities of south China sea sponges *Theonella swinhoei* and *Xestospongia testudinaria*. *FEMS Microbiol Ecol* 90, 935-945.

Johnson, B.K., and Abramovitch, R.B. (2017). Small molecules that sabotage bacterial virulence. *Trends Pharmacol Sci* 38, 339-362.

Ju, K.S., Gao, J., Doroghazi, J.R., Wang, K.K., Thibodeaux, C.J., Li, S., Metzger, E., Fudala, J., Su, J., Zhang, J.K., Lee, J., Cioni, J.P., Evans, B.S., Hirota, R., Labeda, D.P., Van Der Donk, W.A., and Metcalf, W.W. (2015). Discovery of phosphonic acid natural products by mining the genomes of 10,000 actinomycetes. *Proc Natl Acad Sci U S A* 112, 12175-12180.

Junqueira, J.C. (2012). *Galleria mellonella* as a model host for human pathogens: recent studies and new perspectives. *Virulence* 3, 474-476.

Kaeberlein, T., Lewis, K., and Epstein, S.S. (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* 296, 1127-1129.

Kale, R.R., Mcgannon, C.M., Fuller-Schaefer, C., Hatch, D.M., Flagler, M.J., Gamage, S.D., Weiss, A.A., and Iyer, S.S. (2008). Differentiation between structurally homologous Shiga 1 and Shiga 2 toxins by using synthetic glycoconjugates. *Angew Chem Int Ed Engl* 47, 1265-1268.

Kampfer, P., Glaeser, S.P., Busse, H.J., Abdelmohsen, U.R., and Hentschel, U. (2014). *Rubrobacter aplysinae* sp. nov., isolated from the marine sponge *Aplysina aerophoba*. *Int J Syst Evol Microbiol* 64, 705-709.

Kaplan, J.B. (2009). Therapeutic potential of biofilm-dispersing enzymes. *Int J Artif Organs* 32, 545-554.

Kaplan, J.B., Lovetri, K., Cardona, S.T., Madhyastha, S., Sadovskaya, I., Jabbouri, S., and Izano, E.A. (2012). Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. *J Antibiot (Tokyo)* 65, 73-77.

Karmali, M.A., Petric, M., Lim, C., Fleming, P.C., and Steele, B.T. (1983). *Escherichia coli* cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. *Lancet* 2, 1299-1300.

Kersten, R.D., and Dorrestein, P.C. (2009). Secondary metabolomics: natural products mass spectrometry goes global. *ACS Chem Biol* 4, 599-601.

Kim, H.S., Lee, S.H., Byun, Y., and Park, H.D. (2015a). 6-Gingerol reduces *Pseudomonas aeruginosa* biofilm formation and virulence via quorum sensing inhibition. *Sci Rep* 5, 8656.

Kim, S., Jung, U.T., Kim, S.K., Lee, J.H., Choi, H.S., Kim, C.S., and Jeong, M.Y. (2015b). Nanostructured multifunctional surface with antireflective and antimicrobial characteristics. *ACS Appl Mater Interfaces* 7, 326-331.

Kimmitt, P.T., Harwood, C.R., and Barer, M.R. (2000). Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. *Emerg Infect Dis* 6, 458-465.

King, L.A., Loukiadis, E., Mariani-Kurkdjian, P., Haeghebaert, S., Weill, F.X., Baliere, C., Ganet, S., Gouali, M., Vaillant, V., Pihier, N., Callon, H., Novo, R., Gaillot, O., Thevenot-Sergentet, D., Bingen, E., Chaud, P., and De Valk, H. (2014). Foodborne transmission of sorbitol-fermenting *Escherichia coli* O157:H7 via ground beef: an outbreak in northern France, 2011. *Clin Microbiol Infect* 20, O1136-1144.

Kitov, P.I., Sadowska, J.M., Mulvey, G., Armstrong, G.D., Ling, H., Pannu, N.S., Read, R.J., and Bundle, D.R. (2000). Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature* 403, 669-672.

Kleinschmidt, S., Huygens, F., Faoagali, J., Rathnayake, I.U., and Hafner, L.M. (2015). *Staphylococcus epidermidis* as a cause of bacteremia. *Future Microbiol* 10, 1859-1879.

Koenig, J.E., Spor, A., Scalfone, N., Fricker, A.D., Stombaugh, J., Knight, R., Angenent, L.T., and Ley, R.E. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci U S A* 108 Suppl 1, 4578-4585.

Kohler, T., Dumas, J.L., and Van Delden, C. (2007). Ribosome protection prevents azithromycin-mediated quorum-sensing modulation and stationary-phase killing of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 51, 4243-4248.

Konowalchuk, J., Speirs, J.I., and Stavric, S. (1977). Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 18, 775-779.

Korn-Wendisch, F., Kutzner, H.J. (1992). The family *Streptomycetaceae* in the prokaryotes. *Springer-Verlag Inc., New York, USA*, p 921-995.

Kurita, K.L., Glassey, E., and Linington, R.G. (2015). Integration of high-content screening and untargeted metabolomics for comprehensive functional annotation of natural product libraries. *Proc Natl Acad Sci U S A* 112, 11999-12004.

Kwon, H.C., Kauffman, C.A., Jensen, P.R., and Fenical, W. (2006). Marinomycins A-D, antitumor-antibiotics of a new structure class from a marine actinomycete of the recently discovered genus "*Marinispora*". *J Am Chem Soc* 128, 1622-1632.

Lam, K.S. (2006). Discovery of novel metabolites from marine actinomycetes. *Curr Opin Microbiol* 9, 245-251.

Lane, N. (2008). Marine microbiology: origins of death. *Nature* 453, 583-585.

Lang, S., Beil, W., Tokuda, H., Wicke, C., and Lurtz, V. (2004). Improved production of bioactive glucosylmannosyl-glycerolipid by sponge-associated *Microbacterium* species. *Mar Biotechnol (NY)* 6, 152-156.

Leadbetter, M.R., Adams, S.M., Bazzini, B., Fatheree, P.R., Karr, D.E., Krause, K.M., Lam, B.M., Linsell, M.S., Nodwell, M.B., Pace, J.L., Quast, K., Shaw, J.P., Soriano, E., Trapp, S.G., Villena, J.D., Wu, T.X., Christensen, B.G., and Judice, J.K. (2004). Hydrophobic vancomycin derivatives with improved ADME properties: discovery of telavancin (TD-6424). *J Antibiot (Tokyo)* 57, 326-336.

Leal, M.C., Madeira, C., Brandao, C.A., Puga, J., and Calado, R. (2012). Bioprospecting of marine invertebrates for new natural products - a chemical and zoogeographical perspective. *Molecules* 17, 9842-9854.

Leal, M.C., Sheridan, C., Osinga, R., Dionisio, G., Rocha, R.J., Silva, B., Rosa, R., and Calado, R. (2014). Marine microorganism-invertebrate assemblages: perspectives to solve the "supply problem" in the initial steps of drug discovery. *Mar Drugs* 12, 3929-3952.

Leary, J.T., Werger, M.M., Broach, W.H., Shaw, L.N., Santoni, B.G., Bernasek, T.L., and Lyons, S.T. (2017). Complete eradication of biofilm from orthopedic materials. *J Arthroplasty* 32, 2513-2518.

Lee, J.H., Cho, H.S., Joo, S.W., Chandra Regmi, S., Kim, J.A., Ryu, C.M., Ryu, S.Y., Cho, M.H., and Lee, J. (2013). Diverse plant extracts and trans-resveratrol inhibit biofilm formation and swarming of *Escherichia coli* O157:H7. *Biofouling* 29, 1189-1203.

