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The potential of a solid-state fermentation supplement to augment white lupin (*Lupinus albus*) meal incorporation in diets for farmed common carp (*Cyprinus carpio*)

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ABSTRACT

A 10-week feeding trial was conducted to evaluate the effects of partially substituting soya protein concentrate (SPC), with white lupin (*Lupinus albus*) meal in carp (*Cyprinus carpio*) diets. This study further investigated the dietary inclusion of a solid-state fermentation (SSF) product of *Aspergillus niger* in tandem with SPC replacement. Six experimental diets were produced to be isonitrogenous (42%), isolipdic (8%) and isoenergetic (19 MJ kg^{-1}). Four diets were formulated to have 12.5 and 25% substitution of SPC using lupin meal, and with and without a supplement of 0.1% of SSF. An additional two diets were designed to serve as a basal reference with no SPC replacement, but one supplemented with 0.1% SSF inclusion. The results of this study showed that SPC can be replaced with up to 25% white lupin meal in carp diets, without reduction of growth performance, feed utilisation, body composition, gut integrity or health. The addition of SSF to the test diets enhanced growth performance (specific growth rate, $P < 0.05$) and nutrient utilisation (e.g. feed conversion ratio and protein efficiency ratio, $P < 0.05$).

1. Introduction

For decades, plant-derived proteins have been heavily exploited in aquafeeds as they possess suitable nutritional profiles, are readily available on the global feed market, and competitively priced in comparison to fish meal (Hassaan et al., 2019; Davies et al., 2019; El-Husseiny et al., 2018; Goda et al., 2018). One of the major plant proteins being used by aquaculture is soybean meal and its derivatives (e.g. soy protein concentrate, SPC and isolate). However, increasing exploitation of soybean derivatives for both human food and animal feed has resulted in an overall two-fold increase in the global market price between 2000 and 2018 (World Bank, 2019). There are concerns that increasing global soybean production has contributed towards deforestation and displacement of other food production systems in tropical and sub-tropic nations (Byerlee et al., 2014).

Lupin meal could be an attractive alternative source due to its high protein content (up to 44%) and digestible protein and energy (Van Barneveld, 1999; Edwards and Van Barneveld, 1998; Drew et al., 2007; Sweetingham and Kingwell, 2008; Lucas et al., 2015). The suitability of lupin meal has already been tested on farmed fish species. For example, an inclusion of 50% extruded lupin in turbot (*Psetta maxima*) diets supported growth performance as shown by the indicators (i.e. final body weight, daily growth index and feed efficiency) when compared to no inclusion in a control diet formulation (Burel et al., 2000). While, Robaina et al. (1995) reported that using up to 20% treated blue lupin meal (*Lupinus angustifolius*) in gilthead seabream experimental diets produced no significant differences in comparison to the reference diet on final body weight, feed efficiency, protein efficiency and protein productive value.

Like soya meal, lupin meal can possess relatively high levels of carbohydrate fractions that inhibit digestion, e.g. soluble and non-soluble non-starch polysaccharides (NSPs) (Gdala and Buraczewska, 1996; Francis et al., 2001). Furthermore, lupins can also possess several anti-nutritional factors (ANFs), which includes alkaloids, phytate, saponins and tannins, and can contribute towards reduced nutrient availability (Petterson, 2000; Francis et al., 2001). It has been extensively reported that ANF's and especially the NSPs can have a deleterious effect the intestinal morphology and liver of animals, and also

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in fish to varying extents (Baeverfjord and Krogdahl, 1996). This particularly afflicts farmed carnivorous fish species such as salmonids, seabass and seabream. Glencross (2009) in a study on rainbow trout showed that the level and composition of the insoluble and soluble dietary fibres (e.g. NSPs) in relation to digestibility had provided evidence these components affect nutritional bioavailability in the trout diet. Lupins are now being advocated and developed as an important regional grown crop for agriculture in a colder temperate climate (northern Europe). Lupins have the potential to reduce the dependency of imported soybean meal in aquaculture and produce a fish product that has less greenhouse gas emissions (carbon miles) and environmental impact (e.g. deforestation).

Dietary exogenous enzymes could be one effective way of negating the negative impact of ANFs on farmed fish and improve the nutritional digestibility of plant-based aquafeeds. For instance, dietary supplementation of proteolytic and carbohydrase enzyme mixtures in Atlantic salmon diets that contained 34% of soybean meal resulted in improved growth (final weight and SGR) and feed conversion ratio (FCR), in comparison to a test diet without exogenous enzyme inclusion (Carter et al., 1994). In another study, a commercial mixed feed enzyme source (Allzyme Vegpro™, Alltech, Dunboyne, Ireland) was used to pre-treat palm kernel meal, which was subsequently formulated into red hybrid tilapia (*O. mossambicus* x *O. niloticus*) test diets (Ng et al., 2002). The results revealed that growth (final weight, SGR,), feed utilisation (FCR, Protein efficiency ratio and net protein utilisation) and apparent nutrient digestibility (dry matter, lipid and energy) were better (up to 21.6% difference) than no treatment of palm kernel meal at 40% inclusion level. Other studies have explored fermentation processing to make the plant proteins more suitable for aquafeed use. In the study of fermented sunflower meal using *Saccharomyces cerevisiae* and *Bacillus subtilis*, it was found that phytic acid and saponins were significantly reduced from 17.25 mg kg^{-1}, and 0.678 g 100 g^{-1} to 1.78 mg kg^{-1} and 200 mg kg^{-1}, respectively (Hassaan et al., 2018). While, phenolic compounds, chlorogenic acid had decreased by 85.5% and caffeic acid was 86.9% lower compared to no fermentation treatment. In contrast, SSF using fungal species can produce end-products, which contains beneficial functional microbial metabolites and residual enzyme activity. These SSF products have been reported to improve nutrient digestibility and subsequently enhancing growth and feed performances in several different farmed animal species (Graminha et al., 2008). More recently Bowyer et al. (2020) have shown favourable results when an SSF product, Synergen™ was supplemented to diets containing both blue and yellow-flowered lupin meals for tilapia. The product is generated from the solid-state fermentation of cereal bran with *Aspergillus niger* producing a stable complex of various enzymes and bioactive components. SSF type products contain a heterogeneous complex of residual enzymes e.g. xylanases, cellulases, amylases and proteases as well as phytases liberated by the action of *A niger* on the substrate during fermentation. These are also known to have a significant modulating effect on gut function and morphology and integrity (Bowyer et al., 2020).

Common carp (*Cyprinus carpio*) represents one of the world's most cultivated fish species, with over 8 million tonnes being produced in 2017 (FAO, 2019). Far-East Asia dominates carp production by forming > 94% of the global total output. While in Europe, although being the second biggest carp producer is dwarfed in comparison, with the region only representing 4.8% of total production level in 2017 for this species. Carp possess favourable attributes (e.g. tolerate a wide range of environmental conditions, disease resistance) that make them an ideal candidate for freshwater aquaculture (Davis et al., 2009). Carp are also omnivorous and physiologically can tolerate higher dietary inclusions of plant by-products. Therefore, an ideal farmed fish candidate to further develop the application of lupin meal in aquafeeds.

