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Develop a depuration system to control bacteria colonized in Asian clam, *Corbicula fluminea* tissue

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Abstract: In the present study, an Asian clam, *Corbicula fluminea*, depuration system was developed in order to purify and enhance the quality of Asian clam in term of bacterial coliform exposure. Bacterial analysis on Asian clam revealed that the level of bacterial coliform contamination in their tissue is high and proven to be non-suitable for human consumption in general. Therefore, a depuration system was developed in the present study to improve the quality of Asian clam. The depuration system is comprised of a glass aquarium equipped with a sponge filter system and water pump. In the depuration system, there was one container possessed Asian clam (n = 100) used for bacterial analysis on a weekly basis for 4 consecutive weeks. The container was filled with 10 cm thick sand as the substrate. There were 2 treatments namely the control treatment, and fermented soy pulp (FSP) treatment; where 1 % FSP was mixed homogenously with the sand to serve as feed for the clams. Each treatment was made in triplicates. Total bacteria monitoring was carried out by using plate count method. The results of the experiment showed that the depuration system in the present study was able in enhancing the quality of Asian clam from Class B to Class A within 2 weeks period. The findings of the present study were the first to be reported in the literature concerning the development of Asian clam depuration system in enhancing the clam quality to meet the standard for human consumption.

Keywords: depuration system; bacteria; Asian clam; *Corbicula fluminea*

Introduction

Asian clam, *Corbicula fluminea*, is well known rich nutrient shellfish where it was consumed widely in Asia. In Malaysia especially in Kelantan, Asian clam was processed to be smoked Asian clam and served as snack. Shellfish is an important source of seafood throughout the world. The global production of mollusks is twice to that of crustacean. However, as shellfish is widely utilized around the world, there are also risks carried from the consumption regardless of the origin; whether they are harvested in the wild, cultured commercially, or imported from foreign countries. In Malaysia, the regulation specifying the human health safety status for the production and marketing live mollusks is still absent. Local harvesters and traders are free to sell the mollusks without any concern on the safety level of their products. However in European Union (EU), a regulation was introduced where all mollusks industry players need to comply. In the regulation, shellfish or mollusks safety standard is categorized into 4 classes namely Class A, B, C, and Prohibited (Tab. 1). Shellfish products should meet the standard Class A before they could be marketed, as the product is recognized as safe for human consumption. Shellfish products under the Class B and C should undergo depuration procedure before they could be marketed whereas shellfishes under the Class Prohibited are denied completely from being harvested to be used for human consumption. Consuming contaminated shellfish may lead to bacterial and viral infection in the right circumstances, and of which could be deadly for the consumer. Gastroenteritis infection associated with shellfish was extensively reported in the literature (Potasman *et al*., 2002). In Japan, Saitoh *et al*. (2007) reported a large number of gastroenteritis infections cases due to the consumption of Asian clam derived from the freshwater river of Japan. In Malaysia, *Edwardsiella tarda*; a gastroenteritis causative agent, was detected in fresh Asian clam found in Kelantan waters (Lee *et al*., 2013). Therefore, it is necessary to take precautionary steps in order to avoid gastroenteritis infection due to the consumption of Asian clam. Hence, in the present study, an experiment were carried out to reduce total bacteria colonized in Asian clam by using a developed depuration system in order to produce high quality Asian clams for human consumption.

Material and Methods

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Tab. 1: Shellfish Safety Classes for Human consumption (EU standard).

Class	Description	Suggestion
A	≤ 230 *Escherichia coli* / 100g flesh	Safe for consumption
B	≤ 4600 *Escherichia coli* / 100g flesh	Need to undergo purification to meet Class A standard
C	≤ 46000 *Escherichia coli* / 100g flesh	Relaying for at least 2 months
Prohibited	> 46000 *Escherichia coli* / 100g flesh	Harvesting is not allowed for human uses

(Ronald *et al*., 2008; Food Standards Agency Scotland, 2009)

Preparation of Asian clam tissue sample

In the study, Asian clam tissue sample were prepared at Week 0 (initial week), Week 1, Week 2, Week 3, and Week 4. The Asian clam tissue sample preparation was done as described by the study of Ruhil *et al*. (2008). A total of 20 pieces of Asian clam were sampled from the depuration system. The flesh of the clam was removed from its shell using a micro spatula. 10 g of experimental Asian clam flesh were first diluted in 90 ml physiological saline solution. The sample were then homogenized by using Stomacher (Seward, UK). The homogenate samples were subjected to a 10 fold serial dilution using physiological saline solution. 100 µl of sample were pipetted and spread onto selective medium eosin methylene blue (EMB) agar (Merck, Germany) for *Escherichia coli* followed by 24-48 h incubation at 30 ºC in triplicates. After incubation, the total bacterial concentration were calculated for the plates possessing the bacterial colonies ranging from 25-250 single colonies.

