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Effects of *Clostridium autoethanogenum* protein inclusion levels and processing parameters on the physical properties of low-starch extruded floating feed

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ABSTRACT

Clostridium autoethanogenum protein (CAP, RichMore®, China) is a safe and effective alternative protein source. However, the physicochemical properties of CAP and the effects of CAP inclusion on the physical properties of extruded fish feed are still unclear. The extrusion trials were performed to investigate the effects of graded CAP substitution levels (replacing 0%, 25%, 50%, 75% and 100% of fishmeal in the diet), preconditioning moisture content (28% and 32%) and screw speed (200 rpm and 300 rpm) on the physical qualities of low-starch extruded floating feed. The results showed that CAP had high crude protein content (832 g/kg), low crude lipid content (19.0 g/kg) and low ash content (35.0 g/kg). The water-holding capacity, foaming capacity and foaming stability of CAP were significantly higher than those of low-temperature steam dried fish meal (LTFM) ($P < 0.05$). The floatability of all extruded feeds reached 100%. As the replacement level of CAP increased, the peak viscosity of the unprocessed diets increased significantly ($P < 0.05$), whereas the pasting temperature decreased significantly ($P < 0.05$). Specific mechanical energy significantly increased with the increase in CAP substitution level ($P < 0.05$) and decreased with the increase in screw speed and moisture content ($P < 0.05$). Dietary CAP inclusion significantly reduced the bulk density, water solubility index, water solubility and oil leakage ($P < 0.05$), significantly increased the expansion ratio, hardness, water absorption index and oil absorption ($P < 0.05$), and improved the microstructure of the feed. Moisture content and screw speed also had significant effects on the physical qualities of the extruded feed, and the recommended process parameters for the test diets are 28% moisture content and 300 rpm screw speed. Overall, CAP is a promising protein substitute in aquaculture, which can be used to manufacture high-quality low-starch extruded floating feed.

1. Introduction

Aquaculture is the fastest-growing food production sector and plays an important role in providing protein to the global human population (Duarte et al., 2021). As the main component of fish feed, the widespread use of fish meal (FM) has led to its limited supply and inevitably higher costs (Foysal and Gupta, 2022). To maintain the sustainable development of aquaculture, there is an urgent need to find alternative protein sources for FM. Intensive research has been carried out on unconventional nutrient sources, among which single cell protein (SCP), extracted protein or biomass from pure or mixed cultures of microorganisms, is considered a promising alternative to animal- and plant-derived ingredients (Gamboa-Delgado, Márquez-Reyes, 2018). A series of studies reported that SCPs derived from yeast, microalgae and bacteria can improve the growth performance and health of aquatic animals (Abdel-Tawwab et al., 2021; Øverland et al., 2019; Alloul et al.,

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Abbreviations: CAP, *Clostridium autoethanogenum* protein; FM, fish meal; LTFM, low-temperature steam dried fish meal; OHC, oil holding capacity; PDI, protein dispersibility index; SCP, single cell protein; SEM, scanning electron microscopy; SME, specific mechanical energy; WAI, water absorption index; WHC, water holding capacity; WSI, water solubility index.

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Clostridium autoethanogenum is an acetogen that can use carbon monoxide as its carbon and energy source and has been used as a cell factory for industrial-scale gas fermentation (Valgepea et al., 2021). *Clostridium autoethanogenum* protein (CAP, RichMore®, China) is spray-dried biomass derived from the cultures of *C. autoethanogenum* (Chen et al., 2020). Several studies have indicated that CAP is an effective protein source for aquatic animals. Maulu et al. (2021) suggested that dietary CAP inclusion (50–200 g/kg) significantly improved the growth performance of tilapia (GIFT: *Oreochromis niloticus*) juveniles. CAP supplementation in the formula of juvenile Jian carp (*Cyprinus carpio* var. Jian) significantly improved the growth performance and antioxidant capacity of fish (Li et al., 2021). Technologies that enable carbon capture and conversion of carbon monoxide into protein sources can not only reduce the emission of harmful gases but also solve the shortage of feed protein (Fackler et al., 2021). Despite this, the physical quality of aquatic extruded feed is greatly affected by raw materials, which is often neglected when evaluating new feed ingredients (Sørensen, 2012).

The physical properties of extruded feed may affect the feed intake and nutrient digestibility, thus affecting the growth performance of fish (Weththasinghe et al., 2021). The main nutrients of fish feed formula are protein, starch, lipid and so on. Among these, protein not only contributes to the nutritional value of feed but also affects the physical qualities of feed. The physicochemical properties of proteins, such as solubility, emulsifying properties and viscosity enhancement are critical in the determination of their use in feed. The protein undergoes denaturation, degradation, association, and aggregation under the combined action of shear force, heat and pressure during the extrusion process (Chen et al., 2021). In addition, the interaction between molecules is accompanied by changes in molecular conformation in the extruder, thereby forming new conformations and structures (Zhang et al., 2021b). As a new protein source, the physicochemical properties of CAP and the effects of CAP inclusion on the physical properties of aquatic extruded feed are still unclear.

In practice, the production of low-starch (< 10%) extruded floating feed is a big challenge, because the starch content in the formula needs to be kept to a minimum to meet the nutritional needs of carnivorous fish while ensuring a 100% floatability of the feed (Yu et al., 2019). The key to producing low-starch extruded floating feed lies in the use of raw materials with good binding properties. Proteins, as the main components in the formula of carnivorous fish, may act as binders during extrusion process, which is related to their structure and processing history (Sun et al., 2021). To our knowledge, there is no research on the effects of protein sources on the physical properties of low-starch extruded floating feed. In the production of extruded feed, both process parameters and diet formulation have a great influence on the feed qualities (Tyapkova et al., 2016). The process parameters, e.g., moisture content, screw speed, die size, etc., can play their roles in different ways (Luo et al., 2020).

This study aimed to investigate the physicochemical properties of CAP and the effects of the dietary CAP inclusion level, preconditioning moisture content and screw speed on physical qualities of low-starch extruded floating feed.

2. Materials and methods

2.1. Experimental diets and extrusion process

Five formulas were designed, and the starch content of each formula was controlled at about 88.0 g/kg. A formula containing 400 g/kg low-temperature steam dried fish meal (LTFM) was chosen as the control diet (named CAP0). In the other 4 formulas, 25%, 50%, 75% and 100% of the LTFM was replaced with CAP, named as CAP25, CAP50, CAP75 and CAP100, respectively. All the formulas were isoenergetic and isonitrogenous. Due to the different nutrient compositions between FM and CAP, the formulas were balanced by adding microcrystalline cellulose. The formulations and nutrient compositions of the five diets were shown in Table 1. The well-mixed raw materials (200 kg for each diet) were ground by a hammer mill (JYNU30–15, Qingdao Jieyina Machinery Science & Technology Co., Ltd., Qingdao, China) and passed through a 0.180 mm sieve.

