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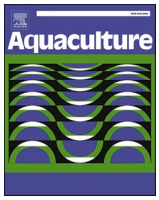

Effect of dietary starch, amylase and ash on nutrient digestibility, faecal waste production and faecal characteristics of rainbow trout, (*Oncorhynchus mykiss*).

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ABSTRACT

Waste management is a critical issue in aquaculture. In this study, we examined nutrient digestibility, faecal waste production, faecal removal efficiency and the faecal characteristics of rainbow trout (*Oncorhynchus mykiss*) in response to the dietary starch level, amylase and ash supplementation and the interaction of dietary starch with amylase and ash. A basal mixture predominantly consisting of plant-based ingredients was formulated. The basal mixture was diluted with 100, 200, 300 and 400 g/kg gelatinised wheat flour, resulting in four diets of varying starch levels, referred to as 10-WF, 20-WF, 30-WF and 40-WF, respectively. Amylase was supplemented at 125 k-novo-α-amylase units (KNU)/kg to the 20-WF and 40-WF diets and referred to them as 20-WFα and 40-WFα. Two high ash diets were prepared by supplementing 20 g diamol/kg to the 10-WF and 30-WF diets and termed as 10-WFA and 30-WFA. Triplicate group of 25 fish (mean weight, 210 g) were restrictively fed one of the eight diets for five weeks. Higher dietary starch level led to increased faecal waste production, lowered faecal removal efficiency and resulted in greater accumulation of non-removed faeces in the system. Moreover, higher dietary starch level also diminished faecal stability, as evident by an increased share of particles <40 μm size in 40-WF upon exposure to mechanical stress. Amylase supplementation in the diet enhanced starch digestibility without influencing faecal waste production, removal efficiency, or the particle size distribution (PSD) of non-stressed faeces. Conversely, dietary ash supplementation increased faecal waste production and altered faecal characteristics by enhancing density and sinking velocity, although without improving its removal efficiency. In summary, this study underscores the potential of dietary manipulations to affect faecal waste production, removal efficiency, and various faecal characteristics, including viscosity, density, sinking velocity, particle size distribution, and stability.

1. Introduction

Increased regulatory concerns over waste production, removal, and discharge from aquaculture systems suggest that waste management will be crucial to the sector's future development (Dalsgaard et al., 2013; Lindland et al., 2019; Van Rijn, 2013). Effective waste management is essential in an intensive production system to achieve maximal productivity and reduce environmental impact. Aquaculture waste can be broadly classified as solid and dissolved waste. Solid waste management is vital in a recirculating aquaculture system (RAS) since it affects not only the growth, welfare, and health of cultured fish but also the efficiency and operational cost of the production system (Davidson et al., 2013; Pedersen et al., 2017; Van Rijn, 2013). As most of the solid waste in aquaculture originates from faeces, which is directly linked to diet, manipulating the dietary factors presents potential avenues for solid waste management.

Diet-based approach to faecal waste management relies on either increasing the digestibility of feed and/or improving the characteristics

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of faeces to favour their quick and efficient removal (Amirkolaie et al., 2006; Letelier-Gordo et al., 2015; Meriac et al., 2014; Prabhu et al., 2019; Tran-Tu et al., 2019). An increase in dietary nutrient digestibility reduces the quantity of faecal waste produced, and their quick and efficient removal reduces the nutrient leaching and accumulation of suspended and dissolved solids in the production system. Various strategies have been explored for managing solid waste in aquaculture, including altering dietary ingredient composition (Prabhu et al., 2019; Prakash et al., 2023), manipulating dietary viscosity (Brinker, 2007; Tran-Tu et al., 2019), and incorporating non-starch polysaccharide (Meriac et al., 2014) and starch (Horstmann et al., 2023b) into the diet. In rainbow trout (*Oncorhynchus mykiss*), dietary inclusion of binders such as guar gum (Brinker, 2007) and insoluble non-starch polysaccharides (Meriac et al., 2014) improved the faecal characteristics and, in turn, the faecal removal efficiency by screen filtration and sedimentation. The improved faecal removal efficiency was shown to be mediated through improved faecal stability and particle size distribution (PSD).

The density of faecal waste is another essential characteristic determining the faecal removal efficiency by sedimentation in RAS, by influencing the density and sinking velocity of faeces (Cortés and Merino, 2020; Wong and Piedrahita, 2000). Additionally, faeces' sinking velocity also affects the dispersion of solid waste in open water systems like cage or raceway farming (Cromey et al., 2012; Pérez et al., 2014). Faecal density depends on its chemical composition and thus can be altered if diet affects the proportion of fractions with differing densities in faeces. An earlier study evaluating the effect of high and low energy (lipid) diets in Atlantic salmon, (*Salmo salar)* did not observe any difference in the density/sinking velocity of faeces and attributed it to the high lipid digestibility, resulting in uniform faecal composition in both treatments (Chen et al., 2003). Most studies investigating the density and sinking velocity of faeces have primarily concentrated on the fate of faecal solid waste within open water systems, such as cages (Chen et al., 1999; Cromey et al., 2002; Magill et al., 2006), with limited attention to its relevance in enclosed systems like RAS. In our previous study (Prakash et al., 2023) aimed at evaluating the effect of different dietary ingredients on faecal removal efficiency by sedimentation in rainbow trout, it was observed that diets resulting in high faecal ash content also had higher faecal removal efficiency. This led us to hypothesise that that altering the dietary ash level by using diamol which is an indigestible ash fraction will result in varying faecal ash content, subsequently impacting the faecal density, sinking velocity and in turn, the faecal removal efficiency by sedimentation. However, this effect of dietary ash content on faecal removal efficiency would also depend on the effect of ash on faecal cohesiveness/stability, which is currently unknown. Accordingly, more detailed studies on the effect of dietary ash content on faecal waste production, faecal characteristics and removal efficiency are warranted.

Starch, often included in the fish diet to serve as a cheap energy source, is shown to impact the quantity and characteristics of faecal waste produced by different fish species. Improvement in faecal removal efficiency was noted in the case of Nile tilapia, (*Oreochromis niloticus)* with increased starch level in diet (Amirkolaie et al., 2006). In contrast, high starch levels in diet reduced faecal removal efficiency in yellowtail kingfish, (*Seriola lalandii)* (Horstmann et al., 2023b), and African catfish, (*Clarias gariepinus)* (Phan et al., 2022). Impact of dietary starch on faecal characteristics of rainbow trout is not known. Starch digestibility in rainbow trout is shown to be influenced by its inclusion level in the diet (Arnesen and Krogdahl, 1993; Bergot, 1979). It is hypothesised that low starch digestibility at high inclusion levels in the case of rainbow trout would result in high faecal starch content with a potential negative impact on the faecal characteristics. Hence, one of the strategies to improve starch digestibility and to mitigate the associated impact on faecal waste production and its characteristics could be to supplement the diet with a starch-digesting enzyme such as α-amylase. However, the negative consequences, such as reduced faecal cohesiveness/stability following amylase supplementation, may also occur, as observed in rainbow trout with supplementation of an enzyme cocktail in the diet (Ogunkoya et al., 2006). Overall, the effect of starch level in the diet on faecal characteristics remains poorly understood and deserves further investigation.

The present study aimed to investigate the effect of starch level, amylase supplementation and ash level in diet on the quantity and characteristics of faeces produced by rainbow trout. To this end, the effect of dietary inclusion of graded levels of gelatinised WF (wheat flour) on nutrient digestibility, faecal removal efficiency and physical characteristics of faeces was assessed. Simultaneously, the potential of amylase to improve starch digestibility, thereby alleviating the impact of dietary starch on faecal characteristics, was also tested. The ability to improve faecal removal efficiency by altering faeces' density and sinking velocity was tested by supplementing the diets with an ash source (diamol).

