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# Influence of strains in development of francisellosis in the blue mussel Mytilus edulis during experimental challenges

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ABSTRACT

The bacterium *Francisella halioticida,* known to induce francisellosis in abalone and Yesso scallop, is suspected of being involved in the blue mussel mortalities observed in France. Recently, several isolates of *F. halioticida* were obtained from moribund mussels and categorized into two type strains, FR21 and FR22. Two other strains, AG1 and AG3, determined to belong to the genus *Francisella* were isolated. To determine the virulence of these isolates, juvenile and adult blue mussels were injected with bacterial solutions at high dose and monitored for 11 days. FR22c and FR22d were found to induce 80% mortality in less than seven days. The isolates AG1 and AG3 led to over 50% mortality in adult mussels but only AG1 led to significant mortality in juveniles (41%). FR22c and FR22d, the most virulent isolates, were selected to determine their respective lethal dose at 50% (LD50) in juveniles and adults. This analysis was performed with bacterial solutions ranging from $10^2$ to $10^6$ CFU/mussel and monitored for 30 days. The isolate FR22c was found to be the most virulent. Observed LD50 for the isolate FR22c was $4.14 \times 10^3$ CFU/juvenile and $3.45 \times 10^3$ CFU/adult and for the isolate FR22d, $1.89 \times 10^4$ CFU/juvenile and $1.52 \times 10^4$ CFU/adult. To confirm Koch's postulate, a selection of moribund, surviving and control animals were plated on specific media. The isolates were reisolated from moribund animals but not from surviving or controls. To confirm the proliferation, a specific real-time PCR was performed. All moribund individuals were positive by PCR. The main Ct values were lower for moribund compared to surviving animals and a dose effect was observed in DNA bacterial load. This study shows that some *F. halioticida* isolates are able to induce francisellosis in mussels and lead to high mortality, highlighting differences in virulence among the strains.

## 1. Introduction

Since 2014, mortality events of farmed and wild blue mussels are observed in France (Béchemin et al., 2015, 2014; Pepin et al., 2019). Similar events have been observed since 2015 in the Netherlands (Capelle et al., 2021). The events vary over the years in term of localization, season and mortality rate inducting that these mortality events cannot be predicted from one year to another and so, no epidemiological pattern was successfully determined yet (Pepin et al., 2019; Pépin et al., 2018, 2017). Some sites were affected repeatedly and never after without any modifications in farming protocols. However, even if events are spread over the coast and can be disseminated, when an event occur in a farming area, consequences can be devastating. In 2020, the hypothesis of the causality of the bacterium *Francisella halioticida* was expressed (Charles et al., 2020). This bacterium was already characterized as a virulent pathogen for the giant abalones (*Haliotis gigantea*) (Kamaishi et al., 2010) and for Yesso scallops (*Mizuhopecten yessoensis*) (Kawahara et al., 2019). Prior to studies in mussels, only three strains were isolated, DSM23729 (LMG26062) from abalone's hemolymph (Brevik et al., 2011; Kamaishi et al., 2010), UTH170823 and 8472–13 A from Yesso scallops muscle lesions (Kawahara et al., 2018, 2019). *F. halioticida*'s DNA has been detected in farmed mussels suffering or

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surviving mortality events in France (Charles et al., 2021, 2020) and in animals collected for studying beds decline in UK (Cano et al., 2022). Despite being extrapolated previously, the presence of *F. halioticida* in mussels does not indicate that this host can suffer francisellosis. In our previous study, several isolates of *F. halioticida* and *Francisella* spp. were obtained from mussels of different ages, location and health status (Bouras et al., 2023).

To our knowledge, the impact and potential pathogenicity of *Francisella* spp. in mussels have never been studied. Wangen et al. (2012) studied the fate of *Francisella noatunensis* in mussel as a potential reservoir risking infecting its original host, the cod *Gadus morhua*. Mussels were able to filtrate and digest the bacterium, but a portion remained alive and infectious in the feces. No information about mortality was reported, thus *F. noatunensis* was not considered as a potential pathogen for mussels. During an hemolymph microbiome analysis, Li et al. (2019) observed that *Francisella* spp. proliferate in the hemolymph of the Korean mussel (*Mytilus coruscus*) during exposition to *Vibrio cyclitrophicus* at 21°C. No identification at the species level was performed in this study. From our knowledge, *F. halioticida* is the only known *Francisella* species to be pathogenic to mollusks. During experimental challenges, the three strains isolated from abalone or scallop were able to produce high mortality rates towards their respective hosts even when injected at low dose such as $10^2$ CFU/mL (Kamaishi et al., 2010; Kawahara et al., 2019).

Isolates of *F. halioticida* obtained from mussels were classified into two strain types, FR21 and FR22 (Bouras et al., 2023). In this previous study, the isolate FR21 was selected for further analysis but did not lead to high mortality in mussels while injected at high dose ($3 \times 10^7$ CFU/mussel) in the given experimental conditions. At that time, it was observed that the four isolates belonging to type FR22 showed limited growth on the current culture media (Bouras et al., 2023), i.e. modified eugon supplemented with 2 mM $FeCl_3$ or 1% hemoglobin (Kamaishi et al., 2010). In opposition to FR21, these isolates were identified with high confidence from MALDI-ToF analysis using the 8472–13 A main spectra profiles (msp). In addition, these isolates corresponded to the proposed Fh-*rpoB* type 1 and showed a perfect identity to the reference strain *F. halioticida* DSM23729 on the 16 S rRNA amplicon. FR21 presented a Fh-*rpoB* type 2 and 99.9% identity to the reference strain on the 16 S rRNA amplicon (Bouras et al., 2023). Due to their similarity to known pathogens and dissimilarities with FR21 type, their implications in mussels' mortality could not be refuted from this study and remained of interest.

The present study relates the investigation of the pathogenicity of the *F. halioticida* and *Francisella* spp. isolates issued from moribund mussels collected during mortality events in France. A first screening was performed by injecting both adult and juvenile blue mussels at high dose ($10^6$-$10^7$ CFU/mussel) with each isolate. Experimental challenge was repeated with the isolates of interest to determine the lethal dose at 50%

