Preliminary investigation on the microbiological quality of edible marine gastropods of the Adriatic Sea, Italy

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Abstract

According to the European Legislation, marine gastropods placed unprocessed on the market must comply with the same requirements established for live bivalve molluscs but, being considered not filter-feeding and unable to concentrate fecal contaminants, they may be harvested outside the classified areas. Despite this statement, little scientific information is available on the microbiological quality of these animals. The aim of the present study was to investigate 28 batches of edible snails of the Adriatic Sea, namely Nassarius mutabilis and Bolinus brandaris, with respect to i) smell and viability, by a method here reported; ii) the bacterial component of the whole body referred to E. coli, Vibrio spp., V. parahaemolyticus, V. vulnificus, V. choleræ and V. alginolyticus. A total of 21 batches of N. mutabilis and 7 batches of B. brandaris were analyzed. Batches of both species retrieved from the primary production were all largely composed of viable animals, had saltwater/neural smell, and showed mean value of Vibrio spp. of 5.34 and 5.79 log_{10} UFC g^{-1} in N. mutabilis and B. brandaris respectively. 47% of the batches of N. mutabilis retrieved from the market, were largely composed of dead animals, had acrid/nasty smell, and showed mean value of Vibrio spp. of 6.53 log_{10} UFC g^{-1}. E. coli, V. vulnificus and V. choleræ were never detected, but all samples were positive for V. alginolyticus. One sample of B. brandaris was positive for V. parahaemolyticus genotyped by PCR at the specie level (ToxR+) and positive for the thermostable direct hemolysin gene (tdh+).

Introduction

Marine gastropods represent a common source of seafood all over the world. Most of the edible species are caught, but some high-value species, as abalone (Haliotis spp.), one of the most expensive of any seafood item worldwide, are farmed reaching over 95% of the total production (FAO, 2018). Fishing of Nassarius mutabilis is by far the most important activity carried out by artisanal fisheries using basket traps in the central and northern Adriatic Sea, yielding from 2000 to 3000 Tonnes of landings each year (Polidori et al., 2015).

Historically, Bolinus brandaris has been a valuable species since the time of the Roman Empire, when it was caught for extracting the purple dye (Spanier and Karmon, 1987), so it is also known as “purple dye murex”. Nowadays, B. brandaris is frequently caught as bycatch by bottom trawlers, but also by artisanal fishing, particularly in southern Europe, including Italy, being considered also as a candidate species for molluscan aquaculture (Vasconcelos et al., 2012).

According to the European Legislation, marine gastropods placed “fresh” (unprocessed) on the market must comply with the same requirements laid down for live bivalve molluscs (European Commission, 2004a), but they may be harvested from non-classified production areas, being considered not filter-feeding, and therefore unable to concentrate fecal contaminants (European Commission, 2010) as E. coli, quantitatively utilized for the classification of the production areas and the suitability for direct human consumption (European Commission, 2004b; European Commission, 2005). For the same reason, the purification process is considered unworkable for these animals (European Commission, 2004a).

Despite these provisions, it must be recognized that the scientific knowledge about the microbiological quality of marine gastropods is poor, and substantially lacking for the edible marine species of the Italian seas. Moreover, the overview report undertaken by the European Commission’s Food and Veterinary Office (European Commission, 2015), pointed out that the Official Controls on live gastropods harvested from non-classified production areas are not yet fully implemented by the Member States, being absent or inappropriate.

Lastly, a standardized method for the assessment of the viability of these animals placed on the market is not currently available, considering that the provisions of European Commission (2004a) is based on bivalve molluscs characteristics, to whom marine gastropods are assimilated.

Marine gastropods are not filter feeding, but most of them are carnivorous, with a degree of predatory activity that varies from actively seeking prey, to grazing on sessile invertebrates, to scavenging (Modica and Holford, 2010).

