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Partial purification and characterization of a bacteriocin produced by *Bacillus subtilis* NCIMB 3610 that exhibits antimicrobial activity against fish pathogens

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The bacteriocin produced by *Bacillus subtilis* NCIMB 3610 was tested for its antimicrobial action against *Enterococcus faecalis*, *Lactococcus lactis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus sphaericus* and the fish pathogens *Vibrio anguillarum* 408 O1 and *Photobacterium damselae* subsp. *piscicida*. Inhibition of growth was observed for all bacteria tested with the exception of *B. sphaericus* which appeared resistant to the bacteriocin treatment. The greatest sensitivity was observed for *Enterococcus,* with an inhibition of 85% compared to control and it was selected as indicator strain. Antimicrobial activity was greater for the cell free supernatant collected after 72 hrs and the bacteriocin appeared sensitive to lipase, chymotrypsin and pepsin but resistant to amylase and proteinase K. The bacteriocin was heat resistant and higher bactericidal activity was observed at pH 6-8.5. Purification was performed by ammonium sulphate precipitation of the culture cell free supernatant, ion exchange chromatography (MacroPrep 25) and solid phase extraction (SPE, C18). SDS electrophoresis revealed that the apparent molecular weight of the bacteriocin was about 16 kDa. Bactericidal activity was retained throughout the purification procedure and appeared significantly increased ($p < 0.001$) for the final SPE product following solvent evaporation, amounting to 98% of control for *Enterococcus*. The strong antibacterial action of the purified bacteriocin against the two fish pathogens examined, renders *B. subtilis* NCIMB 3610 a potential candidate for further evaluation as a probiotic to be used for the prophylactic treatment of fish against bacterial infections.

Key words: *Bacillus subtilis*, bacteriocins purification, fish pathogens.

INTRODUCTION

The potential of *Bacillus subtilis* subsp. *subtilis*, a Gram positive endospore forming bacterium, to produce antimicrobial substances has been well recognized over the last decades, with the predominant class being peptide antibiotics (Stein, 2005). The production of antimicrobials appears to be correlated with the specific strain of *B. subtilis* tested, since some strains are used in human and animal food production, while others have been implicated in food poisoning (Abriouel *et al*., 2011). The production of a variety of antibacterial and antifungal compounds has been reported for various strains of *B. subtilis*. In particular the *B. subtilis* subsp. *spizizenii* ATCC 6633 strain produces lantibiotics (Bierbaum *et al*., 1995; Duitman *et al*., 1999; Stein, 2005), *B. subtilis* LFB112 produces a highly active bacteriocin of 6.3 kDa (Xie *et al*., 2009), while the bacteriocin produced by *B. subtilis* 14B was found to be a molecule of 21 kDa, active against *Agrobacterium tumefaciens* (Hammami *et al*., 2009). However, to the best of our knowledge, there is a lack of evidence on the possible production of bacteriocins by *B. subtilis* NCIMB 3610 as well as on their nature and antimicrobial action.

Probiotics have been proven beneficial for use in human and animal nutrition (Fuller, 1991; Gill & Guarner 2004). Recently, their application in aqua-

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culture has begun (Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000; Gatesoupe, 2008) in accordance with FAO recommendations, on preventive measures against the introduction of pathogens in aquatic animal health (Brugère *et al.*, 2010). The use of probiotics as an alternative to antibiotic treatment may facilitate a reduction in the extensive use of antibiotics, thus minimizing the risk of bacterial resistance (Balcázar *et al*., 2006). The selection of a probiotic bacterium for animal use is a multistep process that must ensure that the potential probiotic is non-pathogenic and beneficial to the host, out-competing potentially pathogenic strains (Verschuere *et al*., 2000) and show adhesion to the intestinal mucus and resistance to the host bile (Nikoskelainen *et al*., 2001). In this context a number of potential probiotics have been tested for use in aquaculture with promising results. Improved growth and survival of sea bream was observed following administration of *Lactobacillus fructivorans* (AS17B), *Lactobacillus plantarum* (Carnevali *et al*., 2006) or six selected bacterial strains (Makridis *et al*., 2008). The administration of probiotics to fish has been reported to offer protection from vibriosis, as in the case of the administration of *Carnobacterium divergens* to Atlantic salmon (Gildberg *et al*., 1997), *Roseobacter* sp. to turbot (Planas *et al*., 2006) and *Vagococcus fluvialis* to sea bass (Sorroza *et al*., 2012). *Bacillus subtilis* BT23 was successfully employed against a *Vibrio harveyi* challenge in *Penaeus monodon* (Vaseeharan & Ramasamy, 2003). However there is a lack of data on the possible effect of *B. subtilis* NCIMB 3610 on fish pathogens, such as *Vibrio (Listonella) anguillarum* and *Photobacterium damsela* subsp. *piscicida*, which are the most prominent pathogenic bacteria in euryaline fish farming (Zorrilla *et al*., 2003; Toranzo *et al*., 2005). Although bacteriocins produced from *B. subtilis* have been considered important in food safety (Zheng & Slavik, 1999) due to their wide antimicrobial spectrum (Shelburne *et al*., 2007), their potential application in animal clinical studies remains to be addressed (Xie *et al*., 2009).

