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The impact of apple cider vinegar on nonalcoholic fatty liver disease in rainbow trout, *Oncorhynchus mykiss*: A study of therapeutic potential and health benefits

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Abstract

In this study, we aimed to experimentally induce fatty liver disease in rainbow trout, *Oncorhynchus mykiss*, and then assessed the illness recovery process, growth, and changes in the expression of FAAH and ACADL genes in both healthy (0 [C2] and 4% apple cider vinegar [T4]) and diseased fish (0 [C1], 1 [T1], 2 [T2], and 4% [T3]) apple cider vinegar. To conduct the study, 180 rainbow trout were randomly assigned to six different experimental treatments, each with three replications. The investigation lasted for 60 days. Growth indices, liver histology, blood biochemical parameters, and transcription of the ACADL and FAAH genes in the liver tissue were measured. The study found no significant differences in the final weights across all the treatments. Apple cider vinegar (ACV) administration resulted in a decrease in AST, ALT, and ALP; however, these values did not show a significant difference from C2. In T3, triglycerides significantly decreased ($p < 0.05$), whereas in T4, triglycerides significantly increased ($p < 0.05$). Hepatocytes from ACV-containing treatments showed reduced fat compared with T4 and the control group (C1). While there

was a significant difference ($p < 0.05$) in the expression of the FAAH gene, there was no significant difference ($p > 0.05$) in the expression of the ACADL gene between experimental treatments. The findings of our study indicate that an inclusion of up to 2% ACV may have positive effects on trout aquaculture and NAFLD treatment.

KEYWORDS
apple cider vinegar, liver health, NAFLD, rainbow trout

1 | INTRODUCTION

The liver, being the largest organ in vertebrates and a vital gland, plays a crucial role in supporting the functions of other organs within the body (Chan et al., 2004). Fatty liver disease is a significant health concern, with the potential to progress to severe conditions such as cirrhosis of the liver. The synthesis of triglycerides resulting from the interaction of free fatty acids and glycerol in the cytoplasm of liver cells leads to the accumulation of triglycerides, culminating in fatty liver disease (Postic & Girard, 2008). Nonalcoholic fatty liver disease (NAFLD) represents a liver ailment with potential implications for advanced liver diseases, including hepatocellular carcinoma. In cases of fatty liver, the liver's fat content can exceed 5% of its total weight, contributing to adverse effects such as impaired lipid homeostasis in fish, reduced growth rate, compromised meat quality, and decreased resistance to pathogens and environmental stressors in rainbow trout farms. These outcomes have significant implications for the aquaculture sector (Oyarzún et al., 2019; Yu et al., 2021). Understanding the impact of fatty liver disease on fish health and its implications for aquaculture is crucial for developing effective management strategies and maintaining sustainable fish farming practices.

Polyphenolic compounds with health-promoting qualities are present in apple cider vinegar (ACV). According to Shahidi et al. (2008), its flavonoid concentration helps lessen the harmful effects of diets rich in cholesterol. Potassium is included in ACV, and it is required for the body to rebuild and repair damaged soft tissue. Minerals, vitamins, amino acids, and peptides are also present in ACV (Kondo et al., 2009; Yamashita et al., 2007).

ACV is a disinfectant that eliminates a wide range of microorganisms found in certain diets. In the body, it decomposes fat deposits, mucus, and sputum. Blood oxidation, detoxification, purification, and coagulation are all aided by this solution. Apple cider vinegar is thought to help treat a number of conditions, including dermatitis, high cholesterol, diabetes, dizziness, ear discharge, and weariness. As ACV boosts immune responses, growth performance, digestive enzyme activity, and the immune characteristics of skin mucus in green terror (*Andinoacara rivulatus*), it can be utilized as a natural growth promoter and immunostimulant (Ahmadniaye Motlagh, Javadmanesh, & Safari, 2020).

The high-fat diet leads to an increase in liver total triglycerides and cholesterol, along with the upregulation of genes involved in lipogenesis, such as sterol regulatory element binding-protein 1 (Srebf1), steraroyl coenzyme A decarboxylase 1 (Scd1), peroxisome proliferator-activated receptor gamma (PPAR), and acetyl-CoA carboxylase 1 (Acaca). However, these effects are mitigated by indole-3-acetic acid treatment. Additionally, the levels of reactive oxygen species (ROS) and malondialdehyde (MDA), as well as the activities of superoxide dismutase (SOD) and glutathione (GSH) in liver tissue, demonstrate that indole-3-acetic acid protects against high-fat diet-induced oxidative stress (Ji et al., 2019).

Fatty Acid Amide Hydrolase (FAAH) encodes a protein that plays a crucial role in hydrolyzing various primary and secondary fatty acid amides, thereby influencing the signaling properties of these compounds. It also acts as a physiological regulator of specific subsets of intracellular N-fatty acyl amino acids (Giang & Cravatt, 1997). On the other hand, Acyl-CoA Dehydrogenase Long Chain (ACADL) is a gene involved in fatty acid and branched chain amino acid metabolism, and reduced gene expression may contribute to nonketotic hypoglycemia (Nandy et al., 1996).

The potential association between the FAAH and ACADL genes and their relevance to NAFLD represents a significant area of investigation. Furthermore, exploring the possible link between these genes and the effects of ACV on NAFLD could yield valuable insights into potential therapeutic interventions.

Several studies have investigated the genetic factors contributing to NAFLD, including the role of FAAH and ACADL genes in lipid metabolism and liver function. For example, a study carried out by Sookoian and Pirola (2011) found that variations in the FAAH gene were associated with an increased risk of developing NAFLD in human. In addition to genetic factors, the potential impact of ACV on NAFLD has attracted research interest. For instance, Kondo et al. (2009) conducted a study demonstrating that acetic acid, a key component of vinegar, may contribute to improved lipid metabolism and reduction of liver fat in animal models.

