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Effect of Licorice on Gene Expression Related to the Growth of Asian Seabass *Lates calcarifer*

Rui Yang [1,2,3,4,†], Wang Zhao [1,2,3,4,†], Yifu Wang [1,2,3,4], Zhengyi Fu [1,2,3,4,5], Jing Hu [1,2,3,4], Shengjie Zhou [1,2,3,4], Minghao Li [1,2,3,4] and Zhenhua Ma [1,2,3,4,5,*]

1 Key Laboratory of Efficient Utilization and Processing of Marine Fishery Resources of Hainan Province, Sanya Tropical Fisheries Research Institute, Sanya 572018, China
2 South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China
3 Hainan Engineering Research Center for Deep-Sea Aquaculture and Processing, Sanya 572018, China
4 International Joint Research Center for Conservation and Application of Fishery Resources in the South China Sea, Sanya 572018, China
5 College of Science and Engineering, Flinders University, Adelaide 5001, Australia
* Correspondence: zhenhua.ma@scsfri.ac.cn
† These authors contributed equally to this work.

Abstract: The Asian seabass (*Lates calcarifer*) has high economic value and is the primary aquaculture species in China. Licorice (*Glycyrrhiza uralensis)* as a feed additive has demonstrated significant immunological benefits in aquaculture. However, its effects on the growth of aquatic animals are largely unexplored. This study explored the influence of licorice on the level of growth-related genes in Asian seabass by conducting an experiment using artificial feed with 0%, 1%, 3%, and 5% licorice. The impact on growth performance and the expression of several genes, including growth hormone–releasing hormone (*GHRH*), growth hormone (*GH*), growth hormone receptor (*GHR*), insulin-like growth factor 1 (*IGF1*), *IGF2*, IGF2 receptor (*IGF2R*), myostatin 1 (*MSTN1*), and myostatin 2 (*MSTN2*), were studied over 56 d. According to the results, the 3% and 5% licorice-supplemented diets significantly improved survival rates and weight gain compared to the control group. Licorice affected the level of growth-associated genes in Asian seabass and significantly increased the levels of *GHR* and *IGF1* in the liver. However, a 5% licorice diet downregulated the expression of *IGF2*. As the licorice content in the diet increased, the levels of *IGF2R* and *MSTN1* in the muscle tissue first decreased and then increased, and licorice addition inhibited the *MSTN2* expression. The inclusion of licorice in the feed led to a significant downregulation of the *GH* and *GHRH* expression ($p < 0.05$). In summary, adding a certain proportion of licorice to the diet can improve the survival rate of the Asian seabass. Moreover, a proper proportion of licorice can increase the expression of related growth genes of fish, effectively increasing their weight gain rate and specific growth rate.

Keywords: traditional Chinese herbs; plant source additive; growth performance; growth-related genes; muscle growth-related genes

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1. Introduction

The Asian seabass is a euryhaline fish found mainly in the Western Pacific, being widely farmed in Australia [1]. Renowned for its taste, rapid growth, and large size, the Asian seabass has a high economic value and is the primary aquaculture species in China [2]. Extensive studies were conducted on the dietary requirements of the Asian seabass, including proteins, fats, carbohydrates, and minerals, as well as the need for additives such as probiotics and antibiotics [3,4]. Studies have shown that some traditional Chinese herbs or extracts, such as *Allium sativum* [5], *Cissus quadrangularis* [6], *Mentha piperita* [7], *Polygonum chinense* [8], can improve the growth performance of Asian seabass. Traditional Chinese herbs, which are rich in nutrients and medicinal components, are characterized by their

abundance, multi-targeting properties, diverse pharmacological effects, low toxicity, and minimal residues, making them ideal green additives for aquaculture feed [9–11]. These additives promote growth, enhance immunity, reduce costs, and improve profitability [12].

