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Original Research Article

Dietary fishmeal replacement by *Clostridium autoethanogenum* protein meal influences the nutritional and sensory quality of turbot (*Scophthalmus maximus*) via the TOR/AAR/AMPK pathways

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ABSTRACT

Clostridium autoethanogenum protein (CAP) is a promising protein source for aquaculture; however, how CAP influences fish quality is worth extensive research. We randomly allocated 630 turbot with initial body weights of about 180 g into 6 groups, with fishmeal-based control diet or diet with CAP replacing 15% (CAP15), 30% (CAP30), 45% (CAP45), 60% (CAP60), or 75% (CAP75) of fishmeal protein. After a 70-d feeding trial, the fillet yield ($P = 0.015$) and content of protein ($P = 0.017$), collagen ($P < 0.001$), hydroxyproline ($P < 0.001$), C20:5n-3 ($P = 0.007$), and ∑n-3/∑n-6 polyunsaturated fatty acids ratio ($P < 0.001$) in turbot muscle was found to decrease linearly with increasing CAP. However, turbot fed CAP15 diet maintained these parameters ($P > 0.05$). By contrast, the muscle hardness increased linearly with increasing CAP ($P = 0.004$), accompanied by linear reduction of muscle fiber area ($P = 0.003$) and expression of myogenesis-related genes, including cathepsin D (*ctsd* $P < 0.001$) and muscle ring finger protein 1 (*murf 1*, $P < 0.001$). Phosphorylation of protein kinase B (Akt, $P < 0.001$), target of rapamycin (TOR, $P = 0.001$), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1, $P < 0.001$), and ribosomal protein S6 (S6, $P < 0.001$) decreased linearly; however, phosphorylation of AMP-activated protein kinase (AMPK, $P < 0.001$), eukaryotic initiation factor 2α (eIF2α, $P < 0.001$), and the abundance of activating transcription factor 4 (ATF4, $P < 0.001$) increased with increasing CAP, suggesting that the TOR signaling pathway was inhibited, and the amino acid response (AAR) and AMPK pathways were activated. Additionally, expression of genes related to protein degradation, including myogenic factor 5 (*myf 5*, $P < 0.001$), myogenic differentiation (*myod*, $P < 0.001$), paired box 7 (*pax 7*, $P < 0.001$), and *ctsd* ($P < 0.001$), decreased linearly with increasing CAP. In conclusion, CAP could be used to replace up to 15% of fishmeal without negatively impacting turbot quality. However, higher levels of CAP decreased fillet yield, muscle protein content, and muscle fiber diameter while increasing muscle hardness, which could be attributed to the inhibition of the TOR pathway and activation of the AAR and AMPK pathways.

1. Introduction

Aquaculture is critical for the global food system, and its production has more than tripled in the last 20 years (Naylor et al., 2021). Fishmeal is the primary protein source; however, production cannot meet the demand (Colombo et al., 2023). Therefore, it is urgent to seek alternatives for fishmeal which are sustainable and renewable. *Clostridium autoethanogenum* protein (CAP) is the by-product of ethanol production by *C. autoethanogenum,* with industrial waste gas carbon monoxide as the only carbon source (Xu et al., 2023). The production process is environmentally

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friendly, reproducible, and has a low environmental footprint with minor health risks (Wang et al., 2023). Furthermore, CAP has high crude protein and vitamin levels and contains fewer anti-nutritional factors (Ritala et al., 2017; Sharif et al., 2021) and could substitute part of fishmeal without compromising growth performance in the largemouth bass *Micropterus salmoides* (Lu et al., 2021; Ma et al., 2022; Yang et al., 2023, 2022a,b; Zhu et al., 2022), grass carp *Ctenopharygoden idella* (Fan et al., 2022; Xue et al., 2023), black sea bream *Acanthopagrus schlegelii* (Chen et al., 2020), large yellow croaker *Larimichthys crocea* (Wu et al., 2022), tilapia *Oreochromis niloticus* (Maulu et al., 2021), and juvenile turbot *Scophthalmus maximus* (Zheng et al., 2022).

Growth performance is the most critical issue for aquaculture farmers in the cultivation stage. However, fish quality directly determines the economic value of the end product and affects consumer purchase behavior (Grigorakis, 2017), hence the usage of a new ingredient cannot be assessed solely by its effect on growth performance. Quality can be defined by nutritional (Mohanty et al., 2019) and sensory quality (Yu et al., 2020). The physiological status of fish is affected by the nutritional profile of the feed, and replacement of fishmeal in the diet may alter fish quality (Glencross et al., 2020). Dietary replacement of fishmeal by CAP altered the proximate composition and amino acid profile of largemouth bass (Yang et al., 2022b), large yellow croaker (Wu et al., 2022), and grass carp (Fan et al., 2022; Xue et al., 2023). For sensory quality, the muscle texture in largemouth bass (Yang et al., 2022b), large yellow croaker (Wu et al., 2022) and grass carp (Fan et al., 2022; Xue et al., 2023), flavor in largemouth bass (Yang et al., 2022b) and grass carp (Xue et al., 2023), and skin color in large yellow croaker (Wu et al., 2022) were affected by CAP inclusion. Although studies investigated the transcription of muscle-related genes altered by CAP (Fan et al., 2022; Huang et al., 2023; Zheng et al., 2022), the molecular mechanisms of how CAP affects fish quality remain unclear.

Turbot is farmed and consumed in Europe and East Asia; however the industry is threatened by over-reliance on fish meal as the primary protein source, and a decline in overall quality (Fernández-González et al., 2023; Guan et al., 2018). The evaluation of CAP on turbot focused on growth performance, digestibility, and nutrient metabolism; only specific quality indicators (fillet yield [FY], muscle amino acid, and fatty acid profile) of juvenile turbot fed CAP were reported (Zheng et al., 2022). There was no report on quality evaluation on the turbot close to market specifications. Therefore, the present study evaluated turbot quality during growth-out phase and investigated the mechanisms from the nutrient-sensing, metabolism, and myogenesis-related signaling pathways.

2. Materials and methods

2.1. Animal ethics statement

The Ethical Scientific Committee for Animal Experimentation of Shandong University approved the fish manipulation and sampling techniques, which complied with the European directive 2010/63/UE.

2.2. Experimental diets

According to our previous study, the basal diet (FM diet) containing 62% fishmeal as the sole protein source was formulated (Bai et al., 2023). Five isonitrogenous (48%) and isolipidic (12%) experimental diets were formulated using CAP to replace fishmeal at levels of 15% (CAP15), 30% (CAP30), 45% (CAP45), 60% (CAP60), and 75% (CAP75). Raw materials were fully ground into powder (<75 μm) and formed into 4-mm particles with a twin-screw extruder (TSE65; Beijing Modern Yanggong Machinery S&T Development Co., Ltd., China). A vacuum oil injection machine (SD1000KG; Zhejiang L&B Machinery Co., Ltd., China) was then used to spray a mixture of soybean oil, fish oil, and soy lecithin evenly on the surface of the particles. The dried diets were stored at 4 °C until use. The diet formulations and proximate composition of FM and CAP are shown in Table 1.

2.3. Feeding trial

Turbot with an initial weight of 180 g were purchased from a local aquafarm (Weihai, China) and transported to the Marine Science and Technology Innovation Center of Shandong University (Weihai, China). Fish were maintained in an indoor plastic tank and fed with the FM diet over the 14 d of acclimation. Fish were individually weighed after 24 h of food deprivation and randomly assigned to 18 fiberglass tanks (volume 600 L, 35 fish per tank, three replicate tanks per diet) within an indoor circulating-water culturing system (Shanghai Haisheng Biological Experiment Equipment Co., Ltd., China) equipped with an oxygen supply system, temperature regulation system, protein separator, UV germicidal lamp, sand filter, and bio-filter. During the 70 d feeding trial, the photoperiod was set at a 2 h light/22 h dark cycle, and 30% water was changed every two days to maintain water quality. The water temperature, salinity, and pH varied from 13 to 15 °C, 28 to 30, and 7.8 to 8.2, respectively. Fish were fed manually to visual satiety twice daily at 08:00 and 17:00. The uneaten diet particles were collected 30 min after feeding, dried at 70 °C, and weighed to calculate feed intake (FI).