We devised a high-fat diet specifically designed to induce fatty liver disease in fish, as there is currently no experimental evidence demonstrating its induction in rainbow trout. This study is the first to evaluate the impact of varying amounts of dietary apple cider vinegar on the growth and hepatosomatic indices (HSI) of rainbow trout. Also, we examined the impact of apple cider vinegar on the expression of two genes, FAAH and ACADL, for the first time in fish with nonalcoholic fatty liver disease.

2 | MATERIALS AND METHODS

2.1 | Experimental diets, experimental design, and breeding

The study involved 300 juvenile rainbow trout with similar weight (49.98 ± 6.71 g), which were initially fed a high-fat diet (Table 1) to induce nonalcoholic fatty liver disease over a one-month period. After this induction phase, numerous samples were taken and compared their liver status with a control group, confirming the accumulation in the liver and a condition similar to fatty liver disease based on clinical observations, hepatosomatic index, and liver histology. Consequently, 160 fish (74.20 ± 5.50 g) were segregated for further experimentation, while 20 additional fish considered healthy and fed a regular farm diet were included. The 180 fish were randomly allocated to six treatments with three replications in pre-divided cages (150 × 50 × 40 cm). Each treatment comprised three cages, each

TABLE 1 The components of the normal and high fat diets for rainbow trou.

Ingredients	Low fat diet (%)	High fat diet (%)
Fish meal	30	30
Meat meal	13	13
Wheat gluten	6	6
Wheat flour	14	10.5
Corn flour	14	10.5
Soybean meal	15	15
Fish oil	2	5.5
Soybean oil	2	5.5
Vitamin C	0.5	0.5
Vitamin premix	1.5	1.5
Mineral premix	1.5	1.5
Salt	0.5	0.5

Note: Mineral premix contains (mg/kg) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; Antioxidant (BHT), 100. 2.Vitamin premix contains (mg kg-1) E, 30; K, 3; Thiamine, 2; Riboflavin, 7; Pyridoxine, 3; Pantothenic acid, 18; Niacin, 40; Folacin, 1.5; Choline, 600; Biotin, 0.7; and Cyanocobalamin, 0.02.

containing 10 fish. Sick fish were provided with an ACV-treated diet (5% acetic acid, Verda Co., Iran) for 60 days at concentrations of 0 (C1), 1 (T1), 2 (T2), and 4 (T3) percent, while healthy fish were fed diets at 0 (C2) and 4 (T4) percent. To prevent the apple cider vinegar from dissolving in water, the diets were sprayed with an 8% gelatin solution and dried for 3 h (Ahmadniaye Motlagh et al., 2017). The animals were fed 2% of their body weight three times daily for 60 days. Tables 1 and 2 provide details of the dietary components and their analyses, respectively.

Throughout the study, the physical and chemical parameters of the rearing water, such as temperature (13 ± 5.6°C), pH (7.3 ± 0.92), dissolved oxygen (7.24 ± 2.0 mg/L), and total hardness (340.68 ± 102.0 mg/L), were carefully monitored. Natural light conditions were maintained throughout the duration of the experiment.

2.2 | Proximate chemical composition of the feed

The proximate chemical composition of the fish feed samples, including both the low fat diet and the high-fat diet for rainbow trout, was analyzed using standard methods. This included determining the crude protein content using the Kjeldahl method, assessing the crude fat content through the Soxhlet extraction method, and evaluating the crude fiber content using the AOAC Official Method 978.10. Additionally, the nitrogen-free extract content was calculated, and the ash content was determined using the AOAC Official Method 942.05. All analyses were conducted in triplicate, and the results were expressed as percentages of dry matter (Horwitz, 2010).

2.3 | Growth parameters

To evaluate the impact of apple cider vinegar (ACV) on the growth of both healthy and infected juvenile fish, a comprehensive biometric assessment was carried out at three intervals (at the start, midpoint, and end of the experiment) using a digital scale with a precision of 0.01 g. All fish within each cage were included in the assessment of growth parameters, while six fish per cage were randomly selected for the calculation of the hepatosomatic index. The growth performance parameters and hepatosomatic index were computed using the following formulas:

$$\text{Specific growth rate (SGR)};\%\text{body weight day}^{-1}) = \{(\ln Wf - \ln Wi)/t\} \times 100.$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed consumed}/\textit{Weight}\text{ gain}.$$

$$\text{Weight gain (g)} = (\text{final weight} - \text{initial weight}).$$

$$\text{Hepatosomatic index (HSI)} = 100 \times \text{Liver weight}/\text{body weight}.$$

TABLE 2 Chemical composition analysis of the normal feed and high fat diet for rainbow trout.

Components	Low fat diet (%)	High fat diet (%)
Crude protein	42.9	42.9
Crude fat	12.38	20.8
Crude fiber	1	0.98
Nitrogen free extract	37.76	29.41
Ash	5.96	5.91
Organic matter	94.04	94.09

2.4 | Analysis of acetic acid in ACV sample

To determine the content of acetic acid in an ACV sample, 4 mL of ACV was combined with 1 mL of 25% metaphosphoric acid and centrifuged for 5 min at 2800 *g* and 4°C. The supernatant was examined by GCFID (Varian, Model CP3800) with a 30 m 0.32 mm 0.5 m Teknokroma TRBFFAP column. The carrier gas was helium and the inlet temperature was 220°C. The oven temperature was increased from 100 to 160°C at a rate of 5°C/min (maintained for 2 min), and the detector temperature was set to 250°C. For the development of calibration curves, acetic acid standards (Sigma, USA) with concentrations of 5000, 2500, 1250, and 625 ppm were utilized (Ahmadniaye Motlagh, Javadmanesh, & Safari, 2020).

