Enumeration of *Escherichia coli* and determination of *Salmonella* spp. and verotoxigenic *Escherichia coli* in shellfish (*Mytilus galloprovincialis* and *Ruditapes decussatus*) harvested in Sardinia, Italy

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Abstract

The aim of the present study was to evaluate the occurrence of *Salmonella* spp., Verotoxigenic *E. Coli* (VTEC) and enumerate *E. coli* in shellfish (*Mytilus galloprovincialis* and *Ruditapes decussatus*) collected before and after depuration from two class B harvesting areas located in Sardinia (Italy). All the samples were analyzed for *Salmonella* spp. detection according to European Commission Regulation (EC) 2073/2005 and examined using the five tube Most-Probable-Number (MPN) method for enumeration of *E. coli* in accordance with the European Union reference method ISO 16649-3:2015. *E. coli* VTEC was investigated following a direct multiplex Polymerase Chain Reaction (PCR) screening test. The enumeration of *E. coli* met the European law limit for Class A areas of 230 MPN/100g. The averaged enumeration of *E. coli* in samples of *M. galloprovincialis* and *R. decussatus* collected at the harvesting time was 39 and 37 MPN/100 g respectively. The average contamination levels in samples collected after purification were 58 MPN/100g (*M. galloprovincialis*) and 32 MPN/100 g (*R. decussatus*). *E. coli* VTEC was not detected, on the contrary, *Salmonella ser. Typhimurium* was detected in one sample of *M. galloprovincialis* and in one sample of *R. decussatus* collected at the harvesting time. No significant associations were observed between *E. coli* levels in shellfish and environmental parameters of water or with the detection of *Salmonella ser. Typhimurium* in *M. galloprovincialis* and *R. decussatus* samples. Nevertheless, the occurrence of *Salmonella ser. Typhimurium*, involved in human infection outbreaks, should be considered a potential risk for consumers.

Introduction

The evaluation of shellfish safety is based entirely on the use of food safety criteria laid down in Commission Regulation (EC) 2073/2005 and Commission Regulation (EU) 2285/2015: absence of *Salmonella* spp. in 25g of flesh and intervalvular liquid and an upper limit of 230 MPN *Escherichia coli*/100 g of flesh and intervalvular liquid in 80% of the samples. The 20% of the samples may contain *E. coli* between 230 and 700 MPN/100g. It is well known that shellfish contamination occurs because bivalve molluscs are filter-feeding animals that selectively filter and accumulate small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the environment (Carella *et al.*, 2010; Leoni *et al.*, 2017). This highlights the role of shellfish as vehicle for several hazards that could result in products that are unsuitable to guarantee the safety of consumers, particularly if live bivalve molluscs are consumed raw or lightly cooked (Rubini *et al.*, 2018; Sferlazzo *et al.*, 2018). Bivalve molluscs transmitted illness can be either due to indigenous bacteria as *Vibrio* species (Smaldone *et al.*, 2014; Leoni *et al.*, 2016) or from non-indigenous bacteria, usually enteric bacteria, derived from fecal contamination (mostly *Salmonella* spp., *E. coli VTEC*, *Shigella* spp.) (Marceddu *et al.*, 2017). In marine environments, *Salmonella* has been detected in coastal waters, molluscs, as well as other seafood products (Bazzoni *et al.*, 2019; Catalao Dionisio *et al.*, 2000). The microorganisms of the genus *Salmonella* spp. are introduced into the aquatic environment via inappropriate disposal of human wastes, agricultural runoffs or sewage discharges (Malham *et al.*, 2014) as well as wildlife (Obiri-Danso and Jones, 2000). Several authors reported a prevalence of *Salmonella* spp. in shellfish samples collected in Italy, ranging from 0 to 3.1% (Bazzoni *et al.*, 2019; Carraro *et al.*, 2015; Marceddu *et al.*, 2017; Prato *et al.*, 2013; Rubini *et al.*, 2018; Sferlazzo *et al.*, 2018;). Although *Salmonella* spp. is considered one of the most common causes of human gastroenteritis (EFSA and ECDC, 2016) and in spite of its presence in marine environments (Catalao Dionisio *et al.*, 2000), the risk of foodborne diseases associated with shellfish consumption is very low (Iwamoto *et al.*, 2010). According to Reg. (EU) 2285/2015, the enumeration of *E. coli* as an indicator of fecal contamination, is the standard way to estimate the associated potential risk to human health from all waterborne enteric pathogens (Balière *et al.*, 2015). Moreover, in addition to being considered as a fecal indicator, *E. coli* includes strains that can be pathogenic to humans (Touchon *et al.*, 2009). Pathogenic *E. coli* are distributed into diarrheagenic pathotypes including Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC) and Enteroaggregative *E. coli* (EAEC) (Baliere *et al.*, 2015). Among EHEC, verotoxigenic *E. coli* (VTEC) are strains that produce Shiga-like toxin (verotoxin). VTEC are not commonly found in seafood, but it is well known that bivalve molluscs harvested in areas contaminated by landfill can carry verotoxigenic *E. coli* (Marceddu *et al.*, 2017). Moreover, VTEC have been isolated in breeding environments from different sites such as soil, manure, sewage, drinking water, irrigation water, crops, and various equipment (Avery *et al.*, 2008). The gastrointestinal tract of ruminants is the main reservoir of VTEC (Caprioli *et al.*, 2005): from agricultural environments, they can be transferred to watercourses, especially during periods of high rainfall, and subsequently spread to coastal areas (Williams *et al.*,

