

1 **How different temperatures and feeding rates impact physiological and**
2 **histological responses of juvenile Asian seabass (*Lates calcarifer*)**

3
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6
7 **ABSTRACT**

8 The current study evaluated the interactive impacts of water temperature and feeding rate on
9 digestive enzymes, intestine histology, growth and stress-related genes, and cultivable intestinal
10 microbiota of Asian seabass (*Lates calcarifer*). For this purpose, 180 fish (85.0±3.0 g) were
11 reared at three different temperatures (20, 27, and 33°C) and two feeding rates (apparent satiation
12 and 2.5% of biomass) with three replications for 6 weeks. The results revealed no significant
13 differences among different treatments regarding the activity of digestive enzymes ($P > 0.05$) of
14 fish reared under different temperatures and feeding rates. The length, width, and thickness of
15 intestinal villi were unaffected by different temperatures and feeding rates ($P > 0.05$). In addition,
16 no variations were found in the total aerobic bacterial count of fish gut from different
17 experimental groups ($P > 0.05$). At the molecular level, *IGF-I* and *HSP70* coding genes were
18 found to be highly expressed in experimental treatments ($P < 0.05$). To conclude, **the present**
19 **study** showed that temperatures between 27 to 33°C are more optimal for Asian seabass, and the
20 different temperatures and feeding rates do not affect digestive enzymes, intestine histology, and
21 gut microbiota **after 6 weeks.**

22 **Keywords:** Temperature, Feeding rate, Digestive enzymes, Gut microbiota, Gene expression,
23 Asian seabass.

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28 **INTRODUCTION**

29 Fish live in a 3-dimensional complex aquatic environment, and since fish are poikilothermic
30 animals, surrounding temperature is a major factor that regulates fish's metabolism and growth
31 rate (Fry, 1971; Groot et al., 1996). Fish are believed to perform best at their optimum
32 temperature, which varies even for different life stages of one species (Pedersen and Jobling,
33 1989), where feed utilization efficiency is maximum. Thus, for the best nutrition management,
34 aquafeed manufacturing companies often provide a range of feeding rates according to water
35 temperature for each specific growing stage of the target species. Therefore, knowing the
36 optimum temperature for each species can help with efficient production and increase the overall
37 yield. It is important to mention that feed accounts for 50 to 80% of the total aquaculture
38 production costs. Moreover, underfeeding or malnourishment might render fish more susceptible
39 to diseases and result in mass mortalities, while overfeeding results in feed loss and water quality
40 deterioration, making aquaculture practice less profitable.

41 The size of the fish and water temperature are important factors for optimizing feeding rates
42 (Kestemont and Baras, 2001; Wang, Xu, and Kestemont, 2009). For instance, the best growth
43 performance of pike perch (*Sander lucioperca*) was obtained when fish were fed to apparent
44 satiation (Ming *et al.*, 2013). Given that, the rearing temperature and feeding rate are closely
45 interlinked and affect various physiological processes of fish. The impacts of feeding rate and
46 different rearing temperatures on various physiological aspects of fish have been reported by
47 other researchers (Fang *et al.*, 2010; Kim *et al.*, 2014; Baloi *et al.*, 2017; Volkoff and Rønnestad,
48 2020). Both the feeding rate and the feeding time greatly influenced the activity of digestive
49 enzymes. Moreover, several factors, such as enough enzyme levels and time for digestion and
50 absorption, can affect the digestion process (Harpaz *et al.*, 2005). Furthermore, water temperature
51 can affect digestive enzyme activities by affecting evacuation time (Temming and Herrmann,
52 2001).

53 Asian seabass (*Lates calcarifer*), an important food fish in tropical regions bordering the Indian
54 Ocean, has been extensively studied due to its economic significance (Jerry, 2013). Asian seabass
55 is cultivated in different aquaculture systems and represents a prospective option for diversifying

56 coastal aquaculture of marine fish. According to previous studies, the fish can reach their growth
57 potential when fed below or at an apparent satiation level, depending on the species (Han *et al.*,
58 2004; Fang *et al.*, 2010). In addition, the increase in temperature can impact the digestive system,
59 intestinal microbiota, and, consequently, growth performance. However, the combined effects of
60 rearing temperature and feeding rate on have not been well studied in Asian seabass. Thus, in this
61 study, we evaluated the effects of different temperatures (20, 27, and 33°C) and feeding rates
62 (2.5% of biomass and apparent satiation) on digestive enzyme activity, gut histology, gut
63 microbiota, growth-regulating gene (*IGF-I*), and stress-related gene (*HSP70*) in a 6-week dietary
64 trial.

