A Solid-State Fermentation (SSF) supplement improved performance, digestive function and gut ultrastructure of rainbow trout (Oncorhynchus mykiss) fed plant protein diets containing yellow lupin meal

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Abstract

A nutritional investigation was conducted to determine whether growth performance, feed efficiency and midgut morphology of rainbow trout (*Oncorhynchus mykiss*) is affected by United Kingdom cultivars of Yellow Lupin (Control, Y) (LC) compared to the addition of a commercial solid-state fermentation product (SSF) in separate dietary treatments 0.1 and 0.5 % (LS0.1 and LS0.5, respectively). At the end of feeding trial, LC- and LS0.1-fed fish did not differ from one another, whilst significant improvement was observed in LS0.5 fed fish ($P < 0.05$). Apparent digestibility of crude protein and crude fibre were significantly higher in fish fed LS0.1 and LS0.5 than LC. No significant differences ($P < 0.05$) in Haemoglobin (Hb) concentrations were observed among dietary treatments. Fish fed the 0.1 % SSF inclusion exhibited significantly ($P < 0.001$) higher haematocrit (Hct) concentration than all other treatments. Significantly ($P < 0.005$) higher serum glucose levels were observed in FMC fed fish compared to fish fed LC and LS0.1, whilst elevation was also observed in LS0.5 over LC and LS0.1. Activity of serum lysozyme was significantly ($P < 0.05$) higher in the LS0.5 treatment compared to LC, S0.1 and FMC groups. Fish fed 0.5 % SSF were observed to have a significant ($P < 0.05$) reduction in goblet cell counts (GC) compared to those fed LC and LS0.1.). Median enterocyte height (EH) of LS0.1 and LS0.5 fed fish did not differ from one another but were both significantly greater than LC fed fish. Mean microvilli length (MVL), diameter (MVD) and enterocyte apical area (EAA) did not differ significantly between dietary treatments ($P > 0.05$). Fish fed 0.5 % SSF displayed a higher microvillar percentage coverage (MVCV) compared to fish fed LC and LS0.1. Total alkaline protease (TAP), trypsin and chymotrypsin activities in the digesta of fish were unaffected by dietary treatment ($P > 0.05$). Activity of alkaline phosphatase (ALP) in digesta was significantly ($P < 0.05$) higher in SSF treatments than LC. Activity of L-leucine aminopeptidase (LAP) in digesta was marginally

significantly (P = 0.045) higher in LS0.1 SSF fed fish than LC fed fish. Activity of LAP was also significantly higher in LS0.5 fed fish than LC fed fish.

Key words: Rainbow trout, Plant proteins, Lupins, SSF, Growth Performance, Gut integrity, Intestinal Enzymes, Haematological indices

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) production is a significant industry throughout the world with continued annual exponential growth attaining approximately 814 thousand tonnes (FAO, 2018). Rainbow trout typically require energy dense diets consisting of high-grade protein and lipid; this means feeding costs are relatively higher than for other species with feed typically contributing well in excess of 60 % of the total operating expenses. Fish meal (FM) and oil (FO) constraints have place tremendous pressure upon the salmonid industry but seen as the animals are naturally carnivorous, reducing dependency upon these ingredients has been a challenge. This was recently reported by Kok et al (2020) who determined the Fish in: Fish out relationships for numerous farmed species highlighting the issue for salmonids in particular. However the use of FM and FO has been greatly reduced in the last 15 years and salmonids are either neutral or net aquatic protein producers due to alternative ingredient use in commercial diets, although this has led to a reduction in their nutritional value with respect to essential fatty acids (Sprague et al. 2016)

In 1990 feeds used in Norwegian salmon production contained, on average, in excess of 65 % FM, by 2013 this figure stood at just over 18 % (Nofima, 2014). With regards to rainbow trout, this metric has been reduced to around 15 % in many diets. Following a wealth of scientific research over the past few decades, soybean products have predominantly filled the space previously occupied by FM, with more minor contributions from the likes of wheat, soybean, rapeseed, sunflower and guar. Bulk ingredient research has now begun to slow down. Attention is now increasingly being turned to solutions such as nutritional programming and genetic selection for carnivorous fish tolerance to plant proteins (Quinton et al., 2007a; Quinton et al., 2007b; Le Boucher et al., 2013; Overturf et al., 2013; Yamamoto et al., 2015). This demonstrates that we are reaching a limit in what can naturally be achieved in ingredient selection but we still have not reached desired goals.

Salmonid fish such as rainbow trout require diets containing an amino acid profile similar to that of FM, as well as a correct balance of essential fatty acids (EFA), again similar to that of FO. Utilisation of carbohydrates is quite restricted in salmonids and their over inclusion may result in the increased utilisation or conversion to stored fat as an energy source (Skiba-Cassy *et al*., 2013). Evidently recreating adequate trout diets with plant-derived products has been somewhat problematic. At present, alternative source of protein in aquafeeds should begin to no longer be viewed, or categorized, as simply alternatives to fish meal; they should be considered as any ingredient with the potential to outperform the sustainability credentials of any other commercially implemented counterpart (Bowyer et al., 2019; Bowyer et al., 2020). Quite simply, we have attained much of what can be done with regards traditional feed manufacture and process technologies, now manipulation of the animal is required in order to optimise feed and cost efficiency. As such lupines are seen to be a great potential competitor to soya bean (Rajeev and Bavitha, 2015). They possess a high protein content and due to the multiple species which are available, there is opportunity for cultivation in different climates and soil types; therefore they can be considered an option for many farmers in both developed and developing countries alike (Yeheyis et al., 2012; Mercedes et al., 2015). This is of considerable relevance in Europe with an increased interest in production of trout and bespoke feed development using home grown crops for animal feeds. This has taken on more momentum since the 2020/21 pandemic.

