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Research Article
Defense Mechanism of the Eared Horse Mussel *Modiolus auriculatus* (Krauss, 1848) (Bivalvia - Mytilidae) with Emphasis on its Associated Microbial Diversity

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Abstract

Background and Objective: Sessile Macro-benthic invertebrates have developed a series of defence strategies against predation and epibiosis based on the synthesis and release of cytotoxic and antimitotic materials. The objectives of this study are to study the secondary metabolites of *Modiolus auriculatus* and to investigate how this mussel defends itself from predators. **Materials and Methods:** *Modiolus auriculatus* was collected from the Arabian Gulf during 2019. Anti-tumour tests of the extract were performed against ATCC mouse cell lines. Nucleic acids of Bacteria, archaebacteria and 18S from the Eukarya Domain were extracted from the mussel using Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE). To determine the chemical structure of the isolated extract, Nuclear Magnetic Resonance (NMR) spectra were obtained using Bruker AC 200. **Results:** All mussel extracts analyzed had high antitumor activity against tumour cell lines but were inactive against brain cancer. The composition of the symbionts associated with the soft bodies has more than 95% similarity. Septamycin, erythromycin, a streptomycin derivative, monensin A, curcutetraol and curcutriolamide were isolated from the extract. **Conclusion:** Symbolization and suppression of microorganisms are a key role in the survival of *Modiolus auriculatus*. About 85% of the total copies analyzed corresponded to Alphaproteobacteria and a few to Gammaproteobacteria, Bacteroidetes and 16S rDNA sequence of Rhodophyta.

Key words: *Modiolus auriculatus*, defence mechanisms, secondary metabolites, tumour cell line, bacteria, archaebacteria, 16S rRNA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Many secondary metabolites were isolated from marine invertebrates like sponges, gorgonians, moss animals, sea squirts, opisthobranchs and bivalves, among others[1]. These natural products have shown effectiveness as human anticancer, anti-inflammatory, antibacterial, antitumor, antibiotics and antiviral among others, with organic structures different from the drugs known so far and with different mechanisms of action[2,3]. Symbiosis with microorganisms from Bacteria and the archaebacteria Domains has been described in many phyla of marine invertebrates[4,5]. For Bacteria Domain, symbiosis of marine invertebrates has been described mainly with microorganisms of the Gammaproteobacteria and Alphaproteobacteria classes[6]. Numerous marine invertebrates such as the gastropod *Bankia setacea* and *Calyptogena soyoae*, the bryozoan *Begula neritina* and the echinoderm *Equinocardium cordatum* are associated in symbiosis with Gammaproteobacteria[7,8]. The marine starfish Many natural products that are extracted from marine invertebrates have structural similarity with metabolites produced by microorganisms[9,10]. The antitumor ET-743 extracted from the ascidian *Ecteinascidia turbinata* shows a very similar molecular structure with the metabolite safranin B produced by *Pseudomonas fluorescens*[11]. The staurosporine metabolite extracted from *Eudistoma toealensis* has a structural similarity with the compound synthesized by the *Streptomyces staurosporea*[12]. Furthermore, this compound has also been isolated from the opisthobranchs *Pseudoceros* sp. and *Eudistoma toealensis*. The biostatins of the bryozoan *Bugula neritina*, with anticancer activity, were synthesized by the symbiotic *Gammaproteobacteria* of the bryozoan[13]. In *Lissoclinum patella* it was found that the symbiote cyanobacterium *Prochloron* sp. synthesizes the natural products patellamides A and C[14].

Over 60% of the drugs that were available on the market or were in the clinical phase during 1981-2002 were of natural origin or were derived from natural products. The alkaloid morphine was isolated from the opium poppy and was used as an analgesic[15]. The alkaloid quinine was isolated and identified as a pharmacologically active component to treat malaria. The further development of environmental chemistry is characterized by the increasing use of powerful physical methods for separation and structure elucidation as well as through close cooperation with biologically oriented fields[16]. In the field of antibiotics, in particular, active substances based on a natural substance have been developed, such as the antibacterial active substances azithromycin and roxithromycin, the development of which was based on erythromycin[17]. Morphine is a strong painkiller and penicillin is an antibiotic to fight bacteria in our bodies. Paclitaxel and Adriamycin are traditionally applied for the treatment of certain neoplasms as ductal carcinoma[18]. Intensively the most examined groups of marine organisms are sponges, followed by tunicates and corals[19]. The bryostatins are protein kinase C inhibitors, immunostimulatory, hematopoietic and prohibit the growth of cancerous cells in leukaemia, ovarian and breast cancer[20]. Ecteinascidin 743 is an alkaloid separated from the tunicate *Ecteinascidia turbinata*, has DNA-interactive substances play an important role in cancer therapeutics. This active metabolite has good *in vitro* results against breast cancer, melanoma, lung and ovarian cancer cell lines were also evident in the *in vivo* clinical studies[21-23].

This study aims to isolate and identify the secondary metabolites of *Modiolus auriculatus,* to investigate the Anti-tumor impact of the extract against ATCC mouse cell lines, identify the micro-symbionts that live in association with the mussel concerned.

MATERIALS AND METHODS

Study area: The study was performed at the Department of Microbiology, Quality Control Laboratory, Imam Abdulrahman University, Saudi Arabia from January, 2019-March, 2020.

Sampling and total extract: Samples of the horse mussel *Modiolus auriculatus* were gathered from the estuarine waters of the Arabian Gulf during 2019. The area presents temperatures between 25.5 and 29.7°C in the water surface of the gulf, decreasing with depth. Salinity ranges from 36.4-38.74% and the oxygen content varies from 4.4-5.8 mL L^{-1}. Species identification was performed at the Department of Biology, Imam Abdulrahman University, Saudi Arabia. The soft bodies of the mussel were dried at lab temperature for 48 hrs and were grounded in a CONDUX-WERK® model LV15M mill (NETZSCH Lohnmahltechnik GmbH Bobingen, Germany). The homogenate material was extracted with solvents of different polarity, ether, dichloromethane and methanol at 45-60°C on Soxhlet system[24].

Determination of the antitumor activity of the samples: Approximately 2 g of fresh soft body weight was homogenized in a mortar in 10 mL of a 1:1 methanol-acetone solution. Centrifugation of the organic phase was done at 4,400 rpm for 1 min to discard the remains of tissues. The supernatant was dried in a vacuum at lab temperature and finally suspended in pure water. Anti-tumour analyzes were

performed against ATCC mouse cell lines Labome® of the colon (CT26.WT), the lung (LL/2-Luc-M38), pancreas (6606PDA), breast (MMTV-PyMT), ovary (ID8-Luc-mCh-Puro) and the brain (GL261-Luc2).

