Microbiology safety of green mussel, *Perna viridis* after treated with boiling and *sous vide*

A S Samsudin and N U Karim

1Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia
2Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.

*Corresponding Author: ulfah@umt.edu.my*

**Abstract.** All samples (treated samples and controls) were subjected to total bacteria, total coliform, *Enterobacteriaceae* sp., *Pseudomonas* sp. and yeast and mould count at interval of 5 day during 20 days of chilled storage. Boiling and *sous vide* were significantly (*p*<0.05) reduced the total bacteria count in green mussel compared to the controls after 20 days of chilled storage. Interestingly, green mussel were free from pathogenic bacteria after treated with *sous vide* as the total coliform, *Enterobacteriaceae* sp and *Pseudomonas* sp were not observed in those samples. In addition, the yeast and mould count were found at amount of 3.23±0.14 log$_{10}$ cfuml$^{-1}$ in green mussel treated with *sous vide* which is lower than in controls (4.10±0.21 log$_{10}$ cfuml$^{-1}$). The shelf life estimation showed that green mussel after treated with *sous vide* had a prolong up to 32 days compared to boiling (~15 days) and controls (~10 days) in chilled storage. Therefore, *sous vide* treatment were effectively in prevent the risk of contamination in green mussel during storage compared to boiling treatment. These emerging preservation technique may widely introduce to the consumer in ensuring the safety of the products.

1. Introduction

Green mussel, *Perna viridis* is the most important aquaculture species in Southeast Asian countries [1,2] and its aquaculture production increases 43% (~650,000 tonnes) over the past two decades [3]. The consumption of bivalve shellfish in Malaysia has increased gradually due to the response of their high availability from wild and cultured conditions. Bivalves are tender, easily digested, minimally processed and additive-free, which make them preference to the consumers [4,5]. In addition, consumption of bivalve shellfish provides an inexpensive source of protein with high biological value and low calorie count, vitamins and essential minerals [6] such as calcium, magnesium, zinc, iron and copper which can combat free-radical induced disorders in the human body [7].

Green mussels are usually cultivated in sheltered waters and intertidal zone which are vulnerable to microbial contamination. Previously, [8,9] stated that marine bacteria such as *Aeromonas* sp., *Shewanella* sp. and *Vibrio* sp. from contaminated water have high tendency to be concentrated within somatic tissues of mussel. Besides that, [10] reported that bacteria such as *Photobacterium* spp, *Vibrio vulnificus* and *Shewanella* spp. were presented in *P. viridis*. The microbial growth and their metabolic activity may leads to the accumulations of amines, sulphides, ketones, aldehydes, alcohols, and...
organic acids which result in unacceptable and unpleasant off-flavors [11]. Chenoll et al. [12] added spoilage also subsequent to sour odours and taste, green discoloration, pack swelling and slime formation of the products.

The risk of bacterial contamination may occur due to the unhygienic handling techniques as well as the tolerance of microorganism to the preservation conditions. Thermal or heat processing is one of the most important technique to maintain the quality of food product. However, excessive heat during processing leads to the detrimental effect which can eliminate all the nutritional and useful properties of the food. Therefore, it is essential to establish the optimum condition of thermal process to guarantee the safety of the food products without causing loss the nutritional value and sensorial qualities of the food.

Boiling involves immersion of food in water that has been heated at boiling point of 100°C. It can maintain the quality of food from spoilage and pathogenic bacteria. Mussel usually boiled until the shell were opened and the colour of mussel change from grey to orange. Sous vide is define as `under vacuum’ which refers to precise slow-cooking of vacuum-packaged food in water held at an appropriate temperature [13]. Sous vide is a combination of barrier technologies which are vacuum packing and cold shock (after thermal processing), which the products are quickly cooled and stored at temperature below 4°C for a few hours or days, based on the nature of the product [14]. A study on the microbiology safety of P. viridis after undergo two different treatments which are boiling (at 100°C for 8 min) and sous vide (at 85°C for 10 min) had been carried out. The shelf life estimation of green mussel from both preservation techniques also had been determined.

