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R-plasmids presence in isolated bacteria from *Tubifex tubifex* (Müller 1974), treated with *Oedogonium capillare* (Linaeus) (Kuetzing 1845) algae.

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ABSTRACT

Tubifex worm is an important aquatic organism's diet component, nevertheless, it has a bacterial load of high ictiopathological risk, compromising the sanitary conditions of culture systems. Purification of *Tubifex* with water baths or different antibiotics, offer a solution but don't eliminate all the pathogenic species important in aquaculture. The proposal to use compounds of natural origin with antibiotic capacity such as algae *Oedogonium capillare*, offers an important solution because it eliminates a significant percentage of ictiopathogens, but it is unknown if it has the capacity to generate R-plasmids. The aim of this study was to prove if algae *O. capillare* has the capacity to generate R-plasmids. For this it was liberated 100 g of *Tubifex* in 40 litter aquariums divided in three treatments with three replicas: a) free *O. capillare*; b) algae contained in a net, and c) common antibiotics used in aquaculture (ampicillin, chloramphenicol, and penicillin). Every seven days, for five weeks, it was made a quantitative and qualitative analysis of bacterial load in each group. After identifying with API 20E, the strains were subjected to: alkaline extraction of R-plasmids, electrophoresis in agarose gel, and diffusion technique in impregnate discs with the mentioned antibiotics. It was made a variance analysis to determine the significant differences between treatments. The results showed a decrease in the number of R-plasmids regarding to initial load and the ones treated with commercial antibiotics.

Key words: pathogenic bacteria, *Oedogonium capillare*, R-plasmids, *Tubifex*.

RESUMEN

El gusano *Tubifex* es importante componente en la dieta para organismos acuáticos, sin embargo, posee carga bacteriana de alto riesgo ictiopatológico, comprometiendo las condiciones sanitarias del cultivo. La purificación del *Tubifex* con baños de agua, o diferentes antibióticos, ofrecen solución, pero no eliminan todas las especies patógenas importantes en acuicultura. La propuesta de utilizar compuestos con capacidad antibióticas de origen natural como el alga *Oedogonium capillare*, ofrece solución importante ya que elimina gran porcentaje de ictiopatógenos, pero se desconoce si posee la capacidad de generar plàsmidos-R. El objetivo de este estudio fue probar si el alga *O. capillare* posee capacidad de generar plásmidos-R. Para lo cual se liberaron 100 g de *Tubifex* en acuarios de 40 litros de capacidad divididos en tres tratamientos con tres replicas: uno en el que se adicionó *O. capillare* libre, otro en donde el alga estaba contenida en una red y el ultimo tratamiento donde se adicionó antibióticos de uso en acuicultura: ampicilina, cloranfenicol y penicilina. Cada siete días y durante cinco semanas se efectuó el análisis cuantitativo y cualitativo de la carga bacteriana de cada grupo. Después de identificarlas con API 20E, las cepas se sometieron a: extracción alcalina de plásmidos-R; electroforesis en gel de agarosa; y a la técnica de difusión en discos impregnados con los antibióticos mencionados. Se efectuó análisis de varianza para la determinación de diferencias significativas entre tratamientos. Los resultados mostraron una disminución en el número de plasmidos-R en comparación con la carga inicial y los tratados con los antibióticos comerciales.

Palabras clave: Bacterias patógenas, *Oedogonium capillare,* plásmidos-R, *Tubifex*.

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INTRODUCCIÓN

Fish production is a productive activity with broad economic growth, nevertheless, it also constitutes a source of bacterial contaminants and resistance genes to antibiotics that are liberated to environment (Baquero et al. 2008; Anderson and Hughes 2012; Ozaktas et al. 2012). To cover the nutrimental needs of cultured fish, the producers use commercial diets and not conventional ones like live food, which also must cover all the quality norms regarding to nutriments and microbiologic quality (Negrete et al. 2001). Despite this, it is frequent that producers obtain live food collected from unhealthy areas such as drainage and industrial waters, so it can be introducing pathogenic bacteria that can affect fish, causing I high mortality percentage, and as consequence, high economic losses. Today the organisms more used as live food for fish are: *Artemia* sp., *Tubifex* sp., *Daphnia* sp., and *Tenebrio* sp. (Luna and Soriano 2001).