Extraction of nucleic acids from the mussel: Soft part samples were cleansed three times with sterile saline water to withdraw particles adhering to the surface. Nucleic acid extractions were made from the entire mussel. Total 1.5 g of soft tissue ground in a mortar with liquid nitrogen and resuspended in 8 volumes of the TE buffer with 0.5% Sodium Dodecyl Sulfate. A similar amount of Phenylic acid, amyl alcohol and trichloromethane was added, homogenized and centrifuged at 4,500 rpm (Labofuge™, 400R, HERAEUS, Thermo Fisher Scientific, Kansas, USA) at 4°C for 6 min. The hydrous phase was placed to another vial and 0.15 volumes of 3 M sodium salt form of acetic acid pH 5.3. Phenolization was repeated until the interface was not cloudy. Two quantities of absolute ethyl alcohol previously cooled to 4°C were added to the aqueous phase and subsequently, it was left at -20°C for a minimum of 90 min. Centrifugation had done at 6000 g at 5°C for 60 min. The liquid above the solid residue was sucked with a sterile micropipette and the precipitate was cleaned with 1.5 mL of 75% ethyl alcohol[25].

Extraction of nucleic acids from bacteria and archaebacteria: For nucleic acid extraction by PCR, biomass was taken directly from the plate culture with the seeding loop and resuspended in 400 µL TE buffer with Sodium Dodecyl Sulfate 0.5%. The phenolization and precipitation of the nucleic acids were carried out. Finally, the nucleic acids were re-postponed in 55 µL of sterilized ultrapure water. Bacteria, Archaebacteria and 18S from the Eukarya Domain with nucleic acids were taken out from the mussel and cultures, the 16S ribosomal RNA genes from Bacteria, Archaebacteria and the Eukarya Domains were performed using PCR[26].

16S ribosomal RNA genes for DGGE amplification: Associated Bacteria Domain was studied by DGGE. The specific primer pair 907R and 341-GC were used, the latter carries a GC tail of the approximately 40 nucleotides at the 5'end[27].

Amplification of the *pufM* gene involved in the aerobic anoxygenic phototrophy: The following amplification conditions were used[28]: 95°C in 4 min (90°C in 2 min, 58°C in 2 min, 73°C for 2 min) 20 cycles. One cycle was performed at 72°C in 10 min.

***nifH* gene of nitrogenase reductase involved in nitrogen fixation:** The amplification conditions of the nitrogenase reductase (*nifH*) gene were previously used by Zehr and McReynolds[29]: 9TC in 4 min, -97°C in 26 sec, -60°C in 37 sec, -70°C in 35 sec, -97°C in 26 sec, -65°C in 37 sec, -70°C in 30 sec. (97°C in 26 sec, 65°C in 35 sec, 70°C in 35 sec) 3 cycles, (97°C in 26 sec, 65°C in 35 sec, 70°C in 40 sec) 4 cycles, (97°C in 25 sec, 50°C in 35 sec, 70°C in 55 sec) 5 cycles, (92°C in 25 sec, 55°C in 40 sec, 70°C in 1 min) 25 cycles. One cycle was applied at 70°C in 15 min.

Products purification of PCR: Purification of the products was performed according to Wang *et al.*[30].

DNase treatment of the sample of nucleic acids: By treating the nucleic acid sample with DNase, the RNA was obtained from the DNA-free sample. The RNA obtained was applied as a strand for amplification by simple reverse transcription. Enzymatic digestion of DNA from nucleic acid extraction was performed from the mussel using the enzyme DNase I (Invitrogen). For the reaction, 10 enzyme units, 50 µL of nucleic acid extraction sample (0.85 µg μL^{-1}) and adjusted to 10×reaction buffer. Incubation at 38°C in 120 min and the digestion was stopped by adding an adjusted amount of stop solution 10×(Invitrogen)[31].

Denaturalizing Gradient Gels Electrophoresis (DGGE): Electrophoresis was used in gradient gels urea and methanamide denaturant (DGGE) to study the symbionts of the mussel, in addition to the construction of libraries of the 16S rRNA genes, For the separation of the different fragments generated in the PCR with the specific primers designed for this technique, a 45-60% gradient of denaturing agents were used (100% is defined as 7 M urea and 40% deionized methanamide). Products of PCR were loaded onto 0.75 mm thick gels made using a Gradient Former 485, Bio-Rad®, California, USA. Electrophoresis was performed at 5 V cm^{-1} at a temperature of 60°C in TAE IX buffer for 16 hrs, Bio-Rad®. Gel staining was done for 15 min with unsymmetrical cyanine dye, Sigma-Aldrich® (100 µL L^{-1} in 0.5X TAE) (Molecular Probes) and washed with TAE IX buffer for 15 min. DGGE gel image was captured with a Canon power shoot (PROGRAF TZ-30000 MFP Z36), Canon®, California, USA. The resulting bands of DGGE were cut with a sterilized knife and re-hold off in 25 µL of sterilized pure water in 5°C for 16 hrs. The eluate was applied as a DNA prototype for the PCR re-exaggeration reactions with the same conditions applied previously.

Laboratory technique applied to establish the taxonomic domains: To determine the microbial domains of mussels, *in situ* hybridization was performed with fluorescent DNA probes that hybridized with specific regions of the 16S rRNA of the microorganisms associated with the mussel, so that depending on the probe used, it was possible to know which phylogenetic group they belonged. This technique not only allows us to visualize the microorganisms *in situ* in the soft body but also allows us to know the structure of the microbial community based on the probes used. The FISH of the soft body samples homogenates were filtered through GTTP isopore filters (Millipore) Sigma-Aldrich®, Darmstadt, Germany. Initially, the soft bodies were washed three times in filtered and sterilized seawater and subsequently fixed in absolute ethanol at 5°C for a minimum of 180 min. After fixation, they were cut with a sterile blade and homogenized by a Vortex-Genie 2 rubber cover, Sigma-Aldrich®, Darmstadt, Germany. The homogenate was filtered through 0.2 µm pore diameter GTTP isopore filters. Finally, the filters were washed with PBS IX with 3.4% NaCl and allowed to air dry. For the hybridization reaction, the filters were dehydrated with 55, 82% and absolute (v/v) ethyl alcohol to 3 min, respectively[32].

Nuclear Magnetic Resonance (NMR): To determine the chemical structure of the isolated extract, NMR spectra were obtained using the following devices: Bruker AC 200 (4.6 Tesla), Sigma-Aldrich®, Darmstadt, Germany operating at 200.1 MHz at the hydrogen frequency (^{1}H), Bruker AC 400 (9.3 Tesla), operating at 400.35 MHz at the hydrogen frequency (^{1}H) and 100.10 MHz at the carbon frequency (^{13}C) in the DQ-UFSCar[33].

Liquid chromatography (HPLC): The equipment used consisted of a Waters® system with two pumps model 600, an in-line Degasser degasser, China Supplier-Gold Member®, China, a UV-Visible spectrophotometric detector, Sigma-Aldrich®, Darmstadt, Germany with reading at two wavelengths model 2487, a model 746 recorder and a Controller 600 control system. A Waters chromatographic system, Sigma-Aldrich®, Darmstadt, Germany consisting of a system control panel Model Waters® 2695 coupled with UV-Visible spectrophotometric detector model Waters® 2996 with a photodiode array detector, which allows observing between wavelengths from 200-800 nm, light scattering detector model Waters® 2424 and mass spectrometric detector (MS) model Waters® Micromass ZQ[34,35].