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2008). Shellfish harvested in these areas can consequently concentrate the pathogen and thereby pose a risk to the health of consumers (Marceddu *et al*., 2017). Sardinia is one of the nationally relevant Italian shellfish production areas: the annual production accounts for 83% of the regional aquaculture, and it almost exclusively rests on *M. galloprovincialis* (Meloni *et al.,* 2015; Sferlazzo *et al.*, 2018). Specific literature in the Sardinian shellfish harvesting areas is limited. Therefore, in this study, we aimed to i) enumerate *E. coli* and detect *E. coli* VTEC and *Salmonella* spp. in two shellfish species (*Mytilus galloprovincialis* and *Ruditapes decussatus*); ii) study the effects of environmental changes on the *E. coli* seasonal distribution considering the interrelations existing among those parameters.

Materials and Methods

Sampling

The study was performed from April 2011 to May 2012 on shellfish samples (n. 68) collected from two class-B harvesting areas (named A and O) located in Sardinia (Italy). Samples of two shellfish species were included in this study: *M. galloprovincialis* (n. 34) and *R. decussatus* (n. 34). Forty-eight total samples were collected at the harvesting time from batches (n. 12). To evaluate the effects of purification on the safety of shellfish, samples (n. 20) from selected batches (n. 5) were collected after purification. The purification centers annexed to both production areas, applied short ''recirculating'' purification protocols (8h) previously described in Sferlazzo *et al.* (2018). The shellfish harvesting areas (Figure 1) were on the central-western coast (production area A) and on the North-Eastern coast of Sardinia (production area O). Sampling was performed by the authors. Environmental conditions (temperature, pH, and salinity) of the water were recorded. The samples were shipped refrigerated to the laboratories of the Department of Veterinary Medicine at the University of Sassari (Italy) and were analyzed within 24h of harvesting. Depending on the size of the *M. galloprovincialis* or *R. decussatus*, 15 to 30 bivalves of each sample were randomly selected for microbiological analysis. Samples were processed for microbiological detection, enumeration and graduated dilutions according to the Reg. (EC) 2073/2005, Reg. (EU) 2285/2015 and ISO 6887-3 method (ISO, 2003).