2.4. Sample collection

After the 70 d feeding trial, fish were food-deprived for 24 h and anesthetized with eugenol (1:10,000 dilution). The total fish weight

Table 1
Ingredients and proximate composition of diets (%, dry matter basis).

Item	Experiment diets[1]					
	FM	CAP15	CAP30	CAP45	CAP60	CAP75
Ingredients						
Fishmeal[2]	62.0	52.7	43.4	34.1	24.8	15.5
CAP[3]	0.0	7.5	15.0	22.5	30.0	37.5
Wheat meal	20.0	20.0	20.0	20.0	20.0	20.0
Fish oil	1.7	2.6	3.5	4.4	5.3	6.2
Soybean oil	1.7	1.7	1.7	1.7	1.7	1.7
Soy lecithin	2.0	2.0	2.0	2.0	2.0	2.0
Premix[4]	1.0	1.0	1.0	1.0	1.0	1.0
Monocalcium phosphate	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	0.1	0.1	0.1	0.1	0.1	0.1
Betaine	0.2	0.2	0.2	0.2	0.2	0.2
Yttria	0.1	0.1	0.1	0.1	0.1	0.1
Microcrystalline cellulose	9.7	10.6	11.5	12.4	13.3	14.2
Proximate composition						
Crude protein	47.7	46.3	46.1	47.3	48.2	47.1
Crude lipid	11.6	11.2	10.7	12.1	11.9	12.2

[1] FM = fishmeal-based basal diet without *Clostridium autoethanogenum* protein; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60% and 75% levels in basal diet, respectively.

[2] Steam-dried fish meal from COPEINCA Group (Lima, Peru); crude protein content of 72.72% and crude lipid content of 9.65% (in dry matter).

[3] *Clostridium autoethanogenum* protein (CAP) from Beijing Shoulang Biotechnology Co., Ltd. (Beijing, China); crude protein content of 90.40% and crude lipid content of 1.36% (in dry matter).

[4] Premix formula (mg/kg diet): vitamin A, 20; vitamin D_3, 10; vitamin K_3, 20; vitamin C, 600; inositol, 150; niacin acid, 80; calcium pantothenate, 40; vitamin B_2, 15; vitamin B_6, 15; vitamin B_1, 10; folic acid, 10; vitamin B_{12}, 8; biotin, 2; wheat middlings, 220; $FeSO_4 \cdot H_2O$, 300; $MgSO_4 \cdot 7H_2O$, 1200; $ZnSO_4 \cdot H_2O$, 200; NaCl, 100; $MnSO_4 \cdot H_2O$, 25; $CuSO_4 \cdot 5H_2O$, 30; $CoCl_2 \cdot 6H_2O$, 5; Na_2SeO_3, 5; KIO, 3; antioxidant, 50; mold inhibitor, 200; zeolite powder, 6382.

was measured to calculate the weight gain (WG). Ten fish were randomly selected from each tank to measure the individual body weight and body length. Three fish underwent skin color measurement and were dissected immediately to remove gills, and the remaining carcass underwent FY calculation (Regost et al., 2001). The other seven fish were dissected to obtain dorsal muscles. The locations of muscle sampling, color measurements, texture, histological, gene expression, and protein analysis are shown in Fig. 1 (Hernández-Urcera et al., 2017). For histological analysis, 5-mm pieces of muscles were sampled and fixed in 4% neutral buffered formalin to conduct histological analysis. Muscle tissues for mRNA and protein analysis were snap-frozen in liquid nitrogen and stored at −80 °C. The remaining muscles were thoroughly chopped, divided into 4 × 6 cm PVC bags (5 g per bag), and stored at −80 °C for proximate composition and fatty acid profile analyses.

2.5. Proximate composition and fatty acid profile

Proximate composition of feed and muscle samples were determined according to standard methods (AOAC, 1995). The moisture content was determined gravimetrically by oven-drying 10 g of muscle samples at 105 °C to constant weight. Crude protein content was determined according to the Kjeldahl method with a semi-automatic Kjeldahl nitrogen determinator (K9804; Shandong Haineng Instrument Co., Ltd., China). Crude lipid was evaluated gravimetrically after petroleum ether extraction with an extraction system (SZC-101; Shanghai Xianjian Instrument Co., Ltd., China). Hydroxyproline (hyp) and glycogen contents were measured using the kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China; product code: A030-2-1) and Bioisco Institute (Lianyungang, China; product code: GMS007), respectively (Zhang et al., 2013). The total collagen content was calculated by multiplying the hyp content by 8 (AOAC, 2005). The muscle fatty acid profile was determined using gas chromatography (GC-2010, Shimadzu, Japan) according to the China National Standard (GB 5009.168-2016).

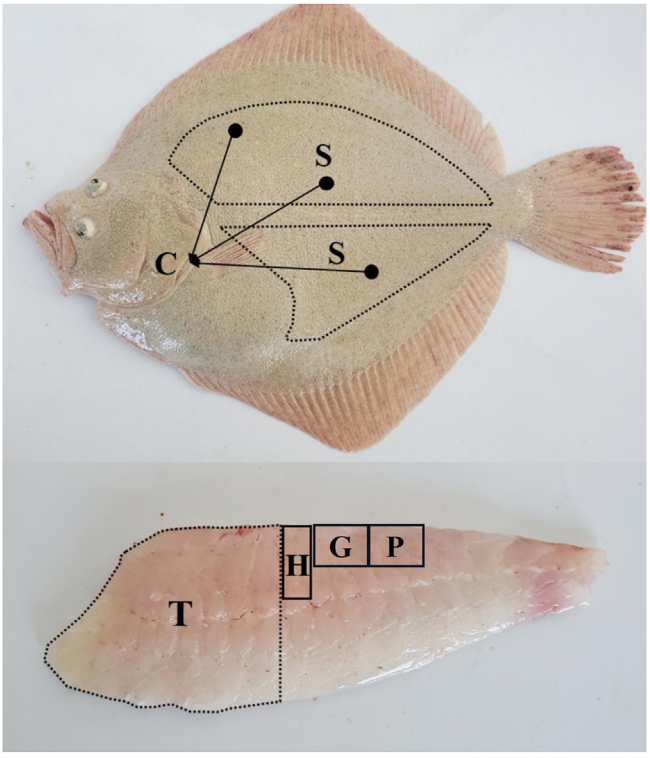

Fig. 1. Locations of the muscle sampling for color measurements, texture analysis, and histological analysis. S = muscle sampling zone; C = color measurements; T = texture analysis; H = histological analysis; G = gene expression analysis; P = protein abundance analysis. The remaining parts of muscle samples were used for nutritional quality analysis.

2.6. Skin color measurements

A chromameter (CR-10, Konica Minolta, Japan) was used to measure surface skin color. The L*a*b* system (L* = lightness; a* = redness; b* = yellowness) was used according to the International Commission on Illumination (McLAREN, 1976), and measurements were performed at three sites to calculate a mean value per fish. The total color difference (ΔE) calculation was performed as follows (Panini et al., 2017):

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2},$$

where ΔL*, Δa*, and Δb* represent the difference between L*, a*, and b* between the FM-fed group and each CAP-fed group, respectively.

2.7. Texture analysis and histological analysis

Texture analysis was performed immediately after dissection (<1 min). Dorsal muscle (without skin) was placed on the platform of a texture analyzer (TMS-PRO, Food Technology Corporation, USA) using the texture profile analysis method. According to previous work (Wijerath Wiriduge et al., 2020), a cylindrical probe (8 mm diameter) was used to vertically compress the muscle with an initial force of 0.3 N and a constant speed of 30 mm/min until deformation of 60% was achieved. Compressions were performed at three locations to calculate an average of the attributes for each fish.

According to the standard histology techniques, the fixed muscle tissues (n = 9 per treatment) were dehydrated with ethanol and xylene and embedded in paraffin. The paraffin blocks were then cut into 5-μm sections and stained with hematoxylin and eosin. A Nikon Eclipse Ci-L microscope (Nikon, Japan) was used to photograph the slides. We chose 200 muscle fibers for each repetition to measure the diameter and area of muscle fibers using NIS-Elements D software.

