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Impacts of rearing techniques on growth, survival and bacterial microbiota of carpet shell clam (*Ruditapes decussatus*) larvae

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Abstract In this study, we investigate the impacts of two water treatment regimes, the closed aquaculture system (CAS) and flow-through system (FTS), on the carpet shell clam (*Ruditapes decussatus*) larval microbiota, growth and survival. A bacteriological barrier (B) was created among breeder stage treated by florfenicol (FF, 8 mg $L^{-1}day^{-1}$ per 5 days). After spawning, larvae were maintained in five different rearing techniques: CAS; FTS; B + CAS; B + FTS and B + CAS + FF (8 mg L^{-1} day^{-1} per 3 days). Significant decrease was observed in both total bacterial count and presumptive *Vibrio* count (PVC) in FF-treated breeders. Besides, larvae reared in B + FTS showed significantly lower PVC than larvae maintained in other rearing techniques. It was also determined that growth and survival of larvae reared in B + CAS + FF and B + FTS were significantly better than those reared in other systems. One hundred and twelve *Vibrio* strains, isolated from breeders, eggs and larvae, were subjected to some bacteriological tests (API 20E strips, vibriostatic agent O/129, growth at different temperatures and salinity). *Vibrio alginolyticus* was the predominant isolated species (36.61 %), followed by *V. fluvialis* (25 %), *V. vunificus* (17.86 %), *V. cholera* (13.39 %) and *V. paraheamolyticus* (7.14 %). These results may contribute to the understanding of the effect of rearing techniques on the bacterial load, survival and growth of early life stages of *R. decussatus* larvae.

Keywords *Ruditapes decussatus* · Larval rearing · Flow-through system · Closed aquaculture system · *Vibrionaceae*

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Introduction

The carpet shell clam, *Ruditapes decussatus* (Linnaeus 1758), is the most abundant and commercially important species of *Veneridae* in different North African estuaries and lagoons. In Tunisia, it is found predominantly in the Gulf of Gabes (Medhioub 1983) and its collecting represents a traditional activity that has a significant socioeconomic impact in terms of both income and employment. The development of this economic sector involves not only the rational management of beds but also the breeding of this species, which requires mass production of juveniles. To this aim, several basic and applied research programs have been conducted to develop new techniques for improvement in *R. decussatus* larval and post-larval reared in the experimental bivalve hatchery of the National Institute of Marine Sciences and Technology.

In general, *R. decussatus* larvae are produced in closed aquaculture systems (CASs), which represent the most common rearing method used in bivalve hatcheries (Castagna et al. 1996; Robert and Gérard 1999; Helm and Bourne 2004; Rico-Villa et al. 2006). However, there are some drawbacks, such as microbial contamination risk and disease transmission. In fact, larval culture tanks water is completely exchanged at least three times per week, allowing microbes to increase their biomass in the rearing water (Andersen et al. 2000; Torkildsen and Magnesen 2004). Despite the use of antibacterial agents as prophylactic therapy, we have been confronted with recurrent epizootic disease outbreaks in larval and early post-larval stages, causing high mortality (Mechri et al. 2015).

Nowadays, large varieties of water circulation systems are used in bivalve hatcheries, such as the flow-through systems (FTSs) and the recirculation aquaculture systems (RASs). The FTS is based on regular water exchange in order to reduce bacteria as well as toxic substances, which accumulate in the rearing water (New and Valenti 2000). This method has been successfully used in the nursery culture of oysters, scallops, clams, mussels and in numerous fish hatcheries (Andersen et al. 2000; Ritar 2001; Tieman and Goodwin 2001; Otoshi et al. 2003; Sarkis et al. 2006; Rico-Villa et al. 2008). Recirculating systems operate by water undergoing treatment, allowing to the hatcheries production system's waters to be partially reused (Rosenthal et al. 1986). This technology has been widely used in fish culture for more than three decades (Rosenthal 1980; Verreth and Eding 1993; Martins et al. 2005), but only recently has it been experimentally tested in nursery culture of some mollusk species such as *Mercenaria mercenaria* (Pfeiffer and Rusch 2000), *Argopecten purpuratus* (Merino et al. 2009) and *Pecten maximus* (Magnesen and Jacobsen 2012).

The aim of this paper is to enhance our understanding about the eventual effect of larvae rearing techniques and antimicrobial treatments at the breeder stage on the bacterial load in the rearing system to fulfill the goal of eliminating the antibacterial use at the larval stage. In this study, we investigate the impacts of the CAS and FTS on the growth, survival and microbiota associated with *R. decussatus* larvae.

Materials and methods

Location of sampling area

The study was carried out at the experimental bivalve hatchery of the National Institute of Marine Sciences and Technology (INSTM), Monastir, Tunisia, which is located on the

edge of Monastir lagoon called Lake of Khniss. This lagoon communicates with the sea by a canal, of which a large part is artificial.

