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Dietary starch level affects nutrient digestibility, faecal waste production and characteristics in yellowtail kingfish (*Seriola lalandi*) depending on the level of fish meal replacement

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ABSTRACT

Yellowtail kingfish have a poor faecal integrity when fed with pelleted diets which may be due to the presence of dietary starch. Moreover, whether dietary starch interacts with plant protein ingredients, is not known. In this study, we investigated the effect of dietary starch level, protein source and their interaction effect on faecal waste production and characteristics (visual appearance, faecal removal efficiency and particle size distribution (PSD)). Four diets were formulated according to a 2 × 2 factorial design (starch level × protein source). The effect of starch level was tested by either including 0% (*LS - low starch*, 4% starch) or 20% gelatinized wheat flour (*HS - high starch,* 20% starch). *FM* diets contained fish meal as protein source, whilst at *FM/P* diets approximately 65% of the fish meal was replaced by plant protein ingredients. Twelve tanks were stocked with 21 fish (mean initial weight 53 g) and fish performance, nutrient digestibility, faecal waste production and characteristics were evaluated over a 36-day experimental period. Both starch level and protein source affected the organic matter digestibility ($p < 0.05$). The effects of starch and protein source were additive regarding macro nutrient digestibility indicated by the absence of an interaction effect ($p > 0.05$). Growth was similar between the *FM* and *FM/P* at *low starch* diets ($p < 0.05$), but was reduced at the *FM/P* at *high starch* inclusion level ($p < 0.05$). The *high starch* and *FM/P* diets resulted in more faecal waste production ($p < 0.05$). Faecal integrity of yellowtail kingfish was adversely affected by starch inclusion. Fish receiving *low starch* diets excreted faecal pellets and short strings, while faecal waste collected from fish receiving *high starch* diets was classified as inconsistent. This was also reflected in a higher faecal removal efficiency and larger faecal PSD for fish receiving the *low starch* diets compared to the *high starch* diets ($p < 0.05$). No protein source or interaction effect was observed for faecal removal efficiency ($p > 0.05$). Consequently, lowering starch level and excluding plant protein ingredients reduced the amount of non-removed faeces by 71.1% and 30.6%, respectively. In summary, our study showed that the reduction of dietary starch offers possibilities to replace fish meal with plant protein ingredients without limiting growth performance in yellowtail kingfish. Moreover, lowering dietary starch level may have the potential to reduce solid loading in recirculating aquaculture systems for yellowtail kingfish.

1. Introduction

Yellowtail kingfish (*Seriola lalandi*) is a relatively recent domesticated fish species in recirculating aquaculture systems (RAS) (EUMOFA, 2020; Moran et al., 2009; Soriano et al., 2018). Culturing fish in RAS has the advantage that water is continuously treated and solid faecal waste is partially removed from the water (Brinker and Rösch, 2005; Schumann et al., 2016; Timmons et al., 2018). In case of yellowtail kingfish, the removal of faecal solid waste is challenging, due to the fine and unstable faecal particles (Moran et al., 2009). A poor faecal waste removal efficiency results in the build-up of total suspended solids (TSS) and eventually release of nutrients in the system and effluent water. This

Abbreviations: NSP, non-starch polysaccharides; PSD, particle size distribution; TSS, total suspended solids..

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would adversely affect animal health, system performance, operating costs and lead to environmental eutrophication when waste streams enter natural water bodies (Amirkolaie, 2011; Brinker et al., 2005; Brinker and Rösch, 2005; Chen et al., 1993; Fernandes and Tanner, 2008; Schumann et al., 2016; Unger and Brinker, 2013). For this reason, controlling the amount of TSS in RAS and effluent water is essential for the success of yellowtail kingfish culture in RAS. Lowering the amount of faecal waste produced through improved nutrient digestibility and improving faecal integrity, such as among others faecal removal efficiency or particle size distribution (PSD), contributes to controlling levels of TSS in RAS (Amirkolaie, 2011; Bureau and Hua, 2010; Cho and Bureau, 1997; Kokou and Fountoulaki, 2018; Tran-Tu et al., 2018).

Research by Horstmann et al. (2023b) has shown that feeding yellowtail kingfish on unprocessed natural feed items (sand eel *(Ammodytes tobianus)*, smelt *(Atherina boyeri)*, krill *(Euphausia superba)* and squid *(Loligo patagonica)*; raw and thawed) resulted in distinct faecal pellets and short strings. However, when these natural feed items were included (freeze dried and ground) in a pelleted diet containing 15% starch, faecal pelleting was not observed. Natural diets of yellowtail kingfish do not contain starch (in large quantities) (Andaloro and Pipitone, 1997; Fielder and Heasman, 2011; Pipitone and Andaloro, 1995). Moreover, yellowtail kingfish are less well able to digest starch compared to other finfish species such as Carp (*Cyprinus carpio*), European sea bass (*Dicentrarchus labrax)* or rainbow trout (*Oncorhynchus mykiss*) (Burel et al., 2000; Horstmann et al., 2023a; Krogdahl et al., 2004; Peres and Oliva-Teles, 2002; Shimeno et al., 1977). Therefore, greater amounts of undigested starch are present along the gastrointestinal tract and ultimately in the faeces. Research by Amirkolaie et al. (2006) with Nile tilapia (*Oreochromis niloticus*) suggested that undigested starch along the gastrointestinal tract can increase faecal waste production and lower its integrity. However, little is known about the effect of dietary starch level on faecal waste production and integrity in yellowtail kingfish.

Currently, yellowtail kingfish diets rely largely on the inclusion of marine ingredients, such as fish meal (Bowyer et al., 2012; Kissinger et al., 2016). Due to a stagnating supply and rising demand of fish meal, its replacement with alternate protein sources, such as plant-based ingredients, is required (Kissinger et al., 2016; Staessen et al., 2020a). However, the inclusion of plant-based ingredients is not without limitations in terms of faecal waste production and its integrity. For instance, recent research with yellowtail kingfish has shown that partial fish meal replacement by plant protein ingredients significantly enhanced the faecal waste production and reduced removal efficiency (Horstmann et al., 2023a). Whether this effect is due to ingredient characteristics or the presence of dietary starch that comes along with plant ingredients, is not known.

