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Comprehensive analysis and assessment of exposure to enteric viruses and bacteria in shellfish

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ABSTRACT

Shellfish species, including oysters, clams, and mussels, are extensively cultured in coastal waters. Its location is determined by factors such as nutrient availability, water temperature, tidal cycle, and the presence of contaminants such as *Escherichia coli* and enteric viruses. With the expansion and intensification of human activities at vicinities, the presence of anthropogenic contaminants has increased, threatening shellfish farms and consumer safety give the prevalent consumption of raw shellfish. This literature review aims to provide a comprehensive analysis of the dietary exposure and assess the risk associated with enteric viruses and bacteria detected in shellfish. The predominant bacteria and viruses detected in shellfish are reported, and the potential interrelation is discussed. The main characteristics of each contaminant and shellfish were reviewed for a more comprehensive understanding. To facilitate a direct estimation of exposure, the estimated daily intake (EDI) of bacteria was calculated based on the average levels of *E. coli* in shellfish, as reported in the literature. The mean daily ingestion of seafood in each of the five continents was considered. Asia exhibited the highest intake of contaminants, with an average of ±5.6 *E. coli* units/day.kg body weight in cockles. Simulations were conducted using recommended shellfish consumption levels established by state agencies, revealing significantly lower ($p < 0.01$) EDI for all continents compared to estimations based on recommended levels. This indicates a higher risk associated with healthy shellfish ingestion, potentially leading to increased intoxication incidents with a change in dietary habits. To promote a healthier lifestyle through increased shellfish consumptions, it is imperative to reduce the exposure of shellfish species to bacteria and enteric viruses. The conventional use of *E. coli* as the sole indicator for consumption safety and water quality in shellfish farms has been deemed insufficient. Instances where shellfish met *E. coli* limits established by state agencies were often found to be contaminated with human enteric viruses. Therefore, a holistic approach considering the entire production chain is necessary to support the shellfish industry and ensure food safety.

1. Introduction

Water quality has been threatened by the expansion and intensification of human activities. The impairment caused by anthropogenic contaminants and pollution is leading to important effects on food chain and environmental sustainability (Fehrenbach et al., 2022; Schweitzer and Noblet, 2018; Vasquez-García et al., 2022). Such effects can be transmitted within the aquatic ecosystem and reaching toxic levels for the host or predator. Shellfish species such as oysters, mussels and clams are constantly exposed to contaminants. Many of these species inhabit areas such as tidal flats, brackish coastal waters, and reefs, based on the nutrient availability, water temperature, tidal cycle, and shelter. Their vulnerability to contaminants is often exacerbated by their feeding behaviours. For example, species such as the bivalve *Mytilus edulis* filters large volumes of water to obtain phytoplankton, or the crab *Procambarus clarkia*, a widely distributed crustacean and benthic feeder; both have

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been reported as bioindicators of environmental contamination (Li et al., 2020; Pastorino et al., 2023). This literature review aims to provide a comprehensive analysis of the dietary exposure and assess the risk associated with enteric viruses and bacteria detected in shellfish.

The placement of shellfish farms is carefully determined by environmental agencies, taking into account factors such as tide patterns and potential pollutant activities nearby in the vicinity. European regulations classify the production areas according to the levels of *Escherichia coli* in the shellfish flesh and intervalvular liquid (EC, 2019; ECR, 2006). *E. coli* serves as a feasible indicator of faecal contamination and is typically linked to the discharge of untreated or poorly treated urban effluents and agricultural runoff. However, relying solely on *E. coli* analysis might not adequately ensure consumer safety. It's imperative to examine other contaminants such as human enteric viruses that can also lead to diseases and not necessarily have its presence dependent on *E. coli*. Dirks et al. (2021), for example, analysed the presence of NOV and hepatitis A virus (HAV) along the food chain in the Netherlands at post-harvest, dispatch centres and retail. The authors reported a positivity for NOV RNA in 53.1% of the mussels and 31% in the oyster samples analysed from 2013 to 2017, with a poor correlation between NOV RNA copies and *E. coli* most probable number (MPN), suggesting that *E. coli* may not serve as ideal biomarker for accurately assessing the true contamination levels in shellfish, highlighting the importance of considering the subsequent stages in the shellfish processing and depuration processing may also contribute to the presence of these contaminants in the market.

Bacteria and human enteric viruses exhibit stability in the environment, particularly when accumulated within shellfish tissues and organs. Even at low concentrations, these contaminants can proliferate within the shellfish, especially if transportation, handling, and commercialization processes are not maintained at low temperatures (Dirks et al., 2021; EFSA, 2012). Based on the concentration of bacteria and human enteric viruses in the shellfish available at market and/or accumulation potential *in vitro* studies, it is possible to estimate the hazard associated with the consumption of raw shellfish. Continuous monitoring of water quality is also a key strategy to prevent the buildup of contaminants in shellfish. This data obtained from monitoring or routine analysis can serve as valuable inputs for prediction models, as demonstrated by Hunt et al. (2023). They presented a Monte Carlo exposure model for norovirus (NOV), which estimates consumer exposure per serving, considering oyster consumption per individual rather than total flesh weight. Estimating the exposure to contaminants is a useful tool to mitigate foodborne diseases and facilitate decision-making. In this paper, the EDI for *E. coli* was calculated based on the average levels in the literature and to facilitate a direct estimation of exposure within the consumption of shellfish. The EDI measures exposure to contaminants through dietary habits, providing a direct exposure diagnosis and insightful data for seafood producers and health agencies. An extensive review of the predominant bacteria and enteric viruses in shellfish is also provided. The main characteristics of each contaminant and shellfish were reviewed to provide an overview to the reader for better comprehension.

2. Contaminants

2.1. Human enteric viruses in shellfish

Human enteric viruses occur globally and are responsible for food and water-borne gastrointestinal diseases. Mainly RNA viruses, these viral genera replicate in epithelial cells of the small intestine, leading to gastroenteritis characterized by diarrhoea and vomiting (Bishop and Kirkwood, 2008). Other symptoms such as nausea, abdominal pain and fever can also be associated to human enteric viruses. The pathways of human enteric viruses from water to shellfish can involve several steps and mechanisms. The contamination of water resources with non-treated urban wastewater and agricultural waste and, consequently, the food chain, is the main route of human infection (Campos et al., 2017; Flannery et al., 2013). With the advent of microscopy and sequencing methods, the identification of source of contamination (e.g. run-off or wastewater discharge) can be done by comparing with viral profiles in surroundings as in taking samples of food or of surface/-groundwater and comparting these to the wastewater profiles. These advances also allow the precise differentiation and classification of human enteric viruses. Nowadays, most gastroenteritis cases in humans are associated with caliciviruses, while rotaviruses (RT) are the main cause of life-threating diarrhoea in children less than 5 years old (Bishop and Kirkwood, 2008).