2. Materials and methods

2.1. Diets

In this experiment, eight dietary treatments were tested and consisted of two parts each having a 2 × 2 factorial design. A basal mixture was prepared, predominantly consisting of ingredients of plant origin such as wheat gluten, soy protein concentrate, soybean meal and pea protein (Table 1). Basal diet was supplemented with crystalline amino acids L-Lysine, DL-Methionine and L-threonine. Additionally, monocalcium phosphate was added to ensure that phosphorus was not a limiting factor. The basal mixture was diluted with gelatinised wheat flour (WF) at 100, 200, 300 and 400 g/kg diet to investigate the effect of dietary starch level, and these diets were referred to as 10-WF, 20-WF, 30-WF and 40-WF respectively. Following dilution of basal diet, the absolute level of protein and energy in resulting diets were lower but the level of specific amino acids expressed as g of amino acid/kg dietary protein would stay similar across dietary treatments as diets were just diluted with WF. The effect of amylase was tested by adding it at 125 k-novo-α-amylase units (KNU)/kg (*Ronozyme HiStarch 900 L*) to the 20-WF and 40-WF diets, which resulted in the first 2 × 2 factorial design. The amylase-supplemented diets were referred as 20-WFα and 40-WFα. To affect the density and sinking velocity of faeces, two high ash diets were

Table 1
The amounts (in g/kg) of ingredients used in the basal mixture.

Ingredients	Inclusion (g/kg, as is)
Wheat gluten	200
Soy protein concentrate	200
Soybean meal	119
Pea protein	200
Fish oil	167
Fish soluble concentrate	32
Mono-calcium-phosphate	39
Chalk ($CaCO_3$)	11
L-Lysine	4
DL-Methionine	7
L-Threonine	2
Premix[†]	19

[†] Premix composition. Vitamins (IU or g kg^{-1} premix): thiamin, 1 g; riboflavin, 1 g; pyridoxine, 1 g; pantothenic acid, 4 g; niacin, 6.5 g; biotin, 0.02 g; cyanocobalamin, 0.017 g, folic acid, 0.33 g; ascorbic acid (as ascorbic acid phosphate), 15 g; DL-alpha tocopherol acetate, 20,000 IU; retinyl palmitate, 300,000 IU; DL-cholecalciferol, 240,000 IU; sodium menadione bisulfite (51%), 1 g; inositol, 40 g; choline, 200 g (given as choline chloride). Minerals (g kg^{-1} premix): iron (as $FeSO_4.7H_2O$), 5 g; zinc (as $ZnSO_4.7H_2O$); 10 g; cobalt (as $CoSO_4.7H_2O$), 0.01 g; copper (as $CuSO_4.5H_2O$), 1 g; Selenium (as Na_2SeO_3), 0.02 g; manganese (as $MnSO_4.4\ H_2O$), 2 g; magnesium (as $MgSO_4.7H_2O$), 50 g; chromium (as Cr Cl3.6H2O), 0.1 g; iodine (as $CaIO_3.6H_2O$) 0.2 g. Preservatives (g kg^{-1} premix): Anti-oxidant BHT (E300-321), 10 g; calcium propionate, 100 g.

prepared by supplementing 20 g diamol/kg to the 10-WF and 30-WF diets, which resulted in the second 2 × 2 factorial design. The ash supplemented diets were referred to as 10-WFA and 30-WFA. Since diets with varying starch levels were selected for estimating the effect of amylase and ash, we could test the effect of three factors, namely dietary starch level, amylase supplementation and ash supplementation and also the interaction effect of amylase and ash with dietary starch levels. Diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) by extrusion using a Clextral BC45 laboratory scale twin-screw extruder (Clextral, Firminy, France) with a 2 mm die, resulting in 3 mm pellets. After extrusion, part of the fish oil (~50 g/kg) in the formula was added to all the experimental diets by vacuum coating (vacuum core coater, Pegasus -10VC, ¼ H/VV nozzle r. 6502) at the Animal Science Group (Wageningen University and Research, The Netherlands). Coating also allowed us to add α-amylase (in liquid form) to the diets together with the fat/oil. For the diets containing amylase, the enzyme was diluted 1:50 with demineralized water to increase the volume and to ensure a homogenous dispersal during coating. To reach the intended enzyme activity (125 KNU/kg diet), 5 mL of diluted α-amylase per kg of feed was added to the oils and coated onto the feed. Averaged over diets, the amylase activity in enzyme-supplemented diet was 94 KNU/kg. The amylase activity was below the detection limit in diets without enzyme supplementation. All experimental diets fulfilled the known nutritional requirements for rainbow trout, according to the NRC, 2011. Yttrium oxide (Y_2O_3) was added (at 0.02%) as an inert marker for digestibility determination (Table 2). Throughout the experimental period, diets were stored at 4 °C. The analysed nutrient composition of experimental diets is provided in Table 2.

2.2. Fish, rearing conditions and housing facilities

The experiment followed the Dutch and European laws on using experimental animals. According to Dutch legislation, the Animal Welfare Body of Wageningen University and Research (The Netherlands) classified this experiment as non-invasive and not an animal-experiment. Juvenile rainbow trout (*Oncorhynchus mykiss*) of mixed sex were obtained from a commercial farm (Mohnen, GmbH, Stolberg, Germany) three weeks before starting the experiment. At the start of the experiment, 600 fish with an average weight of 210 ± 3.8 g (mean ± SEM) were randomly distributed to 24 circular experimental tanks (25 fish per tank) of 0.99 m diameter and 0.48 m depth, resulting in three replicates per dietary treatment. Fish were weighed (Mettler-Toledo ICS429) at the experiment's beginning and end to determine the initial and final weight and calculate growth performance. Fish were starved for a day before being weighed to allow emptying of their gastrointestinal tracts. Water volume in each tank was maintained at 360 L. All tanks were connected to a common RAS, ensuring a similar quality of inlet water. The RAS was equipped with a sump, settling tank, drum filter, protein skimmer, trickling filter and a UV treatment unit identical to that described in Prakash et al. (2023). Each tank was provided with air stones. The outlet was connected to swirl separators (Aqua Optima AS, column height 44 cm; diameter 24.5 cm) to collect faeces and quantify the spilled feed pellets. Water flow rate to each experimental unit was regulated at 7 ± 0.05 L/min using a magnetic inductive flow sensor (SM 6000, IFM electronic, Essen, Germany).

Water quality parameters were monitored daily. Temperature (mean 14.2 °C range 13.9–14.9 °C) and dissolved oxygen (mean 7.8 mg/L, range from 6.5 to 9.1 mg/L) were measured in the outlet water of randomly selected tanks (swirl separator connected to holding tanks) by hand-held digital probe (WTW Multi 3630 IDS - FDO 925). pH (mean 7.4, ranging from 7.0 to 7.7) and electrical conductivity (2.8 millisiemens/cm (mS/cm) ranging between 2.3 and 3.4 mS/cm) (WTW Multi 3630 IDS – Sentix 940) were measured in the sample taken from sump. Other water quality parameters such as TAN, total ammonia nitrogen (Merck, Aquamerck Colorimetric Ammonium test), NO_2-N (Merck Aquamerck, Colorimetric Nitrite test), NO_3-N concentrations (Merck MQuant Nitrate test strips) were maintained below the pre-set range < 2 mg/L, < 1 mg/L, < 80 mg/L, respectively. Water was refreshed to keep the nitrate levels within range. Light intensity was set at 200 lx and photoperiod regime of 12:12 h (light: dark), with lights turning on and off at 8:00 and 20:00 h, respectively.