The micro-organisms that have been approved for use in animal feed in the European Union (EU) are mainly bacterial strains of Gram-positive bacteria including *B. subtillis* (Anadon *et al*., 2006). Although *Bacillus* species are not usually part of the gut microflora, they are considered safe for human consumption (Hong *et al*., 2008; Sorokulova *et al*., 2008) and able to survive in the gut due to their resistant spores (Hamilton-Miller & Gibson, 1999). The production of bacteriocins appears to be species related and the biosynthesis of the lantibiotic subtilin has been established for *B. subtilis* subsp. *spizizenii* ATCC 6633, the bioindicator strain for sterilization control (Stein *et al*., 2004) while other recently isolated strains produce a variety of bacteriocins (Zheng & Slavik, 1999; Bizani & Brandelli, 2004; Hammami *et al*., 2009; Alam *et al*., 2011; Sharma *et al*., 2011).

The present study focused on the effect of cell free extracts of *B. subtilis* NCIMB 3610 on five bacterial isolates and two fish pathogens. In addition, the produced antibacterials were characterized in regard to their sensitivity to pH, heat and enzymes. Partial purification of the antibacterial substances produced by *B. subtilis* NCIMB 3610 was performed, employing ammonium sulphate precipitation, ion exchange chromatography and solid phase extraction and the molecular weight of the isolated antibacterial compound was estimated by SDS-PAGE electrophoresis.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Microbial strains originated from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, Scotland). The strain that was used was *Bacillus subtilis* subsp. *subtilis* NCIMB 3610 and stock cultures were maintained at –80˚C in 20% glycerol, for long term storage. Bacteria were grown in NB broth at 30˚C, for 24 hrs (Papagianni *et al*., 2006). Optical density of cultures was monitored at 630 nm where maximum absorbance was observed. The bacterial strains used [*Enterococcus faecalis* (NCIMB 13280), *Lactococcus lactis* (ATCC 11454), *Staphylococcus aureus* (NCIMB 9518), *Escherichia coli* (NCIMB 8879) and *Bacillus sphaericus* (NCIMB 9370)] were from the collection of the Laboratory of Microbiology (Aristotle University of Thessaloniki, Greece) and were grown in Nutrient agar with the exception of *L. lactis* that was grown in Brain Heart Infusion (BHI) agar, at 37˚C. The fish pathogens used in this study, *Vibrio (Listonella) anguillarum*, 408 serotype O1 was kindly provided by Prof E. Baudin (IFREMER, France) and *Photobacterium damselae* subsp. *piscicida* was a gift from Dr G. Savvidis (Veterinary Research Institute of Thessaloniki, Greece) and they were grown in BHI agar containing 2% NaCl, at 22-24˚C, for 24 hrs (Magarinos *et al*., 1992; Nagano *et al*., 2009).

Preparation of cell free supernatants

Bacteria subjected to a heat shock (70˚C, 25 min) for

the induction of sporulation (Harwood, 1992), were transferred to Nutrient broth at 30˚C, in a rotary shaker at 125 cycles min^{-1} for 4 days. Samples were removed every 24 hrs and *B. subtilis* growth was monitored at 630 nm. The cells were harvested by two sequential centrifugations, one at 8000 × g for 10 min followed by a second at 10000 × g for 5 min. The supernatants were filtered through 0.22 μm membranes and stored in sterile flasks at 4˚C until used for antimicrobial assay.

