In vitro selection of bacteria with potential for use as probiotics in marine shrimp aquaculture

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Abstract – The objective of this work was to isolate strains of lactic acid bacteria with probiotic potential from the digestive tract of marine shrimp (*Litopenaeus vannamei*) and to carry out in vitro selection based on multiple characters. The ideotype (ideal proposed strain) was defined by the highest averages for the traits maximum growth velocity, final count of viable cells, and inhibition halo against nine freshwater and marine pathogens, and by the lowest averages for the traits duplication time and resistance of strains to NaCl (1.5 and 3%), pH (6, 8, and 9), and biliary salts (5%). Mahalanobis distance (D^2) was estimated among the evaluated strains, and the best strains were those with the shortest distances to the ideotype. Ten bacterial strains were isolated and biochemically identified as *Lactobacillus plantarum* (3), *L.* brevis (3), *Weissella confusa* (2), *Lactococcus lactis* (1), and *L. delbrueckii* (1). *Lactobacillus plantarum* strains showed a wide spectrum of action and the largest inhibition halos against pathogens, both Gram-positive and negative, high growth rate, and tolerance to all evaluated parameters. In relation to ideotype, *L. plantarum* showed the lowest Mahalanobis (D^2) distance, followed by the strains of *W. confusa*, *L. brevis*, *L. lactis*, and *L.* delbrueckii. Among the analyzed bacterial strains, those of *Lactobacillus plantarum* have the greatest potential for use as a probiotic for marine shrimp.

Index terms: *Litopenaeus vannamei*, acid lactic bacteria, pathogen inhibition.

Seleção in vitro de bactérias com potencial para uso como probióticos na carcinicultura marinha

Resumo – O objetivo deste trabalho foi isolar cepas de bactérias ácido-~~láticas~~ láticas com potencial probiótico do trato digestório de camarões marinhos e realizar seleção in vitro baseada em múltiplos caracteres. O ideótipo (cepa ideal proposta) foi definido por meio das maiores médias para os caracteres velocidade máxima de crescimento, contagem final de células viáveis e halo de inibição contra nove patógenos de origem continental e marinha, e das menores médias para os caracteres tempo de duplicação e tolerância das cepas a NaCl (1,5 e 3%), pH (6, 8 e 9) e sais biliares (5%). Foram estimadas as distâncias de Mahalanobis (D^2) entre as cepas avaliadas, e as melhores cepas foram aquelas que apresentaram menor distância do ideótipo. Foram isoladas dez cepas de bactérias identificadas bioquimicamente como *Lactobacillus plantarum* (3), *L. brevis* (3), *Weissella confusa* (2), *Lactococcus lactis* (1) e *L. delbrueckii* (1). As cepas de *L. plantarum* apresentaram amplo espectro de ação e os maiores halos de inibição, tanto para patógenos Gram-positivos quanto negativos, alta taxa de crescimento e tolerância a todos os parâmetros avaliados. Em relação ao ideótipo, *L. plantarum* apresentou a menor distância de Mahalanobis (D^2), seguida pelas cepas de *W. confusa*, *L. brevis*, *L. lactis* e *L. delbrueckii*. Entre as cepas bacterianas avaliadas, as de *L. plantarum* apresentam o maior potencial para uso como probiótico para camarões marinhos.

Termos para indexação: *Litopenaeus vannamei*, bactérias ácido-~~láticas~~ láticas, inibição de patógenos.

Introduction

In recent years, the use of probiotic bacteria has attracted the interest of the marine shrimp farming industry. Gatesoupe (1999) defines probiotics as microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health.

There have been several positive reports on the use of probiotics in shrimp culture, such as improved balance of the intestinal microbiota (Li et al., 2009), production/stimulation of the production of digestive enzymes (Liu et al., 2009), improved growth rate (Liu et al., 2009), feed efficiency (Lin et al., 2004), immunostimulation (Chiu et al., 2007), resistance to infection by bacterial (Chiu et al., 2007) and viral pathogens (Tseng et al., 2009). However, the use of probiotics is still controversial due to some negative results (no action on the host) (Meunpol et al., 2003). These reports are usually associated with commercial products, which are often formulated with bacteria not native to the animal under study, including some terrestrial environment species.

