Bacteriological quality of cage-cultured abalone *Haliotis asinina*

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ABSTRACT

Abalone is one of the most highly priced seafood delicacies and prepared in various dishes like breaded, soup, steamed and sashimi. They are susceptible to microbial contamination since it is eaten raw sometimes and pathogenic microorganisms can be hazardous to consumers. The present study was carried out to determine the coliform load and the presence of presumptive pathogenic bacteria in cage-cultured abalone in Taytay, Palawan, Philippines. The study was limited to the detection of coliform and some presumptive pathogenic bacteria in different parts of abalone such as gut, gills and mantle. The result of the study revealed that the count of coliforms present in the mantle and gills of abalone falls within the normal standard limit (7 – 21 MPN $100g^{-1}$ sample). On the other hand, the gut of abalone was beyond the standard limit (460 MPN $100g^{-1}$ sample). Moreover, the gut of abalone harbors *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. and general enteric bacteria. Foodborne infections caused by *Vibrio*, *Salmonella* and *Shigella* are common in Asia.

Keywords: Abalone, Cage culture, Coliform, Microbial load, Most probable number, *Salmonella, Vibrio*

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Introduction

Seafood is one of the most important food components for many people particularly those in coastal communities worldwide (Edun et al., 2016; Bakr et al., 2011). Marine products such as fish and other organisms are not only the cheapest sources of protein but also a significant foreign exchange earner in global trade for a number of countries in the world (Yagoub & Ahmed, 2003). One of the most important fishery products is abalone, a marine vetigastropod that contributes a comparatively low fraction in aquaculture production but considered as one of the most highly priced seafoods worldwide (Cook, 2016). They are marketed as live (US$15-US$200 kg^{-1}), dried (US$156 kg^{-1}), frozen (US$5.5-US$180 kg^{-1}), canned (US$12-US$75 can^{-1}) and steak (US$180 kg^{-1}) (Encena & Bayona, 2010). Countries like China, Hong Kong, Japan, Singapore, Taiwan, Malaysia and USA are the leading importers of abalone products (FAO, 2016).

Abalone is of great importance as food because of its high nutritive value containing Vitamin E (Alpha Tocopherol), Vitamin B12, Iron, Magnesium and Phosphorus as well as bioactive compounds that are antioxidant, anti-thrombotic, anti-inflammatory, antimicrobial and anti-cancer activities (Suleria et al., 2017). However, abalone can be contaminated by various pathogens if the environment is polluted and contaminated during harvesting and handling. The contaminants may include *Vibrio* species, a known foodborne pathogen which are naturally occurring in marine environment and *Escherichia coli* and *Salmonella* spp. which are found in water polluted by sewage (Gnanambal & Patterson, 2005; Chinnadurai et al., 2020).

Consumption of the shellfish which are contaminated by pathogens may cause disease or intoxication to the consumers. *Vibrio cholerae* causes the third-highest number of shellfish-related illnesses, after noncholera *Vibrio* spp. On the other hand, the occurrence of *Salmonella* infections due to seafood consumption is still low compared with salmonellosis associated with other foods (Sanjee & Karim, 2016). Despite this fact, detection of *Salmonella* spp. in seafood should be included as it is responsible for most of the foodborne diseases or gastroenteritis characterized by diarrhea, abdominal cramp, vomiting, nausea, and fever. The Centers for Disease Control and Prevention (CDCP) declared that *Salmonella* is the foremost causative agent of bacterial foodborne diseases resulting in approximately 1.4 million nontyphoidal illnesses, 15,000 hospitalizations, and 400 deaths in the USA annually (Sanjee & Karim, 2016). In addition, fecal coliforms such as *E. coli* are used as monitoring tool of the quality of shellfish-growing waters and bivalve molluscs. There is a need for additional methods to lower coliform aerobic mesophilic count in culture areas and in harvested shellfish (Martinez et al., 2009).

Abalone is prepared in various highly priced dishes like breaded, soup, steamed and sashimi. Abalone is susceptible to microbial contamination and since it is sometimes eaten raw (Surtida, 2000), pathogenic microorganisms can be hazardous to consumers. Thus, this study was conducted to determine the coliform load and the presence of presumptive pathogenic bacteria such as *Vibrio*, *Salmonella*, *Shigella,* and general enteric bacteria in different body parts of cage-cultured abalone. This study showed which part of the abalone is safe to consume raw and which part must be removed or cooked before consumption.