In this study, we investigated how dietary starch level affects faecal waste production and characteristics, such as faeces removal efficiency and PSD. Reducing dietary starch content may allow feeding yellowtail kingfish closer to their natural food source in the future. Furthermore, the type of protein source and their interaction were investigated to identify potential opportunities for replacing fish meal by plant protein ingredients in yellowtail kingfish.

2. Materials and methods

2.1. Diets

To investigate the effect of dietary starch level (SL), protein source (PS) and their interaction effect on nutrient digestibility, faecal waste production and characteristics of yellowtail kingfish, four experimental diets were formulated according to a 2 × 2 factorial design. The effect of the first factor, starch level, was tested by formulating diets either containing 0% or 20% gelatinized wheat flour. Gelatinized wheat flour consists of approximately ±75% starch). This resulted in an average dietary starch content of 3.7% (*LS – low starch* diets) and 19.9% (*HS - high starch* diets). In order to investigate possible interaction effects between the factors starch level and protein source, diets differing in their main protein source were formulated. This was achieved by replacing approximately 65% of the fish meal by plant protein ingredients. *FM* diets contained mainly fish meal as protein source, whilst at *FM/P* diets approximately 65% of the fish meal was replaced by equal amounts of wheat gluten, pea protein concentrate and soy protein concentrate (Table 1). Diets were supplemented with taurine to prevent taurine deficiency. DL-methionine and monocalcium phosphate was added to the *FM/P* diets to ensure a balanced amino acid profile and that phosphorus was not a limiting factor for growth. In all diets, a minimum of 8% fish oil was present to ensure the fulfilment of the requirements for essential fatty acids. Additional fish oil was added to the *LS-FM/P* (3%) *and HS-FM/P* diets (2.4%) in order to achieve a fat content equal to the *FM* diets. All diets contained a minimum of 15% fishmeal to ensure a good palatability and thus feed intake of the experimental diets. *FM* and *FM/P* diets with equal starch contents had a similar nutrient composition, whilst *high starch* diets contained lower levels of crude protein and fat compared to *low starch* diets (Table 1). The analysed nutrient composition is given in Table 1 and pellet quality and particle size distribution of diet mixture prior to extrusion in Table 2.

The diets were produced by cold pelleting (room temperature, approximately 20 °C) by Research Diet Services (Wijk bij Duurstede, The Netherlands) according to Kals et al. (2019) using a Clextral BC45 laboratory scale twin-screw extruder (Clextral, Firminy, France) with a 3 mm die, resulting in 3 mm sinking pellets. After pelleting, the pellets

Table 1
Diet composition and analysed nutrient content of the experimental diets.

Starch level	Low starch		High starch	
Protein source	FM	FM/P	FM	FM/P
Ingredients (g/kg)				
Fish meal	694.175	197.05	555.34	157.64
Wheat gluten	–	150	–	120
Pea protein concentrate	–	150	–	120
Soya protein concentrate	–	150	–	120
Fish oil	100	130	80	104
Gelatinized wheat flour	–	–	200	200
Monocalcium phosphate	–	10	–	8
DL-methionine	–	4	–	3.2
Taurine	6.875	10	5.5	8
Casein	130	130	104	104
Pellet binders[a]	50	50	40	40
Premix[b]	18.75	18.75	15	15
Yttrium oxide	0.2	0.2	0.16	0.16
Analysed nutrient content (g/kg DM)				
Dry matter (g/kg)	935	947	945	947
Crude protein	668	635	561	537
Crude fat	160	185	134	149
Starch and sugars	28	46	204	194
Gross energy (kJ/g DM)	22.5	23.6	21.6	22.4
Crude ash	151	94	122	77
Phosphorus	19.5	13.5	15.9	11.2
Calcium	23.9	9.6	19.5	8.3

FM – Fish meal as protein source; FM/P – 65% fish meal replacement by plant protein ingredients.

[a] Pellet binders – in house composition.

[b] Premix composition. Vitamins (IU or mg/kg complete diet): Vitamin B1–15 mg; Vitamin B2–15 mg; Vitamin B6–15 mg; Vitamin B5–50 mg; Vitamin B3–150 mg; Biotine – 0.7 mg; B-12–0.05 mg; Folic acid – 3 mg; Vitamin C – 500 mg (given as ascorbic acid C, phosphate); Vitamin E – 100 IU; A-vitamin A palmitate – 10,000 IU; D-Rovimix D3–500–2500 IU; K_3 K-menadione sodium bisulphite (51%) – 15 mg; Inositol – 450 mg; Betaine – 500 mg; Choline (given as choline chloride) – 1000 mg; Anti-oxidant BHT (E300–321) – 100 mg; Calcium propionate – 1000 mg. Minerals (mg/kg complete diet); Iron (as $FeSO_47H_2O$) – 50 mg; Zinc (as $ZnSO_47H_2O$) – 80 mg; Cobalt (as $CoSO_47H_2O$) – 0.2 mg; Copper (as $CuSO_47H_2O$) – 8 mg; Selenium (as Na_2SeO_3) – 0.2 mg; Manganese (as $MnSO_47H_2O$) – 30 mg; Magnesium (as $MgSO_47H_2O$) – 750 mg; Chromium (as $CrCl_36H_2O$) – 1 mg; Iodine (as $CaIO_36H_2O$) – 2 mg.

Table 2
Physical pellet characteristics and dietary particle size distribution (PSD, %; prior to extrusion) of the experimental diets.

Starch level	Low starch		High starch	
Protein source	FM	FM/P	FM	FM/P
Physical pellet characteristics				
Hardness (kg)	9.4	12.3	12.1	10.4
Durability (%)[a]	99.3	99.7	99.8	99.8
Bulk density (g/L)	478	519	616	562
Gelatinization degree (%)	BDL[b]	30.8	93.6	87.7
Dietary particle size distribution (%)[c]				
<40 μm	0.7	6.2	3.2	7.5
40–80 μm	20.7	37.1	26.1	38.7
80–150 μm	15.4	15.7	17.0	17.3
150–250 μm	38.6	19.6	32.3	17.7
250–315 μm	7.0	4.9	5.8	4.2
315–425 μm	5.8	5.3	5.2	4.8
425–630 μm	8.2	9.1	6.9	7.6
>630 μm	3.7	2.1	3.5	2.3

FM – Fish meal as protein source; FM/P – 65% fish meal replacement by plant protein ingredients.