2.3. Experimental procedures and sampling

Feeding was done restrictively to keep the amount of feed delivered on dry matter (DM) basis per fish equal. This reduces the variation in faecal characteristics due to variability in feed intake (Bromley, 1994; Horstmann et al., 2023a; Staessen et al., 2020). A feeding level of 14 g/$kg^{0.8}$ BW/day (about 90% of expected satiation) was applied based on the mean initial weight over all diets. Daily feed ration per tank was

Table 2
Analysed nutrient composition and enzyme activity of experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
	10-WF	20-WF	30-WF	40-WF	10-WFA	30-WFA	20-WFα	40-WFα
Diet composition								
Basal mixture	889.8	799.8	699.8	599.8	879.8	679.8	799.8	599.8
Yttrium oxide	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Gelatinised wheat flour	100.0	200.0	300.0	400.0	100.0	300.0	200.0	400.0
Diamol	–	–	–	–	20.0	20.0	–	–
Amylase coating (KNU/kg)	–	–	–	–	–	–	125	125
Analysed nutrient content (g/kg DM)								
Dry matter (DM; g/kg)	945	943	939	943	956	954	941	938
Crude protein	536	489	442	400	527	430	490	399
Crude fat	189	163	158	136	181	141	165	128
Total carbohydrate[a]	201	279	340	411	198	350	276	420
Starch + Sugars	104	195	275	349	117	277	198	353
Non-starch polysaccharides[b]	97	84	64	63	82	73	78	67
Ash	75	69	60	54	93	79	69	54
Phosphorus	13.4	12.2	10.5	9.1	13.0	10.2	12.1	9.4
Calcium	12.6	11.5	10.0	8.6	12.6	9.8	11.4	8.6
Magnesium	2.6	2.4	2.0	1.8	2.7	2.2	2.4	1.8
Measured Enzyme activity (KNU/kg)	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	96	92

Notes. 10-WF, 20-WF, 30-WF and 40-WF refer to diets with 10%, 20%, 30% and 40% gelatinised wheat flour respectively, 10-WFA, 10% gelatinised wheat flour with 2% additional ash (diamol); 30-WFA, 30% gelatinised wheat flour with 2% additional ash (diamol), 20-WFα, 20% gelatinised wheat flour with alpha amylase; 40-WFα, 40% gelatinised wheat flour with alpha amylase; KNU, kilo-novo-α-amylase units; b.d., below limit of detection.

[a] Total carbohydrate was calculated as 1000 – crude protein – crude fat – ash content.

[b] Non-stach polysaccharides was calculated as total carbohydrate – (starch + sugars).

increased based on an expected growth using a FCR of 0.9 for all diets. The daily feed portions was split into two equal portions and fed at 9:00 h and 15:00 h. On first day of experiment feeding was done at 50% of the intended ration and was increased to 100% over next two days. Fish were hand-fed and feeding was completed within 1 h. Fifteen minutes after feeding, uneaten/spilled feed pellets were determined by checking bottles attached to the swirl separators. Mortality was checked twice daily before feeding.

For quantifying the dry matter digestibility, apparent digestibility coefficient (ADC) of nutrients and the faecal removal efficiency, faeces were collected by settling for 48 h (excluding the period during feeding and collection of uneaten pellets) during week 5 (Meriac et al., 2014). To avoid bacterial degradation of faeces, bottles attached to swirl separators were submerged in ice slurry. Faecal samples were pooled per tank and stored at −20 °C until further analysis. For determining faecal particle size distribution (PSD), density, sinking velocity and viscosity, samples were collected in bottles attached to swirl separators during week 4 and 5. Sample collection began after ensuring the left over pellets in the fish tank or swirl separator were removed and was done for 3 h between 10:30 h and 13:30 h for all parameters except for viscosity measurement for which collection was done for 1 h duration (between 10.30 h and 11.30 h). The collected faeces were kept on ice till further analysis. A 100 g-feed subsample was pooled for each diet weekly and used for feed composition analysis.

2.4. Analytical methods

Faecal samples collected for digestibility and faeces removal efficiency were dried in the oven at 70 °C until constant weight. The dried faecal samples were ground (Retsch ZM, 200; Retsch GmbH, Haan, Germany) prior to the analysis. The feed and faeces were analysed for chemical composition as per standard methods. Dry matter was estimated by drying at 103 °C until constant mass (ISO 6496, 1999). Ash was determined gravimetrically in a muffle furnace after 4 h of incineration at 550 °C (ISO 5984, 1978). The ash fraction was dissolved in concentrated sulphuric acid by autoclaving (121 °C, 20 min) to determine minerals such as phosphorus, calcium, magnesium and yttrium by inductively coupled plasma optical emission spectrometry, following Dutch analytical standards (NEN 15510:2017). The total nitrogen content was measured by the Kjeldahl-method (ISO 5983, 1997), calculating crude protein as N × 6.25 (protein conversion factor). Crude fat was determined gravimetrically using acid hydrolysis followed by extraction with petroleum-ether (Soxhlet method; ISO 6492, 1999). Gross energy was measured by bomb calorimetry (C7000; IKA®-Werke GmbH & Co. KG). Starch, including free sugar fraction in feed and faeces were determined enzymatically using amyloglucosidase without ethanol extraction for removing free sugars (Goelema et al., 1998).

The faecal PSD was measured using different mesh size sieves (1600 μm, 850 μm, 300 μm, 100 μm and 40 μm) following the protocol described in Prakash et al. (2023). PSD was determined for undisturbed faecal waste (hereafter termed non-stressed faeces) and faeces exposed to mechanical stress (hereafter termed stressed faeces). Change in faecal PSD following exposure to mechanical stress provided an estimate of faecal stability/consistency. To determine the mass of collected organic matter (OM) fractions on filters, filters were dried and incinerated as described for feed and faeces samples. Faecal PSD data was expressed on mass % basis (on organic matter basis) for each fraction for non-stressed and stressed faecal waste.

To measure density of faecal pellets, salt solutions of varying concentration ranging from 50 to 90‰ with a gradation of 2.5‰ were prepared by dissolving analytical grade sodium chloride in demineralized water. The density of salt solutions was measured with Anton Parr FPH-Density meter DMA5000 and ranged from 1.035 to 1.065 g/cm^3. Faecal pellets collected in the settling bottles were then gently dropped in cups containing solutions of varying density. Five randomly selected faecal pellets were dropped in solutions corresponding to each density, starting from the lowest-density salt solution. Ten seconds after entering the water, the number of floating pellets were counted. Measurement stopped if two consecutive salinities gave all 5 floating pellets. Subsequently, the average density of faecal pellets per tank was calculated as per the formula detailed in the calculations section.

Sinking velocity of faecal particles was measured using a UFT type settling column [see Amirkolaie, 2013 for details on the column features]. The time taken by faecal particles to descend through a depth of 30 cm length in the middle portion of the column was used for calculating sinking velocity. This reduces the possibility of inaccuracy caused by the initial acceleration of faecal particles after dropping. To ensure that the drag effect from the column sidewalls did not affect the results, measurements of faecal particles that fell along the wall of the settling column were excluded. UFT column was filled with 3.5‰ sodium chloride solution at 12 °C and the sinking velocity of 10 randomly selected faecal particles (size range 0.4 cm to 2.1 cm) from each tank was measured.

To measure faeces' viscosity, faecal pellets collected in the settling bottle were gently poured over a 20 μm sieve. The bottom of the sieve was blot-dried with filter paper to remove water droplets sticking to the sieve surface. Following this, a small portion of faeces was centrifuged at 10,000 *g* for 10 min at room temperature. The supernatant obtained was used for viscosity measurement with Brookfield LVDV-I + cone/plate viscometer (Brookfield Engineering laboratories, Middleboro, MA, USA). All viscosity measurements were carried out at 14 °C and a shear rate of 20–100 s^{-1}. Absolute viscosity was expressed in centipoise (cP) at a shear rate of 100 s^{-1}.

2.5. Calculations

Absolute weight gain (WG, g/fish) was estimated as the difference between the mean individual final (W_f) and initial (W_i) body weight per fish. Feed intake per fish per day (FI, g/fish/day) was calculated using the formula:

$$\mathrm{FI} = \frac{\text{Total DM feed offered each day} - \text{Uneaten DM feed each day}}{\text{Fish number}}$$

FI was summed for the whole period to obtain feed intake per fish over the entire experimental period (FI_{tot}).

Specific growth rate (SGR; %BW/d) was calculated as:

$$\mathrm{SGR} = \frac{\left[ln\left(\mathrm{W_f}\right) - ln(\mathrm{W_i})\right]*100}{\mathrm{t}}$$

where t is the duration of trial in days.

The feed conversion ratio (FCR) on dry matter basis was calculated as:

$$\mathrm{FCR} = \frac{\mathrm{FI_{tot}(g\ DM/fish)}}{\mathrm{WG\ (g/fish)}}$$

The dry matter ADC of diets was calculated as follows:

$$\mathrm{ADC_{DM}}(\%) = 100*\left[1 - \left(\mathrm{Y_{diet}}/\mathrm{Y_{faeces}}\right)\right]$$

where Y_{diet} and Y_{faeces} is the concentration of yttrium in diet and faeces respectively expressed on DM basis.