RESULTS

TEM of the soft part: Using Transmission Electron Microscopy (TEM) (Fig. 1a-e), gram-negative bacillary organisms were observed with polyhydroxyalkanoate (PHA) granules associated tunica to the tunica in the soft body of the mussel, approximately 1-3 µm length and 0.3 µm thick. In the soft bodies, two different classes of cocci were observed, although, very sporadically, depending on the ultrastructure of the cell wall. Some, due to the presence of a thick, peptidoglycan layer, could be gram-positive. The other cocci, due to the presence of an external layer similar to the structure of the S layer of Archaebacteria and a high density of material in the periplasmic space, a feature that characterizes the cell wall of some Archaebacteria, could be microorganisms from the Archaebacteria Domain. Most microorganisms were arranged by enveloping the eukaryotic cells and the cells of the internal compartment, with the maximum concentration of microorganisms in the mouth region. The filopodial cells were observed to phagocytose the microorganisms in large quantities, up to 20 phagocytized bacteria/filopodial cells. At the foot region, the presence of diatoms and cellular structures was observed, which, due to their size, would be microorganisms of the Eukarya Domain.

By counting the microorganisms made with the DAPI staining, it was observed that in the soft part of the mussel there were $7.14\times10^{7}+0.5\times10^{7}$ bacteria/cm^3. The cell density of microorganisms is higher in the areas near the mouth region. Within the internal compartment, two zones can be discerned, the stomach where the concentration of microorganisms becomes $1.74\times10^{9}\pm0.8\times10^{9}$ bacteria/cm^3 and filamentous gills $185\times10^{8}\pm0.49\times10^{8}$ bacteria/cm^3. Numerous microorganisms are seen that green pigmentation and the cell size of approximately 5-10 µm could be diatoms (Fig. 1f). Besides, organisms were observed that due to their cell size, pigmentation and red autofluorescence (Fig. 1f-g), could be red algae of the phylum Rhodophyta. The molecular data obtained from the analysis of the 16S rDNA libraries for the Bacteria Domain support these observations since sequences of the rDNA 16S gene from diatom chloroplasts and red algae were found.

Analysis of the antineoplastic activity of *Chicoreus ramosus* extracts: To study whether all the extracts from the mussel had antineoplastic activity, a total of 50 mussels were analyzed. All mussel extracts analyzed had a high anticancer

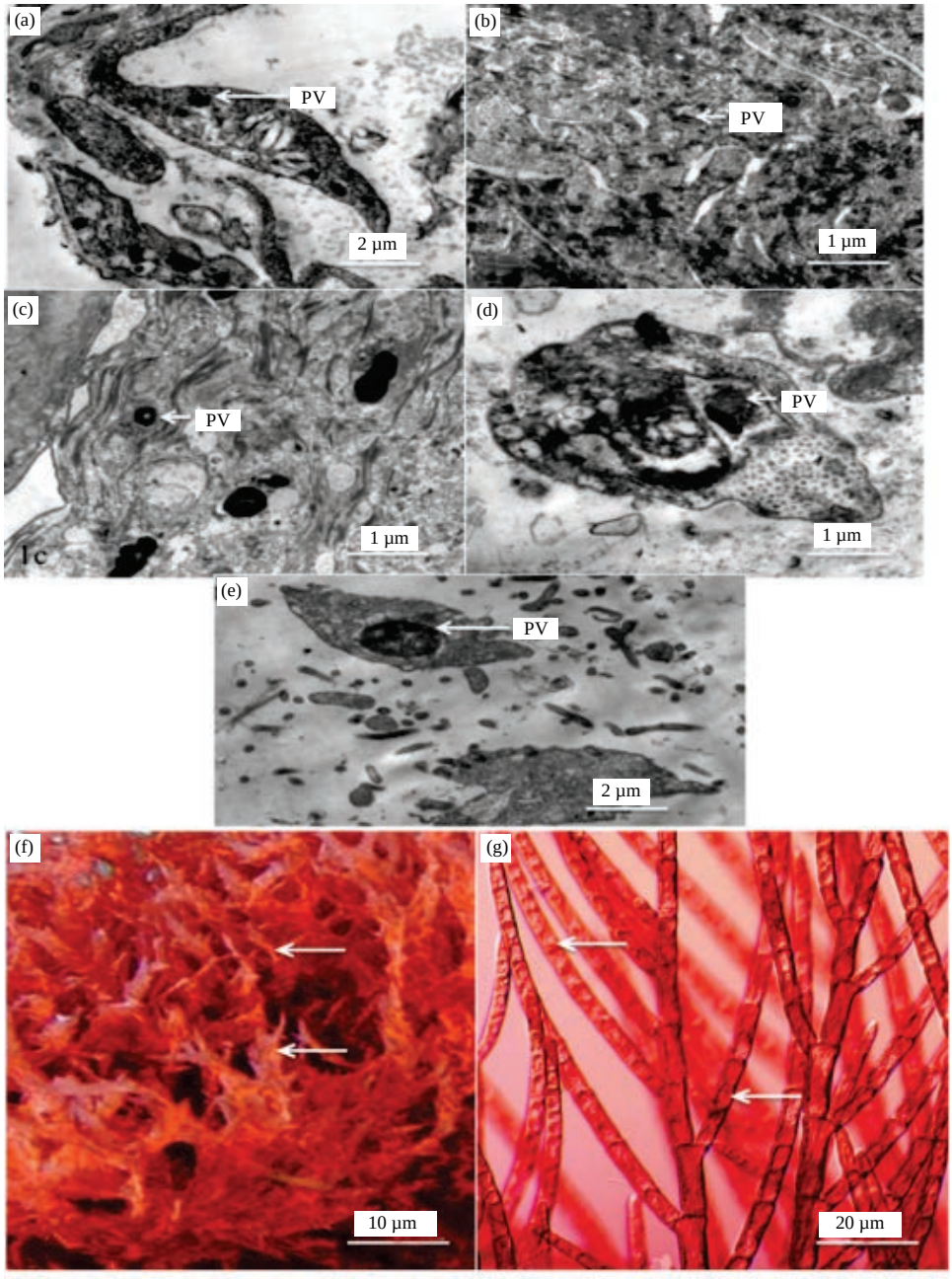

Fig. 1(a-g): TEM of the soft body of the mussel showing microorganisms phagocytosis

Detail of eukaryotic cells that are phagocytizing various microorganisms, showing the projection of the plasma membrane of the eukaryotic cell and different phagolysosomal vacuoles, (a-b) PV, (c) Detail of different stages of lysis of phagocytosed microorganisms in phagolysosomal vacuoles, (d-e) Grouping of bacilli is indicated by arrows, (f) Autofluorescence microscopic image of the foot, arrows point to the red algae and diatoms and (g) Confocal microscopic image to show the red algae and filamentous cyanobacteria on the mussel foot

response contrary to carcinoma lines of the colon metastasis (CT26.WT), lung metastasis (LL/2-Luc-M38), pancreas metastasis (6606PDA), breast metastasis (MMTV-PyMT) and ovary metastasis (ID8-Luc-mCh-Puro) and were inactive against brain cancer (GL261-Luc2). Therefore, it is deduced that the mussel synthesizes the compounds with antitumor activity and that this a character widely extended among the different samples analyzed.

