

Probiotic properties of halophilic *Bacillus* strains enhance protection of *Artemia* culture against pathogenic *Vibrio*

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ABSTRACT: We characterized 3 halophilic *Bacillus* strains isolated from Tunisian hypersaline environments. Sequencing analyses showed that these bacteria corresponded to the genus *Bacillus* sp. Antagonism assay revealed that these strains have an inhibitory effect against tested pathogenic bacteria. Qualitative analysis of biofilm revealed that *Bacillus* strains were unable to produce slime. Furthermore, the investigated strains were fairly adhesive to polystyrene with values ranging from 0.10 to 0.32 at 595 nm. Cell surface hydrophobicity values ranged between 10 and 42%. Pathogenicity and toxicity assays demonstrated that the tested strains were not pathogenic or toxic to the host. Challenge tests performed with *Artemia* larvae provided evidence that the tested *Bacillus* strains enhance the protection of *Artemia* culture against pathogens. These strains are probiotic candidates for *Artemia* culture.

KEY WORDS: 16s RNA · *Bacillus* · *Artemia* · Antagonism · Adhesion · Hydrophobicity · Probiotic

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INTRODUCTION

Infections caused by the *Vibrionaceae* family are an important problem in hatchery environments (Diggles et al. 2000) and a significant constraint to the development of aquaculture (Subasinghe et al. 2001). Infection occurs primarily through the food chain, particularly when *Artemia* are the food source (Muroga et al. 1987).

Different methods of controlling the microbial environment are being investigated. One of the methods gaining recognition is the use of probiotic bacteria (Gatesoupe 1999, Verschuere et al. 2000, Irianto & Austin 2002a). To date the screening of probiotics has been pragmatic, and they are often assessed only on their ability to produce anti-microbial metabolites and reduce the risk of intestinal diseases and eliminate specific microbial disorders (Gaggia et al. 2010).

Probiotics may protect their host from pathogens by producing metabolites which inhibit the colonization or growth of other microorganisms or by competing with them for resources such as nutrients or space (Balcázar & Luna-Rojas 2007). The efficacy of a probiotic application depends on many factors (Gomez-Gil et al. 2000) such as application level, frequency of application and environmental conditions. In their study of trout probiotics, Irianto & Austin (2002a) selected 4 of 11 bacteria which were deemed harmless to the fish following intra-peritoneal or intra-muscular injections. Zhou et al. (2009) reported that probiotic *Bacillus coagulans* SC 8168, supplemented as a water additive at a certain concentration, could significantly increase survival rate and some digestive enzyme activities in shrimp larvae. Mahdhi et al. (2010b) demonstrated that *Pseudomonas stutzeri*, *Candida utilis* and *Bacillus* sp. improve the conditions of

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Artemia culture and enhance protection against pathogenic bacteria. When these bacteria were administered as probiotics to the shrimp *Penaeus monodon*, growth and survival were improved and immunity was enhanced (Deng-Yu et al. 2009).

Various bacteria are able to produce extracellular polysaccharide, termed slime, which can reduce antibiotic susceptibility. These considerations explain the need for diagnostic methods which identify virulent bacteria strains by detecting their ability to produce slime (Christensen et al. 1982). Adhesive ability is considered an important selection criterion for probiotic bacteria, and also a prerequisite, because it offers beneficial microorganisms the capacity to colonize the intestinal tract and compete with micro-organisms (Mahdhi et al. 2010a,b). This property is determined by microbial cell surface hydrophobicity, recognized as one of the determining factors in microbial adhesion (Balebona et al. 1995). Various other factors may influence the processes of adhesion and colonization, reflecting properties related to the bacteria, to the epithelial cell surface, and to exogenous factors in the microenvironment in which the process occurs.

The aim of this study was to find more effective and environmentally-friendly probiotic strains, and obtain insight into their mode of action. This was achieved through the investigation of the probiotic properties of halophilic *Bacillus* strains and of their role in the protection of *Artemia* culture against pathogens.