2. Material and methods

2.1. Samples collection and preparation

Green mussels with commercial size (average 25-28 mussel per kg) were obtained (30 kg) from a local farm in Johor Bahru, Johor, Malaysia. All samples were transported alive to hatchery and undergo depuration for 24 hours. For depuration process, a tank which is filled by treated freshwater and suitable aeration was used to place the mussels. After 24 hours, the mussels were divided randomly into two different experimental treatments. The mussel were cleaned and undergo two different treatments (Table 1).

| Table 1. Two treatments were subjected to green mussel, Perna viridis. |
|---------------------------------------------------------------|
| Treatment             | Description                                                                 |
| Sous vide Cook Chill | Samples were undergo sous vide treatment according to method by [15]. Ziplock bag was used to pack 570g of mussels. Then, it was undergo sous vide treatment (85°C for 10 min in core) using sous vide water oven (Sous Vide Supreme Demi, China). The ziplock bags were chilled immediately at 5°C using blast chiller (Irinox Blast Freezer, USA). |
| Boiling               | Stainless steel pot was used to put the mussels. The mussels were boiled with rapidly boiling water with temperature of 100°C for 8 minutes [16]. Five mussels were added to rapidly boiling water to preserve the temperature of the water upon the addition of cold mussels. |

2.2. Microbiology analysis

Microbiology analysis were carried out using method by [17]. 10±0.1 g of green mussel flesh were aseptically transferred to a sterile stomacher bag. All samples were homogenized in 90 ml of Maximum Recovery Diluent (MRD) (MERCK, Germany) by using homogenizer (Interscience, France). Serial dilution of mussel homogenate were prepared from 10⁻¹ dilution by pipette 1 ml homogenized sample into the 9 ml of MRD. The appropriate dilution were spread accordingly on the
selective agar at instructed incubation hours. Total bacteria count were determined on plate count agar after incubated at 30°C after 48 hours. For total coliform determination, the samples were spread plate on MacConkey agar before incubated at 35°C for 18-24 hours. Meanwhile, Enterobacteriaceae and Pseudomonas sp. count were determined using violet red bile glucose agar and cetrimide agar, respectively after incubated at 35°C for 24 hours. Yeast and mould count were determined on Rose Bengal agar base after incubated at 25°C in the dark for 5 days [18]. All analysis were done in triplicate. Microbiology counts were expressed as log colony forming units per gram of samples (log_{10}CFU g^{-1}). Microbiology analysis were carried out at day 0, 5, 10, 15 and 20 of chilled storage.

2.3. Statistical analysis
The software package IBM SPSS Statistics software (Version 20) was used for statistical analysis. The data were analysed using one way analysis of variance (ANOVA) and the mean value were compared by Tukey’s multiple range test. P values less than 0.05 were considered statistically significant. The results were reported as mean ± standard deviation. The estimation shelf life of all samples was fitted as the response curve to all microbiology analysis. The microbial shelf life was taken as the time to reach 7 log_{10}CFU g^{-1} (for total bacteria) and 2 log_{10}CFU g^{-1} (for pathogenic bacteria) and 3 log_{10}CFU g^{-1} (for yeast and moulds) [19].

3. Result and discussion
Initially, total bacteria count of control samples showed the highest amount (5.12±0.30 log_{10}CFU g^{-1}) compared to other treatments (p<0.05) (Table 2). Sous vide treatment started affect the total bacteria accumulation on day 5 of storage (1.59±0.55 log_{10}CFU g^{-1}) and remained significantly lower (p<0.05) compared with other treatments during 20 days of chilled storage. Meanwhile, total coliform count were recorded at value of 4.64±0.03 log_{10}CFU g^{-1} in controls on day 0 of storage. The value of total coliform in controls and boiling samples were significantly (p<0.05) increased with prolonging storage days (Table 3). Sous vide treatment were effectively eliminate the coliform growth in green mussel from day 0 until day 20 of storage.

Table 2. Total bacteria count (log_{10}CFU g log_{10}CFU g^{-1}) for each treatment at 5°C storage from day 0 until day 20

| Storage (day) | Control | Total bacteria count (log_{10}CFU g log_{10}CFU g^{-1}) |
|--------------|---------|--------------------------------------------------------|
|              | Total   | Sous vide | Boiling |
| 0            | 5.12±0.30 a,A | ND a,B | 0.67 a,B |
| 5            | 6.88±0.09 b,a | 1.59±0.55 ab,B | 5.09±0.27b,A |
| 10           | 6.89±0.92 b,a | 3.12±0.26 bc,B | 6.34±0.14b,A |
| 15           | 8.49±0.09 b,a | 2.69±0.39 bc,B | 3.43±0.21 ab,B |
| 20           | 7.82±0.09 bc,a | 4.01±0.37 c,B | 4.16±0.28 ab,B |

Different superscript (a,b,c) in the same column indicate significant difference (P<0.05) between the storage days. Different superscript (A, B) in the same row indicate significant difference (P<0.05) between treatment (controls, sous vide, boiling). ND indicates no bacteria growth (colonies) detected.

