Escherichia coli and Paralytic Shellfish Poisoning Toxins Contamination of Mussels Farmed in Bulgarian Black Sea Coast

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Abstract

Mussels are filter-feeding organisms that could be a potential source of food-borne diseases and poisonings. Consumption of molluscs, contaminated with bacterial contaminants or marine biotoxins, may lead to severe food-borne diseases. Considering the importance of the health problem connected with Escherichia coli and paralytic shellfish poisoning (PSP) toxins and also the requirements of the European legislation, the aim of this study was: to evaluate the level of E. coli and PSP contamination in cultivated mussels produced in farms along the Bulgarian Black Sea coast.

The study was performed on 55 samples of live mussels (Mytilus galloprovincialis), from 20 aquaculture farms, during the period 2012 - 2015, in the warm season (May - October) and in the cold season (November - April). The determined concentration of E. coli varied between <20 MPN/100 g and 8.2 x 10² MPN/100 g. The level of E. coli in 5 samples tested in the warm season and in 2 samples tested in the cold season was higher than the permissible quantity. All samples met the health standards referring to the content of PSP toxins in bivalve molluscs. No connection was established between the concentration of E. coli and PSP toxins in the tested samples and the level of contamination with E. coli and PSP toxins in cultivated mussels, from the Bulgarian Black Sea coast, was minimal.

Key words: bivalve molluscs, Escherichia coli, PSP toxins

Introduction

The main species of marine aquaculture grown in the coastal waters of the Black Sea is the black mussel – Mytilus galloprovincialis. The production of cultivated mussels in Bulgaria has increased in recent years. There are 25 mussel farms along the Bulgarian Black Sea coast. The annual production of mussels increased by 12 % and reached 18 t in 2016 (Ministry of Agriculture and Food, 2016).
Mussels are filter-feeding organisms that tend to accumulate different biological and chemical agents in their tissues (Oliveira et al., 2011). They could be a potential source of food-borne diseases and poisonings. According to the EFSA report, in 2016 in the EU countries molluscs were the source of 3% of the food-borne outbreaks (EFSA, 2016). The main source of contamination of mussels is marine pollution caused by untreated household and industry sewage in the absence of treatment plants. During a two-year period (2012–2014), the water quality of the Black Sea was evaluated and a presence of pathogenic microorganisms such as *E. coli*, *Vibrio* spp., rotaviruses was detected (Panaiotov et al., 2015).

Stoica (2014) analyzed wild mussels harvested from the north-western coast of the Black Sea (Romania). She proved a close relation between the quantity of *E. coli* in sea water and in the mussel tissues. Her investigations confirmed the interdependence between environmental conditions and the levels of *E. coli* in wild mussels and surface shellfish waters. She detected levels of *E. coli* which exceeded the European requirements in 16.7% of the Romanian shellfish growing coastal zones. A study carried out in the Eastern Aegean Sea showed a maximum fecal coli form count of 2.4 x 10^6 MPN/100 g (Kacar, 2011). Yilmaz et al. (2005) found *E. coli* values between <10 and 5.9 x 10^2 cfu/g in *M. galloprovincialis* harvested in the Marmara Sea. Pomykala et al. (2012) isolated *E. coli* ranging from <2.0 x 10^1 up to >1.8 x 10^4 MPN/100g from raw mussels originated from Spain, the Netherlands and Norway.

Contaminated with bacteria molluscs may lead to severe serious health problems for consumers. Regulation (EC) No 2073/2005 lays down the microbiological criteria for certain microorganisms, including *E. coli* in bivalve molluscs, which must be less than 230 in 100 g of flesh and intra-valvular liquid to be safe for human consumption. Another serious health risk for humans consuming mussels are marine biotoxins. The most common and dangerous is paralytic shellfish poisoning (PSP), which in extreme cases can lead to death due to respiratory distress and muscle paralysis. This type of poisoning is caused by a group of naturally occurring potent neurotoxins produced by Alexandrium, *Gymnodinium* and *Pyrodinium* marine dinoflagellates, which are on the list of established phytoplankton species in the Black Sea. The seawater monitoring performed by the Institute of Oceanology-Varna, included investigation of phytoplankton at 13 stations along the Bulgarian coast during 2015 – 2016. The reports confirmed a presence of *Alexandrium spp.*, *Gymnodinium spp.*, *Prorocentrum spp.* up to 6% in the phytoplankton in Bulgarian Black Sea waters (18). These data suggest a high risk of occurrence of PSP toxins in Black Sea mussels.