2.5 | Sampling

Fish were fasted for 24 h prior to sampling. Six fish were randomly selected from each replication and euthanized using clove powder (0.5 g/L). Using a heparin-soaked syringe, blood was collected from the tail stem. The blood samples were then centrifuged for 5 min at 3000 rpm using a Sigma apparatus. Blood biochemical parameters (cholesterol, triglycerides, alkaline phosphatase [ALP], alanine aminotransferase [ALT], and aspartate aminotransferase [AST]) were measured using an auto-analyzer (Biosystem A15) in accordance with the manufacturer's instructions (Biosystem S.A., Barcelona, Spain). Histopathological examination of the liver was assessed to evaluate the development of fatty liver in the juveniles and to study the recovery process of the treated fish. In compliance with the guidelines for the treatment of laboratory animals at Ferdowsi University of Mashhad, the fish were necropsied and their livers were extracted following anesthesia with clove extract solution. Two slices of liver tissue were set aside for histopathological evaluation and RNA extraction. The part for histopathological examination was placed in a 10% neutral buffered formalin solution. The remaining liver tissue was transferred to a 1.5 mL micro tube containing RNA stabilizer solution (RNAshield, Dena Zist, Iran) and frozen at −80°C until RNA extraction.

2.6 | Liver histopathology

After euthanasia, a part of the livers of six fish were removed, sampled, and fixed in 10% neutral buffered formalin. Following the processing of the liver tissue samples, they were embedded in paraffin waxes. The paraffin-embedded samples were then cut into sections at a thickness of 5 μm. For histological examination, the tissue sections were stained with hematoxylin and eosin (H&E) dyes and examined under a light microscope (Olympus, Japan). To assess the histopathological changes in the liver tissues of all groups, 10 microscopic fields from each slide ($N = 60$ fields per each group) with a magnification of 200× were blindly examined by an expert pathologist. The histopathological changes were scored and statistically analyzed between the studied groups (Liang et al., 2014). These changes included steatosis (score 0: <5%; score 1: 5%–33%; score 2: 34%–66%, and score 3: >66%), hypertrophy and necrosis of hepatocytes (scored like steatosis), and inflammation in the liver tissue. The latter index was scored based on the number of inflammatory foci/field: score 0 for <0.5%; score 1 for 0.5–1; score 2 for 1–2, and score 3 for >2 foci per each filed.

2.7 | RNA extraction and gene expression

To extract RNA from liver tissue, we utilized the Total RNA Extraction Kit (Pars Tous, Iran). The concentration and quality of the extracted RNA were assessed using a microplate reader (Epoch, Biotek, USA). Subsequently, DNase I

(Thermo Fisher Scientific, USA) was employed to eliminate DNA. Following the manufacturer's instructions, we used two micrograms of RNA to synthesize cDNA with the Easy cDNA Synthesis Kit (Pars Tous, Iran).

We analyzed the expression of three reference genes (GAPDH, EF1, and Beta-actin) and two target genes (FAAH and ACADL) using quantitative real-time PCR (qRT-PCR). Each reaction had a volume of 20 µL and underwent two replications. We utilized 10 µL of 2X qPCR master mix (Yekta Tajhiz Azad, Iran) for each reaction. Detailed characteristics of the primers can be found in Table 3. The ABI 7300 System SDS Software RQ Study Application was used to determine the expression of the examined genes (version 1.4.0.27).

We determined the relative expression of the targeted genes using the standard curve method, with a standard curve comprised of five dilutions and three replicates of each dilution. The heat cycling protocol for fragment amplification is outlined in Table 4, followed by an investigation of the melting curve with heating from 60 to 90°C. The efficiency of primer pairs was estimated based on the slope of the standard curve (efficiency = (10^(1/slope − 1) * 100)), with a focus on investigating correlation coefficients ($R^2 \geq 0.99$).

The FAAH and ACADL transcript copy numbers were standardized to the geometric averages of EF1α, Beta-actin, and GAPDH reference genes. We used version 3.550 of the GeNorm software to evaluate the expression stability of the reference genes (Xiang et al., 2014).

2.8 | Statistical analysis

The Shapiro–Wilk and Levene tests were utilized to assess the normality and homogeneity of the data, respectively. One-way ANOVA and Tukey tests were conducted to compare the means at the 5% confidence level. Additionally,

TABLE 3 Sequence of primer pairs used for target and reference genes.

Gene symbol	Sequence (5′ → 3′)	Annealing temp (°C)	Product size (bp)	GenBank accession no
FAAH	GGACGGAACACTAACTCCA ACAGGGACCCCATAAAGGA	60	162	XM_021625264.2
ACADL	TGTATGTCGAGGTGGACAGG CGGACACTCTCCCTGAACAT	60	180	XM_021608460.1
Beta Actin	GATGGGCCAGAAAGACAGCTA TCGTCCCAGTTGGTGACGAT	60	105	NM_001124235.1
EF1α	ACCCGAGGGACATCCTGTG TCCTCTTGGTCGTTTCGCTG	60	159	NM_001124339.1
GAPDH	TTGTAAAGCCCCTGTTCTGG GAAGCAGGTTCAGTGCAACA	60	89	XM_021623341.2

Abbreviations: ACADL, Acyl-CoA dehydrogenase, long chain; Beta-actin, EF1α, Elongation factor 1-alpha; FAAH, Fatty acid amid hydrolase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

TABLE 4 Thermal program used to amplify fragments of the reference and target genes.

Cycle	Time	Temperature (C^0)
Pre-denaturation	5 min	94
Denaturation	30 s	94
Annealing	15 s	60
Elongation	15 s	72

the histopathological scores were analyzed by Kruskal-Wallis and Mann–Whitney *U* tests. The statistical analysis was performed using SAS 9.4 software (SAS Inst., Inc., Cary, NC).

3 | RESULTS

3.1 | Growth parameters

Table 5 displays the impact of various levels of apple cider vinegar on the growth indices of rainbow trout ($n = 6$). The results indicate that the inclusion of this feed additive did not significantly affect the final weight, weight gain, SGR, or FCR. Although the final weight of the experimental treatments was higher than that of C1, this difference was not statistically significant. Moreover, there was no statistically significant difference in HSI among the treatments; however, fish affected by fatty liver had a higher mean liver weight.

3.2 | Biochemical parameters of blood

Table 6 presents the results of liver enzymes (ALP, AST, and ALT), cholesterol, and triglycerides. The data indicate a significant difference among the treatments ($p < 0.05$), with fish affected by fatty liver showing the highest levels of liver enzymes. The cholesterol index exhibited a significant difference ($p < 0.05$), although there were no significant changes among treatments. Triglyceride levels showed a significant difference between C2, T4, and T3 ($p < 0.05$).

