Lactic acid bacteria used as preservative in fresh feed for marine shrimp maturation

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Abstract – The objective of this work was to evaluate the effect of *Lactobacillus plantarum* on the preservation of fresh mussels and its effect on the attractiveness, consumption, and midgut bacterial microbiota of Pacific white shrimp broodstock. The experiment evaluated mussels stored with *L. plantarum* at 4°C. The controls were: mussels stored at -18°C without *L. plantarum*; mussels stored at -18°C with *L. plantarum*; and mussels stored at 4°C without *L. plantarum*. Microbiological analyses on mussels were performed on days 1, 7, 15, 30, 45, and 60 after processing. Additionally, mussels preserved with *L. plantarum* and stored at 4°C, and mussels stored at -18°C without *L. plantarum* were evaluated after 15 days for attractiveness, consumption, and midgut bacterial microbiota of shrimps. Mussels preserved with *L. plantarum* showed higher lactic acid bacteria counts and lower counts of *Vibrio* spp., as well as of total heterotrophic bacteria, after 60 days of storage. No differences were observed for attractiveness or consumption between treatments. The bacterial microbiota of midgut in shrimp fed mussels preserved with *L. plantarum* showed higher lactic acid bacteria count and lower *Vibrio* spp. The use of *L. plantarum* inhibits *Vibrio* spp. and preserves feed without changing attractiveness or consumption for shrimp.

Index terms: *Lactobacillus plantarum*, *Litopenaeus vannamei*, *Vibrio*, probiotic.

Bactérias ácido-lácticas como conservantes do alimento fresco para a maturação de camarões marinhos

Resumo – O objetivo deste trabalho foi avaliar o efeito de *Lactobacillus plantarum* sobre a conservação de mexilhões frescos e avaliar a atratividade, o consumo e a microbiota intestinal de reprodutores de camarão-branco-do-pacífico. O experimento avaliou mexilhões estocados com *L. plantarum* a 4°C. Os controles foram: mexilhões estocados a -18°C sem *L. plantarum*; mexilhões estocados a -18°C com *L. plantarum*; e mexilhões estocados a 4°C sem *L. plantarum*. As análises microbiológicas dos mexilhões foram realizadas nos dias 1, 7, 15, 30, 45 e 60 após o processamento. Além disso, os mexilhões conservados com *L. plantarum* e estocados a 4°C e os mexilhões estocados a -18°C sem *L. plantarum*, após 15 dias, foram avaliados quanto à atratividade, ao consumo e à microbiota bacteriana intestinal dos camarões. Mexilhões conservados com *L. plantarum* tiveram maiores contagens de bactérias ácido-lácticas e menores contagens de *Vibrio* spp., assim como de bactérias heterotróficas totais, após 60 dias de estocagem. Não foram observadas diferenças significativas quanto à atratividade ou ao consumo entre os tratamentos. A microbiota intestinal dos camarões alimentados com mexilhões conservados com *L. plantarum* apresentou maior contagem de bactérias ácido-lácticas e menor contagem de *Vibrio* spp. O uso de *L. plantarum* inibe *Vibrio* spp. e conserva o alimento, sem modificar a atratividade ou o consumo pelos camarões.

Termos para indexação: *Lactobacillus plantarum*, *Litopenaeus vannamei*, *Vibrio*, probiótico.

Introduction

Marine shrimp farming stands out as one of the most important sectors in aquaculture. In particular, *Litopenaeus vannamei*, was responsible for 80% of world shrimp production in 2014 (FAO, 2016). The reproduction of *L. vannamei* in captivity is well established; however, an incomplete or unbalanced

diet can cause poor reproductive performance, or even hinder breeding (Peixoto et al., 2011). Until now, no commercial feed that meets all reproductive needs has been available. Consequently, the use of fresh feed, such as mussels, squid, fish roe and artemia biomass, is essential (Kolkovisk & Kolkovisk, 2011).

In the maturation sector, fresh feed is commonly stored at -18° C (Hoa et al., 2009). However, freezing forms ice crystals which can cause tissue damage. This process results in exudation, fluid loss, reduced nutritional value, and changes in the texture and appearance of feed after defrosting (Cordeiro et al., 2007). Freezing can also affect the structural and chemical properties of meat, such as muscle fibers, lipids and proteins (Pietrasik & Janz, 2009).

