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Effect of Dietary Supplementation of Acidic Calcium Sulfate (Vitoxal) on Growth, Survival, Immune Response and Gut Microbiota of the Pacific White Shrimp, *Litopenaeus vannamei*

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Abstract

Dietary acidifiers have been recognized as beneficial in animal production including aquacultural production of fish where they confer such benefits as improved feed utilization, growth, and resistance to bacterial pathogens. If improvements in growth and immune responses by acidifier supplementation can be confirmed in shrimp, then mortalities due to diseases could be minimized, limiting the emergence of disease-resistant bacterial pathogens as a potential result of antibiotic misuse. With this in mind, a 35-d feeding trial was conducted to evaluate growth, enteric microbiota populations, and nonspecific immune responses of *Litopenaeus vannamei* fed diets containing the commercial acidifier Vitoxal, based on acidic calcium sulfate in an indoor temperature-controlled, recirculating culture system without any natural productivity. Experimental diets were formulated to contain 0 (basal), 0.4, 1.2, 1.6, or 2.0% acidic calcium sulfate (ACS) by weight. Shrimp fed in excess, 15 times a day using automatic feeders. Weight gain and survival among treatments were excellent, but not significantly different ($P > 0.05$). Denaturing gradient gel electrophoresis analysis revealed that the enteric microbial community of shrimp fed the basal diet differed markedly from those fed the acidifier on the basis of 64.9% similarity coefficient. Shrimp fed the commercial acidifier at 1.2 and 2.0% responded significantly ($P < 0.05$) better to reduced stress and displayed enhanced immune responses including hemocyte phagocytic capacity, hemolymph protein concentration, hyaline cell counts, and hemolymph glucose, compared with shrimp fed the basal diet. These results point to an enhanced performance in terms of positive shifts in the composition of enteric microbial communities as well as improved immune performance, with no changes in growth or survival.

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Successful shrimp farming must incorporate strategies to contend with the effects of diseases, which constitute the major problem to large-scale culture of these crustaceans. Antibiotic growth promoters added in the diets of animals for improved weight gain, feed utilization, and disease control have now been criticized because of their potential adverse effects on the environment and the human food chain (Rosen 1996; Ng et al. 2009). In addition, the inappropriate use of antibiotics can bring about the development of antibiotic-resistant bacteria, presence of antibiotic residues in seafood, destruction of microbial populations in the aquacultural environment, and suppression of aquatic animal immune systems (Li et al. 2007; Zhou et al. 2009). Emerging alternatives to antibiotics include the use of probiotics (Burr et al. 2005; Gatesoupe 1999), prebiotics (Zhou et al. 2007), immunostimulants (Chang et al. 2000; Sajeevan et al. 2009), and acidifiers (Lückstädt 2007, 2008; Ng et al. 2009). These compounds enhance the immune system and overall well-being of the animal through various mechanisms including the modification of the gut microbiota by selectively stimulating beneficial bacterial strains, thus reducing the number of potential pathogens (Gibson and Roberfroid 1995; Sritunyalucksana et al. 2005).

Recently, acidifiers have been identified as growth facilitators in aquatic animals (Lückstädt 2007). An acidifier is defined as an inorganic or organic acid and their salts are increasingly receiving attention as agents for the modulation of diseases in cultured fish and shrimp (Lückstädt 2007). In general, dietary acidifiers exert their effects on cultured fish and shrimp in several ways, including changes at the gastrointestinal tract level, in addition to affecting animal metabolism (Freitag 2007).

Acidifiers have also been used as preservatives in diets fed to farm animals and fish (Kemp 2008). These substances have been shown to inhibit microbial growth and the uptake of pathogenic organisms and their metabolites by farm animals (Malicki et al. 2004; Freitag 2007; Ng et al. 2009). Acidifiers also reduce the pH in the stomach and small intestine, thus inhibiting the growth of disease-producing Gram-negative bacteria (Lückstädt 2007). By significantly reducing the duodenal pH, acidifiers enhance pepsin activity, and contribute to improved nitrogen retention and increased nutrient digestibility (Øverland et al. 2000; Lückstädt 2007). In addition, mineral absorption has been shown to be enhanced by acidifiers (Ravindram and Kornegay 1993). Gastric acidity influences the bioavailability of dietary minerals by regulating the chelation and complex formation of the elements, and also by altering the transport mechanisms of minerals (Cross et al. 1990; Wood and Serfaty-Lacrosniere 1992).