Among gastropods, marine snails are considered the source of a variety of toxic compounds, particularly neurotoxins, mainly utilized for predation (West et al., 1996), but it is also well documented that carnivorous species can accumulate toxins through predation on contaminated bivalve molluscs. In fact, Paralytic Shellfish Toxins (PST) and Tetrodotoxin (TTX), and/or related compounds, have been detected in several species of gastropods, including members of the family Nassariidae (Rodiguez et al. 2008; Luo et al., 2012; Silva et al., 2012; Turner et al., 2014; Asakawa, 2017). On the other hand, since the last century, it has been reported that TTX producing bacteria, identified as V. alginolyticus and V. parahaemolyticus, have been isolated from the gastropod Niotha clathrata (Nassarius conoidalis), whose tissues results highly contaminated by bacteria, predominantly Vibrio spp., which resulted 6-8 log_{10} CFU g^{-1} in the muscle, and 6-9 log_{10} CFU g^{-1} in the digestive gland (Cheng et al., 1995).

Vibrio spp. represents a major genus of bacteria inhabiting the marine ecosystem, and within the taxon, serious pathogenic species for both human and marine animals are included (Fukui et al., 2010). The four species most frequently isolated in clinical microbiology laboratories are V. choleræ, V parahaemolyticus, V. vulnificus and V. algi-
nolyticus (Mustafa et al., 2013). V. cholerae, V. parahaemolyticus and V. vulnificus are the most important species in terms of number, distribution, and severity of infections (Boyd, 2007), and are responsible for a dramatic increase of seafood-borne infections worldwide (Bonnin-Jusserand et al., 2019). Notwithstanding the highest densities of vibrios in the environment are frequently found in warm waters ranging from 20°C to 30°C, infections of V. parahaemolyticus and V. vulnificus in humans have recently occurred even in cold high latitude areas where such infection had never been experienced, and these cases are reported to be a result of global warming trends in the ocean (Fukui et al., 2010).

In any case, a multiyear retrospective study conducted on the bivalve Raditula philippinarum in the North west Adriatic Sea, did not reveal a significant correlation between seawater temperature and Vibrio spp. load, resulted 4.71±0.10 and 4.70±0.29 log10 in the warmer months (April-October) and in the cooler month (November- March) respectively, although the prevalence of V. parahaemolyticus and V. vulnificus resulted significantly higher in the warmer months (Serratore et al., 2016).

Infection by V. cholerae and V. parahaemolyticus typically results in gastrointestinal disease, while V. vulnificus can cause more severe illness, including septicemia and death, particularly in individuals with predisposing conditions (Jones et al., 2014). V. alginolyticus is considered the most frequent species living freely in water and sediments (Harriague et al., 2014), being able to survive even under conditions of nutrient stress while maintaining its virulence (Ben Kahla-Nakbi et al., 2007), and it has been found to cause serious seafood-poisoning or fatal extra-intestinal infections in humans (Fu et al., 2016).

The aim of the present study was to investigate the microbiological quality of two edible snails of the Adriatic Sea, namely Nassarius mutabilis and Bolinus brandaris, with respect to E. coli, as safety criterion (European Commission, 2005), Vibrio spp., as the most representative autochthonous bacterial component of the marine environment, and the potentially pathogenic species V. parahaemolyticus, V. vulnificus, V. cholerae and V. alginolyticus. Moreover, the applicability of a simple method to evidence the viability of a batch is presented.

Materials and Methods

A total of 21 batches of N. mutabilis, 17 at retail and 4 from primary production, and 7 batches of B. brandaris, all from primary production, were considered. Samples of N. mutabilis were collected from October to May, that is the fishing season of the species (Poliodri et al., 2015), and samples of B. brandaris from May to June, when N. mutabilis samples were not available on the market. The catch area was the FAO zone 37.2.1 (Adriatic Sea), along the coast from Ravenna to Rimini.

