EXPLORING THE BACTERIOSTATIC POTENTIAL OF MICROALGAE AGAINST PATHOGENIC MULTI-DRUG RESISTANT BACTERIA

Michelon, W.*[1]; Dinnebier, H.C.F.[2]; Da Silva, M.L.B.[3]; Matthiensen, A.[4]; Soares, H.M.[1]

[1]Federal University of Santa Catarina, Florianopolis, SC – Brazil
william@unc.br; soares@enq.ufsc.br
[2]Federal University of Fronteira Sul, Erechim, RS – Brazil
[3]Rice University, Houston, Texas – USA, silva@rice.edu
[4]Embrapa Suínos e Aves, Concórdia, SC – Brazil
alexandre.matthiensen@embrapa.br

ABSTRACT: Microalgae are rich in metabolic compounds with recognized antibiotic properties. In this study, the potential use of the microalgae *Chlorella* spp on the inhibition of several pathogenic antibiotic resistant bacteria was investigated. Microalgae biomass was obtained from a pilot scale reactor simulating phycoremediation of swine wastewater treatment. Microalgae extracts were obtained with solvent extraction using hexane, dichloromethane or methanol. Different concentrations of extracts were tested against the following model pathogenic bacteria known to cause disease in swine, i.e., *Streptococcus suis* (BRMSA 1410), *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus hyicus* (CEDISA 634/15). Results indicated that microalgae extracts obtained with dichloromethane were most efficient to inhibit the growth of *Streptococcus suis*, *Enterococcus faecalis* and *Staphylococcus hyicus* when tested at 0.390 mg mL^{-1}. The microalgae extracts obtained with hexane or methanol showed bactericidal activity against *Enterococcus faecalis* and *Streptococcus suis* at minimum inhibitory concentration of 0.390 and 0.195 mg mL^{-1}, respectively. Overall, the results support the notion that microalgae biomass obtained from swine wastewater treatment holds great promise as raw material for extraction of bacteriostatic compounds. This is of paramount importance nowadays considering the consequences of antibiotic misuse and abuse and its implications on the spread of multi-drug resistant superbug.

Keywords: antimicrobial, solvent extraction, multi-drug resistant bacteria, microalgae

INTRODUCTION

There are several pathogenic bacteria that pose a serious economic threat to animal farming and associated industries. For instance, *Staphylococcus hyicus* can cause exudative epidermis in swine (ANDRESEN, 1998), cutaneous lesions in cattle (HAZARIKA et al., 1991), and conjunctivitis in chickens and turkeys (CHEVILLE et al., 1988). *Streptococcus suis* is another important pathogen that is associated with septicemia (ZHANG, 2017) and meningitis (FOWLER et al., 2013). This same pathogen is also of concern as an emerging zoonotic agent in humans (GOYETTE-DESJARDINS et al., 2014). *Enterococcus faecalis*, an opportunistic pathogen causes nosocomial, urinary, and neonatal infections (ANDERSON et al., 2016). To avoid or minimize the dissemination of infection diseases, antibiotics are broadly utiized. Nonetheless, it is necessary to explore alternative solutions to antibiotics because over the years the misuse and/ or abuse of these drugs resulted in the selection and widespread of several resistant bacteria (BAQUERO et al., 2008). In this regard the antimicrobial and antiviral properties of microalgae extracts (PLAZA et al., 2010) could be an option and as such should be further explored as potential agent against the proliferation of pathogenic bacteria. Interestingly and conveniently, the source of microalgae biomass could be obtained directly from swine wastewater effluents undergoing treatment (i.e., phycoremediation). The strategy to use microalgae to treat different types of wastewaters is not novel with over 75 years of development (ARBIB, 2013). The use of phycoremediation as a tertiary treatment step can yield large amount of microalgae biomass that can be harvested and processed to obtain a wide range of valuable metabolites (YAMAGUCHI, 1997). Therefore, this study aimed to investigate whether the extracts of the microalgae *Chorella* spp. obtained from phycoremediation of swine wastewaters could be effective to inhibit the growth of several model pathogenic bacteria that are known to pose a risk to animal health.