Specifically, *Tubifex* is highly used due to its high protein and fatty acids content but is carrier of large quantity of bacteria such as Salmonella, Shigella, and *Escerichia coli*. Nenoff and Uhlemann (2006), isolated *Microbacterium marinum* from Tubifex, this is bacteria that can cause sickness in fish. Monroy et al. (2013) reported the presence of Salmonella in Tubifex. Due to this, the producers use diverse chemicals for sanitation of this food, but its efficiency has been altered because of its excessive or incorrect use, that has led to the appearing and dissemination of resistant bacteria, because microorganisms develop mechanisms to protect from any substance that can harm them (Sánchez 2006). Also, they can acquire genetic material that gives them the ability to survive against other antimicrobial compounds, which are R-plasmids and transposons that contribute to their dissemination between related bacteria and/or pathogens to non-pathogen bacteria, through transduction, transformation or conjugation, giving them resistance (Acuña et al. 2011; Acevedo et al. 2015).

That's why the aim of this study is to determine if *O. capillare* induce the generation of R-plasmids in its use as bactericide in the purification process of Tubifex bacterial load.

MATERIAL Y MÉTODOS

O. capillare organisms were collected in ponds of Centro de Investigaciones Biológicas y Acuicolas de Cuemanco (CIBAC), Xochimilco, México. The more vigorous organisms were selected with a net, the leaves and organic wastes were removed from water surface before doing the sampling. Algae were identified in the laboratory of Phycology in Universidad Autónoma Metropolitana-Xochimilco, based on the descriptions by Tiffany and Britton (1952), Hirn (1960) and Gauthier-Lievre (1963).

Experimental and control group were placed as follows: in nine aquariums of 40 L capacity, 2L of water free of residual chloride was added and 100 g of Tubifex. Subsequently it was introducing *O. capillare* in a direct way for a treatment and in other it was subject to a net, in the third treatment it was added 2 g of chloramphenicol, ampicillin, and penicillin. Also, it was placed an aquarium for Tubifex only with water, which was considered as control group. It was determined the initial bacterial load of Tubifex quantitative and qualitative (Negrete et al. 2012).

Every eight days during five weeks samples were analyzed qualitative for which ten grams of T. tubifex from each aquarium was extracted with an sterile net, and were homogenized with an homogenizer brand Virtix for three minutes at 3000 rev/min, with 90 mL of sterile distilled water, with an automatic pipette 100 µL were seeded per duplicate in agar plates of specific mediums: Salmonella-Shigella (SS), Eosin methylene blue (EMB), Thiosulfate-citrate-bile salts-sucrose (TCBS) and brain-hearth (BHA). From the tube with the original homogenized content, it was transferred 1 mL of sample to tubes with lactose broth, tetrathionate to which it was added 1000 µL of iodine iodide, and to a tube with peptone water, and were incubated during 24 hours at 36°C. After this, when bacterial growth

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was found in agar plates, it was made successive reseedings in nutritive agar until purifying the strains; when homogeneous grow was confirmed, this strains, were incubated during 24 hours at 36°C, and later on it was made gram stain. Then it was identified the colonies, following the criteria of Merck (1994) manual; and to confirm it, it was used the commercial kit API-20 E (Analytical Profiele Index, 1997). Later it was made the quantitative analysis from the homogenized content mentioned before and with an automatic pipette it was extracted 1000 µL and were made dilutions to the hundredth from 10-1 and up to 10-7, from each flask of dilution, were extracted with an automatic pipette, 1000 µL and were seeded in agar plates of specific medium of Salmonella-Shigella (SS), Eosin methylene blue (EMB), Thiosulfate-citrate-bile salts-sucrose (TCBS) and brain-hearth (BHA). The inoculum was spread in each of the culture medium plates, with layered glass dipstick, all plates were incubating for 24 h at 36°C. After this time, it was made the count of colony forming units (CFU/mL), with a colony count Quebec type.