Hatchery design and operation

Water treatment

The water that feeds the experimental hall of bivalve mollusks and fish is a mixture between water lagoon and seawater at the proportion of 30 and 70 %, respectively. This water undergoes a sequential treatment: first sand filtration followed by a series of filtration pockets 25, 5 and 1 µm. UV treatment was used for algae culture up to 5 L and for larvae culture up to the metamorphosis.

Broodstock conditioning

R. decussatus broodstock from the south of Tunisia (Gabes gulf) was conditioned according to Medhioub et al. (2006) protocol. The reproducers were treated with florfenicol (FF) at the dose of 8 mg L^{-1} day^{-1} during 5 days before the spawning in order to create a barrier which limits the contamination of eggs and larvae. This treatment was incorporated with the food.

Spawning, fertilization and incubation

The spawning was realized according to Medhioub et al. (2006) protocol. Egg suspensions from the different adults are kept separate and should be filtered through 100-µm nylon mesh prior to the addition of the recent dense sperm at the ratio 1–2 mL L^{-1} of egg suspension. Fertilization was achieved at 22–24 °C. All fertilized eggs were pooled after 2 h.

The embryos were transferred to a rectangular flat bottom plastic tank (50 L) at the density of 20×10^3–40×10^3 L^{-1} and at 22–24 °C. After 24–30 h, most larvae reached D-shaped stage; the culture water was well exchanged and sifted through a 30-µm nylon mesh. The D larvae were then counted before being transferred to a culture tank.

Larvae culture method

Two methods with different system of water circulation as described by Helm and Bourne (2004) were used for larvae up to the early pediveliger stage: traditional method with closed aquaculture system (CAS) and flow-through system (FTS). Both technics were carried in 210-L cylindroconic fiberglass tank filled with seawater at 20–21 °C and a mean salinity of 38.

The experiment was conducted in a series of five tanks with three replicates, respectively: six tanks with FTS, three of which contained larvae produced by untreated breeders and the other three contained larvae obtained from florfenicol (8 mg L^{-1} day^{-1} per 5 days)-treated breeders. Nine tanks with CAS, three of which received florfenicol treatment at 8 mg L^{-1} day^{-1} during 3 days and the other six did not received treatment. In the closed aquaculture system, larvae were reared at a density of 5 larvae mL^{-1}, and culture water was 100 % renewed every 2 days where larvae received full food ration. Food ration will be at the half when the water is not renewed the following day. In flow-through

system, 25 larvae mL^{-1} was used with water renewal equivalent at 6–7 time day^{-1} with continuous and constant distribution of feeding. Survival was scored at the end of the cylindroconical experiments.

When larvae reach the early pediveliger stage, they must be transferred from the both system to settling in tube cylinder of (sieves tube) 60 l capacity with a circulating seawater system through 90-µm nylon mesh where water renewal was equivalent to 0.14 $m^3 h^{-1}$.

Feeding

In this study, four species of microalgae were used: *Chaetoceros* sp, *Isochrysis* sp, *Tetraselmis* sp and *Chaetoceros calcitrans*. The first three strains were isolated from Tunisian coast, respectively, in 1998, 2002 and 2008 at INSTM Monastir Center.

In CAS and FTS, larvae were fed a mixture of *Chaetoceros* sp and *Isochysis* sp at the ration of 40 cells μL^{-1} as an initial ration during 3–4 days and 80 cells μL^{-1} as maximum ration up to the end of metamorphosis. This ration was equivalent to 40–80 cells of *Isochrysis* and calculated on a cell volume equivalency basis where 1.0 cell of *Isochrysis* sp = 2.08 cells *Chaetoceros* sp.

From early pediveliger stage to metamorphosis, three species of microalgae were used *Isochrysis* sp, *Cheatoceros calcitrans* and *Tetraselmis* sp. The ration 80 cells of *Isochrysis* μL^{-1} was calculated in a cell volume equivalency basis where 1.0 cell of *Isochrysis* sp = 0.87 *Chaetoceros calcitrans*, 1.0 cell of *Isochrysis* sp = 0.23 *Tetraselmis* sp.

Measurement of larval length

Sixty *R. decussatus* larvae were picked every 2 days from each culture, fixed in formol-saline for 30 min and conserved at 4 °C. Larval specimens were photographed using a microscope (Zeiss, Germany) equipped with a digital camera. The anteroposterior length (µm) was measured using Image J software 1.45.

Collection of samples

Breeders, fertilized eggs and larvae were collected during all three ponds. Samples were immediately packed in UV sterilized oxygen-filled polythene bags separately, placed in an insulated container, and transported to the laboratory for analysis within 2 h.

Bacteriology

Bacterial count

In this study, we used Marine agar (Ma) medium, for the enumeration of the moderately halophiles heterotrophic bacterial count, and thiosulfate citrate bile sucrose agar (TCBS modified agar; Scharlau Microbiogy, Spain) supplemented with 2 % NaCl, specific for the *Vibrionaceae* biota. The enumeration of bacteria was carried out by using serial dilutions and the plating method to establish viable bacterial cell counts.