3.3 | Liver histopathology

Figure 1 illustrates the effect of ACV in different concentrations on histology of rainbow trout liver tissue. Relatively normal hepatocytes with vesicular nuclei containing a prominent nucleolus were visible in the livers of the C2 group. The fish in C1 group showed severe macrovesicular (large vacuoles) steatosis in the liver. Hepatocytes were larger than normal cells and hypertrophied (Figure 1). In addition, the nuclei in hepatocytes were dense and pyknotic, and the nucleolus was not visible. These changes represent cellular necrosis of hepatocytes. Inflammatory foci characterized by infiltration of lymphocytes were seen in the liver parenchyma and portal areas. These lesions were seen to a lesser degree in the T1, T3, and T4 groups. However, the differences in the histopathological scores among these groups were not significant ($p > 0.05$). Compared with the C1 group, the lesions were relatively milder, and the treatment with ACV had improved the lesions to some extent in the T2 group, so that some cells were close to normal status ($p < 0.05$). They had vesicular nuclei with visible nucleolus, and the intensity of inflammation and hepatocyte hypertrophy was reduced ($p < 0.05$). Moreover, in addition to macrovesicular steatosis, some hepatocytes exhibited microvesicular steatosis (Figure 1).

3.4 | Gene expression

Table 7 presents the impact of ACV on the expression of the FAAH and ACADL genes in the liver. All reaction efficiencies ranged between 90% and 110%, and all calculated R2 values exceeded 0.99. The results of amplifying fragments of target genes with specific primers on a 2% agarose gel are illustrated in Figures 2 and 3. The expression of FAAH was notably lower in T2 compared with C1. Additionally, FAAH was expressed at a higher level in the T4 group, although this increase was not significant compared with the C1 group. None of the interventions significantly altered the expression of the ACADL gene ($p > 0.05$).

TABLE 5 Impact of various levels of apple cider vinegar on the growth indices of Rainbow trout (Mean ± SD, $n = 6$).

Treatments	C1	T1	T2	T3	C2	T4
Initial weight (g)	77.71 ± 3.47	78.33 + 1.13	75.96 ± 5.05	78.16 ± 2.04	67.77 ± 1.09	67.53 ± 1.42
Final weight (g)	210.35 ± 21.50	221.83 ± 20.86	223.01 ± 10.25	222.38 ± 18.54	218.90 ± 29.86	201.68 ± 23.24
Weight gain (g)	132.64 ± 21.06	143.05 + 21.42	147.05 + 18.53	144.22 ± 20.51	151.13 ± 30.77	134.15 ± 30.77
SGR	2.00 ± 0.14	1.92 ± 0.03	2.05 ± 0.13	0.46 ± 0.09	1.99 ± 0.13	2.01 ± 0.12
FCR	1.44 ± 0.75	1.37 ± 0.90	1.33 ± 0.95	1.37 ± 0.81	1.23 ± 0.45	1.31 ± 0.59
HSI (%)	1.65 ± 0.25	1.50 ± 1.75	1.51 ± 0.38	1.65 ± 0.31	1.10 ± 0.29	1.49 ± 0.28

Note: 0%ACV (C1), 1% ACV (T1), 2% ACV (T2) and 4% ACV (T3) for sick fish and 0% (C2) and 4% (T4) ACV for healthy fish.

TABLE 6 The effect of different levels of apple cider vinegar on the average activity of biochemical parameters of rainbow trout blood serum (Mean ± SD, $n = 6$).

Treatments	C1	T1	T2	T3	C2	T4
AST (IU/L)	373.47 ± 88.28^{ab}	411.0881.28^{a}	367.07 ± 16.28^{abc}	342.54 ± 107.23^{abc}	209.31 ± 54.14^{c}	241.2 ± 69.91^{abc}
ALP (IU/L)	1156.703 ± 639.47^{ab}	1183.6 ± 381.507^{a}	585.85 ± 486373^{bc}	960.08 ± 686.47^{abc}	278.36 ± 68.72 ^{c}	920.08 ± 586.47^{abc}
ALT (IU/L)	29.07 ± 6.57^{a}	36.04 ± 4.61^{a}	13.40 ± 4.44^{a}	4.1 ± 1.44^{b}	3.0 ± 0.80^{b}	3.8 ± 1.80^{b}
Chol (mg/dl)	194.6 ± 78.54	204.60 ± 42.93	240.605 ± 62.32	187.88 ± 78.54	251.96 ± 56.62	286.84 ± 58.23
TG (mg/dl)	107.8 ± 43.98^{bc}	107.4 ± 27.35^{bc}	168.17 ± 90.94^{ab}	82.692 ± 35.59^{c}	181.03 ± 50.33^{ab}	207.83 ± 60.52^{a}

Note: The lowercase letters above each number indicate statistical significance. 0%ACV (C1), 1% ACV (T1), 2% ACV (T2) and 4% ACV (T3) for sick fish and 0% (C2) and 4% (T4) ACV for healthy fish.

Abbreviations: ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; ASP, Aspartate aminotransferase; Chol, Cholesterol; TG, Triglyceride.