65

66 MATERIALS AND METHODS

67 Fish and experimental design

68 Juvenile Asian seabass (n=180, 85.0±3 g) was procured from Ramoz Company (Bushehr, Iran)
69 and transferred to the laboratory of Marine Aquatic Research, Persian Gulf University (Bushehr,
70 Iran). Before the main experiment commenced, the fish underwent a 2-week acclimatization
71 period to laboratory-rearing conditions. During this period, the fish were fed twice daily with a
72 commercial feed (Beyza, Iran) containing 47% crude protein, 17% crude fat, 2% crude fiber, and
73 14% ash. After this period, fish were distributed into 6 different treatments in triplicates (10
74 fish/300-L tank), including 3 different temperatures (20, 27, and 33°C) and 2 feeding rates (2.5%
75 of biomass or ad libitum). The treatments were designed and named T1 (20°C, 2.5% biomass),
76 T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C,
77 2.5% biomass), and T6 (33°C, apparent satiation). Throughout the 6-week dietary trial, each tank
78 received sand-filtered, dechlorinated, and UV-disinfected seawater, ensuring approximately 60-
79 70% daily turnover of the rearing water. During the experiment, water physiochemical
80 parameters, including salinity (48±2 ppt), pH (7.5±0.5), and dissolved oxygen (70-80%
81 saturation), were monitored and adjusted if necessary. The photoperiod was artificially set as
82 12L: 12D.

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85 **The activity of digestive enzymes**

86 To measure digestive enzyme activity, fish (9 fish per treatment) were anesthetized with (2-
87 phenoxyethanol, (500 ppm, Afkhami *et al.*, 2014; Zeynali *et al.*, 2020), and then the whole gut
88 was removed, washed twice with double distilled water, then homogenized in 100mM Tris-Hcl
89 buffer containing 0.1 mM EDTA and 0.1% Triton X-100 (pH 7.8), and centrifuged at 30000×g
90 for 12 min at 4 °C. The supernatant was then collected and kept frozen at -80°C (Furné *et al.*,
91 2008) until further analysis. A commercial lipase kit (Bionik, Canada) was used to measure the
92 specific activity of lipase. The photometric measurement was based on the hydrolysis of 1,2-o-
93 dilauryl-rac-3-glutaric acid-(6-methyresorufin) ester substrate, resulting in the production of 6-
94 methyresorufin and glutaric acid-6-ethylresorufin ester.

95 Specific activity of amylase was measured using a commercial amylase kit (Bionik, Canada)
96 based on 4, 6 ethylidene-(G7)-p-nitrophenyl-(G1)-alpha-D-maltoheptaside (Eps-G7) as substrate.
97 The method described by (Anson, 1938) was used for measuring total protease activity using
98 casein as the substrate. 1 mL of supernatant samples were added to a reaction mixture (1 mL of
99 1.5% azocasein solution, pH 7.0) and incubated for 10 min at 37°C. After that, 2 mL of 0.4 M
100 trichloroacetic acid was added, the solution was filtered, and 2.5 mL of 0.4 M Na₂CO₃ and 0.5
101 mL of Folin reagent were added.

102 Samples were assessed for protein content using the Bradford method (Bradford, 1976) with
103 bovine serum albumin as the standard (1 mg/mL). Subsequently, digestive enzyme activity was
104 quantified as the change in absorbance per minute per milligram of soluble protein as follows:

105
$$\text{Unit/mg protein} = \frac{\text{Abs (410nm)} \times 1000 \times \text{ml of reaction mixture}}{8800 \times \text{mg protein in reaction}}$$

106
$$\text{mixture}$$

107
108 **Histological studies**

109 At the end of the experiment, two fish (6 fish per treatment) were randomly selected from each
110 tank for histological evaluations. Approximately 0.5 cm segments of the midgut were excised
111 using a sterile scalpel and fixed in 10% formalin solution. Following established histological
112 protocols, the gut samples underwent dehydration in a graded series of ethanol and xylene,
113 followed by embedding in paraffin blocks. Then, 5µm transverse sections were provided, stained

114 with hematoxylin and eosin, and were assessed using light microscopy (Roberts, 2012). The
115 images were processed for length and thickness values using ImageJ software.