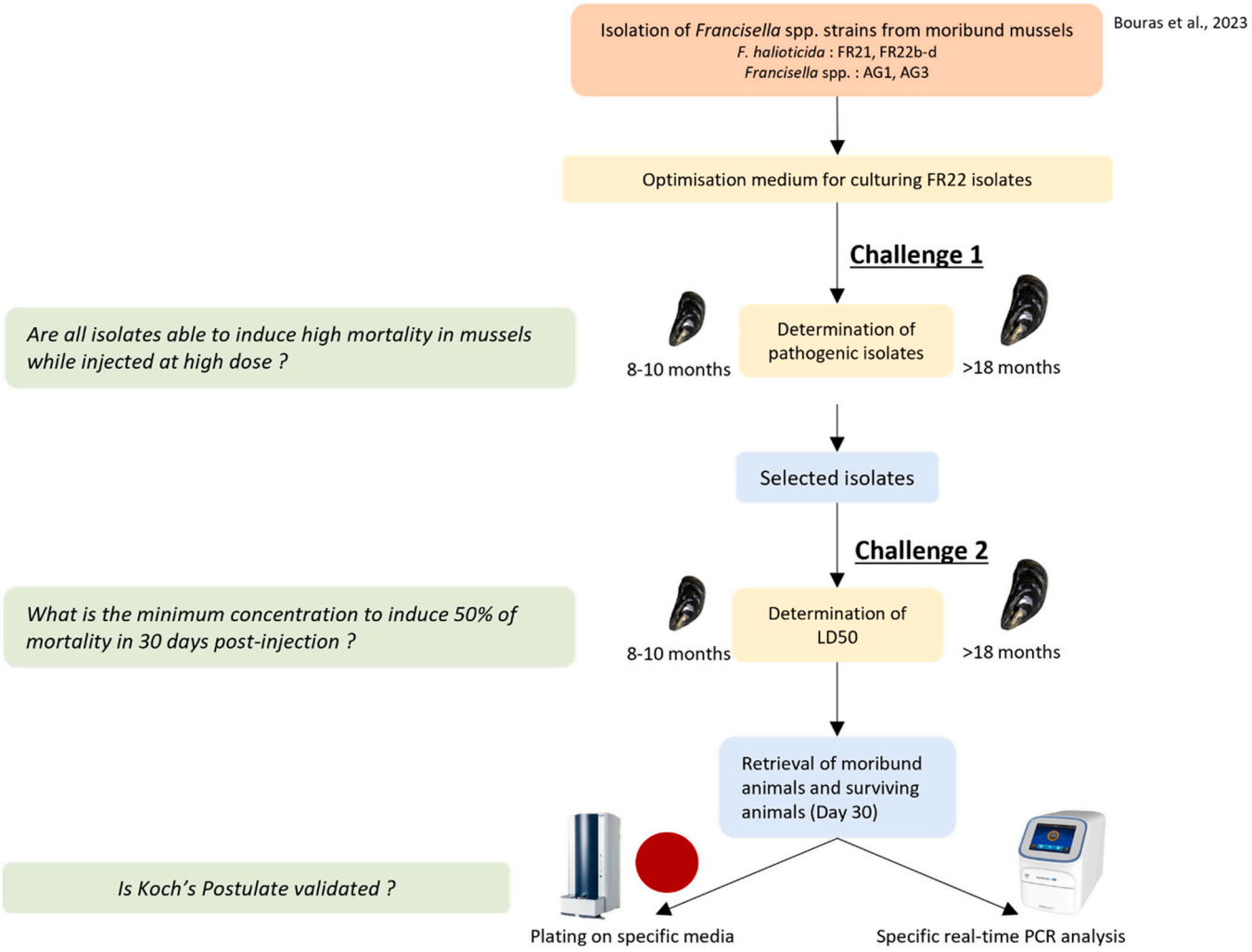


**Fig. 1. Experimental approach of the study**. Previously isolated *Francisella* spp. strains from moribund mussels (orange) were used to fulfill this study. To elucidate if mussels can develop the francisellosis disease, three main experimental steps were performed (yellow) which can be distributed into three main questions (green). Two majors step points (blue) links the studies.

(LD50) and a selection of moribund and surviving animals were randomly chosen to perform Koch's postulate.

## 2. Materials and methods

### 2.1. Experimental approach

The main goal of this study was to determine if some isolates obtained from moribund mussels induce francisellosis in blue mussels. This main goal can be divided into three minor questions that articulated the study (Fig. 1). Due to cultivation issues observed in our previous study, the cultivation media was primarily optimized to allow proper growth of isolates FR22. The isolates were further injected at high concentrations ($>10^6$ CFU/mussel) in juveniles and adults and mussel mortality was monitored (first challenge). Isolates displaying the highest virulence in the current settings were further used in the next challenge, aiming to determine the lethal dose at 50% (LD50, second challenge). Subsequent moribund and surviving animals at the end of the experiment were submitted to plating and specific real-time PCR to approve or refute Koch's postulate.

### 2.2. Strains and animals

The bacterial isolates were issued from a previous study, aiming to isolate *F. halioticida* from mussels (Bouras et al., 2023). Among them, three *F. halioticida* isolates originated from moribund mussels during a mortality event (FR21, FR22b, FR22c) and one *F. halioticida* isolate (FR22d) as well as two unidentified isolates (AG1 and AG3) were isolated from moribund mussels after acclimation in aquariums.

Both juvenile (8–10 months old) and adult blue mussels (>18 months old) were retrieved from the farming area of Agon-Coutainville (Normandy, France). To determine the endemic contamination of the initial batches, the animals harvested for each challenge were pre-screened for the presence of *F. halioticida* by real-time specific PCR based on the DNA-directed RNA polymerase β subunit (*rpoB*) gene (Charles et al., 2021) (Supplementary Table 1). DNA extraction and PCR reactions were performed as described in Bouras et al. (2023). Briefly, gills were collected from each mussel and pooled to produce 20 pools of four animal's gills. 50 mg of each pool was submitted to DNA extraction in an automated way using the NucleoMag tissue kit (Macherey Nagel) on the King-Fischer Flex System (ThermoFischer) following manufacturer's protocols at the exception of eluting with 60 µL buffer. Amplification was performed on a Quant Studio 5 (Applied Biosystems, Life Technologies) thermocycler and the results were analyzed with the associated Quant Studio software. The estimation of genome units (GU) for *F. halioticida* within the samples was achieved by utilizing data from Charles et al. (2021) for both quantification and calibration of the PCR method. Subsequently, this information was employed to formulate the following equation:

$$Genome\ units = 10^{-\frac{Ct-38.856}{3.3302}}$$

### 2.3. Optimisation of FR22 cultivation media

To improve cultivation media and enable correct growth of FR22 isolates, several media recipe were tested. As base, Kliger Iron Agar (Condalab, #1042.00), Bile Esculin Agar (Condalab, #1031.00), Brain Heart Infusion Broth (Condalab, #1400.00) and Columbia Broth (Condalab, #1229.00) were tested in addition to the previously used Modified Eugon Agar (MEA) (Bouras et al., 2023; Kamaishi et al., 2010; Kawahara et al., 2018). Broth media were completed with 15 g/L of bacteriological agar. All media were prepared at 70% artificial sterile seawater (ASSW) and tested in their initial composition or supplemented with 2 mM $FeCl_3$. The MEA was used complemented with 1% hemoglobin or 2 mM $FeCl_3$, as controls, but also with an increase of iron chloride at 4 mM. Pure isolates were subcultured on each recipe and incubated at 17.5°C for 15 days.