2.8. Real-time PCR

The total RNA was extracted from the muscle with RNAiso Plus according to the manufacturer's protocol (Takara, China). The quantity of the total RNA was determined by spectrophotometric analysis at 260 nm and 280 nm, and 1% agarose gel electrophoresis. The total RNA was reverse-transcribed into cDNA using a PrimeScript RT kit (Takara, China). The quantitative real-time PCR system (QuantStudio 5, Applied Biosystems, USA) was applied to analyze the relative expression of genes related to protein degradation and myogenesis. The RNA polymerase II subunit D was used as the reference gene, and the relative mRNA expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method. The primers were designed by the software PrimerPremier (ver. 6), synthesized by Sangon Biotechnology (Shanghai, China) and are listed in Table 2.

2.9. Western blot

Western blot analysis was conducted to analyze the relative protein level of phospho-target of rapamycin (TOR), TOR, phospho-protein kinase B (Akt), Akt, phospho-eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), 4E-BP1, phospho-ribosomal protein S6 (S6), S6, phospho-AMP-activated protein kinase (AMPK), AMPK, phospho-eukaryotic initiation factor 2α (eIF2α), eIF2α, and activating transcription factor 4 (ATF4). Muscle samples were homogenized and

Table 2
Primers used in quantitative real-time PCR.

Item	Forward (5′ to 3′)	Reverse (5′ to 3′)	GenBank no.
myod	AGGTCAPACGACGCCTTCGAGAC	GCAPGGGACTCGATGTAGCTTATGG	MH174948.1
myf 5	GTGGCAPACAPTCTGAACGCAPAACTAC	GGAGAGACAPCTGGAGGCTGGAG	MN072640
myog	CGGCGTCTGAAGAAGGTGAATGAG	TGGAGCCTCTCGATATACTGGATGG	MH174947.1
mrf 4	GACCTCTTCGAGACCAPACGCTTATC	CGTTGTACAPGCGGCAPTGTCCAPG	MN072641
pax 7	CCCAPGACAPTCTACAPCCAPGAGAGGAG	GCCTGTTGCTGAACCAPCAPCCTG	MN072642
murf 1	CAPCGAAGACGAACGCAPTCAPACAPTC	TCTGGCTCTGGTAGACGGCAPAG	MH174966
maf bx	ACGCTCTACCTCTCGCTCTGTG	CGCAPGCCAPGGTGTTGATGTTCC	MH174969
ctsd	CTGCTTGCTTCAPCCAPCAPAATATAA	GACAPAACTGCCAPGATCCAPTACT	KY593341.1
ctsl	GACAPGCTGAGGAGATCAPATGAG	GAGCCAPCTCGCTTCCTTAAA	EU077233.1
rpsd[1]	AACAPCAPGGAAGCAPGCAPGAAC	ACGGCAPGTGATGGTCTCTC	

myod = myogenic differentiation; *myf 5* = myogenic factor 5; *myog* = myogenin; *mrf 4* = myogenic regulatory factor 4; *pax 7* = paired box 7; *murf 1* = muscle ring finger protein 1; *maf bx* = muscle atrophy F box; *ctsd* = cathepsin D; *ctsl* = cathepsin L; *rpsd* = RNA polymerase II subunit D.
[1] The *rpsd* primer sequences referred to Bai et al. (2023).

lysed in ice-cold radioimmunoprecipitation lysis buffer with protease and phosphatase inhibitors (Roche, USA). Lysates were centrifuged (12,000 × *g* at 4 °C for 20 min), and the protein concentrations of supernatant were measured using a BCA Protein assay kit from Beyotime Biotechnology (Nanjing, China; product code: P0012S). Lysates with identical amounts of protein (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45-μm polyvinylidene difluoride membranes (Millipore, USA). The membranes were washed and incubated with a secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG) from Beyotime Biotechnology, developed with Beyo ECL Plus reagents from Beyotime Biotechnology and exposed to X-rays. The band densities were quantified using NIH Image software (ver. 1.63) and normalized to β-tubulin (as an internal control). Based on Jiang et al. (2017) and Xu et al. (2016), primary antibodies against phospho-TOR, phospho-Akt, phospho-4E-BP1, phospho-S6, phospho-AMPK, phospho-eIF2α, TOR, Akt, 4E-BP1, S6, AMPK, eIF2α, and β-tubulin were purchased from Cell Signaling Technology Inc. (Boston, USA). The antibody against ATF4 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). All antibodies were previously validated in turbot (Jiang et al., 2017; Xu et al., 2016). Results were normalized to the data in the FM group.

2.10. Statistical analysis and calculation

The statistical analysis was performed using SPSS (ver. 26.0). All data were tested for normality and homogeneity of variance before one-way analysis of variance (ANOVA), and Tukey's HSD test was performed as a post-hoc test. Data were subjected to orthogonal polynomial contrasts for the trend analysis. Data were subjected to linear, quadratic, or cubic regression models using GraphPad Prism software (ver. 7.00) in orthogonal polynomial contrasts, and the trend between parameter and CAP dietary level was determined based on the lowest *P*-value in orthogonal polynomial contrasts. The level of significance was set at $P < 0.05$.

The following parameters were calculated as follows to evaluate the growth performance, FY, and muscle fat quality:

Survival rate (SR, %) = (Final number of turbot/Initial number of turbot) × 100;

WG (%) = [(Final body weight − Initial body weight)/Initial body weight] × 100;

FI (%/d) = 100 × total feed consumption/[(final body weight + initial body weight)/2]/days;

Condition factor (CF, g/cm^3) = Final body weight/Final body length^3;

FY (%) = [Cooked fillets weight/(Final body weight − Gill weight − Visceral weight)] × 100;

Atherogenicity index (AI) = [C12:0 + (4 × C14:0) + C16:0]/(∑n-3 polyunsaturated fatty acids (PUFA) + ∑n-6 PUFA + ∑mono-unsaturated fatty acids);

Hypocholesterolemic to hypercholesterolemic fatty acids ratio (HH) = (C18:n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3)/(C14:0 + C16:0).

3. Results

3.1. Growth performance and FY

Dietary inclusion of CAP influenced growth performance after 70 d of feeding (Tables 3 and S1). The orthogonal contrasts showed significant ($P < 0.05$) negative linear relationships in WG ($R^2 = 0.5916$), FI ($R^2 = 0.2955$), and FY ($R^2 = 0.2882$) with increasing dietary CAP concentration. However, the WG of turbot fed CAP15 and CAP30, and FY in the CAP15 group was not significantly lower than the FM group ($P > 0.05$). There was no significant difference in FI among groups ($P > 0.05$). The SR and CF were not significantly affected by dietary CAP levels ($P > 0.05$).

3.2. Nutrient content of muscle

3.2.1. Muscle proximate composition

The muscle proximate composition is displayed in Tables 4 and S1. The orthogonal contrasts showed a significant ($P < 0.05$) positive linear relationship in moisture content ($R^2 = 0.3890$) and a significant ($P < 0.05$) negative linear relationship in crude protein ($R^2 = 0.3345$), hyp ($R^2 = 0.5617$), and collagen content ($R^2 = 0.5633$) with increased CAP level. The muscle moisture content in turbot fed CAP diets did not markedly differ from those fed the FM diet ($P > 0.05$). Muscle hyp and collagen content in the CAP15 and CAP30 groups did not significantly differ from the FM group ($P > 0.05$) but were significantly lower in the CAP45, CAP60, and CAP75 groups ($P < 0.05$). Dietary inclusion of CAP did not significantly affect muscle crude lipid or glycogen contents ($P > 0.05$).