[a] Durability expressed as 100% - % feed fines.

[b] BDL – below detection limit; starch and gelatinized starch <10 g/kg.

[c] Calculated as: $I\%_{fish\ meal} \times P\%_{x\ \mu m} + I\%_{wheat\ gluten} \times P\%_{x\ \mu m} + I\%_{pea\ protein} \times P\%_{x\ \mu m} + I\%_{soy\ protein} \times P\%_{x\ \mu m} + I\%_{gelatinized\ wheat\ flour} \times P\%_{x\ \mu m} + I\%_{casein} \times P\%_{x\ \mu m}$, where I% is the inclusion of each ingredient within the diet and P% the fraction (in %) of particles within each of the fractions (e.g. x – 40 μm).

were first dried for 2 h at 45 °C and followed by drying at 70 °C for 3 h. Afterwards, pellets were cooled to room temperature. Diets were produced approximately one week prior to start of the experiment and stored at 4 °C throughout the whole experiment.

2.2. Fish, rearing conditions and housing facilities

The experiment was carried out in accordance with the Dutch and European law on the use of experimental animals. The Animal Welfare Body of Wageningen University and Research (The Netherlands) classified this experiment as non-invasive. Fish were kept and handled in agreement with EU-legislation. Yellowtail kingfish (*Seriola lalandi*) of mixed sex were obtained from a commercial fish farm (Kingfish Zeeland, Kats, The Netherlands). At the beginning and the end of the experiment, fish were weighed (Mettler-Toledo ICS429) to determine initial and final weight and growth. One day prior to weighing, fish were not fed. Per tank, 21 fish with an average initial weight of 53 g were stocked. All fish tanks (370 L water volume, circular) were part of the same RAS, and connected to a sump, settling tank, drum filter (HDF801-1P; Hydrotech, Vellinge, Sweden; mesh size 30 μm), protein skimmer, and trickling filter. The system's refreshment rate was adjusted to keep the NO_3-N concentration below 100 mg/L. The water flow over each tank was controlled (Magnetic-inductive flow sensor, SM 6000; ifm electroic, Essen, Germany) and kept constant at 7.0 ± 0.05 L/min. The outlet of each tank (bottom drain) was directly (~1 m flow distance) connected to an individual swirl separator (column height 44 cm; diameter 24.5 cm; Aqua Optima AS, Pulford, United Kingdom) to quantify feed spillage after feeding and to collect faeces.

Water quality parameters were measured daily. The dissolved oxygen concentration was measured in the outlet water of randomly selected tanks (in the swirl separator connected to the tank) by hand-held digital probes. Oxygen concentrations were always maintained at a level above 5.75 mg/L (>85% saturation). Mean oxygen concentration was 6.9 ± 0.62 mg/L (100 ± 9.0% saturation). Water temperate, salinity, pH, TAN, NO_2-N and NO_3-N were measured from the common outflow of all tanks (before entering the solid removal unit). Temperature was kept within the range 23.1–23.4 °C (mean 23.3 ± 0.05 °C) (WTW Multi 3630 IDS - FDO 925). Salinity was maintained within the range 31.4–35.8 ppt (mean 34.4 ± 0.95 ppt) (WTW Multi 3630 IDS - TetraCon 925) during the experimental period. The pH was maintained within the range of pH 7.1–8.1 (mean 7.5 ± 0.25) (WTW Multi 3630 IDS - SenTix 940). This was done by adding sodium bicarbonate or HCl when necessary to keep the pH between pH 7.3 and pH 7.8. Values for TAN (total ammonium nitrogen, NH_4-N and NH_3-N) concentrations did not exceed the level of 0.8 mg/L (mean 0.2 ± 0.23) (Merck Aquamerck Colorimetric Ammonium test). NO_2-N concentrations were always maintained at concentrations below 0.75 mg/L (mean 0.3 ± 0.17) (Merck Aquamerck Colorimetric Nitrite test) and NO_3-N concentrations were always maintained below 56 mg/L (mean 31.2 ± 18.12 mg/L) (Merck MQuant Nitrate test strips). The photoperiod was set at 20 L:4D during the entire duration of the experiment. Light went on at 7:30 am and switched off at 3:30 am.

2.3. Experimental procedures and sampling

During the 36-day experimental period, the four dietary treatments were tested in triplicate and randomly assigned among 12 tanks. Fish were restrictively fed to maintain an equal amount of feed given per fish per day on dry matter (DM) basis for all treatments. The feeding level was aimed at 23.75 $g/kg^{-0.8}$ BW/d which is approximately 95% of the predicated satiation level. Throughout the experiment, the daily amount of feed was gradually increased based on the average initial fish weight and the predicted daily growth assuming a FCR of 0.9 for all treatments. The daily amount of feed was divided into two equal portions, which were hand fed at 9:00 and 15:00 h. During the first four feeding moments of the experiment, the feeding level gradually increased until the intended feeding level was reached. This allowed the fish to adapt to the diet. Fifteen minutes after finishing feeding, the glass bottles attached to the swirl separators were checked for feed pellets to determine feed spillage. Mortality was checked twice a day before feeding.

Faeces for digestibility analysis were collected overnight (15:30 h – 8:30 h) for 5 days during week 5 by settling (Amirkolaie et al., 2005). Bottles, which were connected beneath the swirl separators, were submerged into ice water to minimize bacterial degradation of the sample. Faecal samples were pooled per tank, where one sample represented the collection throughout one night (17 h). Pooled samples (5 days × 17 h) were stored at −20 °C until further analysis. Faeces collection for determination of faecal removal efficiency was done at the end of the fifth week. The collecting method was the same as for the faecal samples collected for digestibility purposes, except that faecal material was collected continuously for 48 h (including day collection; including feeding moments). Faeces collection for determination of faecal PSD by sieving was done once weekly during the last two weeks of the experiment (3 h collection during the day after morning feeding). After collection, faeces were stored on ice until further analysis. Feed samples were taken by pooling 100 g per experimental diet per week and samples were stored at 4 °C.

