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Marine *Lactobacillus pentosus* H16 protects *Artemia franciscana* from *Vibrio alginolyticus* pathogenic effects

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ABSTRACT: *Vibrio alginolyticus* is an opportunistic pathogen which may affect different aquatic organisms. The aim of this study was to assess the probiotic properties and the protective mode of action of *Lactobacillus pentosus* H16 against *V. alginolyticus* 03/8525, through *in vitro* and *in vivo* studies using *Artemia franciscana* (hereafter *Artemia*). This strain showed antimicrobial activity against *V. alginolyticus* 03/8525 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC33658 possibly related to lactobacilli organic acid production. It was able to survive at high rainbow trout bile concentrations and showed high selective adhesion to rainbow trout mucus ($1.2 \times 10^5 \pm 8.0 \times 10^3$ cells cm^{-2}). H16 outcompeted *V. alginolyticus* 03/8525 and *A. salmonicida* subsp. *salmonicida* ATCC33658, greatly reducing their adherence to rainbow trout mucus (64.8 and 74.1%, respectively). Moreover, H16 produced a cell-bound biosurfactant which caused an important decrease in the surface tension. H16 also protected *Artemia* nauplii against mortality when it was administered previous to *V. alginolyticus* 03/8525 inoculation. Furthermore, H16 bioencapsulated in *Artemia*, suggesting that it is possible to use live carriers in its administration. We conclude that the ability of *L. pentosus* H16 to selectively adhere to mucosal surfaces and produce cell-bound biosurfactants, displacing pathogenic strains, in addition to its antimicrobial activity, confer H16 competitive advantages against pathogens as demonstrated in *in vivo* challenge experiments. Thus, *L. pentosus* H16, a marine bacterium from the intestinal tract of hake, is an interesting probiotic for *Artemia* culture and also has the potential to prevent vibriosis in other aquaculture activities such as larvae culture and fish farming.

KEY WORDS: Probiotic · *Artemia* · Vibriosis · *Lactobacillus pentosus* · Bioencapsulation

INTRODUCTION

Bacterial diseases are a major problem for aquaculture development. When farmed aquatic organisms are exposed to stressful conditions, new pathogens emerge and result in serious economic losses. Diseases caused by *Vibrio* spp. are commonly implicated in episodes of mortality of cultured marine animals (Toranzo et al. 2005, Kesarcodi-Watson et al. 2008, Grześkowiak et al. 2012). Thus, vibriosis had achieved notability in mariculture and in euryaline fish farming, especially in the successful rearing of salmonids for which this disease is a major limiting factor (Denkin & Nelson 1999, Austin & Austin 2007). Furthermore, the high mortality of larval phases of marine fish is frequently attributed to *Vibrio* infections (Vázquez et al. 2005). Particularly, *V. alginolyticus* is recognized as an opportunistic invader of already damaged tissues and a weak pathogen of stressed fish (Austin & Austin 2007).

A probiotic is any microbial cell provided via the diet or rearing water that benefits the host fish condition improving the microbial balance of the fish and the resistance to diseases (Merrifield et al. 2010). Pro-

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biotic application is an interesting alternative to the traditional administration of antibiotics in aquaculture. The excessive use of antibiotics may lead to the emergence and spreading of resistant strains, causing serious sanitary problems (Pérez et al. 2010, Sequeiros et al. 2010). Furthermore, other therapies such as vaccination are not suitable for larval phases or young low immuno-competent individuals (Gram et al. 1999, Balcázar et al. 2008). In contrast, there is evidence that the administration of probiotics bioencapsulated in live carriers such as *Artemia* spp. or rotifers succeed to prevent infections at larval phases (Gomez-Gil et al. 2000, Touraki et al. 2012, Dagá et al. 2013).

