Antimicrobial Activity of Marine Microalgae against Bacteria Causing Skin Diseases of Pet Animals

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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Dedicated to my Parents

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THE AUTHOR

Sharnalika Karmakar

June, 2020

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List of Acronyms and Symbols Used

Abbreviation and symbols	Elaboration
AMR	Antimicrobial
ANOVA	Analysis of Variance
BPW	Buffer peptone water
BFRI	Bangladesh Fisheries Research Institute
	Degree
<	Less than
>	Greater than
%	Percent
°C	Celsius
Ca	Calcium
CFU	Colony Forming Unit
Co	Cobalt
CVASU	Chattogram Veterinary and Animal Sciences University
DMSO	Dimethyl sulphate
DW	Distilled Water
EDTA	Ethylenediamine tetraacetic acid
Fe	Iron
GC	Granulomatous colitis
g	Gram
h	Hour
IBD	Inflamatory Bowel Disease
K	Potasium
L	Liter
MSA	Manitol Salt Agar
SDG	Sustainable Development Goal
MHA	Muller Hinton Agar

WHO	World Health Organization
MIC	Minimum Inhibition Concentration
MRS	Methicillin Resistant Staphylococcus
mg	Milligram
mm	millimeter
ml	milliliter
Mn	Manganese
Mg	Magnesium
mm	millimeter
Min	Minute
Ni	Nickel
NB	Nutrient Broth
Na	Sodium
PUFA	Polysaturated fatty acid
rpm	Rotation per minutes
SAQTVH	S. A. Quadery Teaching Veterinary Hospital
TSAB	Tryptic Soy Agar Base
TSB	Tryptic Soy Agar
Vol	Volume
Zn	Zinc
µm	Nanometer

Abstract

Microalgae play a significant role in the development of new products for medical and pharmaceutical research due to their ability to generate different biologically active metabolites. Microalgae are target organisms in the search for new antibiotic molecules to deal with antibiotic resistance. My results showed antimicrobial activity of three algal species *Tetraselmis* sp., *Nannochloropsis* sp. and *Chlorella* sp. against three pathogenic bacteria from pet dogs by disk diffusion method. *Chlorella* sp. showed maximum inhibition zone (13.88 mm) against *Escherichia coli,* whereas, *Nannochloropsis* sp. exhibited maximum (9.16 mm) against *Streptococcus* sp. and *Tetraselmis* sp. against *Staphylococcus saprophyticus* and *Escherichia coli*. The minimum inhibitory concentration (MIC) value of *Chlorella* was 30 mg/ml against *Escherichi coli* and *Staphylococcus saprophyticus* and The MIC value of *Tetraselmis* was 20mg/ml against *Escherichia coli* and 40 mg/ml against *Staphylococcus saprophyticus*. The current study also reported that *Chlorella* sp. was effective at a constant concentration against both the *Escherichia coli* and *Staphylococcus saprophyticus*. However, lesser amount of *Tetraselmis* sp. was required to suppress growth of Gram-negative *Escherichia coli* compared to Gram-positive *Staphylococcus saprophyticus* that need exactly double. It can be suggested that *Chlorella* sp., *Tetraselmis* sp. and *Nannochloropsi*s sp. could be used as substitutes of antibiotics against pathogenic bacteria of pet animal as they possess bioactive compounds that have antimicrobial properties.

Key words: Microalgae, *Chlorella* sp., *Tetraselmis* sp., *Nannochloropsis* sp., *Escherichia coli, Staphylococcus saprophyticus, Streptococcus* sp.

CHAPTER I

INTRODUCTION

1.1 Background:

Animal diseases, including zoonotic and other infectious diseases, have been considered a major hurdle in the livestock production systems particularly in economic and trade issues. Several recent global and regional events have threatened food security due to emerging or re-emerging animal diseases (Sultana *et al.*, 2016). A cross sectional study on 1070 species of dog, cat and rabbits at Dhaka city reported the highest prevalence of skin diseases 18.69%, 36% and 53.33% respectively (Sultana *et al.,* 2016).Among the emerging and re-emerging zoonoses, bacterial diseases includingpasteurellosis or cat scratch disease, bacteria transmitted by bites or scratches, cutaneous contamination-tuberculosis and kennel cough, as well as gastrointestinal pathogens such as salmonella, campylobacter, leptospirosis etc. have been reported with major human infections (Habiba *et al.*, 2016).

Antimicrobials are used in animals to treat or prevent disease and also to promote growth. Therapeutic, metaphylactic, prophylactic, sub-therapeutic are used as an antimicrobial therapy apply in animal body. The use of antibiotics in any setting contributes to the growing global threat of antibiotic resistance. Antimicrobial resistance (AMR) is the ability of a microorganism like bacteria, viruses, and some parasites to stop an antimicrobial such as antibiotics, antiviral and antimalarials from working against it. As a result, standard treatments become ineffective, infections persist and may spread to others**.** So, it is important to minimize the use of these drugs. This means eliminating unnecessary uses and finding other ways to prevent infections. In animal agriculture, alternative products play a crucial role in allowing farmers and veterinarians to reduce or largely phase out the use of antibiotics. Vaccines are among the most promising and widely used of these alternatives, but prebiotics and probiotics and other innovative products like immune modulators, phages, phytochemicals, organic acid, anti-microbial peptides, etc. are also in use or currently being investigated. Many alternative products enhance animal productivity and prevent infection at the same time, which could make them particularly attractive for commercial operations (Hoelzer *et al.,* 2017). In this regard, microalgal species have potential to benefit health as they contain some bioactive components such as

lutein, β-carotene, fatty acids, phenol, phycocyanin, γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid etc. which also reduce cholesterol and improve skin health (Singh, 2005). Different microalgal species such *as Chondrus crispus., Mastocarpus stellatus, Ascophyllum nodosum, Alaria esculenta, Spirulina plantesis, Nannochloropsis oculata, Chlorella vulgaris* and *Dunaliella salin* which are used to make products such as anti-irritant, antibacterial, antifungal, and anti-virus agent. Moreover, *Chlorella* sp., *Dunaliella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Tetraselmis* sp., *Spirulina*sp. and *Aphanizomenonflos aquae* have been used as antimicrobial agent to cure animal disease (Charoonnart *et al.,* 2018).

A study to explore antimicrobial activities of marine microalgae screened from Moroccan coastlines revealed that ethanolic extracts prepared from *Chlorella* sp. have activities against growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella* sp. The extracts also worked against *Candida albicans* and *Aspergillus niger* that causes human and animal diseases.The highest antibacterial activity was reported in the extract of *Tetraselmis* sp. and *Dunaliella salina* which exhibited an inhibitory effect against the three bacteria *Escherichia coli, Pseudomonas aeruginosa, Staphylococcusaureus* (Dewi, 2018).

1.2 Significance of the study

The current study will make a paradigm in the field of antimicrobial drug design, if successful. This project might discover micro algal bioactive compounds that inhibit bacteria to infect animal body. Marine microalgae might have potential to substitute the expansive antimicrobial agent.

Aims and objectives: The overall aim of this study is to identify microalgae available in marine sources that has/have antimicrobial effects on bacteria causing skin diseases in pet animals. The specific objectives are:

- To study the local marine microalgae whether they have antimicrobial effect on bacteria causing skin disease in the pet animals.
- To analysis the degree of antimicrobial activity of microalgae as potential alternative of currently available antibiotics.

CHAPTER II

REVIEW OF LITERATURE

2.1 Marine microalgae

Algae are simple plants that can range from the microscopic microalgae, to large seaweeds macroalgae, such as giant kelp more than one hundred feet in length. Microalgae include both cyanobacteria; it is similar to bacteria, and formerly called "blue-green algae" as well as green, brown and red algae. Microalgae are small-sized organisms found in fresh and saline waters, in both benthic and littoral habitats, and also throughout the ocean waters as phytoplankton, while the larger macroalgae that is seaweeds occupy the littoral zone (El deen *et al.*, 2011). Microalgae are unicellular to filamentous in form. They lack roots, vascular systems, leaves and stems, and are autotrophic and photosynthetic. Microalgae are generally eukaryotic organisms, although cyanobacteria, such as *Spirulina*, which are prokaryotes, are included under microalgae due to their photosynthetic and reproductive properties (Ravishankar *et al.*, 2012).

