Occurrence of Salmonella enterica subsp. enterica in bivalve molluscs and associations with Escherichia coli in molluscs and faecal coliforms in seawater

Occurrence of *Salmonell*a spp. in bivalve molluscs and associations with *Escherichia coli* in molluscs and faecal coliforms in seawater

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ABSTRACT

The objectives of this study were to present data on the presence of *Salmonell*a spp. and on the enumeration of *Escherichia coli* and faecal coliforms respectively in different species of bivalve

molluscs and seawater and to conduct a retrospective evaluation to assess the capacity of *E. coli* in molluscs and faecal coliforms and *Salmonella* spp. in sea and brackish water to predict the presence of *Salmonella* spp. in bivalve molluscs, and therefore, the risk of exposure for consumers. Data were collected from 4972 seawater samples and 5785 live bivalve molluscs samples (2877 *Ruditapes philippinarum*, 2177 *Mytilus galloprovincialis*, 256 *Chamelae gallina* and 475 *C. gigas* and *O. edulis*) collected in the molluscs production area of Ferrara, Northern Italy, from 1997 to 2015. An overall *Salmonell*a spp. occurrence of 2.2% was reported in water and molluscs, with percentages varying depending on the type of sample and on the classification areas. All the 237 *Salmonella* strains were identified as *Salmonella enterica* and a total of 53 different serovars were observed. Significant associations between the fecal indicators and presence of *Salmonella* spp. were observed both applying EU and USA criteria, but, it should be noted that the EU approach seems to be more stringent achieving the goal of identifying the most critical batches (94 out of the 100) whereas, following the USA approach, a not negligible and higher number of batches compliant for faecal coliforms but contaminated by *Salmonella* spp. has to be mentioned. In any case, the faecal indicators *E. coli* in molluscs and faecal coliforms in seawaters reflect only in part the presence of *Salmonella* spp. in molluscs and the consequent potential risk for consumers. Microbiological evaluation of seawaters seems to have a minor impact into the prediction of *Salmonella* spp. presence in molluscs.

1. Introduction

In Italy bivalve molluscs such as Manila clams (*Ruditapes philippinarum*) and mussels (*Mytilus galloprovincialis*) represent products of great economic importance whereas striped clams (*Chamelae gallina*) and oysters (*Crassostrea gigas* and *Ostrea edulis*) are of less economic importance. Overall they are widely distributed in the food trade and, specifically for *R. philippinarum,* Italy is the second producer after China (Turolla, 2008).

In relation to the sanitary control of shellfish produced and sold for human consumption, two main control systems are in place in the European Community (EC) and United States of America (USA):

53 these are the principal systems used worldwide and countries that trade with EU and/or USA will use
54 either one, or a hybrid of the two systems (Gosling, 2015). In Europe, according to Regulation
55 853/2004, areas in which bivalve molluscs are cultivated in marine or brackish water must be
56 previously classified by veterinary authorities as production area A, B or C depending on the content
57 of *Escherichia coli* in the soft parts and flesh and intravalvular liquid of harvested bivalves, with an
58 upper limit of respectively 230, 4600 and 46000 MPN *E. coli*/100 g sample material is for class A, B
59 and C areas. Bivalve molluscs from class A area can be placed directly on the market provided that
60 they comply with microbiological criteria requirements, whereas molluscs from class B area must be
61 purified by resuspension at class A area, or heat-treated before distribution, and, finally molluscs
62 from class C area, a resuspension at class A area over a long period of time or a sufficient heat
63 treatment is needed.
64 In relation to food safety criteria laid down in EC Regulation 2073/2005 concerning bivalve to be
65 placed on the market, the absence of *Salmonell*a spp. in 25 g of flesh and an upper limit of 230 MPN
66 *E. coli*/100 g sample material are mandatory; according to EC Regulation 2285/2015, from 1st January
67 2017, 20% of the samples may contain *E. coli* between 230 and 700 MPN/100 g sample material
68 while the remaining 80% of the samples must be below 230 MPN/100 g sample material. In the USA,
69 the official controls system is based on the National Shellfish Sanitation Program (NSSP), that is a
70 federal/state cooperative programme recognized by Food and Drug Administration (FDA) and the
71 Interstate Shellfish Conference that is substantially different from the EU one; for example, the
72 microbiological monitoring is based on water testing and considers either total coliforms or faecal
73 coliforms, not *E. coli*, and to achieve 'approved' status the geometric mean faecal coliforms count
74 per water sample from an area must not exceed 14 Most probable Number (MPN)/100 ml, and the
75 ninetieth percentile must not exceed 43 MPN/100 ml (NSSP, 2017).
76 It is well known that live bivalve molluscs, being suspension feeders that gain nourishment by
77 pumping large volumes of water from the environment through their gills, actively filter and retain
78 particles from their surrounding water, including free living or particle bound bacteria, viruses and