Material and Methods

Collection of Samples

Thirty samples of adult cage-cultured abalone *H. asinina* (30-35mm) were collected from Pamantolon, Taytay, Palawan, Philippines (Figure 1) in September 2018. Collection was only done once. The abalone was cultured in floating bamboo cages along the lines of farmed seaweed. The site is near a populated area where majority of the houses are made up of indigenous materials. The average water temperature, salinity and pH of the area were 28°C, 30ppt and 6.5, respectively. Abalone samples were carefully handpicked from the cages while riding a motorless boat. The collected samples were placed in sterile cooler box and were transported live to the Microbiology Laboratory of the Western Philippines University-Puerto Princesa Campus for microbial examination. Upon arrival, the abalone samples were cleaned by immersing it in sterile seawater for 5 minutes followed by another 10 minutes in cold sterile distilled water at 4-6°C to relax the organisms.

Sample Preparation for Microbial Analysis

The abalone samples were soaked in 55°C sterile distilled water. The shell and meat were separated before dissection. Different body parts of abalone; gills (G), foot mantle (M) and the gut (D) were aseptically separated and extracted using sterile dissection tools inside a laminar flow. Ten grams of each of the abalone body parts was blended with 90 mL of sterile distilled water to dilute and to homogenize. The samples of the body parts were processed fresh to maximize inventory of viable organisms.
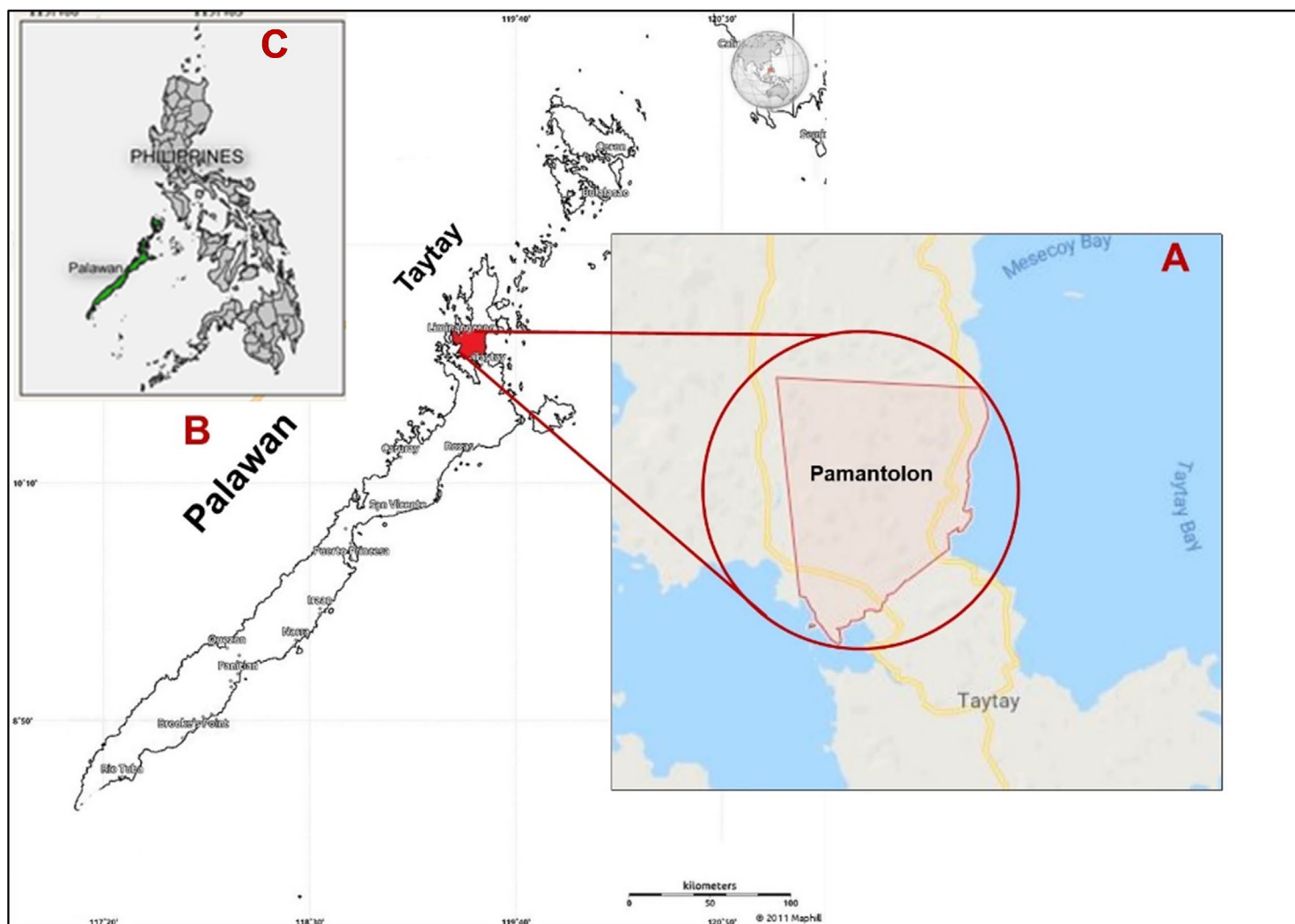
Figure 1. Map showing the site of abalone culture in Pamantolon, Taytay (A), Palawan (B). Philippines (C)