### 2.4. Challenge experiments

#### 2.4.1. Preparation of injected bacterial solutions

The isolates FR21, AG1 and AG3 were cultured on MEA supplemented with 2 mM $FeCl_3$ (Bouras et al., 2023) while the isolates FR22b, c and d were cultured on MEA supplemented with 4 mM $FeCl_3$. For each bacterial isolate, one colony was transferred in modified eugon broth (MEB) supplemented with either 2 (Kamaishi et al., 2010) or 4 mM $FeCl_3$, accordingly. The broth cultures were incubated at 17.5°C with agitation at 190 rpm for three days. To ensure that all solutions injected into mussels contained the same concentration of iron chloride, 100 µL of the isolate cultures of AG1, AG3, FR21 and 500 µL of the isolate culture of FR22b, FR22c and FR22d were transferred to fresh MEB 2 mM $FeCl_3$ and incubated for three days prior to the challenge in the same conditions. The day of the experiment, culture broths were adjusted to optical density at 600 nm ($OD_{600\ nm}$) of 0.3 using sterile MEB 2 mM $FeCl_3$, corresponding to $10^7$ to $10^8$ CFU/mL depending on the isolates. For the first challenge, all strains were used at $OD_{600\ nm}$ 0.3. For the second challenge, the selected strains were prepared in the same manner and diluted 1:10 in series to obtain bacterial solution ranging from $10^3$ CFU/mL to $10^7$ CFU/mL. For both challenges, conventional plate counting was performed for each bacterial solution used for injection to confirm the final concentration (Supplementary Table 2). To ensure proper growth in the *in vitro* setting of conventional plate counting, the isolates were diluted in MEB 2 or 4 mM $FeCl_3$ and spread on adequate MEA, accordingly to the strains as described above. Plates were incubated at 17.5°C for one to 15 days depending on the isolates and read daily for five days after first colony apparition.

#### 2.4.2. Handling of the animals

Prior to each experimentation, mussels were retrieved from the farming area and acclimated within the facilities in large tanks with aeration.

The first challenge was performed in the autumnal period (October-November). As seawater temperature was close to experimental temperature, animals were acclimated only during two to four days at 15°C. The second challenge was performed in the winter period (January-February). Seawater temperature was around 8°C, so mussels were retrieved from farming areas eight days before the experimentation and the water temperature of the tank was gently increased to reach 15°C.

For each experiment, mussels were cautiously separated to reduce the stress and calibrated at 28 ± 3 mm for juveniles and 38 ± 4 mm for adults. The calibration size was selected during the first challenge with the average observed in the mussel batch. To assure uniformity between both challenges, the same calibration settings was maintained in the second challenge. Prior to each injection, mussels were placed in anesthetizing solution (0.2 M $MgCl_2{\cdot}6\ H_2O$, 70% water, 30% seawater) for at least one hour. Once anesthetized, a volume of 50 µL of fresh broth (negative control) or bacterial solution was gently injected in the posterior muscle of each animal with an insulin syringe connected to a 26 G ½ 13 mm needle.

The first challenge (pathogenicity determination) was performed first with the adults (October) and then with the juveniles (November). For each age category, 90 mussels were injected with each strain or with sterile broth for the negative control (720 mussels distributed in eight batches). In the second challenge (LD50 analysis), juvenile and adult mussels were injected in parallel the same day. Due to a limited number of batches, preliminary tests were performed, and it appeared that juveniles might be more sensitive than adults to low concentrations. In consequence, only juveniles were injected with the lowest concentration ($10^3$ CFU/mL). In total, 30 mussels of each age category were injected with a dilution of each strain (540 mussels distributed in 18 batches). Each batch was deposited in a 12 L plastic tank connected to an open circuit seawater maintained at 15°C with a water flux of 12 L/h. Mussels

were fed with culture of *Isochrysis galbana* at a ratio of 1 $\times 10^6$ cells/day/mussel and feeding was launched 12 h after injection. The experiments were monitored twice daily for 11 days in the first challenge and 30 days for the second. In both experiments, moribund mussels were removed from the tank immediately to avoid cross-contamination. In the second challenge, moribund mussels were immediately frozen (-20°C) until further analysis. At the end of the second challenge, all remaining surviving mussels were also frozen (-20°C) until further analysis. Observed LD50 during the challenge was simply performed by plotting the cumulative mortality and observing which concentration led to 50% of mortality and link it to the day it was observed. To determine the exact dose to induce 50% mortality at 30 days post injection, the cumulative mortality at each time point was plotted for each dose. The LD50 was predicted with a non-linear regression curve fitting.

#### 2.4.3. Statistical analysis

For both challenges (virulence determination and LD50 analysis), cumulative mortality was plotted against time to observe the trend. Kaplan-Meier analyses were performed to determine statistical differences between the conditions. P-value were adjusted by Dunn-Sidak correction for multiple comparisons.

The presence of *F. halioticida* and its DNA load determination in samples were performed only for animals retrieved during the second experimental challenge (LD50 analysis). All moribund and surviving animals from conditions producing over 50% mortality at the end of the experiment at the exception of the condition dose 5, i.e., $10^6$ CFU/mussel, were subjected to further analysis. In addition, all control animals, and a selection of 8 surviving mussels for conditions producing less than 50% mortality at the end of the experiment were added to analysis (Supplementary Table 3).

Gills from each selected individual were removed and mashed. DNA extraction and real-time *F. halioticida* specific PCR was performed from 50 mg of tissues as described in 2.2. In addition, the remaining gills were briefly rinsed with artificial sterile seawater (ASSW) and spread onto the specific media for isolation MEA complemented with 1% hemoglobin (Kamaishi et al., 2005), 50 μg/mL ampicillin, 100 units/mL polymyxin B (Soto et al., 2009) and 10 μg/mL erythromycin (Kawahara et al., 2018). Plates were incubated at 17.5°C and monitored daily. Observed colonies with morphology of interest, i.e. grayish-white, circular and slightly convex with entire margins colonies (Brevik et al., 2011), were subcultured in MEA 4 mM $FeCl_3$ and subjected to prior identification with MALDI-ToF on BioTyper MicroFlex LH/SH using the Bruker database 9.0 and the main spectra from the *F. halioticida* 8472–13 A strain (Bouras et al., 2023).

The Ct values were compared with Kruskal-Wallis test followed by a post-hoc Dunn's Test. Analyses were performed for moribund animals for conditions over LD50 (where $n \geq 15$). For surviving mussels, eight randomly sampled surviving mussels were analyzed as well as surviving mussels for conditions having more than 5 surviving mussels. Boxplot were generated with the package ggplot2. All statistical analysis were performed under R version 4.3.0, R studio version 2023.06.0 (R Core Team, 2023).

## 3. Results & discussion

Molluscan diseases are diverse and impact different species of bivalves, mainly oysters and clams (Powell and Hofmann, 2015). Mussels, and especially *Mytilus* spp., are recognized as resistant species to disease and only few diseases have been documented (Lupo et al., 2021). The empirical method for describing disease is to perform Koch's postulate, linking four points to fulfill: the agent should be found in abundance in diseased animals and absent from healthy ones, the agent must be isolated, the agent must re-induce the disease when inoculated in a healthy animal and the same agent must be re-isolated following the challenge (Koch, 1884). For the opportunistic pathogens, the fulfillment of this postulate is even more complicated because their presence in the animal tissues or environment outside of disease outbreak might happen. In the case of fastidious bacteria, the isolation may reveal itself tricky. With the technical advances, some optimization of this protocol using molecular detection have been proposed (Byrd and Segre, 2016; Falkow, 2004; Fredericks and Relman, 1996). To perform reproducible experimental infections, it is crucial to cultivate the isolates in adapted media. At the time of isolation (Bouras et al., 2023), growth of FR22 isolates was unequal and variable in the broth media used (modified eugon complemented with 2 mM $FeCl_3$). The culture media, especially when not being optimal for a given isolate, can impact their potential virulence (Lozoya-Pérez et al., 2020; Pulkkinen et al., 2018). The culture media MEA supplemented with 1% hemoglobin was sufficient to maintain the FR22 isolates but this medium was not compatible with broth use. The species belonging to the genus *Francisella* are known to be fastidious and various type of media are required for each species or subspecies (Colquhoun and Duodu, 2011). The first step to elucidate the potential virulence of *F. halioticida* isolates from French moribund mussels was to optimize the media to ensure proper growth of the FR22 isolates.

