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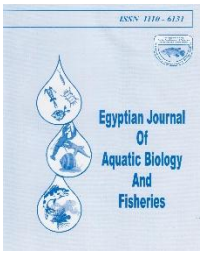

The impact of bacterial depuration on the freshwater bivalve, *Spathopsis rubens arcuata*

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ABSTRACT

Bacterial accumulation causes pathological alterations in shellfish tissues. Bivalve cleaning from bacteria is crucial for safe consumption. The present study aimed to evaluate the effect of bacterial depuration on the gill's histology and gill's apoptosis of freshwater bivalves, *Spathopsis rubens arcuata*. Depuration was applied for one, three, five, and seven days. Microbiological analyses of shellfishes and bacteria species were performed. Furthermore, histological investigations and apoptotic marker caspase-3 were examined in the gills of *S. rubens arcuata*. The results showed that three different bacterial strains; *Aeromona, Streptococcus* and *Bacillus* sp. were found in bivalve tissues. *Aeromonas* and *Streptococcus* were found three days post-depuration, however, on the fifth and seventh days post depuration, they were undetectable. The percentages of the apoptotic cells in gills decreased in a time-dependent manner during depuration. Gills and gill lamellae exhibited morphological deformations before depuration including degeneration of epithelial cells and connective tissues, haemocytes infiltration and necrosis. Nevertheless, seven days post-depuration, the gill lamellae restored their normal appearance as indicated by parallel gill filaments separated with interlamellar spaces. Collectively, depuration for seven days is crucial to clear bacterial contamination and restore the damaged gill cells into normal status.

INTRODUCTION

Bivalves feed by filtering large water volumes and accumulate food particles from their surrounding contaminated environment. Shellfish accumulate pathogenic bacteria during filter-feeding which in turn causes health risk upon consumption **(Jeamsripong *et al.*, 2018)**. Due to the utmost importance of bivalves as a natural food source, therefore, these shellfish must be clear from pathogens. The clearance of contaminants is mediated either by heating or by immersion in clean water tanks to allow depuration **(El-Gamal, 2011)**. The depuration is among the most preferable methodologies in managing risks associated with bivalve contamination **(El-Shenawy, 2004)**.

Gills are target organs for toxicants accumulation due to high water volumes that they filter **(Pal *et al.*, 1990; Au, 2004)**. Histological and histopathological investigations are useful in assessing contaminant-specific alterations in the tissues **(Sunila, 1987)**. In this context, the onset of apoptosis represents a valuable biomarker for the pathogen-host interaction. Apoptosis is considered one of the underlying cellular host defense mechanisms involved in the immune response in mollusks against viral and bacteria pathogens **(Kiss, 2010)**. The caspase-3 protein is a member of the cysteine-aspartic acid protease family that plays a pivotal role in the activation of apoptosis **(Ashida *et al.*, 2011)**. It is considered one of the execution enzymes in apoptosis and used as a marker for apoptotic cells **(Rudel, 1999)**.

Invertebrates are commonly susceptible to several pathogenic bacteria, including *Aeromona*s, *Flavobacterium* and *Pseudomonas* spp. **(Pękala-Safińska, 2018)**. *Aeromonas spp.* are common in river water, irrigation water, freshwater, brackish water, seawater, groundwater and drinking water **(Massa *et al.*, 2001; Soler *et al.*, 2002)**. The prevalence of *Aeromonas* contaminants in aquatic environments mainly causes human pathogenic properties such as septicemia, wound infections and diarrhea **(Hanninen and Siitonen, 1995)**. Therefore, their elimination is an important concern, to prevent the subsequent transmission to humans **(Praveen *et al.*, 2016)**. One of the most crucial bacterial groups related to contamination is *Streptococcus* spp. Streptococcal infections caused marked economic losses in the industrial aquaculture, their control mainly depends on antimicrobials and environmental strategies **(Toranzo *et al.*, 2005; Cheng *et al.*, 2010; Woo and Park, 2014)**. Another important pathogen in aquatic systems is *Bacillus spp*.