Microbiological analysis

Enumeration of E. coli

All the bivalve samples were examined using the five-tube Most Probable Number (MPN) method in accordance with the EU reference method ISO 16649–3 (ISO, 2015). In briefly, 75-100g of flesh and intervalvular liquid were added to 2 parts of Peptone water (BioMérieux, France) and homogenised using a Stomacher for 2.5min. Ten ml of the liquid part of the 1+2 suspension were added to a flask containing 90 ml of Peptone water (BioMérieux, France) resulting in a final 1+9 dilution. Aliquots of 10 ml of the initial suspension (1+2) were transferred to each of five tubes of double-strength Mineral Modified Glutamate Medium (MMGB) (Oxoid, UK). Aliquots

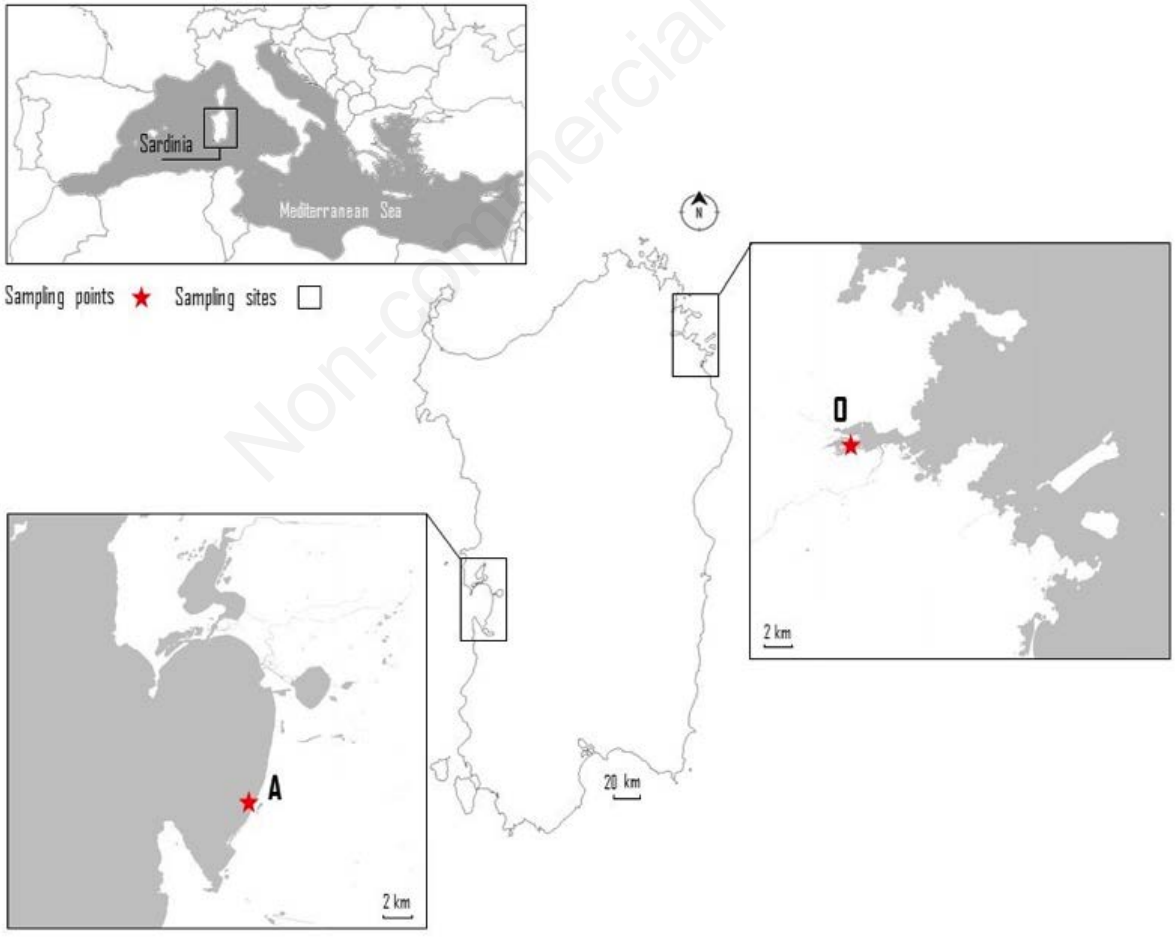

Figure 1. Shellfish harvesting areas located in Sardinia (Italy) included in the study.

