CHAPTER I

INTRODUCTION

1.1 Background of the study

Algae are the base of the entire aquatic food chain, and are called the ‘green gold’ of Bangladesh. Microalgae are phytoplankton that plays a vital role in the rearing of aquatic animals like mollusks, shrimp and fish. Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can be found in all ecosystems both aquatic and terrestrial (Richmond 2004; Mata *et al.,* 2010). Microalgae are defined as unicellular which covert sun's energy into chemical energy by photosynthesis and can process like the mainland plant to produce complex organic compounds (Harun *et al.,* 2010). Filamentous and phytoplankton algae are the two major populations in the algae’s world and the four main classes are diatoms, green algae, blue-green algae and golden algae (Demirbas *et al.,* 2010). There are over thousands or even millions of microalgae species existing in nature (Hannon *et al*., 2010). Species or strain selection is the first and critical step in bioprospecting of microalgae for any commercial application (Barclay & Apt 2013; Borowitzka 2013). Screening of microalgae species involves a series of steps including sample collection, isolation, purification, identification, maintenance and characterization of potential products (Gong & Jiang 2011). They are specially valuable due to their high content of compounds with different biological activities, including both complex organic compounds and primary and secondary metabolites, such as phytopigments (xanthophylls and carotenoids), polyunsaturated fatty acids (PUFAs), phenolic substances, docosahexaenoic acid (DHA), vitamins, carbohydrates, tannins, terpenoids, and peptides, among others (Guzmán *et al.*, 2019).

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. In order to survive, they had to develop tolerance or defense strategies. The variety of these mechanisms resulted in a high diversity of compounds synthesized from diverse metabolic pathways.

It appears that many of these metabolites present very specific chemical structures that are not encountered among terrestrial organisms, and sometimes with a structural complexity that makes often too difficult to reproduce them by heme-synthesis or complete synthesis. Microalgae also have many biologically active compounds (Spolaore *et al.,* 2006). They have the ability to produce some of bioactive compounds such as antioxidants, antibiotics and toxins which are widely used in pharmaceutical industries (García-Casal *et al.,* 2007). Antibacterial, antifungal, antiprotozaol and antiplasmodial properties of microalgae supports produce different type of antibiotics by microalgae extracts or extracellular products. The crucial compounds defined as antimicrobial are fatty acids, acrylic acid, halogebated aliphatic compounds, terpenes, sulphur containing hetero cyclic compounds, carbohydrates and phenols (Kannan *et al.,* 2010).

For a long period of time, marine environments have been a worthwhile fountain for natural products in support of maintaining the human health, especially in the last decade, with the more attention for natural based remedies against animal and human diseases. On the other hand, the microbial communities in seawater and sediments are highly abundant that its concentration may reach 10^6 and 10^9 per milliliter, respectively (Austin, 1988). In reference, marine organisms are exposed in far tougher condition compared with the terrestrial ones. They are continuously exposed to high concentrations of bacteria, fungi and viruses, many of which might be pathogenic. This declares their dependence on successful antimicrobial pathways to protect them against microbial infections. This high diversity provides an excellent opportunity to discover new bioactive compounds (Abubakar *et al.,* 2012). Consequently, the marine environment is considered a rich source of new compounds for the development of new antibacterial medicines. Microalgae have been recognized to use as source of chemical and pharmacological novelty and diversity. Moreover, microalgae act as producers to produce some of highly bioactive compounds found in marine resources. The γ-lactone malyngolide is an antibiotic that isolated from the dichloromethane extract of a shallow-water variety of the blue green alga *Lynbya majuscule* to against *Mycobacterium smegmatis* and *Streptococcus pyogenes* (El Gamal, 2010). Besides that, green algae *Haematococcus pluvialis*can produce the astaxantihin which is a ketocarotenoid.

Astaxanthin is the major carotenoid in *Haematococcus pluvialis*and it is used as a nutraceutical and a medicinal ingredient against degenerative disease such as cancer, inflammation and *Helicobacter pylori* infection (Rao *et al.,* 2010). Kokou and his co-workers (2012) reported that *Nannochloropsis* sp , had the ability to inhibit the growth of six *Vibrio* bacterial strains which are *V. parahaemolyticus*, *V. anginolyticus*, *V. anguillarum*, *V. splendidus*, *V. scophthalmi* and *V. lentus*. That were not affected the antibacterial activity of *Nannochloropsis* sp. with or without the light (Kokou *et al.*, 2012). According to the same researchers, the *Vibrio* bacterial strains were incubated with the *Tetraselmis* sp., cultures for 5 days in natural photoperiod and light condition. The result showed that the ability to inhibit the growth of six *Vibrio* bacterial strains which was *V. parahaemolyticus*, *V. anginolyticus*, *V. anguillarum*, *V. splendidus*, *V. scophthalmi* and *V. Lentus* (Kokou *et al.,* 2012).

Microalgae can be used as sustainable protein sources in the production of peptide-based functional foods for preventing or treating especially cardiovascular disease (CVD). Peptides derived from microalgae have been mainly obtained by enzymatic digestion using proteolytic enzymes. They have been mostly obtained from protein hydrolyzates of *Chlorella vulgaris*, *Chlorella ellipsoidea*, *Tetradesmus obliquus*, *Navicula incerta*, and *Nannochloropsis oculata* (Guzmán *et al.,* 2019). The resulting bioactive peptides have shown to possess different biological functionalities, such as antioxidant, anticancer, antihypertensive, and antimicrobial activities, with beneficial health effects and potential therapeutic applications (Guzmán *et al.,* 2019). The growing number of antibiotic resistant bacteria is becoming a serious problem that affects most of the countries worldwide. Resistance to multiple antibiotics has developed among many common pathogens, such as staphylococci, pneumococci, and pseudomonas organisms (Olofsson and Cars, 2007). Infectious diseases tend to be a limiting factor for public health all over the world. Consequently, a lot of researches have been done to find an effective tool for preventing or curing the disease (Marimuthu *et al.,* 2015). Therefore, the present work has been focused on studying the potentiality of the extracts of marine microalgae as an alternative to antibiotics against bacterial disease of poultry.

1.2. Aims and objectives of the study:

The overall aim of this study is to identify indigenous marine microalgae available in marine sources that have antimicrobial effects on common bacteria causing diseases in poultry. The specific objectives of the study are:

- To demonstrate antimicrobial activity of marine microalgae on bacteria causing diseases in poultry.
- To analyse the degree of antimicrobial activity of specific microalgae as potential alternative to currently available antibiotics.

CHAPTER II

REVIEW OF LITERATURE

2.1 Marine microalgae and their potentials in pharmaceutical field

2.1.1 Marine microalgae

Microalgae are photosynthetic microorganisms adapted to live in very different environments and showing an enormous biochemical and genetic diversity. Microalgae-derived compounds have shown several properties, such as anticancer, antimicrobial, anti-inflammatory, and immunomodulatory. In the last decade, compounds stimulating the immune system, both innate immune response and adaptive immune response, have been used to prevent and fight various pathologies, including cancer thus representing an excellent source of new natural products with possible applications in several biotechnological sectors.

Marine organisms are an exceptional source of bioactive metabolites. The chemical diversity of the molecules they produce as well as the wide range of biological activities they show (e.g., antibiotics, toxins, antibiofilm agents, UV protective compounds, immune modulators) make them very attractive (Zea-Obando *et al.,* 2018). Among marine organisms, bacteria, fungi, microalgae, cyanobacteria and their symbiotic associations have increasingly become sources of such compounds (Zea-Obando *et al.*, 2018). More precisely, a large number of microalgal extracts have demonstrated antibacterial, antifungal, anti-algal and antiprotozoal activities (Zea-Obando *et al.,* 2018). Microalgae contain a high number of active compounds from diverse chemical families such as fatty acids derivatives, peptides, terpenoids, polysaccharides which combat bacterial colonization. Desbois *et al*., (2008) isolated an antibacterial polyunsaturated fatty acid from the marine diatom, *Phaeodactylum tricornutum*, which showed an activity against a range of both Gram-positive and Gram-negative bacteria. Hence, microalgal extracts are particularly interesting to inhibit the development of biofilm and biofouling, especially as they could be obtained in large amounts and optimized by means of biotechnological process (Zea-Obando *et al.,* 2018).

2.1.2 Marine microalgae available in Bangladesh

Worldwide there is a rich biodiversity of over 50,000 species of microalgae exist. In Bangladesh, no updated data is available as we do not have a national microalgae collection and culture centre. However, a total of 200 marine algal taxa (seaweeds) have been reported so far, by several Bangladeshi researchers.

Indigenous species of microalgae reported in Bangladesh:

- *Chlorella vulgaris*
- *Euglena* sp.
- *Scenedesmus* sp.
- *Chaetoceros* sp.
- *Nannochloropsis oculata*
- *Tetraselmis* sp.
- *Isochrysis galvana*
- *Bacillariophyta* sp.
- *Cyanophyta* sp.
- *Chlorococcus* sp.

2.1.3 Characteristics of marine microalgae species

2.1.3.1 *Tetraselmis*

According to the Algae Base, the taxonomy for *Tetraselmis* sp. as below:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Chlorophyta

Class: Chlorodendrophyceae

Older: Chlorodendrales

Family: Chlorodendraceae

Genus: *Tetraselmis*

Availability

Tetraselmis is a flagellated, green colour chloroplast (**Figure 1a**). It has pyrenoid within the chloroplast and a scale-produced the call-wall (Norris *et al.,* 1980; Becker *et al.,* 1994). 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 (Norris *et al.,* 1980). They live in diverse water-environments if enough nutrients and light is available for net photosynthetic activity. Some species of *Tetraselmis* occur in plankton; others are benthic, colonizing sand and a few occur as endosymbionts in metazoans.