Bacteriocin activity assay

The antimicrobial activity was detected in five experimental series, one for each indicator organism. Briefly, 500 μl of the supernatant from a 24, 48, 72 or 96 hrs culture of *B. subtilis* were added in tubes and 500 μl of the indicator organism (approximately 10^6 CFU ml^{-1}) were inoculated. Samples containing 500 μl Nutrient broth instead of cell free supernatant served as controls. Each series was performed in triplicate, samples were incubated in a shaker (220 rotations min^{-1}, 37˚C). Bacterial growth of indicator organisms was daily monitored at 630 nm in a Microplate Autoreader photometer (Bio-Tek Instruments), at 0, 24, 48 and 72 hrs and by morphological identification and counting of the bacterium in culture. For the evaluation of the inhibition of growth in fish pathogens 2% (w/v) NaCl was added in the culture medium and 500 μl of *V. anguillarum* 408, or *P. damsellae* subsp. *piscicida* were inoculated, yielding approximately 10^5 CFU ml^{-1} of the pathogen. As controls served samples in which the pathogen was inoculated in 500 μl of BHI saline broth. The three replicates used for each combination were incubated in a shaker (220 rotations min^{-1}, 30˚C). The pathogen growth was monitored by optical density recordings of 100 μl aliquots at 630 nm and spreading of the culture on BHI agar containing 2% NaCl, morphological identification of the pathogen and counting. Inhibition of growth was calculated as $1- A_m/A_o$, with A_o being the absorbance of the control and A_m the absorbance of the sample (Cabo *et al*., 1999) or following counting as (CFU of control – CFU of sample/CFU of control) × 100 (Kourelis *et al*., 2010).

Effects of enzymes, heat and pH on antimicrobial activity

The proteolytic enzymes pepsin, proteinase K, chymotrypsin, a-amylase and the enzyme lipase were tested on cell-free sterilized *B. subtilis* supernatants collected at 72 hrs of culture. Samples of 1 ml were treated at 37˚C with 2.5 mg ml^{-1} of each enzyme, for a total of 2 hrs. Enzymes were prepared in 0.2 M phosphate buffer pH 7 with the exception of pepsin that was diluted in 0.2 M HCl-glycin buffer pH 3. All supernatants were neutralized prior to use and the appropriate pH adjustments were made for each enzyme tested. Samples were then boiled for 2 min to inactivate the enzyme. Following, the samples were used for antimicrobial activity measurements as described above, with *E. faecalis* as the indicator strain.

To analyze thermal stability, supernatants were exposed to temperatures ranging from 50 to 70˚C for 30 min, 100˚C for 15 to 60 min, or they were autoclaved at 121˚C for 25 min. Two controls were used in this experimental set, one that received no temperature treatment and one with Nutrient broth that was subjected to the same treatment as the sample. After treatment, the indicator organism was added and growth was monitored at 630 nm.

The examination of the effect of pH on the antimicrobial activity of the cell free supernatants, was estimated by adjusting the pH values with 0.1 M HCl or 0.1 M NaOH, to pH 2-10, incubating at 25˚C for 2 hrs followed by neutralization and filter sterilization of the samples. Then the indicator strain was added, incubated as above and its growth was monitored at 630 nm.

Purification of bacteriocin

The crude bacteriocin was precipitated with 30-70% ammonium sulphate, with best precipitation at 60% saturation. The precipitate was collected by centrifugation at 20000 × g for 30 min at 4˚C, resuspended in 5 mM phosphate buffer pH 5 and a sample was filter sterilized and tested for antimicrobial activity. Further purification was performed using ion exchange chromatography (Macro Prep 25S, Strong Cation Exchange Support, Bio Rad Laboratories, USA). A column of 12 cm in length and 1.5 cm in diameter was prepared, activated with 4-5 column volumes of 0.1 M NaOH, equilibrated with 4-5 column volumes of 5 mM phosphate buffer pH 5 and then 40 ml of the sample (without any prior desalting or dialysis) was applied at a flow of 0.5 ml min^{-1}. The column was washed with 4 volumes of phosphate buffer and the bound compounds were eluted with 5 mM phosphate buffer pH 5 containing 1 M NaCl. All column efluents were subjected to antimicrobial activity testing, to ensure that bactericidal activity was retained through

the process. Finally the ion exchange eluate was subjected to reverse solid phase extraction (SPE) employing a column of a total volume of 6 ml packed with Sample Prep C18 (Alltech, USA). The column was activated with 3-4 volumes of methanol (HPLC grade) washed with water and equilibrated with 4 volumes of 5 mM phosphate buffer pH 5. Then 10 ml of the ion exchange pooled fractions were added, the column was washed with 10 ml of 5 mM phosphate buffer pH 5, and the bound substances were eluted with 10 ml of methanol-water (90:10). Following evaporation of methanol in a rotary evaporator, part of the aqueous sample was subjected to testing for antimicrobial activity and the other stored for further characterization. The molecular weight of the bacteriocin was determined by 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. A standard molecular mass marker (Sigma-Aldrich, St. Louis, MO, USA) of 10-200 kDa was used and the gel was stained using Coomasie Brilliant Blue R-250.