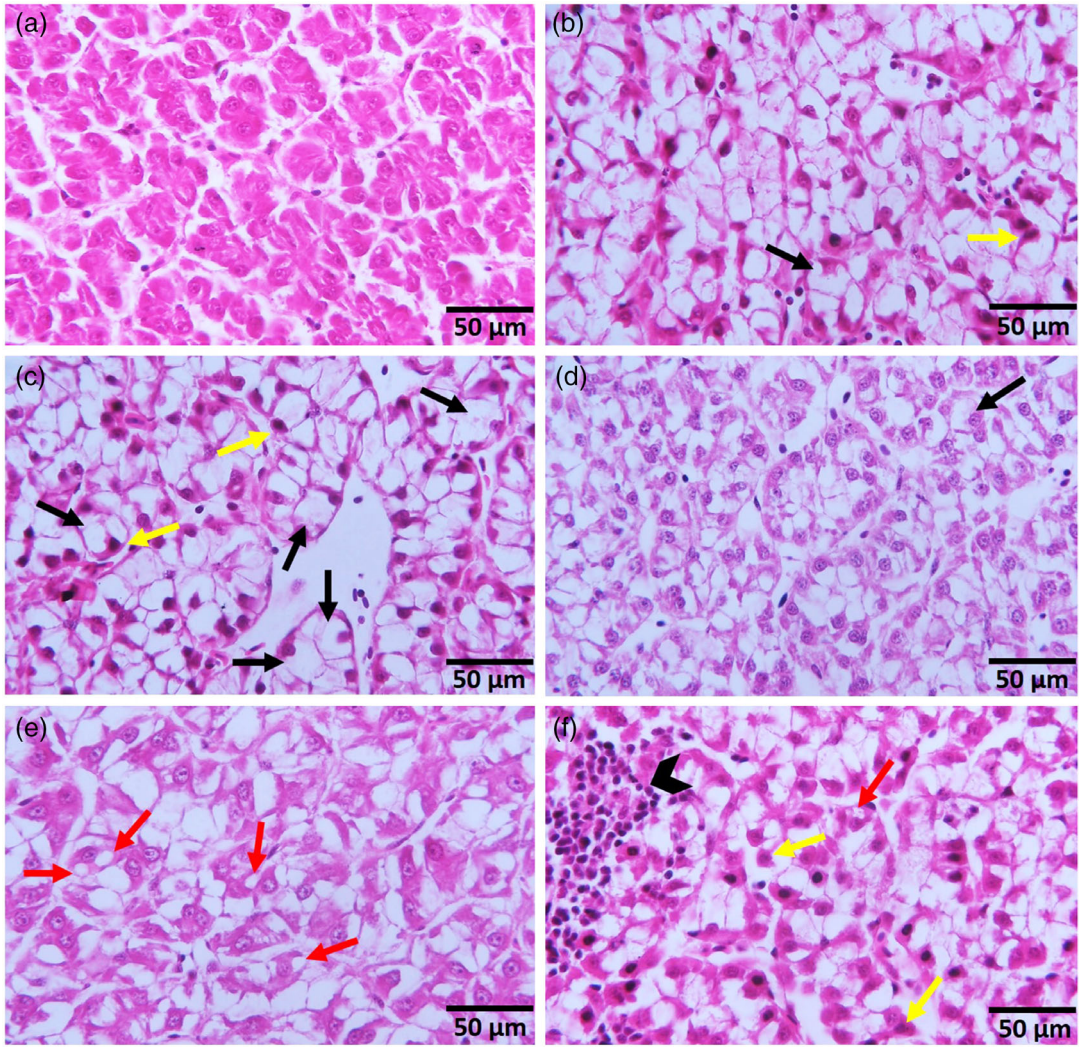

FIGURE 1 High-power photomicrographs of the liver tissues of the fish in the C2 (a), T4 (b), C1 (c), T1 (d), T2 (e), and T3 (f) groups. Lipid vacuoles in hepatocytes have displaced the nucleus to the periphery of the cells. Microvesicular (red arrows) and macrovesicular (causing nucleus displacement) steatosis (most hepatocytes, with the exception of a), hypertrophy of hepatocytes (black arrows), cell necrosis characterized by pyknotic nucleus (yellow arrow), and inflammatory foci with infiltration of lymphocytes (arrowhead) are seen. Hematoxylin and eosin staining. Scale bars = 50 µm for all.

TABLE 7 The effect of different levels of apple cider vinegar on FAAH gene expression in rainbow trout.

Treatments	C1	T1	T2	T3	C2	T4
FAAH	0.91 ± 0.2^{a}	0.28 ± 0.1^{bc}	0.23 ± 0.17^{b}	0.48 ± 0.1^{abc}	0.16 ± 0.1^{c}	0.72 ± 0.2^{ab}
ACADL	0.92 ± 0.15	0.95 ± 0.2	0.90 ± 0.13	0.88 ± 0.3	1.25 ± 0.14	1.08 ± 0.1

Note: GAPDH, EF1α, and Beta actin genes were used as reference genes. Small letters at the top of each column showed a significant difference ($p < 0.05$). 0%ACV (C1), 1% ACV (T1), 2% ACV (T2), and 4% ACV (T3) for sick fish and 0% (C2) and 4% (T4) ACV for healthy fish.

4 | DISCUSSION

Acetic acid, one of the most commonly employed organic acids in the study of aquatic and terrestrial animals, is the primary component in apple cider vinegar, typically ranging from 3% to 9%. This natural byproduct of apple fermentation contains phenoloids, polyphenols, and other organic acids, as well as vitamins and minerals. These components have been found to have various medicinal properties, including antioxidant activity, which may be particularly relevant for aquatic and terrestrial animals exposed to environmental stressors. Additionally, research suggests that apple cider vinegar may contribute to reducing blood pressure and lowering cholesterol levels in animals, offering potential health benefits without negative side effects (Nazıroğlu et al., 2014). These properties make apple cider vinegar an intriguing subject for studies involving the health and well-being of aquatic and terrestrial animals.