Each batch was firstly analyzed for smell and viability. The smell was expressed based on five descriptors: saltwater (very fresh), neutral (freshness), slightly acidic (initial loss of fresh) acidic (loos of freshness), nasty (spoiled). To verify the viability, the whole batch was distributed on a large platter and then dusted by table salt to evidence any sign of reactivity, and only reactive animals were considered viable.

This approach was adopted because any attempt to verify gastropods viability by foot puncture or foot traction, as commonly indicated, resulted time consuming and unsatisfactory compared with the proposed method. For the bacteriological analyses, the sample units (SU) of N. mutabilis were constituted of about 20 individuals, and the SU of B. brandaris of about 10 individuals. Each SU was carefully rinsed off with sterilized seawater and shells were cut aseptically, to remove the whole body, representing the edible part of these animals. Animals bodies were blended with salt solution NaCl 3%, to obtain the first dilution 10¹ and from this the additional ones.

The abundance of Vibrio spp. was checked on thiosulfate-citrate-bile salts-sodium (TCBS) agar (OXOID) NaCl 3% by the spread plate method, and incubation at 20°C for 3–5 days as reported elsewhere (Passalacqua et al., 2016). The results were expressed as Colony Forming Units (CFU) g⁻¹.

The isolation of V. parahaemolyticus, V. cholerae, V. vulnificus and V. alginolyticus was performed on CHROMagar™ Vibrio (CAV) (PBI) incubated at 37°C for 24 h. The colorless colonies were tentatively scored as V. alginolyticus. The mauve and the green blue to turquoise blue colonies were tentatively scored as V. parahaemolyticus and V. cholerae/V. vulnificus respectively. The number of suspected colonies tested for each sample was from 1 to 5 (if available), as is the normal practice. Selected colonies were purified on Tryptone-Soya-Agar (TSA) (OXOID) NaCl 3%, and then tested to confirm the typical traits of the genus as reported elsewhere: Gram negative straight or curved rods, oxidase positive, able to reduce nitrate, dex- trose fermenting and sensitive to the vibrio- static O/129 (150 µg) (Passalacqua et al., 2016).

Results

The sensory evaluation of the batches of N. mutabilis and B. brandaris was successfully achieved. The five descriptors of smell resulted adequate, and the dusting by table salt to evidence reactivity easily applicable to any batch. The viability of the batches was scored as follows: mortality less than 10% (+++), mortality less than 20% (++); mortality 20-40% (+++); mortality >40% (+); mortality 100% (-). The results of the sensory evaluation coupled with the bacteriological findings are reported in Table 2. All the batches from the primary production (N. mutabilis N=4 and B. brandaris N=7), and some batches of N. mutabilis at retail (9 out of 17), had salty or neutral smell, except sample 1323 having slightly acid smell, and were composed of high reactive animals (viability from +++ to ++) when dusted by table salt: extroversion of the foot and active movements (N. mutabilis), or abundant foam production (B. brandaris). The Vibrio spp. load of each sample, expressed as Colony Forming Units (CFU) g⁻¹ have been log-transformed prior to calculate the mean value, that resulted 5,34 log₁₀ (SD=±1,21) in Nassarius batches, and 5,79 log₁₀ (SD=±0,98) in B. brandaris batches. Among the batches of N. mutabilis at retail, the 47% (8 out of 17) had acrid or nasty smell and poor or no reaction when dusted by table salt (viability from ++ to -). This was the case of samples 1324, 1327, 1328, 1332, 1333, 1379, 1406, 1407, with mean value of Vibrio spp. of 6,53 log₁₀ CFU.
Considering the bacteriological results of each batch acquired at retail and the time from the day of packaging and the day of laboratory analyses, controversial aspects were evidenced. Batches 1332, 1333 and 1379, acquired the sixth day from packaging, had nasty smell and were completely composed of dead animals, showing the highest counts of *Vibrio* spp. resulting 7.10, 7.29 and 7.69 log$_{10}$ CFU g$^{-1}$ respectively. On the other hand, batches 1327 and 1328, acquired the same day of packaging, had acrid smell and only low viability (++) as batch 1324 acquired one day from packaging, and batch 1407, also acquired one day from packaging, had nasty smell and very low viability (+).