Acids and their salts have been used with encouraging results in the culture of fish, following their successful use in the rearing of pigs and poultry. Vielma and Lall (1997) cultured rainbow trout, *Oncorhynchus mykiss,* on a fish meal-based diet supplemented with formic acid, and observed that the apparent digestibility coefficients (ADC) of phosphorus, calcium, and magnesium significantly increased in fish that received an acid diet relative to those that were fed the basal diet (no acid). Dietary citric acid has been shown to improve nutrient utilization and growth performance of rohu, *Labeo rohita* (Baruah et al. 2005). Sugiura et al. (1998) also found that the availabilities of magnesium and calcium in fish meal increased when formic acid was supplemented in the diet of rainbow trout. Sodium salts of lactic and propionic acids when supplemented in the diets of the Arctic charr, *Salvenilus alpinus*, significantly increased weight relative to those fed acid-free diets (Ringø 1994). Organic acid salts have also been used with some degree of success in the culture of tilapia, *Oreochromis niloticus*, and Atlantic salmon, *Salmo salar* (Ng et al. 2009; Zhou et al. 2009; Petkam et al. 2008; Christiansen and Lückstädt 2008). Ramli et al. (2005) fed tilapia species a diet supplemented with potassium diformate prior to challenge with *Vibrio anguillarum* and reported that this compound significantly improved feed intake, raised protein efficiency ratio, and increased survival. Vasquez et al.

(2005) found that pathogenic bacteria species in turbot, *Psetta maxima,* were significantly inhibited by lactic and acetic acids when added as supplements in the diet. Inhibition of the pathogenic bacteria was achieved through direct effects of the acids on the bacteria, rather than through improvement of the fish immune responses.

Not much is known of the use of acidifiers in shrimp culture; however, Tung et al. (2006) reported that sodium citrate with inactivated lactobacilli when applied in the diet boosted the growth of Kuruma shrimp, *Masurpenaeus japonicus*. Lückstädt (2007) used the organic acid salt calcium formate to enhance the survival of brackish water shrimp, *Palaemon adspersus*. Kemp (2008) compared the effects of an acidifier, acidic calcium sulfate (ACS 50) added to an experimental diet versus the antibiotic oxytetracycline added to a second set of experimental diet. The diets were fed to the shrimp *Penaeus vannamei* that were infected with *V. anguillarum* in earthen ponds. Results from that study revealed that *P. vannamei* in ACS 50-treated ponds resumed feeding more rapidly than shrimp in ponds treated with the antibiotic. The ACS 50-treated shrimp also exhibited reduced erratic movements consistent with the suppression of disease stress due to bacterial infection and immune suppression (Kemp 2008). Kemp (2008) concluded that ACS 50, when added to the diets of shrimp, was more effective in suppressing vibriosis compared to the standard treatment of infected shrimp with oxytetracycline. No negative effects have been reported for acidifiers unlike the ill effects documented for antibiotics (Lückstädt 2007) or probiotics (Li et al. 2007). If additional research continues to find positive effects on disease resistance and growth enhancement, it is likely that use of acidifiers will become more common in commercial fish and shrimp aquaculture. Therefore, the purpose of this study was to determine the effects of a commercially available acidifier, ACS, on growth, survival, immune responses, and composition of gut microbiota of the Pacific white shrimp, *Litopenaeus vannamei.*

Materials and Methods

Water Quality

To ensure optimal environmental conditions conducive for the growth of shrimp, water quality variables such as temperature, dissolved oxygen (DO), salinity, total ammonia nitrogen (TAN), nitrate-nitrogen, and nitrite-nitrogen were monitored for the duration of the study. Each experimental system for shrimp was monitored daily for DO, salinity, and temperature using YSI 85 meter (Yellow Springs Instrument Company, Yellow Springs, OH, USA). Nitrate, nitrite, pH, and TAN were measured weekly using methods designed for seawater samples (Spotte 1979). More details for nitrite, TAN, and nitrate procedures can be found in Mullin and Riley (1955), Solorzano (1969), and Strickland and Parsons (1972), respectively.