Statistical analysis

The results presented in this study are expressed as means ± SEM of three replicates. Statistical comparisons were performed using Microcal Origin 7.5 software (Microcal Software, Northampton, Maine). One-way analysis of variance (ANOVA) was used for analysis, followed by Tukey test in case the values presented significant differences, with statistical significance set at a level of 0.05.

RESULTS

Bacteriocin production and inhibitory spectrum

The presence of antibacterial activity was tested against a total of seven strains, including Gram-positive and Gram-negative bacteria, as a function of culture time (Fig. 1). Absence of antibacterial activity was observed only against the Gram-positive strain of *B. sphaericus* (Fig. 1E). For all other tested bacteria inhibition of growth was observed, which was greater in the *B. subtilis* supernatants collected at 72 hrs and 96 hrs, with bacterial growth being significantly lower than the control ($p < 0.05$). The inhibition of growth after 72 hrs of culture amounted approximately to 60% for *L. lactis* (Fig. 1B), to 55% for *E. coli* (Fig. 1C), and to about 95% for the *E. faecalis* strain (Fig. 1A). The *S. aureus* strain presented an inhibition of growth of 50% at 48 hrs which however was only 45% at the end of the experiment (Fig. 1D). The strain that showed greater sensitivity was *E. faecalis*, which was therefore used as indicator strain in the following experiments. Inhibition of growth for the fish pathogens tested amounted to about 35% for *V. anguillarum* 408 (Fig. 1F) and 31% for *P. damselae* (Fig. 1G), 72 hrs after the onset of culture of the pathogens in the 72 hrs supernatant of *B. subtilis*.

Sensitivity of bacteriocin to enzymes, heat and pH alterations

The antibacterial activity of supernatants treated with amylase, lipase, proteinase K, chymotrypsin and pepsin, against the indicator strain *E. faecalis*, is presented in Table 1 as a function of culture time. Growth in supernatants treated with lipase, pepsin or chymotrypsin was significantly greater than the relevant growth in untreated *B. subtilis* supernatants ($p < 0.05$), although it did not reach control values. These results indicate a sensitivity of the bacteriocin to these enzymes. Growth of the indicator was inhibited in the amylase and proteinase K treated media, not being significantly different from growth in untreated supernatant ($p > 0.05$), indicating lack of sensitivity of the bacteriocin to these enzymes.

The bacteriocin appeared stable in heat treatments and growth of *E. faecalis* was inhibited even in samples heated at 100°C for the prolonged time of 60 min (Table 1). Only autoclaving at 121°C appears to slightly reduce antibacterial activity, as growth of the indicator strain appeared significantly higher ($p < 0.05$) compared to the growth in the untreated bacteriocin, not reaching however control growth values ($p > 0.05$).

Bacteriocin activity was greatly affected by alteration of the pH (Table 1) and growth of *E. faecalis* in supernatant treated at pH 10 was significantly greater ($p < 0.05$) from growth in untreated medium and not significantly different from the control ($p > 0.05$), indicating loss of antibacterial activity at pH 10. In low pH values the indicator strain growth was greatly inhibited, and the inhibition reached 99% at pH 2 and 4. However, in the corresponding control sample using Nutrient broth with no bacteriocin added and subjected to the same pH treatment, the inhibition of growth of the indicator was also evident. At pH 6 and 8, the inhibition of growth was in both cases at the levels of the inhibition of growth in untreated bacteriocin.