Lee, J.H., Kim, Y.G., Kim, C.J., Lee, J.C., Cho, M.H., and Lee, J. (2012). Indole-3-acetaldehyde from *Rhodococcus* sp. BFI 332 inhibits *Escherichia coli* O157:H7 biofilm formation. *Appl Microbiol Biotechnol* 96, 1071-1078.

Lee, J.H., Kim, Y.G., Lee, K., Kim, C.J., Park, D.J., Ju, Y., Lee, J.C., Wood, T.K., and Lee, J. (2016). *Streptomyces*-derived actinomycin D inhibits biofilm formation by *Staphylococcus aureus* and its hemolytic activity. *Biofouling* 32, 45-56.

Lee, J.H., Kim, Y.G., Ryu, S.Y., Cho, M.H., and Lee, J. (2014). Ginkgolic acids and *Ginkgo biloba* extract inhibit *Escherichia coli* O157:H7 and *Staphylococcus aureus* biofilm formation. *Int J Food Microbiol* 174, 47-55.

Levy, S.B., and Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 10, S122-129.

Lewis, K. (2012). Antibiotics: Recover the lost art of drug discovery. *Nature* 485, 439-440.

Lewis, K., Epstein, S., D'onofrio, A., and Ling, L.L. (2010). Uncultured microorganisms as a source of secondary metabolites. *J Antibiot (Tokyo)* 63, 468-476.

Li, J.W., and Vederas, J.C. (2009). Drug discovery and natural products: end of an era or an endless frontier? *Science* 325, 161-165.

Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schaberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman, V.A., Cohen, D.R., Felix, C.R., Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M., Chen, C., and Lewis, K. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature* 517, 455-459.

Lopez-Gresa, M.P., Cabedo, N., Gonzalez-Mas, M.C., Ciavatta, M.L., Avila, C., and Primo, J. (2009). Terretonins E and F, inhibitors of the mitochondrial respiratory chain from the marine-derived fungus *Aspergillus insuetus*. *J Nat Prod* 72, 1348-1351.

Lopez, E.L., Contrini, M.M., Glatstein, E., Gonzalez Ayala, S., Santoro, R., Allende, D., Ezcurra, G., Teplitz, E., Koyama, T., Matsumoto, Y., Sato, H., Sakai, K., Hoshide, S., Komoriya, K., Morita, T., Harning, R., and Brookman, S. (2010). Safety and pharmacokinetics of urtoxazumab, a humanized monoclonal antibody, against Shiga-like toxin 2 in healthy adults and in pediatric patients infected with Shiga-like toxin-producing *Escherichia coli*. *Antimicrob Agents Chemother* 54, 239-243.

Los, J.M., Los, M., Wegrzyn, A., and Wegrzyn, G. (2010). Hydrogen peroxide-mediated induction of the Shiga toxin-converting lambdoid prophage ST2-8624 in *Escherichia coli* O157:H7. *FEMS Immunol Med Microbiol* 58, 322-329.

Los, J.M., Los, M., and Wegrzyn, G. (2011). Bacteriophages carrying Shiga toxin genes: genomic variations, detection and potential treatment of pathogenic bacteria. *Future Microbiol* 6, 909-924.

Lu, T.K., and Collins, J.J. (2007). Dispersing biofilms with engineered enzymatic bacteriophage. *Proc Natl Acad Sci U S A* 104, 11197-11202.

Macconnachie, A.A., and Todd, W.T. (2004). Potential therapeutic agents for the prevention and treatment of haemolytic uraemic syndrome in shiga toxin producing *Escherichia coli* infection. *Curr Opin Infect Dis* 17, 479-482.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., and Laufs, R. (1996). The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol* 178, 175-183.

Marder, E.P., Garman, K.N., Ingram, L.A., and Dunn, J.R. (2014). Multistate outbreak of *Escherichia coli* O157:H7 associated with bagged salad. *Foodborne Pathog Dis* 11, 593-595.

Martins, A., Vieira, H., Gaspar, H., and Santos, S. (2014). Marketed marine natural products in the pharmaceutical and cosmeceutical industries: tips for success. *Mar Drugs* 12, 1066-1101.

Mayer, A.M., Glaser, K.B., Cuevas, C., Jacobs, R.S., Kem, W., Little, R.D., Mcintosh, J.M., Newman, D.J., Potts, B.C., and Shuster, D.E. (2010). The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol Sci* 31, 255-265.

Mayer, A.M.S. The Global Marine Pharmaceuticals Pipeline. http://marinepharmacology.midwestern.edu/clinPipeline.htm (accessed June 25, 2015).

Mccollum, J.T., Williams, N.J., Beam, S.W., Cosgrove, S., Ettestad, P.J., Ghosh, T.S., Kimura, A.C., Nguyen, L., Stroika, S.G., Vogt, R.L., Watkins, A.K., Weiss, J.R., Williams, I.T., and Cronquist, A.B. (2012). Multistate outbreak of *Escherichia coli* O157:H7 infections associated with in-store sampling of an aged raw-milk Gouda cheese, 2010. *J Food Prot* 75, 1759-1765.

Mcfarland, L.V. (2015). From yaks to yogurt: the history, development, and current use of probiotics. *Clin Infect Dis* 60 Suppl 2, S85-90.

Mcgannon, C.M., Fuller, C.A., and Weiss, A.A. (2010). Different classes of antibiotics differentially influence shiga toxin production. *Antimicrob Agents Chemother* 54, 3790-3798.

Medema, M.H., Kottmann, R., Yilmaz, P., Cummings, M., Biggins, J.B., Blin, K., De Bruijn, I., Chooi, Y.H., Claesen, J., Coates, R.C., Cruz-Morales, P., Duddela, S., Dusterhus, S., Edwards, D.J., Fewer, D.P., Garg, N., Geiger, C., Gomez-Escribano, J.P., Greule, A., Hadjithomas, M., Haines, A.S., Helfrich, E.J., Hillwig, M.L., Ishida, K., Jones, A.C., Jones, C.S., Jungmann, K., Kegler, C., Kim, H.U., Kotter, P., Krug, D., Masschelein, J., Melnik, A.V., Mantovani, S.M., Monroe, E.A., Moore, M., Moss, N., Nutzmann, H.W., Pan, G., Pati, A., Petras, D., Reen, F.J., Rosconi, F., Rui, Z., Tian, Z., Tobias, N.J., Tsunematsu, Y., Wiemann, P., Wyckoff, E., Yan, X., Yim, G., Yu, F., Xie, Y., Aigle, B., Apel, A.K., Balibar, C.J., Balskus, E.P., Barona-Gomez, F., Bechthold, A., Bode, H.B., Borriss, R., Brady, S.F., Brakhage, A.A., Caffrey, P., Cheng, Y.Q., Clardy, J., Cox, R.J., De Mot, R., Donadio, S., Donia, M.S., Van Der Donk, W.A., Dorrestein, P.C., Doyle, S., Driessen, A.J., Ehling-Schulz, M., Entian, K.D., Fischbach, M.A., Gerwick, L., Gerwick, W.H., Gross, H., Gust, B., Hertweck, C., Hofte, M., Jensen, S.E., Ju, J., Katz, L., Kaysser, L., Klassen, J.L., Keller, N.P., Kormanec, J., Kuipers, O.P., Kuzuyama, T., Kyrpides, N.C., Kwon, H.J., Lautru, S., Lavigne, R., Lee, C.Y., Linquan, B., Liu, X., Liu, W., et al. (2015). Minimum Information about a Biosynthetic Gene cluster (MIBiG). *Nat Chem Biol* 11, 625-631.

Mehbub, M.F., Lei, J., Franco, C., and Zhang, W. (2014). Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. *Mar Drugs* 12, 4539-4577.

