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Survival improvement conferred by the *Pseudoalteromonas* sp. NC201 probiotic in *Litopenaeus stylirostris* exposed to *Vibrio nigripulchritudo* infection and salinity stress

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Abstract :

This study aimed to investigate the potential protection conferred by the probiotic strain NC201 against biotic and abiotic stresses in Pacific blue shrimp *Litopenaeus stylirostris* that had received the probiotic throughout their lives. The presence of NC201 in shrimp hemolymph was investigated over the course of 24 h, before exposure to bacterial or physical stress. Results showed that NC201 had invaded the shrimp hemolymph 2 h following administration, but had completely disappeared by 48 h. NC201 identification through morphotype observation was confirmed by MALDI-TOF biotyping and results also indicated that NC201 and *Pseudoalteromonas piscicida* are closely related. A challenge by immersion was carried out on subadults using *Vibrio nigripulchritudo* at 10^5 CFU/ml. Cumulative mortality was two-fold lower in the treated group (24%) than in the control group (48%) at 144 h post infection. The probiotic in the shrimp hemolymph was diminished in infectious conditions compared with non-infectious ones and *V. nigripulchritudo* prevalence was simultaneously lower in animals treated with NC201. The relative expression of genes coding lysozyme and penaeidin 3 was evaluated 24 h post infection and their transcript numbers were found to be lower in probiotic animals than in control animals for both genes. Hyposaline stress was also used to evaluate the benefits of NC201 treatment on early juveniles and subadults. At low salinities, animals showed an increased survival rate when treated with NC201, by 10 and 17.5% at 48 h post stress, respectively. Moreover, in subadults treated with the probiotic, a better recovery of the plasmatic osmolality was observed. All these results confirm that NC201 is a good candidate probiotic for shrimp aquaculture.

Highlights

► Two hours after NC201 probiotic distribution, NC201 was present in the shrimp hemolymph but disappeared at 48 hours. ► In the first hours of infection with *Vibrio nigripulchritudo*, pathogen prevalence was lowered in animals treated with NC201. ► NC201 *Pseudoalteromonas* strain protected *Litopenaeus stylirostris* against biotic and abiotic stresses and mortality was two-fold lower in probiotic animals. ► The genes relative expression coding the lysozyme and the penaeidin 3 were lower in probiotic animals than in control ones.

Keywords : Litopenaeus stylirostris ; Pseudoalteromonas ; Probiotic ; Vibrio nigripulchritudo ; Osmotic stress ; Gene expression

Introduction

Stressful environmental conditions linked to pathogen presence can lead to disease outbreaks in shrimp farming (Kautsky et al., 2000). In New Caledonia, *Vibrio nigripulchitudo* is responsible of the "Summer Syndrome" in grow-out ponds since December 1997 (Goarant et al., 2006); final survival in these ponds are less than 38% while it reachs nearly 60% in unaffected ones (Lemmonier et al., 2006). Mass mortality of 80% in kuruma shrimp ponds caused by *V. nigripulchritudo* was also reported in Japan in december 2005 (Fall et al., 2010). Protection against *V.nigripulchritudo* has be demonstrated experimentally by injecting oxyteracyclin antibiotic in shrimp juveniles (Goarant et al., not published). Instead of using antimicrobial drugs, vibriosis outbreaks can be countered with probiotics (Irianto and Austin, 2002; Verschuere et al., 2000), whose use has become increasingly common over the last decade (Aguirre-Guzmán, 2012; Carnevali et al., 2017; Zorriehzahra et al., 2016). Probiotics are viable cell preparations that, according to the Food and Agriculture Organization and World Health Organization, provide a beneficial effect to the host (Akhter et al., 2015). Many studies have been carried out on the probiotic effects of *Bacillus spp.* and *Lactobacillus spp*. (Balcázar et al., 2007; Luis-Villaseñor et al., 2011; Vieira et al., 2007; Zokaeifar et al., 2012; Ramírez et al., 2017). Several modes of actions of probiotics have been described in literature (Kesarcodi-Watson et al., 2008). Host protection against pathogens is one of the potential roles of probiotics. Castex et al. (2009) have demonstrated that susceptibility of shrimps to *V. nigripulchritudo* was reduced by feeding them with *Pediococcus acidaliciti* probiotic feed. Increasing numbers of studies are looking at the marine bacteria *Pseudoalteromonas*. This bacterial genus, which is specifically marine, is known for its ability to colonize marine animals, such as sponges, mussels, tunicates and pufferfish (Holmström and Kjelleberg, 1999) and belongs to the digestive tract microbial community of Pacific white shrimp *Litopenaeus vanamei* (Tzuc et al., 2014). *Pseudoalteromonas* abilities to protect animals against pathogens were demonstrated in Pacific and flat oysters larvae with *Pseudoalteromonas* sp D41(Kesarcodi-Watson et al., 2012), in *Seriola lalandi* larvae fish with *Pseudoalteromonas sp* SLP1(Leyton et al., 2017) and in the swimming crab larvae with *Pseudoalteromonas aliena* KCCM 11207P (Morya et al., 2014).

Probiotics can inhibit the pathogen's development or propagation as observed in *Pseudoalteromonas flavipulchra* JG1 that can produce an antibacterial protein and five small-molecule compounds, which probably act synergistically against *Vibrio anguillarum* (Yu et al., 2012). Richards et al. (2017) have shown that *Pseudoalteromonas piscicida* released

antimicrobial substances such as proteolytic enzymes and transferred lytic vesicles to destroy vibrios. Another mechanism of action is the probiotic ability to modulate the innate immune system of the host. In kuruma shrimp, survival rate was improved by using probiotic-incorporated diet and significant up-regulation of lyzozyme gene expression was observed (Maeda et al., 2014). In the same way, mortality was lowered in whiteleg shrimp when probiotic *Bacillus subtilis* has been administrated in rearing water for 8 weeks followed by a challenge with *Vibrio harveyi* (Zokaeifar et al., 2014); an up-regulation of several immune genes (prophenoloxydase, peroxinectin, serine protein) was also observed in this experiment.

Penaeid shrimp are exposed to different salinities during their migration, and osmoregulatory mechanisms allow them to tolerate environmental changes. During rainfall season and after a tropical storm, pond water salinity can be close to freshwater salinity in New Caledonia. Osmotic stress was used by Palacios and Racotta (2007) to assess postlarval quality, and osmotic pressure variations can also be a means of estimating the physiological status of shrimp (Lignot et al., 2000). Moreover, other studies have shown lower cumulative mortality in shrimps exposed to sudden salinity changes when probiotics were administered (Franco et al., 2016; Liu et al., 2010). Therefore, probiotics can also enhance an animal's ability to adapt to environmental variations.

Recently, the bacterial strain NC201 belonging to the *Pseudoalteromonas* genus was isolated from the natural environment in New Caledonia (Dufourcq et al., 2014). Previous work has demonstrated that NC201 had no pathogenic effect on *L. stylirostris* larvae and led, in larval rearing conditions, to survival rates equivalent to larvae treated with antibiotics (Pham et al., 2014). The isolated strain, identified as *Pseudoalteromonas* sp. NC201, was studied for its antibacterial potential and its whole genome was sequenced (Sorieul et al., in preparation).

The aim of the present study was to assess the probiotic effect of *Pseudoalteromonas sp.* NC201 in *L. stylirostris* juveniles and subadults when they were confronted with a pathogenic infection or a drastic salinity variation. The probiotic impact on the total culturable heterotrophic flora and the host immune response were evaluated in non-infected and infected conditions, and the plasmatic osmotic pressure was measured during the salinity stress test. Experimental conditions triggering a beneficial effect due to the probiotic constitute a study framework that will allow a deeper exploration of the physiological processes involved in shrimp health status.

Materials and methods

Animals

All the *L. stylirostris* shrimp used in the experiments were reared at the experimental station of Saint-Vincent (Boulouparis, New Caledonia). The breeders were stocked for 8 months in outdoor earthen ponds before transfer to the hatchery. Nauplii and post-larvae were obtained according to a pre-established protocol (Pham et al., 2012) and were stocked in 2-m^3 cylindroconical tanks filled with 5-µm filtered seawater at a density of 180 larvae per liter the first day. The larval production batch was split into two groups immediately after hatching. One group was treated with the NC201 probiotic (P) every other day throughout their life, at a final concentration of 10^5 CFU.L^{-1}, and the other group was reared under standard conditions (C) with erythromycin treatment (2.5 ppm) at days 3, 5, 7 and 9 according Pham et al. (2014). Total culturable heterotrofic flora and vibrios concentrations were checked at days 0, 5 and 9 on Zobell and TCBS culture plates.

