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Control of psychrophilic microbiota in shrimp (*Litopenaeus vannamei*) by *Lactobacillus reuteri*

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As an alternative to traditional methods for controlling microorganisms in food, bioprotection using lactic acid bacteria is promising. *Lactobacillus reuteri* exhibits an ability to produce reuterin, an antimicrobial substance presenting effects against Gram-positive and Gram-negative bacteria, as well as molds, yeasts and protozoa. In the present study, the activity of *L. reuteri* culture and extract over psychrophilic microbiota of *Litopenaeus vannamei* shrimp kept under refrigeration was evaluated. Sterile extract of *L. reuteri* was able to reduce the counts of psychrophiles in 2 log cycles, while *L. reuteri* culture kept the initial count during the whole storage period. *L. reuteri* was efficient in controlling psychrophiles in shrimp suggesting its use as a food bioprotector.

Key words: *Lactobacillus reuteri*, reuterin, antimicrobial activity.

INTRODUCTION

Cultivation of marine shrimps is a new practice in Brazil. It is fast growing because of technical and economic factors in an appropriate price environment and demand for national and international markets besides internal stimulus to the development of this activity. This sector has a strong development potential and became one of the most important agropecuary activity for Brazilian economy, mainly in the Northeastern region (BRDE, 2004).

Processing techniques in the national carciniculture, generally, are not specialized. The operation starts after finishing the shrimp collection (when shrimps are removed from fattening tanks) and before commercialization. The product is processed in order to be proper for buyer demand and, according to specialists, include a simple procedure without aggregating much value (BRDE, 2004).

Because of fish products are very perishable they must be properly stored in order to keep sensorial quality and shelf life. The decreasing in freshness of fish products depends on several factors such as collection, slaughtering and processing conditions.

Food industry always searches for alternative techniques for replacing the traditional methods of microorganisms control in food such as acidification, freezing, drying, salting or using of chemical agents. Bioconservation can increase shelf life and food safety by using a natural or controlled microbiota, mainly lactic acid bacteria (LAB) (Hugas et al., 1995).

Reuterin is produced by *Lactobacillus reuteri*, a heterofermentative species inhibiting the GI tract of humans and animals. It is formed during the anaerobic

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growth of *L. reuteri* by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin. Reuterin has been chemically identified to be 3-hydroxy propanol (β-hydroxyl propionaldehyde) a highly soluble, pH- neutral compound which is in equilibrium with its hydrated monomeric and cyclic dimeric forms. Reuterin exhibits a broad spectrum of antimicrobial activity against certain Gram-positive and Gramnegative bacteria, yeast, fungi and protozoa. Spoilage organisms sensitive to reuterin include species of Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida and Trypanosoma (Mayur and Madhukar, 2014). Reuterin is a hydrosoluble, active in a wide pH range and resistant to proteolytic and lipolytic anzymes (El-ziney and Debevere, 1998). The mode of action is not fully understood, but recent studies have demonstrated that the aldehyde group of 3-HPA is mainly responsible for the antimicrobial activity. Reuterin reacts with sulfhydryl groups of proteins and small molecules inducing oxidative stress responses (Montiel et al., 2014). The aim of present study was to evaluate the control of psychrophilic microbiota from *Litopenaeus vannamei* shrimp kept under refrigeration conditions by *Lactobacillus reuteri* and its sterile extract.

MATERIALS AND METHODS

Microrganisms and growth conditions

L. reuteri ATCC 1428 was kept in MRS Broth with 20% glycerol at -20ºC. For the microorganism reactivation, 1 mL of stock culture was transferred to 10 mL MRS broth and incubated at 37ºC for 24 h. The strain had its antimicrobial activity characterized in a previous study (Silva et al., 2010).

Production of *L. reuteri* cell free supernatant

Production of the cell free supernatant was carried out according to Cleusix et al. (2008) with some modifications. One milliliter of the reactivated culture was transferred to a new tube containing 10 mL of MRS broth and incubated at 37ºC for 6 h. After incubation, the whole volume was added to a flask containing 50 mL of MRS broth and incubated at 37ºC for 12 h. Later, the 61 mL of culture was added to a new flask containing 500 mL of MRS broth and incubated at 37ºC for 24 h. Cells were, then, collected by centrifugation (1500 x *g*, 10 min, 20ºC), washed in potassium phosphate buffer (0.1 M, pH 7.0), resuspended in 300 mL of a sterile aqueous solution containing glycerol (200 mm) and incubated at 37°C for 3 h under anaerobic conditions (Anaerogen®). Cells were collected by centrifugation (8000 x *g*, 10 min) and 150 mL of the supernatant were sterilized by membrane filtration (0.22 µm, Millipore®, Merck, USA).