However, with most terrestrial-derived ingredients (mainly plant by-products), there are inherent nutritional limitations when consumed by most of the world's commercially produced finfish species. In many cases, the finfish digestive system has not evolved to encounter specific compounds found within plant-derived ingredients, thus availability of nutrients is directly impinged or functional gut morphology is aggravated in a manner whereby assimilation of nutrients becomes restricted (Krogdahl et al., 2005). Furthermore, the content

of non-starch polysaccharide (NSP) in lupin is high even after dehulling like many other plant-protein competitors (Van Barneveld, 1999). Furthermore, lupins tend to contain significant quantities of oligosaccharides (Van Barneveld, 1999), notably α-galactosyl homologues of sucrose which have been demonstrated to impede upon the digestive process in fish (Glencross et al., 2014). The expanded utilization of lupins in aquafeed will necessitate breeding and selection of cultivars together with costly processing approaches to address Anti-Nutritional Factors (ANFs) (Mercedes et al., 2015). One solution would be the application of *in vivo* biological strategies aimed at degrading these compounds as a complementary or alternative option (Hassaan et al., 2017; Bowyer et al. 2020) using SSF additives to the feed.

Solid-state fermentation (SSF) is one such option that involves the microbial fermentation of a substrate in the absence of free-flowing water. Subsequent products may present a multi-faceted approach to continued fermentation within the gastro-intestinal tract of livestock, by providing residual bioactive components (including enzymes) theoretically capable of degrading previously indigestible dietary fractions. Enhanced animal performance has previously been observed following inclusion of solid-state fermentation products in more contemporary formulations for poultry (Hooge et al., 2010) and Nile tilapia *Oreochromis niloticus* (Hassaan et al., 2017; 2018) and Bowyer et al. 2020. Diogenes et al. (2018) investigated commercial exogenous enzymes and the SSF product Synergen™ produced by Alltech, USA with very promising results on the performance of turbot (*Scophthalmus maximus*) juveniles fed distillers' dried grains with solubles (DDGS) based diets. Synergen™ has so far provided indications that many of these ANF factors can be directly or indirectly mitigated. This investigation aims to test and quantifiable identify whether this SSF product may improve the availability of specific nutrients in rainbow trout feeds containing high dietary inclusion of yellow lupin. If effective, promotion of performance and feed efficiency is to be expected and increased concentrations of specific nutrients will be present throughout the

carcass if levels of these nutrients exceed maintenance requirements. It was the objective of the present study to evaluate the performance, apparent digestibility, serum glucose and lysozyme levels, intestinal proteolytic activity and gut morphology of rainbow trout (*Oncorhynchus mykiss*) fed diets containing either yellow lupin (*Lupinus luteus*) meals with and without inclusion of a Solid-State Fermentation SSF product.

2. Materials and methods

2.1. Experimental design

The feeding trial was designed to investigate the growth performance, feed efficiency and midgut morphology of rainbow trout (*O. mykiss*) by the evaluation of a specific cultivar of Yellow Lupin compared to a standard trout diet formulation as a control (i.e. Control; LC) and these inclusions compared to the addition of a commercial solid-state fermentation SSF product Synergen™ (SYN) in separate dietary inclusion levels of SYN. These were 0.1 and 0.5 % (LS0.1 and LS0.5, respectively). Synergen™ was graciously provided by Alltech, Nicholasville, Kentucky, USA.

2.2. Experimental animals and housing

The feeding trial was conducted in a closed recirculation research RAS facility. Juvenile triploid rainbow trout (*O. mykiss*) (~ 20 g), of wild phenotype, were obtained from Exmoor Fisheries (Somerset, UK). Upon entering the research facility, the fish were acclimatised for 2 hrs. Following this period, the fish were stocked into circular 120 L tanks on a ~ 6200 L RAS, powered by a 1.50 hp pump (Certikin HPS150M; Oxfordshire, UK). Throughout acclimation, fish were fed BioMar Efico Enviro (BioMar; DK) at 1-2 % BW per day. Throughout conditioning and the trial, adequate water quality was maintained by biological, drum screen (Aquasonic DF100; AUS) and cartridge (HydroClean 105 µm; UK) filtration, supplied by a 0.75 hp pump (Certikin HPS575M; Oxfordshire, UK) at 20 m^3/hr. System temperature was maintained at 12.5 °C ± 1 via external Optipac pool chillers (PSA; FR). Dissolved oxygen was

maintained above 90 % with air supplied via a low pressure side channel blower (Rietschle Ltd.; Hampshire, UK) to perforated piping below biological media and air stones within tanks. A 12 hrs light: 12 hrs dark photoperiod was implemented with AquaRay LED lights and timers (Tropical Marine Center; Hertfordshire, UK). A 2 weeks elevation in salinity (max. 5 ppt), coupled with 2 weekly salt baths (35 ppt, 10 min) and 2 formalin baths (25 mg/l, 30 min), was performed following identification of *Gyrodactylus* sp. Furthermore, a 7 days course of orally-administered florfenicol (Florocol, MSD Animal Health; Buckinghamshire, UK), at 10 mg/kg BW per day, was implemented as a routine precautionary measure. Clinical examination detected no ectoparasites, or clinical symptoms of any other pathogenic threats, for 10 days prior to the start of the trial.