MATERIAL AND METHODS

Microalgae consortia were obtained from a facultative pond at EMBRAPA Swine and Poultry, Concórdia, Brazil. The inoculum was composed by microalgae consortium dominated by *Chlorella* spp. Experiments were conducted in 12-L glass photobioreactors (PBR) filled with non-sterile 6% v v^{-1} UASB digestate effluent diluted in water. PBRs were inoculated with 70 mg dry weight microalgae L^{-1} (or 30 % v v^{-1} of inoculum). PBRs were maintained at room temperature (23ºC) under mixotrophic conditions (44.8 µmol m^{-2} s^{-1}) and continuous agitation. Experiments were also conducted using a pilot scale 500-L reactor placed inside a greenhouse under natural light 321.5 (± 411.4) µmol m^{-2} s^{-1} and temperature controlled (25°C) conditions. The medium was kept under continuous agitation using a submersible aquarium pump. After 11 days following inoculation, the growth medium containing the microalgae biomass was harvested via centrifugation (EVODOS, T10, Netherlands). The harvested biomass was immediately frozen (-40 °C) and lyophilized (Model 030-JJ LJI Scientific) for further analyses. The dried microalgae powder (20 g) was subjected to hexane, dichloromethane or methanol extraction at concentration ratio of 5:1. Using a rotary evaporator the extracts were kept under vacuum at 50 °C to eliminate any residual concentrations of solvents. The extracts were then resuspended in dimethyl sulphoxide (DMSO) at a concentration of 100 mg mL^{-1} for further investigation. The effects of the extracts with antibacterial activity were evaluated by the Kirby Bauer disc diffusion method (LENNETTE et al., 1985). The following bacteria were used as model organisms: *Streptococcus suis* (BRMSA 1410), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus hyicus* (CEDISA 634/15). Filter-paper disc (6.0 mm) were saturated with 20 and 50 µL of the extract solution. To determine the sensitivity of each extract was performed using the Mueller Hinton agar plates (for *S. suis* it was used sheep blood). Plates were incubated at (37°C) for a period of 18–24 h in dark. Florfenicol (30 µg mL^{-1}) was used as positive controls. The minimum inhibitory concentration (MIC) of microalgae extract was determined by micro-dilution. For determination of the minimal bacterial concentration (MBC), plates made with trypticase soy agar (TSA) were used. A 25µL aliquot of diluted extracts was inserted in each well and the plates incubated at 37°C for 18–24 h.

RESULTS AND DISCUSSION

Previous studies reported the use of different microalgae compounds including terpenoids, polyphenols, phenolic acids, phycobiline, hydroxycinnamic acid derivatives, flavonoids and steroids (PINA-PÉREZ et al., 2017) on bacteria inhibition. These compounds previously identified as antimicrobial are fatty acids, halogenated compounds, terpenes and sulphur contain heterocyclic compounds (PRADHAN et al., 2014). Here, the antimicrobial activity of *Chlorella* spp extracts was evaluated against the growth of multi-resistant bacteria (i.e resistant to amoxicillin, ampicillin, ciprofloxacin, enrofloxacin, lincomycin, lincomycin plus spectinomycin, norfloxacin, and penicillin) that causes several diseases in swine. All extracts tested showed antimicrobial activity (Figure 1). Extracts obtained with hexane showed an inhibition halo of 13 mm for *Enterococcus faecalis* and 10 mm for *Streptococcus suis* (Table 1). Extracts obtained with methanol showed an inhibition zone of 16 mm for *Enterococcus faecalis* and 9 mm for *Streptococcus suis* (Table 1). However, only the extract obtained with dichloromethane extraction was efficient to inhibit all the bacteria tested. The zone of inhibition (halo diameter mm) was 15 mm for *Staphylococcus hyicus*, 18 mm for *Streptococcus faecalis*, and 12 mm for *Enteroccoccus sui* (Table 1). The extracts obtained with dichloromethane showed the best antibacterial effect with MIC of 0.390, 0.390 and 0.195 (mg mL^{-1}) for *Staphylococcus hyicus*, *Enteroccoccus faecalis* and *Streptococcus suis*, respectively (Table 2). The effects of the extracts were also tested for its capacity as bacteriostatic (i.e., bacteria are kept alive but the growth inhibited) or bactericidal (i.e., bacteria are killed). The results suggested that dichloromethane-extracted compounds were effective as bacteriostatic at concentrations higher than 0.39 mg mL^{-1} for the gram-positive *Staphylococcus hyicus* and *Enteroccoccus faecalis* and 0.195 mg mL^{-1} for *Streptococcus suis* (Table 3). Most (if not all) antibiotics used today for gram-positive bacteria are also based on similar bacteriostatic effects (PANKEY and SABATH, 2004).