Having regarded the commercial and nutritional importance of bivalve molluscs, Regulation (EU) No 853/2004 has introduced specific requirements for growing, harvesting, storage conditions and set health standards for maximum levels of marine biotoxins in bivalve molluscs. The Health Standards for live bivalve molluscs indicate that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities that exceed 800 micrograms per kilogram for paralytic shellfish poison. The sampling frequency for toxin analysis in molluscs was established in Regulation (EU) No 854/2004.

Considering the importance of the health problem connected with *E. coli* and PSP toxins and also the requirements of the European legislation, the aim of this study was: to evaluate the level of *E. coli* and PSP-contamination in cultivated mussels produced in farms from Bulgarian Black Sea.

**Materials and Methods**

**Sampling**

The study was performed on 55 samples of live mussels (*M. galloprovincialis*). They were originated from 20 aquaculture farms located in Bulgarian waters of the Black Sea. One sample was around 1 kg of mussels. After collection, the samples were immediately delivered to the laboratory at refrigeration temperature. The studies were carried out two seasons: warm (May - October) and cold (November - April) in the period 2015-2016. All of the samples were used for enumeration of *E. coli* and determination of the level of PSP-toxins.

**Sample preparation**

The microbiological analysis started not later than 24 hours after collection. At least ten individual organisms (meat and intra-valvular liquid) were used for preparation of microbiological laboratory samples. The selected mussels were opened with a flame-sterilized shucking knife and the meat and intra-valvular liquid were placed into a sterile stomacher bag.

**Determination of MPN/100g of *E. coli***

The most probable number (MPN/100 g) of *E. coli* was evaluated according to the ISO TS 16649-3/2005 reference method.

**PSP toxin analysis**
The sample preparation and analysis of mussels tissue extract were performed in accordance with the method described in AOAC OMA 2005.6. Chromatographic conditions and equipment

HPLC analysis was carried out by UlitMate 3000 Thermo Fisher Scientific Dionex, equipped with a quaternary pump UltiMate 3000, fluorescent detector Dionex RF 2000 with EX:340 nm and EM:395nm; chromatographic column Kinetex: C18, 150 mm x 4.6 mm, 5μm particle size; flow rate of the mobile phase 1ml/min.

Calibration curves

PSP toxins were identified by comparing chromatograms of certified reference materials (dc-GTX2,3; C1,2, dc-STX, GTX2,3; GTX5, STX, NEO, GTX1,4 and dc-NEO). The quantification of each PSP toxin in the samples was performed by calibration graphs with five concentration levels. Figure 1 shows a chromatogram of 9 certified reference materials obtained by a HPLC-FLD method after periodate oxidation.

The linearity of nine calibration graphs (r > 0.99) was established for the concentrations in the range of (20 to 800) μg STX.2HCL equivalent/kg. The limits of quantitation (LOQ) of the nine toxins were in the range of 20 to 89.3 μg STX.2HCL equivalent/kg. The total amount of paralytic toxins was presented as μg STX dihydrochloride equivalent/kg.

Results

Microbiological analysis

The MPN/100 g of E. coli determined in samples is presented in Table 1. The results varied between less than 2.0 x 10¹ MPN/100 g and 8.2 x 10² MPN/100 g. The samples with a content of E. coli over the limit (> 230 MPN/100 g) dominated in the warm season. The highest result of 8.2 x 10² MPN/100 g was registered in July 2016. The number of E. coli determined in samples during the cold period was in the range less than 2.0 x 10¹ MPN/100 g to 4.0 x 10² MPN/100 g.

Four samples with a content of E. coli higher than the limit were from mussels originated from the north coast of the Bulgarian Black Sea and three samples from the south part of the Bulgarian Black Sea. The distribution of the samples of live shellfish depending on the E. coli content is presented in Table 2.

The majority of samples (48 out of a total of 55 samples) contained E. coli in quantities not exceeding the acceptable limit of 230 MPN/100 g. The numbers of samples (24) with undetectable values of E. coli (below 20 MPN/100 g) were equal with those up to 230 MPN/100 g. The samples with over the limit content of E. coli during the warm season were twice as many as those found in the cold season.

PSP toxin analysis

All 28 samples from 2015 were negative for presence of PSP toxins – the quantities were below
the limit of detection. A small quantity of PSP toxins was identified in July 2016 in a sample from a farm on the northern coast. The chromatogram of the sample is shown in Fig. 2.

The positive sample contained the following PSP toxins:
- gonyautoxin 2,3 (GTX 2,3) with quantity 21.3 µg STX.2HCL equivalent/kg
- saxitoxin (STX) with quantity 29.8 µg STX.2HCL equivalent/kg.

The total toxicity of the sample was 51.1 µg STX.2HCL equivalent/kg. The content of E. coli determined in the sample was < 2.0 x 10^1 MPN/100 g.