MATERIALS AND METHODS

Isolation and identification of bacterial strains

Isolation and screening by plate method

Bacterial strains (n = 20) were isolated from *Artemia salina* cultures obtained from different Tunisian hypersaline environments (Sebkha El Maleh: 33° 32' N, 10° 92' E; Saltworks of Sfax: 34° 72' N, 10° 73' E, and 34° 68' N, 10° 62' E). Water samples were collected and brought to the laboratory aseptically in an insulated container at 4°C. The samples were serially diluted, plated on nutrient marine agar 2216 (Difco) and incubated at room temperature for 24 to 48 h. The colonies from nutrient marine agar were replica plated on the Muller Hinton (MH) agar medium (Difco), swabbed against target bacterial strains and incubated at room temperature for 24 h. The colonies showing a zone of clearance against pathogenic strains (*Vibrio parahaemolyticus* ATCC

17802, *Vibrio alginolyticus* ATCC 17749, *Salmonella typhimurium* ATCC 1408, *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, and *Vibrio alginolyticus* VA, isolated from infected fish; see Ben Kahla-Nakbi et al. 2006) were maintained separately in pure cultures (Hjelm et al. 2004).

Three strains that demonstrated an inhibitory effect against pathogens were retained and preserved at the laboratory and their purity routinely checked during the investigation. Stock cultures were frozen at –80°C with 20% (v/v) glycerol.

DNA extraction, PCR and sequencing

Total DNA was extracted from each strain according to the scheme described by Sambrook et al. (1989) and stored at –20°C. The 2 primers were B-K1/F (5'-TCA CCA AGG CRA CGA TGC G-3') and B-K1/R1 (5'-CGT ATT CAC CGC GGC ATG-3') (Wu et al. 2006). Amplification was carried out using a reaction volume of 50 µl: 2 µl DNA, 0.2 mM of each dATP, dGTP, dCTP and dTTP, 1× buffer solution, 1.5 mM MgCl₂, 1.0 µM of each primer (B-K1/F and B-K1/R1) and 1.0 unit of Taq DNA polymerase (Promega). The PCR program was conducted using a denaturation step of 3 min at 94°C, followed by 25 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 2 min, with an extension step at 72°C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis. The sizes of DNA fragments were estimated using a 100 bp DNA ladder (Promega). PCR products were purified using ExoSAP-IT (USB Corporation) according to manufacturer instructions and then sequenced in 2 directions using dideoxy terminator cycle sequencing (Applied Biosystems 3730). Sequences were automatically aligned using Sequencher 4.1. (GeneCodes) and variable sites were checked visually for accuracy.

Well diffusion agar assay (WDAA)

Selected potential probiotic strains were tested for their antagonistic activity using the well diffusion agar assay (WDAA) against target strains used in the primary screening by the replica plate method. The pathogenic bacteria were grown in 10 ml of nutrient broth and cultured for 24 h on nutrient agar at 30°C. The common colonies from pure culture were suspended in 10 ml of physiological medium, mixed well for 5 min, and 1 ml was spread over the agar plates. Potential probiotic strains were cultured in 10 ml nutrient broth for 24 h, 100 µl of the supernatant was

introduced into the wells of the MH agar medium, and these were then incubated for 24 h at 30°C. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well (Vaseeharan & Ramasamy 2003).

Detection of slime production strain

The ability of *Bacillus* strains to produce slime was tested according to the protocol described by Freeman et al. (1989). All tested bacteria were cultured on Congo red agar plates prepared by adding 0.8 g of Congo red (Sigma) and 36 g of saccharose (Labosi) to 1 l of brain heart infusion agar (Bio-Rad). The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 min and was added when the agar had cooled to 55°C. Plates were incubated at room temperature (37°C) for 24 h under aerobic conditions and subsequently overnight at room temperature. After incubation, pigmented colonies (generally black in color) were considered as slime-positive strains, whereas unpigmented bacteria (pinkish red, smooth colonies with a darkening at the centre) were interpreted as slime-negative (Sechi et al. 2002).