Out of the 5 media recipes tested (BHI, Columbia, Kliger Iron, Bile Esculin and MEA), slow and fastidious growth compared to MEA 1% hemoglobin was observed on three of them, BHI, Columbia and MEA, all of them supplemented with 2 mM $FeCl_3$. Out of the three, the medium MEA 2 mM $FeCl_3$ was the one producing the most efficient growth. Interestingly sustainable and sufficient growth was observed on MEA complemented with 4 mM $FeCl_3$. Even if the media could be further optimized, the isolates belonging to the strain type FR22 were maintained in MEA 4 mM $FeCl_3$ from that time forward.

### 3.1. Determination of virulence

To determine the pathogenicity of the six isolates obtained from moribund animals, challenge experiments were performed on both juvenile (Fig. 2A) and adult mussels (Fig. 2B) using bacterial solution of each isolate at a high dose ($10^{6-7}$ CFU/mussel).

The isolates FR22c and FR22d led to high mortality while injected in both juveniles and adults. The isolate FR22c induced 90% mortality in six days in adults and in nine days in juveniles. The isolate FR22d induced 90% mortality in seven days in adults and ten days in juveniles. For both juveniles and adults, the impact of mortality whether they were injected with FR22c or FR22d was not significant ($p = 0.260$ and $p = 0.940$, respectively). Cumulative mortality induced by all other isolates were significantly different from FR22c and FR22d ($p < 0.0001$). However, the isolate AG1 was the one inducing the highest mortality outside of FR22c and FR22d. It induced 69% mortality in adults and 41% in juveniles and were significantly different from the other mortality curves ($p < 0.05$). The other isolates (AG3, FR21 and FR22b) induced over 40% in adults and were not significantly different from each other ($p > 0.05$). Despite suffering only few mortalities in the control batch (3/90, i.e., 3%), the adults seemed sensitive to all isolates ($p < 0.0001$). The juveniles did not show such sensitivity. No mortality was observed in the control batch and the isolates AG3 and FR21 did not induced significant mortality ($p > 0.05$).

In this first challenge, *F. halioticida* was not detected in the gills prior to the challenge for the adults and the prevalence was of 10% in the juveniles (Supplementary Table 1). The contamination was low (Ct > 38) and could be considering as noise. All isolates were found in mortality cases, with FR21 and FR22b coming from the same mass mortality event (>60% mortality). However, FR21 and FR22b did not lead to high mortality in this study. It was already known that FR21 did not induce high mortality in mussels in summer (Bouras et al., 2023) but it should be noted that, from this study, FR21 induced higher mortality rate compared to the previous study. Indeed, adult animals injected with $10^7$ CFU of FR21/mussel suffered only 36% mortality in 30 days compared to 43% in 11 days in this study. Certain hypotheses can be put forward to explain the fact that FR21 and FR22b were unable to induce mortality at a high rate. As described by Morens et al. (2004), the link between host,

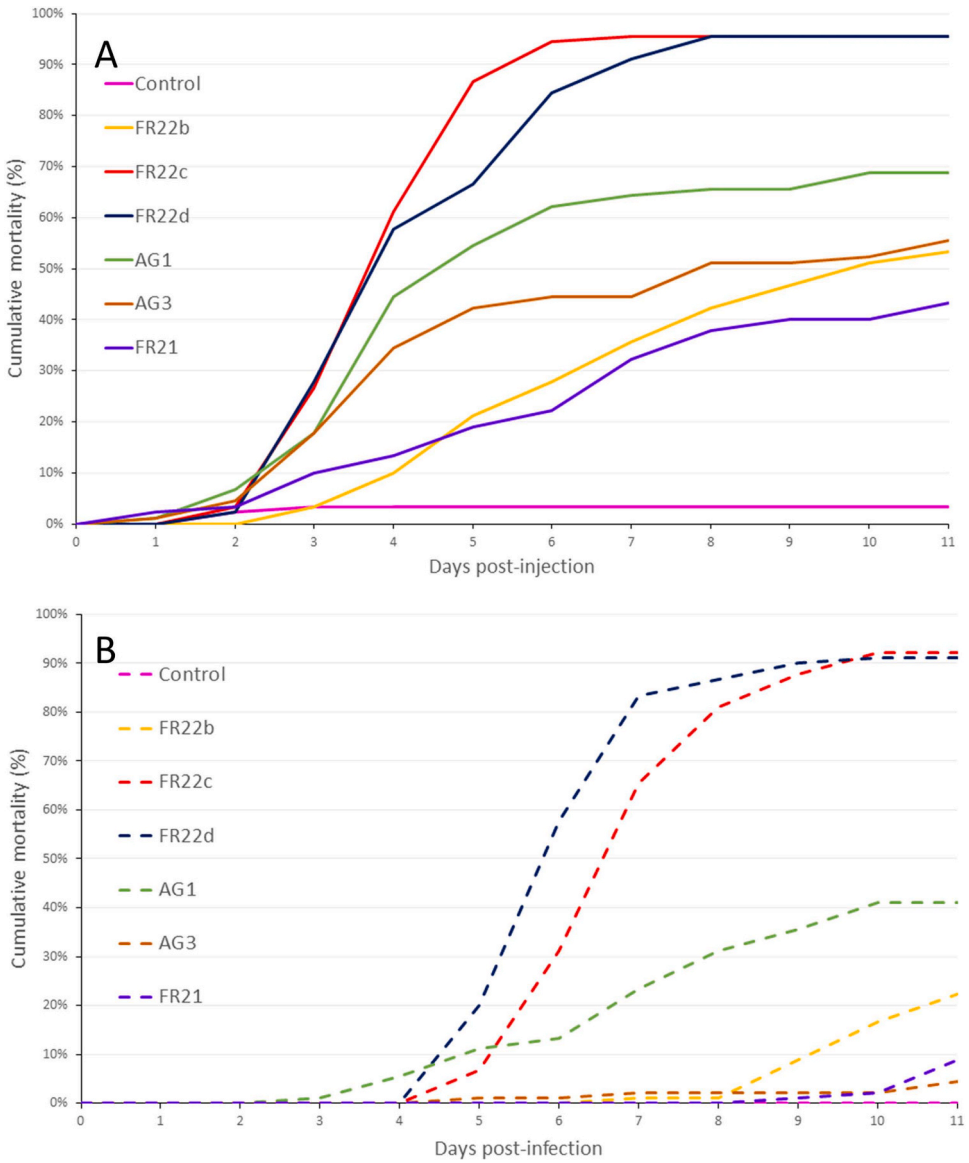


**Fig. 2. Cumulative mortality (%) of adult blue mussels injected with *Francisella* spp. isolated from moribund mussels.** (A) Adult mussels (18–20 months old, 38 ± 4 mm). (B) Juvenile mussels (6–10 months old, 28 ± 3 mm). Mussels were injected in the posterior muscle with bacterial solution at high dose (106–7 CFU/mussel) of *F. halioticida* (FR21, FR22b, FR22c, FR22d) and *Francisella* spp. (AG1, AG3). Negative control batch was injected with sterile broth. The mussels were maintained in circulating water at 15°C for 11 days and mortality was monitored daily.

environment and pathogen is very important to state of the epidemiologic potential of a disease. A model called the epidemiological triangle model must be met to induce a disease: the life stage and physiological state of the host, the pathogenic agent and the environment. As of today, the link between the environment, physiological state of the host and the pathogen is not clearly defined to induce francisellosis in mussels in the field. It might be possible that the conditions chosen in this experiment were not conducive to an induction of mortality by FR22b or FR21. Another hypothesis could be that the isolates need to work in synergy (with each other or with other bacterial species). Azizan et al. (2023) explored causality of summer mortality of the green-lipped mussel, *Perna canaliculus* and showed that *Photobacterium swingsii* and *Vibrio mediterranei* led to higher mortality when injected in synergy rather than alone.