In Egypt, *Spathopsis rubens arcuata* is one of the benthic invertebrates that filter feeds and extracts microorganisms. **El-Khodary *et al.* (2018)** suggested that *S. rubens arcuata* can be a good source of food with high protein and low-fat contents. Nevertheless the impact of bacterial depuration on *S. rubens arcuata* remains elusive. Therefore, the current study aimed to distinguish the various types of bacterial contaminants on *S. rubens arcuata* gills and to evaluate the effect of depuration on the colonization, prevalence of bacteria. In addition the study extended to investigate the improvements of the histological architecture and caspase-3 activity in gills tissues during depuration.

MATERIALS AND METHODS

1. Samples collection

Spathopsis rubens arcuata (freshwater bivalves) were collected from Al-Mahmoudya irrigation canal at Damanhour, El-Beheira Governorate, Egypt. Dead or damaged specimens were eliminated. Specimens with shell sizes extended from 110 to 130 mm in length and 23 to 41 mm in width were utilized to run these investigations.

2. Depuration experiment

The depuration experiment was conducted within 4 hrs post shellfish collection.

Depuration of bacteria was applied for 1, 3, 5 and 7 days for each condition. Ten clams were placed in aquaria containing 10 l of dechlorinated tap water in three replicates under laboratory conditions with continuous aeration. Water was changed and supplanted with new dechlorinated tap water and the aquaria were cleaned every day to prevent contaminations.

3. Microbiological analyses of *S. rubens arcuata*

3.1. Total coliform count using colony count technique

Ten grams of bivalve tissues were weighed and transferred into a sterile stomacher bag containing 90 ml of sterile 1% buffered peptone water. The samples were homogenized in a stomacher followed by two-times dilutions. Then 1 ml of the homogenized extract was inoculated and distributed over a dry surface of sterile violet red bile (VRB) agar (OXOID) using a glass rod. After spreading a cover layer (tempered promptly to about 45°C) of VRB agar was poured over all plates and incubated at 37°C for 24 hrs. Visible purple colonies surrounded by a purple halo were counted. The results were calculated and recorded as total coliforms count per gram. Five colonies of each doubtful type were inoculated into tubes of lactose bile brilliant green broth (OXOID). The tubes were incubated at 30°C for 24 ± 2 hrs. Colonies that showed gas formation in the Durham tube were regarded as coliforms **(ISO, 2006)**.

3.2. Detection of *Aeromonas* spp.

Twenty-five grams of bivalve soft tissues were weighed and homogenized in a sterile stomacher bag containing 225 ml alkaline peptone water. These were then incubated at 37 ºC for 6 hrs. One ml of the pre-enrichment broth was transferred to 225 ml of tryptic soy broth (TSB) (Biolife) and incubated at 37 ºC for 24 hrs. After incubation, 0.1 ml was streaked over Aeromonas selective agar (OXOID) plates supplemented with (5 mg/L) and incubated at 37 ºC for 24 hrs. After that, the plates were examined, and the suspected colonies were stained by the Gram staining method. The Gram-negative, rod-shaped bacteria were selected for additional identification tests. Subsequent identification tests including citrate hydrolysis, motility test and catalase production were performed **(Ashiru *et al.*, 2011).**

3.3. Detection of *Streptococcus* spp.

Twenty-five gram of bivalve soft tissues was weighed and homogenized in a sterile stomacher bag containing 225 ml 1% buffered peptone water. Then 0.1 ml of the homogenized extract was streaked on brain heart infusion agar (OXOID) plate supplemented with 1.5% NaCl. The inoculated plates were incubated at 25 ºC for 24 hrs. Single colonies from plates with dense, virtually pure culture growth was red-streaked on the same media to obtain pure isolates. After that, the plates were examined, and the suspected colonies were stained by the Gram staining method. Typical pure colonies were analyzed for beta-hemolysis on 5% blood agar and colonies displaying beta-hemolysis. The Gram-positive, spherical cocci were selected for additional identification tests. Subsequent identification tests including motility test and catalase production were performed **(Baeck *et al.*, 2006).**