Licorice, a traditional medicinal herb, contains various components, including triterpene saponins, flavonoids, alkaloids, polysaccharides, and amino acids [13–17]. Its pharmacological actions mainly involve antitumor, anti-arrhythmic, antispasmodic, and antitussive effects, mediated by flavonoids; anti-inflammatory, antiviral, antitumor, and detoxifying effects, mediated by glycyrrhizic acid; and immunomodulatory, antiviral, and antitumor effects, mediated by polysaccharides [18–25]. Recently, licorice (*Glycyrrhiza uralensis)* has been studied as a feed additive in aquaculture and has been shown to significantly improve stress resistance, pathogen resistance, survival rate, and antioxidant capacity in fish [12,13]. For instance, adding fermented licorice to the feed of orange-spotted groupers (*Epinephelus coioides*) can reduce liver tissue damage and enhance antioxidative capacity, thereby increasing the survival rates under nitrite stress conditions [26]. In addition, licorice significantly improved the stress resistance of goldfish (*Carassius auratus*) and enhanced the resistance to *Aeromonas hydrophila* in goldfish and Chinese soft-shelled turtles (*Pelodiscus sinensis*) [20,27]. In terms of growth, licorice has a certain promotional effect on freshwater species such as tilapia, sturgeon, grass carp, and koi carp [28–31]. Previous studies have found that licorice can promote the growth of Asian seabass, but no further studies have been conducted [32]. In particular, whether licorice can really promote the expression of growth-related genes is still unexplored.

In this study, juvenile Asian seabass were fed with feed additives containing licorice, and then their growth performance was measured. The qrt-PCR method was used to study the effects on the level of growth-related genes and muscle growth-related genes. The present research aimed to reveal the mechanisms by which licorice influences the growth of Asian seabass at the molecular level, providing a scientific basis for its application in the aquaculture production of Asian seabass and acting as an important reference for the use of traditional Chinese herbal resources.

2. Materials and Methods

2.1. Feed Formulation and Experimental Design

Fish and soybean meals were the main protein sources used. Fish oil and wheat flour were the main fat and carbohydrate sources, respectively. A single-factor concentration gradient method was employed to design four levels of licorice feed with mass fractions of 0%, 1%, 3%, and 5%. The basic feed without licorice served as a control group. According to the nutritional requirements of Asian seabass [33], the experimental feeds were formulated to have a protein level of 41% and a lipid level of 17%, with consistency maintained across all experimental groups. The raw materials were ground and sieved through a 40-mesh sieve, mixed thoroughly using a mixer, pelletized using a small pellet machine (pellet diameter 2.0 mm), and stored at −20 °C for later use. The feed ingredients and nutritional components are listed in Table 1.

2.2. Experimental Method

Juvenile Asian seabass were bred by the Sanya Tropical Fisheries Research Institute Lingshui Experimental Center (Lingshui, Hainan, China). The fish weighed 13.93 ± 0.87 g and had a length of 8.78 ± 0.39 cm. Three hundred and sixty healthy, active, and responsive Asian seabass with smooth skin, no injuries, and good feeding behavior were randomly classified into four experimental groups, with three replicates per gradient and 30 fish per replicate. The feeding trial was conducted in flow-through seawater culture tanks (800 L) at the Sanya Tropical Fisheries Research Institute Lingshui Experimental Center (Lingshui, Hainan, China). During the experiment, the feeding behavior and mortality of the fish, as well as the water quality were monitored and recorded. The key water quality parameters were 26–29 °C, pH 7.3–7.8, and nitrite < 0.02 mg/L. Feeding was performed at 9:00 AM and 3:00 PM every day, using a satiation feeding method until the fish stopped feeding.

Approximately 1 h after feeding, the tanks were siphoned to remove feces and prevent water pollution. The water was changed at 4:30 PM, with two-thirds of the water being replaced. The experiment lasted for 56 d, after which the fish were weighed for calculated growth performance.

Table 1. Feed formula and the list of ingredients.

Ingredients	Diets			
	Control Group (0% *G.*)	Test Group (1% *G.*)	Test Group (3% *G.*)	Test Group (5% *G.*)
Fish meal (Fm)	50	50	50	50
Wheat flour (Wf)	23	22	20	18
Soybean meal (Sm)	12.9	12.9	12.9	12.9
Vitamin premix (Vp) (1)	0.5	0.5	0.5	0.5
Mineral premix (Mp) (2)	0.5	0.5	0.5	0.5
Fish oil (Fo)	13	13	13	13
Glycyrrhiza meal (*G.* m)	0	1	3	5
Choline chloride (Cc)	0.1	0.1	0.1	0.1
Dry ingredients (%)				
Crude protein (Cp)	41.44	41.31	41.06	40.81
Crude lipid (Cl)	17.53	17.51	17.46	17.41
Crude ash (Ca)	9.26	9.22	9.13	9.05
Total energy (Te)	20.28	20.12	19.79	19.46