In hatchery, rearing water was maintained at 29°C and salinity at 35 ppt. Feeding started 24 h after stocking. Microparticles and *Artemia salina* nauplii were then added *ad libitum* throughout the larval rearing period. The water was renewed at a rate of 50% on days 9 and 11, and then every day from day 13 onward. When they had reached postlarval stage 4 (day 19), animals were moved to 2-m^3 outdoor tanks where they were stocked at a density of 20.L^{-1} for 15 days. To obtain juveniles (0.4 g) and subadults (15 g) in each population, postlarvae were kept in 2-m^3 tanks at a density of 2/L and were sampled 1 and 5 months later, respectively. During rearing in 2-m^3 tanks, the water was 100% renewed over 18 h every other day before the probiotic administration. Animals were fed *ad libitum* with local commercial pellets (SICA-NC®) twice a day. The sinking pellets size varied from 1 to 3 mm (according to animals size) and were composed of 40% proteins.

Probiotic production and pathogen culture

Pseudoalteromonas sp. NC201 (Pham et al., 2014) was used as the probiotic strain and *Vibrio nigripulchritudo*, pathogen of *L. stylirostris* (Goarant et al., 2006), was used for the bacterial challenge. All bacterial strains were grown in the same Zobell marine bacteria culture medium, under the same culture conditions: 0.5 L broth medium in 1-L bottles, 16 h at 28°C, and 120 rpm shaking. To obtain a bacterial concentration at 10^5 CFU.mL^{-1} in the rearing water, an absorbance Abs) of a 5-fold diluted culture sample (Abs must be inferior to 1) with growth media was assessed with a colorimeter (Fisher Scientific model 40) at 600 nm to determinate the culture bacteria concentration, considering that 0.5 DO is equivalent to $1.39*10^5$ CFU.mL^{-1} (the correlation was established in previous studies).

Pathogen infection

Pathogen infection with *V. nigripulchritudo* was carried out on 128 subadult shrimps (15 g) from each of the C and P groups. These shrimps were then split up into 200-L tanks at a density of 32 animals per tank in quadruplicate. No feed was added to the tanks during sampling period. Four hours before infection, the probiotic treatment was added to the group P tanks. To carry out the experimental infection, the water volume was lowered to 100 L and inoculated with *V. nigripulchritudo* at a density of 10^5 CFU.mL^{-1}. Two hours following inoculation, the tank water was renewed entirely. During the experimental infection, water was kept around 29°C. Mortality was checked twice a day, Animal was considered as dead when it laid on its side and no movement was detected. The results were monitored as following: non-infected control animals (NIC), non-infected probiotic animals (NIP), infected control animals (IC) and infected probiotic animals (IP). Animal sampling occurred before probiotic administration at T0, 10 animals were taken from the C and P populations. Prior to haemolymph sampling, shrimp were rinsed with sterile seawater. Then, about 250 µL hemolymph was withdrawn from the ventral sinus using a 1-mL sterile syringe and needle (23-gauge) was inserted at 3 mm depth. A volume of 20 µL hemolymph was used in bacterial counts. Similar samplings were performed on 10 animals from NIC, NIP, IC and IP animals at 4, 10 and 24 h post infection (T4, T10 and T24). At T24, 150 µL of hemolymph were used for RNA extraction.

Resistance to salinity stress

Two in vivo experiments were carried out comparing the control group (C) with the probiotic group (P). Different salinity media (3 ppt, 5 ppt and 55 ppt) were prepared from 5µm-sterilized seawater (32 to 34 ppt according the experiments period) by adding either freshwater or marine salt (Reef Crystals, Aquarium Systems). Media were prepared 2 days before the challenge to allow evaporation of any potential chlorine residue from the tap water before shrimp transfer. In the first trial, early juveniles (mean weight 0.4 g) were transferred directly from seawater at 34.3 ppt to 3 ppt (C3 and P3 treatments) or 55 ppt (C55 and P55 treatments) for 72 h. A control treatment was also carried out by transferring from seawater to seawater. The water temperature was 21.7°C. Each treatment was tested in triplicate with 12 animals per 20-L aquarium. In a second experiment conducted on subadults (15 g), only hyposaline conditions (5.0 ppt ± 0.0) were compared with control conditions (32.7 ppt ± 0.1) in 30-L aquaria. Forty animals of each group (C and P) were distributed among five aquaria, at a density of eight animals per aquarium, from seawater to seawater (C33 and P33 treatments) or to desalted water (C5 and P5 treatments). Shrimp survival was monitored twice a day over 48 h and water

temperature was maintained at 25.1°C. All aquaria were constantly aerated and no feed was provided during the stress test. Water salinity was checked with an osmometer (WESCOR Vapro 5600, USA) and osmolality was expressed in $mOsm.kg^{-1}$. The hemolymph osmolality of the subadults was measured on 10 µL sampled from 10 animals of each treatment.

Bacterial count and identification

The total culturable heterotrophic flora (THF) in the shrimp hemolymph was determined on juvenile animals (8 g) using a plate count method after 75 days of impregnation with the probiotic and the infection challenge. A hemolymph volume of 20 µL from each of 10 shrimp was plated in duplicate on glycerol containing Zobell agar. The THF was expressed in $CFU.mL^{-1}$. NC201 was counted based on morphological identification (round, shiny, and bright egg-yolk-like orange-yellow) and *V. nigripulchritudo* was identified by color (colonies appear dark-grey/black when glycerol is present in the medium). Eight randomly selected colonies showing a similar morphology to NC201 were isolated and stored for MALDI-TOF identification.

Using a MALDI-TOF Microflex coupled to Biotyper 3.0 software (Brüker Daltonik), a standard protein profile of NC201 was established after reading eight spots of NC201 in triplicate. Profiles of *Pseudoalteromonas flavipulchra* (DSM 14401), *P. peptidolytica* (DSM 14001) and *P. piscicida* (DSM 6809) were also established in the same conditions and added to the reference bank used for further analysis. The range of masses observed was set between 2 and 20 kDa. An α-cyano-4-hydroxycinnamic acid (HCCA) matrix was used on samples directly spotted on the reflective plate without any further extraction steps. Eight strains (six and two from the P shrimp and C shrimp groups, respectively) presenting the NC201 morphotype were analyzed. The profiles of these randomly isolated colonies were compared to the database for identification. Biotyper 3.0 software was used to establish distances scores between the measured spectra.

Relative gene expression of antimicrobial peptide genes

Hemolymph was collected from subadults (15 g) 24 h post infection. Ten non-infected and ten infected animals from each group (C and P) were sampled and 150 µl of hemolymph of each animal were preserved in an equal volume of sodium citrate prior to hemocyte pelleting by centrifugation. Total RNA was extracted using RNeasy columns (Qiagen) according to the manufacturer's instructions. RNA quantity, purity and integrity were verified spectrophotometrically (A260/A280) and by electrophoresis on 1% agarose gels. The extracted

total RNA (200 ng) was linearized through incubation at 70°C for 5 min then reverse transcribed.

Expression levels of the genes *Litsty pen3* and *lys* (GenBank accession numbers AY351655 and CV699332), coding for the antimicrobial peptides (AMPs) penaeidin 3 and lysozyme, respectively, were measured by quantitative real-time PCR (RT-qPCR). This analysis was performed on an ABI7300 Real-Time PCR System (Applied Biosystems). Amplification efficiencies of all qPCR primers were determined using a previously described method (Pfaffl, 2001). Melting curves were used to verify the specificity of the amplification. The results were normalized with the elongation factor EF1-α (accession no. AY117542.1), a housekeeping gene already used as a reference standard in *L. stylirostris* (Pham, 2011). The real-time PCR primer sequences of antimicrobial peptides were previously published in de Lorgeril et al., (2008). Each run included the cDNA control, negative controls (total RNA treated with DNase I), and blank controls (water). The relative expression of *Litsty pen3* and *lys* to EF1-α at each condition were calculated with the 2^{-DDCt} method (Livak and Schmittgen, 2001).

Statistical analysis

Cumulative mortality data were normalized through an arcsine square root transformation before analysis with a two-way analysis of variance (ANOVA) followed by Tukey's tests.

The data obtained from the RT-PCR analysis were analyzed by two-way analysis of variance (ANOVA), followed by Tukey's tests. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using Excel Stat.

Results

Effect of NC201 administration on THF evolution in the hemolymph

The bacterial flora in the shrimp hemolymph in C (control) and P (probiotic) groups was assessed. The average count of total heterotrophic flora (THF) was between 2 and 3 log $CFU.mL^{-1}$ (Figure 1A). No significant difference of THF between C and P groups was noted over the time course of the experiment, but a slight increase of THF was observed 6 h post administration in the probiotic group. The percentage of NC201 in the THF (orange-yellow colonies) among cultivable flora in the hemolymph was evaluated on morphological characteristics (Figure 1B). Before administration of NC201 in the P group, no orange colonies were observed in either group. In the C group, a small proportion of orange-yellow colonies,

below 5% of the THF, was detected in the first 12h, while in the P animals, the percentage of orange-yellow colonies reached 47% two hours after administration and slowly decreased to 22% over 24 h.