3.2.2. Muscle fatty acid profile

Dietary fishmeal replacement by CAP significantly affected the muscle fatty acid profile (Tables 5 and S1). Significant ($P < 0.05$) negative linear relationships were found in the C16:n-7 ($R^2 = 0.3027$), C20:5n-3 (EPA; $R^2 = 0.4595$) levels, and ∑n-3/∑n-6 PUFA ratio ($R^2 = 0.4431$) with increased CAP level, and a positive linear relationship was found in the C20:2 ($R^2 = 0.2090$) level. The orthogonal contrasts showed significant ($P < 0.05$) cubic

Table 3
Growth performance and fillet yield of turbot fed experimental diets for 70 d.[1]

Item	FM	CAP15	CAP30	CAP45	CAP60	CAP75	ANOVA (Pr > F [2])	Trend[3]
SR, %	98.1 ± 1.0	99.0 ± 1.0	99.0 ± 1.0	97.1 ± 1.65	98.1 ± 1.0	99.0 ± 1.0	0.771	–
WG, %	55.7 ± 2.0^{a}	63.0 ± 1.6^{a}	54.2 ± 3.9^{a}	36.9 ± 1.0^{b}	36.9 ± 3.3^{b}	40.7 ± 2.7^{b}	0.000	Linear
FI, %/d	0.65 ± 0.02	0.68 ± 0.00	0.65 ± 0.06	0.56 ± 0.03	0.61 ± 0.05	0.52 ± 0.06	0.180	Linear
FY, %	73.9 ± 1.1^{a}	71.1 ± 1.3^{ab}	68.4 ± 0.4^{b}	69.2 ± 0.7^{b}	68.7 ± 2.1^{b}	69.9 ± 0.5^{b}	0.048	Linear
CF, g/cm^{3}	1.88 ± 0.08	1.85 ± 0.03	1.92 ± 0.05	1.86 ± 0.05	1.87 ± 0.06	1.86 ± 0.01	0.954	–

SR = survival rate; WG = weight gain; FI = feed intake; FY = fillet yield; CF = condition factor.
[1] Values are expressed as mean ± standard error ($n = 3$). Different superscript letters within a row indicate significant differences ($P < 0.05$). FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively.
[2] Significance probability associated with the F-statistic.
[3] Based on orthogonal contrasts, details are listed in Table S1.

Table 4
Muscle proximate composition, hydroxyproline, and collagen content in the muscle of turbot fed experimental diets for 70 d.[1]

Item	FM	CAP15	CAP30	CAP45	CAP60	CAP75	ANOVA (Pr > F [2])	Trend[3]
Moisture, %	77.7 ± 0.3^{ab}	77.3 ± 0.3^{b}	77.7 ± 0.1^{ab}	78.5 ± 0.2^{ab}	78.7 ± 0.1^{a}	78.3 ± 0.3^{ab}	0.015	Linear
Crude protein, %	19.4 ± 0.6	19.2 ± 0.1	19.7 ± 0.2	18.9 ± 0.3	18.7 ± 0.4	18.4 ± 0.2	0.127	Linear
Crude lipid, %	5.51 ± 0.84	5.22 ± 0.42	5.99 ± 0.48	4.74 ± 0.86	5.22 ± 0.75	5.45 ± 0.97	0.901	–
Muscle glycogen, mg/g	1.13 ± 0.15	0.94 ± 0.07	0.87 ± 0.03	0.92 ± 0.05	0.91 ± 0.04	0.87 ± 0.04	0.227	–
Hydroxyproline, μg/mg	0.51 ± 0.02^{a}	0.39 ± 0.02^{ab}	0.41 ± 0.03^{ab}	0.35 ± 0.05^{b}	0.33 ± 0.02^{b}	0.33 ± 0.01^{b}	0.006	Linear
Collagen, μg/mg	4.11 ± 0.18^{a}	3.13 ± 0.11^{ab}	3.26 ± 0.29^{ab}	2.80 ± 0.42^{b}	2.63 ± 0.15^{b}	2.62 ± 0.07^{b}	0.006	Linear

[1] Values are expressed as mean ± standard error ($n = 3$). Different superscript letters within a row indicate significant differences ($P < 0.05$). FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively.
[2] Significance probability associated with the F-statistic.
[3] Based on orthogonal contrasts, details are listed in Table S1.

relationships of C20:4n-6 ($R^2 = 0.2008$) and C24:n-9 ($R^2 = 0.4158$) levels. The C20:4n-6 level was significantly lower than the FM group only in the CAP30 group ($P < 0.05$). There was no significant difference in C20:5n-3 level among CAP groups with the FM group ($P > 0.05$). The ∑n-3 PUFA, ∑n-6 PUFA, and ∑MUFA levels were unaffected by CAP ($P > 0.05$). The ∑n-3/∑n-6 PUFA ratio was not significantly altered in turbot fed CAP15 and CAP45 diets ($P > 0.05$).

For health value attributes, the orthogonal contrasts showed a significant ($P < 0.05$) negative linear relationship in AI ($R^2 = 0.4677$) and a significant ($P < 0.05$) positive linear relationship in HH ($R^2 = 0.3110$) with the increase of CAP level. No significant difference among treatments was observed in the HH ($P > 0.05$), but only CAP60 group results significantly lowered AI ($P < 0.05$).

Table 5
Fatty acid profile (% total fatty acids) and lipid quality indexes in the muscle of turbot fed experimental diets for 70 d.[1]

Item	FM	CAP15	CAP30	CAP45	CAP60	CAP75	ANOVA (Pr > F [2])	Trend[3]
C14:0	3.38 ± 0.10	3.44 ± 0.26	3.58 ± 0.33	2.94 ± 0.28	3.01 ± 0.28	3.01 ± 0.17	0.370	–
C16:0	18.4 ± 0.2	18.1 ± 1.1	18.0 ± 1.4	18.6 ± 0.7	17.8 ± 1.0	18.3 ± 0.5	0.998	–
C16:n-7	4.84 ± 0.12	5.09 ± 0.45	5.03 ± 0.50	4.29 ± 0.54	4.09 ± 0.42	3.93 ± 0.26	0.247	Linear
C18:0	4.67 ± 0.06	4.28 ± 0.23	4.16 ± 0.27	4.62 ± 0.08	4.30 ± 0.16	4.43 ± 0.04	0.263	–
C18:n-9	16.2 ± 0.1	17.1 ± 1.4	18.3 ± 1.6	16.5 ± 1.4	19.5 ± 1.6	18.5 ± 0.8	0.430	–
C18:2n-6	14.6 ± 0.4	15.5 ± 1.3	16.3 ± 1.5	14.6 ± 0.9	16.8 ± 1.1	17.2 ± 0.6	0.386	–
C18:3n-3	1.80 ± 0.05	2.05 ± 0.22	2.21 ± 0.19	1.74 ± 0.20	2.39 ± 0.27	2.09 ± 0.11	0.208	–
C20:1	1.40 ± 0.06	1.52 ± 0.15	1.53 ± 0.15	1.61 ± 0.24	1.70 ± 0.20	1.49 ± 0.08	0.823	–
C20:2	0.74 ± 0.03	0.78 ± 0.06	0.93 ± 0.08	0.77 ± 0.05	0.88 ± 0.05	0.92 ± 0.03	0.100	Linear
C20:4n-6	1.47 ± 0.05^{a}	1.31 ± 0.05^{ab}	1.23 ± 0.08^{b}	1.52 ± 0.01^{a}	1.30 ± 0.04^{ab}	1.39 ± 0.03^{ab}	0.009	Cubic
C20:5n-3	9.52 ± 0.31	9.15 ± 0.57	8.79 ± 0.72	8.48 ± 0.62	7.72 ± 0.49	7.62 ± 0.29	0.130	Linear
C24:n-9	0.66 ± 0.06^{ab}	0.52 ± 0.02^{b}	0.55 ± 0.06^{ab}	0.70 ± 0.02^{a}	0.63 ± 0.01^{ab}	0.64 ± 0.01^{ab}	0.026	Cubic
C22:6n-3	22.2 ± 0.7^{ab}	21.2 ± 0.9^{ab}	19.4 ± 1.3^{b}	23.6 ± 0.9^{a}	19.9 ± 0.7^{ab}	20.5 ± 0.5^{ab}	0.041	–
∑SFA	26.5 ± 0.3	25.8 ± 1.6	25.7 ± 2.0	26.2 ± 1.1	25.1 ± 1.4	25.8 ± 0.7	0.984	–
∑MUFA	23.1 ± 0.3	24.2 ± 2.0	25.4 ± 2.3	23.1 ± 2.1	25.9 ± 2.0	24.6 ± 1.1	0.824	–
∑PUFA	50.4 ± 1.5	50.0 ± 3.1	48.8 ± 3.8	50.7 ± 2.6	49.0 ± 2.1	49.6 ± 1.3	0.992	–
∑n-3 PUFA	33.6 ± 1.1	32.4 ± 1.7	30.4 ± 2.2	33.8 ± 1.7	30.0 ± 1.2	30.2 ± 0.8	0.303	–
∑n-6 PUFA	16.1 ± 0.4	16.9 ± 1.3	17.5 ± 1.5	16.2 ± 0.9	18.1 ± 1.1	18.6 ± 0.6	0.486	–
∑n-3/∑n-6 PUFA	2.08 ± 0.02^{a}	1.93 ± 0.06^{ab}	1.74 ± 0.03^{bc}	2.10 ± 0.01^{a}	1.66 ± 0.07^{c}	1.63 ± 0.04^{c}	0.000	Linear
AI	0.44 ± 0.00^{a}	0.43 ± 0.00^{ab}	0.44 ± 0.00^{a}	0.42 ± 0.00^{ab}	0.40 ± 0.02^{b}	0.41 ± 0.00^{ab}	0.011	Linear
HH	3.02 ± 0.03	3.08 ± 0.02	3.07 ± 0.01	3.08 ± 0.04	3.25 ± 0.11	3.15 ± 0.00	0.089	Linear

SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; PUFA = polyunsaturated fatty acids; AI = atherogenicity index; HH = hypocholesterolemic to hypercholesterolemic fatty acids ratio.
[1] Values are expressed as mean ± standard error ($n = 3$). Different superscript letters within a row indicate significant differences ($P < 0.05$). FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively.
[2] Significance probability associated with the F-statistic.
[3] Based on orthogonal contrasts, details are listed in Table S1.

