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Effect of dietary supplementation with apple cider vinegar and propionic acid on hemolymph chemistry, intestinal microbiota and histological structure of hepatopancreas in white shrimp, *Litopenaeus vannamei*

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ABSTRACT

This experiment was conducted to evaluate the effects of dietary supplementation of Apple cider vinegar (ACV) and propionic acid (PA) on biochemical parameters of hemolymph, intestinal microbiota and histology of hepatopancreas in white shrimp (*Litopenaeus vannamei*). Five experimental diets were evaluated in this study including diets supplemented with 1.0, 2.0 and 4.0% of ACV, 0.5% propionic acid, and a control diet with no supplements. Shrimps (initial weight of 10.2 ± 0.04 g) in triplicate groups with the density of 25 shrimps per tank were fed the diets for 60 days. At the end of the feeding trial, shrimps fed with ACV and PA supplemented diets had significantly higher total protein level than those fed the control diet (P < 0.05). The number of *Vibrio* spp., R-cells (lipid storage cells) of hepatopancreas and cholesterol level in shrimps fed the diets containing ACV and PA were lower compared to the control group (P < 0.05). However, there was no remarkable variations in glucose concentration, B-cell number and tubule diameter among the experimental diets (P > 0.05). In addition, shrimps fed the ACV diets had significantly lower total heterotrophic marine bacteria compared to the control or PA groups, and the lowest bacterial number was observed in shrimp fed 4% ACV supplemented diet (P < 0.05). Supplementation of 2 and 4% ACV as well as 0.5% PA in the diet led to a significantly higher calcium concentration than the control treatment (P < 0.05). The lowest triglyceride concentration was observed in the shrimps fed diets containing 2.0 and 4.0% ACV, which resulted in 15 and 20% reduction, respectively (P < 0.05). Overall, the findings indicates that ACV and PA possess antimicrobial activity and demonstrate beneficial effects on health status, so they can be potentially used as feed additive in the feeding of *L. vannamei*.

1. Introduction

The prevalence and spread of various pathogens limit the development of aquaculture programs, and antibiotics have long been used to reduce or remove such issues. However, it is known that inappropriate and extensive use of antibiotics can lead to adverse consequences. Therefore, the use of antibiotics for animal production were banned in many countries because they cause negative effects on growth performance, resistance of bacterial strains and potential harm to human and environment [1–4].

Shrimp culture has received high attention in the past decade, and shrimp industry in Iran is growing quickly. One of the strategies to improve shrimp health is the usage of feed additives such as organic acids and their salts, and short-chain fatty acids including citric, formic, lactic, malic, acetic, butyric, propionic and sorbic acids [1]. Organic

acids, which are generally regarded as safe, are used to inhibit pathogens by acidification of the cytoplasm pH, thus limiting harmful bacteria in gastrointerstetial of aquatic animals [5,6] and consequently enhancing growth and resistance disease in aquatic animals.

Several studies have revealed that the utilization of organic acids and their salts can inhibit the growth of pathogenic bacteria in *Litopenaeus vannamei* [7], *Penaeus monodon* [4], *Oreochromis sp* [8], piglet [9] and *Paralichthys olivaceus* [10]. In addition, in a previous study, it was revealed that organic acid blends had protective effects on hepatopancreas of *Litopenaeus vannamei* against *Vibrio harveyi* challenge [2]. Besides antimicrobial properties, organic acids demonstrated beneficial effects on feed palatability, gut pH, digestive enzyme activity, gut morphology, growth performance and nutrient utilization as well as hepatopancreas morphology [11]. Histological studies on the hepatopancreas have been applied as a practical means for evaluating the

nutritional status in the shrimp culture. Recently, other findings also indicated that diets supplemented with different levels of organic acids significantly influenced the morphology of hepatopancreas cells in *M. rosenbergii* [12].