NC201 identification by MALDI-TOF-MS

The strain NC201 was analyzed by MALDI-TOF-MS (Figure 2). NC201 displayed a profile composed of 11 major peaks at *m/z* 4237, 4491, 4738, 5140, 6119, 7208, 7529, 9477, 9947 and 11337. After comparison with the database and the other *Pseudoalteromonas* analyzed, an additional 10 distinguishable peaks were observed. These peaks were used as a reference for comparison with the spectrum of the closely related *P. flavipulchra* and *P. piscicida*. The comparison with *P. piscicida* DSM6609 (Figure 2) revealed 10 peaks in common, within the *m/z* ± 2, distributed along the mass range: 4237, 4739, 5669, 6120, 6430, 7530, 8449, 9477, 9947, 11337. NC201 was also compared with *P. flavipulchra* NCIMB2033 (data not shown), with which there were seven shared peaks: 4739, 6120, 6430, 7208, 7530, 8449, and 9477, within *m/z* ± 2. Only the peak at 7208 is specific to NC201 and *P. flavipulchra* NCIMB 2033, the six others are common to all three strains.

The six colonies, with the same morphotype (orange-yellow) as NC201, randomly picked from the P animals showed a spectral profile identical to NC201 (Table 1). The two colonies isolated in C animals with a morphotype similar to NC201 showed spectra that did not match with NC201 (data not shown).

Resistance to infectious stress

A pathogenic challenge with *Vibrio nigripulchritudo* was used to determine the sensitivity to pathogens of animals treated with NC201 (P) compared with non-treated ones (C). Non-infected control (NIC) and probiotic treated animals (NIP) displayed no significant mortality over the course of the experiment (Figure 3). In IC animals, mortality started 24 h post challenge. At 48 h post infection, infected control (IC) animals showed a mortality of 17%, whereas infected probiotic (IP) animals did not yet display any mortality. At 68 h post infection, IC animals showed an increased mortality of 28% while the IP showed a significantly lower one of 7%. From then on, the mortality increased gradually in IC animals to reach 48% at 144 h post infection. Mortality also increased gradually in IP animals to reach 24% at 144 h post infection. The cumulative mortality of the IC animals was significantly higher than that of the IP ones from the onset of observed mortality in IC animals at 24 h post infection and throughout the observed period (144 h).

Bacterial count in hemolymph during the infectious challenge

The total heterotrophic flora (THF) present in the hemolymph was counted over the course of the experiment (Table 2). Initial populations in NIC and NIP animals were of 3.12 log (±0.59) and 3.21 log (±0.44) CFU.mL^{-1}, respectively. At 24 h post infection, the THF in NIC, NIP, IC and IP animals amounted to 3.75 log (±0.92), 3.97 log (±0.92), 3.87 log (±0.44) and 3.97 log (±0.44) CFU.mL^{-1}, respectively. THF of all treatments seemed to increase over time, although not significantly. No significant difference in the amount of THF could be observed between treatments.

The presence of the probiotic and the pathogenic bacterium *V. nigripulchritudo* was monitored during the infectious challenge (Table 2 and figure 4). Before infection, 15% of the THF was represented by NC201 in NIP and IP populations (Figure 4A). The NC201 concentration in the NIP group decreased slowly to reach 12%, 9% and 3% at 4 h, 10 h and 24 h post infection, respectively. In the IP group, the NC201 percentage in the hemolymph was only 2% at 4 h post infection, and continued to decrease to reach 0.5% at 24 h.

No *V. nigripulchritudo* was detected in animals of both the control or probiotic groups before the infection challenge (Figure 4B). At 4 h post infection, the proportion of the pathogen in the IC group was 33 % (110 CFU.mL^{-1}), which decreased to 17% (460 CFU. mL^{-1}) at 10 h and 22% (1614 CFU.mL^{-1}) at 24 h. However, from 4 to 24 h, no significant difference was noted in the IC group. In the IP group, *V. nigripulchritudo* represented 3 and 2% (88 and 16 CFU.mL^{-1}) of the THF at 4 and 10 h, respectively, and reached 13% (1218 CFU.mL^{-1}) at 24 h. This represents a significant increase at 24 h in the IP group. At 4 and 10 h, the proportion of *V. nigripulchritudo* found in the THF was significantly lower in IP than in IC animals, whereas the difference observed at 24 h was not significant. A small amount of *V. nigripulchritudo* was detected in shrimps hemolymph 4 h and 10 h after infection in the NIP group (0.41 log ± 0.82 CFU/mL) and the NIC group (0.46 log ± 0.86 CFU/mL), respectively (Table 2). At 24 h, the *V. nigripulchitudo* concentration was 0.95 log ± 1.05 CFU/mL in the NIP population while it reached 2.27 log ± 1.21 CFU/mL in the NIC one.

Expression level of lysozyme and penaeidin 3

The expression levels of two genes linked to the immune response, lysozyme (*lys*) and penaeidin 3 (*pen3*), were measured 24 h after exposure to the pathogenic *Vibrio*. The expression level of *lys* in unchallenged animals was significantly lower in NIP than in NIC animals (Figure 5). The challenged animals showed a lower expression than the unchallenged ones, but this

diminution was not significant for either population. Comparing challenged animals, *lys* relative expression was also lower in the IP group than in the IC group. The number of *pen3* transcripts in unchallenged conditions was lower for the control than for probiotic animals, although this difference was not significant (Figure 5). At 24 h after infection, a non-significant increase of *pen3* expression was observed in IC animals but not in NIC ones, while there was a significant decrease in IP animals compared with NIP ones. With a number of transcripts higher for the IC group than the IP group, the difference between the infected populations was significant.

Resistance to salinity stress

The first osmotic stress experiment was conducted on juvenile stage animals (0.4 g) in hypersaline (55 ppt) and hyposaline (3 ppt) conditions (Figure 6). Three hours after transferring animals to 55 ppt, mortalities of 5% and 3% were observed in the control (C55) and probiotic (P55) groups, respectively. Mortality increased in the C55 group to reach 16% at the end of the trial while the loss in the P55 group was only 6%. In the 3 ppt condition, 10% and 9% of the control population (C3) and the probiotic ones (P3) had died by 12 h, respectively. At 24 h, mortalities reached 23% in the C3 group while no additional dead animals were found in the P3 group. Cumulative mortalities were significantly different at the end of the experiment between C55 (16%) and P55 (6%) on the one hand, and between C3 (23%) and P3 (14%) on the other. At both salinity levels, the final survival rate was significantly improved by the addition of NC201. The control conditions without osmotic stress showed a 100% survival rate over the 72 h of the experiment for both treated and non-treated populations (data not shown).
As subadult animals (15 g) were more sensitive to hyposaline conditions, the experiment was performed at 5 ppt instead of 3 ppt (Figure 7). The transfer to isosaline conditions (33 ppt) induced mortality after 10 h and by the end of the experiment, 7.5 and 10% had been lost in the control and probiotic treated animals, respectively. In hyposaline conditions, mortality started after 6 h of exposure, at 8% for the control and 3% for probiotic animals. In the first 24 h of exposure, a regular increase of mortality was observed in both C and P populations, which reached 43 and 35%, respectively, although the difference was not significant. Twenty-six hours after the stress was applied, a significant difference in mortality was observed between the two groups, with 50% and 35% for C5 and P5 populations, respectively. Final cumulative mortality at 48 h was not significant, at 57% and 40% for the control and the treated groups, respectively.

Plasmatic osmotic pressure

The use of shrimp subadults (15 g) allowed us to measure variations of hemolymph osmolality according to external osmolality, as illustrated in figure 8. Average salinity in the tanks in the isosaline transfer was 32.7 ppt (962 $mOsm.kg^{-1}$). A slight decrease of the osmolality in both control (C33) and probiotic (P33) populations in the first 8 h was observed, from 780 $mOsm.kg^{-1}$ to 740 $mOsm.kg^{-1}$, followed by an increase back to the initial value at 24 h. The transfer to hyposaline conditions at 5 ppt (147 $mOsm.kg^{-1}$) induced a strong drop in the osmolality of the control group (620 $mOsm.kg^{-1}$ at 4h, 520 $mOsm.kg^{-1}$ at 8h) as well as in the probiotic group (595 $mOsm.kg^{-1}$ at 4h, 540 $mOsm.kg^{-1}$ at 8h) with no significant difference. Twenty-four hours post challenge, the osmolality in these animals had increased significantly ($p < 0.05$) to 590 $mOsm.kg^{-1}$ and 640 $mOsm.kg^{-1}$ for the C5 and P5, respectively.