Most probable number (MPN) of bacterial coliform analysis

In the most probable number (MPN) analysis, a series of tests namely presumptive test, confirmation test, completion test, and bacterial identification were carried out as described by Ruhil *et al*. (2008). The tests are as follows:

Presumptive test

In the presumptive test, 3 sets of 5 lactose broth (Himedia, India) possessing Durham tube were used for each Asian clam tissue sample obtained at Week 0 (initial week), Week 1, 2, 3 and 4. The sample aliquot with the total volume of 10 ml, 1 ml, and 0.1 ml were inoculated into the 3 sets of lactose broth followed by 24 to 48h incubation at 37 ºC. The presence of bubble in the Durham tube was interpreted as positive and vice versa. The results of the presumptive test were referred to the MPN Determination Chart (Benson, 2002) to calculate the result in unit of MPN index per gram of flash.

Confirmation test

The positive tubes with bubble formation within the Durham tube were used for further confirmation test. The aliquot from the tube were streaked on EMB agar and incubated for 24 to 48 h at 37 ºC. Bacterial colonies growth on EMB agar; noted with the appearance of metallic green sheen were interpreted as positive for the presence of *Escherichia coli* in the sample. The bacterial colonies were then kept for the completion test.

Completion test

The bacterial isolates from the confirmation test were inoculated into lactose broth carrying Durham tube followed by 24 h incubation at 37 ºC. The results were interpreted as positive if 10 % of gas bubble formation was present. The bacteria were then re-isolated for identification process.

Bacterial identification

The bacteria isolated from the completion test were further identified by using biochemical tests such as Gram staining and Indole Production tests. Bacteria showing positive in Indole production test and Gram negative were further determined using commercial identification kit (BBL Crystal, USA). Based on the results of biochemical test and commercial identification kit interpretation, all bacteria (n = 50) were identified as *E. coli.*

Statistical analysis

The data of the total weight bacteria in the Asian clam of the control treatment and FSP treatment were analyzed by using One Way ANOVA followed by analysis using with $p < 0.05$. The statistical analysis was conducted using SPSS software version 23.

Results

Data obtained in the present study was analysed and calculated with the unit of MPN / 100 g of Asian clam flesh. The series of bacterial analysis performed has shown that the sampled Asian clam was initially within Class B according to the Shellfish Safety Classes for Human Uses (EU standard) (Tab. 1). The most probable number (MPN) index of the sampled clam

before the depuration treatment was more than 1600 MPN / 100 g of Asian clam flesh (Tab. 2).

Tab. 2: Most Probable Number (MPN) index per 100 g of Asian clam flesh.

Week	CONTROL MPN Index / 100 g of Asian clam flesh	FSP MPN Index / 100 g of Asian clam flesh
0	> 1600[a]	> 1600[a]
1	825.0 ± 49.5[b]	825.0 ± 106.1[b]
2	170.0 ± 42.4[c]	170.0 ± 14.1[c]
3	12.0 ± 1.4[d]	15.0 ± 2.8[c]
4	6.0 ± 2.8[d]	5.0 ± 2.8[c]

*Control treatment results were significantly different and based on Tukey's Post Hoc Test, the results can be divided into 4 groups.
*Fermented Soy Pulp (FSP) treatment results were significantly different and based on Tukey's Post Hoc Test, the results can be divided into 3 groups.

Resulted MPN/ 100 g of Asian clam flesh from the control treatment group had significantly improved and based on Tukey's Post Hoc Test, the resulting data can be categorized into 4 groups namely group A, B, C, and D. Week 0 is categorized under group A, Week 1 under group B, and Week 2 under group C, whereas Week 3 and 4 were categorized under group D. On the other hand, FSP treatment group yielded results of significant difference as well and based on Tukey's Post Hoc Test, the resulting data can be categorized under 3 groups namely group A, B, and C. Week 0 is categorized under group A, Week 1 under group B, and Week 2, 3, and 4 were categorized under group C.

The developed depuration system in this study was found to be able to purify the experimental Asian clams within 2 weeks; in which the quality of the clam had improved from Class B to Class A as specified by the Shellfish Safety Classes for Human Uses (EU standard). Based on the series of presumptive, confirmation, completed, and bacterial identification tests using biochemical tests and commercial kit, it was revealed that the isolated bacteria was identified as *E. coli* (Tab. 3).

