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The effect of dietary protein source (fishmeal vs. plant protein) and non-starch polysaccharide level on fat digestibility and faecal bile acid loss in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study investigated in rainbow trout (*Oncorhynchus mykiss*) if diet composition and feeding level affect faecal bile acid loss, and whether this reflects on the apparent digestibility coefficient (ADC) of fat. Six diets were formulated with either fishmeal or plant protein as main protein source. This created a contrast in the supply of bile acids, the bile acid precursor cholesterol, taurine and the taurine precursors (methionine + cysteine) involved in bile acid conjugation. For both protein sources, three diets were formulated with increasing inclusion of a non-starch polysaccharide (NSP)-rich ingredient mixture (0.0, 82.0 and 164.2 g/kg diet). This aimed at enhancing faecal bile acid loss. Fish were fed both restrictively (1.2% BW/day) and to satiation. A similar fat ADC was found when substituting fishmeal with a plant protein mixture, suggesting that the lower content of bile acids, cholesterol, taurine, methionine and cysteine in the plant-based diets did not limit fat digestion. Faecal bile acid loss increased alongside dietary NSP level, however, only during satiation feeding and most strongly for fish fed the fishmeal-based diets. Enhanced faecal bile acid loss was not caused by NSP-bile acid binding/entrapment, but by an increase in faeces production. During satiation feeding, fat ADC negatively correlated with faecal bile acid loss. From this it is concluded that bile acid availability/synthesis can become limiting for fat digestion in rainbow trout under conditions that enhance faecal bile acid loss (i.e. dietary NSP level and feeding level).

KEYWORDS

bile acids, cholesterol, fat digestibility, non-starch polysaccharides, *Oncorhynchus mykiss*, taurine

1 | INTRODUCTION

With the shift from fishmeal-based (FM) to plant-based (PB) diets in the last decades, protein sources used in aquafeeds have diversified. However, possibilities for inclusion of these ingredients are not without limits. Several studies report a decrease in the apparent digestibility coefficient (ADC) of fat in fish fed PB diets (Gu, Kortner, Penn, Hansen, & Krogdahl, 2014; Kumar, Makkar, & Becker, 2011; Sinha, Kumar, Makkar, De Boeck, & Becker, 2011). One possible explanation for the reduction in fat ADC of fish fed PB diets is an altered bile acid metabolism.

Bile acids are synthesized in the liver from cholesterol, and conjugated with taurine/glycine (Russell, 2009). In fish, most bile acids are conjugated with taurine (Hagey, Moller, Hofmann, & Krasowski, 2010). Bile acids are secreted in the proximal part of the intestine, where they emulsify fat and facilitate fat hydrolysis and absorption (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011; Tocher, 2003). In the distal intestine, the majority of bile acids is reabsorbed (e.g. ±95% in humans) and transported via the portal vein to the liver for reuse (Cai, Xiong, Wray, Ballatori, & Boyer, 2007). This process is known as enterohepatic circulation of bile acids. Under homeostatic conditions, the total bile acid pool is maintained relatively constant by compensating faecal bile acid loss with de novo synthesis (Lanzini & Lanzarotto, 2000).

Information for fish is limited, but in humans (Walters et al., 1975), rats (Ide, Horii, Kawashima, & Yamamoto, 1989; Ikegami et al., 1990; Overton et al., 1994) and poultry (Choct, 1999; Matin, Shariatmadari, Torshizi, & Chiba, 2016), non-starch polysaccharides (NSP) present in plant ingredients can sequester bile acids, thus enhancing faecal bile acid loss. Bile acid losses are to our knowledge not yet quantified in fish, but have been suggested to be the cause of hampered fat ADC (e.g. Leenhouwers, ter Veld, Verreth, & Schrama, 2007; Pasquier et al., 1996; Romarheim et al., 2008; Sinha et al., 2011). Next to enhanced faecal bile acid loss, de novo bile acid synthesis might be limiting in fish fed PB diets. PB diets do not contain bile acids, neither the bile acid precursor cholesterol nor the taurine needed for bile acid conjugation in fish. Bile acid synthesis in fish fed PB diets might thus be too low to compensate for NSP-induced faecal bile acid loss.

This study investigated in rainbow trout (*Oncorhynchus mykiss*) whether fat ADC and faecal bile acid loss are related and are negatively affected by dietary NSP level. The effect of NSP level was tested in two diets that differed in main protein source: fishmeal-based (FM) vs. plant-based (PB) diets. This resulted in a contrast in supply of bile acids, the bile acid precursor (cholesterol), taurine and taurine precursors (methionine + cysteine). Both restricted feeding and satiation feeding were applied to test if diet effects were dependent on feeding level.

2 | MATERIALS AND METHODS

The Animal Welfare Body of Wageningen University approved this experiment and evaluated it as not being an animal experiment according to Dutch legislation (Act on Animal Experiments).

2.1 | Feed formulation

All experimental diets were formulated to meet known nutrient requirements of rainbow trout (*Oncorhynchus mykiss*) according to the National Research Council (NRC, 2011). Ingredient composition and analysed nutrient content are provided in Table 1. Six diets were formulated according to a two-by-three factorial design.

Two groups of diets were formulated differing in main protein source, plant-based (PB) diets vs. fishmeal-based (FM) diets. Replacement of fishmeal by a plant protein mixture aimed to create a contrast in the dietary supply of bile acids, the bile acid precursor cholesterol, taurine and the taurine precursors (methionine + cysteine). Bile acids and taurine were provided by fishmeal, while absent in the plant protein mixture. The plant protein mixture was supplemented with methionine, lysine and threonine to avoid deficiencies of essential amino acids. A small amount of salmon oil was added to the plant protein mixture to compensate for the fish oil present in fishmeal, and to meet n-3 LC-PUFA's requirements in the PB diets. This inevitably introduced some cholesterol in the plant protein mixture. For each protein source, three diets were formulated in which an increasing part of wheat feed flour was replaced with an NSP-rich ingredient mixture (0% NSP, 8% NSP and 16% NSP). This NSP-rich ingredient mixture was added to enhance faecal bile acid loss. The main fat source was equal for all diets, and a vegetable oil mixture was chosen to avoid additional introduction of cholesterol into the diets. A vitamin/mineral premix, chalk and monocalcium phosphate were added to meet nutrient requirements for vitamins and minerals. Yttrium oxide was included as inert digestibility marker and for determination of faecal bile acid loss. Extruded pellets were vacuum coated with the vegetable oil mixture (3 mm; produced by Research and Diet Services B.V., The Netherlands). Feed was stored at 4°C throughout the experiment.