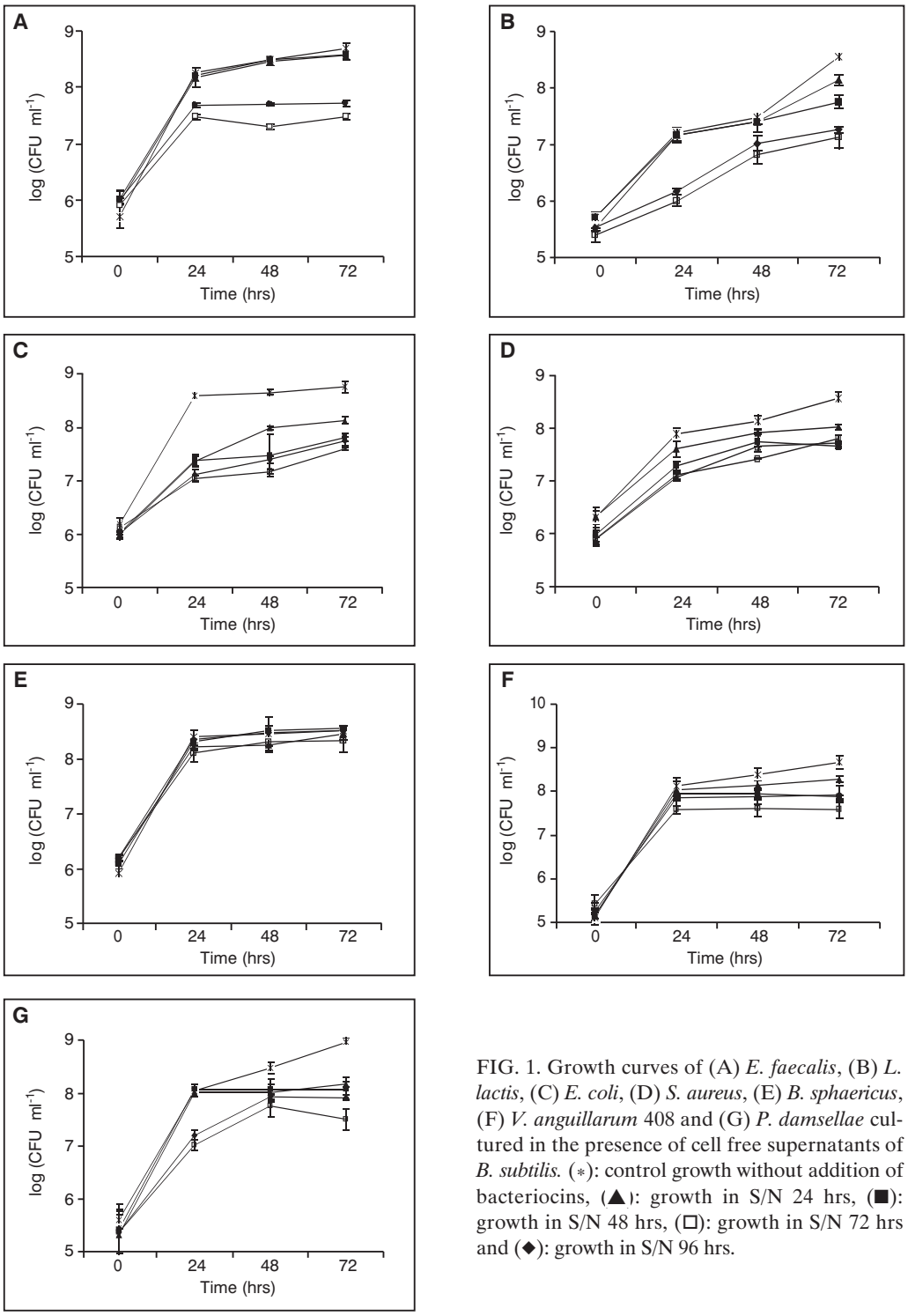

FIG. 1. Growth curves of (A) *E. faecalis*, (B) *L. lactis*, (C) *E. coli*, (D) *S. aureus*, (E) *B. sphaericus*, (F) *V. anguillarum* 408 and (G) *P. damsellae* cultured in the presence of cell free supernatants of *B. subtilis.* (∗): control growth without addition of bacteriocins, (▲): growth in S/N 24 hrs, (■): growth in S/N 48 hrs, (□): growth in S/N 72 hrs and (◆): growth in S/N 96 hrs.

Bacteriocin purification

The antibacterial activity of the bacteriocin throughout the purification procedure is shown in Table 2. In the ammonium sulphate precipitation, trials were made using 40 to 70% saturation with best results obtained at 60% saturation with an inhibition of growth of the indicator strain of 23% after 24 hrs, reaching however 80% inhibition after 72 hrs and not being significantly different ($p > 0.05$) from the inhibition of growth in the 72 hrs crude bacteriocin preparation. The same pattern of inhibition of growth was observed in the sample collected after ion exchange,

TABLE 1. Effect of enzymes, heat and pH on the activity of bacteriocin preparations against *E. faecalis.* Inhibition was calculated at 72 hrs as [100 × (Mean CFU of Control – Mean CFU of Sample) / (Mean CFU of Control)]

