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Probiotic effect of FLOC on *Vibrios* in the pacific white shrimp *Litopenaeus Vannamei*

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Abstract:

The advantages of FLOC over clear water (CW) in rearing juvenile *L. vannamei* and its effects on *Vibrio* communities were evaluated. Survival rate in FLOC and clear water were recorded and a probiotic was tested under both conditions. Daily growth rate (DGR) was higher in FLOC ($p < 0.05$) than in CW. Survival in each system increased significantly when a probiotic was included in the diet ($p < 0.05$). The Vibrionaceae community from the hepatopancreas (HP) and the culture medium did not differ between the two culture media. Nevertheless, a novel group of *Vibrio* strains was found to be unique to FLOC. No high level of lesions was observed in shrimp tissues from the FLOC + probiotic treatment; it suggests that the probiotic contributed to homeostasis and prevented outbreak of opportunistic pathogenic species.

Highlights

► It is described a vibriosis situation in a rearing system for shrimp ► Molecular analysis of *Vibrios* in ‘floc” for shrimp was conducted ► Phylogenetic tree for *Vibrios* in shrimp and seawater is displayed ► The addition of probiotic in clear water and “floc” conditions are examined ► Hepatopancreas histopathology showed that shrimp in floc had a better immune status than in clear water

Keywords : FLOC ; Probiotic ; *L.vannamei* ; Disease ; *Vibrio*

Introduction

BioFLOCs (Avnimelech, 2012) can assimilate pollutants from the water column (Valdes et al, 2013) and provide farmed shrimp with protein during the grow-out period. However, not all *Vibrio* species present in FLOC are beneficial to shrimp (Juis-Villasenor et al, 2013), and use of FLOC may introduce pathogenic *Vibrio* species into CW. The question is whether a FLOC medium will be enhanced by introduction of probiotics (Sapcharaen et al., 2013). Cultivation of penaeid shrimp under FLOC conditions started in the early 1970s as a new system to intensify culture (AQUACOP, 1984) and produced healthy animals (Far et al., 2013). FLOC was defined as a medium rich in organic matter, made of particulate biomass and colonized by bacteria. From a nutritional point of view it helps shrimp to gain weight (more than 2 g per week) owing to an abundance of native protein sources from protozoa, filamentous bacteria, nematodes, ciliates, flagellates, and rotifers (Decamp *et al*., 2002; Ray *et al.,* 2010). Bacteria in FLOC fluctuate and can have some antibiotic activity and similar genetic composition to that observed in wild life communities (Bianchi, 1979). Bacterial ecology in these systems achieves nutrient equilibrium because nitrogen is recycled and transformed by nitrifying bacteria (Burford, 2004). Natural production of some substances (Dinh *et al*., 2010; Iyapparaj et al., 2013) by bacteria in FLOC (Halet *et al*., 2007) has been reported to inhibit growth of co-habiting pathogenic species such as *V. harveyi* (Defoirdt *et al*., 2007). Furthermore, FLOC can improve shrimp gonadal maturity, as shown with females (Emerenciano et al., 2013).

Probiotics can control diseases in ponds (Moriarty, 1999). Also different quality waters affect the bacterial composition of shrimp gut: Moss *et al*. (2000) found higher diversity of Gramm negative aerobic bacteria in eutrophic media compared

to an oligotrophic one; Izquierdo *et al*. (2006) observed better survival and growth of shrimp cultivated green than those form clear water. *Pediococcus* sp. has been coated on pelleted feed for juveniles *L. stylirostris* (Castex *et al*., 2008). These efforts brought in a better control of pathogenic bacterial communities without changing the main physicochemical characteristics of seawater.

The purpose of the present study was to investigate the advantages of using Bio»FLOC» Technology (BFT) over the more traditional CW technique for growing *L. vannamei*, and to evaluate the effects of a probiotic feed supplement on the *Vibrio* community.

Materials and methods

Juvenile shrimp *L. vannamei* were originally caught from ponds at UMDI Sisal (UNAM) where they were kept in FLOC (Emerenciano *et al*., 2013) at 40 shrimp per m^2, with zero water exchange, heterotrophic media, constant aeration, a commercial pellet feed (35% od Crude Protein, Malta Cleyton) and organic fertilization with molasses. For the trial 280 shrimp were used and their initial average weight was 4.8 ± 0.6 g at nearly 2 g biomass per m^2, Water quality was assessed twice a day (08:00 and 16:00 h.): salinity (Vital Sine™ model SR6, Apopka, Florida, USA), temperature, dissolved oxygen (DO) (HACH Co. model hqd40, Loveland, Colorado, USA), pH (pH Testr™ 30, Vernon Hills, Illinois, USA), and ammonium (TAN), nitrates (NO_3^-), and nitrites (NO_2^-) with a saltwater master kit (HACH Co., Loveland, Colorado, USA). FLOC volume (FV) was controlled three times per week with an Imhoff cone. Clear water had closed recirculation with a continuous 30% renewal+aeration. Water quality was maintained through three filters 30, 10 and 5 µm mesh, a biological filter (live rock) and a sand filter, and the temperature was set at 28°C with a chiller Aquabone®; water passed through an