2.4. Analysis

Faeces collected for digestibility and faecal removal efficiency were dried at 70 °C until a dry matter content of >90% was reached. Thereafter, faeces were ground (mixer mill, IKA A11 basic). Feed and faeces were analysed as described by Staessen et al. (2020a). For dry matter determination, faeces and feed were analysed gravimetrically by drying for 4 h at 103 °C until constant weight (ISO 6496, 1999). Ash was determined gravimetrically by combustion for 4 h at 550 °C in a muffle furnace (ISO 5984, 2002) until constant weight. The ash fraction was dissolved in concentrated sulphuric acid by autoclaving (121 °C, 20 min) to determine dietary P, Ca and yttrium content of feed and faeces using ICP-AES (NEN 15510, 2007). Total N was determined according to Kjeldahl's method (ISO 5983-2, 2009); crude protein was calculated

with a protein conversion factor of 6.25. Crude fat was determined gravimetrically using acid hydrolysis (Hydrotherm®, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) followed by petroleum-ether extraction (Soxhlet method; ISO 6492, 1999). Total starch and gelatinized starch were analysed to determine the gelatinization degree of starch in the experimental diets (Nutrilab, Giessen, The Netherlands). Total starch was analysed enzymatically using amyloglucosidase after washing with 40% ethanol to remove sugars. Gelatinized starch was analysed according to the modified glucoamylase method described by Zhu et al. (2016). For digestibility calculations, starch content (including sugars) of pelleted diets and faeces was analysed as described above for total starch analysis, leaving out the ethanol washing step. Gross energy was determined using bomb calorimetry (C7000, IKA werke, IKA analysentechnik, Staufen, Germany).

PSD of the ingredient mixtures of both diets (prior to extrusion) was determined by sieving a 50 g sub-sample through a stack of sieves (mesh sizes: 630 μm, 425 μm, 315 μm, 250 μm, 150 μm, 80 μm and 40 μm; 10 min sieving time, interval of 6 s, amplitude of 2 mm/'g'; Retsch, AS 200 control, Haan, Germany). Pellet hardness was tested using a hardness tester (KAHL Pellet Hardness Tester; AMANDUS KAHL GmbH & Co. KG, Hamburg, Germany). Durability (100% - % feed fines) was determined by sieving a 200 g sub-sample through a sieve (1 mm mesh size; 2 min sieving time, interval of 6 s, amplitude of 2 mm/'g'; Retsch, AS 200 control, Haan, Germany). Bulk density was determined with a 1 L cylinder with slide, fall weight and filling cylinder (Biotechnion, Wageningen, The Netherlands).

Faecal PSD was analysed as a parameter to determine faecal characteristics of the collected faeces by settling. Faecal PSD was determined by sieving (mesh size of 40 μm, 100 μm, 250 μm and 850 μm). Collected faeces were first shortly homogenized (200 rpm, 15 s, MR3000, Heidolph Instruments, Schwabach, Germany) and a sub-sample was poured on an 850 μm sieve. The residue (>850 μm) was collected on a pre-weight 1.5 μm glass fibre filter (90 mm diameter, grade 696, VWR, Radnor, USA) using a vacuum pump. The filtrate (<850 μm) was poured on the next sieve with a smaller mesh size (250 μm). This procedure was repeated twice more using sieves with a mesh size of 100 μm and 40 μm. The residue of the fractions 850–250 μm, 250–100 μm and 100–40 μm was collected on individual pre-weight 1.5 μm glass fibre filters. The filtrate of the fraction <40 μm was finally collected on a pre-weight 1.5 μm glass fibre filter. Filters were stored at −20 °C until further analysis. To determine the collected organic matter (OM) mass of the fractions <40 μm, 40–100 μm, 100–250 μm, 250–850 μm and > 850 μm, filters were dried and combusted as described above.

2.5. Calculations and data analysis

Specific growth rate (SGR; %/d) was calculated as $(\ln W_f - \ln W_i) / t \times 100\%$, where W_i is the individual initial body weight, W_f the individual final body weight and t is the number of days during the experimental period. The absolute feed intake (FI_{abs}; g/d) was calculated as FI_{tot} / t, where FI_{tot} is the total feed intake (g DM). The geometric mean BW (W_G; g) was calculated as $e^{((\ln Wt + \ln W0)/2)}$. Feed conversion ratio (FCR) was calculated on dry matter basis (g/g) as $(FI \times DM_{Diet} / 1000) / (W_f - W_i)$, where DM_{Diet} is the dry matter content of the diet (g/kg). Survival (%) was calculated as $(1 - ((N_i - N_f) / N_i) \times 100$, where N_i is the number of fish at the beginning and N_f the final number of at the end of the experiment.

Apparent digestibility coefficient (ADC, %) of organic matter, crude protein, crude fat, carbohydrate, starch and gross energy were calculated according to Cheng and Hardy (2002) using yttrium as inert marker: $ADC\ (\%) = 100 \times (1 - ((Y_{diet} / Y_{faeces}) \times (N_{faeces} / N_{diet})))$, where Y is the inert marker percentage of the diet or faeces and N is the nutrient percentage (or kJ/g gross energy) of the diet or faeces. Organic matter (g/kg DM) and total carbohydrates in feed and faeces were calculated as 1000 – ash and as 1000 – (crude protein + crude fat + ash), respectively.

Faecal waste production, faecal removal efficiency and non-removed faeces per feed intake were calculated according to Fountoulaki et al. (2022). Faecal waste production (g OM/kg DM FI) was determined on organic matter basis as the amount of non-digested feed per kilogram dry matter feed intake as $(100\% - ADC_{OM}) \times OM_{diet}$, where ADC_{OM} is the organic matter digestibility (in %) during week 5 and OM_{diet} the organic matter (in g/kg) of the diet. Faecal removal efficiency (FR, %) was calculated as the percentage of collected faeces by settling throughout 48 h continuous faeces collection in relation to the total amount of faecal waste production. In detail, this was calculated as the amount of yttrium collected by settling ($Y_{removed}$, g) in relation to the total amount of yttrium given via the fed (Y_{diet}, g) as $Y_{removed} / Y_{diet} \times 100\%$. The non-removed faeces per feed intake (g OM/kg DM FI) was calculated as the difference between the total amount of faecal waste produced and the amount of faeces removed as $((100\% - FR) \times (100\% - ADC_{OM}) \times OM_{diet})$, where FR is the faeces removal efficiency during the 48 h continuous faeces collection and ADC_{OM} the organic matter digestibility during week 5.