Mehlin, C., Headley, C.M., and Klebanoff, S.J. (1999). An inflammatory polypeptide complex from *Staphylococcus epidermidis*: isolation and characterization. *J Exp Med* 189, 907-918.

Mohsin, M., Guenther, S., Schierack, P., Tedin, K., and Wieler, L.H. (2015). Probiotic *Escherichia coli* Nissle 1917 reduces growth, Shiga toxin expression, release and thus cytotoxicity of enterohemorrhagic *Escherichia coli*. *Int J Med Microbiol* 305, 20-26.

Molinski, T.F., Dalisay, D.S., Lievens, S.L., and Saludes, J.P. (2009). Drug development from marine natural products. *Nat Rev Drug Discov* 8, 69-85.

Montaser, R., and Luesch, H. (2011). Marine natural products: a new wave of drugs? *Future Med Chem* 3, 1475-1489.

Moon, D.O., Jin, C.Y., Lee, J.D., Choi, Y.H., Ahn, S.C., Lee, C.M., Jeong, S.C., Park, Y.M., and Kim, G.Y. (2006). Curcumin decreases binding of Shiga-like toxin-1B on human intestinal epithelial cell line HT29 stimulated with TNF-alpha and IL-1beta: suppression of p38, JNK and NF-kappaB p65 as potential targets. *Biol Pharm Bull* 29, 1470-1475.

Muszanska, A.K., Nejadnik, M.R., Chen, Y., Van Den Heuvel, E.R., Busscher, H.J., Van Der Mei, H.C., and Norde, W. (2012). Bacterial adhesion forces with substratum surfaces and the susceptibility of biofilms to antibiotics. *Antimicrob Agents Chemother* 56, 4961-4964.

Nair, S.V., Baranwal, G., Chatterjee, M., Sachu, A., Vasudevan, A.K., Bose, C., Banerji, A., and Biswas, R. (2016). Antimicrobial activity of plumbagin, a naturally occurring naphthoquinone from *Plumbago rosea*, against *Staphylococcus aureus* and *Candida albicans*. *Int J Med Microbiol* 306, 237-248.

Neill, O'.J. (2014). Antimicrobial resistance: tackling a crisis for the health and wealth of nations.https://amr-review.org/sites/default/files/AMRReviewPaper-Tackling crisisforthehealthandwealthofnations_1.pdf (accessed on Mar 20, 2018).

Nett, M., Ikeda, H., and Moore, B.S. (2009). Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat Prod Rep* 26, 1362-1384.

Newman, D.J., and Cragg, G.M. (2007). Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 70, 461-477.

Nichols, D., Cahoon, N., Trakhtenberg, E.M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K., and Epstein, S.S. (2010). Use of ichip for high-throughput in situ cultivation of "uncultivable" microbial species. *Appl Environ Microbiol* 76, 2445-2450.

Nishikawa, K., Matsuoka, K., Watanabe, M., Igai, K., Hino, K., Hatano, K., Yamada, A., Abe, N., Terunuma, D., Kuzuhara, H., and Natori, Y. (2005). Identification of the optimal structure required for a Shiga toxin neutralizer with oriented carbohydrates to function in the circulation. *J Infect Dis* 191, 2097-2105.

Noel, R., Gupta, N., Pons, V., Goudet, A., Garcia-Castillo, M.D., Michau, A., Martinez, J., Buisson, D.A., Johannes, L., Gillet, D., Barbier, J., and Cintrat, J.C. (2013). N-methyldihydroquinazolinone derivatives of Retro-2 with enhanced efficacy against Shiga toxin. *J Med Chem* 56, 3404-3413.

Nowicki, D., Maciag-Dorszynska, M., Kobiela, W., Herman-Antosiewicz, A., Wegrzyn, A., Szalewska-Palasz, A., and Wegrzyn, G. (2014). Phenethyl isothiocyanate inhibits shiga toxin production in enterohemorrhagic *Escherichia coli* by stringent response induction. *Antimicrob Agents Chemother* 58, 2304-2315.

O'brien, A.D., and Holmes, R.K. (1987). Shiga and Shiga-like toxins. *Microbiol Rev* 51, 206-220.

O'connell, K.M., Hodgkinson, J.T., Sore, H.F., Welch, M., Salmond, G.P., and Spring, D.R. (2013). Combating multidrug-resistant bacteria: current strategies for the discovery of novel antibacterials. *Angew Chem Int Ed Engl* 52, 10706-10733.

O'grady, N.P., Alexander, M., Burns, L.A., Dellinger, E.P., Garland, J., Heard, S.O., Lipsett, P.A., Masur, H., Mermel, L.A., Pearson, M.L., Raad, Ii, Randolph, A.G., Rupp, M.E., Saint, S., and Healthcare Infection Control Practices Advisory, C. (2011). Guidelines for the prevention of intravascular catheter-related infections. *Clin Infect Dis* 52, e162-193.

Olson, J.B., Lord, C.C., and Mccarthy, P.J. (2000). Improved recoverability of microbial colonies from marine sponge samples. *Microb Ecol* 40, 139-147.

Otto, M. (2008). Staphylococcal biofilms. *Curr Top Microbiol Immunol* 322, 207-228.

Otto, M. (2009). *Staphylococcus epidermidis*-the 'accidental' pathogen. *Nat Rev Microbiol* 7, 555-567.

Otto, M. (2012). Molecular basis of *Staphylococcus epidermidis* infections. *Semin Immunopathol* 34, 201-214.

Oyanagi, T., Tagami, J., and Matin, K. (2012). Potentials of mouthwashes in disinfecting cariogenic bacteria and biofilms leading to inhibition of caries. *Open Dent J* 6, 23-30.

Oztekin, N., and Erim, F.B. (2005). Determination of cationic surfactants as the preservatives in an oral solution and a cosmetic product by capillary electrophoresis. *J Pharm Biomed Anal* 37, 1121-1124.

Pacheco, A.R., and Sperandio, V. (2012). Shiga toxin in enterohemorrhagic *Escherichia coli*: regulation and novel anti-virulence strategies. *Front Cell Infect Microbiol* 2, 81.

Pagliara, P., and Caroppo, C. (2011). Cytotoxic and antimitotic activities in aqueous extracts of eight cyanobacterial strains isolated from the marine sponge *Petrosia ficiformis*. *Toxicon* 57, 889-896.

Pahlow, S., Kloss, S., Blattel, V., Kirsch, K., Hubner, U., Cialla, D., Rosch, P., Weber, K., and Popp, J. (2013). Isolation and enrichment of pathogens with a surface-modified aluminium chip for Raman spectroscopic applications. *Chemphyschem* 14, 3600-3605.

Palomo, S., Gonzalez, I., De La Cruz, M., Martin, J., Tormo, J.R., Anderson, M., Hill, R.T., Vicente, F., Reyes, F., and Genilloud, O. (2013). Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Mar Drugs* 11, 1071-1086.

Pamp, S.J., Gjermansen, M., Johansen, H.K., and Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to

metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol Microbiol* 68, 223-240.

Park, J.H., Lee, J.H., Kim, C.J., Lee, J.C., Cho, M.H., and Lee, J. (2012). Extracellular protease in actinomycetes culture supernatants inhibits and detaches *Staphylococcus aureus* biofilm formation. *Biotechnol Lett* 34, 655-661.

Park, S.R., Tripathi, A., Wu, J., Schultz, P.J., Yim, I., Mcquade, T.J., Yu, F., Arevang, C.J., Mensah, A.Y., Tamayo-Castillo, G., Xi, C., and Sherman, D.H. (2016). Discovery of cahuitamycins as biofilm inhibitors derived from a convergent biosynthetic pathway. *Nat Commun* 7, 10710.