The water samples were tenfold serially diluted with artificial sterile seawater (ASSW), 1 mL aliquot of each dilution sample was spread out over the two media in duplicate. Colonies were counted after 24–48 h for the TCBS medium and 24–72 h for the marine

agar medium, with incubation at 23 °C. The results were expressed in colony forming units CFU mL^{-1} of water.

The breeder samples were washed thoroughly under running water, sprinkled with 70 % ethanol and allowed to dry. Subsequently, the clams were opened aseptically using a sterile scalpel. Five grams of the intervalvar liquid and soft meat were homogenized with 5 mL of ASSW, and then, 1 g of the mixture was diluted with 4 mL of ASSW to obtain 10^{-1} diluted homogenate. Samples were tenfold serially diluted with artificial sterile seawater (ASSW), 1 mL aliquot of each dilution sample was spread out over the two media in duplicate. Colonies were counted after 24–48 h for the TCBS medium and 24–72 h for the marine agar medium, with incubation at 23 °C. The results were expressed in CFU g^{-1} of clam-wet weight.

The fertilized eggs and larvae were enumerated by cell count under an optical microscope and were crushed in 1 mL of ASSW using an Eppendorf tube sterile pestle. The homogenate samples were serially diluted in the same solution, plated and counted as for the water samples. The count was multiplied by the dilution factor, divided by the number of the larvae and multiplied by 10^5. The results were expressed in CFU $\times$ 10^{-5} eggs or larvae.

Bacterial characterization

Three suspected colonies from typical *Vibrio* colonies on TCBS medium were picked up, purified on TSA agar (+2 % NaCl) and then identified through different physiological and biochemical tests such as Gram nonstaining (KOH) method, cytochrome oxidase activity, motility (Mannitol-Motility agar; Pronadisa, Madrid, Spain), resistance to vibriostatic O129 (10 and 150 μg), salt requirement (growth on 0, 2, 4, 8 and 10 % NaCl medium) and growth at 23 and 37 °C. The species identification of *Vibrio* was performed using API 20 E strips (bioMerieux, Marcy l'Etoile, France). The strains were conserved as frozen stocks at −80 °C in tryptic soy broth (TSB; Bio-Rad, France) with 2 % NaCl plus 15 % (v/v) glycerol.

Statistical analysis

All data were analyzed with SPSS version 21.0 software. Significance differences between bacterial counts were determined by the nonparametric Kruskal–Wallis test and Mann–Whitney test. Tukey test was used to obtain comparison between the larval growth means. Significance levels for all analysis were set at $P < 0.05$.

Results

Control of the bacterial biota

The bacteriological control of *R. decussatus* reproducer showed in all three ponds treated with FF, a significant decrease ($P < 0.05$) in total viable counts (TVC) in Ma medium (Pond1, T_0: $1.80 \times 10^9 - T_{96}$: 3.00×10^5 CFU g^{-1}) as well as in presumptive *Vibrio* counts (PVC) in TCBS medium (Pond1, T_0: $6.10 \times 10^6 - T_{96}$: 5.00×10^2 CFU g^{-1}). However, these bacterial counts increased progressively in untreated ponds, respectively, by a factor of 2.00 and 3.50 in Pond 1. In addition, the fertilized eggs obtained from treated

reproducers were free from any *Vibrio* contamination compared with those produced by untreated breeders (Table 1).

Influence of rearing techniques on larval microbiota

The assessments of the effects of rearing techniques on the bacterial load of *R. decussatus* larvae are presented in Tables 2 and 3, respectively. The total bacterial count for larvae reared in CAS without antimicrobial treatment ranged from 10^4 to 10^8 CFU $\times$ 10^{-5} larvae was significantly higher ($P < 0.05$) on the fourth day (CAS_{D4}: 10^7–10^8 CFU $\times$ 10^{-5} larvae) than in larvae maintained in other rearing systems (D4: 10^3–10^6 CFU $\times$ 10^{-5} larvae). Moreover, a total larval mortality has occurred by the sixth day (Pond 1 and 3) and the eighth day (Pond 2) of rearing in closed system. The bacterial biota of larvae obtained after microbiological breeder barrier and reared in closed-circuit seawater system (B + CAS_{D6}: 10^6–10^8 CFU $\times$ 10^{-5} larvae) was significantly higher ($P < 0.05$) than in larvae reared in CAS and treated three times with FF at 8 mg L^{-1} (B + CAS + FF_{D6}: 10^3 CFU $\times$ 10^{-5} larvae).