Following the conditioning period, the fish were graded by size and visual condition. Fish were stocked into quadruplicate tanks of 37 individuals ($n = 4$). Average initial fish weight was 43.58 g ± 0.41, corresponding to a stocking density of 13.44 kg/m^3 ± 0.13.

2.3. Experimental ingredients and diets

Four experimental diets were formulated, using FeedSoft Pro™ (TX, USA), so as to satisfy all known nutrient requirements of rainbow trout (NRC, 2011) and be isonitrogenous, isolipidic and isocaloric (Table 1). The yellow lupin control diet (LC) was formulated to contain 30 % yellow lupin (*L. luteus* cv. Pootalong), obtained from the same batch (Soya, UK). Two inclusions of Synergen™ (SYN) were incorporated into the basal mix at the expense of corn starch. The experimental inclusion levels of SYN were 0.1 and 0.5 % (LS0.1 and LS0.5, respectively). A FM-based reference diet was also utilised.

All chemical composition analyses were estimated according to AOAC (2002) guideline methods for ingredients, complete diets and in later fish carcasses and faecal material from the trial.

2.4. Feeding regimes

The experimental diets were fed in quadruplicate during the feeding trial (a total of 10 weeks). Daily rations were determined via a predicted daily growth (PG), based on a standardised FCR of 1.00. Growth predictions were reset with actual weights following biomass sampling. Predicted growth was estimated as follows: *Predicted Growth (g)* = *W* + *(((W / 100)* ^{x} *FR) / FCR)*, whereby; *W* = tank weight (actual or predicted) of previous day (g), FR = feeding rate (% BW (g)) of previous day and FCR = standardised FCR of 1.0. Feeding was performed by hand, 4 times per day, amounting to ~ 1.8 % BW with reference to a commercial chart guide from the feed manufacturer. The fish were fed a reduced ration (0.0 - 1.0 % BW) on days prior to biomass sampling.

2.5. Biomass sampling and control

The tanks were weighed in bulk on a bi-weekly basis (to 1 g). During week 6, stocking density was reduced from 37 to 25 fish per tank (average. 28.80 kg/m^3 ± 1.18 to 22.87 kg/m^3 ± 1.04, respectively). This was undertaken on the basis of system carrying capacity constraints, so as to maintain optimum environmental conditions; performance calculations were adjusted accordingly.

2.6. Performance and somatic index calculations

Growth performance of fish, feed utilisation was assessed through calculation of weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR). All mortalities were accounted for in performance calculations. Somatic indices were calculated as indicators of fish condition and health, in accordance with methods described by Rawling *et al.* (2012). Fulton's K-factor (K-F) was used as an indicator of fish condition. All above parameters were defined and calculations were noted in the footnote of Table 2

2.7. Faecal sampling

At the end of day 12 of the feeding trial, the fish were anaesthetised in buffered MS222 (200 mg/L), until loss of equilibria and response to human contact was observed. Manual

stripping of faeces was performed by hand, by lightly applying pressure to the hind portion of the abdomen. Faecal material was collected in aluminium trays over ice and pooled by tank. All of the fish were sampled and reintroduced to their respective tanks. The faecal samples collected were freeze-dried, following this, they were manually homogenised with a synthetic pestle and mortar. Apparent digestibility/bioavailability coefficient (ADC/ABAC) calculations were performed as follow:

Apparent bioavailability coefficient (%) = 100 – (100 ˣ (Y_d / E_f) / (Y_f / E_d)) Whereby; Y_d = YO concentration in the diet, Y_f = YO concentration in the faeces, E_d = element concentration in the diet, E_f = element concentration in the faeces.

2.8 Haematology and serology of rainbow trout

Rainbow trout (140-159g) were anaesthetised using Tricaine methanesulphonate (MS222) (200 mg/L) in the final week of the trial with blood (0.5ml) removed from the caudal vessel using both heparinised and non-heparinized syringes and needles of (20) gauge.

Whole blood haemaglobin (Hb) haematocrit (Hct) and serum glucose and lysozyme were determined according to the protocols described by Rider et al. (2009) and by Owen (2011)

2.9 Intestinal histology

2.9.1 Sampling

Sampling of the posterior intestine (PI), for tissue (n = 8), was scheduled immediately at the end of the 10 weeks for scanning electron microscopy (SEM), followed by ~3 mm for transmission electron microscopy (TEM) and lastly ~7.5 mm for light microscopy (LM). \

2.9.2. Light Microscopy

The specimens were fixed in 10% formalin which was replaced by 70% ethanol after 24 h. The specimens were subsequently dehydrated in graded ethanol concentrations then embedded in paraffin wax. Multiple sections were cut from each sample at 5 µm thicknesses (Leica, RM2235; Buckinghamshire, UK) and stained with haematoxylin and eosin (Leica,

Autostainer XL; Buckinghamshire, UK). The specimens were screened with a Leica DMIRB microscope and Olympus E410 digital SLR camera. Enterocyte heights (µm) were measured throughout intestinal folds. H&E-stained micrographs, at 20 X magnification, were appraised for enterocyte height (µm) (EH) through measurement of the cells at 50 intermittent locations around the intestinal folds of each fish (Fig. 1. A). Lamina propria width (µm) (LPW) was measured in at least 30 locations per fish using H&E-stained micrographs at 20 X magnification (Plate 4.3A). Goblet cell counts (no./mm) (GC) were performed Using PAS-stained micrographs at 20 X magnification. This was conducted in at least 20 intermittent locations, of varying distances (50 – 250 µm), around the epithelial layer of each fish (Fig. 1. B). Using PAS-stained micrographs at 10 X magnification, the thickness (µm) of the total muscularis, stratum longitudinale (SLG), stratum circulae (SCR) and stratum compactum (SCM) was measured at 12 locations around the intestinal cross-sections (Fig. 1. C). SLG, SCR and SCM are expressed as percentage of total muscularis thickness (%MT). Muscularis thickness index (MTI) was calculated as follows: *MTI (AU) = MT / GP,* Whereby; MT = total muscularis thickness (µm) and GP = total intestinal perimeter (µm). All appraisals were conducted using ImageJ 1.45 (National Institutes of Health, USA).