2.2 | Design and management

The experiment was carried out in the aquatic metabolic unit of Wageningen University in the Netherlands. The metabolic unit comprised 12 glass tanks (90 × 60 × 45 cm; 200 L) connected to a common water recirculation system. Water flow into each tank was controlled by a water flow meter (MAGFLO® MAG 5000, Danfoss A/S) and was kept constant at 7.0 ± 0.05 L/min. A trickling filter, sump and drum filter (Hydrotech 500®, Hydrotech Engineering) helped in maintaining water quality parameters within a set range. Maximum allowed values for NH_4^+-, NO_2^--concentrations, and conductivity of the outlet water were < 4 mg/L, < 1.5 mg/L and < 4,000 µS/cm respectively. A partial water change was done when these parameters surpassed their allowed maximum. The pH of the inlet water was kept within the range of 7.0–7.8. Water temperature was maintained at 14.0 ± 0.5°C. The concentration of dissolved oxygen in the outlet was maintained at a level > 4.5 mg/L. Each tank was drained separately, and each outlet, located at the lower point of the sloping tank bottom, was connected to a swirl separator (44 cm in height, 24.5 cm in diameter; Aqua Optima

TABLE 1 Ingredient composition and analysed nutrient content of the experimental diets

	Diets					
	FM			PB		
	0% NSP	8% NSP	16% NSP	0% NSP	8% NSP	16% NSP
Ingredients (g/kg)						
Fishmeal[†]	492.3	492.3	492.3	–	–	–
Plant protein mixture[‡]	–	–	–	492.3	492.3	492.3
Pea protein	–	–	–	**256.0**	**256.0**	**256.0**
Soy protein concentrate	–	–	–	**103.4**	**103.4**	**103.4**
Wheat gluten	–	–	–	**98.5**	**98.5**	**98.5**
Salmon oil[§]	–	–	–	**27.1**	**27.1**	**27.1**
L-lysine HCL	–	–	–	**4.4**	**4.4**	**4.4**
DL-methionine	–	–	–	**1.5**	**1.5**	**1.5**
L-threonine	–	–	–	**1.5**	**1.5**	**1.5**
Vegetable oil mixture[¶]	200.0	200.0	200.0	200.0	200.0	200.0
Palm oil	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
Rapeseed oil	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
Wheat feed flour	261.3	179.3	97.2	261.3	179.3	97.2
NSP-rich ingredient mixture[††]	–	82.0	164.2	–	82.0	164.2
Soy hulls	–	**41.0**	**82.1**	–	**41.0**	**82.1**
Wheat bran	–	**41.0**	**82.1**	–	**41.0**	**82.1**
Monocalcium phosphate	30.8	30.8	30.8	30.8	30.8	30.8
Vitamin/mineral premix[‡‡]	10.3	10.3	10.3	10.3	10.3	10.3
$CaCO_3$	5.1	5.1	5.1	5.1	5.1	5.1
Yttrium oxide	0.2	0.2	0.2	0.2	0.2	0.2
Analysed nutrient content (g/kg DM)						
DM (g/kg)	970	982	963	974	989	975
Ash	116	120	123	56	60	63
Crude protein (*N* × 6.25)	408	411	410	415	423	423
Crude fat	243	249	256	246	262	270
Starch + sugars	217	168	115	232	167	129
NSP[§§]	16	50	96	51	88	115
Gross energy (kJ/g DM)	23.4	23.1	23.0	24.7	24.8	24.7
Calcium	26	26	28	9	10	10
Phosphorous	19	19	20	11	11	12
Yttrium	0.2	0.2	0.2	0.2	0.2	0.2
Bile acids (μmol/kg DM)	474	490	485	BDL	BDL	BDL
Cholesterol	2.3	2.2	2.5	1.4	1.7	1.3

(Continues)

A/S, UK) to collect faeces. A photoperiod of 12-hr light–12-hr dark was maintained for the entire duration of the experiment.

2.3 | Experimental procedures and sampling

Each diet was tested in duplicate and assigned randomly to the tanks. Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial fish farm (Mohnen Aquaculture GmbH). Upon arrival, fish were randomly stocked at a density of 30 fish/tank. The fish underwent four weeks of restricted feeding, followed by three weeks of satiation feeding. During restricted feeding, fish were fed 1.2% BW/day, except the first week when the feed ration was stepwise increased from 20% to 100% of the intended feeding level to allow habituation to the diets. The daily amount of feed was increased throughout the restricted feeding period by

TABLE 1 (Continued)

	Diets					
	FM			PB		
	0% NSP	8% NSP	16% NSP	0% NSP	8% NSP	16% NSP
Taurine	2.6	2.6	2.6	0.0	0.0	0.0
Methionine	11.4	11.6	11.1	6.6	6.0	6.6
Cysteine	3.7	3.8	3.7	5.8	5.4	5.9

Note: Abbreviations: BDL, below the detection limit (< 30 μmol/kg DM); PB x% NSP, plant-based diet with x% inclusion of the NSP-rich ingredient mixture; FM x% NSP, fishmeal-based diet with x% inclusion of the NSP-rich ingredient mixture.

[†]LT fishmeal – crude protein 72%, Triple Nine Fish protein, Esbjerg, Denmark.

[‡]The plant protein mixture consisted of pea protein, soy protein concentrate, wheat gluten, salmon oil, L-lysine HCL, DL-methionine and L-threonine. The inclusion levels for these ingredients in the final diets are highlighted in bold and sum up to the inclusion level given for the plant protein mixture in the respective diets.

[§]INVE Salmon oil, Belgium.

[¶]The vegetable oil mixture consisted of palm oil and rapeseed oil. The inclusion levels for these ingredients in the final diets are highlited in bold and sum up to the inclusion level given for the vegetable oil mixture in the respective diets.

[††]The NSP-rich ingredient mixture consisted of soy hulls and wheat bran. The inclusion level for these ingredients in the final diets are highlited in bold and sum up to the inclusion level given for the NSP-rich ingredient mixture in the respective diets.

[‡‡]Vitamin/mineral premix.

