Journal of Applied Phycology (2020) 32:2061–2070
https://doi.org/10.1007/s10811-020-02072-w

# Changes on the intestinal bacterial community of white shrimp *Penaeus vannamei* fed with green seaweeds

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Received: 22 November 2019 / Revised and accepted: 11 February 2020 / Published online: 16 March 2020


## Abstract

In recent years, development of sustainable and ecological food production has gained worldwide interest. It seems clear that this phenomenon is causing changes in aquaculture-focused research, with the development of new integration systems. However, it is still necessary to understand different aspects involved in integrated systems, including co-culture systems such as shrimp and seaweed. This study evaluated the effect of green seaweeds as food source on white shrimp *Penaeus vannamei* intestinal bacterial communities. Shrimp were evaluated after a 4-week experimental trial under different diet treatments: fed with only pellet (P), only *Ulva clathrata* (UC), *U. clathrata* + pellet (UCP), only *Ulva lactuca* (UL), and *U. lactuca* + pellet (ULP). In terms of growth and survival, no significant differences ($P > 0.05$) were found between ULP and UCP treatments compared with the control (P). Analysis of the bacterial biota of shrimp intestine revealed significant differences on community composition in ULP, UL, and UC compared with the control (P) ($P < 0.05$). We found that *Proteobacteria* is the most abundant phylum in all treatments, followed by *Bacteroidetes* for UC, UCP, and UL and *Actinobacteria* for P and ULP treatments. Shrimp fed only with seaweed *U. lactuca* (UL, ULP) had a significantly higher abundance of *Rubritalea*, *Lysinibacillus*, *Acinetobacter*, and *Blastopirellula*, and for *U. clathrata* treatments (UC, UCP), it was *Litoreibacter*. Relative abundance of *Vibrio* was higher in the control (P), showing a decrease in UC and UL treatments. Our findings provide a better understanding of integrated aquaculture systems, specifically those utilizing seaweed as natural feed source.

**Keywords** *Ulva lactuca* · *Ulva clathrata* · Chlorophyceae · Macroalgae · Shrimp feed · Bacteria community · Aquaculture

## Introduction

Aquaculture is one of the fastest-growing food production industries in the world. This is particularly true for farmed shrimp; worldwide production in 2015 was 4.8 million tonnes, representing a value of US$24.9 billion (FAO 2017). However, growth in this industry has led to many challenging factors, including increasing the demand for balanced feed that can reduce water pollution. Global production of seaweed has rapidly increased; 29.4 million tonnes were produced in 2015 (FAO 2018); seaweed has a great value in different applications including food, pharmaceutical, and cosmetics and in integrated aquaculture (Thuy et al. 2015; Couteau and Coiffard 2016; Elizondo-González et al. 2018).

The integration of seaweed into shrimp monoculture systems has been proposed as an effective and environmentally friendly expansion of aquaculture (Neori et al. 2004; Troell et al. 2009). Seaweeds are excellent feed additives that provide a good source of protein, carotenoids, minerals, vitamins, and polysaccharides (Kumar et al. 2011; Peña-Rodríguez et al. 2011; Syad et al. 2013). In the case of Chlorophyta, fresh *Ulva clathrata* and *Ulva lactuca* have been shown to be a natural food with a potential to partially replace pelleted feed in shrimp (Cruz-Suárez et al. 2010; Pallaoro et al. 2016; Peña-Rodríguez et al. 2017a). Additionally, integrated culture has

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10811-020-02072-w) contains supplementary material, which is available to authorized users.

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been shown to increase water quality when compared with shrimp cultured in a monoculture system (Khoi and Fotedar 2011; Brito et al. 2014; Elizondo-González et al. 2018; Ge et al. 2019). Seaweeds produce secondary metabolites which could have antibacterial activity potentially impacting the bacterial communities (Kandhasamy and Arunachalam 2008). In the case of Ulvales different studies have described their antibacterial properties against pathogen *Vibrio* species, with a significant decrease of mortality of shrimp (Selvin et al. 2011; Sivakumar et al. 2014). Seaweeds could also impact the intestinal microbial community of shrimp, which in turn affects several physiological processes of its host, such as modulation of immune responses, nutrient absorption, vitamin synthesis, prevention of the establishment of pathogenic microorganism, and others.

Previous studies have shown that the gut microbiota of *P. vannamei* were different between wild and cultured shrimp (Cornejo-Granados et al. 2017), mostly attributed to the different environmental factors that influence changes in the microbiome. In addition to environmental changes, other factors including developmental stage, antibiotics, and changes on the diet can induce modifications in gut bacterial composition in different species (Harris 1993; David et al. 2014; Zhang et al. 2014; Kim et al. 2017). Li et al. (2007) demonstrated that dietary supplementation in *P. vannamei* with short-chain fructooligosaccharides modifies gastrointestinal microbiota composition. Moreover, the source of the lipid and carbohydrate can modify the microbiological community in *P. vannamei* intestine (Zhang et al. 2014; Qiao et al. 2017).

Despite many aspects of integrated shrimp/seaweed culture systems have been described, including growth performance of shrimps and seaweed, nutrient uptaking, and water quality improvement (Brito et al. 2014; Elizondo-González et al. 2018), few studies are focused on the influence of seaweeds as food source on the white shrimp intestinal microbiota, limiting the understanding and importance of the microorganisms present in these systems. Therefore, the aim of this research is to determine changes on intestinal microbiome of white shrimp *P. vannamei* upon ingesting of the green seaweeds *U. lactuca* and *U. clathrata*.