The isolation of bacteria from the digestive tract of the animal species of interest is the first step to successfully obtain a new probiotic (Balcázar et al., 2006). This stage normally involves the isolation of dozens or even hundreds of strains. Following isolation, several in vitro selection tests are usually performed, such as pathogen inhibition (Guo et al., 2009), production of digestive enzymes (Ochoa-Solano, 2006), production of antimicrobial substances (Vázquez et al., 2005; Sugita et al., 2007), growth rate (Vine et al., 2004a), and ability to adhere to the intestinal epithelium (Vine et al., 2004b), in order to select certain strains for in vivo assays.

Among the bacteria used as probiotics, lactic acid strains stand out because they are easy to manipulate, produce antimicrobial compounds (organic acids, lactic acid, bacteriocins, and hydrogen peroxide), and stimulate nonspecific immune response in hosts (Gatesoupe, 2008).

The objective of this work was to isolate strains of lactic acid bacteria with probiotic potential from the digestive tract of marine shrimp (*Litopenaeus vannamei*) and to carry out in vitro selection based on multiple characters.

Materials and Methods

The experiments were carried out at the microbiology sector of the Laboratório de Camarões Marinhos at the Universidade Federal de Santa Catarina (UFSC), between January and December 2008.

The strains were isolated from the digestive tract of adult shrimp obtained from the hatchery of the Laboratório de Camarões Marinhos at UFSC. The digestive tracts of collected shrimp were removed under sterile conditions, macerated in 2% NaCl saline solution, sprayed on plates with de Man, Rogosa, and Sharpe (MRS) growth media, and incubated for 48 hours at 30°C.

After incubation, the colonies grown in culture media were identified morphologically using Gram's method. Colonies of interest were spread in Petri dishes containing MRS growth media using the streaking method for isolation.

To evaluate growth kinetics, the strains were incubated in triplicate in a test tube containing 10 mL of liquid MRS growth medium and kept at 30°C under constant agitation during 24 hours. Bacterial growth was measured every two hours by reading a 100 µL sample from each tube in a microplate reader at 630 nm. Inoculum concentration was converted to colony forming units (CFU)/mL from a standard curve previously devised for each strain. From these results, maximum growth rate (μ_{max}) and doubling time (t_{dup}) of strains were calculated, according to the following equations (Madigan et al., 2004): $\mu_{max} = \ln(Z) - \ln(Z_0)/dt$ in which μ_{max} is the maximum growth rate, Z is the inoculum concentration (CFU/mL), Z_0 is the initial inoculum concentration (CFU/mL), and dt is the culture time (hours); and $t_{dup} = \ln(2)/\mu_{max,}$ in which t_{dup} is the doubling time (hours) and μ_{max} is the maximum growth rate.

After 24 hours of growth, samples from all flasks were sprayed in MRS agar growth medium using the serial dilution technique and incubated at 30°C for 48 hours. After that period, colony forming units (CFU mL^{-1}) were estimated.

For bile salt tolerance, the strains were incubated (30°C) for 24 hours in tubes containing 10 mL MRS broth with 5% (w/v) bile salts (using bovine bile) and with no added bile salts, in triplicate. Next, 100 µL samples from each culture, from each tube, were analyzed using a microplate reader at 630 nm. The tolerance of each isolated bacterial strain to bile salts was determined as the percentage reduction in absorbance in relation to the growth medium without added bile salts.

The bacterial strains were incubated in tubes containing 10 mL of MRS broth growth medium with added salt (0%, 1.5%, and 3%) and incubated (30°C) during 24 hours in triplicate to order study NaCl

tolerance. Then, 100 µL samples from each culture were analyzed using a microplate reader at 630 nm. The tolerance of each bacterial strain to the different concentrations was determined as the percentage reduction in absorbance in relation to the growth medium without added salt.

To evaluate pH tolerance, the bacterial strains were incubated in MRS broth growth medium at different pH (6, 7, 8, and 9) and placed in an incubator (30°C) during 24 hours in triplicate. After that, 100 µL samples from each culture were analyzed using a microplate reader at 630 nm. The tolerance of each bacterial strain to the different pH was determined as the percentage reduction in absorbance in relation to the growth medium with pH 7, the neutral pH.