Adherence to polystyrene surface

The ability of *Bacillus* strains to form a biofilm on an abiotic surface was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc), as described previously (Mahdhi et al. 2010a,b). An overnight culture grown in Trypticase Soy Broth (TSB, Bio-Rad) at 37°C was diluted to 1:100 in TSB with 2% (w/v) glucose. A total of 200 µl of these cell suspensions was transferred to a U-bottomed 96-well microtiter plate (Nunc). Each strain was tested in triplicate. The plates were incubated aerobically at 37°C for 24 h. The cultures were removed and the microtiter wells were washed twice with phosphate-buffered saline (PBS) (7 mM Na₂HPO₄, 3 mM NaH₂PO₄ and 130 mM NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck) for 5 min. The excess stain was rinsed off and the wells were washed 3 times with sterile distilled water. The water was then cleared and the microplates were air-dried. The optical density of each well was measured at 595 nm (OD₅₉₅) using an automated Multiskan reader (GIO. DE VITA

E C). Adhesion ability was interpreted as strong (OD₅₉₅ ≥ 1), fair (0.1 ≤ OD₅₉₅ < 1) or slight (OD₅₉₅ < 0.1) (Knobloch et al. 2001).

Cell surface hydrophobicity analysis

The hydrophobicity of *Bacillus* strains was evaluated by the microbial adhesion to solvent (MATS) test, which evaluates the affinity of the cells towards apolar solvents (hexadecane), as described by Bellon-Fontaine et al. (1996). Bacterial cells were harvested by centrifugation at 7000 × *g* for 5 min and resuspended in a buffered salt solution (pH 7.0) containing Na₂HPO₄ and NaH₂PO₄ (0.2 mol l⁻¹) to a final density of 10⁹ CFU ml⁻¹ (OD = 0.4 at 600 nm). This bacterial suspension was mixed with a solvent in the proportion of 1:6 (0.4/2.4 v/v) by vortexing for 3 min in order to form an emulsion. This mixture was then left for 30 min until the separation of 2 phases. The aqueous phase was recovered with a Pasteur pipette and the turbidity at 600 nm was measured. The percentage of adhesion was expressed as: % adhesion = [(OD₆₀₀ before mixing – OD₆₀₀ after mixing)/OD₆₀₀ before mixing] × 100 (Wang & Han 2007).

Pathogenicity/toxicity towards *Artemia*

Preparation of probiotic bacteria

Potential probiotic strains were cultured in 30 ml of nutrient broth for 24 h at 37°C. The broth was then transferred to a sterile 45 ml centrifuge tube and centrifuged at 2000 × *g* for 10 min. The supernatant was poured into a sterile flask and kept at 4°C, while the bacterial pellet was resuspended in sterile seawater (Niall 2004).

Artemia gnotobiotic culture

Experiments were performed with *Artemia salina* cysts collected from the Saltworks of Sfax (34° 43' N, 10° 44' E). Bacteria-free cysts and nauplii were obtained via decapsulation as described by Sorgeloos et al. (1986). Decapsulated cysts were washed with filtered and autoclaved sea water (FASW) over a 50 µm net sterile filter. This procedure was repeated 9 times, using new FASW. After this step, washed decapsulated cysts were transferred to a sterile Falcon container with 30 ml of FASW. The capped containers were placed and exposed to the constant incandescent

light of a shaker incubator (28°C, 120 rpm). After 18 to 20 h of incubation, *Artemia* culture containing newly hatched nauplii was obtained.

Pathogenicity/toxicity assay

From the *Artemia* culture, 100 µl volume containing 20 newly hatched nauplii was added to each well of a 96-well microtitre plate. 100 µl of either probiotic bacterial suspension or the supernatant from each probiont was added to the *Artemia*. The control for the bacterial suspension was sterile sea water (CSSW), while sterile marine broth was used as the control for the supernatant (CSMB). After incubating the plate at 25°C for 24 h, the number of dead *Artemia* in each well were counted. Then, 5 µl of concentrated formalin (100%) was added to each well to kill the live nauplii and the total number of *Artemia* per well were counted. All manipulations were carried out in triplicate under sterile conditions (Jann-Para et al. 2004).