parasites. This highlights the role of bivalve molluscs as vehicle for several hazards that could result in potential health risks for consumers, particularly if live bivalve molluscs are eaten raw or lighted cooked. With specific regard to bacteria, the microbiota found in bivalve molluscs include indigenous bacteria that naturally occur in marine or estuarine environments (mostly *Vibrio* spp.), non-indigenous bacteria, usually enteric bacteria, derived from faecal contamination (mostly *Salmonell*a spp., *E. coli*, *Shigella* spp. and rarely *Campylobacter* spp. and *Yersinia enterocolitica*), and bacteria from contamination during food preparation and processing by the distribution industry or consumers (*Bacillus cereus, Stapylococcus aureus* and *Clostridium perfringens*) (Anacleto, Pedro, Leonor, Rosa, & Marques, 2013).

Salmonella is the second most common cause of human gastroenteritis (EFSA & ECDC, 2016): the risks of foodborne illness associated with *Salmonella* in molluscs are low compared to viruses and *Vibrio* spp. (Iwamoto, Ayers, Mahon, & Swerdlow, 2010; NACMCF, 1992) but there is a considerable amount of epidemiological data regarding the presence of *Salmonella* in seafood and related illness. The EU summary report on zoonoses, zoonotic agents and food-borne outbreaks in 2015 reports that, for the causative agent of foodborne outbreaks by food vehicle, in "fish, shellfish, molluscs, crustaceans and products thereof", histamine was the leading cause of strong-evidence outbreaks (52.5%) followed by calicivirus including Norwalk-like virus (norovirus) (25%) and *Salmonella* (12.5%) (EFSA & ECDC, 2016).

Even if *Salmonella* spp. are not natural inhabitant of the acquatic environment, several *Salmonella* serovars are widely distributed in water (sea, estuarine, river) and in a variety of seafood, with the highest prevalence in molluscs, shrimp, clams, and various fish species (Novoslavskij et al., 2016). *Salmonella* spp. and, in general, faecally derived enteric pathogens, are introduced into the aquatic environment via anthropogenic activities like inappropriate disposal of human wastes, agricultural runoffs or sewage discharges (Malham et al., 2014) as well as wildlife (Obiri-Danso & Jones, 2000), and, given the reported ability to survive long periods in different aquatic environments, these microrganisms could pass into new hosts (Amagliani, Brandi, & Schiavano, 2012). Yet the use of

faecal bacterial indicators in bivalve monitoring was based on their relation to the probable occurrence of human faecal pathogens: it has been well remarked that no correlation was between faecal microorganisms and both *Vibrio* spp. and viruses for humans, but, the true evidence of this correlation in relation to *Salmonella* spp. is still a matter of debate.

The current publication presents data on the presence of *Salmonell*a spp. and on the enumeration of *E. coli* in different species of bivalve molluscs, namely Manila clams (*R. philippinarum*), mussels (*M. galloprovincialis*), striped clams (*C. gallina*) and oysters (*C. gigas* and *O. edulis*), and on faecal coliform levels in water samples from seashore and inland channels of the Ferrara province, Emilia-Romagna Region, Northern Italy with a continuous sampling history from 1997 to 2015. In addition, a retrospective evaluation was conducted to assess: i) the capacity of the faecal indicator *E. coli* to predict the presence of *Salmonella* spp. in bivalve molluscs, and therefore, the risk of exposure for consumers; ii) the efficacy of sea and brackish water analysis for faecal coliforms and *Salmonella* spp. to predict the presence of *Salmonella* spp. in live bivalve molluscs iii) the comparison of the regulations currently in force in USA versus Europe.