The bacterial strains isolated from shrimp were evaluated for their ability to inhibit Gram-negative (*Vibrio harveyi* ATCC 14126, *Vibrio alginolyticus* BCCM 2068, *Vibrio anguillarum* ATCC 19264, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* D363, and *Aeromonas hydrophila* ATCC 7966) and Gram-positive (*Enterococcus durans* ATCC 19432, *Micrococcus luteus* A270, and *Yersinia enterocolitica* ATCC 23715) pathogenic bacterial strains in vitro. To that end, Petri dishes containing MRS agar growth medium were sprayed with the bacterial strains isolated from marine shrimp and incubated at 30°C for 48 hours. After that period, new Petri dishes were sprayed with one of the pathogenic strains in Tryptone Soy Agar (TSA) growth medium for freshwater pathogenic strains and in TSA supplemented with 1.5% salt for saltwater strains. Agar disks were removed (1 cm in diameter) from the Petri dishes containing the initially isolated and grown bacteria. These agar disks were placed on the growth medium of the dishes just sown with pathogens and incubated at 30°C for 24 hours. Pathogen growth inhibition was determined by the diameter of the halo produced around the agar disk.

The initial identification of the selected strains was performed phenotypically using the carbohydrate fermentation test (API 50 CHL, bioMérieux, Inc., Marcy l'Etoile, France). The strain with the best results in in vitro selection was identified molecularly by sequencing and phylogenetic analysis of fragments from gene RNAr 16S at the Universidade Estadual de Campinas (Unicamp). Its sequences were compared to those of organisms on file at Genbank.

The results of the evaluated characters were submitted to one-way analysis of variance, in a completely randomized design with three replicates. The means of the variables were compared using the Student-Newman-Keuls (SNK) test, at 5% probability (Zar, 1984).

The ideotype (ideal proposed strain) was developed using the highest means among the evaluated strains for the characters maximum growth rate, final viable cell count, and inhibition halo against pathogens, and the lowest means for the characters doubling time, loss of viability of strains to NaCl (1.5% and 3%), pH (6, 8, and 9), and bile salts (5%). The Mahalanobis distances (D^2) were calculated from standardized data, between all evaluated strains and the ideotype, using the Genes software (Cruz, 2001). The strains were classified according to the selection index of distance to the ideotype (the distance of each strain to the ideotype), and the best ones were considered those with the shortest distances.

Results and Discussion

A total of ten lactic acid bacterial strains were isolated from the digestive tract of marine shrimp, of which three were biochemically identified as *Lactobacillus plantarum*, three as *L. brevis*, two as *Weissella confusa*, one as *Lactococcus lactis*, and one as *L. delbrueckii*. The strain *L. plantarum* 1 was identified molecularly and kept at the microorganism collection of Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) of Universidade Estadual de Campinas (Unicamp), under access number CPQBA 007-07 DRM01. To develop an efficient probiotic for use in aquaculture, it is essential that the microorganism used as a probiotic come from the animal of interest (Balcázar et al., 2006). The results obtained in the present work show that lactic acid bacteria fulfill this requirement.

The evaluated strains showed great phenotypic variability, as evidenced by the significant difference observed through the analysis of variance in all studied characters (Tables 1 and 2). The majority of evaluated characters showed wide variability, represented by the great breadth between the highest and lowest means. The inhibition growth zone against *V. anguillarum* was the character that showed the greatest variability, as evidenced by the higher number of different classes

(eight) obtained through the SNK multiple comparison test. A wide variability was also observed among the evaluated strains, ranging from those unable to inhibit the growth of *V. anguillarum* (*L. brevis* 1) to those that inhibited it (*L. plantarum* 1) (Table 1).

Strain *L. plantarum* 1 averaged the largest growth inhibition halos against Gram-negative strains (*V. harveyi*, *V. alginolyticus*, *V. anguillarum*, *P. aeruginosa*, *E. coli*, and *A. hydrophila*), followed by *L. plantarum* 2, and *L. plantarum* 3. For Gram-positive bacteria (*M. luteos*, *E. durans*, and *Y. enterocolitica*), the three *L. plantarum* strains averaged the largest inhibition halos. These results show that *L. plantarum* strains feature a wide spectrum of action against pathogens, both Gram-positive and negative.