2.2 Characteristics of marine microalgae species

The important properties of marine microalgae species are;

i) Optimum temperature and temperature tolerance: Geographical location and its climate is an important factor for production of microalgae. The average temperature of Bangladesh varies from 23.9°C to 31.1°C during summer and from 7.2°C to 12.8°C in winter (Shahid, 2010). It is to be kept in mind that, the lethal temperature for microalgae is generally slightly higher than optimum temperature. Strain with broad optimum range usually shows better growth (Goldman *et al.*, 1977). Respiration during night time causes loss of biomass significantly (Grima *et al.*, 1996). So, length of day is also needed to be considered.

ii) CO_2 supply, pH and O_2 tolerance: For high rates of photosynthesis, efficient uptake of inorganic Carbon by the cell is very important. All microalgae can take up CO_2 and some can take up carbon in the form of HCO_3^- (Korb *et al.*, 1997). Generally, inorganic carbon exists in 4 forms in water; CO_2, H_2CO_3, HCO^{3-} , CO_3 Uncontrolled pH can be fatal for microalgae, as CO_2 is converted into $HCO3^-$ with increasing pH.

Most of the microalgae cannot uptake HCO^{3-} as their nutrient. Moreover, photosynthesis nearly ceases at pH 9. It should be noted that, CO_2 diffusion from air to the medium is greater at more alkaline pH (Lee, 1984).

iii) Respiration rate: Respiration rate is usually dependent on conditions like temperature, nitrogen content, light etc.

iv)Salinity: For production of lipid enriched microalgae, salinity of water is an important factor. Use of saline water reduces the pressure on fresh water requirement for cultivation. It also reduces the possibility of contamination by local freshwater strains in the medium.

v) Competitive strain: The selected strain must outlive other weaker strains for successful cultivation. Some selected species can outcompete contaminating organisms by surviving in high pH and producing DMSO (dimethylsulphoxide).This can act as an antibiotic and help in successful long term outdoor production.

2.3 Marine microalgae available in Bangladesh

The biodiversity of microalgae is enormous and they represent an almost untapped resource. It has been estimated that about 200,000-800,000 species in many different genera exist of which about 50,000 species are described(Starckx, 2012). They are also known as phytoplankton in the coasts of lakes and oceans, which include diatoms, dinoflagellates, green and brownish flagellate, and blue-green algae (Durmaz 2007).

i. Green algae or diatoms are the most commonly found microalgae as an alternate for production of an energy source. They are cultivated for basically high-value components, that is, pigments and proteins. Examples include: *Chlorella* sp., *Spirulina* sp., *Dunaliella* sp. and *Haematococcus* sp. Only *Dunaliella* sp. is a dominant sea species.

ii. Blue-green algae are also known as cyanobacteria and have similar features to bacteria. Their features include: anticancer and cytotoxic activities, antibacterial activity, antifungal activity, immunosuppressive activity.

iii. Dinoflagellatesare also known as Pyrrhophyta. They are found in oceans and can cause death of fish. They are a toxic form of algae.

iv. Bacillariophyceae are most frequently used algae because of their short doubling time and easy growth (Durmaz, 2007).

2.3.1 *Chlorella* sp.

Chlorella is a type of algae that grows in fresh water. The whole plant is used to make nutritional supplements and medicine.

Properties:

The pharmaceutical importance of *Chlorella* is attributed to its medicinal properties. There is ample experimental evidence of its antitumor, anticoagulant, antibacterial, antioxidant, and antihyperlipidemia effects in addition to a hepatoprotective property and the immune-stimulatory activity of enzymatic protein hydrolyzate(Korb*et al*., 1997; Lee *et al.,* 2012; Ordog*et al.*, 2004).

Bioactive compound:

Many antioxidant compounds are thought to be responsible for *Chlorella* functional activities. Antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid, and α-tocopherol, which are active against free radicals, have been identified. Some of these compounds not only are important as natural colorants or additives but also may be useful in reducing the incidence of cancer and in the prevention of macular degeneration (Korb *et al.*, 1997; Kokou *et al.*, 2012).

Uses:

- The biomass of *Chlorella* is used in aquaculture as feed, growth enhancers and immunostimulants.
- *Chlorella vulgaris* is an important species with a good bimolecular composition. Commercially, it is one of the most commonly used microalgae in aquaculture.
- Several studies confirmed its ability to improve nutrition, immunity, aquatic bioremediation, amelioration of stress, disease resistance of fish and inhibits bacterial quorum sensing when used appropriately.
- Despite claims of its benefits, *Chlorella vulgaris* is reported to have unfavourable effects when incorporated in diets at higher inclusion levels.
- In addition, its rigid cell wall might restrict the access of digestive enzymes to the intracellular components for proper digestion and assimilation.

- The role of *Chlorella vulgaris* and its importance in aquaculture with emphasis on its environmental requirements, morphology, pigments, digestibility, dynamics on growth performance, antibacterial activity, bacterial quorum sensing, immunomodulatory effect, anti-stress effect, gut microbiome, aquatic bioremediation and its safety as food or feed (Ahmed *et al.,* 2016).

2.3.2 *Tetraselmis* sp.

Tetraselmis is a green algal genus within the order Chlorodendrales, and they are characterised by their intense green coloured chloroplast, their flagellated cell bodies, the presence of a pyrenoid within the chloroplast, and a scale-produced thecal-wall. Species within this genus are found in both marine and freshwater ecosystems across the globe; their habitat range is mainly limited by water depth due to their photosynthetic nature. *Tetraselmis* species are found in both marine and freshwater ecosystems, and they occupy niches as primary producers in benthic and planktonic food webs. They can be found in many global waters, and their main enforcer of habitat range is light availability which restricts cells to the photic zone of the water column.

Properties:

The methanolic extracts of *Tetraspora cylindrica* present antibacterial activity against *Corynebacterium diphtheria*, *Klebsiella pneumoniae* and *Shigella boydii*, among others. These extracts also present antifungal activity against: *Curyularialunata, Fusarium sporotrichoids, Macrophominaphaseaolina, Rhizoctonia solani, Sclerotium rolfsii*and *Trichoderma harziamm* (Ghazala *et al.,* 2004). Use of cultured microalgae is common in the rearing of larvae of marine fish, crustaceans and bivalves. It has been observed that the addition of microalgae has a positive effect on the bacterial load of larval rearing systems by decreasing the numbers of opportunistic bacteria (Kokou *et al.,* 2012). It has been observed that five cultures of microalgae (*Chlorella minutissima, Tetraselmischui, Nannochloropsis* sp., *Arthrospira platensis* and *Isochrysis* sp.) with no culturable bacteria were tested for their ability to inhibit the growth of six *Vibrio* bacterial strains (*Vibrio parahaemolyticus, Vibrioanguillarum, Vibriosplendidus, Vibrioscophthalmi, Vibrioalginolyticus* and *Vibriolentus*) (Kokou *et al.,* 2012). The promising use of microalgae and microalgae compounds as sources of natural antibiotics against human

pathogens but also about their potential to limit microbial infections in aquaculture. An alternative to conventional antibiotics is needed as the microbial resistance to these drugs is increasing in humans and animals.

Bioactive compounds:

Tetraselmis is a prasinophyte commonly used in feeding of marine animals in aquacultures due to its nutritional value. Different Biochemical compound extracted from *Tetraselmis* like phthalic acid, 1,2-benzenedicarboxylic acid, 9-octadecenamide, oleanitrile, hexadecanoic acid, 2-hexadecanol etc (Kokkali *et al.*, 2020).

Use:

Tetraselmis and other microalgae species are used as food in aquaculture, and for biotechnological uses. *Tetraselmis* species, along with other microalgae are a promising source for biofuel use due to their fast growth rate, high lipid content, cheaper photosynthetic mechanisms, less need for agricultural land, useful by-products, and for being environmentally friendly. Research is currently being performed into specific microalgae species for biofuel use. *Tetraselmis* has a very high lipid level; their amino acids stimulate feeding in marine organisms (Alonso *et al.*, 2012).

2.3.3 *Nannochloropsis* sp.

*Nannochloropsis*is a genus of algae comprising six known species. The species have mostly been known from the marine environment but also occur in fresh and brackish water. All of the species are small, nonmotile spheres which do not express any distinct morphological features that can be distinguished by either light or electron microscopy.

Properties:

*Nannochloropsis*is widely distributed in ocean worldwide. It plays a significant role in global carbon and mineral cycles. These microalgae contain high rich protein, pigment and polyunsaturated fatty acid. It is commonly used in aquaculture as feed. These microalgae used as an excellent candidate for biodiesel production (Ma *et al.*, 2016).

Bioactive compound:

Nannochloropsis has six species, namely *Nannochloropsis gaditana, Nannochloropsis. granulata, Nannochloropsis limnetica, Nannochloropsis oceanica, Nannochloropsis salina,* and *Nannochloropsis oculata* (Ma *et al.,* 2014). *Nannochloropsis* is one type of microalgae that has been widely used as food for cultivation. *Nannochloropsis* is a microalgae that has been widely cultivated and is rich in benefits, especially in terms of health. The nutrients contained in it include protein, carbohydrates, fats, some minerals such as Ca, K, Na, Mg, Zn, Fe, Mn, Cu, Ni, and and Co (Rebolloso *et al*., 2001), fiber (Fithriani *et al.,* 2020) and vitamins such as tocotrienols (Durma, 2007). Some beneficial pigments such as chlorophyll-a, zeaxanthin, canthaxanthin, astaxanthin and violaxanthin are found (Lubian *et al.*, 1992). *Nannochloropsis* is very potential as a source of bioactive compound because it has high growth rates, and easy to cultivate even in unfavorable environmental conditions (Borges *et al.,* 2011). The nutrients contained in *Nannochloropsis* are also beneficial to the body, such as protein, carbohydrates, fats, minerals (Rebolloso *et al.*, 2001), and vitamins (Durmaz, 2007).