Table 3. Total coliform count (log_{10}CFU g^{-1}) for each treatment at 5°C storage from day 0 until day 20

| Storage (day) | Control | Total coliform count (log_{10}CFU g log_{10}CFU g^{-1}) |
|--------------|---------|--------------------------------------------------------|
|              | Total   | Sous vide | Boiling |
| 0            | 4.64±0.03 a,a | ND B | ND a,B |
| 5            | 5.82±0.20 b,a | ND B | 4.86±0.24 b,c |
| 10           | 6.42±0.22 c,a | ND B | 5.86±0.26 b,c |
| 15           | 6.30±0.36 b,c,a | ND B | 6.86±0.49 ab,AB |
Different superscript (a,b,c) in the same column indicate significant difference (P<0.05) between the storage days. Different superscript (A, B, C) in the same row indicate significant difference (P<0.05) between treatment (controls, sous vide, boiling). ND indicates no bacteria growth (colonies) detected.

At initial of storage day, Enterobacteriacea sp. count in controls were recorded at 3.22±0.13 \( \log_{10} \text{CFU g}^{-1} \) and significantly increased (p<0.05) to 5.61±0.39 \( \log_{10} \text{CFU g}^{-1} \) after 20 days of storage (Table 4). Meanwhile, Enterobacteriacea sp. count in boiled samples were recorded at value of 4.71±0.27 \( \log_{10} \text{CFU g}^{-1} \) on 5th day of storage. On day 15 of storage, the Enterobacteriacea sp. count showed significant different between controls and boiled treatment. Apart from that, no bacteria growth detected in sous vide green mussel throughout 20 days of storage.

The Pseudomonas sp. count in controls and boiling treatment showed no significant (p>0.05) different from day 0 until day 20 of storage (Table 5). On day 10 of storage, Pseudomonas sp. count in boiled green mussel showed a value of 1.77±0.49 \( \log_{10} \text{CFU g}^{-1} \) which significantly lower than (p<0.05) the value in controls (5.16±0.41 \( \log_{10} \text{CFU g}^{-1} \)). On last day of storage, Pseudomonas sp. count in controls recorded 5.60±0.09 \( \log_{10} \text{CFU g}^{-1} \) which significantly higher compared to boiled green mussel (2.10±0.17 \( \log_{10} \text{CFU g}^{-1} \)). Besides that, sous vide treatment did not affect the Pseudomonas sp. growth in green mussel from day 0 until day 20 of storage.

The yeast and mould count in boiling green mussel were higher than control green mussel but not significantly difference (p>0.05) with the value 2.42±0.89 \( \log_{10} \text{CFU g}^{-1} \) and 1.13 \( \log_{10} \text{CFU g}^{-1} \) respectively (Table 6). Yeast and mould count in controls showed no significant (p>0.05) different from day 0 until day 20 of storage. Sous vide treatment successfully eliminated the yeast and mould growth until day 10 of storage. On day 10 of storage, yeast and mould count in sous vide green mussel recorded the value 2.67±0.41 \( \log_{10} \text{CFU g}^{-1} \) but remained significantly (p<0.05) lower compared to other treatments value.

### Table 4. Enterobacteriacea sp. count (\( \log_{10} \text{CFU g}^{-1} \)) for each treatment at 5°C storage from day 0 until day 20

| Storage (day) | Control \( \log_{10} \text{CFU g}^{-1} \) | Sous vide \( \log_{10} \text{CFU g}^{-1} \) | Boiling \( \log_{10} \text{CFU g}^{-1} \) |
|--------------|---------------------------------|-------------------|-------------------|
| 0            | 3.22±0.13\(^{a,A}\)            | ND\(^{B}\)        | ND\(^{a,B}\)      |
| 5            | 4.95±0.05\(^{b,c,A}\)         | ND\(^{B}\)        | 4.71±0.27\(^{b,A}\) |
| 10           | 4.40±0.46\(^{c,A}\)           | ND\(^{B}\)        | 4.43±0.35\(^{b,A}\) |
| 15           | 5.20±0.17\(^{b,A}\)           | ND\(^{B}\)        | 4.10±0.35\(^{b,c}\) |
| 20           | 5.61±0.39\(^{b,c,B}\)         | ND\(^{B}\)        | ND \(^{a,B}\)     |

Different superscript (a,b,c) in the same column indicate significant difference (P<0.05) between the storage days. Different superscript (A, B, C) in the same row indicate significant difference (P<0.05) between treatment (controls, sous vide, boiling). ND indicates no bacteria growth (colonies) detected.