116

117 **Gut microbiota**

118 In aseptic conditions, the intestine samples (9 fish per treatment) were washed homogenized in
119 sterile saline solution (0.9% NaCl), and the homogenate was diluted as required. The fish's outer
120 layer was disinfected using 70% ethanol before dissection. The suspensions were serially diluted
121 (10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7}) and 0.1 ml of the solution was spread onto TSA (Tryptic Soya
122 Agar) plates. Total aerobic bacterial colonies were counted after incubation at 27°C for 48-72
123 hours (Rawling *et al.*, 2009).

124

125 **Gene expression**

126 The expression of genes of interest was evaluated in liver samples. A portion of liver tissue
127 samples were removed, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis.
128 An RNA extraction kit (Cinnagen Iran) was used to extract the total RNA content of samples
129 according to the manufacturer's instructions. The extracted RNA was quantified using a
130 spectrophotometer (ND-1000, Nanodrop). The quality of extracted RNA was assessed on 1%
131 agarose electrophoresis. The complementary DNA (cDNA) strand was subsequently synthesized
132 using 1µg of total RNA and 10 pmol Random Hexamer primer and RevertAid™ First Strand
133 cDNA Synthesis Kit (Fermentas, K1622). Specific primers (*HSP70*, *IGF-I*, and *Efla*) for real-
134 time PCR analysis of Asian seabass were designed with Primer3Plus software (Table 1). The
135 *Efla* gene served as the housekeeping gene. RT-PCR was performed using a real-time PCR
136 machine (RotorGene, RG-2000, Sydney, Australia) in total volume of 12.5 µl containing 6.25 µl
137 of 2X SYBR Green qPCR Master Mix (Cinnagen, Iran), 0.5 µl of cDNA, 0.5 µl of each primer,
138 0.1 µl Tag polymerase and 4.65 µl of double distilled and DNase free water (DEPC water). The
139 amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 30
140 s, 60°C for 45 s and 72°C for 45. The average threshold cycle (Ct) was calculated for each
141 sample and normalized to the housekeeping gene. The relative expression of genes was
142 calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

143 **Table 1.** The sequences of the primers used in this experiment for *IGF-I* and *Hsp70* genes of
144 Asian seabass (*Lates calcarifer*).

Gene name	Function	Sequences of primers	Accession number	Efficiency
IGF-I	Growth	F: ACGCTGCAGTTTGTATGTGG	XM_018697285.1	98%
		R: CCTTAGTCTTGGGAGGTGCA		
Hsp70	Stress	F: AAGGCAGAGGATGATGTC	XM_018672747.1	94%
		R: TGCAGTCTGGTTCTTGTC		
Efla	Housekeeping	F: AAATTGGCGGTATTGGAAC	GQ507427.1	97%
		R: GGGAGCAAAGGTGACGAC		

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146 **Statistical analysis**
147 The data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences
148 among the groups were evaluated through a two-way analysis of variance (ANOVA), followed
149 by Tukey's multiple range post hoc test. Results are presented as means±standard error (n= 3),
150 and statistical significance was determined at P< 0.05.

151
152 **RESULTS**

153 **The activity of digestive enzymes**

154 Table 2 showed no significant difference among treatments regarding total protease, amylase, and
155 lipase enzyme activity (Table 2; P> 0.05). The interactive influence of temperature and feeding
156 rate did not statistically affect the normal function of the digestive system. However, individuals
157 from the T5 treatment exhibited the highest specific activities of lipase, amylase, and total
158 protease, suggesting that elevated temperature (33°C) and a feeding rate of 2.5% biomass
159 enhanced the activity of digestive enzymes.