Faecal PSD was determined by sieving as $P_{fraction} / P_{total}$, where $P_{fraction}$ is the collected organic matter within a respective fraction (<40 μm, 40–100 μm, 100–250 μm, 250–850 μm or > 850 μm) and P_{total} is the total collected organic matter of all fractions.

2.6. Statistical analysis

Tanks were used as the experimental unit ($n = 12$). Normality of data and equality of variance was assumed. A two-way ANOVA was used to investigate the effect of protein source, starch level and their interaction. In the case of a significant interaction effect ($p < 0.05$), a Tukey HSD test

Table 3
Fish performance of yellowtail kingfish fed the experimental diets restrictively (3 replicates) for 36 days.

Starch level	Low starch		High starch			*p-value*		
Protein source	FM	FM/P	FM	FM/P	SEM	SL	PS	SL × PS
Survival (%)	98	100	100	100	0.8	ns	ns	ns
Initial body weight (g)	53	53	54	53	0.7	ns	ns	ns
Final body weight (g)	247^{c}	253^{c}	216^{b}	206^{a}	1.8	***	ns	**
FI_{abs} (g DM/d)	3.66	3.75	3.73	3.74	0.000	–	–	–
FI_{DP} (g/d)	2.34^{d}	2.30^{c}	1.96^{b}	1.89^{a}	0.01	***	***	*
FI_{DF} (g/d)	0.55^{c}	0.64^{d}	0.45^{a}	0.49^{b}	0.00	***	***	***
FI_{DE} (kJ/d)	77.5^{b}	80.3^{c}	72.7^{a}	73.0^{a}	0.42	***	**	*
Growth (g/d)	5.4^{c}	5.6^{c}	4.5^{b}	4.3^{a}	0.04	***	ns	**
SGR (%/d)	4.27^{b}	4.36^{b}	3.85^{a}	3.78^{a}	0.029	***	ns	*
FCR	0.68^{a}	0.67^{a}	0.83^{b}	0.88^{c}	0.008	***	*	*

FM – Fish meal as protein source; FM/P – 65% fish meal replacement by plant protein ingredients; FI_{abs} – feed intake absolute; FI_{DP} – digestible protein intake; FI_{DF} – digestible fat intake; FI_{DE} -digestible energy intake; SGR – specific growth rate; FCR – feed conversion ratio (on DM basis). SL – starch level; PS – protein source; SL × PS – starch level × protein source; Values are means and pooled standard error of the means (SEM); in case of a significant interaction effect, means within the same row not sharing a common letter are different ($p < 0.05$); ns - not significant $p > 0.05$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

(honest significant difference; 95% significance) was performed to compare treatment means. Statistical analyses were performed by using the statistical program SPSS Statistics 27 (IBM, New York, United States of America).

3. Results

3.1. Fish performance

Fish performance data is presented in Table 3. Survival was high (99.6%) and unaffected by treatments ($p > 0.05$). Fish fed *high starch* diets had a smaller nutrient intake compared to fish fed *low starch* diets ($p < 0.001$). An interaction between starch level × protein source on growth was observed ($p < 0.05$). *High starch* inclusion negatively affected daily growth (g/d) and SGR (%/d) ($p < 0.001$). In particular, the daily growth of fish fed the *FM/P* diet was negatively affected by *high starch* inclusion (*HS-FM* versus *LS-FM/P;* SL × PS $p < 0.01$). However, when starch was not included in the *FM/P* diet, daily growth was unaffected by the factor protein source (e.g., *LS-FM/P* versus *LS-FM*). An interaction effect was observed for the SGR (SL × PS $p < 0.05$). In line with the daily growth, the SGR at fish fed the *HS-FM* diet was higher compared to fish fed the *HS-FM/P* diet, although not being statistically different.

3.2. Digestibility

Apparent digestibility coefficients (ADC, %) of organic matter and energy were reduced by both *high starch* and plant protein ingredient inclusion (Table 4, $p < 0.001$). Crude protein digestibility increased by plant protein ingredient inclusion ($p < 0.01$), while *high starch* levels reduced crude protein digestibility ($p < 0.001$). Crude fat digestibility was in absolute numbers 4% lower for the *high starch* diets ($p < 0.001$). Plant protein ingredient inclusion tended to reduce crude fat digestibility ($p < 0.1$). Starch digestibility was negatively affected by plant protein ingredient inclusion ($p < 0.001$), while starch level had no effect ($p > 0.05$). An effect of starch level, protein source and an interaction between starch level × protein source on phosphorus digestibility was observed ($p < 0.01$). In detail, fish fed the *FM/P* diets showed a higher phosphorus ADC compared to fish fed the *FM* diets. *High starch* levels negatively affected the phosphorus ADC at fish fed the *FM* diet, while not affecting it at the *FM/P* diet.

3.3. Faecal characteristics and removal efficiency

Image 1 shows the overnight collected faecal waste. Visually, large differences appeared between treatments differing in dietary starch inclusion levels. Clear faecal pellets and short strings were observed for the *low starch* diets, while faecal waste of fish fed *high starch* diets had a disintegrated appearance. Faecal PSD of collected faeces by sedimentation is shown in Table 5. Fish fed diets containing *low starch* excreted larger particles compared to fish receiving diets with *high starch* level (>850 μm, $p < 0.05$). PSD was unaffected by the interaction effect between starch level and protein source ($p > 0.05$). The fraction of particles between 40 and 100 μm was influenced by dietary protein source, but the differences were small being 3.4% versus 1.5% at the *FM* versus *FM/P* diets respectively.

The total amount of faecal waste produced, faecal removal efficiency and the amount of non-removed faeces are shown in Fig. 1 and Fig. 2. No interaction effect between starch level and protein source was observed for any of these parameters ($p > 0.05$). The amount of faecal waste was influenced by both starch level and protein source ($p < 0.001$). *High starch* diets gave more faecal waste than *low starch diets.* Plant protein ingredient inclusion also increased the amount of faecal waste. Faecal removal efficiency was only affected by the starch level ($p < 0.001$). The combined effects of faecal waste production and faecal removal efficiency resulted in an increased amount of non-removed faeces by 245.8% for diets containing *high starch* versus *low starch* levels and by 44.1% for diets containing plant protein ingredients compared with diets without plant proteins. These effects of starch level and protein source on the amount of non-removed faeces were additive, indicated by the absence of their interaction effect.