Table 6
The skin color of turbot fed experimental diets for 70 d.[1]

Item	FM	CAP15	CAP30	CAP45	CAP60	CAP75	ANOVA (Pr > F [2])	Trend[3]
L*	27.8 ± 0.8	26.8 ± 0.9	26.5 ± 0.3	28.7 ± 0.3	27.2 ± 0.2	26.6 ± 0.4	0.104	Cubic
a*	0.41 ± 0.02^{a}	0.39 ± 0.07^{a}	0.12 ± 0.02^{b}	0.45 ± 0.05^{a}	−0.63 ± 0.05^{c}	−0.58 ± 0.01^{c}	0.000	Linear
b*	5.24 ± 0.56	4.88 ± 0.09	4.34 ± 0.35	5.40 ± 0.15	4.90 ± 0.28	4.72 ± 0.13	0.253	–
ΔE	0.00	1.15	1.63	0.83	1.26	1.69		

L* = lightness; a* = redness; b* = yellowness; ΔE = total color difference calculated by L*a*b* system.
[1] Values are expressed as mean ± standard error ($n = 3$). Different superscript letters within a row indicate significant differences ($P < 0.05$). FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively.
[2] Significance probability associated with the F-statistic.
[3] Based on orthogonal contrasts, details are listed in Table S1.

3.3. Sensory quality

3.3.1. Skin color

Skin color gives consumers a preconceived notion of fish quality. As shown in Tables 6 and S1, the orthogonal contrasts showed a significant ($P < 0.05$) cubic relationship in the L* value ($R^2 = 0.2802$) and a significant ($P < 0.05$) negative linear relationship in the a* value ($R^2 = 0.6535$) with increased CAP level. There was no significant difference in the a* value in turbot fed FM, CAP15, and CAP45 diets ($P > 0.05$), but it was significantly lower in fish fed CAP30, CAP60, and CAP75 diets ($P < 0.05$). Dietary inclusion of CAP had no significant effect on the b* value ($P > 0.05$).

3.3.2. Texture and histological analysis of dorsal muscle

Dietary CAP significantly affected the texture of the dorsal muscle (Tables 7 and S1). The orthogonal contrasts showed a significant ($P < 0.05$) positive linear relationship in hardness ($R^2 = 0.3588$) and a significant ($P < 0.05$) negative linear relationship in adhesiveness ($R^2 = 0.7164$) with increased CAP level. Significant ($P < 0.05$) cubic relationships in chewiness ($R^2 = 0.6105$), springiness ($R^2 = 0.5103$), and cohesiveness ($R^2 = 0.4669$) were observed. Turbot fed CAP75 diets showed more significant hardness than FM-fed turbot ($P < 0.05$).

The present work also showed that dietary CAP induced significant changes in muscle histology (Table 8, Fig. 2, and Table S1). The orthogonal contrasts showed significant ($P < 0.05$) negative linear relationships in muscle fiber area ($R^2 = 0.4508$) and diameter ($R^2 = 0.4424$) with increased CAP level; however, these parameters in CAP15 and CAP30 groups were not significantly lower than the FM group ($P > 0.05$). For the muscle fiber frequency parameters, the orthogonal contrasts showed a significant ($P < 0.05$) positive linear relationship in the frequency of muscle fibers with a diameter under 60 μm ($R^2 = 0.5337$) and a significant ($P < 0.05$) negative linear relationship in those with diameter between 60 and 80 μm ($R^2 = 0.4882$) with increased CAP level. The frequency of muscle fibers under 60 μm was significantly higher in the CAP45, CAP60, and CAP75 groups than in the FM group ($P < 0.05$).

3.4. Expression of genes related to myogenesis and protein degradation

At the transcriptional level, dietary CAP affected the expression of genes related to myogenesis and protein degradation (Fig. 3 and

Table 7
Texture analysis of dorsal muscle of turbot fed experimental diets for 70 d.[1]

Item	FM	CAP15	CAP30	CAP45	CAP60	CAP75	ANOVA (Pr > F [2])	Trend[3]
Hardness, N	8.57 ± 0.24^{b}	9.58 ± 0.16^{ab}	9.40 ± 0.33^{ab}	9.21 ± 0.29^{ab}	9.40 ± 0.19^{ab}	10.13 ± 0.20^{a}	0.016	Linear
Chewiness, mJ	2.14 ± 0.23^{b}	3.08 ± 0.42^{ab}	3.30 ± 0.03^{a}	2.27 ± 0.13^{ab}	2.46 ± 0.19^{ab}	3.36 ± 0.27^{a}	0.011	Cubic
Springiness, mm	1.39 ± 0.10^{bc}	1.69 ± 0.09^{ab}	1.74 ± 0.04^{a}	1.31 ± 0.06^{c}	1.47 ± 0.06^{abc}	1.61 ± 0.07^{abc}	0.007	Cubic
Adhesiveness, N · mm	0.30 ± 0.00^{a}	0.25 ± 0.04^{ab}	0.18 ± 0.03^{bc}	0.18 ± 0.02^{bc}	0.11 ± 0.01^{c}	0.12 ± 0.01^{c}	0.001	Linear
Cohesiveness	0.18 ± 0.00	0.20 ± 0.01	0.20 ± 0.00	0.19 ± 0.00	0.18 ± 0.01	0.21 ± 0.01	0.084	Cubic

[1] Values are expressed as mean ± standard error ($n = 3$). Different superscript letters within a row indicate significant differences ($P < 0.05$). FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively.
[2] Significance probability associated with the F-statistic.
[3] Based on orthogonal contrasts, details are listed in Table S1.

Table 8
Histological analysis of dorsal muscle of turbot fed experimental diets for 70 d.[1]

Item	FM	CAP15	CAP30	CAP45	CAP60	CAP75	ANOVA (Pr > F [2])	Trend[3]
Muscle fiber area, μm^2	3648 ± 123^{a}	3317 ± 121^{ab}	3327 ± 100^{ab}	3065 ± 56^{b}	3180 ± 163^{ab}	3076 ± 100^{b}	0.033	Linear
Muscle fiber diameter, μm	67.6 ± 1.2^{a}	64.5 ± 1.3^{ab}	64.6 ± 1.0^{ab}	61.9 ± 0.6^{b}	63.1 ± 1.7^{ab}	61.9 ± 1.1^{b}	0.045	Linear
Muscle fiber frequency, %								
Diameter < 60 μm	20.0 ± 1.1^{c}	29.5 ± 10.6^{abc}	27.6 ± 5.1^{bc}	42.0 ± 4.0^{ab}	43.8 ± 6.9^{ab}	48.7 ± 4.8^{a}	0.043	Linear
60 μm ≤ diameter < 80 μm	77.2 ± 2.5	67.9 ± 11.3	67.6 ± 6.2	56.0 ± 4.8	53.0 ± 5.2	50.0 ± 6.2	0.085	Linear
Diameter > 80 μm	2.78 ± 2.08	2.65 ± 1.72	4.78 ± 2.89	1.96 ± 1.01	3.24 ± 1.69	1.39 ± 1.39	0.885	–

[1] Values are expressed as mean ± standard error ($n = 3$). Different superscript letters within a row indicate significant differences ($P < 0.05$). FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively.
[2] Significance probability associated with the F-statistic.
[3] Based on orthogonal contrasts, details are listed in Table S1.