Use:

Nannochloropsis species have been used for several decades to produce nutraceuticals and feed supplements. Genetic engineering tools for nuclear transformation have been recently developed for this species.
The aquaculture potential for microalgae cultivated in is mostly to enhance the nutritional content (proteins, carotenoids, fatty acids, etc.), which positively affects the health and physical condition of the produced organisms. There exist a number of different modes in which microalgae are utilized for the larvae of molluscs, crustaceans, fish hatchings, or rotifers in aquaculture that are all sources of biomass – directly as unprocessed cells (live feed), to build up the respective food chain, or in the form of processed biomass to be added to the diet.

2.4 Marine microalgae and their potentials in pharmaceutical field

Pharmaceutically valuable products from microalgae and its industrial commercialization today is still in its infancy and can be seen as a gateway to a multibillion-dollar industry. Microalgae generally grow autotrophically and are

ubiquitous in nature. They represent a major untapped resource of genetic potential for valuable bioactive agents and fine biochemical. This proven ability of microalgae to produce these compounds places these microorganisms in the biotechnological spotlight for applications and commercialization as in the pharmaceutical industry. The immense chemical diversity of microalgae provides numerous applications in the food, feed and pharmaceutical industries. Microalgae are cultivated for the production of whole biomass and valuable substances such as nutraceuticals, carotenoids, phycocyanin and poly-unsaturated fatty acids (PUFAs), which are utilised in the food and feed (notably aquaculture) industry. The production of biofuel from lipid- or carbohydrates-rich microalgae is under way (Ravishankar *et al.,* 2012).Microalgae comprise a vast group of photosynthetic, heterotrophic organisms which have an extraordinary potential for cultivation as energy crops. They can be cultivated under difficult agro-climatic conditions and are able to produce a wide range of commercially interesting by-products such as fats, oils, sugars and functional bioactive compounds. Many valuable compounds can be extracted from microalgae, including pigments, lipids, proteins, polysaccharides, vitamins or minerals (Encarnacao *et al.,* 2015). The variety of compounds generated by microalgae can serve a broad spectrum of applications such as pharmaceuticals, cosmetics, human and animal nutrition, environmental restoration and protection or bioenergy (Priyadarshani *et al.,* 2012). Some strains of microalgae can produce metabolites with antibiotic activity aimed at killing or inhibiting bacterial growth. In some cases, this activity has only been identified in general extracts from the algal culture, without properly determining the chemical identity of the active compounds (Ordog*et al*., 2004; Chu *et al.*, 2004). There are indications that antibiotics are more likely to occur in strains isolated from environments polluted by bacteria than in strains isolated from cleaner environments. Several compounds have shown potent biological activities, such as antioxidants, anticoagulants, anti-inflammatory, antimicrobial or antitumoral. The possible use of these compounds as a source of prebiotics, nutraceuticals, chemopreventive agents or antimicrobial drugs was investigated and has demonstrated promising results (Talero *et al.,* 2015; Amaro *et al.,* 2011)

2.5 Antimicrobial activity of marine microalgae

Microalgae are present in almost all ecosystems around the world. They evolved in extreme competitive environments, are largely grazed by highly diverse consumers

and exposed to microbial pathogens such as bacteria, viruses and fungi. Marine microalgae constitute attractive sources of novel and active metabolites, comprising proteins, enzymes, pigments and polyunsaturated fatty acids (PUFA) that could be exploited in pharmaceutical, food, feed and cosmetic industries. Compounds with pharmaceutical characteristics, as antioxidative anti-inflammatory, antimicrobial or antitumoral properties, have been identified; some of them have been in the clinical trial state (Maadane *et al.,* 2020).

Microalgal species have potential to benefit health as they contain some bioactive components such aslutein, β-carotene, fatty acids, phenol, phycocyanin, γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid etc are also reduce cholesterol and improve skin health (De jesus *et al.,* 2013). Different microalgae species such as *Chondrus crispus, Mastocarpus stellatus, Ascophyllum nodosum, Alariaesculenta, Spirulina plantesis, Nannochloropsis oculata, Chlorella vulgaris, Dunaliellasalin*which are used to make products such as anti-irritant, antibacterial, antifungal and antivirus agent. Moreover, *Chlorella* sp., *Dunaliella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Tetraselmis* sp., *Spirulina* sp., *Aphanizomenonflos aquae* have been used as antimicrobial agent to cure animal disease (Maadane *et al.,* 2020). Eight marine microalgae *Chaetoceros* sp., *Chlorella* sp., *Dicrateria* sp., *Dunaliella* sp., *Isochrysis* sp., *Nannochloropsis* sp., *Synechococcus* sp., *Tetraselmis* sp. showed species specific activity in inhibiting the growth of bacteria such as *Salmonella paratyphi, Pseudomonas fluorescens, Shigella boydi, Klebsiella pneumoniae* and *Escherichia coli* (Krishnika *et al.,* 2017).

2.6 Marine microalgae as potential alternative to antibiotics in livestock production

Animal diseases, including zoonotic and other infectious diseases, have been considered as a major hurdle in the livestock production systems particularly in economic and trade issues. Several recent global and regional events have threatened food security due to emerging or re-emerging animal diseases (Tomley and Shirley, 2009). Antimicrobials are used in animals to treat or prevent disease and also to promote growth. Therapeutic, metaphylactic, prophylactic, sub-therapeutic etc. are used as an antimicrobial therapy apply in animal body. The use of antibiotics in any setting contributes to the growing global threat of antibiotic resistance (Milic *et al.,*

2013). Antimicrobial resistance (AMR) is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial such as antibiotics, antiviral from working against it. As a result, standard treatments become ineffective; infections persist and may spread to others. So, it is important to minimize the use of these drugs. This means eliminating unnecessary uses and finding other ways to prevent infections. In animal agriculture, alternative products play a crucial role in allowing farmers and veterinarians to reduce or largely phase out the use of antibiotics. Vaccines are among the most promising and widely used of these alternatives, but prebiotics and probiotics and other innovative products like immune modulators, phages, phytochemicals, organic acid, anti-microbial peptides are also in use or currently being investigated. Many alternative products enhance animal productivity and prevent infection at the same time, which could make them particularly attractive for commercial operations (Hoelzer and Talkington, 2017).

2.7 Bacterial diseases of pet animals in Bangladesh

In rural areas of Bangladesh, very few people keep dog as a pet animal but in urban areas dog rearing is getting popularity day by day. Like other domestic animals, dog is not free from diseases. Rural dogs are abundant in the ecosystem of area and interact with other species of wild carnivores and domestic animals in ways that could encourage disease transmission. Domestic dogs pose a significant risk as reservoirs for infectious diseases, especially for wild canids (Bronson *et al.,* 2008). The threat of disease transmission from domestic animals to wildlife has become recognized as an increasing concern within the wildlife community in recent years (Hossain *et al.*, 2017). Bacteria is everywhere and while most microorganisms do not have much impact on our daily lives, some types can cause disease. Our pets are exposed to bacteria daily and most of the time their immune system is able to fight it off without showing any signs of sickness. Bacterial disease occurs when a dog's immune system is weakened and the bacteria is able to replicate and spread in the dog's body. Common types of pathogenic bacteria in pet include *Salmonella* sp., *Leptospirosis* sp., *Campylobacter* sp., *Helicobacter* sp., *Streptococcus* sp., *Staphylococcus* sp.,*Clostridia*sp., *Bordetella* sp., *Escherichia coli*. The source of bacteria is usually contaminated water, dairy, feces or undercooked meat. Bacterial infections are common in dogs that come from rescue situations or spend a lot of time in boarding

kennels where many dogs are housed in the same area. Puppies and geriatric dogs are much more at risk of becoming sick because their immune systems are not as strong as adult dogs in their prime. Pets are sick with a bacterial infection usually have digestive upset (diarrhea or vomiting), a fever and are lethargic or cranky since they do not feel well. Cleanliness practices when handling dog food (particularly raw meats) and picking up stool are key to keeping bacteria from spreading (Sultana *et al.*, 2016)

Diseases caused by *Escherichia coli*:

Eecherichia coli is a gram-negative bacterium that can cause diarrhea in dogs. It is most often linked to a disease called granulomatous colitis. However, it is not a common cause of disease; it is often associated with hemolytic-uremic syndrome in dogs (Weese, 2008).