Notes: (1) Vp: VA 900,000 IU, VB_1 320 mg, VB_2 1090 mg, VB_5 2000 mg, VB_6 500 mg, VB_{12} 116 mg, VC 5000 mg, VD 250,000 IU, VE 50 IU, VK_3 60 IU, niacin 40 mg, folic acid 5 mg, phaseomannite 150 mg, calcium pantothenate 20 mg, biotin 0.2 mg. (2) Mp: $MgSO_4 \cdot 7H_2O$ 3.0 g·100 g^{-1}, KCl 0.7 g·100 g^{-1}, KI 0.015 g·100 g^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.14 g·100 g^{-1}, $MnSO_4 \cdot 4H_2O$ 0.03 g·100 g^{-1}, $CuCl_2$ 0.05 g·100 g^{-1}, $CoCl \cdot 6H_2O$ 0.005 g·100 g^{-1}, $FeSO_4 \cdot 7H_2O$ 0.15 g·100 g^{-1}, $KH_2PO_4 \cdot H_2O$ 45.0 g·100 g^{-1}, $CaCl_2$ 28.0 g·100 g^{-1}. The dietary energy was calculated as protein (23.64 MJ·kg^{-1}), carbohydrate (17.15 MJ·kg^{-1}), and lipid (39.54 MJ·kg^{-1}). Source of materials: fish meal (Changsheng fishmeal factory, Cangzhou, China), wheat flour (Shandong developed face industry Co., Ltd., Dezhou, China), soybean meal (China Textile grain and oil Co., Ltd., Rizhao, China), vitamin premix (Henan Fangmu Shanze biological technology Co., Ltd., Nanyang, China), mineral premix (Henan Fangmu Shanze biological technology Co., Ltd., Nanyang, China), fish oil (Rongcheng City sea source fish oil aquatic products Co., Ltd., Rongcheng, China), glycyrrhiza meal (Longzhilin Medicine Store, Lingshui, China), Choline chloride (Taian Havay Group Co., Ltd., Tai'an, China).

2.3. Experiment Sampling

At the end of the feeding trial, three fish were sampled from each tank (i.e., nine fish per treatment) and placed in seawater containing 7 mg/L eugenol (provided by Changshu Shangchi Dental Materials Co., Ltd., Changshu, China) for anesthesia. Once anesthetized, the fish were quickly dissected to collect the brain tissue for *GHRH* and *GH*; the liver tissue for *GHR*, *IGF1*, and *IGF2*; and the muscle tissue for *IGF2R*, myostatin 1 (*MSTN1*), and Myosta2tin 2 (*MSTN2*). The tissues were then placed in cryogenic vials, flash-frozen, and stored at −80 °C.

2.4. RNA Extraction

The stored tissues were ground, and the total RNA was extracted using the previous method [34]. The concentration of the extracted RNA was detected using an ND 5000 micro-volume spectrophotometer (Beijing Baitake Biotechnology Co., Ltd., Beijing, China) at 260 and 280 nm to analyze the RNA integrity and purity.

2.5. qrtPCR Experiment

The extracted RNA was used for cDNA synthesis based on the PrimeScript™ Mix (Takara Bio Engineering Company) protocol and was then stored at −20 °C. The reaction mix for reverse transcription included 2 µL RT Master Mix. The conditions for the reverse transcription reaction were 37 °C for half an hour, followed by 85 °C for 5 s. The growth-related gene sequences of Asian seabass, including *GHRH*, *GH*, *GHR*, *IGF1*, *IGF2*, *IGF2R*, *MSTN1*, and *MSTN2* were obtained from the NCBI database, and primers were designed with a reference gene as the control (Table 2). The qPCR was performed on an rt-PCR

device (Langji Scientific Company). The 20 µL reaction mix included 10 µL 2 × Real PreMix, 0.6 µL 10 µM primers, and 2 µL diluted cDNA. The PCR program was as follows: 95 °C for 15 min; 95 °C for 10 s; 58 °C for 20 s; 72 °C for 30 s; 40 cycles. A melt curve was applied to ensure the specificity of the products and the absence of primer–dimer formation. A no-DNA template control was used to confirm the absence of contamination during PCR.