Tab. 3: Bacterial coliform analysis from Asian clam tissue sample from the developed depuration system.

Test	Observation
Presumptive test	Refer to Tab. 2
Confirmation test	Green metallic sheen colonies were present on eosine methylene blue (EMB) agar plate.
Completion test	Gas production was detected
Bacterial identification	*E. coli* was successfully identified

Discussion

There are a few existing studies that have reported on the prevalence of bacteria in Asian clam tissue. For instance, Lee *et al*. (2013) has revealed the antibiogram, heavy metal resistance profile, and genetic diversity of *Edwardsiella tarda* colonizing the Asian clam meat. However, the study did not carry out the research on the MPN index of Asian clam. Hence, the present study is the first investigation on the MPN index of Asian clam in the literature along with the effort to determine the safety class of Asian clam sold in the market of Kelantan, Malaysia.

All shellfishes should be in the Class A category before being sold to the end users regardless of the source; whether they are harvested in the wild, cultured commercially, or imported from foreign countries (WHO, 2010). Based on the findings of the present study, all of the sampled Asian clam tissue were initially categorized under class B according to the Shellfish Safety Classes for Human Uses (EU standard); and considered to be unsafe for human consumption. This means that the risk of *E. coli* infection is high for the consumer after ingesting ill-prepared Asian clam. Therefore, it has to be a critical requirement in ensuring that the Asian clams meant for human consumption has been subjected to depuration process before it can be marketed (Lee *et al*, 2012).

In the European countries, a bill has been passed where it is required by law that shellfishes only under the Class A category could be marketed (Food Standards Agency Scotland, 2009). Traders and harvesters are required to officially declare and present proofs to the relevant governing body that their shellfish product is under the Class A category. In Malaysia, depuration process are being used for two types of bivalve namely *Crassostrea iredalei* (slipper cupped oyster) and *Crassostrea belcheri* (lugubrious cupped oyster) by using water recirculating system equipped with a ultraviolet (UV) light treatment for seawater disinfection (Ronald *et al*., 2008). Hence, we strongly suggest the usage of depuration system to monitor and enhance the safety level of Asian clam throughout the country, as the findings of the present study has evidently proved that local Asian clam was categorized under Class B; where they should have underwent depuration process before being sold at the market.

Based on the findings of the present study, it was shown that the developed depuration system took about one week to enhance the clam safety category from Class B to Class A. The developed system was observed to require a longer time in purifying the experimental clams compared to the studies on other species of bivalves. For instance, Ming *et al*. (2018)

reported that the developed depuration system for their study could effectively purify oysters from *Vibrio parahaemolyticus* in 5 days. Another study that has also reported shorter period of purifying shellfishes is performed by Barile *et al*. (2009); where it was claimed that the depuration system developed for the study was able to purify bivalve mollusks (*Chamelea gallina* and *Mitylus galloprovincialis*) from *Salmonella typhimurium* and *V. parahaemolyticus* in merely 72 to 84 h and 36 to 48 h, respectively.

Lewis *et al*. (2010) reported that the Atlantic oyster (*Crassostrea virginica*) depuration system was found to be capable in enhancing the quality of oyster from Class C to Class A within 6 days. Furthermore, the depuration system for wedge clam (*Donax trunculus*) and carpet shell (*Tapes decussatus*) in the study of Colakolu *et al*. (2014) was able to reduce 40 % of bacterial load in both clams within a 12 h period. In order to enhance the quality of shellfish in such a short period, there were suggestions that a depuration system must be equipped with UV light treatment (Correa *et al*., 2012), lower stocking density (Sorio and Peralta, 2017), while increasing the water flow rate (Sorio and Peralta, 2017; Ming *et al*., 2018).

Based on the biology and anatomy of all bivalves including Asian clam, it is apparent that shellfishes are a natural biological filter or biofilter; where they could filter out fine particle materials from their environment even as miniscule as bacteria. Ergo, numerous and various number of bacterial species could accumulate in the bivalve tissue; in which the accumulated bacterial cells will also be used as feed in addition to other organic matter. In European countries, although Asian clam is designated as one of the worst invasive aquatic species, studies have however shown that the *Corbicula fluminea* is indeed an excellent and effective bacterial biofilter, where it was proved that this species could remove *E. coli* in waste water effectively (Gomes *et al*., 2018).

Conclusion

The depuration system developed for the present study took about a week to enhance the clam safety quality; where it is comparatively slower than the studies on other bivalves from the literature. Hence for future works, it is highly suggested that the UV light treatment, stocking density of shellfish, and increment of water flow rate would be the prime aspect to consider in developing a commercially viable depuration system in order to improve the effectiveness of the current set up.

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