The extrusion trials were performed by using a pilot twin-screw extruder with a screw diameter of 56 mm and a length-to-diameter ratio (L/D) of 20:1 (SJPS56 × 2, Muyang, Jiangsu, China). The screw of the extruder was divided into 4 zones, and the temperature of each zone can be adjusted independently. The temperature profile used in this study was list in Table 2. The flow rate of the material was constant at 70 kg/h. The pre-conditioning was carried out for 60 s by adding steam (0.3 Mpa) and water into the pre-conditioner, where the temperature was greater than 95 °C. The moisture content of the material was adjusted by changing the water flow rate. The extrusion parameters were set following the experimental design (Table 2). Two three-millimetre dies were used in all trials. A six-blade knife installed in front of the die rotated at a speed of 900 rpm. The sample (5 kg) was collected after the processing parameters reached the set value and remained stable for 15 min. The torque (N·m), the flow rate of material (kg/h) and screw speed (rpm) were used to calculate the specific mechanical energy (SME; kJ/kg) (Riaz, 2000). All treatments were performed in triplicate. The extrudates were cooled and dried at room temperature (25 °C) for about 48 h to achieve a moisture content of 4–6% (wet basis). All the soybean oil in the formula was added to the extruded feeds by using a vacuum coater (ZJB-40, New Profit Food Machinery Co., Ltd, Weifang, China). The samples were stored at 4 °C until analysis.

2.2. Chemical analyses and physicochemical properties of the raw protein materials

Crude protein, crude lipid and ash were measured in duplicate according to AOAC (2006). The starch content of the feeds was measured

Table 1
Formulation and nutrient compositions of the experimental diets (g/kg).

Ingredients[a]	CAP0	CAP25	CAP50	CAP75	CAP100[b]
Fish meal	400	300	200	100	0
Clostridium autoethanogenum protein	0	90	180	270	345
Cottonseed protein concentrate	148	148	148	148	148
Soybean protein concentrate	148	148	148	148	148
Wheat gluten	30	30	30	30	30
Spray-dried blood cell powder	30	30	30	30	30
$Ca(H_2PO_4)_2$	10	10	18	18	18
Soybean oil	85	89	97	102	111
Cassava	120	120	120	120	120
Kelp meal	15	15	15	15	15
Microcrystalline cellulose	14	20	14	19	35
Total	1000	1000	1000	1000	1000
Nutrient compositions (g/kg, dry matter basis)					
Crude protein	530	536	533	534	524
Crude lipid	124	115	117	120	122
Starch content	88.2	88.3	88.1	88.4	88.0

[a] Fish meal: Triple Nine Fish Protein Co. Ltd (Denmark); Clostridium autoethanogenum protein (CAP): Beijing Shoulang Biotechnology Co. Ltd (China); Cottonseed protein concentrate: Xinjiang Jinlan Plant Protein Co. Ltd (China); Soybean protein concentrate: Yihai Kerry Investment Co. Ltd (China); Wheat gluten and soybean oil: Bohai Oil Co., Ltd (Qingdao, China); Spray-dried blood cell meal: Beijing Hongshun Source Biotech Co., Ltd; Ca(H2PO4)2: Yunnan Phosphate Group Co., Ltd (Yunnan, China); Cassava: Haid Group Co., Ltd (Guangdong, China); Kelp meal: Qingdao Hisea Imp.& Exp. Co., Ltd (Qingdao, China); Microcrystalline cellulose: Huzhou City Linghu Xinwang Chemical Co., Ltd.

[b] CAP0 was the control diet. In the other 4 diets, 25%, 50%, 75% and 100% of the fish meal was replaced with CAP, named as CAP25, CAP50, CAP75 and CAP100, respectively.

Table 2
Extrusion parameters used during the production of the experimental feeds.

Diets[a]	Moisture content, %	Screw speed, rpm	Extrusion zone 1, °C	Extrusion zone 2, °C	Extrusion zone 3, °C	Die temperature, °C
CAP0	28	200	98	130	132	145
	28	300	99	134	131	149
	32	200	99	126	130	145
	32	300	98	134	131	147
CAP25	28	200	96	132	134	142
	28	300	97	132	130	143
	32	200	98	128	133	145
	32	300	98	128	128	145
CAP50	28	200	97	128	133	144
	28	300	95	130	130	143
	32	200	98	129	135	144
	32	300	98	131	135	146
CAP75	28	200	99	120	134	147
	28	300	98	120	128	144
	32	200	98	121	133	145
	32	300	98	138	132	146
CAP100	28	200	99	125	127	142
	28	300	98	120	129	143
	32	200	98	124	132	140
	32	300	98	124	128	143

[a] CAP: Clostridium autoethanogenum protein. CAP0 was the control diet. In the other 4 diets, 25%, 50%, 75% and 100% of the fish meal was replaced with CAP, named as CAP25, CAP50, CAP75 and CAP100, respectively.

using the Megazyme Starch Assay Kit (Megazyme International, Wicklow, Ireland) following the manufacturer's instructions. The oil holding capacity (OHC) and water holding capacity (WHC) were determined by using the method described by Zhang et al. (2019). The flow figure and loose bulk density were determined following the method of Samuelsen et al. (2013). The protein dispersibility index (PDI) was measured using the method described by Rafiee-Yarandi et al. (2016). The foaming properties of protein materials were determined following the method of Lee et al. (2006) but with some modifications. The protein materials (1%, w/v) were dispersed in 30 mL of deionised water. The dispersion was whipped at 15,000 rpm for 2 min using a high-speed disperser (IKA® ULTRA-TURRAX® dispersers, IKA-Werke GmbH, Staufen, Germany). The volume (mL) of the foam was recorded at 0 min (V_0) and 30 min (V_1) to calculate foaming capacity (%, $V_0/30 *100$) and foam stability (%, $V_1/30 *100$). Emulsifying properties were measured according to the method of Lee et al. (2021).

2.3. Pasting properties of raw material

The pasting properties of the unprocessed diets were measured following the method used by Ma et al. (2021). Each treatment was tested three times.