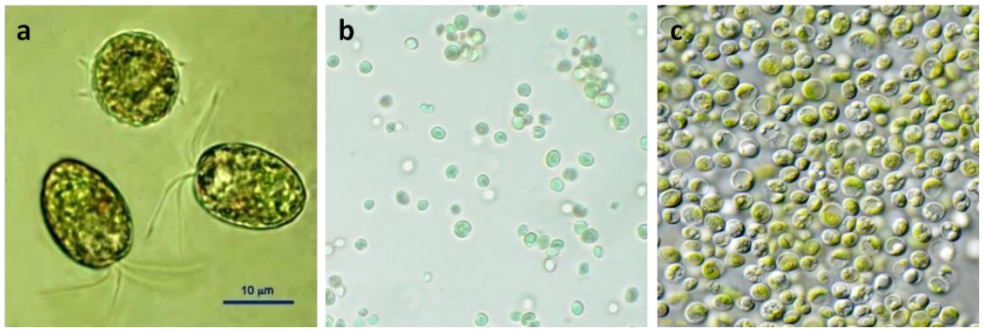

Figure 1. Common microalgae available in Bangladesh. a) *Tetraselmis* sp. has 4-flagella at one end, source: Planktonnet; b) *Nannochloropsis* sp. are small, nonmotile spheres, source: Culture Collection of Autotrophic Organisms (CCALA); c) *Chlorella* sp. is spherical with no flagella, source: Wageningen University.

Properties

Tetraselmis sp. is unicellular flagellate phytoplankton that is elliptical or almost spherical in shape and it is green motile cells. The motile cells of *Tetraselmis* sp. are generally laterally compressed, and it form 4 equal and homodynamic flagella in an anterior pit of the cell. It usually grows to 10 µm long × 14 µm wide. Thin cell wall formed by extracellular fusion of scales cover the cells which it is called theca. The hairs and pentagonal scales will cover the flagella. *Tetraselmis* sp. has single chloroplasts which comprise a single eyespot and a pyrenoid that only can be found in *Tetraselmis* (Arora *et al.,* 2013). It reproduces by asexual division when in the non-motile stage. During the non-motile stage, the new walls develop and a stalk formed by old walls accumulating as concentric rings around the cell or being polarized on one side.

Bioactive components

Tetraselmis sp. possess chlorophylls, carotenoids and starch that have potential to inhibit certain bacteria because it contains antioxidative substances that important source used in pharmacological studies (Lee & Hur, 2009).

Use

Tetraselmis sp. is used in aquaculture for decades as source of nutrition for juvenile molluscs, shrimp larvae and rotifers (Laws *et al.,* 2011; Arora *et al.,* 2013). *Tetraselmis* sp. has proven to be useful for both research and industry. *Tetraselmis* sp. has been studied for understanding plankton growth rates, and recently a colonial colony species is being used to gain an understanding of multicellularity evolution (Norris *et al.*, 1980, Arora *et al*, 2013). Additionally, many species are currently being examined for their use as bio-fuels due to their high lipid content.

2.1.3.2 *Nannochloropsis*

According to the Algae Base, the taxonomy for *Nannochloropsis* sp. as below:

Domain: Eukaryota

Super phylum: Heterokonta

Phylum: Ochrophyta

Class: Eustigmatophyceae

Family: Eustigmataceae

Genus: *Nannochloropsis*

Availability

Nannochloropsis are six known species and have mostly been known from the marine environment but also occur in fresh and brackish water (Hibberd 1981; Fawley 2007). All of the species are small, non-motile spheres which do not express any distinct morphological feature, and cannot be distinguished by either light or electron microscopy (**Figure 1b**). The characterisation is mostly done by rbcL gene and 18S rRNA sequence analysis.

Properties

Nannochloropsis differ from other related microalgae in that they have chlorophyll-a and completely lack chlorophyll-b and chlorophyll-c. In addition they are able to build up high concentrations of a range of pigments such as astaxanthin, zeaxanthin anad canthaxanthin (Lubián *et al.,* 2000). They are 2-3 µm in diameter and have a very simple ultra structure with reduced structural elements compared to neighbouring taxa (Kandilian *et al.,* 2013).

Bioactive components

Terpenoids is an active compound contains in the *Nannochloropsis* sp. and it is most powerful antioxidants in the body and it has antibacterial activity as well. The functions of terpenoids are organic synthesis process and regain of body cells (Yanuhar *et al.,* 2011).

Use

Nannochloropsis is considered a promising algae for industrial applications because of its ability to accumulate high levels of polyunsaturated fatty acids (Sukenik, 1989). Moreover, it shows promising features that can allow genetic manipulation aimed at the genetic improvement of the current oleaginous strains. Various species of *Nannochloropsis* indeed are transfect able and there has been evidence that some strains are able to perform homologous recombination (Kilian *et al.,* 2011). It is mainly used as an energy-rich food source for fish larvae and rotifers. Nevertheless, it has raised growing interest also for the investigation of bio-fuel production from photosynthetic organisms. *Nannochloropsis* is also used as food additive for human nutrition and it is also served at restaurant "A Poniente" of El Puerto de Santa María (Cádiz, Spain) close to the natural environment where *Nannochloropsis gaditana* was first isolated and still grows (Lubián, 1982).

2.1.3.3. *Chlorella*

According to the Algae Base, the taxonomy for *Chlorella* sp. as below:

Phylum: Chlorophyta

Class: Trebouxiophyceae

Family: Chlorellaceae

Genus: *Chlorella*

Availability

Chlorella sp. is green algae found either singly or clustered in fresh or salt water and in soil.

Properties

Chlorella sp. is spherical in shape, about 2-10 μm in diameter, and is without flagella. It contains the green photosynthetic pigments chlorophyll-a and chlorophyll-b in its chloroplast. In ideal conditions it multiplies rapidly, requiring only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce.

Bioactive components

Chlorella sp. is one of the main sources of natural bioactive compounds used in the food and pharmaceutical industries.

Use

Chlorella sp. has been extensively used in photosynthetic studies, in mass cultivation experiments, and for purifying sewage effluents. Because the algae multiply rapidly and are rich in proteins and B-complex vitamins, several species have also been studied as a potential food product for humans both on Earth and in outer space. *Chlorella* is sometimes used as a vegan nutritional supplement. *Chlorella* is consumed as a health supplement primarily in the United States and Canada and as a food supplement in Japan (Becker, 1994). Manufacturers of *Chlorella* sp. products assert that it has a number of purported health effects, including an ability to treat cancer. According to the American Cancer Society, "available scientific studies do not support its effectiveness for preventing or treating cancer or any other disease in humans". *Chlorella* sp. has been considered as a potential source of food and energy because its photosynthetic

efficiency can, in theory, reach 8% which exceeds that of other highly efficient crops such as sugar cane (Boussiba *et al.*, 1987).

2.1.4 Antimicrobial activity of marine microalgae

Microalgae have been recognised to use as source of chemical and pharmacological novelty and diversity. The γ-lactone malyngolide is an antibiotic that isolated from the dichloromethane extract of a shallow-water variety of the blue green alga *Lynbya majuscule* to against *Mycobacterium smegmatis* and *Streptococcus pyogenes* (El Gamal, 2010). Besides that, green algae *Haematococcus pluvialis* can produce astaxantihin which is a keto-carotenoid. Astaxanthin is the major carotenoid in *Haematococcus pluvialis* and it used as a nutraceutical and a medicinal ingredient against degenerative disease such as cancer, inflammation and *Helicobacter pylori* infection (Rao *et al.,* 2010). Superior antioxidant properties to β-carotene had showed by astaxanthin in a number of in vitro studies (Jyonouchi, 1994).

In an earlier study of 3000 of algae species reported that a few percentages have potential bioactive compound (Bhakuni & Rawat, 2005). *Nannochloropsis oculata* is a unicellular marine alga belonging to the class Eustigmatophyceae (Demirbas, 2010). *N. oculata* that contains fats, proteins, carbohydrates, chlorophylls, carotenoids, minerals and trace elements (Sukenik *et al.*, 1989). *Haematococcus pluvialis* contains bioactive compound astaxanthin (Rao *et al.*, 2010), *Nannochloropsis* sp. has terpenoids (Yanuhar *et al.,* 2011), *and Tetraselmis* sp. has antioxidant substances (Lee & Hur, 2009). The functions of terpenoids are organic synthesis process and regain of body cells (Yanuhar *et al.,* 2011). Kokou and his colleagues (2012) reported that *Nannochloropsis* sp. has the ability to inhibit the growth of six *Vibrio* bacterial strains which were *V. parahaemolyticus*, *V. anginolyticus*, *V. anguillarum*, *V. splendidus*, *V. Scophthalmi* and *V. lentus*.

Tetraselmis sp. also has potential to inhibit certain bacteria because it contains antioxidative substances that are used in pharmacological studies (Lee & Hur, 2009). Kokou and his colleagues incubated *Vibrio* bacterial strains with *Tetraselmis* sp. cultures

for 5 days in natural photoperiod and light condition. The result showed that the *Tetraselmis* sp. has the ability to inhibit the growth of six *Vibrio* bacterial strains; *V. parahaemolyticus*, *V. anginolyticus*, *V. anguillarum*, *V. splendidus*, *V. Scophthalmi* and *V. Lentus* (Kokou *et al.*, 2012).

Chlorella sp. has antimicrobial activity against bacteria and fungi. Wang and his colleagues found that *Chlorella* sp. pre-treated by nitrogen starvation performed amazingly in ammonia removal, assimilating NH3–N at 19.1 mg/L/d in wastewater with 160 mg/L NH3–N (Wang *et al.*, 2015).

2.2 Marine microalgae as potential alternative to antibiotics in poultry industry

2.2.1 Bacterial diseases of poultry in Bangladesh

Poultry birds that have been domesticated to reproduce and grow in captivity and that render products of economic value such as meat, eggs, feathers, fertiliser, animal food, and pharmaceuticals. Chickens, ducks, muscovies, geese, Guinea fowl, quail, pigeon, pheasants, and turkeys are generally considered as poultry birds. In the current study, only chicken was focused as it is the mostly consumed poultry in Bangladesh having significant economic value.