The ADC of macronutrients and macro-minerals of diets were calculated according to the formula described by Cheng and Hardy (2002):

$$\mathrm{ADC}\ (\%) = 100*\left[1 - \left(\mathrm{Y_{diet}}*\mathrm{N_{faeces}}\right)/\left(\mathrm{Y_{faeces}}*\mathrm{N_{diet}}\right)\right]$$

Where N_{diet} and N_{faeces} represent the nutrient percentage (g/kg DM or kJ/g DM gross energy) of the diet and faeces respectively.

Carbohydrate content in feed and faeces was determined by the difference as [1000 – (crude protein + fat + ash)]. NSP level is calculated as the difference between the carbohydrate and starch content.

Faecal waste production (g DM/kg DM FI) was calculated on dry

matter basis as the amount of non-digested feed per kilogram feed intake as:

$$\text{Faecal waste production (g DM/Kg DM FI)} = (100\% - \text{ADC}_{\text{DM}})*1000$$

Faecal removal efficiency (FR, %) was estimated as the percentage of total faeces collected by settling in proportion to total faecal waste produced. In detail, this was calculated based on yttrium collected in settled faeces ($Y_{removed}$, g) in relation to the amount of yttrium supplied by diet (Y_{diet}, g) as:

$$\text{FR } (\%) = (Y_{\text{removed}}/Y_{\text{diet}})*100\%$$

Non-removed faeces (g DM/kg DM FI) was calculated as the difference between the total amount of faeces produced and faeces removed as:

$$\text{Non} - \text{removed faeces} = [(100\% - \text{ADC}_{\text{DM}})*(100\% - \text{FR})]*1000$$

Average density of faecal pellets was calculated as:

$$\text{Average density} = \frac{\sum_{i=1}^{n} P_i * D_i}{\sum_{i=1}^{n} P_i}$$

$P_i = (x_{i+1}) - (x_i)$, wherein x_i is the number of faecal pellets floating in i_{th} solution

$$D_i = \frac{d_i + d_{i+1}}{2}$$

wherein d is the density of solution.

2.6. Statistical analysis

Tanks were the experimental units ($n = 24$) in the statistical analysis. SAS 9.4, SAS Institute, North Carolina, USA was used for all statistical analysis. Preliminary, data of the diets 10-WF, 30-WF, 10-WFA and 30-WFA were tested for the effect of WF, ash and their interaction by two-way ANOVA ($n = 12$) and data of diets 20-WF, 40-WF, 20-WFα and 40-WFα for the effect of WF, amylase and their interaction also by two-way ANOVA (n = 12). This preliminary analysis showed that for only 5 of the 94 tested parameters, an interaction effect was present. As all eight diets were tested at the same time (i.e., same rearing conditions, genetics, etc.) it was decided to test the main effects of WF, ash and amylase supplementation at once ($n = 24$) without any interaction effect and start the results sections first with the relevant interactions effect followed by presenting the main effect. The following model was used (by PROC GLM):

$$Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl}$$

where, Y_{ijkl} is the measure value; μ is overall mean; WF_i = fixed effect of wheat flour supplementation i ($i = 1,.., 4$); Ash_j is fixed effect of ash supplementation j (j = 1, 2); $Amylase_k$ is fixed effect of amylase supplementation k (k = 1, 2); and e_{ijkl} is error term being the variation between replicates within diets (k = 1, 2, 3). When the effect of WF was significant ($P < 0.05$) pair wise comparison of WF means was done by Tukey's multiple comparison test.

3. Results

Interaction effects between WF inclusion levels and amylase supplementation and WF inclusion levels and ash supplementation (Supplementary Table S1, S4): For all performance traits (growth, SGR, FCR), no

Table 3
Main effect of gelatinised wheat flour (WF) level in the diet on performance parameters, nutrient digestibility and faecal characteristics of rainbow trout fed restrictively during the experimental period (35 days).

	10-WF	20-WF	30-WF	40-WF	Pooled SEM	*P*-value
Growth performance						
Initial body weight (g/fish)	207	208	208	211	3.1	ns
Final body weight (g/fish)	403^{b}	397^{b}	385^{a}	373^{a}	4.1	***
Weight gain (g/fish)	197^{c}	189^{c}	177^{b}	163^{a}	2.0	***
Feed intake (g DM/fish/day)	4.35	4.35	4.35	4.35		
Digestible energy intake (kJ/fish/day)	91^{d}	89^{c}	84^{b}	80^{a}	0.3	***
Growth (g/day)	5.6^{c}	5.4^{c}	5.0^{b}	4.6^{a}	0.06	***
SGR	1.91^{c}	1.84^{c}	1.75^{b}	1.63^{a}	0.021	***
FCR	0.77^{a}	0.81^{a}	0.86^{b}	0.93^{c}	0.009	***
Survival (%)	100	100	100	100		
Digestibility (%)						
Dry matter	82.3^{c}	82.3^{c}	79.7^{b}	78.5^{a}	0.31	***
Organic matter	86.9^{c}	86.4^{c}	83.3^{b}	81.7^{a}	0.29	***
Crude protein	96.1	96.0	95.7	95.6	0.13	#
Crude fat	95.2^{b}	94.6^{b}	93.7^{ab}	93.2^{a}	0.25	***
Total carbohydrate	54.4^{a}	65.0^{b}	63.0^{b}	65.5^{b}	0.76	***
Starch + sugar	90.1^{b}	89.8^{b}	82.5^{a}	81.0^{a}	0.74	***
Ash	33.0	34.1	33.2	32.4	0.79	ns
Phosphorus	48.9	49.5	49.8	49.4	0.56	ns
Calcium	6.6^{a}	8.4^{b}	9.4^{b}	11.2^{b}	1.10	**
Magnesium	51.7^{a}	54.4^{b}	54.2^{b}	55.6^{b}	0.54	**
Energy	89.9^{c}	89.2^{c}	86.4^{b}	84.8^{a}	0.28	***
Faecal characteristics						
Density (g/cm^3)	1.053^{ab}	1.054^{a}	1.051^{b}	1.053^{ab}	0.0008	*
Sinking velocity (cm/s)	3.56	3.32	3.23	3.12	0.148	ns
Viscosity (cP)	1.58^{a}	1.78^{b}	1.83^{b}	1.96^{c}	0.025 s	***

Notes. 10-WF, 10% gelatinised wheat flour; 20-WF, 20% gelatinised wheat flour; 30-WF, 30% gelatinised wheat flour; 40-WF, 40% gelatinised wheat flour; FCR, feed conversion ratio (on DM basis); SGR, specific growth rate; pooled SEM, pooled standard error of the mean.
Values are least square means of the main effect of gelatinised WF level in the diet from the model $Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl}$. ns, not significant, $P > 0.1$; #, tendency, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Means along a row lacking common superscript letter differ significantly, $P < 0.05$ (Tukey's post-hoc test).