Vinegar has recently been used in medicine as a food additive, and it was shown to have some impacts on pharmacological functions such as hyperlipidemia, reduced blood sugar, hypercholesterolaemia, nutritional support, antioxidant defense and lowering blood pressure in human and animal models [13–16]. Apple cider vinegar (ACV) is an acidic solution produced by fermenting apples. It also contains organic acids, flavonoids, polyphenols, vitamins and minerals. Furthermore, the main class of organic acid in ACV is acetic acid (3–9%) [17]. Some studies investigated the inhibitory effects of ACV and bamboo vinegar against bacteria under in vitro conditions [14,18]. However, little information exists about the dietary application of ACV in aquatic animals. Previous studies showed that ACV supplemented diets can regulate some immune-related genes in *Litopenaeus vannamei* [1]. Similarly, Safari et al. [19], found that dietary supplementations of ACV impacted some immune-related and antioxidant genes, total immunoglobin concentration and lysozyme activity in *Cyprinus carpio*. Its impacts on immune system has led to be considered as a natural immunostimulant.

There is limited knowledge regarding the dietary application of ACV in shrimp feeding and its effects on physiological factors. Therefore, the present study aimed to investigate the effect of diets contaning ACV and PA, on total viable bacterial and *Vibrio* counts, hepatopancreatic histology and some hemolymph constituents of whiteleg shrimp (*Litopenaeus vannamei*), in order to evaluate their potential use as feed additives in aquaculture.

2. Material and method

2.1. Feed preparation

The experimental diets were prepared according to a previous study [1]. The basal diet was considered as control diet and four diets were supplemented with different levels of ACV (1, 2 and 4%) and 0.5% PA (Sigma Aldrich St. Louis, Mo, USA, pure ≥ 99.5%). Experimental diets were stored in polythene bags at $-20\,^{\circ}C$ until use.

2.2. Experimental design

Three hundred and seventy-five healthy individuals of *L. vannamei* (10.2 ± 0.04 g) obtained from the local commercial shrimp farm in Gomishan, Golestan province, Iran. They were transported to the aquaculture lab at Gorgan University of Agricultural Sciences and Natural Resources. The shrimps were then adapted to the experimental conditions for 14 days, and fed with commercial diet (Beyza Feed Mill, Iran). After the adaptation period, shrimps were transferred into 15 round fiberglass tanks (400 L) with constant aeration, according to a completed random design with 3 replicate tanks for experimental diet (25 shrimps/tank). Water quality parameters were measured daily (20.27 ± 1.21 ppt salinity and 25.84 ± 1.64 $^{\circ}C$ temperature), and kept at optimum levels for this shrimp species. Each morning, approximately 15% of the total volume of water from each tank was exchanged with new sea water for removing any feces and uneaten food. Shrimps were fed to apparent satiation with experimental diets four times (07:00, 11:30, 15:30 and 22:00) a day for 60 days.

2.3. Hepatopancreas histology

After being fed for 60 days, a total of three shrimps' hepatopancreas from each tank were dissected for histological examination. The hepatopancreas was fixed in 10% buffered formalin [20], and then transferred to 70% ethanol until processing. The histological sections (5–7 μm) were stained with hematoxylin-eosin and examined by a light microscope (SX-30 Olympus, Japan) and photographed with digital camera coupled to a microscope. The number of the R-cells (Restzellen or Resorptive cell) and B-cells (Blasenzellen or Blister cell), and tubule diameter were quantified within 20 randomly selected tubules from each treatment [2].

2.4. Intestinal bacterial count

At the end of feeding trial, five shrimps from each replication were sampled randomly and the intestinal tracts were aseptically dissected with forceps and scalpel and then weighed. About 100 mg of intestinal tract for each replication was pooled and stored in crucible. Then, the samples were homogenized (~2 min) with sterile saline in a mortar. The samples were serially diluted (10-fold) in sterile saline and 100 μl of the supernatant was spread onto a thiosulfate-citrate-bile-sucrose agar (TCBS; a selective medium for *Vibrio* spp.) and Marine agar (nonselective medium for total heterotrophic marine bacteria) plates in triplicate. Finally, plates were incubated at 28 °C and controlled after 24 h. The total count of the colony-forming units (CFU) per gram in the intestinal tract was expressed as a logarithm of CFU per gram of intestine ($Log_{10}\,CFU\,g^{-1}$) [4,21,22].

2.5. Hemolymph sampling

At the end of the feeding trial, hemolymph from five shrimps per experimental unit was extracted from ventral sinus with sterile syringes of 2 mL, needle 25G. The hemolymph was immediately centrifuged at $12,000 \times g$ for 20 min, and supernatants were kept at $-20\,^{\circ}C$ until biochemical analysis [23].