Microorganism's cultivation and analysis of the antitumoral activity of the extracts: A total of 27 plate culture soft bodies were selected taking into account their morphological

characters, size, shape and pigmentation. Nucleic acid extractions were performed, the 16S rDNA genes were amplified and the resulting digestion patterns were analyzed by ARDRA. Subsequently, the 16S rDNA gene from one or more isolates was sequenced from each restriction profile and the sequence was compared by nucleotide BLAST, GenBank, USA. Most of the isolates obtained in pure culture, 22 out of a total of 25, belonged to the Alphaproteobacteria class and the other 3 isolates belonged to the Gammaproteobacteria class. All isolates obtained are typical of marine environments, except for food *Halomonas*, isolated from fermented salted seafood, a typical Korean food. The sequence of the 16S rDNA gene from isolate E showed greater than 97% similarity with the H30, AI and FU sequences of the soft body. None of the extracts from the pure cultures showed antitumor activity against the analyzed tumour cell lines. Furthermore, the antitumor activity of the extracts was also not obtained from mixed cultures.

Antibiotic treatment of the mussel maintained in the aquaria: Through this experiment, the aim was to observe if there was a change in the antitumor activity of the mussel kept in the aquarium due to antibiotic treatment. Furthermore, a parallel change in the associated prokaryotic community was tested. The results indicated that all mussels treated with antibiotics and the controls, presented high anti-carcinoma activity against the metastasis, with no change in activity levels being observed during the experiment. However, in the bacterial community associated with the treated mussel for four weeks, a change compared to untreated controls was observed by DGGE (Fig. 2a). With the DGGE result, a presence and absence of bands were constructed and the dendrogram of similarity was calculated based on the Jaccard index between the different samples (Fig. 2b), which estimates the similarity of the composition of the symbionts associated with the different samples analyzed. The composition of the symbionts associated with the sample treated for 4 weeks (T_4) is considerably different from the other samples, presumably as a consequence of treatment with antibiotics. On the other hand, the results indicate that the symbionts associated with the mussel remain stable after keeping the mussel artificially in the aquarium out of their environment for a month since the composition of the microbots of the mussel kept in the aquarium shows a similarity greater than 95% with the microbots of sample 6 freshly extracted from the sea, used to construct libraries of the rDNA 16S gene. The resulting bands of DGGE were reamplified and sequenced and in the T_4 sample, sequences of Betaproteobacteria and Alphaproteobacteria that did not appear in any of the other samples were obtained: *Janthinobacterium agaricidamnosus* with a percentage of similarity of 98% (band 1) and *Stappia* sp. with similarity percentages of 94 and 98% (bands 6 and 7). Furthermore, bands 4 and 5 corresponding's to phylogenetically close microorganisms *Mesorhizobium* sp. with 98 and 99% similarity.

Analysis of the prokaryotes diversity through molecular techniques

Extraction of total nucleic acids and treatment with DNase: Approximately 170 µg of nucleic acids were obtained per gram of fresh weight of the soft body (Fig. 2c). For the RT-PCR reactions, a nucleic acid sample from the soft body enzymatically treated with DNase I was used as a template (Fig. 2d). After treatment of the total nucleic acid sample, approximately 100 µg of RNA were obtained per gram of fresh weight from the soft body.

Amplification by PCR and RT-PCR of bacteria and archaebacteria 16S ribosomal RNA genes: Amplification products had been obtained separately by the primary transcripts specific for the Bacteria and Archaebacteria Domains. Different amounts of template DNA (from 170-1360 ng of acids) were tested with the primers for the Archaebacteria domain.

Analysis of the prokaryotes community using DGGE: The diversity of the prokaryotic community of the Bacterial Domain associated with the mussel was analyzed. For this, seven consecutive samples of different collection times were analyzed: (sample 7), (sample 2) and (samples 3, 4, 5 and 6). Besides, a soft body was studied whose foot was scraped with a sterile blade to eliminate debris and mucus as much as possible. Therefore, from the analysis of the results, it can also be deduced if there is a change in the community of the symbionts associated with the mussel between cold and warm months. Nucleic acid extractions were made from the seven samples, amplified by PCR with the DGGE-specific primers and the products were separated on a denaturing gradient gel (Fig. 2e). With the DGGE results, a presence and absence of bands matrix was constructed and, using the Jaccard index (common characters/total characters), the similarity dendrogram between the different samples was calculated. This allowed estimating the differences of symbionts allied with the different samples analyzed. From the DGGE analysis, it is observed that the composition of the symbionts associated with the soft bodies (samples 3, 4, 5 and 6) is very similar, with more than 95% similarity. Furthermore,