The procedure of isolated species identification as well as the extraction of plasmids -R and susceptibility to antibiotics, was carried out simultaneously with collection strains: *Aeromonas hydrophila* (ATCC356), Vibrio alginolyticus (ATCC177), *Vibrio parahaemolyticus* (ATCC178)., Escherichia coli (ATCC11775), *Klebsiella pneumoniadae* (ATCC13833).

The susceptibility to different antibiotics of the identified strains, was determined through the use of plate diffusion method (Bauer et al. 1966), for which the pure and identified strains were seeded in gar plates of Luria Bertani (LB), and were incubated at 36°C, during 24 h, later the obtained colonies were moved to a tube with 5000 µL of LB broth, until obtaining turbidity of 0.5 of Mc Farland (Hindler 1992), promptly with a sterile hyssop the strains were seeded in agar plates of Hüller-Milton, after 15 min, little discs of filter paper cut with a paper hole punch (Sanofi, Mexico) were placed, this included the following antibiotics in the indicated concentrations; Chloramphenicol (CL) 30 µg, Ampicillin (AM) 10 µg, and Penicillin (PENI) 30 µg. Additionally it were placed petri boxes with agar (HM), filter paper discs, with the same characteristics, impregnate with extract of the algae *Oedogonium capillare*, prepared as mentioned before. Those were incubated at 30°C during 24 hrs. after which with a Vernier the inhibition halos were measured. The strains were classified in Resistant (R), Intermediate (I), or Susceptible (S), depending on the halos diameter, including the discs diameter (6 mm) (Barry et al. 1985; Giono 1983; Stanley 1983).

The extraction of R-plasmids was made using the alkaline lysis technique (Birnboin and Dolly 1979). Again the strains were seeded in agar plates of Luria Bertani (LB), and were incubated at 36°C, during 24 h, later on the obtained colonies were seeded in tubes with 5 mL of LB broth, incubated in water bath with agitation at 36°C during 24 h, 2000 µL of this culture was transferred to a sterile Eppendorff tube and was centrifuged at 14000 rev/min during 30 seg, the supernatant was removed, the pellet that remains in the bottom of the Eppendorff tube was resuspended with 100 µL of lysozyme solution, this was resuspended by shaking it with a vortex during 1 min, later on it was incubated on ice during 30 min, it was added 200 µL of Duodecyl Sodium Sulfate, mixed gently by inversion and incubated during 5 min on ice, after it was added 150 µL of Sodium Acetate 3 M, mixed gently again by inversion and incubated during 60 min, again it was centrifuged during 5 min at 14000 rev/min, the supernatant was transferred to another Eppendorf tube, adding 1000 µL of cold Ethanol, was incubated during 30 min and then centrifuged during 30 min at 14000 rev/min, the supernatant was removed, the pellet was dissolved with 100 µL of Sodium Acetate 0.1 M and Tris 0.05 M pH 8, re-precipitating in 300 µL of cold Ethanol (Bitrnboim and Doly 1979). The supernatant was eliminated and added 10 µL of buffer solution of 5X sample.

RESULTADOS

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Isolated bacterial load from *Tubifex* worm (control group) were formed by 15 different bacterial species: *Enterobacter cloacae, Escherichia coli* I (ATCC11775), *Escherichia coli* II (ATCC23716), *Escherichia coli* III (ATCC194) *Klebsiella pneumonidae, Klepsiella* spp, *Proteus vulgaris, Salmonella typhimurium, Salmonella enteritidis, Serratia plymuthica, Shiguella flexnerii, Vibrio parahaemolyticus, Vibrio algynolyticus, Vibrio fluviales*, and *Aeromonas hydrophila.* In the last two weeks of experiment it was identified and isolated *S. plymuthica.*

For antibiotic commercial treatments, penicillin, ampicillin, and chloramphenicol, were register inhibition halos from first week until fifth week of experimental treatment. However, for those cases of free O. capillare (L) and net O. capillare (N), inhibition halos were no registered (Table 1). After applying the ANOVA analysis to strain number, which shown inhibition halos, showed significant differences (P=0.001) between groups, treated with different antibiotics.