The present study aimed to evaluate the effects of dietary white lupin as a soybean alternative and a commercially available SSF end product Synergen™ to assess growth performance, feed utilisation and general health in common carp. The principal objective was to further our knowledge on whether these feed ingredients are more effective for fish feeds with the addition of exogenous enzyme sources under *in vivo* conditions commensurate with production conditions and feed. Such studies will serve to help diversify the over-reliance of a select group of plant proteins currently being used to feed farmed carp and other fish species.

2. Materials and methods

2.1. Diet formulation and experimental design

Experimental diets were formulated to the NRC (2011) guidelines on the nutritional requirements for carp. Two diets had the SPC component substituted with white lupin (*L. albus*) meal (Inveja Sas- Lup'Ingredients, Martigne-Ferchaud, France) at a replacement level of 12.5 % (or 8.33% SPC replacement, WL12.5) and 25% (16.67% SPC replacement, WL25, Table 2). A further two diets were formulated with the same replacement levels of white lupin but with the addition of 0.1% SSF supplementation (Synergen ™ Alltech, USA) (WL12.5 s and WL25 s). As a basal reference to the dietary treatments, two SPC based diets (without lupin meal) were produced, one with (SPCs), and one without (SPC) supplemental inclusion of SSF. All six diets were formulated to be isonitrogenous (42%), isolipidic (8%) and isoenergetic (19 MJ kg^{-1}).

Diets were manufactured by initially dry mixing ingredients before homogenising through a commercial food mixer. Diet pellets were extruded through a cold press extruder (PTM P6 Extruder System, Plymouth, UK) using a 2 mm aperture die. Feeds were subsequently dried in a dehumidifying oven for 24 h at 40 °C. Test diet formulations and proximate composition are presented in Table 1.

2.2. Experimental facilities and fish

The feeding trial was carried out in a freshwater recirculation aquaculture system, which was comprised of 12 fibreglass tanks (72 L) with a total system water capacity of 2200 L. Each tank had a flow rate of 4 L min^{-1}, with dissolved oxygen (6.82 ± 0.19 mg L^{-1}), temperature (25 ± 0.20 °C) and pH (6.62 ± 0.24) being measured daily (HQ 40d, HACH Company, Loveland, USA). Total ammonia nitrogen (0.11 ± 0.06 mg L^{-1}), nitrite (0.09 ± 0.05 mg L^{-1}) and nitrate (26.84 ± 9.64 mg L^{-1}) were measured weekly (Hach Lange, DR 2800, Loveland, USA). A 12 h light: 12 h dark photoperiod was maintained throughout the feeding trial period.

Common carp (*Cyprinus carpio L.*) was sourced from Bowlake fish farm (Hampshire, UK). Fish acclimated to the aquarium system for 75 days before the feeding trial. During that time, fish were fed on a maintenance diet (Sigma 50, EWOS/Cargill, Bergen, Norway). Fish were graded and randomly distributed into the tanks (n = 25, 15.35 ± 0.57 g). Experimental diets were given

three times daily at a ration level of ~3% of the fish body weight. Fish were weighed weekly after feeds were withheld for 24 h to allow gut clearance, and the amount of diet was adjusted accordingly to the weight. The present research study was carried out following the European Union's Animal research Directive 2010/63/EU and United Kingdom's Animal Scientific Procedures Act (1986). Furthermore, appropriate ethical review committee approval has been received.

2.2.1. Growth performance indices and feed utilisation calculations

Fish body weight (BW) and fork tail length (FL) measurements was carried out in order to determine the dietary effects has on the common carp growth performance. Growth performance indices and feed utilisation were calculated with the following formulae:

Weight Gain, **WG** g $fish^{-1}$ = Final BW (g) - Initial BW (g)

Specific Growth Rate; **SGR** % day^{-1} = (((ln (Final BW (g)) - (ln (Initial BW (g))) / Time) × 100

Table 1
Formulation and proximate composition of the experimental carp diets with partial inclusion of lupin meal and supplementation solid state fermentation product (%, dry weight).

	Diet					
Diet composition	SPC/0	SPCs	WL12.5/0	WL12.5s	W L25/0	WL25s
Soy protein concentrate[a]	49.81	49.81	41.48	41.48	33.14	33.14
White lupin[b]	–	–	12.50	12.50	25.00	25.00
Corn Starch[c]	28.32	28.22	25.52	25.42	22.72	22.62
Fish meal[d]	10.00	10.00	10.00	10.00	10.00	10.00
Fish Oil[e]	3.00	3.00	30.00	3.00	2.64	2.64
Corn Oil[f]	2.37	2.37	1.00	1.00	–	–
Corn gluten[g]	2.00	2.00	2.00	2.00	2.00	2.00
Pea protein[h]	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin and mineral Premix[i]	2.00	2.00	2.00	2.00	2.00	2.00
Carboxylmethyl-cellulose[j]	0.50	0.50	0.50	0.50	0.50	0.50
SSF[k]	–	0.10	–	0.10	–	0.10
Proximate composition						
Moisture	6.53	5.27	6.64	5.12	6.45	6.18
Protein	41.02	42.26	41.74	42.16	41.66	41.62
Lipid	7.52	7.32	7.39	7.62	7.43	7.72
Ash	6.20	6.27	6.12	6.09	5.73	6.00
NFE	39.72	37.87	38.09	39.00	38.72	38.45
Gross Energy; MJ kg^{-1}	19.29	19.40	18.90	19.38	19.2	19.07

[a]Hamlet Protein A/S, Horsens, Denmark.
[b]White lupin, Inveja Sas- Lup'Ingredients, Martigne-Ferchaud, France.
[c]Corn starch, Sigma-Aldrich, St. Louis, Missouri, USA.
[d]LT94, United Fish industries, Grimsby, UK.
[e]Fish oil, United Fish industries, Grimsby, UK.
[f]Corn oil, KTC, Wednesbury, UK.
[g]Glutalys®, Roquette Frères, Lestrem, France.
[h]Lysamine®, Roquette Frères, Lestrem, France.
[i]Fish PNP 2 %, Premier Nutrition, Rugeley, UK.
[j]Carboxylmethyl-cellulose (CMC), Sigma-Aldrich, St. Louis, Missouri, USA.
[k]Synergen™, Alltech, Nicholasville, Kentucky, USA.

Feed Intake, **FI** g $fish^{-1}$ = Feed given per tank (g) / number of fish

Feed Conversion Ratio; **FCR** = Feed intake (g) / weight gain (g)

Protein Efficiency Ratio, **PER** % =Weight gain / Protein intake × 100 (Wilson, 1989)

Apparent Net Protein Utilisation, **ANPU** % = ((FBP-FBW) - (IBP-IBW)) / PI × 100

Condition Factor, **K** = (Final BW / FL^3) × 100

Survival, % = 100 - Mortality (%) = (Final population / Initial population) × 100.

2.3. Proximate analysis

Finished test diets and fish samples (initial and final) from the feeding trial were analysed according to AOAC (2012) standard methods. Samples were homogenised using a food blender prior to analysis. All samples were analysed in triplicate. Moisture content was determined after drying material at 105 °C with a fan assisted oven until a constant weight was achieved. Similarly, ash levels in the samples were measured by incineration in a muffle furnace at 550 °C for period 16 h. Crude protein ($N \times 6.25$) was performed by the automated Kjeldhal method after acid digestion (Kjeldahltherm microsystem 40, C. Gerhardt GmbH, KG, Germany). Lipid content was determined through a Soxhlet gravimetric method using petroleum ether (Soxtec extractor HT 1043, Foss Tecator AB, Hoganas, Sweden). Gross energy was carried out by adiabatic bomb calorimetry (1356, Parr Instrument Company, IL, and the USA).