An alternative for the preservation of fresh feed is the use of lactic acid bacteria. These bacteria can control the spread of pathogenic ones of the *Vibrio* genus, which are commonly found in squid and mussels (Prado et al., 2010). Lactic acid bacteria could also help in the prevention of viral contamination as that caused by White Spot Syndrome Virus (WSSV), which is sensitive to low pH (Gao et al., 2011). As final products, lactic acid bacteria generate lactic acid and other organic acids, hydrogen peroxide, and bacteriocins, which act as biopreservatives controlling the dissemination of various microbes and pathogenic bacteria. For these reasons, lactic acid bacteria have been used in the preservation of different raw foods, such as meat, milk, and vegetables (Fadda et al., 2010; Udhayashree et al., 2012; Newaj-Fyzul et al., 2014).

In addition, using lactic acid bacteria as a feed preservative can have probiotic effects. Several studies have shown the probiotic benefits for marine shrimp by improving the balance of midgut microbiota (Vieira et al., 2008; Zhang et al., 2009), survival (Pham et al., 2014), resistance to infection (Chiu et al., 2007; Vieira et al., 2007), immunostimulation (Tseng et al., 2009; NavinChandran et al., 2014), and digestibility (Buglione-Neto et al., 2013).

The objective of this work was to evaluate the effect of *Lactobacillus plantarum* on the preservation of fresh mussels, and its effect on the attractiveness, consumption, and midgut bacterial microbiota of Pacific white shrimp broodstock.

Materials and Methods

The experiment was conducted from May to October 2012. The shrimp species used was *Litopenaeus vannamei* (Bonne, 1931) from a specific-pathogen-free (SPF) lineage Speedline SPF (Aquatec Aquacultura Ltda., Canguaretama, RN, Brazil). They were kept in biofloc system tanks (50,000 L) at the Laboratório de Camarões Marinhos (LCM), Universidade Federal de Santa Catarina (UFSC). Fresh mussels (*Perna perna*, Linnaeus, 1978) were obtained from the Laboratório de Moluscos Marinhos (LMM/UFSC).

The lactic acid bacterium strain *Lactobacillus plantarum* (CPQBA 007 07 DRM01) was used as a probiotic. This bacterium was isolated from adult *L. vannamei* shrimp (Vieira et al., 2007) and maintained in the Microbiology Laboratory of the LMC/UFSC at -20°C, in MRS culture medium (De Man et al., 1960) supplemented with 25% glycerol (Ramírez et al., 2006).

The preservation trial evaluated the mussel preserved with *L. plantarum* stored at 4°C. The following controls were used: a standard method for shrimp broodstock by which mussels were stored at -18°C, without *L. plantarum*; freezing control by which mussels were preserved with *L. plantarum* and stored at -18°C; and a bacterial control by which mussels were stored at 4°C, without *L. plantarum*.

At first, an inoculum of *L. plantarum* was prepared by growing the bacterium in MRS liquid medium supplemented with 3% NaCl, for 24 hours at 35°C, until reaching a concentration of 10^9 colony forming units (CFU) per milliliter (Vieira et al., 2008). Then, 500 g of fresh mussels were stored in 500 mL glass bottles and, then, they were immersed in *L. plantarum* inoculum suspension (10^9 UFC mL^{-1}) for 6 hours at room temperature (28°C). Next, excess of the inoculum suspension was removed by sieving (1 mm mesh) during 5 min, and mussels were packed in 50 plastic vacuum-sealed bags containing 10 g mussels each. One half of the bags was stored at 4°C, and the other half was frozen at -18°C. Similarly, 500 g of mussels were placed in 50 plastic bags with 10 g each, without inoculum and, then, they were vacuum packed and stored under two different conditions: 4°C and -18°C.

Microbiological analyses of mussels were performed on days 1, 7, 15, 30, 45, and 60, after processing (all in triplicate). To perform the analysis, 1 g from each sample was removed, macerated in a mortar, and 10-fold serially diluted up to 10^{-8} dilution in sterile saline solution (SSE) at 3% NaCl. The dilutions were cultured in the following media: Marine Agar medium (Difco, Leeuwarden, The Netherlands) for total count of

heterotrophic bacteria; Thiosulfate Citrate Bile Sucrose agar - TCBS (Difco), for total count of *Vibrio* spp.; and MRS Agar, for count of lactic acid bacteria. Colony forming units (CFU) were determined 24 hours after incubation at 30°C in Marine Agar and TCBS agar, and 48 hours after incubation at 35°C in MRS.