Discussion

In *Litopenaeus vannamei*, shrimp microbiota can depend on larval developmental stages (Nimrat et al., 2012; Zheng et al., 2016), overall health status (Zheng and Wang, 2016), diet (Zhang et al., 2013) or probiotic administration (Sha et al., 2016). In our study, the total culturable heterotrophic flora (THF) in subadult *L. stylirostris* hemolymph showed no quantitative difference in noninfectious conditions, but this bacteria population may only represent a small proportion of the bacteria present in the shrimp hemolymph. However, animals that had received the probiotic showed a peak proportion of NC201 in the first hours of administration, representing up to 47% of the THF. In *L. vannamei*, Nimrat et al. (2012) showed that the quantity of THF found in whole postlarvae was not impacted by the use of *Bacillus spp* as a probiotic administered through feed; however, the *Bacillus* proportion in the THF increased from PL1 to PL22 to reach 10 fold higher in treated animals than in control ones. The authors suggested that these results might be due to a progressive substitution of a part of the gut microflora by the administered probiotic. Similar results were reported in *Fenneropenaeus indicus* postlarvae by Ziaei-Nejad et al. (2006), where a commercial *Bacillus spp*. probiotic mixture could represent 88% of the total bacteria, while THF quantity was similar in control and treated groups. These results agree with ones obtained for *Pseudalteromonas* NC201 and support the hypothesis that the hemolymph microflora is tightly regulated (Wang and Wang, 2015).

Orange-yellow strain pigmentation was used as a primary tool to distinguish and quantify NC201 in the THF of treated animals. However, some colonies (less than 5%) observed in the THF of control animals showed a morphotype similar to NC201. Then, this quick identification

method was completed with the analysis of the proteic spectrum, as NC201 displayed a unique pattern of peaks in MALDI-TOF analysis, and allowed us to conclude that yellow colonies in control treatment was different from NC201. In addition, the profile of NC201 represents another profile of *Pseudoalteromonas* to add to the MALDI-TOF biotyper profile database established by Emami et al. (2016). NC201 displayed one of the two peaks common to all *Pseudoalteromonas* at *m/z* 4236, the other common peak at *m/z* 5095 could not be distinguished (Dieckmann et al., 2005). It is possible that the peak at *m/z* 5095 is present but not distinguishable due to the intensity of the *m/z* 5140 peak. Comparison of the NC201 mass spectrum to other published *Pseudoalteromonas* (Emami et al., 2016), revealed numerous shared peaks with other species. The NC201 strain showed three peaks similar to those found in *P. ulvae* and *P. aurantia* and four peaks similar to those of *P. citrea*. The profile of NC201 was established without any extraction step prior to MALDI-TOF analysis. This process could help refine the profile of the probiotic strain and allow a finer comparison with the spectra of other *Pseudoaltermonas* obtained after an extraction step (Dieckmann et al., 2005; Emami et al., 2016). *Pseudoalteromonas flavipulchra* NCIMB2033 and *P. piscicida* DSM14401 profiles showed that the biotyping method could be reliably used for the *Pseudoalteromonas* genus. Closely related species show profiles similar enough to be classified as belonging to the same genus, but with enough distinctive peaks to tell these species apart and avoid misidentification (Emami et al., 2016). As 16S ribosomal RNA sequences proved insufficient for species identification of *Pseudoalteromonas* as demonstrated by Beurmann et al. (2017), the MALDI-TOF identification method provides a reliable alternative (Carbonnelle et al., 2012). Moreover, the comparison of the NC201 spectrum to the closely related *P. flavipulchra* and *P. piscicida* confirmed the greater distance between *P. flavipulchra* NCIMB 2033 and NC201 than between *P. piscicida* and NC201. Studies on NC201 genome sequence have already been conducted for identification at species level (Sorieul et al., in preparation).

We observed both an rapid invasion of the hemolymph of *L. stylirostris* by NC201 and an overall tendency for high bacterial presence (up to 0.9 log in the hemolymph) in our study, as reported in *L. vannamei* juveniles (Gomez-Gil et al., 1998), which is over 1 log higher than levels of bacteria found in healthy *M. japonicus* (Fagutao et al., 2009; Wang et al., 2014). Although NC201 represented half the bacteria present in the hemolymph at 2 h post administration, the colonization of the shrimp hemolymph was transient, since it disappeared completely between 24 h and 48 h. There could be several potential causes of this disappearance. We should ask whether NC201 was targeted by the immune system, whether it migrated to other tissues, or whether these bacteria simply died out because they are not adapted

for persistence in the hemolymph. Another explanation for the disappearance of NC201 from the hemolymph could be the autolytic activity observed in some *Pseudoalteromonas* strains (Holmström and Kjelleberg, 1999). More studies must be conducted to evaluate these hypothesis.

NC201 belongs to the *Pseudoalteromonas* genus and was selected for its antibacterial potential towards pathogenic *Vibrio* (Pham et al., 2014). A number of studies have shown the interest of using strains of this genus in aquaculture, including two *Pseudoalteromonas sp.* (Goulden et al., 2012; Leyton et al., 2017) and *P. undina* (Banerjee and Ray, 2017). Due to their synthesis of antibacterial compounds, these marine bacteria can have a beneficial (Kesarcodi-Watson et al., 2012) or detrimental (Neu et al., 2014) effect on marine organisms and are therefore a source of potential probiotics.

MALDI-TOF results showed NC201 to be related to *P. piscicida* and *P. flavipulchra*, two species with high antibacterial potential belonging to the pigmented clade (Offret et al., 2016). In our study, it appears that NC201 has a protective effect on *L. stylirostris* against a *V. nigripulchritudo* challenge, as survival rates were significantly improved by the probiotic treatment in subadults confronted with infectious stress. Animals treated with probiotics and then infected with *V. nigripulchritudo* showed a delay of 24 h in the onset of mortality compared with infected controls. Probiotics have been reported to improve survival rates in *L. vannamei* infected with *V. parhaemolyticus* (Kongnum and Hongpattarakere, 2012) or *V. harveyi* (Vieira et al., 2007). The delay of mortality in the probiotic group in our experiment could be explained either by a direct antibacterial action of NC201 against *V. nigripulchritudo*, as shown by studies of NC201 inhibitory potential (Sorieul et al., in preparation), or by an indirect effect such as an improvement in the overall shrimp health or the stimulation of its immune system. Four hours after infection, the pathogen prevalence in our study was much higher in animals that had not received a probiotic treatment. The NC201 presence in the hemolymph and its previously described antibacterial effect against *V. harveyi, V. nigripulchritudo* and *V. penaeicida* (Pham et al. 2014, Sorieul et al., in preparation) could explain this delay in appearance of the pathogen. Similar results were observed by different authors, who have demonstrated that probiotics could lower the prevalence of pathogens such as bacteria (Vieira et al., 2007) or viruses (Leyva-Madrigal et al., 2011) in *L. vannamei*. The experimental infection was performed four hours after probiotic administration, at the time corresponding to the peak presence of NC201 in the hemolymph. A direct antibacterial effect would therefore have been possible, in a similar way as seen in hemolymph of *Marsupenaeus japonicas*, which maintains a low bacterial presence (Wang et al., 2014). Surprisingly, *V. nigripulchitudo* was also observed in non-infected

populations, but in lower concentration than in infected control ones. Three virulence patterns have been described in *V. nigripulchritudo* in New Caledonia: non-pathogenic, moderately pathogenic and highly pathogenic stains (Labreuche et al., 2012). The transfer and handling could have stressed the animals and favoured the non-pathogenic *V. nigripulchitudo* settlement, associated with weak mortalities in NIC and NIP populations. Whereas this phenomenon could be prevented in IC and IP populations by the experimental infection with highly pathogenic strains. Molting stage of animals sampled is another issue that must be pointed out when experimenting with crustaceans as it could affect the bacteria invasion in hemolymph and explain the large fluctuations in individual data.

Wang et al. (2014) have shown that the hemolymph microflora is highly controlled, mainly through antimicrobial peptides (AMP) synthesized by the hemocytes. In our study, two AMP markers, lysozyme (*lys*) and penaeidin 3 (*pen3*), were used for their involvement in the immune response in previous works (Fall et al., 2010; Muñoz et al., 2002). In our experiment, 24h after the *V. nigripulchritudo* challenge, lower *lys* and *pen3* expression levels were observed in shrimp treated with NC201 compared to control ones. A lower *lys* gene expression was also observed in selected line compared to control line in *L. stylirostris* (de Lorgeril et al., 2008) but these data cannot clearly explain even if some authors suggest a migration of AMP expressing haemocytes towards infected tissues (Bachère at al., 2004). In contrast, *pen3* expression level was higher in selected animals when challenging with *V. penaeicidae*. In another study, overexpression of *pen3* in *L. vannamei* hemocytes has been reported following injection of a mix of pathogens, including *V. alginolyticus* (Destoumieux et al., 2000). As the onset of mortality was delayed by 24 h between the control and IP in our study, it would have been interesting to include further time points to monitor the subsequent evolution. As demonstrated by Burge et al. (2007), *lys* expression level in *L. vannamei* exposed to *V. campbellii* over a 48 h time course was dependent sampling time.