Diseases caused by *Staphylococcus saprophyticus*:

Staphylococcus saprophyticus is a Gram-positive coccus belonging to the genus *Staphylococcus saprophyticus* is a common cause of community-acquired urinary tract infections (Levinson, 2010). Staphylococci are found on the skin of both humans and dogs. In the latter, however, subspecies of this pathogen can lead to bacterial dermatitis. The bacteria particularly enjoy the mucous membranes or open wounds, and can be transmitted by other animals or humans. The skin infection caused by these bacteria usually occurs on the torso, between the toes or on the elbows. In puppies, signs of bacterial infection are usually visible on the stomach. Symptoms include itching, red skin, hair loss, ulcers and boils. Inflammation can spread to the heart, bones and joints (Widerstrom *et al.*, 2012).

Diseases caused by *Streptococcus* sp.:

Streptococcus sp. are opportunistic pathogens that normally reside in the upper respiratory, intestinal, lower urinary and genital tracts but can cause localized infection or septicemia in dogs of all ages (Lamm *et al.*, 2010). Streptococcal infection in dogs has been associated with abortion, pneumonia, septicemia, endocarditis, necrotizing fasciitis, keratitis, lower urinary tract infections, cholangio-hepatitis, arthritis, and meningo-encephalitis (Lappin *et al.*, 2019).

2.8 Common antimicrobials used in livestock production

Antimicrobials are used in animals to treat or prevent disease and also to promote growth. Therapeutic, metaphylactic, prophylactic, sub-therapeutic etc. are used as an antimicrobial therapy apply in animal body. The use of antibiotics in any setting contributes to the growing global threat of antibiotic resistance (Milic *et al.*, 2013). Different antimicrobial drugs used in various purposes such as cefalexina, moxiolin, amitriptyline, cefpodoxime, cefliofur, chloramphenicol, ciprofloxacin, clavamox, clavaseptin, clavulanic acid, clindamycin, dichlorophene, doxcycline, enrofloxacin, neomycin, oxytetracycline, pirlimycin, etc are use as antibiotic for bacterial infection. On the other hand, different fungicide like dichlorophene, nystatin and pesticide like clamoxiquine, fenbendazole, fipronil, levamisole, praziquantel, pyrantel etc. used in livestock (Milic*et al.*, 2013).

2.9 Antimicrobial resistance as a major obstacle in livestock sector

Antimicrobial resistance (AMR) is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial such as antibiotics, antiviral from working against it. As a result, standard treatments become ineffective; infections persist and may spread to others. There is concern about how antimicrobial resistance emergence in livestock will impact sustainable development goal (SDG), i.e. ensuring healthy lives and promoting wellbeing for all, at all ages. With meat production set to increase from 200 million tons to 470 million tons by 2050, it is likely that farmers will rely even more on antibiotics to prophylactically prevent disease in their livestock to meet this expected demand. The high proportion of poor-quality veterinary medicine for therapeutic use in livestock compounds the problem of antibiotic overuse, particularly in low- and middle-income countries. Numerous cases of antimicrobial resistance in humans have been traced to resistant microbes suspected of originating in livestock, which is particularly concerning as infected livestock, can be asymptomatic. Transmission of resistant bacteria from livestock to humans can occur through the consumption of meat, direct contact with colonized animals or manure spread in the environment. The strongest correlation between interspecies pathogen transmissions is observed in countries with policies to reduce agricultural

antibiotic use. So, it is important to minimize the use of these drugs. This means eliminating unnecessary uses and finding other ways to prevent infections.

2.10 Spread of AMR and risks to public health

Every year, antimicrobial resistance causes the death of around 700 000 people, and this number is expected to rise to an estimated 10 million deaths annually by 2050 (Hoelzer *et al.*, 2017). Antimicrobial resistance has the potential to affect almost all sustainable development goals (SDGs), particularly those targeting poverty, hunger, health and economic growth. Although the reduction and eradication of antimicrobial resistance is not included as an individual SDG, paragraph 26 of Transforming our world: the 2030 agenda for sustainable development states: "We will equally accelerate the pace of progress made in fighting malaria, human immunodeficiency virus/acquired immunodeficiency syndrome AIDS, tuberculosis, hepatitis, Ebola and other communicable diseases and epidemics, including by addressing growing antimicrobial resistance and the problem of unattended diseases affecting developing countries. Antimicrobial resistance is a public health threat. Because antimicrobial consumption in food-producing animals contributes to the problem, policies restricting the inappropriate or unnecessary agricultural use of antimicrobial drugs are important. However, this link between agricultural antibiotic use and antibiotic resistance has remained contested by some, with potentially disruptive effects on efforts to move towards the judicious or prudent use of these drugs. The types of evidence available for each step in the causal pathway from antimicrobial use on farms to human public health risk, and to evaluate the strength of evidence within a 'Grades of Recommendations Assessment, Development and Evaluation '(GRADE) framework (Hoelzer *et al.*, 2017).

2.11 Alternatives to antibiotics

In animal agriculture, alternative products play a crucial role in allowing farmers and veterinarians to reduce or largely phase out the use of antibiotics. Vaccines are among the most promising and widely used of these alternatives, but prebiotics and probiotics and other innovative products like immune modulators, phages, phytochemicals, organic acid, anti-microbial peptides are also in use or currently being investigated. Many alternative products enhance animal productivity and

prevent infection at the same time, which could make them particularly attractive for commercial operations (Hoelzer and Talkington, 2017).

2.12 Marine microalgae as potential alternative to antibiotics:

Microalgal species have potential to benefit health as they contain some bioactive components such aslutein, β-carotene, fatty acids, phenol, phycocyanin, γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid etc. are also reduce cholesterol and improve skin health (De jesus *et al.,* 2013). The resistance of pathogenic bacteria to existing antibiotics has become a global epidemic. Marine microalgae algae derivatives have shown promise as candidates in novel, antibacterial drug discovery. The efficacy of these compounds, their mechanism of action, applications as antibiotics, disinfectants, and inhibitors of food borne pathogenic and spoilage bacteria are reviewed (Shannon and Abu Ghannam, 2016). Different microalgae *Chaetoceros* sp., *Chlorella* sp., *Dicrateria* sp., *Dunaliella* sp., *Isochrysis* sp., *Nannochloropsis* sp., *Synechococcus* sp., *Tetraselmis* sp. showed species specific activity in inhibiting the growth of bacteria such as *Salmonella paratyphi*, *Pseudomonas fluorescens, Shigella boydi*, *Klebsiella pneumoniae* and *Escherichia coli*. Previous study reveals that the extracts of *Chlorella* sp., *Nannochloropsis* sp., *Dunaliella* sp., *Tetraselmis* sp. and *Isochrysis* sp. have optimal activity against all the pathogenic bacteria studied (Krishnika *et al.,* 2017).However, there is very limited or no published report of available microalgae in marine sources and their activities against the microbial agents causing aquaculture, human or animal diseases in Bangladesh. So the present work provides the eligibility of marine algae commonly found in Bangladesh as a prominent natural antibiotic against various pathogens of pet animals.

CHAPTER III

MATERIALS AND METHODS

Experimental design: My experiments were divided into three phases:

3.1 Culture of marine microalgae

3.2 Isolation, identification and culture of bacteria

3.3 Antimicrobial activity test

3.1 Culture of marine microalgae

Tetraselmis sp., *Chlorella* sp., and *Nanochloropsis* sp. were supplied by Bangladesh Fisheries Research Institute (BFRI), Cox's Bazar.

3.1.1 Conway medium preparation

Conway medium was used as a culture medium for the marine microalgae. Preparation of Conway medium needs to prepare the three stock solutions which were macronutrients (A), trace metal solutions (B) and vitamins (C). Each of the vitamins was dissolved separately in 100 ml distilled water and stored in a refrigerator. Different reagent bottles were prepared for the solution A, B and C. For each 1ml of (A), 0.5 ml of (B), and 0.1 ml of (C) was added into 1000 ml of filtered and sterilized sea water (James, 1996). Composition of Conway medium shown in Table 3.1.