2.4. Extruded feed quality measurement

The feed bulk density, expansion ratio, floatability, hardness, water solubility, water absorption index (WAI) and water solubility index (WSI) were measured following the method of Ma et al. (2021). The oil absorption capacity of the extruded feed was determined following the method of Liu et al. (2021) but with some modification. The samples (100 g) and excess soybean oil (250 mL) were put into a lab-scale vacuum coater (Rocker410, Rocan Scientific Co., Ltd, Xiamen, China), and the pressure was reduced to 0.9 bar. After vacuuming for 5 min to exhaust all air in the feed samples, the pressure was slowly released to atmospheric pressure. The samples were poured into a cylinder mould to drain out free oil and then used kitchen paper to wipe off the oil on the surface of the extrudates. The oil absorption (in relative %) was calculated by dividing the weight increase of extrudates (g) by the initial weight of the extrudates (100 g). Oil leakage (in relative %) was determined as the loss of oil from 100 g of extrudates at room temperature (25 °C) for 24 h and calculated by dividing the weight loss of feeds (g) by the initial weight of the extrudates (100 g). Scanning electron microscopy (SEM) was used for examining the microstructure of the cross-section and surface of uncoated extrudates from the five diets processed under 28% of moisture content and 300 rpm of screw speed. The extrudates were cut in half using a razor blade, then sputter-coated with gold (JEOL FineCoat Ion Sputter, JFC-1100, Jeol, Japan) and examined with a scanning electron microscope (JEOL JSM 840 SEM, Jeol, Japan). Micrographs were obtained at a magnification of × 15 for cross-section and × 300 for the surface of extrudates and an accelerating voltage of 3.0 kV. Unless otherwise stated, the above-mentioned test was performed in triplicate for each treatment.

2.5. Statistical analysis

The physicochemical properties of the two protein materials were compared with an independent t-test. When $P < 0.05$, it indicated that there was a significant difference. The data of pasting properties of the five unprocessed diets were analyzed by One-Way ANOVA, while the data of the physical properties of the feeds and SME were analyzed by a two-way ANOVA. Homogeneity of variance was confirmed by Levene's test before ANOVA. Tukey's multiple range test was performed to analyze the differences among the means. When $P < 0.05$, it demonstrated that there was a significant difference among the treatments. Pearson's correlation was used to assess relationships between the physical properties of the feeds. All the analysis was performed using the SPSS statistics 19 (SPSS Inc., USA).

3. Results

3.1. Nutritional components and physicochemical properties of protein material

The nutritional components and physicochemical properties of LTFM and CAP were shown in Table 3. Compared with LTFM, CAP had higher crude protein content, lower crude lipid content and lower ash content. The WHC, foaming capacity and foaming stability of CAP were significantly higher than those of LTFM ($P < 0.05$). The OAC, PDI, flow figure and emulsifying stability of CAP were significantly lower than those of LTFM ($P < 0.05$).

Table 3
Nutritional components and physicochemical properties of the protein materials.

Items	LTFM	CAP
Nutritional components, g/kg		
Crude protein	720	832
Crude lipid	88.4	19.0
Ash	150	35.0
Physicochemical properties (Means ± SEM)		
Water-holding capacity, g/g	2.79 ± 0.01^{a}	3.88 ± 0.01^{b}
Oil absorption capacity, g/g	0.78 ± 0.02^{b}	0.56 ± 0.03^{a}
Loose bulk density, kg/m^{3}	569 ± 4.98	596 ± 4.01
Protein dispersibility index, %	16.1 ± 0.47^{b}	6.04 ± 0.92^{a}
Flow figure, cm	6.80 ± 0.26^{b}	1.20 ± 0.06^{a}
Foaming capacity, %	16.7 ± 1.92^{a}	120 ± 3.85^{b}
Foaming stability, %	0.00 ± 0.00^{a}	38.9 ± 1.11^{b}
Emulsifying activity, m^{2}/g	0.86 ± 0.08	0.25 ± 0.02
Emulsifying stability, min	61.2 ± 3.33^{b}	18.8 ± 0.86^{a}

Within the same row, means with different superscripts are significantly different (t-test;
$P < 0.05$).
CAP: *Clostridium autoethanogenum* protein; LTFM: Low-temperature steam dried fish meal; SEM: Standard error of treatment means.

3.2. Pasting properties of raw material

The pasting properties of the five unprocessed diets were shown in Table 4. Dietary CAP inclusion significantly increased the peak viscosity ($P < 0.05$) and significantly reduced the setback viscosity and pasting temperature ($P < 0.05$). The trough viscosity of the CAP25 group was significantly lower than that of the CAP0 group ($P < 0.05$). As the replacement level of CAP increased from 50% to 100%, the trough viscosity of the raw material increased significantly ($P < 0.05$). As the replacement amount of CAP increased from 0% to 75%, the breakdown viscosity of the raw material increased significantly ($P < 0.05$), and when the replacement amount reached 100%, the breakdown viscosity decreased significantly ($P < 0.05$). The substitution amount of CAP in the formulas had no significant effect ($P > 0.05$) on the final viscosity of the raw material.

3.3. Specific mechanical energy

CAP substitution level, moisture content, screw speed and their interaction results (P values) on SME were presented in Table 5. SME varies between 10.6 and 12.1 kJ/kg. SME was significantly affected by the CAP substitution level, moisture content, screw speed and their interaction terms ($P < 0.001$). SME significantly increased with the increase in CAP substitution level ($P < 0.05$). The screw speed and moisture content had a negative influence on SME ($P < 0.05$). The highest SME (12.1 kJ/kg) appeared in the CAP100 group with low screw speed and low moisture content.

3.4. Physical qualities of the feeds

The physical qualities of the feeds were shown in Table 5. The bulk density and expansion ratio of the extruded feeds were significantly affected by the CAP substitution level, moisture content, screw speed and their interaction terms ($P < 0.001$). As the substitution level of CAP increased from 0% to 75%, the bulk density showed a downward trend ($P < 0.05$), and when the substitution level reached 100%, the bulk density increased ($P < 0.05$). The bulk density decreased as the screw speed and moisture content increased ($P < 0.05$). With the increase of CAP substitution level, the expansion ratio showed an opposite trend to bulk density. The expansion ratio of feeds significantly decreased with the increase of moisture content ($P < 0.05$). As the screw speed increased, the expansion ratio increased significantly ($P < 0.05$). The floatability of all feeds reached 100%, which met the requirement of extrusion floating feed.

The CAP substitution level ($P < 0.001$), screw speed ($P < 0.001$), moisture content ($P < 0.001$) and their interaction terms ($P < 0.05$) had significant effects on the hardness. The hardness increased with the increase in CAP substitution level from 0% to 75% and increased with an increase in screw speed ($P < 0.05$). Low moisture content led to higher hardness (averaging 64.2 N) compared to high moisture content (averaging 51.6 N). The three-way interaction among CAP substitution levels, moisture content and screw speed for hardness revealed that 75% CAP substitution level, high screw speed and low moisture content led to the highest hardness (90.8 N).