Experimental Diets

Diets used in the study were formulated as shown in Table 1. The basal diet included cholesterol, vitamin, and mineral premixes to meet or exceed all known nutritional needs of penaeid shrimp (D'Abramo et al. 1997). Acidic calcium sulfate (Vitoxal) was incrementally added in the basal diet to obtain concentration levels of 0.4, 1.2, 1.6, and 2.0%. According to the manufacturer, Vitoxal is comprised of acidic calcium sulfate manufactured from inorganic materials using proprietary technology. Diets were prepared by the method of Gong et al. (2000). Briefly, to obtain the base mix, dry ingredients (excluding alginate and sodium hexametaphosphate) were added to a Hobart L-800 mixer (Hobart Corporation, Troy, OH, USA) and mixed for approximately 10 min. A portion of this mix was then combined with the remaining dry ingredients (excluding alginate and sodium hexametaphosphate) in a V-mixer (3 L, Patterson-Kelley, East Stroudsburg, PA, USA) for approximately 10 min. Menhaden fish oil and soybean oil were added and the resulting mixture was thoroughly blended for additional 15 min. Ingredients in the V-mixer were combined with those in the Hobart mixer and further mixed for a total of 60 min. The base

TABLE 1. *Composition of experimental diets used in the feeding trials.*

Ingredient	Basal	0.4%	1.2%	1.6%	2.0%
Menhaden fish meal[a]	8.0	8.0	7.9	7.9	7.8
Squid muscle[a]	30.0	29.9	29.6	29.5	29.4
Soybean[a] (90%)	5.7	5.7	5.6	5.6	5.6
Phospholipid[b] (97%)	4.0	4.0	4.0	3.9	3.9
Wheat starch[c]	29.5	29.4	29.2	29.0	28.9
Mineral/Vit premix[d] #1	0.3	0.3	0.3	0.3	0.3
Mineral/Vit premix[d] #2	0.2	0.2	0.2	0.2	0.2
Vitamin C[d]	0.04	0.04	0.04	0.04	0.04
Diatomaceous earth[e]	2.8	2.8	2.8	2.8	2.8
Alginate[f]	2.0	2.0	2.0	2.0	2.0
Sodium chloride	0.7	0.7	0.7	0.7	0.7
Potassium chloride	1.9	1.9	1.9	1.9	1.9
Magnesium oxide	1.6	1.6	1.6	1.6	1.6
Calcium carbonate	2.5	2.5	2.5	2.5	2.5
Dicalcium phosphate	4.2	4.2	4.2	4.1	4.1
Vitoxal[g]	0.0	0.4	1.2	1.6	2.0
Sodium hexametaPO$_4$	1.0	1.0	1.0	1.0	1.0
Cholesterol	0.2	0.2	0.2	0.2	0.2
Cellulose[e]	3.2	3.2	3.2	3.2	3.14
Oil, menhaden[h]	0.6	0.6	0.6	0.6	0.6
Oil, soybean[i]	0.6	0.6	0.6	0.6	0.6
Chromic oxide	1.0	1.0	1.0	1.0	1.0
Analyzed proximate composition of two experimental diets (% as fed)					
Moisture		9.0			9.3
Ash		17.4			18.9
Crude protein		37.7			36.5
Crude fat		6.7			6.5
Crude fiber		1.7			1.4
Mineral analysis					
Phosphorus		1.7			1.7
Calcium		2.5			2.4

[a]Oceanic Institute, Waimanalo, HI, USA.
[b]Cargill, Nutrena feeds, Giddings, TX, USA.
[c]MP Biomedicals, Solon, OH, USA.
[d]Zeigler Bros., Gardeners, PA, USA.
[e]Sigma- Aldrich, St. Louis, MO, USA.
[f]ChemPoint, Bellevue, WA, USA.
[g]Mionix Corp, Round Rock, TX, USA.
[h]OmegaProtein, Houston, TX, USA.
[i]IGA Family Center, Port Aransas, TX, USA.