The runoff of slurry and cattle manure plays a major role in contamination of shellfish waters. They can increase the levels of nutrients in water, promoting the eutrophication and potentially leading to a reduction of dissolved oxygen. This imbalance can also cause the overgrowth of toxic species and accumulation of human enteric viruses. In a simulation reported by Ramos et al. (2006) studying the effect of application of slurry to remediate erosion, the soil detachment was reduced by 70% but the runoff volume increased by 30%, having rivers and coastal areas as the final destination of surface waters. Porcine circovirus type 2 (PCV2), a pathogen that had only been detected in pigs, was also detected in *M. edulis*, indicating a decrease on water quality and rise of shellfish exposure to in land activities (Krog et al., 2014). The authors also tested for rotaviruses (RV) and hepatitis E virus (HEV) which are commonly detected zoonotic viruses in pig slurry, however the analysed samples were negative, confirming the stability of PCV2 in the environment. Not perceived as posing a risk to public health, the transmission however flags a contamination route for other enteric viruses transmitted from human to pig and shellfish, consequently. The stability of PCV2, attributed to its diminutive size and compact structure, implies its potential utility as a surrogate for assessing the presence of other viruses. This suggestion arises from the notion that PCV2, with its resilience, could serve as an indicator for the existence of other viruses that may be present at lower concentrations or beneath the limit of detection. Another study, Fusco et al. (2019), screened for the presence of viruses in shellfish harvest areas in the Gulf of Naples (Italy) to assess water quality over a three-year period. In 289 samples from Class A (16%), B (82.1%) and C (0.3%)[1] installations classified according to EC (2019), the most commonly detected virus was norovirus genogroup 2 (NOVg2) (39.7%), followed by astrovirus (ASTRO) (20.8%), sapovirus (SaV) (18.8%), norovirus genogroup 1 (NOVg1) (10.8%), rotavirus (ROTA) (9%), hepatitis A virus (HAV) (8.9%), aichivirus (AIC) (5.6%) and adenovirus (ADEN) (5.6%). Of the Class A and B samples, 20 and 136 tested positive for at least one enteric virus, respectively. Suffredini et al. (2020) assessed the prevalence of enteric viruses in *Crassostrea gigas* and *Meretrix lyrata* over a period of nine months in samples from fish markets and supermarkets in Vietnam. NOVg2 was detected in 79.3% of the 121 samples, followed by NOVg1 (50.4%), AsV (12.4%), AiV (11.6%), hepatitis E virus (11.6%) and HAV (1.7%). A relationship between AiV genotype detected in *C. gigas* and *M. lyrata* and the epidemiological profile in Vietnam was established as both belonged to genotype A. Sequencing also revealed the presence of NOVg2 swine genotype in 6 samples analysed, indicating a potential contamination of water with slurry from pig farms.

2.1.1. Norovirus

The norovirus (NOV) genus constitutes caliciviruses of primary concern for shellfish farmers and consumers (Fusco et al., 2019). They are widely detected in shellfish, whose filter-feeding capacity allows the

[1] Class A: ≤230 *E. coli* per 100 g of flesh and intravalvular liquid in 80% of the samples and should not exceed 700 *E. coli* per 100 g in the remaining 20% of samples.Class B: ≤4600 *E. coli* per 100 g of flesh and intravalvular liquid in 90% of the samples and 46,000 *E. coli* per 100 g in the remaining 10%.Class C: ≤46,000 *E. coli* per 100 g of flesh and intravalvular liquid.

shellfish such as oysters to bioaccumulate up to 100 times the level of viruses in seawater (Richards, 2016). In a systematic review describing 32 years of shellfish-related outbreaks, NOV was involved in 83.7% of the cases, followed by HAV, which was associated with 12.8% of outbreaks (Bellou et al., 2013). Fresh shellfish consumption also favours direct exposure to NOV, which has a short incubation time of 1–2 days, when the first symptoms as nausea, diarrhoea, abdominal cramps, vomiting and fever are experienced, potentially leading to dehydration. The infectivity of NOV differs depending on its genogroup: GI, GII and GIV infect humans; GIII infects cattle; and G5 infects mice (Bishop and Kirkwood, 2008; Campos et al., 2017). Outbreaks are often associated with GI and GII genotypes. NOVg2 prevalence in shellfish is usually higher than NOVg1 (Dirks et al., 2021; Fusco et al., 2019; Suffredini et al., 2020).

NOV tends to accumulate in shellfish tissues and digestive tract where it may be phagocytized by haemocytes in the same way as other calciviruses. The accumulation of NOV in shellfish and high number of human cases demand a routine analysis of NOV levels in shellfish. Seasonality of NOV is observed with increased detection in autumn and a peak during winter months (Dirks et al., 2021; Lowther et al., 2018). Rincé et al. (2018) reported higher levels of NOV at lower water temperature during winter, with 22% positivity for NOV in shellfish (oyster, mussel and cockle) mainly harvested during autumn and winter from harvesting areas in Brittany and Normandy, in France. Of the 22% positive samples, just over 8% were under the limit of quantification (LOQ) of the method (35 RNA copies per gram of digested tissue). This fact was also observed by Campos et al. (2017), with significantly higher levels of NOV in shellfish water catchments in England and Wales at <5 °C than in samples >10 °C. In that study, water temperature, catchment area versus urban area and the combined volume of continuous sewage discharges were found to predict NOV presence - potentially linked to oyster metabolic function or seasonal prevalence. Water temperature and volume of sewage discharge were found to predict the levels of total NOV, with temperature as a key variable.