Vitamins (IU or mg/kg diet): thiamin, 10 mg; riboflavin, 10 mg; pyridoxine, 10 mg; pantothenic acid, 40 mg; niacin, 20 mg; biotin, 0.2 mg; cyanocobalamin, 0.015 mg; folic acid, 2 mg; ascorbic acid, 100 mg; DL-alpha tocopherol acetate, 100 IU; retinyl palmitate, 3,000 IU; DL-cholecalciferol, 2,400 IU; sodium menadione bisulphate (51%), 10 mg; inositol, 400 mg; choline, 2,000 mg; butylhydroxytolueen, 100 mg; calcium propionate, 1,000 mg; anti-oxidant BHT (E300-321), 100 mg.

Minerals (mg/kg diet): iron (as $FeSO_4 \cdot 7H_2O$), 50 mg; zinc (as $ZnSO_4 \cdot 7H_2O$), 30 mg; cobalt (as $CoSO_4 \cdot 7H_2O$), 0.1 mg; copper (as $CuSO_4 \cdot 5H_2O$), 10 mg; selenium (as Na_2SeO_3), 0.5 mg; manganese (as $MnSO_4 \cdot 4H_2O$), 20 mg; magnesium (as $MgSO_4 \cdot 7H_2O$), 500 mg; chromium (as $CrCl_3 \cdot 6H_2O$), 1 mg; calcium (as $CaIO_3 6H_2O$), 2 mg.

[§§]NSP = 1,000 – ash – crude protein – crude fat – (starch + sugars).

predicting fish growth and weight, using the initial body weight (117 ± 1.6 g/fish) and an expected FCR of 1.1. The daily feed ration was divided into two equal portions and was hand-fed fed at 9:00 and 15:30 hr. During satiation feeding, fish were fed twice daily at 9:00 and 15:30 hr until they stopped feeding, with a maximum duration of 1 hr per feeding moment. Uneaten pellets were collected for accurate determination of feed intake. Tanks were checked for mortality twice daily, and dead fish were removed. In case of mortality during restrictive feeding, the feeding list for the respective tank(s) was adjusted to maintain equal feed intake for all treatments. Faeces were collected using swirl separators the last week of both the restricted and satiation feeding periods. Faecal collection bottles were submerged into ice water to minimize bacterial decomposition. Faeces were pooled per week and per tank and stored at −20°C for analysis. At the beginning and end of both feeding periods, fish were starved 24 hr and batch weighed for determination of total biomass.

2.4 | Analytical methods

Faecal samples were dried at 70°C until constant weight. Dried faeces were pulverized using a mixer mill with a 1 mm fixed screen opening set at 12,000 RPM (MM 2000 Retsch®, Brinkmann). After one week of acclimatization to ambient air in the laboratory, diets and faeces were analysed for dry matter content (DM), ash, minerals (calcium, phosphorous and yttrium), crude protein, crude fat, starch + sugars, energy, bile acid content and cholesterol content. Total carbohydrates (g/kg DM) were calculated as 1,000 – ash – crude protein – crude fat, and NSP (g/kg DM) as 1,000 – ash – crude protein – crude fat – (starch + sugars), with ash, crude protein, crude fat and starch + sugars expressed in g/kg DM. Additionally, diets were analysed for taurine, methionine and cysteine.

Dry matter was determined gravimetrically by drying samples in an oven at 103°C (ISO 6496, 1999). Ash content was determined gravimetrically by incineration of dried samples in a muffle furnace at 550°C (ISO 5984, 2002). Minerals were dissolved in concentrated sulphuric acid by autoclaving at 121°C for 20 min, and calcium, phosphorous and yttrium content were measured by ICP-AES (NEN 15510, 2007). Crude protein content was measured according to Kjeldahl's method (ISO 5983–2, 2009), using a protein conversion factor of 6.25. Crude fat was determined gravimetrically using acid hydrolysis (Hydrotherm®, C. Gerhardt GmbH & Co. KG) paired with Soxhlet extraction (Soxtherm®, C. Gerhardt GmbH & Co. KG) (ISO 6492, 1999). Starch and free sugars were determined using an enzymatic digestion method (Nutrilab, the Netherlands). Starch was determined after washing away free sugars with 40% ethanol. The dried residue was digested with Termamyl®, after which starch was hydrolysed using amyloglucosidase. The formed glucose units were determined using the Luff-Schoorl reagent. Starch + sugars was measured as described above, leaving out the washing step with 40% ethanol.

Gross energy was measured using a bomb calorimeter (C7000 IKA®, IKA analysentechnik) (ISO 9831, 1998). The total bile acid concentration was determined enzymatically after extraction with diethyl ether. Extraction was performed according to Porter et al. (2003), and total bile acid concentration in the extracts was measured using a commercial kit (TBA assay, Dialab®) with the use of methanol as a reagent blank. Cholesterol was determined enzymatically on the crude fat extract, using a commercially available kit (Cholesterol liquid colour, Human GmbH). Taurine, methionine and cysteine in the diets were analysed by Evonik Nutrition and Care GmbH (AMINOLab®, Germany), using ion exchange chromatography and post-column derivatization with ninhydrin. Taurine, methionine and cysteine were oxidized with performic acid, which was neutralized with sodium metabisulfite. Amino acids were liberated from the protein by hydrolysis with 6 N HCl for 24 hr at 110°C and quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 hr at 110°C.

2.5 | Calculations

Calculations of performance indicators, apparent digestibility coefficients, faecal bile acid loss and faecal cholesterol loss were made for both the restricted and satiation feeding periods separately.

Mortality (%) was calculated as $(N_0-N_t)/N_0 \times 100\%$, where N_0 and N_t are the number of fish at the beginning and end of each feeding period respectively. Feed conversion ratio on dry matter basis (FCR; g/g) was calculated as $(FI \times dmF)/(W_t-W_0)$, where FI is feed intake (g), dmF is the dry matter content of the feed (%), and W_0 and W_t are the initial and final BW (g) for each feeding period respectively. Feed intake (FI; % BW/day) was calculated as $FI/t/W_g \times 100\%$, where FI is feed intake (g), t is the number of days, and W_g is the geometric mean BW (g) of each feeding period respectively. The W_g was calculated as $e^{((\ln W_t + \ln W_0)/2)}$, where W_0 and W_t are the initial and final BW (g) for each feeding period respectively. Relative faeces production on dry matter basis (RFP; % BW/day) was calculated as $FI \times (dmF) \times (D_i/F_i)/t/W_g \times 100\%$, where FI is feed intake (g), dmF is the dry matter content of the feed (%), D_i is the percentage inert marker of the diet, F_i is the percentage inert marker of the faeces, t is the number of days, and W_g is the geometric mean BW (g) of each feeding period respectively. Specific growth rate (SGR; % BW/day) was calculated as $(\ln W_t - \ln W_0)/t \times 100\%$, where W_0 and W_t are the initial and final BW (g), and t is the number of day of each feeding period respectively.