Table 2
Growth performance, feed utilisation and survival of common carp (*Cyprinus carpio*) after being fed with experimental diets for 10 weeks.

Parameters	Diet	0	S	*P*-value
IW, g $fish^{-1}$	SPC	15.68 ± 0.36^{a1}	15.4 ± 0.1^{a1}	1.08
	L12.5	15.2 ± 0.09^{a1}	15.4.0.06^{a1}	
	L25	15.36 ± 0.27^{a1}	15.06 ± 0.54^{a1}	
FBW; g $fish^{-1}$	SPC	43.92 ± 1.01^{a1}	53.84 ± 0.73^{a2}	3.18
	L12.5	44.2 ± 0.76^{a1}	62.24 ± 0.41^{b2}	
	L25	42.12 ± 0.94^{a1}	52.36 ± 1.17^{a2}	
WG; g g $fish^{-1}$	SPC	28.24 ± 0.64^{a1}	38.44 ± 0.62^{a2}	2.34
	L12.5	29 ± 0.85^{a1}	46.84 ± 0.34^{b2}	
	L25	26.76 ± 0.66^{a1}	37.3 ± 0.63^{a2}	
SGR; % day^{-1}	SPC	1.47 ± 0.00^{a1}	1.78 ± 0.00^{a2}	0.05
	L12.5	1.52 ± 0.03^{b1}	2.00 ± 0.00^{b2}	
	L25	1.44 ± 0.00^{a1}	1.78 ± 0.01^{a2}	
FI; g $fish^{-1}$	SPC	60.71 ± 1.54^{a1}	68.48 ± 1.01^{ab2}	3.74
	L12.5	58.38 ± 0.61^{a1}	71.79 ± 0.06^{b2}	
	L25	57.32 ± 1.25^{a1}	64.80 ± 0.98^{a2}	
FCR	SPC	2.14 ± 0.00^{b2}	1.77 ± 0.00^{b1}	0.05
	L12.5	2.01 ± 0.03^{a2}	1.53 ± 0.01^{a1}	
	L25	2.14 ± 0.00^{b2}	1.73 ± 0.00^{b1}	
FCE; %	SPC	46.51 ± 0.11^{a1}	56.13 ± 0.06^{a2}	1.56
	L12.5	49.64 ± 0.94^{b1}	65.24 ± 0.42^{b2}	
	L25	46.67 ± 0.14^{a1}	57.55 ± 0.10^{a2}	
PI; g $fish^{-1}$	SPC	24.29 ± 0.61^{a1}	29.62 ± 0.44^{ab2}	1.54
	L12.5	24.36 ± 0.25^{a1}	30.26 ± 0.02^{b2}	
	L25	23.88 ± 0.52^{a1}	26.97 ± 0.41^{a2}	
PER	SPC	1.15 ± 0.00^{ab1}	1.29 ± 0.00^{b2}	0.05
	L12.5	1.18 ± 0.02^{b1}	1.52 ± 0.02^{c2}	
	L25	1.11 ± 0.00^{a1}	1.37 ± 0.00^{a2}	
ANPU	SPC	17.86 ± 0.04^{a1}	20.06 ± 0.02^{a2}	0.55
	L12.5	18.69 ± 0.33^{b1}	24.06 ± 0.13^{c2}	
	L25	17.32 ± 0.05^{a1}	21.27 ± 0.04^{b2}	
LER	SPC	6.19 ± 0.01^{a1}	7.67 ± 0.00^{b2}	0.21
	L12.5	6.72 ± 0.12^{b1}	8.57 ± 0.06^{c2}	
	L25	6.28 ± 0.017^{a1}	7.45 ± 0.014^{a2}	
ER; %	SPC	2.48 ± 0.00^{a1}	2.98 ± 0.00^{a2}	0.07
	L12.5	2.59 ± 0.04^{b1}	3.43 ± 0.01^{c2}	
	L25	2.46 ± 0.01 ^{a1}	3.05 ± 0.00^{b2}	
K; %	SPC	2.26 ± 0.05 $^{a\ 1}$	2.29 ± 0.02 ^{a1}	0.14
	L12.5	2.21 ± 0.02 $^{a\ 1}$	2.30 ± 0.02 ^{a1}	
	L25	2.17 ± 0.02 ^{a1}	2.28 ± 0.06 ^{a1}	
Survival; %	SPC	100	100	
	L12.5	100	100	
	L25	100	100	

± S.D. IBW, initial body weight; FBW, final body weight; WG, weight gain, SGR: specific growth rate; FI, feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; ANPU, apparent net protein utilisation; K, condition factor. Data in the same row with different superscript are significantly different ($P < 0.05$).

2.4. Tissue collection

Three fish per tank were euthanised with overdose buffered tricaine methane sulphate (MS222, Pharmaq, Norway) followed by the destruction of the brain. The intestinal and liver samples were taken washed with cold phosphate buffered saline (pH 7.3) to remove debris and faecal matter. An intestinal section from the posterior region and mid-gut were used in light and electron microscopy analysis. Liver samples were analysed using light microscopy.

2.4.1. Light microscopy

Tissue samples for light microscopy were fixed at 4% formaldehyde (10% v/v from 40% stock) in neutral buffered saline (pH 7.0) for 24 h, and subsequently processed for embedding in paraffin wax. Sections were stained in haematoxylin and eosin for mucosal fold length, mucosal fold width and lamina propria width measurements. Alcian blue and periodic acid Schiff staining were used for goblet cell determination. Hepatocyte size, nucleus size and the ratio of nucleus diameter to hepatocyte diameter were measured (Omar, 2011).

Table 3
Proximate body composition of common carp (*Cyprinus carpio*) after being fed with test diets for 10 weeks (% live weight, ± SD).

Parameters	Diet	Initial		0	S	*p*-value
Moisture	SPC	77.97	± 0.13	76.02 ± 0.19^{a1}	75.64 ± 0.08^{a1}	0.80
	L12.5			76.09 ± 0.21^{a1}	75.36 ± 0.19^{a1}	
	L25			76.40 ± 0.20^{a2}	75.58 ± 0.35^{a1}	
Crude protein	SPC	14.47	± 0.02	15.25 ± 0.09^{a1}	15.17 ± 0.03^{a1}	0.42
	L12.5			15.25 ± 0.09^{a1}	15.28 ± 0.12^{a1}	
	L12.5			15.02 ± 0.20^{a1}	15.09 ± 0.08^{a1}	
Crude lipid	SPC	4.74	± 0.02	7.03 ± 0.20^{a1}	7.62 ± 0.09^{a1}	
	L12.5			6.65 ± 0.11^{a1}	7.65 ± 0.15^{a2}	0.71
	L25			6.58 ± 0.22^{a1}	7.49 ± 0.30^{a2}	
Ash	SPC	2.45	± 0.02	1.96 ± 0.05 ^{a2}	1.72 ± 0.02 ^{a1}	0.12
	L12.5			1.90 ± 0.03 ^{a1}	1.82 ± 0.02 ^{ab1}	
	L25			1.95 ± 0.01 ^{a1}	1.92 ± 0.04^{b1}	
Gross energy; MJ kg^{-1}*	SPC	23.76	± 0.01	25.09 ± 0.14^{b1}	25.22 ± 0.07^{a1}	0.50
	L12.5			24.39 ± 0.15^{a1}	25.06 ± 0.21^{a2}	
	L25			24.76 ± 0.12^{ab1}	24.87 ± 0.05^{a1}	

Data in the same row with different superscript are significantly different ($P < 0.05$). *Gross energy of dried material.