Pennington, H. (2010). *Escherichia coli* O157. *Lancet* 376, 1428-1435.

Peoples, A.J., Zhang, Q., Millett, W.P., Rothfeder, M.T., Pescatore, B.C., Madden, A.A., Ling, L.L., and Moore, C.M. (2008). Neocitreamicins I and II, novel antibiotics with activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. *J Antibiot (Tokyo)* 61, 457-463.

Peoples, A., Ling, L.L., Lewis, K., and Zhang , Z. (2011). Novel Antibiotics. NovoBiotic Pharmaceuticals. U.S. Patent 20110136752 A1.

Peoples, A., Zhang, Q., Moore, C., Ling, L., Rothfeder, M., and Lewis K. (2012). NovoBiotic Pharmaceuticals. US patent 8097709 B2.

Percival Sl, M.S., Cruz H, Williams Dw (2011). Introduction to biofilms. *Biofilm Vet Med* 6:, 41e68.

Percival, S.L., Suleman, L., Vuotto, C., and Donelli, G. (2015). Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. *J Med Microbiol* 64, 323-334.

Periasamy, S., Joo, H.S., Duong, A.C., Bach, T.H., Tan, V.Y., Chatterjee, S.S., Cheung, G.Y., and Otto, M. (2012). How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proc Natl Acad Sci U S A* 109, 1281-1286.

Piel, J. (2006). Bacterial symbionts: prospects for the sustainable production of invertebrate-derived pharmaceuticals. *Curr Med Chem* 13, 39-50.

Pietiainen, M., Francois, P., Hyyrylainen, H.L., Tangomo, M., Sass, V., Sahl, H.G., Schrenzel, J., and Kontinen, V.P. (2009). Transcriptome analysis of the responses of *Staphylococcus aureus* to antimicrobial peptides and characterization of the roles of *vraDE* and *vraSR* in antimicrobial resistance. *BMC Genomics* 10, 429.

Pifer, R., and Sperandio, V. (2014). The interplay between the microbiota and enterohemorrhagic *Escherichia coli*. *Microbiol Spectr* 2.

Pogodin, S., Hasan, J., Baulin, V.A., Webb, H.K., Truong, V.K., Phong Nguyen, T.H., Boshkovikj, V., Fluke, C.J., Watson, G.S., Watson, J.A., Crawford, R.J., and Ivanova, E.P. (2013). Biophysical model of bacterial cell interactions with nanopatterned cicada wing surfaces. *Biophys J* 104, 835-840.

Preidis, G.A., Hill, C., Guerrant, R.L., Ramakrishna, B.S., Tannock, G.W., and Versalovic, J. (2011). Probiotics, enteric and diarrheal diseases, and global health. *Gastroenterology* 140, 8-14.

Pringle, J.H., and Fletcher, M. (1983). Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Appl Environ Microbiol* 45, 811-817.

Rahman, H., Austin, B., Mitchell, W.J., Morris, P.C., Jamieson, D.J., Adams, D.R., Spragg, A.M., and Schweizer, M. (2010). Novel anti-infective compounds from marine bacteria. *Mar Drugs* 8, 498-518.

Rajivgandhi, G., Vijayan, R., Maruthupandy, M., Vaseeharan, B., and Manoharan, N. (2018). Antibiofilm effect of *Nocardiopsis* sp. GRG 1 (KT235640) compound against biofilm forming Gram negative bacteria on urinary tract infections (UTIs). *Microb Pathog* 118, 190-198.

Rajput, A., Thakur, A., Sharma, S., and Kumar, M. (2018). aBiofilm: a resource of anti-biofilm agents and their potential implications in targeting antibiotic drug resistance. *Nucleic Acids Res* 46, D894-D900.

Rampioni, G., Leoni, L., and Williams, P. (2014). The art of antibacterial warfare: deception through interference with quorum sensing-mediated communication. *Bioorg Chem* 55, 60-68.

Rani, S.A., Pitts, B., Beyenal, H., Veluchamy, R.A., Lewandowski, Z., Davison, W.M., Buckingham-Meyer, K., and Stewart, P.S. (2007). Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol* 189, 4223-4233.

Rappe, M.S., and Giovannoni, S.J. (2003). The uncultured microbial majority. *Annu Rev Microbiol* 57, 369-394.

Rappuoli, R., Mandl, C.W., Black, S., and De Gregorio, E. (2011). Vaccines for the twenty-first century society. *Nat Rev Immunol* 11, 865-872.

Rasko, D.A., Moreira, C.G., Li De, R., Reading, N.C., Ritchie, J.M., Waldor, M.K., Williams, N., Taussig, R., Wei, S., Roth, M., Hughes, D.T., Huntley, J.F., Fina, M.W., Falck, J.R., and Sperandio, V. (2008). Targeting QseC signaling and virulence for antibiotic development. *Science* 321, 1078-1080.

Rateb, M.E., and Ebel, R. (2011). Secondary metabolites of fungi from marine habitats. *Nat Prod Rep* 28, 290-344.

Richards, J.J., and Melander, C. (2009). Controlling bacterial biofilms. *Chembiochem* 10, 2287-2294.

Riordan, J.T., Dupre, J.M., Cantore-Matyi, S.A., Kumar-Singh, A., Song, Y., Zaman, S., Horan, S., Helal, N.S., Nagarajan, V., Elasri, M.O., Wilkinson, B.J., and Gustafson, J.E. (2011). Alterations in the transcriptome and antibiotic susceptibility of *Staphylococcus aureus* grown in the presence of diclofenac. *Ann Clin Microbiol Antimicrob* 10, 30.

Ritchie, J.M., Greenwich, J.L., Davis, B.M., Bronson, R.T., Gebhart, D., Williams, S.R., Martin, D., Scholl, D., and Waldor, M.K. (2011). An *Escherichia coli* O157-specific engineered pyocin prevents and ameliorates infection by *E. coli* O157:H7 in an animal model of diarrheal disease. *Antimicrob Agents Chemother* 55, 5469-5474.

RKI (2011). Abschließende Darstellung und Bewertung der epidemiologischen Erkenntnisse im EHEC O104:H4 Ausbruch. *Abschlussbericht* (Berlin).

Rodriguez-Marconi, S., De La Iglesia, R., Diez, B., Fonseca, C.A., Hajdu, E., and Trefault, N. (2015). Characterization of bacterial, archaeal and eukaryote symbionts from Antarctic sponges reveals a high diversity at a three-domain level and a particular signature for this ecosystem. *PLoS One* 10, e0138837.

Roemer, T., Davies, J., Giaever, G., and Nislow, C. (2011). Bugs, drugs and chemical genomics. *Nat Chem Biol* 8, 46-56.

Rohde, H., Burdelski, C., Bartscht, K., Hussain, M., Buck, F., Horstkotte, M.A., Knobloch, J.K., Heilmann, C., Herrmann, M., and Mack, D. (2005). Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol Microbiol* 55, 1883-1895.

Romero, D., and Kolter, R. (2011). Will biofilm disassembly agents make it to market? *Trends Microbiol* 19, 304-306.

Rund, S.A., Rohde, H., Sonnenborn, U., and Oelschlaeger, T.A. (2013). Antagonistic effects of probiotic *Escherichia coli* Nissle 1917 on EHEC strains of serotype O104:H4 and O157:H7. *Int J Med Microbiol* 303, 1-8.

Sauleau, P., Moriou, C., and Al Mourabit, A. (2017). Metabolomics approach to chemical diversity of the Mediterranean marine sponge *Agelas oroides. Nat Prod Res* 31, 1625-1632.

Scallan, E., Griffin, P.M., Angulo, F.J., Tauxe, R.V., and Hoekstra, R.M. (2011). Foodborne illness acquired in the United States-unspecified agents. *Emerg Infect Dis* 17, 16-22.