From this study, in the given environment, it seemed that in the autumnal season, adult individuals seemed to be more sensitive to the isolates than juveniles at equivalent dose injected ($p < 0.0001$). The challenge including several isolates on two ages of mussels was not performed at another period of the year. The autumn was selected to suit the observation that *F. halioticida* is less detected at this period compared to spring and summer. One limit of the study is that no preliminary contamination of AG1 or AG3 could be performed as these isolates are not detected by the *F. halioticida* specific real-time PCR. These isolates are not yet identified but from 16 S rRNA sequencing, the closest relative to AG1 is *F. halioticida* with 98.2% identity and AG3 is believed to belong to the Thiotrichales order (Bouras et al., 2023). The identity value for AG1 is lower than the proposed threshold of 98.65% (Kim et al., 2014) which might indicate that it is a novel species in the *Francisella* genus. 16 S rRNA identity is not sufficient to support the description of a novel species and further in-depth genomic analyses are required.

Even if the isolate AG1 did not met the criteria of this study (over 80% mortality), its ability to induce mortality in both juvenile and adult mussels raise the question of its potential pathogenicity to other hosts and in more optimized conditions for its proliferation. The isolate AG3 should also be potentially further studied to determine if it can be deleterious to other hosts.

### 3.2. Serial dilution challenge

The selected strains FR22c and FR22d were used to perform LD50 analysis in the same conditions than the previous challenge. Only a single individual suffered mortality in the control batch both in juveniles and adults, at day 3 and day 7, respectively. In opposition to the first challenge performed in the autumn, the detection prevalence of *F. halioticida* in the control batch of the second challenge were high with 80% in adults and 90% in juveniles (Supplementary Table 1). Such detection was already observed in our primary study performed in the summer (Bouras et al., 2023). However, no living cells could be obtained from this control batch. It is known that isolation of *F. halioticida* can be challenging, especially when present in low number (Bouras et al., 2023; Kawahara et al., 2018) so from this study it cannot be stated if the signal comes from remaining DNA or living cells.

Overall, mortality trends induced by FR22c (Fig. 3A) and FR22d (Fig. 3B) were similar in both juveniles and adults for the two highest concentrations injected, i.e., dose 4 ($3\times10^5$ CFU/mussel) and dose 5 ($3\times10^6$ CFU/mussel) ($p = 1$). In comparison to the first challenge in adults (Fig. 2), mortality with the dose 5 was similar with 50% of mortality reached at day 4 post-injection. In juveniles, mortality was observed slightly earlier with 50% at day 4 in the serial dilution challenge (Fig. 3) opposed to day 6 in the first challenge (Fig. 2). As the animal reaction towards dose 4 and 5 was similar, it was decided to not analyze further dose 5 ($3\times10^6$ CFU/mussel) and use dose 4 ($3\times10^5$ CFU/mussel) as the highest dose.

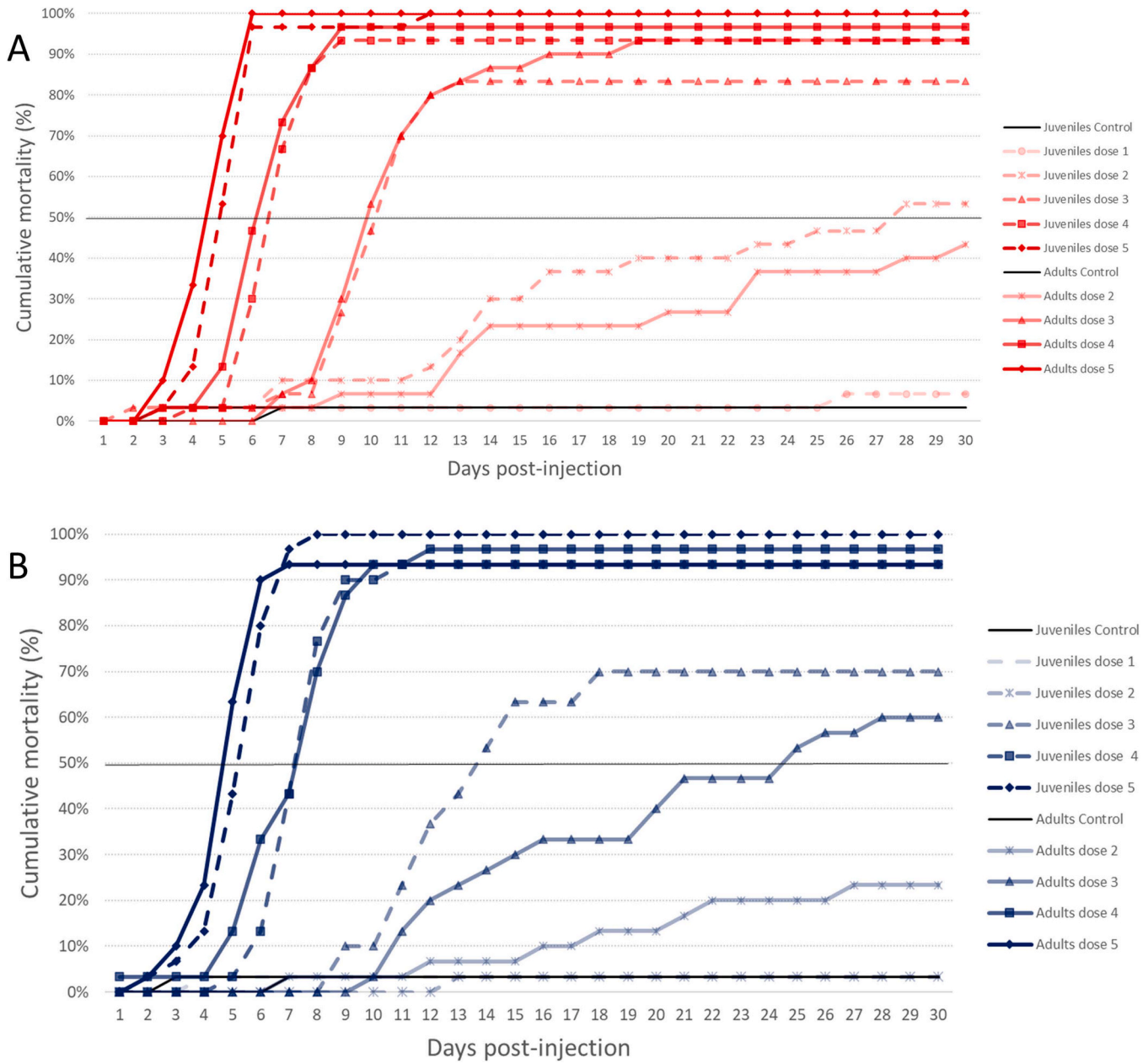


**Fig. 3. Serial dilution experimental challenge of *Francisella halioticida* towards juvenile and adult blue mussels**. Cumulative mortality of mussels injected with *F. halioticida* FR22c (A) and FR22d (B). Juvenile and adult anesthetized blue mussels were injected in the posterior muscle with solutions of *F. halioticida* from dose 2 ($3\times10^3$ CFU/mussel) to dose 5 ($3\times10^6$ CFU/mussel). In addition, one batch of juveniles were also injected with a solution at dose 1 (3 ×102 CFU/mussel). Animals were injected per batch of 30 individuals and maintained in open-circuit water tanks at 15°C for 30 days. Mortality was monitored daily. Negative control was performed by injected sterile broth. Juvenile are represented with dotted lines and adult with full lines. The black line represents the delimitation of the 50% of cumulative mortality.