WAI, WSI and water solubility were significantly affected by CAP substitution level, moisture content, screw speed and their interaction terms ($P < 0.001$). With the increase in the CAP substitution level and screw speed, WAI increased significantly ($P < 0.05$). Dietary CAP inclusion reduced the WSI significantly ($P < 0.05$). WSI decreased with an increase in screw speed ($P < 0.05$). WAI decreased as the moisture content increased ($P < 0.05$), while WSI increased with the increase in moisture content ($P < 0.05$). The increase in CAP substitution level ($P < 0.05$) and moisture content ($P < 0.05$) significantly reduced the water solubility of feed, while the increase in screw speed significantly increased the water solubility ($P < 0.05$).

Except for moisture content, the replacement amount of CAP, screw speed and their interaction terms had significant effects on the oil absorption ($P < 0.001$). As the replacement amount of CAP and screw speed increased, the oil absorption of feed increased significantly ($P < 0.05$). The CAP substitution level ($P < 0.001$), moisture content ($P < 0.001$), screw speed ($P = 0.001$) and their interaction terms ($P < 0.001$) had significant effects on the oil leakage. As the replacement amount of CAP increased from 0% to 75%, the oil leakage decreased significantly ($P < 0.05$), and as the replacement amount reached 100%, the oil leakage of feed increased ($P < 0.05$). The oil leakage decreased with the increase in moisture content ($P < 0.05$) and decreased with the decrease in screw speed ($P < 0.05$).

The SEM photographs (Fig. 1) showed that the numbers of homogenous pores in the extruded feeds increased as the CAP substitution level increased from 0% to 75%. When the replacement amount of CAP was

Table 4
Pasting properties of the five unprocessed diets (means ± SEM).

Diets[1]	Peak viscosity, cp	Trough viscosity, cp	Breakdown viscosity, cp	Final viscosity, cp	Setback viscosity, cp	Pasting temperature, °C
CAP0	431 ± 2.08^{a}	325 ± 1.20^{b}	106 ± 1.86^{a}	521 ± 19.7	197 ± 20.8^{c}	94.8 ± 0.11^{d}
CAP25	425 ± 5.93^{a}	308 ± 3.53^{a}	118 ± 2.40^{a}	503 ± 8.14	195 ± 10.2^{c}	82.1 ± 0.54^{c}
CAP50	509 ± 0.67^{b}	337 ± 1.53^{b}	172 ± 1.45^{b}	500 ± 1.15	163 ± 1.00^{bc}	78.5 ± 0.26^{b}
CAP75	600 ± 11.8^{c}	370 ± 5.69^{c}	230 ± 6.94^{c}	503 ± 10.0	133 ± 4.37^{ab}	76.1 ± 0.27^{a}
CAP100	575 ± 13.4^{c}	403 ± 8.67^{d}	172 ± 5.17^{b}	525 ± 12.2	122 ± 3.53^{a}	76.9 ± 0.26^{a}

In the same column, values with no letter or the same letter superscripts mean no significant difference (Turkey test; $P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).
SEM: Standard error of treatment means.
[1] CAP: *Clostridium autoethanogenum* protein. CAP0 was the control diet. In the other 4 diets, 25%, 50%, 75% and 100% of the fish meal was replaced with CAP, named as CAP25, CAP50, CAP75 and CAP100, respectively.

Table 5

Effects of the increasing dietary inclusion of *Clostridium autoethanogenum* protein (CAP), moisture content and screw speed on extrudate physical properties and specific mechanical energy (means ± SEM).