## Material and methods

### Seaweeds and pelleted feed

*Ulva lactuca* seaweed was collected from La Paz bay in Baja California Sur, Mexico (Permit for collecting, Conapesca #PRMN/DGOPA-019/2015), and *Ulva clathrata* was provided by Algal tech SAPI de CV. The seaweeds were washed with sterilized marine water to remove epiphytes and then were placed in laboratory conditions in 5-L marine water tanks at 23 °C with a photoperiod of 12 h:12 h light/dark controlled by artificial light (75-W fluorescent light tubes) and using a sterile medium solution (12 g $L^{-1}$ $NH_4NO_3.P_2O_5$; 2 g $L^{-1}$ $NH_4.H_2PO_4$; 1.1 g $L^{-1}$ $FeCl_3.6H_20$; 1 g $L^{-1}$ $ZnCl_2$; 0.2 g $L^{-1}$ $MnSO_4$; 0.5 g $L^{-1}$ $Cu_2SO_4.5H_20$; 3 mg $L^{-1}$ vitamin B12; 2 mg $L^{-1}$ vitamin B1; 0.1 mg $L^{-1}$ biotin). Seaweeds were kept under laboratory conditions during the 2 weeks prior to shrimp feeding trial.

A balanced pelleted feed was manufactured in the Aquaculture nutrition laboratory at CIBNOR (Table 1). All dry ingredients (≤ 250 μm) were homogenized in a 3.2-L mixer (KitchenAid, USA) and then oil-based ingredients, and water were added and mixed again. The mixture was then passed through 2-mm die in a meat grinder. Finally, the pelleted feed was dried in a forced air oven at 45 °C for 12 h and stored at 4 °C until feeding time.

The proximal composition of the pelleted feed and seaweeds is presented in Table 2. The pelleted feed had 38.1% protein and 9.3% lipids. *Ulva clathrata* had a higher content of protein (23.4%) and crude fiber (4.2%) than *U. lactuca* (16.5 and 3.3%, respectively). Both seaweeds had low lipids (< 1%) and high ash content (> 36%).

**Table 1** Ingredient composition of pelleted diet (g $kg^{-1}$ diet)

| Ingredients | |
|---|---|
| Fish meal[a] | 200 |
| Soybean meal[b] | 304 |
| Wheat meal[c] | 370 |
| Corn gluten[d] | 34.5 |
| Soy lecithin[e] | 41 |
| Fish oil[a] | 30 |
| CMC[f] | 10 |
| Vitamin mineral premix[g] | 9 |
| Vitamin C[h] | 1 |
| Antioxidant BHT[i] | 0.5 |

[a] Proteinas Marinas y Agropecuarias SA de CV, Jalisco, MX

[b] Promotora industrial acuasistemas SA de CV (PIASA), Baja California Sur, MX

[c] Molino San Cristobal, Sonora, MX

[d] Agro Insumos Basicos, SA de CV, Jalisco, MX

[e] Suministros AZ, Baja California Sur, MX

[f] Carboxymethyl cellulose (CMC), IMSA SA de CV, Mexico City, MX

[g] Vitamins: Vit. A, (20,000 UI $g^{-1}$) 90 mg $kg^{-1}$; Vit. B1, 9 mg $kg^{-1}$; Vit. B2, 54 mg $kg^{-1}$; Vit. B5, 90 mg $kg^{-1}$; Vit. B6, 18 mg $kg^{-1}$; Vit. B12, 0.04 mg $kg^{-1}$; Vit. K3, 36 mg $kg^{-1}$; Vit. D3, (850,000 UI $g^{-1}$) 144 mg $kg^{-1}$; Vit. H, 1 mg $kg^{-1}$; folic acid, 3.24 mg $kg^{-1}$; Inositol, 90 mg $kg^{-1}$. Minerals: $CoCl_2$, 20 mg $kg^{-1}$; $H_2MnO_5S$, 3.3 g $kg^{-1}$; $H_{14}O_{11}SZn$, g $kg^{-1}$; $CuH_{10}O_9S$, 1.3 g $kg^{-1}$; $FeSO_4$, 20 g $kg^{-1}$; $Na_2SeO_3$, 50 mg $kg^{-1}$; KI, 330 mg $kg^{-1}$. Sigma Aldrich, USA

[h] Rovimix Stay C 35%, DSM, NL

[i] Sigma Aldrich, USA

**Table 2** Proximate composition of pelleted feed, *U. clathrata*, and *U. lactuca* during the experiment (% dry basis)

| Composition | Pelleted feed | *Ulva lactuca* | *Ulva clathrata* |
|---|---|---|---|
| Protein | 38.1 ± 0.1 | 16.5 ± 2.3 | 23.4 ± 1.7 |
| Ash | 8.2 ± 0.1 | 36.5 ± 2.5 | 37.5 ± 2.3 |
| Lipids | 9.3 ± 0.2 | 0.6 ± 0.2 | 0.7 ± 0.1 |
| Crude fiber | 1.2 ± 0.1 | 3.3 ± 1.2 | 4.2 ± 0.6 |
| NFE | 43.1 | 43.1 | 34.2 |

Values are given as mean ± SD of triplicate determinations. In the case of seaweeds, determinations were made from samples collected every 7 days during the experiment

*NFE* nitrogen-free extract

## Feeding trial

A 4-week feeding trial was conducted to determine the effect on shrimp gut microbiota of a pelleted diet based or supplemented with fresh seaweed in white shrimp *P. vannamei*. Shrimp were donated by Larvas Gran Mar, SA de CV (La Paz, BCS, Mexico) and maintained for 1 week prior the experiment in a 2000-L tank at 28 ± 1.1 °C with constant aeration and fed ad libitum twice a day with commercial feed (Purina, 35% protein and 8% lipids). For the experiment trial, we evaluated five different treatments: only pelleted feed as a control (P), *Ulva lactuca* + pelleted feed (ULP), *Ulva clathrata* + pelleted feed (UCP), only *Ulva lactuca* (UL), and only *Ulva clathrata* (UC). All treatments were evaluated in triplicate, where each replicate consisted of a 50-L fiberglass tank that was aerated and temperature controlled, containing ten juvenile *P. vannamei* (initial weight 0.79 ± 0.06 g) obtained from a commercial hatchery (Larvas Gran Mar, SA de CV) and acclimated for 2 weeks to laboratory conditions (28 °C and 37‰ salinity).

