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Gastrointestinal survival and adaptation of antibiotic-resistant enterococci subjected to an *in vitro* digestion model

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A B S T R A C T

The ability of four enterococcal strains to survive human digestion and maintain their antibiotic resistance (AR) traits was investigated to determine the health risk posed by seafood-borne streptomycin-, erythromycin-, tet- racycline- and gentamycin-resistant enterococci. After demonstrating *ant(6)-I*, *ermB*, *tetO* and *aac(6')-aph(2")* gene transferability by mating assays, strains were inoculated into a mussel homogenate which underwent *in vitro* digestion. Digestion reduced plate counts by 2–3 log; qPCR counts decreased by 1.5 log (*E. faecium* 22571/ 2), 2.5 log (*E. faecalis* 113324) or did not decrease (*E. faecium* 125745 and 6767/2). The much lower plate than qPCR counts seen in *E. faecium* suggested a viable-but-non-culturable (VBNC) subpopulation. Replica plating on antibiotic-supplemented agar and qPCR demonstrated that maintenance of AR traits was antibiotic-dependent, with a variable amount of erythromycin-, tetracycline- and gentamycin-resistant colonies and no streptomycin- resistant colonies. The seeking of unexpressed/lost AR genes in cells turned antibiotic-susceptible after digestion, identified *aac(6')-aph(2")* in all colonies of *E. faecalis* 113324 and *E. faecium* 6767/2, in the 40% of 125745 and in none of the *E. faecium* 22571/2. In conclusion, after digestion the AR traits of antibiotic-resistant (also VBNC) enterococci contained in seafood may be maintained and transferred to the human microbiota and from it to intestinal pathogens.

1. Introduction

Foodborne pathogens cause enteritis and other infections which in developing countries involve a high rate of mortality (Crim et al., 2015; Henao, Jones, Vugia, & Griffin, 2015). The outbreaks of foodborne infections around the world are triggered by several bacterial species (Kirk et al., 2015; Scallan et al., 2011). According to the One-Health concept, whereby the health of humans, animals and the environment is closely related, bacteria are also vehicles for ARs and contribute to their environmental dissemination. Enterococci are commonly found in the gut of humans and animals, in food and in the environment, including marine coastal areas (Lebreton, Willems, & Gilmore, 2014; Pieniz, Andreazza, Anghinoni, Camargo, & Brandelli, 2014). Although they rarely cause disease in healthy individuals, they are leading causes of a variety of hospital infections – including sepsis and endocarditis – whose treatment is being hampered by the emergence of hospital-adapted multidrug-resistant (MDR) strains (Arias, Contreras, & Murray, 2010; Babady, 2016; Fisher & Phillips, 2009; Tacconelli & Cataldo, 2008). Gut colonisation is underpinned by several factors, including tolerance of physicochemical changes, which enables enterococci to survive the digestion process, and biofilm production (Fisher & Phillips, 2009; Mohamed & Huang, 2007). Moreover, exposure to environmental stress results in the induction of viable but non-culturable (VBNC) cells: in this state enterococci are not susceptible to antibiotics and are undetectable by routine microbiological assays, yet the preservation of basic metabolic activity and of virulence and antibiotic resistance (AR) genes entails that in favourable conditions they can recover full metabolic activity, gene expression and culturability (Lleò, Benedetti, Tafi,