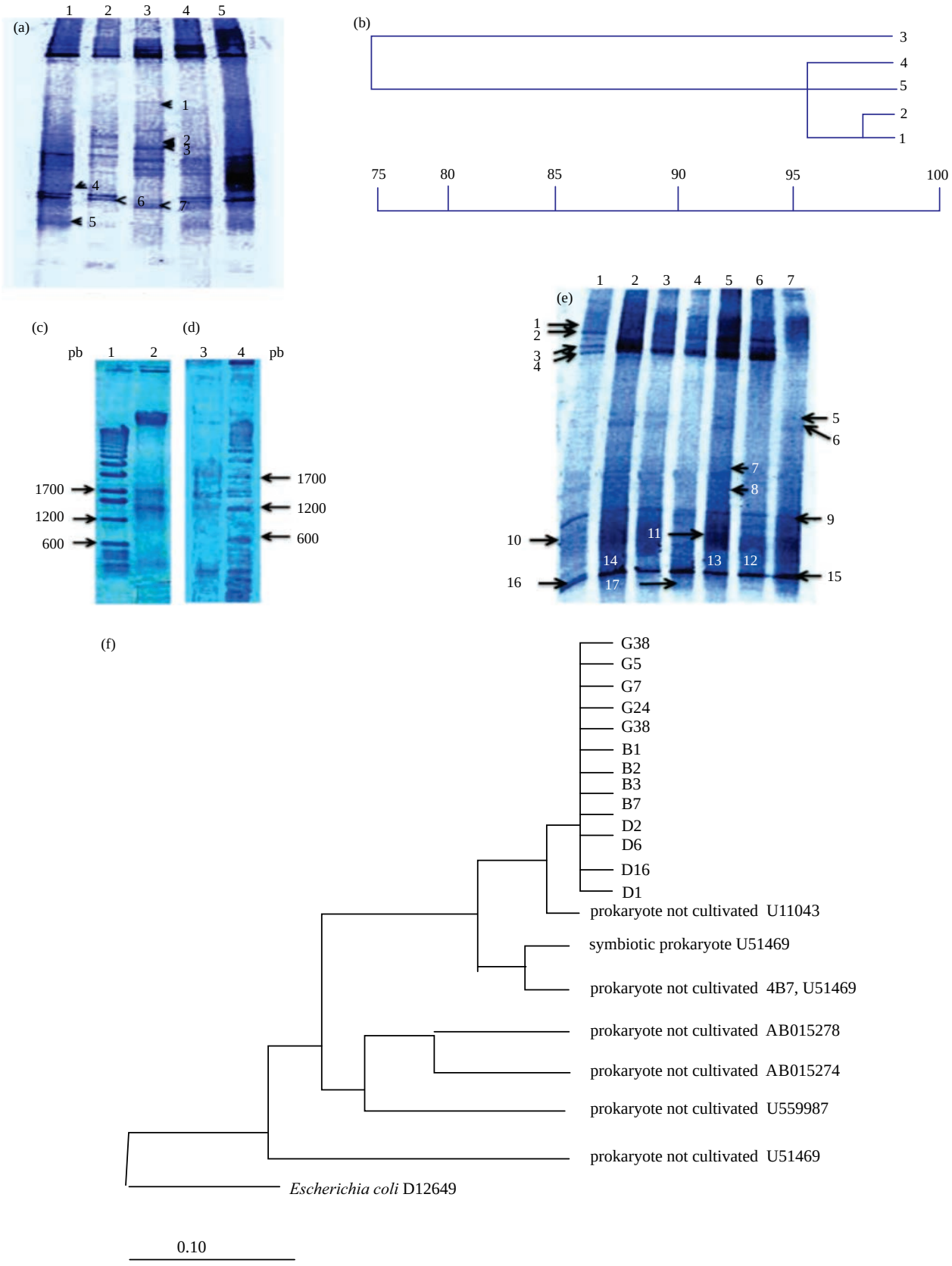

Fig. 2(a-l): Continued

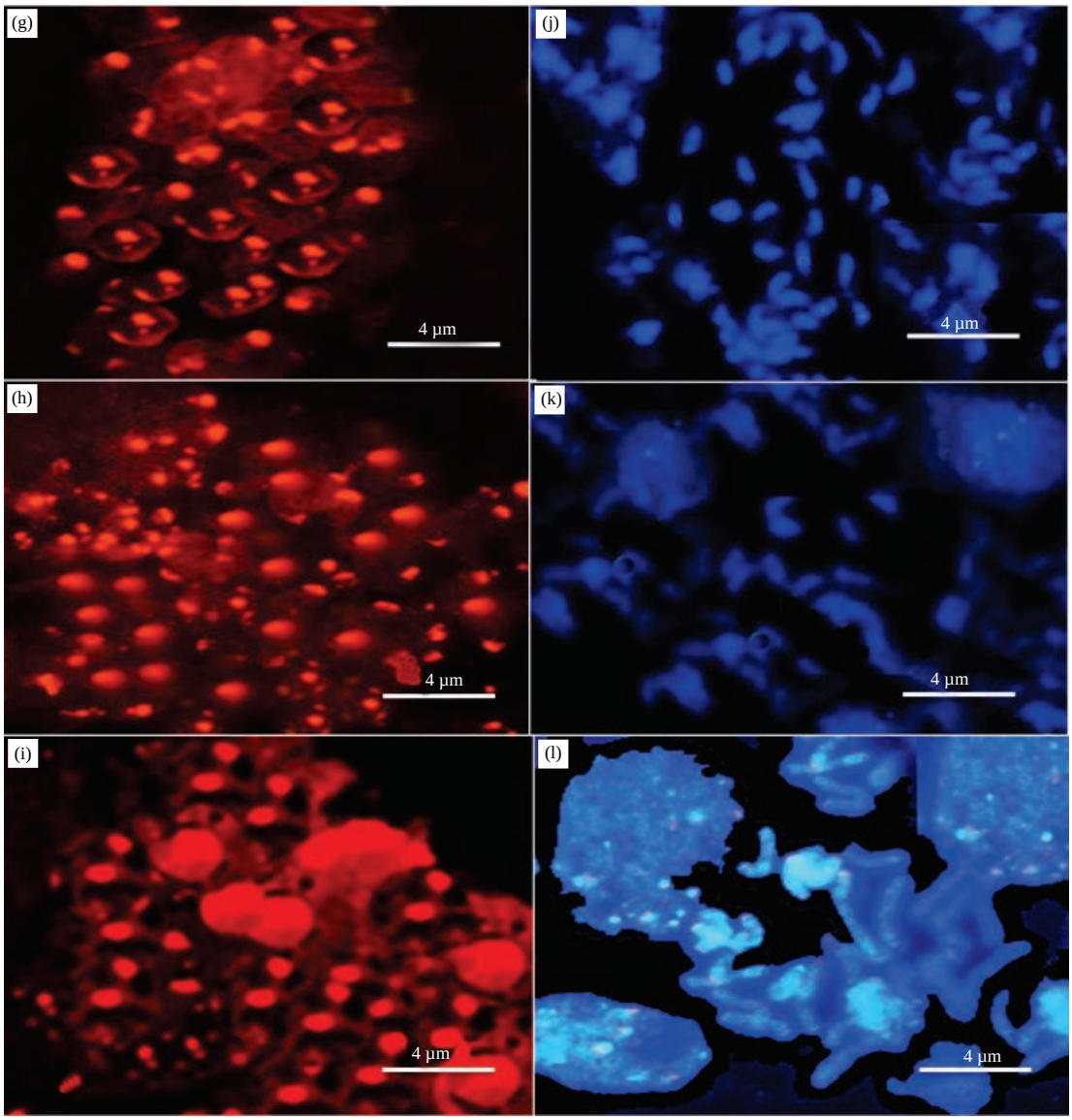

Fig. 2(a-l): Fluorescent *in situ* hybridizations of the soft body to show the amplified products for the bacterial Domain

(a) DGGE gel of the amplified products for the Bacterial Domain of the analyzed samples: untreated control sample (1), treated 1 week (2), the sample treated for 4 weeks (3), the sample treated for 2 weeks (4) and sample 6, the sample treated for 3 weeks (5). Arrows point to the cut and sequenced bands. (b) Dendrogram of similarity based on the Jaccard index (common characters/total characters) between the different aquaria samples analyzed by DGGE. (c) A 0.8% LE agar gel showing the nucleic acids of the soft body of cold months (lane 2) (A), (d) A 0.8% LE agar gel showing DNA-free RNA from the soft body of warm months, (e) DGGE gel of the amplified products for the Bacterial Domain of samples 1-7. Arrows point to trimmed and sequenced bands. (f) A tree was constructed by maximum likelihood with the complete sequences of the library B of the soft body for the Archaebacteria Domain. The partial sequences of libraries G, of the entire soft body in cold months and D, of the soft body in warm months, were introduced by parsimony. The figures on the left (g-i) show hybridization with the NON (g), EUB338 (i) and Alf968 probes, labeled with Cy3 fluorophore and the right show the DAPI staining (j-l)

there is no change in the community between the extracted in different collection times (samples 3, 4, 5 and 6). On the contrary, greater differences in the composition of the symbionts are observed between sample 2 and the summer samples. Besides, differences in the community of the soft body with the scraped base (sample 1) with the samples of winter are observed. The prokaryotic community (sample 7) presents a percentage of similarity with the symbionts associated with winter samples of 75%. The different sequences were obtained from the DGGE bands. Within the Bacteria Domain, sequences were obtained from Alphaproteobacteria (lanes 5-17) and Bacteroidetes (lane 3).

