Effect of dietary prebiotic inulin and probiotic Bacillus subtilis and Lactobacillus sp., on the intestinal microbiota of white shrimp Litopenaeus vannamei

Efecto de la inclusión de inulina (prebiótico) y Bacillus subtilis y Lactobacillus sp. (probiótico) en el alimento, sobre la microbiota intestinal del camarón blanco Litopenaeus vannamei

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ABSTRACT
Juvenile Litopenaeus vannamei (1.05 ± 0.1 g) were fed during a 4-week period with four experimental diets: control (Ctrl), inulin as prebiotic (5 g kg⁻¹) (Pre), Bacillus subtilis and Lactobacillus sp. as probiotic (1 x 10⁵ CFU g⁻¹) (Pro), and a mix of inulin + B. subtilis and Lactobacillus sp. (5 g kg⁻¹ + 1 x 10⁵ CFU g⁻¹) (Syn). Syn diet fed shrimps showed a significantly better utilization of feed and higher growth than those in control diet (P < 0.05). The probiotic employed induced higher intestinal bacterial richness, whereas inulin induced higher bacterial diversity in shrimp intestine. The most dominant bacterial phylum in the shrimp intestine among treatments was Proteobacteria with an abundance ranging between 80 and 84 %. Prebiotic diet (Pre) increased relative abundance of Firmicutes in shrimp intestine (2 %) compared to the rest of the treatments (0.6 %). When probiotics were included in the feed (Pro and Syn), a reduction between 3 and 13 % in the relative abundance of Vibrio sp. in shrimp intestine was observed with respect to the control treatment, which represent an advantage to control potential pathogens of this genus.

Keywords: Functional feed, Shrimp, Bacterial modulation.

INTRODUCTION
The rapid worldwide expansion of farmed shrimp has faced different challenges in terms of pathogen outbreaks and demand for functional feeds that promote optimal growth and health. In the past decades, the control of shrimp diseases has been through chemicals and antibiotics; however, their indiscriminate use, as preventive strategy, resulted in the resistance by pathogens and environmental problems (Martinez, 2009). In different studies, the use of probiotics, natural prebiotics and synbiotics (combination of probiotics and prebiotics) added to the feed or in the culture water conferred health benefits to the host (Hai, 2015; Riung et al. 2010; Olmos et al., 2020). Furthermore, this has been proposed to replace the use of chemicals and antibiotics to control and prevent shrimp diseases (Luna-González et al., 2012; Jamal et al., 2019; Partida-Arangure et al., 2013).

In the case of probiotics, some criteria to select beneficial microorganisms are the mode of action to enhance the immune response, production of inhibitory compounds to exclude pathogens, growth enhancement and improvement of water quality (Kesarcodi-Watson et al., 2008; Verschuere et al., 2000). Another determinant factor in the effectiveness of probiotics in aquaculture, is the origin of the microorganisms employed, where non-native bacteria from the marine environment may have poor success (Ninawe and Selvin, 2009) or represent a risk for the marine microbial ecology (Vargas-Albores et al., 2017). It has also been suggested that multiple probiotic strains have better results than a single probiotic strain in relation to growth and health of shrimp (Wang et al., 2019). Two of the most studied bacterial genus employed as probiotics for farmed shrimp are Bacillus and Lactobacillus, which have been described to improve shrimp growth when supplemented in the feed (Kongnum and Hongpattarakere, 2012; Zheng et al., 2017; Wang et al., 2019). This is attributed in part to an increase in digestive enzymes activity (Liu et al., 2009; Zheng and Wang, 2017; Zhou et al., 2009). In addition, the administration of Bacillus sp. to water has been considered to improve survival rate and growth of shrimp larvae and water quality (Liu et al., 2010; Zhou et al., 2009). The use of
**MATERIAL AND METHODS**