Signoretto, & Canepari, 2007). The high genetic plasticity of enterococci facilitates the acquisition of AR genes through horizontal genetic transfer (HGT) events (Torres et al., 2018; Werner et al., 2013). *Enterococcus faecalis* and *Enterococcus faecium* are the species most frequently involved in human infections. *E. faecalis* carries a wider spectrum of virulence genes and is more frequently isolated from humans, whereas *E. faecium* is more prone to acquire (through HGT) genes conferring resistance to different antibiotic classes (Guzman Prieto et al., 2016) and antibiotic-resistant *E. faecium* has been found more frequently than *E. faecalis* in coastal marine environments (Cittterio et al., 2017; Di Cesare, Vignaroli, Luna, Pasquaroli, & Biavasco, 2012). MDR *E. faecium* and *E. faecalis* strains can be found in freshwater, seawater, marine sediments, fish and shellfish (Byappanahalli, Nevers, Staley, & Harwoodc, 2012; Cittterio et al., 2017; Vignaroli et al., 2018). Their ubiquitous nature entails that enterococci can reach the human gut through a variety of routes, including the food chain. Seafood, which is often eaten raw or undercooked, is a typical route, since filter-feeding organisms like bivalves can accumulate and concentrate MDR bacterial strains found in their environment (Cittterio et al., 2017; Vignaroli et al., 2016). Although foodborne enterococci are not currently considered as direct causes of severe human infection, they can transfer resistance genes to the human gut microbiota (Hammerum, Lester, & Heuer, 2010).

To date, the ability to survive the human digestion process has been evaluated only for organisms used as probiotics (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011; Nueno-Palop & Narbad, 2011; Veljović et al., 2017). To the best of our knowledge, there are no data about the effect of human digestion on the viability of enterococci or the stability of their resistance traits. In this work the effects of the human gastrointestinal digestion on enterococcal survival, the maintenance of antimicrobial resistance traits and the possible development of VBNC forms were evaluated to provide additional information on the health risks associated with the presence of antibiotic-resistant enterococci (ARE) in seafood.

2. Materials and methods

2.1. Bacterial strains and preparation of inocula

Four enterococcal strains, 2 from the marine environment and 2 from human blood were used in *in vitro* digestion experiments. All four strains had previously been characterised for their carriage of AR genes (Cittterio et al., 2017) as follows: the environmental strains included *E. faecium* 6767/7, resistant to tetracycline (TET) and gentamycin (CN), and *E. faecium* 22571/2, resistant to erythromycin (ERY), streptomycin (STR) and CN; the blood strains included *E. faecium* 125745, resistant to ERY, STR and CN and *E. faecalis* 113324, resistant to TET and CN.

Strains were grown in Brain Heart Infusion broth (BHIB, Oxoid, Basingstoke, UK) or agar (BHIA, Oxoid) and stored as stock cultures in BHIB supplemented with 20% glycerol at - 80 °C.

The bacterial inocula for *in vitro* digestion were grown in BHIB for 18 h at 37 °C, centrifuged at 4500 g for 15 min at 4 °C, washed twice in sterile saline (0.85% NaCl) and diluted to OD_{550} 0.1.

2.2. In vitro conjugation experiments

Conjugal transfer was performed by filter mating using the four strains as donors and *E. faecalis* JH2-2 and *E. faecium* 64/3, both resistant to fusidic acid and rifampin, as recipients (Vignaroli, Zandri, Aquilanti, Pasquaroli, & Biavasco, 2011). The transconjugants were selected on BHIA plates containing 10 μg/ml fusidic acid, 10 μg/ml rifampicin, 10 μg/ml TET, 500 μg/ml CN, 10 μg/ml ERY and 1000 μg/ml STR (all from Sigma Aldrich, St. Louis, MI, USA). Conjugation frequency was expressed as the number of transconjugants per donor cell. Species-specific qPCR targeting the *ddl* gene *of E. faecalis* and *E. faecium* (Cittterio et al., 2017) and Pulsed Field Gel Electrophoresis (PFGE) of Sma I- (Takara Bio Inc., Goteborg, Sweden) -digested total DNA were used to check the correspondence of transconjugants to the recipient.