The apparent digestibility coefficient (%) of each nutrient was calculated using the formula described by (Halver & Hardy, 2002): $1-((F/D) \times (D_i/F_i)) \times 100$, where D is the percentage nutrient (or kJ/g gross energy) of the diet, F is the percentage nutrient (or kJ/g gross energy) of the faeces, D_i is the percentage inert marker of the diet, and F_i is the percentage inert marker of the faeces.

Faecal bile acid loss (μmol kg^{-1} BW day^{-1}) was calculated as $((FI \times (D_i/F_i))/t)/W_g) \times F_b$, where FI is feed intake (g), D_i is the percentage of inert marker in the diet, F_i is the percentage of inert marker in the faeces, t is the number of days, W_g is the geometric mean BW (kg), and F_b is the faecal bile acid content on wet weight (μmol/g) for each feeding period respectively. Faecal cholesterol loss (mg kg^{-1} BW day^{-1}) was calculated as $((FI \times (D_i/F_i))/t)/W_g) \times F_c$, where FI is feed intake (g), D_i is the percentage of inert marker in the diet, F_i is the percentage of inert marker in the faeces, t is the number of day, W_g is the geometric mean BW (kg), and F_c is the faecal cholesterol content on wet weight (mg/g) of each feeding period respectively.

2.6 | Statistical analysis

Tanks (n = 12) were considered as experimental units. Combined data of restricted feeding and satiation feeding were first analysed using a mixed model ANOVA to look at the effect of feeding level (i.e. feeding period), protein source (PS), NSP level, their interaction effects and the random effect of tank nested within PS and NSP level. The effect of PS, NSP level and their interaction were tested against the random effect of tank nested within PS and NSP level. The effect of feeding level and its interactions with PS and NSP level were tested against the random error of the whole model (i.e. random variation within tanks between feeding levels). Data were tested for sphericity using Mauchly's test. Since for many parameters interaction effects between feeding level and both PS and NSP level were present, data of restricted feeding and satiation feeding were also analysed separately using a two-way ANOVA for the effect of PS, NSP level and their interaction. All data were tested for homogeneity of variance by Levene's test prior to ANOVA. Normal distribution of residuals was checked using the Kolmogorov–Smirnov test. ANOVA was followed by a Tukey's test for pairwise comparison of means. The correlations were tested using Pearson's correlation test. Statistical significance was tested at the 0.05 probability level. All tests were performed using the statistical program SPSS statistics 23, IBM Statistics Inc.

3 | RESULTS

3.1 | Performance

Performance indicators were calculated to judge the quality of the experiment. Full results on fish performance are given in the Table S1. Mortality was low, and no effect of treatment occurred. Fishmeal-based (FM) diets had significantly better feed conversion ratios (FCR) compared to the plant-based (PB) diets ($p < .05$), and NSP level negatively affected the FCR during both feeding periods ($p < .01$). Growth (SGR) followed a similar trend, except that, during satiation feeding, higher NSP levels significantly increased SGR ($p < .01$). The latter

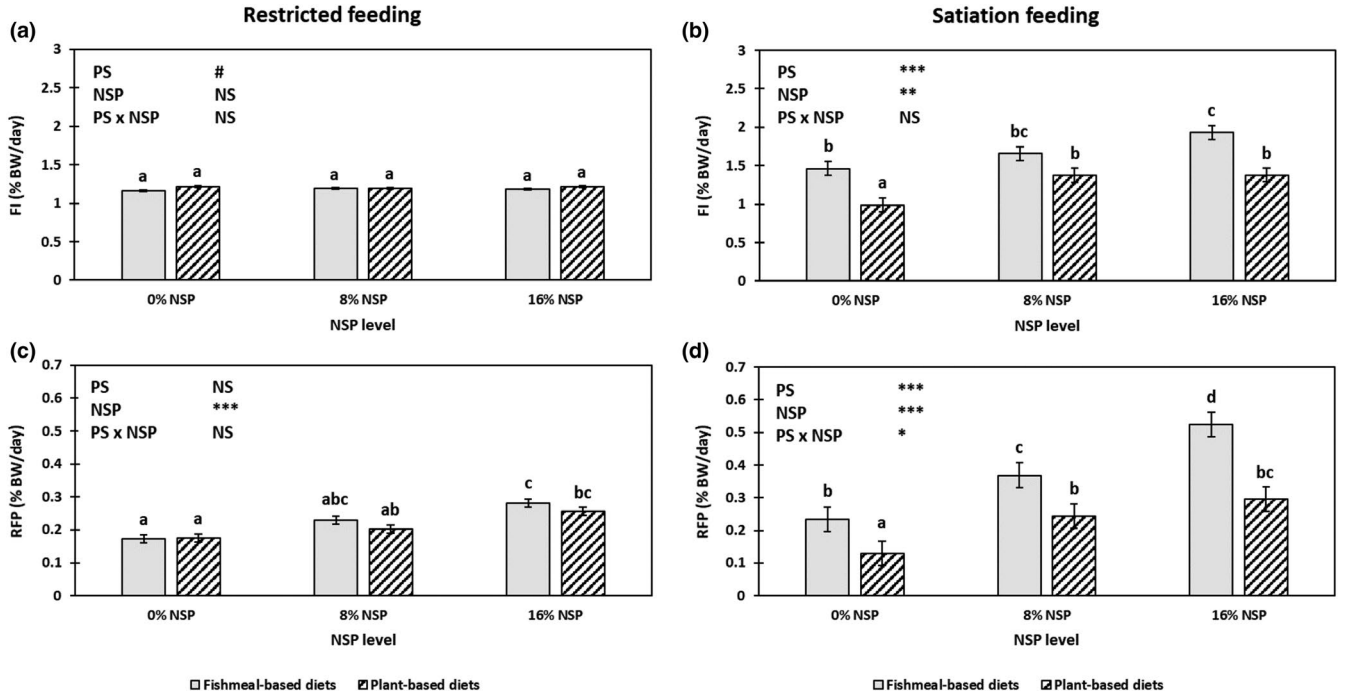

FIGURE 1 Feed intake (FI, % BW/day) during (a) restricted feeding and (b) satiation feeding of diets differing in protein source (PS) and non-starch polysaccharide (NSP) level; Relative faeces production (RFP, % BW/day) during (c) restricted feeding and (d) satiation feeding of diets differing in PS and NSP level; BW, body weight; Error bars indicate standard error of means; NS, not significant; #: $p < .1$; *: $p < .05$; **: $p < .01$; ***: $p < .001$; Treatments in the same panel lacking common letters are statistically different ($p < .05$) according to Tukey's multiple comparison test