Coliform Detection (MPN Method)

The number of coliform in the samples was determined using the conventional three-tube MPN (most probable number) method (Brown, 2005). Ten mL of the homogenized sample was added in test tube containing 10 mL volume of double strength lactose broth (DSLB). One mL and 0.1 mL of the sample were added separately in test tube containing 10 mL volume of single strength lactose broth (SSLB). The total sets of tubes were incubated at 35°C for 24 h and examined for the presence of growth accompanied by gas production. Those cultures positive for gas formation were inoculated into Eosin Methylene Blue (EMB) agar and were incubated at 35°C for 24 h. After incubation, EMB Agar plates were examined. *Escherichia coli* colonies grow with a metallic sheen with a nucleated center, *Aerobacter aerogenes* colonies have a brown center, and nonlactose-fermenting Gram-negative bacteria appear pink. A loopful of sample from positive EMB agar were inoculated in DSLB tubes and incubated for 24 h. at 35°C. Gram staining followed for verification. Quantification was done using the standard MPN table and coliform was reported as MPN 100 g^{-1} sample.

Enumeration of Presumptive Pathogenic Bacteria

The pour-plate method was used in this study as adopted from the study of Sanders (2012). Different selective culture media were used to enumerate presumptive pathogenic bacteria from cage-cultured abalone *H. asinina.* The Thiosulfate Citrate Bile Salts Sucrose (TCBS) was used for total *Vibrio* species, *Salmonella-Shigella* (SS) agar for total *Salmonella* and *Shigella* species and McConkey agar for total enteric bacteria. Each medium was prepared according to the suggested ratio and proportion of the manufacturer found in the labels. One mL of each previously homogenized sample was added

to the prepared medium, mixed gently, and poured into the petri dish and allowed to solidify. There were three replicates prepared for each body part and each selective culture medium. All plates with different culture media were incubated at 35 °C for 24 hours. After incubation, all plates were examined. Colonies growing on each plate were examined for individual characteristics, counted as colony forming units (CFU) and recorded. Rapid lactose fermenting colonies such as *E. coli* appear pink in color on MacConkey agar. Colonies of *Salmonella* species appear red with black centers while *Shigella* species are red to pink colonies without black center on SS agar. *Vibrio* colonies appear yellow and green on TCBS agar.

Statistical Analyses

The data on the number of presumptive pathogenic bacteria at different parts of abalone were analyzed using one-way analysis of variance (ANOVA) to test the significant differences. The data were subjected to Post hoc test (Tukey's Test) to compare the means ($p < 0.05$).