Table 2. Primer of growth-related genes in barramundi were used in qPCR.

Gene Classification	Gene	Sample	Sequence (5′-3′)	Amplicon Size (bp)	Accession No.
Growth-related gene	*GHRH*	brain	F: GCGTGTTGTTGCACAGGCC R: CTACAGGCCGGTGTTGTTTA	121	XM018681526
	GH		F: AGGTGTTGTTGACAGGCAC R: AACTCCCAGGTGTTGTTCAA	86	X59378
	GHR	liver	F: AAGGTGTTGTTAACAGGCAGC R: GCACGTGTTGTTGACAGGCGC	206	XM_018702498
	IGF1		F: TGACAGGCGGTGTGTTGTTCT R: TGGTGTTGTTTACTAACCT	144	EU136176
	IGF2		F: AGACAGGCAAGTGTTGTTGTG R: GAAGATAACCTGCTCCTGTG	131	XM_018664155
	IGF2R	muscle	F: AGCTGGAAACCCCGAATT R: GAGCGAGACAGGCTGGATA	150	XM_018687313.1
	MSTN1		F: AACTGCGAATGAAAGAAGCTC R: CTTGGACGATGGACTCAGGT	204	XM_018696695
	MSTN2		F: GTCTGTTCAGCCTCAGTCCA R: CGGGTGTTGTTTCCCTCTTT R: GACGTCCAATGGGCTTTCT R: CAAACAGGGTGATGGGGTA	145	XM_018661271
	β-actin		F: AACCAAACGCCCAACAACT R: ATAACTGAAGCCATGCCAATG	112	XM_018667666

Notes: Hormone–releasing hormone (*GHRH*), growth hormone (*GH*), growth hormone receptor (*GHR*), insulin-like growth factor 1 (*IGF1*), insulin-like growth factor 2 (*IGF2*), *IGF2* receptor (*IGF2R*), myostatin 1 (*MSTN1*), and myostatin 2 (*MSTN2*). The PCR efficiency of the primers listed in the table has been verified to be 90–110%.

2.6. Calculation and Statistical Analysis

The calculation formulas of survival rate (SR), weight gain (WG), feed intake (FI), and specific growth rate (SGR) are as follows:

SR (%) = 100 × number of fish at the end of the test/number of fish at the beginning of the test:

$$\text{WG (g fish}^{-1}) = W_t - W_0$$

$$\text{FI (g fish}^{-1}\ \text{d}^{-1}) = (\text{feed consumed per tank/fish})/t$$

$$\text{SGR (\%/d)} = 100 \times [(\text{Ln}W_t - \text{Ln}W_0)]/t$$

W_t—final average weight (g); W_0—initial average weight (g); t—the number of days the feeding text lasted.

The relative levels of gene mRNA were detected based on the $2^{-\Delta\Delta Ct}$ approach, with the *β-actin* as the reference gene for normalization. Experimental data were presented as mean ± SD. The SPSS 19.0 tool was applied for statistical treatment data. The Shapiro–Wilk test was employed to check the data for normal distribution, and the Levene test was used to assess the homogeneity of variance. Comparisons among the groups were conducted using the ANOVA (LSD test), with a statistical level of 0.05.

3. Results

3.1. Effects of Licorice in Feed on the Growth Performance of Asian Seabass

After adding different levels of licorice to the feed and feeding for 56 days, the survival rate, weight gain rate, body-length growth rate, and specific growth rate of the seabass are shown in Figure 1. The survival rate of the 3% and 5% licorice-supplemented diet group was significantly higher than that of the control group ($p < 0.05$) (Figure 1A). The survival rate reached the maximum in the 3% licorice-supplemented diet group, and the value was (98.89 ± 1.93) %. The WG was significantly increased in all the licorice treatment groups compared to the control group ($p < 0.05$) (Figure 1B). The FI was not significant among the groups ($p > 0.05$) (Figure 1C). The SGR of the 5% licorice-supplemented diet group was significantly higher than that of the control group ($p < 0.05$) (Figure 1D).