**Table 1. Phenotypical traits utilized to characterize the suspected colonies grown on CHROMagar™ Vibrio: *V. alginolyticus* colorless, *V. parahaemolyticus* mauve, *V. cholerae*/*V. vulnificus* green blue to turquoise blue. Expected results.**

| Sample Number | Species         | Origin | Smell/Viability | O129/150 µg | SIM | A/L/O | KIA |
|---------------|-----------------|--------|-----------------|-------------|-----|-------|-----|
| 1321          | *N. mutabilis*   | C      | S/+/+           | 3.20        |     |       |     |
| 1323          | *N. mutabilis*   | C      | S/+/+           | 5.95        |     |       |     |
| 1324          | *N. mutabilis*   | C      | A/++           | 6.25        |     |       |     |
| 1327          | *N. mutabilis*   | C      | A/++           | 6.41        |     |       |     |
| 1328          | *N. mutabilis*   | C      | A/++           | 5.88        |     |       |     |
| 1332          | *N. mutabilis*   | C      | NV/-            | 7.10        |     |       |     |
| 1333          | *N. mutabilis*   | C      | NV/-            | 7.29        |     |       |     |
| 1337          | *N. mutabilis*   | C      | S/+/+           | 6.31        |     |       |     |
| 1338          | *N. mutabilis*   | C      | S/+/+           | 6.23        |     |       |     |
| 1339          | *N. mutabilis*   | C      | S/+/+           | 6.95        |     |       |     |
| 1340          | *N. mutabilis*   | C      | S/+/+           | 6.09        |     |       |     |
| 1378          | *N. mutabilis*   | C      | S/+/+           | 6.00        |     |       |     |
| 1379          | *N. mutabilis*   | C      | NV/-            | 7.69        |     |       |     |
| 1382          | *N. mutabilis*   | C      | S/+/+           | 5.20        |     |       |     |
| 1384          | *N. mutabilis*   | C      | S/+/+           | 6.42        |     |       |     |
| 1386          | *N. mutabilis*   | C      | S/+/+           | 3.45        |     |       |     |
| 1389          | *N. mutabilis*   | PP     | N/+/+           | 5.92        |     |       |     |
| 1406          | *N. mutabilis*   | C      | A/++           | 6.32        |     |       |     |
| 1407          | *N. mutabilis*   | C      | NA/+            | 5.26        |     |       |     |
| 1408          | *N. mutabilis*   | PP     | S/+/+           | 5.81        |     |       |     |
| 1409          | *B. brandaris*   | PP     | S/+/+           | 5.67        |     |       |     |
| 1414          | *N. mutabilis*   | PP     | S/+/+           | 5.43        |     |       |     |
| 1415          | *B. brandaris*   | PP     | S/+/+           | 3.54        |     |       |     |
| 1416          | *B. brandaris*   | PP     | S/+/+           | 5.54        |     |       |     |
| 1417          | *B. brandaris*   | PP     | S/+/+           | 3.86        |     |       |     |
| 1418          | *B. brandaris*   | PP     | S/+/+           | 6.06        |     |       |     |
| 1419          | *B. brandaris*   | PP     | S/+/+           | 6.96        |     |       |     |
| 1420          | *B. brandaris*   | PP     | S/+/+           | 6.51        |     |       |     |