Bacterial diseases of chicken in Bangladesh were studied by several researchers (Giasuddin *et al.*, 2002; Islam *et al*., 2003; Rahman *et al.*, 2004; Rahman *et al.*, 2007; Kamaruddin *et al*., 2007; Ahmed *et al.*, 2009; Hasan *et al.*, 2010; Uddin *et al.*, 2010; Islam *et al.*, 2012). According to them the important bacterial diseases include collibacillosis, salmonellosis, fowl cholera, nectrotic enteritis, infectious coryza, gangrenous dermatitis, and staphylococcosis. In a study by Rahman and colleagues reported that in chicks salmonellosis was 53.90%, colibacillosis 13.36%, necrotic enteritis 1.18% and infectious coryza 0.59% (Rahman *et al.*, 2007). The study also reported that in birds, salmonellosis, colibacillosis, infectious coryza and necrotic enteritis were detected at the rate of 55.96, 11.93, 29.91 and 2.20%, respectively. Furthermore, in adult chicken salmonellosis was found at a rate of 53.32% followed by

infectious coryza 6.11% and necrotic enteritis 1.48%. They did not find any colibacillosis infection in adult chicken. Another study by Giasuddin and colleagues reported salmonellosis 5.56%, collibacillosis 4.42% and fowl cholera 3.08% (Giasuddin *et al.*, 2002).

2.2.1.1 Diseases caused by *E. coli*

Colibacillosis is a localised or systemic infection caused by avian pathogenic *Escherichia coli* (APEC). It manifests in diverse ways, including as acute fatal septicaemia, sub-acute pericarditis, airsacculitis, salpingitis, peritonitis, and cellulites. It is one of the most commonly occurring and economically devastating bacterial diseases of poultry worldwide. Signs are nonspecific and vary with age, organs involved, and concurrent disease. Young birds dying of acute septicaemia have few lesions except for an enlarged, hyperemic liver and spleen with increased fluid in body cavities.

Omphalitis or navel infection is characterised with reddening and tissue oedema in the umbilical region. When the amount of egg white is bigger, it impedes the absorption during hatching, resulting in subcutaneous jelly-like oedemas that are an excellent media for the development of *E. coli* infection.

Salpingitis or inflammation of the oviduct could also be observed in growing birds. The oviduct is dilated, with thinned wall and filled with caseous exudates all along its length. Salpingites are among the commonest causes for death in layer hens. *E. coli* penetrates from the cloaca via an ascendant route. Predisposing factors are the intense egg laying and the associated estrogens activity.

Cellulites or inflammation of the subcutaneous tissue that affects also the overlying skin is predominates in broilers and is detected mainly in slaughter-houses. Macroscopically, the lesions are with a yellowish-brown colour.

Panophthalmitis or inflammation of all tissues of the eyeball generally develops secondary to *E. coli* septicaemia and is usually unilateral.

Arthritis, osteomyelitis and osteonecrosis (inflammation of joints, bone marrow and bone necrosis, respectively) are common sequel to *E. coli* septicaemia. Clinically, lameness, prolonged lying down, dehydration and retarded growth rate are observed. The coxofemoral joints, the femur and tibiotarsal joints are most commonly affected. The bacteria colonize the physes of growing bones and provoke an inflammatory response that is further causing osteomyelitis. Pathoanatomically, fractures of the femoral head are usually discovered.

2.2.1.2 Diseases caused by *Stenotrophomonas maltophilia*

Water quality in the drinking water system (DWS) plays an important role in the general health and performance of broiler chickens. Conditions in the DWS of broilers are ideal for microbial biofilm formation. Since pathogens might reside within these biofilms, they serve as potential source of waterborne transmission of pathogens to livestock and humans. The most identified dominant species from TAC were *Stenotrophomonas maltophilia, Pseudomonas geniculata* and *Pseudomonas aeruginosa*. Overall, 92% of all tested microorganisms were able to form biofilm under lab conditions. *Stenotrophomonas maltophilia* considered as opportunistic pathogens and could consequently be a potential risk for animal health. Additionally, the biofilm-forming capacity of these organisms could promote attachment of other pathogens such as *Campylobacter* sp. and *Salmonella* sp.

2.2.1.3 Diseases caused by *Staphylococcus saprophyticus*

Staphylococcus saprophyticus is a common cause of acute uncomplicated urinary tract infection and bovine mastitis. *S. saprophyticus* has become one of the most serious human pathogens during recent decades owing to its acquisition of antibiotic resistance. A study in the UK showed a high prevalence of macrolides antibiotics resistance in *S.*

saprophyticus, indicating that this species might be a potential reservoir of macrolides antibiotics resistance genes. Facing with the increasing number of multidrug-resistant (MDR) bacteria, new strategies to combat resistant pathogens are extremely needed. What's worse, the rate of antibiotic discovery has dropped substantially (Laxminarayan, 2014). Francolin is a common bird in southern China and its meat was reported to be tastier, juicier, more palatable, and richer in protein than domestic chicken meat (Ukoha *et al.,* 1992). Francolins ophthalmia is often caused by resistant conditional pathogenic bacteria. Conditional pathogenic *Staphylococcus saprophyticus* is a potential reservoir of macrolides antibiotics resistance gene (Wang *et al.*, 2019).

2.2.2 Common antimicrobials used in poultry industry

Antimicrobials are used in poultry industry to treat clinical diseases caused by bacteria, virus, fungus, and parasites etc. The antibiotics commonly used in human are frequently used in poultry (**Table 1**). Furthermore, antibiotics are also used as growth promoter to produce healthy chickens.

Table 1. List of antimicrobials used to treat selected bacteria in poultry.

Antimicrobials	**Target pathogen**
Ceftiofur	*E. coli, P. multocida, Staphylococcus* sp., *R. Anatipestifer*
Amoxicillin	*E. coli, O. Rhinotracheale*
Ampicillin	*B. avium, E. Coli*
Apramycin	*E. coli*
Erythromycin	*E. coli, Staphylococcus* sp.
Gentamicin	*E. coli, Staphylococcus* sp.
Neomycin	*E. coli, E. rhusiopathiae, Staphylococcus* sp.
Penicillin-G	*O. rhinotracheale, E. coli, Staphylococcus* sp.
Neomycin- tetracycline	*O. rhinotracheal*
Oxytetracycline	*O. rhinotracheale, E. coli, Staphylococcus* sp.

2.2.3. Antimicrobial resistance (AMR)

The World Health Organization (WHO) defines antimicrobial resistance as a microorganism's resistance to an antimicrobial drug that was once able to treat an infection by that microorganism. A person cannot become resistant to antibiotics. Resistance is a property of the microbe, not the person or other organism infected by the microbe. Resistance can be described in two ways:

a) Intrinsic or natural whereby microorganisms naturally do not possess target sites for the drugs and therefore the drug does not affect them or they naturally have low permeability to those agents because of the differences in the chemical nature of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to affect their action, or

b) Acquired resistance whereby a naturally susceptible microorganism acquires ways of not being affected by the drug.

Various factors are responsible for acquired resistance:

- Presence of an enzyme that inactivates the antimicrobial agent.
- Presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent.
- Mutation in the antimicrobial agent’s target, which reduces the binding of the antimicrobial agent.
- Post-transcriptional or post-translational modification of the antimicrobial agent’s target, which reduces binding of the antimicrobial agent.
- Reduced uptake of the antimicrobial agent.
- Active efflux of the antimicrobial agent.
- Overproduction of the target of the antimicrobial agent.
- Expression or suppression of a gene in vivo in contrast to the situation in vitro.
- Previously unrecognized mechanisms

2.2.4. Transmission of antimicrobial resistant and risks to public health

Antibiotics are crucial in human healthcare. They are used in the treatment of bacterial infectious diseases, supporting surgical interventions, and in cancer and prophylactic treatment. Antibiotics are also used widely in livestock and domestic animal veterinary treatments and as growth promoters in aquaculture. Global production of antibiotics for human use is valued at $40 billion a year illustrating their societal and economic importance. Antibiotic consumption is on the rise and between the years 2000 and 2010 there was an estimated 36% increase in use globally for human healthcare. Microorganisms exposed to antibiotics at low, sub-lethal or sub-inhibitory exposure concentrations can develop, or acquire, antimicrobial resistance (AMR) and this has been identified as a major threat to public health (Coast and Smith, 2002). AMR is likely to persist and disseminate in diverse environments, including in aquatic ecosystem. Where the benefit of possessing and expressing the resistance gene outweighs the fitness costs of carriage, antibiotics in the environment may select for and enrich resistance genes in bacterial populations/communities which can then harbour these resistance determinants and transfer them to human pathogens. Antimicrobial resistance (AMR) is now a global problem, and resistance in *Enterobacteriaceae*, specifically *E. coli* and *Klebsiella pneumoniae*, is a critical threat to human health. There are numerous studies that highlight the occurrence of antimicrobial resistant *E. Coli* in cattle, horses, pigs, sheep, goats, and companion animals such as dogs. Microbiological studies show that several types of integrons (DNA element involved in antimicrobial resistance) are shared among *E. coli* that are isolated from humans, dogs, and domestic livestock like cattle, pigs, and poultry, making the transmission between species likely. Thus, it is possible that resistant bacteria are transmitted to humans from animals. People who are involved in livestock farming have been shown, in some studies, to have higher rates of carriage of antimicrobial resistant bacteria than non-farming controls (Siegel *et al.,* 1975). Poultry workers in the United States were significantly more likely to carry *E. coli* resistant to gentamicin, an antibiotic of limited human use, than community controls who were not involved with poultry production (Price *et al.,* 2007). Similarly, in Saudi Arabia, poultry farmers had a higher prevalence of gentamicin resistant *E. coli* than hospitalized patients