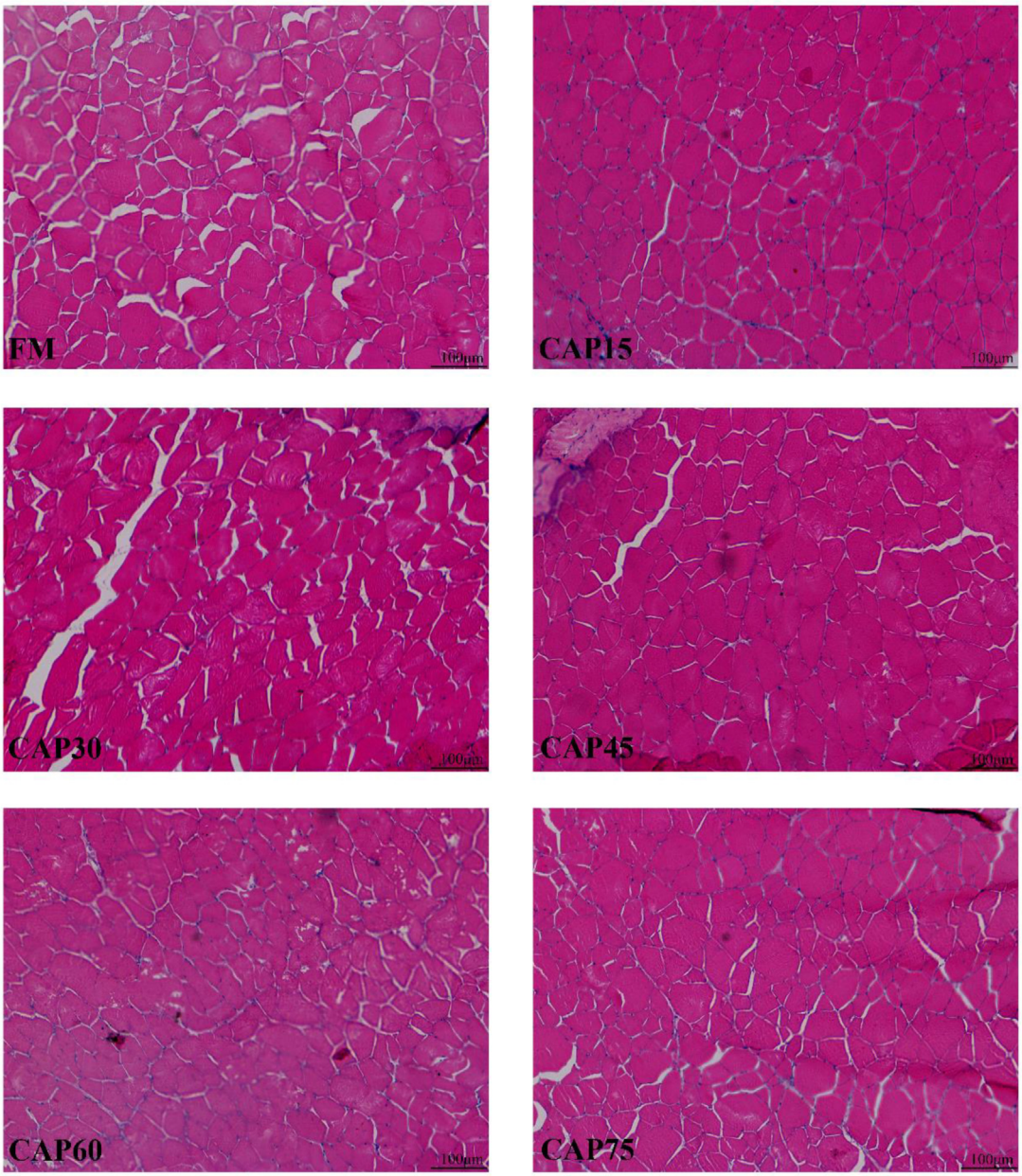

Fig. 2. Representative pictures of hematoxylin/eosin-stained histological sections of the dorsal muscle of turbot fed diets with different *Clostridium autoethanogenum* protein levels for 70 d. FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively. Bar represents 100 μm (magnification, 10×).

Table S1). For myogenesis-related genes, the orthogonal contrasts showed significant ($P < 0.05$) cubic relationships in the expression levels of myogenic regulatory factor 4 (*mrf 4*; $R^2 = 6995$) and myogenin (*myog*; $R^2 = 0.8363$) and significant ($P < 0.05$) negative linear relationships in the expression of myogenic factor 5 (*myf 5*; $R^2 = 0.1987$), myogenic differentiation (*myod*; $R^2 = 0.0916$), and paired box 7 (*pax 7*; $R^2 = 0.7665$) with increasing CAP level. The CAP significantly reduced the expression of *myod* at any level, and CAP at levels higher than 45% significantly reduced the expression of *pax 7* ($P < 0.05$). For protein degradation-related genes, the orthogonal contrasts showed significant ($P < 0.05$) negative linear relationships in the expression levels of cathepsin D (*ctsd*; $R^2 = 0.3732$) and muscle ring finger protein 1 (*murf 1*; $R^2 = 0.3831$) and significant ($P < 0.05$) quadratic relationships in the expression level of cathepsin L (*ctsl*; $R^2 = 0.4158$) with increased CAP level; *ctsl* and *murf 1* expression were significantly lower in all CAP-groups ($P < 0.05$), and dietary CAP at 45% and 60% levels significantly down-regulated the *ctsd* expression compared with FM group ($P < 0.05$).

3.5. Changes in the TOR, amino acid response (AAR), and AMPK pathways

At the protein level, dietary CAP significantly affected the pathways (Fig. 4 and Table S1). The orthogonal contrasts showed significant ($P < 0.05$) negative linear relationships in the