Lactic acid bacteria are known as producers of antimicrobial compounds (Balcázar et al, 2008). Bacteriocins are among the most studied of these compounds, with action particularly against Gram-positive bacteria (Gillor et al., 2008). *Lactobacillus plantarum* produces a bacteriocin known as plantaricin (Hernández et al., 2005), which may be related to the formation of the growth

Table 1. Inhibition growth zone (mm) against Gram-negative bacteria (*Vibrio harveyi*, *V. alginolyticus*, *V. anguillarum*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Aeromonas hydrophila*) and Gram-positive bacteria (*Enterococcus durans*, *Micrococcus luteus*, and ~~*Yersenia*~~ *enterocolitica*) from ten lactic acid bacteria strains isolated from the digestive tract of marine shrimp (*Litopenaeus vannamei*)[(1)].

Strains[(2)]	*Vibrio harveyi*	*Vibrio alginolyticus*	*Vibrio anguillarum*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Aeromonas hydrophila*	*Enterococcus durans*	*Micrococcus luteus*	*Yersenia enterocolitica*
Lpl1	16.33±0.58a*	13.00±1.00b	17.67±0.58a	16.67±1.53a	26.33±0.58a	9.67±0.58cd	14.67±0.58ab	24.00±1.00a	16.00±1.00a
Lpl2	13.33±0.58b	20.00±2.00a	10.33±0.58c	13.33±0.58cd	16.33±1.15c	13.33±1.53a	12.67±1.15ab	24.33±0.58a	16.67±0.58a
Lpl3	16.00±0.00a	13.33±0.58b	14.00±0.00b	15.67±0.58abc	20.00±2.00b	8.67±0.58cd	14.67±0.58ab	22.33±0.58b	15.00±1.73ab
Lbr1	9.00±0.00cd	9.67±1.15cd	0.00±0.00g	13.67±1.53bcd	14.00±1.00d	8.00±0.00d	6.00±5.20d	15.67±1.53d	10.67±2.08cd
Lbr2	8.33±0.58d	11.67±0.58bc	7.33±0.58e	8.67±0.58e	10.33±0.58cd	10.33±0.58bc	7.67±0.58cd	13.67±0.58ef	8.00±1.00d
Lbr3	0.00±0.00f	9.00±0.00d	9.00±0.00d	12.33±0.58d	10.67±0.58cd	8.00±0.00d	10.67±0.58bc	15.00±1.00de	12.33±2.08bc
Wco1	9.67±0.58c	11.33±0.58bc	7.67±0.58e	8.67±0.58e	11.33±0.58e	8.33±0.58d	5.67±0.58d	11.33±0.58g	8.33±0.58d
Wco2	0.00±0.00f	13.67±1.15b	10.00±0.00c	16.00±2.00ab	9.00±0.00de	11.33±1.15b	9.00±0.00cd	19.67±0.58c	16.67±0.58a
Lla1	6.33±0.58e	6.33±0.58e	6.33±0.58f	14.67±0.58abcd	8.33±0.58e	8.67±0.58cd	6.00±1.00d	13.00±1.00f	8.67±0.58d
Lde1	0.00±0.00f	11.67±1.15bc	9.00±0.00d	0.00±0.00f	19.00±1.00b	9.00±0.00cd	0.00±0.00e	19.00±1.00c	14.67±2.31ab
Ideotype	16.33±0.58	20.00±2.00	17.67±0.58	16.67±1.53	26.33±0.58	13.33±1.53	14.67±0.58	24.33±0.58	16.67±0.58

[(1)]Mean values followed by equal letters, in the columns, do not differ by the SNK multiple comparison test, at 5% probability. [(2)]Lpl, *Lactobacillus plantarum*; Lbr, *Lactobacillus brevis*; Wco, *Weissella confusa*; Lla, *Lactococcus lactis*; Lde, *Lactobacillus delbrueckii.*

Table 2. In vitro evaluation of viable total bacteria count after 24 hours (VTB), maximum growth rate (MGS), doubling time (DT), and percentage of reduction in growth by using culture medium with 1.5% NaCl (NaCl15), 3% NaCl (NaCl30), pH 6, pH 8, pH 9, and 5% of bile salts (BS), compared with a medium with pH 7 and no salt addiction, from ten lactic acid bacteria strains isolated from the digestive tract of marine shrimp (*Litopenaeus vannamei*)[(1)].

