Journal of Sustainability Science and Management
Volume 19 Number 1, January 2024: 74-86
eISSN: 2672-7226
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THE ANTIBACTERIAL POTENTIAL OF EUKARYOTIC MARINE MICROALGAE AGAINST PATHOGENS FROM CHICKEN, DOG AND AQUACULTURE

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http://doi.org/10.46754/jssm.2024.01.007
Submitted final draft: 28 June 2023 *Accepted: 13 September 2023* *Published: 15 January 2024*

Abstract: Antibiotic resistance is an emerging concern, leading to the search for alternative antibacterial agents. Scientists looking for potential alternatives to antibiotics see promise in the antimicrobial propensity of microalgae. We investigated the antibacterial potentials of *Tetraselmis*, *Chlorella,* and *Nannochloropsis* against pathogenic bacteria from chicken, dogs, and fish. We identified *E. coli, Stenotrophomonas* sp., *Streptococcus* sp., *Staphylococcus* sp., *Aeromonas* sp. and *Lysinibacillus* sp. by colony characteristics, Gram staining and VITEK-2 tests. Results demonstrated that *Tetraselmis* was highly sensitive against *Stenotrophomonas* sp. ($p < 0.0001$) and *E. coli* ($p < 0.001$) from chicken, and *Staphylococcus* sp. ($p < 0.01$) from dogs. Moreover, *Chlorella* was highly sensitive against *E. coli* ($p < 0.0001$) from dogs. All the bacteria isolated from chicken were moderate to highly sensitive to *Chlorella*. *Nannochloropsis* was marginally sensitive to all the bacteria isolated from chickens and dogs. The minimum inhibitory concentration values indicate that a minimum of 10 mg/ml of *Tetraselmis* can suppress the growth of *Aeromonas* sp. from fish and *Chlorella* can suppress *E. coli* and *Stenotrophomonas* sp. from chicken. Results indicate that native microalgae may have an active somatic or secretory components that prevent bacterial cell division and/or induce lysis. Advanced studies should be performed to identify the active component for the development of newer and sustainable antimicrobial drugs.

Keywords: Antibacterial sensitivity, microalgae, sustainability.

Introduction

Microalgae are of growing interest to the scientific community because of medically and commercially promising bioactive compounds (Saha *et al.,* 2022). The marine environment is rich in nutrients and chemical compounds for the growth of microalgae. Common microalgae species identified off Bangladesh's coast are *Chlorella miminutissima, Tetrselmis chuii, Nannochloropsis* sp., *Arthrospira platensis, Isochrysis* sp., *Chondruscrispus, Mastocarpus stellallatus, Ascophyllum nodosum, Alaria esculentus, Sprirulina platensis, Chlorella esculentus, Nannochloropsis oculata,* and *Dunaliella salina* (Islam *et al.,* 2021). There are a variety of mechanisms resulting from the metabolic pathways of microalgae, including the synthesis of diverse bioactive components such as fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, alkaloids, phytol, astaxanthin, lutein, sulphur-containing heterocyclic compounds, carbohydrates, and phenols etc. (de Morais *et al.,* 2015; Olguin *et al.,* 2022). Previous studies showed that the bioactive compounds have properties as potential antioxidants, antibiotics, and toxins widely used in pharmaceutical industries (Sathasivam *et al.,* 2019; Khavari *et al.,* 2021; Xia *et al.,* 2021). For example, *Chlorella* was found to effectively suppress *Streptococcus mutans* biofilm production to prevent dental caries formation (Hwang *et al.,* 2021). The γ-lactone malyngolide isolated from *Lyngbya majuscule* microalgae was found to be effective against *Mycobacterium smegmatis* and *Streptococcus pyogenes* (Cardllina *et al.,* 1979). Majuscuiamide C is another component that inhibits fungal plant pathogens (Carter *et al.,* 1984). Microcolins A and B have immunosuppressive activities and are found to be effective against murine P3888 leukaemia (Koehn *et al.,* 1992). Water-soluble polysaccharides from *Tetraselmis* sp. were found to have antioxidant, antifungal, and tyrosinase inhibitory activities (Amna *et al.,* 2018).