Experimental series	Survival (log CFU ml^{-1})				Inhibition
	0 hrs	24 hrs	48 hrs	72 hrs	(%)
Control Untreated S/N (37°C, pH 8.4)	5.30 ± 0.21	8.15 ± 0.15	8.55 ± 0.12	8.70 ± 0.11	–
	5.10 ± 0.26	7.17 ± 0.21*	7.20 ± 0.30*	7.18 ± 0.10*	95.98
Treatment with enzymes					
Lipase	5.58 ± 0.18	8.14 ± 0.11	8.10 ± 0.17a	8.21 ± 0.16*	68.46
Pepsin	5.42 ± 0.04	8.15 ± 0.05	8.05 ± 0.36	8.47 ± 0.10*	41.11
Chymotrypsin	5.39 ± 0.13	8.13 ± 0.08	8.04 ± 0.13a	8.19 ± 0.19*	68.86
Amylase	5.48 ± 0.38	7.49 ± 0.29*	7.38 ± 0.18*	7.38 ± 0.18*	95.14
Proteinase K	5.39 ± 0.01	7.53 ± 0.13*	7.42 ± 0.19*	7.47 ± 0.25*	94.11
Temperature effect					
50°C	5.08 ± 0.20	7.39 ± 0.16*	7.35 ± 0.18*	7.26 ± 0.17*	96.98
60°C	5.12 ± 0.27	7.46 ± 0.14*	7.36 ± 0.13*	7.30 ± 0.15*	96.40
70°C	5.15 ± 0.15	7.42 ± 0.13*	7.30 ± 0.12*	7.28 ± 0.15*	96.02
100°C/15 min	5.29 ± 0.20	7.53 ± 0.16*	7.30 ± 0.18*	7.25 ± 0.17*	96.20
100°C/30 min	5.22 ± 0.27	7.51 ± 0.24*	7.42 ± 0.13*	7.35 ± 0.15*	96.46
100°C/60 min	5.35 ± 0.20	7.49 ± 0.26*	7.58 ± 0.18*	7.42 ± 0.17*	95.54
120°C/25 min	5.48 ± 0.27	7.68 ± 0.32*	8.18 ± 0.32*	8.25 ± 0.21a*	94.75
pH Effect					
2	5.02 ± 0.20	6.00 ± 0.16a*	6.09 ± 0.18a*	6.12 ± 0.12a*	99.73
4	4.99 ± 0.27	5.82 ± 0.29a*	6.26 ± 0.22a*	6.30 ± 0.30a*	99.60
6	5.09 ± 0.15	7.36 ± 0.13*	7.41 ± 0.11*	7.35 ± 0.15*	95.54
8	5.10 ± 0.20	7.51 ± 0.16*	7.30 ± 0.08*	7.23 ± 0.07*	96.62
10	4.98 ± 0.27	8.07 ± 0.19a	8.15 ± 0.12a	8.40 ± 0.15a	49.80

* significant difference from control values in same column

a denotes significant difference from untreated supernatant value in same column at $p < 0.05$.

TABLE 2. Purification of bacteriocin from cell free supernatants of *B. subtilis* NCIMB 3610. Inhibition was calculated at 72 hrs as [100 × (Mean CFU of Control – Mean CFU of sample) / (Mean CFU of Control)]

Experimental series	Survival (log CFU ml^{-1})				Inhibition
	0 hrs	24 hrs	48 hrs	72 hrs	(%)
Control	5.47 ± 0.21	8.20 ± 0.15	8.50 ± 0.12	8.70 ± 0.06	–
Untreated S/N	5.21 ± 0.27	7.40 ± 0.30	7.30 ± 0.32*	7.20 ± 0.10*	95.98
Ammonium sulphate precipitation	5.12 ± 0.30	8.06 ± 0.16a	8.20 ± 0.14a*	8.00 ± 0.15a*	79.86
Ion exchange chromatography	5.08 ± 0.19	8.04 ± 0.13a	8.32 ± 0.12a	8.28 ± 0.10a*	61.67
Solid Phase Extraction	5.19 ± 0.17	7.18 ± 0.19*	7.11 ± 0.15*	6.84 ± 0.11a*	98.62

* significant difference from control values in same column

a denotes significant difference from untreated supernatant value in same column at $p < 0.05$