Most of the sequences of microorganisms in this group (bands 5, 6, 8, 10, 11, 13-17) show different percentages of similarity (91-99%) with the microorganism *Mesorhizobium* sp. associated with the marine dinoflagellate *Gymnodinium catenatum*. A sequence (lane 9) was phylogenetically close to *Roseobacter* sp. (97% similarity) and two sequences (bands 7 and 12) that showed different percentages of similarity (90-91%) with Mucus bacterium. In both the summer and winter samples, a sequence (band 3) phylogenetically close to *Tenacibaculum maritimum*, a microorganism from the phylum Bacteroidetes and three sequences (bands 1,2 and 4) of the rDNA 16S gene of diatom chloroplasts and rhodophytic algae were obtained. The sequences obtained by DGGE were introduced by parsimony in the tree constructed by maximum likelihood with the complete sequences of the library of the mussel.

Comparison of the results of the gene library and the DGGE analysis: Using DGGE it was found that the composition of the symbionts associated with the different soft bodies analyzed was very similar in the composition of the community was observed between collection times. In this study with the sequences of DGGE and the libraries related to Alphaproteobacteria, a high diversity of sequences was found that were grouped into seven different groups. Group I consists of 28 copies and four different ARDRA profiles represented by copies A13, Cl, C7 and F20. The sequences obtained were related to *Hyphomonas polymorpha* (92-93% similarity). However, no sequences associated with a group I was found in the library and the prokaryotic community analysis by DGGE. About 14 copies and four different restriction profiles represented by copies F25, A15, C15 and F13, which were not homogeneously sampled in a group although they were phylogenetically related to *Hansenula polymorpha* and *Rhodothalassium Imhoff*. Group 2 consists of 13 copies and three restriction profiles represented by Al copies, FU and H30. The three sequences were related to *Erythrobacter ramosum* and *Agrobacterium sanguineum*. cladogram II sequences were also 96-98% related to *Erythrobacter luteolus*, aerobic anoxygenic phototrophic marine microorganism. Group III consists of DGGE band 9 and 8 copies with three different restriction profiles represented by copies A3, A5 and H16. The four sequences analyzed were related to *Roseobacter* sp., which performs aerobic anoxygenic phototrophy. Group IV is made up of 27 copies and seven different restriction profiles and was related to *Agrobacterium kieliense*. Group V consists of 5 DGGE sequences (DGGE 11, 13, 14, 16 and 17) and 30 copies with four different restriction profiles represented by copies A7, H9, H38 and H48. DGGE sequences were related to *Mesorhizobium* sp., While copies were 94-95% similar to *Bartonella* sp. not cultivated, associated with aquatic invertebrates. However, by aligning the sequence between bases 274 and 775 of the complete sequences of copies A7, H9, H38 and H49, it turned out that they were related, like the DGGE sequences, with the microorganism *Mesorhizobium* sp. Group VI consists of 7 DGGE sequences (DGGE bands 5, 6, 7, 8, 10, 12 and 15) and 18 copies with five different restriction profiles represented by copies F7, F9, AlO, H13 and H49. All the sequences analyzed were 92% related to the aerobic anoxygenic phototrophic microorganism *Rodhobium orientis*. Group VII consists of 7 copies with two different restriction profiles represented by copies A24 and F48. Both sequences were related 95-96% with *Hyphomicrobium* sp. Within the sequences of the Gammaproteobacteria class, 10 copies were found with five different restriction profiles. Sequences F8 and F22 of the entire soft body were related to *Oceanospirillum kriegii* and sequences A19, A23 and C5 were related to uncultivated Gammaproteobacteria. Within the Bacteroidetes phylum, sequences were found both by DGGE, band 3 and by libraries, 4 copies with three different restriction profiles. The three sequences, distant from each other, corresponding to phylogenetically related microorganisms with *Cytophaga* sp. and *Flexibacter maritimus*. Within the Eukarya Domain, sequences (bands 1 and 2 of DGGE and copy C15 of library C) of photosynthetic microorganisms of the Heterokonta group, a complex phylogenetic group composed mainly of diatoms, brown or brown algae, green-yellow algae, single-cell flagellates, another unicellular and multicellular protist were found. The three sequences analyzed corresponded to diatom chloroplasts from the phylum Bacillariophyta, phylogenetically related to *Odontella sinensis* and *Skeletonema costatum*. The E9 sequence obtained in the Cyanobacteria library was associated with chloroplasts from *Rhopalodia gibba* diatom, which possesses intracellular spherical bodies capable of nitrogen fixation under light conditions. These spherical bodies are probably the result of an ancestral endosymbiosis between the diatom and a free-living diazotrophic cyanobacterium. The second group of sequences, band 4 of DGGE and copies A17 and E2, would correspond to chloroplasts of multicellular algae of the phylum Rhodophyta. Within the Bacterial Domain, sequences (copies E3, El2, E15 and E25) of photosynthetic microorganisms of the phylum Cyanobacteria were found. The E3, E12 and E15 sequences were related to *Spirulina* sp. and the E25 sequence with cyanobacteria of the order Pleurocapsales. In summary, in the samples of the soft body, both by the library and by DGGE, most of the analyzed sequences were related to

microorganisms of the Alphaproteobacteria class, although sequences from the Gammaproteobacteria, Bacteroidetes and chloroplast groups of photosynthetic microorganisms were also found.

Archaebacteria domain gene libraries: All the analyzed sequences of the three libraries built for the Archaebacteria Domain were related to 92-93% similarity with *Cenarchaeum symbiosum*, Archaebacteria endosymbiont of sponges of the genus *Axinella* sp. They are present within the group of uncultivated planktonic marine archaebacteria of the phylum Crenarchacota. In the phylogenetic tree for the copies of the Archaebacteria Domain of the B, D and G libraries (Fig. 2f), all the sequences analyzed were grouped within the same group, in which a high micro-diversity sequence was observed.