**Bacterial strains and encapsulation**

The probiotic bacteria *Bacillus subtilis* (BSB) (Balcázar and Rojas-Luna, 2007) and *Lactobacillus* sp. (Cb-Lta) (García-Rodríguez 2003) were previously isolated from *L. vannamei*. Both strains were grown on marine agar (MA) plates (seawater at 0.5 % meat peptone, 0.1 % yeast extract, and 1.7 % agar), at 35 °C during 24 h. The optical density of each bacteria was adjusted in a 2.5 % NaCl buffer to 1 at 600 nm (OD600 = 1) using a BioPhotometer (Eppendorf, D30, NY, USA), to standardize the number of bacteria at ~1 x 10^7 CFU mL^-1. The probiotics encapsulation was performed by mixing equal parts of both bacteria in a sterile solution of 2 % low viscosity alginic sodium (Sigma Aldrich). The mixture was added dropwise using a syringe (0.55-mm diameter) into a 2 % CaCl₂ solution while stirring magnetically. After filtered and washed with distilled water, capsules were stored at 4 °C in a sterile flask.

**Experimental diets**

Four balanced experimental diets were formulated for shrimp: a control diet (Ctrl), a prebiotic diet with inulin included at 0.5 % (Pre), the third diet with the encapsulated probiotic at a final concentration of 1 x 10^5 CFU g^-1 of each bacteria (Pro), and finally a symbiotic diet containing the same ratio of both probiotic and prebiotic (Syn) (Table 1). Manufactured diets were prepared as follows: all dry ingredients, including probiotic and prebiotic if applicable, were mixed (KitchenAid® 4.7 L mixer, Michigan, US) to obtain a homogeneous blend, followed by the incorporation of the oil-based ingredients and remixed. Then, distilled water was incorporated.

![Table 1. Ingredients and proximate composition of experimental diets.](image_url)
rated and homogenized (~350 mL kg⁻¹), and finally passed through a 2-mm die (Torrey® M-12-FS, Nuevo Leon, MX). The pelleted diets were dried in an oven at 35 °C for 12 h (~10 % moisture) and kept in plastic bags at 4 °C until further use. Proximate composition analysis of shrimp diets was conducted as follows: dry matter was estimated by gravimetric analysis using a force-air oven at 100 °C for 24 h (Method 930.15; AOAC, 2005). The crude protein content was estimated by the Dumas combustion method (Ebeling, 1968) with a LECO® FP-528 analyzer (LECO Inc., Michigan, US). The ether extract content was determined using a micro Foss Sixtec® Avanti 2050 (Foss, Hóganás, SE) (Method 2003.05; AOAC, 2005). Ash content was analyzed gravimetrically at 550 °C for 6 h with a furnace (Method 942.05; AOAC, 2005). Crude fiber (Method 978.10; AOAC, 2005) was determined using a Fibre Tec® M6 System (Foss, Hóganás, SE). Nitrogen-free extract (NFE) was estimated by difference (100 % less the percentages of lipids, crude protein, ash and crude fiber). Gross energy was analyzed with an adiabatic calorimeter (Parr Instruments, model 1261, Illinois, US). All experimental diets presented similar protein, lipid, and ash contents with a slight increase of crude fiber when inulin was included (Pre and Syn diets) (Table 1).

**Feeding trial**

Juvenile shrimp *L. vannamei* were kindly donated by Larvas Gran Mar, S.A. de C.V. (Baja California Sur, Mexico), acclimated to laboratory indoor conditions (28 ± 0.6 °C, > 4 mg L⁻¹ DO, 16:8 h dark:light photoperiod and 37 %o salinity) for one week prior feeding trial. Experimental dietary treatments were evaluated by triplicate during 28 days; each replicate corresponded to a 50-L aerated fiberglass tank containing 10 shrimp (initial avg. wt. of 1.05 ± 0.1 g). Shrimps were fed to satiety for all treatments with two initial rations corresponding to 5 % of shrimp biomass (09:00 and 15:00 h). Every day, the feed ration was adjusted according to consumption in each tank. Every day 50 % water was exchanged, to keep water parameters of dissolved oxygen (4 ± 1.1 mg L⁻¹) and temperature (28 ± 0.5 °C) which were measured daily with a multiparameter YSI model 85. Twice a week, pH (7.9 ± 0.1) and temperature (28 ± 0.5 °C), which were kept in plastic bags at 4 °C until further use. Proximate composition analysis of shrimp was conducted as follows: dry matter was estimated by gravimetric analysis using a force-air oven at 100 °C for 24 h (Method 930.15; AOAC, 2005). The crude protein content was estimated by the Dumas combustion method (Ebeling, 1968) with a LECO® FP-528 analyzer (LECO Inc., Michigan, US). The ether extract content was determined using a micro Foss Sixtec® Avanti 2050 (Foss, Hóganás, SE) (Method 2003.05; AOAC, 2005). Ash content was analyzed gravimetrically at 550 °C for 6 h with a furnace (Method 942.05; AOAC, 2005). Crude fiber (Method 978.10; AOAC, 2005) was determined using a Fibre Tec® M6 System (Foss, Hóganás, SE). Nitrogen-free extract (NFE) was estimated by difference (100 % less the percentages of lipids, crude protein, ash and crude fiber). Gross energy was analyzed with an adiabatic calorimeter (Parr Instruments, model 1261, Illinois, US). All experimental diets presented similar protein, lipid, and ash contents with a slight increase of crude fiber when inulin was included (Pre and Syn diets) (Table 1).

