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The Effects of a Natural Polyphenol Extract from Sugarcane (*Saccharum officinarum*) on the Growth, Survival, and Feed Conversion Efficiency of Juvenile Black Tiger Shrimp (*Penaeus monodon*)

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Abstract: Farmed shrimp feeds are under continuous development to maximise shrimp growth, health, and feed efficiency. There is evidence in aquaculture species that botanical ingredients may be capable of improvements in each of these traits. However, the full potential of these ingredients remains largely unexplored. We investigated the effect of dietary additions of a polyphenol-rich sugarcane extract (PRSE) at the rate of 0, 2, 4, or 6 g kg^{-1} of diet, on the growth, survival, feed conversion ratio, and pathogen loading of black tiger shrimp (*Penaeus monodon*) over a 10-week feeding period in a small-scale intensive RAS system. Shrimp fed the highest level of PRSE compared to the control were 54% heavier (15.4 vs. 10.0 g; $p < 0.05$), had 39% lower feed conversion ratios (1.9 vs. 3.1; $p < 0.05$), had a higher survival rate (50 vs. 26%), and an increased carapace length (27.5 vs. 23.3 mm; $p > 0.05$). These findings demonstrate that polyphenol-rich sugarcane extracts have the potential to improve shrimp growth and FCR when added into feed formulations. Further research should be conducted in commercial rearing conditions for validation.

Keywords: antioxidants; black tiger shrimp; botanical extracts; *Penaeus monodon*; polyphenols; sugarcane

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1. Introduction

Marine shrimp are important aquaculture species, with the combined global production of the two main farmed species, the Pacific whiteleg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*), reaching nearly five million tonnes in 2016 [1]. As feed is the biggest economic cost in shrimp farming, continual measures are being undertaken to improve shrimp diets [2]. For aquaculture feeds, the ultimate goal is to produce diets that produce favourable growth rates, animal health, and feed efficiencies that delivers a socially responsible and affordable product for the consumer. As such, more environmentally and economically sustainable ingredients such as plant bioactives are being increasingly utilised in aquafeed formulations [3].

Shrimp farming is faced with a range of additional animal welfare challenges that are negatively affecting productivity around the world. This includes increased disease burden and spread, higher stocking densities, and suboptimal water quality, all cited as contributing factors [4]. The common factor present in each of these conditions is an increase in oxidative stress. For example, a range of anti-oxidant enzymes are linked to the immune response in shrimp and infection with viruses such as white-spot syndrome virus, or the use of antibiotics such as enrofloxacin, all shown to stimulate the production of reactive oxygen species [5–7]. This increased oxidative stress leads to interference with

the immune response and, ultimately, reduced production outcomes and total biomass yields [7].

In an attempt to increase biomass yields, the use of antibiotics for growth promotion has been adopted in aquaculture in a similar manner to their use in agriculture [8]. This dependence on antibiotics for both agriculture and aquaculture promotes antibiotic resistance in bacterial pathogens that cause disease [9]. As a consequence, increased regulatory and consumer pressure is encouraging the industry to limit the use of antibiotic growth promoters. In addition, some antibiotics have also been demonstrated to increase the production of reactive oxygen species in shrimp, which further strains the immune response [5]. There is now a need to identify novel ingredients and additives that maximise shrimp growth and health, whilst also aiding in the sustainable expansion of global shrimp aquaculture.

Natural antioxidant compounds, such as polyphenols, are a potential option for enhancing production outcomes as they are abundant in plants and have a long evolutionary history of consumption in omnivorous/herbivorous species. Plant polyphenols are a broad range of secondary metabolites containing one or more aromatic rings and at least two hydroxyl groups [10]. Polyphenols are associated with a variety of positive effects on health, through a diverse range of proposed mechanisms. These mechanisms include modulation of metabolism, interaction with gut bacteria, and as a scavenger of free radicals [11]. Furthermore, evidence generated by animal and human studies shows that the anti-oxidant, anti-inflammatory, and anti-microbial properties [12–14] (of polyphenols may be beneficial as preventative agents against many non-communicable and microbial diseases in animals, including aquatic species [15].

The inclusion of a wide range of polyphenol-rich ingredients into standard basal diets has been studied in shrimp nutrition with observed mixed effects, suggesting that the response of the shrimp may be specific to the specific polyphenol composition of the diet [16–23]. Many studies have linked plant polyphenol intake with an increased resistance to infectious disease [24–28]. However, the specific effect of sugarcane-derived compounds has not been explored in black tiger shrimp. A sugarcane extract (*Saccharum officinarum*), known commercially as Polygain™, has shown promising growth results in the giant freshwater prawn (*Macrobrachium rosenbergii)* [29], justifying further investigation into the effect of this extract in influencing productivity outcomes in black tiger shrimp.

In this trial, the effect of dietary addition of the polyphenol-rich sugarcane extract (PRSE) Polygain™ obtained from sugarcane was evaluated on the growth, survival, feed conversion ratio, and pathogen loading of black tiger shrimp (*Penaeus monodon*) in a small-scale intensive rearing system.