(38% versus 22%, respectively) (Al-Ghamdi *et al.,* 1999). In Holland, poultry farmers had a higher prevalence of carriage of ciprofloxacin resistant *E. coli* (17%) than subjects participating in other studies (<1% to 3%) in the 1990s (Bruinsma *et al., 2002).*

2.2.5. Alternatives to antibiotics

To meet the increasing threat of antibiotic resistance, a paradigm shift in the treatment of infectious disease is needed and alternatives to antibiotics ought to be considered. Until today, there are already several non-antibiotic approaches to the treatment and prevention of infection. Antibiotics do unfortunately not discriminate between pathogenic bacteria and bacteria of the normal flora. Side effects, like diarrhea, are therefore common since a disrupted normal flora provides opportunistic bacteria with a chance to colonize. Probiotics are products containing live microorganisms that can help to establish or maintain the normal flora and thus prevent or treat mild infections, for example of the gut**.** Bacteriocins are a type of antimicrobial peptide with a low toxicity to mammalian cells, but highly effective against bacteria. There are different groups of bacteriocins. Generally, they kill bacteria by inserting themselves into the cell walls and opening them up, leading to disruption or death of the affected bacteria. A group of lactic-acid bacteria known to be harmful are used in over 50 countries as a food-preservative. Another bacteriocin produced by Enterococcus faecium has been effective against multi-drug resistant bacteria. Finding potential alternatives to antibiotics is to some extent related to identifying natural enemies of pathogenic bacteria. Plants and animals produce substances known as antimicrobial peptides as a defense against intruding pathogens. The usability of antimicrobial peptides in human medicine is being evaluated, as are the applicability of what is known as bacteriao phage therapy. Some of the alternatives to conventional antibiotics may also be used in conjunction with antibiotics. This combination can increase the efficiency of the antibiotic or extend the time an antibiotic can be used before it becomes obsolete. This is important, because the rate of discovery of antibiotics has significantly slowed while bacteria continue to develop resistance.

2.2.6 Marine microalgae as potential alternative to antibiotics

Microalgae are introduced to poultry diets mainly as a rich source of n-3 long chain polyunsaturated fatty acids (LCPUFA n-3), including docohexaenoic and eicosapentaenoic acid, but they also serve as a protein, microelement, vitamin and antioxidants source, as well as a pigmentation agent for skin and egg yolks. The majority of experiments have shown that microalgae, mainly *Spirulina* and *Chlorella* sourced as a defatted biomass from bio fuel production, and can be successfully used as a feed ingredient in poultry nutrition. They can have beneficial effects on meat and egg quality, i.e., via an increased concentration of LCPUFA n-3 and carotenoids, and in regards to performance indices and immune function. Positive results were obtained when fresh microalgae biomass used to replace antibiotic growth promoters in poultry diets. As a rich source of nutrients and biologically active substances, including protein, amino acids, LCPUFA n-3, microelements, vitamins, antioxidants, and carotenoids, they have a long history of instance *Nannochloropsis gaditana*, *Schizochytriumli macinum*, *Phaeodactylum tricornutum*, and application as a food for humans (Belay *et al.*, 1996). The use of some microalgae species, for *Isochrysis galbana*, in poultry nutrition could be of interest not only as a source of nutrients, but also as an alternative way of enriching of eggs with LCPUFAs n-3. The blue-green algae *Spirulina* is cultivated worldwide for use in the food and feed Industries. Dried *Spirulina* biomass has a high nutritional value for human and animals as it contains about 60-70% protein, as well as being a good source of essential fatty acids, vitamins and minerals (Khan *et al.*, 2005). *Spirulina* is a rich source of carotenoids and contains around 6 gm total xanthophylls and 7 gm total carotenoids/kg in freeze-dried biomass. A very early study with chickens Combs, 1952) demonstrated that dried *Chlorella*, included into the diet at 10% could serve as a rich source of certain nutrients, i.e., carotene, riboflavin and vitamin B12, and increased performance in birds when the diet was deficient in these nutrients. Kang and colleagues studied the effects of the replacement of antibiotic growth promoter with different forms of *Chlorella* on performance, immune indices and the intestinal microfloral population (Kang *et al.*, 2013). They found that *Chlorella* in its fresh liquid form included at a 1% dietary level beneficially affected BWG, some immune characteristics (e.g., number of white blood

cells and lymphocytes, plasma IgA, IgM, and IgG concentrations) and the intestinal production of *Lactobacillus* bacteria. The results of several experiments have shown that microalgae, as a rich source of LCPUFAs n-3. Poultry products enriched with n-3 long chain polyunsaturated fatty acids are good examples of a functional food, i.e., food that, in addition to possessing traditionally understood nutritional value, can beneficially affect the metabolic and health status of consumers, thus reducing the risk of various chronic lifestyle diseases (Pietras and Orczewska-Dudek, 2013.

CHAPTER III

MATERIALS AND METHODS

3.1 Culture of microalgae

Pure isolate seeds of *Tetraselmis* sp., *Nannochloropsis* sp. and *Chlorella* sp. were collected from Live Feed Research corner of Department of Aquaculture, CVASU. Three replicates of each species of microalgae were cultured in a 500 ml conical flask with the 150 ml of Conway Medium. Three replicates of each microalgae were incubated at 28°C±2, 3000 Lux of illumination provided by warm-white fluorescent in 16:9 h light-dark cycle and aeration was supplied (Ghasemi *et al*., 2003).

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 0.1ml of C was added into 1000 ml of filtered and sterilized sea water (James, 1996). The compositions of Conway medium shown in **Table3.1.1**

Table 3.1.1 Composition and preparation of Conway medium Tompkins et al., (1995)

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 B_{12} (cyanocobalamine)	10mg

3.1.2 Mass culture of microalgae

Five millilitres of pure microalgae species from pure stock were added into 15 ml Conway culture medium in a 50 ml conical flask and was cultured in the constant environmental condition. Stopper with non-absorbent cotton wool was wrapped around a glass pipette to act as aerating tube and the top of pipette was inserted an air filter. When reached stationary phase of microalgae's growth curve, 50 ml of microalgae was transferred into 200 ml of culture medium in a 500 ml conical flask. The steps were repeated to transfer into the large-scale medium until got the 20 L of biomass. Then, the *Tetraselmis* sp. was harvested on the tenth day while *Nannochloropsis* sp. and *Chlorella* sp. were harvested on the twelve day by centrifugation at 4000 rpm for 10 min at 4°C to get rid of the water content.

3.1.3 Preparation of microalgae crude extracts

The algal paste of *Tetraselmis* sp. was dried at 60°C for 12 h in a hot air oven while *Nannochloropsis* sp. and *Chlorella* sp. were air dried at 40°C for 24 h by using hot air oven. About 7g of dried microalgae was obtained from 20 L culture of *Nannochloropsis* sp. The dry microalgae were then soaked in 70 ml of methanol solvent

(10ml/g of microalgae) in sterile screw-capped bottles for two days at room temperature (Arun *et al.,* 2012). After that, the extract was filtered through sterile Whatman no. 1 filter paper to remove all unextractable matter such as cellular material. The filtrate was then concentrated under reduced pressure in a rotary evaporator (SIMIĆ *et al.,* 2012). The dry extract was stored at -20°C until use.

3.2 Isolation and identification of bacteria from chicken samples

3.2.1 Sample collection from poultry

Samples were collected from dead chickens from different types of commercial poultry farms supplied to the S. A. Quadery Teaching Veterinary Hospital (SAQTVH) and Pathology Lab of Chattogram Veterinary and Animal Sciences University (CVASU) for post-mortem examinations. While a carcass was opened up, a portion of liver sample was collected aseptically by sterile scissors. Inoculums collected from a liver inoculated in a test tube containing Buffer peptone water.

3.2.2 Preparation of bacterial culture media

Buffered peptone water

One gram of peptone bacteriological powder and 1g peptone powder were weighed and mixed in 1000ml distilled water in conical flask. The mixture was heated for proper mixing, autoclaved at 121^0C for 15 min for sterilization and then cooled to room temperature. 5 ml of buffered peptone water was poured into the test tube for collection of bacterial samples.

MacConkey's agar

An amount of 51.53g MacConkeys agar was suspended into 1000ml distilled water and heated to boil in a hot plate until dissolved completely. Then the media was sterilized by autoclave at 121^0C for 15 minutes. The medium was poured gently into Petri dish when

60°C under laminar flow. One plate was incubated at 37^0C for 24 hours to check sterility (no bacterial growth). The plates were stored at 4°C in refrigerator until use.

XLD agar

An amount of 56.58g XLD agar was suspended into 1000ml distilled water and heated to boil in a hot plate until dissolved completely. After boiling, the agar media was transferred to water bath at 45-50^0C for 2 minutes. After autoclave, the media was poured into sterile Petri-dish when 60°C in a laminar flow. To check sterility one plate was incubated at 37^0C for 24-48 hours at inverted position and confirmed by no growth of bacteria. The plates were stored at 4°C in refrigerator for further study.

EMB agar

An amount of 35.96g of EMB agar was suspended into 1000ml distilled water and heated to boil in a hot plate. After autoclave, the media was poured into Petri-dish when 60°C in a laminar flow. Following sterility check the agar plates were stored at 4°C in refrigerator for further study.

Blood agar preparation

Eight gram of blood agar powder was mixed with 200ml distilled water (40g/L). The mixture was heated while stirring to fully dissolve all components. After autoclave, the mixture was placed in a water bath at 55^0C.Then 10ml of sheep blood was added and mixed gently (5ml blood/100ml agar medium). Then the prepared blood agar poured into the Petri-dish. To check sterility one plate was incubated at 37^0C for 24-48 hours at inverted position and confirmed by no growth of bacteria. The plates were stored at 4°C in refrigerator for further study.