Main effect			The physical properties of the extruded feed and specific mechanical energy								
CAP substitution levels, %	Moisture content, %	Screw speed, rpm	Bulk density, g/L	Expansion ratio	Hardness, N	WAI	WSI, %	Water solubility, %	Oil absorption, %	Oil leakage, %	SME, kJ/kg
0	28	200	386 ± 0.33^{jk}	1.70 ± 0.01^{cd}	39.9 ± 0.88^{a}	2.51 ± 0.05^{a}	18.6 ± 0.26^{m}	4.71 ± 0.01^{jk}	52.5 ± 0.14^{a}	4.97 ± 0.01^{hi}	10.7 ± 0.01^{bc}
	28	300	392 ± 0.58^{l}	1.76 ± 0.01^{fg}	50.6 ± 1.53^{cd}	3.06 ± 0.06^{efg}	11.3 ± 0.29^{e}	4.85 ± 0.06^{jk}	62.2 ± 0.70^{bc}	7.24 ± 0.09^{j}	10.8 ± 0.02^{cd}
	32	200	358 ± 0.58^{f}	1.67 ± 0.01^{c}	39.2 ± 0.81^{a}	2.60 ± 0.06^{abc}	16.7 ± 0.21^{l}	5.09 ± 0.16^{kl}	69.0 ± 0.94^{cde}	10.5 ± 0.12^{k}	10.8 ± 0.01^{bc}
	32	300	382 ± 0.33^{i}	1.54 ± 0.01^{a}	36.6 ± 1.09^{a}	2.54 ± 0.01^{ab}	19.3 ± 0.16^{m}	5.61 ± 0.01^{l}	55.4 ± 1.86^{ab}	4.59 ± 0.10^{ghi}	10.7 ± 0.01^{b}
25	28	200	393 ± 0.33^{l}	1.75 ± 0.01^{ef}	48.6 ± 0.64^{bc}	2.98 ± 0.12^{def}	9.89 ± 0.08^{c}	3.69 ± 0.02^{gh}	69.1 ± 2.11^{cde}	3.53 ± 0.04^{efg}	11.1 ± 0.01^{f}
	28	300	382 ± 0.67^{i}	1.81 ± 0.01^{gh}	64.2 ± 1.68^{fgh}	3.31 ± 0.13^{gh}	10.5 ± 0.13^{cd}	3.52 ± 0.03^{fgh}	62.2 ± 1.58^{bc}	3.02 ± 0.36^{bcdef}	10.8 ± 0.01^{bc}
	32	200	386 ± 0.01^{jk}	1.62 ± 0.01^{b}	48.9 ± 0.91^{bc}	2.61 ± 0.01^{abc}	14.2 ± 0.07^{h}	4.38 ± 0.09^{ij}	69.6 ± 0.13^{de}	2.13 ± 0.05^{abcd}	10.8 ± 0.01^{bc}
	32	300	360 ± 0.67^{fg}	1.67 ± 0.01^{bc}	42.0 ± 1.66^{ab}	2.74 ± 0.01^{abcd}	15.9 ± 0.03^{jk}	2.75 ± 0.08^{cde}	52.1 ± 0.08^{a}	4.68 ± 0.05^{ghi}	10.6 ± 0.01^{a}
50	28	200	385 ± 0.88^{ij}	1.76 ± 0.01^{f}	59.0 ± 1.34^{efg}	2.80 ± 0.01^{bcde}	16.4 ± 0.05^{kl}	3.02 ± 0.14^{def}	64.4 ± 0.68^{cde}	1.07 ± 0.03^{a}	11.4 ± 0.01^{g}
	28	300	361 ± 0.67^{g}	1.77 ± 0.01^{fg}	75.7 ± 2.23^{ij}	3.59 ± 0.11^{ij}	10.7 ± 0.27^{de}	4.48 ± 0.07^{ij}	70.1 ± 2.01^{def}	5.66 ± 0.66^{i}	10.9 ± 0.01^{e}
	32	200	376 ± 0.67^{h}	1.73 ± 0.01^{def}	57.7 ± 1.69^{def}	2.72 ± 0.01^{abcd}	16.8 ± 0.09^{l}	3.28 ± 0.09^{efg}	70.0 ± 1.14^{def}	4.17 ± 0.04^{fgh}	11.1 ± 0.01^{f}
	32	300	373 ± 0.01^{h}	1.67 ± 0.01^{bc}	51.3 ± 0.99^{cd}	2.61 ± 0.02^{abc}	14.8 ± 0.01^{hi}	3.65 ± 0.15^{gh}	67.2 ± 0.76^{cde}	2.03 ± 0.03^{abc}	10.9 ± 0.01^{de}
75	28	200	362 ± 0.67^{g}	1.84 ± 0.01^{hi}	66.5 ± 1.64^{h}	2.81 ± 0.01^{bcde}	15.4 ± 0.06^{ij}	2.25 ± 0.07^{bc}	55.1 ± 0.43^{a}	2.34 ± 0.59^{abcde}	11.7 ± 0.02^{j}
	28	300	325 ± 1.15^{c}	1.93 ± 0.01^{j}	90.8 ± 1.88^{k}	3.60 ± 0.02^{ij}	10.3 ± 0.18^{cd}	3.95 ± 0.21^{hi}	78.3 ± 0.32^{gh}	3.47 ± 0.11^{defg}	11.5 ± 0.02^{h}
	32	200	353 ± 0.58^{e}	1.78 ± 0.01^{fg}	65.3 ± 1.47^{gh}	2.80 ± 0.01^{bcde}	13.3 ± 0.05^{g}	1.40 ± 0.06^{a}	63.6 ± 1.54^{cd}	2.61 ± 0.18^{bcde}	11.5 ± 0.01^{h}
	32	300	312 ± 0.33^{a}	1.88 ± 0.01^{i}	54.3 ± 1.61^{cde}	2.84 ± 0.01^{cde}	14.3 ± 0.14^{h}	1.85 ± 0.10^{ab}	76.5 ± 1.58^{fg}	1.77 ± 0.23^{ab}	11.1 ± 0.01^{f}
100	28	200	389 ± 0.67^{k}	1.76 ± 0.01^{fg}	68.9 ± 1.95^{hi}	3.22 ± 0.02^{fgh}	10.1 ± 0.01^{cd}	1.49 ± 0.17^{a}	71.3 ± 2.32^{ef}	3.24 ± 0.14^{cdef}	12.1 ± 0.02^{l}
	28	300	320 ± 0.33^{b}	1.94 ± 0.01^{j}	77.8 ± 1.82^{j}	2.86 ± 0.02^{cde}	12.2 ± 0.12^{f}	2.50 ± 0.10^{cd}	84.0 ± 0.98^{h}	8.41 ± 0.29^{j}	11.5 ± 0.01^{i}
	32	200	386 ± 0.33^{jk}	1.70 ± 0.01^{cde}	65.4 ± 1.35^{gh}	3.79 ± 0.01^{j}	7.33 ± 0.06^{a}	1.83 ± 0.04^{ab}	62.4 ± 1.86^{bc}	4.25 ± 0.28^{fgh}	11.8 ± 0.01^{k}
	32	300	336 ± 0.33^{d}	1.86 ± 0.01^{hi}	55.5 ± 1.35^{cde}	3.44 ± 0.02^{hi}	9.10 ± 0.05^{b}	1.89 ± 0.04^{ab}	76.5 ± 0.31^{fg}	1.83 ± 0.16^{ab}	11.5 ± 0.02^{hi}
Mean CAP substitution levels											
0			380^{d}	1.67^{a}	41.6^{a}	2.68^{a}	16.5^{e}	5.07^{d}	59.8^{a}	6.82^{d}	10.7^{a}
25			381^{e}	1.71^{b}	50.9^{b}	2.91^{b}	12.6^{b}	3.58^{c}	63.2^{ab}	3.34^{b}	10.8^{b}
50			374^{c}	1.73^{c}	60.9^{c}	2.93^{b}	14.7^{d}	3.61^{c}	67.9^{bc}	3.23^{b}	11.0^{c}
75			338^{a}	1.86^{e}	69.2^{e}	3.01^{c}	13.3^{c}	2.36^{b}	68.4^{bc}	2.55^{a}	11.4^{d}
100			358^{b}	1.82^{d}	66.9^{d}	3.33^{d}	9.68^{a}	1.93^{a}	73.5^{c}	4.43^{c}	11.7^{e}
Mean moisture content											
28			370^{b}	1.80^{b}	64.2^{b}	3.07^{b}	12.5^{a}	3.45^{b}	66.9	4.29^{b}	11.2^{b}
32			362^{a}	1.71^{a}	51.6^{a}	2.87^{a}	14.2^{b}	3.17^{a}	66.2	3.85^{a}	11.1^{a}
Mean screw speed											
200			377^{b}	1.73^{a}	55.9^{a}	2.88^{a}	13.9^{b}	3.11^{a}	64.7^{a}	3.88^{a}	11.3^{b}
300			354^{a}	1.78^{b}	59.9^{b}	3.06^{b}	12.8^{a}	3.50^{b}	68.5^{b}	4.27^{b}	11.0^{a}
Statistical analysis											
CAP			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
MC			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.237	< 0.001	< 0.001
SS			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001	< 0.001
CAP × MC			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
CAP × SS			< 0.001	< 0.001	0.012	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
MC × SS			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
CAP × MC × SS			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

In the same column, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference (Turkey test; $P < 0.05$).

MC: Moisture content; SS: Screw speed; WAI: water absorption index; WSI: water solubility index; SEM: Standard error of treatment means; SME: specific mechanical energy.