2. Material and Methods

2.1. Preparation of Polyphenol-Rich Sugar Cane Extract

The polyphenol-rich sugarcane extract (PRSE) used in this study (Polygain™, The Product Makers, Melbourne, Australia) originated from sugarcane (*Saccharum officinarum*) and was made using a patented process. Briefly, clarified sugarcane juice was vacuum-evaporated and crystallised to produce molasses. The molasses was then fermented in the presence of baker's yeast (*Saccharomyces cerevisiae*), and ethanol was removed by vacuum evaporation and condensation. The remaining vinasse was diluted in water and sieved through a series of filters (5, 1, 0.5, and 0.1 µm). The vinasse was then further concentrated under vacuum and hot-filled (80 °C) into plastic drums.

The resulting PRSE had the following specifications provided by the manufacturer: 55° brix, pH 4.4, density of 1.28 g cm^{-3}, ≥38,000 mg total polyphenols L^{-1} (as gallic acid equivalents), ≥10,000 mg flavonoids L^{-1} (as catechin equivalents), 20–40 g K kg^{-1}, 30–50 g Mg kg^{-1}, 3–5 g Ca kg^{-1}, 0.5–2 g Na kg^{-1}, 100–150 mg Fe kg^{-1}, 40–46 g total amino acids kg^{-1} (≈20% were essential amino acids), and organic acids at 4–5% *w*/*w* of sugars.

According to the provided specification, the following polyphenols and flavonoids have been identified in PRSE: chlorogenic acid, caffeic acid, sinapic acid, syringic acid, vanillin, homoorientin, orientin, vitexin, swertisin, diosmin, apigenin, tricin, and dios-

metin. The LC-MS analysis allowed the tentative identification of 7 apigenin-C-glycosides, 3 methoxyluteolin-C-glycosides, and 3 tricin-O-glycosides.

2.2. Diets

Four diets containing 0, 2, 4, or 6 g kg^{-1} PRSE (Section 2.1) were formulated (Table 1). Diets were made using a dry shrimp mash (Ridley Aquafeeds, Narangba, QLD, Australia). This dry mash is a proprietary formulated dry powder blend of multiple plant, animal, and marine-based ingredients and additives. To make the feeds, PRSE and/or sucrose (Chem-supply, Port Adelaide, SA, Australia) were measured gravimetrically and then dissolved in water. This solution was then added to the dry mash. The wet mixture was mixed on a Hobart Planetary Mixer (Model A200-B1HE, Hobart Food, Silverwater, Australia) for 10 min at the highest speed. The moist mix was then extruded through a 3 mm die on a Hobart mixer meat grinder attachment. The extruded strands were removed from the meat grinder when approximately 20 cm long, loosely separated from each other, and steamed for 5 min. The steamed strands were placed in aluminium trays and dried in an oven at 60 °C for 20 h. Dried strands were placed in a blender (Vitamix model VM0109, Vita-Mix Corp., Cleveland, OH, USA) and blended in pulses until the strands were broken into pellets ranging from 2 to 6 mm in length. Diets were then sieved (1 mm ø mesh size) to remove dust and stored at −20 °C until use. The PRSE addition rates were chosen as these levels have previously been reported to have beneficial effects on the growth and health of the giant freshwater prawn *Macrobrachium rosenbergii* (Rubel, Shimul & Nahid, 2018). Sucrose was chosen as a substitute to PRSE in diets with <0.6% PRSE to maintain a homogenous concentration of nutrients across the diets produced. Proximate analysis was carried out by the National Measurement Institute. The results of the proximate analysis indicated that diets consisted of 40.6% protein, 6.2% fat, 28% carbohydrate, 9.4% ash, and 15.9% moisture.

Table 1. Experimental diet compositions used to evaluate the effect of adding a polyphenol-rich sugarcane extract (Polygain™) to the diet of the black tiger shrimp, *Penaeus monodon*.

Diet	Polygain (g kg^{-1} Diet) [1]	Sucrose (g kg^{-1} DM) [2]	Dry Mash (g kg^{-1} DM) [3]	Total Additional Dietary Polyphenol Addition (mg kg^{-1} DM)
Control	0	3.6	996.4	0
0.2% Polygain	2	2.4	995.6	60.8
0.4% Polygain	4	1.2	994.8	121.6
0.6% Polygain	6	0	994.0	182.4

[1] The Product Makers, Melbourne, Vic, Australia. Polygain additions were of the unaltered liquid supplied by the manufacturer; [2] Chem-supply, Port Adelaide, SA, Australia. Sucrose additions as a filler for Polygain were based on Polygain's estimated 60% DM content by weight; [3] Shrimp dry mash, Ridley Aquafeeds Ltd., Narangba, QLD, Australia.

2.3. Shrimp Husbandry

Post-larvae shrimp (PL 15) were obtained from a commercial farm in northern Queensland. Animals (≈220–250 individuals, ≈15 animals L^{-1}) were transported for 2 h in ≈35 ppt seawater in a plastic bag (15 L volume) with an oxygen-enriched atmosphere. The bag was contained within a polystyrene box to stabilise the water temperature. Upon arrival at the experimental facility, the animals and the transport water were treated with formalin (0.2 g L^{-1} for 1 h in an aerated bucket) as a quarantine requirement. At the time of the experiment, no animal ethics approval was required for shrimp trials in Australia.