3.2.3 Preparation of Gram staining solutions

Crystal violet

- Two gram of certified crystal violet was dissolved into 20 g 95% ethyl alcohol
- Dissolved 0.8 g ammonium oxalate into 80 ml distilled water

- The two solutions were mixed together and allowed to stand overnight at room temperature (25^0C)
- Filtered through coarse filter paper before use
- Stored at room temperature

Gram's iodine

- One gram of crystalline iodine and 2 g potassium iodine were grinded in a mortar
- Added to 300 ml of distilled water
- Stored at room temperature in a foil covered bottle

Decolourizer

- Acetone 500ml
- Ethanol or methanol 475 ml
- Distilled water 25 ml

  Mixed thoroughly and stored at room temperature.

Safranin

- Added 2.5g certified safranin-O to 100 ml 95% ethyl alcohol
- Added 10 ml mixture of safranin and ethyl alcohol solution to 90 ml distilled water
- Stored at room temperature

3.2.4 Isolation and identification of bacteria

E. coli

Collected sample was put in 10 ml buffered peptone water in a test tube and incubated at 37^0C for 24 hours. The next day, the sample from buffered peptone water was streaked onto MacConkey agar plate and EMB agar plate by using inoculating loop and incubated overnight. Bright pink colour large colonies in MacConkey agar and green metallic sheen colonies in EMB agar confirmed growth of *E. coli*. Single colony was stained with Gram's stain and observed rod-shaped Gram-negative bacilli under microscope. Identification of *E. coli* was further confirmed by applying standard biochemical test using a VITEK 2 system.

Stenotrophomonas maltophilia

Samples from overnight incubated buffered peptone water was streaked onto XLD agar plate by using inoculating loop and incubated overnight. Black colour large colonies indicated the presence of *Stenotrophomonas maltophilia*. Single colony was stained with Gram's stain and observed rod-shaped Gram-negative bacilli with polar flagella under microscope. Further confirmation was done by applying standard biochemical test using a VITEK 2 system.

Staphylococcus saprophyticus

Samples from overnight incubated buffered peptone water was streaked onto MacConkey agar and blood agar using inoculating loop and incubated overnight. Colourless large colonies on MacConkey agar and round, greyish, shiny & non-haemolytic colonies yielded on blood agar. Single colony was stained with Gram's stain and observed Gram positive round bacteria arranged in grapes-like groups under microscope. Further confirmation was done by applying standard biochemical test using a VITEK 2 system.

The bacterial stocks were prepared by mixing glycerine at a final volume of 3% in eppendorf tubes and sealed with paraffin paper and stored at -20°C for further use.

3.2.5 Gram staining procedure

Preparation of slide smear

A clean, grease-free slide was taken and a minute amount of a bacterial colony from the petri dish was transferred by inoculation loop. The culture was spread with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter, approximately the size of a dime. Heat fixed the sample to the slide by carefully passing it through a Bunsen burner three times.

Staining procedure

- Primary stain crystal violet was added to the sample/slide and incubated for 1 minute. Rinsed the slide with a gentle stream of water for a maximum of 5 seconds to remove unbound crystal violet.

- Added Gram's iodine for 1 minute- this is a mordant, or an agent that fixes the crystal violet to the bacterial cell wall.
- Rinsed the slide with acetone or alcohol for ~3 seconds and rinsed with a gentle stream of water
- Added the secondary stain, safranin, to the slide and incubated for 1 minute. Washed with a gentle stream of water for a maximum of 5 seconds.
- Air dried and observed under microscope
  Gram positive: **Blue/purple colour**
  Gram negative: **Red colour**

3.2.6 Biochemical tests

Biochemical tests were done using VITEK 2 system in Marine Biotechnology Laboratory at the University Malaysia Terengganu, Malaysia. The VITEK 2 system was used for accurate identification of *E. coli*, *Stenotrophomonas maltophilia*, and *Staphylococcus saprophyticus.*

VITEK 2 biochemical identification method

Principle

The VITEK 2 system is an automated microbial identification system that utilizes the VITEK 2 Gram-positive (GP) identification card for the identification of most significant Gram-positive organisms. The VITEK 2 GP card is based on 43 biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities. Identification results are attained in approximately 8 h or less.

Apparatus and reagents

- VITEK 2 system
- VITEK 2 GP test cards
- Vortex
- Incubators set to 30°C and 35–37°C
- VITEK 2 DENSICHEK kit
- DENSICHEK calibrator

- VITEK 2 cassette
- Sterile saline: aqueous 0.45 to 0.50% NaCl, pH 4.5 to 7.0)
- Disposable test tubes 12 mm × 75 mm clear plastic (polystyrene)
- Sterile sticks or swabs
- Brain Heart Infusion (BHI) slants
- Culture media: Columbia agar with 5% sheep blood (CBA), trypticase soy agar (TSA), or trypticase soy agar with 5% sheep blood (TSAB) plates

Preparation of test suspension

- Aseptically transferred 3.0 ml sterile saline (aqueous 0.45 to 0.50% NaCl, pH 4.5–7.0) into polystyrene test tubes (12 × 75 mm). Did not use glass tubes.
- Using a sterile stick or swab, transferred a sufficient number of colonies from a 24 h culture on recommended culture medium to the saline tube to achieve a density equivalent to McFarland 0.50 to 0.63 with the VITEK 2 DENSICHEK.
- Tested the cultures by the VITEK 2 GP method within 30 min of preparation of the suspended culture.
- Inserted the culture tube and the VITEK 2 GP card into the VITEK 2 cassette and referred to the User Manual (provided with the instrument) for instructions on use of the instrument.
- Reported identification results from the VITEK 2 system.
- As indicated in the VITEK 2 GP product information provided to end-users, slash line or low discrimination identifications are acceptable results for the VITEK 2 GP method that require supplemental tests to further resolve the organism identification.

Table 3.2.6 Probability of identifying bacteria using VITEK 2 system

Bacteria	**Probability** (%)
Escherichia coli	96
Stenotrophomonas maltophilia	99
Staphylococcus saprophyticus	99

Results and Interpretation

The results are interpreted by the VITEK 2 system. Printed results indicated a high probability match to a single species if a unique identification pattern is recognized. If a unique pattern is not recognized, the system would suggest supplemental tests to distinguish between two or three closely related organisms, or indicate the result as an unidentified organism (either >3 organisms can exhibit the observed pattern, or the bio pattern is very atypical and is not represented in the database) (Reference: *J. AOAC Int.* 95, 1427(2012)).

3.3 Antimicrobial activity test

The agar well diffusion method was used for the determination of antimicrobial activity (Kumar *et al*., 2010). Fifty milligram of dried microalgae extract was dissolved into 1 ml of extraction solvents (Al-Wathnani *et al.,* 2012). Bacterial suspension was adjusted by adding 0.85% physiological saline to match turbidity of a 0.5 McFarland standard approximately 1.5 x 10^8 CFU/ml (Yilmaz, 2012). Each of bacteria species was inoculated in five replicates into the Muller-Hinton agar (MHA) plate for the use of antimicrobial activity by using cotton swabs. Five wells (6 mm) were made on the MHA surface by using sterilized cork borer. 6mm paper discs were made by sterile Whatman no. 1 filter paper, then sterile them again. After that, the sterile discs were soaked in the extraction solvents of algae then placed three discs of algae on the MHA plate. Methanol socked by sterile filter paper discs were placed into separate wells as negative control. Meanwhile, standard antibiotic disc was placed onto the MHA agar surface as positive control. The 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 of slide callipers from one edge of the zone to other edge. The relative percentage inhibition was the comparison of the antimicrobial activity of the extracts and control were used the following formula (Kannan *et al*., 2002):

$$\frac{\text{Inhibition zone of sample}}{\text{Inhibition zone of the standard}} \times 100$$

The total area of inhibition was calculated by using area = πr2; where, r = radius of inhibition zone.

3.4 Minimal inhibitory concentration

Minimal inhibitory concentration (MIC) of microalgae was determined by using a tube dilution technique which measured sensitivity of bacteria to microalgae suspension. A 96 well microtitreplate was used for this test.

Procedure

- Microalgae stock solution preparation: 6 ml Nutrient Broth+ 600 mg Microalgae (concentration: 100mg/ml)
- 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 TSB 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

In this study, we cultured *Tetraselmis*, *Nannochloropsis* and *Chlorella* in 20 L culture jar. After harvest by centrifugation and drying, we achieved about 7 grams of dry *Nannochloropsis*, 7gm of *Tetraselmis* and 6.2gm of *Chlorella* from each 20 L bulk culture. The dry extracts were further processed by addition of methanol, filtration etc. before stored at -20°C.

Figure 4.1 Culture of microalgae. Small-volume culture of *Chlorella* (left), dried microalgae after harvest (right).

4.2 Isolation and identification of bacteria

Following collection of samples in buffer peptone water (BPW), growth of bacteria was observed on next day when the clear BPW turned into cloudy (**Figure 4.2**) before inoculation into selective media.

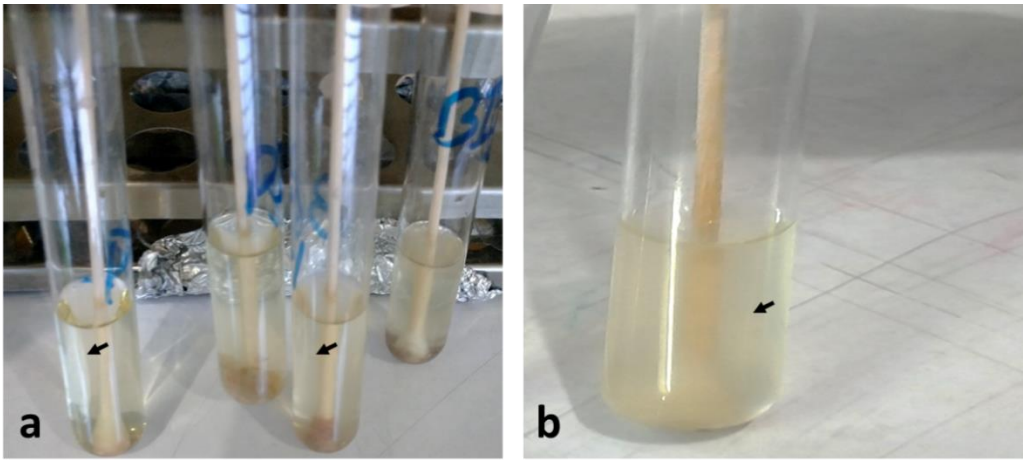

Figure 4.2 Sample collection and primary culture in buffer peptone water. a) Samples were collected using sterile cotton-swab inserted directly into the buffer peptone water (BPW, arrows indicate clear media). b) Cloudy appearance of BPW indicates growth of bacteria (arrow indicates cloudy medium).