The presumptive *Vibrio* spp. count for larvae obtained from FF-treated breeders and reared in FTS (B + FTS_{D12}: 10^2–10^3 CFU $\times$ 10^{-5} larvae) was significantly lower ($P > 0.05$) than in FF-treated *R.decussatus* larvae and maintained in CAS (B + CAS + FF_{D12}: 10^5 CFU $\times$ 10^{-5} larvae). On the eighth day of rearing, a total mortality was recorded in larvae produced in closed-circuit water system. In addition, larvae obtained from treated reproducers and reared in FTS (BB + FTS_{D12}: 10^2–10^3 CFU $\times$ 10^{-5} larvae) showed significantly lower ($P > 0.05$) PVC than in larvae maintained in FTS (FTS_{D12}: 10^4–10^5 CFU $\times$ 10^{-5} larvae).

Influence of rearing techniques on larval growth and survival

The results showed that larval length was significantly affected by the rearing techniques (Table 4). In fact, larvae grew successfully and completed their metamorphosis with 100 % survival (data not presented) only when they were reared in B + FTS and B + CAS + FF. *R. decussatus* larvae maintained in FTS showed significantly better length (Pond 2, B + FTS_{D10}: 181.10 μm) than those produced in closed-circuit seawater system (Pond 2, B + CAS_{D10}:145.10 μm). Moreover, Larvae produced by untreated breeders and reared in FTS showed significantly lower length (Pond 3, FTS_{D12}: 168.40 μm) than those obtained from FF-treated reproducers (Pond 3, B + FTS_{D12}: 201.90 μm). Growth of larvae maintained in B + FTS did not differ from growth of larvae reared in CAS and treated with FF (Table 4). Besides, survival was better in larval group reared in B + CAS + FF (up to 69 %) compared to those maintained in other rearing systems (0–63 %).

Bacterial characterization

A total of 112 strains were isolated from breeders ($n = 42$) and larvae ($n = 70$) collected from the INSTM clam hatchery and biochemically identified as members of the family *Vibrionaceae* by API 20 E miniaturized system (Table 5). *Vibrio alginolyticus* was the most predominant species (41/112) followed by *V. fluvialis* (28/112), *V. vunificus* (20/112), *V. cholera* (15/112) and *V. paraheamolyticus* (8/112). High phenotypic heterogeneity was observed among the tested isolates. In fact, the majority of *Vibrio* strains were positive for

Table 1 Bacteriological control of *Ruditapes decussatus* reproducers biota by florfenicol treatment (8 mg L^{-1})

	Bacterial count (CFU g^{-1})										Spawning	
	T_{0h}		T_{24h}		T_{48h}		T_{72h}		T_{96h}		FE	
	TCV	PVC	TCV	PVC	TCV	PVC	TCV	PVC	TCV	PVC	TCV	PVC
Pond 1												
T	1.80×10^{9}	6.10×10^{6}	6.00×10^{8}	1.79×10^{5}	2.10×10^{7}	6.90×10^{4}	1.60×10^{6}	3.30×10^{3}	3.00×10^{5}	5.00×10^{2}	3.10×10^{2}	0.00
NT	$4.50 \times 10^{9(a)}$	2.70×10^{6}	$4.50 \times 10^{9(a)}$	6.50×10^{6a}	$5.20 \times 10^{9(a)}$	7.20×10^{6a}	$6.00 \times 10^{9(a)}$	8.70×10^{6a}	9.10×10^{9}	9.40×10^{6a}	1.20×10^{5}	4.50×10^{2}
Pond 2												
T	5.20×10^{9}	8.00×10^{6}	5.00×10^{8}	8.20×10^{4}	8.00×10^{7}	4.40×10^{3}	1.50×10^{7}	7.00×10^{2}	5.60×10^{6}	3.20×10^{2}	5.50×10^{2}	0.00
NT	$1.20 \times 10^{9(a)}$	6.40×10^{6a}	$2.00 \times 10^{9(a)}$	6.60×10^{6a}	$3.50 \times 10^{9(a)}$	7.80×10^{6a}	$3.00 \times 10^{9(a)}$	8.00×10^{6a}	$4.90 \times 10^{9(a)}$	8.80×10^{6a}	7.00×10^{5}	1.00×10^{3}
Pond 3												
T	2.00×10^{9}	2.70×10^{6}	7.00×10^{8}	2.90×10^{5}	1.20×10^{8}	1.70×10^{4}	3.20×10^{7}	5.00×10^{3}	7.90×10^{6}	9.60×10^{2}	8.00×10^{2}	0.00
NT	$6.30 \times 10^{9(a)}$	5.50×10^{6a}	$7.10 \times 10^{9(a)}$	6.00×10^{6a}	$7.80 \times 10^{9(a)}$	6.60×10^{6a}	$8.50 \times 10^{9(a)}$	6.60×10^{6a}	$9.20 \times 10^{9(a)}$	7.90×10^{6a}	2.00×10^{6}	4.70×10^{3}

The count for breeder samples (CFU g^{-1} wet weight soft tissues) is the average of 5 clam samples per test. The count for fertilized eggs (CFU $\times 10^{-5}$ eggs) is the average of 3 samples per pond

TVC Values in the same row with the same superscript in parentheses are not significantly different ($P > 0.05$)