of 1.0 ml of the 1+9 dilution were transferred to each of five tubes of single-strength MMGB. Further dilutions were prepared in the same way. All the double and single-strength MMGB were incubated aerobically at 37°C for 24h. Subcultures from positive MMGB tubes that changed colour from purple to yellow, were plated on chromogenic Tryptone Bile Glucuronide Agar (TBX) plates (Oxoid, UK) incubated aerobically at 44°C for 20h. At the end of incubation, the number of positive tubes of double or single-strength MMGB tubes were counted in order to estimate the level of *E. coli*/100g using the MPN table, generated with the MPN calculator referenced in ISO 7218 (ISO, 2007). The enumeration of *E. coli* in bivalve molluscs by the MPN technique has limits of detection of fewer than 18 *E. coli* cells.

Detection of E. coli VTEC

For the direct detection of *E. coli* VTEC, 10 g of each sample were subjected to selective enrichment in 90 ml of modified-Tryptone Soya Broth (m-TSB) containing novobiocin (20 mg/L) and incubated at 37°C for 18–20h. Subsequently, an aliquot was frozen at -20°C (Angelantoni Industrie Spa, Massa Martana, Italy) for Immuno-Magnetic Separation (IMS) screening test by using the protocol for the *E. coli* O157, O26, O103, O111, and O145 Dynabeads capture (Dynal, Oslo, Norway), as described by the manufacturer. Another aliquot equal to 1.0mL was used for DNA extraction using the Chelex 100 (BioRad, Hercules, CA, USA) resins. VTEC detection was carried out by a Polymerase Chain Reaction (PCR) one-step method for the detection of *stx*1 and *stx*2 genes using the primer sets MK1/MK2 (Karch and Meyer, 1989). A negative control (NCTC 12900) and a positive control (ATCC 35150) were included at each PCR test. All PCR amplifications were performed by using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). All PCR-positive samples (VTEC presence) were subjected to qualitative detection of *E. coli* O157, O26, O103, O145, and O111 serogroups by IMS using Dynabeads anti-E. coli (Invitrogen, Carlsbad, CA, USA). Each Dynabeads-microorganism complex was streaked on CT-SMAC (MacConkey agar cefixime tellurite sorbitol, Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) for the detection of serogroup O157, CT-RMAC (MacConkey agar cefixime tellurite rhamnose, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, UK) for O26, and EHLY (Enterohemolysin agar, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, UK) for serogroups O103, O111, and O145. All the plates were incubated at 37°C for 24 h. Isolates with typical morphological characteristics were subjected to biochemical identification by the API 20E identification system (bioMérieux, Marcy l'Etoile, France). All the isolated *E. coli* were subjected to a multiplex PCR for the detection of *stx*1, *stx*2, *hly*A, and *eae* genes (Paton and Paton, 1998).

Detection of Salmonella spp.

All the samples were analyzed for *Salmonella* spp. detection according to the ISO 6579 method (ISO, 2002). Briefly, 25 g of each sample were added to 225 ml of Buffered Peptone Water (BPW) and incubated at 37°C for 18 h (Thermo Fisher Scientific, Waltham, MA, USA). One-hundred µl of the BPW enrichment were inoculated in 10 ml of Rappaport-Vassiliadis Soya enrichment broth (RVS) and incubated at 42°C for 24 h while 100 µL of the BPW enrichment were streaked over the surface of a Modified Semi-Solid Rappaport-Vassiliadis agar plate (MSRV) and incubated at 42°C for 24 h. Finally, 1 ml of the BPW enrichment was transferred to 10 ml of Muller-Kaufmann tetrathionate-novobiocin broth (MKTTn) and incubated at 37°C for 24h. RVS, MKTTn, and MSRV were sub-cultured onto the surface of one Xylose-Lysine-Desoxycholate (XLD) agar plates and incubated at 37°C for 24h. Presumptive *Salmonella*-like colonies were submitted to phenotypic identification with the API ID 32E identification system (bioMérieux, Marcy l'Etoile, France). One colony from each positive sample was selected and sent to the laboratories of the *"Centro Nazionale di Referenza per le Salmonellosi"* in Legnaro (Padua, Italy), serotyped by agglutination tests with specific O and H antisera (Staten Serum Institute, Copenhagen, Denmark) and classified according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007).