4.2.1 *Escherichia coli*

All the samples collected from chicken were positive for *E. coli*, and on MacConkey agar observed characteristics 2-3 mm, circular, moist, smooth and of entire margin, and pink or red or colorless colonies (**Figure 4.3**). Gram negative rod-shaped bacilli were observed in Gram staining. *E. coli* species was confirmed by series of biochemical tests using a VITEK 2 system.

Figure 4.3 Isolation and identification of *E. coli* by culture and Gram staining. a) On MacConkey agar, *E. 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 *Stenotrophomonas maltophilia*

To identify *Stenotrophomonas*, bacterial culture from BPW was inoculated ontoXLD agar and characteristics black colour large colonies were observed (**Figure 4.4**). In Gram staining, Gram negative rod- shaped bacilli were observed under microscope. *Stenotrophomonas maltophilia* species was confirmed by series of biochemical tests using a VITEK 2 system.

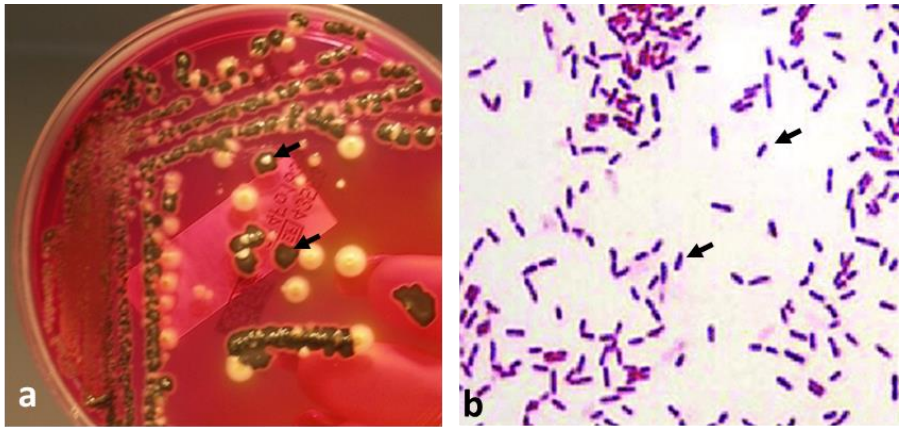

Figure 4.4 Isolation and identification of *Stenotrophomonas* sp. by culture and Gram staining. a) On XLD agar, *Stenotrophomonas* was observed as black colour large colonies indicted by arrows. b) Gram negative rod-shaped bacilli indicated by arrows were observed under light microscope (400×) after Gram staining.

4.2.3 *Staphylococcus saprophyticus*

Staphylococcus sp. was identified by bright white, creamy, non-hemolytic colonies on blood agar (**Figure 4.5**). On Gram staining, Gram positive uniform cocci with grapes like arrangements were observed. *Staphylococcus saprophyticus* species was confirmed by several biochemical tests using a VITEK 2 system.

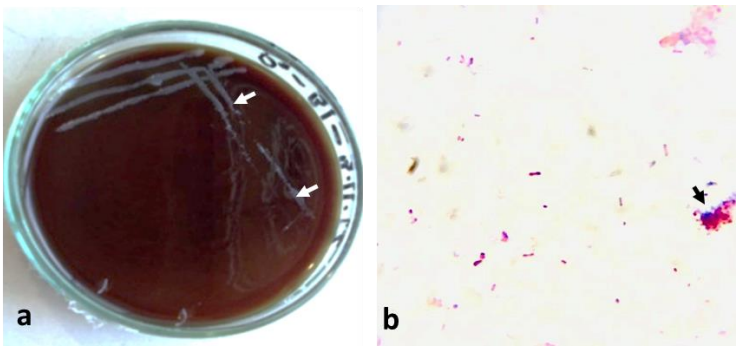

Figure 4.5 Isolation and identification of *Staphylococcus saprophyticus* by culture and Gram staining. a) On blood agar, *Staphylococcus* was observed as white colored creamy non-hemolytic colonies indicted by arrows. b) Gram positive circular cocci with grapes-arrangement indicated by arrow were observed under light microscope (400×) after Gram staining.

4.3 Antimicrobial activity test

4.3.1 Antimicrobial activity of microalgae against *E. coli*

Antimicrobial activity of methanol extracts of all three microalgae *Tetraselmis*, *Chlorella* and *Nannochloropsis* was demonstrated at variable extents against *E. coli* in the current study (**Figure 4.6**). The largest inhibition zone appeared around the disc loading of extract of *Chlorella* with a diameter of 8.30mm (p<0.05).

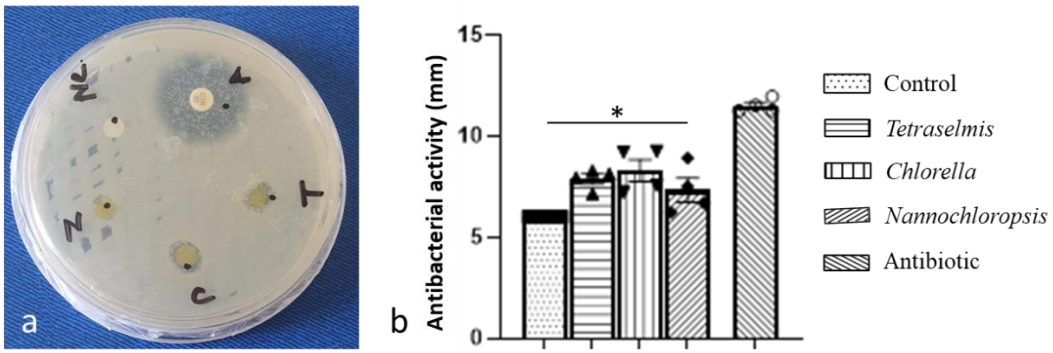

Figure 4.6 Antimicrobial activity of microalgae against *E. coli*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), *Chlorella* (Ch), and *Nannochloropsis* (N) with no zone of inhibition around control disc (Ne). b) All three microalgae showed significantly higher zone of inhibition with *Chlorella* the highest. Statistical tests done by ANOVA, error bars represent standard error of means, p*<0.05.

4.3.2 Antimicrobial activity of microalgae against *Stenotrophomonas maltophilia*

The antimicrobial activity of methanol extracts of *Tetraselmis*, *Chlorella*, *Nannochloropsis* was observed against *Stenotrophomonas maltophilia* (**Figure 4.7**). Among the microalgae, *Nannochloropsi s*had minimum effect on the bacteria, however, the largest inhibition zone appeared around the disc loading of extract of *Tetraselmis* with a diameter of 9.39 mm (p<0.01).

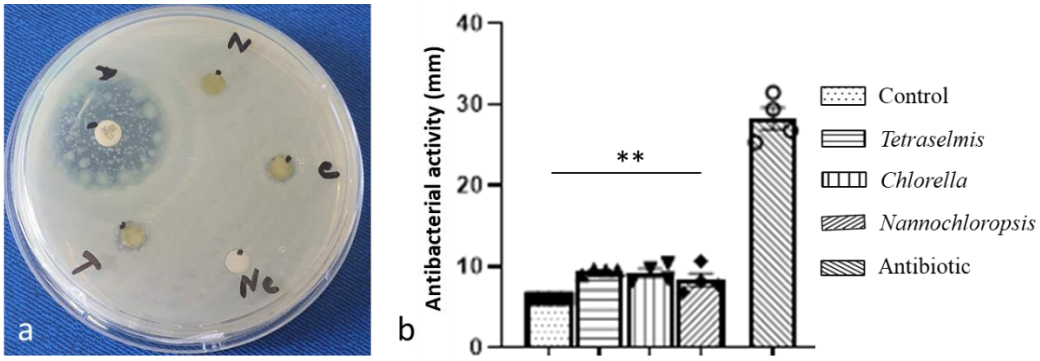

Figure 4.7 Antimicrobial activity of microalgae against *Stenotrophomonas*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), and *Chlorella*(C) with minimum zone around *Nannochloropsis* (N), and no zone of inhibition around control disc (Ne). b) All three microalgae showed significantly higher zone of inhibition with *Tetraselmis* the highest. Statistical tests done by Kruskal-Wallis test, error bars represent standard error of means, p**<0.01.

4.3.3 Antimicrobial activity of microalgae against *Staphylococcus saprophyticus*

Against *Staphylococcus saprophyticus*, *Chlorella*showed greater activity compared to *Tetraselmis* and *Nannochloropsis* with a diameter of 9.72mm (p<0.001) (**Figure 4.8**). However, *Tetraselmis* showed minimum activity against *Staphylococcus*.