The results indicated that the application of ACV had no effect on the weight FCR, SGR, and HSI of the fish. In comparison with other farmed animals, such as chickens and pigs, the use of organic acids has led to greater growth. Numerous studies have shown that organic acids can increase growth and improve nutritional efficiency in aquatic species, such as Nile tilapia (Elala & Ragaa, 2015; Romano et al., 2015), common carp (Safari et al., 2017), and western white leg shrimp (*Litopenaeus vannamei*) (Pourmozaffar et al., 2017). However, other studies have shown that adding these substances to the diet did not increase the growth of red tilapia (Ng et al., 2009), rainbow trout (Gao

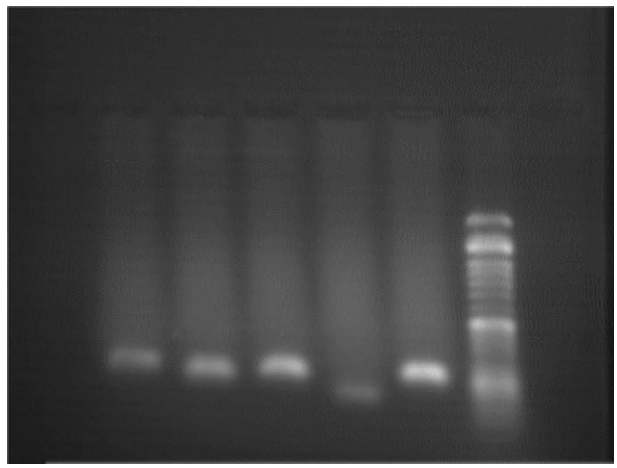

FIGURE 2 Examining the polymerase chain reaction product; electrophoresis gel to examine the product of the PCR reaction. From right to left: Ladder, Beta-Actin, FAAH, ACADL, GAPDH, efiα.

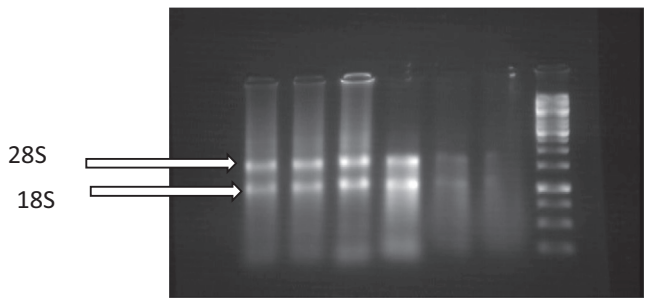

FIGURE 3 Checking the quantity and quality of extracted RNA.

et al., 2011), and catfish (*Clarias gariepinus*) (Mdegela et al., 2006). Results may vary due to variations in apple cider vinegar usage or species-specific biological factors.

ALP and aminotransferases, such as ALT and AST, are used as criteria for evaluating and diagnosing liver damage. Fatty liver is characterized by an enzyme increase of more than the normal level (Eidi et al., 2016). Fish with nonalcoholic fatty liver had the highest levels of these enzymes, as determined by this study. Several investigations have indicated that there was no significant difference in the level of ALT and AST in *C. carpio* and green terror (*Andinoacara rivulatus*) treated with ACV compared with the control groups (Ahmadniaye Motlagh, Sarkheil, et al., 2020; Nekoubin et al., 2020).

Several studies on the effect of ACV on blood lipids have yielded similar findings. In a study examining the impact of ACV on blood lipids in rats, a significant difference was observed between the ACV-treated group and the control group. The rats receiving ACV for 8 weeks showed a positive effect, leading to a substantial reduction in detrimental blood lipids. This suggests that regular use of ACV can improve the blood lipid profile and reduce hepatic TG accumulation. Furthermore, the results of ACV consumption demonstrated a significant decrease in cholesterol and total blood lipids in diabetic rats (Omar et al., 2016). In our study, we observed a decrease in LDL cholesterol and an increase in HDL cholesterol, indicating an improvement in the blood lipid profile.

Examination of organic acids on tiger shrimp revealed that nonorganic acid treatments caused higher histological damage than organic acid treatments (Ng et al., 2015). In experiments demonstrating the effect of ACV on diabetic mice, ACV-treated animals had normal liver tissue; mice fed a high-fat diet with apple cider vinegar had a low proportion of inflammatory cells and a moderate proportion of fibrosis (Bouazza et al., 2016). ACV improves the liver tissue of nicotine-poisoned mice, as histological scans revealed the liver's normal structure (Omar et al., 2016).

Various genes, including those encoding antioxidant enzymes, are associated with the metabolic functions of the liver in rainbow trout. However, we initially evaluated the two genes FAAH and ACADA, which are responsible for fat metabolism. Long-chain acyl-CoA dehydrogenase is a crucial enzyme in the oxidation of long-chain fatty acids, which is encoded by the ACADL gene. This enzyme is one of three dehydrogenases that facilitate fatty acid oxidation (Indo et al., 1991). The effect of ACV on the expression of this gene did not differ substantially among the tested treatments, as shown by statistical analysis.

The gene that encodes fatty acid amide hydrolase is responsible for producing a membrane enzyme that catalyzes the conversion of fatty acid amide hydrolase to the corresponding acids. These lipids are rapidly deactivated through hydrolysis mediated by FAAH. FAAH, a serine membrane hydrolase, breaks down several active fatty amides, including the endocannabinoid anandamide (Long et al., 2011). Endocannabinoids are substances that can cause liver cell damage and contribute to liver disease. To protect liver cells from endocannabinoid-induced cell death, the liver expresses the enzyme FAAH. Studies have shown that the liver exhibits the highest level of FAAH gene expression and that oxidative stress increases the expression of this gene in the liver (Biernacki et al., 2016). Statistical analysis revealed that C1 had the highest gene expression, which is consistent with the function of this gene in the liver.

ACADL is a crucial enzyme in the oxidation of mitochondrial fatty acids as it catalyzes the initial phase of beta-oxidation of long-chain fatty acids (Zhao et al., 2020). ACADL-deficient mice with hepatic lipidosis exhibited severe heart issues, elevated blood sugar, increased serum-free fatty acid concentrations, and insulin resistance-induced liver impairment (Zhang et al., 2007). In the current study, the expression of this gene in liver tissue did not show statistical significance; however, the highest expression of this gene was observed in healthy fish treated with T3. Given that this enzyme is involved in beta-oxidation of fatty acids in the liver and contributes to liver tissue breakdown, the higher expression of this gene in treatment can explain how ACV reduces fat.

Researchers have hypothesized that acetic acid may enhance fat metabolism in cells and reduce fat storage (Li et al., 2018). The current study's results also showed that treating fish with fatty liver using apple cider vinegar can significantly decrease fat accumulation in the liver and surrounding the organ. These findings suggest that apple cider vinegar, at a concentration of no more than 2%, can have a positive impact on fat metabolism and improve the health

of diseased fish by enhancing liver function. The expression of these two genes was examined to support the conclusion that apple cider vinegar enhances fat metabolism in the liver.