2.4.2. Scanning electron microscopy (SEM)

Collected intestinal samples from the posterior region (ca. 2 mm) were washed in 1% scarboxymethyl-L-cysteine to remove epithelial mucus and fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1: 1 vol., pH 7.2, 3% NaCl). Samples were critically point dried with ethanol as the intermediate fluid and CO_2 as the transition fluid. The dried intestinal samples were mounted on aluminium stubs and gold coated. SEM imaging was carried out on a JSM 6610 L V electron microscope at 15 kV (Jeol, Tokyo, Japan) and ImageJ (V1.52o, U. S. National Institutes of Health, Bethesda, Maryland, USA) processing software was used to calculate microvilli density (MD). A thresholding technique was used to differentiate the ratio between the microvilli covered area (M, foreground) to the background (B, background), MD = M / B, and the result was expressed as an arbitrary unit (AU). Three different images per sample were used to evaluate the MD.

2.5. Haematological parameters

At the end of the trial, fish were euthanised, and blood collected from 6 fish per treatment. Fish were anaesthetised with buffered tricaine methane sulphate (MS222, Phamaq, Norway) at 200 mg L^{-1} followed by the destruction of the brain. Blood was sampled from the caudal vein using a 25-gauge heparinized needle and 1-ml syringe. Haematocrit is used as an indicator of animal health and is the percentage of packed blood cells to plasma volume (Rao and Deshpande, 2005). Haemoglobin (Hb) concentration was calculated based on Drabkin's cyanide-ferricyanide solution as described by Rao and Deshpande (2005). Differential leukocyte measurements were performed by blood smears using fresh whole blood (Dacie and Lewis, 1995). Smears were air-dried and stained with May Grunwald Giemsa stain. Two hundred leukocytes were counted per slide to determine leukocyte population groups.

2.6. Statistical analysis

All results are expressed as mean values with the corresponding standard deviation (± SD). Data analysis was performed using two-way ANOVA with post-hoc LSD test was used to identify significant differences between dietary treatments.

3. Results

3.1. Growth and feed utilisation

All fish had readily accepted the experimental diets during the feeding trial. From the 10 week feeding period, substituting dietary SPC with up to 25% of white lupin meal (WL12.5 and WL25) did not negatively affect growth performance (final weight, FBW; weight gain, WG) or feed utilisation (feed intake, FI; protein efficiency ratio, PER; apparent net nitrogen utilisation, ANPU) when compared to the reference diet (SPC, $P > 0.05$, Table 2). Although, mean FCR was found to have decreased by 6.3% in 12.5% lupin meal dietary treatment in comparison to the SPC/0 group.

The dietary inclusion of 0.1% SSF significantly enhanced carp SGR and FCR utilisation in SPCs, WL12.5s and WL25s treatment groups ($P = 0.05$). The most significant improvement in SGR and FCR were carp that received WL12.5s diet In comparison to the dietary control (SPC/0), WL12.5s diet had increased 35% in the final weight, 50% weight gain, 31% SGR, 17% FI, 28% PER and 30% ANPU. For FCR, there was a decrease of 33% in WL12.5s in contrast to SPC dietary treatment group. The inclusion of SSF into the SPC basal diet was also observed to have an improved effect on growth performance and feed utilisation. For instance, SPCs fed fish had a weight gain that was 31% higher than without the inclusion of SSF.

Based on the final mean weights, the dietary treatment groups could be ranked to the following effectiveness: WL25 < SPC < WL12.5 < WL25s < SPCs < WL12.5s. In general, these ranking trends are also observed in WG, SGR, FI, FCR, PER and ANPU. The condition factor (K) value for all groups ranged from 2.30 (WL12.5s) to 2.17 (WL25). No significant differences in K were found between dietary treatment groups.

3.2. Body composition

Both the initial and end of feeding trial body composition are presented in Table 3. The assessment of final carp proximate composition showed that moisture levels were lowered marginally when SSF was present in the diet. The lowest moisture content was 75.36% in fish that received WL12.5s, when compared to 76.02% in SPC dietary group ($P = 0.80$). Carp that received dietary inclusion of lupin meal and/or SSF was found not influence body protein levels ($P = 0.42$).

The replacement of SPC with lupin meal did not significantly affect body lipid levels ($P = 0.71$). However, the addition of SSF into the diets did increase mean body lipid content regardless of the presence of lupin meal in the diet. The highest lipid concentrations were in carp that received WL12.5s diet, with an 8% increase in comparison to the dietary control. Both ash and energy levels showed no apparent changes between the dietary treatments ($P = 0.12$ and $P = 0.50$, respectively).

3.3. Gut morphology

The results of the histological examinations on the mucosal fold

Table 4
Common carp (*Cyprinus carpio*) intestinal and liver morphology after being fed with experimental diets for 10 weeks (± S.D.).

Parameters	Gut Region	Diet	0	S	*P*-value
Villi length; μm	Posterior	SPC	507.21 ± 26^{a1}	608.41 ± 80^{a1}	16.35
		L12.5	509.08 ± 15^{a1}	644.56 ± 64^{a1}	
		L25	602.06 ± 64^{a1}	603.09 ± 24^{a1}	
	Mid	SPC	440.71 ± 12^{a1}	612.78 ± 14^{a2}	10.76
		L12.5	530.50 ± 23^{a1}	596.86 ± 34^{a1}	
		L25	517.53 ± 44^{a1}	598.04 ± 30^{a1}	
Goblet cells; 100 μm^{-1}	Posterior	SPC	3.60 ± 0.26^{a1}	3.77 ± 0.28^{a1}	0.99
		L12.5	3.08 ± 0.40^{a1}	3.19 ± 0.27^{a1}	
		L25	2.98 ± 0.09^{a1}	3.59 ± 0.21^{a1}	
	Mid	SPC	3.7 ± 0.30^{a1}	3.11 ± 0.23^{a1}	0.73
		L12.5	3.77 ± 0.16^{a1}	4.31 ± 0.16^{b1}	
		L12.5	4.61 ± 0.14^{b1}	4.61 ± 0.14^{b1}	
Villi width; μm	Posterior	SPC	111.42 ± 3.19^{a1}	111.72 ± 2.83^{a1}	13.08
		L12.5	111.99 ± 5.43^{a1}	123.08 ± 2.36^{ab1}	
		L25	106.91 ± 4.15^{a1}	125.32 ± 3.86^{b2}	
	Mid gut	SPC	106.90 ± 3.14^{a1}	120.85 ± 4.73^{a1}	18.47
		L12.5	117.53 ± 3.51^{a1}	119.53 ± 6.91^{a1}	
		L25	107.10 ± 7.28^{a1}	128.53 ± 4.99^{a2}	
Lamina propria Width (μm	Posterior gut	SPC	27.44 ± 1.87^{a1}	31.03 ± 1.47^{a1}	5.92
		L12.5	26.37 ± 1.53^{a1}	35.22 ± 1.81^{a2}	
		L25	26.76 ± 1.57^{a1}	32.68 ± 1.92^{a2}	
	Mid gut	SPC	29.99 ± 1.13^{a1}	35.99 ± 0.46^{a2}	4.54
		L12.5	33.54 ± 1.48^{a1}	40.64 ± 0.97^{b2}	
		L25	31.28 ± 1.44^{a1}	38.97 ± 1.62^{ab2}	
Microvilli density*	Posterior	SPC	1.52 ± 0.04^{a1}	2.21 ± 0.34^{a1}	0.72
		L12.5	1.85 ± 0.2^{a1}	1.59 ± 0.19^{a1}	
		L25	1.18 ± 0.08^{a}	1.66 ± 0.18^{a1}	
Hepatocyte size; μm		SPC	11.76 ± 0.24^{a1}	12.27 ± 0.44^{a1}	1.13
		L12.5	12.45 ± 0.16^{ab1}	13.65 ± 0.30^{b2}	
		L25	12.94 ± 0.29^{b1}	13.02 ± 0.41^{ab1}	
Nucleus size; μm		SPC	5.42 ± 0.34^{a1}	5.66 ± 0.13^{a1}	0.71
		L12.5	5.9 ± 0.14^{a1}	5.94 ± 0.11^{a1}	
		L25	5.85 ± 0.18^{a1}	5.76 ± 0.17^{a1}	