Statistical analysis

Statistical analysis was performed with R software (version 1.0.153, R Foundation for Statistical Computing, Vienna, Austria). The enumeration of *E. coli* in *M. galloprovincialis* and *R. decussatus* collected in the two harvesting areas in relation to season, temperature, pH, and salinity were compared and analyzed with a linear regression model. The model was defined as follows: y= site + species + period + pH + temperature + salinity + e, where y is the contamination levels of *E. coli* expressed as MPN/100g; site is the effect of the harvesting area; species is the effect of the shellfish; period is the effect of the month and year of sampling; temperature (°C), pH, and salinity (%) express the effects of environmental parameters; and e is the error term. The mean values were compared with Tukey's honestly significant difference test. The results were considered statistically significant when $p<0.05$ for all the tests performed.

Results and Discussion

Environmental parameters of water

The environmental parameters of the two harvesting areas were averaged and were as follows (mean ± standard deviation): temperature, 17 ± 4.20°C; salinity, 36 ± 1.50 %; pH, 8.21 ± 0.26. Data are summarized in Table 1.

Table 1. Effects of purification on the levels of contamination by *E. coli* in shellfish (MPN/100g). Data from the two harvesting areas were averaged.

Month	*Mytilus galloprovincialis*		*Ruditapes decussatus*	
	Before purification	After purification	Before purification	After purification
May 2011	<18	40	<18	<18
July 2011	40	40	40	40
November 2011	40	40	61	40
March 2012	130	130	40	40
May 2012	61	40	40	40
Total	54	58	36	32

Microbiological analysis

Enumeration of E. coli and detection of E. coli VTEC

The results of enumeration of *E. coli* conducted in shellfish samples are summarized in Table 2. *E. coli* was found in 100% of the samples. As reported in Table 2, the averaged values of *E. coli* load were respectively 39 and 37 MPN/100g in samples of *M. galloprovincialis* and *R. decussatus* collected at the harvesting time. The average contamination levels in samples collected after purification were 58 MPN/100g (*M. galloprovincialis*) and 32 MPN/100g (*R. decussatus*). The purification treatment allowed a slight reduction in *E. coli* loads ($p<0.01$) only in the latter species. On the contrary, in Sferlazzo *et al.* (2018), purification allowed a progressive and significant reduction in *E. coli* loads ($p<0.01$) in 10 batches of *M. galloprovincialis*. However, the results of *E. coli* load were consistently in agreement with the legislation limits for class A harvesting areas (EU 2285/2015). As previously reported by Sferlazzo *et al.* (2018), this evidence, with a moderate initial contamination, allowed the Food Business Operator to effectively reduce the purification times (~8h). The results of the present study have been used by the Food Business Operators to support requests for re-classification of the harvesting areas.

Statistical analysis showed no significant difference ($p>0.05$) between *E. coli* levels and environmental parameters of water, seasonality, site and shellfish species. As reported by Bazzardi *et al.* (2014), Sardinian seawaters had stable salinity, temperature, dissolved oxygen, and pH during the year, without significant variations. However, recent studies showed a positive correlation between *E. coli* levels and seasonality: Bazzoni *et al.* (2019) found *E. coli* in three of the four seasonal mollusc samples, with the highest counts in autumn and winter (270 and 330 MPN/100 g, respectively). *E. coli* was not found in the summer. The same results were previously obtained by Sferlazzo *et al.* (2018), who found significantly lower ($p<0.05$) levels of *E. coli* contamination during the summer period. *E. coli* VTEC was not detected in samples of *M. galloprovincialis* and *R. decussatus* collected before as well after purification. In a recent study carried out in Sardinia, the prevalence of *E. coli* VTEC was 6.6% in samples of *Cerastoderma* spp. and *R. decussatus* (Marceddu *et al.*, 2017). The ten *E. coli* VTEC strains belonged to the serogroups O157, O26, O103, O145 and O11 and showed a complete pathogenicity profile *(stx1 +, stx2 +, eae +, hlyA +)*. As previously reported by Rodriguez-Manzano *et al.* (2014), the prevalence of *E. coli* VTEC in bivalve molluscs is low and could be related to the presence of competitive bacterial flora, and to the decreased vitality of these microorganisms *in vitro*.

