# Possible role of intestinal obligate anaerobic bacteria in the digestive process of Pacific white shrimp *Litopenaeus vannamei*

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## ABSTRACT

The important role of the normal anaerobic intestinal microbiota has been elucidated in humans and some domesticated animals, however, the presence of intestinal anaerobes, let alone their importance in invertebrates are largely unknown. The purpose of this research was to isolate and to identify obligate anaerobic members of the intestinal microbiota of the Pacific white shrimp, *Litopenaeus vannamei*. Intestinal samples for microbial isolation were taken from shrimp maintained at three salinities (3, 11, 31 ppt) and on a diet containing different protein concentrations. Members of the anaerobic intestinal microbiota were successfully isolated and cultivated in a Bactron II anaerobe chamber. Many of these isolates could not be identified using available biochemical and molecular-level techniques and databases, and consequently, may represent new, yet undescribed taxa. Of the isolates identified, the majority belonged to the genus *Clostridium*. Strains of the species, *C. subterminale* and *C, beijerinckii* were delineated by biochemical tests, while two species, *C. butyricum* GC subgroup A and C. *bifermentans* GC subgroup A were identified by whole-cell fatty acid methyl ester analysis. Isolates were tested for the expression of constitutive enzymes using the APIZYM™ system. The anaerobic bacteria demonstrated enzyme activities of acid and alkaline phosphatases, C4 and C8 esterases, C14 lipases, arylamidases, and glycosidases. Enzyme activities differed with isolates. Generally, strains identified as *C. butyricum* expressed

enzymes for the breakdown of carbohydrates, while strains identified as *C. bifermentans* produced enzymes for the breakdown of proteins. Most isolates formed phosphatases possibly used for absorption of nutrients.

Keywords: Shrimp: Anaerobic bacteria: Bacterial enzymes
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## 1. INTRODUCTION

The important role of the normal obligate anaerobic intestinal microbiota has been elucidated in humans and domesticated animals such as ruminants, pigs, and chickens. The presence of anaerobes let alone their importance in lower vertebrates and invertebrates is largely unknown. The role of obligate anaerobic microorganisms in digestion, nutrition, disease production or prevention has never been thoroughly investigated in lower animals. Moreover, the normal anaerobic microbiota colonizing the gut of important food-fish and shellfish maintained in aquaculture largely remains unknown. Identifying members of the diverse microbial populations in the intestinal ecosystem may favorably impact the propagation of species in aquaculture: once identified, understanding the physiological interactions between the normal microbiota and host may have important implications. For example, new dietary formulations that include members of the normal intestinal anaerobic microbiota as probiotics may effectively combine enhanced nutrition with disease prophylaxis.

Studies from vertebrates have provided evidence of the bacterial influence on digestion. For example, protozoans residing in the stomach of cows break down cellulose from ingested plant material. Bacteria and fungi also provide useful nutrients such as volatile fatty acids, microbial proteins, and B vitamins (McSweeney et al., 1999). Intestinal microbes found in symbiotic relationships with humans are involved in the synthesis of vitamin K and the manufacturing of bile acids, bilirubin, cholesterol, short-chain fatty acids, biotin, and folic acid (Neish, 2002; Wallace, et al., 1996). Crustaceans, like other animals, have

intestinal systems that are heavily colonized by microorganisms. The microbial influence on crustacean digestion and nutrient absorption has not been as thoroughly investigated in invertebrates.

## 2. MATERIALS AND METHODS

### 2.1 Shrimp Culture - Diet Study

Shrimp intestines for bacterial isolation and characterization were obtained from The Oceanic Institute, Waimanalo, Hawaii. Specific pathogen-free (SPF) Pacific white shrimp, *Litopenaeus vannamei*, were raised in biosecure 58-$m^2$ covered raceways in the summer of 2001. Water in the raceways was recirculated through a bead filter designed for clarification and biofiltration. The raceways were stocked with juvenile shrimp at a density of 302/$m^2$ and an average stocking weight of 2.14 g per shrimp. Shrimp received a commercial 35% protein feed with 2.5% squid *ad libitum.* After the first seven weeks of the study the diet was changed to 25% protein with 2.5% squid for three weeks. At week ten, shrimp were returned to the original commercial diet. Shrimp were monitored for growth and survival for a total of 12 weeks.