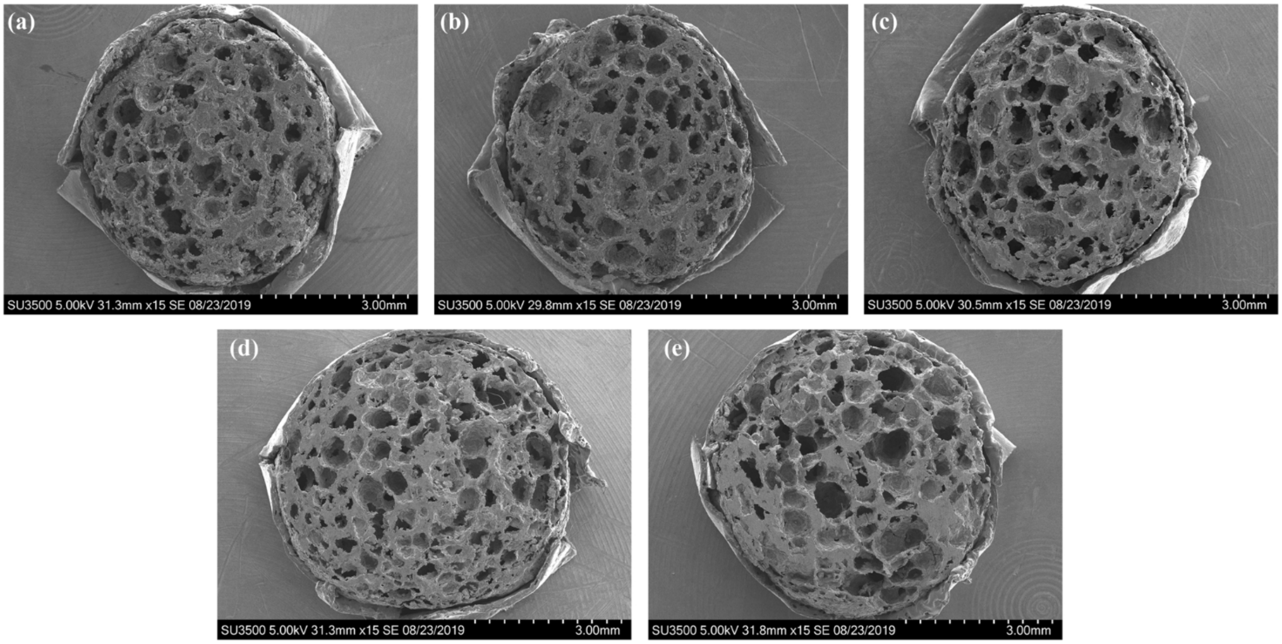

Fig. 1. Cross-section of extruded pellets with different *Clostridium autoethanogenum* protein (CAP) substitution levels (0%, 25%, 50%, 75% and 100%) processed under 28% of moisture content and 300 rpm of screw speed: (a) CAP0, (b) CAP25, (c) CAP50, (d) CAP75 and (e) CAP100.

100%, there were many large pores in the cross-section of the extrudates. Besides, the surface of the extrudates became smoother (Fig. 2) when the CAP replacement levels increased from 0% to 100%.

3.5. Correlation among the physical qualities of the feeds

A correlation analysis (Table 6) showed that bulk density was negatively correlated with expansion ratio ($r = -0.68$, $P < 0.01$) and oil absorption ($r = -0.59$, $P < 0.01$). Expansion ratio was positively correlated with hardness ($r = 0.74$, $P < 0.01$), WAI ($r = 0.46$, $P < 0.05$) and oil absorption ($r = 0.61$, $P < 0.01$), and negatively correlated with WSI ($r = -0.49$, $P < 0.05$) and water solubility ($r = -0.50$, $P < 0.05$). Hardness was positively correlated with WAI ($r = 0.67$, $P < 0.01$) and oil absorption ($r = 0.54$, $P < 0.05$), and negatively correlated with WSI ($r = -0.59$, $P < 0.01$). WAI was negatively correlated with WSI ($r = -0.87$, $P < 0.01$). WSI was negatively correlated with oil absorption ($r = -0.46$, $P < 0.05$). Water solubility was positively correlated with oil leakage ($r = 0.45$, $P < 0.05$).

4. Discussion

The physicochemical properties of protein sources could determine their application and acceptability in feed systems (Lu et al., 2020). WHC and OAC respectively refer to the amount of water and oil that the

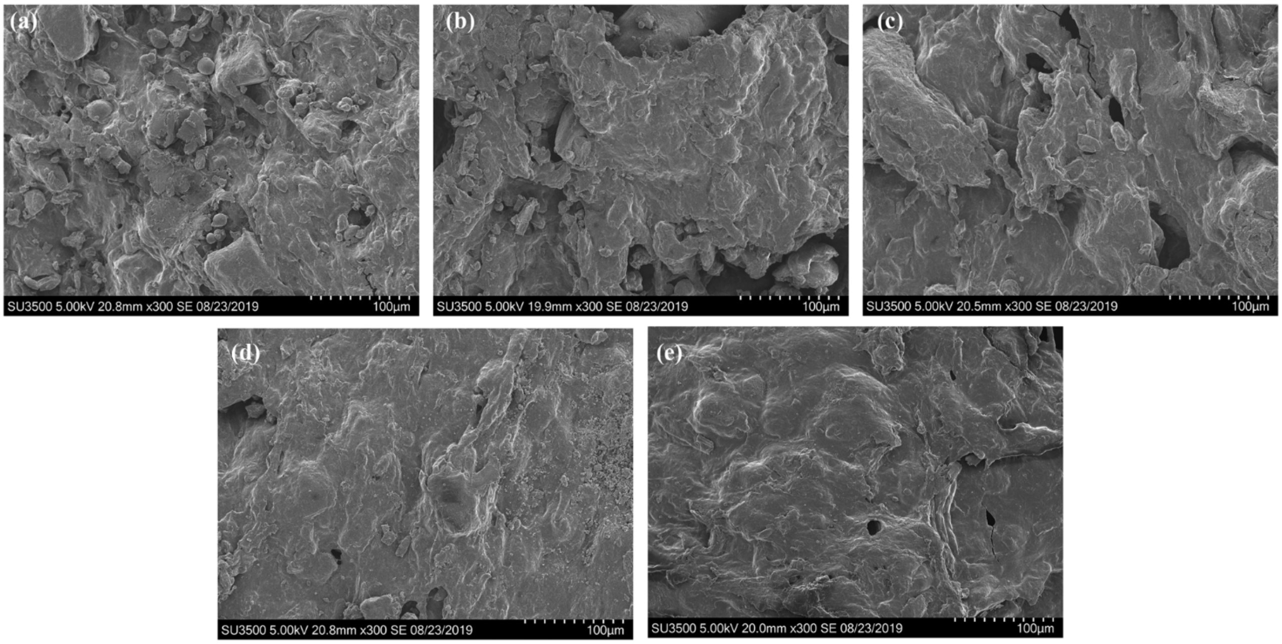

Fig. 2. Surface morphology of extruded pellets with different *Clostridium autoethanogenum* protein (CAP) substitution levels (0%, 25%, 50%, 75% and 100%) processed under 28% of moisture content and 300 rpm of screw speed: (a) CAP0, (b) CAP25, (c) CAP50, (d) CAP75 and (e) CAP100.