**Bioinformatic analyses**

The raw reads were quality-trimmed using the Galaxy version 20.09 (The Institute for CyberScience at Penn State, and Johns Hopkins University) (http://usegalaxy.org) and aligned using the SILVA v138 database (Yilmaz et al. 2014). The sequences were randomly subsampled to normalize the number of sequences and assigned to operational taxonomic units (OTUs) based on 97 % similarity. The structural diversity of the microbial communities was calculated with three indices, including Chao, Shannon, and Simpson, calculated by a binary matrix using PAST software (Hammer et al., 2001) (http://palaeo-electronica.org).

**RESULTS**

After 28 days of the feeding trial, all treatments resulted in at least 1.0 g weight gain per week, nevertheless shrimp fed Syn diet resulted in significantly higher growth rate, and SGR compared to shrimp under Ctrl and Pre feeding treatments (P < 0.05). The Syn diet showed a significant improvement in feed utilization in terms of FCR and PER compared to the Ctrl diet (P < 0.05), but it was not significantly different from Pre and Pro treatments. Survival of shrimp was ≥ 93 % for all treatments (P > 0.05) (Table 2).

Each library had 36,278–59,857 raw reads, and after quality control, 33,199–53,250 reads remained per library.
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Table 2. Growth, feed efficiency and survival of Litopenaeus vannamei shrimps fed experimental diets after 4-weeks.

|                      | Ctrl | Pre | Pro | Syn |
|----------------------|------|-----|-----|-----|
| Growth rate (%)      | 381±4* | 378±11* | 390±12ab | 403±3b |
| SGR (% day⁻¹)        | 5.61±0.03b | 5.58±0.08b | 5.68±0.09ab | 5.77±0.02 |
| FCR                  | 1.56±0.03ab | 1.50±0.05ab | 1.47±0.02ab | 1.45±0.03ab |
| PER                   | 1.84±0.03b | 1.94±0.07ab | 1.97±0.02ab | 2.01±0.04b |
| Survival (%)         | 97±6 | 93±6 | 93±6 | 97±6 |

Values are given as mean ± SD of triplicate determinations. Values in the same row with different superscripts are significantly different (P < 0.05). Growth rate = (final wt – initial wt) / initial wt × 100.
SGR: Specific growth rate = 100 (ln avg final wt – ln avg initial wt)/ d.
FCR: Feed conversion ratio = dry weight of pelleted feed consumed (g) / wet wt gain (g).
PER: Protein efficiency ratio = wt gain/feed protein intake.
Survival = final number of shrimp/initial number shrimp × 100.