Animals were housed in rectangular plastic tanks within an isolated environmentally controlled recirculating aquaculture system (RAS). The system consisted of a total of 40 rectangular plastic tanks (working volume ≈ 43 L), two biofilters (≈100 L each), two UV filters (Emperor Aquatics 25 W UVC filter, Pentair, Florida, USA), and two sumps (≈350 L each). The total system volume was ≈ 2600 L. A 12:12 h light regime was used throughout the trial, with four red fluorescent tubes used to dimly light the room during the 12 h light period. Water quality parameters were measured daily using commercial

kits for NH_3/NH_4^+, NO_2^-, NO_3^- (Aquasonic Pty Ltd., Wauchope, NSW, Australia), and pH (API high range pH test kit, Mars Fishcare North America Inc., Chalfont, PA, USA), or a probe (YSI 556 MPS, Yellow Springs, OH, USA) for DO, salinity, and temperature. Water parameters over the length of the trial were within the optimal range for *P. monodon*; 29.2 ± 0.04 °C, pH 8.2 ± 0.02, 96.5 ± 6.3 DO (% saturation), <0.01 mg NH_3/NH_4^+ L^{-1}, <0.05 mg NO_2^- L^{-1}, 10.5 ± 4.4 mg NO_3^- L^{-1} (mean ± SEM, n = 75). Salinity was gradually decreased from 31 to 20 g L^{-1} over a 10-day period after the introduction of the shrimp to the system, from which time it was maintained at 20.1 ± 0.02 g L^{-1} (mean ± SEM, n = 75) for the trial period. Filtered (1 µm) seawater was obtained from the open ocean (AIMS, Townsville, QLD, Australia), while the freshwater used to reduce the salinity was carbon-filtered tap water. A commercial blend of cultured bacteria (Stability, Seachem Laboratories Inc., Madison, GA, USA; Batch 77,975 4/20) was added to the system at the rate of 250 mL/day (0.1 mL L^{-1}) for two consecutive days starting on the day that the shrimp were added to the system. Shrimp were stocked at 25–30 animals per tank (≈0.6 shrimp L^{-1}). The density was decreased to ≈ 15 shrimp per tank from PL 40 to minimise cannibalism during moulting. Shrimp were fed at a rate of >10% body weight day^{-1}, with commercial shrimp crumble (Shrimp MR SRT #0, Ridley Aquafeeds Ltd.) from PL 15 to PL 50, and with the control experimental diet from PL 51 to PL 57. No health or disease concerns were observed during the mandatory 2-week quarantine period (PL 15 to PL 28).

The feeding trial started when shrimp (PL 58) reached a size of 1.04 ± 0.02 g (mean ± SEM, n = 80). Twenty shrimp were selected at random and measured for carapace length. Five shrimp were selected at random, group weighed, and then stocked into each experimental tank. Treatments were assigned randomly to each tank (4 tanks $treatment^{-1}$, 16 tanks in total for the trial). The daily feed ration was fed in three portions (divided ≈ 25:25:50%) at ≈ 09:30, 13:00, and 16:30 h. The feed amounts fed per tank were weighed and recorded daily. Each day, the uneaten feed amount was syphoned, dried in an oven, and weighed. Feeding rates were subsequently adjusted for each tank based on the uneaten feed amount, with the aim of feeding all shrimp to satiation. Mortalities were recorded daily.

2.4. Sampling

Individual shrimp were sampled for weight and carapace length after 0 (Section 2.2), 35, and 70 days on the experimental diets. At day 0 and day 35, all prawns were weighed and then restocked into their respective tanks. In addition, at 70 days, pleopods were sampled from each shrimp for analysis of viral loading (see Section 2.6). To sample, shrimp were collected with nets, patted dry with paper towel, measured, and then returned to their respective tanks.

Shrimp pathogen and dicer-1 mRNA detection and quantification was conducted by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and PCR.

Samples were collected for pathogen screening on day 70 of the experiment. The pair of pleopods proximal to the cephalothorax were excised using medical scissors from individual *P. monodon* (n = 31, 128 DOC). Pleopod pairs were immediately placed in 2 mL matrix-D tubes (MP Biomedicals™, Thermo Fisher Scientific, Foster City, CA, USA) containing 350 µL MagMAX™ CORE Lysis Solution (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). Tubes were then maintained at room temperature (≈28 °C) for 24 h. Total nucleic acids (TNA) were extracted using a MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems) on a Thermo Scientific KingFisher Flex magnetic particle processor following the manufacturer's instructions.