2.3. Mussel samples

Mediterranean mussels (*Mytilus galloprovincialis*) of average size (5–7 cm in length) were provided by Istituto Zooprofilattico Sperimentale of Umbria and Marche (Ancona, Italy). After removing mud, encrustations, epiphytes and epizoa, mussels were rinsed with sterile artificial seawater (ASW), salinity 37 psμ (g/kg), pH 8.0 ± 0.5, at 18 ± 1 °C (Croci, Suffredini, Cozzi, & Toti, 2002) and placed into three 5 L ASW tanks (25 mussels each). The tanks were aerated to obtain an oxygen saturation > 94%, corresponding to natural en- vironmental conditions. The water was changed on alternated days for 2 weeks; then 3 mussels per tank were homogenised using a Potter homogeniser with a glass pestle (Steroglass S.r.l. Perugia, Italy) and analysed for the presence of enterococci, spreading 1 mL of homogenate on Slanetz Bartley Agar (SBA, Biolife) plates, incubated at 37 °C for 24 h before the CFU/ml counting. Twelve bacteria-free mussels (4/tank) were opened in sterile conditions using disposable scalpels; the byssus was removed and the soft tissue was extracted and divided into 12 aliquots of 4 g. The aliquots were exposed to germicidal UV light for 4 h, to kill occasional microbial contaminants that may be transferred during handling, and then homogenised using the Potter homogeniser.

2.4. In vitro gastrointestinal digestion

For each enterococcal strain, 12-g of mussel homogenate (MuH) were prepared and divided in three 4 g-aliquots. Each of them was then processed as described: the first aliquot was inoculated with 4 ml of saline containing 1 × 10^8 CFU/ml of the tested enterococcal strain and maintained at 4 °C (UNDIG); the second aliquot was inoculated with 4 ml of saline containing 1 × 10^8 CFU/ml of the tested enterococcal strain and then digested (DIG); the third aliquot wasn't inoculated and used as negative control, to verify the absence of enterococci in the original homogenate.

The *in vitro* digestion model was performed as previously described (Oomen et al., 2003; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005), with few modifications. Briefly, the MuH aliquot, inoculated with each enterococcal strain and treated with artificial digestive juices at 37 ± 2 °C, were incubated with constant head-over-heels shaking (55 rpm), mimicking peristaltic movements, to maximise enzymatic activity (Table 1), as described by Desideri, Roselli, Feduzi,

Table 1
Steps of the *in vitro* digestion process (Versantvoort et al., 2005).

Digestion	Sample	Digestion juice	Temperature (°C)	Time (min)
Salivary (1st step)	4 g homogenate inoculated with enterococci (10^8 CFU)	Salivary 6 ml	37 °C	5
Gastric (2nd step)	Sample obtained from salivary digestion	Gastric 12 ml	37 °C	120
Duodenal (3rd step)	Sample obtained from gastric digestion	Duodenal 12 ml + 6 ml bile	37 °C	120

Ugolini, and Meli (2018). The enterococcal content of each aliquot (uninoculated, inoculated-undigested, and inoculated-digested) was evaluated by plate count after spotting 10 μl of the undiluted and diluted (up to 1:10,000) sample on BHIA plates, followed by incubation at 37 °C for 24 h.

The digestion solutions were prepared shortly before use, sterilised in an autoclave, with UV light or using a 0.22 μm membrane filter (Millipore, Burlington, MA, USA), depending on their heat resistance and physicochemical properties, and maintained at 4 °C.

At the end of *in vitro* digestion, samples were centrifuged at 6000 rpm for 10 min at 4 °C and the pellet was resuspended in 10 ml sterile saline solution.

All experiments were carried out twice in triplicate.

2.5. Culture-based enterococcal counts and detection of antibiotic-susceptible subpopulations

The homogenate 10 ml-aliquots, either uninoculated or inoculated with the enterococcal culture, were diluted in saline at 10^2 (negative control), 10^6 (digested aliquots) or 10^7 (undigested aliquots); 100 μl of each dilution was then spread on BHIA plates and incubated at 37 °C for 24 h before the CFU/ml counting.

To verify the maintenance of the resistance phenotype, growing colonies were replica-plated onto BHIA supplemented with TET and CN (respectively 10 μg/ml and 500 μg/ml) in the case of *E. faecium* 6767/7 and *E. faecalis* 113324 and onto BHIA supplemented with ERY, CN and STR (respectively 10, 500 and 1000 μg/ml) in the case of *E. faecium* 22571/2 and *E. faecium* 125745.