2. Material and methods

2.1. Sample and data collection

Microbiological records for *E. coli,* faecal coliforms and *Salmonella* spp. analyses of live bivalve molluscs and water were collated from official monitoring performed by the regional Veterinary Authority from 1997 to 2015 and from a shellfish monitoring program that has been carried out since 1997 in the province of Ferrara, Emilia Romagna region, Italy. A total of 10757 samples were collected, respectively 4972 seawater samples (of which 1237 in class A area and 3735 in class B area) and 5785 live bivalve molluscs samples; the examined bivalve molluscs comprise 2877 Manila clams (*R. philippinarum,* all in class B area), 2177 mussels (*M. galloprovincialis,* 969 and 1208 in class A and B areas), 256 striped clams (*C. gallina,* all in class A area) and 475 oysters (*C. gigas* and *O. edulis,* respectively 62 and 413 in class A and B areas). A total of 4815 paired samples of bivalve

mollusc and seawater were collected. Each mollusc sample comprised at least 10 live individuals (commercial size or adult product) that were analyzed for *Salmonella* spp. detection and *E. coli* enumeration. Seawater samples were collected in from 0.2 m below the water surface using 1.2 L polypropylene or glass bottle for *Salmonella* spp. detection and for faecal coliforms count. All samples were transported under chilled conditions to the laboratory and processed within 24 h after collection.

2.2. Sampling site

All the samplings were performed in the north-western area of the Adriatic Sea facing the Po river delta, the major Italian river which, from spring to estuary, flows through the Po Valley (Pianura Padana) for a total of 652 Km. The Po Valley is a high populated area with abundant large animals intensive farms. The Po River, near to its end in the Adriatic sea, creates a wide delta with a surface area of 31 Km^2 and an average depth of 1.5 m; its hydrographic network is for the most part artificially regulated, and, as a consequence, freshwater flows are partially independent of rain events. More than one third of the lagoon surface is exploited for clam farming, with an annual production that reached a maximum of 87000 t y-1 in 2011 (Bison, 2012).

The areas of molluscs production have been divided into 5 sub-areas (see Figure 1): i) long-line: the marine class A area used to breed mostly mussels and secondary oysters; ii) Lupini: the coastal marine area including seawaters between 1 and 2 nautical miles that is classified as class A area in which natural banks of striped clams are present and harvested; iii) B-Out: the narrow sea coastal area and inland waters classified as class B area; iv) B-In: class B area that includes the inner channels directly connected to the sea, together with internal waters; v) Sacca: the class B area included between the Po river and the marine coastline. All these last three sub-areas were used to breed mussels, Manila clams and oysters.

2.3. Microbiological analyses

157 All samples were analyzed at the Experimental Institute for Zooprophylaxis of Lombardy and Emilia
158 Romagna in Ferrara. For live bivalve molluscs, the isolation of *Salmonella* spp. was performed using
159 the official International Organization for Standardization (ISO) cultural methods, UNI EN ISO
160 6579:1993 still 2002, the ISO 6579:2002 since 2003 with its technical corrigendum and amendments,
161 whereas, *E. coli* was enumerated using a 5 tube 3 dilutions MPN procedure based on ISO 16649-3.
162 For water analysis, the isolation of *Salmonella* spp. was carried out filtering a sample volume of 1000
163 mL through 0.45 µm-pore size membrane filters according to APAT CNR IRSA 7080 procedure
164 (APAT, 2003); the enumeration of faecal coliform and *E. coli* from 1998 to 2014 were performed by
165 a five tube three dilutions MPN methods according respectively to APAT CNR IRSA 7020 and 7030
166 procedures (APAT, 2003), whereas from August 2014, a membrane filtering method was applied
167 (APAT, 2003).
168 Cultures displaying a reaction typical of *Salmonella* (an alkaline slant and acid butt, with or without
169 production of H_2S) were confirmed by biochemical tests using miniaturized galleries, e.g. API-20E
170 strip (bioMérieux, Marcy l'Etoile, France) or MID (Microgen Bioproduct Ltd, Camberley, United
171 Kingdom) (Anacleto et al. 2013; APAT, 2003). The serotyping of *Salmonella* strains was performed
172 using commercial antisera (BBL Becton Dickinson, Franklin Lakes, USA; Statens Serum Institut,
173 Copenhagen, Denmark); following the White-Kauffman-Le Minor serotyping scheme (Baudart,
174 Lemarchand, & Brisabois, 2000).
175
176 *2.4. Statistical analysis*
177 Fisher's exact test were used to study the association between the occurrence of *Salmonella* spp. in
178 molluscs and: i) *E. coli* levels in molluscs; ii) faecal coliform levels in paired seawater; iii) presence
179 of *Salmonella* spp. in paired seawater.
180 To perform the statistical analysis the *E. coli* counts in molluscs were divided based on the *E. coli*
181 contamination level laid down into EC Regulation 2073/2005 as food safety criteria applicable for
182 products from class A areas, namely collected for direct human consumption and placed on the