Strains[(2)]	VTB	MGS	DT	Percentage of reduction in growth					
	(CFU mL^{-1} x 10^8)	(h^{-1})	(h)	NaCl15	NaCl30	pH 6	pH 8	pH 9	BS
Lpl1	21.67±10.41b	0.11± 0.02b	6.35±1.23ab	0.00±0.00a	33.97±2.27ab	11.88±6.77a	0.00±0.00a	0.00±0.00a	68.28±12.12bcd
Lpl2	49.67±33.20a	0.15±0.01a	4.56±0.38a	19.49±9.07bc	43.02±6.05ab	11.00±1.00a	3.00±1.00a	16.32±4.61ab	30.33±3.21a
Lpl3	27.90±2.59b	0.13±0.01b	5.50±0.23ab	8.57±8.73ab	48.82±24.45ab	11.29±3.52a	5.83±0.06a	17.63±3.18ab	64.44±7.51bcd
Lbr1	7.57±1.91b	0.12±0.02b	6.19±1.27ab	35.49±9.90c	45.64±5.32ab	5.75±2.26a	0.00±0.00a	16.62±4.56ab	63.79±14.69bcd
Lbr2	4.87±1.60b	0.11±0.00b	6.03±0.02ab	36.56±10.48c	64.16±9.77b	55.35±9.54d	48.30±9.63b	17.79±1.90ab	73.20±0.99cd
Lbr3	2.10±0.95b	0.10±0.01b	7.25±1.09b	34.29±13.40c	47.18±1.98ab	36.37±4.44c	0.00±0.00a	34.59±6.07de	74.03±3.87cd
Wco1	5.33±1.90b	0.10±0.00b	6.68±0.10b	20.20±0.89bc	26.73±1.61a	27.71±5.19b	24.21±3.29b	26.77±6.10cd	71.36±5.25cd
Wco2	6.93±1.90b	0.11±0.01b	6.54±0.76b	29.68±4.79c	32.57±3.70ab	1.64±2.29a	0.00±0.00a	17.55±8.43ab	60.24±3.10ab
Lla1	3.06±2.25b	0.10±0.00b	6.71±0.15b	23.28±5.38bc	42.91±6.34ab	61.12±0.60d	20.81±3.05b	36.80±2.17e	49.02±15.92b
Lde1	1.73±1.27b	0.13±0.01b	5.52±0.39ab	77.19±4.45d	80.27±2.56c	89.72±0.47e	89.98±0.72d	74.33±3.48f	86.56±2.15e
Ideotype	49.67±33.20	0.15±0.01	4.56±0.38	0.00±0.00	26.73±1.61	1.64±2.29	0.00±0.00	0.00±0.00	30.33±3.21

[(1)]Mean values followed by equal letters, in the columns, do not differ by the SNK multiple comparison test, at 5% probability. [(2)]Lpl, *Lactobacillus plantarum*; Lbr, *Lactobacillus brevis*; Wco, *Weissella confusa*; Lla, *Lactococcus lactis*; Lde, *Lactobacillus delbrueckii*.

inhibition halos against the Gram-positive bacteria analyzed in the present study. However, bacteriocins do not offer significant action against Gram-negative bacteria (Vázquez et al., 2005). The growth inhibition halos formed against Gram-negative bacteria are associated with other compounds produced by lactic acid bacteria, such as hydrogen peroxide (Sugita et al., 2007), organic acids, and acetic acid (Vázquez et al., 2005). A similar pathogen inhibition in vitro was reported for other potential probiotics isolated from aquatic organisms, such as *Lactobacillus* sp. isolated from *Salmo salar* (Balcázar et al., 2008), *M. lutueus* and *Pseudomonas* sp. (El-Rhman et al., 2009), and *L. plantarum* (Jatobá et al., 2008) isolated from tilapia (*Oreochromis niloticus*).

The faster growth rate found for strain *L. plantarum* 2 indicates it has superior performance compared to the others (Table 2). Faster growth rate and lower doubling time of bacteria make commercial production processes more efficient and may result in greater competitiveness in vivo of the strain (Vine et al., 2004a). The growth kinetics results obtained from the strains isolated from shrimp were greater for maximum growth rate and final viable cell count, and lower for doubling time, in comparison with the strains of probiotics with aquaculture potential isolated by Vine et al. (2004a).