Escherichia coli is a multi-antibiotic-resistant bacterium and is a primary cause of morbidity and mortality, and is associated with major economic loss to the poultry industry (Kabir, 2010; Millman *et al.,* 2013; Nakayama *et al.,* 2022). *Stenotrophomonas maltophilia* is another poultry pathogen that causes biofilm production in the respiratory system and its resistance to multiple antibiotics has been reported (Sanchez, 2015; Blanco *et al.,* 2019; Flores-Trevino *et al.,* 2019). *Staphylococcus saprophyticus* is zoonotic and has been known to cause serious urinary tract infections (Sommers *et al.,* 2017). *Escherichia coli, Streptococcus,* and *Staphylococcus* cause necrotising fasciitis, pyoderma, and dermatitis in dogs, respectively (Worth *et al.,* 2005; De Martino *et al.,* 2012). Multi-drug resistant strains of these bacteria have been isolated from the skin of dogs (Deb *et al.,* 2020). *Aeromonas hydrophila* is another zoonotic bacterium that causes a broad range of diseases such as gastroenteritis, soft tissue and muscle infections, septicemia, and skin diseases in humans and animals (Igbinosa *et al.,* 2012). About 69% of strains of *A. hydrophila* have been identified as multidrug-resistant (Saleh *et al.,* 2021). Based on our previous study (Islam *et al.,* 2021), we investigated the potential antibacterial properties of *Tetraselmis*, *Chlorella*, and *Nannochloropsis* from local marine sources and pathogenic antibacterial potentials against virulent bacterial species isolated from chicken, dog, and aquaculture. Interestingly, we found that all three microalgae have bacteriostatic and bactericidal activity of variable degrees with minimum inhibitory concentrations identified.

Materials and Methods

Experimental Design

The study was performed according to the guidelines of Chattogram Veterinary and Animal Sciences University Animal Ethics Committee [approval no. CVASU/Dir(R&E) EC/2020/165(5)]. Samples from chicken (n = 4) and dog (n = 4) were collected at the Teaching Veterinary Hospital of Chattogram Veterinary and Animal Sciences University (CVASU). A small sample (1-3 cm^3) of liver was collected from dead chicken aseptically by sterile scissors and put into a sterile test tube containing buffer peptone water (BPW, Figure 1). The skin and anal mixed swabs from dogs were collected and put into BPW. Intestinal and gill samples from Tilapia and Bata fish (n=5) were collected at the local fish market. The samples in BPW were incubated at 37°C for 24 hours to enrich the bacterial population. The sample from BPW was smeared onto different selective media for isolation and identification of bacteria by colony characteristics. Gram staining was performed on single colonies and examined under a light microscope. Bacteria were confirmed by VITEK-2 tests. Crude methanol extracts of *Tetraselmis*, *Chlorella,* and *Nannochloropsis* were used to determine sensitivity against the isolated bacteria. Minimum Inhibitory Concentration (MIC) were determined to further assess the antibacterial potency.

(i) Anal and skin mixed swabs were collected from dogs.

(ii) Liver swabs from chicken.

(iii) Gill and gut mixed swabs from Bata and Tilapia fish.

(iv) Swabs were placed into buffer peptone water and incubated at 37ºC overnight for bacterial enrichment.

(v) Bacteria were isolated by growing in selective media and identified by colony characteristics, Gram staining and VITEK-2 tests.

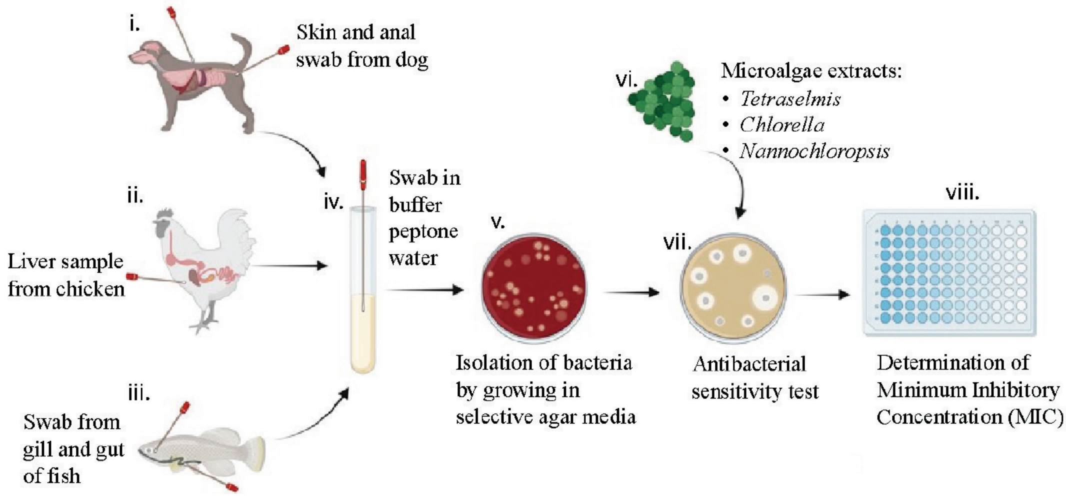

Figure 1: Experimental design for the determination of Minimum Inhibitory Concentration (MIC)