ozonizer Ikal-HA^{TM} model 1000, Cuernavaca, Morelos, Mexico. Meanwhile, the FLOC treatment had closed recirculation from a mesocosm main tank to feed experimental units, with aeration and zero water exchange. The trial lasted 45 days under a 12:12 light-dark photoperiod. The commercial feed was fed to the juveniles at 2% of body weight. A carbon source (molasses) maintained C:N ratio at ~20:1 (Avnimelech, 2012). The food was placed on a feeding tray at 08:00, 14:00 and 20:00 h. Remaining food was siphoned out each morning. Every 15 days, the weight gain was measured and the feed was adjusted. A commercial probiotic was added, containing a mixture of *Bacillus subtilis, B. natto, B. megaterium, Lactobacillus acidophilus, L. plantarum, L. brevis, L. casei and Saccharomyces cerevisiae* (Altai^{TM}, Providencia, Santiago, Chile) at 10^9 CFU g^{-1} or 2.3 g kg^{-1} feed. Daily growth coefficient (DGC) was expressed as mg day^{-1}((final weight$^{1/3}$-initial weight$^{1/3}$)/t, days)*100 and survival percentage was calculated as final minus initial number of shrimp* 100.

Water quality in the tanks was sampled at days 1, 15, 30 and 45. Ten shrimp were taken at day one (control) and on day 45 for each treatment. For histological studies samples of the HP in Davidson solution were processed at the Research Centre for Food and Development (CIAD, A.C in Mazatlán, Sinaloa, Mexico). Samples were dehydrated (Tissue-Tek^{TM} II model 4640-B, Tokyo, Japan), paraffin-embedded (Leica^{TM} EG1160, Nussloch, Germany) and sliced in a microtome Leica^{TM} 820, Nussloch, Germany (Lightner, 1996). Sections of the HP were stained with hematoxylin & eosin for identification of lesions observed with Olympus^{TM} CH30, Melville, NY and classified according to 1=less severe, 2=severe, 3=more severe. Tissues with the greatest severity of lesions were sent

to the APL (U o Arizona) and a fluorescent *in situ* hybridization (FISH) assay was performed to confirm a Necrotizing Hepatopancreatitis (NHP) disease. FISH assays used paraffin blocks previously prepared for histology, and a gene probe specific to the detection of NHP-causing bacteria; consecutive sections from each block were stained with Mayer-Bennett hematoxylin & eosin-phloxine.

All bacteria samples were cultured on TCBS agar selective for *Vibrio*. The colonies were differentiated according to their morphology and isolated for DNA extraction. Primes 357F (5 'CCTACGGGAGGCAGCAG 3') and 907R (5 'CCGTCAATTCCTTTGAGTTT 3') corresponding to variable regions V3-V5 were used for 16S ribosomal RNA gene amplification. PCR products were purified and sent for sequencing to the Biotechnology Institute (IBT-UNAM). Sequences were aligned with MUSCLE through the MEGATM 5 interphase along with the 16S ribosomal gene sequences from *Vibrio* strains identified with the BLAST EzTaxonTM Server 2.1 platform (Chun et al., 2007) as the nearest matches to our sequences. Phylogenetic tree (Fig 1) was constructed on alignments using 470 bp. The phylogenetic tree was generated using the Neighbor-Joining statistical method and a Jukes-Cantor substitution model. All trees were validated by a bootstrap method as a support test of their phylogeny. All data were analyzed with the Statistica^{TM}5.0 bioinformatic program. Bifactorial ANOVA of 2 x 2 (CW *vs* FLOC; and presence/ absence of probiotic) was applied on DGR and survival (previously arcsine-transformed), at significance level 0.05 (Tukey test).

Results

DGC and survival of shrimp all showed the significant advantage of FLOC over CW (Table 2). 16S ribosomal RNA gene sequences of the representative vibrios showed >98% of identity for *V. rotiferianus, V. owensii, V. plantisponsor* and *V.*

vulnificus. HP histology allowed a comparison between animals grown in CW or FLOC, and between the presence/absence of a probiotic. Shrimp sampled as a control group on day 1 showed level 1 lesion in HP. Regardless of presence of feed probiotic, at day 45 severe lesions (level 2 and 3) were more common on shrimp in CW than in FLOC (Table 3, Fig 1).