3.4. Detection of *Bacillus* spp.

Twenty-five gram of bivalve soft tissues was weighed and homogenized in a sterile stomacher bag containing 225 ml 1% buffered peptone water. Then 0.1 ml of the homogenized extract was inoculated in nutrient agar (OXOID) plates by streaking at 30 ºC for 24 hrs. After that, the plates were examined, and the suspected colonies were

stained by the Gram staining method. The Gram-positive, rod-shaped, spore-forming bacilli were selected for additional identification tests. Subsequent identification tests including citrate hydrolysis, motility test and catalase production were performed **(Amin *et al.*, 2015).**

4. Detection of caspase-3 by flow cytometry

The proapoptotic marker caspase-3 was detected in the gills of *S. rubens arcuata* after depuration for one, three, five, seven and 15 days. The fluorochrome is conjugated to the primary antibody (PE and FITC conjugate). Aliquot of 100 µl of cell suspension (1×10^4 cell/ml), 10 µl of the dilution antibody (FITC rabbit anti-active caspase-3, solid as, material No.559341, catalog No. 554714, from BD Pharmingen) were mixed well and incubated at room temperature for 30 min. Cells were washed with 2 ml of PBS/BSA, and then centrifuged at 1500 rpm for 5 min and the supernatants were discarded. The cells were re-suspended in 0.2 ml of PBS/BSA. The data were acquired by using FACS (flow activated cell sorter) Calibur Flow Cytometer (Becton Dickin-son, Sunnyvale, CA, USA) equipped with a compact air-cooled low power 15 mW Argon ion laser beam (488 nm) at Mansoura University Hospital.

5. Histological investigation

Gills were dissected out from 5 randomly selected individuals from each group. Samples were fixed in aqueous 10% formalin and processed for light microscopy. Serial sections were cut at 5 µm and stained with Mayer's and eosin. Slides were examined and photographed under an Olympus microscope (CX31) equipped with an image analyzing system.

6. Statistical analysis

Data were presented as mean ± SD and were analyzed using one–way analysis of variance (ANOVA) followed by Dunnet test. P values lower than 0.05 were considered significant. All statistical analysis was performed using SPSS 23.0 for Windows (IBM Corp. 2015).

RESULTS

1. Levels of coliform contamination and pathogenic bacterial incidence in freshwater bivalve

Freshwater bivalve exposed to depuration time ranged from one to seven days. The results showed that the levels of coliforms were 1.21×10^6, 5.8×10^5 and 1.2×10^2 CFU/ml following one, three, and five days of depuration respectively. Meanwhile, coliforms were not detected seven days post-depuration.

Furthermore, the levels of coliforms in water collected from the El-Mahmoudya canal was 3.52×10^6 CFU/ml, whereas the CFU in treated water was undetectable **(Fig. 1)**. The presence of pathogenic bacteria was also reported one to seven days post depuration. However, the increasing depuration time reduced the counts dramatically. The biochemical tests of suspected *Aeromonas*, *Streptococcus* and *Bacillus* isolates are present in **Table 1**.

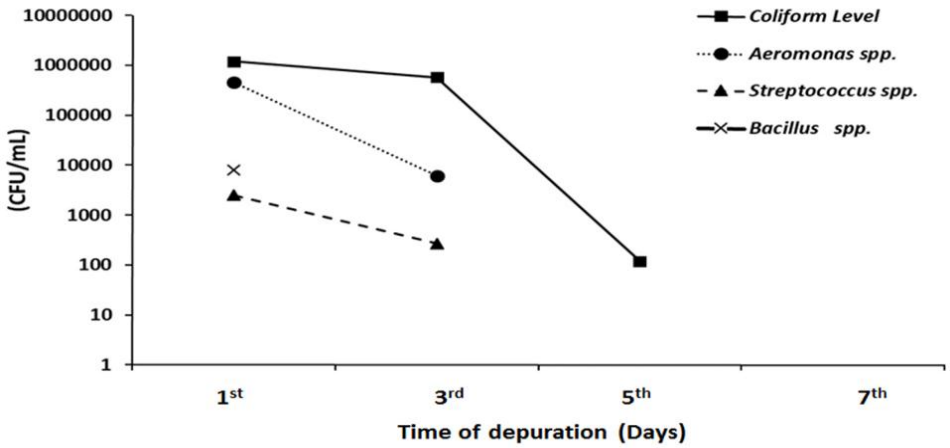

Fig. 1. Counts of pathogenic bacteria (log count/ml) in soft tissues of *S. rubens arcuata* during the depuration time.