Table 3.1 Preparation of Conway medium and constituents

Constituents	Quantities
Solution A: Chemicals	
• Potassium nitrate	100g
• Sodium orthophosphate	20g
• Sodium EDTA	45g
• Boric acid	33.4g
• Ferric chloride	1.3g
• Manganese chloride	0.36g
• Distilled water	1000ml
Solution B: Trace metals	

- Zinc chloride 4.2g
- Cobalt chloride 4.0g
- Copper sulphate 4.0g
- Ammonium molybdate 1.8g
- Distilled water 1000ml

Acidify with HCl to obtain a clear solution

Solution C: Vitamins

- Vitamin B (Thiamin) 200mg
- Vitamin B12 10mg

3.1.2 Mass culture of microalgae

Mass culture of selected potential isolates was done in large scale in tank using Conway medium. The culture was gradually scaled up from an initial starter culture volume of 20 ml to 20 L. Initially, 20 ml of microalgal stock cultures were mixed with 30 ml medium in each flask (total culture volume 50 ml), with batch cultures of increasing volume (250, 500) ml, 1L, 10 L) as inocula for the next step after which they were transferred to bigger container 20 L culture medium. After that, *Nannochloropsis* sp. and *Chlorella* sp. were harvested at their stationary phase on the day-12 and *Tetraselmis* sp. was harvested on the day-10. The microalgae were harvested by centrifugation at 5000 rpm for 5 minutes to get rid of the water content.

3.1.3 Preparation of microalgae extracts

The collected algae paste was dried at 40^0C for 24h using a hot air oven. The dried biomass was taken in clean 70 ml volume screw-crapped bottles and immersed in methanol solvents for 48h at room temperature (Arun *et al.*, 2012). Via a sterile funnel and sterile Whatman filter paper No.1 the supernatants were purified. Using a 0.2μm membrane filter paper, the filter paper was sterilized. In a rotary evaporator the filtrate was then concentrated under decreased pressure. The dry extract was stored at 4^0C until use.

3.2 Isolation, identification and culture of bacteria

3.2.1 Sample collection

Skin and anal swabs from dogs visiting SAQ Teaching Veterinary Hospital of Chattogram Veterinary and Animal Sciences University were collected by using sterile swabs and inoculated into Buffered Peptone Water (BPW). The samples were incubated at 37°C overnight. Turbid broth indicates bacterial growth. Bacteria was streaked onto different agar media for isolation and identification.

a **b**

Figure 3.1:a) Buffer peptone water (arrows indicate clear media) before bacteria inoculation **b)** Cloudy appearance of Buffer peptone water indicates growth of bacteria after 24 h of bacteria inoculation (arrow indicates cloudy media).

3.2.2 Agar preparation

Mannitol salt agar:

Suspended 111 gm of MRS media into 1000 ml of distilled water. The mixture was boiled to dissolve the medium completely. Autoclaved at 121°C for 15-20 minutes. Dispensed into sterile plates while liquid.

MacConkey agar:

Suspended 49.53 gm of agar powder into 1000 ml distilled water. Heated to boil to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes. Cooled to 45-50°C. Mixed well before pouring into sterile plates.

Blood agar:

Suspended 28 g of nutrient agar powder in 1000 ml of distilled water. Heated the mixture while stirring to fully dissolve all components. Agar cooled to 45-50°C in

water bath. Then added 5% (vol/vol) sterile defibrinated blood and mixed gently. Dispensed into sterile plates while liquid**.**

3.2.3 Isolation and identification of bacteria by culture and staining

Bacterial culture from buffered peptone water was streaked onto macconkey agar, mannitol salt agar, and blood agar and incubated at 37°C for 24 hrs. Bright-pink large colonies in macconkey agar, medium-sized yellow colonies in mannitol salt agar and round, smooth, and glistening, looking like dewdrops with/without haemolysis in Blood agar indicated growth of *Escherichia coli*, *Staphyloccus saprophyticus* and *Streptococcus* sp. respectively. Identification of bacteria was further confirmed by Gram's staining.

Gram staining of bacterial samples

Preparation of Gram's stain

Crystal violet

Firstly, 95% ethyl alcohol was dissolved into 20ml of 2g marked crystal violet. Then 8g ammonium oxalate dissolved into 80ml of purified water**.** The two solutions were combined together and allowed to stand at room temperature (25°C) overnight then filtered in a coarse filter paper and stored at room temperature.

Gram's iodine

One gram of crystalline iodine and 2 g potassium iodide were grinded in a mortar and mixed in 300 ml distilled water. After that, stored at room temperature (25°C) in a foil-covered bottle (to protect solution from light).

Decolorizer

To prepare decolorizer, 500 ml acetone, 475 ml ethanol or methanol and 25 ml distilled water were mixed thoroughly and stored at room temperature.

Safranin

Firstly, 2.5g certified safranin added into 95% ethyl alcohol. Then added 10 ml mixture of safranin and ethanol alcohol solution to 90 ml distilled water.Then stored at room temperature.

Gram staining procedure

Preparation of slide smear

One loop full of bacterial broth culture was taken onto a glass slide using sterile inoculating loop. A drop of water was taken before bacterial samples taken from agar plates and mixed well. The smear was left to air dry at room temperature. The smear was fixed by holding over flame for few seconds.

Staining protocol

In the sample/slide, primary stain crystal violet was applied for 1 min. To wash out the unbound crystal violet, rinsed the slide with a soft stream of water for up to 10 seconds.Added Gram's iodine for 1 min, this is a mordant or argent that fixes the crystal violet to the bacterial cell wall. Rinsed the slide with acetone for 3 seconds and rinsed with a gentle stream of water. After that, secondary stain safranin was added to the slide for 1 min and washed with a gentle stream of water. After air dried, slides were examined under light microscope. Gram-positive bacteria resembled blue/purple colour while Gram-negative bacteria as pink colour.

For preservation of bacteria, single colonie swere streaked onto macconkey agar slant, manitol salt agar slant, blood agar slant according to bacteria and incubated at 37°C overnight. Following addition of glycerol, the bacterial stocks were preserved in -20°C for later use.

3.2.4 Identification of bacteria by Vitek-2 System:

Vitek-2 system is a fully automated microbiology system for the identification of bacteria and yeasts (clinical and industrial applications) and susceptibility testing (clinical or veterinary applications). Identification of microorganisms is accomplished by biochemical methods. A turbidometrically controlled suspension of pure colonies in saline is inoculated into identification cards. These cards contain 29 different biochemical broths in reaction cells and one negative control cell to assess growth and viability of the suspension. Conventional catalase, coagulase, and oxidase tests (where appropriate) and the results of a Gram stain are required before inoculation of cards. To perform this test, bacterial isolates in slant agar were shipped to University Malaysia Terrenganu, Malaysia. Homogenous organism suspension was prepared from isolated and fresh pure colonies in 3ml of saline solution. Inoculum's density

was checked according to McFarland (McFarland depending of the card with the use of the densicheck). Age of suspension was not exceeded 30 min before inoculating card. Automatic Cards filled with vacuum and incubated all cards in same incubator Cards reading every 15.Incubation times vary from 2-15 hrs depending on the growth rate of the organism. The Vitek programmed computer determined whether each well was positive or negative by measuring light attenuation with an optical scanner. When the incubation period was completed, the reactions are analyzed automatically and the identification was printed.

3.3 Antimicrobial activity test

Fifty milligrams of dried extracts were dissolved in 1ml of extraction solvents (Al-Wathnani *et al*., 2012); the agar well diffusion method was used as determination of the antimicrobial activity (Ajay Kumar *et al*., 2010). Bacterial suspension was adjusted by adding 0.85% physiological saline to match turbidity of a 0.5 McFarland standard approximately 1.5×10^8 CFU/ml (Yilmaz, 2012). Each of bacteria species were inoculated in three replicates into the Muller-Hinton agar (MHA) plate for the use of antimicrobial activity by using cotton swabs. Wells (6 mm) were made on the MHA surface by using sterilized cork borer. 30µl of the extraction solvents and methanol (negative control) were pipette into separate wells. Meanwhile, standard antibiotic disc was placed in the MHA surface as positive control. The antimicrobial agent used for *Escherichia coli*, *Streptococcus* sp. and *Staphylococcus saprophyticus* were ceftriaxone as standard disc. Those plates were incubated at 37°C for overnight. Clear and circular zones were the inhibition zones that produced by the extracts and the zone of inhibitions were measured by using digital slide calipers (Robotics BD shipment) from one edge of the zone to the other edge. The relative percentage inhibition was the comparison of the antimicrobial activity of the extracts and control were used the following formula (Ajay Kumar *et al*., 2002).

$(X-Y) \div (Z-Y) \times 100$

Where,

X: total area of inhibition of the microalgae extracts

Y: total area of inhibition of the solvent

Z: total area of inhibition of the standard antimicrobial agents

For the total area of inhibition was calculated by using area = πr^2; where, r = radius of inhibition zone.