4. Discussion

4.1. Nutrient digestibility and fish performance

Despite the high gelatinization degree of the experimental diets (Table 2), starch digestibility ranged between 81.3% and 87.3% during our study (Table 4). This suggests that yellowtail kingfish are not as efficient in digesting starch compared to other finfish species. For instance, studies with Carp, European sea bass or rainbow trout report starch digestibility of above 98% (Burel et al., 2000; Krogdahl et al., 2004; Peres and Oliva-Teles, 2002; Shimeno et al., 1977). It is expected that the low α-amylase activity in the gastrointestinal tract of yellowtail kingfish is responsible for the low starch digestibility (Chen et al., 2006; Kaushik et al., 2022; Shimeno et al., 1977). However, it is surprising that the starch digestibility was not negatively affected by increasing starch level during the current study. This is contradictory to previous studies with Atlantic salmon (*Salmo salar*), Nile tilapia and yellowtail kingfish, where increasing starch levels resulted in a reduced starch digestibility (Amirkolaie et al., 2006; Krogdahl et al., 2004; Shimeno et al., 1977). This could be explained by the higher gelatinization degree of starch in the *high starch* diets (Table 2) (Amirkolaie et al., 2006). The gelatinization of starch changes its crystalline structure to a gel structure, being more accessible for hydrolysis by the α-amylase (Amirkolaie et al., 2006; Englyst and Cummings, 1987). Therefore, the higher gelatinization degree of the *high starch* diets might have counteracted the negative effect of higher starch level on starch digestibility.

Despite the higher crude protein and fat intake, *low starch* diets were found to increase on absolute numbers the crude protein and fat digestibility on average by 2% and 4%, respectively (Table 4). This is in line with findings by Hemre et al. (1995) and Krogdahl et al. (2004) who

Table 4
Apparent digestibility coefficient (ADC, %) of yellowtail kingfish fed the experimental diets restrictively (3 replicates) for 36 days.

Starch level	Low starch		High starch			*p-value*		
Protein source	FM	FM/P	FM	FM/P	SEM	SL	PS	SL × PS
Organic matter	92.9	88.4	88.3	84.1	0.43	***	***	ns
Crude protein	95.5	96.5	93.7	94.3	0.18	***	**	ns
Crude fat	93.4	91.9	89.4	87.9	0.71	***	#	ns
Starch and sugars	86.8	81.3	87.3	81.7	0.68	ns	***	ns
Energy	94.0	90.8	90.0	87.1	0.48	***	***	ns
Phosphorus	53.2^{b}	62.1^{c}	43.2^{a}	60.2^{c}	1.22	***	***	**

FM – Fish meal as protein source; FM/P – 65% fish meal replacement by plant protein ingredients; SL – starch level; PS – protein source; SL × PS – starch level × protein source. Values are means and pooled standard error of the means (SEM); in case of a significant interaction effect, means within the same row not sharing a common letter are different ($p < 0.05$); ns – not significant $p > 0.1$; # – tendency $p < 0.1$; ** - $p < 0.01$; *** - $p < 0.001$.

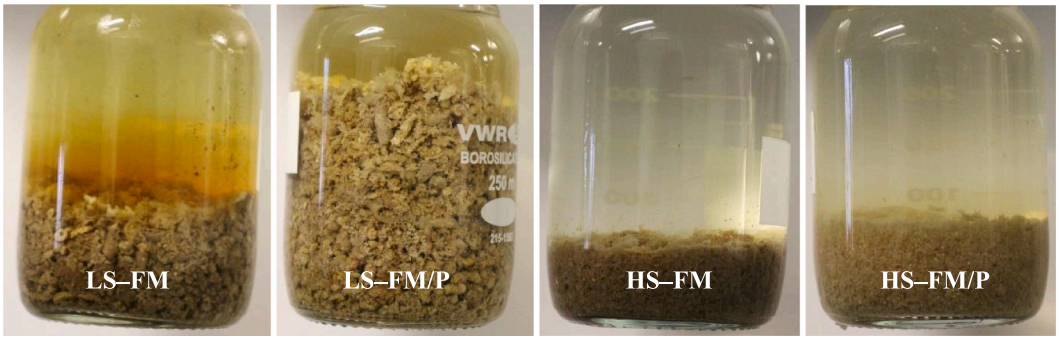

Image 1. Faeces collected (each collection bottle represents one replicate per treatment) from yellowtail kingfish fed the experimental diets restrictively for 36 days; LS-FM – low starch level and fish meal as protein source; LS-FM/P – low starch level and 65% replacement of fish meal by plant protein ingredients; HS-FM – high starch level and fish meal as protein source; HS-FM/P – high starch level and 65% replacement of fish meal by plant protein ingredients.

Table 5
Faecal particle size distribution (PSD; %) of collected faeces by sedimentation of yellowtail kingfish fed the experimental diets restrictively (3 replicates) for 36 days.

Starch level	Low starch		High starch			*p-value*		
Protein source	FM	FM/P	FM	FM/P	SEM	SL	PS	SL × PS
<40 μm	3.2%	3.0%	2.9%	5.3%	1.17%	ns	ns	ns
40–100 μm	3.2%	1.3%	3.7%	1.7%	0.49%	ns	**	ns
100–250 μm	5.5%	3.9%	7.1%	6.7%	0.74%	*	ns	ns
250–850 μm	24.0%	25.9%	35.5%	37.9%	2.71%	**	ns	ns
>850 μm	64.2%	65.9%	50.8%	48.5%	4.21%	**	ns	ns

FM – Fish meal as protein source; FM/P – 65% fish meal replacement by plant protein ingredients; SL – starch level; PS – protein source; SL × PS – starch level × protein source. Values are means and pooled standard error of the means (SEM); ns - not significant $p > 0.05$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