183 market, irrespective of the fact that samples were collected from class A or B areas. Therefore, two
184 groups were considered using 230 MPN/100g as cutoff. Similarly results of fecal coliforms count in
185 water were divided in three categories identified according to the classification approach used in USA
186 with standards set for categories ranging from waters with low contamination levels, ≤ 14, between
187 14 and ≤ 43 and > 43 MPN/100 ml water. Data on faecal coliforms was available since 1999.
188
189 **3. Results**
190 A total of 237 out of 10757 samples (2.2%) were positive for *Salmonella* spp., respectively 137
191 seawater samples (2.8%) and 100 bivalve molluscs (1.7%): the presence of *Salmonella* spp. ranged
192 from 0 to 3.4% depending on the type of sample and on the classification areas (Table 1).
193 The overall *Salmonella* spp. prevalence differs considerably through the years and between water
194 class areas and species of molluscs: water prevalences were of 0 to 4.2% and of 0 to 13.8%
195 respectively for class A and B areas; in molluscs, *R. philippinarum* (collected only in class B area)
196 reported higher prevalences of 0 to 8.2% followed by *M. galloprovincialis* (0 to 4.3% and of 0 to
197 2.4% respectively for class A and B areas), *C. gallina* (collected only in class A area) with prevalences
198 of 0 to 4.3% and lastly *C. gigas* and *O. edulis* with prevalences of 0 to 2.1%.
199 All the 237 *Salmonella* strains were identified as *Salmonella enterica* and a total of 53 different
200 serovars were observed (Figure 2), respectively 43 and 32 serovars from the 137 and 100 seawater
201 and molluscs positive samples. *Salmonella* ser. Typhimurium resulted the dominant serotype
202 (26.9%), followed by its monophasic variant 4,[5],12:i:- (11.8%), *Salmonella* Derby (6.3%),
203 *Salmonella* Newport (5.5%), and *Salmonella* Thompson (4.6%).
204 A significant association was observed between *E. coli* levels in molluscs and presence of *Salmonella*
205 spp. in molluscs samples, both from class A and B areas. In more details, an association was observed
206 in *R. philippinarum* from class B area (no *R. philippinarum* were on class A area) and in *M.*
207 *galloprovincialis* collected only from class A area, not for class B area (table 2).

208 A significant association was observed between faecal coliforms levels in seawater and presence of
209 *Salmonella* spp. both in total molluscs and *R. philippinarum* samples (table 3).
210 A significant association between presence of *Salmonella* spp. in molluscs and paired seawater was
211 observed only for *R. philippinarum* samples (see table 4), where a co-presence of *Salmonella* spp. in
212 13 samples was observed. In 5 samples the same serovar was isolated in the two different matrices,
213 respectively *S. enterica* serovars Typhimurium (3 samples), its monophasic variant 4,[5],12:i:- (1
214 sample) and *Salmonella* Thompson (1 sample).
215