PVC Values in the same row with the same superscript are not significantly different ($P > 0.05$)

TVC total viable counts g^{-1} wet weight soft tissues (10^{-5} eggs), PVC presumptive *Vibrio* counts g^{-1} wet weight soft tissues (10^{-5} eggs) TVC Values in the same row with

T treated, *NT* non-treated, *FE* fertilized eggs, *h* hours

Table 2 Effect of *Ruditapes decussatus* larval rearing techniques on total bacterial count

	Total viable count (CFU × 10^{-5} larvae)									
	POND 1					POND 2				
	CAS	FTS	B + CAS	B + FTS	B + CAS + FF	CAS	FTS	B + CAS	B + FTS	B + CAS + FF
D0	2.00×10^{6}	5.20×10^{6}	$5.10 \times 10^{4(a)}$	2.20×10^{4a}	$6.10 \times 10^{4(a)}$	6.70×10^{5}	7.10×10^{5a}	$7.30 \times 10^{4(b)}$	3.50×10^{4}	$6.00 \times 10^{4(b)}$
D2*	4.50×10^{7}	7.00×10^{6}	6.50×10^{5}	3.90×10^{4a}	2.00×10^{5}	8.20×10^{6}	7.50×10^{5a}	4.00×10^{5}	6.00×10^{4}	2.70×10^{5}
D4*	5.50×10^{8}	9.80×10^{6}	8.20×10^{6}	8.20×10^{4b}	3.50×10^{4}	7.00×10^{7}	9.00×10^{5}	9.60×10^{5}	8.80×10^{4}	7.00×10^{4}
D6*	X	3.60×10^{7}	1.70×10^{8}	9.80×10^{4b}	9.00×10^{3}	4.9010^{8}	3.20×10^{6}	3.20×10^{6}	1.60×10^{5}	8.10×10^{3}
D8		6.20×10^{7}	X	3.20×10^{5}	5.40×10^{3}	X	5.70×10^{6}	5.10×10^{7}	4.50×10^{5}	3.30×10^{3}
D10		8.10×10^{7}		6.90×10^{5c}	8.90×10^{5}		8.80×10^{6}	3.50×10^{8}	7.70×10^{5}	5.00×10^{5}
D12		1.00×10^{8}		8.00×10^{5c}	5.50×10^{7}		1.70×10^{7}	X	9.10×10^{5}	7.10×10^{7}

	Total viable count (CFU × 10^{-5} larvae)				
	POND 3				
	CAS	FTS	B + CAS	B + FTS	B + CAS + FF
D0	5.00×10^{5}	1.60×10^{5}	$1.70 \times 10^{3(c)}$	4.20×10^{3a}	$1.50 \times 10^{4(c)}$
D2*	7.10×10^{7}	3.70×10^{5}	9.50×10^{4}	5.50×10^{3a}	7.00×10^{5}
D4*	1.20×10^{8}	6.00×10^{5}	5.00×10^{5}	8.00×10^{3}	8.20×10^{4}
D6*	X	8.80×10^{5}	3.40×10^{6}	2.00×10^{4}	7.10×10^{3}
D8		2.10×10^{6}	7.20×10^{8}	5.10×10^{4}	2.30×10^{3}
D10		4.60×10^{6}	X	7.30×10^{4b}	3.70×10^{5}
D12		7.70×10^{6}		9.80×10^{4b}	2.10×10^{7}

The count for larvae samples (CFU × 10^{-5} larvae) is the average of duplicate samples for each rearing technique values in the same column with the same superscript are not significantly different ($P > 0.05$)

Values in the same row with the same superscript in parentheses are not significantly different ($P > 0.05$)

FF florfenicol, *BB* breeders barrier, *CAS* closed aquaculture system, *FTS* flow-through system, *D* day

*Florfenicol treatment (8 mg L^{-1})

Table 3 Effect of *Ruditapes decussatus* larval rearing techniques on presumptive *Vibrio* count

	Presumptive *Vibrio* count (CFU × 10^{-5} larvae)				
	POND 3				
	CAS	FTS	B + CAS	B + FTS	B + CAS + FF
D0	8.80×10^{3}	5.00×10^{3a}	$6.20 \times 10^{1(d)}$	2.20×10^{1a}	$6.00 \times 10^{1(d)}$
D2*	6.70×10^{5a}	6.20×10^{3a}	5.10×10^{2}	2.80×10^{1a}	4.20×10^{3}
D4*	4.20×10^{6}	8.00×10^{3}	2.60×10^{3}	4.40×10^{1}	8.90×10^{2}
D6*	X	9.50×10^{3}	5.40×10^{4}	7.50×10^{1}	8.00×10^{1}
D8		2.80×10^{4}	3.80×10^{6}	1.20×10^{2b}	5.10×10^{1}
D10		4.70×10^{4}	X	2.70×10^{2b}	3.20×10^{3}
D12		8.20×10^{4}		5.00×10^{2}	5.30×10^{5}