(vi) Methanolic extracts of microalgae *Tetraselmis*, *Chlorella,* and *Nannochloropsis* were used to investigate.

(vii) Antibacterial potential by disc diffusion method on Muller Hinton agar.

(viii) The minimum inhibitory concentration of the microalgae was determined using the microplate dilution method.

Preparation of Microalgae Crude Extracts

Pure microalgae isolate seeds of *Tetraselmis*, *Chlorella,* and *Nannochloropsis* were collected from the Live Feed Research Corner of the Department of Aquaculture, CVASU, Bangladesh. Three replicates of each species of microalgae were cultured in Conway Medium (Gachon *et al.,* 2007) at 28 ± 2°C, 3000 Lux of illumination provided by warm-white fluorescent in 24 hours light-dark cycle (Younes *et al.,* 2004). The culture was centrifuged at 4,000 rpm for 10 minutes at 4°C to obtain large-scale biomass. The algal paste of *Tetraselmis* was dried at 60°C for 12 hours. The dry microalgae were then soaked in methanol at 10 ml/gm of microalgae for two days at room temperature. The extract was filtered through sterile Whatman no. 1 filter paper (Merck, Germany) and concentrated under reduced pressure in a rotary evaporator. The dry extract was stored at -20°C until use.

Isolation and Identification of Bacteria from Chicken, Dog, and Fish

Bacterial samples from BPW were smeared onto selective media. After 24 hours of incubation at 37°C, bacterial colonies were examined for morphological properties of size, shape, elevation, edges, surface, and colour. A single colony was smeared on microscope slides and Gram staining was performed for the microscopic identification of bacteria (Frobose *et al.,* 2020). *E. coli* was identified as having characteristics 2-3 mm in diameter, circular, moist, smooth and of entire margin, and pink or red or colourless colonies on MacConkey agar (Merck, Germany). Upon Gram staining, *E. coli* was observed as Gram-negative, rod-shaped bacilli with no specific arrangements. *Stenotrophomonas* sp. on XLD agar (Merck, Germany) was identified as large, dispersed, black-colour colonies of rod-shaped bacilli and Gram negative on Gram staining. The *Staphylococcus* sp. was identified as colourless or yellow, glossy, large colonies on mannitol salt agar (Merck, Germany), and Gram-positive uniform cocci with grapes-like arrangements under a microscope. *Streptococcus* sp. was identified as greyish colonies on blood agar (Merck, Germany) and Gram-positive cocci of typical chain arrangement were observed under a microscope. *Aeromonas* sp. was identified as creamy-white colonies on trypticase soy agar (Merck, Germany) and rod-shaped Gram-negative bacilli were observed on microscopic examination. *Lysinibacillus* sp. was identified by the purple colour of large colonies on blood agar and rod-shaped Gram-positive bacilli under microscope. To confirm bacterial identification, pure colonies were shipped to the Marine Biotechnology Laboratory at the Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu and subjected to standard biochemical tests such as indole, Voges Proskauer, methyl red, and citrate utilization tests followed by VITEK-2 microbial identification system (Biomerieux, USA).