Evaluation of reuterin production

A fresh culture of reuterin-producing *L. reuteri* ATCC 1428 was inoculated at 1% in 1 L of MRS broth and incubated anaerobically at 37°C overnight. After growth, cells were harvested by centrifugation (4500 × *g*, 5 min) and gently washed in sterile aqueous solution of glycerol (100 mM). In order to produce reuterin from glycerol, the obtained cell biomass was resuspended into 250 ml sterile aqueous solution of glycerol (100 mM), and resting cells were incubated under anaerobic conditions at 37°C for 3 h. After centrifugation (6600 × *g*, 5 min), the resulting supernatant was collected, filter-sterilized (0.22 µm) and maintained at −40°C for subsequent experiments. The concentration of reuterin in the supernatant was determined by a colorimetric method as described by Lüthi-Peng et al. (2002). Acrolein (Fluka; Sigma-Aldrich Quimica SA, Madrid, Spain) was used for obtaining the standard curve, since 3-hydroxypropionaldehyde dehydrates in equimolar concentrations to acrolein. Standards were made diluting acrolein in distilled water. Supernatants containing reuterin were diluted with distilled water if necessary before the colorimetric reaction. All determinations were carried out in triplicate.

Sample of shrimp

Samples of 1000 g of fresh and deshelled pacific white shrimp were bought in the Public Market of Florianopolis, Brazil. Samples were placed in isothermal boxes containing ice and took to the Food Microbiology Laboratory (Florianopolis, Brazil) for analysis.

Treatment of samples with *L. reuteri*

Each sample was divided in portions of 300 g and each portion was labeled according to the treatment: Treatment 1: portion added with 0.1 mL/g of *L. reuteri* culture; Treatment 2: portion added with 0.1 mL/g of *L. reuteri* cell free supernatant; Control: portion added with 0.1 mL/g of sterile distilled water. Each sample was analyzed in relation to psychrotrophic count and further *L. reuteri* count for T1 samples: right after treatment (T0), 3 h after treatment (T3 h), 6 h after treatment (T6 h), 24 h after treatment (T24 h) and 48 h after treatment (T48 h). Samples were kept under refrigeration (±7ºC) until analysis. All analyses were carried out in triplicate.

Microbiological determinations

Representative shrimp samples (25 g) were homogenized with 250 mL of sterile 0.1% (w/v) peptone solution in a Stomacher 400 (A. J. Seward Ltd, London, UK). Decimal dilutions of shrimp homogenates were prepared in sterile 0.1% (w/v) peptone solution. *L. reuteri* counts were determined on duplicate plates of Rogosa agar (Difco) and incubated at 37°C for 48 h under anaerobic conditions, and psychrotrophic counts on Plate Count agar (Oxoid) for five days at 7°C. Psychrotrophic counts in the T1 samples were expressed as the difference between the total count of psychrotrophic and Lactobacillus reuteri counts.

Statistical analysis

Results of psychrotrophic counts were submitted to analysis of variance (ANOVA) and averages of treatments were analyzed by Tukey's test in order to verify differences among the treatments. Confidence level was of 95%. Tests were performed using STATISTICA 9.0 software (StaSoft).

RESULTS AND DISCUSSION

Standard count carried out at zero time showed that samples present an initial contamination of about 10^6 CFU/g. According to Vanderzant et al. (1971), shrimp collected in tropical waters contains counts ranging from 10^6 to 10^7 CFU/g. This result was confirmed by

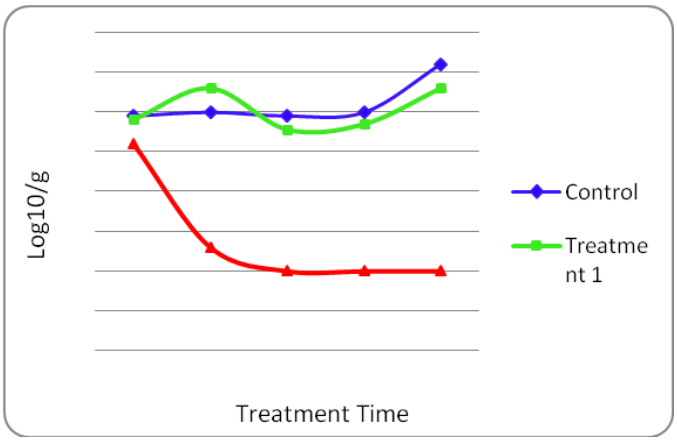

Figure 1. Count of psychrophilic microrganisms (Log 10) in shrimp samples treated with *L. reuteri* culture (treatment 1) and *L. reuteri* extract (treatment 2) for 48 h at 7°C.

Jeyasekaran et al. (2006) which evaluated the increasing of the microbiota of pacific white shrimp stored under different refrigeration and modified atmosphere conditions. Moura et al. (2003), evaluating the microbiological quality of pink shrimp commercialized in São Paulo, found counts ranging from 1.1 x 10^4 to 3.0 x 10^7 CFU/g.