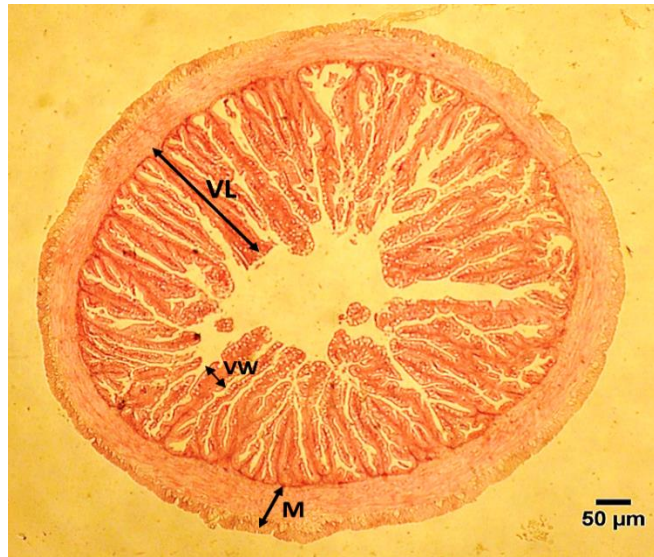
160
161 **Table 2.** The activity of digestive enzymes of Asian seabass (*Lates calcarifer*) reared
162 at different temperatures and feeding rates for 6 weeks.

Treatment	Lipase (U/mg protein)	Amylase (U/mg protein)	Protease (U/mg protein)
T1	4.43±1.19	8.76±2.87	1.90±0.70
T2	4.36±0.15	7.76±2.17	1.90±0.34
T3	5.70±0.20	9.16±1.44	2.40±0.10
T4	4.93±1.78	8.56±1.10	2.20±0.10
T5	6.00±1.70	10.50±3.50	2.60±0.52
T6	4.25±0.05	7.40±1.00	2.23±0.35
Two-way ANOVA			
Temperature	0.747	0.805	0.529
Feeding rate	0.675	0.811	0.181
Temperature × Feeding rate	0.095	.0244	0.235

163 T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C,
164 apparent satiation), T5 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Data are presented as
165 mean±SE. The absence of superscript letters indicates no significant difference (P> 0.05).

166 **Histological studies**

167 Histological studies were carried out to identify any significant changes in intestine tissue
168 morphology where muscularis thickness, villi height, and villi width were measured, as depicted
169 in Figure 1.



170
171 **Figure 1.** Details of intestinal histological structure of Asian seabass (*Lates calcarifer*) cultured
172 in different temperatures and feeding rates. VL: villi length; VW; villi width; M: muscular width
173 (H & E, 400X, Scale bar= 50 μm).

174
175 Results from histological evaluations are depicted in Table 3, which indicates no considerable
176 changes in muscular thickness, villi height, and width between different treatments ($P > 0.05$).
177 However, the results were complex and variable; thus, no definitive conclusions could be drawn
178 regarding the influence of temperature, feeding rate, or their combined effects on the measured
179 morphometric values of fish intestines.

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186**Table 3.** Intestinal morphology of rainbow of Asian seabass (*Lates calcarifer*) reared at different temperatures and feeding rates for 6 weeks.

Treatment	Muscularis thickness (μm)	Villi height (μm)	Villi width (μm)
T1	44.08 \pm 6.38	160.20 \pm 16.19	84.46 \pm 1.96
T2	40.33 \pm 6.76	142.52 \pm 13.82	74.45 \pm 6.22
T3	47.96 \pm 8.99	135.67 \pm 84.56	62.73 \pm 3.66
T4	55.81 \pm 7.90	192.91 \pm 19.49	84.46 \pm 8.94
T5	55.70 \pm 9.70	159.75 \pm 11.28	74.14 \pm 4.55
T6	41.34 \pm 3.49	154.84 \pm 84.56	67.86 \pm 3.88
Two-way ANOVA			
Temperature	0.778	0.095	0.054
Feeding rate	0.286	0.064	0.303
Temperature \times Feeding rate	0.324	0.095	0.676

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T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Data represent mean \pm SD (n= 3). The absence of superscripts in each column indicates no significant difference ($P > 0.05$).

192 **Gut microbiota**

193 Figure 2 displays variations in bacterial counts in the intestines of fish subjected to different
194 temperature and feeding rate conditions. The results indicate minor fluctuations in the total
195 number of culturable bacteria across fish intestines; however, statistical analysis revealed no
196 significant differences ($P > 0.05$). The highest bacteria were observed in T5, where fish were
197 reared at high temperatures and fed 2.5% of biomass.