2.9.3. Transmission electron microscopy

The samples were processed in accordance with methods detailed by Mercedes et al. (2015). In brief, specimens were fixed in sodium cacodylate buffered 2.5% glutaraldehyde then post-fixed in sodium cacodylate buffered 1% osmium tetroxide (OsO4). Dehydration was conducted in graded levels of ethanol; thereafter infiltration, curing and polymerization were conducted with low viscosity resin premix (AGR1078, Agar Scientific; Essex, UK). Ultra-thin sectioning (~80 nm) was conducted using a Reichert- Jung Ultracut E ultratome (Leica; Buckinghamshire, UK) with a diamond knife (Microstar Tech.; TX, USA). Staining was conducted on copper grids using saturated uranyl acetate with secondary contrasting using lead

citrate solution. The specimens were screened with a 120 kV JEOL JEM-1400 transmission electron microscope (Tokyo, Japan) with a Gatan Orius 830 imaging system (CA, USA). Microvilli heights (MVH) (µm) and microvilli diameters (MVD) (µm) were measured using micrographs orientated in a cross-section fashion (Fig. 1. D. E. F). Micrographs providing an aerial view of the brush border were used to measure microvilli counts (MVCT) (no. /µm2) using 1 µm2 quadrants placed upon standardized co-ordinates. Estimated total absorption surface area (TAS), expressed as µm2 per 1 μm^2 foot-print, was calculated using the previous parameters as follows: $ETAS\ (\mu m^2) = ((2 \times \pi \times 1/2\ MVD \times MVL) + (\pi \times 1/2\ MVD^2)) \times (MVCT) \times EAA;$ whereby; π = Pi, MVD = mean microvilli diameter (µm), MVL = mean microvilli length (µm), MVCT = mean microvilli counts (no. / 1 μm^2) and EAA = enterocyte apical area (μm^2).

2.10. Endogenous intestinal protease activity

2.10.1. Sample collection

At the end of feeding trial, sampling for intestinal proteolytic enzyme activities was performed, following an overnight starvation period, tanks were fed four times to satiation, at 80 min intervals, over a period of 4 hrs. Exactly 80 min after their final feed, 2 fish per tank were manually euthanized and immediately immersed in ice. Sampling was promptly undertaken on glass trays, over ice and under aseptic conditions. The intraperitoneal cavity was opened and the hindmost portion of the intestine was clamped and detached at the anus. Fat was then cleared from the outer mucosal surface of the intestine. The anterior intestine (AI) was clamped at both ends and carefully removed away from the pyloric caeca and posterior intestine (PI). The clamps were removed and the AI was gently squeezed to remove digesta; remaining mucosa was rinsed with cold distilled water. Collected digesta and mucosa samples were immediately sealed in cryogenic tubes and immersed in liquid nitrogen. Samples were then stored at -80 °C until enzyme extraction. Each individual was treated as replicate (n=8).

300 *2.10.2. Enzyme activities*

301 Total alkaline protease (TAP) activity was measured in the digesta of fish as an
302 indicator of digestive capacity, following procedures described by Alarcón *et al.* (1998). One
303 unit of TAP activity (U) was defined as the amount of enzyme that released 1 µg of tyrosine
304 per min, considering an extinction coefficient of 0.008 µg/ml/cm. Trypsin activity was
305 measured in the digesta of fish as an indicator of digestive capacity following procedures
306 described by Alarcón *et al*. (1998) and modified by Alarcón *et al*. (1998). Chymotrypsin
307 activity was measured in the digesta of fish as an indicator of digestive capacity following
308 procedures described by DelMar *et al*. (1979) and modified by Alarcón *et al*. (1998). One unit
309 of trypsin and chymotrypsin activity (U) were defined as the amount of enzyme that released
310 1 µmol of *p*NA per minute, considering an extinction coefficient of 8800 M/cm. L-leucine
311 aminopeptidase (LAP) activity was measured in the digesta and mucosa of fish as an indicator
312 of absorptive capacity following procedures described by Bergmeyer (1974). One unit of LAP
313 activity (U) was defined as the amount of enzyme that released 1 µmol of *p*NA per minute,
314 considering an extinction coefficient of 8800 M/cm. Alkaline phosphatase (ALP) activity was
315 measured in the digesta and mucosa of fish as an indicator of absorptive capacity following
316 procedures described by Bergmeyer (1974). One unit of ALP activity (U) was defined as the
317 amount of enzyme that released 1 µg of nitrophenyl per minute. All data sets were $n = \geq 6$.
318 Values were expressed as U/g digesta or U/g tissue.

319 *2.11. Statistical analysis*

320 All statistical analyses were performed using Sigma Plot 13.0 (SyStat Software Inc.; IL,
321 USA). Data expressed as percentages were arcsine transformed prior to statistical analysis. All
322 tests on normally-distributed data were conducted via ANOVA with post-hoc Fisher's LSD
323 with significance accepted at $P \leq 0.05$.