### 2.2 Shrimp Culture - Salinity Study

SPF Pacific white shrimp, *L. vannamei*, were raised in a 337-$m^2$ round pond lined with a substrate of pea gravel. At an initial salinity of 34 ppt the pond was stocked with juvenile shrimp with a density of 48/$m^2$ and an average stocking weight of 6.5 g per shrimp from June 3 to September 16, 2002. Salinity was decreased gradually over a period of eleven days from 34 ppt to 2 ppt to acclimate the shrimp. More specifically, salinity was reduced from 34 ppt to 15 ppt over a period of three days, from 15 ppt to 6 ppt over a three day period, from 6 ppt to 3 ppt in three days, and finally from 3 ppt to 2 ppt over a two day period. Salinity remained at 2 ppt for 13 weeks, was increased to 11 ppt by week 15, and finally raised to 31 ppt by week 16. The transition to higher salinity was accomplished by adding a continuous inflow of seawater at week 14. Shrimp

were fed a commercial 35% protein feed with 2.5% squid *ad libitum* throughout the entire study.

2.3 Bacterial Sampling

**Diet Study:** Ten shrimp were harvested weekly for 12 weeks. Shrimp were weighed prior to the removal of intestines. Mid- and hindguts were aseptically excised, placed in Anaerobic Transport Medium (Anaerobe Systems, Morgan Hill, CA)(one shrimp intestine per tube) and sent to the Fish Disease Laboratory at California State University Hayward (CSUH) by overnight courier. Intestines were sampled for anaerobic bacteria.

**Salinity Study:** Nine samples, each composed of ten individual shrimp intestines, were collected in approximately two-week intervals. The mid- and hindguts of harvested shrimp were aseptically excised, individually placed in Anaerobic Transport Medium and sent by overnight courier to the Fish Disease Laboratory at CSUH. In addition, an initial baseline sample, composed of ten individual shrimp intestines was collected from a holding tank maintained at a salinity of 34 ppt. Over the course of the study, seven samples were collected at 2 ppt (weeks 1 through 13), one at 11 ppt (week 15), and one at 31 ppt salt concentration (week 16).

2.4 Bacterial Isolation and Biochemical Analysis

Traditional light microscopy and biochemical analyses were performed to differentiate microbes to the genus level. Sample preparation and microbial isolation followed established procedures (Dixon and Ramirez, 2001; Ramirez and Dixon, 2003). Media used were commercially available as pre-reduced anaerobically sterilized (PRAS; Anaerobe Systems, Morgan Hill, CA). Intestinal samples were individually placed in thioglycollate broth and incubated for 48 h in an anaerobic atmosphere (5% $CO_2$, 5% $H_2$ and 90% $N_2$) in a Bactron II Anaerobic Chamber (Anaerobe Systems, Morgan Hill, CA) maintained at 28°C. Microorganisms from the thioglycollate broth were plated onto Phenylethyl Alcohol Agar (PEA) for 48 h to inhibit the growth of Gram-negative bacteria.

Isolated colonies from the PEA plates were plated onto Egg Yolk Agar (EYA) for 48 h for enzyme analysis for lipase, lecithinase and protease. At the time of the EYA plate inoculation, the same cultures were plated onto Chocolate Agar for aerotolerance testing to identify obligate anaerobes. The Chocolate Agar plates were aerobically incubated supplemented with 5% $CO_2$. Isolates that did not grow on Chocolate Agar but grew on EYA were considered to be obligate anaerobes. Obligate anaerobic microorganisms from the EYA plates were stained by the Gram method. Pure cultures were plated on Brucella Blood Agar (BBA) to determine sensitivity to kanamycin (1,000 µg), colistin (10 µg), and vancomycin (5 µg). BBA was used to test for nitrate reduction, urease, and tryptophanase production. Periodically, colonies were checked for purity and endospore formation. Slides for microscopic staining were initially prepared and dried inside the anaerobic chamber (Sutter et al., 1986; Engelkirk et al., 1992; Summanen et al., 1993).