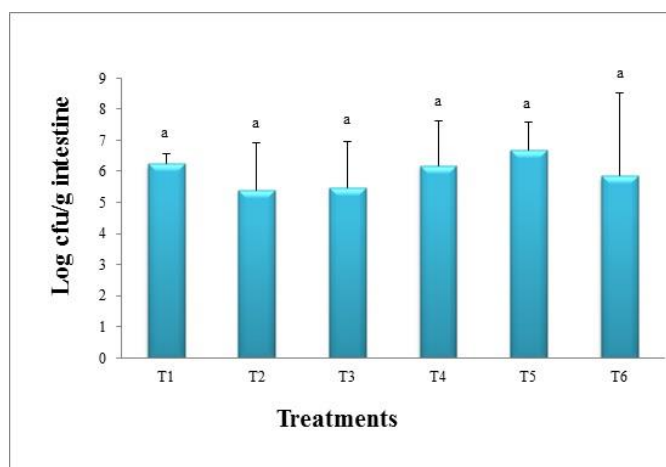
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Figure 2. Bacterial colonies cultured from intestine tissue of Asian seabass (*Lates calcarifer*) after 6 weeks. T1 (20°C, apparent satiation), T2 (20°C, 2.5% biomass), T3 (27°C, apparent satiation), T4 (27°C, 2.5% biomass), T5 (33°C, apparent satiation), and T6 (33°C, 2.5% biomass). Data are presented as mean \pm SE. The same superscripts indicate no significant differences ($P > 0.05$).

205 **Gene expression**

206 The relative expression of *IGF-I* and *HSP70* genes was evaluated in fish reared under different
 207 temperatures and feeding rates, and the results are shown in Table 4. In contrast, temperature
 208 significantly affected the expression of *IGF-I* and *HSP70*, which is evident when comparing low-
 209 temperature treatments (T1 and T2) with other treatments. From the statistical point of view, the
 210 transcription levels of *IGF-I* and *HSP70* were the lowest in T1 and T2 ($P < 0.05$) and the highest
 211 in T5 and T6 in comparison with other treatments ($P < 0.05$).

212
 213 **Table 4.** Relative expression of *IGF-I* and *HSP70* genes in liver tissue of Asia seabass (*Lates*
 214 *calcarifer*) reared under different temperatures and feeding rates for 6 weeks.

Treatment	IGF-I	HSP70
T1	1.00±0.04 ^b	1.00±0.24 ^c
T2	1.14±0.24 ^b	1.13±0.02 ^c
T3	3.27±0.61 ^a	4.72±0.44 ^b
T4	3.77±0.24 ^a	4.62±0.62 ^b
T5	3.56±0.24 ^a	9.29±1.69 ^a
T6	3.69±0.76 ^a	10.20±1.50 ^a
<i>Two-way ANOVA</i>		
Temperature	0.00	0.00
Feeding rate	0.354	0.532
Temperature × Feeding rate	0.723	0.676

215 T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5
 216 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Data represent mean±SE (n= 3). Different superscripts in
 217 each column indicate significant differences ($P < 0.05$).

218

219 **DISCUSSION**

220 The current study investigated the interactive effects of different water temperatures and feeding
 221 rates on digestive enzyme activity, histology, and gut microbiota of Asian seabass. The present
 222 results revealed that different temperatures and feeding rates or their interaction (Two-way
 223 ANOVA analysis) do not significantly affect the activity of digestive enzymes and gut
 224 morphometrics. However, higher activity of digestive enzymes was found in individuals from T5
 225 (33°C and 2.5% biomass) and T3 (27°C and 2.5% biomass), respectively. Furthermore, at
 226 molecular levels, significant changes were observed at the same water temperature but at
 227 different feeding rates. It is worth mentioning that no mortality was observed during the trial;
 228 however, 10-fold higher transcription levels of HSP-70 were
 229 Other researchers have investigated similar hypotheses. For instance, Baloi et al. (2017)
 230 evaluated the effects of different feeding rates, ranging up to satiation levels, on juvenile

231 Brazilian sardine (*Sardinella brasiliensis*). Their study revealed a notable decline in total protease
232 and amylase activities as feeding rates increased, while lipase activity demonstrated no
233 significant variation in response to feeding rate adjustments. This is most likely associated with
234 excessive food for the capacity of the target species' digestive system. It is also possible that a
235 higher temperature will speed up metabolism and increase the digestive system's capacity;
236 however, the present results did not support this (Table 2). In addition, Harpaz et al. (2005)
237 observed elevated activity of brush border enzymes in Asian seabass under reduced feeding rates,
238 coinciding with a decline in growth performance, which may be attributed to limited food
239 availability. The discrepancy between results among studies might be attributed to the type of
240 enzymes, various temperature ranges, tested feeding rate, duration of experiment, and fish
241 species.