As the shrimp were not specific-pathogen-free (SPF), it was likely that they would be carrying background levels of viral and/or bacterial infection. To detect pathogen load, hepatopancreas parvovirus (HDV/HPV), infectious hypodermal and hematopoietic necrosis/*Penaeus stylirostris* densovirus (IHHNV/PstDV), a bacterial toxin gene, *Photorhabdus* insect-related toxin-like gene component-A (Pir-A), white spot syndrome virus (WSSV), gill-associated virus (GAV), whenzhou shrimp virus 2 (When-2), yellow head virus 1 and

7 (YHV-1 and YHV-7), and *P.monodon* dicer-1 gene, qPCR was conducted on each nucleic acid extract. The primer and probe sequences of each assay are presented in Table 2. DNA-targeting assays were analysed using a SensiFAST™ Probe Lo-ROX Kit (Bioline, Meridian Bioscience, Cincinnati, OH, USA). RNA-targeting assays were prepared using an AgPATH-ID™ One-step RT-PCR Kit (Thermo Fisher Scientific). All qPCRs consisted of 17.5 µL of master mix containing the manufacturer's supplied reagents, primer (800 nM each), probe (250 nM), and 2.5 µL of eluted extract. Real-time PCR analysis was conducted with a Quant Studio 5 real-time PCR machine (Applied Biosystems) for 40 cycles. The cycle conditions of the DNA-targeting assay consisted of 95 °C × 10 min; 45 × 95 °C × 15 s, 60 °C × 30 s. The cycle conditions of the RNA-targeting assay consisted of 45 °C × 10 min; 95 °C × 4 min; 40 × 95 °C × 15 s, 60 °C × 30 s. Raw qPCR data were processed in QuantStudio™ Design and Analysis Software (Applied Biosystems) and converted to a calculated copy number of positive detections prior to analysis in Excel with linear regression against the assay positive-control standard curves.

Table 2. Primer and Probe sequences used in this study.

Pathogen Target	Primer Detail (5′-3′)	DNA/RNA Genome	Reference
HDV/HPV	qF (4440)-CTA CTC CAA TGG AAA CTT CTG AGC qR (4571)-GGC ACT TCC TGT ATT TTT CCC G qProbe (4471)-TAC CGC CGC ACC GCA GCA GC	DNA	[30]
IHHNV/PstDV	qF-CCT AAA GAA AAC AGT GCA GAA TAT GAC qR-TCA TCG TCA AGT TTA TTG ACA AGT TC qProbe-CTC CAA CAC TTA GTC AAA	DNA	[31]
Pir-A	qF (Han)-TTG GAC TGT CGA ACC AAA CG qR (Han)-GCA CCC CAT TGG TAT TGA ATG qProbe (Han)- AGACAGCAAACATACACCTATCATCCCGGA	DNA	[32]
WSSV	qF (OIE)-TGG TCC CGT CCT CAT CTC AG qR (OIE)-GCT GCC TTG CCG GAA ATT A qProbe (OIE)-AGC CAT GAA GAA TGC CGT CTA TCA CAC A	DNA	[33]
GAV	qF-GGG ATC CTA ACA TCG TCA ACG T qR-AGT AGT ATG GAT TAC CCT GGT GCA T qProbe-TCA GCC GCT TCC GCT TCC AAT G	RNA	[34]
When-2	qF-GGC TCT TTAGCC TGA CTT TAT CT qR-GCA GAG GAC AGG AAG TGA TTA qProbe-ACC TCA CTG TCT GAG TTC TGC ACA	RNA	This publication
YHV-7	qF-CAT CCA ACC TAT CGC CTA CA qR-TGT GAA GTC CAT GTG AAC GA qProbe-CAA CGA CAG ACA CCT CAT CCG TGA	RNA	[35]
Dicer-1	qF-TGG TAC CAA AGT CAC CCA TTA G qR-ACC TTC CCA TCA ACA AGA CGT T qProbe-AAC CAG AAA CAG CCA AAT	RNA	[36]

2.5. Calculations

Feed conversion ratios were calculated over the trial period using; FCR = feed fed (g)/increase in animal weight (g). The condition factor was calculated using K = wet mass (g)/(length (cm))^3. The specific growth rate was calculated using SGR = ($\log_e$(final weight)−$\log_e$(initial weight))/time (d) × 100.

2.6. Data Analysis

Results were analysed as a function of dietary PRSE level using regression analysis in Graphpad Prism (GraphPad Software, San Diego, CA, USA, V. 7.03). Data were tested against increasingly complex regression models, starting with the null hypothesis that

PRSE had no effect on the outcome (horizontal line). Models were checked with inbuilt options for normality of residuals using the D'Agnostino and Pearson omnibus test, for curve deviation from data points using the replicates test, and for homoscedasticity. Models that failed one or more of these checks were rejected in favour of more complex models. Models with an adjusted $R^2 < 0.2$ were rejected in favour of simpler models.

3. Results

3.1. Shrimp Growth, Survival, and FCR after 35 Days

Shrimp weight, carapace length, and specific growth rates increased linearly in response to dietary PRSE levels over the first 35 days of feeding the experimental diets (Figure 1a–c). The shrimp FCR decreased linearly in response to increasing dietary levels of PRSE over the same period (Figure 1f). No relationship was found between shrimp condition factor, survival, and dietary PRSE level after 35 days of feeding the diets (Figure 1d,e). Overall, the fitted linear models found that shrimp fed the highest level of PRSE (6 g kg^{-1} diet) were 65% heavier (6.1 vs. 3.7 g), 11% longer (23.4 vs. 21.0 mm), had an SGR 32% higher (5.0 vs. 3.8% day^{-1}), and an FCR 39% lower (1.9 vs. 3.1) than shrimp fed control diets without PRSE.