Challenge tests with *Artemia* nauplii

To evaluate the effect of the bacterial strains on *Artemia* culture, 4 challenge tests were performed in triplicate under sterile conditions during 6 d. A total of 10 axenic nauplii instars (II) obtained from *Artemia* gnotobiotic culture, as described above, were transferred to sterile Falcon containers with 30 ml of FASW, together with the amount of feed scheduled for Day 1. All manipulations were carried out under a laminar flow hood and all necessary tools were previously autoclaved at 120°C for 20 min. Test 1 was *Artemia* with pathogenic bacteria *Vibrio alginolyticus* VA (A+VA), Test 2 was *Artemia* with beneficial and pathogenic bacteria: S1 and *V. alginolyticus* VA (A+S1+VA). Test 3 was made with beneficial bacteria S2 and pathogenic bacteria strain *V. alginolyticus* VA (A+S2+VA). Test 4 was *Artemia* with strain S3 and *V. alginolyticus* VA (A+S3+VA). During challenge tests, beneficial bacteria S1, S2 and S3 were added during the first 3 d and the pathogenic bacterial strain was provided on Day 3 only. The *V. alginolyticus* (VA) strain used for comparative purposes was isolated from infected fish (Ben Kahla-Nakbi et al. 2006). Potential probiotic and pathogenic bacterial suspensions were added at a density of 10^7 CFU ml⁻¹ and 10^6 CFU ml⁻¹, respectively. The concentration of each bacterial strain was estimated through a regression analysis of the optical density of the pure culture. The number of CFU ml⁻¹ was determined using Petri

plates with marine agar. The number of swimming larvae was determined daily and the survival percentage was calculated. At the beginning and the end of each run, the absence of bacteria was monitored by transferring larvae and 100 µl of water to Petri plates in triplicate with marine agar as described in Mahdhi et al. (2010a).

Statistical analysis

Survival percentages were arcsine transformed to satisfy normal distribution and homoscedasticity requirements. Differences in survival of *Artemia* cultured under different conditions were investigated with ANOVA and Duncan's test. Correlation between cell surface hydrophobicity and semi quantitative adherence assay was assessed using multivariate ANOVA. All statistical analyses were carried out at $\alpha = 0.05$, using the software Statistica (version 5.5).

RESULTS

Bacterial identification

Based on the results of the amplification of the 16S RNA gene of *Bacillus* sp., the 3 tested strains yielded a PCR product with an amplicon around 1114 bp long. These active probiotic bacterial isolates were subjected to the 16S rDNA sequence analysis. After comparison with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool), they were identified as *Bacillus* sp. These sequences were deposited in GenBank under the accession numbers: HM117832 (S1); HM117833 (S2); HM117834 (S3).

Antagonism assay

The selected *Bacillus* strains had an antibacterial activity on Gram-positive and on Gram-negative bacteria with diameter of the inhibition zones ranging between 12 and 18.6 mm (Table 1).

Slime production, adherence to polystyrene and hydrophobicity

The isolated strains were non-slime producers and were fairly adhesive to polystyrene with values rang-

Table 1. Antagonistic activity (mean \pm SD, measured as diameter [mm] of the inhibition zone) of the selected *Bacillus* strains against Gram-negative and Gram-positive pathogens in the well diffusion agar assay