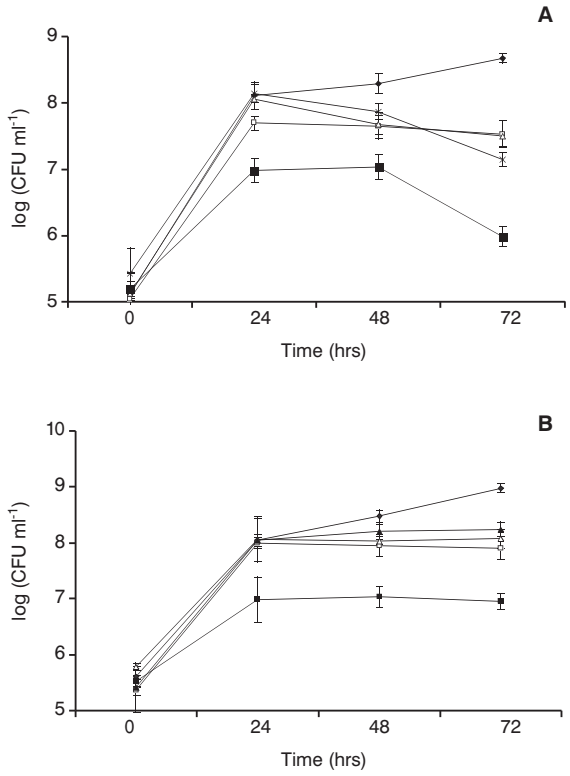

FIG. 2. Antimicrobial activity of bacteriocins during purification from crude extracts of *B. subtilis* 72 hrs culture supernatants against (A) *V. anguillarum* 408 and (B) *P. damselae* subsp. *piscicida*. (●): control-growth of pathogen without addition of bacteriocins, (□): crude untreated bacteriocin preparation, (△): following ammonium sulphate 60% precipitation (◆): following precipitation and ion exchange chromatography, (■): following precipitation, ion exchange and SPE.

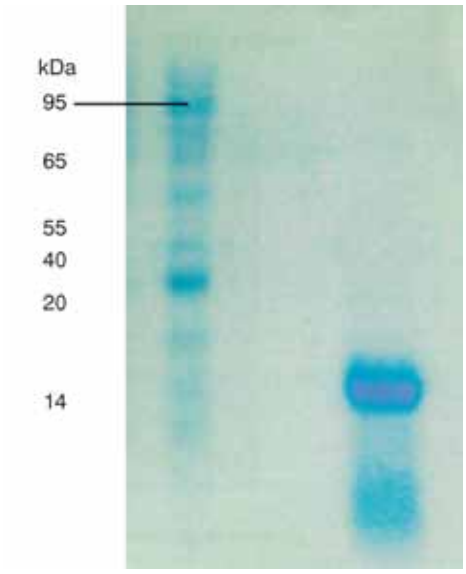

FIG. 3. SDS-polyacrylamide gel electrophoresis of purified bacteriocin from *B. subtilis* NCIMB 3610.

while following the subsequent SPE procedure inhibition of growth amounted to 95% compared to the control value and was significantly higher ($p < 0.05$) than the inhibition of growth in the crude bacteriocin sample. Other purification protocols employed, namely precipitation and ion exchange chromatography or precipitation and SPE resulted in lower recovery of antimicrobial activity, amounting to 43% and 36%, respectively. The purified bacteriocin was also active against the two fish pathogens and inhibition of growth amounted to 75% for *V. anguillarum* 408 and to 70% for *P. damselae,* values significantly higher ($p < 0.05$) than the initial inhibition of growth of crude bacteriocins preparations (Fig. 2). The SDS - PAGE results (Fig. 3) showed that the bacteriocin has an apparent molecular weight of 16 kDa.

DISCUSSION

Bacteriocins, the antimicrobial peptides produced by bacteria, exhibit a relatively wide range of activity. Subtilosin, a bacteriocin isolated from *B. subtilis* ATCC 6633, was found active against a wide range of Gram-positive and Gram-negative bacteria, but inactive against certain human pathogens (Shelburne *et al*., 2007). Although the *B. subtilis* strain NCIMB 3610 used in the present study has been reported to produce an anti-cyanobacterial and phytotoxic product (Wright *et al*., 1991) and the biocatalyst phenolic acid esterase (Donaghy *et al*., 1998), there is no report on the production of antibacterials or their possible inhibitory action against animal pathogens. The antimicrobial activity spectrum determined in the present study for the bacteriocin produced by *B. subtilis* NCIMB 3610 was relatively wide, since it inhibited *E. faecalis*, *L. lactis* and *S. aureus* which are Gram positive bacteria as well as *E. coli*, *V. anguillarum* 408 and *P. damselae* which are Gram-negative bacteria. In fact, the only case in which this bacteriocin did not exhibit inhibitory activity, was against *B. sphaericus*, a Gram positive spore forming strain that is closely related to *B. subtilis* (Harwood, 1992). The greater inhibitory activity was observed in the supernatants of *B. subtillis* cultures collected after 72 and 96 hrs, when sporulation occurs. These findings are in accordance with the fact that under unfavourable growth conditions that lead to sporulation, there is an induction of the production of bacteriocins (Stein, 2005). However, the different rates of inhibitory activity that were observed for the bacteria tested in the present study, indicate a selective manner of action for bacteriocins,

which appear to act only against certain bacteria, and are inactive against *B. sphaericus*. The inhibition pattern observed against *S. aureus*, with inhibition being higher at 48 hrs compared to the inhibition at 72 hrs, indicates a possible ability of recovery of this bacterium in the presence of bacteriocins. Since the most sensitive strain in our study was *E. faecalis*, it was selected as the indicator strain.