To determine the enzyme profile, bacterial isolates were inoculated into an APIZYM rapid detection test system consisting of 19 substrates and processed according to manufacturer's recommendations (bioMerieux, Vitek, Inc., Hazelwood, MI). This colorimetric assay was incubated for 4 h at 35°C. Following the addition of reagents the intensity of color development showed the presence and concentration of enzyme activity.

## 2.5 Specimen Preparation and Identification by Whole-cell Fatty Acid Methyl Ester Analysis

Species-level differentiation of the isolates was based on whole-cell fatty acid methyl ester (FAME) analysis following manufacturer's recommendations (MIDI, Newark, DE). FAME analysis was only performed on bacterial isolates from the salinity study. In short, pure isolates were first inoculated in thioglycollate broth for 48 h at 28°C then sub-cultured three times 48 h apart on BBA (Anaerobe Systems, Morgan Hill, CA). Of the third subculture ~50 mg biomass was transferred into a glass tube and capped with a Teflon-lined cap. Whole-cell lipids were saponified at 95°C using sodium hydroxid prepared in

aqueous methanol. Fatty acids were methylated at 80°C in aqueous methanol acidified with hydrochloric acid and extracted with a 1:1 hexane/methyl-*tert*-butyl ester mixture. Finally, the methyl ester derivatives were washed with a diluted sodium hydroxide solution and injected into a Model 6790 HP gas chromatograph using a flame ionization detector (FID). Bacteria were identified by the Sherlock software package, Version 4.0 (MIDI, Newark, DE). The chromatograms were compared using the dendrogram and the 2D plot subroutines of the same program.

## 3. RESULTS

### 3.1 Shrimp in Diet Study

During the 12 weeks of the diet study, shrimp grew to an average weight of 17.75 g. The average shrimp weight at harvest was 19.9 g. (THIS DOESN'T MAKE SENSE!) Overall, shrimp biomass production was 5.2 kg/m$^2$ with survivorship reaching 86%. The amount of water used to produce one kilogram of whole shrimp was 352 l. Results from these trials indicated that high shrimp biomass was maintained under biosecure conditions at zero-water exchange.

### 3.2 Bacteria Recovered in Diet Study

Facultative and obligate anaerobic bacteria were detected in this study, however, only obligate anaerobes were further investigated. All isolates of interest were identified as Gram-positive bacteria. The majority of these isolates (93%) formed endospores (Fig. 4) and was presumptively identified as *Clostridium* spp. The other 7% were non-endospore forming Gram-positive bacteria (5% Gram-positive rods and 2% Gram-positive cocci).

### 3.3 Bacterial Enzymes Produced in Diet Study

Isolated obligate anaerobic bacteria produced enzymes including proteases, phosphohydrolases, lipases, carbohydrases, and phosphatases . Initially, the number of isolates producing esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase was highest in week 1 but then decreased to

only one or two isolates producing these enzymes by week 2. Leucine arylamidase production followed a similar trend though the highest number of isolates producing this enzyme was detected in week 10. By week 6 the number of isolates producing esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, and leucine arylamidase increased. Acid phosphatase initially was produced by two isolates, then the number decreased to one isolate by week 3 to increase later with some fluctuations and to peak in week 6. Then, it decreased in weeks 7 and 8 with no bacterial isolates recovered in the remainder of the study. In addition, one bacterial isolate produced the enzyme alkaline phosphatase in week 1, two in week 4 and one in week 8. Alkaline phosphatase was not detected at any other times in this study.