The routine determination of NOV is not established by United States and European Union regulations, for example, but indirectly assessed using *E. coli* as an indicator. *E. coli* is employed due to its natural presence in the human digestive tract and warm-blooded animals. Its detection in water serves as an indicative measure, suggesting potential faecal contamination. However, many authors reported a poor correlation or lack of correlation between *E. coli* levels and NOV levels in shellfish. Dirks et al. (2021) for example, reported a low correlation (Pearson's R2 <0.1 at $p = 0.6$) between the most probable number (MPN) of viable *E. coli* in bivalve molluscs from dispatch centres and retail facilities, and detection levels of NOV. In a one-year survey of NOV in oysters at the retail level, Lowther et al. (2018) reported a positivity for NOV RNA in 68.7% of analysed samples, while the great majority 76.5% tested negative for *E. coli*.

2.1.2. Sapovirus

Other calciviruses such as sapovirus (SaV) can also accumulate in shellfish and lead to similar symptoms to those from NOV infections. First detected in 1977 in a Japanese orphanage, five SaV strains have been identified until now, with genogroup 1 (GI), GII, GIV and GV detected in humans, while GII has been found in swine (Varela et al., 2016a). The similarity between NOV and SaV symptoms is likely one of the reasons that SaV was not prioritized for monitoring, with NOV by far the most often detected human enteric virus in shellfish. Recently, SaV have been included in these analyses in shellfish most likely by the wide access to reverse-transcriptase polymerase chain reaction (RT-PCR) standard and research methods, increasing the reporting of this virus in shellfish. Fusco et al. (2019), for example, reported an increase in SaV detection from 11% in 2015 to 26.5% in 2017 in 20 shellfish farms in Italy, with an average positivity of 18.8% (54 of 289 samples) over the three years of study. Varela et al. (2016b) detected SaV in 37.5% of the mussels from Class B and C areas, close to a densely populated area, with most of the positive samples collected in the cold season from November to March. They also reported higher levels of SaV in waters with lower circulation/renewal, a potential factor in increasing SaV persistence in the environment.

2.1.3. Hepatitis viruses

Hepatitis A virus (HAV) and Hepatitis E virus (HEV) are transmitted through the consumption of contaminate food and water. HAV is associated with serious diseases, and outbreaks have often been associated with the consumption of contaminated bivalve molluscs produced in areas affected by sewage, wastewater and presence of faecal material (Dirks et al., 2021; Fusco et al., 2019). Its persistence in environmental conditions is noteworthy, with its presence positively correlated to the mean rainfall, as reported by Boussettine et al. (2023). The study reported the occurrence of HAV exclusively during the winter months, likely associated with the overflow of septic tanks due to elevated rainfall levels. Its estimated that 1.5 million people are infected by HAV per annum with the genotypes I, II and III the major ones associated with human infection (WHO, 2017). HAV is persistent in the environment, and its seasonality has been HEV is a significant public health concern in many parts of the world. Cases have increased in Europe and it is estimated that 20 million individuals annually are affected worldwide (ECDC, 2017). Of the eight HEV genotypes identified, only I-IV and VII are associated with human infection (Upfold et al., 2021).

2.1.4. Detection and quantification of human enteric viruses in shellfish

HAV, HEV, NOV and SaV quantification in shellfish are carried out mainly prior to commercialization by reverse-transcriptase polymerase chain reaction (RT-PCR). For bivalve molluscan shellfish, digestive glands are dissected and treated with proteinase K, followed by RNA extraction and RT-PCR. In water samples, ultrafiltration using positively charged membranes is used to retain the virus through adsorption and elution (ISO, 2017). A similar procedure with minor modifications was reported by Varela et al. (2016b) for SaV quantification in *M. galloprovincialis* sampled from Ria do Burgo, Spain. Fusco et al. (2019) identified HAV in Class A ($n = 1$) and B ($n = 3$) areas in winter and early summer, indicating risk of direct ingestion of HAV if shellfish are consumed raw in Class A areas. Dirks et al. (2021) detected NOV RNA in 53.1% of mussel and 31.6% of oyster samples collected at different stages of the food chain at post-harvest, dispatch centres and retail centres. NOV levels in undepurated mussels collected from Class B areas were higher than undepurated mussels collected from Class A areas, at the dispatch centre and retail level, suggesting that depuration effectively reduced *E. coli* in the mussels and was less efficient in reducing viral load. Not only detection and quantification, but the infectivity of human enteric viruses is also necessary to be addressed. Molecular methods are unable to predict the infectivity of NOV in the shellfish.

Some improvements such as the pretreatments with enzymes (e.g Proteinase K), staining differentiation of permeable and impermeable capsid are being investigated. However, an inconsistency is observed in the inactivation step for analyses, damaging NOV by different mechanisms, leading to inaccuracy and deviation on RT-qPCR assays (Gyawali et al., 2019). Cell culture has been highlighted as a potential alternative for assessing NOV infectivity, however, the absence of a standardized cell-based assay for NOV prevents a direct and universally accepted method for measuring infectivity. Other significant challenges include the extraction of NOV from shellfish, and inaccuracy of amplification from RT-PCR and infectivity reported by cell culture assays (Evans et al., 2023).

The SARS – CoV-2 pandemic has raised attention regarding distribution of the virus in water and marine environment. Its infection was proved to alter the human intestinal microbiota and faecal-oral transmission was reported (Guo et al., 2021). Bivalves were vulnerable to SARS – CoV-2 due to filter feeding and number of cases during the pandemic. To address this concern, Mancusi et al. (2022) applied a

droplet digital RT-PCR method to analyse 179 bivalve molluscs collected between September 2019 and April 2021 from different production sites and illegal harvesting. The authors reported an average positivity of 15.1% in the samples analysed from this period, with higher frequency between January and March 2021. However, bivalves should not be considered vectors of SARS – CoV-2 as infectivity cannot be assumed with only the presence of viral RNA in the absence of an infection assay. Polo et al. (2021) reported a high degree of RNA degradation and altered capsid indicating a non-infective viral state of SARS-CoV-2 RNA in clam and estuarine sediment samples after analysing the capsid integrity by PMAxx-triton viability RT-qPCR.

2.1.5. Levels of human enteric viruses in shellfish

Table 1 presents a comprehensive overview of human enteric viruses in various shellfish species as reported by multiple studies. This compilation of data offers insights into the human enteric viruses contamination levels across a range of shellfish species and main findings reported by authors.