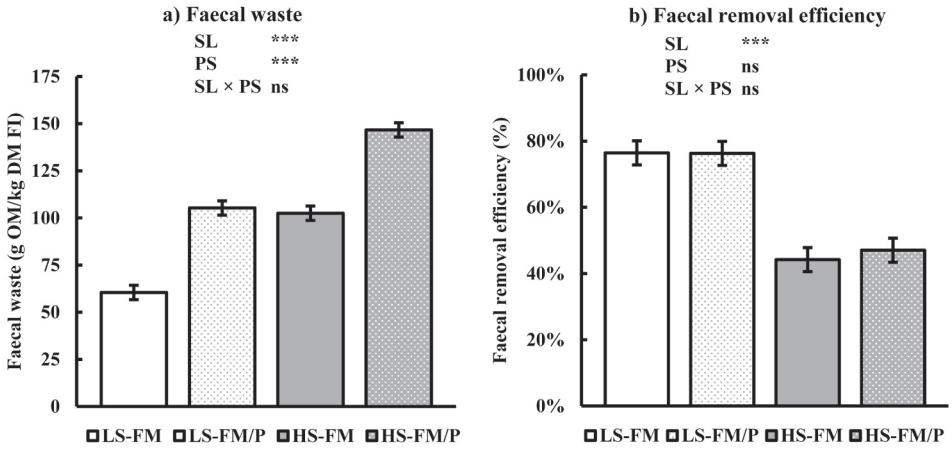

Fig. 1. Faecal waste per feed intake (g OM/kg DM FI; graph a) and faeces removal efficiency by settling (%; graph b) of yellowtail kingfish fed the experimental diets restrictively (3 replicates) for 36 days; OM - organic matter; DM- dry matter; FI - feed intake; LS-FM – low starch level and fish meal as protein source; LS-FM/P – low starch level and 65% fish meal replacement by plant protein ingredients; HS-FM – high starch level and 100% fish meal as protein source; HS-FM/P – high starch level and 65% fish meal replacement by plant protein ingredients; SL – starch level; PS – protein source; SL × PS – starch level × protein source; error bars indicate standard error of means; means not sharing a common letter are different ($p < 0.05$); ns – not significant $p > 0.05$; *** - $p < 0.001$.

observed a reduced nutrient digestibility for Atlantic salmon fed with increasing starch levels. However, contradictory results were observed for rainbow trout and Nile tilapia, where starch level did not negatively affect digestibility of other nutrients (Amirkolaie et al., 2006; Krogdahl et al., 2004). This suggests that fish species with a low starch digestibility potential, such as Atlantic salmon and yellowtail kingfish, are more susceptible to the negative effects of starch. During our study, the negative effects of an increasing starch level on the digestibility of other nutrients are expected due to the higher presence of undigested starch, which is suggested by Hemre et al. (1995) may have behaved like fibre within the gastrointestinal tract. In case of similar effects of starch in regard to fibre, it is expected that *high starch* diets negatively affected gut physiology and morphology. Moreover, *high starch* diets may increase the digesta viscosity, resulting in a reduced mixing of chyme and enzymes, ultimately impairing the nutrient digestion and absorption (Amirkolaie et al., 2006; Sinha et al., 2011). Besides a negative effect on digesta viscosity, gut physiology and morphology, it is also suggested that carbohydrates can affect the gut microflora, induce microbial fermentation and lead to osmotic imbalances (Booth et al., 2013; Hung et al., 1990; Kokou and Fountoulaki, 2018; Refstie et al., 2005; Sinha et al., 2011; van Barneveld, 1999). In summary, it was shown that the addition of starch in form of gelatinized wheat flour negatively affects the crude protein and lipid digestibility. It might be worthwhile to investigate the underlying mechanisms of dietary starch on digestion kinetics. Moreover, future research should focus on minimizing the effect of dietary starch on nutrient digestibility in yellowtail kingfish.

Apart from a dietary starch level effect on nutrient digestibility, it was observed that the inclusion of plant protein ingredients resulted in a lower nutrient digestibility (Table 4). The observed negative effects of plant protein inclusion are in line with findings from previous experiments with yellowtail kingfish performed at the same research facility (Horstmann et al., 2023a) and expected due to the presence of non-starch polysaccharides (NSP) (Maas et al., 2020; Sinha et al., 2011; Staessen et al., 2020a, 2020b). Even though this experiment was not

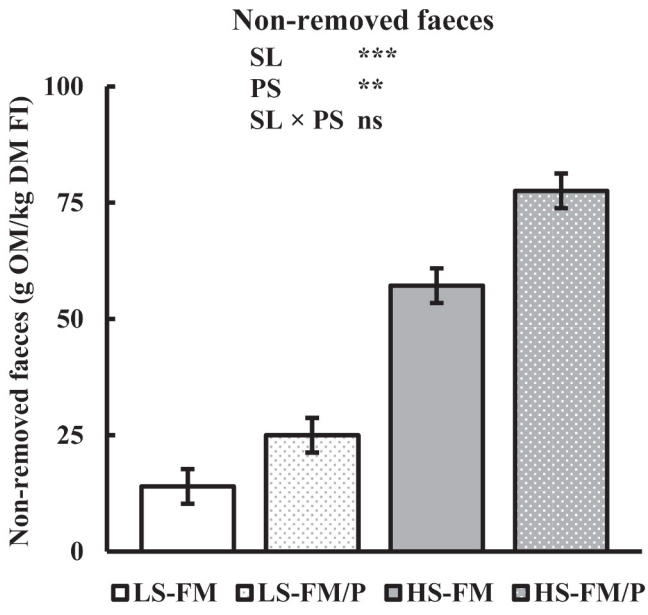

Fig. 2. Non-removed faeces per feed intake (g OM/kg DM FI) of yellowtail kingfish fed the experimental diets restrictively (3 replicates) for 36 days; OM – organic matter; DM – dry matter; FI – feed intake; LS-FM – low starch level and fish meal as protein source; LS-FM/P – low starch level and 65% fish meal replacement by plant protein ingredients; HS-FM – high starch level and fish meal as protein source; HS-FM/P – high starch level and 65% fish meal replacement by plant protein ingredients; SL – starch level; PS – protein source; SL × PS – starch level × protein source; error bars indicate standard error of means; means not sharing a common letter are different ($p < 0.05$); ns – not significant $p > 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