A weak *Vibrio* concentration in treated shrimp hemolymph in the first 24 h of infection could explain the lower AMP expression levels. The probiotic might inhibit pathogenic *Vibrio* to such an extent that the immune system of the shrimp did not register any threat markers and lowered the expression levels of its effectors. This effect could be compared with the usual consequences of antibiotic treatment on shrimp immune status, lowering expression levels of key immune genes (Fagutao et al., 2009). Thus, the lowered expression of the AMP markers in shrimp hemolymph treated with NC201 could be linked to the lower exposure to *V. nigripulchitudo*.

Hemolymph osmolality is a direct marker of the impact of a salinity change on the physiology of an animal (Lignot et al., 2000; Péqueux, 1995). The impact of different factors on this

physiological parameter has already been studied in *L. stylirostris* (Mugnier and Soyez, 2005; Wabete et al., 2006). In both hyper and hyposaline challenges, early juveniles treated with probiotics showed an improved survival rate compared with controls. We noted that hyposaline stress had a stronger impact on survival rate than hypersaline stress, as already reported in *L. vannamei* postlarvae (Liu et al., 2010). The faster effect of hyposaline stress was therefore chosen for experiments on subadult stages. Survival rates were also improved by the probiotic treatment in shrimp subjected to hyposaline stress. This result is quite interesting for shrimp industry in New Caledonia as drastic salinity variations can be observed in growout ponds during the rainy seasons. NC201 showed not only a protective effect against *vibrio* infection, but also during a salinity challenge, probably due to a general improvement of the shrimp's health status (Lignot et al., 2000). We can hypothesize an indirect effect of the probiotic, as all energy not consumed to face a biotic stress is available to deal with abiotic variations. To confirm this idea, plasmatic osmotic pressure was measured in probiotic and control populations. A slight variation in osmotic pressure was observed in the isosaline transfer and could be due to transfer stress (Wabete et al., 2008). In our study, the strong disturbance observed at low salinity was not significantly different between the populations until 8 h post challenge, but probiotic animals had recovered faster than the control population by 24 h post challenge. Stressors could induce alterations of osmoregulatory transport (Lignot et al, 2000), with greater cell damage in control animals. However, monitoring over a longer period would allow us to see whether treated animals are able to make a full recovery in a shorter time than control ones. Overall improvement of shrimp health is suggested by the increased survival rate during the abiotic challenges and confirms the probiotic effect of NC201.

This study confirms the safety of NC201 administration for subadult shrimp and highlights the protective effect of this bacterial strain against both biotic and abiotic stresses. However, the basis of this beneficial effect has yet to be explained. We will focus our next study on the observation of additional immune parameters as well as oxidative stress markers and their evolution over a time course. The same experimental approach could be used to measure expression level of other genes involved in the response to stresses like immune response, but also homeostasis and osmotic balance.

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Table 1: Biotyping scores and identification of bacterial colonies with NC201 morphotypes isolated in *L. stylirostris* treated with NC201.

Time post probiotic administration (h)	Tissue	Identification	Score
2	Hemolymph	NC201	2,513
2	Hemolymph	NC201	2,582
2	Hemolymph	NC201	2,59
6	Hemolymph	NC201	2,55
6	Hemolymph	NC201	2,598
12	Hemolymph	NC201	2,27

Table 2: Evolution (log) of the total culturable heterotrophic flora (THF), probiotic (NC201) and pathogens (Vn) in the *L. stylirostris* hemolymph during *V. nigripulchritudo* infection, of non infected control animals (NIC), non infected animals treated with NC201 (NIP), infected control animals (IC) and infected animals treated with NC201 (IP). On the same line, values with different letters are statistically different ($p < 0.05$).

		Time post infection (h)			
Treatment	Flora	0	4	10	24
NIC	THF	3.12 ± 0.59	2.95 ± 1.25	3.17 ± 0.88	3.75 ± 0.92
	NC201	0	0	0	0
	Vn	0^{a}	0^{a}	0.46^{b} ± 0.86	2.27^{c} ± 1.21
NIP	THF	3.21 ± 0.44	2.98 ± 0.90	2.82 ± 1.35	3.97 ± 0.92
	NC201	1.28 ± 1.49	1.74 ± 1.80	1.48 ± 1.58	2.37 ± 1.21
	Vn	0	0.41 ± 0.82	1.26 ± 1.29	0.95 ± 1.05
IC	THF	3.12 ± 0.59	2.54 ± 0.43	3.43 ± 0.65	3.87 ± 0.66
	NC201	0	0	0	0
	Vn	0^{a}	2.11^{b} ± 2.17	2.58^{b} ± 1.66	3.14^{b} ± 1.48
IP	THF	3.21 ± 0.44	2.47 ± 1.13	2.90 ± 0.82	3.97 ± 0.81
	NC201	1.28 ± 1.49	1.60 ± 1.38	0.36 ± 1.07	0.61 ± 1.08
	Vn	0^{a}	1.89^{b} ± 1.35	0.79^{b} ± 1.25	2.05^{b} ± 1.02

Figure 1: Evolution of the total heterotrophic flora (log) in the *L. stylirostris* hemolymph (A), orange colonies percentage in the THF of *L. stylirostris* hemolymph (B). Control treatment (C), animals treated with NC201 (P). Bars sharing different letters are significantly different ($P < 0.05$)

Figure 2: Protein profile of NC201 (top) and *P. piscicida* DSM 6609 (bottom) analyzed by MALDI-TOF.

Figure 3: Cumulative mortality in subadult shrimp (15g) challenged with *V. nigripulchritudo*. Non infected control shrimp (NIC), non infected shrimp treated with probiotic (NIP), infected control shrimp (IC) and infected shrimp treated with probiotic (IP). Statistical difference ($P < 0.05$) are marked with an asterisk (*).

Figure 4: Proportion of NC201 (A) and *V. nigripulchritudo* (B) in the THF of subadult shrimp (15g) challenged with *V. nigripulchritudo*. Non infected shrimp treated with probiotic (NIP), infected control shrimp (IC) and infected shrimp treated with probiotic (IP). Bars sharing different letters are significantly different ($P < 0.05$).

Figure 5: Relative expression of lysozyme and penaeidin 3 in hemocytes of subadult shrimp (15g), at 0 and 24 hours after challenge with *V. nigripulchritudo*. Non infected control shrimp (NIC), non infected shrimp treated with probiotic (NIP), infected control shrimp (IC) and infected shrimp treated with probiotic (IP). Bars sharing different letters are significantly different ($P < 0.05$).

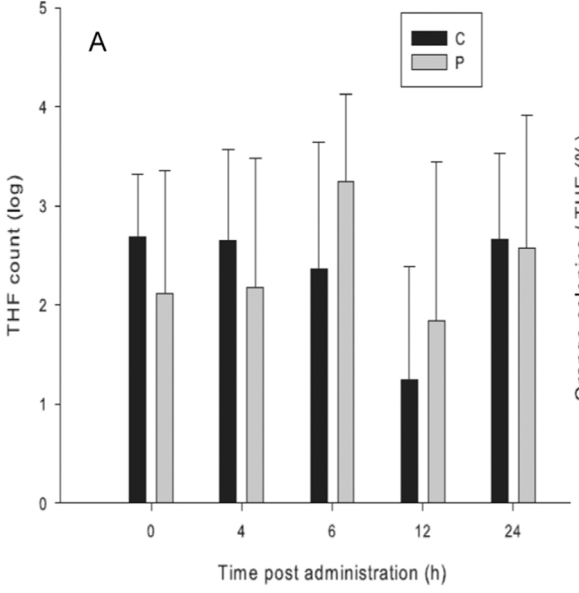
Figure 6: Cumulative mortality in juvenile shrimp (0,5g) challenged by salinity stress. Control shrimp in hyposaline conditions (C 3ppt), shrimp treated with NC201 in hyposaline conditions (P 3ppt). Control shrimp in hypersaline conditions (C 55ppt), shrimp treated with NC201 in hypersaline conditions (P 55ppt). Statistical difference ($P < 0.05$) are marked with an asterisk (*).

Figure 7: Cumulative mortality rate in subadult shrimp (15g) challenged by a hyposaline stress. Control shrimp in isosaline conditions (C 33ppt), shrimp treated with NC201 in isosaline conditions (P 33ppt), shrimp in hyposaline conditions (C 5ppt) and shrimp treated with NC201 in hyposaline conditions (P 5ppt). Statistical difference ($P < 0.05$) are marked with an asterisk (*).

Figure 8: Hemolymph osmolality of the in subadult (15g) L. stylirostris following isosaline (33 ppt) and hyposaline (5ppt). Control shrimp in isosaline conditions (C 33ppt), shrimp treated with NC201 in isosaline conditions (P 33ppt), control shrimp in hyposaline conditions (C 5ppt) and shrimp treated with NC201 in hyposaline conditions (P 5ppt). Statistical difference ($P < 0.05$) are marked with an asterisk (*).