2.2. Bacteria in shellfish

The marine environment harbours a vast complexity and variety of micro-organisms. Constant fluctuations in water parameters such as pH, temperature, salinity, and the impact of precipitation on these factors,

Table 1
Occurrence and concentration of human enteric viruses in shellfish species at different locations.

Virus	Shellfish	Concentration or positive samples	Location and comments	Ref.
NOV GI	*C. gigas, M. lyrata*	4.7×10^2 copies/g	Hanoi - Vietnam. Detected in 50.4% of total samples.	Suffredini et al. (2020)
	M. galloprovicialis, R. philippinarum	<LQ	Campania - Italy. 31% of total positivity over a 3-year studying period. Less prevalent than GII.	Fusco et al. (2019)
	Oysters	586 copies/g	Vendors directly available to consumers - United Kingdom. 15.7% positive for GI only.	Lowther et al. (2018)
NOV GII	*M. galloprovicialis, R. philippinarum*	1.1×10^6 copies/g*	Campania - Italy 39.7% of total positivity over the 3-year studying period.	Fusco et al. (2019)
	Cerastoderma spp	25% positive for GII	Sardinia - Italy. Not generally related to the presence of faecal bacteria.	Marceddu et al. (2017)
	Oysters	1802 copies/g	Vendors directly available to consumers - United Kingdom. Highest concentration detected.	Lowther et al. (2018)
	M. falcata and *C. Brasiliana*	21% positive for GII	Lagunar Complex - Brazil. Strains with >94.9% of similarity to isolated from clinical cases in Brazil.	Vasquez-García et al. (2022)
NOV GI and NOV GII	Oysters, mussels, cockles	22% positive for GI and GII	Brittany and Normandy - France. 150 samples processed.	Rincé et al. (2018)
	C. gigas, M. lyrata	3.8×10^3 copies/g	Hanoi - Vietnam. Detected in 79.3% of samples. Higher prevalence in autumn.	Suffredini et al. (2020)
	C. gigas	1.76 log	Post harvest, dispatch and retail - Netherlands. 27% of oysters were positive for both GI and GII.	Dirks et al. (2021)
	M. edulis	2.04 log	Post-harvest, dispatch and retail - Netherlands. 31.6% of mussels analysed. Undepurated post-harvest Class B samples were significantly higher than other groups.	Dirks et al. (2021)
	Oysters	39% positive for GI and GII	Vendors directly available to consumers - United Kingdom. Majority (85.9%) had levels lower than 100 copies/g.	Lowther et al. (2018)
HAV	*M. galloprovicialis, R. philippinarum*	4.2×10^2 copies/g	Campania - Italy. Only one sample above the limit of quantification.	Fusco et al. (2019)
	C. gigas, M. lyrata	1.3×10^2 copies/g	Hanoi - Vietnam. 1.7% of the samples.	Suffredini et al. (2020)
	M. edulis	0.25% positive for HAV	Netherlands. 1/392 tested positive from 2013 to 2017.	Dirks et al. (2021)
	M. galloprovincialis, C. gigas	11.03% and 5.13% positive for HAV, respectively	Harvesting Areas Class A, B, and C - Morrocco. A non-significant positive correlation between HAV-positive samples and mean rainfall.	Boussettine et al. (2023)
HEV	*C. gigas, M. lyrata*	1.2×10^2 copies/g	Hanoi - Vietnam. 11.6% of the samples.	Suffredini et al. (2020)
RV	*M. galloprovicialis, R. philippinarum*	1.9×10^3 copies/g	Campania - Italy. 26% total positivity over the 3-year studying period.	Fusco et al. (2019)
AsV	*M. galloprovicialis, R. philippinarum*	1.4×10^3 copies/g	Campania - Italy. 20.8% of the samples.	Fusco et al. (2019)
	C. gigas, M. lyrata	1.5×10^3 copies/g	Hanoi - Vietnam. 12.4% of the samples.	Suffredini et al. (2020)
SaV	*M. galloprovicialis, R. philippinarum*	5.3×10^2 copies/g	Campania - Southern Italy. 18.8% of samples.	Fusco et al. (2019)
	M. galloprovincialis V. philippinarumm, V. decussata, C. edule	1.9×10^3-1.4×10^5 copies/g	Galicia - Spain. 17.9% of samples tested positive. Highest prevalence in *C. edule*.	Varela et al. (2016a)
	M. galloprovincialis	2.2×10^3-2.1×10^5 copies/g	Ria do Burgo - Spain. 37.5% of the samples tested positive. Cold and rainy seasons.	Varela et al. (2016a)
AiV	*M. galloprovicialis, R. philippinarum*	3.4×10^2 copies/g	Campania - Southern Italy. 5.6% of samples. Possibly influenced by weather conditions.	Fusco et al. (2019)
	C. gigas and M. lyrata	11.6% of the samples	Hanoi - Vietnam. Three AiV-1 genotype A sequences were obtained.	Suffredini et al. (2020)
SARS-CoV-2	*M. galloprovincialis*	$1.1–1.4 \times 10^2$ copies/g	Campania - Italy. 27 of 179 samples tested positive.	Mancusi et al. (2022)
	R. philippinarum, R. decussatus	<LOQ to 4.48 Log copies/g	Galicia - Spain. 9/12 samples tested positive.	Polo et al. (2021)
PCV2	*M. edulis*	$1.02–92.3 \times 10^2$ genome copies/g	Denmark. 12/29 samples tested positive. PCV2 is a potential faecal indicator of porcine waste in mussels.	Krog et al. (2014)

NOVg1: Norovirus genogroup 1; NOVg2: Norovirus genogroup 2; HAV: Hepatitis A virus; HEV: Hepatitis E virus; AdV: Adenovirus; AsV: Astrovirus; SaV: Sapovirus; AiV: Aichivirus; RV: Rotavirus; SARS-CoV-2: Severe-acute respiratory syndrome coronavirus 2; PCV2: Pig-specific porcine circovirus type 2. LQ: limit of quantification *S. constricta: Sinovacula constricta; M. meretrix: Meretrix meretrix*; MPN: most probable number; *T. granosa: Tegillarca granosa; M. falcata: Margaritifera falcata*; VTEC: Verotoxigenic; PCR: polymerase chain reaction; *R decussatus: Ruditapes decussatus; C. brasiliana: Crassostrea brasiliana; M. edulis: Mytilus edulis; PCV2: Porcine circovirus type 2; M. arenaria: Mya arenaria; D. trunculus: Donax trunculus; S. Plana: Scrobicularia plana; C. gigas: Crassostrea gigas; O. edulis: Ostrea edulis; V. parahaemolyticus: Vibrio parahaemolyticus; M. galloprovincialis: Mytilus galloprovincialis; V. philippinarumm: Venerupis philippinarumm; C. edule: Cerastoderma edule;* *: Average concentration.