Data presented as mean ± S.D.; a, b data with the same superscripts with the same column are not significantly different ($P > 0.05$) and data with the different superscripts with the same column are significantly different ($P < 0.05$). 1, 2 data with the same superscript with the same row are not significantly different ($P > 0.05$) and data with the different superscript with the same row are significantly different ($P < 0.05$). *Arbitrary unit.

length, width, number of goblet cells and lamina propria width of the midgut and posterior gut are presented in Table 4, Figs. 1 and 2. The extensive gut morphology assessment showed no significant changes ($P > 0.05$). However, the substitution of SPC with 12.5% lupin meal showed an increase in the midgut mean mucosal fold length by 19% compared to no replacement (SPC/0 diet). However, increasing to 25% lupin meal inclusion did not show a further increase in mean mucosal fold length. Furthermore, villus found in the midgut was the widest in white lupin inclusion diets with SSF supplementation (WL12.5s and WL25s) than the dietary control treatment (SPC/0). The highest goblet cell count was found in the midgut of carp that were fed with 25% lupin meal replacement and SSF inclusion. The increase was from 3.70 goblet cells 100 μm^{-1} in the SPC diet to 4.61 goblet cells 100 μm^{-1} in the WL25s. In general, the posterior gut had wider mean values of the lamina propria in fish that fed on SSF supplemented diets. The inclusion of SSF into the SPC based diet produced fish that had denser microvilli enterocyte surfaces at the ultrastructural level from 1.52 ± 0.10 (SPC/0) to 2.21 ± 0.84 ABU (SPCs, Fig. 3).

However, the inclusion of SSF in conjunction with lupin meal into the diet did not show a similar mean value increase.

3.3.1. Liver histology

Atrophy and necrosis of hepatic cells, vascular and fatty degeneration were generally observed. Mean hepatocyte diameter size increased by 10% in the 25% SPC replacement dietary group, however, this trend was not present in the 12.5% SPC substitution fish (Table 4). The addition of SSF into the carp diet further increased mean values of hepatocyte diameter size. Although, no significant differences were observed in hepatic nuclei diameter ($P = 1.13$) or the ratio of the nucleus to hepatocyte diameter ($P = 0.71$) between the dietary groups.

3.3.2. Blood parameters

Haematological measurements of the carp fish after the feeding trial showed that packed cell volume (PCV) was increased by SPC substitution with white lupin meal. The rise in PCV level in the 12.5 and 25% SPC dietary replacement group in comparison to the control treatment (SPC) was 24 and 31%, respectively (Table 5). However, like the other diets with SSF inclusion, this increase did not show a significant difference ($P = 8.49$). The addition of SSF into the carp diet also increased mean haemoglobin content, but again, no significant difference was found when compared to the control diet (SPC/0). For differential leukocyte counts, the four types of cells: lymphocytes, neutrophils, monocytes, eosinophil and basophils, showed no significant differences between the dietary treatments ($P > 0.05$).

4. Discussion

4.1. Growth performance

The present study has demonstrated that SPC could be effectively substituted with up to 25% white lupin meal in common carp diet, without loss in growth performance or feed utilisation. The results from the current study were comparable to those reported by Hernández and Roman (2016). The authors had tested 25% dietary lupin meal inclusion on common carp and reported that there were no statistical differences in growth performance response (e.g. weight gain and FCR) when compared to the reference diet group. The knowledge on the effects of dietary lupin meal has on common carp is so far limited; however, there are reported studies on other farmed fish species. For example, the omnivorous tilapia fish (*Oreochromis niloticus* × *O. aureus*) was fed with test diets that had partially replaced defatted soybean