A wide range of microorganisms (Gram-negative and positive bacteria, yeasts and microalgae) have been proposed as aquaculture probiotics, but lactic acid bacteria (LAB) are the most deeply characterized group due to its extensive application in animal and human probiotic formulations (Ringø & Gatesoupe 1998). Particularly, lactobacilli are present in the natural microflora of the gastrointestinal and urogenital tracts of humans and animals, and they are recognized as important microorganisms in the prevention of colonization by pathogen strains (Soccol et al. 2010). The mechanisms related to the protective role of lactobacilli against pathogens include the production of inhibitory compounds (e.g. hydrogen peroxide, organic acids, bacteriocins, and biosurfactants) and the competition for adhesion sites mediated by their ability to adhere to the epithelial cells and mucosal surfaces (Pascual et al. 2008, Siciliano et al. 2008, Gołek et al. 2009). Adhesion to fish mucus layers is considered a prerequisite for microbial colonization, which contributes to protect skin surface, gills and the gastrointestinal tract (Collado et al. 2007a, Grześkowiak et al. 2012, Jensen et al. 2012). Highly adhesive *Lactobacillus brevis* JCM 1170 induced changes in the gut bacterial community and protected juvenile hybrid tilapia against *Aeromonas hydrophila* infection, suggesting that the degree to which *Lactobacillus* strains adhere to the gut may be a favorable criterion in selecting probiotic strains for aquaculture (Liu et al. 2013). *Lactobacillus plantarum* 7-40 (NTU102) increased the resistance of white shrimp (*Litopenaeus vannamei*) to *V. alginolyticus*, enhancing its cellular and humoral immune responses (Chiu et al. 2007). Other studies have also observed positive effects of lactobacilli in Nile tilapia, carps, eel, sea bass, and rainbow trout (Nikoskelainen et al. 2003, Aly et al. 2008, Frouël et al. 2008, Giri et al. 2013, Lee et al. 2013).

Even though the use of marine microorganisms for biotechnological purposes is currently blooming (Dionisi et al. 2012), most of the studies using lactobacilli as aquaculture probiotics have employed non-marine strains. Nevertheless, marine strains may present the advantage of being adapted to salinity, and they are able to compete with pathogenic strains in euryaline fish farming and mariculture. Regarding *L. pentosus*, numerous studies focused on different aspects of isolates from dairy, meat and vegetable ferments as well as from human and terrestrial animal gastrointestinal and urogenital tracts (Todorov & Dicks 2007, Liu et al. 2008, Jensen et al. 2012, Lee et al. 2012, Anukam et al. 2013). On the other hand, studies on wild *L. pentosus* strains from marine sources are scarce (Lee et al. 2010).The aim of this study was to assess the probiotic properties and the protective mode of action of the marine strain *L. pentosus* H16 against *V. alginolyticus* 03/8525, through *in vitro* and *in vivo* studies using *Artemia franciscana* (hereafter *Artemia*). *Artemia* spp. are commonly used live feeds in fish and crustacean larvae aquaculture and they are also important aquatic models to study host-microbial interactions under gnotobiotic conditions (Verschuere et al. 2000, Gunasekara et al. 2011). The encapsulation of *L. pentosus* H16 in *Artemia* was also investigated, which is an interesting but scarcely explored alternative to administer probiotics to farming aquatic animals as larval phases of marine fish and crustaceans (Gomez-Gil et al. 2000, Van Hai et al. 2010).

MATERIALS AND METHODS

Isolation and characterization of strain H16

Strain H16 was isolated from the intestinal tract of hake *Merluccius hubbsi* during a screening to select marine bacteria showing antimicrobial activity against fish pathogens. Fish were collected at the northeast coast of the Chubut Province, Argentina. For isolation, the intestinal tract was aseptically dissected and homogenized in 10 ml of sterile saline solution. Aliquots (0.1 ml) of the suspensions were plated in De Man, Rogosa and Sharpe Agar (MRS; Laboratorios Britania) supplemented with 2.0% (w/v) NaCl. Plates were incubated at 25°C for 7 d.

DNA from strain H16 was extracted using the Wizard Genomic DNA Purification kit (Promega). The 16S rRNA gene sequence (corresponding to positions 27-1492 in the *Escherichia coli* gene) was PCR-amplified as described by Olivera et al. (2005), using a Multigene Gradient thermal cycler (Labnet International). Sequencing on both strands of the PCR

fragment was performed using the dideoxy chain termination method by the commercial services of the CENPAT Molecular Biology Laboratory (Argentina). 16S rRNA gene sequence similarity searches were carried out using the EzTaxon-extended database (Kim et al. 2012) and BLAST at the NCBI database (Altschul et al. 1990). H16 16S rRNA gene sequence was deposited in the GenBank database under the accession number KC921993.