2.6. Hemolymph biochemical analysis

The biochemical parameters of hemolymph were determined by using standard analytical techniques. Total proteins (mg/ml) were determined by method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard [23]. Glucose, triglyceride, cholesterol, calcium concentrations (mg/dl) were assayed using detection kits (Co, Pars Azmon kit, Iran) with an autoanalyzer (Hitachi 917, Japan) [24,25].

2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) and differences among means were determined by post hoc LSD test. Normality was assessed by the Kolmogorov-Smirnov test. Data homoscedasticity was evaluated by the test of Bartlett [7]. A significance level of 5% was considered for all the statistical tests.

3. Result

3.1. Histology of hepatopancreas

Star like hepatopancreatic tubules of shrimp fed the supplemented diets are presented in Fig. 1(a–e). The hepatopancreas of shrimp fed control diet had significantly more R-cells than those fed the supplemented diets (P < 0.001). The lowest number of R-cells was observed in shrimps fed 1% (18.92 cells/tubule) and 2% (19.07 cells/tubule) ACV supplemented diets respectively (Table 1). There were no differences in B-cell number among the experimental groups (P > 0.05). As for tubules diameter, no significant differences were observed between the treatments, although it is slightly larger in shrimps fed 2 and 4% ACV and PA enriched diets compared to control group (Table 1).

3.2. Intestinal bacterial count

The total bacterial count was lower (P < 0.05) in intestinal tract of shrimps fed with diets supplemented with ACV compared with other
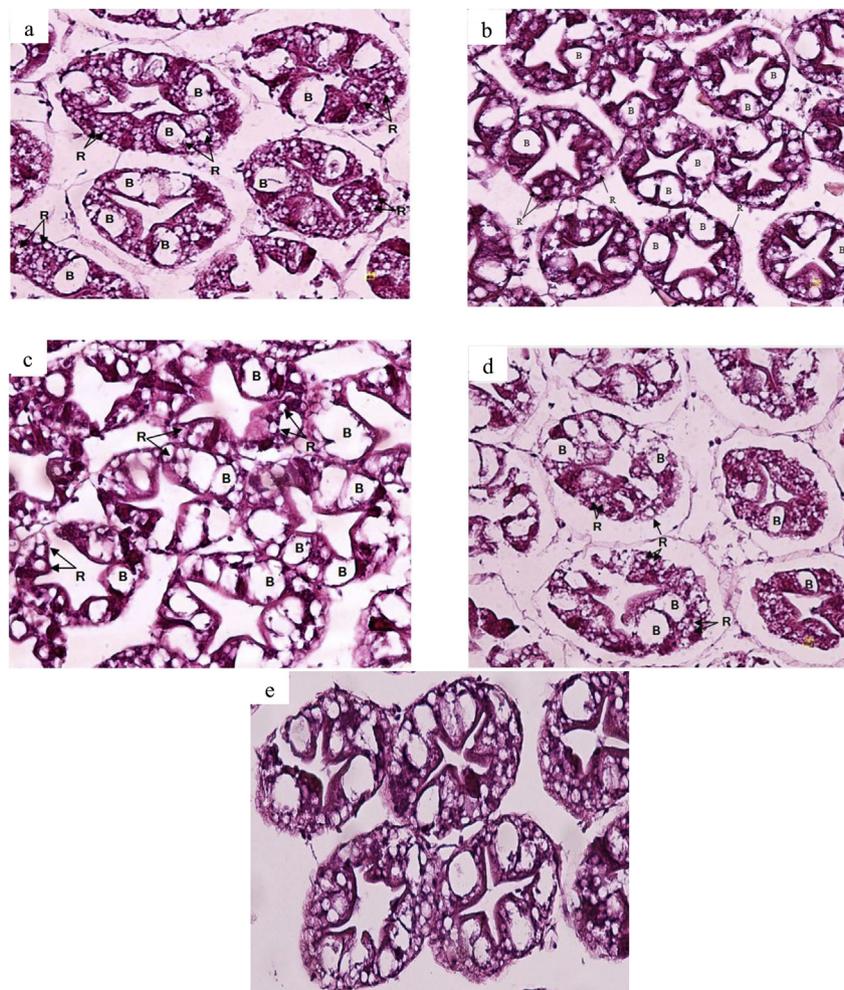
Fig. 1. Histological features of the hepatopancreas from *L. vannamei* fed the control diet (a), 1% ACV (b), 2% ACV (c), 4% ACV (d) and 0.5% PA supplemented diets (e). The hepatopancreas cells include B (B) and R (R). Note, a decrease in R-cells number was observed in the ACV and PA treatments. Magnification × 20.