324

325 *2.12. Ethical statement*

326 Fish protocols were approved by the Institutional Animal Care and Welfare Committee and
327 conforming to European Union statutory regulations for Animal Scientific Procedures and the
328 UK Animal Scientific Procedures Act 1986 operating under Home Office project license PPL
329 30/2644 and personal license PIL 30/10402

330 ***2.* Results**

331 *3.1. Fish, feed performance and somatic indices*

332 At the end of the feeding trial, the experimental animals had exceeded a 3-fold average
333 increase in body weight. Significant differences in FW ($P < 0.001$), WG ($P < 0.001$), FCR (P
334 < 0.001), and SGR ($P < 0.001$) and PER ($P < 0.001$) was observed between the dietary
335 treatments (Table 2). Consistently, LC- and LS0.1-fed fish did not differ from one another,
336 whilst significant improvement was observed in LS0.5 fed fish ($P < 0.05$). FMC fed fish
337 performed significantly better than the lupin-based treatments in all instances ($P < 0.05$). No
338 significant differences ($P > 0.05$) were found in K-F and HIS between the dietary treatments.

339 *3.2. Apparent macronutrient digestibility*

340 Apparent digestibility coefficients are displayed in Table 3. The apparent digestibility of
341 total dry matter and crude lipid was unaffected by dietary treatment of SYN. Apparent
342 digestibility of crude protein was significantly different between the lupin-based diets ($P <$
343 0.001); significant incremental increases were observed between LC, LS0.1 and LS0.5. Crude
344 fibre apparent digestibility was significantly different between treatments ($P < 0.001$), being
345 significantly higher in LS0.5 than LC and LS0.1. Gross energy apparent digestibility was
346 significantly ($P < 0.05$) higher in LS0.5 than other treatment diets fed to rainbow trout.

347 *3.3. Carcass composition*

348 Carcass composition results are displayed in Table 4. Carcass moisture, crude lipid (CL)
349 and ash content were unaffected by dietary treatment. However, crude protein (CP) content of

whole carcasses was significantly different between fish fed the respective diets ($F = 36.24$, $P < 0.001$). Carcass CP was significantly higher in fish fed LS0.5 and FMC than LC and LS0.1; values for fish fed LS0.5 and FMC did not differ from one another. Gross energy (GE) of whole carcass was found to be significantly affected by diet ($P = 0.001$), being significantly higher in SYN-treated and FMC diets than LC.

3.4. Haematological and serological parameters

The data obtained for the haematological and serological parameters of rainbow trout fed the experimental diets are viewed in Table 5. It can be observed that no differences in the haemoglobin Hb and haematocrit Hct were detected amongst the respective treatment groups. However serum glucose concentrations were significantly different ($P<0.05$) with the SFF SYN additive in diets for lupin meal but were also high in the FMC fishmeal group. Likewise serum lysozyme activity was significantly increased with SYN above other diet groups.

3.5. Intestinal morphology

Results of the quantitative appraisal of posterior intestinal morphology, by light and electron microscopy techniques, are displayed in Table 6. Enterocyte height (EH) was observed to differ significantly between treatments ($P < 0.05$). Median Enterocyte height (EH) of LS0.1 and LS0.5 fed fish did not differ from one another but were both significantly greater than LC fed fish. Fish fed 0.5 % SYN were observed to have a significant ($P < 0.05$) reduction in goblet cell counts (GC) compared to those fed LC and LS0.1. No significant difference was observed in GC between LC and LS0.1 fed fish. Lamina propria width (LPW) was unaffected by dietary treatment ($P > 0.05$). Muscularis thickness index, corrected to total intestinal diameter (MTI), did not differ among dietary treatments, nor did proportional contributions of muscle layers ($P > 0.05$). Mean microvilli length (MVL), diameter (MVD) and enterocyte apical area (EAA) did not differ significantly between dietary treatments ($P > 0.05$). Microvilli counts (MVCT) were significantly different between treatments ($P = 0.001$), due to significantly lower counts

375 in LS0.1 than other treatments. Estimated total absorption surface areas per enterocyte were
376 significantly different between treatments ($P < 0.05$). Fish fed 0.5 % SYN displayed a higher
377 microvillar percentage coverage (MVCV) compared to fish fed LC and LS0.1. Qualitative
378 appraisal observed a reduction in areas of conformational irregularity or where denuding of
379 microvilli had occurred in LS0.5 SSF group, and evidence of higher microvilli density,
380 uniformity and improved surface structure (Fig. 4). Further, qualitative assessment appeared to
381 show tighter assembly of enterocytes and a higher degree of regularity in the structure of the
382 brush border within the LS0.5 group, compared with both the LC and LS0.1 group (Fig. 2 and
383 Fig. 3). Qualitative appraisal did appear to show greater spaces between enterocytes within the
384 LS0.1 group (Fig. 3).

385 *3.6. Intestinal proteolytic enzyme activity*

386 Anterior intestinal protease activity results are displayed in Table 7. Total alkaline
387 protease (TAP), trypsin and chymotrypsin activities in the digesta of fish were unaffected by
388 dietary treatment ($P > 0.05$). Activity of alkaline phosphatase (ALP) in digesta was
389 significantly ($P < 0.05$) higher in SYN treatments than LC. Activity of L-leucine
390 aminopeptidase (LAP) in digesta was significantly ($P = 0.045$) elevated in LS0.1 fed fish than
391 LC fed fish. Activity of LAP was also significantly higher in LS0.5 fed fish than LC fed fish.