Based on the preservation trial, mussels preserved with *L. plantarum* and stored at 4°C, and mussels stored at -18°C, both during 15 days, were evaluated for attractiveness, consumption, and midgut bacterial microbiota of shrimp.

Attractiveness was evaluated according to Nunes et al. (2006). In the present work, we used a Y-shaped glass aquarium equipped with a Y-maze 1.3 x 0.3 x 0.4 m (length x width x height). Shrimp (24±1.47 g) used for attractiveness analysis were kept in a 6 Mg tank with 30-35 ‰ salinity, at 28-29°C , above 5 mg L^{-1} dissolved oxygen, and 7.5-8.0 pH. To stimulate faster feeding response, shrimp were left to fast 24 hours prior to analysis. Shrimp behavior was evaluated in three ways, as described by Nunes et al. (2006): detection time, orientation time/locomotion, and feeding activity. When the chosen diet was consumed, it was classified as a positive choice, and when it was refused, it was recorded as rejection. The number of positive choices was calculated as a percentage, to finally compare each diet. Before each behavioral assessment, aquarium water was changed to prevent the influence of contamination from the diet previously used, and shrimp were kept in a chamber for acclimatization for 10 min to allow adaptation to the system. Subsequently, the two experimental diets were offered separately in equal amounts of 2 g, and placed individually on the perimeter of the arms of the Y-maze. Thirty-three tests were performed (one shrimp per test). Timeout was set at 7 min; thus, in the case of nondetection, shrimp specimen was changed.

Mussel consumption test was conducted in six 50 L aquaria stocked with four shrimp each (24±1.47 g), using the two diets above described (in triplicate). Feeding was carried out twice a day, at 9 am and 3 pm, at 6% of shrimp biomass. After one hour, feed leftovers were collected and frozen at -18°C. Samples were collected for seven days. For dry weight determination, samples were placed in an oven at 105°C until constant weight was achieved, which required about 27 hours. Feed consumption was calculated by "ingestion of feed per hour", a formula described by Hernandez-Cortes et al. (1999) as L=Mi-Mf-Mp/T, in which: L, is the ingestion of feed per hour; Mi, is the initial weight of dry feed; Mf is the final dry weight of feed; Mp is the weight of dry feed dissolved in water; and T is the elapsed time of feed in water.

After testing consumption, shrimp remained on their respective treatments until the 18th day of feeding. Subsequently, 24 shrimp (12 of each treatment) were collected, disinfected with alcohol at 70%, and their midguts were extirpated in a laminar flow chamber. The midguts were weighed, homogenized in a mortar, serially diluted in 3% NaCl SSE (1/10), and seeded in culture medium as described by Vieira et al. (2008).

The values of bacterial counts on mussels and shrimp midguts were transformed to $\log_{10}(x+1)$ before analysis. Data were analyzed by the Bartlett test to verify homoscedasticity. Data were also subjected to analysis of variance with repeated measures supplemented by the Tukey´s test. Meanwhile, data from mussel consumption and midgut microbiota were analyzed by Student's t test. For percentages of choice and rejection of feed, chi-square test was used, whereas detection time, locomotion, and feeding were all analyzed by Student's t test. All tests were performed at 5% probability.

Results and Discussion

Mussels preserved with *L. plantarum*, regardless of the storage temperature, had higher lactic acid bacterial counts. No difference was observed in the count of total heterotrophic bacteria for the different treatments until the 30th day (Table 1). On day 60th, mussels preserved with *L. plantarum* and stored at both 4°C and -18°C had lower total heterotrophic bacteria count than mussels without *L. plantarum*. For the count of *Vibrio* spp., mussels preserved with *L. plantarum* and stored at -18°C showed lower values on the seventh day, in comparison to the other treatments; and, from the 15th day, mussels preserved with *L. plantarum*, at both temperatures, had lower counts of *Vibrio* spp. Lactic acid bacteria count in mussels preserved with *L. plantarum* indicates that these bacteria remained viable in the mussels. This group of bacteria is used as biopreservatives to improve sensory quality, color, and texture of feed by decreasing the feed pH and producing bacteriocins, organic acids, and hydrogen peroxide, which have bacteriostatic or bactericidal activity (Fadda et al., 2010). Reduced heterotrophic bacteria count in mussels preserved with *L. plantarum*

and stored at -18°C, and lower counts of *Vibrio* spp. in the mussels preserved with *L. plantarum* could be attributed to these substances (Fadda et al., 2010).