For both juveniles and adults, the cumulative mortality induced by either FR22c or FR22d at a given dose was similar ($p > 0.05$) at the exception of the juveniles injected at $10^3$ CFU/mussel (dose 2, $p = 0.004$) and the adults injected at $10^4$ CFU/mussel (dose 3, $p < 0.0001$). In the same way, whether FR22c or FR22d was injected into mussels, juveniles and adults suffered similar mortality for a given dose ($p > 0.05$).

Both isolates at high dose led to fast and high morbidity with 80% cumulative mortality in less than eight days with the highest mortality rate observed at day 9 and day 12 when injected with FR22c or FR22d, respectively. When injected with FR22c at the dose 3 ($3\times10^4$ CFU/mussel), the similar cumulative mortality curve trend was observed. Both juveniles and adults reacted in the same manner up to day 13 (over 80% mortality), then only adults continued to suffer slow mortality to reach 90% at day 19 post-injection. A change of mortality curve trend was observed since dose 2 ($3\times10^3$ CFU/mussel) when injected with FR22c and since dose 3 ($3\times10^4$ CFU/mussel) with FR22d. When injected with FR22c at the dose 2 only juveniles crossed 50% of mortality at day 28. Despite being slightly delayed, the cumulative mortality of adults reached 43% at the end of the experiment. When injected with FR22d, juveniles reached a maximum of cumulative of mortality at day 18 (70%) while adults achieved 60% cumulative mortality at day 28. A similar phenomenon was observed in infection of Nile tilapia with *Francisella noatunensis* subsp. *orientalis* LADL 07–285 A at $10^5$ CFU/mL after intraperitoneal injection. LD50 were observed at $1.8\times10^4$/fish at day 20 post-injection (Soto et al., 2009). At concentrations under this shift, only fews moribunds animals were observed, which was not significant compared to the control batch ($p > 0.05$).

A follow-up experiment of 30 days may be demanding in term of costs and maintenance. To determine if the analysis could have been reduced to 15 days, the $LD50_{15\ days}$ and $LD50_{30\ days}$ were generated. Independently of the age, the animals injected with FR22c showed a LD50 between 3.45 and $7.97\times10^3$ CFU/mussel while those injected with FR22d ranged from 1.52 to $5.26\times10^4$ CFU/mussel. At the exception of $LD50_{15\ days}$ of adults injected with FR22d, predicted LD50 values, either at 15 or 30 days showed similarities among a given strain and a given time post-injection (Table 1). There was a decrease around 2-fold in LD50 of animals injected with FR22c between 15 and 30 days. The LD50 of juvenile animals injected with FR22d only slightly decrease between 15- and 30-days post-injection ($2.17–1.89\times10^4$ CFU/mussel), while adults showed a decrease of near 3.5-fold in 15 days.

The $LD50_{30days}$ of adults injected with FR22c was lower than the one obtained for juveniles even though only the latter conditions crossed the 50% mortality in this study. This could be explained by the fact that predictive LD50s were obtained from non-linear regression and the dose 2 ($3\times10^3$ CFU/mussel) was the lowest dose injected to adults. If some data were gathered for the impact of *F. halioticida* FR22c at $3\times10^2$ CFU/mussel (dose 1) in adults, the output of prediction would have been different. We emphasize to take the predictive LD50 carefully because, in a general manner, mussel origin, farming area, a potential previous encounter with the isolate type and the experimental conditions might strongly influence this value. A change in sensibility in the animal batch was seen for the dose 3 ($3\times10^3$ CFU/mussel), where FR22c was still able to reach 80% rapidly for both adults and juveniles whilst FR22d showed a slower mortality rate. In fact, the animals used in this study originated from the same area of Agon-Coutainville as those from which FR22d was isolated. The isolate FR22c was isolated from animals farmed in the south of Britany. Dégremont et al. (2019) highlighted that mussel that already survived mortality events presented a better resistance the following year. This observation suggests a hypothesis that mussels exposed to the bacterium at low concentrations may exhibit greater resistance compared to those with no prior exposure. It would also englobe that FR22c and FR22d might not be clones and present distinct differences which should be investigated at the genome level. It would be interesting to improve the current detection tool to determine if the isolate FR22c have already been detected in animals in Normandy to re-enforce or question this hypothesis. In this study, $LD50_{30\ days}$ was slightly lower than the $LD50_{15\ days}$ despite being in the same log range. Even if it could still represent a good indicator if a challenge could not be performed for an extended period, at low concentration, 30 days might be a minimum to study *F. halioticida* pathogenicity.

**Table 1**
Determination of predicted LD50 at 15 days ($LD50_{15\ days}$) and 30 days ($LD50_{30\ days}$) post injection of Francisella halioticida infections of blue mussels Mytilus edulis. The isolates FR22c and FR22d were injected in both juveniles (8-10 months) and adults (22 months) with serial dilution concentrations ranging from $3\times10^2$ (only in juveniles) to $3\times10^6$ CFU/mussel. LD50 values are indicated in CFU/mussel.

| | $LD50_{15\ days}$ | $LD50_{30\ days}$ |
|---|---|---|
| FR22c - Juveniles | $7.78\times10^3$ | $4.14\times10^3$ |
| FR22c - Adults | $7.97\times10^3$ | $3.45\times10^3$ |
| FR22d - Juveniles | $2.17\times10^4$ | $1.89\times10^4$ |
| FR22d - Adults | $5.26\times10^4$ | $1.52\times10^4$ |

### 3.3. Detection of F. halioticida

A selection of individuals from several conditions (Supplementary Table 3) were plated on specific media and incubated at 17.5°C. Colonies being identified as *F. halioticida,* in mass spectrometry using MALDI-TOF, were obtained only from injected samples but none in the control animals whether moribund or surviving. Colonies were observed from 28 to 32 days of incubation while the plates were already colonized by various bacterial species and moistures. In comparison, few bacterial species and few molds were observed in the control animal plates. The isolation media based on three antibiotics and hemoglobin was previously described to isolate *F. halioticida* from frozen abalone's hemolymph (Kamaishi et al., 2010) or Yesso scallop lesions (Kawahara et al., 2018). Its limitation in isolation of the bacterium from mussel was already highlighted in our previous study (Bouras et al., 2023). As stated before, the culture medium was optimized by the increase of iron chloride from 2 to 4 mM. Despite the successful re-isolation of strains, no counting could be performed due to primary heavy contamination. When the condition cannot be met to plate the tissues fresh, the need for freezing is crucial to avoid the proliferation of bacteria after death. However, the passage to freeze complicates the isolation of *F. halioticida*. For further studies, it is crucial to adapt and optimize the isolation media to the mussel matrix. However, even in the context of difficulty of growth it could be proposed that the re-isolation of isolates from the moribund animals and not in the surviving and controls ones allow to fulfill the Koch's postulate. Since several years, molecular tools are used to support Koch's postulate with fastidious bacterial species (Byrd and Segre, 2016; Lagier et al., 2017; Paillard et al., 2004). The Ct values obtained from specific real-time PCR (Charles et al., 2021) for moribund or surviving animal at the end of the experiment were analyzed.