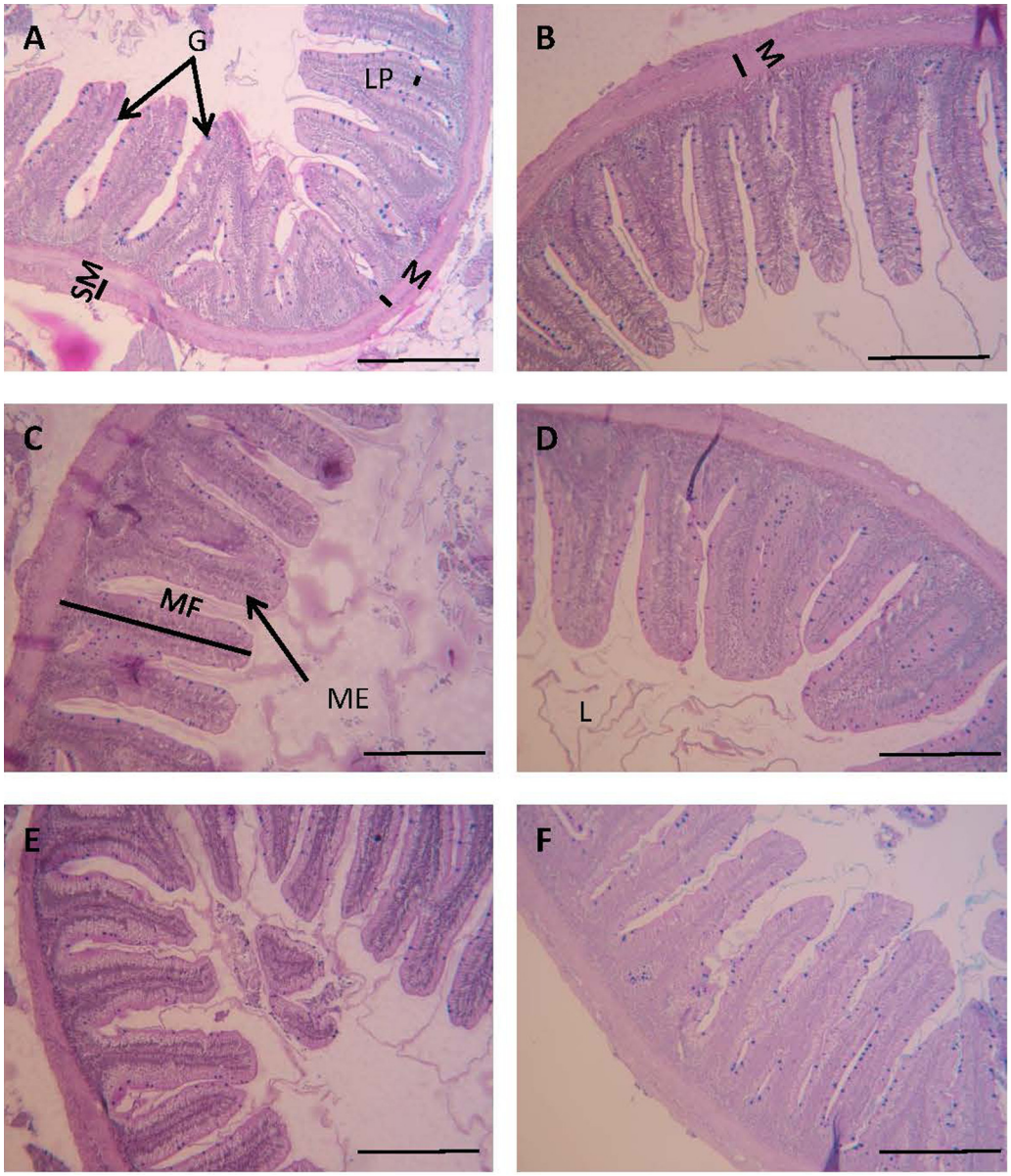

Fig. 1. Mid intestinal section of common carp stained with Alcian blue and Periodic acid–Schiff after been fed for 10 weeks on experimental diets. Scale bar represents 50 µm. Dietary treatments: (A) SPC, (B) SPCs, (C) WL12.5, (D) WL12.5s, (E) WL25, and (F) WL25s. L: Lumina, LP: Lamina propria, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells.

meal with up to 67% of blue lupin (*L. angustifolius*) seed meal. The response after the feeding trial demonstrated that the fish growth performance (i.e. final fish weight, weight gain, specific growth rate and feed conversion ratio) indicators were unaffected by the change in the diet composition (Chien and Chiu, 2003). Similarly, the replacement of soymeal meal up to 30% lupin meal in gilthead seabream (*Sparus aurata*) did not yield differences in growth performance or feed utilisation when statistically tested against the basal reference diet (Robaina et al., 1995).

The suitability of lupin meal in aquafeeds could have a significant impact on commercial aquaculture, as it would mitigate and de-risk the over-reliance of imported plant-based proteins, i.e. soybean by-products. Like many other plant-based proteins, the presence of high NSP levels in lupin meal is a major barrier in formulating in fish diets. It has been reported that lupin kernel meal (405 g kg^{-1}) can contain nearly twice the amount of NSP than soybean meal (217 g kg^{-1}, van Barneveld, 1999).

The use of exogenous enzymes to aid the breakdown NSP in aquafeeds has been widely reported. Sardar et al. (2007) stated that microbial phytase supplementation could improve growth, weight gain, feed utilisation and survival in common carp soybean-based diet. Lin et al. (2007) reported that supplement 0.1% commercially exogenous enzyme (neutral protease, b-glucanase and xylanase) into plant-based diets for hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) can significantly enhance growth performance and feed utilisation. Moreover, the authors indicated that exogenous enzyme supplementation can promote the secretion of the endogenous enzymes in fish. The current study also found that SSF supplementation into the SPC diet formulation, and in test diets with white lupin meal yielded significantly elevated growth performance (i.e. specific growth rate) and feed utilisation (i.e. feed conversion ratio) parameters in common carp. Furthermore, these improvements were higher than the carp that were fed with diets having lupin meal alone. The residual enzyme activity in SSF could be breaking down the high NSP in the diets, thereby increasing feed digestibility and growth performance under the conditions of the trial. Reported studies of SSF use in aquafeeds is limited, but