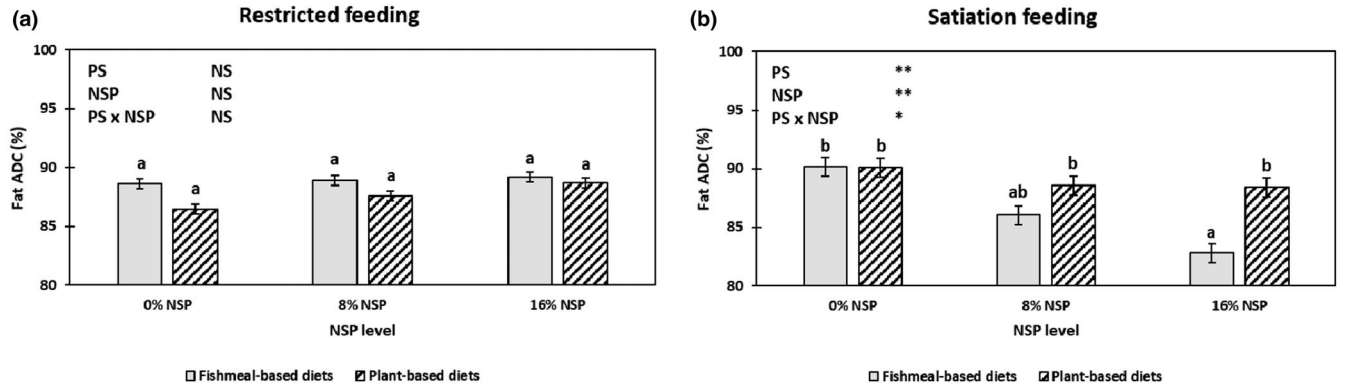

FIGURE 2 Fat apparent digestibility coefficient (ADC, %) during (a) restricted feeding and (b) satiation feeding of diets differing in protein source (PS) and non-starch polysaccharide (NSP) level; Error bars indicate standard error of means; NS, not significant; #: $p < .1$; *: $p < .05$; **: $p < .01$; ***: $p < .001$; Treatments in the same panel lacking common letters are statistically different ($p < .05$) according to Tukey's multiple comparison test

occurred in parallel with higher feed intake (FI) of the high-NSP diets during satiation feeding ($p < .01$). Since the results of fat apparent digestibility coefficients (ADC) and faecal bile acid loss are later in the discussion related to feed intake and relative faeces production (RFP), the latter parameters are further focused on in this section. Results on feed intake during both feeding periods are shown in Figure 1 a and b. Feed intake was equal for all diets during restricted feeding and averaged 1.20 ± 0.017% BW/day. During the satiation feeding period, feed intake of the FM diets (averaged 1.67 ± 0.231% BW/day) was higher compared with the PB diets (averaged 1.25 ± 0.225% BW/day) ($p < .01$). Moreover, feed intake increased with dietary NSP level ($p < .01$). An interaction effect between NSP level and protein source (PS) on feed intake was not present ($p > .05$). Faeces production (RFP; % BW/day) is shown in Figure 1 c and d. Relative faeces production increased alongside dietary NSP level during both feeding periods ($p < .001$) but the increase was more pronounced during satiation feeding, in particular for the FM diets ($p < .05$).

3.2 | Nutrient Apparent digestibility

Full results on nutrient ADC are given in the Table S2. Because of the focus in this study, effects on fat ADC are described in more detail below and are also shown in Figure 2 a and b. The significant

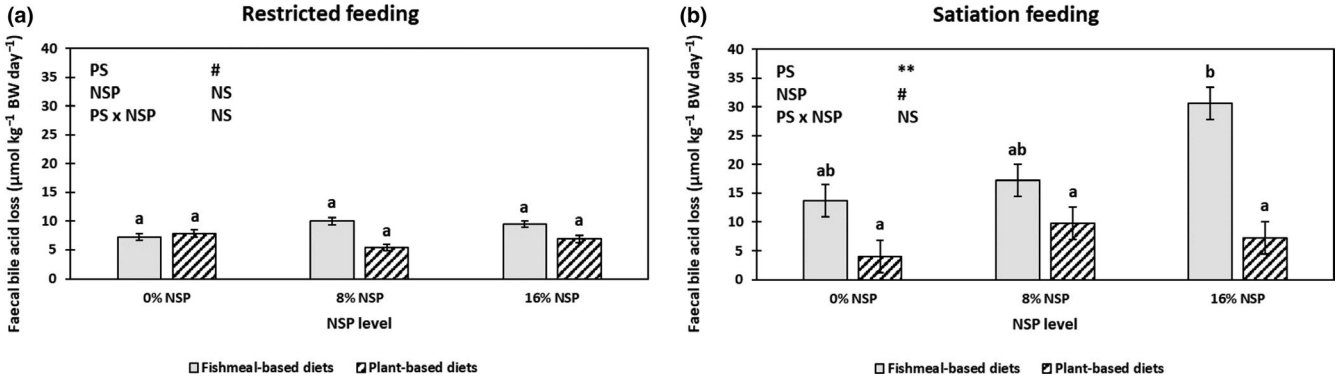

FIGURE 3 Faecal bile acid loss (µmol kg^{-1} BW day^{-1}) during (a) restricted feeding and (b) satiation feeding of diets differing in protein source (PS) and non-starch polysaccharide (NSP) level; BW, body weight; Error bars indicate standard error of means; NS, not significant; #: $p < .1$; *: $p < .05$; **: $p < .01$; ***: $p < .001$; Treatments in the same panel lacking common letters are statistically different ($p < .05$) according to Tukey's multiple comparison test

interaction effect between feeding level and NSP level showed a decrease in fat ADC with increasing feeding level ($p < .01$), the latter being strongest for the high-NSP diets. Furthermore, the decrease in fat ADC with feeding level was more pronounced for the FM diets compared with the PB diets ($p < .01$). Neither PS nor NSP level had a significant effect on fat ADC during restrictive feeding ($p > .05$). In contrast, satiation feeding showed both main effects of PS and NSP level on fat ADC ($p < .01$), as well as an interaction effect between PS and NSP level ($p < .05$). The fat ADC of the FM diets decreased from 90.2% to 82.8% with increasing dietary NSP level, while being unaffected for the PB diets.