Results and Discussion

Samples from different body parts of abalone showed gas formations after 24 h of incubation in multiple tube test indicating the presence of gas-forming lactose fermenters which implied the presence of coliform bacteria. When confirmation test was done, it was confirmed that the coliform present in this study was *E. coli*. Results of this study showed that the gut of abalone exceeded the acceptable limit of *E. coli* for shellfish with a count of 460 MPN $100g^{-1}$ (Table 1). The acceptable limit of *E. coli* for shellfish is 230 MPN $100g^{-1}$ based on several references enumerated in Table 1. *Escherichia coli* is frequently used as an indicator of fecal contamination because it lives naturally in human feces and can survive in water (Duncan et al., 2009). The high level of *E. coli* in the gut could be due to the probable high count of fecal coliforms in their growing water areas. It was observed that the culture areas in Pamantolon, Taytay were surrounded by houses built

with low-cost materials with comfort rooms that don't have septic tank and very near the shore so runoff from terrestrial area could have contributed to the presence of coliforms. Chinnadurai et al. (2020) proved that bacterial concentrations in shellfish correlate strongly with those in the waters. Their sampling sites (growing sites of shellfish) receive high levels of contaminants from drainage channels, open toilet drain, non-functional septic tank and livestock production areas, and they found similar high contamination in the shellfish from the areas. Another study examined the concentration of coliforms in oysters in the River Blackwater Estuary in the UK where they found that the main source of *E. coli* and *Streptococci* to the oyster beds are sewage and agricultural sources, respectively (Florini et al., 2020).

Microorganisms that can be found in marine environment and most commonly encountered by marine species are free-living forms found in water and sediment and rarely include any species of mammalian pathogens (ICMSF, 1986). Hence, fish and shellfish that are handled properly during harvest from waters not polluted by human or animal wastes are often free from intrinsic microbiological hazard. Fish and other marine animals do not usually carry *Escherichia coli*, the 'fecal coliforms', and enterococci as these microorganisms are generally considered to be typical mammalian microflora. The presence of human enteric organisms on marine food products is clear evidence of contamination from a terrestrial source (ICMSF, 1986). It is important to understand the origin of fecal contamination in shellfish farms to assess the associated health risks as well as the actions needed to address the problem (Florini et al., 2020). In addition, since the abalone samples of this study were also cultured along the lines of farmed seaweeds, the water current and mixing may be obstructed resulting in possible accumulation of microorganisms around the area. On the other hand, the gills and mantle of the abalone had *E. coli* number lower than the microbial limit for shellfish. This is reassuring to note as the part of abalone mostly consumed is the mantle.

Table 1. Most probable number (MPN) of coliform bacteria present in different parts of adult abalone *Haliotis asinina* and the microbial limit (*Escherichia coli*) for shellfish based on European Communities (EC) (2007) and Philippine National Standards - Bureau of Agriculture and Fisheries Product standards (PNS-BAFPS) (2011).

Sample	*E. coli* MPN/100g	Microbial Limit
Gut	460	
Gills	21	230 MPN/100g according to PNS-BAFPS (2011) and EC (2007)
Mantle	7	