	Presumptive *Vibrio* count (CFU × 10^{-5} larvae)									
	POND 1					POND 2				
	CAS	FTS	B + CAS	B + FTS	B + CAS + FF	CAS	FTS	B + CAS	B + FTS	B + CAS + FF
D0	6.20×10^{4}	3.00×10^{4}	$3.50 \times 10^{2(a,b)}$	$2.50 \times 10^{2a(a)}$	$4.00 \times 10^{2(b)}$	4.40×10^{3}	7.20×10^{3a}	$2.10 \times 10^{2(c)}$	$1.50 \times 10^{2a(c)}$	$2.20 \times 10^{2(c)}$
D2*	3.70×10^{5}	5.90×10^{4a}	5.20×10^{3}	3.50×10^{2ab}	7.40×10^{3}	6.90×10^{3}	8.00×10^{3ab}	3.50×10^{3}	2.20×10^{2a}	1.20×10^{3}
D4*	6.60×10^{6}	6.30×10^{4a}	7.00×10^{5}	4.30×10^{2b}	1.40×10^{2}	2.70×10^{4}	9.60×10^{3b}	9.90×10^{3}	5.50×10^{2b}	8.90×10^{2}
D6*	X	8.80×10^{4}	9.90×10^{6}	6.90×10^{2c}	7.20×10^{1}	2.00×10^{6}	2.20×10^{4}	3.20×10^{4}	6.70×10^{2b}	6.70×10^{1}
D8		3.30×10^{5}	X	8.20×10^{2c}	3.60×10^{1}	X	5.00×10^{4b}	7.50×10^{5}	9.30×10^{2}	4.70×10^{1}
D10		6.00×10^{5}		2.10×10^{3d}	5.10×10^{3}		6.60×10^{4b}	4.10×10^{6}	3.00×10^{3c}	6.20×10^{3}
D12		9.10×10^{5}		3.90×10^{3d}	6.30×10^{5}		8.20×10^{4}	X	4.60×10^{3c}	8.00×10^{5}

The count for larvae samples (CFU × 10^{-5} larvae) is the average of duplicate samples for each rearing technique Values in the same column with the same superscript are not significantly different ($P > 0.05$)

Values in the same row with the same superscript in parentheses are not significantly different ($P > 0.05$)

FF florfenicol, *B* breeders barrier, *CAS* closed aquaculture system, *FTS* flow-through system, *D* day

*Florfenicol treatment (8 mg L^{-1})

Table 4 Effect of rearing techniques on *Ruditapes decussatus* larval growth and survival

	Mean ± SD (μm)									
	POND 1					POND 2				
	CAS	FTS	B + CAS	B + FTS	B + CAS + FF	CAS	FTS	B + CAS	B + FTS	B + CAS + FF
D0	94.10 ± 2.2^{a}	94.90 ± 3.5^{a}	94.10 ± 3.8^{a}	94.10 ± 4.4^{a}	94.10 ± 3.8^{a}	93.10 ± 2.5^{a}	93.10 ± 5.2^{a}	93.10 ± 3.4^{a}	93.10 ± 2.1^{a}	93.10 ± 4.0^{a}
D2*	96.20 ± 3.5^{a}	97.21 ± 5.0^{a}	97.00 ± 4.8^{a}	96.90 ± 3.5^{a}	97.20 ± 4.8^{a}	95.50 ± 3.8^{a}	96.00 ± 4.8 ^{a}	95.20 ± 5.0^{a}	100.43 ± 4.2^{b}	105.10 ± 4.9^{b}
D4*	97.00 ± 3.1^{a}	100.88 ± 2.1^{a}	98.12 ± 3.5^{a}	106.00 ± 5.0^{b}	108.56 ± 3.5^{b}	96.90 ± 6.0^{a}	99.90 ± 4.0^{a}	99.50 ± 6.3^{a}	117.53 ± 5.1^{b}	120.31 ± 3.5^{b}
D6*	X	106.44 ± 4.0	99.45 ± 2.7	126.50 ± 11.3^{b}	129.23 ± 2.7^{b}	104.00 ± 5.5^{a}	108.20 ± 7.4^{a}	106.10 ± 6.9^{a}	131.46 ± 9.4^{b}	132.97 ± 2.7^{b}
D8		128.40 ± 5.5	X	156.70 ± 12.0^{b}	159.21 ± 5.0^{b}	X	125.40 ± 8.2^{a}	123.00 ± 8.0^{a}	161.00 ± 12.6^{b}	162.18 ± 5.8^{b}
D10		150.00 ± 8.0		183.40 ± 10.0^{b}	185.90 ± 5.9^{b}		149.10 ± 7.5^{a}	145.10 ± 10.2^{a}	181.10 ± 11.6^{b}	185.00 ± 5.9^{b}
D12		172.20 ± 10.2		201.15 ± 12.6^{b}	205.00 ± 11.1^{b}		169.00 ± 9.0	X	200.10 ± 14.2	206.50 ± 11.1
S (%)	0	15	0	50	69	0	10	0	56	70