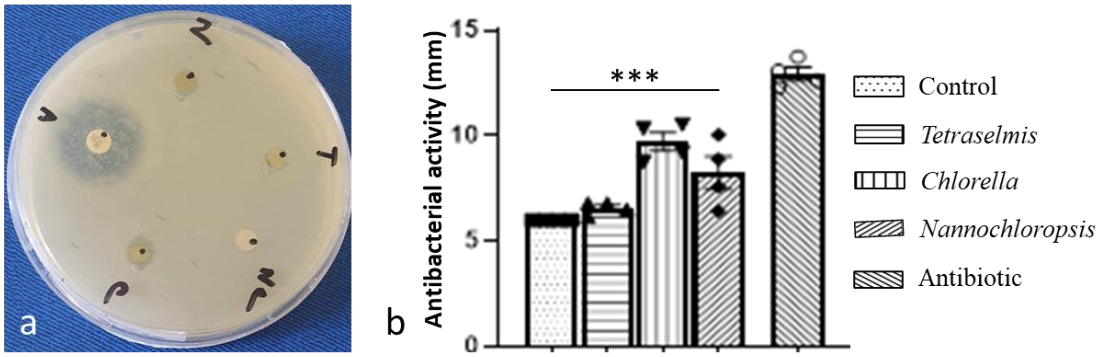

Figure 4.8 Antimicrobial activity of microalgae against *Staphylococcus saprophyticus*. a) Agar plate indicates clear zone of bacterial growth surrounding the antibiotic (A), *Tetraselmis* (T), *Chlorella* (C) and *Nannochloropsis* (N) with no zone around the control disc (Ne). b) All three microalgae showed significantly higher antibacterial activity with *Chlorella* the highest. Statistical tests done by ANOVA, error bars represent standard error of means, $p^{***}<0.001$.

4.4 Antimicrobial index of microalgae extract

Antimicrobial index of microalgae extract was calculated by using the formula that stated at above, this formula used to compare the antimicrobial effect of the microalgae with the standard antibiotic agents. The results showed that *Tetraselmis* extract exhibited maximum relative percentage inhibition against *E.coli* with minimum against *Staphylococcus saprophyticus* while *Chlorella* and *Nannochloropsis* extracts exhibited maximum relative percentage inhibition against *Staphylococcus saprophyticus* followed by *E. coli* and *Stenotrophomonas maltophilia* (**Table 4.1**).

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

Name of microalgae	Relative percentage		
	E. coli	*Stenotrophomonas maltophilia*	*Staphylococcus saprophyticus*
Tetraselmis	26.92	6.8	4.8
Chlorella	34	6.3	44.59
Nannochloropsis	18	4.4	24.4

4.5 Minimum inhibitory concentration (MIC)

The MIC of the extracts from *Chlorella* and *Tetraselmis* grown under different conditions were determined against *E. coli* and *Stenotrophomonas maltophilia*. Due to insufficient amount, it was not possible to determine MIC value of *Nannochloropsis* against these bacteria and any of these three microalgae against *Staphylococcus*. 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 *Stenotrophomonas maltophilia*, however, *Chlorella* has against both *E.Coli* and *Staphylococcus* (**Table 4.2**).

Table 4.2 MIC value of microalgae against *E. coli* and *Stenotrophomonas*

Microalgae	MIC value (mg/ml)	
	E. coli	*Stenotrophomonas maltophilia*
Chlorella	10	10
Tetraselmis	30	20

CHAPTER V

DISCUSSION

The present study demonstrated the bacterial loads in the liver samples of dead broiler chickens and whether the specific marine microalgae have antibacterial effects against them. It was observed that all samples collected from liver were positive for *E. coli*, *Stenotrophomonas maltophili a*and *Staphylococcus saprophyticus.* All these three identified bacteria are clinically important in chicken and cause a wide variety of infections such as gastro-enteritis, perihepatitis, pericarditis, peritonitis, cellulitis, air sacculitis, arthritis, meningitis, endocarditis, respiratory tract infections, fatal septicemia and death. Although most of the bacterial infections are treated successfully with antibiotics, antimicrobial resistance is a major growing threat 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 infections in chickens.

Methanolic solvent was used to extract the *Tetraselmis, Chlorella* and *Nannochloropsis* to study antibacterial activity against pathogenic bacteria from chickens in the current study. The highest activity against all the microorganisms tested was present in case of methanolic extract in previous studies (Abubakar *et al.,* 2012; Lapornik *et al.,* 2005).

In this study, it was observed that *Tetraselmis, Chlorella* and *Nannochloropsis* have antimicrobial properties against *E. coli*, *Stenotrophomonas maltophilia* and *Staphylococcus saprophyticus*in variable extents. *Chlorella* showed stronger activities against both *E. coli* and *Staphylococcus saprophyticus* than the others. However, *Tetraselmis* exhibited as a major inhibiting alga against *Stenotrophomonas maltophilia*.The resilience of marine biota to survive in a wide range of marine environment and conditions, as well as lack of physical defense or adaptive immunity against pathogens and parasites might have turned them as natural sources of the bioactive substances (Bragadeeswaran *et al.,* 2013). Like other marine microalgae

Tetraselmis, *Chlorella* and *Nannochloropsis* have some active bioactive compound that have antimicrobial activity (Zea-Obando *et al.*, 2018). Previous study reported that *Tetraselmiss uecica* extracted by using the methanol and chloroform in ratio 1:1 had able to show antimicrobial activities against the Gram-negative *Proteus* sp. and Gram-positive *Streptococcus pyogenes* (Bai & Krishnakumar, 2013). Besides that, crude extract of *Tetraselmis chuii* had proved that it had antimicrobial properties to against the *Vibrio* bacterial strains *V. parahaemolyticus*, *V. splendidus*, *V. anguillarum*, *V. scophthalmi*, *V. alginolyticus* and *V. Lentus* (Kokou *et al.*, 2012).

Furthermore, methanolic extract of *Chlorella* showed antimicrobial activity toward the *E. coli, Staphylococcus saprophyticus* and *Stenotrophomonas maltophilia*.This indicates that *Chlorella* is more effective against Gram-negative bacteria than the Gram-positive. The results are in agreement with a previous study (Rao & Parekh, 1981). The difference in cell wall structures of the Gram-positive and Gram-negative bacteria might control the penetration, binding and antimicrobial activity of the compound (Maligan *et al.*, 2013). Previous study stated that *Enterobacter, Proteus* and *E. coli* were resistant to crude active compounds extracted from *Chlorella vulgaris* at low concentrations (10 mg/ ml) and sensitive at high (100mg/ml) concentrations. However, *Klebsiella* was found resistant to all active compounds present in *Chlorella vulgaris* at low and high concentrations. The results also revealed that *Enterobacter* was more sensitive to crude active compounds in compare with *Proteus* and *E. coli* (Hussein *et al.*, 2018). Moreover, *Staphylococcus aureus*, *Lactobacillus acidophilus* and *Streptococcus pyogenes* bacteria were reported as resistant to the crude active compounds extracted from *Chlorella vulgaris* at low concentrations (10mg/ml) and sensitive at high (100mg/ml) concentrations. The previous studies also revealed that *Staphylococcus aureus* were more sensitive to the crude active compounds in compare with *Lactobacillus acidophilus* and *Streptococcus pyogenes* (Hussein *et al.*, 2018).

Minimum inhibitory concentration (MIC) of the *Chlorella* and *Tetraselmis* was determined in the present study. It was observed that *Chlorella* was able to inhibit growth of both *E. coli* and *Stenotrophomonas maltophilia*at a concentration of 10mg/ml.

However, *Tetraselmis* showed better antimicrobial capacity against *Stenotrophomonas maltophilia* compared to *E. coli*. The chemical nature and quantity of bioactive metabolite compounds present in the extracts and their mode of action to test organism might be the reason behind the difference of MIC values. Different microalgae species may achieve the peak amounts of bioactive compounds during different phase of growth that mean some of the microalgae species will reach the maximum of production of bioactive compounds at exponential phase and some of them at stationary phase (Skulberg, 2000).

CHAPTER VI

CONCLUSION

The present study demonstrated antibacterial activity of three-species of marine microalgae against three-species of bacteria isolated from dead broiler chicken under diagnosis by postmortem. It was observed that *Chlorella* has the greatest propensity to suppress *E. coli* and *Stenotrophomonas maltophilia.* However, *Tetraselmis* was very suppressive against *Stenotrophomonas maltophilia Nannochloropsis* showed variable degrees of sensitivity against all three-species of bacteria. Further studies are directed to identify the specific components of microalgae that are responsible for the sensitivities against bacterial infections.

CHAPTER VII

RECOMMENDATION AND LIMITATIONS

The bioactive compounds of the microalgae will need to study and identify the chemical structures and benefits effect for inhibition of pathogenic bacteria. The bioactive compounds of the microalgae may be suitable used for the other pathogenic bacteria. Besides that, identification of microalgae species is more preferable. As we know, there have a lot of the species in the Genus of *Tetraselmis, Chlorella* and *Nannochloropsis* we recommend further studies about the specific species.

Isolation and identification of microalgae directly from the marine sources was one of the limitations of the current study. We used the microalgae that we already have in our laboratory preserved. A larger bacterial sample size could increase the sensitivity and specificity of the results. We were also unable to grow enough microalgae sufficient for the antibacterial sensitivity testing and determination of MIC values.