Table 1. Biochemical tests of suspected *Aeromonas*, *Streptococcus* and *Bacillus* isolates.

Biochemical Reaction	*Aeromonas* spp.	*Streptococcus* spp.	*Bacillus* spp.
Gram stain	-	+	+
Motility	+	-	+
Catalase	+	-	+
Citrate	V	*	+

V: Variable *: Not tested

The results showed that there was a presence of *Aeromonas* spp., at day one and three post depuration at 4.5×10^5 and 6.2×10^3 CFU/ml, respectively. However, after five and seven days of depuration *Aeromonas* spp. were undetectable. *Aeromonas* spp. was detected at 1.6×10^5 CFU/ml from the water collected from El-Mahmoudya canal and not detected from water treated *in vitro*. *Streptococcus* spp. incidence in the freshwater bivalves was confirmed after one and three days of depuration at 2.5×10^3 and 2.7×10^2 CFU/ml, respectively and not detected after five and seven days. *Streptococcus* spp. was also detected at 5.4×10^3 CFU/ml from the water collected from El-Mahmoudya lake and not detected from water treated *in vitro*. *Bacillus* spp. was absent after three, five, and seven days of depuration and water treated *in vitro*.

2. Effects of depuration time on the gill cells apoptosis

The flow cytometry analyses of caspase-3 performed 1–7 days post gills depuration are present in **Figure 2**. The percentages of the apoptotic cells were decreased in a time-dependent manner; ranging from 41.1% to 27.9% at one and seven days post-depuration, respectively After 15 days of depuration, the percentages of apoptotic cells of gills of *S. rubens* was 25%.

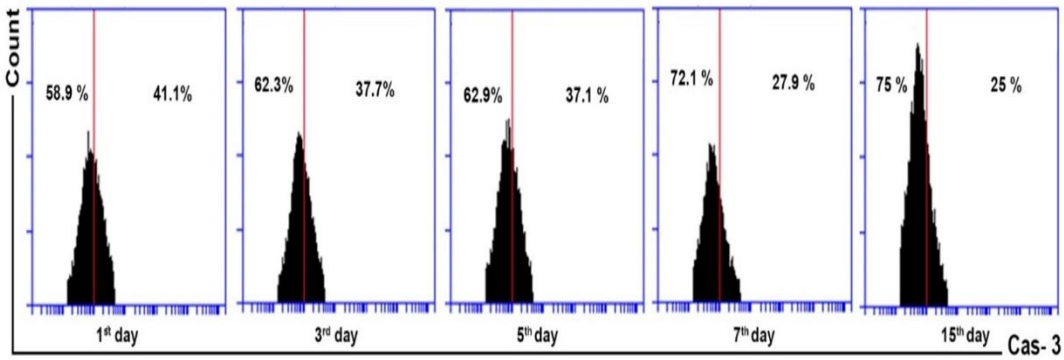

Fig. 2. Flow cytometry analysis of caspase-3 in gills cells harvested from shellfish bivalve *S. rubens arcuata* after 1, 3, 5 , 7 and 15 days post depuration.

3. Histological alterations of *S. rubens arcuata* gills post depuration time

According to light micrographs of *S. rubens arcuata* gill lamellae, there were morphological deformations before depuration including degeneration of epithelial cells and connective tissues, haemocytes infiltration and necrosis. After seven days of depuration, gill lamellae showed normal appearance consisting of parallel gill filaments which are equal in length and separated by interlamellar space. These gill filaments consist of ciliated columnar epithelial cells with well developed connective tissue **(Fig. 3)**.