mix plus acidified calcium sulfate were subsequently combined in the V-mixer for 15 min in different proportions to provide the desired ACS level in the diet designated for each treatment. The mix was then transferred to a Hobart C-100 mixer (Hobart Corporation) after which alginate and sodium hexametaphosphate were added and mixed for 20–30 s to obtain a uniform dough. The pasty mass was pelletized through a 2-mm die using a Hobart A-200 meat grinder (Hobart Corporation). Strands of pelletized diets were crumbled and allowed to dry at 30 C (with air circulation) for a period of 16–18 h. Diets were subsequently ground to sizes appropriate for juvenile shrimp, packed in sealed plastic bags, and stored in the refrigerator at −10 C until required for feeding or appropriate analyses.

Experimental Shrimp and Feeding

Disease-free *L. vannamei* post larvae obtained from Harlingen Farms (Harlingen, TX, USA) were reared at the Texas AgriLife Shrimp Mariculture Project, Texas A&M University System, Port Aransas, Texas, USA. Husbandry conditions were maintained at 30 ± 2 C, 26–36 ppt salinity, and 5.2 ± 0.2 mg/L DO. While mean value of nitrite-nitrogen was 0.078 mg N/L in the experimental units, mean values of nitrate-nitrogen and TAN in the experimental tanks were 0.807 mg N/L and 0.089 mg N/L, respectively. Continuous aeration in the experimental units was achieved through the use of air pumps.

Shrimp were fed a commercial post-larval diet (Rangen, Inc., Angleton, TX, USA). The commercial diet was supplemented with live *Artemia* nauplii twice daily until the feeding trials were initiated. This study was conducted in an indoor temperature-controlled, recirculating aquaculture system (RAS) without natural productivity. There were 100, 30-L rectangular tanks (bottom area 0.1 m^2) in the RAS but only 40 tanks were used for the feeding trial. Seawater was pumped through a sand filter, biological filter, 50-μm cartridge filters, and UV disinfection units to achieve an average recirculation rate of 4114%/tank/d. The RAS operated with a daily exchange rate of 147%/d with new seawater. The level of control was such that the culture environment was near optimum for the growth of shrimp. Groups of six shrimp of similar size (0.2 ± 0.014 g) were blotted dry and weighed before being stocked into each tank. Also, all shrimp from each tank were blotted dry and weighed as a group when the study was terminated. There were eight replicate groups of shrimp per dietary treatment. Mean initial

weights of shrimp did not differ more than 3% in the various treatments. Shrimp were fed the experimental diets 15 times/d using automatic feeders. The feeding rate was maintained so as to slightly exceed apparent satiation. Shrimp were monitored daily for molting activity as well as to assess mortality. Uneaten diets and molted exuviae were siphoned out daily prior to the first morning feeding. The feeding trials lasted for a period of 35 d.

Intestinal Tract Samples

After the feeding trial, the intestinal tract contents immediately posterior to the cephalothorax from three shrimp per tank were aseptically removed and pooled to characterize enteric microbial populations. The intestinal tract contents were expressed into sterile RNA-free centrifuge tubes and stored at −80 C until analyzed.

DNA Isolation and PCR

Genomic DNA was isolated from 0.2 g of freeze-dried digesta with QIAGEN DNA mini kit (Cat# 51304, Qiagen, Valencia, CA, USA), using the manufacturer's method with the following modifications: The pellets were suspended in 300 µL of the lysis solution. Lysozyme (L-687, Sigma, St. Louis, MO, USA) was added to a final concentration of 20 mg/mL and mixed with a sterile pestle. The solution was incubated at 37 C for 2 h after which it was centrifuged at 20,800 *g* for 3 min; the supernatant was removed and placed into a clean 1.5-mL microcentrifuge tube. Then, 20 µL of the RNAse solution (final concentration 20 mg/mL) was added and the mixture was incubated at 37 C for 45 min. After DNA precipitation, hydration, and quantification, polymerase chain reaction (PCR) was subsequently conducted using the method of Hume et al. (2003). Bacteria-specific PCR primers (forward and reverse) were used to target conserved regions flanking the variable V3 region of 16 rDNA (Muyzer et al. 1993).