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APPENDIX

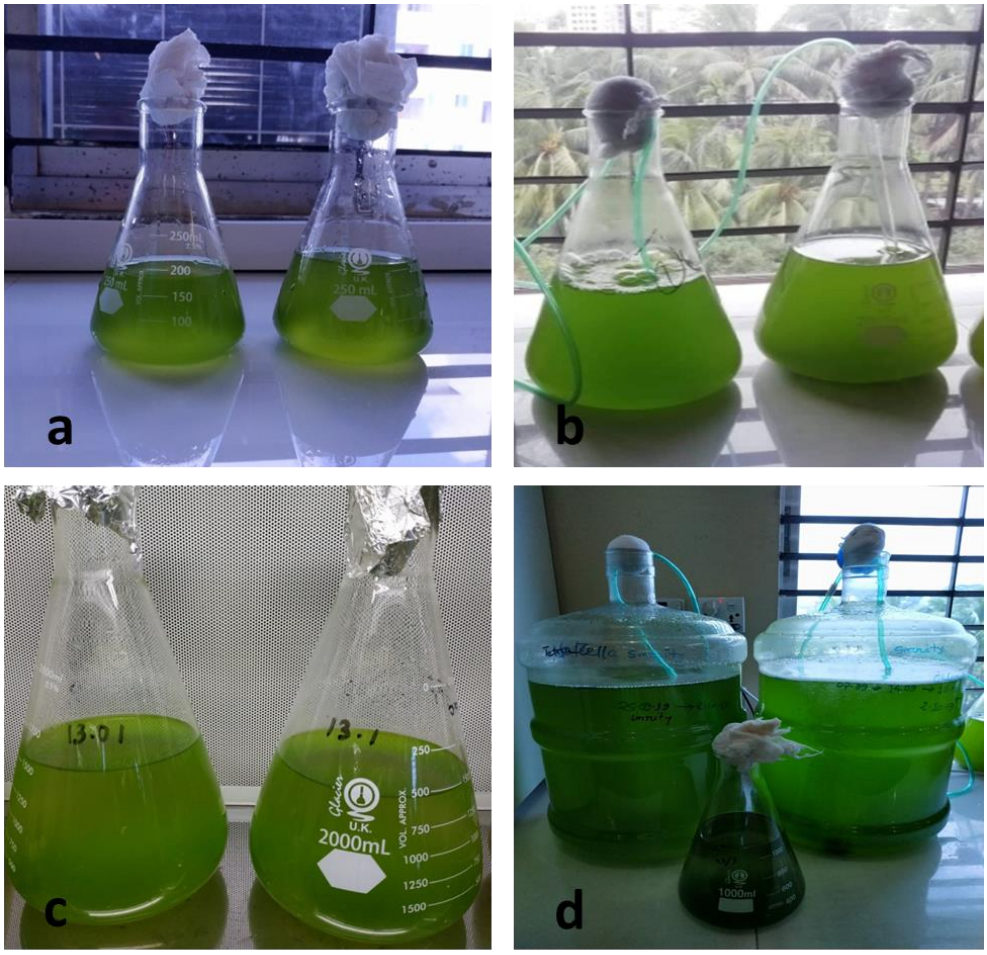

Figure 1 Culture of microalgae. Small-volume culture of a) *Chlorella*, b) *Nannochloropsis* and c) *Tetraselmis*. d) Mass-culture of microalgae in 20L jars.

Figure 2 Laboratory activities regarding culture of microalgae. (Left) mass-culture of microalgae and addition of culture media,(middle)drying microalgae extracts in a hot air oven at 60^0C for 12h, (right) harvesting microalgae by centrifugation.

(a)

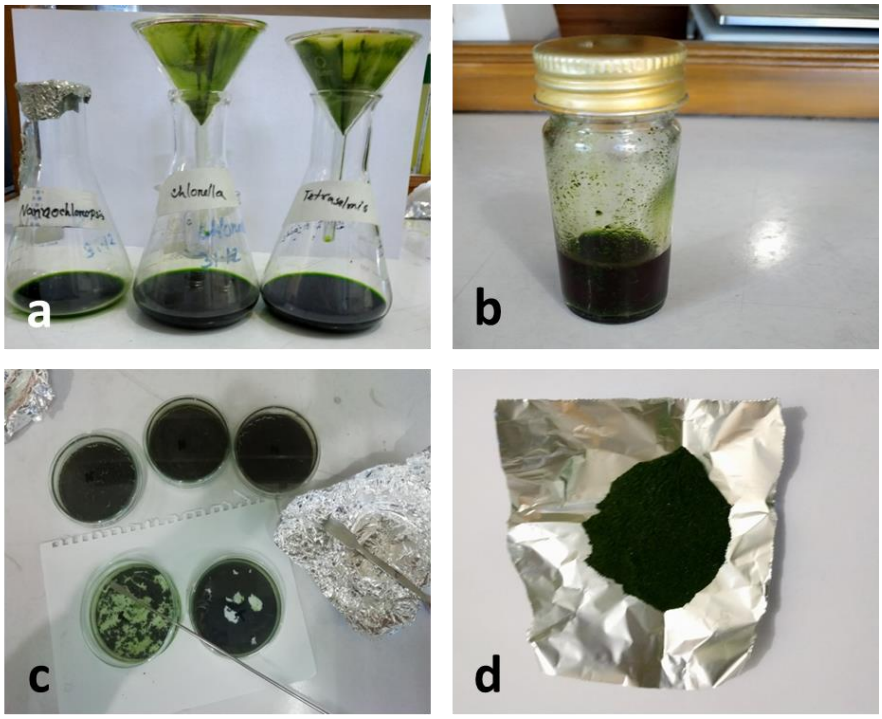

Figure 3 Harvesting cultured microalgae and preparation of dry extracts. a) Filtration using Whatman filter paper 1, b) microalgae soaked in methanol, c) evaporated microalgae, d) dry extract ready to use.

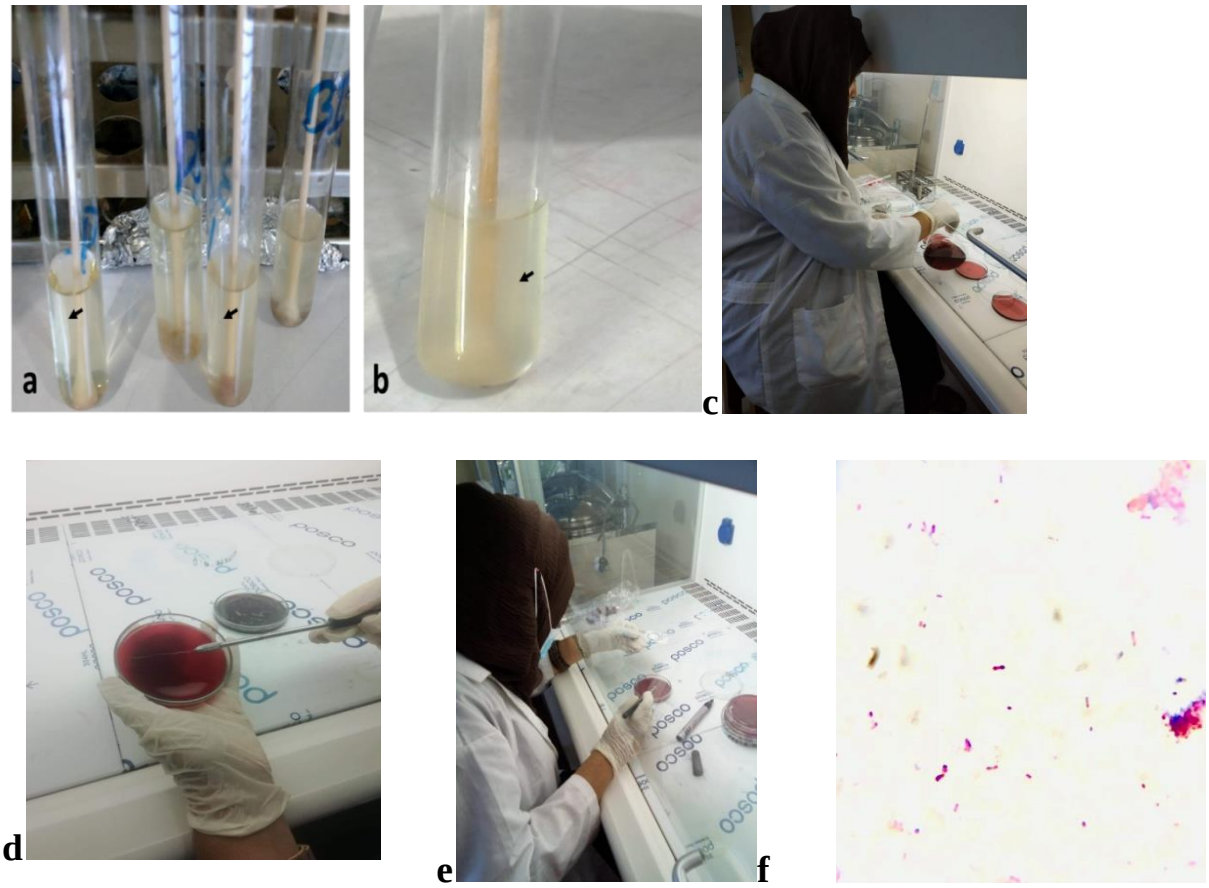

Figure 4: Sample collection and isolation of bacteria. a) sample collection from poultry, b) bacterial samples in buffered peptone water, c) preparation of agar plates, d)streaking agar plates, e) slide preparation for Gram staining, f) microscopic view.

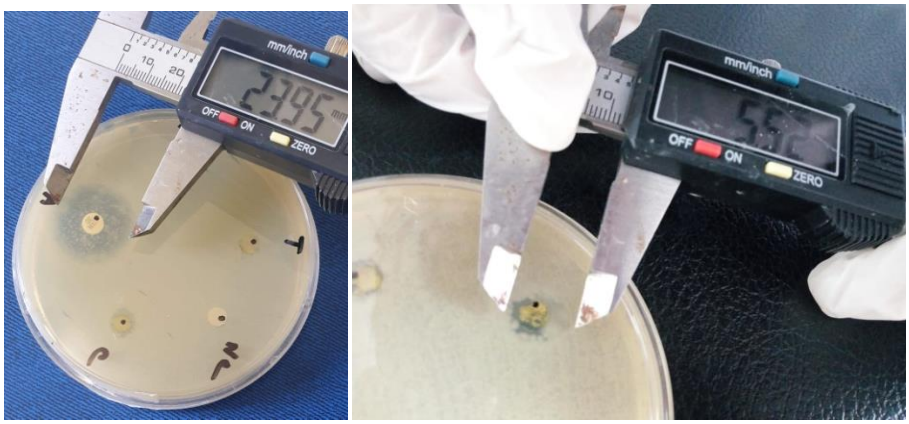

Figure 5: Measurement of zone of inhibition. (left) Measuring bacterial inhibition zone surrounding antibiotic, (right) measurement around microalgae.

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