All experiments were performed twice in triplicate.

2.6. qPCR enterococcal counts

Genus-specific qPCR targeting 23S rDNA was performed as described previously (Di Cesare et al., 2013), except that total DNA was extracted from 500 μl of each sample using the GeneAll® Exgene™ Soil DNA mini (GeneAll Blgd, Dongnam-ro, Songpa-gu, Seoul, Korea) following the manufacturer's instructions. *E. faecalis* ATCC 29212 DNA and RNase-free water were used respectively as the positive and the negative control. The abundance of enterococcal cells was calculated by dividing the amount of amplified DNA (ng) first by the weight of one copy of the 23S rDNA amplicon (0.0996×10^{-9} ng) and then by the gene copy number (4) found in one enterococcal cell; the value thus obtained was multiplied by 500 (undigested aliquots) or 5000 (digested aliquots), using the cell abundance determined in 1 ml of the initial sample as the reference.

2.7. Detection of resistance genes

The presence of genes *aac(6')-aph(2")*, *tet(O)*, *ermB* (Garofalo et al., 2007) and *ant(6)* (Kobayashi et al., 2001) was investigated by PCR, as previously reported.

2.8. Statistical analysis

Among-group comparisons were performed by one-way ANOVA followed by post-hoc Scheffé test. All analyses were performed using SPSS 23.0 and a dedicated Excel spreadsheet. Significance was set at $p < 0.05$.

3. Results

3.1. In vitro conjugal transfer of antibiotic resistance traits

The four ARE, two from human blood and two from the marine environment (Cittterio et al., 2017), were analysed for their ability to transfer their resistance traits in conjugation assays (Table 2).

The resistance determinants of all strains were transferred in both intra- and interspecific matings with the only exception of *ermB* of *E. faecium* 125745, which failed to be transferred in interspecific matings.

3.2. Enterococcal survival after in vitro gastrointestinal digestion

The ability of enterococci to survive human digestion was investigated by inoculating 4-g aliquots of mussel homogenate with 1×10^8 CFU/g of each of the four ARE and digesting them *in vitro* using a model of human gastrointestinal digestion. Digested and undigested aliquot pairs inoculated with the same bacterial strain were investigated for their content in culturable (plate count) and total viable (qPCR) enterococci and compared (Fig. 1).

The efficiency and reliability of the adopted qPCR protocol were demonstrated by the reaction R^2 value (0.99) and its Limit Of Detection (LOD), quantified as 4.66×10^{-9} ng/reaction.

The undigested aliquots consistently showed comparable plate and qPCR counts, suggesting that the whole cell population was culturable. In contrast, the digested aliquots exhibited a discrepancy between plate and qPCR counts, with a similar reduction (2–2.5 log) in CFU counts but variable qPCR results. In particular, the qPCR counts were higher than the plate counts in the *E. faecium* strains (about 1 log in *E. faecium* 22571/2 and about 2 log in *E. faecium* 125745 and 6767/2), whereas they were comparable to the plate counts in *E. faecalis* 113324.

3.3. Antibiotic resistance determinants and bacterial fitness

The maintenance or loss of the resistant phenotype after digestion was determined by plating the inoculated and digested aliquots on BHIA and replica-plating grown colonies on antibiotic-supplemented BHIA.

The bacterial counts found in BHIA without and with antibiotic supplementation are shown in Fig. 2.

Significantly fewer ($p < 0.005$) colonies grew on plates supplemented with each antibiotic than on BHIA. *E. faecium* 22571/2 and 125745 uniformly lost STR resistance (Fig. 2A), whereas ERY and TET resistance exhibited a uniform 3.5 log reduction. CN resistance, the only trait shared by all isolates, showed a strain-specific pattern ranging from a 2 log loss in the environmental isolate *E. faecium* 6767/2 to the absence of resistant cells of the blood isolate *E. faecium* 125745 (Fig. 2 A and B).