The effect of temperature and NaCl concentration on the H16 growth kinetics was investigated. Strain H16 was grown in MRS broth with 1.0% (w/v) NaCl at 15°, 25°, 30°, 35°, 40°, and 45°C. To examine the NaCl concentration effect, H16 was cultured in MRS broth, prepared with 0.0, 0.5, 1.0, 1.5, 2.0, and 3.0% NaCl (w/v), as well as with natural sterile seawater, and incubated at 30°C. The maximum specific growth rate was calculated during the exponential growth phase as $\mu_{max} = \ln (X_2/X_1)/(t_2 - t_1)$, where X_2 and X_1 are the culture absorbance (λ = 600 nm) at times t_2 and t_1, respectively. Then, the generation time was calculated: $t_g = \ln 2/\mu_{max}$. Assays were performed in triplicate.

Haemolytic activity was tested in blood-agar plates (adding 1.5% NaCl). Plates were incubated at 25°C for 5 d and checked for clearing zones that indicated haemolytic activity.

H16 antimicrobial activity and biosurfactant production

H16 antimicrobial activity was determined by the agar well diffusion assay described by Parente et al. (1995). The indicator pathogens used were *Yersinia ruckeri* ATCC 29473, *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, *Lactococcus garvieae* 03/8460, *Carnobacterium piscicola* 4020 and *Vibrio alginolyticus* 03/8525. H16 was grown in MRS broth supplemented with 1.5% (w/v) NaCl at 25°C for 48 h. Aliquots of 50 µl of cell-free supernatant (7000 × *g* for 10 min) and 50 µl of 1 M NaOH neutralized supernatant (pH 6.5) were pipetted into 6 mm wells performed in plates inoculated with the different indicator strains. Wells filled with MRS broth were added as controls to determine possible inhibitory activity of the medium. *L. garvieae* plates were prepared with MRS agar. *Carnobacterium piscicola, Y. ruckeri* and *A. salmonicida* subsp. *salmonicida* were cultured in Triptycase soy agar (TSA; Laboratorios Britania), and *V. alginolyticus* in TSA with NaCl 2.0% (w/v). Before incubation, plates were placed at 4°C for 2 h to allow the diffusion of the supernatant components into the agar. Except for *A. salmonicida* subsp. *salmonicida* which was incubated at 25°C, indicator strains were incubated at 30°C for 24 h. After incubation, the diameter of the inhibition zone was observed.

Probiotic microorganisms can produce surface-active molecules (biosurfactants) which have antimicrobial/antiadhesive properties against microbial pathogens (Gudiña et al. 2010, Saravanakumari & Mani 2010). H16 cells grown on MRS agar 1.5% (w/v) NaCl, at 25°C for 48 h, were recovered from Petri dishes (~30 mg dry weigh) taking care not to collect solid media, washed twice with saline solution, and resuspended in 15 ml of phospate-buffered saline (PBS, 150 mM NaCl, 5 mM KH_2PO_4, 5 mM K_2HPO_4, pH 7.2). The suspensions were stirred in an orbital shaker at 120 rpm and 25°C for 2 h to promote biosurfactant release. Bacteria were removed by centrifugation (7000 × *g*, 10 min) and the surface tension was determined with a du Noüy ring tensiometer (Decalab) at 25°C as an indication of cell-associated biosurfactant production.

H16 was also cultured in MRS broth with 1.5% (w/v) NaCl, at 25°C for 48 h, and the surface tension of the culture supernatants and of controls without inocula was determined in order to assess extracellular biosurfactant production.

H16 tolerance to rainbow trout bile

H16 tolerance to rainbow trout bile was tested according to Nikoskelainen et al. (2001). After 48 h of the last feeding, bile was obtained from healthy rainbow trout by gallbladder puncturing. A H16 cell suspension was adjusted to an absorbance (λ= 600 nm) of 0.25 (~1.0×10^8 CFU ml^{-1}), then a 500 µl aliquot was centrifuged (7000 × *g*, 10 min) and the cells suspended in PBS and PBS buffer with 10% (v/v) rainbow trout bile, in triplicate. After incubation for 1.5 h at 25°C, samples were serially diluted and plated on MRS agar with 1.5% NaCl (w/v). Plates were incubated at 25°C for 48 h and H16 colony forming units (CFU) counted.