Souza et al. (2006) conducted a study on the antimicrobial activity of *Lactobacillus sakei* in fermented striped bonito (*Euthynnus pelamis*) showing the inhibitory effect of *L. sakei* strain against spoilage bacteria. Additionally, this strain caused an effective reduction of pH by the lactic acid production. The same results were observed by Espírito Santo et al. (2003), who utilized *L. sakei* in the fermentation process of Brazilian sardinella (*Sardinella brasiliensis*). These results support the hypothesis that low pH (3.8) combined with antimicrobial compounds generated by *L. plantarum* sufficiently preserved fresh mussels at both temperatures.

Using *L. plantarum* combined with storage at 4°C can be advantageous for shrimp reproduction in commercial laboratories, which have mainly used storage at -18°C (Hoa et al., 2009). Since feed in this case does not undergo freezing, the formation of ice crystals can be prevented. As noted previously, this condition can cause tissue damage, and such damage, in turn, can result in exudation, loss of liquids, reduction of nutritional value, and change of the texture and appearance of feed (Cordeiro et al., 2007). Additionally, feed stored at 4°C reduces energy cost compared to that stored at -18°C.

The attractiveness and consumption tests showed no difference between the two offered diets, – that is, mussels preserved with *L. plantarum* and stored at 4°C, and mussels stored at -18°C without *L. plantarum* –, which suggests that *L. vannamei* had no preference for either diet (Table 2).

Lactic acid bacteria count on the midgut of shrimp fed mussels preserved with *L. plantarum* and stored

Table 1. Lactic acid bacteria, total heterotrophic bacteria and *Vibrio* spp. counts in mussels preserved with *Lactobacillus plantarum* and stored at 4°C (*L. plantarum* +4°C), mussels stored at 4°C without *L. plantarum* (control +4°C), mussels frozen at -18°C without *L. plantarum* (control -18°C), and mussels preserved with *L. plantarum* and stored at -18°C (*L. plantarum* -18°C) for 60 days[(1)].

Treatment	Days of analysis					
	1	7	15	30	45	60
	Count of lactic acid bacteria in agar MRS (log CFU g^{-1})					
L. plantarum +4°C	8,08±0,21a	8,09±0,15a	7,94±0,03a	7,43±0,38a	7,29±0,49a	6,62±0,33b
Control +4°C	3,72±0,12b	2,39±0,09b	2,74±0,26b	3,19±0,59b	2,39±0,09c	3,15±1,93a
L. plantarum -18°C	4,66±0,26b	7,76±0,28a	7,76±0,28a	7,80±0,76a	7,40±0,17a	6,87±0,15b
Control -18°C	2,15±0,15c	2,15±0,15b	0,00±0,00c	3,40±0,35b	4,35±0,17b	3,08±0,46a
	Count of total heterotrophic bacteria in agar Marine (log CFU g^{-1})					
L. plantarum + 4°C	3,51±3,04a	4,97±2,57a	5,15±1,20a	2,95±2,59a	2,90±2,59ab	4,43±1,50bc
Control + 4°C	6,79±3,08a	7,05±2,42a	5,76±0,11a	5,68±3,38a	5,70±3,39a	9,05±2,95a
L. plantarum -18°C	4,33±1,84a	4,14±1,25a	4,36±0,14a	4,12±1,05a	0,00±0,00b	0,00±0,00c
Control -18°C	2,86±0,42a	5,95±2,88a	5,32±0,17a	4,81±3,44a	4,38±0,74a	8,46±1,89ab
	Count of *Vibrio* spp. in agar TCBS (log CFU g^{-1})					
L. plantarum + 4°C	4,45±1,55ab	2,38±0,43a	0,77±1,33b	0,00±0,00c	0,00±0,00b	0,00±0,00b
Control + 4°C	5,14±1,52a	4,33±1,25a	3,65±2,31a	2,65±1,33b	2,30±1,91a	2,30±1,91a
L. plantarum -18°C	4,30±1,15ab	0,00±0,00b	1,00±0,30b	0,00±0,00c	0,00±0,00b	0,00±0,00b
Control -18°C	2,87±0,97b	3,56±1,45a	4,63±2,17a	5,34±1,57a	3,83±2,00a	3,09±2,00a

[(1)]Means followed by equal letters, in the columns, do not differ by Tukey´s test, at 5% probability.