392 **4. Discussion**

393 *4.1. Fish performance and feed efficiency*

394 The inclusion of a commercial SSF, solid state fermentation product (SYN) in yellow
395 lupin-based diets for rainbow trout returned clear evidence of performance enhancement at
396 12.5 °C within this investigation; however, this was only apparent following an inclusion rate
397 of 0.5 %, with the 0.1 % inclusion returning negligible effects. Previously, the 0.1 % inclusion
398 was deemed effective in lupin-based diets for warm water species such as Nile tilapia (Bowyer
399 et al. 2020) and also for common carp (Cyprinus carpio) Anwar et al. (2020) but it is evident

400 that functionality of SYN was less efficient at this level within a typical salmonid species, in
401 this case rainbow trout. Lower rearing temperature will inevitably lead to a reduction in the
402 activation energy available for bioactive components to work effectively under *in vivo*
403 conditions. Although perhaps crude in its basis, simply increasing inclusion rate has been
404 demonstrated as an effective means of maintaining the functionality of phytase applications in
405 salmonid diets (Vandenberg et al., 2012). Indeed, in this case a five-fold increase was highly
406 effective in promoting growth performance and general feed utilisation. Most promisingly, the
407 LS0.5-fed fish were closer in performance to those fed a high-grade fishmeal (FM) diet, which
408 could be considered cost-ineffective due its ingredient matrix. However, in this study the FM-
409 diet served as a reference, semi-purified diet for achieving maximum allowable performance
410 under the experimental conditions. Rainbow trout fed phytase supplemented diets have
411 previously been reported to express performance characteristics closer to a FM-based diet than
412 the original basal diet (Vandenberg et al., 2011). Recently, the Yellow LC diet of Nile tilapia
413 resulted in numerically inferior SGR, FCR and PER values compared to the Black LC but diets
414 supplemented with SFF were improved (Bowyer et al., 2020). The potential of the same
415 commercial solid-state fermentation supplement (SFF) Synergen^{TM} to augment white lupin
416 (*Lupinus albus*) meal incorporation in diets for common carp (*Cyprinus carpio*) was also
417 confirmed by Anwar et al. (2020). This is not surprising since the SSF of wheat with
418 *Aspergillus niger* is a natural complex containing an array of residual enzymatic activity
419 following fermentation of a substrate with a microorganism (e.g. fungus) in the absence of free-
420 flowing water. The microorganism can secretes its vast natural complement of digestive
421 enzymes that can greatly augment animal performance in commercial practice.

422 *4.2. Apparent digestibility*

423 Quantification of nutrient digestibility coefficients for trout revealed many
424 improvements in both SYN inclusion levels for rainbow trout fed the lupin-based diets. The

425 increase in apparent digestibility of crude protein, by 2.27 % between the LC and LS0.5 groups,
426 serves a likely contributor to enhanced growth, protein efficiency and also an increase in
427 carcass crude protein content. In support of these findings, augmented protein efficiency,
428 leading to increased performance has been observed in broilers fed commercial diets
429 supplemented with a similar product, Allzyme®SSF (Yadava *et al.*, 2009). Improved
430 availability of protein is also extensively regarded as one of the main benefits of phytase
431 additions, with observations across many species (Kumar *et al.*, 2012). Gross energy
432 digestibility was similarly improved following SYN supplementation, increasing by 8.25 %
433 between the LC and LS0.5 groups. Once again, increased gross energy was expressed within
434 the carcasses of sampled fish. Improved gross energy availability has been extensively
435 observed following supplementation of a range of purified exogenous enzyme preparations in
436 monogastrics; including endo-β-glucanase and endo-β-xylanase in pigs (O'Connell *et al.*,
437 2006), multi-enzymes and xylanase in poultry (Danicke *et al.*, 2000; Zhang *et al.*, 2012) and
438 multi-enzymes in tilapia (de Oliveira *et al.*, 2007; Guimaraes *et al.*, 2009). Increased gross
439 energy provision has also been reported as possible following phytase supplementation in
440 rainbow trout diets (Cheng and Hardy, 2004). The mode of action in this instance is widely
441 regarded to consist of degradation of chelates and reduction of intestinal viscosity which
442 augments enzyme-substrate interactions. With the latter of the explanations, one would expect
443 to see some form of improved carbohydrate degradation.
444 Although the LS0.1 diet also induced significant improvements in digestible protein and
445 energy, this was not observed in the digestibility of crude fibre in the present investigation.
446 Only the LS0.5 dietary treatment induced an effect on this carbohydrate component, with an
447 increase of approximately 31.5 %. It is recognised that limitations exist in determination of
448 crude fibre, considering it is not a comprehensive definition of all non-starch polysaccharides.
449 Its analytical quantification does lose substantial quantities of soluble hemicelluloses during

450 the procedure. However, it is fair to suggest that some exogenous carbohydrase enzymes,
451 derived from SYN, particularly cellulose may impart efficacy within the luminal environment
452 of the GIT. Hemicellulase action is also likely since industrial fermentation processes with *A.*
453 *niger* used in the SFF process are regarded to produce potent activities of xylanases,
454 mannanases and galactosidases (Laerke *et al.*, 2015; Inoue *et al.*, 2015; Manzanares *et al.*,
455 1998; Magalhaes *et al.*, 2014). Consequently, polysaccharide chain length would have been
456 reduced to a level which caused monosaccharides to be more available to the animals, or to a
457 level capable of significantly increasing short-chain oligosaccharides which could be
458 fermented by the intestinal microbiota. Either way, a protein sparing effect is highly likely to
459 have occurred, considering the significant decrease of carbohydrate fractions from the diet.