Adhesion capability of strain H16

Selective adhesion to rainbow trout mucus

Mucus was obtained from the whole body of healthy Patagonian rainbow trout by carefully scraping the skin with a rubber spatula. The mucus was collected into a small volume of PBS buffer and then

centrifuged to remove the particulate and cellular material. Mucus suspensions were homogenized and the protein concentration adjusted to 0.5 mg ml^{-1} (Sica et al. 2012). Proteins were determined by the Bradford method (1976) using bovine serum albumin (Sigma) as standard. H16 adhesion to rainbow trout mucus was assessed according to Sica et al. (2012). Briefly, H16 was incubated in MRS broth with 1.5% (w/v) NaCl for 48 h at 25°C. Thereafter, the cultures were centrifuged (2500 × *g*, 15 min) and the pellets washed twice with PBS buffer. The cells were suspended in the same buffer to an absorbance (λ = 600 nm) of 0.25, and then the suspension was diluted to 1.0×10^7 CFU ml^{-1}. Rainbow trout mucus was immobilized on clean and sterile microscope glass coverslips by overnight incubation at 4°C, and the excess of mucus was removed by washing with PBS. A 100 µl aliquot of H16 suspension was added to each coverslip containing the immobilized mucus. After incubation for 1 h at 18°C, the coverslips were washed twice with 250 µl of PBS to remove the unbound bacteria. To assess if H16 mucus adhesion was due to non-specific adhesion, its adhesion to glass was also measured (Sica et al. 2012). Adhesion was assessed by light microscopy (Standard RA, Carl Zeiss) after Gram staining with a Britania kit (Laboratorios Britania). Results from 3 independent replicates were expressed as the average logarithm units of cells adhered cm^{-2} after counting at least 20 fields by coverslip.

Competitive exclusion assay against fish pathogens

The capability of H16 to avoid the adherence of *V. alginolyticus* 03/8525 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 to rainbow trout mucus was assessed. H16 suspension and rainbow trout mucus were prepared as described above. *V. alginolyticus* was cultured in Trypticase soy broth (TSB) with 2.0% (w/v) NaCl, and *A. salmonicida* subsp. *salmonicida* in TSB at 25°C during 48 h. Cells were harvested by centrifugation, washed twice with PBS and suspended in the same buffer to an absorbance (λ = 600 nm) of 0.25. H16 suspension was mixed with each pathogen suspension (1:1) and 100 µl aliquots were added onto the coverslip immobilized mucus. After incubation for 1 h at 18°C, the coverslips were washed twice with 250 µl PBS to remove the unbound bacteria. Adhesion was assessed by light microscopy as indicated above. Gram positive H16 was distinguished from the Gram negative pathogens by morphology and Gram staining. The results from 3 independent replicates were expressed as the average units of cell number of pathogens adhered cm^{-2} and compared with the pathogen adhesion without H16 addition (control). The control values were taken as 100% of adhesion, and the inhibition of pathogen adherence was calculated by subtracting each adhesion percentage in H16 presence from the corresponding control value (Sica et al. 2012).

Bioencapsulation of H16 strain in *Artemia*

The experiment was carried out using bacteria-free cultures of *Artemia* (INVE Aquaculture). In order to obtain sterile cysts and subsequently sterile nauplii, *Artemia* cysts were hydrated and decapsulated following the procedures described by Martínez-Díaz & Hipólito-Morales (2013). Seawater was filtered through a Millipore membrane filter (0.45 µm) and autoclaved at 121°C for 20 min (Olivera et al. 2000). After 19 h of incubation of the cysts, the nauplii were harvested and distributed at a density of 100 nauplii per tube with 10 ml of sterile seawater. Immediately, the tubes were inoculated with H16 strain, in a single dose. Three final H16 concentrations in the tubes were tested (1.0×10^5, 0.5×10^6 and 1.0×10^6 CFU ml^{-1}). Controls consisting of sterile seawater and nauplii (without the addition of H16) were also performed. The assay was performed in triplicate. Then, the tubes were incubated at 26°C for 24 h. Following incubation, H16 encapsulation in the nauplii was confirmed after thorough washing of the nauplii with sterile seawater, aseptically homogenization, and H16 colony counts on MRS agar with 1.5% (w/v) NaCl.

In vivo assessment of H16 probiotic capability

Two challenge experiments using *Artemia* nauplii infected with *V. alginolyticus* 03/8525 were performed to assess *in vivo* H16 probiotic activity. Bacteria-free nauplii were obtained as previously described. After 19 h of incubation of the cysts, nauplii were distributed at a density of 100 individuals per vessel containing 100 ml of sterile seawater. In the first experiment, each treatment included 3 vessels containing the bacteria-free nauplii and (1) H16 (4.6×10^5 CFU ml^{-1}), (2) *V. alginolyticus* (5.3×10^5 CFU ml^{-1}), and (3) H16 and *V. alginolyticus* simultaneously co-inoculated at those concentrations. Both, H16 and *V. alginolyticus* were inoculated immediately after distributing the nauplii in the vessels, in a single dose. After 48 h of