Table 6
Pearson's correlation coefficients (r) for the relationships among feed quality parameters.

Variables	Bulk density	Expansion ratio	Hardness	WAI	WSI	Water solubility	Oil absorption	Oil leakage
Bulk density	1							
Expansion ratio	-0.68 * *	1						
Hardness	-0.42	0.74 * *	1					
WAI	-0.13	0.46 *	0.67 * *	1				
WSI	0.09	-0.49 *	-0.59 * *	-0.87 * *	1			
Water solubility	0.34	-0.50 *	-0.42	-0.30	0.41	1		
Oil absorption	-0.59 * *	0.61 * *	0.54 *	0.33	-0.46 *	-0.24	1	
Oil leakage	-0.06	-0.08	-0.12	-0.08	0.09	0.45 *	0.07	1

* $P < 0.05$, * * $P < 0.01$.
WAI: water absorption index; WSI: water solubility index.

sample can absorb (Lee et al., 2021). Protein materials with high WHC could compete with other biopolymers to absorb water during the extrusion process. In the preliminary experiments, we found that the production of feeds containing high content of CAP ($\geq$ 270 g/kg) at a low moisture content (24%) could cause extruder blockage, which could be due to that CAP absorbing more water and other biopolymers were cured and burnt under the condition of low moisture content. Therefore, the moisture content set in the present study (28% and 32%) was higher than normal levels (22–26%), which could inevitably cause an increase in drying energy consumption. Zielińska et al. (2018) reported that more hydrophobic protein showed better oil absorption capacity, which meant that the side chains of non-polar amino acids were combined with the paraffin chains of fats. Compared with CAP, LTFM had higher OAC and lower WHC, implying that LTFM contained more available non-polar side chains on its protein molecules. PDI varies inversely with nutritional quality and with the severity of heat treatment (Offiah et al., 2019). Shen et al. (2021) also suggested that higher PDI showed lower degeneration. The lower PDI of CAP could be attributed to greater denaturation during the process of producing and thus aggregation of protein molecules (Chen et al., 2020). The flow figure reflects the particle size of the protein materials (Samuelsen et al., 2013). In this study, the lower flow figure of CAP was due to its smaller particle size and lower content of lipid. Foams are formed when the protein unfolds to create an interfacial skin at the air-water interface, which reduces the surface tension and keeps the bubbles in suspension, thereby preventing them from collapsing (Lee et al., 2021). In the present study, CAP showed higher foaming capacity and foaming stability, which could be related to its low lipid content. Consistent with the present study, Bratosin et al. (2021) reported that SCP was the main surfactant, which helped to form and stabilize the dispersed gas phase during the foam preparation. Some proteins could be used as emulsifiers to reduce the interfacial tension to form emulsions due to their amphiphilic molecular structure (Wang et al., 2019). Deng et al. (2019) documented that high solubility of protein was essential for good emulsifying ability. The poor emulsion properties of the two test protein materials may be due to their low protein solubility.

The RVA results gave the information on the change in the viscosity of the mixture with temperature. The increase in peak viscosity indicates that the ability of starch to swell and/or gelatinize is enhanced (Zhang et al., 2021a). In the present study, the peak viscosity varied from 425 cP to 600 cP, which was the most sensitive to the CAP substitution level. The results indicated that when the CAP substitution level is $\geq$ 50%, CAP could increase the water binding and swelling of starch. The breakdown viscosity is the difference between the peak viscosity and the trough viscosity. High breakdown viscosity indicates a low ability to withstand shear stress and heating during cooking (Wang et al., 2021b). The increased breakdown viscosity with the increase in CAP substitution level meant that CAP weakened the resistance of swollen starch granules to shearing. Low setback viscosity indicates higher resistance to retrogradation (Zhang et al., 2021a). During the cooling and low-temperature holding stage, the CAP inclusion reduced the setback viscosity of the mixture, indicating that the interaction of protein and starch inhibited the realignment of molecular chains (Wang et al., 2021b). Besides, CAP inclusion was beneficial to starch gelatinization by reducing the pasting temperature.

SME is a quantitative description of mechanical energy in the extrusion process, which determines the degree of macromolecular transformation and the rheological properties of the melt (Alves Cayres et al., 2021). In this study, the SME decreased with the increase in screw speed and moisture content, which were caused by the shear-thinning behaviour of melt and the plasticization of water (Baidoo et al., 2019). The SME increased with the increase in CAP substitution level, suggesting that the CAP could increase the viscosity of the melt and flow resistance during the extrusion process, which was consistent with the partial results of the pasting properties of the unprocessed diets. Kristiawan et al. (2017) documented that in the same extrusion system, a formulation with a higher viscosity corresponded to a higher SME. The positive effect of CAP on the SME could be due to its higher WHC and lower lipid content compared with LTFM (Draganovic et al., 2011; Espinosa-Ramírez et al., 2021). Besides, Espinosa-Ramírez et al. (2021) reported that the smaller particles in the feed mixture could increase the interactions both between particles and with the barrel, thereby increasing the shear stress and SME. Thus, the smaller particle size of CAP could be contributed to the increased SME.

Bulk density, expansion ratio and floatability are the critical and easily available indicators for evaluating the quality of extruded floating feed. In the present study, high screw speed led to a higher expansion ratio and lower bulk density. The higher screw speed could increase the thermal input through viscous dissipation, thereby increasing the gelatinization of starch, further improving the expansion of the feed (Leonard et al., 2020). The increase in moisture content resulted in a decrease in bulk density in this study. Consistent with the present study, Wang et al. (2021a) reported that the bulk density of commercial sinking fish feed decreased as the moisture content increased from 27% to 32%. Under high temperature and high moisture content conditions, the superheated water in the extruder promoted bubbles formation and decreased the viscosity of the melt, resulting in a decrease in the bulk density (Samray et al., 2019). However, the expansion ratio decreased with the increase in the moisture content in this study, which may be due to a substantial shrinking during drying of the feeds with high initial moisture content (Jangchud et al., 2018). Dietary CAP inclusion could reduce the bulk density and increase the expansion ratio of the feed. Consistent with the present study, Øverland et al. (2006) reported that dietary bacterial protein meal (the bacteria culture of *Methylococcus capsulatus*) inclusion led to the increase of expansion ratio of dog food. D'mello and Acamovic (1976) and Plavnik et al. (1981) suggested that the SCP has cohesiveness after hydration and may be used as the binder in feed processing. Thus, dietary CAP inclusion resulted in the production of more porous extrudates could be due to the plastic behaviour of the melted dough inside the extruder, which needed to be further studied.