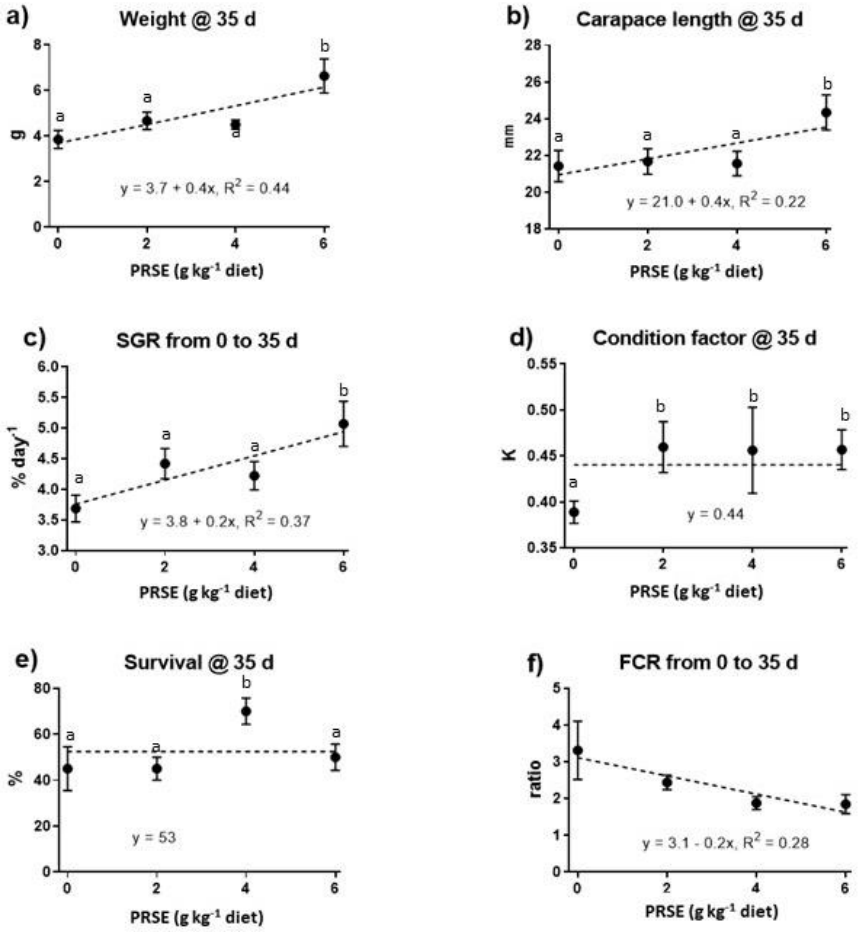

Figure 1. (**a**) Weight; (**b**) carapace length; (**c**) specific growth rate; (**d**) condition factor; (**e**) survival; and (**f**) feed conversion ratios of juvenile black tiger shrimp *Penaeus monodon* fed diets with increasing levels of a polyphenol-rich sugarcane extract (PRSE) (Polygain^{TM}) for 35 days. Dashed lines represent best fit models for the data. Equations and adjusted R^2 values represent the relationship between the measured parameter and dietary PRSE level. Horizontal lines indicate no relationship was present. Data are mean ± SEM, $n = 4$.

3.2. Shrimp Growth, Survival, and FCRs after 70 Days

After 70 days on the diets, the shrimp weights, carapace lengths, and survival continued to increase linearly in response to dietary PRSE levels (Figure 2a,b,e). The shrimp FCR decreased linearly in response to increasing dietary levels of PRSE over the same period (Figure 2f). No relationship was found between the shrimp SGR, condition factor, and dietary PRSE level after 70 days of feeding the diets (Figure 2c,d). Overall, the fitted linear models found that shrimp fed the highest level of PRSE (6 g kg^{-1} diet) were 54% heavier (15.4 vs. 10.0-g), 18% longer (23.3 vs. 27.5 mm), had a 92% higher survival (50 vs. 26%), and an FCR 39% lower (1.9 vs. 3.1) than shrimp fed the control diet.