Colonies unable to grow on the antibiotic-supplemented plates were picked from plates without the antibiotic, amplified and assessed for any unexpressed resistance genes. Whereas *ant(6)-I*, *ermB* and *tetO* were uniformly lost, *aac(6')-aph(2")* showed a variable pattern: it was never detected in the CN-susceptible colonies of *E. faecium* 22571/2 and consistently detected in those of *E. faecium* 6767/2 and *E. faecalis* 113324; of the *E. faecium* 125745 colonies, 40% maintained and 60% lost the gene.

4. Discussion

The presence of AR genes reservoirs in the marine environment (Citterio et al., 2017; Vignaroli et al., 2016) represents an underestimated risk factor for the human health. Since the exceptional ability to acquire AR of some bacterial genera, such as enterococci, in the human gut has been reported (Torres et al., 2018), the consumption of raw or undercooked seafood containing ARE risks spreading AR by transferring antibiotic-resistant genes to stable as well as transient intestinal microorganisms.

In this work, three antibiotic-resistant strains of *E. faecium* and one strain of *E. faecalis* – the two main enterococcal species associated with human infections – were tested for their ability to overcome the human gastrointestinal barrier by assessing their survival and the maintenance of key resistance determinants after digestion. To do this, ARE were inoculated into a mussel homogenate that was subsequently subjected

Table 2
Donors, recipients, antibiotics used for the selection, conjugation frequencies and transferred genes.

Donor	Recipient	Selection on	Conjugation frequency	Transferred genes
E. faecium 22571/2	*E. faecalis* JH2-2	ERY	8.30E-06	*ermB*
		CN	5.90E-09	*aac(6')-aph(2")*
		STR	8.80E-07	*ant(6)-I*
	E. faecium 64/3	ERY	2.35E-08	*ermB*
		CN	2.40E-08	*aac(6')-aph(2")*
		STR	1.80E-09	*ant(6)-I*
E. faecium 125745	*E. faecalis* JH2-2	ERY	ND	/
		CN	1.58E-09	*aac(6')-aph(2")*
		STR	1.58E-09	*ant(6)-I*
	E. faecium 64/3	ERY	2.27E-7	*ermB*
		CN	1.27E-07	*aac(6')-aph(2")*
		STR	6.27E-08	*ant(6)-I*
E. faecium 6767/2	*E. faecalis* JH2-2	TET	1.10E-07	*Tet(O)*
		CN	1.50E-07	*aac(6')-aph(2")*
	E. faecium 64/3	TET	7.20E-09	*Tet(O)*
		CN	8.90E-04	*aac(6')-aph(2")*
E. faecalis 113324	*E. faecalis* JH2-2	TET	8.33E-10	*Tet(O)*
		CN	3.30E-09	*aac(6')-aph(2")*
	E. faecium 64/3	TET	1.13E-08	*Tet(O)*
		CN	2.27E-09	*aac(6')-aph(2")*

ERY = erythromycin, CN = gentamycin, STR = streptomycin, TET = tetracycline, ND = not detected.

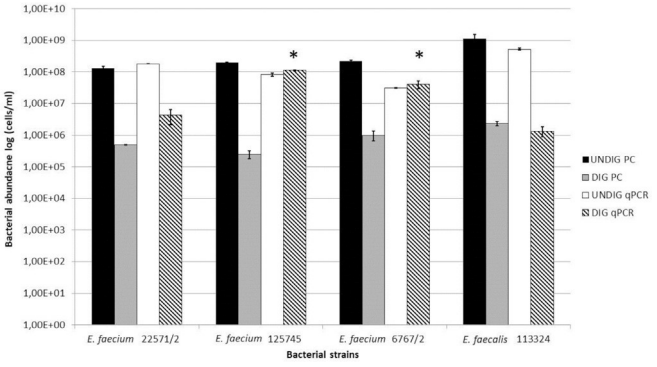

Fig. 1. Enterococcal count in undigested/digested aliquots of mussel homogenates. The abundance of culturable (plate count, PC) and total viable (qPCR) enterococcal cells was determined in paired undigested (UNDIG) and digested (DIG) aliquots. Results are mean of three biological replicates ± standard deviation. *p < 0.0.5.

to an *in vitro* human digestion model (Versantvoort et al., 2005). This approach obviates the use of experimental animals while enabling large numbers of strains to be tested in standardised conditions.