	Mean ± SD (μm)				
	POND 3				
	CAS	FTS	B + CAS	B + FTS	B + CAS + FF
D0	93.90 ± 4.5^{a}	93.90 ± 5.8^{a}	93.90 ± 3.7^{a}	93.90 ± 2.6^{a}	93.90 ± 5.5^{a}
D2*	96.00 ± 5.0^{a}	97.90 ± 6.4^{a}	97.10 ± 4.5^{a}	98.10 ± 4.3^{a}	100.87 ± 3.0^{a}
D4*	98.20 ± 7.2^{a}	100.00 ± 4.0^{a}	98.90 ± 4.9^{a}	108.00 ± 3.5^{b}	110.64 ± 4.2^{b}
D6*	X	104.00 ± 5.3^{a}	103.50 ± 6.2^{a}	130.10 ± 7.7^{b}	133.63 ± 6.0^{b}
D8		129.30 ± 7.8	121.00 ± 7.0	160.30 ± 9.8^{a}	164.10 ± 8.0^{a}
D10		141.90 ± 9.0	X	180.00 ± 10.8	186.87 ± 9.7
D12		168.40 ± 10.5		201.90 ± 11.5	208.31 ± 10.3
S (%)	0	18	0	63	75

The measurement of larval length (μm) is the average triplicate for each rearing technique and for each spawning. Values in the same row with the same superscript are not significantly different ($P < 0.05$)

S survival, *FF* florfenicol treatment, *BB* breeders barrier, *CAS* closed aquaculture system, *FTS* flow-through system, *SD* standard deviation, *D* day

* Florfenicol treatment (8 mg L^{-1})

lysine decarboxylase, indole production and glucose fermentation. Only, *V. fluvialis* isolates gave positive results with adenine dehydrolase and tryptophan deaminase tests. About 75 % (6/8) of the *V. paraheamolyticus* strains gave positive results with amygdalin and arabinose. *Vibrio* strains were able to tolerate low and moderate concentrations of NaCl (2–8 %), while only *V. alginolyticus* isolates were able to grow over a wide range of salinities (0–10 % NaCl).

Discussion

Members of the genus *Vibrio* are commonly found as part of the normal flora of molluscan intestinal tract (Oxley et al. 2002; Davis et al. 2013), thus making broodstock one of the principal sources of contamination in marine bivalve hatcheries. Several studies have been carried out on the gonads of reared mollusks revealed that they may be strongly infested with *Vibrio* (Lodeiros et al. 1987; Llanos et al. 2002; Sainz-Hernández et al. 2005) which leads to the hypothesis of their vertical transmission between reproducers and gametes. Widman (2001) suggested that *Vibrio* contamination into bivalve molluscan hatcheries can be controlled by relaying pre-spawning breeders for 24 h at 17–20 °C in filtered seawater. Conversely, Sainz-Hernández et al. (2005) revealed that this technique would not be sufficient for avoiding the circulation of vibrios into the clam system, since they found *Vibrio* in the gonadal tissue far from the digestive tract. In this study, we managed to create a bacteriological barrier among the breeder stage by FF treatments (8 mg L^{-1}) for five successive days before spawning in order to reduce *Vibrio* dissemination and to eliminate the use of the antimicrobial agents for *R. decussatus* larval rearing. TCBS data showed a significant decreasing of PVC in broodstock after antibiotic treatments. Moreover, fertilized eggs originated from treated reproducers presented relatively better microbiological quality than those obtained from control breeders.

This study clearly demonstrated that *R. decussatus* larvae obtained from FF-treated breeders and reared in FTS showed significantly lower bacterial load, especially for vibrio count, than other techniques. These findings are in agreement with Andersen et al. (2000) who showed that great scallop larvae rearing water in flow-through tanks had fewer bacterial counts than both rearing water in antibiotic-treated and untreated static tanks. In addition, only larvae maintained in both CAS + FF and B + FTS succeeded to develop beyond the stage of metamorphosis. In the literature, it is reported that antibiotic treatments during larval rearing prevent bacterial contamination, which increases successful larval rearing (Robert et al. 1996; Uriarte et al. 2001; Torkildsen et al. 2005). However, the overuse of antibiotics in treatment of mollusk aquaculture may contribute to an increase in the level of antimicrobial resistance (Mechri et al. 2013, 2015). The introduction of a flow-through system instead of closed-circuit water system in larval rearing tanks eliminated the use of antibiotics by improving water quality (Southgate and Ito 1998; Magnesen et al. 2006).