Bacteria domain gene libraries: From library A, 17 copies were completely sequenced for the Bacteria Domain and most of the sequences obtained in the library, 85% of the total of copies analyzed corresponded to Alphaproteobacteria microorganisms. Copies of microorganisms of the Gammaproteobacteria class and the phylum Bacteroidetes and a 16S rDNA sequence of chloroplasts from algae of the phylum Rhodophyta were also obtained. From library C, 9 copies were completely sequenced. The Nucleotide BLAST results of the analyzed sequences, in which it is observed that 65% of the sequenced copies belong to the Alphaproteobacteria class. Total 4 sequences of phylogenetically related microorganisms were also obtained with *Reichenbachia agariperforans* from the phylum Bacteroidetes and 2 phylogenetically related copies with uncultivated microorganisms of the class Gammaproteobacteria. Besides, 9 copies were obtained -not found in any of the other libraries for the Bacterial Domain, represented by copy C3, which were related to 89% similarity with uncultivated bacteria found in areas contaminated with compounds derived from explosive material and were also associated with 89% similarity to uncultivated bacteria found in phosphate-activated sludge reactors. Furthermore, the alignment of this sequence showed that the first 12 organisms with the highest percentage of 16S rDNA similarity were uncultivated microorganisms found from environmental samples. Due to the low percentage of similarity obtained in the alignment of the 16S rDNA and the size of the sequence (approximately 425 bp), it could not be assigned to a phylogenetic group. All the sequenced copies from library H were associated with microorganisms of the Alphaproteobacteria class and no sequences from other phylogenetic groups were found. A total of 78% of the copies of the F library, constructed from the 16S rRNA genes from the entire soft body sample, belonged to the Alphaproteobacteria class, although Bacteroidetes and Gammaproteobacteria sequences were also obtained. Three phylogenetic trees were made with all the sequences obtained from the libraries for the Bacteria Domain, one for the sequences related to Alphaproteobacteria, a second tree for the sequences related to the Bacteroidetes and Gammaproteobacteria groups and a third tree with the sequences related to photosynthetic organisms.

Gene library of *pufM*: Following the results of the concatenation of the 16S ribosomal DNA gene from the library for Cyanobacteria, a library of sample 6 was constructed from the mRNA of the *nifH* gene amplified by RT -PCR. More than 1000 copies were obtained in the library, of which 26 were analyzed by enzymatic digestion, obtaining 5 different restriction patterns. The concatenation was analyzed nucleotide BLAST, GenBank and the five orders were found to resemble the *nifH* gene of the cyanobacterium *Nostoc* sp. with percentages of similarities greater than 99%. Therefore, it can be concluded that cyanobacteria are expressing the *nifH* gene.

Fish of the mussel soft body: The soft body presented an autofluorescence in the red, orange and green, although to a lesser extent in the orange stripe, so *in situ* hybridization was performed with probes labelled with the Cy3 fluorophore. Autofluorescence at the soft body was so intense that it masked the signal from the probes. All the microorganisms associated with the mussel gave a high sign. with the Eub338 investigation to the Bacteria Domain and, besides, all of them hybridized with the Alf968 and Alflb probes for the Alphaproteobacteria class (Fig. 2g-l). No signal was obtained with the Arc915 probes for microorganisms from the Archaebacteria domain or with the probes for other phylogenetic groups in the Bacteria domain.

Isolation of secondary metabolites

Septamycin and erythromycin: The infrared spectroscopy of septamycin (Fig. 3) shows strong broadband at 3306 cm^{-1}, which indicates at least one NH or OH stretching vibration as well as two other bands at 2926 and 2852 cm^{-1}, which are characteristic of the CH stretching vibrations of alkyl groups. At 1460 and 1374 cm^{-1} there are bands for CH3 deformation vibrations. Further bands are at 1164 and 1100 cm^{-1}. Since the UV spectrum of septamycin shows no absorption, the presence of a chromophore can be excluded. The ^{1}H-NMR

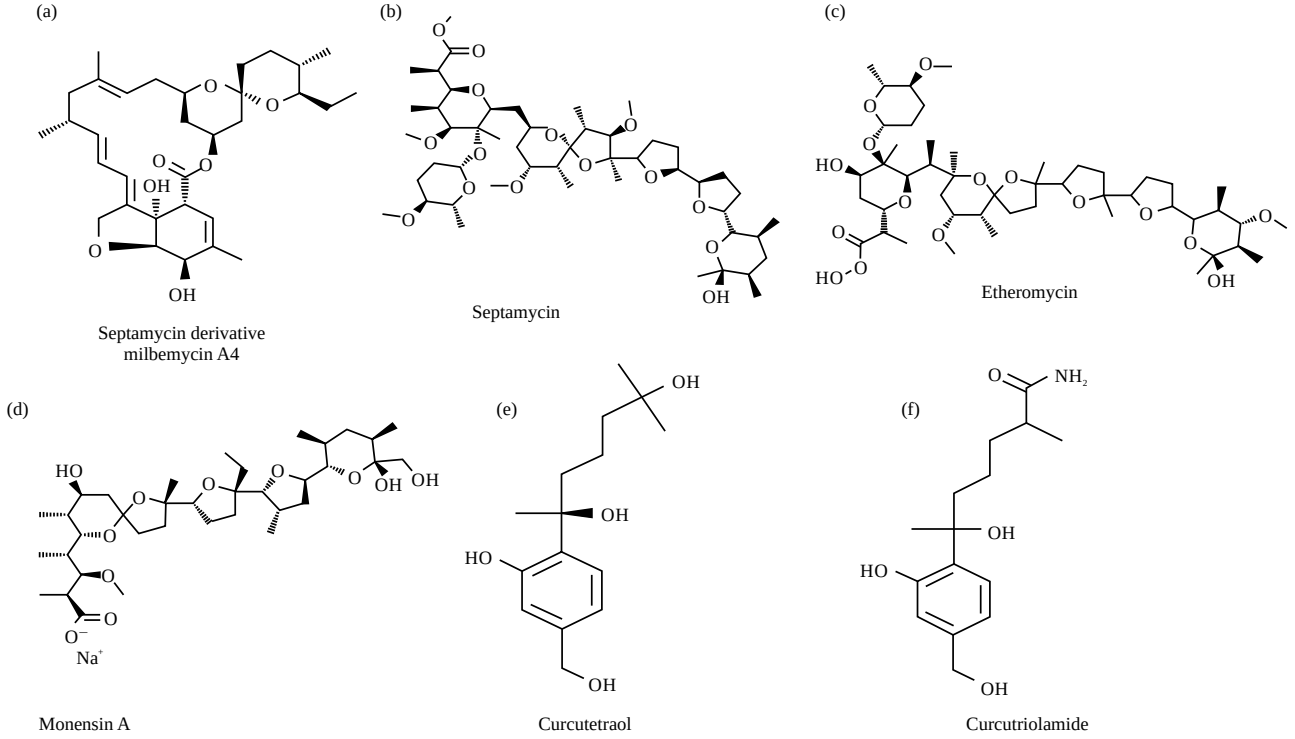

Fig. 3(a-f): Structure of the (a) Septamycin derivative Milbemycin A4, (b) Septamycin, (c) Erythromycin, (d) Monensin A, (e) curcutetraol and (f) Curcutriolamide

spectrum of septamycin shows four singlets at δ_H = 3.35, 3.41, 3.47 and 3.54, the integration of which corresponds to three protons. The down field shift indicates methyl groups that are each bound to heteroatoms.