<i>Bacillus</i> strains	<i>Salmonella typhimurium</i> ATCC 1408	<i>Escherichia coli</i> ATCC 35218	<i>Staphylococcus aureus</i> ATCC 25923	<i>Vibrio alginolyticus</i> ATCC 17749	<i>Vibrio alginolyticus</i> VA	<i>Vibrio parahaemolyticus</i> ATCC 17802
S1	13.3 \pm 0.05	14.6 \pm 0.05	16 \pm 0.14	18.6 \pm 0.11	14.6 \pm 0.05	13.6 \pm 0.05
S2	12.6 \pm 0.05	14.5 \pm 0.07	15 \pm 0.05	16 \pm 0.05	12.3 \pm 0.05	17.3 \pm 0.05
S3	13 \pm 0.14	12 \pm 0.0	13 \pm 0.0	12.5 \pm 0.07	12.6 \pm 0.05	14.3 \pm 0.05

ing from 0.10 to 0.32 at 595 nm. The affinity to hexadecane (apolar solvent) was low, suggesting a hydrophilic character for the strain S1. Conversely, strains S2 and S3 were relatively hydrophobic (Table 2).

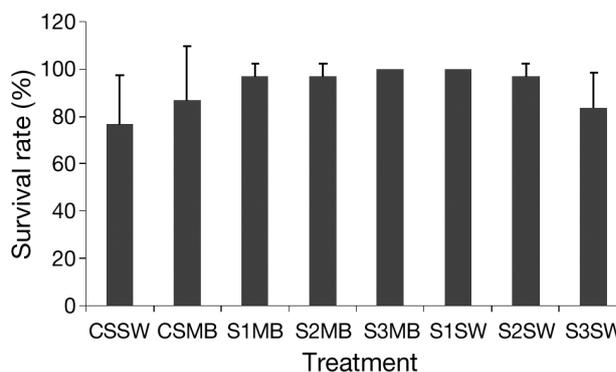
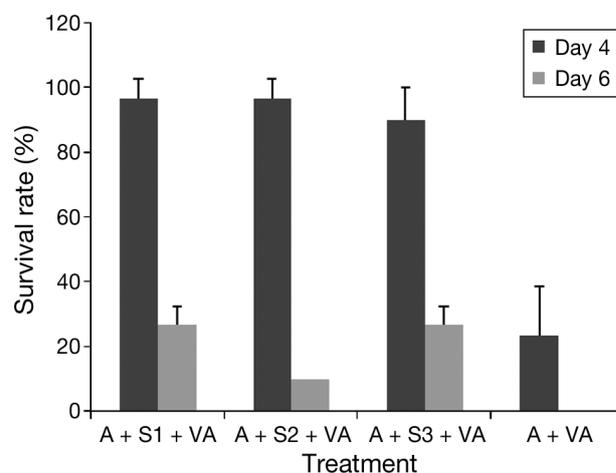
Effect on *Artemia* culture

The tested strains were not pathogenic or toxic to *Artemia* nauplii; survival rate of *Artemia* in the control treatments sterile sea water (CSSW) and sterile marine broth (CSMB) were 76% and 86%, respectively. In treatments where *Artemia* nauplii were treated with probiotic bacterial suspension (S1SW, S2SW and S3SW) and probiotic culture supernatant (S1MB, S2MB and S3MB), survival rate ranged from 83% to 100%. There was no significant difference between those treatments and the controls (Fig. 1).

Survival rate of *Artemia* larvae in treatment *Artemia* with pathogenic *Vibrio alginolyticus* VA (A+VA) was 0% at the end of treatment (Day 6) (Fig. 2). In the presence of potential probiotics (S1, S2 and S3), *Artemia* nauplii challenged with VA resulted in higher survival rate (90 to 100%) 1 d after addition of pathogenic bacteria (Day 4). However, at the end of the treatment (Day 6), survival rate ranged between 10 and 26%. Compared to treatment (A+VA), a significant difference was found for Strains S1 and S3.