216 **4. Discussion**

217 Only safe food should be placed on the market (Regulation EC 178/2002). With regards to bivalve
218 molluscs, the climate change, the pollution and the anthropogenic factors as well as the food
219 distributions and consumers behaviour changes (undercooked or raw seafood consumption, mainly
220 bivalve molluscs) have created a cunning environment in which each element may have a potential
221 impact on food safety. In this context, it appears evident that the acquatic environment and its quality
222 and/or safety is a critical point for live bivalve molluscs that, being excellent bio-samplers, reflect the
223 quality of the surrounding water in a given location. This study, reporting data on the presence of
224 *Salmonell*a spp. and on the enumeration of *E. coli* in different species of bivalve molluscs and of fecal
225 coliform in water samples, with a continuous sampling history over 19-year-period (1997 through
226 2015), is useful to present the real scenario in which bivalve molluscs are bred in the molluscs
227 production area of Ferrara, leading us to perform some considerations.
228 An overall occurrence of *Salmonell*a spp. of 2.2% was reported in water and molluscs collected in
229 the considered production area: this rate could be considered low, but, at the same time poses a not
230 negligible threat for the assessment of the risks of faecal pollutions in the aquatic environment. The
231 significance of *Salmonella* spp. as human pathogen and as a leading cause of food-borne illness is
232 well known, but, the observed occurrence suggests to reconsider the role and the ecology of this
233 allochthonous pathogen at the base of the marine and estuarine environment and ecosystem; in fact,

234 although allochthonous organisms are historically viewed as transient member of the microbial
235 community of coastal waters, the coastal environment may serve as an important niche for these
236 microrganisms to persist, exchange genetic material, and grow (Bienfang et al., 2011).
237 The observed *Salmonella* spp. occurrence in seawater and bivalve molluscs is in agreement with
238 available data in literature, in which the prevalence rates considerably vary depending on climate
239 conditions, the season and area of samplings, the type of considered seafood and similar or different
240 environmental conditions (estuarine or seawater, after rainfall, low solar radiation) (Amagliani et al.,
241 2012; Novoslavskij et al., 2016); in Italy, *Salmonella* spp. prevalence rates from 0 to 3.1% were
242 reported (Normanno, Parisi, Addante, Quaglia, & Dambrosio, 2006; Mazzette, Virgilio, Piras,
243 Tempesta & Serra, 2010; Serracca et al., 2010; Prato et al., 2013; Fusco, Aprea, Galiero, Guarino, &
244 Viscardi, 2011; Carraro et al., 2015; EFSA & ECDC, 2016). In our 19-years period of monitoring in
245 live bivalve molluscs, from class A areas (products that can be placed directly on the market) only 6
246 out of the 1287 (0.5%) samples collected resulted *Salmonella* spp. positive, showing a good insight
247 the official monitoring usefulness. In the other hand, a total of 94 samples resulted *Salmonella* spp.
248 positive among the 4498 (2%) molluscs collected from class B areas (molluscs that may be placed on
249 the market only after treatment in a purification centre or after relaying); no food safety considerations
250 could be gathered from these data, but, again, the EU classification areas approach achieves the goal
251 of identifing the most critical batches (94 out of the 100).
252 Lastly, it should be noted that 87 out of total 100 *Salmonella* spp. positive molluscs were *R.*
253 *philippinarum* and, therefore, an higher *Salmonella* spp. prevalence in Manila clams than the other
254 bivalve molluscs species considered in this study has to be reported, according with the studies of
255 Anacleto et al. (2013), that reported an occurrence of 17% and 25%, respectively for *V. pullastra* and
256 *R. philippinarum*, and of Carraro et al. (2015) that observed the presence of *Salmonella* spp. in only
257 one sample of Manila clam among the 540 samples analyzed.
258 In literature, other studies investigated the correlation between the presence of *Salmonella* spp. and
259 the bacterial indicator of faecally pollution *E. coli* in bivalve molluscs, even if with contrasting results,

but, as far as we known, no other studies were on the comparison of bacterial indicators of faecal pollution *E. coli* in molluscs and faecal coliforms in water (representative of EU and USA monitoring approaches) in relation to the power of prediction of the presence of *Salmonella* spp. in bivalve molluscs.