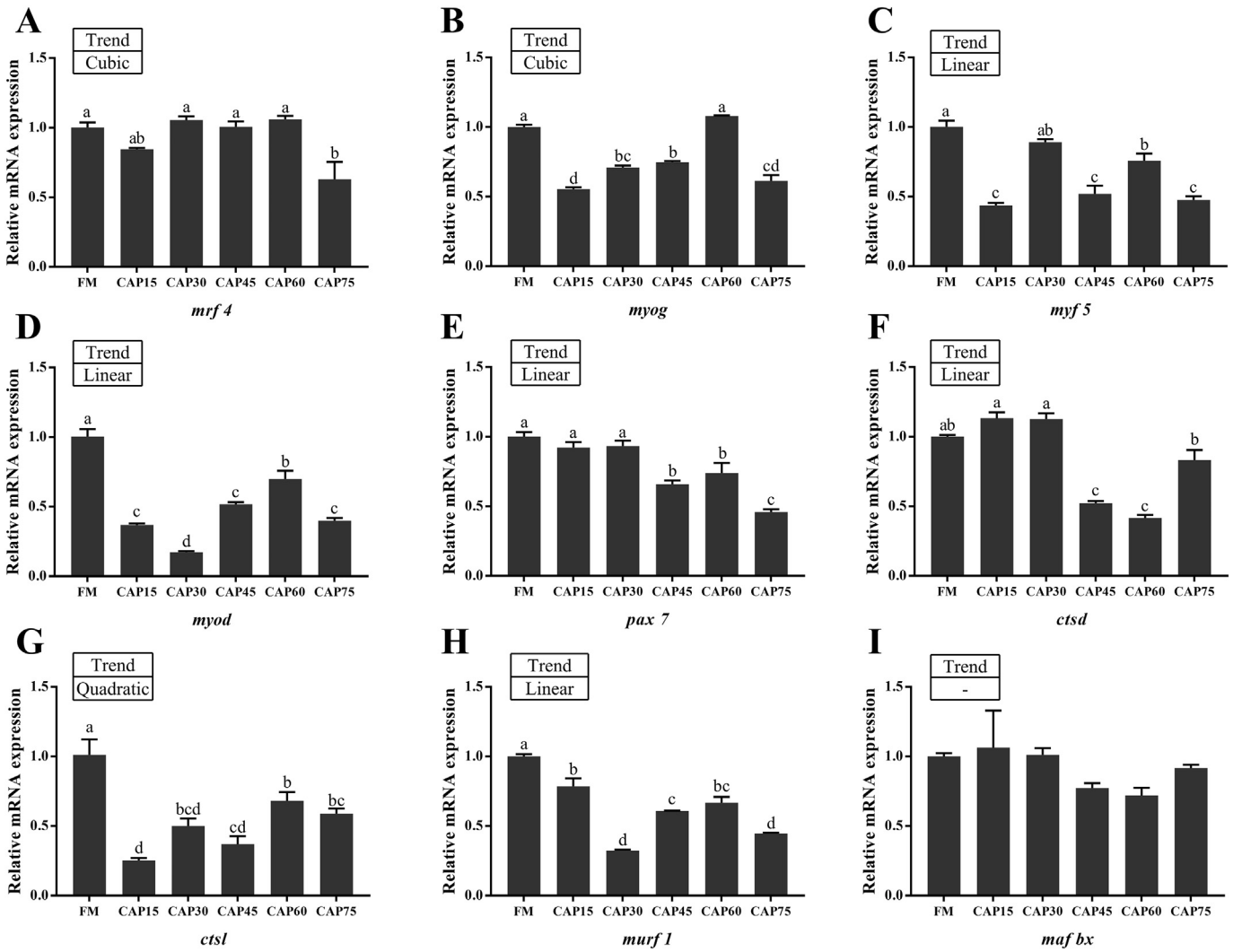

Fig. 3. Relative mRNA expression related to (A to E) myogenesis and (F to I) protein degradation in the muscle of turbot fed diets with different levels of *Clostridium autoethanogenum* protein for 70 d. Based on orthogonal polynomial contrasts, the expression of (A) *mrf 4*, (B) *myog* fit cubic model, (C) *myf 5*, (D) *myod*, (E) *pax 7*, (F) *ctsd*, and (H) *murf 1* fit linear model, and (G) *ctsl* fit quadratic model; details are listed in Table S1. FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively. *mrf 4* = myogenic regulatory factor 4; *myog* = myogenin; *myf 5* = myogenic factor 5; *myod* = *myogenic differentiation*; *pax 7* = paired box 7; *ctsd* = cathepsin D; *ctsl* = cathepsin L; *murf 1* = muscle ring finger protein 1; *maf bx* = muscle atrophy F box. Values are expressed as mean and standard error of the mean ($n = 3$). $^{a–d}$Different letters above the columns indicate significant differences ($P < 0.05$).

phosphorylation levels of TOR ($R^2 = 0.6169$), Akt ($R^2 = 0.8673$), 4E-BP1 ($R^2 = 0.6580$), and S6 ($R^2 = 0.8975$), and significant ($P < 0.05$) positive linear relationships in the phosphorylation levels of AMPK ($R^2 = 0.5843$), eIF2α ($R^2 = 0.5048$), and the total protein expression level of ATF4 ($R^2 = 0.8270$) with increased CAP level. For the TOR pathway, the phosphorylation level of TOR was significantly lower in the CAP60 and CAP75 groups, the phosphorylation level of Akt was significantly lower in all CAP groups, the phosphorylation level of 4E-BP1 was significantly lower in the CAP75 group, and the S6 phosphorylation level was significantly lower in the CAP30, CAP45, CAP60, and CAP75 groups than the FM group ($P < 0.05$). The phosphorylation level of AMPK was more significant in the CAP30, CAP45, CAP60, and CAP75 groups ($P < 0.05$). For the AAR pathway, CAP60 treatment significantly increased the eIF2α phosphorylation level, and the protein level of ATF4 was significantly higher in the CAP45, CAP60, and CAP75 groups ($P < 0.05$).

4. Discussion

4.1. Growth performance and FY

CAP could replace up to 30% of fishmeal in turbot diet without negatively impacting SR, WG, or CF. This finding was similar to a juvenile turbot study in which CAP replaced up to 45% of the dietary fishmeal without compromising growth performance (Zheng et al., 2022). As one of the primary traits affecting the economic benefits of fish (Sae-Lim et al., 2012), FY in the 15% CAP substitution level was similar to control. However, this parameter significantly decreased when the substitution level further increased. The other research team also observed a similar linear downward trend on juvenile turbot, but FY was not significantly affected until the dietary CAP level higher than 80% (Zheng et al., 2022). This discrepancy may be related to muscle growth patterns and dietary nutrient utilization between the juvenile and growing-out phases (Valente

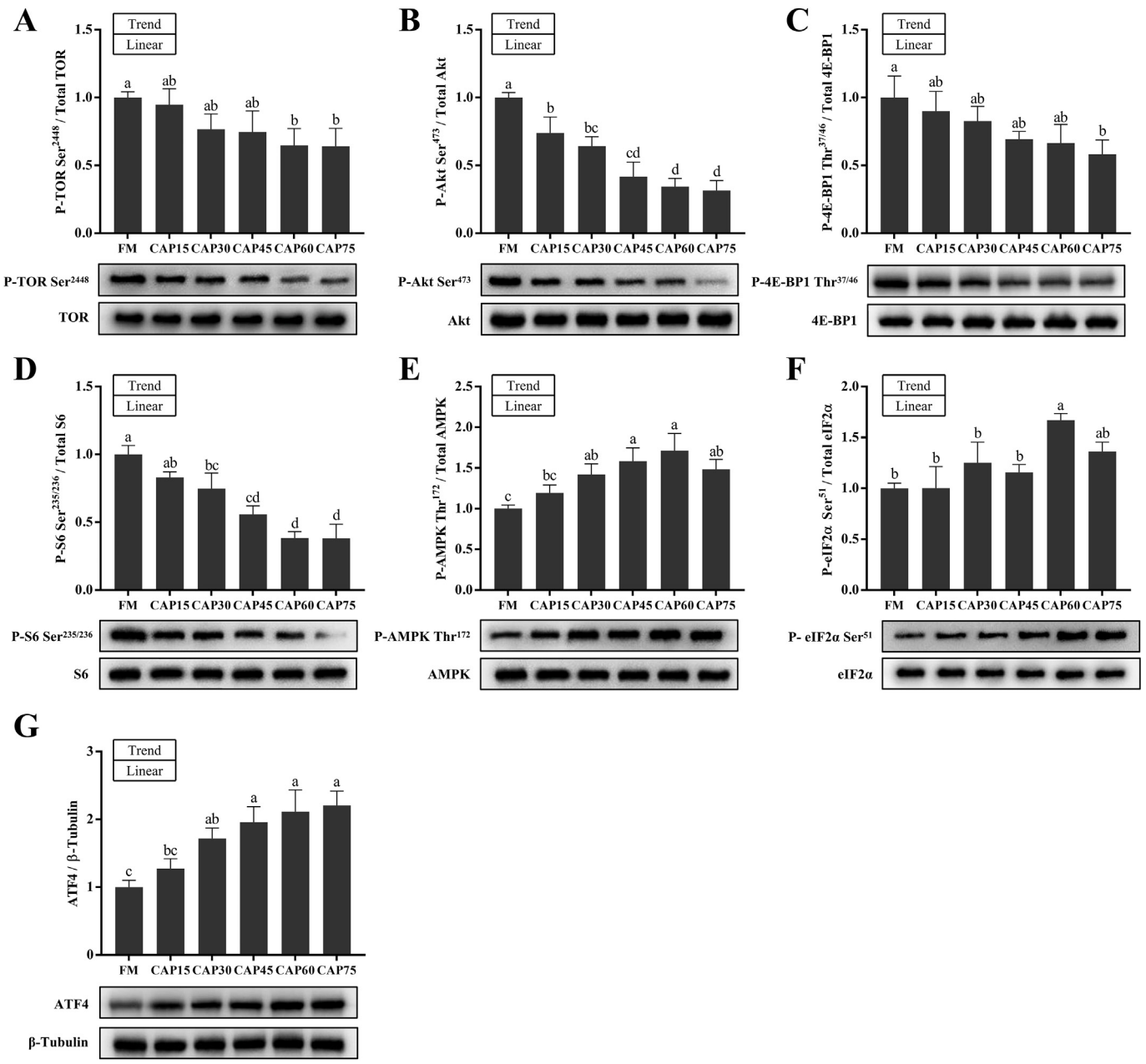

Fig. 4. The modulation of nutrition-sensing signaling pathways, including the (A to D) target of rapamycin, (E) AMP-activated protein kinase, and (F and G) amino acid response in the muscle of turbot fed diets with different levels of *Clostridium autoethanogenum* protein for 70 d. Based on orthogonal polynomial contrasts, the phosphorylation of (A) TOR, (B) Akt, (C) 4E-BP1, (D) S6, (E) AMPK, (F) eIF2α, and the expression of (G) ATF4 all fit the linear model; details are listed in Table S1. FM = basal diet with fishmeal as a primary protein source; CAP15, CAP30, CAP45, CAP60 and CAP75 = *Clostridium autoethanogenum* protein meal replaces fishmeal at 15%, 30%, 45%, 60%, and 75% in basal diet, respectively. TOR = target of rapamycin; Akt = protein kinase B; 4E-BP1 = eukaryotic initiation factor 4E-binding protein 1; S6 = ribosomal protein S6; AMPK = AMP-activated protein kinase; eIF2α = eukaryotic initiation factor 2α; ATF4 = activating transcription factor 4. Values are expressed as mean and standard error of the mean ($n = 3$). $^{a-d}$Different letters above the columns indicate significant differences ($P < 0.05$).

et al., 2013). The mechanism of FY reduction by dietary CAP in turbot is discussed below.