selectively favour the most adaptable strains. The survival of these strains depends on developing unique features to overcome environment changes. Bacteria are widely present in the marine environment. They are responsible for such important processes as decomposition in nutrient recycling (e.g carbon, nitrogen, phosphorus, sulphur and iron), photosynthesis, detoxification and production of vitamins. While most marine bacteria are harmless to shellfish or humans, anthropogenic activities can disrupt this environment leading to imbalances that favour harmful strains, eutrophication, and interference in bacteria equilibrium. Johnston and Roberts (2009) reported this disruption, identifying a strong association between anthropogenic contamination and reduction of species diversity and evenness in the marine environment. Industrial and agricultural faecal waste, and urban effluent discharges, can introduce invasive species or stimulate imbalance in the native microbiota. A geospatial model developed by Tuholske et al. (2021) to measure the impact of human sewage on 135,000 watersheds globally reported the addition of 6.2 Teragram (Tg) of nitrogen in coastal waters, 63% coming from sewer systems, 32% from direct input and 5% from septic sources. Climate change also poses a significant threat to marine ecosystems by disrupting the delicate equilibrium within these environments. One of the primary concerns is the potential for severe alterations in water temperature, nutrient availability, and salinity levels (Pontavice et al., 2020). Collectively, these changes threaten to disrupt the complex interactions and ecological processes that underpin marine ecosystems, ultimately compromising their resilience and long-term viability.

Shellfish species can exhibit varying levels of bacteria accumulation. In a study by Jin et al. (2016), the accumulation of *E. coli* in *Sinonovacula constricta*, *Meretrix meretrix* and *Tegillarca granosa* was assessed. The results showed that *E. coli* and aerobic colony counts were significantly higher in *S. constricta* compared to *M. meretrix* and *T. granosa*, with a slightly stronger accumulation of *E. coli* in *M. meretrix*. The authors also noted lower levels of *E. coli* in *S. constricta* during persistent rainfall and higher levels under sunny or cloudy conditions. In another study Vásquez-García et al. (2019) detected *E. coli* in 32% of *Mytella falcata* and *Crassostrea brasiliana* samples collected in different seasons from natural banks of the Lagunar Estuary (25° 0′ 54″ S, 47° 55′ 37″ W), São Paulo, Brazil, a region characterized by megathermal super humid climate with excessive rain in summer. The authors also confirmed the presence of virulence genes, specifically eaeA, in *E. coli* colonies isolated from shellfish samples.

The simultaneous evaluation of different contaminants can improve the assessment of water quality in production areas. For example, in a study by Rincé et al. (2018), the correlation between *Salmonella* and *Campylobacter jejuni* and/or *Campylobacter coli* as well as with *E. coli* concentration was reported. Whereas the current legislation establishes *E. coli* as marker of contamination, other important bacteria of higher risk can also be present in water (EC, 2019; Krog et al., 2014). The authors also detected a correlation between the presence of *Campylobacter lari* and the detection of human noroviruses. Marceddu et al. (2017), however, did not detect a correlation between faecal bacteria and positive results for NOV (25% GII-positive and 10% GI positive) in *Cerastoderma* spp.

2.2.1. Escherichia coli

E. coli is a type of bacteria that when present in shellfish can pose risks to consumers. It is the main indicator of faecal contamination and has been used as an indicator to verify water and shellfish quality. The presence of *E. coli* also plays a crucial role in designating areas for shellfish production, representing a key strategy to mitigate the impact of anthropogenic contaminants. In European waters, faecal contamination is the foremost parameter considered when defining production zones for bivalve molluscs. This often precedes a comprehensive sanitary risk assessment and the subsequent establishment of a monitoring program. Class A are the cleanest areas and in bivalves harvested for direct consumption, *E. coli* levels should not exceed 230 *E. coli* per 100 g of flesh and intravalvular liquid in 80% of the samples collected and should not exceed 700 *E. coli* per 100 g in the remaining 20% of samples. Class B and C are more lenient and may suggest an external source of human or animal pollution. Class B establishes a limit of 4600 *E. coli* per 100 g in 90% of the samples and 46,000 *E. coli* per 100 g in the remaining 10%. Class C area is the most contaminated, with a limit of 46,000 *E. coli* per 100 g (EC, 2019). As they are not adequate for consumption, bivalves harvested from Class B and C facilities must be treated to meet the health standards of Class A areas prior marketing and raw consumption. Bivalves are kept for long periods in depuration plants with recirculation of clean seawater to promote the purging of accumulated faecal bacteria. Alternatively, live bivalve molluscs can be consumed after sterilisation process in sealed containers or heat treatments (EC, 2004).

In a study published by Campos et al. (2017), factors associated with *E. coli* contamination in oysters were cumulative over seven days of rainfall before sampling. Verotoxigenic and enterotoxigenic *E. coli*, VTEC and ETEC, respectively, are present in the gastrointestinal tracts of ruminants and are responsible for a significant number of human infections, mostly caused by serotype O157 (Chalmers et al., 2000; Marceddu et al., 2017). The characterization of virulence genes and antimicrobial resistance by PCR and disk diffusion, respectively, can also assist in the quality assessment of production areas. Mass spectrometry provides an alternative method for speciation.