Antibacterial Sensitivity Test

The disk diffusion method was performed according to the published guideline (Balouiri *et al.,* 2016) using Mueller-Hinton agar (MHA, Merck, Germany) plates. The bacterial suspension was adjusted to match the turbidity of a 0.5 McFarland standard approximately 1.5×10^8 CFU/ml using 0.85% physiological saline (Hombach *et al.,* 2015). Each bacterial species was inoculated with four to five replicates onto the MHA plate using sterile cotton swabs. Sterile filter paper discs of 6 mm in diameter were immersed into the microalgae extraction solvents (50 mg microalgae in 1 ml of methanol). The microalgae discs were dried by evaporation and placed on the MHA plate using sterilized forceps. A third-generation cephalosporin antibiotic ceftriaxone disc was used as a positive control. A methanol-treated dried sterile filter paper disc was used as negative control (Blank). The plates were incubated in an inverted position at 37°C overnight. The diameter of the clear and circular zones surrounding the discs produced by the extracts or antibiotics was measured using digital slide callipers (Robotics, Bangladesh). In initial experiments, *Nannochloropsis* was found to have no effect on the bacteria isolated from aquaculture (data not shown in the results section) and therefore, not used for the sensitivity test.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of the microalgae extract against bacteria were determined using a microplate dilution technique (Salem *et al.,* 2011). The bacterial suspension was prepared to match the 0.5 McFarland standards in nutrient broth and 200 µl was added to the 96F well microplate. Microalgae extracts were added at 10 mg/ml, 20 mg/ml, 30 mg/ml, and 40 mg/ml (final concentration) and incubated overnight at 37°C. Bacterial suspensions with PBS or ceftriaxone were used as controls. The growth of bacteria in each well was determined by colour and turbidity. The MIC value was determined as the minimum concentration of microalgae extract capable of inhibiting bacterial growth.

Statistical Analysis

Confirmation of normal distribution of data sets was checked using the Pearson normality test in GraphPad Prism 8 statistical software. All the data sets from different groups passed the normality test and therefore were compared using a t-test. A *p-value* of ≤ 0.05 was considered significant.

Results and Discussion

Chlorella has the Highest Antibacterial Potential Against Bacteria Isolated from Chicken

We identified *E. coli, Stenotrophomonas* sp., and *Staphylococcus* sp. from liver samples of chicken. We found that *E. coli* was significantly suppressed by *Tetraselmis* ($p < 0.001$) and *Chlorella* ($p < 0.01$) [Figure 2 (i) and Supplementary Figure 1 (i)]. All three microalgae have antibacterial potential against *Stenotrophomonas* sp. [Figure 2 (ii) and Supplementary Figure 1 (ii)] and *Staphylococcus* sp. [Figure 2 (iii) and Supplementary Figure 1 (iii)].

Colibacillosis is a common poultry disease caused by avian pathogenic *E. coli* and is communicable to humans (Kabir, 2010). It causes simple cellulitis to acute fatal septicemia

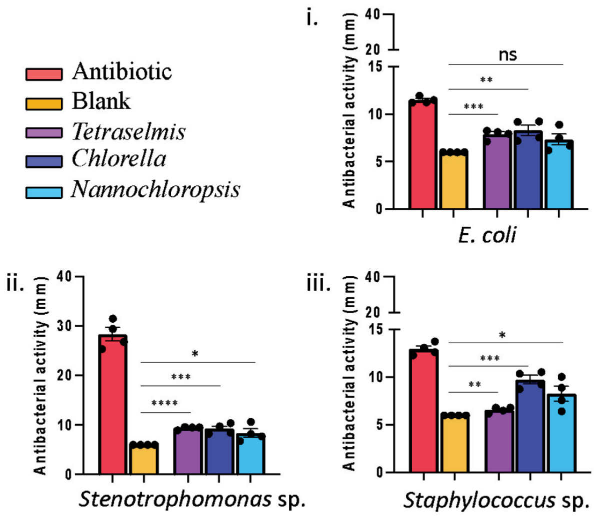

Figure 2: Antibacterial sensitivity of microalgae against bacteria isolated from chicken. (i) *Tetraselmis* and *Chlorella* showed significantly higher antibacterial sensitivity against *E. coli*. (ii) All three microalgae have antibacterial potential against *Stenotrophomonas* sp. (iii) *Chlorella* has the maximum antibacterial potential against *Staphylococcus* sp. Statistical analysis by unpaired t-test, p * ≤ 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, ns: not significant

in chicken (Kabir, 2010) and bacteremia with multisystemic infection in humans (Mead *et al.,* 1999). We also identified non-pathogenic *Stenotrophomonas* sp. from chicken which, however, can transmit to humans and cause nosocomial respiratory infection (Denton & Kerr, 1998; Yamamoto *et al.,* 2020).