FISH analyses suggested that the level 1 and 2 lesions found in the tissues from shrimp fed probiotics have been a result of “Septic Hepatopancreatitis Necrosis” (SHPN), an infection caused by a pathogenic *Vibrio* sp. (APL *pers. com*,). There was no evidence of *Candidatus hepatobacter penaei.*

Discussion

The growth results confirmed previous reports related to significant improvement in growth and health of shrimp cultured in FLOC (Emerenciano, 2013). In this study the addition of a probiotic to the diet, whether in CW or in FLOC, had a beneficial effect on survival. Shrimp grown in CW were significantly smaller ($p>0.05$), and survival rates lower ($p<0.05$) than those grown in a FLOC. Furthermore, more shrimp had severe (level 2 and 3) lesions in HP when grown in CW. The type of lesions suggested a disease, SHPN or Vibriosis (Soto-Rodríguez *et al*., 2010), caused by a *Vibrio peneicidae*. (Saulnier *et al*., 2000; Goarant and Merien, 2006) or V. alginolyticus (Hsieh *et al*., 2007).

Actually, probiotics are extensively used in aquaculture (Vine *et al*., 2006), and they enhance the growth and survival of shrimp (Supamattaya *et al*., 2005); this was confirmed in the present study for survival in each rearing medium, and for weight gain in FLOC. Previous observations suggest that the resulting pH decrease due to transformation of sugars into lactic acid by the *Lactobacillus* spp. can, at the same time, avoid the proliferation of pathogens (Ma et al., 2009), as

has been shown for pathogenic *Vibrio* species in shrimp aquaculture (Griffith, 1995). However, the effect of probiotics on the composition of the Vibrionaceae community in rearing system and shrimp gut has not been established, nor their effects on the severity of the disease. The probiotic altered the species composition of the V*ibrio* community in a similar way in each rearing system. The relative severity of disease suggests a synergistic effect between the probiotic and FLOC that creates an advantage over CW to prevent a pathogenic outbreak. This could very well explain an increase in average growth and survival rates observed in FLOC tanks where probiotic was added, as well as the low number of severe lesions observed in the shrimp tissues. In addition, FLOC provides shrimp with nutrients (Crab, 2010) and increases their nutrition thanks to molasses (Schneider *et al*., 2006). Survival rates also improved not only because of a better nutrition (Burford *et al*., 2004), but also due to a stable bacterial community able to control pathogenic outbreak (Thompson *et al*., 1999). Furthermore, it has been suggested that FLOC benefits the shrimp immune system (Hsieh et al., 2007), since bacteria isolated from FLOC produced carotenoids, retinoids, poly-β-hydroxybutyrate (Defoirdt *et al*., 2007; Nhan *et al*., 2010) and exoenzymes (Bairagi et aal., 2002). In FLOC, although some shrimp developed severe tissue lesions, caused by bacterial infections, these were fewer than in CW. The exclusiveness of the novel group of vibrios found in FLOC was confirmed during this trial when five more sequences that belonged to isolates recovered from FLOC and to the shrimp obtained from this system cladded within it. Among the identified species, *V. harveyi* has been known to cause pathogenic outbreaks (Lightner, 1996) that have highly increased mortalities in shrimp. These outbreaks disturb the equilibrium among the bacterial communities present in the rearing medium, making it easier to cause

massive infections that will create lesions in shrimp tissues and eventually lead to death. Another explanation for the changes in the Vibrionaceae community is the effect of quorum-sensing molecules. *B. subtilis* present in the probiotic is known to produce communication molecules to alternate between competence for DNA uptake and sporulation (Kumar and Singh, 2013). These molecules could in turn interfere with the communication between *vibrios* and therefore prevent their proliferation.

This study revealed the presence of a unique group of *vibrios* exclusively found in FLOC, and shrimp showed better health status than those grown in CW. It suggests not only the strengthening of the shrimp immune system by the molecules described above, but also a structuring of the microbial community that may be keeping in equilibrium and prevent an outbreak from unidentified opportunistic pathogenic *Vibrio.*

Conclusion

This study showed a synergistic effect of probiotic and Bio»FLOC» for the juveniles of *L. vannamei* that prevented the progression of lesions in HP caused by pathogenic vibrios present in the water. Bio»FLOC» was initially developed to mitigate the water quality problem that can often related to mortality; it can limit contamination in natural water, thereby preventing a spread of pathogens. Under such conditions of zero water exchange, antibiosis can occur naturally; growth and survival would be similar with probiotic. The differences in *vibrio* species observed between CW and FLOC resided in the fact that some microorganisms in FLOC particles play a key role on bacterial communities. Histopathology showed that shrimp in FLOC had better immune status than in CW, where more lesions occurred with or without probiotic; therefore, although probiotic effected on survival

by stabilizing to some extent the shrimp digestive flora, this was not enough to enhance growth rate. A dose-response should confirm an absence of effect.