International Commission on Microbiological Specification for Food (ICMSF) (1998) established a limit of 10^7 CFU/g of aerobic standard plate counts for frozen raw crustaceae, with no limit for psychrotrophic microorga-nisms. Brazilian legislation (Kirschink and Viegas, 2004; ANVISA, 2010) did not present limits for mesophilic aerobic and psychrotrophic plate counts in fish products. However, it is well known that elevated populations can reduce the shelf life of fish products (Ruch, 1974).

Counts of psychrophile in control treatment ranged from 6 to 7 log cycles during storage of shrimp at ±8ºC. In can be noticed that refrigeration temperature was efficient in controlling the microbial growth during 48 h, once averages during the whole storage period were not statistically different ($p > 0.05$).

According to Jeyasekaran et al. (2006), storage under refrigeration in temperatures lower than 6ºC is sufficient for controlling microbial growth for 72 h. After this period, psychrotrophic bacteria grow fast, leading to the spoilage of the product. In shrimps, such psychrotrophic microbita includes *Pseudomonas* sp. and *Aeromonas* sp.

Analyzing the effect of *L. reuteri* culture (Treatment 1) over psychrotrophiles in shrimps, it was observed an increasing on counts of these microorganisms after 3 h of treatment. *L. reuteri* culture added to the sample could influence this count. A slight decrease in psychrophile counts was observed after 6 h, although results did not present statistical differences ($p>0.05$) when comparing the test sample and the control. After 48 h, microbial counts were similar to those found for control. It can be explained by a probable inability of *L. reuteri* in growing and producing antimicrobial substances in temperatures under 10°C. In this way, the effect observed at 6 and 24 h can be due to a small amount of an antimicrobial substance produced during the growth, once *L. reuteri* can produce small quantities of reuterin at anaerobic conditions (Ruch, 1975; Tomé, 2006). The effect of treatment 1 compared to the control during 48 h is presented in the Figure 1.

Reuterin concentration in cell free supernatant from *L. reuteri*, obtained after incubation in 100 mM glycerol, was approximately 65 mM, as determined by the method described by Lüthi-Peng et al. (2002). The activity of *L. reuteri* cell free supernatant over shrimp microbiota (Treatment 2) presented singular positive results (Figure 1), with statistical analysis demonstrating differences between the averages of treatment and control for T6 ($p = 0.002$), T24 ($p = 0.0002$) and T48 ($p = 0.0009$). This result shows the efficiency of the *L. reuteri* cell free supernatant in reducing and keeping the counts of psychrophiles low and suggesting an extension in product shelf life.

Several authors suggest that species of *Lactobacillus* genera are the principal antagonists of pathogenic and spoiling microorganisms in fish products (Lyhs, 2001; Suárez, 2008). Suárez et al. (2008) evaluated the effect of the bacteriocin produced by *Lactobacillus plantarum* over mesophilic and psychrotrophic bacterial populations, coliforms at 35°C and thermotolerant coliforms present in *Piaractus brachypomus* fillets.

Authors verified that the initial population of psychrotrophic cycles regarding to control. Souza et al. (2006) evaluated the reduction of total mesophiles during fermentation of bonito-da-barriga-listrada using *L. sakei* as starter culture. Authors reported a decreasing from 10^8 CFU/g to 10^4 CFU/g for total mesophiles.

The inhibitory effect of reuterin produced by *L. reuteri*

has been investigated in several *in vitro* studies. Also, studies applying this antimicrobial substance in food have been reported. El-ziney and Debevere (1998) reported the inhibitory effect of reuterin against *Listeria monocytogenes* and *Escherichia coli* O157:H7 in milk and cottage cheese artificially contaminated.

Arques et al. (2004) reported that reuterin added to milk at 8 UA/mL exhibited a bacteriostatic effect on *L. monocytogenes* in treated milk at 37°C with no regrowth of the pathogen, fact already observed in a previous study. *L. monocytogenes* was completely inactivated within five days at ±7°C in milk with reuterin at 150 UA/mL (*5*). According to these authors, the inactivation rate depends on the concentration of reuterin.

Arques et al. (2007) reported that the presence of reuterin in milk leads to a decreasing in *S. enteric* and *Y. enterocolitica* counts after 12 days while *C. jejuni* and *A. hydrophila* were completely inactivated after 76 and 12 days, respectively. In this work, authors suggest that reuterin is very effective when applied to foodstuffs stored under refrigeration.

According to Spinler et al. (2008), reuterin is produced during fermentation of glycerol. In this way, it can be suggested that the inhibitory action of the *L. reuteri* extract produced through glycerol fermentation (treatment 2) is due to the presence of reuterin.

The use of reuterin in food can be more explored considering its efficiency and the safety for human health, once *L. reuteri* is an autochthon bacterium from gastrointestinal tract and known a safe for ingestion as probiotic (Spinler et al. (2008).

Conclusions

L. reuteri exhibited the ability to reduce psychrotrophic microbiota in pacific white shrimp stored under refrigeration and can be used as a bioprotector in food.

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Conflict of interest

The authors did not declare any conflict of interest.

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