The presence of *E. coli* in food or water implies that there could be other pathogens present like *Klebsiella* and *Vibrio* and other clinically important bacterial pathogen (WHO, 2001). In this study, presumptive pathogenic bacteria were detected in different body parts of abalone. The analysis of variance (ANOVA) proved that there were significant differences in the number of presumptive pathogenic bacteria ($p < 0.05$) at different parts of abalone. Tukey's test showed that the total enteric bacteria had the highest number in the gills of abalone with a count of 29 CFU g^{-1} sample followed by *Salmonella-Shigella* and then *Vibrio* (Figure 2). In the gut, ANOVA proved significant differences among the different groups of presumptive pathogenic bacteria and Tukey's test showed that *Vibrio* and enteric bacteria were higher in terms of total number of colonies with a count of 101 CFU g^{-1} sample and 93 CFU g^{-1} sample respectively, than the number of *Salmonella* and *Shigella* (Figure 2). On the other hand, *Vibrio* was found to be significantly highest in the mantle with 22 CFU g^{-1} (Figure 2). Among the three body parts of abalone that were tested, the gut harbors the highest number of presumptive pathogenic bacteria. In addition, the *Vibrio* group had the highest number found in abalone. According to PNS-BAFPS (2011), *Salmonella* species should be absent in 25 g sample and *Vibrio* should not exceed 100 MPN/100g sample.
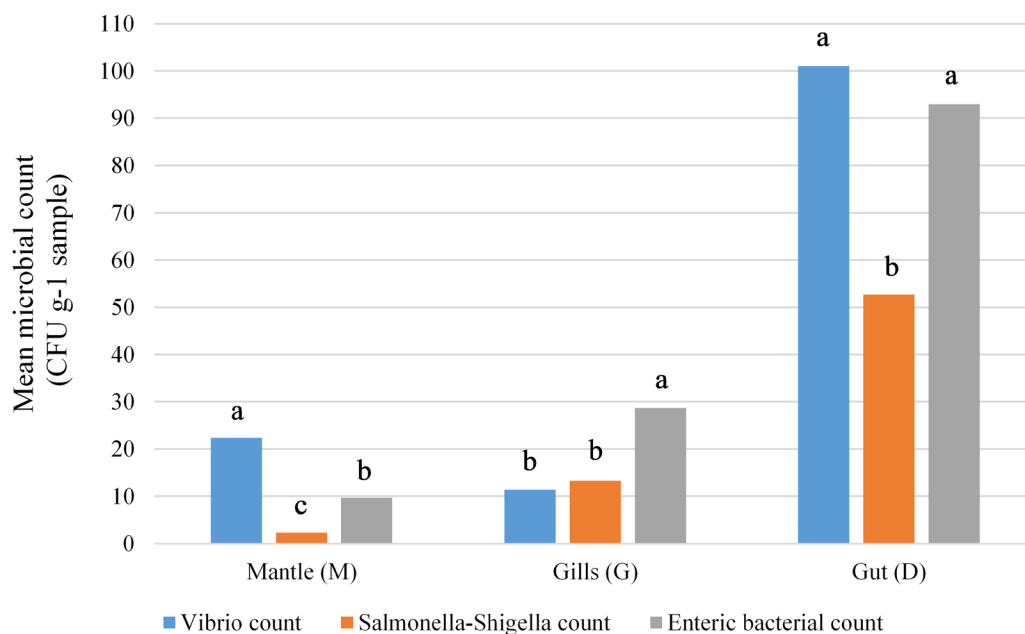
Figure 2. Mean microbial count of presumptive pathogenic bacteria from the gills, gut and mantle of adult cage-cultured abalone *Haliotis asinina* Linn. Different letters signify significant differences at p< 0.05.

The outbreak of seafood infections from contaminated waters are caused by variety of bacteria, viruses and parasites have been reported worldwide (Florini et al., 2020). Centers for Disease Prevention and Control (CDC) reported to the Food-borne Disease Outbreak Surveillance System (FDOSS) 188 outbreaks of seafood-associated infections, causing 4,020 illnesses, 161 hospitalizations, and 11 deaths from 1973 to 2006. A total of 76.1% of these seafood-associated outbreaks were due to a bacterial agent (CDC, 2010). It was recorded that *Vibrio* and *Salmonella* were the most commonly reported bacteria that cause seafood contamination outbreaks (Iwamoto et al., 2010).

Salmonella species is one of the most important food-borne pathogens and have been detected in seafoods (Edun et al., 2016). In this study, *Salmonella* was present in the mantle, gills and gut of abalone. This species can cause wide range of illness. Example is the common typhoid fever caused by *Salmonella typhi* with common symptoms of fever, headache, malaise, anorexia and red spots on the trunk (WHO, 1996). In Brazil, the absence of *Salmonella* spp. in 25 g of oyster flesh is required (Brazilian Regulations, 2019). Similar microbial limit in *Salmonella* spp. is also imposed in the Philippines by PNS-BAFPS (2011). In the study conducted by Lameira Silva et al. (2020), *Salmonella* spp. was present in the flesh of oyster in all sampling sites in Amazon estuaries in Pará, Brazil irrespective of the seasonal period. In contrast, the study conducted by Sorio and Peralta, (2018) revealed that *Salmonella* spp. was not detected in any samples of oysters growing in selected production areas in Dumangas, Iloilo, Philippines. Similar result was presented by Martinez et al. (2009) wherein all molluscan shellfish samples (mussel,

clams and cockles) in their study were negative for the presence of genes encoding virulence factors in *Salmonella*. Another study was conducted in South Korea to analyze the microbiota of abalone to improve awareness on outbreaks and causes of food poisoning and to help the management of seafood products (Lee et al., 2016). In this study, there were over 2700 species of microorganisms detected in the samples but only five species were potentially pathogenic and did not include either *Salmonella* or *Vibrio* species.