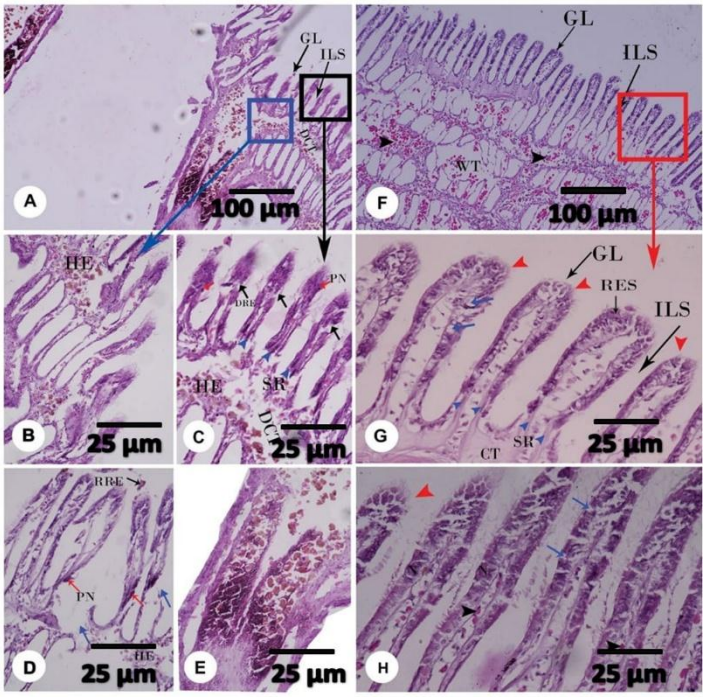

Fig. 3. Light micrographs of gills' and gills lamellaes' sections of *S. rubens arcuata* showing gill structure at the day of collection (A-E) and post 7 th day of depuration period (F-H). GL: Gill lamellae; ILS: Interlamellar space; WT: Water tube; DCT: Degenerated connective tissue; HE: Haemocytes; CT: Connective tissue; N: Necrosis; DGL: Degenerated gill lamellae; RGL: Ruptured gill lamellae.

DISCUSSION

Bivalve mollusks are susceptible to numerous infectious diseases because of the high exposure to bacteria and waterborne pathogens that can cause several human diseases **(Santos *et al.*, 2017)**. Bivalves feed by filtering large volumes of water and accumulating food particles from their surrounding polluted environment, which may lead to accumulating pathogenic bacteria during filter-feeding. This in turn causes health risk when bivalves are lightly cooked or consumed raw by humans. The results of the present study showed that the collected bivalves, *S. rubens arcuata* were infected with three different species of bacteria, namely *Aeromonas, Streptococcus* and *Bacillus* spp. This finding was in agreement with previous examinations which reported that most bacterial diseases of bivalves are caused by a large range of *Vibrio* species and *Aeromonas* as gram-negative bacteria **(Sugumar *et al.*, 1998)**. Consistent with our findings, the prevalence of *Aeromonas hydrophila* strains has been reported previously in sewage and wastewaters **(Varela *et al.*, 2016)**. Also *Aeromonas spp.* demonstrates a higher prevalence in intestinal samples and body surface of prawn **(Vivekanandhan *et al.*, 2005)**. Furthermore, *Aeromonas spp.* was isolated from the gills, stomach and ventral muscles of freshwater bivalves **(Zeaur and Aziz, 1994)**.

Depuration methodology is widely used to treat bivalve mollusks harvested from the polluted sites. It is effective for the removal of bacterial pathogens from the contaminated water. A recent study reported that the eight-day long depuration of *S. rubens arcuata* was an excellent strategy to reduce heavy metals to an acceptable level for human consumption **(El-Khodary *et al.*, 2018)**. The current study showed that depuration for seven days eliminated the bacterial infection from the gill tissues of the freshwater bivalves. This finding is in line with a previous study that reported that the depuration effect on *Aeromonas salmonicida* transmission between the freshwater bivalves **(Starliper, 2001)**. A further study by **Othman *et al.* (2015)** demonstrated the biological control of streptococcal infection in filter-feeding bivalve mussel. The results of **Van Slooten and Tarradella (1994)** and **El-Gamal (2011)** also confirmed the present data where the bacterial contamination is usually reduced by 85% after 4 days of depuration,