Literature reports that bacterial indicators of faecal pollution in water provide an adequate indicator of *Salmonella* spp. presence in waters (Efstratiou, Mavridou, & Richardson, 2009; Ferguson, Coote, Ashbolt, & Stevenson, 1996; Morinigo, Castro, Balebona, Munoz, & Borrego, 1992) and in bivalve molluscs, particularly in *R. philippinarum*, or in other bivalve species like oysters (Anacleto et al., 2013), but, on several occasions or no *Salmonella* spp. was detected in samples with high indicator counts or *Salmonella* spp. was detected in samples with low indicator counts (Dionisio, Joao, Ferreiro, Fidalgo, & Garc, 2000; Efstratiou, Mavridou, & Richardson, 2009; Hood, Ness, & Blake, 1983; Mannas, Mimouni, Chaouqy, Hamadi, & Martinez-urtaza, 2014; Morigligo, Cornax, Oz, Romero, & Borrego, 1990). In this context, despite our monitoring was not drawn with the specific aim of testing these associations, and therefore, it could be biased by several factors, our study results is a useful and robust tool for assessing the *E. coli*, faecal coliform and *Salmonella* spp. parameters all together in a long period of time and in a specific area and considering both water versus molluscs and mollusc versus mollusc and some considerations could be performed from our findings.

A significant associations between fecal indicators and presence of *Salmonella* spp. was observed both applying EU and USA criteria prevalently for *R. philippinarum*, whereas, the weight carried for the other observed associations resulted of less importance or partially affected by *R. philippinarum* results. Considering that the *R. philippinarum* data we analyzed belongs exclusively from class B area, it seems evident that or this fact may has biased our findings or *R. philippinarum* really has a different behavior with respect with other bivalve species.

It should be noted that, following EU regulations and its official monitoring system, only two batches (0.2%) with *E. coli* levels ≤230 MPN/100 g in molluscs from class A area showed the presence of *Salmonella* spp. in molluscs, whereas, following the NSSP, a total of 58 batches (1.6%) with faecal

coliforms ≤14 MPN/100 ml in seawaters, were contaminated by *Salmonella* spp. Shellfish from "approved" waters, namely waters in which the MPN faecal coliforms median does not exceed the considered value of ≤14 MPN/100 ml water, can be sold directly on the market without prior treatment (Gosling, 2015). In this specific case, a not negligible and higher number of batches compliant for the faecal indicators but contaminated by *Salmonella* spp. has to be mentioned. This scenario is not representative of the current situation for two reasons: i) the sampling plan was not designed to assess the microbiological quality of waters according to NSSP; ii) the statistical evaluation could not be performed calculating the geometric mean of fecal coliforms enumeration but only singular values were considered. On the other hand calculating the gemetric median of all data collected in this study on fecal coliform contamination of water a level of 12 MPN/100 ml water was obtained, finding that could be considered compliant with NSSP. Despite our attempts, in one hand, this estimation should be considered anyway a forced parameter which remains an important data gap having some implications in the final outputs of this association, but in the other, it should be noted that, in general, concentrations of faecal coliform bacteria under steady-state conditions are approximately 10 to 100 times higher in bivalves than the surrounding seawater (Strubbia, Lyons, & Lee, 2016; Bernard, 1989). This demonstrates the fact that the occurrence of faecal bacteria in waters is highly variable and depending on several factors including pollution sources as well as environmental conditions that are not easily identifiable and testable. This observation should be taken into account in relation to the different approaches used for faecal indicators, that in the EU are measured in shellfish flesh while in the USA in shellfish-growing waters.

5. Conclusion

This study has reported the presence of *Salmonella* spp. in seawaters and in bivalve molluscs collected in the Ferrara area from 1997 to 2015 and has demonstrated that its presence varied by bivalve species considered, classification areas in which molluscs were collected and sampling occasion. The faecal indicators *E. coli* in molluscs and faecal coliforms in seawaters reflect only in part the presence of

Salmonella spp. in molluscs and the consequent potential risk for consumers. Microbiological evaluation of seawaters seems to have a minor impact into the prediction of *Salmonella* spp. presence in molluscs.

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