Schmidt, H. (2001). Shiga-toxin-converting bacteriophages. *Res Microbiol* 152, 687-695.

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., Perez, T., Rodrigo, A., Schupp, P.J., Vacelet, J., Webster, N., Hentschel, U., and Taylor, M.W. (2012). Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J* 6, 564-576.

Scholl, D., Cooley, M., Williams, S.R., Gebhart, D., Martin, D., Bates, A., and Mandrell, R. (2009). An engineered R-type pyocin is a highly specific and sensitive bactericidal agent for the food-borne pathogen *Escherichia coli* O157:H7. *Antimicrob Agents Chemother* 53, 3074-3080.

Selvin, J., Ninawe, A.S., Kiran, G.S., Lipton, A.P. (2010). Sponge-microbial interactions: ecological implications and bioprospecting avenues. *Critical reviews in microbiology* 36, 82-90.

Seyedsayamdost, M.R. (2014). High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. *Proc Natl Acad Sci U S A* 111, 7266-7271.

Shakhnovich, E.A., Hung, D.T., Pierson, E., Lee, K., and Mekalanos, J.J. (2007). Virstatin inhibits dimerization of the transcriptional activator ToxT. *Proc Natl Acad Sci U S A* 104, 2372-2377.

Shaw, T., Winston, M., Rupp, C.J., Klapper, I., and Stoodley, P. (2004). Commonality of elastic relaxation times in biofilms. *Phys Rev Lett* 93, 098102.

Sheng, L., Rasco, B., and Zhu, M.J. (2016). Cinnamon Oil Inhibits Shiga Toxin Type 2 Phage Induction and Shiga Toxin Type 2 Production in *Escherichia coli* O157:H7. *Appl Environ Microbiol* 82, 6531-6540.

Sianglum, W., Srimanote, P., Taylor, P.W., Rosado, H., and Voravuthikunchai, S.P. (2012). Transcriptome analysis of responses to rhodomyrtone in methicillin-resistant *Staphylococcus aureus*. *PLoS One* 7, e45744.

Silva, L.N., Zimmer, K.R., Macedo, A.J., and Trentin, D.S. (2016). Plant natural products targeting bacterial virulence factors. *Chem Rev* 116, 9162-9236.

Silver, L.L. (2011). Challenges of antibacterial discovery. *Clin Microbiol Rev* 24, 71-109.

Simister, R.L., Deines, P., Botte, E.S., Webster, N.S., and Taylor, M.W. (2012). Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environ Microbiol* 14, 517-524.

Skropeta, D., and Wei, L. (2014). Recent advances in deep-sea natural products. *Nat Prod Rep* 31, 999-1025.

Sleytr, U.B. (1997). Basic and applied S-layer research: an overview. *FEMS Microbiol Rev* 20, 5e12.

Smith, J.L., Fratamico, P.M., and Gunther, N.W.T. (2014). Shiga toxin-producing *Escherichia coli. Adv Appl Microbiol* 86, 145-197.

Sommer, M.O. (2014). Microbiology: Barriers to the spread of resistance. *Nature* 509, 567-568.

Sood, A., Mathew, R., and Trachtman, H. (2001). Cytoprotective effect of curcumin in human proximal tubule epithelial cells exposed to shiga toxin. *Biochem Biophys Res Commun* 283, 36-41.

Sousa, S., Mesquita, F.S., and Cabanes, D. (2015). Old war, new battle, new fighters! *J Infect Dis* 211, 1361-1363.

Speziale, P., Pietrocola, G., Foster, T.J., and Geoghegan, J.A. (2014). Protein-based biofilm matrices in staphylococci. *Front Cell Infect Microbiol* 4, 171.

Stallforth, P., and Clardy, J. (2014). Atlas for drug discovery. *Proc Natl Acad Sci U S A* 111, 3655-3656.

Stamatios Perdicaris, T.V., Athanasios, V. (2013). Bioactive natural substances from marine sponges: new developments and prospects for future pharmaceuticals. *Natural products chemistry and research* 1, 1:115.

Stanford, K., Bach, S., Baah, J., and Mcallister, T. (2014). A mixture of *Lactobacillus casei*, *Lactobacillus lactis*, and *Paenibacillus polymyxa* reduces *Escherichia coli* O157:H7 in finishing feedlot cattle. *J Food Prot* 77, 738-744.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. *Science* 292, 2285-2289.

Stearns-Kurosawa, D.J., Collins, V., Freeman, S., Tesh, V.L., and Kurosawa, S. (2010). Distinct physiologic and inflammatory responses elicited in baboons after challenge with Shiga toxin type 1 or 2 from enterohemorrhagic *Escherichia coli. Infect Immun* 78, 2497-2504.

Stevens, N.T., Sadovskaya, I., Jabbouri, S., Sattar, T., O'gara, J.P., Humphreys, H., and Greene, C.M. (2009). *Staphylococcus epidermidis* polysaccharide intercellular adhesin induces IL-8 expression in human astrocytes via a mechanism involving TLR2. *Cell Microbiol* 11, 421-432.

Stewart, P.S., and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135-138.

Subramani, R., and Aalbersberg, W. (2012). Marine actinomycetes: an ongoing source of novel bioactive metabolites. *Microbiol Res* 167, 571-580.

Subramani, R., and Aalbersberg, W. (2013). Culturable rare actinomycetes: diversity, isolation and marine natural product discovery. *Appl Microbiol Biotechnol* 97, 9291-9321.

Sugimoto, N., Tada, A., Kuroyanagi, M., Yoneda, Y., Yun, Y.S., Kunugi, A., Sato, K., Yamazaki, T., and Tanamoto, K. (2008). Survey of synthetic disinfectants in grapefruit seed extract and its compounded products. *Shokuhin Eiseigaku Zasshi* 49, 56-62.

Supong, K., Suriyachadkun, C., Pittayakhajonwut, P., Suwanborirux, K., and Thawai, C. (2013a). *Micromonospora spongicola* sp. nov., an actinomycete isolated from a marine sponge in the Gulf of Thailand. *J Antibiot (Tokyo)* 66, 505-509.

Supong, K., Suriyachadkun, C., Suwanborirux, K., Pittayakhajonwut, P., and Thawai, C. (2013b). *Verrucosispora andamanensis* sp. nov., isolated from a marine sponge. *Int J Syst Evol Microbiol* 63, 3970-3974.

Suter, L.G., Paltiel, A.D., Rome, B.N., Solomon, D.H., Golovaty, I., Gerlovin, H., Katz, J.N., and Losina, E. (2011). Medical device innovation-is "better" good enough? *N Engl J Med* 365, 1464-1466.

Sutherland, I.W. (2001). The biofilm matrix-an immobilized but dynamic microbial environment. *Trends Microbiol* 9, 222-227.

Takeda, T., Yoshino, K., Adachi, E., Sato, Y., and Yamagata, K. (1999). *In vitro* assessment of a chemically synthesized Shiga toxin receptor analog attached to chromosorb P (Synsorb Pk) as a specific absorbing agent of Shiga toxin 1 and 2. *Microbiol Immunol* 43, 331-337.

Takizawa, M., Colwell, R.R., and Hill, R.T. (1993). Isolation and diversity of actinomycetes in the chesapeake bay. *Appl Environ Microbiol* 59, 997-1002.

Tang, X., Li, J., Millan-Aguinaga, N., Zhang, J.J., O'neill, E.C., Ugalde, J.A., Jensen, P.R., Mantovani, S.M., and Moore, B.S. (2015). Identification of thiotetronic acid antibiotic biosynthetic pathways by target-directed genome mining. *ACS Chem Biol* 10, 2841-2849.