In the present study, the oil absorption of feed is highly positively correlated with the expansion ratio. The oil absorption of feed increased with the increase in screw speed and CAP substitution level, which could

be attributed to the increase of physical retention of oil in the extrudate (Arivalagan et al., 2018). The scanning electron micrographs of the cross-section of the particles could explain the increased oil absorption. CAP inclusion facilitated the formation of the microstructure of the extrudates, which enabled the extrudates to infuse more oil. As the substitution levels of CAP increased from 0% to 75%, the oil leakage of feeds reduced. Øverland et al. (2006) reported that bacterial protein meal (the bacteria culture of *Methylococcus capsulatus*) inclusion in the dog diets decreased the fat leakage by increasing the extrudate expansion. The decreased oil leakage with the increase in expansion ratio may be due to the low interconnectivity between the pores and the less fragmented structure of extrudates (Alcaraz et al., 2021). However, when the CAP replacement level reached 100%, the oil leakage of feeds increased, which could be attributed to the high content of large pores. Banjac et al. (2021) reported that oil leakage mainly occurred in the large pores.

High-quality extruded feed should have the characteristics of high hardness to resist mechanical stress during pneumatic conveying, transportation, storage and feeding. In this study, the hardness of feeds increased with the decrease in moisture content. Consistent with our study, Wang et al. (2021a) reported that the moisture content had an adverse effect on the hardness of extruded commercial sinking fish feed. The above discussion mentioned that at high temperatures, the high moisture content could promote extrudate expansion in our study. The expanded extrudate had larger pores with thinner cell walls, resulting in lower hardness (Leonard et al., 2020). The hardness of the feeds increased with the increase in the screw speed in our study. This may be because the materials in the extruder were severely mechanically sheared at higher screw speed, which led to the formation of harder products (Gupta et al., 2008). Dietary CAP inclusion could improve the hardness of the feed in the present study, which confirmed that the protein source is the main factor determining the physical properties of the extruded feed (Irungu et al., 2018). CAP inclusion improved the hardness and expansion ratio of feeds simultaneously indicating that CAP could increase the toughness of the structure and degree of expansion of the feeds. Øverland et al. (2006) reported that dietary bacterial protein meal (the bacteria culture of *Methylococcus capsulatus*) inclusion increased the breaking force of the extruded salmon feeds. Aarseth et al. (2006) reported that the inclusion of 5 g/kg red yeast cells (*Xanthophyllomyces dendrothous*) in the diet for salmonids could increase the breaking force of extruded feeds, where the red yeast cells acted as a binder to improve the binding strength of feeds. Li et al. (2018) documented that protein cross-linking induced the formation of dense protein networks, which could help improve the texture properties of proteins. Thus, the increased hardness may be attributed to the binding property of CAP and the formation of denser protein networks during the extrusion process, which needed to be further studied. However, when the CAP substitution level reached 100%, the hardness had a slight decrease, which was due to the larger pores in the feeds (Fig. 1). Aarseth and Prestløkken (2003) showed that pellets with higher content of larger pores had lower strength because the stress was concentrated around the larger pores, which was expanding due to the applied stress.

WAI represents the amount of water absorbed by the extrudate, whereas WSI refers to the number of small molecules dissolved in water (Leonard et al., 2020). The degree of protein denaturation and starch gelatinization of extrudates determines the value of WAI and WSI (Singha and Muthukumarappan, 2018). The high moisture content resulted in lower WAI and higher WSI of extrudates in our study, which may be owing to the dextrinization of the starch. In the extrusion process, there was a physical competition between protein coagulation into a continuous network and starch gelatinization. The formation of protein networks could limit the starch gelatinization and lead to greater dextrinization (Ding et al., 2006). The increase in WAI with the increase in screw speed in our study could be contributed to the increase in starch gelatinization (Shameena Beegum et al., 2019). In this study, the WAI of the extrudates increased with the increase in CAP substitution level, which could be due to the high WHC of CAP. Consistent with the previous study, the trend of WAI was opposite to that of WSI in this study (Xie et al., 2021). Dietary CAP inclusion decreased the WSI of extrudates, which may be resulted from the lower soluble protein fraction in CAP.

Water solubility could reflect the number of substances leached into the water after the feeds immerse in water for a while (Ma et al., 2021; Wang et al., 2021a). In the present study, the water solubility decreased with the increase in moisture content, suggesting that sufficient moisture content was conducive to the formation of intermolecular forces and denser microstructure of the extrudates (Kaliyan and Morey, 2009). Consistent with our study, Wang et al. (2021a) found that water solubility of the extruded commercial sinking fish feed increased with the increase in the screw speed, which may be due to that the higher shear rate promoted the degradation of macromolecular substances. In our study, CAP inclusion reduced the water solubility of feeds, which could be attributed to the modification and the formation of cross-links of proteins. In addition, another possible explanation for the decreased water solubility was that the dense and smooth surface of the extrudates (Fig. 2) allowed them to interact with water for a longer time before they started to dissolve.

5. Conclusion

Compared with LTFM, CAP has higher WHC, lower PDI and flow figure, and could increase the viscosity of the unprocessed diets. Dietary CAP inclusion could improve the expansion degree, texture, oil absorption capacity and water stability of the feeds. Processing feeds containing high content of CAP ($\geq$ 270 g/kg) requires more moisture content, which could cause an increase in drying energy consumption. Dietary CAP inclusion could increase the SME during the extrusion process. To save drying energy consumption and mechanical energy, the recommended process parameters for the test diets are 28% moisture content and 300 rpm screw speed. These process parameters are only applicable to the specific extruder used in our experiment. Overall, CAP is an excellent protein source for the production of high-quality low-starch extruded floating feed. However, the mechanism by which CAP inclusion improves the physical qualities of extruded feed and the optimization of processing parameters need to be further studied.

CRediT authorship contribution statement

Shifeng Ma: Conceptualization, Data curation, Methodology, Formal analysis, Writing – original draft. **Hao Wang:** Methodology, Writing – review & editing. **Jie Yang:** Methodology, Visualization. **Junguo Li:** Validation, Resources. **Min Xue:** Conceptualization, Data curation, Supervision, Funding acquisition, Writing – review & editing. **Hongyuan Cheng:** Validation, Software. **Fangqi Zou**: Validation, Resources. **Christophe Blecker:** Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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