3.3 | Faecal NSP content, faecal bile acid content, faecal bile acid loss and faecal cholesterol loss

Results on faecal NSP content, faecal bile acid content, faecal bile acid loss, faecal cholesterol content and faecal cholesterol loss are given in Table S3. Feeding level did not affect the faecal NSP content ($p > .05$). During both feeding periods, faecal NSP content was significantly higher for fish fed the PB diets ($p < .001$). Moreover, faecal NSP content increased alongside dietary NSP level ($p < .001$), and during satiation feeding this increase was more pronounced for fish fed the FM diets ($p < .05$). Faecal bile acid content ($p > .05$) was unaffected by feeding level and NSP level ($p > .05$). Faecal bile acid content was unaffected by PS during restricted feeding ($p > .05$), while satiation feeding resulted in a slightly higher faecal bile acid content of fish fed the FM diets ($p < .05$). Results on faecal bile acid loss are also shown in Figure 3. Feeding level did significantly increase the faecal bile acid loss ($p < .05$). Furthermore, the interaction effect between feeding level and NSP level indicates that the observed increase in faecal bile acid loss alongside dietary NSP level was stronger during satiation feeding compared to restricted feeding ($p < .05$). However, looking at the individual feeding periods, NSP level tended to increase faecal bile acid loss only during satiation feeding ($p < .1$). Combined data of restricted feeding and satiation feeding also showed this increase in faecal bile acid loss with increasing NSP level ($p < .05$). PS had an effect on faecal bile acid loss during satiation feeding, showing higher faecal bile acid losses for fish fed the FM diets compared with the PB diets ($p < .01$). Faecal cholesterol loss was higher during satiation feeding compared with restricted feeding ($p < .05$), and the difference in faecal cholesterol loss between feeding periods was biggest for the FM diets ($p < .05$). No effects of dietary treatment on faecal cholesterol loss were found during restricted feeding ($p > .05$). Satiation feeding showed an

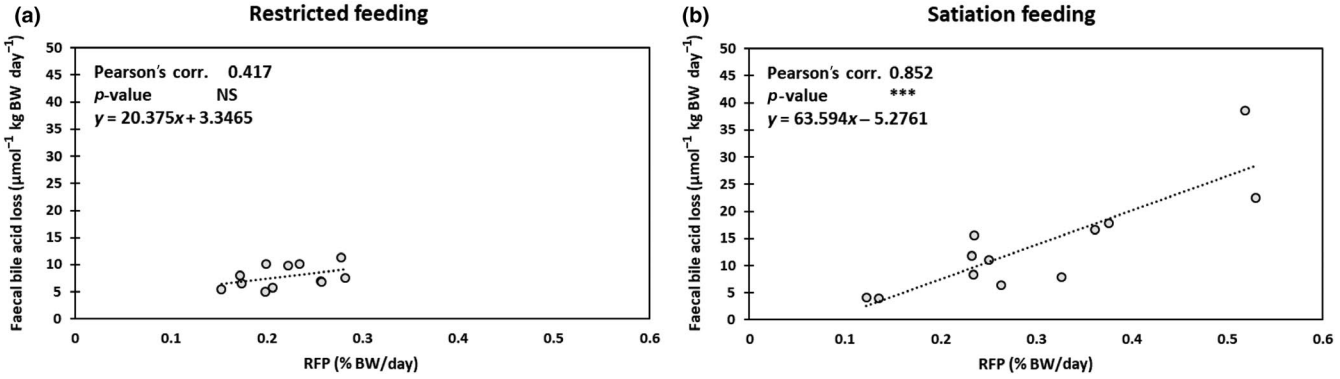

FIGURE 4 Relationship between relative faeces production (RFP; % BW/day) and faecal bile acid loss (µmol kg^{-1} BW day^{-1}) during (a) restricted feeding and (b) satiation feeding of diets differing in protein source (PS) and non-starch polysaccharide (NSP) level; BW, body weight; NS, not significant; #: $p < .1$; *: $p < .05$; **: $p < .01$; ***: $p < .001$

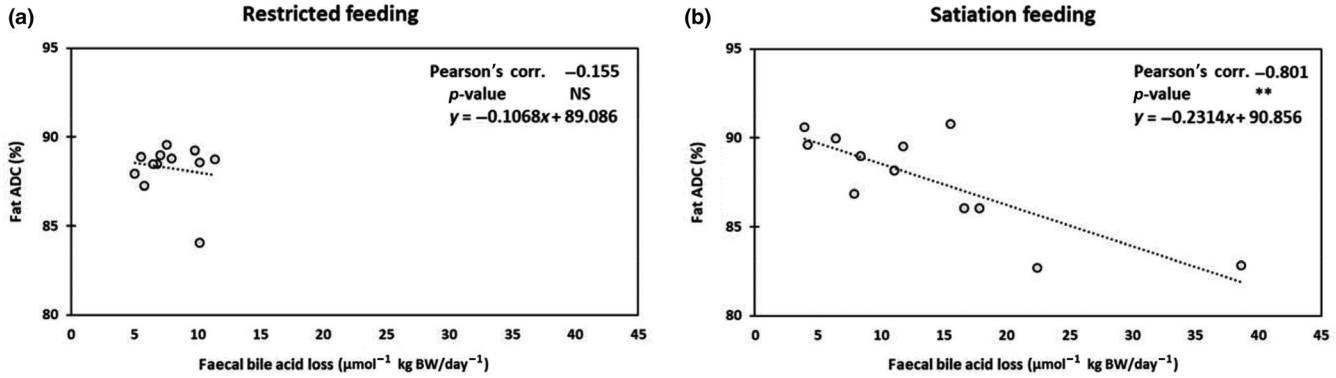

FIGURE 5 Relationship between faecal bile acid loss (µmol kg^{-1} BW day^{-1}) and fat apparent digestibility coefficient (ADC, %) during (a) restricted feeding and (b) satiation feeding of diets differing in protein source (PS) and non-starch polysaccharide (NSP) level; BW, body weight; NS, not significant; #: $p < .1$; *: $p < .05$; **: $p < .01$; ***: $p < .001$

increase in faecal cholesterol loss alongside dietary NSP level ($p < .01$). Furthermore, satiation feeding of the FM diets compared with the PB diets resulted in more faecal cholesterol loss ($p < .001$).