### Table 5. Pseudomonas sp. count (\( \log_{10} \text{CFU g}^{-1} \)) for each treatment at 5°C storage from day 0 until day 20

| Storage (day) | Control \( \log_{10} \text{CFU g}^{-1} \) | Sous vide \( \log_{10} \text{CFU g}^{-1} \) | Boiling \( \log_{10} \text{CFU g}^{-1} \) |
|--------------|---------------------------------|-------------------|-------------------|
| 0            | 3.67±0.17\(^{a,A}\)            | ND\(^{B}\)        | ND\(^{a,B}\)      |
| 5            | 5.36±0.27\(^{a,A}\)           | ND\(^{B}\)        | ND\(^{a,B}\)      |
| 10           | 5.16±0.41\(^{a,A}\)           | ND\(^{B}\)        | 1.77±0.49\(^{a,B}\) |
| 15           | 3.82±0.17\(^{a,A}\)           | ND\(^{A}\)        | 0.67\(^{a,A}\)    |
Different superscript (a,b,c) in the same column indicate significant difference (P<0.05) between the storage days. Different superscript (A,B,C) in the same row indicate significant difference (P<0.05) between treatment (controls, sous vide, boiling). ND indicates no bacteria growth (colonies) detected.

Table 6. Yeast and mould count (log$_{10}$CFU g$^{-1}$) for each treatment at 5°C storage from day 0 until day 20

| Storage (day) | Control  | Yeast and mould count (log$_{10}$ CFU g$^{-1}$) | Sous vide | Boiling |
|--------------|----------|-----------------------------------------------|-----------|---------|
| 0            | 1.13$^{a,A}$ | ND$^{a,A}$                                  | 2.42±0.89$^{b,A}$ |
| 5            | 3.44±0.43$^{a,A}$ | ND$^{a,B}$                             | 3.56±0.07$^{a,A}$ |
| 10           | 3.44±0.16$^{a,A}$ | 2.67±0.41$^{b,B}$                        | 3.58±0.22$^{a,A}$ |
| 15           | 4.56±0.07$^{a,A}$ | 1.59±0.55$^{ab,B}$                       | 3.74±0.13$^{a,A}$ |
| 20           | 4.10±0.21$^{a,A}$ | 3.23±0.14$^{b,A}$                        | ND$^{b,A}$          |

Different superscript (a,b,c) in the same column indicate significant difference (P<0.05) between the storage days. Different superscript (A, B, C) in the same row indicate significant difference (P<0.05) between treatment (controls, sous vide, boiling). ND indicates no bacteria growth (colonies) detected.

3.1. Shelf life prediction

In regards to the total bacteria count, the shelf life of green mussel were predicted to extend at approximately up to 32 days compared to boiling (~15 days) and controls (~10 days) in chilled storage.

Similarly, [20] reported that total aerobic mesophilic count in raw mussel was 5.59±0.02 log$_{10}$ CFU g$^{-1}$. Besides that, [21] also indicated the similar value of total aerobic count for mussel samples with the value 5.00 log$_{10}$ CFU g$^{-1}$. However, the previous study conducted [15] stated the different value of total bacterial count in raw mussel (Mytilus galloprovincialis) during chilled storage at 3°C was 2.20±0.33 log$_{10}$ CFU g$^{-1}$. Total bacteria count is influence by the temperature of storage and species of mussel [22]. The spoilage rate of fish and shellfish is temperature dependent. Previous study [23,24] stated that the bacterial growth in the product were reduced during cold storage as it can prolong the bacterial lag phase thus can improve the shelf life of the product.

The increasing trend of bacteria count with a prolong storage days are due to low residual oxygen in vacuum packed sous vide that supported the growth of microaerophilic bacteria than aerobic or anaerobic bacteria [25]. Microaerophilic bacteria refers to those microbes which requires small amount of oxygen for growth and unable to grow at normal atmospheric oxygen tensions [26]. Besides that, temperature used (85°C) were only cause the bacteria thermally injured but they are capable to recover throughout storage [27,28]. Previous study [29,27,30,31] stated that at high temperature, mesophilic and psychrophilic bacterial counts in sous vide fish products were significantly low during storage. However, these may cause the loss of sensorial acceptability of the products [32]. Unlike sous vide, boiling treatment with temperature (100°C) promote the favourable conditions for aerobic bacteria to grow. Besides that, the presence of the spores by spore-forming bacteria that survived after treatment enhance their survival in stressed environment [33]. Although the product were stored at 5°C during storage, the microflora might able to tolerate the preservation conditions by changing their abilities. The upper acceptability of total bacteria count for fresh water fish and seafood recommended by ICMSF [19] were 7 log$_{10}$ CFU g$^{-1}$.