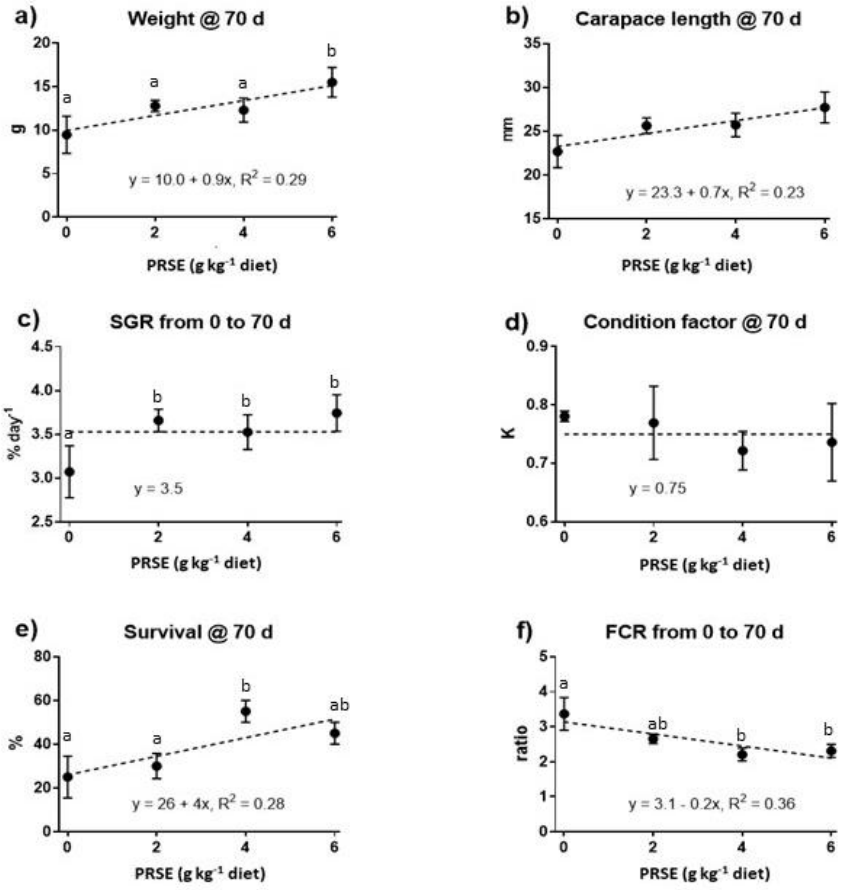

Figure 2. (**a**) Weight; (**b**) carapace length; (**c**) specific growth rate; (**d**) condition factor; (**e**) survival; and (**f**) feed conversion ratios of juvenile black tiger shrimp *Penaeus monodon* fed diets with increasing levels of a polyphenol-rich sugarcane extract (PRSE) (Polygain^{TM}) for 70 days. Dashed lines represent best fit models for the data. Equations and adjusted R^2 values represent the relationship between the measured parameter and dietary PRSE level. Horizontal lines indicate no relationship was present. Data are mean ± SEM, n = 4 (or 3 for the control group data other than for survival as one replicate was lost).

3.3. Shrimp dcr1 mRNA Expression and Pathogen Levels after 70 Days

After 70 days of feeding the PRSE experimental diets, no differences were found between treatments in *dcr1* expression (average expression of 6.6 × 10^5 copies), or the copy number of hepatopancreatic parvovirus or gill-associated virus (Figure 3). The other pathogens were not detected in any of the analysed shrimp (refer to the legend of Figure 3).