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List of Tables

Table 1.

	CW	**FLOC**	CW	**FLOC**
UPS	36.6 ±0.5	36.8 ±0.3	37.2±.8	40.6±1.4
T°C	26.4±1.8	26.5 ±0.6	27.2±1.1	28.0±1.1
DO mg L^{-1}	5.1 ± 0.4	4.4 ± 0.2	5.4±0.4	4.8±0.2
pH	7.7±0.2	7.9 ± 0.1	7.5±0.2	7.9±0.2
N-NH_3 mgL^{-1}	0.6	0.6	0.6	0.6
N-NO_2 mg L^{-1}	0.2	0.2	0.2	0.2
N-NO_3 mg L^{-1}	10	10	10	10
FLOC volume ml L^{-1}	-	6.7	-	7.3

Table 2.

	CW w /	CW w /o	FLOC w /	FLOC w /o
IWT	4.76±0.20	4.77±0.28	4.67±0.15	4.88±0.26
FWT	6.69±1.05 ^{b}	7.25±0.85 ^{b}	12.54±0.31^{a}	12.41±0.66 ^{a}
DGR mg d^{-1}	0.44 ^{b}	0.55 ^{b}	1.42 ^{a}	1.35 ^{a}
survival(%)	71±17^{b*}	54±15^{b**}	92±8^{a*}	77±7^{a**}

Superscripts indicate significant differences between treatments. Letters indicate significant differences due to the rearing system (CW vs. *FLOC*). Asterisc indicate significant differences due to the probiotic presence (w /or w/o): Tukey test with $p<0.05$.

Table 3.

SYMPTOMS	SEVERITY				
		CW w/o	CW w/	FLOC w/o	FLOC w/
Hydropic degeneration	1	10	10	10	
	2	10			
	3	10			
Cellular detachment	1				
	2		10		
	3		10		
Tubular atrophy	1	10	40	20	20
	2	10			
	3	10	20		
Decrease of B-cells	1		10		
	2	10	10		
	3	10	10		
Inflammation	1				10
	2	10			
	3		20		
Focal necrosis in tubules	3	10			
Hemocitic nodules	1		10	20	
	2		10		
Hemocytic melanized nodules	1	10	10		
	2		10		
	3		10		

Caption for Figures

Fig 1: Phylogenic tree with *Vibrio* species present in CW and *FLOC*. Probiotic present (w/) or absent (w/o) in feed. *V. rotiferianus*, *V. owensii*, *V. plantisponsor*, *V.vulnificus* strains in red possess <98% identity with selected strains. *Photobacterium phosphoreum* was used as *outgroup*.

Fig 2. HP histology, shrimp raised in CW (w/ or w/o probiotic). (bar=50µm) except for A (bar=20µm). A: tubules w/ hydropic degeneration, arrows for cell detachment. B: atrophied tubules w/ a decrease of B-cell; C: arrow for hemocytic nodules around tubules; E: tubule necrosis, arrow for melanization; F: inflammation, arrows for melanized nodules. (hematoxylin & eosin staining).