Tarr, P.I., Gordon, C.A., and Chandler, W.L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* 365, 1073-1086.

Tasdemir, D., Topaloglu, B., Perozzo, R., Brun, R., O'neill, R., Carballeira, N.M., Zhang, X., Tonge, P.J., Linden, A., and Ruedi, P. (2007). Marine natural products from the Turkish

sponge *Agelas oroides* that inhibit the enoyl reductases from *Plasmodium falciparum*, *Mycobacterium tuberculosis* and *Escherichia coli*. *Bioorg Med Chem* 15, 6834-6845.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev* 71, 295-347.

Thacker, R.W., and Freeman, C.J. (2012). Sponge-microbe symbioses: recent advances and new directions. *Adv Mar Biol* 62, 57-111.

Then, R.L., and Sahl, H.G. (2010). Anti-infective strategies of the future: is there room for species-specific antibacterial agents? *Curr Pharm Des* 16, 555-566.

Thomas, T.R., Kavlekar, D.P., and Lokabharathi, P.A. (2010). Marine drugs from sponge-microbe association-a review. *Mar Drugs* 8, 1417-1468.

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem J* 474, 1823-1836.

Tilden, J., Jr., Young, W., Mcnamara, A.M., Custer, C., Boesel, B., Lambert-Fair, M.A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., and Morris, J.G., Jr. (1996). A new route of transmission for *Escherichia coli*: infection from dry fermented salami. *Am J Public Health* 86, 1142-1145.

Tillotson, G. (2015). Antimicrobial resistance: what's needed. *Lancet Infect Dis* 15, 758-760.

Tyler, J.S., Mills, M.J., and Friedman, D.I. (2004). The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. *J Bacteriol* 186, 7670-7679.

Van Soest, R.W.M., Boury, E.N., Hooper J.N.A., Rützler K., De Voogd N.J., Alvarez B., Hajdu E., Pisera A.B., Manconi R., Schönberg C., Klautau M., Picton B., Kelly M., Vacelet J., Dohrmann M., Díaz M.C., Cárdenas P., Carballo J. L. (2018). WoRMS Porifera: World Porifera database (version 2018-03-01) (accessed on April 2, 2018).

Van Tilburg Bernardes, E., Lewenza, S., and Reckseidler-Zenteno, S. (2015). Current research approaches to target biofilm Infections. *Postdoc J* 3, 36-49.

Varankovich, N.V., Nickerson, M.T., and Korber, D.R. (2015). Probiotic-based strategies for therapeutic and prophylactic use against multiple gastrointestinal diseases. *Front Microbiol* 6, 685.

Versluis, D., Mcpherson, K., Van Passel, M.W.J., Smidt, H., and Sipkema, D. (2017). Recovery of previously uncultured bacterial genera from three Mediterranean sponges. *Mar Biotechnol (NY)* 19, 454-468.

Villa, F.A., and Gerwick, L. (2010). Marine natural product drug discovery: Leads for treatment of inflammation, cancer, infections, and neurological disorders. *Immunopharmacol Immunotoxicol* 32, 228-237.

Vinh, D.C., and Embil, J.M. (2005). Device-related infections: a review. *J Long Term Eff Med Implants* 15, 467-488.

Vojdani, J.D., Beuchat, L.R., and Tauxe, R.V. (2008). Juice-associated outbreaks of human illness in the United States, 1995 through 2005. *J Food Prot* 71, 356-364.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., Deleo, F.R., and Otto, M. (2004). A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* 279, 54881-54886.

Waksman, S.A. (1950). The actinomycetes: their nature, occurrence, activities and importance. *Chronica Botanica Company. Mass. USA*, p 3-21.

Wang, K.L., Wu, Z.H., Wang, Y., Wang, C.Y., and Xu, Y. (2017). Mini-review: antifouling natural products from marine microorganisms and their synthetic analogs. *Mar Drugs* 15.

Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., Deleo, F.R., and Otto, M. (2007). Identification of novel cytolytic peptides as key virulence determinants for community-associated methicillin resistant *Staphylococcus aureus* (MRSA). *Nat Med* 13, 1510-1514.

Webster, N.S., Luter, H.M., Soo, R.M., Botte, E.S., Simister, R.L., Abdo, D., and Whalan, S. (2012). Same, same but different: symbiotic bacterial associations in GBR sponges. *Front Microbiol* 3, 444.

Webster, N.S., Wilson, K.J., Blackall, L.L., and Hill, R.T. (2001). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl Environ Microbiol* 67, 434-444.

Wei, R.B., Xi, T., Li, J., Wang, P., Li, F.C., Lin, Y.C., and Qin, S. (2011). Lobophorin C and D, new kijanimicin derivatives from a marine sponge-associated actinomycetal strain AZS17. *Mar Drugs* 9, 359-368.

Weller, M.G. (2012). A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. *Sensors (Basel)* 12, 9181-9209.

WHO (2001). Global Strategy for Containment of Antimicrobial Resistance. www.who.int/drugresistance/WHO_Global_Strategy_English.pdf (accessed on Mar 29, 2018).

WHO (2014). Antimicrobial resistance: global report on surveillance 2014. http://www.who.int/drugresistance/documents/surveillancereport/en/ (accessed June 27, 2015).

WHO (2015). Antibiotic resistance fact sheet. http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/(accessed June 21, 2016).

Widerstrom, M. (2016). Significance of *Staphylococcus epidermidis* in health care-associated infections, from contaminant to clinically relevant pathogen: this is a wake-up call! *J Clin Microbiol* 54, 1679-1681.

Wilson, M.C., and Piel, J. (2013). Metagenomic approaches for exploiting uncultivated bacteria as a resource for novel biosynthetic enzymology. *Chem Biol* 20, 636-647.

Winn, M., Goss, R.J., Kimura, K., and Bugg, T.D. (2010). Antimicrobial nucleoside antibiotics targeting cell wall assembly: recent advances in structure-function studies and nucleoside biosynthesis. *Nat Prod Rep* 27, 279-304.

Wong, C.S., Jelacic, S., Habeeb, R.L., Watkins, S.L., and Tarr, P.I. (2000). The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N Engl J Med* 342, 1930-1936.

Wright, A.E., Killday, K.B., Chakrabarti, D., Guzman, E.A., Harmody, D., Mccarthy, P.J., Pitts, T., Pomponi, S.A., Reed, J.K., Roberts, B.F., Rodrigues Felix, C., and Rohde, K.H. (2017). Dragmacidin G, a bioactive bis-indole alkaloid from a deep-water sponge of the genus *Spongosorites*. *Mar Drugs* 15.

Yamagami, S., Motoki, M., Kimura, T., Izumi, H., Takeda, T., Katsuura, Y., and Matsumoto, Y. (2001). Efficacy of postinfection treatment with anti-Shiga toxin (Stx) 2 humanized monoclonal antibody TMA-15 in mice lethally challenged with Stx-producing *Escherichia coli*. *J Infect Dis* 184, 738-742.

Yamanaka, K., Reynolds, K.A., Kersten, R.D., Ryan, K.S., Gonzalez, D.J., Nizet, V., Dorrestein, P.C., and Moore, B.S. (2014). Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. *Proc Natl Acad Sci U S A* 111, 1957-1962.

Yarwood, J.M., Bartels, D.J., Volper, E.M., and Greenberg, E.P. (2004). Quorum sensing in *Staphylococcus aureus* biofilms. *J Bacteriol* 186, 1838-1850.

Young, D., Morton, R., and Bartley, J. (2010). Therapeutic ultrasound as treatment for chronic rhinosinusitis: preliminary observations. *J Laryngol Otol* 124, 495-499.