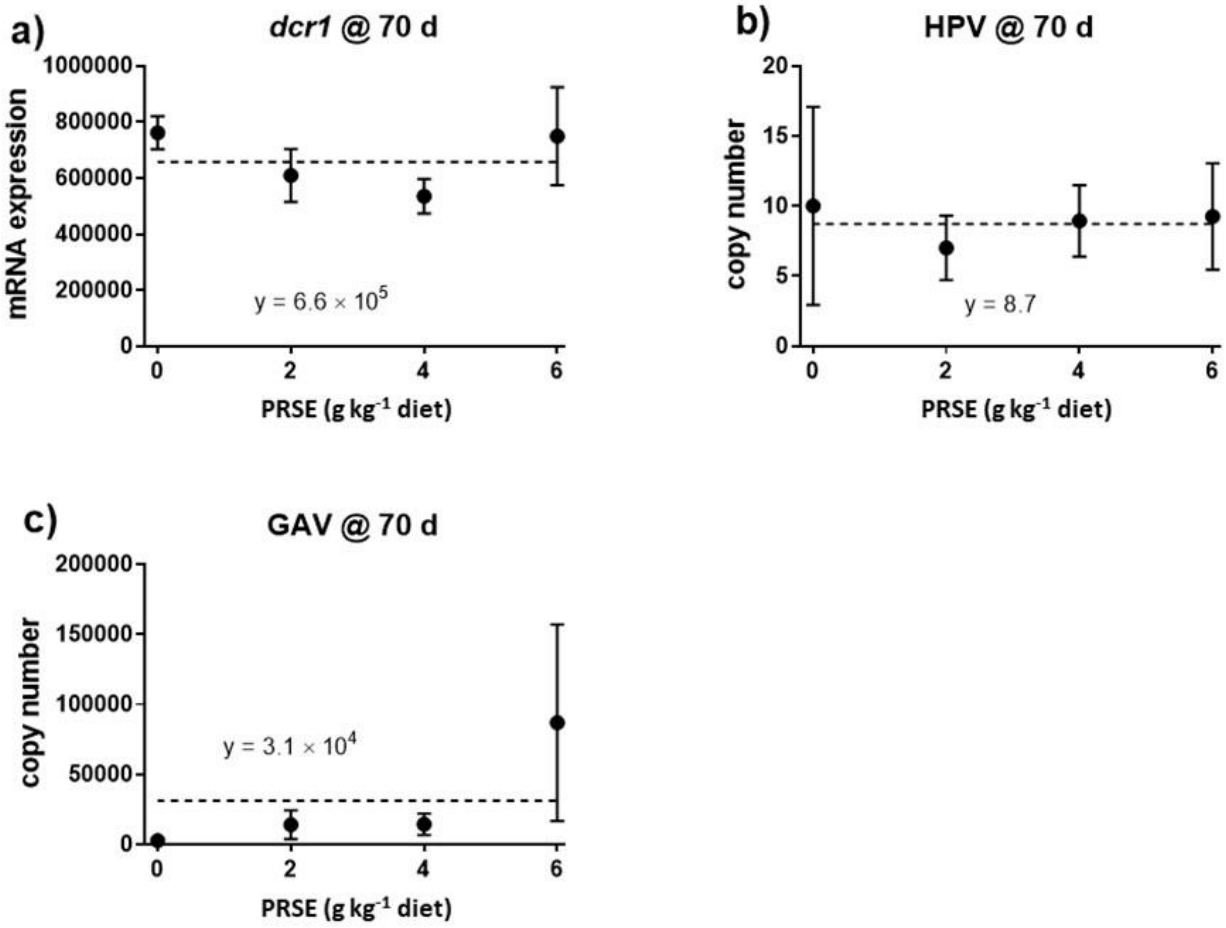

Figure 3. (**a**) The expression of dcr1 and (**b**) the viral loads (viral copies/μL pleopod extract) of hepatopancreas parvovirus (HPV) and (**c**) gill-associated virus (GAV) in juvenile black tiger prawns fed diets with increasing levels of a polyphenol-rich extract derived from sugarcane (PPE) for 70 days. Horizontal lines indicate no relationship was present. The other prawn pathogens tested for—infectious hypodermal and hematopoietic necrosis virus (IHHNV), yellow head virus genotype 7 (YHV7) and genotype one (YHV1), white spot syndrome virus (WSSV), whenzhou uncharacterised prawn virus (When-2), and Vibrio parahaemolyticus (Pir-A)—were not detected. Data are mean ± SEM, n = 4 (or 3 for the control group, as one replicate was lost), where the results for all animals were pooled for each tank.

4. Discussion

The data demonstrate that PRSE had a positive effect on growth, survival, and feed conversion efficiency in juvenile black tiger shrimp. This effect on growth appeared to begin from a very early stage of the trial (from 0 to 35 days of feeding, 58 to 93 post-larvae (PL) shrimp), with shrimp mass differences between treatments decreasing over the following 35 days of feeding (35–70 days of feeding, 94 to 128 PL). However, the overall shrimp weight and length remained larger in all treatments with PRSE included. This suggests that shrimp fed the PRSE diets would be likely to reach their harvest weight quicker, improving the turnover of shrimp receiving dietary PRSE in a production scenario.

One of the most significant findings of the trial was the improvement of FCR. This dose-dependent trend was observable at both the 35- and 70-day marks of the trial. In addition, improvements from the control diet were also observed at every dose of PRSE studied at both of these time points, with the most pronounced difference observed with the 6 g/kg dosage. Due to the FCR continuing to show a linear regression alongside the increasing inclusion of PRSE, it is possible that the optimal dosage for FCR improvement has not been reached in this trial. However, the improvement in FCR following a relatively

minor change in the feed formulation is a result that justifies further examination of the potential mechanisms contributing to this finding.

The effect of PRSE on survival was one of the key findings of this trial. Over the first 35 days, there was no clear trend in survival due to amount of PRSE included in the diet. This may be due to interference derived from cannibalism and aggression between individuals in the early moulting phases. However, over the entire 70-day timeframe of the trial, there was a positive relationship between the levels of PRSE included in the diet and the increased rate of survival across the population. As a result of these significant differences in survival correlated with the intake of extract, it is expected that the total biomass yield would be further increased beyond the improvements to FCR and growth rate already discussed. It should be noted, however, that the test conditions of this study placed the animals in close proximity, which could have caused undue stress and likely impacted survival throughout the study. In fact, shrimp on a non-PRSE diet had a lower survival than seen in many feed additive trials (e.g., [37], although survival in experimental tank systems for feed trials can vary dramatically from study to study [38]. For a more substantial representation of the impact of PRSE on survival, this study should be replicated in a commercial rearing environment.

This trial demonstrates that the inclusion of the PRSE sugarcane extract has a range of benefits, but it is difficult to hypothesise the exact mechanism that has produced these results and the molecular mechanisms need to be examined further. One of the main polyphenols identified in the sugarcane extract is chlorogenic acid. This phenolic acid has been included in shrimp diet formulations previously, with significant improvements to survival and FCR analogous to what was observed in the present trial [39]. Whilst the chlorogenic acid trial was performed on a different species of saltwater shrimp (*Litopenaeus vannamei*), the similarities of the effects add evidence to the possibility that chlorogenic acid is one of the active ingredients present in the sugarcane extract. Wang et al. (2015) hypothesised that the increased antioxidant capacity and transcription of antioxidant enzymes observed was the likely mechanism that led to the improved production outcomes, including stress resistance and increased total biomass yield. Chlorogenic acid has been observed to be involved in the regulation of nuclear factor erythroid 2-related factor (*Nrf-2)* and inflammatory markers such as nuclear factor kappa-light chain enhancer of activated B-cells (*NF-κB)* in vertebrates [40]. However, the activation of *Nrf-2* or regulation of *NF-κB* have not been directly studied with chlorogenic acid-enriched diets in shrimp.