Tetraselmis has the Highest Sensitivity Against Bacteria Isolated from Dog

We identified *E. coli*, *Staphylococcus* sp., and *Streptococcus* sp. from dogs. The highest sensitivity against *E. coli* was observed in *Chlorella* ($p < 0.0001$), followed by *Tetraselmis* and *Nannochloropsis* [Figure 3 (i) and Supplementary Figure 1 (iv)]. *Streptococcus* sp. was moderately sensitive to *Tetraselmis* and *Nannochloropsis* [Figure 3 (ii) and Supplementary Figure 1 (v)]. However, *Staphylococcus* sp. was only sensitive to *Tetraselmis* [Figure 3 (iii) and Supplementary Figure 1 (vi)]. *Chlorella* does not have any effects on *Streptococcus* sp. and *Staphylococcus* sp.

Extraintestinal pathogenic *E. coli* has been reported in dogs in a recent study (Valat *et al.,* 2020). We identified *E. coli* from chicken, and skin and anal mixed swabs from dogs. We identified *Staphylococcus* sp., *Streptococcus* sp., *Aeromonas* sp., and *Lysinibacillus* sp. *Staphylococcus* sp. causes urogenital tract infection in humans and has reports of zoonoses (Hovelius & Mardh, 1984; Han *et al.,* 2016). Horizontal transmission of *Streptococcus* sp. between dogs and humans is reported because of close contact (Handl *et al.,* 2011; Wetzels *et al.,* 2021; Garrigues *et al.,* 2022).

Sensitivity of Microalgae Against Bacteria Isolated from Fish

Aeromonas sp., *Staphylococcus* sp., and *Lysinibacillus* sp. were identified from fish samples. Both *Aeromonas* sp. and *Staphylococcus* sp. were highly sensitive to *Tetraselmis* and *Chlorella* [Figures 4 (i) & (ii) and Supplementary Figure 1 (vii) & (viii)]. Non-pathogenic *Lysinibacillus* sp. was found sensitive to *Tetraselmis* only [Figure 4 (iii) and Supplementary Figure 1 (ix)]. However, *Nannochloropsis* had no effects on any bacteria isolated from fish (data not shown).

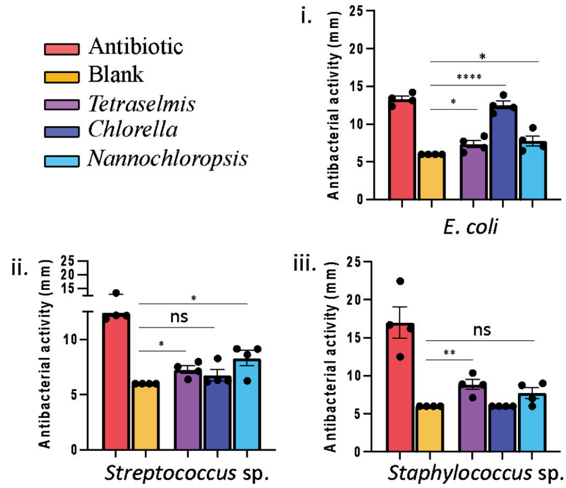

Figure 3: Antibacterial sensitivity of microalgae against bacteria isolated from dogs. (i) *Chlorella* has the highest antibacterial sensitivity against *E. coli*. (ii) *Tetraselmis* and *Nannochloropsis* have moderate sensitivity against *Streptococcus* sp. (iii) Only *Tetraselmis* is sensitive against *Staphylococcus* sp. Statistical analysis by unpaired t-test, p * ≤ 0.05, ** < 0.01, **** < 0.0001, ns: not significant

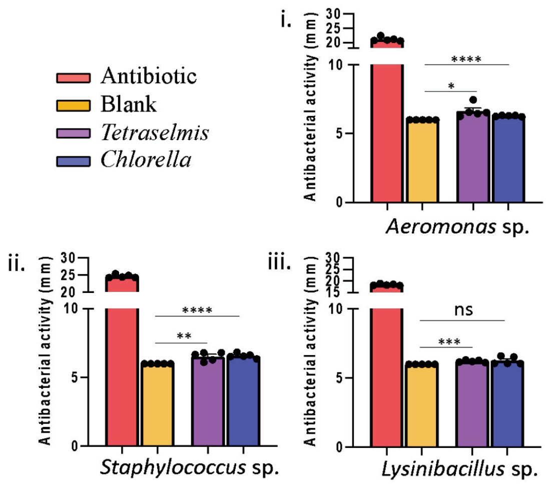

Figure 4: Antibacterial sensitivity of microalgae against bacteria isolated from aquaculture. *Tetraselmis* and *Chlorella* have significantly higher antibacterial sensitivity against (i) *Aeromonas* sp. and (ii) *Staphylococcus* sp. (iii) Only *Tetraselmis* was sensitive to *Lysinibacillus* sp. Statistical analysis by unpaired t-test, p * ≤ 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, ns: not significant