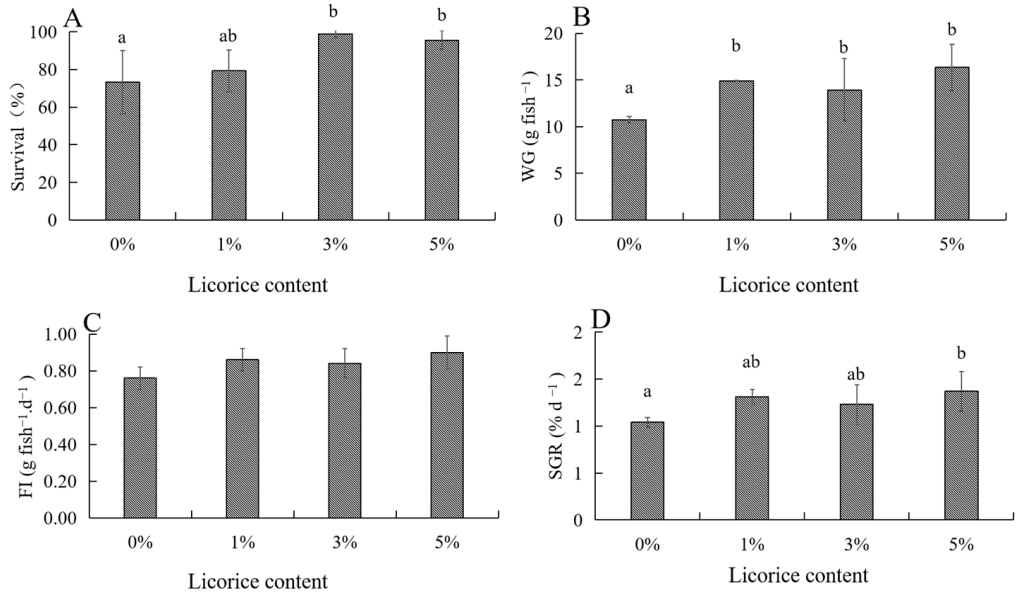

Figure 1. Effects of licorice on survival rate and growth of Asian seabass. (**A**) survival, (**B**) weight gain (WG), (**C**) feed intake (FI), (**D**) specific growth rate (SGR); means within rows with the same superscript are not significantly different ($p > 0.05$), while the different letters mean significant differences ($p < 0.05$).

3.2. Effects of Licorice on the Expression of Growth-Related Genes in the Asian Seabass Liver

As shown in Figure 2, adding licorice to the feed significantly affected the level of the *GHR* and IGF genes in the livers of Asian seabass. According to the comparison result, the relative expression level of the *GHR* gene in the liver increased significantly after the addition of licorice ($p < 0.05$), and the influence of licorice on the *GHR* gene's relative expression level varied with its concentration, with the highest increase of 362% observed in the 5% group, followed by the 1% group, and the lowest in the 3% group. The relative expression level of the *IGF1* gene in the livers of the experimental groups significantly increased with the increase in licorice content in the feed ($p < 0.05$), with the 5% group showing the max level of the *IGF1* gene, which was 751% of the control group's level. The *IGF2* gene level showed an initial increase, followed by a decrease with increasing licorice content, with significant differences between the two groups ($p < 0.05$). The 1% group had the highest level, followed by the 3% group, with both levels significantly increased ($p < 0.05$). The *IGF2* level in the 5% group was significantly lower ($p < 0.05$).