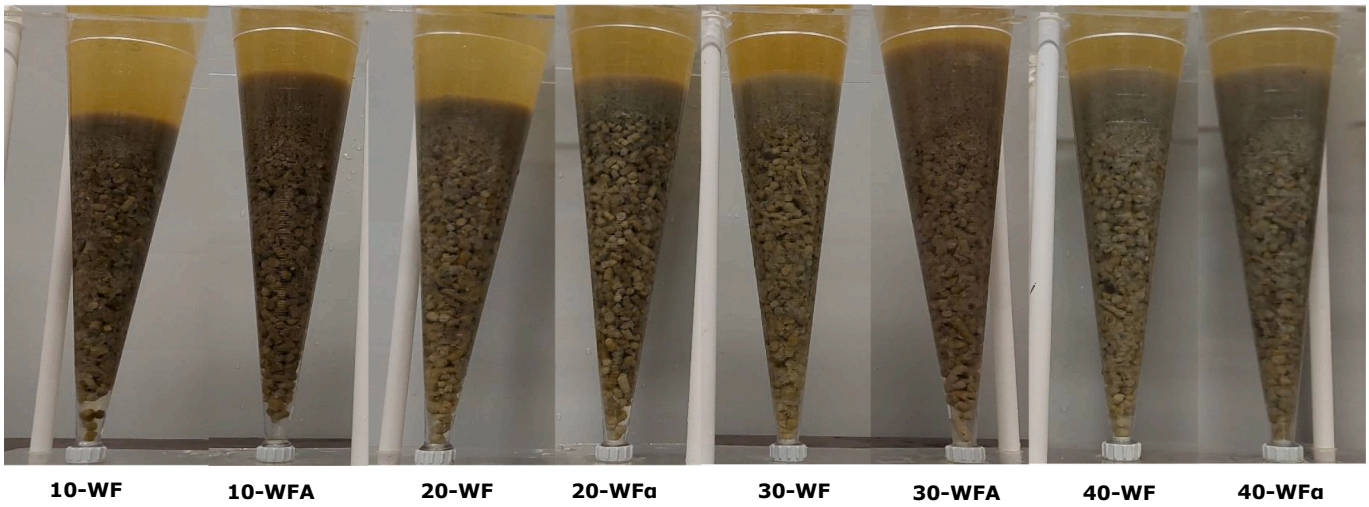

Plate 1. Appearance of overnight collected faeces of rainbow trout following transfer to see-through Imhoff cones.
Each cone represents faecal waste collected from one treatment of each experimental diet. 10-WF, 10% gelatinised wheat flour; 20-WF, 20% gelatinised wheat flour; 30-WF, 30% gelatinised wheat flour; 40-WF, 40% gelatinised wheat flour; 10-WFA, 10% gelatinised wheat flour with 2% additional ash (diamol); 30-WFA, 30% gelatinised wheat flour with 2% additional ash (diamol), 20-WFα, 20% gelatinised wheat flour with α-amylase; 40-WFα, 40% gelatinised wheat flour with α-amylase.

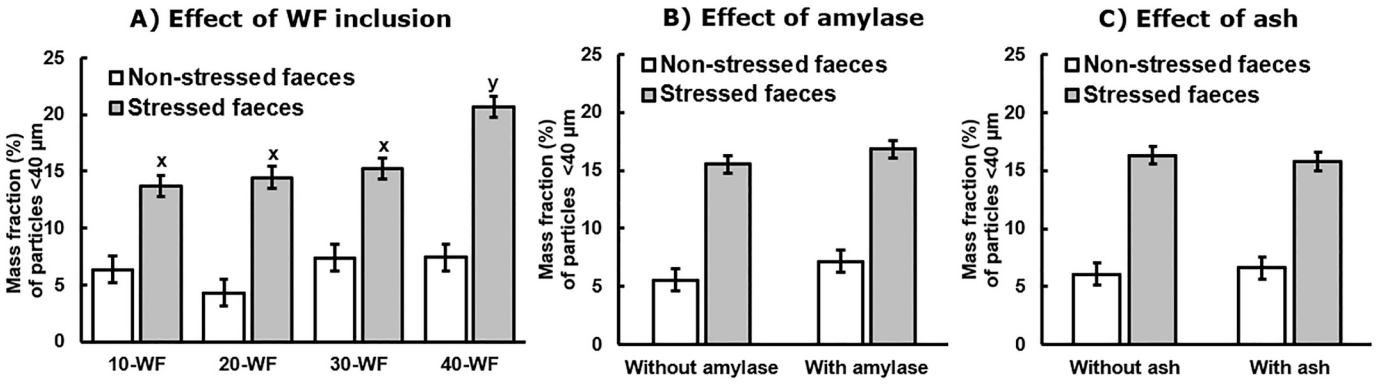

Fig. 1. Main effect of wheat flour (WF) inclusion (A), amylase supplementation (B) and ash level (C) on mass fraction (%) of faecal particles <40 µm in non-stressed and stressed faeces of rainbow trout fed restrictively during the experimental period (35 days).
10-WF, 10% gelatinised wheat flour; 20-WF, 20% gelatinised wheat flour; 30-WF, 30% gelatinised wheat flour; 40-WF, 40% gelatinised wheat flour. Values are least square means of the main effects (A. WF; B. amylase; C. ash) in the diet from the model $Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl.}$. Error bars are pooled standard error of the mean (pSEM). Different superscripts (lower case, x or y, above the bars) indicate dietary differences for mass fraction (%) of faecal particles <40 µm in stressed faeces of rainbow trout for varying levels of gelatinised wheat flour ($P < 0.001$) in the diet.

significant interaction was noted between WF and amylase supplementation (Supplementary Table S1) or WF and ash supplementation (Supplementary Table S4). Crude protein digestibility was negatively affected by 40-WF inclusion when no amylase was supplemented in the diet, while not being affected when amylase was included in the diet (Supplementary Table S1). This resulted in an observed interaction effect between starch and amylase for crude protein digestibility. Starch and calcium ADC was influenced by an interaction between WF inclusion level and ash supplementation (Supplementary Table S4). Ash supplementation increased Ca digestibility at 10% WF inclusion diets but did not influence Ca ADC when 30% WF was included in the diet.

*Effect of WF inclusion (*Table 3*,* Plate 1*,* Fig. 1A, 2A, 3A*):* No fish died during the experiment. Fish weight almost doubled during the experimental period of 35 days. Increasing WF inclusion in the diet had a negative impact on growth (Table 3). Since the feed intake on dry weight basis was similar across the treatments, reduced growth at high WF inclusion (30-WF and 40-WF) resulted in a higher FCR and a lower SGR ($P < 0.001$). FCR at 40-WF (0.93) was 20% poorer than at 10-WF (0.77).

The ADC of DM, protein, fat, starch and energy declined as the WF inclusion increased ($P < 0.01$; Table 3). Compared to the decline in digestibility of fat (2.0% points) and crude protein (0.5% points), the drop in starch digestibility (9.1% points) was much larger between 10-WF and 40-WF. Ash and phosphorus digestibility were unaffected by increasing WF inclusion ($P > 0.1$). Similar feed intake but declining energy digestibility with increasing WF inclusion resulted in 12% lower digestible energy intake at 40-WF compared to 10-WF.

Density of faecal particles was affected by the WF inclusion level ($P < 0.05$; Table 3) but no particular trend was evident. Numerically lower but statistically similar ($P > 0.1$; Table 3) sinking velocity of faecal pellets was observed with the increasing WF inclusion level in the diet (Table 3). Faecal viscosity increased with increasing WF inclusion level in the diet ($P < 0.001$; Table 3).

From visual observation of faeces in settling cone (representing stressed faeces, Plate 1), 40-WF appeared to have greater share of faecal fines, pulpy and less firm. However, this did not reflect in the particle size distribution analysis of faecal waste under non-stressed scenarios for any size class (Table S7) across varying WF inclusion levels ($P > 0.1$). Nevertheless, in line with our observation of increased faecal fines in settling cones at high WF inclusion, significantly higher proportion of particles of small size faecal particles (<40 µm size) was found at 40-WF

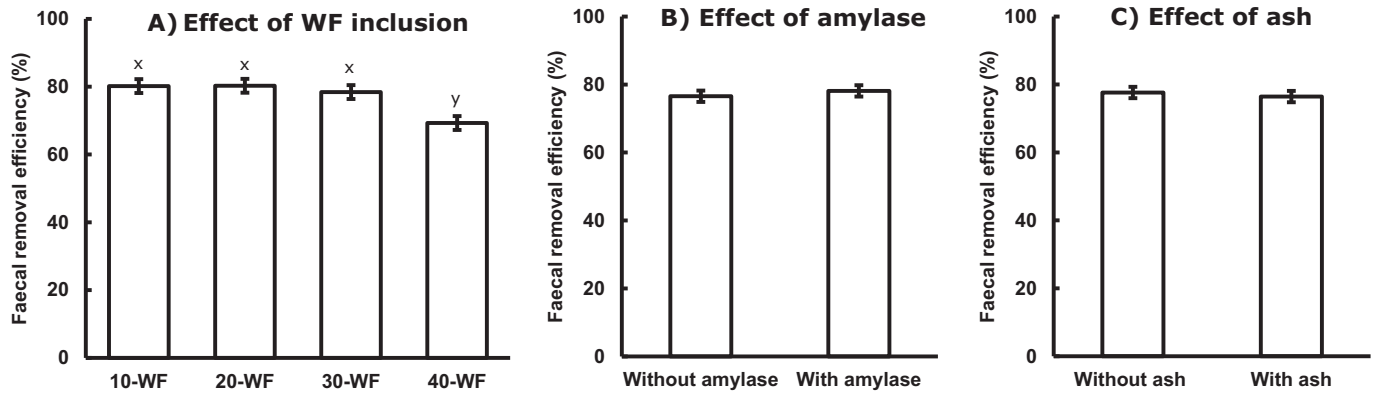

Fig. 2. Main effect of wheat flour (WF) inclusion (A), amylase supplementation (B) and ash level (C) on faecal removal efficiency (%) of rainbow trout fed restrictively during the experimental period (35 days).
10-WF, 10% gelatinised wheat flour; 20-WF, 20% gelatinised wheat flour; 30-WF, 30% gelatinised wheat flour; 40-WF, 40% gelatinised wheat flour.Values are least square means of the main effects (A. WF; B. amylase; C. ash) in the diet from the model $Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl}$. Error bars are pooled standard error of the mean (pooled SEM). Effect of WF was significant at $P < 0.01$, and different letters (lower case, x or y, above the bars) indicate statistically significant dietary differences (Tukey's post-hoc test).