Table 2. Qualitative and quantitative estimation of biofilm formation on polystyrene microtiter plates by slime-negative *Bacillus* strains. OD595 \geq 1: highly adherent, 0.1 \leq OD595 < 1: fairly adherent, OD595 < 0.1: slightly adherent

Strain	Phenotype on CRA	Adherence OD595 (mean \pm SD)	Cell surface hydrophobicity (%)
S1	Orange	0.29 \pm 0.28	41.99 \pm 0.49
S2	Pink	0.32 \pm 0.09	36.21 \pm 1.38
S3	White	0.11 \pm 0.07	10.11 \pm 1.76

Fig. 1. *Artemia salina*. Survival rate of *Artemia* nauplii after 24 h exposure to bacterial pellet and their culture supernatant (mean \pm SD). Controls were incubated in sterile sea water (CSSW) and sterile marine broth (CSMB). S1MB: culture supernatant of strain S1; S2MB: culture supernatant of strain S2; S3MB: culture supernatant of strain S3. S1SW: probiotic bacterial suspension of strain S1; S2SW: probiotic bacterial suspension of strain S2; S3SW: probiotic bacterial suspension of strain S3Fig. 2. *Artemia salina*. Survival rate (mean \pm SD) of *Artemia* larvae after different treatments on Day 4 and Day 6: *Artemia* with strain S1 and *Vibrio alginolyticus* (A + S1 + VA); *Artemia* with strain S2 and *Vibrio alginolyticus* VA (A + S2 + VA); *Artemia* with strain S3 and *Vibrio alginolyticus* VA (A + S3 + VA); *Artemia* with *Vibrio alginolyticus* VA (A + VA)

DISCUSSION

Our results indicate that *Bacillus* strains isolated from hypersaline environments have an inhibitory effect against the tested pathogenic bacteria and are able to enhance the protection of *Artemia* culture against pathogens. These findings can be explained by the antagonist ability of *Bacillus* strains to inhibit pathogenic bacteria, which can result from their artificial and temporary dominance when they are present in high concentrations. Also, the adhesive capacity of some microorganisms helps them to adhere to the intestinal tract and their effect on the digestive processes of aquatic animals can improve *Artemia* culture and protect against pathogens. *Bacillus* sp. are antagonistic against other microorganisms including fish and shellfish pathogenic bacteria (Gatesoupe 1999, Zhou et al. 2009, Deng-Yu et al. 2009), and significantly increase survival rate and some digestive enzyme activities in shrimp larvae (Zhou et al. 2009). The important role played by slime is further increased by its frequent association with reduced antibiotic susceptibility (Costeron et al. 1999). These considerations explain the need for diagnostic methods which identify virulent bacterial strains by correctly detecting their ability to produce slime. In this study, all the tested strains were non-slime producers and were fairly adherent to polystyrene microplates. Adherence ability to abiotic surfaces helps *Bacillus* strains to remain in the bacterial flora of the gut for several days and to be active during intestinal transit and participate in digestive processes, and thus aids in the elimination of potential pathogens and creation of a healthy environment for *Dicentrarchus labrax* larvae (Larpen et al. 1994). Selected probiotic bacteria, like *Lactobacillus* strains, have adhesive properties that enable them to inhibit and/or prevent the colonization of pathogens (Servin & Coconnier 2003). Also, following adhesion to a surface, spores may germinate and vegetative cells may multiply. This can help this bacterium to compete with pathogens and ensure the necessary protection for the host (Zottola & Sasahara 1994).

Pseudomonas stutzeri and *Candida utilis* are fairly adherent and can be used as probiotic candidates for *Artemia* culture (Mahdhi et al. 2010). Microbial cell surface hydrophobicity is one of the determining factors in microbial adhesion to bioremediation surfaces, a phenomenon commonly observed in natural and engineering systems (Wang et al. 2005a). It is generally accepted that hydrophobic interactions play an important role in the bioadhesion phenome-