It was also noted that growth of larvae reared in B + CAS + FF and B + FTS was significantly better than those maintained in the other rearing systems. Phenicol are common broad-spectrum antibiotics used for aquacultural practice, as chemotherapeutic agents to manage and prevent diseases (Uriarte et al. 2001; Campa-Cordova et al. 2006; Mechri et al. 2012). Some countries have restricted the use of chloramphenicol and thiamphenicol in aquaculture because of their highly toxic potential on consumers (Van de Riet et al. 2003; Sarmah et al. 2006). FF is a fluorinated derivative of thiamphenicol with

an expanded spectrum of activity and enhanced potency but with less harmful effects (Samuelsen and Bergh 2004; Xu et al. 2005). Thus, FF is often used to control bacterial disease outbreaks in mollusk larval hatcheries (Torkildsen et al. 2002; Torkildsen and Magnesen 2004; Miranda et al. 2013). However, several reports have demonstrated that the extensive use of antibiotics in aquaculture could facilitate the emergence and the selection of resistant bacteria (Laganà et al. 2011; Mechri et al. 2015). Previous studies of antimicrobial resistance in fish pathogenic bacteria have reported the occurrence of the *floR* gene as the major facilitator superfamily and code for efflux proteins that export florfenicol out of the cell (Miranda et al. 2013; Schwarz et al. 2004). Recently, Miranda et al. (2015) found that most of gram-negative bacteria associated with scallop hatcheries carried the *floR* gene.

Sarkis et al. (2006) showed that calico scallop growth for FT-reared larvae was comparable or significantly higher than in the static system, which corroborate with our findings. Similarly, Rico-Villa et al. (2008) reported significant growth and competence improvements in *Crassostria gigas* larvae reared in the flow-through system compared to static system. These authors also revealed that the use of FTS with constant flows of high-quality water and a daily cleaning of the sieve clogs facilitated the circulation in the rearing tank and removal of metabolic waste products providing favorable conditions for larval development. Several studies suggested an association between the stocking density and the water exchange rate (Magnesen et al. 2006; Rico-Villa et al. 2008). Furthermore, Andersen et al. (2000) and Sarkis et al. (2006) reported a reduction in growth performance at high stocking density when they reared scallop (*Pecten maximus*) and oyster (*C. gigas*) larvae in FTS.

Survival of *R. decussatus* larvae reared in B + CAS + FF was better than for those maintained in other rearing techniques. These findings are in agreement with Andersen et al. (2000) who showed that in spite of low bacterial counts in great scallop larvae, increased larval survival was not demonstrated in the FTS compared to the antibiotic-treated CAS. Torkildsen and Magnesen (2004) reported that *P. maximus* larval survival in FTS was approximately similar to survival in CAS with prophylactic addition of chloramphenicol. Besides, Temperature and algal concentration were the most important factors that affect growth and survival (Le Pennec et al. 2003; Pernet and Tremblay 2004).

The predominant species isolated from almost all of broodstock and larvae samples was *V. alginolyticus* (36.6 %). This species has become increasingly recognized as an important opportunistic pathogen causing epizootic outbreaks in cultured fish (Ben Kahla-Nakbi et al. 2006), shellfish (Beaz Hidalgo et al. 2008) and crustaceans (Gomez-Gil et al. 1998). In our previous study, we found that *V. alginolyticus* species was the most commonly isolated (55 %) from the different compartments of *R. decussatus* hatchery (Mechri et al. 2012). Castro et al. (2002) characterized 123 *Vibrio* isolates from cultured *Ruditapes philippinarum* using phenotypic and genotypic methods and showed that *V. harveyi, V. splendidus and V. tubiashii* were the most abundant species. Moreover, Beaz Hidalgo et al. (2008) reported that 67.7 % of the *Vibrio* species associated with cultured carpet shell clams (*R. decussatus*) and Manilla clams (*R. philippinarum*) belonged to *V. splendidus*, *V. alginolyticus* and *V. diazotrophicus*.

Phenotypic characteristics of *Vibrio* isolates recovered from INSTM hatchery revealed high levels of identity. In fact, 112 *Vibrio* strains isolated on TCBS agar were further biochemically identified using the commercial miniaturized Api 20E kit and showed biochemical profiles similar to those reported earlier by Mechri et al. (2012). Conversely, these finding disagree those of Al-Sunaiher et al. (2010) who showed that the majority of *V. fluvialis* strains, isolated during fish outbreak, were positive to indole test and were able

to ferment mannitol. These authors also reported that all *V. vulnificus* strains gave negative result with lysine decarboxylase and were unable to ferment glucose and amygdalin.

Conclusion

In this paper, we demonstrate that the prophylactic use of FF at breeder stage improve the bacteriological quality of *R. decussatus* eggs. Despite considerable variability of the *Vibrio* load, no significant differences in growth performance were noted among larvae reared in B + FTS and B + CAS + FF. Thus, FTS appears to be an alternative that can eliminate the need for antibiotic treatments during larval rearing. However, several important parameters, such as larval densities, temperature and alimentation, need optimization to improve *R. decussatus* rearing performance. We also reported that *V. alginolyticus* strains were predominantly isolated from breeders, eggs and larvae. Further research is needed to understand the boundaries between commensalism and pathogenicity in this species.

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