Highlights

- Two hours after NC201 probiotic distribution, NC201 was present in the shrimp hemolymph but disappeared at 48 hours.
- In the first hours of infection with *Vibrio nigripulchritudo,* pathogen prevalence was lowered in animals treated with NC201.
- NC201 *Pseudoalteromonas* strain protected *Litopenaeus stylirostris* against biotic and abiotic stresses and mortality was two-fold lower in probiotic animals.
- The genes relative expression coding the lysozyme and the penaeidin 3 were lower in probiotic animals than in control ones.


A
C
P
THF count (log)
0
1
2
3
4
5
0
4
6
12
24
Time post administration (h)

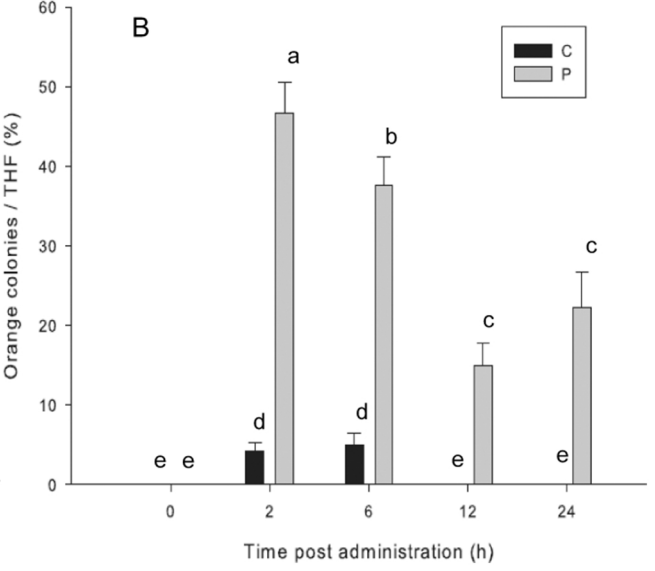

B
C
P
Orange colonies / THF (%)
0
10
20
30
40
50
60
a
b
c
c
c
d
d
e
e
e
e
0
2
6
12
24
Time post administration (h)

Figure 1

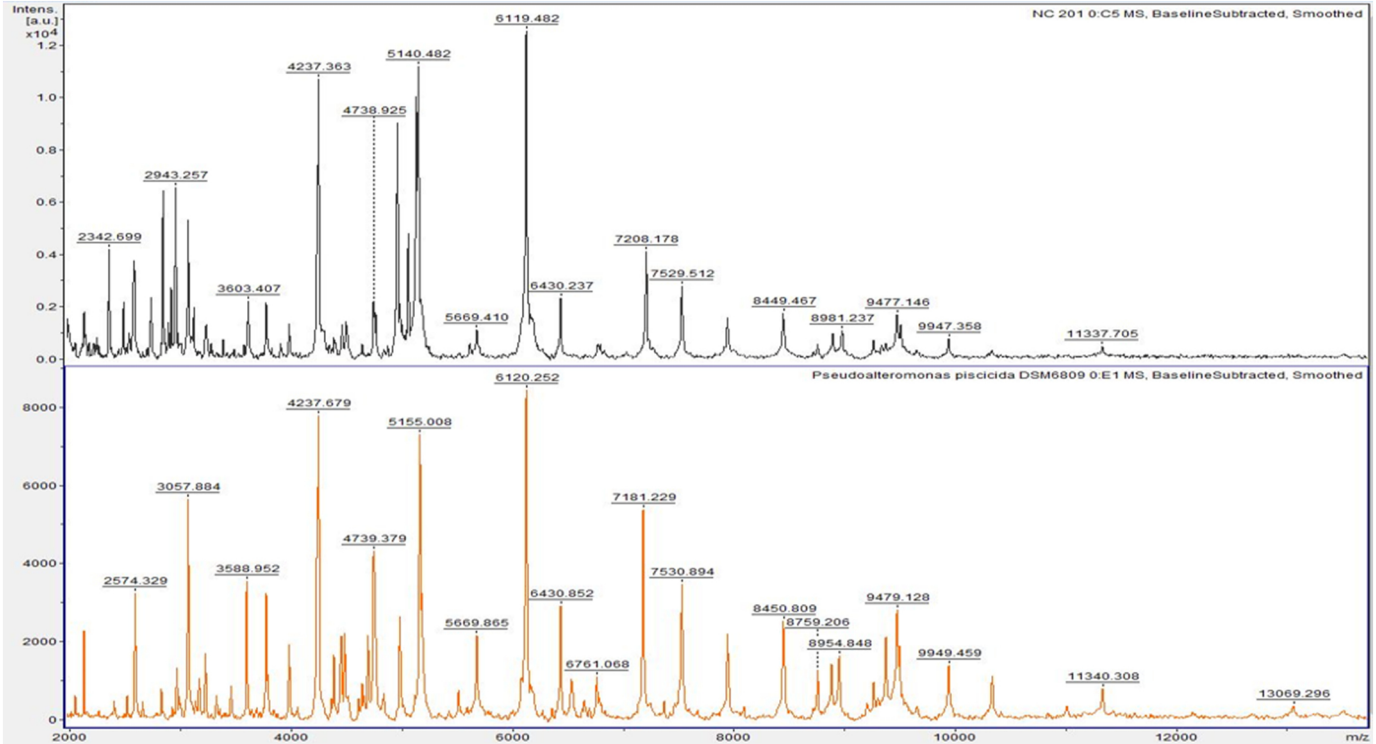

Intens.
[a.u.]
x10^4
NC 201 0:C5 MS, BaselineSubtracted, Smoothed
6119.482
5140.482
4237.363
4738.925
2943.257
2342.699
3603.407
5669.410
6430.237
7208.178
7529.512
8449.467
8981.237
9477.146
9947.358
11337.705
Pseudoalteromonas piscicida DSM6809 0:E1 MS, BaselineSubtracted, Smoothed
6120.252
4237.679
5155.008
3057.884
7181.229
4739.379
2574.329
3588.952
6430.852
7530.894
5669.865
8450.809
8759.206
8954.848
9479.128
6761.068
9949.459
11340.308
13069.296
m/z

Figure 2

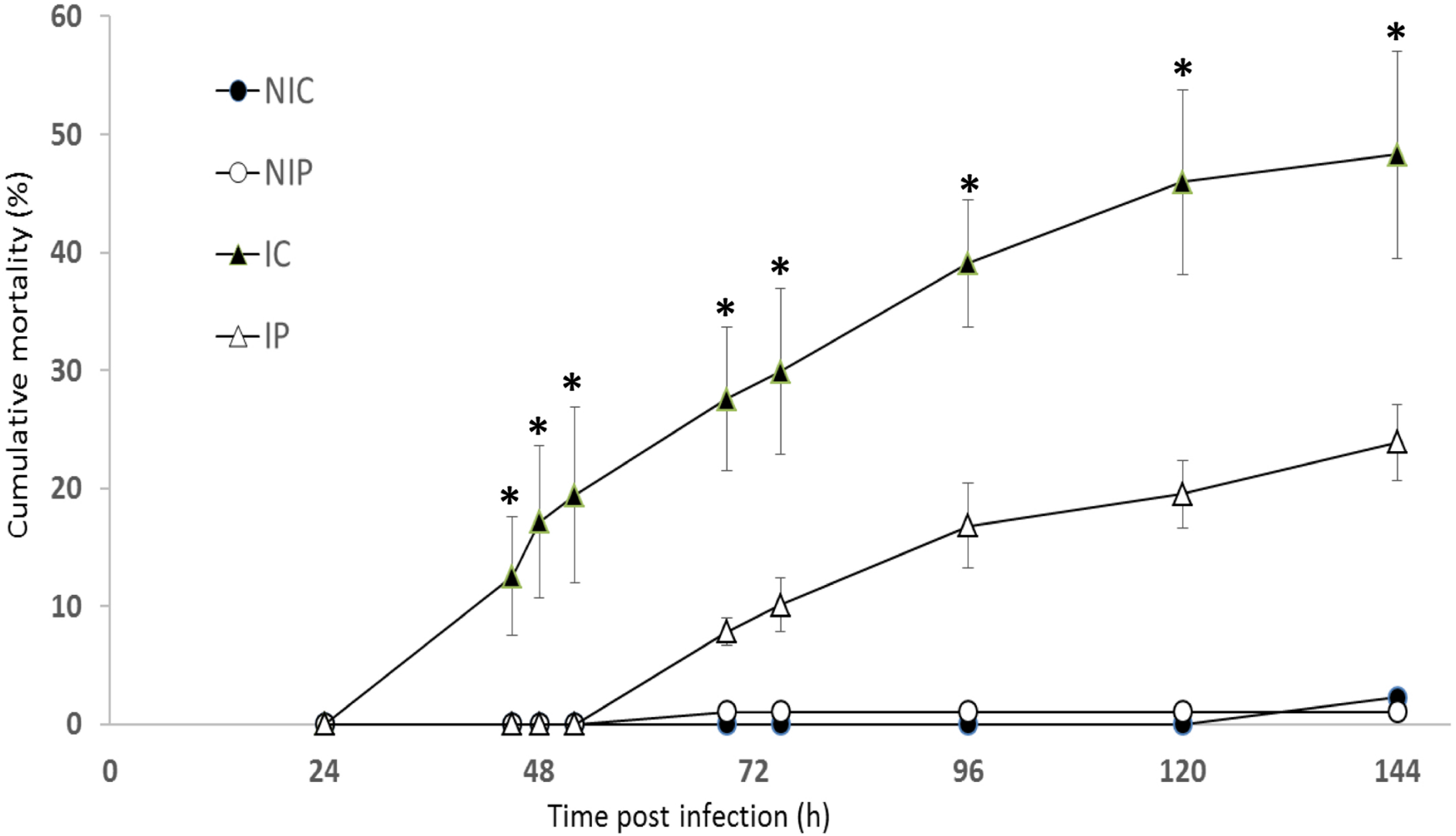

60
50
40
30
20
10
0
Cumulative mortality (%)
NIC
NIP
IC
IP
*
0
24
48
72
96
120
144
Time post infection (h)