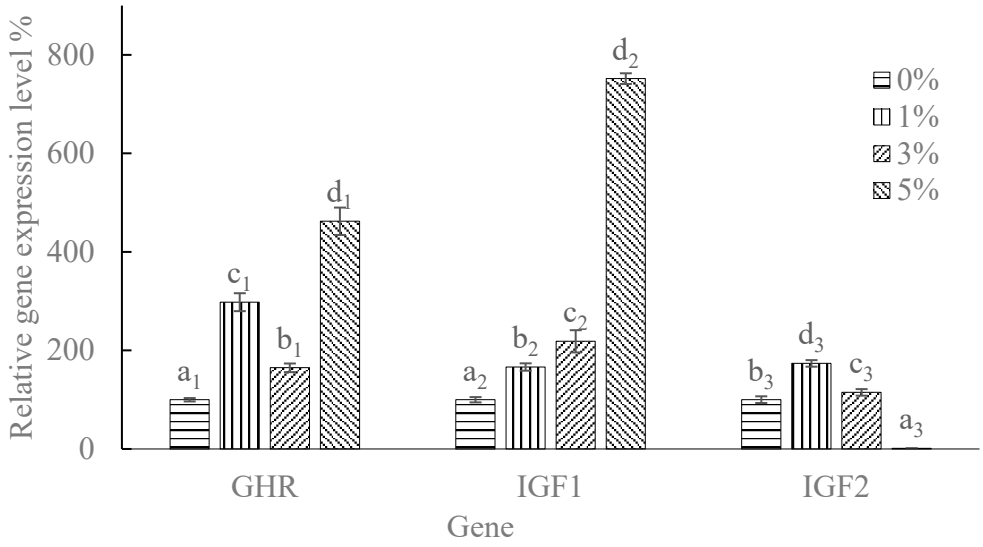

Figure 2. The relative effect of licorice on the level of growth-related genes in the liver tissue. Note: Different letters indicate significant differences. Subscripts 1, 2, and 3 represent *GHR*, *IGF1*, and *IGF2*, respectively.

3.3. Effects of Licorice on Level of Muscle Growth-Related Genes in Asian Seabass

As indicated in Figure 3, licorice significantly affected the level of the muscle growth-associated genes *IGF2R*, *MSTN1*, and *MSTN2* in Asian seabass. With an increase in the licorice content in the feed, the expression level of the *IGF2R* gene initially decreased and then increased; however, the expression levels of *IGF2R* in all experimental groups were significantly lower than those in the other group ($p < 0.05$). Similar to the results of the *IGF2R* gene, the *MSTN1* gene level also initially decreased and then increased, with the lowest level observed in the 1% group. The level increased in the 3% group and was significantly higher compared to the other groups ($p < 0.05$) and continued to increase in the 5% group, reaching 333% of that in the control group. The addition of licorice to the feed caused a significant reduction in the *MSTN2* gene level at all levels ($p < 0.05$).

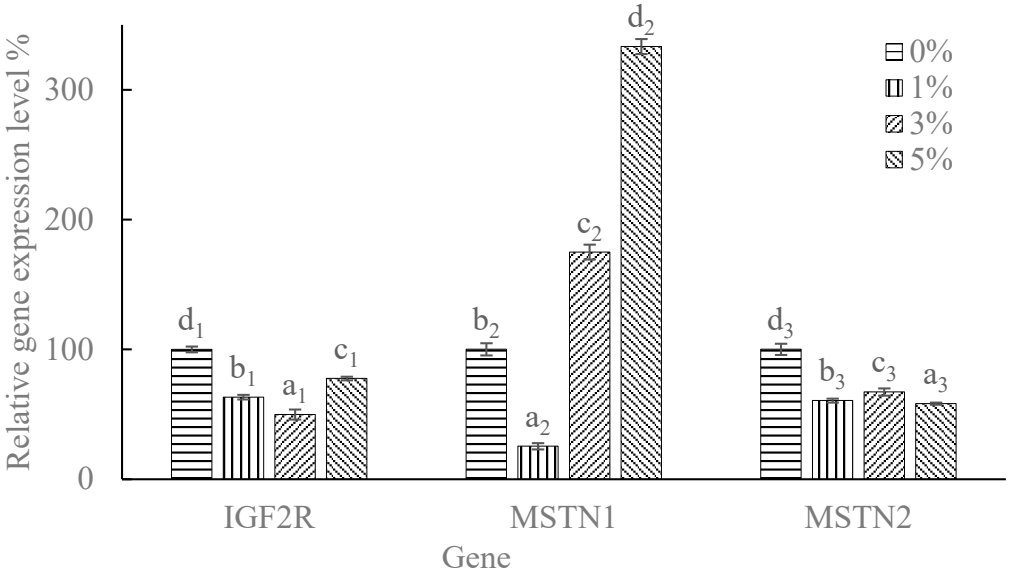

Figure 3. The relative effect of *Glycyrrhiza uralensis* on the level of growth-associated genes in the muscle tissue. Note: Different letters indicate significant differences. Subscripts 1, 2, and 3 represent *IGF2R*, *MSTN1*, and *MSTN2*, respectively.