designed as a growth performance trial, the observed interaction effect between starch level and protein source is of interest. The combined negative effects of plant protein ingredient inclusion and *high starch* level on nutrient digestibility, in combination with the lower crude protein and fat intake of fish receiving the *high starch* diets (Table 1), resulted in the lowest observed growth performance for fish fed the *HS-FM/P* diet (Table 3). Contradictory results were observed for fish receiving the *low starch* diets. A numerical higher growth performance was observed for fish fed the *LS-FM/P* diet ($p > 0.05$), although fish receiving the *LS-FM* diet had a higher digestible crude protein intake (Table 4). On one hand, fish fed the *LS-FM/P* diet had a higher digestible fat intake which may resulted in the numerically higher growth. On the other hand, it could be that fish fed the *LS-FM/P* diet had a better nutrient utilization compared to fish receiving the *LS-FM* diet. This improved nutrient utilization for fish receiving the *LS-FM/P* diet could be expected due to a protein-sparing effect by carbohydrates. Fish fed the *LS-FM/P* diets received greater amounts of starch, sugars and NSP compared to fish fed the LS-FM diet (Table 1). This assumption is supported by findings of Booth et al. (2013), who proved that juvenile yellowtail kingfish are able to partly utilize carbohydrates to support fish growth. In summary, it is expected that both the higher carbohydrate and digestible fat intake supported growth at fish fed the *LS-FM/P* diet. Overall, in the current study it was shown that *high starch* level negatively affect nutrient digestibility, in particular when fed in combination with plant protein ingredients. Moreover, it was shown that under conditions of *low starch* inclusion, high quality plant ingredients could be used as a substitute for fish meal without growth limitation in yellowtail kingfish. Overall, excluding or reducing starch from aquaculture diets could allow the transition to more plant-based diets, in particular for fish which are sensitive to starch such as yellowtail kingfish or Atlantic salmon.

4.2. Faecal quantity and characteristics

In practice, both the amount of faecal waste production and faecal removal efficiency affect the TSS concentration in RAS (Amirkolaie, 2011; Bureau and Hua, 2010; Cho and Bureau, 1997; Kokou and Fountoulaki, 2018; Tran-Tu et al., 2018). During the current study, both starch level and plant protein ingredient inclusion adversely affected the organic matter digestibility (Table 4). Since the faecal waste production follows the amount of non-digested feed, this resulted in greater amounts of faecal waste produced by fish fed the *high starch* and *FM/P* diets, being highest for fish fed the *HS-FM/P* diet (Fig. 1).

Besides differences in the amount of faeces produced, it was also observed that *high starch* diets resulted in smaller faecal PSD and inconsistent faeces (Table 5 and Image 1), while fish fed *low starch* diets excreted distinct faecal pellets and short strings (Image 1). This was as well reflected in the faecal removal efficiency by settling (Fig. 1b), as in general larger faecal particles result in an increased faecal removal efficiency (Reid et al., 2009; Timmons et al., 2018; Tran-Tu et al., 2018). In particular, *low starch* diets resulted in a higher faecal removal efficiency by 67.4% compared to fish fed the *high starch* diet. Thus, lowering starch in yellowtail kingfish diets improves the faecal characteristics. According to literature, it is suggested that indigested carbohydrates, in particular disaccharides can induce a higher osmolality in the distal intestine, due to a high water binding capacity (Amirkolaie et al., 2006; Hung et al., 1990; Refstie et al., 1999). As previously mentioned, it is suggested that the presence of undigested starch and its breakdown products can induce microbial fermentation processes (Amirkolaie et al., 2006; Hung et al., 1990; Sinha et al., 2011; van Barneveld, 1999). Moreover, microbial fermentation of carbohydrates could result in gas production. This gas could be entrapped in faecal strand (Amirkolaie et al., 2006; Hung et al., 1990; Kokou and Fountoulaki, 2018; van Barneveld, 1999). On one hand, the gas could break the faecal pellet apart when excreted in the water, consequently alter the ease of removal from the system water. On the other hand, gas might also cause more floating faeces when remaining entrapped in the faecal pellet. However, no floating faecal pellets and thus floating faeces were observed at *high starch* diets during our experiment (Image 1).

In this study, it was shown that both *FM* and *low starch* diets resulted in a lower amount of non-removed faeces by 26.9% and 70.5%, respectively (Fig. 2). This effect is greater for the latter, due to both a positive effect of *low starch* on faecal waste production and faecal characteristics, while plant protein ingredient exclusion only resulted in a lower faecal waste production (Fig. 1 and Fig. 2). In practice, lower amounts of non-removed faeces would potentially improve animal health and system performance, while reducing the operation cost and consequently result in a reduced pressure on the environment when discharging effluent water into natural waters (Amirkolaie, 2011; Brinker et al., 2005; Brinker and Rösch, 2005; Chen et al., 1993; Fernandes and Tanner, 2008; Schumann et al., 2016; Unger and Brinker, 2013). Overall, with the current study, the potential of reducing the TSS load in RAS by lowering dietary starch levels is clearly shown. This might offer new possibilities of RAS-based farming for fish species which are less well able to digest starch such as yellowtail kingfish or Atlantic salmon.

5. Conclusion

It was shown that the reduction of dietary starch offers possibilities to replace fish meal with plant protein ingredients in regard to growth performance. At the same time, lowering the dietary starch level improved the nutrient digestibility, thereby reducing the faecal waste production. *Low starch* level resulted in faecal pelleting in yellowtail kingfish, improving the faecal removal efficiency by settling. Consequently, this resulted in lower amounts of non-removed faeces. Overall, lowering dietary starch level may have the potential to reduce solid loading in RAS for yellowtail kingfish.

CRediT authorship contribution statement

Peter Horstmann: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Roel M. Maas:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Jeroen Kals:** Conceptualization, Methodology, Writing – review & editing. **Satya Prakash:** Formal analysis, Investigation, Writing – review & editing. **Thomas W.O. Staessen:** Conceptualization, Writing – review & editing, Funding acquisition, Project administration. **Fotini Kokou:** Conceptualization, Writing – review & editing. **Johan W. Schrama:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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Data availability

Data will be made available on request.

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