Figure 3

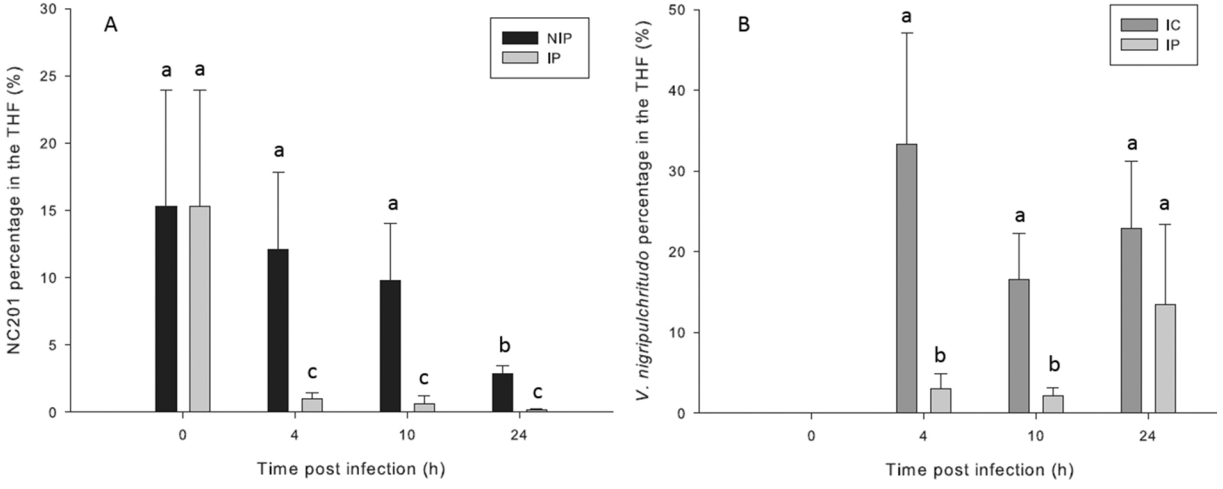

A
NIP
IP
NC201 percentage in the THF (%)
30
25
20
15
10
5
0
a
a
a
c
a
c
b
c
0
4
10
24
Time post infection (h)
B
IC
IP
V. nigripulchritudo percentage in the THF (%)
50
40
30
20
10
0
a
b
a
b
a
a
0
4
10
24
Time post infection (h)

Figure 4

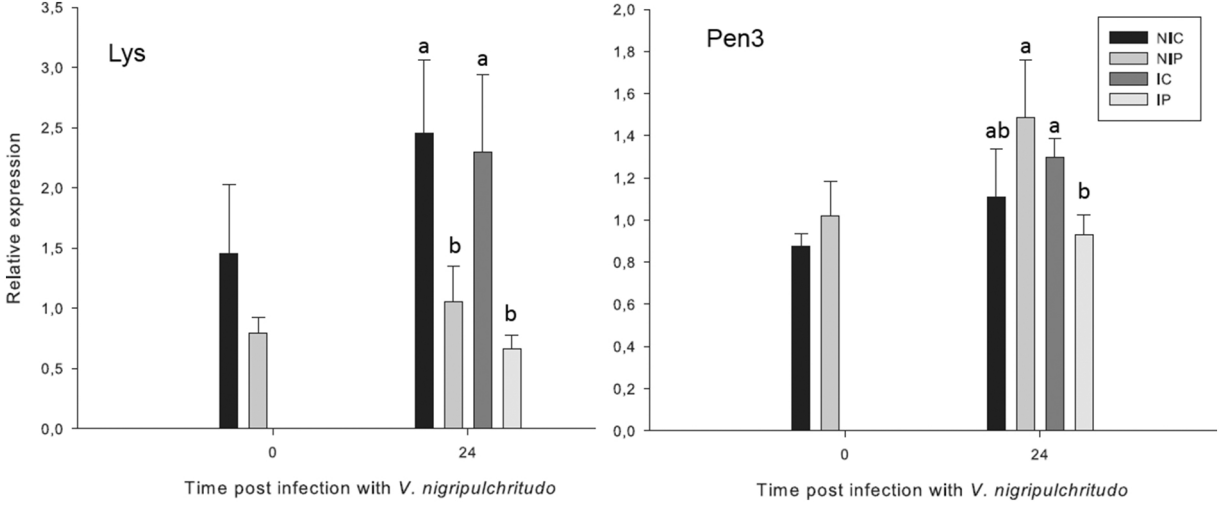

Lys
Relative expression
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
a
a
b
b
0
24
Time post infection with V. nigripulchritudo
Pen3
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
ab
a
a
b
NIC
NIP
IC
IP
0
24
Time post infection with V. nigripulchritudo

Figure 5

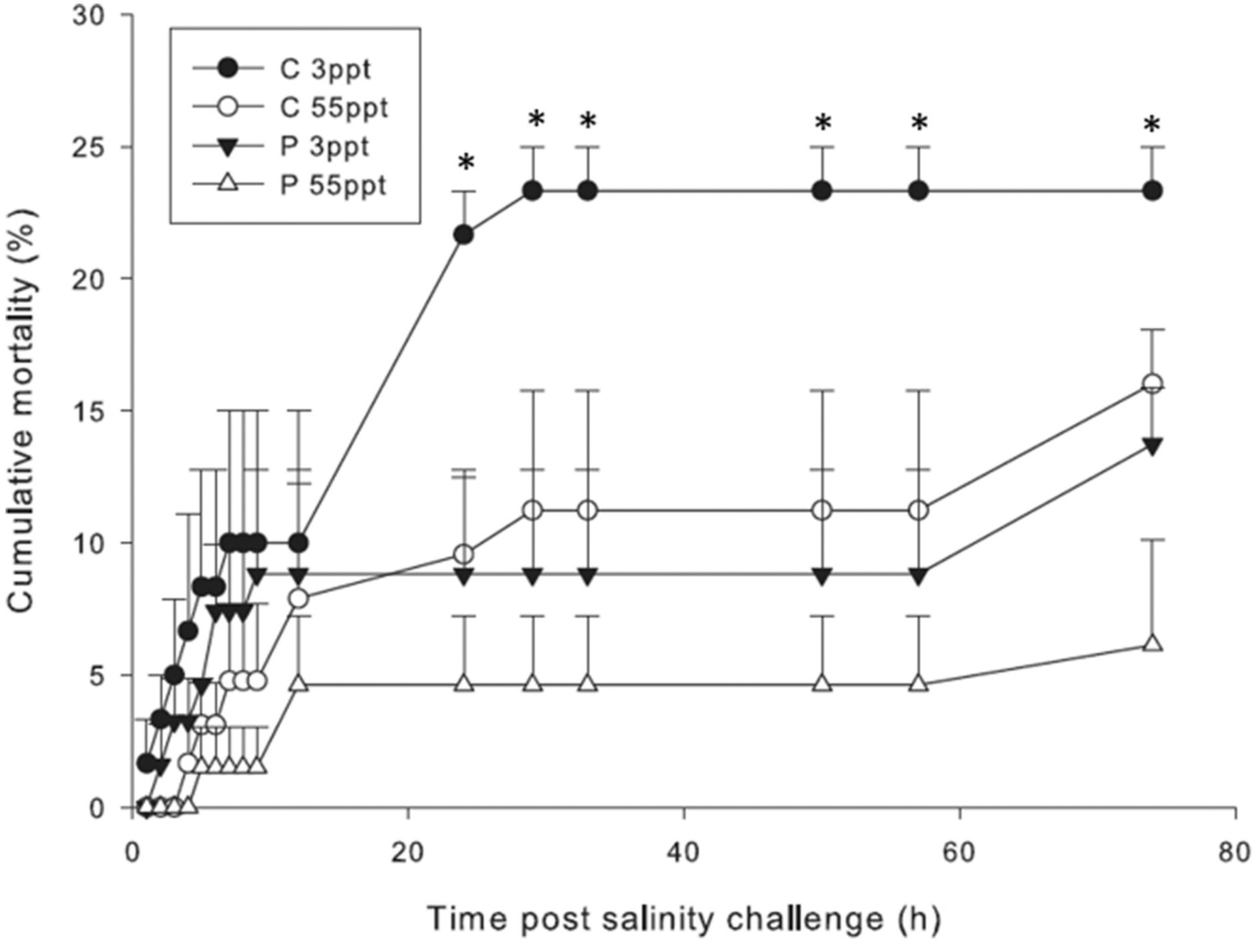

C 3ppt
C 55ppt
P 3ppt
P 55ppt
Cumulative mortality (%)
Time post salinity challenge (h)
0
5
10
15
20
25
30
0
20
40
60
80
*
*
*
*
*