Polygain™ (PRSE) extract contains a diverse range of compounds beyond polyphenols, including; organic acids, minerals, phytosterols, fatty acids, and amino acids. Some of these have been quantified, such as chlorogenic acid, but many others have only been putatively identified in the extract or are known to be abundant in sugarcane and fermentation products, such as organic acids. Some of these qualitatively identified or even unidentified compounds could have a major role in the mechanism of action of this extract. For example, organic acids are abundant in sugarcane production streams [41]. Succinic acid has been shown to have a positive effect on growth and immune function in *Litopenaeus vannamei* [42]. As a result of the variety of compounds present in the PRSE sugarcane extract, the potential of non-phenolic components playing an active role in the improved performance should not be overlooked. The most appropriate hypothesis at this stage is that a range of these components are acting synergistically to produce the beneficial results observed in this trial.

Other studies on the addition of polyphenol-rich plant extracts to shrimp diets focus on the effect on shrimps' resistance to specific diseases. For example, an aqueous extract containing polyphenols from the leaves of spurred mangroves (*Ceriops tagal*) prevented infection with white spot syndrome virus (WSSV), when coated onto the diet at the rate of 10 g kg^{-1}, for whiteleg shrimp [28]; while an extract from Bermuda grass (*Cynodon dactylon*) added at 20 g kg^{-1} to diets prevented infection by WSSV in black tiger shrimp [25]. However, the shrimp used in the current study were free of WSSV and most of the other shrimp pathogens tested. The pathogens present, hepatopancreas parvovirus (HPV) and gill-associated virus (GAV), were present at low levels and were not different between

treatments (Figure 3). The mRNA expression of *dcr1*, which codes for the endoribonuclease Dicer-1 protein that plays an antiviral defence role in *P. monodon* [36], was also not different between treatments. While the presence of unknown pathogens cannot be ruled out, overall the pathogen data in this study suggest that differences in shrimp growth and survival were not limited to the PRSE-induced-inhibition of infectious disease. Further work on other damaging pathogens to the shrimp industry, such as *Enterocytozoon hepatopenaei*, and under challenge conditions would help in understanding the impact this extract may have on disease defence.

Dietary polyphenols are associated with positive effects on animal health and growth performance, through a diverse range of other mechanisms not targeted in this experimental design, including interaction with gut bacteria, modulation of metabolic pathways, and as a direct immunomodulator [43]. However, for some of these factors, such as the microbiome, there may be species differences that may alter what would be expected to be observed in vertebrates. Since vertebrates are used for the majority of the research into the interaction between polyphenols and the microbiome, key physiological differences that would impact microbiome development require consideration.

The digestive tract of shrimp differs substantially from that of vertebrates; therefore, the interaction with the microbiome may differ as a consequence. For example, shrimp secrete a chitin sheath in the midgut that surrounds the digesta. This sheath is thought to protect the hindgut from bacteria, but it also limits nutrient uptake [44]. Furthermore, the entire digestive process in shrimp occurs at a neutral pH and at a lower temperature than in mammals. In addition to this, the overall gut transit times for food are between 2 and 4 h for penaeid shrimp (reviewed by [45]), versus around 8 h in mammals such as mice [46]. While data are limited, there is some evidence that plant polyphenols can positively affect specific bacteria in shrimp and could potentially play a role in modulating the gut microbiome. For example, the polyphenols present in a green tea extract can inhibit the growth and lethality of the pathogenic bacteria *Vibrio harveyi* in kuruma shrimp (*Marsupenaeus japonicas*) [47].

Some studies have found a direct effect on immune response and function with the addition of a range of plant sources that was linked to improved survival [43]. Given the positive effects on survival, in addition to other production outcomes such as growth rate and FCR, a follow-up study that directly measures any immunomodulating effects would be useful. A further unexplored area in aquaculture nutrition is the ability of polyphenols to chelate iron, which may have a positive effect on iron homeostasis and therefore growth and survival. A study that tracked iron bioavailability in the presence of the sugarcane extract would provide further understanding if this mechanism was involved in the observations collected during this trial [48] It will also be important to assess the performance of this sugarcane extract in the later stages of the animals' development.

Small additions of PRSE (~6 g kg^{-1} of diet) led to increases in the growth, survival, and feed conversion efficiency of shrimp. Overall, this study demonstrates that plant polyphenolics have potential as additives to shrimp feed and validates the need for further research in this area, including research conducted in commercial rearing environments rather than test conditions. Due to the sourcing of the raw material from the existing sugarcane industry, this extract provides a prospective opportunity to extend the sustainability of the aquaculture industry.

5. Conclusions

This trial indicates a number of positive results from the inclusion of sugarcane extracts in the diet of tiger shrimp. Further work is needed to translate these initial results into full-scale farm trials and to further understand the underlying mechanisms of action. However, the results collected in this trial demonstrate that these further investigations are warranted.

Author Contributions: S.P., M.F. and B.K. drafted the manuscript. All authors were involved in the conception of the study and editing of the manuscript, and the critical evaluation of the results. S.P., T.A., and K.C. ran the trial. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: At the time of the experiment, no animal ethics approval was required for shrimp trials in Australia.

Data Availability Statement: Data and material will be made available on request.

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