with a total of 538,446 reads (n = 12). One library of the prebiotic treatment was identified as an outgroup replicate according to a principal component analysis, and was eliminated from subsequent analyses. The analyses clustered the sequences into 9,247 OTUs. At phylum level, shrimps fed with the four treatments (Figure 1) revealed that the most dominant bacterial phylum of the shrimp intestine was Proteobacteria with 80 % in Ctrl diet, 82 % in Pre and Pro diets, and the highest percentage was in Syn diet with 84 %. Within Proteobacteria phylum, control diet showed the highest relative proportion (42 %) in Gammaproteobacteria class compared to the rest of treatments, in which Pro diet presented a significant reduction of this bacteria group (23 %). In contrast, Alphaproteobacteria in control diet resulted in the lowest relative proportion (34 %) respect to the other diets (41-53 %). The second dominant bacterial phylum was Bacteroidetes, with the highest proportion in control diet (16 %), and the rest of the treatments resulted in the range of 11 to 13 %. The third dominant phylum was Actinobacteria with 2-3 % among treatments. Firmicutes phylum includes the probiotics used, where the highest phylum was present in the prebiotic-treatment group with 2 %, whereas the rest of treatments showed 0.6 %.

At Order taxa level (Figure 2), Rhodobacteriales was the most abundant bacteria in shrimp among treatments, and mostly represented by Rhodobacteraceae bacterial family (> 97 %). Shrimps fed with Pro diet probiotic showed the highest proportion of Rhodobacteraceae (53 %), followed by prebiotic and symbiotic treatments (41 and 40 %, respectively), and the rest of the treatments resulted in the range of 11 to 13 %. The most abundant genus present in Rhodobacteraceae among treatments was Octadecabacter with 11 % of abundance for control, 12 % for prebiotic, 14 % for symbiotic, and 19 % for probiotic treatment.

The second most abundant bacteria order among treatments was Vibrionales (Figure 2), manly from Vibrionaceae family. In this regard, the Pro treatment presented the lowest proportion among treatments with 16 %, followed by the symbiotic with 26 %, and prebiotic and control with 28 and 29 %, respectively; in all cases, this family was represented entirely by the Vibrio genus. In the case of Flavobacteriales order level, represented by the Flavobacteriaceae family, control treatment showed 15 % relative abundance in contrast to 13 % in Pro, and 11 % in Pre and Syn treatments.

In the case of probiotic bacteria related to diets, at class taxonomic level, Bacilli bacteria were more abundant in probiotic treatment (2 %) than in probiotic, symbiotic (0.5 % each), and control (0.4 %). In terms of genus abundance, Lactobacillus was absent in shrimp fed with control treatment, whereas those fed with probiotic represented 0.92 % of total bacteria, and < 0.01 % in probiotic and symbiotic treatments. In the case of Bacillus, probiotic and symbiotic treatments.
showed 0.05 and 0.04% abundance, prebiotic 0.03%, and control 0.01%. Among different treatments, 20 species were identified including five species of *Vibrio* *furnissii*; *Vibrio* *furnissii* was the most abundant for control treatment (3%); whereas *Vibrio* *antiquarius* was the dominant species present for the rest of treatments (3-4%).

According to the Chao index, bacterial richness estimator index values among treatments ranged from 1,567 to 2,123, where Pro and Syn treatments, both with probiotics in the feed, resulted with higher values than those presented in Ctrl and Pre dietary treatments (Table 3). In terms of diversity assessed by the Shannon index among treatments, values ranged from 3.03 to 3.57. Simpson index values ranged from 0.14 to 0.23, where Pre and Syn treatments showed higher diversity among treatments.

### Table 3. Number of OTUs, richness estimation (Chao1), and diversity estimation (Shannon and Simpson) of intestinal community bacteria, of shrimp fed experimental diets after 4-weeks.

| Diets   | OTUs   | Richness estimator | Diversity estimators |
|---------|--------|--------------------|---------------------|
|         | Ctrl   | Pre    | Pro    | Syn    |
| OTUs    | 340±80 | 496±12 | 687±115| 650±30 |
| Chao1   | 1,567±0.57 | 1,719±0.57 | 1,938±0.57 | 2,123±1.15 |
| Shannon | 3.03±0.14 | 3.52±0.07 | 3.30±0.40 | 3.57±0.12 |
| Simpson | 0.17±0.12 | 0.20±0.03 | 0.19±0.08 | 0.23±0.09 |