460 4.3. *Serum glucose and lysozyme*

461 Serum glucose levels were significantly elevated in the LS0.5 and FMC compared with
462 LC and LS0.1. Elevated blood glucose in salmonids is often regarded as a stress response
463 (Benfey and Biron, 2000) or volatile glucose homeostasis but clearly in this case there are no
464 grounds on which to attribute stress or poor regulation as factors since the elevation was
465 observed in the two highest performing treatments, including one which was fed a relatively
466 optimal dietary formulation. Therefore, attribution to dietary factors is most likely. The first,
467 and simplest, explanation is that of increased provision of dietary glucose. It may perhaps be
468 surprising that the FMC fed fish expressed the highest levels but it is important to remember
469 that although FM-based, this semi-purified diet contained appreciable quantities of starch,
470 which can be considered a relatively non-complex carbohydrate matrix. The anterior intestine
471 of salmonids is known to contain activity of α-amylase as well as mucosal maltase, sucrase and
472 lactase (Furne *et al.*, 2005; Furne *et al.*, 2008; Geurden *et al.*, 2007; Santigosa *et al.*, 2008) so
473 glucose can be obtained from the hydrolysis of dietary starch (Krogdahl *et al.*, 2005).
474 Furthermore, oligosaccharides have been shown to impair the intestinal uptake of glucose in

475 rats (Sone *et al*., 1992) whilst fibre in general was observed to do so in fish (Shiau 1997); thus
476 possible degradation of long- and short-chain polysaccharides in LS0.5 may have facilitated
477 sugar absorption. All things considered, the higher serum glucose levels observed may be a
478 result of improved availability due to reduced substrate complexity in the FMC group and
479 exogenous carbohydrase activity in LS0.5. Indeed, in the case of LS0.5, increased crude fibre
480 digestibility indicates that a significant absorption of cellulose-derived glucose was achieved.
481 Conversely to these suggestions, chickens and quails supplemented with exogenous enzymes
482 have been reported to display no effect on blood glucose levels (Moharrery, 2006; Jozefiak *et*
483 *al*., 2011; Sahin *et al*., 2007). However one must consider the slow glucose turnover time and
484 metabolic assimilation of glucose in carnivorous fish, compared to birds and mammals
485 (Polakof et al., 2012); as well as the fact that digesta was still present in the intestine of sampled
486 fish showing that digestion and assimilation of nutrients was on going at the time of
487 measurement. It is also worth considering that proteinaceous dietary factors may have
488 influenced the observed results.

489 The LS0.1 and LS0.5 exhibited lysozyme activity modulation within the blood.
490 Lysozyme is an integral part of the innate immune system, being involved in the hydrolysis of
491 predominantly Gram-positive bacterial cell walls. Studies have examined the stimulation of
492 lysozyme activity in fish following dietary supplementation of Gram-positive probiotics such
493 as *Lactobacillus acidophilus* (Talpur *et al*., 2014) and *Enterococcus faecium* (Kim *et al*., 2012),
494 confirming substantial elevation in activity when doing so. Similarly, prebiotics may stimulate
495 lysozyme activity through associated microbial proliferation, as has been observed with
496 galacto-oligosaccharides, mannan-oligosaccharides and most extensively, β-glucans (Kim *et*
497 *al*., 2012; Aramli *et al*., 2015; Dawood *et al*., 2015; Ghaedi *et al*., 2015).
498 Although lysozyme is typically associated with defence against bacteria, it can also catalyse
499 the breakdown of 1,4-β-*N*-acetylglucosaminyl oligosaccharides, which are found within

chitodextrin of fungal cell walls. Residual *A. niger* from the SSF procedure will inevitably be present within the diet, so theoretically lysozyme activity could respond to the presence of these fungal polysaccharides, regardless of their non-pathogenic threat. Indeed, lysozyme derived from insects and plants alike is believed to play a pivotal role in defence against common fungal infections (Javar *et al*., 2015; Manikandan *et al*., 2015) and its use in fungicidal drugs is of interest in human pharmacology (Woods *et al*., 2012). Furthermore, a recent study which supplemented polysaccharides from the mycelia of caterpillar fungus (*Cordyceps sinensis*), into the diets of white shrimp (*Litopenaeus vannamei*), stimulated a prolific increase in haemato-lysozyme activity (Deng *et al*., 2015). This theoretical potential is of worthy of consideration. Future studies should consider whether lysozyme activity can be modulated by fungal residues from fermentation procedures, to determine whether this possibility is indeed correct. A functionality of this kind could be highly beneficial in priming the immune system for possible pathological threats. Efforts have already been made to investigate the benefits of bacterial cell wall derivatives in salmonid diets, with pronounced immunomodulation observed in doing so (Skalli *et al*., 2013). However, efficacy through a product such as SYN would present a more multi-faceted and cost-effective approach.