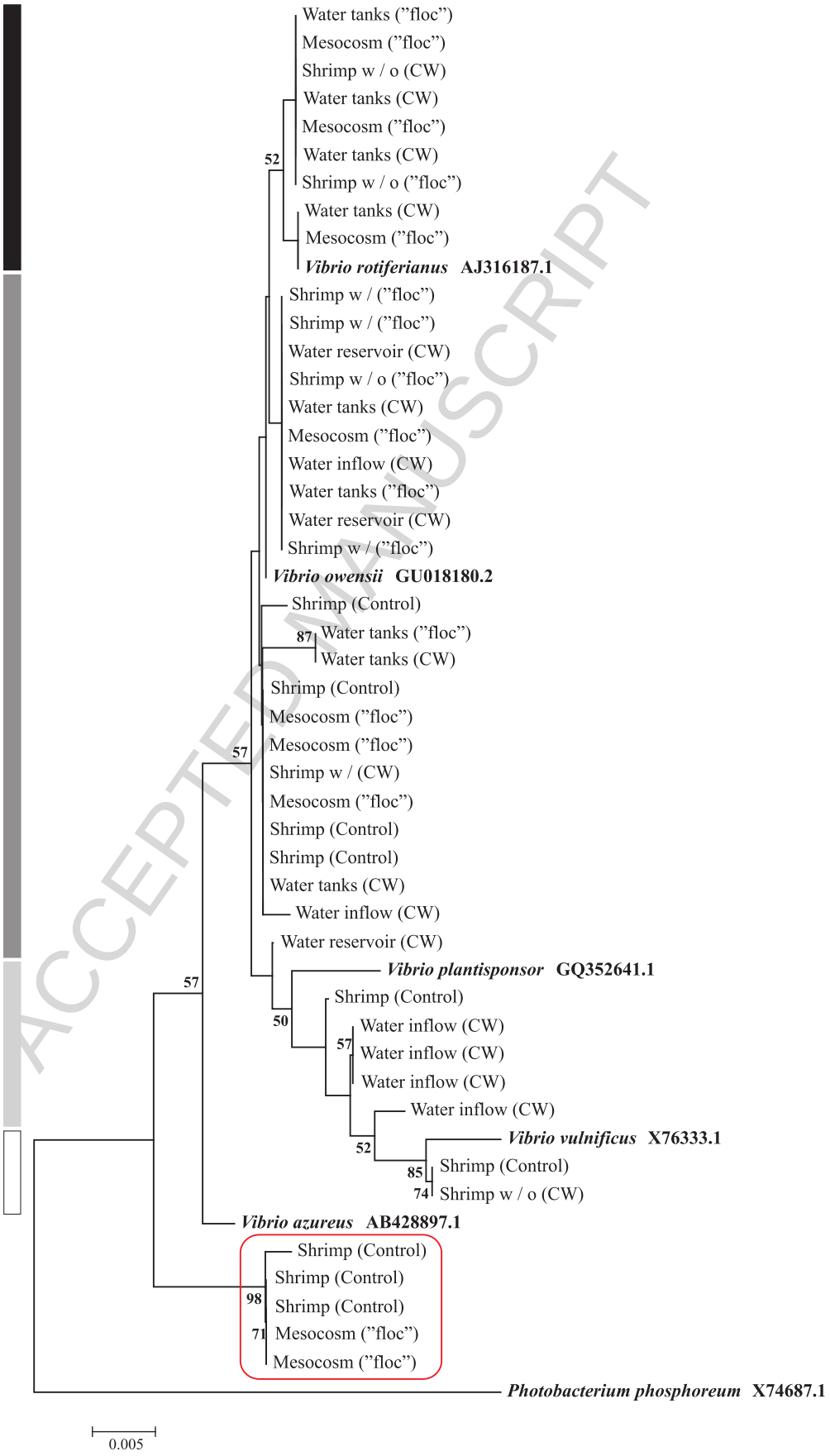

Fig 1:

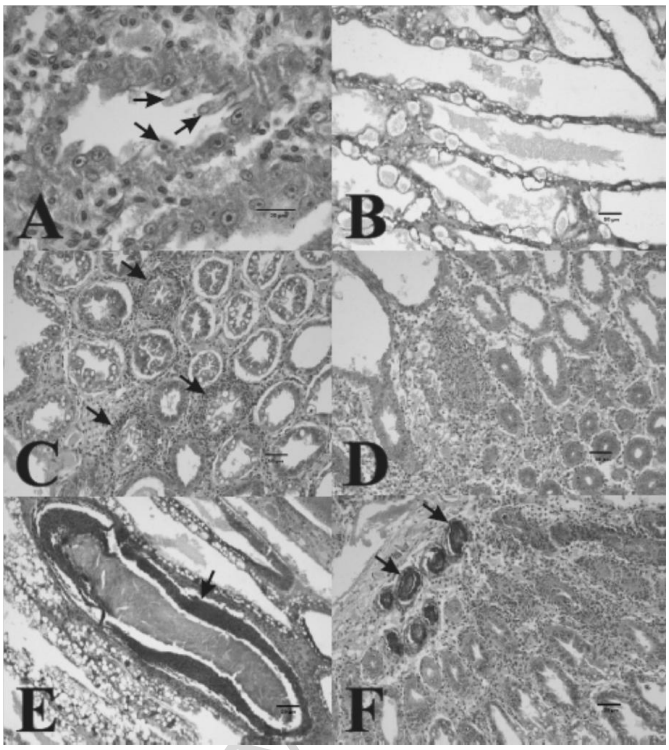

Fig 2.

Referees proposed: Avnimelech Yoram; Lightner Donald; Gatesoupe Joel; Gabaudan Jacques; Diaz Bruno