3.3.1 Minimal inhibitory concentration

Minimal inhibitory concentration (MIC) of microalgae was determined by using a tube dilution technique which measured sensitivity of bacteria to methanol extracts of microalgae suspension (Salem *et al*, 2011). For preparation of *Tetraselmis* and *Chlorella* stock solutions, mixed 600 mg microalgae into 6 ml nutrient broth in an eppendorf tube. Bacterial suspension was prepared and diluted to match the 0.5 McFarland standards. 200 µl bacterial suspension was put in each well of a 96 well microtiter plate down the column (1-7). In each of the two-replicates, microalgae stock solution was put to make the concentration as 10mg/ml, 20mg/ml, 30mg/ml, and 40mg/ml. Bacterial suspensions with the nutrient broth act as the positive control and bacterial suspensions with antibiotic act as a negative control. Incubated the 96 well plate overnight at 37^0C temperature. The presence of bacteria in each well was determined by colour and turbidity.MIC value was determined as the minimum concentration of microalgae extract capable of inhibition of bacterial growth.

CHAPTER IV

RESULTS

4.1 Culture of marine microalgae

Total 20L microalgae cultured of each species.

Table 4: Amount of harvested microalgae

Microalgae	Amount of harvested microalgae(g/20L)
1.*Chlorella* sp.	6.2g
2. *Tetraselmis* sp.	7g
3.*Nannochloropsis* sp.	6.7g

4.2 Isolation and identification of bacteria

4.2.1 *Escherichia coli*

In the current study, all 4-samples collected from dog skin were positive for *Escherichia coli* as observed characteristics pink colored colonies on MacConkey agar (Figure 4.1). Gram negative rod-shaped bacilli were observed in Gram staining. *Escherichia coli* species was confirmed by series of biochemical tests using a VITEK 2 system.

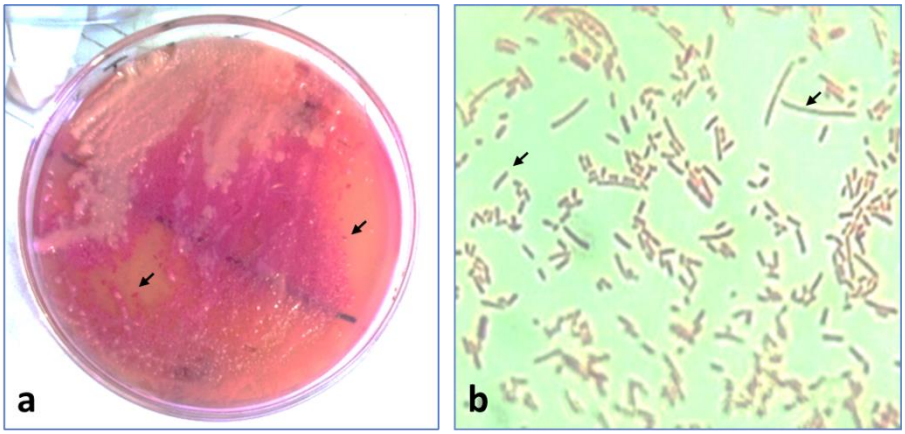

Figure 4.1 Isolation and identification of *Escherichia coli* by culture and Gram staining. a) On Mac Conkey agar, *Escherichia coli* was observed as pink colored colonies indicted by arrows. b) Gram negative bacilli indicated by arrows were observed under light microscope (400×) after Gram staining.

4.2.2 *Streptococcus* sp.

To identify *Streptococcus* sp., the crude bacterial culture was grown in blood agar and characteristics greyish colored colonies were observed (Figure 4.2). In Gram staining, Gram positive typical chain-arrangement of cocci were observed under microscope. Although several biochemical tests in a VITEK 2 system did not match with any specific bacteria, based on the colony characteristics and Gram staining it was considered as *Streptococcus* sp.

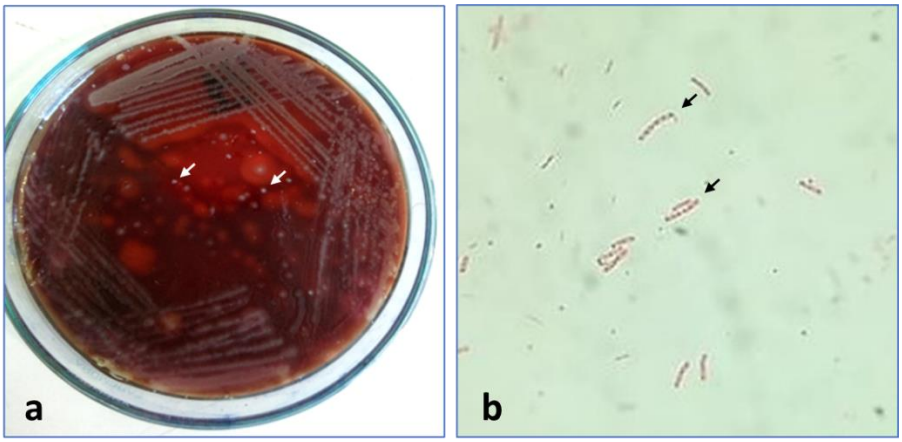

Figure 4.2 Isolation and identification of *Streptococcus* sp. by culture and Gram staining. a) On blood agar, *Streptococcus* sp. was observed as gray colored colonies indicted by arrows. b) Gram positive circular cocci with chain-arrangement indicated by arrows were observed under light microscope (400×) after Gram staining.

4.2.3 *Staphylococcus saprophyticus*

We targeted *Staphylococcus* sp. and was identified by pink color colonies with no color change of Mannitol salt agar (Figure 4.3). On Gram staining, Gram positive uniform cocci with grapes like arrangements were observed. The species was confirmed by several biochemical tests using a VITEK 2 system.

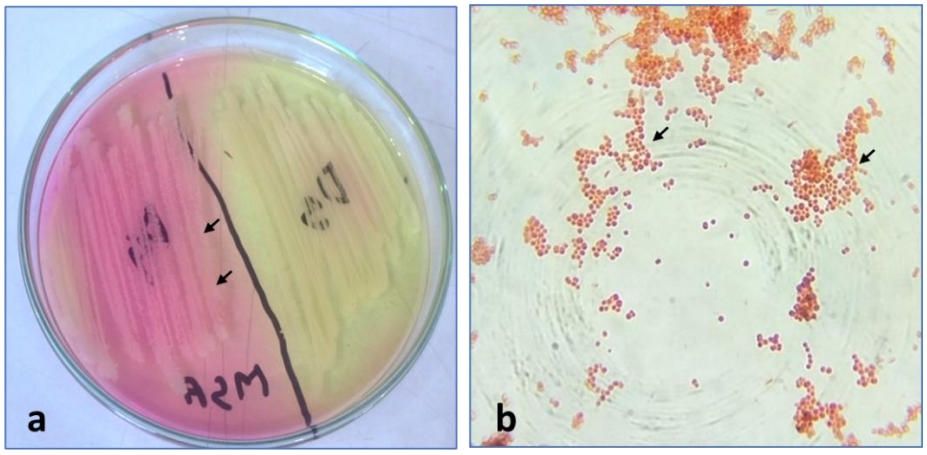

Figure 4.3 Isolation and identification of *Staphylococcus saprophyticus* by culture and Gram staining. a) On Mannitol Salt agar, *Staphylococcus* was observed as pink colored colonies indicted by arrows with unchanged color of medium (yellowish). b) Gram positive circular cocci with grapes-arrangement indicated by arrows were observed under light microscope (400×) after Gram staining.

4.3Antimicrobial activity test

4.3.1 Antimicrobial activity of microalgae against*Escherichia coli*

Variable degrees of antimicrobial activity of methanol extracts of *Tetraselmis*, *Chlorella* and *Nannochloropsis* was demonstrated against *Escherichia coli* in the current study (Figure 4.4, Appendix I).The largest inhibition zone appeared around the disc loading of extract of *Chlorella* with a diameter of 13.88mm (p<0.0001).

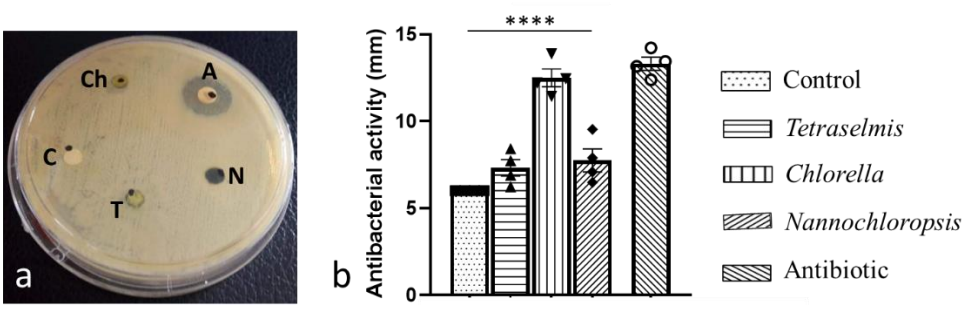

Figure 4.4 Antimicrobial activity of microalgae against *Escherichia coli*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), *Chlorella* (Ch), and *Nannochloropsis* (N) with no zone of inhibition around control disc (C). b) All three microalgae showed significantly higher zone of inhibition with *Chlorella* the highest. Statistical tests done by Kruskal-Wallis test, error bars represent standard error of means, p****<0.0001.