treatments (Fig. 2). However, no significant differences were detected among the PA received group and control group ($P > 0.05$). The total presumptive *Vibrio* spp. counts in shrimps fed with diets supplemented with ACV and PA showed lower concentrations compared to that in the control group ($P < 0.05$) (Fig. 3). The lowest of number of *Vibrio* spp. was observed in shrimps fed 4% ACV-supplemented diet. In addition, increasing dietary levels of ACV led to a reduced intestine population of *Vibrio* bacteria.

3.3. Biochemical parameters of the hemolymph

Total protein level of *Litopenaeus vannamei* treated with dietary supplementation of ACV and PA is summerized in Fig. 4. As it is observed, protein levels significantly increased in treated shrimps ($P < 0.05$). The highest protein level was observed under 1 and 2% ACV and PA diets (45, 58 and 63%, respectively). Dietary intake of 2

and 4% ACV and PA significantly enhanced calcium concentration compared with the control group and the group received 1% ACV. Hemolymph cholesterol concentration was lower in shrimp fed with ACV and PA diets, compared to the control group ($P < 0.05$). Furthermore, 4% ACV group indicated the lowest value (26.07 mg/dl) for cholesterol concentration compared to control group (33 mg/dl). Triglyceride level was approximately 15 and 20% lower in shrimps fed 2 and 4% ACV supplemented diets respectively, compared to control group ($P < 0.05$). The concentrations of glucose however showed no significant differences between the groups ($P > 0.05$).

4. Discussion

In this study, the effect of dietary supplementation of AVC and PA were investigated on *Litopenaeus vannamei*, biochemical parameters of hemolymph, intestinal microbiota and histology of hepatopancreas, and

Table 1
B-cell and R-cell prevalence (number/tubule) and tubule diameter (μm) from the hepatopancreas of shrimp *L. vannamei* fed the control diet, 1% ACV, 2% ACV, 4% ACV and 0.5% PA supplemented diets for 60 days.

	Experimental Diets				
	Control	1% ACV	2% ACV	4% ACV	0.5% PA
B-cells	4.72 ± 0.95	3.93 ± 0.75	5.17 ± 0.65	5.4 ± 1.2	4.65 ± 0.2
R-cells	34.29 ± 1.63[a]	18.92 ± 3.19[c]	19.07 ± 4.30[c]	25.67 ± 3.7[b]	21.22 ± 2.29[cb]
Tubule diameter	69.47 ± 5.82	67.99 ± 1.66	75.54 ± 4.57	73.25 ± 3.28	72.87 ± 2.24
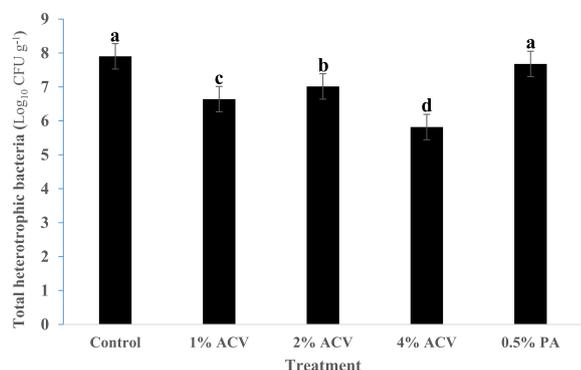
Fig. 2. Total heterotrophic marine bacteria of shrimp *L. vannamei* fed control diet, 1% ACV, 2% ACV, 4% ACV and 0.5% PA supplemented diets for 60 days. Values are presented as the mean ± SD (n = 15 in each group). Different lowercase letters indicate statistically significant *differences* between different experimental groups (P < 0.05).
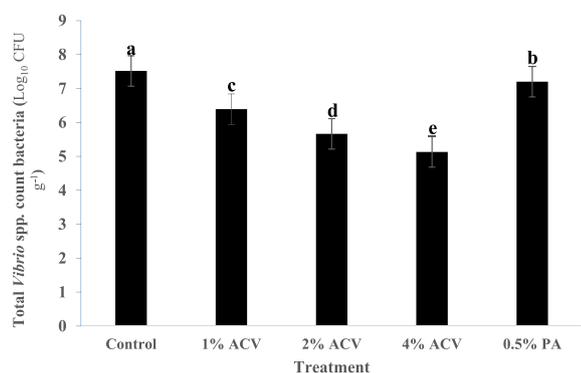
Fig. 3. The mean *Vibrio* spp. count of shrimp *L. vannamei* fed with diets supplemented with Apple Cider Vinegar (ACV) and Propionic acid (PA) after 60 days. Values are presented as the mean ± SD (n = 15 in each group). Different lowercase letters indicate statistically significant *differences* between different experimental groups (P < 0.05).

the findings provided useful information given the important role of dietary additives in shrimp feeding.