The number of coliform counts found in raw green mussel were recorded exceed the microbiological standard documented in Malaysia Food Act 1983 (ACT 281) [34] and Regulation (2014) which is 1.70 log$_{10}$ CFU g$^{-1}$. The high value of coliform count could be explained by the handling factor, water conditions and temperature [35]. Apart from that, there were no coliform count detected on green mussel treated with sous vide treatment on day 0 until day 20 of storage time. Most
of the coliform are mesophilic bacteria which grow well in 20-45°C and have an optimal growth temperature range of 30-40°C [36]. For example, *E. coli* is a typical mesophile can tolerate wide range of pressure of pressure (1–400 atm) [37] and have optimum temperature for grow is near 39°C, the maximum is 48°C and the minimum is 8°C [38]. Thus, the vacuum condition of *sous vide* and the temperature (85°C) applied in this experiment may inhibited the growth of coliform in the green mussel. From previous study, the absence of *Salmonella* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, *S. aureus*, coliform bacteria, *E. coli*, sulphite reducing *Clostridium* spp. and *B. cereus* during storage of *sous vide* bonito at 4°C and 12°C samples were recorded [31]. Besides that, other previous studies indicated that the growth of these microbes was not observed in *sous vide* packaged fish [27,29,32].

The initial *Enterobacteriaceae* sp. count of raw mussels in the present study were higher than reported by Goulas *et al.* [39] in mussels (*Mytilus galloprovincialis*) stored under modified atmosphere packaging (1.5 log CFUg⁻¹). Besides that, [15] indicated that the *Enterobacteriaceae* sp. count in raw mussel were 2.00±0.23 logCFUg⁻¹. *Enterobacteriaceae* are considered by food manufacturers as hygiene indicators and these microorganisms are used to monitor the effectiveness of implemented preventive pre-requisite measures such as Good Manufacturing Practices and Good Hygiene Practices (GMP/GHP) [40]. There were no *Enterobacteriaceae* found in green mussel treated by *sous vide* not until 20 days of storage. This result were supported by the previous study [27] which indicated that the Enterobacteriaceae sp. were only detected in *sous vide* trout processed at 70°C after 45 days of storage at 10°C with the value (2.84±0.21 log₁₀CFUg⁻¹). The *Enterobacteriaceae* sp. is a facultative anaerobes psychrotrophic that may grow in refrigerated foods irrespective of the specific packaging condition such as vacuum packaged as shown in the present study [41]. However, the combination of vacuum packaging, low temperature storage and *sous vide* cooking allowed the destruction of *Enterobacteriaceae* [25,42]. In boiling treatment, *Enterobacteriaceae* sp. count on day 5 of storage were recorded with the value of 4.71±0.27 log₁₀CFUg⁻¹. In aquatic environment, various members of the *Enterobacteriaceae* can be found and some of them acts as indicator of fecal pollution [43]. The temperature applied in boiling treatment were insufficient to inhibit the growth of *Enterobacteriaceae* sp. in the green mussel.

*Pseudomonas* is common spoilage psychotrophic microorganism that cause spoilage in chilled food [44]. Due to the prototrophic character of *Pseudomonas*, it have ability to tolerate with different forms of stress [45]. However, there were no bacterial growth in *sous vide* green mussel recorded until the end of the storage day. [46], the growth of *Pseudomonas* are inhibited by vacuum packaging in *sous vide* foods. This is because *Pseudomonas* sp. grow aerobically thus vacuum package characteristic of the *sous vide* prevent the growth of these microorganisms. Philippine National Standard [47] indicated that the limit level of yeast and mould count are at 3 log₁₀CFUg⁻¹. From the present study, the yeast and mould count in controls and boiling were exceed the limit level on 4th day of storage whereas for *sous vide* on 16th of storage.

4. Conclusion

A combination of *sous vide* and chilled storage can control the bacterial growth in the green mussels. *Sous vide* treatment were more effective in prevent the biological deterioration in green mussel during storage compared with boiling treatment. At the end of the 20 storage days, the bacteria count and yeast mould count reached a value <5 log₁₀CFUg⁻¹ whereas the coliform count, *Enterobacteriaceae* sp. count and *Pseudomonas* sp. count showed no bacteria growth. The heat treatment and storage temperature play a key role to ensure the quality and safety of the products.

5. References

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