2.2.2. Vibrio

Other than *E. coli*, *Vibrio* and *Salmonella* are also monitored as threats to consumer safety. *Vibrio* species are widely distributed in estuaries and coastal waters where they accumulate in the water column, which facilities their uptake by filter feeders such as bivalves (Froelich and Noble, 2016). *Vibrio* species are temperature-dependant and are often detected during summer when water temperatures rise. Rincé et al. (2018) reported the presence of *Vibrio* species when water temperatures were higher than 15 °C and the peak of precipitation exceeded 10 mm per 48 h, probably associated with the higher temperature on the surface and/or run off nutrients into analysed waters. *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* were detected in seawater and shellfish batches by the authors, where *V. parahaemolyticus* was the most abundant. Harrison et al. (2022) recently reported oscillation in *Vibrio* levels with temperature in English and Welsh coastline areas at Chichester Harbour, Lyme Bay, Osea Island and Whitstable Bay. A variety of *Vibrio* species such as *V. rotiferianus*, *V. jasicida* and *V. parahaemolyticus* were detected, supported by the increase in sea-surface temperatures. Park et al. (2018) also reported a higher occurrence of *V. parahaemolyticus* in seawater and sampled shellfish from the Gyeongnam coast (South Korea) during summer, when waters reached 25.5 °C and <6% of isolates were positive for virulence genes tdh and trh. The levels of *V. parahaemolyticus* were higher during summer and statistically significant for the entire investigation period from 2004 to 2016, followed by an increase in seafood-borne outbreaks, with 138 outbreaks for the same period (KMFDS, 2017). Studies assessing the presence of *Vibrio* in market have played a crucial role in confirming its persistence. In a study conducted by Siriphap et al. (2024), 33 non-pathogenic *Vibrio parahaemolyticus* isolates were obtained from shrimp, shellfish and squid from wet markets and supermarkets in Northern Thailand. The *V. parahaemolyticus* isolates were screened for five virulence markers, with all shellfish and shrimp isolates positive for T3SS1, and one of two squid positive for the same gene. The isolates were also screened for antimicrobial resistance, and 5 were resistant to kanamycin-streptomycin (1) carrying *sul*2 and ampicillin-kanamycin-streptomycin (4) carrying *tetA* (2), *tetA*-sul2 (1).

2.2.3. Salmonella

Salmonella is not a natural inhabitant of aquatic environments and can cause foodborne illnesses in humans. Its responsible for approximately 1.35 million infections, 26,500 hospitalizations and 420 deaths

in United States every year (CDCP, 2023). First symptoms are observed from 6 h to 6 days after ingestion, and are usually stomach cramps, vomit, diarrhoea and fever, and in some cases paratyphoid and typhoid fever. *Salmonella* reaches the shellfish by faecal contamination, and it is widely diverse as reported by Rubini et al. (2018), which identified a total of 237 *Salmonella* strains and 53 different serovars in water and bivalves after analysing the data collected from 1997 to 2015 in a production area in the Northern Italy. *Salmonella* ser. *Typhimurium* was the dominant serovar and this is a common inhabitant of gastrointestinal tract of ruminants, suggesting contamination from farms, whereas a positive association was observed by the authors between faecal coliforms in seawater and the presence of *Salmonella* in total molluscs and *Ruditapes philippinarum*.

2.2.4. Levels of Escherichia coli, Salmonella and Vibrio in shellfish species

Table 2 presents a comprehensive overview of bacterial concentrations in various shellfish species as reported by multiple studies. This compilation of data offers insights into the microbial contamination levels across a range of shellfish species and main findings reported by authors.

3. Assessing the risks and exposure to *Escherichia coli* and risk mitigation strategies

Assessing risks associated with an activity or hazard is a process that integrates an extensive analysis of all knowledge and data available to support a predictive model, that allows the further monitoring of the entire chain (Fehrenbach et al., 2022; Oscar, 2012). Different approaches are available to a risk assessment study. Here, the *E. coli* levels in shellfish presented in Table 2 were used to determine the estimated daily intake (EDI) of bacteria with the consumption of shellfish. EDI was calculated as reported by Fehrenbach et al. (2022). Initially, the EDI is determined considering the contaminant concentration in shellfish, the daily per capita consumption of shellfish in the region or area where it is predominantly consumed (referred to as daily mean ingestion or DMN), and the average body weight of the consumers (Equation (1)). EDI was estimated based on the Food Balance report published by the Food and Agriculture Organization of the United Nations (FAO, 2010), where Europe, Asia, Africa, America and Oceania consume mean values of 0.16 ± 0.23, 16.24 ± 1.77, 0.55 ± 0.2, 1.56 ± 2 and 14.24 ± 1.8 g day^{-1}, respectively, and the mean body weight of males and females as 70.8, 57.7, 60.7, 74.3 and 74.1, respectively (Walpole et al., 2012). The average body weight between males and females in each continent were used in the calculation to estimate the exposure to bacteria and provide a simple approach for its assessment. To obtain a precise evaluation of consumers based on their region and dietary habits, it is essential to consider the local consumption patterns of shellfish, considering factors such as age and gender, and correlating this information with the average body weight within each demographic category.

Equation (1). Estimated daily intake.

$$EDI = \frac{\text{Concentration of contaminant x daily mean ingestion}}{\text{Body weight}} \quad \text{Equation 1}$$

The guidelines from the United States Department of Agriculture (USDA) and the United States Department of Health and Human Services (USDHHS) in 2020, recommends that species with low mercury levels can be consumed in 2–3 servings per week, not exceeding 340 g per week. The aim of this consumption advice is to encourage the consumption of seafood as part of a balanced diet due to its health benefits. As an example, considering the consumption of 2 servings, equivalent to

Table 2
Occurrence, concentration and main findings reported by authors in studies of bacteria in shellfish species at different locations.