Figure 6

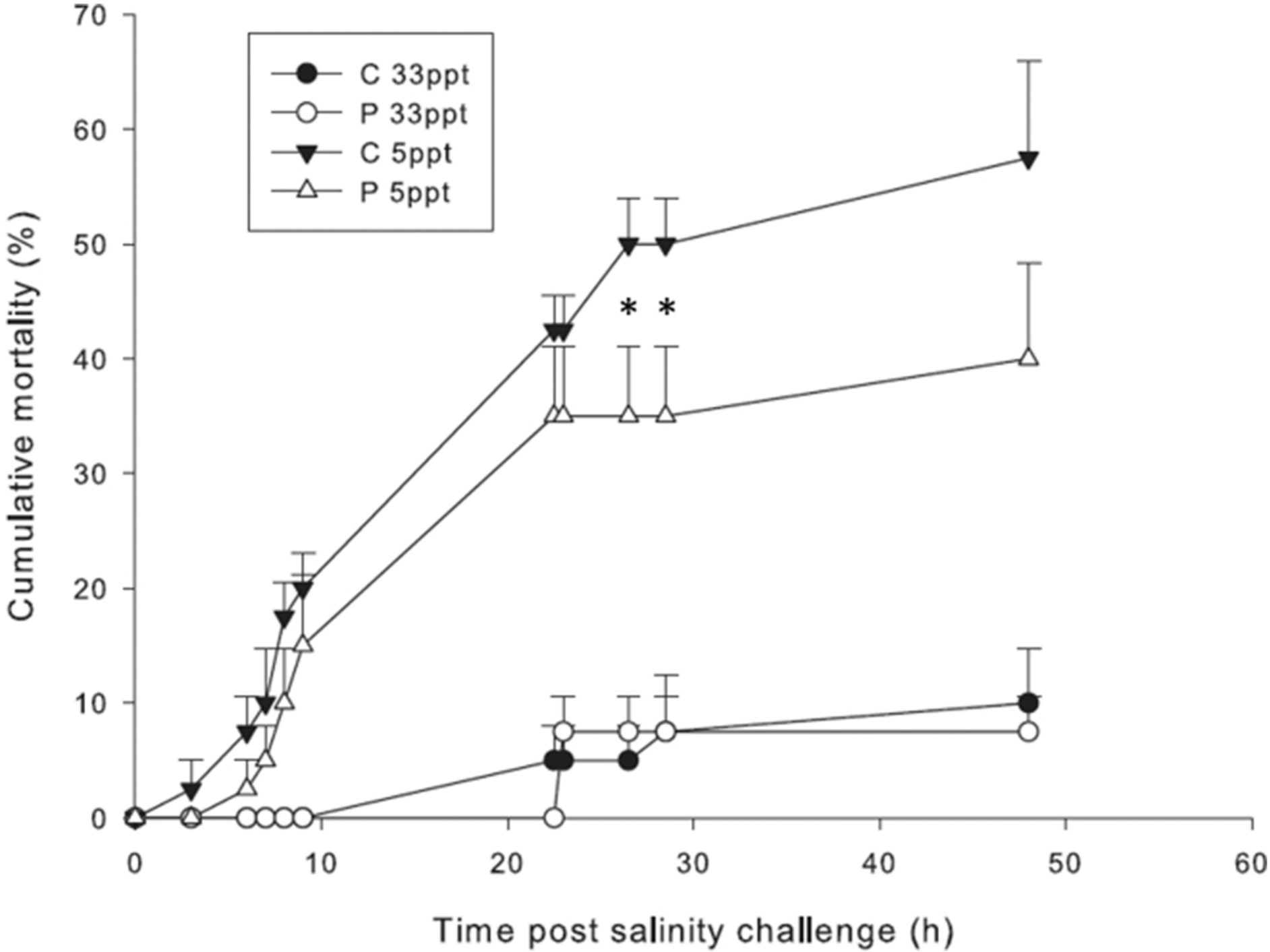

C 33ppt
P 33ppt
C 5ppt
P 5ppt
Cumulative mortality (%)
Time post salinity challenge (h)
0
10
20
30
40
50
60
70
* *

Figure 7

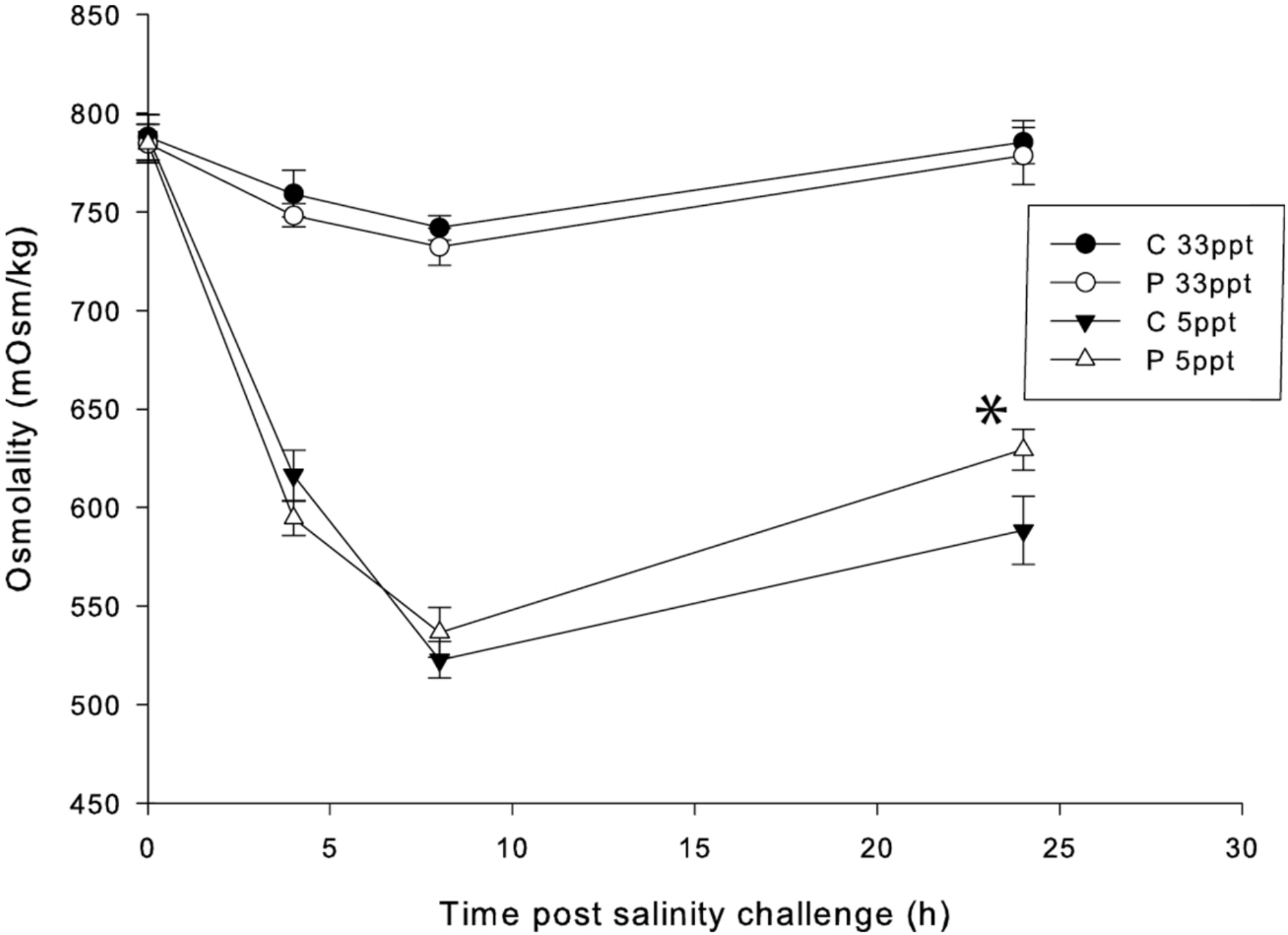

850
800
750
700
650
600
550
500
450
Osmolality (mOsm/kg)
0
5
10
15
20
25
30
Time post salinity challenge (h)
C 33ppt
P 33ppt
C 5ppt
P 5ppt
*

Figure 8