4.2. Nutritional quality

Muscle is the edible part of fish, and its nutrient composition and fatty acid profiles are indicators of fish quality (Landry et al., 2023). Fish collagen-based biomaterials have been used in wound healing, drug delivery, cell culture, and tissue engineering (Subhan et al., 2021). When humans ingest fish collagen, the hyp peptides stimulate the skin, joints, and bone cells, leading to collagen synthesis (Subhan et al., 2015). The present work demonstrated that turbot tolerated up to 30% of fish meal substitution by CAP without affecting muscle hyp and collagen contents; however, higher CAP levels significantly decreased hyp and collagen contents. Similar phenomena were observed in large yellow croakers (Wu et al., 2022) and largemouth bass (Yang et al., 2022b). The reason could be attributed to the deficiency of hyp in CAP protein (Ramshaw et al., 2019) and Zhang et al. (2013) proved that turbot muscle hyp and collagen contents positively correlated with dietary hyp content.

Aquatic products provide a healthier food source for humans, primarily because of their higher content of long-chain n-3 PUFA

(especially EPA and C22:6n-3 [DHA]) when compared to terrestrial meat products, and consumers prefer products with high levels of long-chain n-3 PUFA for health reasons (Oliver et al., 2020). The consumption of EPA and DHA reduces the risk of cardiovascular disease, cancer, and Alzheimer's disease (Tacon et al., 2020). An increased ∑n-3/n-6 PUFA ratio may enhance inflammatory processes (Liput et al., 2021). Although CAP linearly decreased EPA and the ∑n-3/n-6 PUFA ratio, turbot maintained the EPA level and ∑n-3/n-6 PUFA ratio when the substitution level of fishmeal by CAP was no more than 15%. Similar phenomenon was also observed on juvenile turbot (Zheng et al., 2022). Furthermore, the health value of fatty acids in turbot muscle was improved by CAP. The HH and AI are essential parameters for evaluating the healthiness of food fats for human consumption (Chen and Liu, 2020). The lower AI values represent the more significant protective potential against coronary artery disease; the HH reflects the influence of specific fatty acids on cholesterol metabolism, with high HH values being desirable for human health (Chen and Liu, 2020). In the present study, the AI of each group was within the recommended dietary value ranges (Ulbricht and Southgate, 1991) and CAP optimized both AI and HH. In a word, thirty percent or less CAP replacement had no adverse effect on turbot nutritional quality.

4.3. Muscle texture and muscle cellularity

Texture characteristics are essential parameters of fish quality; producers, processors, and consumers prefer firm textures (Cheng et al., 2014). From this view, CAP improved the muscle texture. Muscle cellularity is associated with texture characteristics (Listrat et al., 2016). The negative relationship between muscle fiber diameter and muscle firmness was reported for turbot (Wiriduge et al., 2020) and other fish (Hu et al., 2022; Periago et al., 2005; Zhang et al., 2022a,b). In the present work, dietary CAP made the muscle fibers smaller, and the improved muscle texture in the present work could be attributed partly to the decreased muscle fiber size.

We analyzed some myogenesis-related genes, including myogenic regulatory factors (*mrfs*) and *pax 7*, to explore how CAP reduces muscle fiber diameter. The *mrfs* regulate muscle cell differentiation and fusion, which are critical for the increase of muscle fiber diameter (Valente et al., 2013); *pax 7* is a marker for satellite cells, which are capable of proliferation and differentiation into muscle cells (Seale et al., 2000). Dietary CAP down-regulated *mrfs* (*myog*, *myod*, and *myf 5*) and *pax 7*, contributing to the smaller muscle fiber. Grass carp also reported similar results (Fan et al., 2022).

4.4. Nutritional sensing and metabolism pathways

The current investigation revealed that fishmeal substitution with CAP reduced FY, muscle protein content, and muscle fiber diameter. Protein deposition in muscle is the foundation for the increase of muscle biomass and muscle fiber diameter, and it is determined by nutrient-sensing and equilibrium between protein synthesis and degradation (Johnston et al., 2011). TOR and AAR are complementary signaling pathways for protein synthesis that regulate downstream metabolism (Goodman et al., 2011). The TOR pathway is activated by dietary amino acid availability to promote protein translation (Wullschleger et al., 2006) and induces *mrfs* expression to promote muscle cell differentiation (Miyabara et al., 2010; Sun et al., 2004; Zanou and Gailly, 2013). Previous works on grass carp and grouper found that CAP may employ TOR pathways to regulate muscle protein deposition (Fan et al., 2022; Huang et al., 2023). The present study confirmed that CAP suppressed the TOR pathway, evidenced by reduced Akt, TOR, 4E-BP1, and S6 phosphorylation. In contrast, the AAR pathway was activated due to an imbalance of amino acids in the diet, inhibiting protein synthesis (Düvel et al., 2010). The AMPK pathway inhibits the TOR pathway and metabolic processes such as protein synthesis to maintain energy homeostasis in energy and nutrient scarcity (Hardie et al., 2012). In the present study, increased phospho-eIF2α, phospho-AMPK, and ATF4 expression were observed with increased CAP levels, indicating stimulated AAR and AMPK pathways; this finding could explain why excess CAP inhibited muscle protein synthesis in turbot. Previous work on turbot demonstrated that fishmeal substitution led to TOR inhibition and the activation of the AAR and AMPK pathways, even though the dietary amino acid content met the requirements (Song et al., 2016; Xu et al., 2016). Thus, we hypothesized that the changes in the TOR, AAR, and AMPK pathways could be attributed to unbalanced amino acid profile of CAP, which is rich in lysine and isoleucine but deficient in arginine, histidine, and tryptophan (Wang et al., 2023). These amino acids modulate protein synthesis and quality traits in fish by regulating the TOR and AAR pathways (Habte-Tsion, 2020; Jiang et al., 2021; Miao et al., 2021; Wang et al., 2020; Wu et al., 2020).

The protein degradation pathway was also negatively correlated with fish muscle histology, texture, and protein deposition (Liu et al., 2020; Wang et al., 2018). The autophagy-lysosomal and ubiquitin-proteasome systems are significant pathways for protein degradation in fish muscle (Matias et al., 2020). The current investigation found that CAP down-regulated the expression of genes in these pathways, evidenced by down-regulated *ctsd*, *ctsl*, and *murf 1*. Similar results were reported in juvenile turbot fed diets with relatively higher levels of CAP (Zheng et al., 2022) and methanotroph bacteria meal (Zheng et al., 2023). These works and the present study indicated a homeostatic response of turbot to balance the protein synthesis and degradation to maintain amino acid balance in various nutritional states.

5. Conclusion

CAP can replace up to 15% of fishmeal without negatively impacting turbot quality. However, higher levels of CAP decreased the FY, muscle protein content, and muscle fiber diameter, and increased muscle hardness, which could be attributed to the inhibition of the TOR pathway and CAP's activation of the AAR and AMPK pathways.

Author contributions

Zezheng Qi: Investigation, Formal analyses, Visualization, Software, Writing - original draft. **Nan Bai**: Conceptualization, Funding acquisition, Project administration, Investigation, Writing - original draft. **Qing Li**: Investigation. **Shihui Pan**: Resources. **Min Gu**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing-review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.012.

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