A lower protein content diet was administered to shrimp in week 7 of the study. This change coincided with a change in the production of bacterial enzymes; some previously detected enzymes such as lipases, esterases, a protease, carbohydrase, and phosphatases were not detected after the diet change. Specifically, leucine arylamidase ceased to be produced by any isolate in week 8 but the number of isolates producing it increased in week 10. In addition, the number of isolates producing this enzyme continued to decline towards the end of the study with only one isolate producing it in week 12. Production of naphthol-AS-BI-phosphohydrolase was briefly interrupted at week 7, continued to be produced by fewer isolates in weeks 9, 10, and 11. Isolates did not produce naphthol-AS-BI-phosphohydrolase at week 12. Acid phosphatase and alkaline phosphatase both ceased to be produced from week 9 on. The production of alkaline phosphatase was sporadic. The enzyme lipase (C14) was only produced by one isolate from weeks 5 and 6 and was not detected in the remainder of the study. Esterase lipase (C8) was produced by isolates until week 6, while esterase (C4) continued to be produced until week 7. Neither enzyme was produced by any isolates beyond week 7. This pattern continued for the duration of the study even though the initial diet was resumed at week 10.

3.5 Shrimp in Salinity Study

After a period of about 4 months, shrimp grew with a growth rate of 1.27 g per week to a final average weight of 19.08 g. The average shrimp weight at harvest was 26 g (THIS DOESN'T MAKE SENSE!). The overall shrimp biomass production reached 1.1 kg/$m^2$ and survivorship 88.5%. Results from these trials indicated a high survivorship and high growth rate in a low salinity environment.

3.6 Bacteria Recovered in Salinity Study

Both facultative and obligate anaerobic bacteria were detected in this study, however, only obligate anaerobes were investigated. All isolates of interest were initially identified as Gram-positive bacteria. The majority of the obligate anaerobic isolates (85%) formed endospores and was presumptively identified as *Clostridium* spp. The other 15% were non-endospore forming Gram-positive bacteria (14% Gram-positive rods and 1% Gram-positive cocci). The initial baseline shrimp sample collected at 34 ppt salt concentration showed no evidence of obligate anaerobic bacteria but facultative anaerobes were detected. Few obligate anaerobic bacteria were isolated until weeks 11 and 13 when there was an increase in the number of isolates and the largest numbers of obligate anaerobes were detected. This number declined by week 15 when salinity was increased to 11 ppt. Furthermore, no obligate anaerobic bacteria were found in week 16 when the salinity reached 31 ppt, although facultative anaerobes were still detected.

Identification of bacterial isolates made it possible to detect changes in the microbial community structure present in *L. vannamei* throughout this study. In week 1 (2 ppt salt concentration), *Clostridium subterminale*, strains identified as *Clostridium* spp., and unidentified isolates were detected. Three isolates identified as *Clostridium beijerenckii*, some as *Clostridium* spp., and some unidentified isolates were found in week 3 at 2 ppt salt concentration. In week 5 (2 ppt salt concentration), two main bacterial groups were detected, *C. butyricum*-GC-subgroup A and *C. bifermentans*-GC-subgroup A, along with some unidentified isolates. In week 7, only strains of *C. bifermentans*-GC-subgroup A were identified. A number of isolates from week 7 originally detected on WHAT

PLATE was not subjected to FAME analysis because they could not be kept viable. *Clostridium butyricum* GC-subgroup A and *C. bifermentans* GC subgroup A, *Clostridium* spp., and a few unidentified isolates were detected in week 9 at 2 ppt salt concentration. A similar trend continued in weeks 11 and 13 (2 ppt salt concentration) when the largest numbers of isolates were detected. The same trend continued for week 15 when the salinity was increased to 11 ppt, but the overall number of isolates decreased with only *C. bifermentans* GC-subgroup A and *C. butyricum* GC-subgroup A being identified. Finally, for week 16 at 31 ppt salt concentration, no obligate anaerobic isolates were detected. Of all 115 strains isolated 38% were identified to the genus level (24% to species level) by FAME analysis, 52% remained unidentified (though their FAME fingerprint could still be used for comparison), and 10% died prior to FAME analysis.

### 3.7 Bacterial Enzymes Produced in Salinity Study

Bacterial isolates were found to produce different types of enzymes. Overall, the isolates produced enzymes including glycosidases (carbohydrases), esterases, lipases, phosphatases and proteases.