Aeromonas sp. is a septicaemic bacterium that was recovered from Bata and Tilapia, and causes significant losses in the aquaculture industry (Plumb & Hanson, 2010). *Lysinibacillus* sp. identified in the current study is non-pathogenic for fish, however, it has an effect on insect control (Kellen *et al.,* 1965; Ahmed *et al.,* 2007).

Although most of the current commercial antibiotics are effective in the treatment of the bacterial infections investigated in the present study, there are increasing frequencies of Anti-Microbial Resistance (AMR) and Multi-Drug Resistance (MDR). Multidrug-resistant avian pathogenic and extra-pathogenic *E. coli* were recovered from workers associated with the poultry industry (Vounba *et al.,* 2019; Aworh *et al.,* 2021). A few antibiotics are suggested to treat *Stenotrophomonas* sp. infections due to resistance (Cikman *et al.,* 2016). A recent study reported higher rates of biofilm formation by *Staphylococcus* sp. due to higher rates of AMR (Hashemzadeh *et al.,* 2021). Multiple AMR has also been reported against *Streptococcus* sp. in different cohorts (Passali *et al.,* 2007; Alves-Barroco *et al.,* 2020; Johnson & LaRock, 2021). *Aeromonas* sp. was reported to be MDR from aquatic sources and in humans (Odeyemi & Ahmad, 2017; Ugarte-Torres *et al.,* 2018). Due to the increase in disease outbreaks and the development of AMR, alternatives to antibiotics are in demand (Dadgostar, 2019). Microalgae might be a potential alternative because of its bioactive components that have antibacterial properties (Alsenani *et al.,* 2020; Rojas *et al.,* 2020). In this study, we investigated *Tetraselmis*, *Chlorella,* and *Nannochloropsis* as available native microalgae.

The findings of the study revealed that *Tetraselmis* has the highest potential to prevent the growth of all bacteria isolated from chicken, dogs, and fish (Figures 2, 3 & 4). The current results are in agreement with previous studies that reported that crude components of *Tetraselmis* contributed to the prevention of *Staphylococcus*, *Vibrio anguillarum*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Lactobacillus* sp. (Duff *et al.,* 1966; Austin & Day, 1990; Austin *et al.,* 1992). The findings are further supported by Makridis *et al.* (2006) who found that *Tetraselmis* prevented the growth of *Vibrio* strains. In relation to this, Guzman *et al.* (2019) identified the AQ-1766 peptide (LWFYTMWH) from *Tetraselmis suecica* to have antibacterial effects against *E. coli, Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bacillus cereus*, methicillin-resistant *Staphylococcus aureus*, *Listeria monocytogenes,* and *Micrococcus luteus*. However, it is necessary to identify the active antibacterial components of the *Tetraselmis* used in the current study.

Minimum Inhibitory Concentration (MIC)

We investigated the minimum concentration of microalgae capable of inhibiting bacterial growth. The results indicated that *Tetraselmis* with 10 mg/ml was able to prevent the *Aeromonas* sp. of fish (Figure 5). However, Maadane *et al.* (2017) reported that the ethanolic extracts of *Tetraselmis* had the highest antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa,* and *S. aureus* with a MIC of 2.6-3 mg/ml. The extraction solvent, source and species of microalgae, and bacterial species might have contributed to the differences in the MIC values. *Chlorella* was able to prevent the growth of *E. coli* and *Stenotrophomonas* sp. from chicken. However, a higher concentration of *Tetraselmis* and *Chlorella* (40 mg/ml) was required to suppress *Staphylococcus* from chicken and *Aeromonas* from fish, respectively.