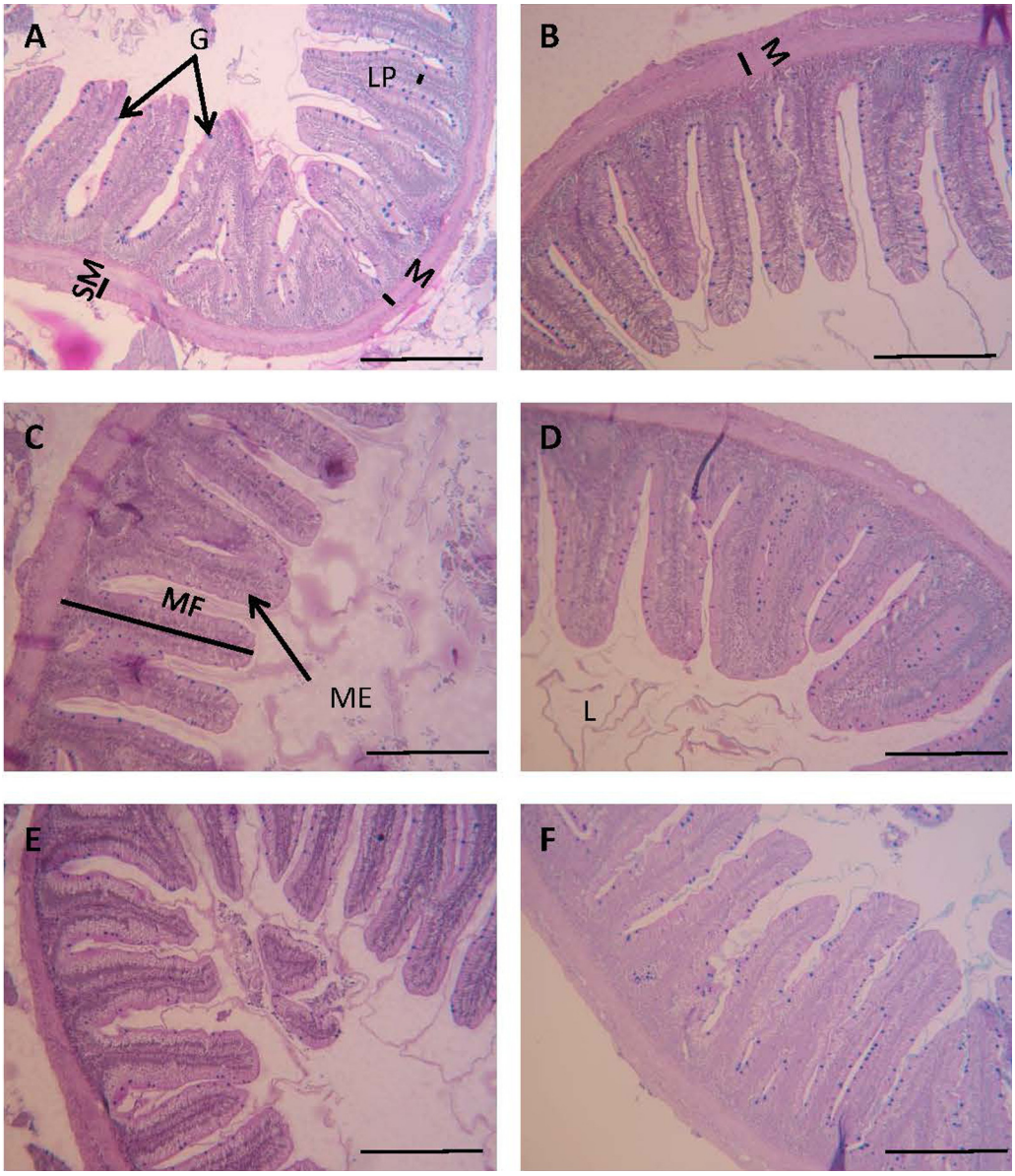

Fig. 2. Posterior intestinal section of common carp stained with Alcian blue and Periodic acid–Schiff after been fed for 10 weeks on experimental diets. Scale bar represents 50 µm. Dietary treatments: (A) SPC, (B) SPCs, (C) WL12.5, (D) WL12.5s, (E) WL25, and (F) WL25s. L: Lumina, LP: Lamina propria, ME: Mucosal epithelium, MF: Mucosal fold, M: Muscularis, SM: Serous membrane, G: Goblet cells.

a study carried out on European seabass (*Dicentrarchus labrax*) using 0.2 and 0.4% SSF (Synergen™, Alltech, Dunboyne, Ireland) gave increased apparent digestibility coefficient values by up to 18% compared to no inclusion (Magalhães et al., 2018). Although, the increase in feed digestibility was further enhanced when SSF inclusion was doubled in the diet. The use of 0.04% SSF (Synergen™) in turbot (*Scophthalmus maximus*) diets had only produced limited dry matter apparent digestibility improvements of up to 6% when compared to the control diet (Diógenes et al., 2018). It is possible that this may be due to the comparatively shorter intestinal tract in this latter species and faster transit time.

4.2. Body composition

Analysis on the end of trial carp body composition showed dietary white lupin meal inclusion of up to 25% did not influence moisture, protein or lipid content. Similar trends were found in other studies where white lupin meals were included into the diet for rainbow trout (Burel et al., 1998; Borquez et al., 2011; Zhang et al., 2012) and turbot (*Psetta maxima*) (Burel et al., 2000).

In comparison, the supplementation of SSF to 12.5% lupin meal inclusion diets produced an increase in gross energy and ash content. While body moisture, protein and lipid remained unchanged for any of the SSF supplement fish groups. While compared with other commercial exogenous enzymes feeding studies, such as Farhangi and Carter (2007). The unchanged whole-body composition was also found when rainbow trout (*Oncorhynchus mykiss*) was fed with up to 50% of dehulled blue lupin (*L. angustifolius*) inclusion diet along with either Energex™, Bio-Feed ™ Pro, alpha galactosidase™ or a mixture.

4.3. Gut and liver morphology

It should be noted that soybeans contain antinutritional factor(s) that induce enteritis in the distal intestine of salmonid fishes. The resulting inflammation causes widening and stunting of the mucosal folds and disappearance of the supranuclear vacuolisation of the absorptive cells in the intestinal epithelium. It also produces increased amounts

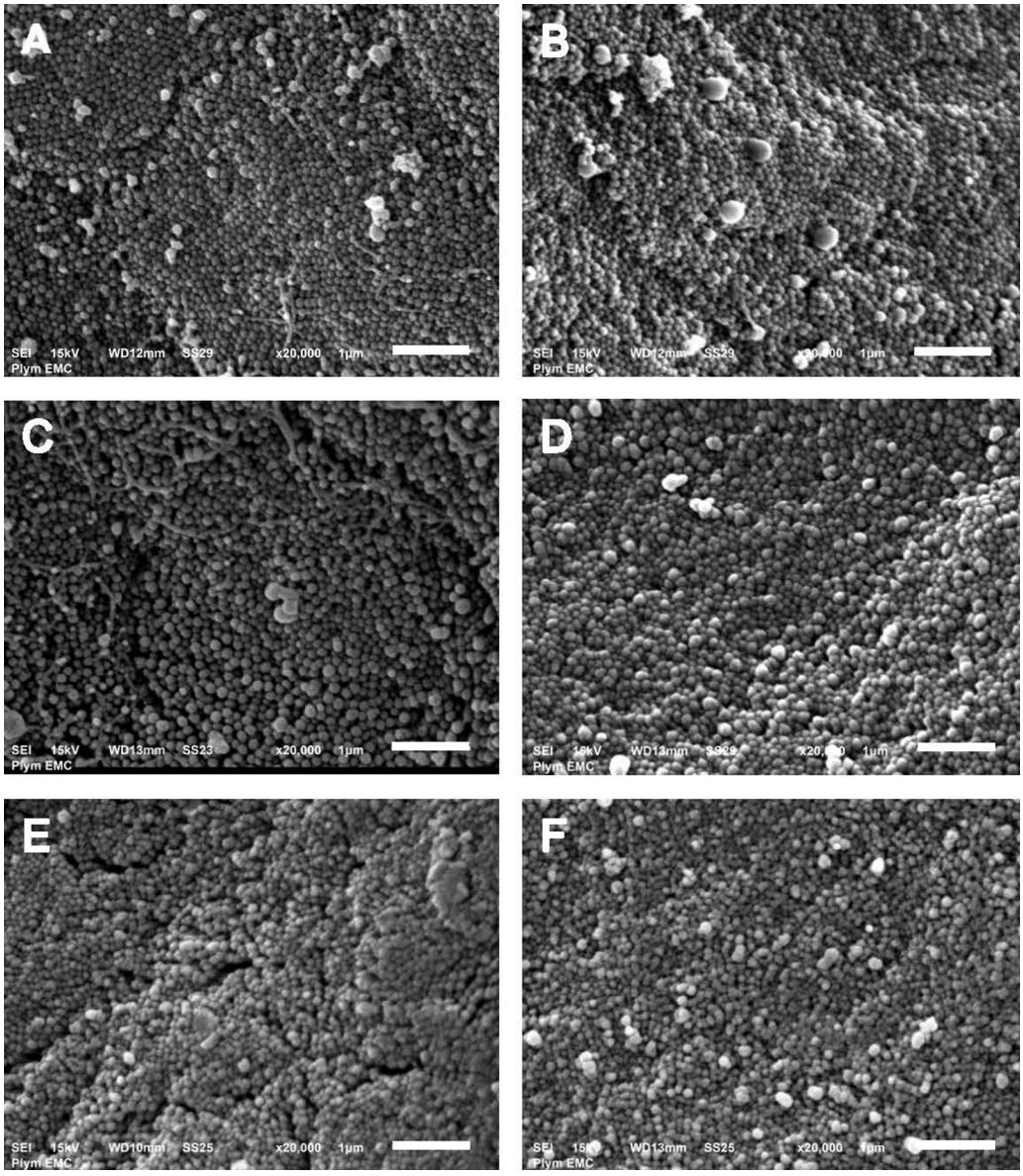

Fig. 3. Posterior intestinal section of the common carp after been fed for 10 weeks on experimental diets under scanning electron microscopy. Scale bar represents 50 µm. Dietary treatments: (A) SPC, (B) SPCs, (C) WL12.5, (D) WL12.5s, (E) WL25, and (F) WL25s.