Bacteria	Shellfish	Concentration or %positive samples	Location and main findings	Ref.
Escherichia coli	Cockle	3.3 log/100 g	La Fresnaye – France. 7452 *E. coli* strains were isolated in the study.	Rincé et al. (2018)
	Oyster	2.6 log/100 g	La Fresnaye – France. Oysters were less contaminated than cockles and mussels.	Rincé et al. (2018)
	Mussel	2.9 log/100 g	La Fresnaye – France.	Rincé et al. (2018)
	S. constricta	2.1–3 log MPN/100 g	*In vitro*. Higher accumulation potential than that for *M. meretrix* and *T. granosa*.	Jin et al. (2016)
	M. meretrix	1.56–2.36 log MPN/100 g	*In vitro*. *E. coli* accumulation slightly higher than that of *T. granosa*.	Jin et al. (2016)
	T. granosa	1.56–2.3 log MPN/100 g	*In vitro*. Lowest capability of accumulating *E. coli* compared to *M. meretrix* and *S. constricta*.	Jin et al. (2016)
	M. falcata	3.6–15.5 (winter), 17 (spring), 927 (summer) MPN/g	Canaréia - Brazil. *E. coli* levels were seasonally dependant, with peak in summer.	Vásquez-García et al. (2019)
	Cerastoderma spp, *R. decussatus*	VTEC total prevalence of 6.6%	Sardinia - Italy. All of the isolates showed complete pathogenicity profile.	Marceddu et al. (2017)
	C. brasiliana	3–7.1 (winter), 48.6 (spring), 14.3–15.8 (summer) MPN/g	Canaréia - Brazil. *E. coli* levels were lower than in mussels.	Vásquez-García et al. (2019)
	M. edulis	20-310 MPN/100 g	Denmark. Flesh and liquid samples. Detected along with PCV2.	Krog et al. (2014)
Salmonella	Bivalve molluscs	1.7% of bivalves analysed	Ferrara - Italy. *S. enterica* subsp. *enterica*	Rubini et al. (2018)
	C. gigas, M. arenaria	0.15–6.4 MPN/100 g	East Coast - Canada. Levels in naturally contaminated shellfish. *Salmonella* detected in oyster after 30 min of exposure.	Tamber et al. (2020)
	D. trunculus, S. plana	5% of the isolates were positive for *Salmonella* spp.	Agadir beach - Morocco. Positive correlation between faecal indicator bacteria and *Salmonella*.	Chahouri et al. (2022)
Vibrio	*Cerastoderma* spp.	3.19 log CFU/g	Class B area. A 90% prevalence of naturally occurring *Vibrio*.	Marceddu et al. (2017)
	R. decussatus	2.84 log CFU/g	Class B area. Mean bacterial load after purification.	Marceddu et al. (2017)
	C. gigas	2385 CFU/g	Osea Island - UK. August. Species isolated: *V. jasicida, V. alginolyicus*.	Harrison et al. (2022)
	C. gigas	3480 CFU/g	Chichester Harbour – UK. August. Species isolated: *V. rotiferianus, V. jasicida, V. alginolyicus*.	Harrison et al. (2022)
	O. edulis	1573 CFU/g	Chichester Harbour - UK. July. Species isolated: Photobacterium, *V. jasicida, V. rotiferianus, V. alginolyicus*.	Harrison et al. (2022)
	C. gigas and M. galloprovincialis	<30-11,000 MPN/100 g	Gyeongnam coast - Korea. 2.1–28.6% *V. parahaemolyticus* detection rate. Seasonally dependent with high levels in summer to early autumn.	Park et al. (2018)

E. coli: Escherichia coli; S. constricta: Sinovacula constricta; M. meretrix: Meretrix meretrix; MPN: most probable number; *T. granosa: Tegillarca granosa; M. falcata: Margaritifera falcata;* VTEC: Verotoxigenic; PCR: polymerase chain reaction; *R decussatus: Ruditapes decussatus; C. brasiliana: Crassostrea brasiliana; M. edulis: Mytilus edulis;* PCV2: Porcine circovirus type 2; *M. arenaria: Mya arenaria; D. trunculus: Donax trunculus; S. plana Scrobicularia plana; C. gigas: Crassostrea gigas; O. edulis: Ostrea edulis*; *V. parahaemolyticus: Vibrio parahaemolyticus; M. galloprovincialis: Mytilus galloprovincialis.*

283 g in a week, the reference for daily consumption is obtained dividing 283 g per 7 days, resulting in 40.4 g of seafood each day. Therefore, the *E. coli* intake associated with the consumption of 40.4 g of shellfish was also calculated, considering the average body mass globally of 62 kg (Walpole et al., 2012), and the results are presented in Fig. 1 as recommended consumption of seafood (RCS).

The highest level of intake of contaminants from all continents was in Asia with ±5.6 *E. coli* units/day.kg body weight with levels reported in cockle (Rincé et al., 2018). However, when incorporating RCS in the comparison, the EDI calculated in this study is significantly lower ($p < 0.01$) compared to the consumption recommended by the Food and Drug Administration (FDA), as depicted in Fig. 1. This comparison was conducted considering a global average body mass and the same shellfish. The statistical significance of this difference was determined by Student's t-test, demonstrating a disparity in contaminant intake levels. This observation points to a higher risk associated to the consumption of recommended shellfish levels, potentially leading to increased intoxication incidents with the change in dietary habit to align with the consumption suggested by the FDA. It is necessary to reduce the exposure of shellfish to bacteria and human enteric viruses. Innovative strategies such as real time monitoring of contaminants in seawater and effective purification technologies offer promising routes to ensure the safety of shellfish consumption. Enhanced safety measures could increase the consumer confidence, possible leading to higher daily shellfish intake and supporting growth of this industry sector. Consensus on the exposure to bacteria and viruses remains elusive, given the variability associated with species/strains, host factors, and immune response. The FDA established a limit of 230 MPN per 100 g of shellfish. In the European Union, the limit is set at 230 *E. coli* per 100 g of flesh and intravalvular liquid (EC, 2019). There is no permissible level of *Salmonella* contamination in food products, including live shellfish. The analytical reference method for detecting *Salmonella* is described in ISO 6579 and the absence of *Salmonella* in 25 g sample is required to ensure food safety. In the case of *Vibrio* bacteria, it is essential to adhere to best hygiene practices, as specific scientific criteria are not yet established (EC, 2005). Similarly, when it comes to viral standards, neither EU food legislation nor the FDA have limits for viruses in shellfish. It remains the responsibility of the food business operator to prioritize and ensure food safety standards.

The exposure to human enteric viruses and bacteria by consuming shellfish can be mitigated by several ways. A holistic approach is presented in Fig. 2. It includes a pre-assessment of local conditions (analyses of water quality, environment conditions, potential contaminants), production (handling, harvest, and transport), processing (classification, depuration and packaging) and commercialization (retail market/restaurants/direct consumption, time to consumption and storing conditions). With all the risks identified and measured, a monitoring strategy (continuous assessment) is recommended. If not managed at the first stages, the only alternatives to mitigate the exposure is an extensive depuration stage and/or cooking process.