In marine shrimp farming, the variation in the salinity of cultures, depending on region, time of year and rainfall, can vary from values close to zero (Araneda et al., 2008) up to levels higher than ocean salinity, such as in salt works areas. Therefore, it is important that a given commercial probiotic bacterial ~~specie~~ can withstand the wide variation in salinity. *Lactobacillus plantarum* 1 was the only strain that did not show reduced growth when exposed to 1.5% NaCl. At 3% NaCl, a loss in growth was observed for all bacterial strains in relation to the 1.5% rate. The strain with the lowest growth loss at 3% NaCl was *W. confusa* 1 although it did not differ significantly from strains: *L. plantarum* 1, 2 and 3; *L. brevis* 1 and 3; *W. confusa* 1; and *L. lactis* 1 (Table 2). Ricciardi et al. (2009) and Papamanoli et al. (2003), respectively, also observed that strains of *W. cibaria* and *L. plantarum* showed low loss of viability in media with 3% NaCl.

Like salinity, culture water pH is not constant, ranging from values close to 6 in super-intensive shrimp cultures under bioflake (Vinatea et al., 2010) up to 9 in an ~~eutrophied~~ culture pond (Momoyama, 2004). Therefore, it is important that a strain selected to be used as a probiotic resist a wide range of pH. At pH 6, there was growth loss in all evaluated strains; however, strains *L. plantarum* 1, 2 and 3; *L. brevis* 1, and *W. confuse* 2 had the lowest growth loss compared to the others. At pH 8 and 9, *L. plantarum* 1 was the only strain that did not show growth loss (Table 2). Papamanoli et al. (2003) observed that *L. plantarum* features low loss of viability in growth media with pH up to 5.

Bile salts have an emulsifying function, increasing the solubility of fats and fat-soluble vitamins to aid in their adsorption (Lehninger et al., 2008). This detergent effect is microbicidal, as it can affect phospholipids and fatty acids in the walls of microorganisms. However, some bacteria are able to hydrolyze bile salts using specific enzymes, reducing the detergent effect (Erkkilä & Petäjä, 2000). For a probiotic to efficiently colonize the digestive tract of an animal, it is important that it be able to resist bile salts. Bile salts diminished the growth of all evaluated strains. *Lactobacillus plantarum* 2 and *L. lactis* 1 had the lowest growth losses. Strains of *L. plantarum* and *L. lactis* isolated from rainbow trout also had low viability loss from bile salts (Balcázar et al., 2008).

Considering all characters studied in vitro, strain *L. plantarum* 1 achieved the shortest Mahalanobis distance (D^2) in relation to the ideotype (Table 3). It was followed by the two other *L. plantarum* strains, the two *W. confusa* strains, the three *L. brevis* strains, and *L. lactis*. *Lactobacillus delbrueckii* was the strain with the longest distance to the ideotype (Table 3).

Table 3. Selection index of distance to the ideotype by Mahalanobis distance (D^2) from ten lactic acid bacteria strains isolated from the digestive tract of marine shrimp (*Litopenaeus vannamei*).

Strain	Distance to ideotype	Ranking
Lactobacillus plantarum 1	304	1st
Lactobacillus plantarum 3	1693	2nd
Lactobacillus plantarum 2	3163	3rd
Weissella confusa 2	8304	4th
Weissella confusa 1	9635	5th
Lactobacillus brevis 3	10975	6th
Lactobacillus brevis 1	12493	7th
Lactobacillus brevis 2	13922	8th
Lactococcus lactis 1	15110	9th
Lactobacillus delbrueckii 1	21469	10th

These results evidence that, among the evaluated bacterial strains, those of *L. plantarum* showed the best potential for use as probiotics in shrimp culture, due to the positive results for all evaluated characters, as evidenced by the shortest distance to the ideotype.

Conclusion

Among the strains isolated from the digestive tract of *Litopenaeus vannamei*, *Lactobacillus plantarum* strains show the greatest potential for use as a probiotic for marine shrimp.

Acknowledgements

To professor Elpídio Beltrame (in memoriam) from the Department of Aquaculture from Universidade Federal de Santa Catarina (UFSC), for his inestimable support in this study; to Ministério da Agricultura e Pesca (MPA), to Financiadora de Estudos e Projetos (Finep), to Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (Fapesc), and to Aquabrasil, for financial support.

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Received on July 29, 2012 and accepted on May 20, 2013