vessel incubation, the nauplii survival rate was recorded. In the second experiment, the effect of H16 inoculation previously to *V. alginolyticus* infection was assessed. After 19 h of incubation of the cysts, nauplii were distributed in the vessels as explained in the first experiment. Each treatment included 3 vessels containing the bacteria-free nauplii and (1) H16 (4.6×10^5 CFU ml^{-1}) inoculated immediately after distributing the nauplii in the vessels, (2) *V. alginolyticus* (5.3×10^5 CFU ml^{-1}) inoculated after 24 h of vessel incubation, and (3) H16 (4.6×10^5 CFU ml^{-1}) inoculated immediately after distributing the nauplii in the vessels and *V. alginolyticus* (5.3×10^5 CFU ml^{-1}) inoculated after 24 h of vessel incubation. Both H16 and *V. alginolyticus* inocula were administered in a single dose. The duration of the test was 72 h and then the nauplii survival rate was recorded. In both experiments, 3 sterile units (bacteria-free nauplii + sterile seawater) were used as controls.

Statistical analyses

Test results are expressed as the mean ± SE. We performed a non-parametric Mann-Whitney rank sum test to compare median values of adhesion and bile tolerance tests. At the bioencapsulation and *in vivo* experiments, the significance of the differences among treatments was evaluated by a non-parametric Kruskal-Wallis test. Statistical analyses were carried out with SPSS 7.0 package (Norusis 1997).

RESULTS

Identification, physiological properties, and antimicrobial activity of strain H16

The closest match of the 16S rRNA sequence (~1400 bp) of strain H16 was to that of *Lactobacillus pentosus* JCM 1558T (100% homology). *L. pentosus* H16 grew at temperatures between 5° and 45°C, while the optimal growth (generation time, t_g = 44 min) was obtained at 35°C (Table 1). Moreover, optimal growth (t_g = 46 min) was obtained at 0.5% (w/v) NaCl, although *L. pentosus* H16 was able to grow with and without NaCl addition to the medium (Table 2).

L. pentosus H16 showed extracellular antimicrobial activity against *Vibrio alginolyticus* 03/8525 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 (Fig. 1). Such activity was not observed when the culture supernatant was neutralized (Fig. 1). On the other hand, no antimicrobial activity was registered against *L. garvieae*, *Carnobacterium piscicola* and *Yersinia ruckeri.*

Table 1. Maximum specific growth rate (μ_{max}) and generation time (t_g) of *Lactobacillus pentosus* H16 cultures growing at different temperatures

Temp. (°C)	μ_{max} (h^{-1})	t_g (min)
15	0.22	189
25	0.36	115
30	0.82	50
35	0.90	46
40	0.80	52
45	0.58	71

Table 2. Maximum specific growth rate (μ_{max}) and generation time (t_g) of *Lactobacillus pentosus* H16 cultures growing at different NaCl concentrations

NaCl (% w/v)	μ_{max} (h^{-1})	t_g (min)
0.0	0.86	48
0.5	0.94	44
1.0	0.82	50
1.5	0.77	54
2.0	0.72	58
3.0	0.62	67
Seawater	0.55	75

Interestingly, after being in contact with H16 cells, PBS surface tension decreased from 71.5 ± 0.3 to 46.4 ± 0.4 mN m^{-1}, indicating that *L. pentosus* H16 has the capability of releasing surface-active compounds associated to the cells. No surface tension reduction of the culture supernatants was observed when H16 was grown in MRS broth, confirming no extracellular biosurfactant production.

In addition, H16 exhibited high tolerance to rainbow trout bile as no significant difference ($p < 0.05$)

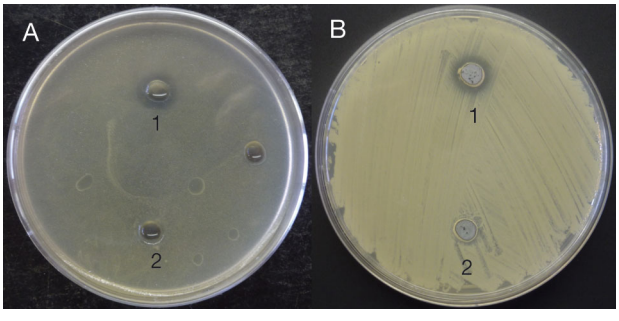

Fig. 1. Agar well diffusion assay of cell-free supernatant of *Lactobacillus pentosus* H16 against (A) *Vibrio alginolyticus* 03/8525 and (B) *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. Supernatants (1) without treatment or (2) neutralized to pH 6.5

was detected in the viable recovery of cultures treated with 10% (v/v) rainbow trout bile ($5 \times 10^8 \pm 5 \times 10^7$ CFU ml^{-1}) and the controls without bile ($1 \times 10^8 \pm 6 \times 10^6$ CFU ml^{-1}). Besides, H16 did not show haemolytic activity.