In the second challenge, the pre-screening batch was positive with mean Ct values of 34.6 ± 2.1 and 35.0 ± 1.8 for adults and juveniles, respectively. This corresponds to an estimate of 4–81 GU and 4–32 GU, respectively. The single moribund individual for each control condition showed a Ct of 34.3 (≈ 34.3 GU) and 31.3 (≈ 185 GU) for juvenile and adult, respectively. For the conditions that produced over 50% cumulative mortality, all moribund mussels (Fig. 4) had a positive detection to the real-time PCR specific to *F. halioticida* with Ct values ranging from 18 (≈ $1.8\times10^6$ GU) to 34 (≈ 28 GU) and means from 21 to 25 (≈ $1.4\times10^4$ – $2.3\times10^5$ GU). In animals injected with dose 4 ($3\times10^5$ CFU/mussel), adults mean Ct values were similar between both strains ($p = 1.0$) while juveniles presented a significative difference ($p = 0.003$) with FR22c showing the lowest mean Ct. The opposite is observed when injected with dose 3 ($3\times10^4$ CFU/mussel), juveniles showed similar mean Ct between both strains while adults injected with FR22c showed lower Ct values, but it was not significative ($p = 0.09$). It needs to be noted with

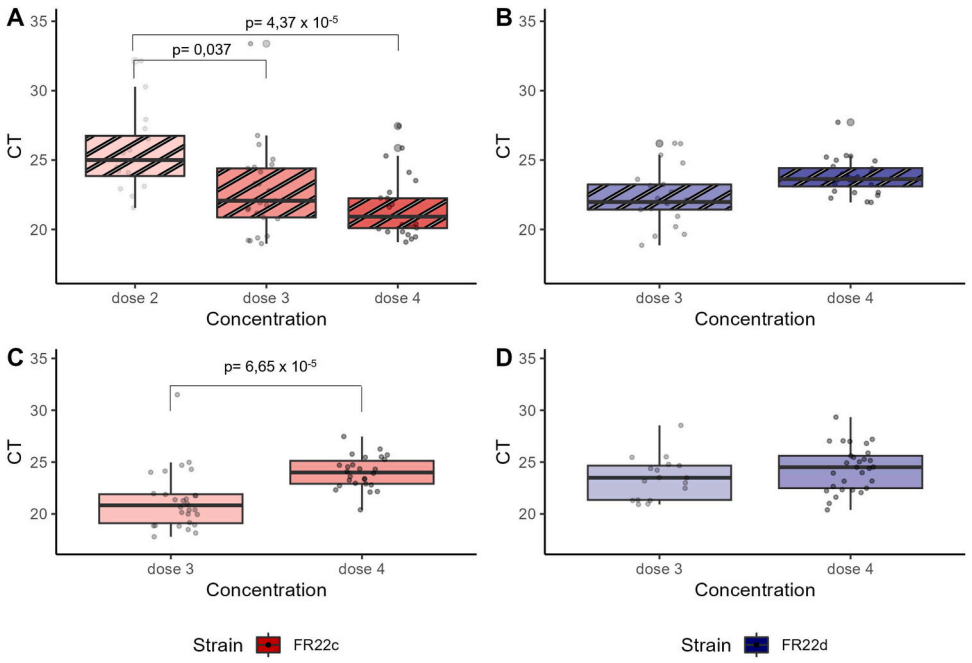


**Fig. 4. Detection of *Francisella halioticida* in moribund mussels.** For each condition that have led to over 50% mortality, each moribund animals were submitted to specific real-time PCR. Cycle threshold (CT) for each animal was retrieved. Dark dots represent the Ct of each individual used for calculation. Significance was obtained by Kruskal-Wallis test followed by a Dunn's test. A) Ct values of juvenile animals injected with FR22c. B) Ct values of juvenile animals injected with FR22d. C) Ct values of adult animals injected with FR22c. D) Ct values of adult animals injected with FR22d. Dose 2: 3 ×103 CFU/mussel, Dose 3: $3\times10^4$ CFU/mussel, Dose 4: $3\times10^5$ CFU/mussel.

less moribund animals were obtained when injected with FR22d at dose 3 ($3\times10^4$ CFU/mussel, n = 17) than with FR22c at the same dose (n = 28). For animals injected with the isolate FR22d, the mean for a given dose was similar between adults and juveniles (p = 1.0). When injected with FR22c, a significative difference between adults and juveniles was observed for dose 4 ($3\times10^5$ CFU/mussel, $p < 0.001$) but not for dose 3 ($3\times10^4$ CFU/mussel, p = 1.0). Juveniles showed similar mean Ct values whether they were injected with FR22c at the dose 3 or 4. Overall, the condition with the lowest mean Ct values was adults injected with FR22c at dose 3 ($3\times10^4$ CFU/mussel) and the one showing the highest Ct mean