The greatest number of isolates producing esterase (C4) was found in the initial 2 ppt salt concentration sample. No isolate produced esterase (C4) during week 3. In subsequent weeks the number of esterase (C4) producing isolates remained low until week 13, when the number increased. Once salinity increased to 11 ppt in week 15, the number of esterase (C4) producing bacterial isolates decreased. Similarly, both lipase (C14) and esterase lipase (C8) displayed almost the same expression trend, except for the finding that lipase (C14) was not produced by any isolates in weeks 1, 3, and 5. In week 15 (11 ppt salt concentration), no bacteria were found to produce either enzyme.

The variety of enzymes produced by bacterial isolates increased at 2 ppt salt concentration during the weeks 11 and 13. During this time the number of isolates producing acid phosphatase, alkaline phosphatase and naphthol-AS-BI-phosphohydrolase also increased. At week 15, salinity was elevated to 11 ppt.

Subsequently, the number of isolates producing acid phosphatase and naphthol-AS-BI-phosphohydrolase substantially decreased. Alkaline phosphatase producing bacterial isolates were not detected at this salinity.

A sharp increase was seen in the number of bacterial isolates producing leucine arylamidase in weeks 11 and 13. This number decreased dramatically in week 15 when salinity was increased to 11 ppt. Similarly but not as pronounced, the number of isolates producing the proteases α-chymotrypsin, trypsin and valine arylamidase increased in weeks 11 and 13. No bacteria were found to produce these proteases in week 15 when salinity increased to 11 ppt.

The diversity of carbohydrases produced by bacteria remained relatively constant ranging from a maximum of six carbohydrases in week 5 to only two in week 15. In contrast, the number of isolates producing these carbohydrases fluctuated during the study. The number α-galactosidase producing isolates peaked in week 5, decreased to zero in week 7, and fluctuated the rest of the sampling weeks with another large increase in weeks 11 and 15. Similar fluctuation was observed with regards to α-glucosidase and β-glucosidase with the highest number of producers present at weeks 3 and 11 and 11 and 15, respectively. N-acetyl-β-glucosaminidase was only produced by a few isolates in weeks 5, 7, 9, and 13. No α-fucosidase producing strains were detected until week 11 with the greatest number of isolates in week 13. α-fucosidase production ceased in week 15 altogether.

The overall increase in enzyme production during weeks 11 and 13 at 2 ppt salt concentration coincided with the marked increase in the average number of isolates obtained from each intestinal sample during the same periods. Over the course of the study, the variety of bacterial enzymes increased until the salinity was raised to 11 ppt (week 15) when a large variety of carbohydrases, proteases, lipase (C14) and alkaline phosphatase ceased to be produced. No enzymatic tests were performed with samples at 31 ppt since no obligate anaerobes were isolated.

3.8 Fatty Acid Methyl Ester Analysis from Salinity Study

Species-level identification for most isolates was based on fatty acid methyl ester analysis. From most shrimp intestines several bacterial strains could only be identified to genus level, *Clostridium* spp., a total of 16 strains. One isolate was identified as *C. subterminale*, three as *C. beijerinckii*, nine as *C. bifermentans* GC-subgroup A, and fifteen as *C. butyricum* GC-subgroup A. For the majority of the isolates (61 out of 100) no match was found in the database. All 100 bacterial isolates analyzed contained 16:0 palmitic acid. Myristic acid (14:0) was found in 49% of all isolates.

3.9 Dendrogram Generated from Salinity Study

A dendrogram generated by the Sherlock program (MIDI, Newark, DE) compared several features of the separated fatty acid methyl esters, such as their retention time, peak area over peak height, and equivalent carbon length in a pair wise fashion and grouped the isolates. Paired strains having less than 10 units of arbitrarily computed Euclidian distance were considered to belong to the same species.