H16 *in vitro* inhibition of pathogen adhesion to rainbow trout skin mucus

Our results revealed that *L. pentosus* H16 selectively adhered to rainbow trout skin mucus as its adhesion to glass ($3.8 \times 10^4 \pm 3.9 \times 10^3$ cells cm^{-2}) was significantly lower ($p < 0.05$) than to mucus ($1.2 \times 10^5 \pm 8.0 \times 10^3$ cells cm^{-2}). Moreover, it was capable of successfully inhibiting the attachment of *Vibrio alginolyticus* 03/8525 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 to rainbow trout skin mucus. The adhesion capability of *V. alginolyticus* and *A. salmonicida* subsp. *salmonicida* to the mucus significantly decreased (64.8% ± 4.7 and 74.1% ± 3.9, respectively; $p < 0.05$) when they were co-incubated with *L. pentosus* H16 (Fig. 2).

Bioencapsulation and *in vivo* challenge of *Artemia* enriched with *L. pentosus* H16 and *V. alginolyticus*

Results indicated that *L. pentosus* H16 encapsulated in *Artemia* after 24 h of exposure and that the encapsulation can be enhanced by increasing H16 inoculum size (Table 3). Moreover, no negative effects of H16 over *Artemia* survival rate were observed. After 48 and 72 h of H16 administration, *Artemia* survival rate was not significantly different ($p < 0.05$) from that of the control without H16 (Figs. 3 & 4, respectively).

In order to evaluate a possible protection offered to *Artemia* by *L. pentosus* H16, the nauplii were challenged with *V. alginolyticus* 03/8525 after 0 and 24 h of H16 administration (Figs. 3 & 4). Our results showed that at the same H16 inoculum concentration, a protective effect of the nauplii due to the probiotic was observed when H16 was administered previously to *Vibrio* infection (Fig. 4).

DISCUSSION

Vibrio alginolyticus is a known *Artemia* pathogen which may cause a lower rate of growth and/or survival, and hence a decrease of biomass production (Rico-Mora & Voltolina 1995, Verschuere et al. 1999).

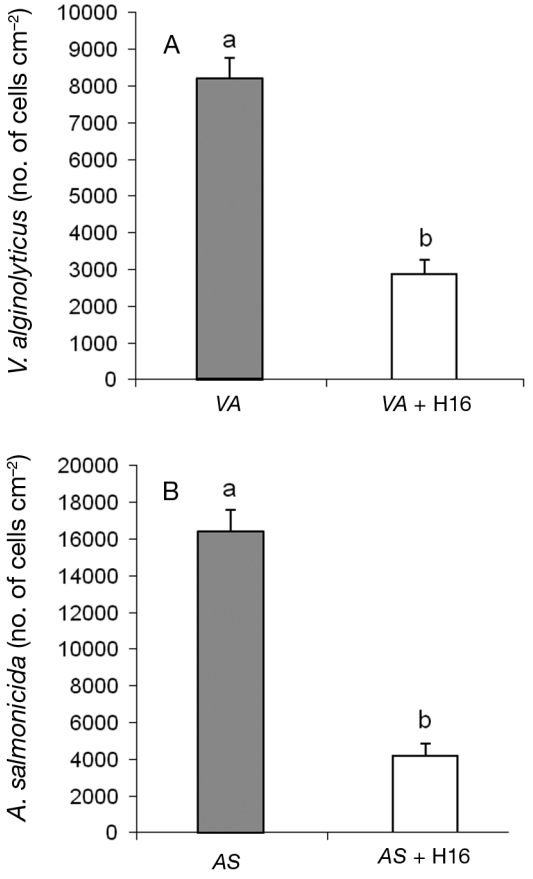

Fig. 2. Competition for selective adhesion to rainbow trout skin mucus between *Lactobacillus pentosus* H16 and different pathogen species. Mucus adhesion of (A) *Vibrio alginolyticus* 03/8525 (*VA*) and (B) *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 (*AS*) in absence and presence of H16. Data: mean ± 1 SE. Different lowercase letters: significant differences among mean values ($p < 0.05$)