**DISCUSSION**

The importance of gastrointestinal microbiota on host health and growth has been documented. The objective of probiotics and prebiotics in the feed is to induce favorable intestinal bacteria to promote growth, improve pathogen resistance and the immune response. The inclusion of 0.2-0.8% inulin has proved to enhance shrimp growth (Li et al., 2020; Zhou et al., 2020), however, in the present work no difference on shrimp growth was evident, when included without probiotic, compared to Ctrl diet as reported by other authors with inulin inclusion levels between 0 and 1% (Luna-González et al., 2012; Partida-Arangure et al., 2013). This discrepancy may be associated to microorganism community present in the culture system. The use of inulin has been proven to be a good source of nutrients to promote probiotic bacteria from *Lactobacillus* and *Bacillus* genus (Savedboworn et al., 2018; Zherebtsov et al., 2002), providing a wide range of exoenzymes, including inulinas, that allow using inulin as source of carbon (Saha, 2006; Zherebtsov et al., 2002). *Bacillus* sp. and *Lactobacillus* sp. included in feed may also enhance shrimp protease, lipase, amylase, and cellulase activities, which suggests a better use of the nutrients contained in the feed and reflected in improvement of growth and feed efficacy utilization (Kongnum and Hongpattarakere, 2012; Zheng et al., 2017).

In the present study, the final weight of shrimps fed Pro treatment was higher than shrimps fed the control diet (Ctrl), but not significantly different (*P > 0.05*). Wang et al. (2019) described that the inclusion of *Lactobacillus pentosus* or *Bacillus subtilis* at ~1 × 10^6 CFU g^-1 in feed, improved weight gain in white shrimp. Similar effect was shown when the inclusion of probiotic *Bacillus* (*B. subtilis* and *B. licheniformis*) in the diet at 1 × 10^6 and 1 × 10^4 CFU g^-1 significantly improved growth performance and feed utilization efficiency in *L. vannamei* (Sadat Hoseini Madani et al., 2018). In the case of the synbiotic treatment (Syn), shrimp growth and feed utilization efficiency improved in contrast to the Ctrl diet. Similar results were shown by Huynh et al. (2018), when shrimp fed with a synbiotic diet composed of *Lactobacillus plantarum* and galactooligosaccharide improved shrimp weight gain; nevertheless, the use of the probiotic alone showed no significant differences compared to the control diet, highlighting the importance of the presence of the prebiotic in the diet. The synergism between prebiotics and probiotics to improve shrimp growth (Boonanuntanasarn et al., 2016; Munaeni et al., 2014) has been suggested by the indirect and direct release of extracellular bacterial digestive enzymes and bioactive compounds that, in conjunction with the activation of the digestive enzymes of the host, improve the efficiency of feed utilization (Huynh et al., 2017).

After sequencing analysis, between 35 and 40% of the reads were classified within a genus or species. The use of the probiotic in the feed (Pro and Syn treatments) increased the shrimp intestinal bacterial richness according to the Chao index. Inclusion of prebiotic (Pre and Syn) in the feed promoted higher bacterial diversity according to both Shannon and Simpson indices. The increase of species richness in intestinal microbiota in *L. vannamei* has been also reported with the use of synbiotic composed of galactooligosaccharide and *L. plantarum* (Huynh et al., 2019), *Bacillus* sp. probiotic, honey prebiotic or a mix of them (synbiotic) (Hasyimi et al., 2020), suggesting that synbiotics in feed may modulate intestinal bacterial community.

The dominant bacterial group in shrimps fed the four treatments was the phylum *Proteobacteria*, which has been reported to be highly abundant in both healthy or diseased shrimp (Dai et al., 2018; Zheng et al., 2017), and also in shrimp fed probiotics and synbiotics included in the diet (Hasyimi et al., 2020). Differences in the *Proteobacteria* abundance due to the diet effect may also relate to shrimp size as observed by Dai et al. (2018), who found a temporal variation in shrimp gut microbiota composition (between 8 to 9 g). Microbiome changes also occur between culture stages, as observed in *L. vannamei* larvae, being *Proteobacteria* the most abundant phylum (Zheng et al., 2017). Some bacteria from the *Proteobacteria* phylum serve as a direct source of nutrients (Moss,
2002), and could improve feed digestion due to their digestive enzymes that may enhance nutrient utilization in shrimp (Wainwright and Mann, 1982). A diverse microbial community has been suggested to improve the degradation of low or indigestible substrates, which may increase the efficiency of feed utilization (Cottrell and Kirchman, 2003).