(width) of connective tissue in the central stroma within the mucosal folding together with and infiltration of a mixed leukocyte population in the lamina propria and submucosa (Baeverfjord and Krogdahl, 1996).

Liver and intestinal histological analysis carried out in the present study showed that there were no observable changes amongst the white lupin treatment groups (WL12.5 and WL25) compared to the reference dietary group. While the results were not significant, the addition of SSF to the SPC treatment diet and diets with 12.5 and 25% white lupin meal inclusion showed better gut morphology through an increase in mucosal fold length, width, the number of goblet cells and lamina propria dimension. These results may suggest that dietary supplementation of SSF can lead to an increase of the absorptive area in the mid and hindgut could improve nutrient assimilation, feed utilisation and growth performance.

Recently Bowyer et al. (2020) showed a beneficial effect of SSF on the gut ultrastructure and enhanced microvilli absorptive capacity (enterocyte microvilli length) in a study with tilapia also testing both blue and yellow lupin cultivars included as an alternate feed ingredient. This is in contrast with the findings of Marković et al. (2012) who reported an inverse relationship between mucosal fold length and growth rate in fish fed an SSF product. Several authors have observed histological alterations in the intestine of fish fed a high level of a plant-based diet. Uran et al. (2008) found that common carp show signs of enteritis when fed high levels of soybean. In the case of lupin meal, Farhangi and Carter (2001) observed that increasing dietary inclusions of blue lupin (*L. angustifolius*) for rainbow trout (*Oncorhynchus mykiss*) diet can shorten the mean villus length by up to 15%. Furthermore, Borquez et al. (2011) found that inclusion of 40 and 50% of white lupin to rainbow trout diet led to histological changes in the mid intestine such as decrease the number of basophil granulocytes, distal displacement of enterocyte nucleus and an increment in lipid drops. It is possible to suggest that sparteine, a common hepatoxic lupinine alkaloid could be interfering with the intestinal morphology. Rainbow trout (*Oncorhynchus mykiss*) fed diets that had up to 100 mg kg^{-1} showed a decrease in absorptive vacuoles size (Serrano et al., 2012). While dietary groups fed with > 1000 mg kg^{-1} sparteine displayed fewer mucosal and smaller sized folds at the mid intestine.

There was some evidence of minor atrophy and necrosis of hepatic cells, vascular and fatty degeneration in all treatment groups in this

Table 5
Haematological parameters of Common carp (*Cyprinus carpio*) after been fed with test diets for 10 weeks (± S.D.).

Parameters	Diet	0	S	*p*-value
Haematocrit; %	SPC	33.83 ± 2.89^{a1}	32.33 ± 3.13^{a1}	8.49
	L12.5	43.00 ± 1.83^{b1}	43.33 ± 0.65^{b1}	
	L25	46.33 ± 2.98^{b1}	41.5 ± 1.38^{b1}	
Haemoglobin; g dL^{-1}	SPC	7.66 ± 0.26^{a1}	7.95 ± 0.41^{a1}	1.43
	L12.5	8.89 ± 0.43^{ab1}	8.45 ± 0.43^{ab1}	
	L25	9.2 ± 0.48^{b1}	9.45 ± 0.24^{b1}	
Lymphocytes; %	SPC	87.16 ± 1.81^{a1}	87.66 ± 1.40^{b1}	2.21
	L12.5	88.83 ± 1.91$^{a;2}$	84.16 ± 1.99^{a1}	
	L25	87.16 ± 1.02^{a1}	88.11 ± 1.71^{b1}	
Neutrophils; %	SPC	3.25 ± 0.88^{a1}	3.91 ± 0.66^{a1}	3.04
	L12.5	4.16 ± 1.16^{a1}	5.08 ± 0.89^{a1}	
	L125	3.33 ± 0.65^{a1}	4.08 ± 0.62^{a1}	
Monocytes; %	SPC	4.75 ± 0.89^{a1}	4.66 ± 0.62^{a1}	2.41
	L12.5	3.25 ± 0.35^{a1}	4.75 ± 0.47^{a1}	
	L25	4.5 ± 0.85^{a1}	3.16 ± 0.6^{a1}	
Eosinophils; %	SPC	3.58 ± 0.61^{a1}	2.08 ± 0.26^{a1}	2.45
	L12.5	2.00 ± 0.44^{a1}	4.08 ± 1.00^{a1}	
	L25	3.41 ± 0.80^{a1}	3.05 ± 0.64^{a1}	
Basophils; %	SPC	1.25 ± 0.30^{a1}	1.66 ± 0.42^{a1}	1.65
	L12.5	1.75 ± 0.60^{a1}	1.91 ± 0.59^{a1}	
	L25	1.58 ± 0.35^{1a}	1.58 ± 0.35^{a1}	

Data in the same row with different superscript are significantly different ($P < 0.05$).

study with carp. This was also observed by Borquez et al. (2011) which investigated the dose-response relationship of dietary white lupin in rainbow trout (*Oncorhynchus mykiss*) diets. These authors reported there was evidence of lipid infiltration into hepatocytes and enterocytes at 40% dietary inclusion level of lupin. However, in another study, no such alterations in lipid and glycogen storage in the hepatocytes was found in gilthead seabream (*Sparus aurata*) when fed with 30% de-hulled blue lupin (*L. angustifolius*) seed meal (Robaina et al., 1995). Although it was found not to be significant, the present investigation found a general trend of increased hepatocyte size (diameter) when fish are fed with SSF supplemented lupin inclusion diets.

4.4. Blood parameters

Haematological evaluation can be a useful tool in monitoring fish health status and physiological function (Clauss et al., 2008). Fish fed with experimental feeds that had SPC replaced with white lupin meal and with or without SSF supplementation did not compromise the carp basic blood parameters or health status, i.e. total leukocyte cell count and leukocyte differentiation. The lack of change in the fish health parameters found in the present study was comparable to Bransden et al. (2001). The authors reported that up to 40% dietary inclusion of de-hulled blue lupin (*Lupinus angustifolius*) did not have significant adverse effects on growth, immune function or blood chemistry and disease resistance in Atlantic salmon (*Salmo salar*).

5. Conclusion

In conclusion, the use of white lupin meal can be included by up to 25% in carp diets, without showing adverse effects on growth performance, feed utilisation, body composition, physiological status and gut integrity. Reducing SPC in aquafeeds using lupin meal can substantially decrease the over-reliance on soybean meal use in aquaculture. This could have a significant effect on decreasing global soybean demand in aquaculture, which would lead to a reduction in the environmental impact of its production (e.g. deforestation). The inclusion of SSF into a plant-based carp diet formulation improved growth performance and feed utilisation for this species. It also has the potential to reverse the negative effects of plant proteins in carp diets through improvements in gut morphology. This can benefit the exploitation of plant protein concentrates for other farmed fish species that would have high economic importance, e.g. salmonids, seabass and sea bream.

CRediT authorship contribution statement

Ayub Anwar: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft. **Alex HL Wan:** Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Samad Omar:** Funding acquisition, Project administration, Supervision, Validation, Writing - review & editing. **Ehab El-Haroun:** Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Simon J Davies:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100348.

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