The four ARE used in the experiments, which had previously been isolated from different sources (Cittterio et al., 2017), were able to transfer their resistance determinants to susceptible recipients. Each strain did so with a different conjugations frequency that most likely depends on the type of genetic element carrying the gene and on possible rearrangements occurring during the transfer process (Manson, Hancock, & Gilmore, 2010; Palmer et al., 2012).

The count of culturable bacteria in undigested and digested aliquots differed by 2–2.5 log (from 10^8 to $5x10^5$-10^6 CFU/ml) regardless of bacterial species, strain origin and resistance phenotype. Such limited reduction is not surprising given the intestinal habitat of this bacterial genus (Lebreton et al., 2014).

Our findings also highlighted the ability of the four strains of *E. faecalis* and *E. faecium* (which are part of the intestinal microbiota and at the same time infectious agents) to survive digestion and reach the intestine regardless of their clinical or environmental origin. This agrees with the reported ability of *Enterococcus durans* LAB 18s to survive to a combination of simulated gastric juice and bile salts (Pieniz et al., 2014).

Although the ability of *E. faecalis* to develop VBNC forms is amply documented (Gin & Goh, 2013; Lleò et al., 2007; Signoretto, Burlacchini, Pruzzo, & Canepari, 2005; Wery et al., 2006), little is

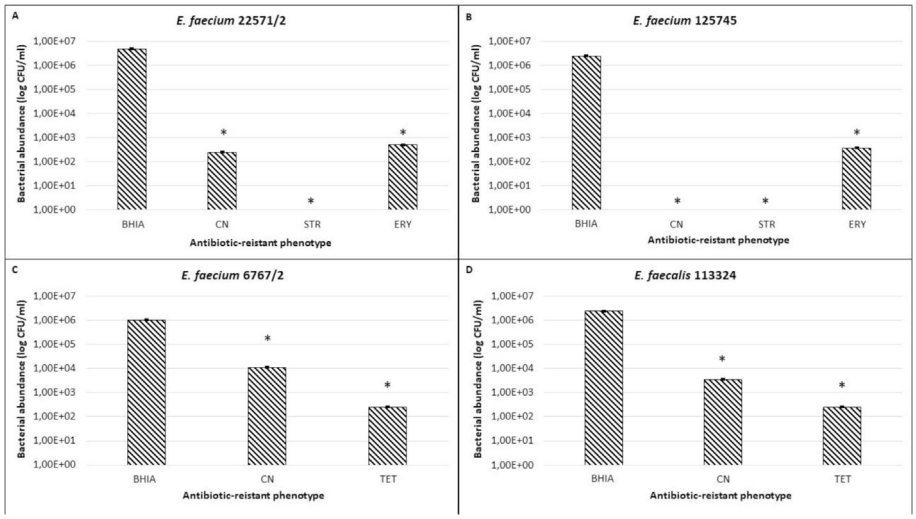

Fig. 2. Maintenance of phenotypic resistance after digestion. Plate counts in antibiotic-supplemented/unsupplemented BHIA plates. Results are mean of three biological replicates ± standard de- viation. BHIA = brain hearth infusion, CN = gentamycin, STR = streptomycin, ERY = erythromycin, TET = tetracycline.
*p < 0.05.

known about *E. faecium*. The significantly higher (1–2.5 log) qPCR counts found in *E. faecium* 6767/2 and *E. faecium* 125745 after digestion compared with the plate counts suggest the involvement of VBNC forms in their survival strategy, likely as a consequence of the exposure to stressors such as bile salts and low pH. The fact that, at variance with the data reported by Lleò et al. (2001), *E. faecalis* 113324 showed similar qPCR and plate counts could be related to a strain- and/or stress-specific response, although our small sample prevents drawing any firm conclusions.