Since the phenotype of an organism is always genetically determined, this comparison ultimately has taxonomic and phylogenetic value. The dendrogram results were indirectly confirmed by the observation that closely related bacteria appeared to produce similar enzymes. The two larger groups identified as *C. butyricum* GC-subgroup A and *C. bifermentans* GC-subgroup A generally were found to produce different enzymes. All isolates identified as *C. butyricum* GC-subgroup A produced mostly carbohydrases, with the exception of one isolate, which produced a protease in addition to the carbohydrases. Other isolates identified as *C. bifermentans* GC-subgroup A produced mostly proteases, except for two isolates that produced one or two carbohydrases, respectively in addition to proteases. One isolate identified as *C. bifermentans* GC-subgroup A produced three different carbohydrases and no proteases. Three isolates identified as *C. beijerenckii* were found to produce carbohydrases, while only one isolate identified as *C. subterminale* produced an esterase. Isolates identified

only at genus level, *Clostridium* spp., were found to produce mainly proteases, esterases, esterase lipases and phosphohydrolases. The majority of the isolates produced either acid phosphatase or alkaline phosphatase or both. Finally, unidentified isolates (no match found in the MIDI database) produced a wide variety of enzymes, such as carbohydrases, proteases, esterases, lipases, phosphohydrolases and phosphatases.

## 4. DISCUSSION

Overall, high numbers of Gram-positive spore-forming obligate anaerobic bacteria were isolated from the intestines of *L. vannamei* that were determined to belong to the genus *Clostridium*. Some of the isolates recovered in the salinity study were identified to the species level through whole-cell fatty acid methyl ester analysis. Strains of *Clostridium butyricum* GC-subgroup A, *C. bifermentans* GC-subgroup A, *C. beijerenckii* and C*. subterminale* were identified to the species level. In addition, many more isolates were confirmed to belong to the genus *Clostridium*, while for the majority of isolates no matches were found in the available database. Combined results from a variety of methods used to characterize and describe the isolates suggested that *C. butyricum* GC-subgroup A, *C. bifermentans* GC-subgroup A, and numerous unidentified *Clostridium* species were consistently present throughout the study. They may be members of the resident normal microbiota of the *L. vannamei* intestinal gut. In contrary, the presence of *C. beijerenckii* and *C. subterminale* in the *L. vannamei* intestinal gut is inconclusive for these isolates were only detected in the initial weeks of the study.

The low number of bacterial isolates identified to the species-level may be due to more than one factor. First, samples were collected from a unique environment, the intestines of *L. vannamei*. To date, knowledge of microorganisms present in such an environment is limited. Second, the microorganisms were removed from their natural niche and placed in an artificial laboratory environment. Third, it is unlikely that the proprietary MIDI database has any input on Gram-positive, obligate anaerobic, spore-forming bacteria from

the intestines of shrimp. Therefore, not unexpectedly, the FAME analysis resulted in 61% unidentified strains.

Results suggested nonetheless the presence of diverse microbial populations composed of more than one taxon in the shrimp intestines. Other studies have found similar trends in gastrointestinal microbial diversity. A study by Gomez-Gil et al. (1998) concluded that healthy *Penaeus vannamei* may have a range of different bacterial populations in their tissues and digestive tract. In addition, Moss et al. (2000) also found diverse microbial populations present in the gut of *L. vannamei* that were raised in pond water. This study focused only on obligate anaerobic bacteria. The results pointed mostly to Gram-positive endospore formers and therefore, it underestimated the actual intestinal microbial diversity because other microbes such as facultative anaerobes were not included in the study. Furthermore, the procedures for isolating bacteria may also favor certain anaerobic species over other species.

Although the study environments where the shrimp were raised were different, similar trends were observed in the intestinal bacterial community. First, the obligate anaerobic bacteria from both studies were identified predominantly as Gram-positive endospore-forming bacteria. It is possible that these findings are similar because the shrimp were of the same source and genetic lineage. Moreover, in a study by Dixon et al. (1998) members from the genus *Clostridium* spp. were detected in the intestines of shrimp raised in Ecuador. The study also found other groups of obligate anaerobic non-spore forming strains such as *Bacteroides*, *Fusobacterium*, and *Peptostreptococcus* spp. in the gut of *L. vannamei* and *L. stylirostris*. The large diversity of bacteria found by Moss et al. (2000) and here in the salinity experiment suggests that there are diverse assemblages of microbial communities in the gut of *L. vannamei.*