Total initial R-plasmids at the beginning of treatment from 15 bacterial identified species were 93 plasmids (Table 2), which have different molecular weights from 2,916 until 12,500 base pairs (bp), only Aeromonas hydrophila carried the biggest quantity of them (14), corresponding to 15% of total plasmids. The 11 enterobacteria species were: *Enterobacter cloacae, Escherichia coli* I, II, and III, *Klepsiella peumonidae, Klepsiella* spp., *Protus vulgaris, Salmonella thyphimurium, Salmonella enteritidis,* and *Shiguella flexnerii*, and they have: 74 R-plasmids (79.5%) with molecular weights of 6,000 to 12,500 bp with a mean value of 6,878 bp. The four species from Vibrionavceae family only has the 3.2% with: *Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio algynolyticus* and *Vibrio fluviales*, they obtained nine R-plasmids with molecular weights of 2,915,7,500 and 9,500 bp.

The quantity of R-plasmids in isolated bacteria species from the five treatment weeks were significant different (P=0.001) after ANOVA analysis. In Table 3 it is shown the change in molecular weights quantities (bp) from each 15-experimental species. The experiment began with 21 different molecular weights, which decrease in quantity at 1-2 weeks (14 and 18 respectively), and in week three to 39, to decrease to 22 different molecular weights at penultimate week. At fifth and last treatment week it only was found 12 different molecular weights. However, although the quantity of different molecular weights and different quantity of species, significantly decreases, the quantity of bp increase. At the initial of experiment it was registered 67,090 bp in 31 plasmids and 15 distinct species, and in last week it was registered 24,923 bp in 18 plasmids from five species. The ANOVA shows significant differences with a P=0.05 value.

Panel analysis between different molecular weights obtained from initial and last week of Tubifex sp. experimental treatment for five weeks with different antibiotics, were obtained that R-plasmids molecular weights show significant differences, with P=0.001 value.

Table 1: Mean values of number of strains that showed resistance halo to antibiotics for five experimental weeks.

	Penicillin	Ampicillin	Chloramphenicol	*O. capillare* Free	O. *capillare* Red
Initial	12	0	14	4	6
Week 1	8	0	8	2	2
Week 2	29	6	26	0	0
Week 3	42	2	35	0	0
Week 4	32	8	20	0	0
Week 5	18	0	15	0	0

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Table 2: Number of R. plasmids carried from different isolated bacteria from *Tubifex* sp. treated with different antibiotics for five experimental weeks.

Identified species	Initial	Week 1	Week 2	Week 3	Week 4	Week 5
Aeromonas hydrophila	14	0	0	3	6	0
Escherichia coli I	7	0	4	8	4	4
Escherichia coli II	7	1	2	7	4	2
Escherichia coli III	6	1	2	9	0	2
Enterobacter cloacae	11	1	11	3	0	4
Klebsiella pneumonidade	9	1	7	9	10	0
Klebsiella spp	10	4	4	2	0	0
Proteus vulgaris	0	1	8	0	0	0
Salmonella thyphimurium	7	2	7	0	4	4
Salmonella enteritidis	5	0	5	0	0	0
Serratia plymuthica	0	0	0	0	0	0
Shiguella flexnerii	8	1	6	7	7	0
Vibrio vulnificus	3	0	0	0	0	0
Vibrio algynolyticus	6	0	0	0	0	0
Vibrio fluvialis	0	1	2	0	0	0
Vibrio parahaemolyticus	0	0	0	0	0	0
Number of species	12	8	10	7	6	5
Extracted plasmids	93	13	58	48	35	16

DISCUSSION

The results of this study show that when live food was treated with antibiotics like: penicillin, chloramphenicol, and ampicillin, was obtained an important decrease of quantity of species in bacterial load, and always of quantity of CFU mL^{-1} from each one. However, it was necessary to consider the high cost involved in chemical supply (Buschmann 2001), and not less important, their use as environmental water pollutant of containers from treatment lots, and finally was discharge to water fluxes as mentioned by Espinosa y Bermudez (2001), and for bacterial resistance what was generated by antibiotic use, which were incorporated to environment at the same moment that water was discharge to treatment water containers (Cabello 2011).