3.4. Effects of Licorice on Level of Growth-Related Genes in Brain Tissue of Asian Seabass

The influence of licorice on the level of the growth-related genes *GH* and *GHRH* in the brain tissue of Asian seabass is illustrated in Figure 4. After adding licorice, the level of *GH* decreased significantly ($p < 0.05$). The level of the *GHRH* gene exhibited a trend of initially decreasing and then increasing with increasing licorice content. The lowest level appeared in the 3% licorice group, followed by the 5% licorice group, with the highest level observed in the 1% licorice group. However, the expression levels of *GHRH* in all experimental groups were significantly lower than those in the control group ($p < 0.05$).

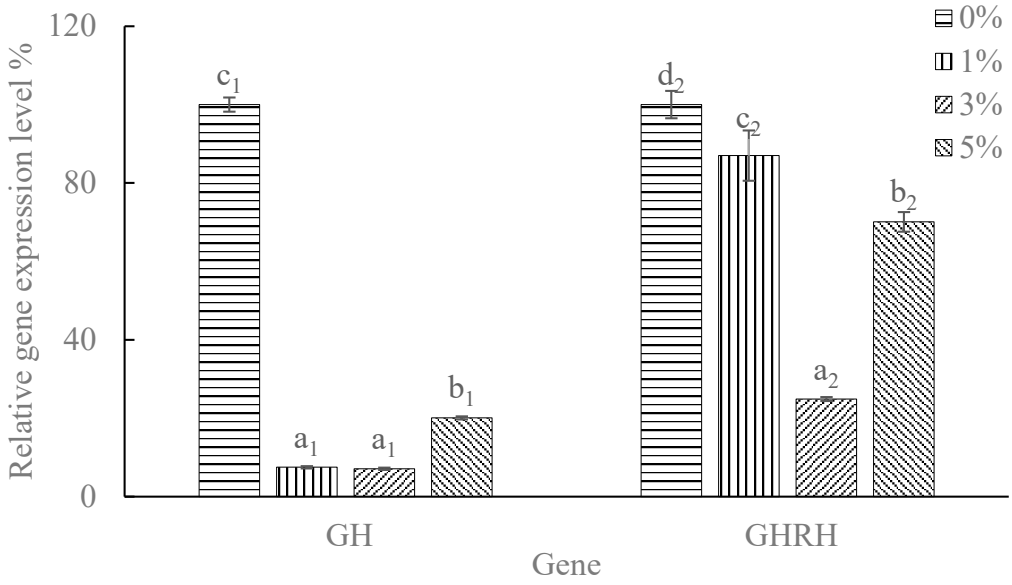

Figure 4. The relative effect of *Glycyrrhiza uralensis* on the level of growth-associated genes in the brain tissue. Note: The letters indicate significant differences. Subscripts 1 and 2 represent *GH*, and *GHRH*, respectively.

4. Discussion

We found that adding a proper concentration of licorice in the feed significantly promoted the expression of genes related to *GHR*, *IGF1*, and *IGF2* in the liver of Asian seabass. The growth of teleost fish is primarily adjusted by the *GH*/IGF system and other endocrine factors. *GH* binds to *GHR* on the surface of target organs, stimulating the liver to secrete IGF [35,36]. IGF binds to IGF receptors in target tissues, initiating a series of cellular processes related to growth, such as cell proliferation and differentiation, leading to overall growth [37]. In the present research, it was found that adding licorice to Asian seabass feed significantly upregulated the level of *GHR*. The downstream genes of the *GH*/IGF axis in the liver, *IGF1*, and *IGF2* showed significant changes, confirming that *GH* can exert biological effects through *IGF1* and *IGF2* after binding to *GHR* [38]. When the addition of licorice did not exceed 3%, the levels of *IGF1* and *IGF2* significantly increased, suggesting that a 3% licorice addition could significantly promote processes related to cell growth. The max level of *IGF1* appeared in the 5% licorice group but the significant downregulation of the *IGF2* expression indicates that high levels of licorice in Asian seabass feed might affect the functions related to *IGF2*.