This study the reduction of R-cell following the shrimp feeding with ACV and PA supplemented diets. A decrease in energy reserve in hepatopancreas could be linked to the decrease of R-cells (Restzellen or Resorptive cell) which are responsible for lipid storage in hepatopancreas gland. Acetate could inhibit the activity of carbohydrate-responsive element-binding protein (ChREBP), which regulates several genes required for changing glucose to fatty acids in the digestive gland. The transcripts of lipogenic genes such as ACC, FAS, ACL, ME G6PD and LPK in the liver were down-regulated in rats fed acetate [26]. Moreover, Khalil et al. [12], reported the negative effect of dietary sodium lactate at 1% concentration on the number of lipid vacuoles and deformation of R-cells in the freshwater prawn *Macrobrachium rosenbergii*. In agreement with our study, the level of lipid droplet storage was significantly decreased in the hepatopancreas of *L. vannamei* fed with 13.5 and 22.5 g/kg oxytetracycline [27]. Johnston et al. [28] also demonstrated that increase in the number of R-cells resulted in faster growth in rock lobster (*Jasus edwardsii*). In contrast, a previous study revealed that ACV and PA enriched diets had no effect on growth parameters in *Litopenaeus vannamei* [1]. The B-cells (Blasenzellen or Blister cell) are large cells responsible for enzyme storage, and this study indicated that there was no significant difference in the number of B-cells among shrimps submitted to the experimental dietary treatments.

ACV-supplemented diets used in the present study were able to markedly reduce both total viable bacterial and *Vibrio* spp in the

shrimp. In addition, *Vibrio* spp. counts in the intestine of198 shrimps fed diet supplemented with PA significantly decreased. In this regard, Ng et al. [8], found that the use of different dietary levels of organic acid blends and 0.3% potassium diformate caused a marked reduction in the total bacterial counts in the faces of red hybrid tilapia, *Oreochromis* sp. In *Litopenaeus vannamei* the dietary supplementation with different levels of butyrate reduced *Vibrio* spp. count after 27 and 47 days [7]. In addition, dietary usage of organic acid blends significantly increased the survival of *Litopenaeus vannamei* after challenge with *Vibrio harveyi* [2] and *Vibrio parahaemolyticus* [29]. Mine and Boopathy [30] in vitro investigated the growth inhibitory effects of organic acids on *Vibrio harveyi*, and the result showed that minimum inhibitory concentrations of acetic, propionic, butyric acids were 0.04, 0.03 and 0.06%, respectively. Total bacterial count in the intestine of olive flounder (*Paralichthys olivaceus*) fed with organic acid blends for 10 weeks showed significant decrease compared to control treatment [10]. It is generally accepted that the reduction in the bacterial population by organic acids can be primarily due to their ability of diffusion into the bacterial cells, and decreased pH in neutral cytoplasm, thereby causing inhibition of bacterial cells' metabolism and subsequent cell death [11]. Further, Yagnik et al. [14], demonstrated the antimicrobial potential of ACV against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The minimum concentration of ACV required for growth inhibition was 1/2 ACV for *C. albicans*, 1/25 for *S. aureus* and 1/50 for *E-coli*. In this case, ACV activity has been related to damage cell integrity, structural and metabolic proteins as well as nuclear material. Hindi et al. [31] verified the inhibitory potential against both gram positive and gram negative bacteria when exposed to ACV.