CONCLUSIONS

The extracts obtained from *Chlorella* spp biomass collected from swine wastewater phycoremediation showed antimicrobial activity against several multi-drugs resistant bacteria. Depending on the chemical characteristics of the solvent used, different compounds are selectively extracted. The extracts obtained using hexane, dichloromethane and methanol showed antimicrobial activity with a zone of inhibition (halo) >10 mm. The use of broth dilution method as a quantitative method to estimate the minimum inhibitory bacteriostatic concentration indicated values of 0.390 mg mL^{-1} for *Staphylococcus hyicus* and *Enteroccoccus faecalis*, and 0.195 mg mL^{-1} for *Streptococcus suis*. Overall, extracts of microalgae *Chlorella* spp. were effective as a bacteriostatic agent against the proliferation of pathogenic multi-drug resistant bacteria known to threaten animal health and are thus of great concern in the animal industry.

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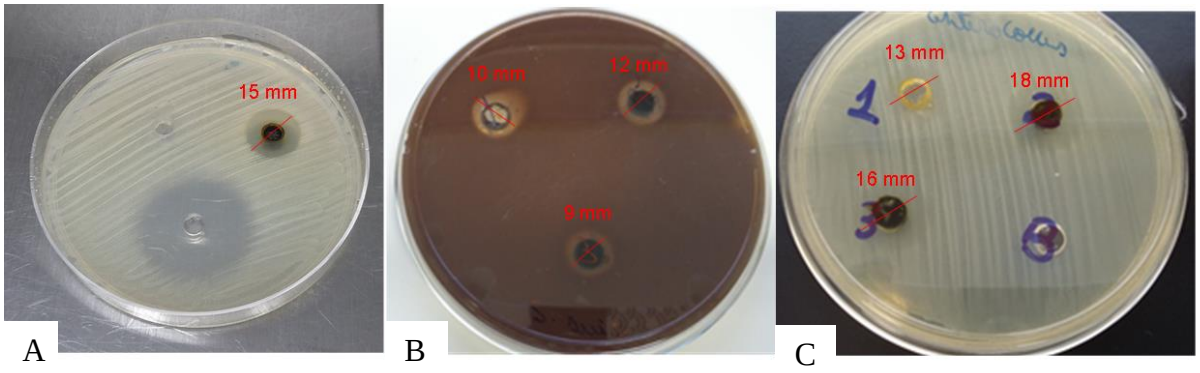

Figure 1. Effects of *Chlorella* spp. extracts on the inhibition of *Staphylococcus hyicus* (A), *Streptococcus suis* (B) and *Enteroccoccus faecalis* (C). The larger the diameter (i.e., inhibition halo) surrounding the wells containing the extracts, the higher is the bacteriostatic effect.

Table 1. Antimicrobial activity of microalgae extracts against pathogenic bacteria.

Test organism	Mean diameter of inhibition zone (mm)				Concentration (100 mg mL^{-1})
	Florfenicol (30 µg mL^{-1})	Hexane	Dichloromethane	Methanol	
Staphylococcus hyicus	24	-	15	-	50 µL
		-	12	-	20 µL
Enteroccoccus faecalis	26	13	18	16	50 µL
		-	10	9	20 µL
Streptococcus suis	20	10	12	9	50 µL
		7	9	7	20 µL

Table 2. The minimum inhibitory concentration (MIC) of dichloromethane extract.

Test organism	Minimum Inhibitory Concentration (mg mL^{-1})
	Dichloromethane
Staphylococcus hyicus	0.390
Enteroccoccus faecalis	0.390
Streptococcus suis	0.195

Table 3. Effect of dichloromethane-extracted compounds from *Chlorella* spp. as bacteriostatic or bactericidal.

Test organism	Bacteriostatic	Bactericidal
Staphylococcus hyicus	+	-
Enteroccoccus faecalis	+	-
Streptococcus suis	+	-