Table 3. *Lactobacillus pentosus* H16 bioencapsulation in *Artemia franciscana*. Data: means (n = 3) ± 1 SE. Lowercase letters: significant differences among treatments ($p < 0.05$)

H16 inoculum (CFU ml^{-1})	CFU per *Artemia* nauplii
1.0×10^5	$1.2 \times 10^3 \pm 3.6 \times 10^2$ ^{a}
0.5×10^6	$3.5 \times 10^3 \pm 2.6 \times 10^2$ ^{b}
1.0×10^6	$6.0 \times 10^3 \pm 5.7 \times 10^2$ ^{c}

Artemia mortality after *V. alginolyticus* infection may vary depending on the *Vibrio* strain and the different *Artemia* species. Some authors reported 100% *Artemia* mortality after 24 to 48 h of *V. alginolyticus* administration (Rico-Mora & Voltolina 1995, Mahdhi et al. 2011, Lamari et al. 2014), while other studies revealed no *Artemia* mortality in the same period (Gomez-Gil et al. 1998). In this study, *V. alginolyticus*

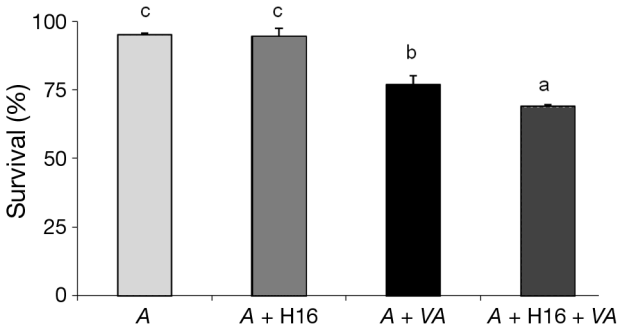

Fig. 3. Effect of co-inoculation of *Lactobacillus pentosus* H16 and *Vibrio alginolyticus* 03/8525 on *Artemia franciscana* nauplii survival after 48 h. *A*: sterile *Artemia* control; *A* + H16: *Artemia* + *L. pentosus* H16; *A* + *VA*: *Artemia* + *V. alginolyticus*; *A* + H16 + *VA*: *Artemia* co-inoculated with *L. pentosus* H16 and *V. alginolyticus*. Data: means ± 1 SE. Different lowercase letters: significant differences among mean values ($p < 0.05$)

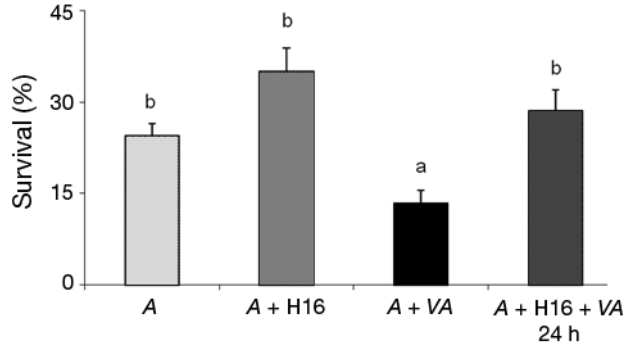

Fig. 4. Effect of *Lactobacillus pentosus* H16 administration, 24 h before to *Vibrio alginolyticus* 03/8525 inoculation, on the survival of *Artemia* nauplii. *A*: sterile *Artemia* control; (*A* + H16: *Artemia* + *L. pentosus* H16; *A* + *VA* 24 h: *Artemia* + *V. alginolyticus* after 24 h of incubation; *A* + H16 + *VA* 24 h): *Artemia* inoculated with H16 at the beginning of the experiment and *V. alginolyticus* after 24 h of incubation. Duration of the tests was 72 h. Data: mean ± 1 SE (vertical lines). Different lowercase letters: significant differences among mean values ($p < 0.05$)