4.3.2 Antimicrobial activity of microalgae against *Streptococcus* sp.

The antimicrobial activity of methanol extracts of *Tetraselmis*, *Chlorella*, *Nannochloropsis* were observed against *Streptococcus* sp. (Figure 4.5, Appendix I). Among the microalgae, *Chlorella* had minimum effect on the bacteria, however, the largest inhibition zone appeared around the disc loading of extract of *Nannochloropsis* with a diameter of 9.16mm (p<0.05).

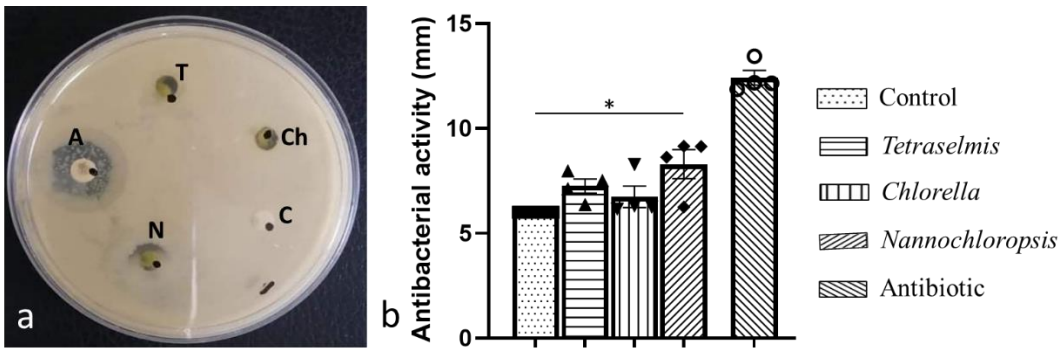

Figure 4.5 Antimicrobial activity of microalgae against *Streptococcus*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), and *Nannochloropsis* (N) with minimum zone around *Chlorella* (Ch), and no zone of inhibition around control disc (C). b) All three microalgae showed significantly higher zone of inhibition with *Nannochloropsis* the highest. Statistical tests done by ANOVA, error bars represent standard error of means, p*<0.05.

4.3.3 Antimicrobial activity of microalgae against *Staphylococcus saprophyticus*

Against *Staphylococcus saprophyticus*, *Tetraselmis* showed greater activity compared to *Nannochloropsis* with a diameter of 10.39 mm (p<0.001) (Figure 4.6, Appendix I). However, *Chlorella* showed no activity against *Staphylococcus saprophyticus*.

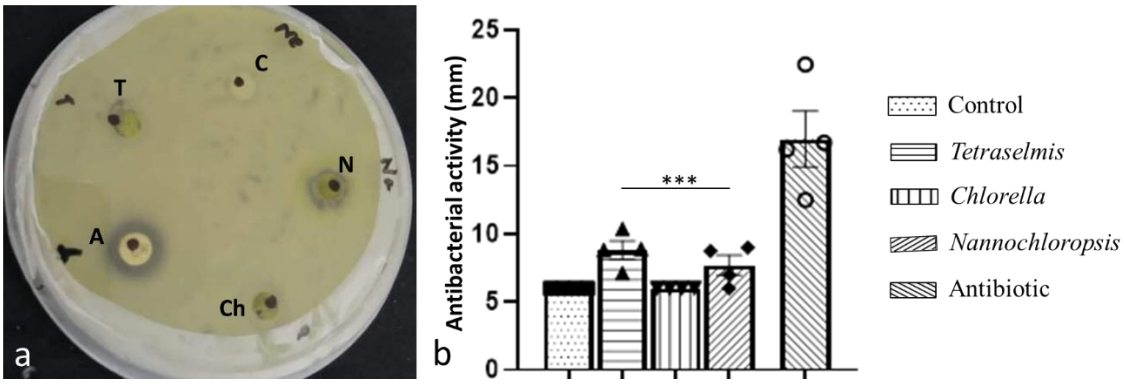

Figure 4.6 Antimicrobial activity of microalgae against *Staphylococcus saprophyticus*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), and *Nannochloropsis* (N) with no zone around *Chlorella* (Ch) and the control disc (C). b) *Tetraselmis* and *Nannochloropsis* showed significantly higher zone of inhibition with *Nannochloropsis* as higher. Statistical tests done by Kruskal-Wallis test, error bars represent standard error of means, p^{***}<0.001.

4.4Antimicrobial index of microalgae extracts

Antimicrobial index of microalgae extracts was calculated by using the formula that stated at above which used to compare the antimicrobial effect of the microalgae extracts with the effect of known standard antibiotic agents. The results of antimicrobial activity of microalgae extracts were compared to the antibiotic agents (positive control) are showed in **Table 4.1**. According to the table *Chlorella* had the highest relative percentage of inhibition (84.85%) compared to *Nannochloropsis*(16.9%) and *Tetraselmis* (12.32%) against *Escherichia coli*. But *Nannochloropsis*showed the highest relative percentage of inhibition (27.72%) against *Streptococcus* sp. and *Tetraselmis* exhibited the highest relative percentage of inhibition (17.68%) against *Staphylococcus saprophyticus.*

Table 4.1 Relative percentage of inhibition of the microalgae extracts compared to antibiotics

Name of microalgae	Relative percentage		
	Escherichia coli	*Streptococcus* sp.	*Staphylococcus saprophyticus*
Tetraselmis	12.32	14.03	17.68
Chlorella	84.85	7.98	11.90
Nannochloropsis	16.9	27.72	----

4.5 Minimal inhibitory concentration (MIC)

The MIC of the extracts from *Chlorella* and *Tetraselmis*grown under different conditions were determined against *Escherichia coli* and *Staphylococcus saprophyticus.* Due to insufficient amount, it was not possible to determine MIC value of *Nannochloropsis* against these bacteria and any of these three microalgae against *Streptococcus*. The MIC value was determined as the minimum concentration of microalgae capable to prevent bacterial growth. It was observed that *Tetraselmis* has strong antimicrobial activity against *Escherichia coli*, however, *Chlorella* has against *Staphylococcus* (**Table 4.2**).

Table 4.2 MIC value of microalgae against *Escherichia coli* and *Staphylococcus saprophyticus*

Microalgae	MIC value (mg/ml)	
	Escherichia coli	*Staphylococcus saprophyticus*
Chlorella sp.	30	30
Tetraselmis sp.	20	40

CHAPTER V

DISCUSSION

The current study demonstrated the bacterial loads in the cases of skin infections in dogs and whether the specific marine microalgae have antibacterial effects against them. It was observed that all samples collected from dermatological lesions were positive for *Escherichia coli, Streptococcus* sp. and *Staphylococcus saprophyticus.* All these three identified bacteria are clinically important in dogs and cause a wide variety of skin infections such as pyoderma, septic wound etc. In most cases, these bacteria cause secondary infection with previous malassezia dermatitis, allergy or wounds. Although most of the bacterial infections are easily checked by regular dressing with antiseptics and application of antibiotics there are some bacteria for example, methicillin resistant *Staphylococcus* (MRS) are very hard to treat. Therefore, the current study was designed to determine whether the marine microalgae have antibacterial effects on the common bacteria causing skin infections in dogs.

Antibiotic resistance in bacteria and fungi is one of the principal emerging health care related complications in the world; it developed into a bigger problem of providing treatment against resistant pathogenic bacteria (Sieradzki *et al.,* 1999). The majority of clinically used antimicrobial drugs have flaws like toxicity, absence of effectiveness, inhibiting cost and their persistent use in prompting to the evolution of immune strains. One technique to minimize antibiotic resistance is the design of novel antimicrobial compounds for scientific study (Desbois *et al.,* 2008 and 2009). Algal organisms are valuable source of structurally unique and biologically active secondary and primary metabolites which may be potential bioactive compounds of concern in the pharmaceutical industry (Tuney *et al.,* 2006). Microalgae and cyanobacteria offer various conveniences for antimicrobial investigations because of their enormous biodiversity and rapid production time (Pulz and Gross, 2004).