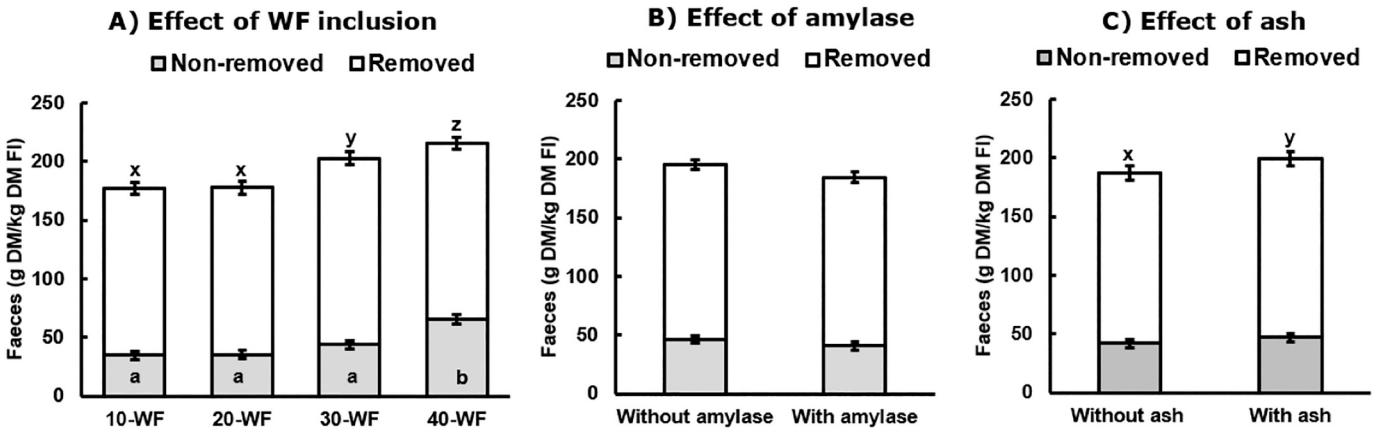

Fig. 3. Main effect of wheat flour (WF) inclusion (A), amylase supplementation (B) and ash level (C) on total faeces produced, removed and non-removed faeces (g DM/kg DM feed intake) in rainbow trout fed restrictively during the experimental period (35 days).
10-WF, 10% gelatinised wheat flour; 20-WF, 20% gelatinised wheat flour; 30-WF, 30% gelatinised wheat flour; 40-WF, 40% gelatinised wheat flour. Total amount of faeces produced (entire bar), removed (white) and non-removed (grey) faeces per kg dry matter feed intake (g DM/kg DM feed intake).
Values are least square means of the main effects (A. WF; B. amylase; C. ash) in the diet from the model $Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl}$. Error bars are pooled standard error of the mean (pooled SEM). Different superscripts (lower case, a or b) in grey bars indicate dietary differences for non-removed faeces ($P < 0.01$) for varying levels of gelatinised wheat flour. Different superscripts (lower case, x, y and z) labelled above the bars indicate dietary differences for total faeces produced for varying levels of gelatinised wheat flour ($P < 0.001$) and ash level ($P < 0.01$) in the diet.

upon exposure to mechanical stress (Fig. 1A). The proportion of large size faecal particles (>1600 μm) reduced almost to half at 40-WF (19%) compared to 10-WF (35%) under stressed scenario ($P < 0.1$, Supplementary Table S7), implying that high WF inclusion in diet has a negative impact on cohesiveness/stability of faecal pellets.

WF inclusion in the diet reduced removal efficiency ($P < 0.01$; Fig. 2A) and increased the amount of faecal waste produced ($P < 0.001$, Fig. 3A) and the amount of non-removed faeces ($P < 0.001$, Fig. 3A) accumulating in the system. Amount of faecal waste produced was 22% higher at 40-WF (215 g DM/kg DM FI) than at 10-WF (177 g DM/kg DM FI). Lower faecal removal efficiency ($P < 0.05$) was observed at highest inclusion level (40-WF). Due to the combined effect of increased faecal waste production and reduced removal efficiency, the amount of non-removed faeces at 40-WF (66 g DM/kg DM FI) was almost twice as that of at 10-WF (35 g DM/kg DM FI).

*Effect of amylase (*Table 4, Plate 1, Fig. 1B, 2B, 3B*):* No effect of amylase supplementation in diet on growth performance and FCR were found (Table 4). Amylase supplementation enhanced the digestibility of starch fraction of the diet by 4 percentage points ($P < 0.001$, Table 4). Digestibility of crude protein, fat, total carbohydrate and ash fraction remained unaffected by the amylase supplementation ($P > 0.05$, Table 4). PSD of faecal waste was not altered by amylase supplementation in the diet under non-stressed scenario ($P > 0.1$, Supplementary Table S8). Following exposure to mechanical stress, larger proportion of particles in 40–100 μm size range and smaller proportion of particles in 850–1600 μm size range were found in enzyme supplemented group ($P < 0.05$, Supplementary Table S8), indicating that amylase supplementation has a negative impact on faecal stability. No interaction effect ($P > 0.1$) between WF and amylase for the PSD of faeces under stressed or non-stressed scenarios was observed (Supplementary Table S2). Amylase supplementation in the diet did not alter the faecal characteristics such as density, sinking velocity or viscosity of faeces (Table 4). Despite an improvement in starch digestibility with amylase supplementation, non-significant effect of amylase supplementation on dry matter digestibility, resulted in statistically similar amount of faecal waste production at un-supplemented and supplemented group

Table 4
Main effect of amylase supplementation in diet on performance parameters, nutrient digestibility and faecal characteristics of rainbow trout fed restrictively during the experimental period (35 days).

	Without amylase	With amylase	Pooled SEM	*P*-value
Growth performance				
Initial body weight (g/fish)	210	207	2.7	ns
Final body weight (g/fish)	390	389	3.6	ns
Weight gain (g/fish)	180	182	1.7	ns
Feed intake (g DM /fish/day)	4.35	4.35	–	–
Digestible energy intake (kJ/fish/day)	85	86	0.2	*
Growth (g/day)	5.1	5.2	0.05	ns
SGR	1.77	1.81	0.018	ns
FCR	0.85	0.84	0.008	ns
Survival (%)	100	100		
Digestibility (%)				
Dry matter	80.5	80.9	0.25	ns
Organic matter	84.3	84.8	0.24	ns
Crude protein	95.8	95.9	0.09	ns
Crude fat	94.2	94.1	0.20	ns
Total carbohydrate	61.4	62.5	0.62	ns
Starch + sugar	83.8	87.8	0.55	***
Ash	32.9	33.5	0.61	ns
Phosphorus	49.2	49.6	0.44	ns
Calcium	9.2	8.6	0.80	ns
Magnesium	53.9	54.0	0.47	ns
Energy	87.4	87.8	0.22	ns
Faecal characteristics				
Density (g/cm^3)	1.053	1.052	0.0007	ns
Sinking velocity (cm/s)	3.23	3.39	0.116	ns
Viscosity (cP)	1.80	1.77	0.022	ns

Notes. FCR, feed conversion ratio (on DM basis); SGR, specific growth rate; Pooled SEM, pooled standard error of the mean; Values are least square means of the main effect of amylase supplementation in the diet from the model $Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl}$. ns, not significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 3B). Amylase supplementation in diet did not alter the faecal removal efficiency ($P > 0.1$, Fig. 2B) or the amount of non-removed faeces accumulating in the system ($P > 0.1$, Fig. 3B).