**Discussion**

Data from our study showed that 12.7% of all 55 analysed samples exceeded the microbiological criteria for E. coli, listed in Commission Regulation (EC) No 2073/2005. The highest levels of contamination were in the mussels tested in the warm season. Significantly better was the hygiene status of molluscs during the cold period – 93% of the samples complied with the requirements.

Our results are in agreement with the findings of other authors. Panaiotov et al. (2012) identified toxigenic E. coli in the Black Sea waters during summer and early autumn. Stoica (2014) established that 16.7% of the mussel samples from the Romanian Black Sea coast exceeded the levels permitted by the European legislation. She registered the highest concentrations (up to 490 MPN/100 g) during the summer months. Soegianto and Supriyanto (2008) found a very low MPN of E. coli in October and December and a greater content of these bacteria during the summer months. Contrary to our results, Kacar (2011) reported a maximum faecal coliform level in mussels from Eastern Aegean Sea during the cold months (November and March) and the lowest value in July.

The summarised results for E. coli content in tested mussels during the period 2015–2016 showed a minimum risk for consumers of cultivated mussels, especially in the summer months, when the pollution of the sea water was greater. Food

| Table 1. Quantity of E. coli (MPN/100 g) in Black Sea cultivated mussels, 2015 – 2016 |
| Season | Number of the tested samples | MPN/100g |
|        |                              |          |
|        |                              | < 2.0 x 10^1 | Up to 2.3 x 10^2 | > 2.3 x 10^2 |
| Warm   | 28                            | 6          | 17           | 5 |
| Cold   | 27                            | 18         | 7           | 2 |
| Total  | 55                            | 24         | 24          | 7 |

<p>| Table 2. Distribution of the samples cultivated mussels according to the content of E. coli |</p>
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business operators have to perform strictly the Hygiene Requirements for The Production and Harvesting of Live Bivalve Molluscs Set in Regulation (EC) 853/2004, Annex III, Section VII.

The results of the analyses for the detection of biotoxins in cultivated mussels from Bulgarian Black Sea for the period 2015 – 2016 showed that there was no risk of paralytic shellfish poisoning for consumers. PSP toxins were detected only in one sample of all tested mussels. The detected quantities were close to the limits of quantitation of PSP toxins. The total toxicity (51.1 µg STX.2HCL equivalent/kg) of the sample was significantly lower than the health standard for PSP toxins – 800 µg STX.2HCL equivalent/kg.

The toxic profile of the sample from the present study, which contained saxitoxin (STX) and gonyautoxin 2,3 (GTX 2,3), complied with the Alexandrium spp. profile. This corresponds fully to the type of phytoplankton found in the Bulgarian Black Sea waters during the monitoring in 2015 - 2016. The presence of dinoflagellates of the genus Alexandrium was proven by Vershinin et al. (2006) in a 2001–2004 monitoring of the northeast Black Sea coast. They identified Alexandrium tamarense and Alexandrium minutum in the waters of the Caucasus coast and also small amounts of saxitoxin (STX), gonyautoxin 2,3 (GTX 2,3) and gonyautoxin 5 (GTX 5) were determined in extracts of mussel hepatopancreas, applying HPLC method and fluorescence detection.

The established lack of contamination with PSP toxins of Bulgarian cultivated mussels for the studied period 2015 – 2016 confirmed the results of our previous investigation (Kalinova et al., 2015). Of all 57 analysed samples for the period 2011-2014, PSP toxins were determined in 5 samples in quantities 2 – 3-fold less than the legal limit.

Regardless of the minimum risk, it was assumed that these results do not fully reflect the actual level of contamination with biotoxins, as manufacturers control the mussels once a year and there were no data for contamination in the remaining months. They do not perform the inspection required by Regulation (EC) No 854/2004, Annex II, Chapter II, point B (5): “The sampling frequency for toxin analysis in the molluscs is, as a general rule, to be weekly during the periods at which harvesting is allowed”.

**Conclusion**

This was the first parallel study of the amount of *E. coli* and paralytic toxins in mussels farmed along the Bulgarian Black Sea coast. No connection was established between the concentration of *E. coli* and PSP toxins in the tested samples. The level of contamination with *E. coli* and PSP toxins in cultivated mussels from the Bulgarian Black Sea
coast was minimal and their concentrations met the requirements of the European legislation.

Considering the conclusion of the EFSA report (2016) that bacteria and marine biotoxins were the causative agents in 11.9% and 2.9%, respectively, in food-borne outbreaks in EU countries, the Bulgarian competent authorities should reinforce the hygiene control of cultivated mussels.

Reference
Regulation (EC) № 2073/2005 of microbiological criteria for foodstuffs.
http://phyto.bss.ibss.org.ua/wiki/List_checked
http://www.bgodc.io-bas.bg/Monitorig