3.4 | Relationship between relative faeces production and faecal bile acid loss, and between faecal bile acid loss and fat ADC

Figure 4 a and b show the relationship between relative faeces production (RFP) and faecal bile acid loss. Positive relationships between faeces production and faecal bile acid loss were found for both restricted feeding and satiation feeding, however, the correlation was only significant during satiation feeding ($p < .001$). Increasing faeces production with 0.1% BW/day resulted in an increase in faecal bile acid loss of 2.04 and 6.36 µmol kg^{-1} BW day^{-1} during restricted feeding and satiation feeding respectively. The increase in faecal bile acid loss in response to higher faeces production was thus stronger during satiation feeding (Pearson's correlation coefficient: .852) compared with satiation feeding (Pearson's correlation coefficient: .417). Figure 5a and b show the relationship between faecal bile acid loss and fat ADC. Fat ADC was negatively correlated with faecal bile acid loss during both restricted feeding and satiation feeding; however, the correlation was only significant for the satiation feeding period ($p < .01$). Increasing faecal bile acid loss with 10 µmol/kg BW/day corresponded with a decrease in fat ADC of 1.1% during restricted feeding and 2.3% during satiation feeding. The response of fat ADC to an increase in faecal bile acid loss was thus much stronger during satiation feeding (Pearson's correlation coefficient: −.801) compared with restricted feeding (Pearson's correlation coefficient: −.155).

4 | DISCUSSION

The current study with rainbow trout showed that the fat apparent digestibility coefficient (ADC) averaged over all dietary treatments was negatively affected by increased feeding level (Figure 2). This is in contrast with the finding of Windell, Foltz, and Sarokon (1978), who did not detect an effect of increased feeding level on fat ADC in rainbow trout. On the other hand, Haidar, Petie, Heinsbroek, Verreth, and Schrama (2016) and Schrama et al. (2012) did find a decline in fat ADC with increased feeding level for Nile tilapia. Comparing these studies shows that the impact of feeding level on absolute decline in fat ADC can strongly differ. This confirms the statement in the review of Olsen and Ringø (1997), that adequate information about the effect of feeding level on fat ADC in fish is limiting and often inconclusive. This variability in impact of feeding level on fat ADC might be related to differences in diet formulation. In this study, the decline in fat ADC with feeding level was dependent on both NSP level and protein source. During restricted feeding, minor differences in fat ADC were present, whereas during satiation feeding, fat ADC ranged between 90.2% and 82.8% for the fishmeal-based (FM) diets and between 90.1% and 88.4% for the plant-based (PB) diets. This study thus shows that the effect of feeding level on fat ADC is dependent on dietary composition (i.e. NSP level and PS). As a consequence of the chosen experimental design, the observed effect of feeding level on ADC during satiation feeding might partially be due to an age or weight effect of the fish. However, no studies in literature report a decrease in ADC with increasing age of weight in fish. The observation in the current study of a decrease in ADC for most nutrients suggests that age or weight is unlikely to have been the cause of the decline in ADC. However, if an age or weight effect was present, it will most likely have led to an underestimation of the feeding level effect.

Diets in the current study were formulated with either fishmeal or a plant protein mixture as main PS, which aimed at creating a contrast in supply of bile acids, the bile acid precursor cholesterol, taurine and methionine + cysteine (Table 1). Lower supply these compounds in the PB diets compared with the FM diets were hypothesized to result in lower fat ADC, the latter because rate-limiting steps exist in bile acid synthesis from cholesterol (Chiang, 2013), endogenous cholesterol synthesis (Deng et al., 2013) and taurine synthesis from methionine and cysteine (Gaylord et al., 2007).

Endogenous synthesis alone might be too low to compensate faecal bile acid loss and maintain a sufficiently large bile acid pool to support proper fat digestion. However, the current study showed that lower bile acid, cholesterol, taurine and cysteine + methionine levels in the PB diets did not result in lower fat ADC (Figure 2). On the contrary, averaged over NSP level, fat ADC was lowest for fish fed the FM diets. Although taurine was completely absent from the PB diets (Table 1), fat ADC was not significantly lower compared with the control FM diet. This result is not in line with the observation of Richard, Colen, and Aragao (2017) in Senegalese sole that fat ADC of a taurine-free PB diet significantly improved after taurine supplementation. However, taurine synthesis capacity is highly dependent on fish species. Many marine fish species have a low taurine synthesis capacity, creating a need for exogenous taurine, while exogenous taurine seems not required for many freshwater fish species (Divakaran, Ramanathan, & Ostrowski, 1992; El-Sayed, 2014). Nevertheless, Gaylord et al. (2007) showed for rainbow trout cultured in freshwater that taurine supplementation to PB diets was beneficial for growth (fat ADC was not reported), however, only when no crystalline methionine + cysteine was provided. In the current study, a minimal amount of methionine was supplemented to the PB diets in order the meet both individual and combined cysteine + methionine requirements (NRC, 2011). If dietary methionine + cysteine levels of the PB diets would have been lower, taurine synthesis might have become limiting to support proper fat ADC. Besides taurine requirement, little attention has been given towards the dietary requirement of cholesterol of most farmed fish. Kortner, Bjorkhem, Krasnov, Timmerhaus, and Krogdahl (2014) did not find an improvement of fat ADC in Atlantic salmon fed PB diets after cholesterol supplementation. The dietary cholesterol level in the PB diets in this study was lower than those in the aforementioned study, but still seems sufficient to support proper fat ADC in rainbow trout. Availability of cholesterol for bile acid synthesis might have becoming limiting for fat ADC of fish fed the FM diets to satiation because of a significant increase of faecal cholesterol loss for those fish (Table S3). Increased cholesterol loss due to NSP-rich diets has been demonstrated by Moundras, Behr, Remesy, and Demigne (1997) in rats. The outcome of this study suggests that intake of bile acids, cholesterol, taurine and cysteine + methionine in rainbow trout fed PB diets was sufficient to support proper fat digestion. In contrast, fat ADC of the FM diets fed to satiation was lowered compared with restrictive feeding, especially for the high-NSP diets. This suggests that intake of bile acids, cholesterol, taurine and cysteine + methionine and/or other dietary factors can limit fat ADC in fish fed FM diets to satiation.