The two path-ways to apply the algae *O. capillare* treatment were effective, because it reduced bacterial load, and decrease quantity of R-plasmids presence in the isolated strains from the worm even in 58%. Using free *O. capillare* that allowed contact with the worm, resulted more effective because it achieved the maximum R-plasmid reduction in less time lapse, probably because the worm ingest the algae. Although chloramphenicol gave satisfactory results in experimental treatment, it does not eliminate completely R-plasmids presence.

Recent studies show than more than 90% of bacteria from marine origin were resistant to one or more antibiotics (Acevedo et al. 2015), at present study even if isolated bacteria were from freshwater origin, the 67% of isolated species ported more than five R-plasmids, mostly enterobacteria, indicating that were exposed to antibiotics in various occasions. The species from Vibrionaceae family were ported by two plasmids maximum, with exception of *Aeromonas hydropila* and *Vibrio fluvialis*, which

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Table 3: Molecular weight (bp) from R-plasmids ported by isolated bacteria from *Tubifex* sp. worm for five experimental weeks.

	Control	Semana 1	Semana 2	Semana 3	Semana 4	Semana 5
Aeromona hydrophyla	5163	22500	5000			
	7853	21000	7500			
	7083		22500			
	7853		27000			
Enterobacter cloacae	8750	10000		37000		
Escherichia coli I	8750	22500	23500	10000		20000
				19000		26000
						45000
Escherichia coli II	7083	22500	2500	15000		27500
			19500	20000		45000
			35500			
Escherichia coli III	6250	22500	22500	17500	10000	20000
			29000	19000	15000	45000
				28000	16500	
					20000	
					25000	
Klebsiella pneumonidade	10416	20000	17500	1750	10000	20000
	11000		19000	22500	15000	26000
	11750		23000	27000	17500	25000
	12500		29000	37500	20000	26000
Kleibsella spp.		10416	20000	17500	10000	
		11000		19000	15000	25000
		12500		29000	20000	26500
Proteus vulgaris		9100	22500	17500		
		12000		19000		
				23000		
				29000		
Salmonella thyphimurium	7500	22500	19000	17500	12500	
			29500	22500	17500	
Salmonella enteritidis	6000	20000	19500	17500		
				37500		
Shiguella flexnerii	9000	21000	17500	7500	10000	
				10000	12500	
				15000	20000	
				27500		
Vibrio vulnificus	7500					
Vibrio parahaemolyticus	9500			15000		
Vibrio algynolyticus	7500			20000		
Vibrio fluvialis	2916		22500		12500	
			25000		17500	
			29000		17500	

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were extracted from six or eight different plasmids. Having therefore, according Acevedo (2015), a multi resistance case. This show that *Tubifex* sp. worm was previously maintained in environmental contact with different antibiotics, involving a considerable environmental pollution.

It is important to know about the increase of molecular weights against the decrease of quantity of R-plasmids, possibly because of the exposing time (five weeks) where survival bacteria strains were maintained in permanent contact with commercial antibiotics which produce plasmids, as mentioned in several studies (Angulo 2000; Negrete et al. 2004; FAO 2008), continuous application of antibiotics were reflected in quantity plasmids and molecular weights increase as microorganisms developed strategy against antibiotic substances that can damage them (Baires 2012).

Different treatments applied to *Oedogonium capillare* were significant different between other treatments with respect to their capacity to produce R-plasmids, so it can be confirm that *Odeogonium capillare* can be an effective strategy as natural purifier of Tubifex sp. worm, because bacterial load can be decrease and do not encourage R-plasmid production. That's why this method can be applied safely in production or collecting centers of *Tubifex* sp. worm and fish production.

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