The biochemical composition of hemolymph can be induced by environmental conditions such as food availability, salinity and temperature [32]. Hemolymph metabolites such as cholesterol, triglycerides and proteins have been displayed to reflect nutritional status and health in shrimp [22,33]. In our study, glucose concentration in shrimp fed with diets containing ACV and PA did not significantly differ from that of the control group. The physiological role of glucose in aquatic animals includes response to stress and it is also the main energy source during starvation [34,35]. Similarly, previous study has illustrated that shrimp fed with β-glucan plus *Bacillus subtilis* and β-glucan plus *Pediococcus acidilactici* supplementations had no effect on the glucose concentration in pacific white shrimp [22]. Iman et al., demonstrated that dietary ACV significantly reduced blood glucose level in diabetic mice [17]. The protein level in hemolymph could be related to immune function. Here, shrimps fed with of diets containing ACV and PA showed an increased level of proteins after 60 days. Similar to our study, He et al. [29] found positive effect of organic acid blends (citric and sorbic acid) on protein concentration of *Litopenaeus vannamei*. In addition, dietary supplementation with succinic acids significantly increased immune response of *Litopenaeus vannamei* [36]. Pourmozaffar et al., previously reported a positive effect of dietary ACV and PA on the expression of antimicrobial peptide genes such as prophenoloxidase and lysozyme in white shrimp [1]. Moreover, oral administration of ACV plus two type of probiotics remarkably increased total immunoglobulin and lysozyme activity in skin mucus and serum of common carp [19].

Cholesterol is a major biomembrane component that is important in preserving cellular function and structure. In addition, cholesterol is the main precursor of steroid hormones and the major sterol in shrimp especially during the moult cycle [37]. Triglycerides play an important role in primary metabolic reserve and production of energy [32]. The results of the present study showed that cholesterol in shrimps fed dietary ACV and PA was significantly lower than those in the control group. In addition, shrimp fed with 2 and 4% ACV supplementations had the lowest triglyceride level compared to the control group. In this respect, the use of different levels of ACV in diets caused significant decrease in cholesterol and triglyceride contents in rats [26,38–40] and human [16]. Furthermore, Maita et al. [41] concluded that a decrease in plasma lipid such as cholesterol and triglycerides led to higher
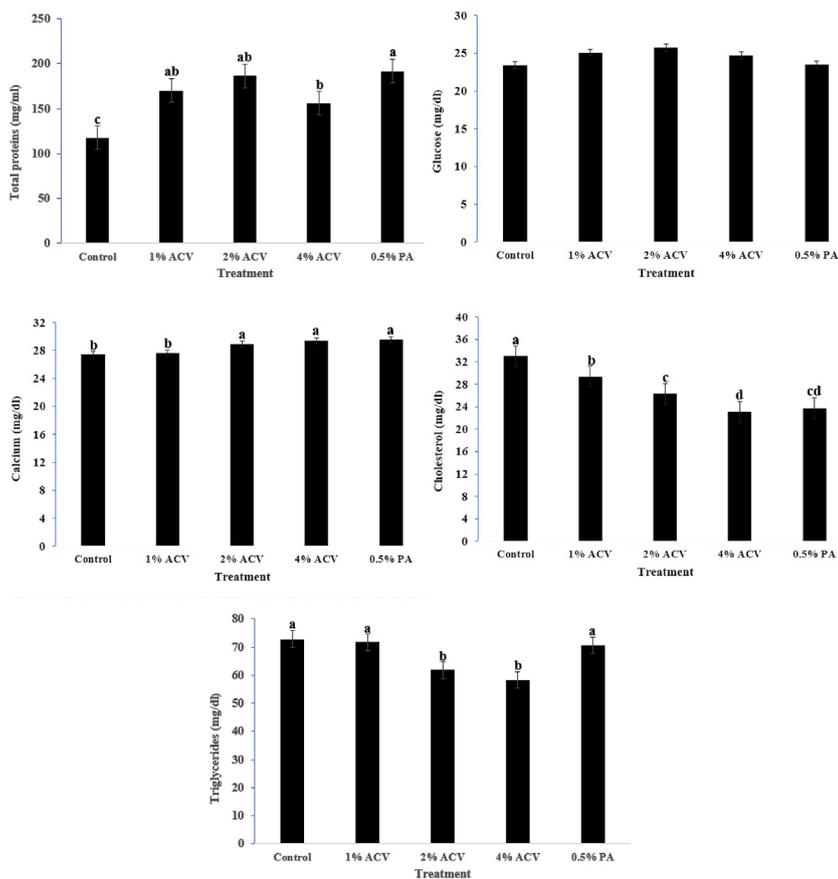
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