Pelleted feed and seaweed were supplemented ad libitum. In the case of pelleted feed, ratio was adjusted everyday according to the rest of unconsumed feed. Seaweed treatments were supplemented daily with 5 g of fresh seaweed. Every morning, the remaining feed and seaweeds were collected and weighed to determine consumption. Following removal of feed, a 60% water exchange was performed in all experimental tanks. During the experimental period, water temperature (28.4 ± 0.4 °C) and dissolved oxygen (5.2 ± 1.2 mg $L^{-1}$) were monitored daily with a multiparameter YSI 556 (YSI, USA). Every 3 days, total ammonia (0.80 ± 0.4 mg $L^{-1}$), nitrites (<0.25 mg $L^{-1}$), and nitrates (1.5 ± 0.5 mg $L^{-1}$) were analyzed with a colorimetric API saltwater kit, and pH (7.8 ± 0.2) was measured with a Bluelab pH meter pen.

At the end of the feeding experimental period, shrimp performance was determined in terms of final weight, weight gain, specific growth rate (SGR), feed consumption (FC) and seaweed consumption (SC), feed conversion ratio considering only pelleted feed (FCR), total feed conversion ratio including both seaweed and pelleted feed, and survival. The pelleted feed and seaweeds were analyzed for dry matter (Method 930.15; AOAC, 2005), protein (Ebeling 1968), lipids (Method 2003.05; AOAC, 2005), ash (Method 942.05; AOAC, 2005), and crude fiber (Method 978.10; AOAC, 2005). Nitrogen-free extract (NFE) was estimated by difference (calculated as: 100% – protein% – lipid% – ash% – moisture%).

## DNA extraction

After 4 weeks, shrimp intestines were excised from the carcass with sterile scissors and forceps and washed with sterile nuclease-free water to remove fecal matter. The intestines were placed in 2-mL tube with 1 mL 90% ethanol and stored at − 80 °C. before DNA extraction. A total of 5 DNA samples per treatment, composed of 4 different shrimp intestines, were extracted with the UltraClean Microbial DNA isolation kit following the manufacturer's procedures (Mo Bio Laboratories, USA).

## Sequencing and sequence processing

In order to study the impact of a diet supplemented with seaweed on the shrimp gut microbiome, amplicon sequencing of the intestinal microbial community was performed by the Next Generation Sequencing Core at Argonne National Laboratory, Argonne, IL, USA. Briefly, the 16S rRNA gene V4 regions were amplified using primer set 515F (5′-GTGC CAGCMGCCGCGGTAA-3′) and 806R (5′-GGAC TACHVGGG TWTCTAAT-3′) following the method described by Kozich et al. (2013). Amplicons for 16S (pair-ended: 150 × 150 bp) were sequenced using Illumina MiSeq 500-cycle kit with the Illumina MiSeq sequencing system.

Pair-ended bacterial 16S rRNA gene sequences were assembled using Ribosomal Database Project (RDP) paired-end reads assembler (Cole et al. 2014) with assembled read length without primers between 250 and 280 bases (−1250 – L 280). Assembled sequences outside of this range were non-microbial by BLAST. Assembled sequences with an expected maximum error adjusted Q score less than 25 over the entire sequence were eliminated. VSEARCH (2.4.3, 64bit) (Rognes et al. 2016) was used to remove chimeras de novo, followed by removing chimeras by reference using RDP 16S rRNA gene training set sequences (No15). High-quality and chimera-free sequences were then clustered at 97% sequence similarity by CD-HIT (4.6.1) (Fu et al. 2012), resulting in the identification of unique operational taxonomic units (OTUs) and their abundance in each sample. We used CD-HIT because it is fast and produces clusters highly similar to true number of OTUs from simulated complex data (Bonder et al. 2012; Chen et al. 2013). The taxonomy of each

representative OTU sequence was identified using RDP Classifier (Wang et al. 2007) with a confidence cutoff at 50% (− c 0.5).

## Data analyses

The results of shrimp performance under different feeding treatments were analyzed for normality and homoscedasticity with a Shapiro-Wilk and Levene's test, respectively. Data were subjected to one-way analyses of variance (ANOVAs), followed by Tukey's multiple comparison tests if applicable ($\alpha = 0.05$). All statistical analyzes were performed using SPSS statistics 17.0 software.

The alpha diversity index, Chao 1, and Shannon estimators were calculated in R using the packages vegan and the function plot_richness from phyloseq. Good's coverage was calculated to evaluate the sampling depth. Distance matrix was calculated using Bray-Curtis dissimilarity metric and visualized using NMDS. The estimation of microbial diversity coming from microbial community analysis was realized with the chao1 estimator. The differential abundance for each treatment group when compared with the control group was estimated in R package DESeq2, as denoted by log2_fold change. All the above analyses were conducted by R (version 3.2.2; http://www.r-project.org/).

# Results

## Shrimp performance

After 28 days of feeding trial, the treatment with *U. clathrata* + pelleted feed (UCP) showed significantly higher final weight, weight gain, and SGR when compared with the other treatments ($P < 0.05$), except for shrimp fed with only pellet (P) (Table 3). Shrimp seaweed consumption was significantly higher for *U. clathrata* compared with *U. lactuca*; nevertheless, weight loss was observed in shrimp fed only with seaweeds (UL or UC).