Another trend seen in both studies was related to the enzymes produced by the microbial isolates. The types of enzymes detected in both studies have been indicated to play a role in the digestive processes of other crustaceans. In a study of the white shrimp, *L. schmitti,* trypsin and chymotrypsin were extracted

from whole homogenized larval, postlarval stages, and from the adult digestive gland, the hepatopancreas (Lemos et al., 1999). These results suggest that bacteria can produce extracellular enzymes similar to those of the host, which could facilitate the breakdown of proteins, carbohydrates, and lipids. While there is much information on mammalian ∝-glucosidases, few studies have focused on the presence of this enzyme in aquatic organisms. Chuang et al. (1992) reported ∝-glucosidase isolated from the shrimp *P. japonicus*, the majority of which was found in the hepatopancreas. Le Chevalier and Van Wormhoudt (1998) reported that in *L. vannamei* oligosaccharides, branched ∝-dextrins, and maltose were hydrolyzed by the enzyme ∝-glucosidase found in the hepatopancreas. Furthermore, Le Chevalier and Van Wormhoudt (1998) and Rosas et al. (2000) suggested that the ∝-glucosidase in *P. monodon* could be involved in the final intracellular digestion of carbohydrates. In these studies obligate anaerobes from the intestinal tract of *L. vannamei* were found to produce ∝-glucosidase, an enzyme that contributes to the breakdown of carbohydrates. A similar enzyme was produced by the obligate anaerobes cultured in the salinity and diet experiments of this study further suggesting that these microbes may contribute to the breakdown of carbohydrates.

Bacterial isolates from both diet and salinity studies were found to produce esterases, similar to those produced by crustaceans during the molting process. The esterases found in these studies also have been found in the hepatopancreas of the American lobster, *Homarus americanus*, the penaeid shrimp, *Sicyonic ingentis*, and two species of crab (Homola and Chang, 1996). Additionally, esterases from the lobster *Callianassa californiensis* were found to hydrolyse methyl farnesoate, a compound involved in the molting cycle (Homola and Chang, 1996; Wilder et al., 1995). Another molting enzyme, N-acetyl-β-glucosaminidase, was found in the hepatopancreas and epidermis of the fiddler crab, *Uca pugilator* (Zou and Fingerman, 1999). This important enzyme was also present in the hepatopancreas of the crabs *Cancer borealis* and *C. irroratus*, as reported by Brun and Wojtowicz (1976). The enzyme N-acetyl-β-glucosaminidase was found, though not extensively, in both diet and salinity

studies. To grow, crustaceans must molt their exoskeleton, which is composed of the polysaccharide chitin. Many crustaceans eat their exoskeleton to re-absorb stored calcium. Bacteria producing these enzymes may facilitate the breakdown of the exoskeleton releasing calcium for absorption. The molting of the exoskeleton may present an opportunistic situation for the bacteria to covert macromolecules such as the polysaccharides in the chitin.

Matusiewicz and Dabrowski (1995) suggested that the enzyme alkaline phosphatase may catalyze a reaction that makes ascorbic acid (Vitamin C) in the phosphorylated form more bioavailable in the digestive tract of salmonids. Phosphorylated ascorbic acid is hydrolyzed by a reaction catalyzed by alkaline phosphatase. Both acid and alkaline phosphatases, previously found in the hepatopancreas of shrimp *Metapenaeus monocerus*, are involved in the production and secretion of digestive enzymes (Bojan, 1988). His study found acid and alkaline phosphatases in the muscle, cuticle, and hemolymph of *M. monocerus*. Baker and Gibson (1978) found that acid and alkaline phosphatases were involved in extracellular digestion and active transport in cell membranes of the mud crab, *Scylla serrata.* Nilsen et al. (2001) found alkaline phosphatase in the hepatopancreas of northern shrimp, *Pandalus borealis*. Phosphatases were also produced by most of the bacterial isolates detected in both diet and salinity studies. This further suggests that phosphatases may be playing a role in the digestive process of *L. vannamei*, especially since studies in the past have determined the involvement of this enzyme in such processes.