non. This finding can be explained by the significant correlation that we detected between the hydrophobicity of cells and the semi-quantitative adherence assay. Pathogenicity and toxicity assay show that the tested strains enhance *Artemia* nauplii resistance to pathogenic *Vibrio*. Marine *Streptomyces* strains and *Aeromonas hydrophila* also have a probiotic effect on the survival and growth of *Artemia* nauplii and are able to protect them from *Vibrio* sp. in culture (Das et al. 2010, Gunasekara et al. 2010). Also, it is possible that when pathogenicity is suppressed or lost, other factors such as growth rate or attachment ability may influence the microflora to the benefit of its host (Gomez-Gil et al. 2002, Liu et al. 2010). Mahdhi et al. (2010b), using mixture design have constructed a consortium of potential probiotic *Bacillus* strains able to protect gnotobiotic *Artemia* against pathogenic *Vibrio*. The same strategy was adopted by Avella et al. (2010) and demonstrated that the administration of a *Bacillus* probiotic mixture has a benefit for sea bream larvae in terms of stress response and growth.

In conclusion, the isolated bacterial strains are potential probiotic organisms in *Artemia* culture, and possibly for shellfish or fish larvae. Further studies, such as purification and characterization of antibacterial substances and extracellular products, would help to elucidate the mode of action of the beneficial effects of *Bacillus* strains.

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LITERATURE CITED

- Gunasekara RAYSA, Rekecki A, Baruah K, Bossier P, Van den Broeck W (2010) Evaluation of probiotic effect of *Aeromonas hydrophila* on the development of the digestive tract of germ-free *Artemia franciscana* nauplii. *J Exp Mar Biol Ecol* 393:78–82
- Avella MA, Gioacchini G, Decamp O, Makridis P, Bracciatelli C, Carnevali O (2010) Application of multi-species of *Bacillus* in sea bream larviculture. *Aquaculture* 305:12–19
- Balcázar JL, Luna-Rojas T (2007) Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). *Curr Microbiol* 55:409–412
- Balebona MC, Morinigo MA, Faris A, Krovacek K, Mansson I, Bordas MA, Borrego JJ (1995) Influence of salinity and pH on the adhesion of pathogenic *Vibrio* strains to *Sparus aurata* skin mucus. *Aquaculture* 132:113–120