Microalgae constitute one of the commercially important living and sustainable sources. Algae are an extremely attractive natural source of new compounds and many of them possess antioxidant, antimicrobial, and antiviral activities (Plaza *et al.*, 2010). An extensive number of algal extracts have been discovered to have antimicrobial action (Plaza *et al.*, 2010). In the current study, all three microalgae *Chlorella*, *Tetraselmis* and *Nannochloropsis* showed variable degrees of sensitivity against *Escherichia coli*, *Streptococcus*sp. and *Staphylococcus saprophyticus*. Najdenski *et al.,* (2013) stated that ethanolic extract of *Scenedesmus obliqus, Chlorella* sp. and *Nostoc* sp. has antibacterial effect against *Staphylococcus aureus* and *Bacillus cereus*. In the same manner Sanmukh *et al.*, (2014) explored bioactive compounds of a group of microalgae with emphasizing on the *Chlorella sp.* which showed antibacterial effect against *Staphylococcus* sp. Krishnika, (2017) tested antibacterial activity of *Scenedesmus* sp. isolated from a natural pond against three pathogenic bacteria with different solvents, the aqueous and methanol extracts gave further results. Sanmukh *et al.,* (2014) affirmed promising applications encompassing antibacterial, antiviral, and antifungal activities; also, he declared that the application of bioactive compounds derived from algae will turn out useful and often more efficient as measured with traditional treatment methods.

The antimicrobial activity of *Tetraselmis*, *Chlorella* and *Nannochloropsis* was driven out to detect inhibition against one of the commonest pathogens like *Escherichia coli* which are pathogenic to humans and animals. The highest inhibition zone was observed by *Chlorella*. Syed *et al.*, (2015) recorded similar results with the highest inhibition zone when using ethanol extracted *Chlorella vulgaris* against pathogenic bacteria *Escherichia coli, Klebsiella* sp., and *Bacillus* sp. The antibacterial property of *Chlorella* could attribute to the fact that they may contain useful bioactive compounds such as flavonoids, tannins, phenolic compounds, terpenes, cardiac glycosides, saponins, and carbohydrates (Alghanmi and Omran, 2020). Substantial evidence of the existence of these seven bioactive compounds indicated that *Chlorella vulgaris* shows a significant part in achieving various bioactive compounds as an effective precursor. The drugs extracted from these algae species must get some specific function to contain bacterial growth, which rises in further specialized control of vector infections without any side effects (Alghanmi and Omran, 2020). In another

survey, five species (four cyanobacterial and one green algae), namely *Nostoc caeruleum, Spirulina platensis, Cylindrospermum majus, Oscillatoria formosa* and *Chlorella vulgaris* were analyzed for their antibacterial action against three gram-positive bacteria *(Staphylococcus aureus, Staphylococcus epidermidis,* and *Streptococcus pyogenes),* three gram- negative bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Escherichia coli*), as closely as for their antifungal activity against *Aspergillus fumigatus, Candida albicans, Geotricum candidumn,* and *Trichophyton mentagrophytes* using the agar well diffusion method (Ahmed, 2016). The results pointed out that *Chlorella vulgaris* extract was more potent against bacteria and fungi strains investigated (Ahmed, 2016). However, contradictory to our findings Maadane *et al.,* (2020) noted that *Chlorella* sp. did not demonstrate any inhibitory activity against *Escherichia coli* even at the concentration of 5.0 mg extract per ml.

Like *Escherichia coli* the antimicrobial activity of *Tetraselmis, Chlorella* and *Nannochloropsis* was carried out to determine inhibition against *Streptococcus* sp.The largest inhibition zone developed around the disc loading of extract of *Nannochloropsis*. The methanolic extracts of *Nannochloropsis* displayed highest degree of antimicrobial activity against *Streptococcus* compared to *Chlorella* and *Tetraselmis*. The displayed results were identical to that recorded on the antimicrobial activity of different solvent extracts of *Nannochloropsis.*In a comparable study antimicrobial activity of the methanol extracts of *Nannochloropsis oculata* exhibited varying degree of antimicrobial activities against test microorganisms *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis* (Sangeetha and Anuradha, 2020).

In the present investigation, methanolic extracts of *Tetraselmis* sp. was found significantly sensitive against *Staphylococcus* sp. The antimicrobial index of *Tetraselmis* sp. showed *Staphylococcus* more sensitive to *Tetraselmis* extract compared to *Chlorella*. It can be thought that *Tetraselmis* sp. present antibacterial activity against Gram-positive bacteria. In a previous study, the highest inhibition zone was recognized in methanol + chloroform (1:1) extract of *Tetraselmis suecica* against Gram-negative bacteria *Proteus* and Gram-positive bacteria *Streptococcus pyogens*(Austin *et al.,* 1992). The antimicrobial activities of *Tetraselmis* might be

dueto different compounds, comprising those previously determined (Maadane *et al.*, 2015): fatty acids, carotenoids and phenolic compounds. Fatty acids, which constitute major parts of the extracted biomasses, are particularly considered because their antimicrobial effects have been long recognized (e.g., Galbraith *et al.*, 1971; Desbois*et al.*, 2009; Cakmak*et al.*, 2014).The extract from *Tetraselmis* contains high amounts of oleic acid (48.8 % of the ethanol-extracted fatty acids), linoleic acid (36.4%) and palmitic acid (18.6%). These fatty acids were determined as major components in the ethanolic-extract or hexanic-extract of *Dunaliella salina* (Herrero *et al.*, 2006), and reported to be responsible, in a main part, of the antimicrobial activity, exercised against *Escherichia coli*, *Staphylococcus aureus* and *Chlorella albicans*. Furthermore, the antibacterial activities observed in the algal species, *Nostocspongiforme*, *Oscillatoria tenius* and *Chlorococcus* sp. were linked to their contents in fatty acids (Maadane *et al.*, 2020). Considering these literature-data, the antibacterial activity of *Tetraselmis* sp. can be linked, for a major part, to its content in palmitic, oleic and linoleic acids.

The current study also reported that Chlorella is effective at a constant concentration against both the *Escherichia coli* and *Staphylococcus saprophyticus.* However, lesser amount of *Tetraselmis* species is required to supress growth of Gram-negative *Escherichia coli* compared to Gram-positive *Staphylococcus saprophyticus* that needs exactly double. This might be due to the thicker cell wall of Gram-positive bacteria necessitating more concentrated microalgae extracts of *Tetraselmis* sp.

CHAPTER VI

CONCLUSION

The current study demonstrated antibacterial activity of three-species of marine microalgae against three-species of bacteria isolated from dermatological cases of dog skin. It was observed that *Chlorella* has the greatest propensity to supress E*scherichia coli*, however, *Nannochloropsis* was very suppressive against *Streptococcus* and *Tetraselmis* showed sensitivity against *Staphylococcus saprophyticus*. Further studies are directed to identify the specific components of microalgae that are responsible for the sensitivities against bacterial infections.

LIMITATIONS

I was unable to isolate and identify microalgae directly from the marine sources. I used the microalgae that we already have in our laboratory stored. A larger bacterial sample size could increase the sensitivity and specificity of the results. I also unable to grow enough microalgae sufficient for the antibacterial sensitivity testing and determination of MIC values.

CHAPTER VII

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Appendix I

Table 1 Inhibition zones against *Escherichia coli*(diameter in mm)

Sample	Ceftriaxone	*Tetraselmis*	*Chlorella*	*Nannochloropsis*	Negative
1	13.45	6.89	12.45	7.89	6
2	12.38	7.73	11.43	7.08	6
3	14.22	8.42	13.88	9.53	6
4	13.15	6.23	12.21	6.48	6
Mean	13.32	7.31	12.49	7.74	6

****p<0.0001

Table 2 Inhibition zone of *Streptococcus*(diameter in mm)

Sample	Ceftriaxone	*Tetraselmis*	*Chlorella*	*Nannochloropsis*	Negative
1	13.45	6.38	6.25	6.27	6
2	12.16	7.52	8.28	9.16	6
3	11.87	7.13	6.31	8.63	6
4	12.18	7.98	6.25	9.14	6
Mean	12.41	7.25	6.74	8.29	6

*p<0.05

Table 3 Inhibition zone of *Staphylococcus saprophyticus*(diameter in mm)

Sample	Ceftriaxone	*Tetraselmis*	*Chlorella*	*Nannochloropsis*	Negative
1	12.51	10.39***	6	8.75	6
2	16.71	8.83	6	7.02	6
3	16.22	8.91	6	7.23	6
4	20.46	7.13	6	9.03	6
Mean	16.47	8.81	6	8.00	6

***p<0.001

BIOGRAPHY

SharnalikaKarmakarwas born in Saidpur, Nilphamari, Bangladesh in 1996. She is the elder daughter of Vambal Nath Karmakar and Shanchita Karmakar. She passed the Secondary School Certificate Examination from Sunflower School and College Gaibandhar Govt College, Gaibandha in 2011 with GPA 4.88 followed by Higher Secondary Certificate Examination from Gaibandhar Govt College, Gaibandha in 2013 with GPA4.80. She completed her graduation degree on BSc in Fisheries from Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh in 2018 with CGPA 3.47. As an intern student she received practical Knowledge from University of Malaysia Terrenganu, Malaysia. She has a great enthusiasm in research and has done some aquaculture base research works. Now, she is a Candidate for the degree of MS in Aquaculture, Dept. of Aquaculture, Faculty of Fisheries, CVASU