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DG GE) was conducted following the method of Hume et al. (2003) and Muyzer et al. (1993), as modified by Li et al. (2007). The fragment analysis pattern relatedness was determined with Molecular Analysis fingerprinting Software (v1.6, Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA).

Dendogram Analysis

The unweighted pair group method with arithmetic mean (UPGMA) algorithm was used to construct a dendogram of DGGE band profiles. The dendogram analysis compares DGGE profiles based on numbers, positions, and intensities of bands. The Dice similarity coefficient (SC) was used for computing sample similarity based on band position and intensity. Comparison between sample band patterns are expressed as a percentage SC (%SC) (Fig. 1).

Immunological Parameters

At the end of the feeding trial, eight shrimp per treatment group fed either 0, 0.4, 1.2% or 2.0% ACS (Vitoxal) were sampled for immunity evaluations. For immunological parameters, hemolymph (ca.100 µL) was drawn from the ventral sinus of each shrimp into a 1 mL sterile syringe using a 25-gauge needle containing 100 µL of anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, pH 7.6, osmolarity 780 mOsm/kg). For physiological parameters, a second batch of hemolymph (ca. 100 µL) was obtained using the same procedure, but in this case it was not mixed with any anticoagulant.

Immediately after bleeding, 25 µL of hemolymph was removed and thoroughly mixed with 25 µL of trypan blue in a sterile microcentrifuge tube. This solution was then placed in a hemocytometer for cell counting. Hemocytes were identified following the guidelines provided by Braak (2002). Total hemocyte count (THC) and differential hemocyte count (DHC) were obtained thereafter.

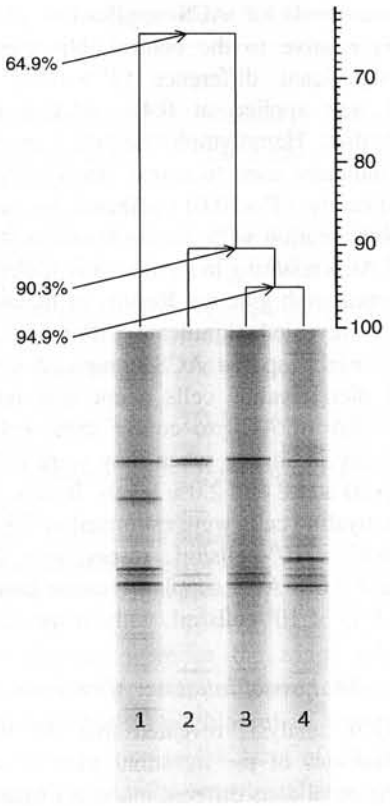

FIGURE 1. *Dendogram of the microbial luminal communities of shrimp fed the basal diet (Lane 1), the diets containing acidic calcium sulfate (ACS; Vitoxal) at 0.4, 1.2, and 2.0%, Lanes 2, 3, and 4, respectively. The community from shrimp fed the basal diet was different from that of shrimp fed the ACS diets (SC = 64.9%). The luminal communities in shrimp fed the ACS diets were very similar, but the communities were not identical (SC, 90.3 and 94.9%).*

Phagocytic activity of the macrophages was determined via a microscopic counting technique described by Brown et al. (1996) and further modified by Mustafa et al. (2000). This assay determines the proportion of hemocytes that are able to take up formalin-killed bacteria, *Bacillus megaterium*. Briefly, a *B. megaterium* culture was grown in the laboratory and then killed with formalin. One hundred microliters of this culture were added to double-etched microscope slides (Eric Scientific, Portsmouth, NH, USA) containing attached hemocytes, which had been previously isolated from the sampled shrimp and incubated for 90 min at room temperature. Upon bacterial addition, slides were further incubated for 60 min. Following incubation, the slides were washed with phosphate buffered saline (PBS, P4417, Sigma, St. Louis, MO, USA), air dried, fixed in methanol, and stained with Wright-Giemsa stain (Sure-stain, Fisher Diagnostics, Middletown, VA, USA) for microscopic examination at 100× magnification (Enane et al. 1993). At least 100 cells per treatment were examined to determine the phagocytic capacity of hemocytes – the percent of hemocytes containing five or more bacteria.