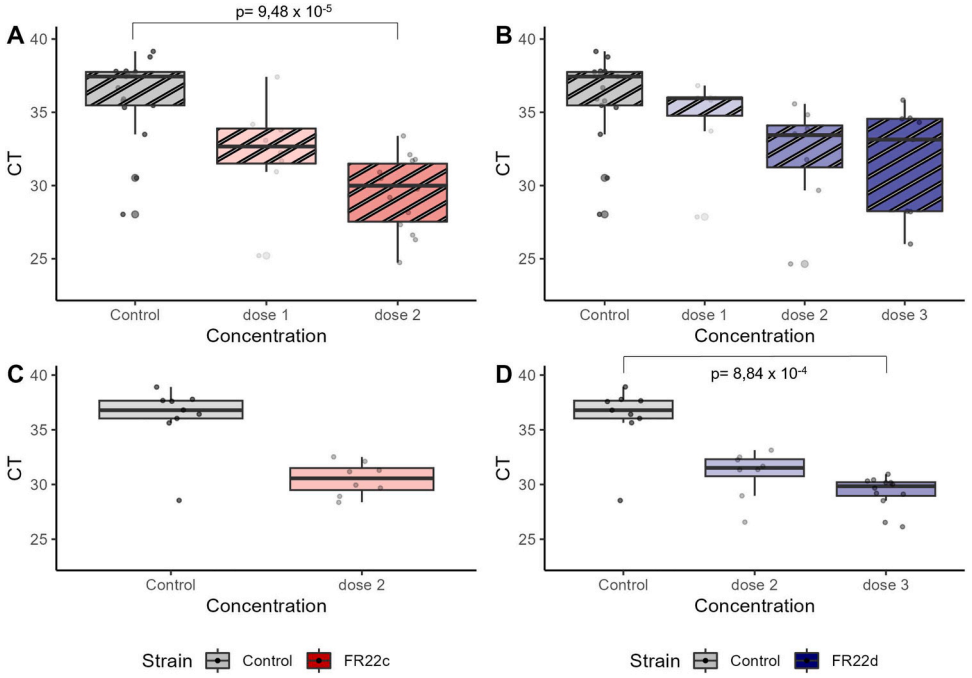


**Fig. 5. Detection of *Francisella halioticida* in surviving mussels.** All positive control animals, surviving individuals of conditions above LD50 if n >5 and eight randomly selected surviving animals from condition that did not reach 50% mortality were analyzed by specific real-time PCR. Cycle threshold (CT) for each animal was retrieved. Significance was obtained by Kruskal-Wallis test followed by a Dunn's test. In each figure, the control animals are represented on the left, in grey. A) Ct values of juvenile animals injected with FR22c. B) Ct values of juvenile animals injected with FR22d. C) Ct values of adult animals injected with FR22c. D) Ct values of adult animals injected with FR22d. Dose 1: 3 ×102 CFU/mussel, Dose 2: $3\times10^3$ CFU/mussel, Dose 3: $3\times10^4$ CFU/mussel.

was juveniles injected with FR22c at dose 2 ($10^3$ CFU/mussel). The estimation of genome units should be approached with caution as it may not necessarily represent the viable fraction of cells. Nevertheless, it is noteworthy that in the condition with the lowest Ct values (adults injected with FR22c at dose 3), the DNA load ranged from $5.9\times10^4$ to $9.1\times10^5$ GU/2 µl/50 mg of tissues, while only $3\times10^4$ CFU were introduced into the mussels, with an initial load of less than 100 GU/2µl/50mg.

In surviving mussels (Fig. 5), the number of isolates analyzed were either 8 (conditions not leading to 50% mortality) or the total amount of surviving mussels at the end of the experiment for controls (n = 29) and conditions leading to over 50% mortality (if n >5). Among the surviving control individuals, 17 out of 29 juveniles and 9 out of 29 adults had a positive detection. Only positive individuals were included in the analysis. The mean detection Ct stayed stable between the start (Supplementary Table 1) and the end of the experiment, but the proportions of positive animals decreased more in adults (0.80–0.31) than in juveniles (0.90–0.59). As no living cells could be isolated and that no further test such as RNA PCR detection was conducted, it cannot be clearly stated if the preliminary contamination originates from DNA or whole cells. It has been observed that field animals tend to be positive from December to September with the highest detection rate and lowest Ct values being in the spring. Recent studies on juvenile and adult Yesso scallops in Japan highlighted that DNA detection is also observed outside of mortality event. Even if some minor changes could be observed through the years, a stable cycle of contamination in Yesso scallops could be proposed (Furumoto et al., 2023; Kawahara et al., 2024).

Ct values of individuals injected with bacterial solutions were mainly comprised between 25 ($\approx 1.4\times10^4$ GU) and 35 ($\approx$ 14 GU) but none were detected under 25. When possible (juveniles injected with FR22c at dose 4 ($3\times10^5$ CFU/mussel), juveniles injected with FR22d at dose 3 ($3\times10^4$ CFU/mussel) and adults injected with FR22d at dose 3 ($3\times10^4$ CFU/mussel), differences between moribund and surviving mussels were analyzed. For conditions injected with FR22d, mean Ct values were significantly different ($p < 0.001$). For the condition injected with FR22c, the mean was less distant, but difference was still significative ($p = 0.011$).

The fact that the bacteria were injected in the muscle and were detected in the gills highlighted that the bacteria or its DNA circulated through the tissues. *F. halioticida* is a facultative intracellular pathogen (Kawahara et al., 2024; Meyer et al., 2017) and other *Francisella* spp. have been demonstrated to proliferate heavily in macrophages (Bakkemo et al., 2011; Clemens et al., 2009; Nano et al., 2004). From this knowledge, it could also be hypothesized that *F. halioticida* may circulate in the mussel tissues *via* the hemocytes circulation. In adults infected with FR22c, the fact that the Ct values increase as the injected concentration increased is surprising. In comparison with the other conditions tested, it may seem that FR22c in adults proliferated more when injected with dose 3 than dose 4 before death. When animals were injected with FR22d, no significant differences were observed between dose which could indicate that a given number of cells could be necessary to provoke the death of the individual. This hypothesis cannot be set for FR22c as trend are inversed between juveniles and adults.

### 3.4. Perspectives

It appears from this study as well as from the previous studies in mussels (Bouras et al., 2023; Charles et al., 2021) and in scallops in Japan (Furumoto et al., 2023; Kawahara et al., 2024) that this bacterium seem to be present nearly throughout the year in their respective hosts. It would be interesting to determine if this presence could be characterized as *F. halioticida* being part of the microbiota. This would encourage the exploration of a potential disease caused by an opportunistic pathogen. To understand better the disease, it is crucial to apprehend and comprehend the mechanisms leading to the proliferation of *F. halioticida* in field animals leading to francisellosis and mortality event. By its extended presence in animals, francisellosis in bivalves could be related to a long-time development disease. We observed a delay in mortality for the juveniles at the dose 5 between the first experimental injection (October for Adults and November for juveniles) and the LD50 analysis (February). The animals suffered high mortality two days earlier in January than in October. It is necessary to test the pathogenicity of the isolates at LD50 throughout the year to determine the highest season risk.

The isolate AG1 and AG3 despite not being selected for further analysis in this study in mussel should be further studied in other potential marine hosts. Other bivalve species are often farmed near mussels. We observe that the different isolates originated from different localizations. As of today, the spatiality of each isolate type in the French coast is not known. To prevent mass mortality of another species, it is necessary to test all isolates of this study in other farmed animal species. It would be also interesting the test the strains pathogenic to scallops (8472–13 A, UTH170823) or abalone (DSM23729) in mussels to see if the pathogenicity of *F. halioticida* is host-specific. Primary testing was performed in mussel with 8472–13 A which did not lead to subsequent mortality (data not published). However, proper testing with similar conditions as performed in this study should be conducted to verify these primary findings.

## 4. Conclusion

This study increased the knowledge of the pathogenicity of *F. halioticida* in mussels. The virulence of this bacterium seemed to be isolate-dependent, in the current tested conditions. Out of six *Francisella* spp. isolates tested, only two, belonging to the species *F. halioticida*, led to a high mortality when injected at high dose. The LD50 were similar between 15- and 30-days post-injection with $3.45–7.78\times10^3$ CFU/mussel when injected with FR22c and $1.52–5.26\times10^4$ CFU/mussel with FR22d. The two currently unidentified isolated showed pathogenic capacities for bivalves and should be further studied. Increased knowledge of francisellosis in mussel should be considered to understand the dynamic and the potential farming optimization that could be applied to manage the disease.

## CRediT authorship contribution statement

**Helene Bouras:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Maryline Houssin:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Manuel Savary:** Project administration, Funding acquisition. **Céline Zatylny-Gaudin:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Jean-Louis Blin:** Resources, Methodology, Investigation. **Yann Quesnelle:** Investigation. **Suzanne Trancart:** Investigation.

## 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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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102135.

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