We identified the antibacterial potential of *Chlorella* against *E. coli* from dogs and chicken, *Stenotrophomonas* sp. and *Staphylococcus*

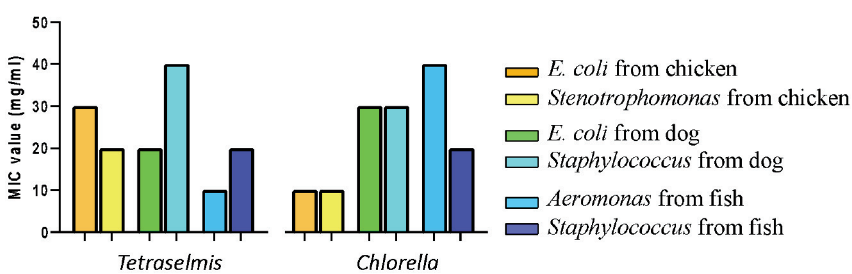

Figure 5: MIC value of microalgae against bacteria. The lowest concentration of *Tetraselmis* was found highly sensitive against *Aeromonas* sp. from fish (opposite to *Chlorella*, right panel). However, the highest 40 mg/ml concentration is needed to suppress *Staphylococcus* sp. from dogs (left panel). A low concentration of *Chlorella* is equally effective against *E. coli* and *Stenotrophomonas* sp. from chicken (right panel)

sp. from chicken, and *Aeromonas* sp. and *Staphylococcus* sp. from fish, with the highest activity against *E. coli* from dogs in the current study. Our findings are supported by Sedighi *et al.* (2019), who found that *Chlorella* hydrolysate was highly effective in preventing the growth of *E. coli* CECT 434. Another study suggests that *Chlorella* pepsin hydrolysates could prevent *S. aureus* and *E. coli* (Tejano *et al.,* 2019). However, Maadane *et al.* (2017) did not find any effects of *Chlorella* against *E. coli*, *Pseudomonas aeruginosa,* and *S. aureus*. We used methanol to extract the *Chlorella* crude solution as it has high polarity to produce high extraction yields (Sultana *et al.,* 2009). Chlorellin is an important antimicrobial metabolite of *Chlorella* that has been reported to have an antimicrobial capacity equal to ampicillin and oxacillin to inhibit *Staphylococcus* sp. (Acurio *et al.,* 2018). The active component of the *Chlorella* in the current study was not identified and this is an urgent focus for future study. We also found that the lowest concentration of *Chlorella* 10 mg/ml was able to prevent *E. soli* and *Stenotrophomonas* from chicken (Figure 5). However, Alsenani *et al.* (2020) reported that 1 mg/ml MIC could prevent the growth of several Gram-positive and Gram-negative bacteria, including *E. coli* and *Staphylococcus aureus*. Shaima *et al.* (2022) extensively studied *Chlorella* against a significant number of bacteria and found MICs as low as 0.39 mg/ml against methicillin-resistant *Staphylococcus aureus* and as high as 6.25 mg/ml against *Serratia marcescens*. Assessment of MIC values of *Chlorella* with further dilutions is suggested in future studies.

In the current study, *Nannochloropsis* had limited effects on *E. coli* and *Stenotrophomonas* sp. from chicken and *E. coli* and *Streptococcus* sp. from dogs, with no effects on *E. coli* from chicken and all the bacteria from fish (data not shown for fish). *Nannochloropsis* was previously reported to be effective against *Lactococcus garvieae* and *Yersinia ruckeri* (Cagatay *et al.,* 2021). Li and Tsai (2009) suggested that the antimicrobial peptide from transgenic *Nannochloropsis oculate* with bovine lactoferrin could potentially prevent *Vibrio parahaemolyticus* infection in medaka fish. Another study suggests that the *Nannochloropsis* extract-mediated silver nanoparticles were found effective in stimulating apoptosis in cancer cells (Gnanakani *et al.,* 2019). We were not able to determine the MIC values of *Nannochloropsis* due to poor antibacterial activity during agar diffusion studies. However, Maadane *et al.* (2017) suggest that the *Nannochloropsis gaditana* is susceptible at 2.6-4.3 mg/ml MIC values against *E. coli*, *Pseudomonas aeruginosa,* and *S. aureus*.

Conclusion

The current pilot study identified *Tetraselmis*, *Chlorella,* and *Nannochloropsis* to have significant antibacterial potential. Larger sample sizes and molecular detection of microalgae and bacterial species could potentially improve the sensitivity and specificity of the results. Advance studies are suggested to identify the active components of the microalgae and their delivery system to improve sustainable human and animal health.

Acknowledgements

We thank the attendants at the Aquaculture Laboratory, Teaching Veterinary Hospital, CVASU and Marine Biotechnology Laboratory, Universiti Malaysia Terengganu for their support in laboratory work and sample collection.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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