Physiological Parameters

For glucose analysis, a drop of hemolymph (not mixed with anticoagulant) was placed on a glucose strip and inserted into a standard glucometer (Precision Xtra, Abbot Laboratories, Abbot Park, IL, USA). Hemolymph glucose was measured following protocols previously described by Gensic et al. (2004) and Schreck and Moyle (1990), and validated for use in fish by Wedemeyer et al. (1990). For packed hemocyte volume, hemolymph was collected in capillary tubes centrifuged in a Critspin Micro-Hematocrit Centrifuge (StatSpin Inc, Westwood, MA, USA) and read using a Micro-Hematocrit Capillary Tube reader (Monojet Scientific, St. Louis, MO, USA). Hemolymph protein was estimated using a hand held Protein Refractometer (VEEGEE, Lab safety Supply Inc., Janesville, WI, USA).

Statistical Analysis

Growth and other production data were subjected to analysis of variance (ANOVA) using the general linear model of SAS (version 9.2, Cary, NC, USA). Data obtained for immune studies were statistically analyzed using Mintab 15.1.0 2007. Means and SEM were calculated for each assay by one-way ANOVA and differences were considered significant at $P < 0.05$. In addition, immune responses of *L. vannamei* were compared via regression analysis to detect differences in the responses to ACS levels in the diet.

TABLE 2. *Growth performance of* Litopenaeus vannamei *fed four levels of ACS (Vitoxal).*[a]

Diet (%ACS)	Survival (%)	Weight gain (g)	Growth (g/wk)	FCR (g fed/g gain)	Biomass (g/m^2)
Control	93.8	7.8	1.6	1.7	454.4
0.4	97.9	7.8	1.6	1.5	471.1
1.2	95.8	8.0	1.6	1.5	470.6
1.6	90.5	8.2	1.6	1.6	452.8
2.0	90.5	7.9	1.6	1.6	441.1
ANOVA					
$Pr > F$	0.5375	0.8398	0.8778	0.8400	0.8973
Pooled SE	3.684	0.312	0.062	0.107	24.816

ACS = acidic calcium sulfate; FCR = feed conversion ratio.

[a]Values are means of eight replicate tanks with six shrimp per tank. Mean responses of shrimp fed the various diets were not significantly different ($P > 0.05$).

Results

Water quality variables in all treatment groups remained within optimal ranges for the culture of *L. vannamei* (Saoud et al. 2003) and appeared to support excellent shrimp growth. Dietary treatments had no significant effect ($P > 0.05$) on these variables.

Weekly growth rates (Table 2) were very high and ranged between 1.55 and 1.64 g in all treatments starting with an initial size of 0.2 ± 0.014 g. Shrimp survival was also very high and varied between 90.4% in animals fed ACS at 1.6% and 93.7% in shrimp fed the basal diet. Growth and other production parameters appeared unaffected by dietary supplementation of ACS as there were no significant differences in growth parameters among treatments ($P > 0.05$).

In contrast, the acidifier appeared to have potentiated the immune responses of *L. vannamei* as ACS fed at 0.4 to 2.0% significantly ($P < 0.05$) affected hemolymph protein, whereas ACS fed at 1.2 and 2.0% significantly ($P < 0.05$) affected hemolymph glucose, hemocyte phagocytic capacity, and hemolymph packed cell volume (Table 3). Total hemocyte counts were 7.74 and 12.04×10^6/mL for shrimp fed the basal and 2.0% ACS diets, respectively. There were significant differences ($P < 0.05$) in total hemocyte count at two levels of ACS application (1.2 and 2.0%) relative to the control, but there was no significant difference ($P > 0.05$) when ACS was applied at 0.4% relative to the basal diet. Hemolymph glucose – an important indicator used to assess stress – also was significantly ($P < 0.01$) affected by acidifier supplementation with dietary levels of 1.2 and 2.0% ACS resulting in significantly lower levels of hemolymph glucose. Results of hemolymph protein revealed significant differences ($P < 0.01$) in shrimp fed ACS compared with the basal diet. Hyaline cells count also reflected the ability of ACS to confer some enhanced immunity in shrimp when they were fed ACS (Vitoxal) at 1.2 and 2.0% levels. In this experiment, hyaline cells were estimated at 7.2×10^6 and 6.87×10^6 cells/mL, respectively, for the 1.2 and 2.0% ACS supplementation compared with 5.35×10^6 cells/mL without the acidifier.