Bacterial proteases may play an important role in the digestive processes of *L. vannamei* that are commercially fed a high percentage of protein in their diet. Several bacterial isolates were found to produce proteases in both the diet and salinity studies. Maeda-Martinez et al. (2000) found trypsin-like enzymes in the digestive tube of the terrestrial crustacean *Triops.* Trypsin activity in the hepatopancreas was studied in *L. vannamei* post-larvae by Brito et al. (2001). Previous studies in our laboratory also found trypsin producing anaerobic bacteria in the intestines of *L. vannamei* from Ecuador (Dixon et al., 1998).

Fluctuations were detected in the number of obligate anaerobes producing individual enzymes in both the salinity and diet studies. It is not clear though how important these fluctuations were since this observation was based on the number of isolates producing enzymes and not on a direct detection of enzyme concentrations. The pronounced increases in the number of obligate anaerobes cultured in the salinity study during weeks 11 and 13 may have been due to environmental factors or physiological changes such as molting during their life cycle. During these weeks, the shrimp were at a size when they were expected to molt every two weeks instead of every week (Wyban and Sweeney, 1991). Therefore, these fluctuations may have been due to the microbiota having more time to become established in the shrimp intestine in between molts, since part of the lining of the hindgut would also be discarded during molting (Brusca and Brusca, 1990).

A plateau in shrimp mean weight in the diet study after week 7 suggests that changing to a diet composed of lower protein content was not as conducive for shrimp growth. This plateau coincided with a decline in the variety of enzymes produced and the number of obligate anaerobes isolated. The change in diet supplied to the shrimp may have disrupted the microbial community, which could then have affected the growth of the host. A possible approach for preventing such a disruption would be adding probiotics, specific bacteria to aid in digestion, to the diet.

The preliminary nature of these findings is especially obvious in the light of the study focus: isolation and identification of obligate anaerobic microorganisms, while other members of the shrimp gut microbiota were considered outside of the focus area. Moreover, general caution is appropriate when discussing the role of the microbiota present in the shrimp intestines; results by published authors were obtained without much separation of the gut colonizing microbiota and the host tissues, while our enzyme assays were carried out with pure bacterial cultures *in vitro*.

## 5. CONCLUSION

Relevant information has been gained from this study on the obligate microbiota of Pacific white shrimp, *Litopenaeus vannamei,* and the potential role of the microbiota in the digestive processes. Overall, the data reflect that the obligate anaerobic bacterial population in the intestines of *L. vannamei* is diverse, with occasional shifts in the type of species present. Microbial diversity in the shrimp intestines was affected by the change in diet and exposure to different salinities. In the diet study the diversity of microorganisms decreased when the percentage of protein being fed to shrimp was lowered. A plateau in the shrimp growth was observed when protein content in the diet was altered. The decrease in the growth rate may have been affected not only by the change in protein content, but also by the resultant decrease in the bacterial population and subsequent decrease in detectable enzymes. A change in diet affected the types of enzymes produced by intestinal bacteria.

In the salinity study the diversity of the microbial community was affected by the abrupt increase in salinity. Initially at low salinity (2 ppt) few obligate anaerobes were recovered. By weeks 11 and 13 the number of anaerobic isolates increased with a resultant increase in enzyme production. As the salinity increased (11 ppt) the number of anaerobes recovered from the intestines decreased. No obligate anaerobic bacteria were detected at the highest salinity of 31 ppt. Based on these data it is submitted that abrupt increases in salinity negatively impacted the number of obligate anaerobic bacteria.

Based on fatty acid methyl ester analysis, clostridial species were identified in the shrimp intestines. The most common species identified were *C. butyricum* GC-subgroup A and *C. bifermentans* GC-subgroup A. In the salinity study both microorganisms were consistently present producing carbohydrases and proteases, respectively. Clostridia were also recovered in the diet study. These isolates were identified to the genus level.

Further *in vivo* research needs to be done to address to what extent the enzymes produced in the gut of shrimp are of an endogenous nature and if the microbiota plays a direct role in the digestive processes of shrimp. The enzymes

produced by bacteria could potentially enhance the digestive process by supplementing the endogenous enzymes produced in the intestinal tract of shrimp. The latter has important practical implications in aquaculture.

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