A septamycin derivative: After RP-18 HPL chromatographic purification, a natural product septamycin derivative could be obtained in a yield of 0.1 mg. In the poor-quality infrared spectroscopy, it had a mass/charge number = 952.5. Another observed signal at mass/charge number = 966.5 suggests that mass/charge number = 952.6 is $[M+Na]^+$ and mass/charge number = 967.6 is $[M+K]^+$, since both signals are differ by 16 mass units. Thus, the undetected molecular ion $[M]^+$ would correspond to a mass of mass/charge number = 928. The ^{1}H-NMR spectrum (Fig. 3) of the isolated natural product septamycin derivative is similar to septamycin $C_{48}H_{82}O_{16}$. At δ_H = 3.20, in addition to δ_H = 3.54, 3.41, 3.35 and 3.32, an additional singlet can be detected whose integration corresponds to three protons and which could be another methoxy group. The shift at δ_H = 4.82 corresponds to a proton as does δ_H = 4.12, 4.05, 3.96, 3.77, 2.81, 2.62, 2.36 and 2.21. The doublet at δ_H = 1.10 corresponds to a methyl group. Other methyl groups are in the ranges δ_H = 0.96-1.00 and δ_H = 0.84-0.90. The interpretation of the ^{13}C-NMR spectrum turned out to be very difficult due to the small amount of isolated natural products. The ^{13}C signals were identified by evaluating the HSQC and HMBC spectrum.

Biosynthesis of monensin A: The polyether antibiotics are characterized by the presence of tetrahydrofuran and/or pyran rings and are descendants of polyketide biosynthesis. Initial biosynthetic studies using ^{13}C-labeled precursors and the subsequent degradation of the monensin A $C_{36}H_{61}NaO_{11}$ (Fig. 3) showed that the carbon structure is made up of five acetates, seven propionates and one butyrate molecule. After the basic structure has been formed, it is epoxidized three times with molecular oxygen. A cascade-like reaction, starting from the hydroxyl group in C-5-C-9, finally leads to the ring closures, which result in five ether rings.

Curcutetraol and curcutriolamide: After cleansing of the extract by column chromatography, two new natural substances, which are to be named curcutetraol and curcuttriolamide were identified (Fig. 3). The purification was carried out by HPL-chromatographic separation on RP-18 with the solvent mixture water/acetonitrile. The cleansing of fragment 12 was done by High-performance liquid chromatography on RP-2. 40 and 41 μm are brownish oils that

occur in different concentrations in the microorganism. The main compound is Curcutetraol $C_{15}H_{24}O_4$ with a yield of 14.8 mg. Curcutriolamide $C_{15}H_{23}NO_4$ could be isolated in an amount of 2.4 mg. 7.2 TLC and HPLC analysis. In thin-layer chromatograms, both compounds show absorption at 254 nm but no fluorescence at 366 nm.

DISCUSSION

Secondary metabolites of the mussel and its associated microbionts had high antitumor activity against tumour lines. The prokaryotic community associated with the mussel is Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes and Cyanobacteria. Greater diversity within the Alphaproteobacteria class was found in the soft body. The complex formation usually takes place by chelating cations via the lone pairs of electrons. Monensin A and septamycin prefer Na^+ ions, but monensin A with a higher affinity than septamycin.

Sessile marine invertebrates harbour symbionts like cyanobacteria, germs, Photoheterotrophs, Chemoheterotrophs. single-celled seaweeds and fungi[36,37]. These microorganisms inhabit the extra and intracellular spaces and sometimes constitute an important part of the host biomass as is the case of the *Aplysina aerophoba* in which 40% of its total biomass is made up of microorganisms[38]. The visualization of pelagic marine Archaebacteria of the phylum Crenarchaeota was achieved by using polyribonucleotide probes instead of oligonucleotide probes so that it was possible to increase the signal of the probe[39]. The benefits that these symbionts can provide to the sessile invertebrates are a nutritional improvement due to the incorporation of organic matter released in seawater, transport of metabolites, improved structural rigidity and chemical defence against dangerous agents[40,41]. The number of bacteria in the *Aplysina aerophoba* tissue and the extract of *Rhopaloeides odorabile* has been estimated (6.4×108 g^{-1} and 1.5×108 mL^{-1}, respectively). This interesting dichotomy in microbial fauna and sessile invertebrates associations has received little attention, possibly because most recent studies have focused on evolutionary aspects[42] and on species that produce pharmacologically active secondary metabolites[43]. In the natural environment, the secondary metabolites produced by microorganisms fulfill multiple functions related to their survival[44]. Associated microorganisms secrete secondary metabolites to repel hemocytes thus prevent digestion[45]. Numerous species of marine invertebrates are in symbiosis with the single-celled cyanobacterium *Prochloron didemni*, which is specifically associated with the epidermis and internal cavities, where it actively performs photosynthesis using phycobiliproteins and chlorophylls a and b[46]. The biological relationship between bacteria and marine invertebrates has obtained considerable interest as a source of natural[47]. Due to the similarity between compounds isolated from macro and microorganisms, it has been speculated that symbionts secrete their metabolites in marine invertebrates. Symbiotic microorganisms in marine invertebrates are rich in natural products[48]. The main reason for ascribing a microbial origin to a particular compound may be the structural similarity, partial or almost total, between the isolated compound and other compounds of microbial origin[49,50]. Some aspects that can be constituted as indications that the production occurs by the symbiotic microorganisms are: That the abundance of the microorganism in question is correlated with the amount of the isolated natural product, that the microorganism is found in the same portions or tissues of several hosts of the same species or several specimens collected in different or at least distant locations and when different species harbour the same metabolite. Although it is speculated that marine invertebrates contribute the secondary metabolism, it is still difficult to prove it[51]. The sesquiterpenes, herbadysiolide and isodysidenine were isolated from specimens of this cyanobacteria[52]. The analyzes of the extracts of the bacteria through HPLC and NMR showed that the cytotoxic tetraketide A was common in the single-celled microorganism, while the antifungal peptides was found in the Actinomycetes. Muyzer *et al.*[53] concluded that the cultivable heterotrophic bacterial community presented only 0.1% of the total microbial community of *Rhopaloides odorabile* and 0.15% of the total population of *Aplysina aerophoba* bacteria. Combinatorial biology is a strategy of random mixing of multiple genes from more than one species that produce metabolites, which has been generated by the development of vectors to transfer DNA in bacteria using cosmids to transfer 30-40 kb DNA. This allows the genetic information for 40-500 enzymes to be transported in a single mutation to a commercial vector.

CONCLUSION

The secondary metabolites of the mussel and its associated microbionts had high antitumor activity against tumour lines. Bacillary organisms are symbionts with *Modiolus auriculatus*. Alphaproteobacteria live in the different tissues of the mussel of the Gammaproteobacteria class, Bacteroidetes and algae of Rhodophyta. *Cenarchaeum symbiosum* from Archaebacteria Domain was associated. Secretion of secondary metabolites by the mussel is not influenced by

captivity. Septamycin, erythromycin, a septamycin derivative, monensin A, curcutetraol and curcutriolamide were isolated from the extract.

SIGNIFICANCE STATEMENT

This study discovers the possible role of the secondary metabolites of the mussel and its associated microbionts in biological defence. This study will help the researcher to uncover the critical area of defence strategies of marine invertebrates against predation and epibiosis based on the synthesis and release of cytotoxic and antimitotic materials. Thus, a new theory on these defence mechanisms and possibly other combinations, may be arrived at.

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