Detection of Salmonella spp.

Salmonella spp. were detected only in one sample of *R. decussatus* (April 2011) and in one sample of *M. galloprovincialis* (November 2011) collected at the harvesting time. In a recent study carried out in Sardinia, *Salmonella* spp. were only present in samples of *M. galloprovincialis* collected in spring (Bazzoni *et al.*, 2019). On the contrary, in Marceddu *et al.* (2017) *Salmonella* spp. were not detected in any of *Cerastoderma* spp. and *R.decussatus* samples. As previously reported by Rubini *et al.* (2018), these results were not surprising: the presence of *Salmonella* spp. is strongly related to the shellfish species, the production areas and the sampling period. All the *Salmonella* spp. strains were identified as S. *enterica* subsp. *enterica* and *Salmonella ser. Typhimurium* resulted the only serovar (100%). In a recent Italian study (Rubini *et al.*, 2018), *Salmonella ser. Typhimurium* resulted the dominant serovar (26.9%). No significant associations ($p>0.05$) were observed between the detection of *Salmonella ser. Typhimurium* and the *E. coli* levels in *M. galloprovincialis* and *R. decussatus* samples.

Conclusions

In our study, we applied a combined microbiological and biomolecular approach to enumerate *E. coli* and detect *E. coli* VTEC and *Salmonella* spp. in two shellfish species harvested in Sardinia (Italy). Our results provided useful information regarding the health risks associated with the consumption of *M. galloprovincialis* and *R. decussatus*. Although the enumeration of *E. coli* was consistently within the legal limits (EU Regulation 2285/2015) in 100 % of the samples, this study has reported the presence of *Salmonella ser. Typhimurium* in *M. galloprovincialis* and *R. decussatus* samples. As it happens in the rest of South Italy, in Sardinia these shellfish species are usually consumed raw or slightly cooked (Sferlazzo *et al.,* 2018). *Salmonella ser. Typhimurium* is involved in most of human infection outbreaks and its recovering could

Table 2. Enumeration of *E. coli* (MPN/100g) in *Mytilus galloprovincialis* and *Ruditapes decussatus*. Data from the two harvesting areas were averaged.

Month	*Mytilus galloprovincialis*	*Ruditapes decussatus*	Temperature (°C)	Salinity (‰)	pH
April 2011	<18	<18	16.45	36.45	8.19
May 2011	<18	<18	20.00	35.40	8.41
June 2011	<18	78	22.00	36.61	8.27
July 2011	40	40	23.65	36.60	8.11
September 2011	40	40	24.10	37.90	7.81
October 2011	40	40	20.64	39.05	8.26
November 2011	40	61	16.90	33.25	7.94
December 2011	40	40	12.92	37.65	8.91
January 2012	40	40	11.07	35.70	8,17
March 2012	130	40	13.83	35.50	8.11
April 2012	40	40	16.20	36.00	8.15
May 2012	61	40	18.20	35.10	8.18
Total	39	37	17±4.20*	36±1.50*	8.21±0.26*

*mean±sd.

lead to products that are unsuitable for guaranteeing the safety of Sardinian consumers. Recent studies reporting collateral surveys carried out in *M. galloprovincialis* and *R. decussatus* harvested in the same period from the same production areas, highlighted the presence of potentially pathogenic *V. parahaemolyticus* isolates (*tdh* + or *trh* +) too (Lamon *et al*., 2019a; Lamon *et al.,* 2019b).

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