03/8525 significantly reduced *Artemia* survival after 48 and 72 h of the administration, whereas the preventive colonization of *Artemia* with *Lactobacillus pentosus* H16 protects the nauplii against mortality. In contrast, when *L. pentosus* H16 is administered together with *V. alginolyticus* 03/8525, it cannot protect *Artemia* nauplii. This suggests that *Artemia* gut colonization and persistence is required for an effective *L. pentosus* H16 probiotic action. Adhesion to mucosal surfaces is considered a prerequisite for the gut colonization not only by probiotics strains but also by pathogens (Collado et al. 2007b). *L. pentosus* H16 showed a high specific adhesion to rainbow trout mucus, possibly related to the presence of mucus-binding proteins and other proteins involved in adherence in this species (Anukam et al. 2013). This high mucus adhesion capability allowed H16 to outcompete with *V. alginolyticus* 03/8525 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, greatly reducing their adherence to rainbow trout mucus (64.8 and 74.1%, respectively). Moreover, H16 produced a cell-bound biosurfactant (extractable with PBS). In other *L. pentosus* strain, CECT-4023T, a biosurfactant associated with the cell that could be a glycoprotein or a glycolipopeptide was also found (Bustos et al. 2007, Moldes et al. 2013). Biosurfactants are involved in the microbial adhesion and desorption processes and could contribute to the prevention of the colonization of urogenital and gastrointestinal tracts by pathogenic microorganisms (Reid 2000, Gudiña et al. 2010). Thus, the selective adhesive properties and the cell-bound biosurfactant produced by *L. pentosus* H16 could contribute to its rapid colonization of *Artemia* gut, explaining the observed protective effects when it was administered before to *V. alginolyticus* 03/8525.

The genus *Lactobacillus* is strictly fermentative, producing large amounts of lactic acid and small amounts of other compounds as a result of its carbohydrate metabolism (Hammes & Hertel 2006). Lactic acid production lowers the pH of the substrate and suppresses the growth of many other bacteria (Hammes & Hertel 2006). *L. pentosus* H16 exerted extracellular antimicrobial activity associated to pH decrease, as it was not observed after neutralizing the culture supernatant. This was possibly related with the production of organic acids characteristic of LAB metabolism. *L. pentosus* K34, isolated from the small intestine of Korean native chicken, showed strong inhibitory activity against gastro-intestinal pathogenic bacteria attributed to organic acid production, mainly lactic acid, oleic acid, succinic acid, pyruvic acid and phenyllactic acid (Lee et al. 2012). Undissociated weak organic acids may cross cell membranes and become dissociated inside the cell, causing intracellular acidification and promoting the expulsion of H^+ ions from the cell and uncoupling of the Na^+–K^+ (ATPase) pump (Huang et al. 1986, Vázquez et al. 2005).

L. pentosus H16 also showed other positive characteristics for its application in aquaculture. Tolerance to acid medium is not required for the culture of marine larvae whose digestive system is alkaline during the live feed period (Fjellheim et al. 2010). However, for fish farming, resistance to bile and acid medium is important to persist in the intestinal tract (Nikoske-

lainen et al. 2001). H16 tolerated a concentration of rainbow trout bile as high as 10% (v/v), which could permit its survival in the intestine. The growth of H16 in a range of 0 to 3% (w/v) NaCl at moderate temperatures pointed out its potential application in mariculture and also in freshwater aquaculture. In line with these results, *L. pentosus* PL11 showed value for Japanese eel aquaculture as it enhanced the growth performance and the resistance to *Edwardsiella tarda* infection (Lee et al. 2013). Furthermore, previous studies demonstrated that H16 produced conjugated linoleic acid (CLA), compounds with well-known health benefits (Vela Gurovic et al. 2014). This is considered a desirable property of starter cultures and probiotic strains.

Finally, *L. pentosus* H16 bioencapsulated in *Artemia*, suggesting that it is possible to use live carriers in H16 administration, for example to prevent infections at larval phases. A positive correlation was observed between H16 inoculum size and its bioencapsulation in *Artemia*; this could be exploited to set the desired H16 dose.

CONCLUSION

Overall our results indicated that *Lactobacillus pentosus* H16 selectively adheres to mucosal surfaces, displacing pathogenic strains such as *Vibrio alginolyticus* 03/8525 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. This, in addition to its antimicrobial activity possibly related with lactobacilli organic acid production and its capability to produce cell-bound biosurfactants, confer H16 competitive advantages against pathogens. *In vivo* experiments confirmed that H16 preventive administration protects *Artemia* nauplii of mortality caused by *V. alginolyticus* 03/8525 infection. *L. pentosus* H16, a marine bacterium from the intestinal tract of hake, is an interesting probiotic for *Artemia* culture and has also the potential for other aquaculture activities such as larvae culture and fish farming.

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