242 Volkoff and Rønnestad (2020) have suggested a direct effect of temperature on energy
243 requirement. Temperature influences food intake, consumption, nutrient absorption, protein
244 synthesis, and growth rate (Fauconneau, 1985). A temperature higher than optimal increases the
245 gastrointestinal tract evacuation rate, which leaves less time for the digestion process and might
246 reduce growth. In line with our results regarding the length and width of the villi and thickness of
247 the intestinal muscular layer, Bowyer *et al.* (2012) have reported no significant histological
248 changes.

249 Our findings suggest that rearing temperature and feeding rate do not significantly influence the
250 number of cultivable bacteria in the fish guts across different treatments. Fish intestinal
251 microbiota contributes to various physiological processes. Sugita *et al.* (1989) suggested that
252 temperature can significantly affect bacterial populations in the water but not fish microflora.
253 Liston (1957) has reported that in the intestine of skates (*Raja* spp) and lumen of Sole
254 (*pleuronectes microcephalus*), *Vibrio* spp. was dominant during all seasons. According to the
255 literature, ecological and environmental factors can selectively promote the dominance of
256 particular microbial populations (Ley *et al.*, 2008; Wong and Rawls, 2012). Fish can experience
257 stress, reduced growth, and disruption in intestinal microbial communities at temperatures higher
258 than optimal temperatures (Jobling, 1981). Moreover, bacterial growth in the fish intestine may

259 increase under elevated water temperatures (Huyben *et al.*, 2018). According to Hagi *et al.*
260 (2004) a study involving three carp species and channel catfish revealed that lactic acid bacteria
261 (LAB) populations in the fish gut showed no significant variation between summer temperatures
262 (23–28°C) and winter temperatures (4–10°C). Soriano *et al.* (2018) have reported a notable
263 alteration in gut microbiota in response to acclimation temperatures, while Huyben *et al.* (2018)
264 documented dysbiosis in the intestinal microbiota in response to seasonal fluctuations. The
265 interactive effects were also investigated at molecular levels, and as per the expression of *IGF-1*
266 and *HSP70*, there were significant differences among different treatments. It is acknowledged
267 that investigating *IGF-1* might help understand the correlations between temperature feeding rate
268 and fish growth. The present study aligns with reports from rainbow trout (Chauvigné *et al.*,
269 2003) and chinook salmon (Beckman *et al.*, 1998), which experienced an increase in *IGF-1*
270 correlated with the increased water temperature. Since fish did not experience any growth
271 retardation, we suggest that the increased *HSP70* may not have occurred due to stress-mediated
272 protein damage but rather as enhanced cytoprotection (Deane and Woo, 2005) which requires
273 further research. *HSP70* gene expression was affected in different groups. Research has
274 demonstrated that small heat shock proteins play a role in responding to temperature fluctuations
275 (Podrabsky and Somero, 2004). Additionally, studies have reported that chronic acclimation of
276 silver sea bream (*Sparus sarba*) to cold temperatures (12°C) led to an upregulation of *HSP70*
277 compared to warmer temperatures (25°C) (Deane and Woo, 2005). This phenomenon may be
278 attributed to stress-induced protein damage, as documented in prior studies (Ananthan *et al.*,
279 1986) and increased levels of *HSP70* have been associated with enhanced cytoprotection and
280 prevention of cell apoptosis (Deane and Woo, 2005).

281
282 **CONCLUSIONS**

283 The present findings suggest that variations in temperatures and feeding rates do not significantly
284 impact digestive enzymes, intestinal histology, and gut microbiota of Asian seabass over 6 weeks.
285 However, temperature and feeding rate did influence the transcription of genes related to growth
286 and stress responses, such as *IGF-1* and *HSP70*. These results underscore the need for further

287 investigation into how different temperatures and feeding rates affect the immune response and
288 antioxidant capacity of Asian seabass at the molecular level.

289

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294

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