*Effect of ash (*Table 5*,* Fig. 1C, 2C, 3C*):* Increased dietary ash had a negative effect on the growth performance and feed utilisation parameters of rainbow trout (Table 5). Ash supplementation in diet reduced the dry matter digestibility of diet, but the organic matter digestibility remained unchanged. Ash supplementation resulted in a tendency towards reduced protein digestibility ($P < 0.1$), but did not influence the digestibility of fat and phosphorus ($P > 0.1$). Mean ash content in faeces of group with ash (298 g/kg DM) was 34% higher than in group without ash (222 g/kg DM) (Supplementary Table S12). Manipulation of faecal ash content by diamol inclusion in diet resulted in a higher density and sinking velocity of faeces ($P < 0.001$; Table 5). Under both stressed and non-stressed scenarios, proportion of faecal particles <40 μm size did not change in response to ash supplementation in the diet ($P > 0.1$, Fig. 1C). Ash supplementation resulted in higher faecal waste production ($P < 0.01$). Despite the effect on density and sinking velocity, dietary ash supplementation did not influence the faecal removal efficiency ($P > 0.1$, Fig. 2C) or the amount of non-removed faeces accumulating in the system ($P > 0.1$, Fig. 3C).

4. Discussion

The present study focused on quantifying the effect of different dietary starch levels, amylase supplementation and ash supplementation and the interaction of dietary starch with amylase supplementation and ash supplementation on faecal waste production and faecal characteristics in rainbow trout. Consequently, the results on growth, FCR and nutrient digestibility are discussed briefly or discussed in parlance with their relevance to faecal waste production and faecal characteristics.

4.1. Effect of dietary starch level

In this study, the dietary starch level was varied by increasing the inclusion of WF in the basal mixture. Since the major nutrient fraction varied by the WF inclusion level was the dietary starch content, the effect obtained by WF inclusion is discussed as the effect of dietary starch levels.

Among the several factors regulating growth and FCR in cultured fish species, one of the most critical parameters is the diet's nutrient composition. Increasing dietary WF inclusion level reduced dietary energy content and the digestibility of lipid, protein and starch. This effect is reflected in lower digestible energy intake with increasing dietary starch levels (Table 3). These observations, combined with the earlier report of lower utilisation efficiency of carbohydrate as an energy source by rainbow trout at high inclusion levels (Schrama et al., 2018), explain the reduced growth and increased FCR at high dietary starch levels. Higher FCR with increased inclusion of starch means that a greater quantity of feed will be required to achieve the same production target, simultaneously enhancing the amount of faecal waste produced. Apart from the environmental consequences of waste discharge into natural waters, increased faecal waste production has financial implications for production systems, as waste management practices incur additional costs.

Concerns regarding waste production and discharge from aquaculture production systems coupled with the interest to explore potential usage of the sludge is stimulating the adoption of innovative strategies for waste management within this industry (Aas, 2021; Krogli, 2023; Lindland et al., 2019; Olaussen, 2018). The amount of faecal waste produced follows the dry matter digestibility of the diet. In this study, the decline in dry matter digestibility by 4.8% from 10-WF (82.3%) to 40-WF (78.5%) resulted in 22% higher faecal waste production at 40-WF (216 g DM/kg DM FI) than at 10-WF (177 g DM/kg DM FI). Since diets in this study were diluted with gelatinised WF without altering the ratio

Table 5
Main effect of ash supplementation in diet on performance parameters, nutrient digestibility and faecal characteristics of rainbow trout fed restrictively during the experimental period (35 days).

	Low ash	High ash	Pooled SEM	*P*-value
Growth performance				
Initial body weight (g/fish)	210	207	2.7	ns
Final body weight (g/fish)	394	385	3.6	#
Weight gain (g/fish)	184	178	1.7	*
Feed intake (g DM /fish/day)	4.35	4.35		
Digestible energy intake (KJ/fish/day)	86	85	0.2	**
Growth (g/day)	5.3	5.1	0.05	*
SGR	1.80	1.77	0.018	ns
FCR	0.83	0.86	0.008	*
Survival (%)	100	100		
Digestibility (%)				
Dry matter	81.3	80.1	0.25	**
Organic matter	84.4	84.7	0.24	ns
Crude protein	96.0	95.7	0.09	ns
Crude fat	94.1	94.3	0.20	ns
Total carbohydrate	60.7	63.2	0.62	*
Starch + sugar	84.4	87.3	0.55	**
Ash	36.8	29.5	0.61	***
Phosphorus	49.8	49.0	0.44	ns
Calcium	7.9	9.8	0.80	ns
Magnesium	54.7	53.2	0.47	*
Energy	87.4	87.8	0.22	ns
Faecal characteristics				
Density (g/cm^3)	1.049	1.057	0.0007	***
Sinking velocity (cm/s)	2.910	3.705	0.116	***
Viscosity (cP)	1.80	1.77	0.022	ns

Notes. FCR, feed conversion ratio (on DM basis); SGR, specific growth rate; pooled SEM, pooled standard error of the mean; Values are least square means of the main effect of ash level in the diet from the model $Y_{ijkl} = \mu + WF_i + Ash_j + Amylase_k + e_{ijkl}$. ns, not significant, $P > 0.1$; #, tendency, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

between the other dietary ingredients, the variability in faecal waste produced could be attributed to the increasing WF supplementation and the declining starch digestibility. The drop in crude fat and protein digestibility may have partially contributed to the enhanced faecal waste production. However, their effect would be less pronounced since their concentration in diets following dilution diminished. Reduced starch ADC at inclusion above 20-WF aligns with the earlier reported inverse relationship between the inclusion levels of starch source and the salmonids' ability to digest it (Hua and Bureau, 2009). Overall (including all 8 diets) starch ADC values (84%), as observed in this study, are much lower than the previously reported values (> 95%) for rainbow trout (Groot et al., 2021; Groot et al., 2022). Variability in starch digestibility values across studies has been reported earlier and linked to the variation in feed processing settings like extrusion temperature, source, inclusion level, and degree of gelatinization of starch (Hua and Bureau, 2009). In summary, the findings of this study demonstrate that high dietary starch level negatively affects nutrient digestibility, reduces dry matter digestibility and thus leads to increased faecal waste production in rainbow trout.

Next to the amount of faecal waste produced, the removal efficiency of egested faeces regulates the quantity of non-removed faeces accumulating in the production system. The decline in faecal removal efficiency with high starch inclusion level at 40-WF aligns with the previously reported negative impact of starch level in the diet on faecal removal efficiency in carnivorous yellowtail kingfish (Horstmann et al., 2023b) and omnivorous African catfish (Phan et al., 2022). Starch and its digestion products can alter the chyme osmolality and consequently, water balance in the gut (Elesho et al., 2022; Harter et al., 2013) which may have consequences for faecal characteristics (Hu et al., 2016). For instance, we observed increased faecal starch content, leading to a subsequent elevation in faecal viscosity as the dietary starch level increased (Table 3, Fig. S1). In line with the findings of our study regarding faecal removal efficiency, an increase in dietary viscosity with guar gum lowered faecal removal efficiency in case of Pangasius, (*Pangasionodon hypophthalmus)* but the faecal viscosity was not reported in that study (Tran-Tu et al., 2020). In contrast, Brinker (2007) documented a simultaneous increase in the viscosity of faeces and an improvement in their stability with increasing supplementation of viscous guar gum in rainbow trout diet. This discrepancy suggests a potential variation in the influence of faecal viscosity on faecal removal efficiency, contingent upon the source and strength of the viscosity and fish species investigated. An increase in chyme water content in response to higher chyme osmolality may also result in poor faecal quality, ultimately affecting the faecal removal efficiency. However, the results from studies investigating the effect of dietary starch levels on faecal dry matter were often conflicting, and the variability in diet composition between those studies complicates drawing a definitive conclusion (Ciavoni et al., 2023; Elesho et al., 2022; Harter et al., 2013). Recently, Ciavoni et al. (2023) measured the DM in the chyme from distal intestine of rainbow trout and found higher DM in groups fed with high-starch high-fat diet than in groups receiving a low-starch low-fat diet. Thus, it is unclear whether starch's impact on faecal removal efficiency results from the change in chyme DM. Another hypothesis concerns microbiota-induced fermentation of undigested starch leading to trapping of gases in the faecal strand, thus impacting the binding of faeces and generating more faecal fines (Amirkolaie et al., 2006). In our earlier study utilizing the same RAS set up, faecal PSD data of undisturbed faeces showed strong correlation ($r = 0.88$) with faecal removal efficiency in rainbow trout (Prakash et al., 2023). In contrast, the faecal PSD of non-stressed faeces in the current study did not show any difference between WF inclusion level (Supplementary Table S7), making it unlikely that fermentation and, thus faecal PSD was the determining factor for lower removal efficiency at 40-WF. Apart from the faecal PSD, faeces' density and sinking velocity can influence their removal efficiency while using sedimentation. Differences in density of faeces could not explain the sinking velocity of faeces as there was no particular trend

discernible when looking at the main effect of WF inclusion level (Table 3). Overall, the increased dietary starch level negatively impacts faecal removal efficiency, but delineating the exact mechanism involved would need further investigation.