For treatments fed with only pelleted feed or in combination with seaweed, no significant differences ($P > 0.05$) were found for FCR or total FCR. In terms of survival, treatments P, ULP, and UCP showed significant higher survival (≥ 97%) compared with shrimp fed with UL (23%) and UC (65%).

## Comparison of gut microbial composition

### Bacterial diversity

An average of 20,650, reads per sample were obtained (25 samples with 5 replicates per treatment). The sequences were clustered into operational taxonomic unit (OTUs) at 97% similarity. Chao 1, Shannon, and InvSimpson indices presented in Fig. 1 were higher In the UL treatment than the other treatments, while the lowest index was obtained with UCP treatment (Individual replicate results of diversity are presented in Online Resource 1).

Figure 2 shows the NMDS plot of the bacterial intestinal communities from the different treatments after the 4-week feeding trial. Samples from shrimp fed with the pelleted feed grouped closest to those fed with ULP or UCP, suggesting that the shrimp intestines from the three treatments share similar microbial features. A highly contrasting difference was observed in microbial communities found in shrimps fed with UC or UL compared with the control (P).

### Taxonomic distribution of shrimp gut bacteria

The 16S rRNA profile of relative abundances at phylum level is shown in Fig. 3 (Individual replicate results of abundances are presented in Online Resource 2). *Proteobacteria* was the most abundant phylum in all treatments, followed by *Bacteroidetes* in UC, UCP, and UL and *Actinobacteria* for P and ULP treatment. The greatest difference in microbial composition was observed for the treatment UL, with only an average of 32% relative abundance of *Proteobacteria* comparing to an average of 87% in control (P). Additionally, increases in relative abundances of the phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Planctomycetes*, and *Cyanobacteria* were observed in UL. The relative abundances of Firmicutes and *Bacteroidetes* on UC and ULP treatments were lower in contrast with the control (P).

Figure 4 showed the general distribution of the top 10 bacterial genus, obtaining a different distribution with the inclusion of seaweed treatment compared with control. The relative abundance of *Vibrio* was higher in the control (P) and obtained a decrease when shrimp fed only with seaweed (UC and UL treatments). The higher difference on abundance at genus level compared with control was obtained with UL treatment, with a significant increase of *Rhodococcus*, *Spongiimonas*, unclassified *Clostridiales* and unclassified *Enterobacteriaceae* ($P < 0.01$) while a decrease on *Vibrio* and *Aliiroseovarius* genus. The abundances of the pathogenic bacteria genus *Flavobacterium* and *Pseudomonas* were higher on UL treatment, while their relative abundance was similar between UC, UCP, ULP, and P control. In the case of genus frequently employed as probiotics, *Bacillus* and *Streptococcus* were higher on UL treatment and *Lactobacillus* genus for UC and ULP treatments, however not significantly higher compared with the control (P). There were no significant differences on *Paracoccus* genus for all the treatments evaluated. Additionally, in the case of shrimp fed with seaweeds, we found significantly higher abundances of *Rubritalea*, *Lysinibacillus*, *Acinetobacter*, and *Blastopirellula genera* for *U. lactuca* treatments (UL, ULP) and *Litoreibacter* for

**Table 3** Growth performance, feed utilization, and survival after 4-week experimental trial with shrimp *P. vannamei* fed only pellet (P), *U. lactuca* + pellet (ULP), *Ulva clathrata* + pellet (UCP), only *U. lactuca* (UL), and only *U. clathrata* (UC)

| | P | UC | UCP | UL | ULP |
|---|---|---|---|---|---|
| Final weight (g) | $3.63 \pm 0.14^{bc}$ | $0.78 \pm 0.01^{a}$ | $3.92 \pm 0.03^{c}$ | $0.77 \pm 0.01^{a}$ | $3.51 \pm 0.18^{b}$ |
| Weight gain (%) | $359 \pm 18^{bc}$ | $-2 \pm 1^{a}$ | $395 \pm 5^{c}$ | $-2 \pm 1^{a}$ | $345 \pm 22^{b}$ |
| SGR (% $day^{-1}$) | $5.44 \pm 0.3^{bc}$ | $-0.07 \pm 0.09^{a}$ | $5.71 \pm 0.2^{c}$ | $-0.08 \pm 0.46^{a}$ | $5.32 \pm 0.4^{b}$ |
| FC (g) | $3.62 \pm 0.14^{ab}$ | – | $3.73 \pm 0.08^{b}$ | – | $3.34 \pm 0.19^{a}$ |
| SC (g) | – | $6.42 \pm 0.13^{d}$ | $2.97 \pm 0.10^{c}$ | $0.85 \pm 0.05^{b}$ | $0.57 \pm 0.11^{a}$ |
| FCR | $1.28 \pm 0.08$ | – | $1.19 \pm 0.02$ | – | $1.23 \pm 0.07$ |
| Total FCR | $1.28 \pm 0.08$ | ND | $1.29 \pm 0.02$ | ND | $1.25 \pm 0.07$ |
| Survival (%) | $100^{c}$ | $65 \pm 7^{b}$ | $97 \pm 3^{c}$ | $23 \pm 6^{a}$ | $100^{c}$ |

Values are given as mean ± SD by triplicate determinations. Means with the same superscript are not significantly different ($P < 0.05$). Weight gain (%) = (final weight-initial weight)/initial weight × 100, SGR (% $day^{-1}$) = 100 (ln(average final weight) – ln(average initial weight))/number of days

FC = pelleted feed consumed per shrimp

SC = seaweed consumed per shrimp (fresh weight)

FCR = pelleted feed consumed (g)/wet weight gain (g)