5 | CONCLUSIONS

The decreased expression of the FAAH gene, reduced AST, ALT, and ALP and reduced hepatocyte fat accumulation in the ACV treated fish indicated that 2% ACV can enhance fatty acid oxidation and improve overall liver function. However, the use of 4% ACV resulted in detrimental consequences, including elevated FAAH expression, increased fat deposition in hepatocytes, higher levels of AST, ALT, ALP, and triglycerides even in healthy fish. Consequently, it appears that an inclusion of up to 2% ACV may have positive effects on trout aquaculture and NAFLD treatment. However, further studies are recommended to explore the effects of additional concentrations, including 3%, to provide a more comprehensive understanding of ACV's impact.

AUTHOR CONTRIBUTIONS

Salimeh Asadi was responsible for project administration and writing, while Hamidreza Ahmadniaye Motlagh contributed to conceptualization and formal analysis. Omid Safari was involved in conceptualization and validation, and Ali Javadmanesh handled validation, writing, and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Ahmadniaye Motlagh, H., Javadmanesh, A., & Safari, O. (2020). Improvement of non-specific immunity, growth, and activity of digestive enzymes in Carassius auratus as a result of apple cider vinegar administration to diet. *Fish Physiology and Biochemistry*, *46*(4), 1387–1395. https://doi.org/10.1007/s10695-020-00797-6

Ahmadniaye Motlagh, H., Sarkheil, M., Safari, O., & Paolucci, M. (2020). Supplementation of dietary apple cider vinegar as an organic acidifier on the growth performance, digestive enzymes and mucosal immunity of green terror (Andinoacara rivulatus). *Aquaculture Research*, *51*(1), 197–205.

Biernacki, M., Luczaj, W., Gęgotek, A., Toczek, M., Bielawska, K., & Skrzydlewska, E. (2016). Crosstalk between liver antioxidant and the endocannabinoid systems after chronic administration of the FAAH inhibitor, URB597, to hypertensive rats. *Toxicology and Applied Pharmacology*, *301*, 31–41.

Bouazza, A., Bitam, A., Amiali, M., Bounihi, A., Yargui, L., & Koceir, E. A. (2016). Effect of fruit vinegars on liver damage and oxidative stress in high-fat-fed rats. *Pharmaceutical Biology*, *54*(2), 260–265. https://doi.org/10.3109/13880209.2015.1031910

Chan, D. F. Y., Li, A. M., Chu, W. C. W., Chan, M. H. M., Wong, E. M. C., Liu, E. K. H., Chan, I. H. S., Yin, J., Lam, C. W. K., & Fok, T. F. (2004). Hepatic steatosis in obese Chinese children. *International Journal of Obesity*, *28*(10), 1257–1263.

Eidi, A., Nateghi, Z., Mortazavi, P., & Zarringhalam Moghadam, J. (2016). Effects of ethanol extracts in licorice root (Glycyrrhiza glabra L.) on activity of liver enzymes in normal and alloxan-induced diabetic rats. *ISMJ*, *19*(4), 526–535.

Elala, N. M. A., & Ragaa, N. M. (2015). Eubiotic effect of a dietary acidifier (potassium diformate) on the health status of cultured Oreochromis niloticus. *Journal of Advanced Research*, *6*(4), 621–629.

Gao, Y., Storebakken, T., Shearer, K. D., Penn, M., & Øverland, M. (2011). Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate. *Aquaculture*, *311*(1–4), 233–240.

Giang, D. K., & Cravatt, B. F. (1997). Molecular characterization of human and mouse fatty acid amide hydrolases. *Proceedings of the National Academy of Sciences*, *94*(6), 2238–2242.

Horwitz, W. (2010). *Official methods of analysis of AOAC international. Volume I, agricultural chemicals, contaminants, drugs/edited by William Horwitz* (p. 1997). AOAC International.

Indo, Y., Yang-Feng, T., Glassberg, R., & Tanaka, K. (1991). Molecular cloning and nucleotide sequence of cDNAs encoding human long-chain acyl-CoA dehydrogenase and assignment of the location of its gene (ACADL) to chromosome 2. *Genomics*, *11*(3), 609–620.

Ji, Y., Gao, Y., Chen, H., Yin, Y., & Zhang, W. (2019). Indole-3-acetic acid alleviates nonalcoholic fatty liver disease in mice via attenuation of hepatic lipogenesis, and oxidative and inflammatory stress. *Nutrients*, *11*(9), 2062.

Kondo, T., Kishi, M., Fushimi, T., & Kaga, T. (2009). Acetic acid upregulates the expression of genes for fatty acid oxidation enzymes in liver to suppress body fat accumulation. *Journal of Agricultural and Food Chemistry*, *57*(13), 5982–5986.

Li, L., He, M., Xiao, H., Liu, X., Wang, K., & Zhang, Y. (2018). Acetic acid influences BRL-3A cell lipid metabolism via the AMPK signalling pathway. *Cellular Physiology and Biochemistry*, *45*(5), 2021–2030.

Liang, W., Menke, A. L., Driessen, A., Koek, G. H., Lindeman, J. H., Stoop, R., Havekes, L. M., Kleemann, R., & van den Hoek, A. M. (2014). Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PLoS One*, *9*(12), e115922.

Long, J. Z., LaCava, M., Jin, X., & Cravatt, B. F. (2011). An anatomical and temporal portrait of physiological substrates for fatty acid amide hydrolase [S]. *Journal of Lipid Research*, *52*(2), 337–344.

Mdegela, R., Myburgh, J., Correia, D., Braathen, M., Ejobi, F., Botha, C., Sandvik, M., & Skaare, J. U. (2006). Evaluation of the gill filament-based EROD assay in African sharptooth catfish (Clarias gariepinus) as a monitoring tool for waterborne PAH-type contaminants. *Ecotoxicology*, *15*(1), 51–59.