Yue, W.F., Du, M., and Zhu, M.J. (2012). High temperature in combination with UV irradiation enhances horizontal transfer of *stx2* gene from *Escherichia coli* O157:H7 to non-pathogenic E. coli. *PLoS One* 7, e31308.

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E.J., Short, J.M., and Keller, M. (2002). Cultivating the uncultured. *Proc Natl Acad Sci U S A* 99, 15681-15686.

Zhang, H., Lee, Y.K., Zhang, W., and Lee, H.K. (2006). Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Antonie Van Leeuwenhoek* 90, 159-169.

Zhang, X., Mcdaniel, A.D., Wolf, L.E., Keusch, G.T., Waldor, M.K., and Acheson, D.W. (2000). Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. *J Infect Dis* 181, 664-670.

Zhou, X., Liu, J., Yang, B., Lin, X., Yang, X.W., and Liu, Y. (2013). Marine natural products with anti-HIV activities in the last decade. *Curr Med Chem* 20, 953-973.

Ziemert, N., Podell, S., Penn, K., Badger, J.H., Allen, E., and Jensen, P.R. (2012). The natural product domain seeker NaPDoS: a phylogeny based bioinformatic tool to classify secondary metabolite gene diversity. *PLoS One* 7, e34064.

8. Appendix

List of abbreviations and symbols

EHEC	Enterohemorrhagic *Escherichia coli*
HUS	hemoytic uremic syndrome
Stx	Shiga toxin
ELISA	Enzyme Linked Immunosorbent Assay
NMR	nuclear magnetic resonance
1D, 2D, 3D	one dimensional, two dimensional, three dimensional
ESI	electrospray ionization
HR-MS	high resolution mass spectrometry
SBT	SeaBiotech
Fr	fraction
HPLC	high pressure liquid chromatography
BIC_{90}	90% biofilm inhibitory concentration
MIC	minimum inhibitory concentration
MBIC	minimum biofilm inhibitory concentration
Da	dalton
min	minute
h	hour
d	day
WHO	World Health Organization
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
iChip	isolation chip
HGT	horizontal gene transfer
pH	power of hydrogen
US-FDA	United States Food and Drug Administration
GI	gastro-intestinal
IBD	inflammatory bowel disease
CFUs	colony forming units
Gb3	Globotriaosylceramide
STEC	Shiga toxin producing *E. coli*

TGN	trans Golgi network
ER	endoplasmic reticulum
DRIs	device-related infections
nm	nanometer
cm	centimeter
UV	ultraviolet
CoNS	coagulase negative staphylococci
CoPS	coagulase positive staphylococci
AtlE	autolysin
Aap	accumulation-associated protein
Bap	biofilm-associated protein
PNAG	poly-N-acetylglucosamine
GlcNAc	N-acetylglucosamine
FnBP	fibronectin binding protein
WTA	wall teichoic acid
LTA	lipoteichoic acid
QS	quorum sensing
PSM	phenol soluble modulins
CW	cell wall
CM	cell membrane
PG	peptidoglycan
μm	micrometer
NPs	natural products
MNPs	marine natural products
kg	kilogram
l	liter
rRNA	ribosomal RNA
DGGE	denaturing gradient gel electrophoresis
FISH	Fluorescent *In Situ* Hybridization
GC	Guanine-cytosine
NCBI	National Center for Biotechnology Information
BD	bloody diarrhea
m/z	mass to charge ratio
MAbs	monoclonal antibodies

δ_C	chemical shift (ppm), ^{13}C-NMR
δ_H	chemical shift (ppm), ^{1}H-NMR
^oC	degree Celsius
rpm	revolutions per minute
ASW	artificial sea water
^{13}C-NMR	carbon nuclear magnetic resonance spectroscopy
COSY	correlation spectroscopy
DMSO	Dimethyl sulfoxide
HMBC	heteronuclear multiple bond correlation
^{1}H-NMR	proton nuclear magnetic resonance spectroscopy
HSQC	heteronuclear single quantum coherence
Hz	hertz
MHz	megahertz
MeOH	methanol
EtOAc	ethylacetate
PC	positive control
µM	micromolar
LB	Luria broth
NIH	National Institute of Health
eDNA	extracellular DNA
TSB	Tryptic soy broth
v/v	volume per unit volume
w/v	weight per unit volume
g/l	gram per liter
µg/l	microgram per liter
%	percentage
mM	millimolar
ml	milliliter
ISP2	International *Streptomyces* project medium 2
OD	pptical density at a particular wavelength
µg	microgram
PBS	phosphate buffer saline
ATCC	American type culture collection
mm	millimeter

HCEC	human corneal epithelial cells
U/ml	units/millilter
µl	microliter
BIC_{50}	50% biofilm inhibitory concentration
A_{260}/A_{280}	absorbance at 260 nm to absorbance at 280 nm
qPCR	quantitative real time PCR
PCR	polymerase chain reaction
Ct	cycle threshold
DHFR	dihydrofolate reductase
DCM	dichloromethane
MeCN	acetonitrile
kV	kilovolts
LC-MS	liquid chromatography-mass spectrometry
SEM	scanning electron microscopy
CLSM	confocal laser scanning microscopy
IOLs	intraocular lenses
mRNA	messenger RNA
Rt	retention time
FTIR	fourier transform infra-red spectroscopy
MRSA	methicillin resistant *S. aureus*
MSSA	methicillin sensitive *S. aureus*
VRSA	vancomycin resistant *S. aureus*
VRE	vancomycin resistant enterococci
ND	not determined
NC	no cytotoxicity
ABC	ATP binding cassette
SMR	small molecule resistance
CSSSI	somplicated skin and skin structure infections
ABSSI	acute bacterial skin and skin structure infections
ISP2_F	International *Streptomyces* project medium 2_filamentous
BLAST	Basic Local Alignment Search Tool
antiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
MIBiG	Minimum Information about a Biosynthetic Gene Cluster
NaPDoS	Natural Product Domain Seeker

LPS	lipopolysaccharide
sRNA	small RNA
PCA	principal component analysis
MC	medium control
UT	untreated
t	time
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
M	molecular weight ladder
bp	base pair
HT	heat treated
TT	trypsin treated
PKT	proteinase K treated
DOSY	Diffusion Ordered Spectroscopy
ns	not significant (statistically); $p > 0.05$
*, **, ***, ****	$p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$

List of figures (chapter-wise)

List of tables (chapter-wise)

Statement of author contributions

Author contributions to the manuscripts

Cheng C.*, Balasubramanian S.*, Fekete A., Krischke M., Mueller M.J., Hentschel U., Oelschlaeger T.A., and Abdelmohsen U.R. (2017). Inhibitory potential of strepthonium A against Shiga toxin production in enterohemorrhagic *Escherichia coli* (EHEC) strain EDL933. *Nat Prod Res.* 31(23):2818-2823. doi: 10.1080/14786419.2017.1297443.

Participated in	Author Initials, Responsibility decreasing from left to right				
Study Design Methods Development	CC, SB	URA, TAO, UHe			
Data Collection	CC, SB	FA, KM			
Data Analysis and Interpretation	CC, SB	FA, KM	URA, TAO, UHe		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	CC, SB	URA, TAO, UHe	FA, KM, MMJ		

Explanation: * CC and SB contributed equally to this manuscript. Shared first authorship.