Total FCR = pelleted feed consumed (g) + seaweed consumed (g in dry basis) / wet weight gain (g)

Survival (%) = final number of shrimp/initial number of shrimp X 100

ND: not determined

*U. clathrata* treatments (UC, UCP) compared with the control (P). Moreover, in the present work, control treatment (P) resulted in a significantly higher abundance of *Agarivorans* and *Pseudoalteromonas* compared with treatments with *U. clathrata* (UC, UCP) and also higher abundance of *Demequina* and *Shimia* compared with only seaweed treatments (UL, UC) and *Planctomicrobium* for all seaweed treatments (UL, UC, UCP, and ULP) ($P < 0.01$). On the other hand, the cellulose-degrading bacteria such as *Actinomyces* had a higher abundance on UCP and UL against control. On *Anoxybacillus*, there are no significant differences between treatments, and *Clostridium*, *Citrobacter*, and *Leuconostoc* were not detected in all treatments.

## Shared microbial population

The Venn diagrams showed specific and common OTUs of all treatments. In the case of all treatments, the diagrams indicated 45 OTUs overlap, while 11, 15, 9, and 11 OTUs were specific of P, UC, C, and ULP treatments, respectively. Besides, 211

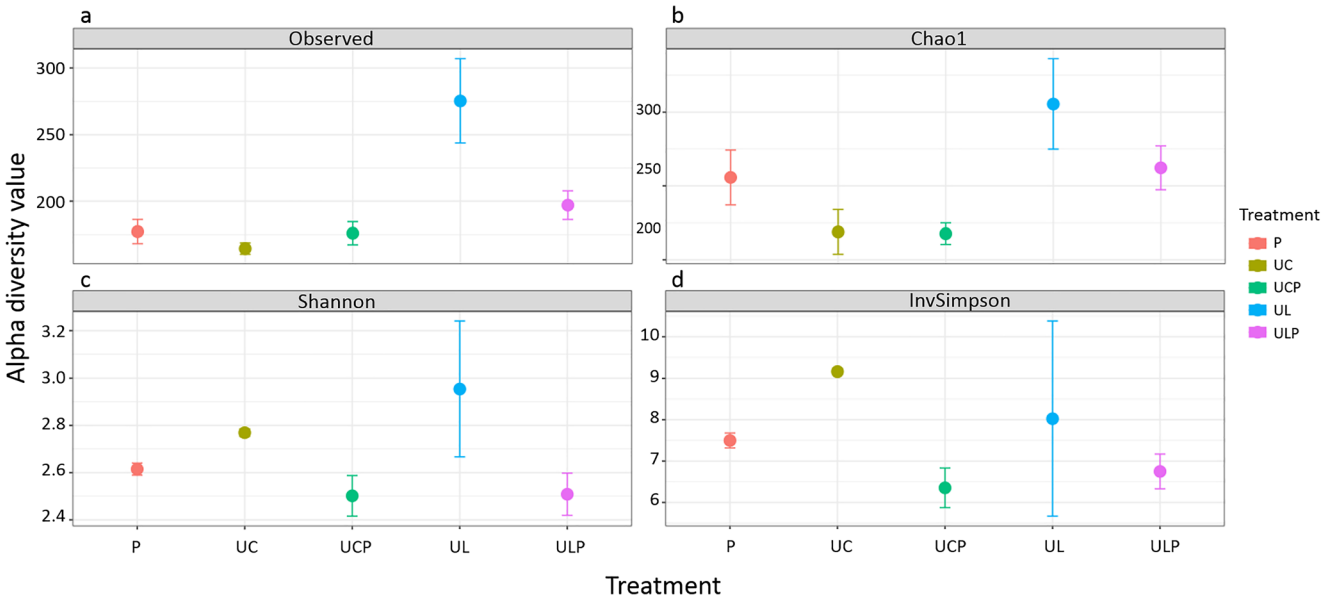


**Fig. 1** Boxplot of richness and bacterial diversity. Observed OTUs (**a**), Chao 1 (**b**), Shannon (**c**), and InvSimpson (**d**) indices used to estimate bacterial diversity. Treatments: only pellet (P), only *U. clathrata* (UC), *U. clathrata* + pellet (UCP), only *U. lactuca* (UL), and *U. lactuca* + pellet (ULP)