Vibrio species are problems in molluscan shellfish hatcheries including abalone (Lee et al., 2001; Handlinger et al., 2005; Kua et al., 2011). According to Romalde et al. (2014), *Vibrio parahaemolyticus, V. harveyi, V. splendidus, V. aglinolyticus, V. anguillarum* and *V. vulnificus* (Lee et al., 2001; Handlinger et al., 2005; Cai et al., 2006; Pitchon et al., 2013) are major species infecting abalone species. Aside from outbreaks of diseases caused by *Vibrio* species that leads to mass mortalities and economic losses in cultured species, they are also associated with live seafood as they form part of the indigenous microflora of the marine environment. Foodborne infections caused by *Vibrio* spp. are common throughout the world so proper precautionary measures are also important (FAO & WHO, 2020). In the USA, consumption of raw oysters with contamination of *V. vulnificus* and *V. parahaemolyticus* causes septicemia and other infection (FAO & WHO, 2005). In Japan, *V. parahaemolyticus* infections results from consumption of raw seafoods (FAO & WHO, 2011). On the other hand, bacterial infection is low in Thailand and other Southeast Asian countries including Philippines because shellfish are generally consumed after cooking (FAO & WHO, 2011). Although in one particular event in Cebu City, Philippines, *V. parahaemolyticus* has been linked to fish and shellfish contamination causing foodborne disease wherein 97 people were hospitalized (Borromeo, 2007). This bacterium is a common cause of bloody diarrhea, abdominal cramps, nausea, vomiting, and fever worldwide that occur about 4–96 h from the time of ingestion (FSIS, 2014). Undercooking could explain the presence of *Vibrio* in fish and shellfish commodities that leads to infection and disease (FAO & WHO, 2020). On the other hand, some countries like Japan, France, Australia, New Zealand, China and Taiwan isolated several species of *Vibrio* such as *V. campbellii, V. harveyi, V. parahaemolyticus, V. alginolyticus and V. splendidus* from different species of *Haliotis* where these *Vibrio* species caused mass mortality in cultured abalone (Bower, 2017).

In this study, results showed that most of the pathogenic bacteria were found in the gut of abalone. This result supports the previous studies (Mabuhay-Omar et al., 2019; Santiago & Mabuhay-Omar, 2019) wherein the gut of abalone harbored the highest number of microorganisms compared to gills and mantle. Mantle is the part of abalone usually consumed by human and so it is good to note that the number of microorganisms is very small compared to the maximum limit but since fecal coliform and some presumptive pathogenic bacteria are present, it is important to depurate and properly prepare the abalone before eating. The presence of fecal coliform and presumptive pathogenic bacteria in the mantle of abalone can be due to contamination during handling and lack of proper cleaning protocol. In addition, removal of gut and gills of abalone before cooking or preparing uncooked menu is needed since presumptive pathogenic microorganisms are found in these parts of abalone.

Conclusion

This study proved the presence of coliform such as *E. coli* and some presumptive pathogenic microorganisms in abalone such as *Salmonella*, *Shigella*, *Vibrio* and total enteric bacteria. With this information, it is recommended for the abalone farmers to optimize the culture management practices such as monitoring of the physico-chemical parameters of water since the presumptive pathogenic bacterial species detected are also opportunistic pathogens and could cause massive losses in abalone production under favorable conditions. Also, these species are considered to be human pathogens and could cause various infections among human population. It is also important to properly cook the abalone before eating. In addition, removal of gut and gills of abalone before preparing uncooked menu is needed since microorganisms are found in these parts of abalone. Prior to selling cultured abalone to consumers, depuration methods may be applied to minimize possible contamination.

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