For both protein sources, three diets were formulated with increasing inclusion level of an NSP-rich ingredient mixture (Table 1). Some NSP can sequester bile acids (Li, Mense, Brewer, Lau, & Shi, 2017), and increasing the dietary NSP level aimed at enhancing faecal bile acid loss. Enhanced faecal bile acid loss was hypothesized to reduce the amount of bile acids available for micelle formation, resulting in a reduction of fat ADC. The results of this study showed no effect of dietary NSP level on faecal bile acid loss during restricted feeding, whereas satiation feeding showed a trend for increased faecal bile acid loss alongside NSP level (Figure 3). Furthermore, the observed increase in faecal bile acid loss during satiation feeding was more pronounced for fish fed the FM diets compared with the PB diets, the former having the highest feed intake. In literature, a reduced fat ADC of diets with high-NSP content is often related to direct binding or entrapment of bile acids by NSP, which is mainly associated with soluble NPS (Adam et al., 2001; Dongowski, 2007; Ide et al., 1989; Ikegami et al., 1990; Matin et al., 2016). However, in none of these studies, faecal bile acid content was reported. In the current study, the increase in faecal bile acid loss with NSP level was not caused by bile acid-NSP binding or entrapment. This was substantiated by the absence of an NSP level effect on both the faecal bile acid content and the faecal bile acid loss in the PB diets (despite equal amounts of NSP were included in both FM and PB diets) (Table S3). Rather than direct binding or entrapment of bile acids by NSP, the data of the current experiment indicate that in the interpretation of the effect of dietary NSP level on faecal bile acid loss, also feeding level/faeces production needs to be considered. Dietary NSP level affected both feed intake and faeces production (Figure 1). Feed intake increased with NSP level during satiation feeding. Literature shows that nutrient dilution due to presence of NSP can be compensated by higher feed intake (Dias, Huelvan, Dinis, & Metailler, 1998; Lekva, Hansen, Rosenlund, Karlsen, & Hemre, 2010; Sinha et al., 2011). The higher feed intake of the FM diets compared with the PB diets in this study might be explained by higher palatability of diets containing fishmeal for rainbow trout (Gomes, Rema, & Kaushik, 1995). Evidently, higher feed intake results in higher faeces production. This is especially the case for NSP-rich diets, since NSP are mostly indigestible to monogastrics (Choct, Dersjant-Li, McLeish, & Peisker, 2010; Sinha et al., 2011) and can cause faecal bulking (Davidson & McDonald, 1998). As faeces production increased, so did the amount of faecal bile acid loss. This is also indicated by the positive correlation between faeces production and faecal bile acid loss during satiation feeding, while absent for the restricted feeding period (Figure 4). An increase in faecal bile acid loss because higher faeces production has to our knowledge not been reported for fish so far.

Besides dietary NSP level, also presence of saponins in soy hulls in the NSP-rich ingredient mixture could have contributed to the increase in faecal bile acid loss and decrease in fat ADC observed in this study. The majority of saponins forms non-absorbable complexes with bile acids (Kregiel et al., 2017), inhibit pancreatic lipase activity (Han, Xu, Kimura, Zheng, & Okuda, 2000) and can cause enteritis in salmonids (Iwashita, Suzuki, Matsunari, Sugita, & Yamamoto, 2009; Knudsen et al., 2008; Kortner, Penn, Björkhem, Måsøval, & Krogdahl, 2016; Krogdahl et al., 2015). In this study, the increase in faecal bile acid loss and reduction in fat ADC only occurred for the FM diets. Since soy hulls were included in the same levels in both FM and PB diets, soy saponins are unlikely to have had a major contribution to the adverse effects on faecal bile acid loss and fat ADC. Also, enteritis is unlikely to have occurred since Krogdahl et al. (2015) found that a minimum level of 2 g soy saponins/kg diet was needed to induce

enteritis in Atlantic Salmon, and the maximum level in the FM diets in this study was estimated to be around 1.6 g soy saponins/kg diet, based on 20 g saponins/kg soy hulls (Shi et al., 2004).

The effects of dietary treatment on fat ADC followed a trend similar to faecal bile acid loss (Figure 2). No effect of NSP level and PS on fat ADC occurred during restricted feeding. Satiation feeding resulted in a decrease in fat ADC alongside NSP level, but the latter was only significant for the FM diets. Negative effects of NSP on fat ADC in fish have been described in literature. Storebakken (1985) and Amirkolaie, Leenhouwers, Verreth, and Schrama (2005) showed that the increase in digesta viscosity caused by guar gum reduced mixing of fat and digestive enzymes, resulting in lowered fat ADC in respectively rainbow trout and Nile tilapia. In the current study, the NSP-rich ingredient mixture (Table 1) consisted mainly of the non-viscous NSP cellulose and hemicellulose (Englyst, 1989; Knudsen, 1997). Increased digesta viscosity was thus unlikely the cause for the decrease in fat ADC in this study, since this would otherwise also have resulted in a decreased fat ADC for fish fed the PB diets during both restricted feeding and satiation feeding. NSP composition of the diets in this study was more similar to those used in the study by Hansen and Storebakken (2007). They did not find adverse effects on fat ADC of rainbow trout fed diets rich in the NSP cellulose, which is not in line with the outcome of the current study. Keeping in mind that the effect of NSP on fat ADC was dependent on feeding level in this study, differences in feeding level between the study of Hansen and Storebakken (2007) (±0.9% BW/day) and the current study (±1.9% BW/day) might be an explanation for the difference in effect of NSP level on fat ADC.

The similar trend of faecal bile acid loss and fat ADC in response to the dietary treatments might imply a connection between both. Indeed, a significant negative correlation between faecal bile acid loss and fat ADC was found for the satiation feeding period, while this correlation was absent during restricted feeding (Figure 5). This connection between fat ADC and faecal bile acid loss was reported by Overton et al. (1994) for rats, but is to our knowledge not reported for fish so far. The correlation between faecal bile acid loss and fat ADC might mean that that the total bile acid pool available for fat is limiting when faecal bile acid loss becomes too high in trout, the latter under a combination of dietary characteristics (i.e. high palatability and high-NSP level) and high feeding levels.

5 | CONCLUSION

The similar/higher fat ADC when substituting fishmeal with a plant protein mixture suggests that the lower intake of bile acids, the bile acid precursor cholesterol, taurine and the taurine precursors (methionine + cysteine) of fish fed the plant-based diets did not hamper fat digestion. The enhanced faecal bile acid losses at higher dietary NSP levels during satiation feeding were not related to bile acid binding/entrapment by NSP, but were linked to an increased amount of faeces produced. During satiation feeding, fat ADC was negatively correlated with faecal bile acid losses. This indicates that bile acid synthesis/uptake in rainbow trout is insufficient for proper fat digestion under conditions (i.e. dietary NSP level and feeding level) that strongly enhance faecal bile acid losses.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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