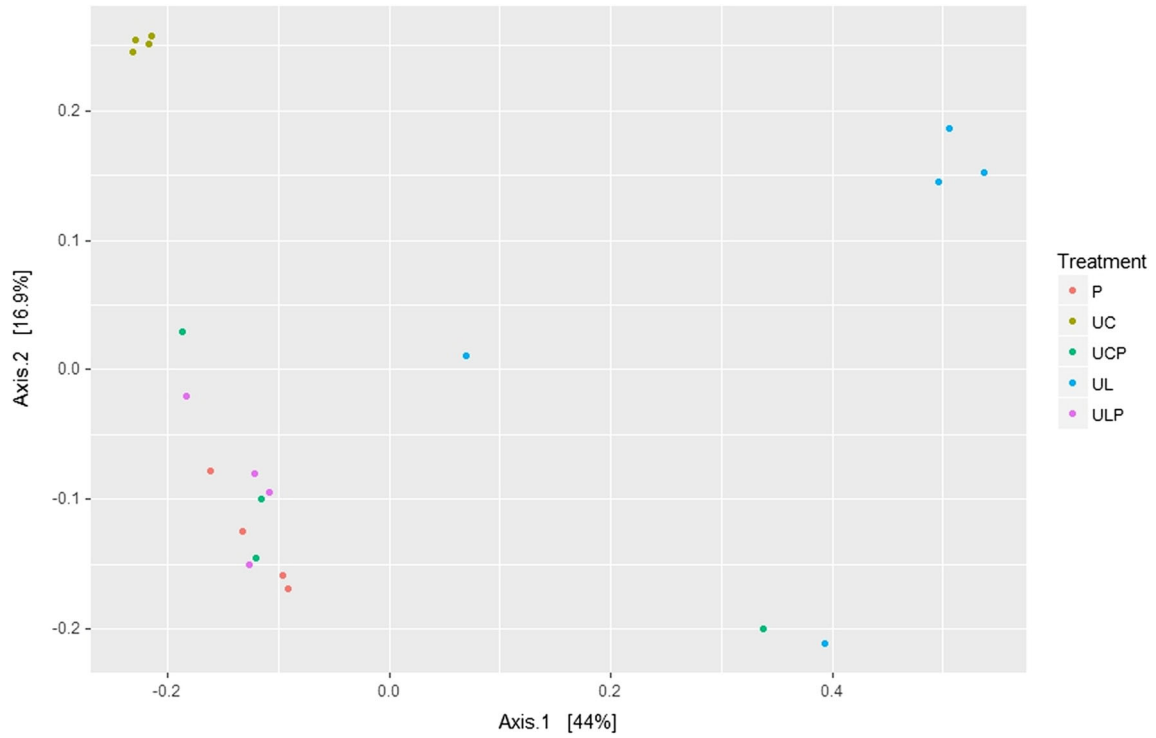


**Fig. 2** Nonmetric multidimensional scaling (NMDS) ordination based on a distance matrix using Bray-Curtis dissimilarity metric and of the pelleted feed as a control (P), *U. lactuca* + pelleted feed (ULP), *U. clathrata* + pelleted feed (UCP), only *U. lactuca* (UL), and only *U. clathrata* (UC) treatments

OTUs were found for UL treatment, showing the highest number of OTUs that were specific (Fig. 5).

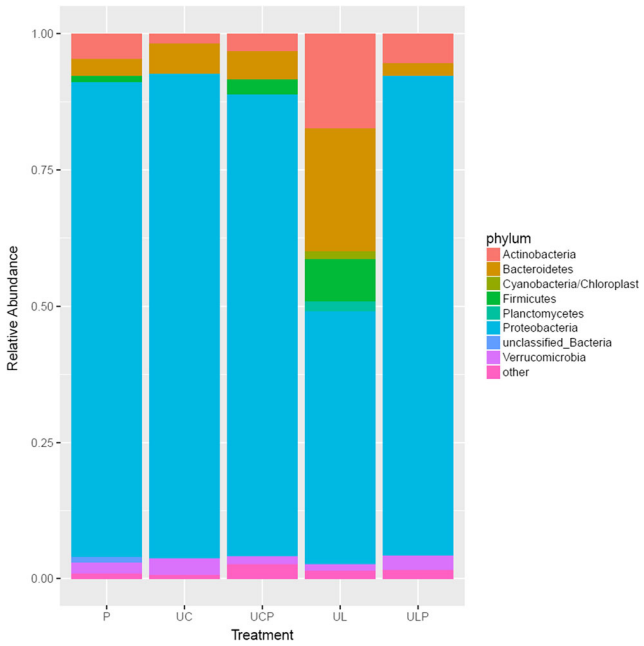


**Fig. 3** Relative read abundance of different bacterial phyla in each treatment (P, ULP, UCP, UC, UL). Sequences that could not be classified into any know group were assigned as "unclassified_Bacteria" or "other")

## Discussion

The proximal composition of seaweeds may vary according to geographic distribution, seasonal variations, and nutrient availability in water, among other factors (Lahaye et al. 1995; Marinho-Soriano et al. 2006; Peña-Rodríguez et al. 2011). The seaweeds employed in the present work (Table 2) were in a close range of proximal composition presented by other authors in seaweeds cultured under control systems or in integrated aquaculture systems. For *U. clathrata*, dry weight composition has been reported in a range between 20 to 26% protein, 38 to 49% ash, 0.4 to 1.5% lipids, and 4.2 to 5.6% crude fiber (Cruz-Suárez et al. 2010; Peña-Rodríguez et al. 2011, 2017b), meanwhile for *U. lactuca*, 13 to 25% protein, 24 to 37% ash, 1 to 1.5% lipids, and 3.3 to 5.3 crude fiber (Khoi and Fotedar 2011; Santizo et al. 2014; Pallaoro et al. 2016; Omont et al. 2019).

In terms of shrimp performance, *U. lactuca* did not improve shrimp growth when consumed with pelleted feed, as also described for western king prawn (*Penaeus latisulcatus*) in a closed recirculating system (Khoi and Fotedar 2011). Brito et al. (2014) described a similar effect, with no differences observed in growth in an intensive system of *P. vannamei* without seaweed compared with shrimp cultured with *U. lactuca*. However, in that same study, an improvement of weight gain was shown when biofloc and seaweed were evaluated compared with only biofloc, but no significant