**Table 2. Smell, viability, *Vibrio* spp. and *E. coli* enumeration in *N. mutabilis* e *B. brandaris* and origin of the batches.**

| Sample Number | Species         | Origin | Smell/Viability | Vibrio spp. log$_{10}$ CFU g$^{-1}$ | Positivity for *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* | *E. coli* |
|---------------|-----------------|--------|-----------------|-------------------------------------|-------------------------------------|-----------|
| 1321          | *N. mutabilis*   | C      | S/+/+           | 6.31                                | VA+                                 | nd        |
| 1323          | *N. mutabilis*   | C      | S/+/+           | 6.23                                | VA+                                 | nd        |
| 1324          | *N. mutabilis*   | C      | A/++           | 6.41                                | VA+                                 | nd        |
| 1327          | *N. mutabilis*   | C      | A/++           | 5.88                                | VA+                                 | nd        |
| 1332          | *N. mutabilis*   | C      | NV/-            | 7.10                                | VA+                                 | nd        |
| 1333          | *N. mutabilis*   | C      | NV/-            | 7.29                                | VA+                                 | nd        |
| 1337          | *N. mutabilis*   | C      | S/+/+           | 6.31                                | VA+                                 | nd        |
| 1338          | *N. mutabilis*   | C      | S/+/+           | 6.23                                | VA+                                 | nd        |
| 1339          | *N. mutabilis*   | C      | S/+/+           | 6.95                                | VA+                                 | nd        |
| 1340          | *N. mutabilis*   | C      | S/+/+           | 6.09                                | VA+                                 | nd        |
| 1378          | *N. mutabilis*   | C      | S/+/+           | 6.00                                | VA+                                 | nd        |
| 1379          | *N. mutabilis*   | C      | NV/-            | 7.69                                | VA+                                 | nd        |
| 1382          | *N. mutabilis*   | C      | S/+/+           | 5.20                                | VA+                                 | nd        |
| 1384          | *N. mutabilis*   | C      | S/+/+           | 6.42                                | VA+                                 | nd        |
| 1386          | *N. mutabilis*   | C      | S/+/+           | 3.45                                | VA+                                 | nd        |
| 1389          | *N. mutabilis*   | PP     | N/+/+           | 5.92                                | VA+                                 | nd        |
| 1406          | *N. mutabilis*   | C      | A/++           | 6.32                                | VA+                                 | nd        |
| 1407          | *N. mutabilis*   | C      | NA/+            | 5.26                                | VA+                                 | nd        |
| 1408          | *N. mutabilis*   | PP     | S/+/+           | 5.81                                | VA+                                 | nd        |
| 1409          | *B. brandaris*   | PP     | S/+/+           | 5.67                                | VA+                                 | nd        |
| 1414          | *N. mutabilis*   | PP     | S/+/+           | 5.43                                | VA+                                 | nd        |
| 1415          | *B. brandaris*   | PP     | S/+/+           | 3.54                                | VA+                                 | nd        |
| 1416          | *B. brandaris*   | PP     | S/+/+           | 5.54                                | VA+                                 | nd        |
| 1417          | *B. brandaris*   | PP     | S/+/+           | 3.86                                | VA+                                 | nd        |
| 1418          | *B. brandaris*   | PP     | S/+/+           | 6.06                                | VA+                                 | nd        |
| 1419          | *B. brandaris*   | PP     | S/+/+           | 6.96                                | VA+                                 | nd        |
| 1420          | *B. brandaris*   | PP     | S/+/+           | 6.51                                | VA+AP+                               |           |

C, commerce; PP, primary production; S, saltwater; NE, neutral; SA, slightly acrid; A, acrid; NA, nasty; ++++, mortality less than 10%; ++, mortality less than 20%; +, mortality 20-40%; -, mortality >40%; -. mortali-
ty 100%. VA, *V. alginolyticus*; VC, *V. cholerae*; VP, *V. parahaemolyticus*; VV, *V. vulnificus*; nd, not detected.
Discussion

According to European Legislation (European Commission, 2004a) live gastropods, together with live echinoderms and live tunicates intended for human consumption, must comply with the requirements laid down for live bivalve molluscs, so they must “have organoleptic characteristics associated with freshness and viabilitiy, including shells free of dirt, an adequate response to percussion and normal amounts of intravralcular liquid”. This provision, clearly based on bivalve molluscs characteristics, is obviously unsuitable for marine gastropods, and therefore a standardized method to verify their viability is lacking for the Competent Authorities and for the Food Operators.