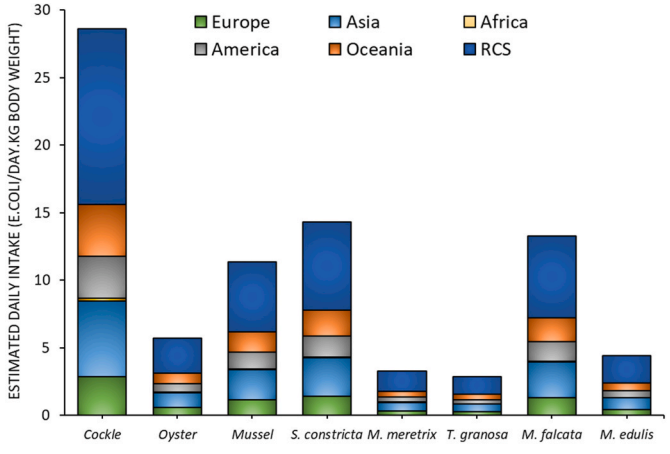

Fig. 1. Estimated daily intake (*E. coli* units/day.kg body weight) derived from *E. coli* reported by authors in Table 2 for various shellfish species. RCS: Recommended consumption of seafood. *Average EDI calculated for each continent; values below 0.4 *E. coli* units/day.kg bw were excluded from the graph.

Understanding the challenges experienced by the shellfish industry and the need for alternatives to improve the decontamination of shellfish, we reported in our previous work the development of a prototype based on pulsed ultraviolet-light prototype to inactivate important foodborne pathogens such as *S. aureus* (5.63 log), *C. albicans* (5.15 log), *S. typhimurium* (5 log), *B. cereus* (4.59 log) and *E. coli* (4.55 log) (Fehrenbach et al., 2023). Shellfish depuration is the last step where contaminants accumulated from the previous steps 1 and 2 (Fig. 2) can be removed before commercialization. The risk mitigation, however, must include a continuous assessment of every process in the shellfish production chain. As a fresh food, oyster for example is susceptible to bad practices at handling or commercialization, creating conditions for opportunistic micro-organisms such as *E. coli* to multiply and increase the risk related to food intoxication. Aware of the real complexity of a shellfish farm, the methodology presented here is a simplified approach to support the shellfish industry to assess its production risks. The key-information provided aimed to support the risk monitoring and controlling in all stages of production.

4. Conclusion

- Shellfish farms are constantly challenged by environmental changes, water contamination, and human activity. This review delves into the impact of these factors on bacteria and human enteric viruses levels in shellfish.
- Shellfish exhibit a broad spectrum of bacterial accumulations. *E. coli, Vibrio,* and *Salmonella* were predominantly associated to illnesses and widely detected in shellfish. High levels were reported in different waters and environments, proven its persistency and adaptability.
- When assessing the EDI of *E. coli* based on the levels presented in the literature, Asia has the highest exposure with ±5.6 *E. coli* units/day.kg body weight. However, the EDI was significantly lower ($p < 0.01$) than observed for FDA suggested consumption presented by RCS.
- A change in dietary habits to increase the ingestion of shellfish to health levels might increase the exposure potentially leading to increased intoxication incidents.
- It's imperative to invest in mitigation technologies to safeguard shellfish and support a health increase of shellfish consumption.
- There is an urgent need for standardising the detection and quantification of human enteric viruses and its infectivity.
- A holistic analysis of shellfish production chain is a promising alternative to reduce the exposure to bacteria and human enteric viruses associated with the consumption of shellfish.

CRediT authorship contribution statement

Gustavo Waltzer Fehrenbach: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Emma Murphy:** Conceptualization, Data curation, Project administration, Resources, Supervision, Writing – review & editing. **Robert Pogue:** Conceptualization, Data curation, Investigation, Supervision, Writing – review & editing. **Frank Carter:** Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Eoghan Clifford:** Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Ian Major:** Conceptualization, Formal analysis, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

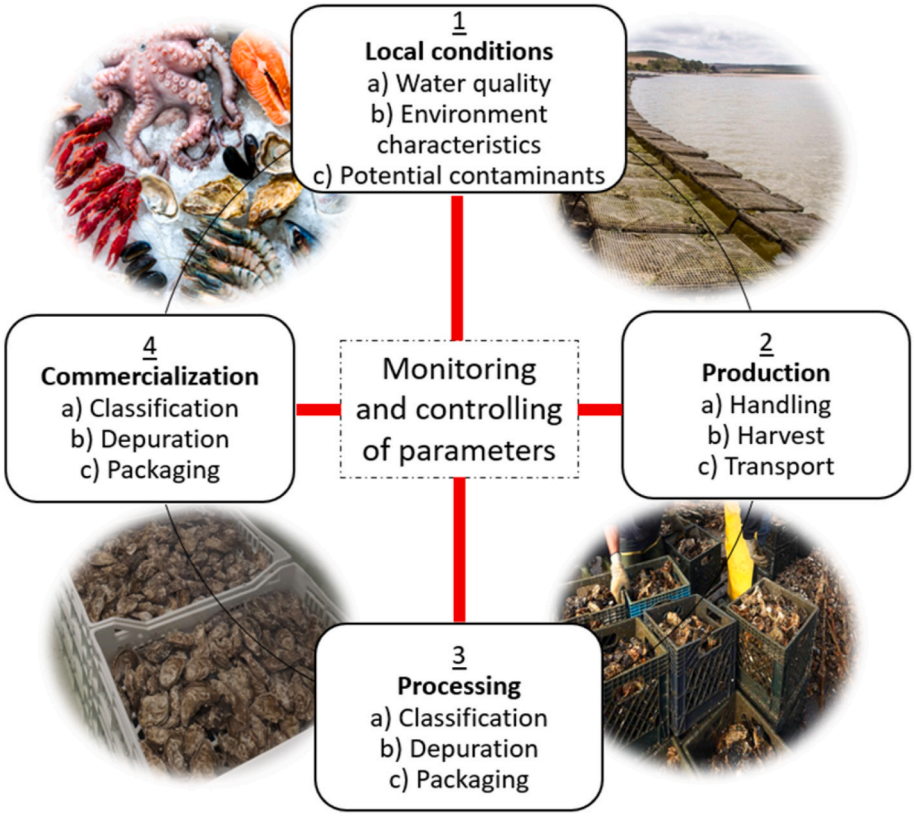

Fig. 2. An example of a holistic approach to mitigate the exposure of shellfish to bacteria and human enteric viruses, improving consumers safety and shellfish quality.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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