Analysis of Intestinal Microbiota

DGGE analysis revealed that the luminal communities of the intestinal tract of shrimp fed the basal diets differed markedly from those fed the acidifier on the basis of SC (64.9%, Fig. 1). The luminal communities within the intestinal tracts of shrimp fed ACS exhibited similar characteristics (SC = 90.3 and 94.9%), although DNA from individual bacterial species within the gastrointestinal tracts of shrimp fed different levels of ACS was not sequenced, thus no species were identified.

Discussion

Although Kemp (2008) reported improved growth and survival of shrimp when acidifiers comprising mostly of acidic calcium sulfate were applied to the diets of shrimp, no significant differences were obtained in this study for survival, growth and feed conversion ratio (FCR) of shrimp fed ACS (Vitoxal) compared with animals fed the basal diet (Table 2). It is possible that effects of ACS on growth were not obvious because of the quality of post larvae and diets used, and the near optimal conditions of the culture medium. Water quality variables such as DO, pH, salinity, nitrate-nitrogen,

TABLE 3. *Immune response data of* Litopenaeus vannamei *fed ACS-supplemented diet.*[a]

Stress/immune response	Control	ACS (0.4)	ACS (1.2)	ACS (2.0)	Analysis of variance (P value)	Regression coefficient (R^2)
Total hemocyte count ($\times 10^6$/mL)	7.74 ± 0.64	9.50 ± 1.05	12.27 ± 1.08*	12.04 ± 0.45*	0.02	0.358
Hyaline cells ($\times 10^6$/mL)	5.35 ± 0.49	4.78 ± 0.70	7.20 ± 0.57*	6.87 ± 0.49*	0.01	0.183
Granular cells ($\times 10^6$/mL)	0.79 ± 0.25	1.39 ± 0.24	1.76 ± 0.29	1.87 ± 0.24*	0.02	0.256
Semi-granular cells ($\times 10^6$/mL)	1.60 ± 0.20	3.32 ± 0.56	3.31 ± 0.70	3.30 ± 0.48	0.06	0.133
Hemolymph glucose (mg/dL)	76.00 ± 4.48	62.75 ± 5.25	45.37 ± 3.96*	45.12 ± 4.77*	<0.001	0.477
Hemolymph packed cell volume (%)	36.50 ± 1.29	34.12 ± 1.54	30.87 ± 1.38*	30.00 ± 1.52*	0.01	0.304
Hemolymph protein (mg/mL)	157.25 ± 6.04	130.87 ± 4.11*	124.62 ± 3.22*	115.12 ± 1.96*	<0.001	0.605
Hemocyte phagocytic capacity (%)	23.87 ± 0.72	24.50 ± 0.56	27.37 ± 0.87*	28.50 ± 0.73*	<0.001	0.471

ACS = acidic calcium sulfate.
[a]Values are means ± SE (n = 8).
*Significantly different than control.

nitrite-nitrogen were in the range adjudged favorable for the culture of shrimp. It is important to note that the addition of ACS to the diet of *L. vannamei* up to the level of 2% had no adverse effects on the growth and other production parameters of shrimp.