The antibacterial activity of the bacteriocin was greatly reduced after lipase, pepsin and chymotrypsin treatment, thereby revealing the lipo-proteinaceous nature of the compound. Similar results have been reported for bacteriocins produced by other probiotics such as *Pediococcus acidilactici* and *L. lactis* (Millette *et al*., 2007), although resistance to lipase treatment has been reported for a bacteriocin produced by *B. subtilis* EC1524 (Narbutaite *et al*., 2008). The pH spectrum of the antimicrobial activity of the bacteriocin in the present study was broad ranging from pH values of 2 to about 9, with loss of antibacterial activity at pH 10 and optimum pH 8. Our results are contradictory with the pH range of 2 to 12 reported for the bactericidal activity of a bacteriocins produced by *B. subtilis* 14B (Hammami *et al*., 2009). However, Bizani & Brandeli (2004) reported a pH range similar to the range reported in our study for the antimicrobial activity of a bacteriocin produced by *B. cereus* 8A. Moreover, the inhibition of growth of the indicator strain at low pH values of 2 and 4 can not by any means be solely attributed to bacteriocin activity, since similar inhibition was found in controls subjected to pH treatment in the absence of bacteriocin. These results indicate that the nutrients might suffer severe modifications due to pH alterations and the inhibitory effect at these low pH values, might be due to the exhaustion of nutrients. The ability of the bacteriocins produced by *B. subtilis* NCIMB 3610 to withstand heat treatment, is in accordance with the proposed nature of bacteriocins as thermo-tolerant proteins (Hammami *et al*., 2009), although heat sensitive bacteriocins such as bacteriocins produced by the probiotic *L. lactis* have also been reported (Lee *et al*., 2001; Todorov *et al*., 2006).

During purification of the bacteriocins from crude extracts, various protocols were applied in the present study, employing combinations of ammonium sulphate precipitation at various saturation levels, ammonium sulphate precipitation followed by ion exchange chromatography or ammonium sulphate precipitation followed by SPE. Higher antibacterial activity was recovered only by the combined application of 60%, ammonium sulphate precipitation followed by ion exchange chromatography and finally SPE with evaporation of the methanolic phase. In all other cases a lower antibacterial activity was observed. A similar low recovery of about 26% was reported for the bacteriocins purified from *L. lactis* cultures using ammonium sulphate and ion exchange chromatography (Rajaram *et al*., 2010). The purification protocols previously applied for other bacteriocins include precipitation and size exclusion chromatography (Sharma *et al*., 2011) or gel filtration using Sephadex G-50 (Hammami *et al*., 2009), while SPE using StrataX columns (Fuchs *et al*., 2011; Saavedra & Sesma, 2011) was also reported. The purification protocol presented in our study resulted in satisfactory recovery of antibacterial activity amounting to 98%. The molecular weight determination of the bacteriocins revealed that it is a protein of about 16 kDa. An active molecule of 6.3 kDa was reported for a bacteriocin isolated from *B. subtilis* LFB112, resistant to heat and pH stable but sensitive to proteinase K (Xie *et al*., 2009). A similar molecular weight of 21 kDa has been reported for a bacteriocin produced by *B. subtilis* 14B, which presented great similarities to the bacteriocin produced by *B. subtilis* NCIMB 3610 in the present study, in regard to heat tolerance and sensitive to proteases, however with an optimum pH 7 (Hammami *et al*., 2009).

In conclusion, our data show that *B. subtilis* NCIMB 3610 produces a bacteriocin that is of a lipo-proteinaceous nature, heat resistant and pH stable and of a molecular weight of about 16 kDa. The purification protocol described resulted in excellent recovery of antimicrobial activity of the bacteriocin. The fact that antimicrobial activity against the two fish pathogens was present in cell free supernatants as well as following purification renders *B. subtilis* NCIMB 3610 a suitable candidate for further evaluation studies as a potential probiotic in aquaculture.

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