Another aspect of faecal characteristics impacting faecal removal or its dispersion in closed and open systems respectively is its stability, i.e. the ability to withstand exposure to currents/turbulence (mechanical force). In the current study, the diet 40-WF had reduced faecal stability, evident with an increased proportion of particles <40 μm upon exposure to mechanical stress (Fig. 1A, Supplementary Table S7). This aspect of faecal waste has implications for system design, as the breakdown rate would depend on the intensity of exposure to turbulent hydrodynamic forces before reaching the removal unit. For example, a lower solid removal efficiency was reported in a raceway system where breakdown of faecal solids occurred due to turbulence induced by aeration or waterfall (Brinker and Rösch, 2005). Faecal stability is also critical for facilitating the collection of faeces from tarpaulin-covered cages namely, closed containment system (Nilsen et al., 2020).

From a management perspective, minimizing the amount of non-removed faeces is desirable in a RAS. In this study, the combined effect of higher faecal waste production and lower removal efficiency at 40-WF resulted in a greater amount of non-removed faeces at 40-WF than at other dietary starch levels (Fig. 3A). Our finding aligns with earlier report on the effect of dietary starch level on the amount of non-removed faeces in yellowtail kingfish (Horstmann et al., 2023b). In conclusion, the study showed that lowering dietary starch levels offers efficient solid waste management prospects.

4.2. Effect of amylase

Amylase supplementation in the diet increased the starch digestibility but did not influence growth. The growth did not improve despite an improvement in digestible energy intake with amylase-supplemented diets (Table 4). Similarly, Carter et al. (1992) did not observe any effect of amylase supplementation on growth and feed utilisation in Atlantic salmon, (*Salmo salar)*. Kumar et al. (2006) reported improvement in dry matter digestibility of Rohu, (*Labeo rohita)* following amylase supplementation in diets with non-gelatinized corn but not with gelatinized corn. Increased starch digestibility with amylase supplementation did not alter the faecal waste production or its characteristics, such as viscosity, density, sinking velocity and particle size distribution of unstressed faeces. This finding seems in conflict with our argumentation in the previous section that the increased quantity of undigested starch fraction was the main driver for increased faecal waste production or its impact on faecal characteristics. However, it is pertinent to mention that due to the combined effect of higher starch inclusion and lower digestibility, faecal starch content increased 6-fold between the 10-WF and 40-WF diet groups (Supplementary Table, S10). In contrast, the difference in faecal starch content of amylase-supplemented and non-supplemented groups was only 1.3 times (Supplementary Table, S11). Therefore, it is suggested that the ability of starch to impact faecal removal efficiency depends on the faecal starch content and not merely on the dietary starch levels. The inability of amylase supplementation to impact the faecal waste production and its removal efficiency explains our observation of similar amount of non-removed faeces for amylase-supplemented and non-supplemented groups. An increased proportion of small particles (40–100 μm) and a decreased proportion of large particles (850–1600 μm) under a stressed scenario (Supplementary Table S8) for the supplemented group suggested that amylase supplementation lowered faecal stability. This finding aligns with the previously reported adverse effect of supplementation of enzyme cocktail (containing xylanase, amylase, cellulase, protease, and β-glucanase) on faecal cohesiveness in rainbow trout (Ogunkoya et al., 2006). Overall, this study demonstrated that dietary amylase supplementation increases starch digestibility. However, observed differences were not large enough to influence the amount of faecal waste produced, its removal rate or the non-removed faeces accumulating in the system.

4.3. Effect of ash

We had observed higher faecal removal efficiency by sedimentation with dietary ingredients producing faeces with high ash content in our earlier study (Prakash et al., 2023). Based on that finding, we hypothesised that ash supplementation in diet could be an effective way to increase faecal density and sinking velocity and thereby the removal efficiency of faeces by sedimentation. Since the supplemented ash fraction was indigestible, it resulted in a decline in DM digestibility of diet and thus, faecal waste production increased (Fig. 3A). Reduced dry matter digestibility with high ash diets corresponded with a reduction in digestibility of ash fraction (36.8% at high ash diet vs. 29.5% at low ash diet). As hypothesised, dietary supplementation of insoluble ash in the diet increased the faecal density and sinking velocity. A significant positive correlation was observed between the faecal ash content and density of faeces ($r^2 = 0.51$, $P < 0.001$, Fig. S2). A plausible reason could be a higher proportion of the denser inorganic over less dense organic fraction of faeces as reported for composts (Al-Bataina et al., 2016; Khater, 2015; Mamo et al., 2021). Our findings demonstrate that the composition of the diet can steer faecal density and sinking velocity. Contradictorily, a clear impact of dietary nutrient or ingredient composition on density and sinking velocity of faeces was not noted in a few of the earlier studies (Chen et al., 2003; Fountoulaki et al., 2022). Nevertheless, the improved density and sinking velocity of faeces following ash supplementation did not increase faecal removal efficiency or reduce the amount of non-removed faeces. Apparently, in rainbow trout reared in fresh water, faecal particle size is the primary determinant of the faecal removal rate by settling rather than the faecal particle density/sinking velocity. Alternatively, the short depth (depth of settling column = 44 cm) over which the particles had to settle suppresses any potential impact, the density/sinking velocity of faeces might have on faecal removal efficiency. Further, one should interpret the sinking velocity data presented in this study cautiously as they were obtained using water of 35 gL^{-1} salinity, whereas the sedimentation column for estimating faecal removal efficiency was operated with ambient culture water of <1.5 gL^{-1}. Nevertheless, the findings may still be interesting for salmonid open cage culture systems where sedimentation depth extends over several meters and the high density of ambient sea water creates more resistance in settling of faeces, thereby providing an opportunity to regulate the removal or dispersal of solid waste through manipulation of faecal density/sinking velocity. In summary, it is concluded that while ash content of diet increased the density/sinking velocity of faeces, it did not result in increased faecal removal efficiency in case of rainbow trout reared in fresh water.

Overall, our study demonstrated that high dietary starch level increased faecal waste production in rainbow trout by reducing the digestibility of starch and other macronutrients such as protein and fat. Starch supplementation reduced removal efficiency of faeces and increased the quantity of faecal waste accumulating in the system. Amylase supplementation in the diet enhanced the starch digestibility but did not impact the faecal waste production, removal efficiency and the PSD of non-stressed faeces. High ash level in the diet increased faeces' density and sinking velocity but did not improve faecal removal efficiency. The increase in faecal density and sinking velocity of faeces with ash supplementation can be of interest to open cage culture operators, as it opens up possibilities for more effective faeces collection and a potential reduction in spatial dispersion of faecal waste.

CRediT authorship contribution statement

Satya Prakash: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roel M. Maas:** Writing – review & editing,

Validation, Methodology, Data curation, Conceptualization. **Peter Horstmann:** Writing – review & editing, Investigation, Formal analysis. **Jan Jules Elbers:** Methodology, Investigation, Formal analysis. **Fotini Kokou:** Writing – review & editing, Conceptualization. **Johan W. Schrama:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Antony J. Prabhu Philip:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.740612.

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