Nandy, A., Kieweg, V., Kräutle, F.-G., Vock, P., Küchler, B., Bross, P., Kim, J.-J. P., Rasched, I., & Ghisla, S. (1996). Medium-long-chain chimeric human acyl-CoA dehydrogenase: Medium-chain enzyme with the active center base arrangement of long-chain acyl-CoA dehydrogenase. *Biochemistry*, *35*(38), 12402–12411.

Nazıroğlu, M., Güler, M., Özgül, C., Saydam, G., Küçükayaz, M., & Sözbir, E. (2014). Apple cider vinegar modulates serum lipid profile, erythrocyte, kidney, and liver membrane oxidative stress in ovariectomized mice fed high cholesterol. *The Journal of Membrane Biology*, *247*(8), 667–673. https://doi.org/10.1007/s00232-014-9685-5

Nekoubin, H., Hajimoradloo, A., & Hoseinifar, H. (2020). Effect of different levels of apple cider vinegar on some parameters of non-specific immune system of blood serum on common carp (*Cyprinus carpio*) fingerlings. *Journal of Animal Environment*, *12*(3), 323–328.

Ng, W.-K., Koh, C.-B., Sudesh, K., & Siti-Zahrah, A. (2009). Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, Oreochromis sp., and subsequent survival during a challenge test with Streptococcus agalactiae. *Aquaculture Research*, *40*(13), 1490–1500. https://doi.org/10.1111/j.1365-2109.2009.02249.x

Ng, W.-K., Koh, C.-B., Teoh, C.-Y., & Romano, N. (2015). Farm-raised tiger shrimp, Penaeus monodon, fed commercial feeds with added organic acids showed enhanced nutrient utilization, immune response and resistance to Vibrio harveyi challenge. *Aquaculture*, *449*, 69–77.

Omar, N. A. A., Allithy, A. N. E. A., & El Sayed, S. M. (2016). Hepatoprotective and antidiabetic effects of apple cider vinegar (a prophetic medicine remedy) on the liver of male rats. *The Egyptian Journal of Hospital Medicine*, *62*(1), 95–104.

Oyarzún, R., Martínez, D., Soto-Davila, M., Munoz, J. L. P., Dantagnan, P., & Vargas-Chacoff, L. (2019). Effect of ration level on growth performance, body composition, intermediary metabolism and serum parameters in juvenile Patagonian blennie Eleginops maclovinus. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *230*, 122–130.

Postic, C., & Girard, J. (2008). Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: Lessons from genetically engineered mice. *The Journal of Clinical Investigation*, *118*(3), 829–838.

Pourmozaffar, S., Hajimoradloo, A., & Miandare, H. K. (2017). Dietary effect of apple cider vinegar and propionic acid on immune related transcriptional responses and growth performance in white shrimp, Litopenaeus vannamei. *Fish & Shellfish Immunology*, *60*, 65–71. https://doi.org/10.1016/j.fsi.2016.11.030

Romano, N., Koh, C. B., & Ng, W. K. (2015). Dietary microencapsulated organic acids blend enhances growth, phosphorus utilization, immune response, hepatopancreatic integrity and resistance against Vibrio harveyi in white shrimp, Litopenaeus vannamei. *Aquaculture*, *435*, 228–236. https://doi.org/10.1016/j.aquaculture.2014.09.037

Safari, R., Hoseinifar, S. H., Nejadmoghadam, S., & Khalili, M. (2017). Apple cider vinegar boosted immunomodulatory and health promoting effects of lactobacillus casei in common carp (Cyprinus carpio). *Fish & Shellfish Immunology*, *67*, 441–448. https://doi.org/10.1016/j.fsi.2017.06.017

Shahidi, F., McDonald, J., Chandrasekara, A., & Zhong, Y. (2008). Phytochemicals of foods, beverages and fruit vinegars: Chemistry and health effects. *Asia Pacific Journal of Clinical Nutrition*, *17*, 380–382.

Sookoian, S., & Pirola, C. J. (2011). Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (*PNPLA3*) on the susceptibility and histological severity of nonalcoholic fatty liver disease. *Hepatology*, *53*(6), 1883–1894. https://doi.org/10.1002/hep.24283

Xiang, R., Lee, A. M., Eindorf, T., Javadmanesh, A., Ghanipoor-Samami, M., Gugger, M., Fitzsimmons, C. J., Kruk, Z. A., Pitchford, W. S., & Leviton, A. J. (2014). Widespread differential maternal and paternal genome effects on fetal bone phenotype at mid-gestation. *Journal of Bone and Mineral Research*, *29*(11), 2392–2404.

Yamashita, H., Fujisawa, K., Ito, E., Idei, S., Kawaguchi, N., Kimoto, M., Hiemori, M., & Tsuji, H. (2007). Improvement of obesity and glucose tolerance by acetate in type 2 diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats. *Bioscience, Biotechnology, and Biochemistry*, *71*(5), 1236–1243.

Yu, K., Huang, K., Jiang, S., Tang, X., Huang, X., Sun, L., Pang, L., & Mo, C. (2021). Protective function on liver and proteomic analysis of the improvement mechanism of Sedum sarmentosum Bunge extract on nonalcoholic fatty liver disease in Nile tilapia. *Aquaculture*, *531*, 735977.

Zhang, D., Liu, Z.-X., Choi, C. S., Tian, L., Kibbey, R., Dong, J., Cline, G. W., Wood, P. A., & Shulman, G. I. (2007). Mitochondrial dysfunction due to long-chain acyl-CoA dehydrogenase deficiency causes hepatic steatosis and hepatic insulin resistance. *Proceedings of the National Academy of Sciences*, *104*(43), 17075–17080.

Zhao, X., Qin, W., Jiang, Y., Yang, Z., Yuan, B., Dai, R., Shen, H., Chen, Y., Fu, J., & Wang, H. (2020). ACADL plays a tumor-suppressor role by targeting hippo/YAP signaling in hepatocellular carcinoma. *Npj Precision Oncology*, *4*(1), 1–11.

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