Shrimps fed the four treatments presented a high abundance of Bacteroidetes (8-19 %) and Actinobacteria (2-3 %), the last reported as abundant in healthy shrimps (5.6 %), compared to diseased shrimps (0.1 %) (Dai et al., 2018). Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes were found in all treatments, and are considered natural microbiota in the penaeid shrimp gut (Rungrassamee et al., 2014; Tzuc et al., 2014).

At family taxonomic level, a similar abundance of Flavobacteriaceae was found among treatments, which has been described as abundant in healthy farmed shrimp (Xiong et al., 2015; Zheng et al., 2017). Likewise, some specialized bacteria belonging to this family can degrade complex compounds including structural polysaccharides such as chitin from shrimp exoskeleton (Kirchman, 2002; Williams et al., 2013), implying a beneficial effect on pond water quality.

The nutrition strategy, including diets with prebiotics, probiotics, and synbiotics, represents an alternative to the use of antibiotics to control pathogenic bacteria including some Vibrio species (Johnson, 2013; Kewcharoen and Srisapoome, 2019). According to our results, shrimps fed with probiotics decreased the abundance of Vibrio sp. compared to the control treatment, as reported in the freshwater prawn Macrobrachium rosenbergii fed with the inclusion of the probiotic Lactobacillus that showed an inhibitory effect on the gram-negative bacteria in the intestine (Venkat et al., 2004). Moreover, a previous study with the inclusion in feed of B. subtilis at 10^7 CFU g^-1, produced antimicrobial activity against pathogenic Vibrio species, with a reduction on shrimp mortality (Balcázar and Rojas-Luna, 2007). In the same way, the inoculation of 10^6 CFU ml^-1 of Lactobacillus sp. in water reduced the prevalence of pathogenic Vibrio in brine shrimp culture (Quiroz-Guzmán et al., 2018). The Vibrioaceae reduction in shrimps fed the probiotic diet (Pro) resulted in an increase of Rhodobacteraceae family, which has been reported at higher abundance in healthy shrimp as compared to diseased shrimps (Dai et al., 2018).

In the case of the Syn treatment, a reduction in Vibrio sp. abundance compared to the control treatment was observed. This result agrees with that observed in the same species, when L. plantarum and galactooligosaccharide synbiotic was included in the diet (Huynh et al., 2019), as well as for Peneaus japonicus (Zhang et al., 2011) fed a Bacillus sp. and isomaltooligosaccharides supplemented diet. The use of a mix of B. subtilis with β-glucan as prebiotic included in feed, also reduced the prevalence of Vibrio sp. in shrimp intestine, in contrast to β-glucan alone (Boonanuntanasarn et al., 2016). The reduction of Vibrio abundance by the use of synbiotics in the diet (oligosaccharides from sweet potato and Bacillus sp. NPS) has been related to an increase of total bacterial count in L. vannamei (Munaeni et al., 2014). Nevertheless, in the present work, Vibrio in the prebiotic treatment was similar to the control treatment. Vibrioaceae abundance may be affected by the source of the prebiotic employed; in the case of β-glucan, it has been reported that Vibrio abundance in shrimp gut is not affected when included alone in feed (Boonanuntanasarn et al., 2016). In turn, some Vibrio species may efficiently use inulin as a source of carbon to proliferate, as reported by Mahious et al. (2006) in turbot larvae. In this regard, the potential benefits of including inulin to the shrimp diet seems to be closely related to the natural microbial community and the introduced microorganism (probiotics) in the culture system.

CONCLUSIONS

In conclusion, we found that the synbiotic included in the diet improved shrimp growth, and the probiotic employed (B. subtilis and Lactobacillus sp.) reduced the relative abundance of Vibrio sp. in the shrimps’ intestine; nevertheless, when combined with a prebiotic (inulin), this effect was reduced.

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