- Bellon-Fontaine M, Rault J, Van Oss C (1996) Microbial adhesion to solvents: a novel method to determine the electron-donor/electron acceptor or Lewis acid-base properties of microbial cells. *Colloids Surf B Biointerfaces* 7:47–53
- Ben Kahla-Nakbi A, Chaieb K, Besbes A, Zmantar T, Bakhrouf A (2006) Virulence and enterobacterial repetitive intergenic consensus PCR of *Vibrio alginolyticus* strains isolated from Tunisian cultured gilthead sea bream and sea bass outbreaks. *Vet Microbiol* 117: 321–327
- Christensen GD, Simpson WA, Bisno AL, Beachey EH (1982) Adherence of slime-producing *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun* 37:318–326
- Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–1322
- Das S, Ward LR, Burke C (2010) Screening of marine *Streptomyces* spp. for potential use as probiotics in aquaculture. *Aquaculture* 305:32–41
- Diggles BK, Moss GA, Carson J, Anderson CD (2000) Luminescent vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*. *Dis Aquat Org* 43:127–137
- Freeman DJ, Falkiner FR, Keane CT (1989) New method for detecting slime production by coagulase negative staphylococci. *J Clin Pathol* 42:872–874
- Gaggia F, Mattarelli P, Biavati B (2010) Probiotics and prebiotics in animal feeding for safe foods production. *Int J Food Microbiol* 141:S15–S28 doi:10.1016/j.ijfoodmicro.2010.02.031
- Gatesoupe FJ (1999) The use of probiotics in aquaculture. *Aquaculture* 180:147–165
- Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* 191:259–270
- Gomez-Gil B, Roque A, Velasco-Blanco G (2002) Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium, with the microalga *Chaetoceros muelleri*. *Aquaculture* 211:43–48
- Hjelm M, Bergh Ø, Riaza A, Nielsen J and 6 others (2004) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Syst Appl Microbiol* 27:360–371
- Irianto A, Austin B (2002a) Probiotics in aquaculture. *J Fish Dis* 25:633–642
- Jann-Para G, Schwob I, Feuillade M (2004) Occurrence of toxic *Planktothrix rubescens* blooms in Lake Nantua, France. *Toxicon* 43:279–285
- Knobloch JK, Bartscht K, Sabottke A, Rohde H, Feucht HH, Mack D (2001) Biofilm formation by *Staphylococcus epidermidis* depends on functional *RsbU*, an activator of the *sigB* operon: differential activation mechanisms due to ethanol and salt stress. *J Bacteriol* 183:2624–2633
- Larpernt JP, Chateau N, Castellanos MI, Larpernt JL (1994) La microflore intestinale et son rôle. In: *Les probiotiques en Alimentation Animale et Humaine*. Techniques at Documentation. Lavoisier, Paris
- Liu KF, Chiu CH, Shiu YL, Cheng W, Liu CH (2010) Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. *Fish Shellfish Immunol* 28:837–844
- Mahdhi A, Chaieb K, Kamoun F, Bakhrouf A (2010a) Use of *Pseudomonas stutzeri* and *Candida utilis* in the improvement of the conditions of *Artemia* culture and protection against pathogens. *Braz J Microbiol* 41:107–115
- Mahdhi A, Harbi B, Angeles Esteban M, Chaieb K, Kamoun F, Bakhrouf A (2010b) Using mixture design to construct consortia of potential probiotic *Bacillus* strains to protect gnotobiotic *Artemia* against pathogenic *Vibrio*. *Biocontrol Sci Technol* 20:983–996
- Muroga K, Higashi M, Keetoku H (1987) The isolation of intestinal microflora of farmed sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schelegeli*) at larval and juvenile stages. *Aquaculture* 65:79–88
- Niall GV (2004) Towards the development of a protocol for the selection of probiotics in marine fish larviculture. PhD thesis, Rhodes University, Grahamstown
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sechi LA, Deriu A, Falchi MP, Fadda G, Zanetti S (2002) Distribution of virulence genes in *Aeromonas* spp. isolated from Sardinian waters and from patients with diarrhoea. *J Appl Microbiol* 92:221–227
- Servin AL, Coconnier MH (2003) Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Pract Res Clin Gastroenterol* 17: 741–754
- Sorgeloos P, Lavens P, Leger P, Tackaert W, Versichele D (1986) *Manual for the culture and use of brine shrimp Artemia in aquaculture*. Laboratory of Mariculture, State University of Ghent, Belgium
- Subasinghe RP, Bondad-Reantaso MG, McGladdery SE (2001) Aquaculture development, health and wealth. In: Subasinghe RP, Bueno P, Phillips MJ, Hough C, McGladdery SE, Arthur JR (eds) *Aquaculture in the third millennium*. Tech Proc Conf Aquaculture in the Third Millennium. NACA, Bangkok and FAO, Rome, Italy
- Tseng DY, Ho PL, Huang SY, Cheng SC, Shiu YL, Chiu CS, Liu CH (2009) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei* by the probiotic, *Bacillus subtilis* E 20. *Fish Shellfish Immunol* 26:339–344
- Vaseeharan B, Ramasamy P (2003) Abundance of potentially pathogenic micro-organisms in *Penaeus monodon* larvae rearing systems in India. *Microbiol Res* 158:299–308
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* 64:655–671
- Wang YB, Han JZ (2007) The role of probiotic cell wall hydrophobicity in bioremediation of aquaculture. *Aquaculture* 269:349–354
- Wang YB, Xu ZR, Xia MS (2005a) Bacteria attached to suspended particles in northern white shrimp (*Penaeus vannamei* L.) ponds. *Aquaculture* 249:285–290
- Wu XY, Walker MJ, Hornitzky M, Chin J (2006) Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. *J Microbiol Methods* 64:107–119
- Zhou X, Wang Y, Li W (2009) Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. *Aquaculture* 287:349–353
- Zottola EA, Sasahara KC (1994) Microbial biofilms in the food processing industry — should they be a concern. *Int J Food Microbiol* 23:125–148