Table 2. Percentages (%) of positive choices, detection time, locomotion, and feeding activity, for *Litopenaeus vannamei* shrimp held in a Y-maze apparatus, and offered diets consisting of mussels preserved with *Lactobacillus plantarum* stored at 4°C (*L. plantarum* +4°C), and mussels stored at -18°C without L. *plantarum* (control -18°C).

Diet	Consumption (g per shrimp)	Detection time (s)	Locomotion (s)	Feeding activity (s)	Positive choices (%)
Control -18°C	0.69±0.19	144.03±174.32	338.05±267.60	576.52±278.41	57,57
L. plantarum +4°C	0.53±0.21	82.78±55.21	384.21±239.47	551.64±243.85	42,43

at 4°C was higher than the count for the group fed mussels stored at -18°C without *L. plantarum* (Figure 1). *Vibrio* spp. count was lower in the midgut of shrimp fed mussels on a diet of *L. plantarum* and stored at 4°C. For total heterotrophic bacteria count, no difference was observed between groups.

The largest number of lactic acid bacteria was found in the midgut of shrimp fed mussels preserved with *L. plantarum* and stored at 4°C, which indicates that *L. plantarum* remains viable in shrimp midgut. The same result was observed in *L. vannamei* fed ration supplemented with *L. plantarum* (Vieira et al., 2008; Ramírez et al., 2013). In addition, *L. plantarum* isolated from *L. vannamei* midgut has potential as a probiotic, inhibiting the growth of pathogenic bacteria, and enhancing the immunological system (Vieira et al., 2007, Vieira et al., 2013).

In addition, *Vibrio* spp. count was reduced in the guts of shrimp fed mussels on a diet of *L. plantarum* and stored at 4°C, possibly by the ability of *L. plantarum* to produce antimicrobial substances (Deegan et al., 2006). Similar results were observed in *L. vannamei* fed ration supplemented with *L. plantarum* (Vieira et al., 2007, 2008; Ramírez et al., 2013). These studies show that *L. plantarum* has a probiotic activity, thus improving immune parameters and resistance to infection (*Vibrio* sp.) in marine shrimp. Therefore, the utilization of fresh mussels preserved with *L. plantarum*, in shrimp breeding laboratories, could be a promising alternative to frozen feed, since it can act as a biopreservative and as a probiotic for shrimp broodstock.

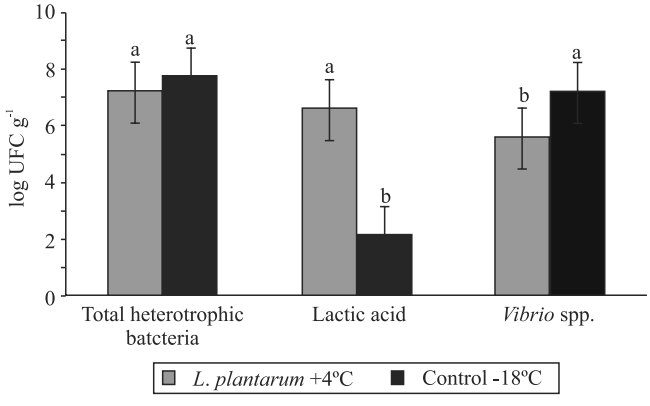

Figure 1. Count of total heterotrophic bacteria, lactic acid bacteria, and *Vibrio* spp., in the midgut of shrimp fed mussels preserved with *Lactobacillus plantarum* and stored at 4°C (*L. plantarum* +4°C), and mussels stored at -18 °C (control -18°C). Different letters indicate differences between treatments, in the columns, by Student´s t test, at 5% probability.

Conclusions

1. Fresh mussels can be preserved with the use of *Lactobacillus plantarum*, and they can be stored at 4°C for up to 60 days, without changing attractiveness and consumption by *Litopenaeus vannamei* broodstock.

2. *L. plantarum* modifies the midgut microbiota of *L. vannamei* shrimp fed mussels which consumed these bacteria; this fact increases the number of lactic acid bacteria and decreases *Vibrio* spp. in shrimp.

Acknowledgements

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process numbers PQ 309868/2014-9 and 308292/2014-6), for fellowships granted; to Ministério da Pesca e Aquicultura (MPA), for financial support; to the Southern Oceans Education & Development Project (SOED), for the scholarship granted; and to Dr. Aimê Rachel Magenta Magalhães of the Laboratório de Moluscos Marinhos (LMM/USFC), for supplying mussels for the experiment.

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Received on September 29, 2015 and accepted on July 29, 2016