According to European Legislation (European Commission, 2004a), a batch of gastropods may be placed on the market by a Dispatch Center after repackaging of a batch received from another Dispatch Center and so on, moving the product further and further away from the day and place of landing. In this way, the date of packaging is not indicative of the time between landing and placing on the market of the product at retail. Consequently, it is not surprising that 47% of the batches at retail considered in this study, had acidic or nasty smell and were largely or completely composed of dead animals after 6 days from packaging.

In the present study, the bacteriological component of N. mutabilis and B. brandaris tissues was checked without eviceration, considering that the edible part of these animals is the whole body, as opposed to the Argentine legislative requirements, establishing that the commercialization of unevcerated snail is not allowed (Turner et al., 2014).

Despite it is well documented in literature the ability of marine gastropods to accumulate toxins through predation (Rodriguez et al. 2008; Luo et al., 2012; Silva et al., 2012; Turner et al., 2014; Asakawa, 2017), the possibility to harbor high concentration of bacteria in their tissues, including potentially pathogenic species, is poorly investigated, and to our knowledge, it is actually unrecognized as safety issue.

Only few reports on this matter have been published, and among them the investigation on disease outbreaks of Abalone, reporting bacterial contamination of tissues also in healthy animals (Shi et al., 2017), and the study of Cheng et al. (1995) reporting an high a contamination of N. clathrata muscle by Vibrio spp. (6-8 $\log_{10}$ CFU g$^{-1}$), coupled with positivity for V. alginolyticus and V. parahaemolyticus producing TTX.

Accordingly, our study evidenced high Vibrio spp. value in gastropods tissues, exceeding $5 \log_{10}$ CFU g$^{-1}$ also in viable batches, and positivity for V. alginolyticus (100% of the batches) and V. parahaemolyticus (1 out of 28, corresponding to the 3.6% of the batches).

Interestingly, it should be noted that the abundance of Vibrio spp. here reported is higher than the abundance registered on the bivalve Ruditapes philippinarum belonging to the same sea-area, showing a mean value of Vibrio spp. less than $5 \log_{10}$ CFU g$^{-1}$ (Serratore et al. 2016), but with a positivity for pathogenic V. parahaemolyticus of 6.7%. Our data seem to confirm that marine gastropods may accumulate in their tissues bacteria present in their feed, just like bivalves do by their filter-feeding from seawater, and this represents a food safety issue actually underestimated.

Conclusions

To our knowledge, this is the first study on the bacterial component associated to edible marine gastropods of the Adriatic Sea, namely N. mutabilis and B. brandaris, together with the evaluation of sensory characteristics of the batches both from primary production and commerce.

Even if this may be considered only a preliminary investigation, two specific findings are of concern: 1) the concrete possibility to evaluate viability and freshness of the gastropods batches to avoid the placing on the market of products with poor or no signs of viability, and therefore not conforming to the European legislative requirements (European Commission, 2004a); 2) the high concentration of bacteria as Vibrio spp. in the whole body, that is the edible part, and the positivity for pathogenic bacteria as V. alginolyticus and V. parahaemolyticus.

The failure to evidence E. coli, seems to justify the provision of the European Commission (2010), laying down that marine gastropods may be harvested from non-classified production areas, but only hypothesizing an extremely low concentration of E. coli in the areas of harvesting, or a different kinship of E. coli towards gastropods tissues with respect to Vibrio spp.

To confirm our achievements, more data are needed, and obviously other gastropods species should be tested. Finally, regarding food safety, the study suggests the need to investigate the biogenic amine content of edible gastropods, because the catalytic activity of a high bacterial load makes not far-fetched the production of these natural anti-nutritional factors in their tissues.

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