The search for the resistance genes in strains turned susceptible after *in vitro* digestion highlighted the uniform loss of *ant(6)-I*, *ermB* and *tet*O in all isolates. This can be explained with the fitness cost of extrachromosomal elements and AR genes (Andersson & Hughes, 2010) and suggests that their loss threatens population survival. Moreover, *ant(6)-I*, *ermB* and *tet*O are generally carried by plasmids, and the absence of selective pressure (San Milan & MacLean, 2017; Baltrus, 2013) and exposure to stress conditions other than antibiotic pressure (Vogwill & MacLean, 2015) are known to induce the loss of resistance plasmids. In contrast, *aac(6′)-aph(2″)* was detected in three of the four ARE. Notably, whereas this gene was carried by all *E. faecium* 6767/2 and *E. faecalis* 113324 colonies tested, it was detected in only 40% of *E. faecium* 125745 colonies. It may be hypothesised that this subpopulation – and the whole population of *E. faecium* 6767/2 and *E. faecalis* 113324 – arises from a cell where recombination events led to a physical association between *aac(6′)-aph(2″)* and genes essential for survival in an unfavourable environment (such as the gastric milieu) or a tox/antitox system (Clewell et al., 2014). Moreover, *aac(6′)-aph(2″)* and *tet(O)* were probably carried by different genetic elements, as suggested by the loss of *tet(O)* in all four strains and the preservation of *aac(6′)-aph (2″)* in *E. faecium* 6767/2 and *E. faecalis* 113324. It is conceivable that in the marine environmental strain, *E. faecium* 22571/2 – the only isolate that lost all three genes – *aac(6′)-aph(2″)*, *ant(6)-I* and *ermB* are carried by the same element, likely a plasmid involving a fitness cost. Indeed, in the absence of selective pressure it may be convenient for marine environmental isolates – which are more exposed to multiple physical and chemical stressors compared with human isolates – to lose the AR genes. The maintenance of an additional extrachromosomal element involves itself a fitness cost (San Milan & MacLean, 2017; Baltrus, 2013). Indeed, it is well established that plasmid-carrying strains (Vogwill & MacLean, 2015) and those expressing plasmid-encoded resistance genes (Humphrey et al., 2012) have a reduced reproduction rate and that exposure to stressors other that those they are able to contrast, can induce the loss of genes that are not required for survival [Vogwill & MacLean, 2015]. In any case, *aac(6′)-aph(2″)* was the resistance gene recovered most frequently after gastrointestinal digestion. This is a cause for additional concern, since high-level CN resistance can hamper the treatment of severe enterococcal infections (Sparo, Delpech, & García Allende, 2018).

5. Conclusions

In a previous study we described the health threats posed by clams as reservoirs of MDR enterococci. We now provide further evidence of the risks associated with the consumption of contaminated seafood. In this work we demonstrated that three clinical and one marine environmental ARE survived gastrointestinal digestion while preserving their viability and AR traits. ARE also seemed to develop persistent (i.e. VBNC) forms, which in favourable conditions like those provided by some body districts, including the lower intestinal tract, can probably regain a full metabolic state. The ability of our ARE to transfer AR traits to human strains and survive *in vitro* digestion while maintaining high-level resistance to gentamycin underscore the involvement of seafood in the spread of AR to humans and in difficult to treat ARE infections.

Author contribution

BC and GM contributed equally to design the study; BC, CR and MAM performed *in vitro* digestion experiments; GM and NC performed microbiological and molecular assays; CV performed microbiological assays; MR performed the statistical analyses; FB discussed the results and coordinated all the work. All authors discussed the results and contributed to the final version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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