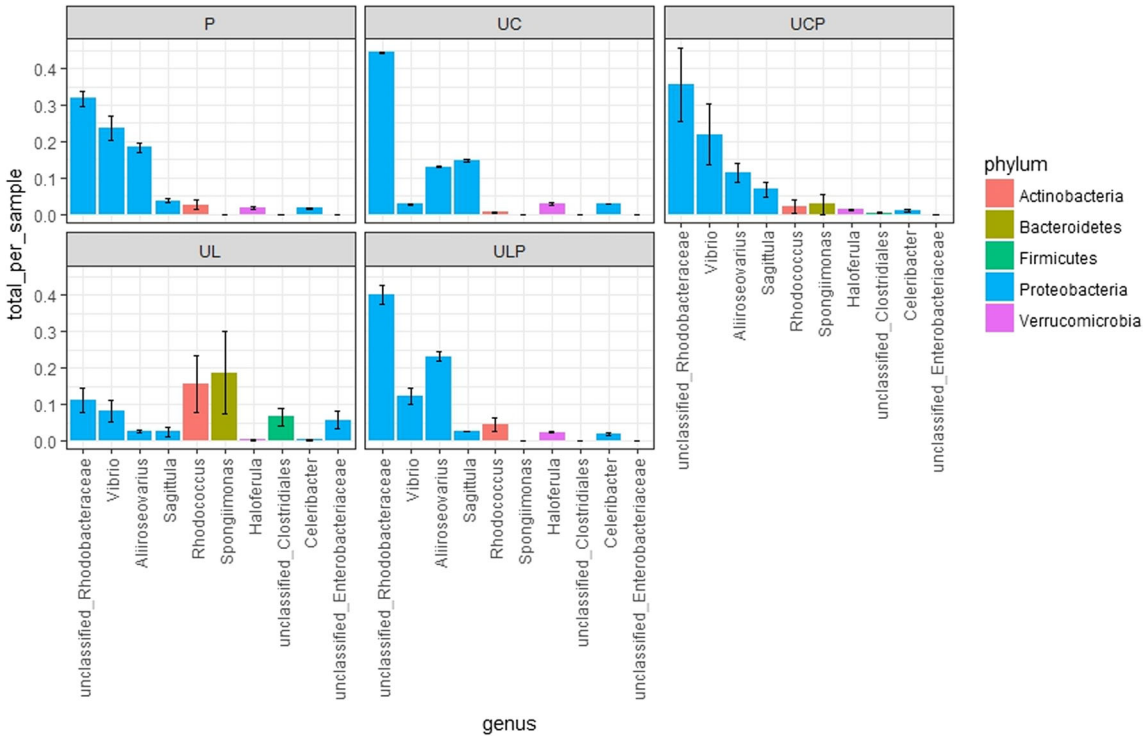


**Fig. 4** Comparative bacterial composition with the top 10 most abundant genera in each treatment (P, UC, UCP, ULP, and UL)

differences were shown in FCR. Pallaoro et al. (2016) found that *U. lactuca* may partially replace commercial feed up to 50% without significant differences on growth compared with *P. vannamei* fed only with commercial feed, whereas Laramore et al. (2018) found that only up to 25% replacement was possible. In contrast with *U. lactuca*, shrimp co-fed with *U. clathrata* and pelleted feed resulted in a 10% higher growth compared with shrimp fed only with pelleted feed, but without presenting significant differences. This growth enhancement has also been observed in outdoor co-culture systems of *P. vannamei* and *U. clathrata*, where up to 45% reduction of pelleted feed ratio resulted in a significant increase of weight gain compared with shrimp under monoculture system (Cruz-Suárez et al. 2010). In a clear water indoor experiment, white shrimp *P. vannamei* and brown shrimp *Farfantepenaeus californiensis* fed with fresh *U. clathrata* and 50% less pelleted feed resulted in similar growth than shrimps fed with pelleted feed ad libitum without seaweed (Gamboa-Delgado et al. 2011; Peña-Rodríguez et al. 2017a).

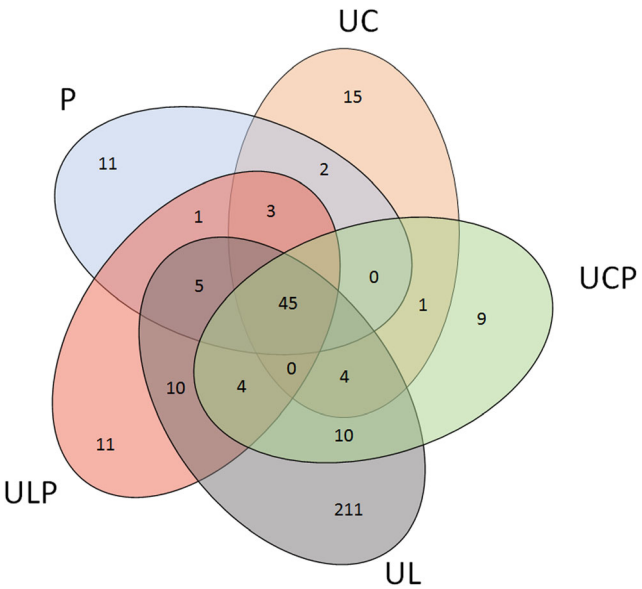


**Fig. 5** Shared OTU analysis of the libraries. Venn diagram showing the distribution of all operational taxonomic units (OTUs) unique and shared by P, UC, UCP, ULP, and UL treatments

On the other hand, when comparing the treatments with the supplementation of the two different seaweeds, shrimp fed with *U. clathrata* + pelleted feed (UCP) obtained a significantly ($P < 0.05$) higher final weight, weight gain, SGR, and seaweed consumption than treatment with *U. lactuca* + pelleted feed (ULP). These effects on growth may be due to differences on seaweed consumption attributed to the shape as thin filaments of *U. clathrata* that facilitate the ingestion by shrimp. In the case of shrimp fed only with seaweeds, the poor growth and survival of shrimp is attributed to the lower content in lipids and protein compared with a balanced feed as reported by Cruz-Suárez et al. (2010).

Several factors contribute to the composition of intestinal bacterial communities, including diet, genetics, and environment (Zhang et al. 2014), and it has been shown that they are key on different metabolic process in their host, including nutrient absorption, degradation, and vitamin production (Hooper et al. 2002; Bäckhed et al. 2004; Turnbaugh et al.