The dendogram analysis indicated that the luminal communities in shrimp fed ACS had similar microbial communities. These communities were different from those shrimp fed the basal diet on the basis of obtained SC. It has been established that acids and their salts modulate the intestinal microbiota of farm animals including fish, and this modulation can be achieved through a shift in dominant hierarchies of bacteria through the lysing of Gram-negative bacteria (Hellweg et al. 2006; Owen et al. 2006; Encarnacao 2008; Zhou et al. 2009). Although individual bacterial species were not identified in this study, results obtained from DGGE confirmed that microbial populations were similar in shrimp that received all levels of ACS which is consistent with earlier observations by Owen et al. (2006) and Encarnacao (2008) of variations in microbiota upon dietary supplementation with acidifiers. Such variations, which are typically accompanied by the predominance of "good" microbiota, (Encarnacao 2008; Lückstädt 2008) have been suggested as responsible for equipping shrimp with the ability to withstand the attack of pathogens, in addition to ensuring improved growth and survival. Production of "good" microbiota may not always be the case when acidifiers are added in the diets of aquatic organisms. For example, the addition of potassium diformate (KDF) in the diet of hybrid tilapia by Zhuo et al. (2009) affected the intestinal allochthonous bacterial community by selectively improving the relative bacterial abundance of some bacteria such as *Mycobacterium* sp., *Pseudomonas* sp., and six uncultured bacterium-like species while depressing others such as *alpha Proteobacterium* IMCC1702- like, *Rhodococcus* sp. and three uncultured bacterium-like species. Zhuo et al. (2009) noted that some of these bacteria appeared to be organisms known to be pathogenic to fish and humans. To the extent that ACS was able to modify microbial communities in shrimp, which in turn may account for its ability to initiate an immune response in *L. vannamei*, ACS would appear to exhibit some characteristics already identified in other prebiotics. For example, Li et al. (2007) observed short chain fructooligoscharides causing changes in the gut microbiota of shrimp,

which in turn benefited the crustacean, possibly by increasing nonspecific immune responses.

There are no previous reports in published literature about the ability of acidifiers to influence stress and immune response in shrimp. Results obtained in this study highlight for the first time the immunopotentiating action of dietary acidifiers in *L. vannamei*. ACS (Vitoxal) supplemented at 1.2% significantly ($P < 0.05$) increased the phagocytic activity in hemocytes as well as elevated total hemocyte counts, which was noted in shrimp fed diets with 2.0% ACS supplementation. Although culture conditions were near optimal, the acidifier appeared to have increased stress tolerance as indicated by significant effects on both hemolymph protein and glucose for shrimp fed ACS at dietary inclusion levels of 0.4 and 1.2%, respectively, under the conditions of this study. Therefore, ACS may become an important dietary component in the culture of shrimp under intensive conditions as more research documents its beneficial effects on disease resistance.

The complete action of ACS on stress tolerance and immune responses in shrimp needs to be further elucidated, but this study provides strong indications as to potential enhancements of shrimp immunity. Lopez et al. (2003) observed that probiotics and prebiotics, some of which have been documented to enhance growth as well check the menace of diseases in some fish species may replace antibiotics in the culture of shrimp and fish. Together with probiotics and prebiotics, acidifiers may trigger the release of alarm molecules, helping to activate the immune response of invertebrates such as shrimp. Another possibility is that by modulating microbial communities in the gastrointestinal tract, acidifier-fed shrimp may benefit from the proliferation of beneficial microorganisms such that pathogenic bacteria may be excluded from the enteric environment. In addition, it is also possible that acidifiers may directly enhance several individual components of the crustacean immune system as documented in this study with *L. vannamei*.

Because under intensive culture conditions shrimp are often presented with a variety of stressors and pathogenic microorganisms, the capacity to upregulate several mechanisms of the immune response may prove fundamental in the future of shrimp culture, particularly as problems associated with the use of antibiotics such as residual toxicity as well as regulatory issues may be avoided. Nevertheless, more studies need to be conducted to validate and expand results obtained in this experiment. Specifically, controlled pathogenic challenges involving *L. vannamei* fed diets with or without the acidifier should provide further insight as to the effectiveness of acidifier supplementation.

Under the experimental conditions of this study, ACS (Vitoxal) did not affect growth and other performance parameters which were excellent for shrimp fed with and without the acidifier. It is likely that shrimp fed diets more limited in nutrient availability may experience performance improvements upon ACS supplementation. However, more trials with borderline formulations are needed to substantiate this hypothesis.

In conclusion, dietary ACS (Vitoxal) supplementation was shown to significantly modify luminal communities of *L. vannamei*. Some stress indicators and other immune response mechanisms significantly improved in shrimp fed ACS-supplemented diets, although the specific physiological action through which these changes were obtained need further investigation.

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