The current study shows that the bacterial contamination leads to some pathological alterations in the gills of the freshwater bivalves *S. rubens arcuata*. This finding is in line with earlier reports that investigated the gills of the polluted bivalves **(Al-Hashem, 2017 and Ben Cheikh *et al.*, 2017)**. Bacterial infection can induce host cell death through several distinct modalities including necrosis and apoptosis that are critical defense mechanisms of bivalves against microbial infection **(Ashida *et al.*, 2011)**. **Chang *et al.* (2008)** reported an increase of caspase-3 activity expression in crustaceans *Litopenaeus vannamei* after *Vibrio alginlolyticus* infection. Depuration for seven days decreased the level of caspase-3 in the gill cells suggesting a reduction in the percentage of the apoptotic cells. Moreover, no substantial differences were found in the percentage of the apoptotic cells after 15 days of depuration. The micrographs of gills after seven days of depuration reveal restoration of the normal morphological characteristics. This could be due to the elimination of the bacterial species by the depuration process as suggested by **Guzmán-Guillén *et al.* (2015)**. In this context, the animals are able to reprogram their cell response once transferred to an unpolluted site, thus achieving a recovery of the

normal status and elimination of the toxins. **Alvarez-Muñoz *et al.* (2009)** found a decrease in all the histopathological responses in gills of fish exposed to a surfactant after being depurated for three days.

Guzmán-Guillén *et al*. (2017) also reported that the effectiveness of short depuration periods (three or seven days) on the recovery of different organs of tilapia (*Oreochromis niloticus*) following the cylindrospermopsin-induced damage. Surprisingly, the gills achieved a recovery and revert to the normal structure after three days of depuration. In contrast, **Stara *et al*. (2020)** detected a slight recovery of histological conditions of *Mytilus galloprovincialis* after ten days in uncontaminated seawater.

CONCLUSION

Filter-feeding bivalves, *S. rubens arcuate* collected from El-Mahmoudya canal were infected with three different kinds of bacterial species. Depuration for seven days eliminated the bacterial infection, decreased the gill cell apoptosis and mitigated the histopathological alternations. Finally, these results validate the depuration process as an effective practice in freshwater bivalves contaminated with bacteria.

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ARABIC SUMMARY

تأثير التطهير البكتيري على محارالماء العذب (*Spathopsis rubens arcuata*)

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يؤدي التراكم البكتيري في الرخويات الصدفية إلى حدوث تغيرات مرضية في أنسجتها. حيث أن التطهير للمحاريات ذات المصراعين من البكتيريا أمر ضروري للاستهلاك الآمن. ولذلك تهدف الدراسة الحالية إلى تقييم تأثيرعملية التنقية على إزالة البكتريا من نسيج وخلايا خياشيم محار الماء العذب *Spathopsis rubens arcuata*. حيث تم التطهير لمدة ١و ٣ و ٥ و ٧ أيام. وقد تم تقييم أنواع البكتريا وعددها في المحار. علاوة على ذلك، تم فحص التحاليل النسيجية ونشاط كاسبيز-٣ في *ثباثوبسس روبنز أركيوتا*.وقد أظهرت النتائج وجود ثلاثة سلالات بكتيرية مختلفة هي *Aeromona* و *Streptococcus* و *Bacillus* sp. حيث تم العثور على *Aeromonas* و *Streptococcus* حتى اليوم الثالث من عملية التطهير، ولكن في اليوم الخامس والسابع لم يتم العثورعليهم. وقد لوحظ انخفاض النسب المئوية للخلايا الميتة في الخياشيم معتمدة علي زمن التعرض لعملية التطهير. كما وجدت تشوهات مورفولوجية حادة في الخياشيم قبل عملية التطهير بما في ذلك انحلال الخلايا الطلائية والأنسجة الضامة وتخثر خلايا الدم ونخرها. بينما، بعد مرور ٧ أيام من عملية التطهير، استعادت صفائح الخياشيم مظهرها الطبيعي الذي يتكون من خيوط الخياشيم المتوازية مفصولة بمسافات بين خيشومية واضحة. ولذلك تعتبرعملية التطهير لمدة ٧ أيام عملية مهمة لإزالة التلوث الجرثومي البكتيرى واستعادة خلايا الخياشيم التالفة بنيتها الطبيعية.