2006; Daniel et al. 2014). However, despite the important role of the intestinal bacteria in their host, research focused on the shrimp gut microbiome and the changes generated by growing and feeding conditions is relatively sparse. We analyzed the differences on gut microbial community between shrimp feed with commercial pellet and/or green seaweed (*U. lactuca* and *U. clathrata*) to determinate the influence on the complementation of the green macroalgae in shrimp diet. Results suggested that all pair treatments contributed significantly to community variations, except when compared P and UCP. The most significant difference in community structure was observed in the treatments UC and UL ($P < 0.01$). Even when both seaweeds are feasible to be consumed by shrimp (Table 3), the higher consumption of pelleted feed in terms of dry weight reflects a higher impact on bacterial communities in the shrimp intestine (Fig. 2). *Proteobacteria* was the dominant phylum in all treatments, in agreement with past research (Zhang et al. 2014; Cardona et al. 2016; Huang et al. 2016). *Proteobacteria* are widely distributed in the marine environment and important for nutrient cycling process (Kersters et al. 2006). The second most abundant phylum in UC, UCP, and UL treatments was *Bacteroidetes*, which agrees with Huang et al. (2016) and Cardona et al. (2016). However, some authors (Zhang et al. 2014; Cornejo-Granados et al. 2017; Qiao et al. 2017) have reported a relative low abundance of *Bacteroidetes*. This difference in *Bacteroidetes* abundances could be a result of differences on culture system and diet. In the case of P and ULP treatment, the second most abundant phylum was *Actinobacteria*, which has also been reported as the second most abundant phylum in shrimp by Qiao et al. (2017).

Bacteria belonging *Vibrio* are part of the microflora of most aquatic habitants but represent a potential pathogen for shrimp culture (Sung et al. 1999, 2001; Liu et al. 2004), with serious economic losses in the aquaculture industry during vibriosis outbreaks. Our results show a decline of *Vibrio* in UC, UL, and ULP treatments compared with the control with only pellet (P) feed, with the lowest detection in the UC treatment (Fig. 4). This reduction in *Vibrio* was also observed by Niu et al. (2018) who found a reduction on abundance of harmful bacteria as *Vibrio* in *P. vannamei* fed with feed with different levels of inclusion of *Porphyra haitanensis*. However, future research is required to determine if changes in *Vibrio* species belong to pathogenic strains in these systems. In addition, we observed an increase of *Bacillus* with UL treatment, and *Lactobacillus* is the UC and ULP treatments compared with the control (P), which have been reported as beneficial in the cultivation of shrimp (Zokaeifar et al. 2012; Swapna et al. 2015).

The significant changes in abundances of genera such as *Rubritalea*, *Lysinibacillus*, *Acinetobacter*, *Blastopirellula*, and *Litoreibacter* increase in shrimp fed with *Ulva* sp., and the significant abundance reduction in other genera like *Planctomicrobium*, *Agarivorans*, *Pseudoalteromonas*, *Demequina*, and *Shimia* indicates that seaweeds have a high influence in the intestinal community composition. In the case of *Actinomyces*, *Anoxybacillus*, *Leuconostoc*, *Citrobacter*, and *Clostridium,* associated previously on cellulose degradation (Wu et al. 2012), we found an increase in *Actinomyces* in UL and UCP treatments compared with the control (P). This could be due to the important role of these bacteria in the degradation of food in the intestine, especially after increasing the cellulose contents in the diet with the addition of macroalgae. The genera *Citrobacter*, *Clostridium*, and *Leuconostoc* were not detected in any of the treatments in the present work.

The Venn diagram obtained in the OTUs overlapping showed that 45 OTUs are shared by the five treatments analyzed, while 11 OTUs were specific to control (P), 15 for UC, 9 for UCP, 211 for UL, and 11 for ULP treatment (Fig. 5). In all cases, the OTUs specific for the different treatments were very similar, except for the UL treatment. This was possibly due to the large changes in the microbiome of the shrimp intestine associated with the stress caused by the low nutritional coverage provided by the feed based only with *Ulva lactuca*. To our knowledge, this is the first report of intestinal microbiome of shrimp associated with the presence of green seaweed as partial and completed replacement of commercial feed. Further research will help to understand in detail the effect caused by changes on intestinal bacterial community during aquaculture multitrophic integration.

In summary, our results showed no significant differences in survival, growth performance, or feed utilization of *P. vannamei* fed with pellet and fresh seaweeds *U. clathrata* or *U. lactuca* compared with pellet feed. Shrimp fed only with seaweed showed no growth and significantly lower survival compared with the rest of the treatments. Intestinal bacterial communities of shrimp were modified by the incorporation of seaweeds in the diet, with a decrease of *Vibrio* except for UCP. The present study strengthens our understanding of the microbial communities necessary for integration of green seaweed in shrimp aquaculture.

**Acknowledgments** We are thankful to Gran Mahr laboratory, especially Samuel Echeverria and Omar Camorlinga for kindly donating the shrimp juveniles; Armando León from Algal Tech SAPI de CV for providing *U. clathrata* seaweed; Sandra de la Paz-Reyes from Laboratory of Aquaculture Nutrition, Pablo Monsalvo-Spencer, and Gabriel Robles Villegas from the laboratory for acclimatization and maintenance of aquatic organisms; and Gabriela Mendoza-Carrión from Genomics and Bioinformatics Laboratory at CIBNOR for all the facilities and technical support during the experiment development. This work was performed within the framework of a UBO/CIBNOR agreement to receive Master's degree candidates from UBO to complete their MS thesis project.

**Data availability statement** Data available from corresponding author on request.

**Funding information** This work was supported by a grant to Maxence Gemin by the "Laboratoire d'Excellence" LabexMER (ANR-10-LABX-19) and co-funded by a grant from the French government under the program "Investissements d'Avenir".

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

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