In Nile tilapia (*Oreochromis niloticus*), *GH* is expressed only in the pituitary gland, whereas *GHR* is expressed in various tissues and organs, including muscle, hypothalamus, and thymus, with the highest expression in the liver, indicating that *GH* has multiple physiological functions [39]. In teleosts, *MSTN1* primarily inhibits muscle hyperplasia but not hypertrophy, which is mainly achieved through the downregulation of *MSTN2* expression [40]. This research showed the variable influence of licorice on *MSTN1* expression, indicating the stages of rapid muscle hyperplasia in Asian seabass [41]. The lower expression of *MSTN1* and *MSTN2* with 1% licorice suggests reduced inhibition of muscle

hyperplasia and promotion of muscle hypertrophy, while the increased *MSTN1* expression with 3% and 5% licorice indicates an enhanced inhibition of muscle hyperplasia and the promotion of muscle hypertrophy.

This study found that after adding licorice, the level of *GH* in the brain samples of Asian seabass was downregulated, and the levels of *GHR*, *IGF1*, and *IGF2* in the liver tissue were upregulated, possibly due to a delayed regulatory effect of *GH* on the growth rate and its negative feedback regulation with *IGF1* [42]. The hypothalamus-secreted *GHRH* physiologically regulates the generation and release of *GH* in the pituitary gland, which is an important hormone for regulating fish growth, development, reproduction, and immunity [43]. In the present research, the *GHRH* and *GH* expression was significantly downregulated and did not show a linear relationship, which may be related to the multifactorial influence on *GH* secretion that maintains a dynamic balance between promoting and inhibiting factors [44].

The beneficial effects of licorice may be attributed to glycyrice polysaccharides (GPS). Dietary supplementation of GPS has been found to enhance growth performance, body size, and the relative expression of the growth-related gene IGF-1 in broilers [45]. Furthermore, it can improve serum and intestinal immune status, promote the expression of immune-related genes in the spleen, and enhance broiler immunity. The optimal supplemental concentration is 600 mg/kg [46,47]. In weaned piglets, GPS supplementation significantly promotes the mRNA expression levels of the IGF-1 gene in the liver, as well as the IGF-1 and IGF-2 genes in the dorsal longus muscle ($p < 0.05$) [48]. Additionally, the dietary addition of GPS improves growth performance, reduces diarrhea rate, enhances humoral immunity, promotes the related growth gene expression, and even exhibits a certain level of resistance against PRRSV infection in piglets. Supplementation with 1000 mg/kg GPS alleviates stress response, reduces diarrhea rate, and improves growth performance by enhancing the intestinal mucosal barrier effect, immune function, and intestinal microflora structure in weaned piglets [49]. Moreover, GPS exhibits inhibitory effects on the TLR4/MyD88/NF-κB signaling pathway, thereby reducing the excessive expression of immune and inflammatory, apoptosis, and tight junction protein genes induced by LPS in IPEC-J2 cells [50]. Additionally, they enhance the cell's antioxidant capacity and decrease ROS accumulation and the apoptosis rate, ultimately alleviating the inflammatory damage caused by LPS in the IPEC-J2 cells. It also enhances antioxidant capacity, reduces ROS accumulation and cell apoptosis rates, and mitigates inflammatory damage induced by LPS in IPEC-J2 cells. GPS serves as an effective immune enhancer to enhance the integrity of the intestinal barrier [51]. The addition of licorice to animal feed can effectively stimulate the growth of terrestrial animals such as chickens and pigs, as well as that of aquatic species such as Asian seabass. Moreover, it exhibits immune-enhancing properties, thereby positioning licorice as a promising feed additive with excellent application prospects.

5. Conclusions

In conclusion, the inclusion of licorice in the diet of Asian seabass significantly enhances growth performance and survival rates. Specifically, diets supplemented with 3% and 5% licorice showed notable improvements in weight gain and survival compared to the control group. Licorice supplementation positively influenced the expression of growth-related genes, particularly increasing the *GHR* and *IGF1* levels in the liver. However, higher levels of licorice (5%) resulted in the downregulation of *IGF2* expression and complex effects on the *IGF2R* and *MSTN1* levels in the muscle tissue. Additionally, licorice inhibited the expression of *GH*, *GHRH*, and *MSTN2*. These findings suggest that incorporating an optimal proportion of licorice into the diet can effectively enhance the growth and health of Asian seabass in aquaculture.

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Institutional Review Board Statement: This animal study was reviewed and approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, the Chinese Academy of Fishery Sciences. The ethical code is 2020TD15, approved on 10 March 2020.

Informed Consent Statement: Not applicable.

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

Conflicts of Interest: The authors declare no conflicts of interest.

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