Balasubramanian S., Othman E.M., Kampik D., Stopper H., Hentschel U., Ziebuhr W., Oelschlaeger T.A., and Abdelmohsen U.R. (2017). Marine sponge-derived *Streptomyces* sp. SBT343 inhibits staphylococcal biofilm formation. *Front Microbiol*. 1-8: 236. doi: 10.3389/fmicb.2017.00236.					
Participated in	**Author Initials, Responsibility decreasing from left to right**				
Study Design Methods Development	SB	TAO	URA		
Data Collection	SB	EMO			
Data Analysis and Interpretation	SB	EMO	URA		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	SB	EMO	URA	TAO, UHe, WZ	DK, HS

Balasubramanian S., Skaf J.S., Holzgrabe U., Bharti R., Förstner K.U., Ziebuhr W., Hentschel U., Abdelmohsen U.R., and Oelschlaeger T.A. (2018). A new bioactive compound from the marine sponge-derived *Streptomyces* sp. SBT348 inhibits staphylococcal growth and biofilm formation. *Front Microbiol*. (under review)					
Participated in	**Author Initials, Responsibility decreasing from left to right**				
Study Design Methods Development	SB	TAO	WZ, URA		
Data Collection	SB	JS, RB			
Data Analysis and Interpretation	SB	JS, RB	TAO, KUF		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	SB	JS, RB	UHo, KUF, WZ, UHe, URA, TAO		

Author contributions to the figures and tables

Cheng C.*, Balasubramanian S.*, Fekete A., Krischke M., Mueller M.J., Hentschel U., Oelschlaeger T.A., and Abdelmohsen U.R. (2017). Inhibitory potential of strepthonium A against Shiga toxin production in enterohemorrhagic *Escherichia coli* (EHEC) strain EDL933. *Nat Prod Res*. 31(23):2818-2823. doi: 10.1080/14786419.2017.1297443.

Figure	Author Initials, Responsibility decreasing from left to right				
Graphical abstract	SB	CC			
1	CC	SB			
2 (A, B)	SB				

Explanation: * CC and SB contributed equally to this manuscript. Shared 1st authorship.

Balasubramanian S., Othman E.M., Kampik D., Stopper H., Hentschel U., Ziebuhr W., Oelschlaeger T.A., and Abdelmohsen U.R. (2017). Marine sponge-derived *Streptomyces* sp. SBT343 inhibits staphylococcal biofilm formation. *Front Microbiol*. 1-8: 236. doi: 10.3389/fmicb.2017.00236.

Figure	Author Initials, Responsibility decreasing from left to right				
1 (A, B, C)	SB	WZ			
2 (A, B)	SB				
3 (A, B)	SB				
4 (A, B, C)	SB				
5 (A, B)	SB				
6 (A, B)	SB	URA			
Table 1	SB				
Table 2	SB				
Table 3	SB	EMO			
Table 4	SB	URA			

Balasubramanian S., Skaf J.S., Holzgrabe U., Bharti R., Förstner K.U., Ziebuhr W., Hentschel U., Abdelmohsen U.R., and Oelschlaeger T.A. (2018). A new bioactive compound from the marine sponge-derived *Streptomyces* sp. SBT348 inhibits staphylococcal growth and biofilm formation. *Front Microbiol.* (under review)				
Figure	**Author Initials, Responsibility decreasing from left to right**			
1 (A, B, C, D)	SB				
2 (A, B, C)	SB	JS	UHo		
3 (A, B, C)	SB				
4	SB				
5	SB				
6 (A, B, C, D)	SB, RB	KUF			
Table 1	SB				
Table 2	SB				
Table 3	SB				
Table 4	SB, RB	KUF			

The doctoral researcher confirms that he has obtained permission from both the publishers and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above-mentioned assessment.

_____   _____

Srikkanth Balasubramanian   Dr. Tobias Ölschläger

Würzburg,   Würzburg,

List of publications

1. **Balasubramanian, S.**, Skaf, J., Holzgrabe, U., Bharti, R., Förstner, K.U., Ziebuhr, W., Hentschel, U., Abdelmohsen, U.R., and Oelschlaeger, T.A. A new bioactive compound from the marine sponge-derived *Streptomyces* sp. SBT348 inhibits staphylococcal growth and biofilm formation. *Front. Microbiol.* (under review).

2. **Balasubramanian, S.**, Othman, E.M., Kampik, D., Stopper, H., Hentschel, U., Ziebuhr, W., Oelschlaeger, T.A., and Abdelmohsen, U.R. (2017). Marine sponge-derived *Streptomyces* sp. SBT343 inhibits staphylococcal biofilm formation. *Front. Microbiol.* 8, 236.

    doi: 10.3389/fmicb.2017.00236.

3. Cheng, C.*, **Balasubramanian, S.***, Fekete, A., Krischke, M., Mueller, M., Hentschel, U., Oelschlaeger, T.A., and Abdelmohsen, U.R. (2017). Inhibitory potential of strepthonium A against Shiga toxin production in Enterohemorrhagic *E. coli* (EHEC) strain EDL933. *Nat. Prod. Res.* 31(23), 2818-2823.

    doi: 10.1080/14786419.2017.1297443.

    * CC and SB contributed equally to this manuscript (shared first authorship).

4. Skaf, J., Hamarsheh, O., Berninger, M., **Balasubramanian, S.**, Oelschlaeger, T.A., and Holzgrabe, U. (2018). Improving anti-trypanosomal activities of alkamides isolated from *Achillea fragantissima*. *Fitoterapia*. 125,191-198.

    doi: 10.1016/j.fitote.2017.11.001.

5. Abdelmohsen, U.R., **Balasubramanian, S.**, Oelschlaeger, T.A., Grkovic, T., Pham, N.B., Quinn, R.J., and Hentschel, U. (2017). Potential of marine natural products against drug-resistant fungal, viral and parasitic infections. *Lancet. Infect. Dis.* 17(2), e30-e41.

    doi: 10.1016/S1473-3099(16)30323-1.

6. Abdelmohsen, U.R., Grkovic, T., **Balsubramanian, S.**, Kamel, M.S., Quinn, R.J., and Hentschel, U. (2015). Elicitation of secondary metabolism in actinomycetes. *Biotechnol. Adv.* 1(33), 798-811.

    doi: 10.1016/j.biotechadv.2015.06.003.

Poster presentations at symposia and conferences

Best of SFB630, conclusion-the final symposium, Würzburg, Germany (2015)
Title: Antagonistic effects of sponge-associated actinomycetes on biofilm formation and Shiga toxin production

67 Jahrestatung der DeutschenGeschellschaft für Hygiene und Mikrobiologie (DGHM), Münster, Germany (2015)
Title: Elicitation of secondary metabolism in sponge-associated actinomycetes

Eureka, 10th International GSLS students' symposium, Würzburg, Germany (2015)
Title: Antagonistic potential of marine sponge-derived *Streptomyces* sp. D56 on staphylococcal biofilm formation

Biofilms7, International scientific meeting, Porto, Portugal (2016)
Title: Inhibition of staphylococcal biofilm formation by marine sponge-derived *Streptomyces* sp. D56

ASM Microbe 2017, New Orleans, USA (2017)
Title: Antibiofilm potential of marine sponge-derived *Streptomyces* sp. SBT343 against staphylococci

Eurobiofilms 2017, 5th European congress on microbial biofilms, Amsterdam, The Netherlands (2017)
Title: Antibiofilm activities of marine *Streptomyces* against staphylococci

Selected workshops

2014

- Scientific writing for PhD students
- Good scientific practice
- Workshop on kinetoplastida infections

2015

- Intercultural communication and cooperation
- Poster design
- Introduction to biotech industries
- EndNote for scientists

2016

- Analyzing your market potential as a scientist
- Quality management in biotech industries

2017

- Scientific image processing and analysis
- Oral presentation skills

Additional activities

- Mentor of DAAD-RISE intern student Ms. Brinley Raynor from North Carolina State University, USA (2016)
- Organizing committee member, Eureka!2016 International GSLS Symposium, Würzburg, Germany (2016)

Curriculum Vitae