4.4.4 Intestinal morphology

Goblet cell numbers appeared greatly reduced in the LS0.5 treatment, suggesting a decreased level of mucus secretion. Elevated fibre content, including soyabean hulls, has been demonstrated to augment goblet cell abundances in the duodenum of piglets (Pascoal *et al*., 2015). Similarly, high fibre dietary components have been observed to stimulate goblet cell proliferation and activity in rodents (Ito *et al*., 2009; Hino *et al*., 2012, Hino *et al*., 2013). Such occurrences have also been associated with the increase in digesta viscosity by soluble NSPs (Piel *et al*., 2005). Furthermore, fibre-induced goblet cell proliferation has been demonstrated

525 to occur independently of microflora, in germ-free rats, but effects may be attenuated by
526 colonising bacteria (McCullough *et al*., 1998). Lectins are another causative agent behind
527 goblet cell proliferation due to the manner in which they bind to oligosaccharides and the
528 mucosal surface (Menghi *et al*., 1989); therefore, a reduction in carbohydrates could reduce
529 their potency. It is of interest that goblet cell numbers appear to follow the same trend as those
530 observed in the digestibility of fibre. Considering existing evidence that cellulosic and
531 hemicellulosic polymers increase goblet cell proliferation, be it directly or indirectly, it is
532 suggested that increased hydrolysis of such compounds in LS0.5, as indicated by crude fibre
533 digestibility and perhaps also the microbiota, could contribute to the morphological results
534 observed. Alternatively, it is also worthy to note that goblet cell proliferation and thus mucus
535 production has been suggested as a response to sloughing off pathogenic bacteria within the
536 gut of fish species, such as Arctic charr (Lodemel *et al*., 2001). So, the reduced abundance of
537 goblet cells in LS0.5 may also be linked to its microbiome characteristics. In any case, the
538 reduced presence of goblet cells in the LS0.5 group appears to indicate a reduced investment
539 in mitigating the effects of stressors within the lumen.
540 Mucosal duodenal layers have been observed to become thicker following probiotic application
541 in fish (Batista *et al*., 2015) and there is theoretical potential for these muscular tissues to
542 exhibit morphological responses if digesta viscosity, thus ease of passage, is drastically altered.
543 However, no such observations were noted under the current study.
544 The LC group generally exhibited healthy gut ultrastructure, in correspondence with previous
545 findings regarding the application of lupins in salmonid diets (Borquez *et al*., 2011; Serrano *et*
546 *al*., 2012). However, microvilli lengths were numerically lower, whilst counts were
547 significantly lower in the LS0.1 group, which resulted in a significantly reduced estimated total
548 absorption area per enterocyte. It is therefore apparent that some form of intermediary product
549 or process of nutrient digestion by SYN prompted the depression of beneficial morphology, as

the LS0.5-fed fish showed similar, even perhaps improved morphology compared with those fed the basal diet. Out of the macronutrients which were altered, no intermediary products of protein hydrolysis are likely to cause such effects, again leaving carbohydrate fractions to be scrutinised.

4.5. Intestinal proteolytic activity

Exogenous-endogenous enzyme interactions in animal nutrition are poorly understood and sparsely investigated. Although the introduction of exogenous sources usually aims to supplement the digestive system with previously absent components, it has been highlighted that interactions are a possibility, reducing the cost of hydrolytic investment by the animal (Bedford and Partridge, 2010). The activity of trypsin and chymotrypsin (the indicators of digestive capacity) appeared not to be altered by the SYN inclusions, suggesting no endo-exogenous interactions or any noticeable reduction in inhibitors. This is somewhat supported by Vandenberg *et al.*'s (2011) findings, where phytase-supplemented rainbow trout were effective in reducing protein-limiting phytate yet they did not affect trypsin activity within the intestine of exposed fish. However, ALP and LAP activity were indicated to be elevated following SYN inclusion within the digesta; whilst activity within their predominant site, the mucosa, appeared slightly reduced. Few reports exist but xylanase and phospholipase have been demonstrated to have no effect upon ileal LAP activity in pigs (Sileikiene *et al.,* 2006). Considering significant increases of ALP and LAP activity within the digesta may be coupled with numerical decreases in expression within the mucosa, it cannot be discounted that the results observed are simply a consequence of secretion into the lumen; despite highly rigorous efforts to standardise feeding, intestinal transit and sampling times. However, a number of causative agents must still be considered for explaining the elevated LAP and ALP activity within the lumen.

574 Firstly, nutrient profiles within the lumen may influence expression levels of ALP. Of
575 particular note, high luminal Ca concentrations have been demonstrated to increase the
576 expression of ALP in the intestine of rats (Brun *et al*., 2012). Meanwhile, intestinal Na has
577 been reported to decrease mucosal aminopeptidase in broilers (Zdunczyk *et al*., 2012).
578 Supplementing rainbow trout diets with LPS has indeed shown stimulation of intestinal ALP
579 secretion (Nya and Austin, 2011).

580 **Conclusion**

581 Yellow lupin-based diets provided adequate performance and nutrient availability to
582 juvenile rainbow trout and may hold certain benefits over other plant proteins with higher
583 ANF's. Synergen™ SSF inclusion, at 0.5 %, was highly effective in augmenting the macro-
584 nutritional profile of the diet containing 30 % yellow lupin, attributable to its potent bioactive
585 components. This evidently led to substantial improvements in production and feed efficiency
586 parameters, equating to performance which was more comparable with a high-grade FM diet
587 than the original lupin-based counterpart. It is also recognised that the observed results are
588 highly attractive for improving environmental impact, due to the likely reduction of both
589 organic and inorganic waste. Additionally, rainbow trout fed a 0.5 % inclusion of SSF
590 displayed some marked evidence of improved morphological determined gut integrity. It was
591 also noted that Synergen™ SSF may hold potential for immunomodulation due to anti-oxidant
592 and cell wall properties of constituent microorganisms. The application of optimal inclusions
593 of commercial SSF products in contemporary salmonid and other fish diets therefore appears
594 a promising avenue for further investigation. This will enable a more efficient use of raw
595 materials in meeting the protein and energy 'gap' for aquaculture diets.

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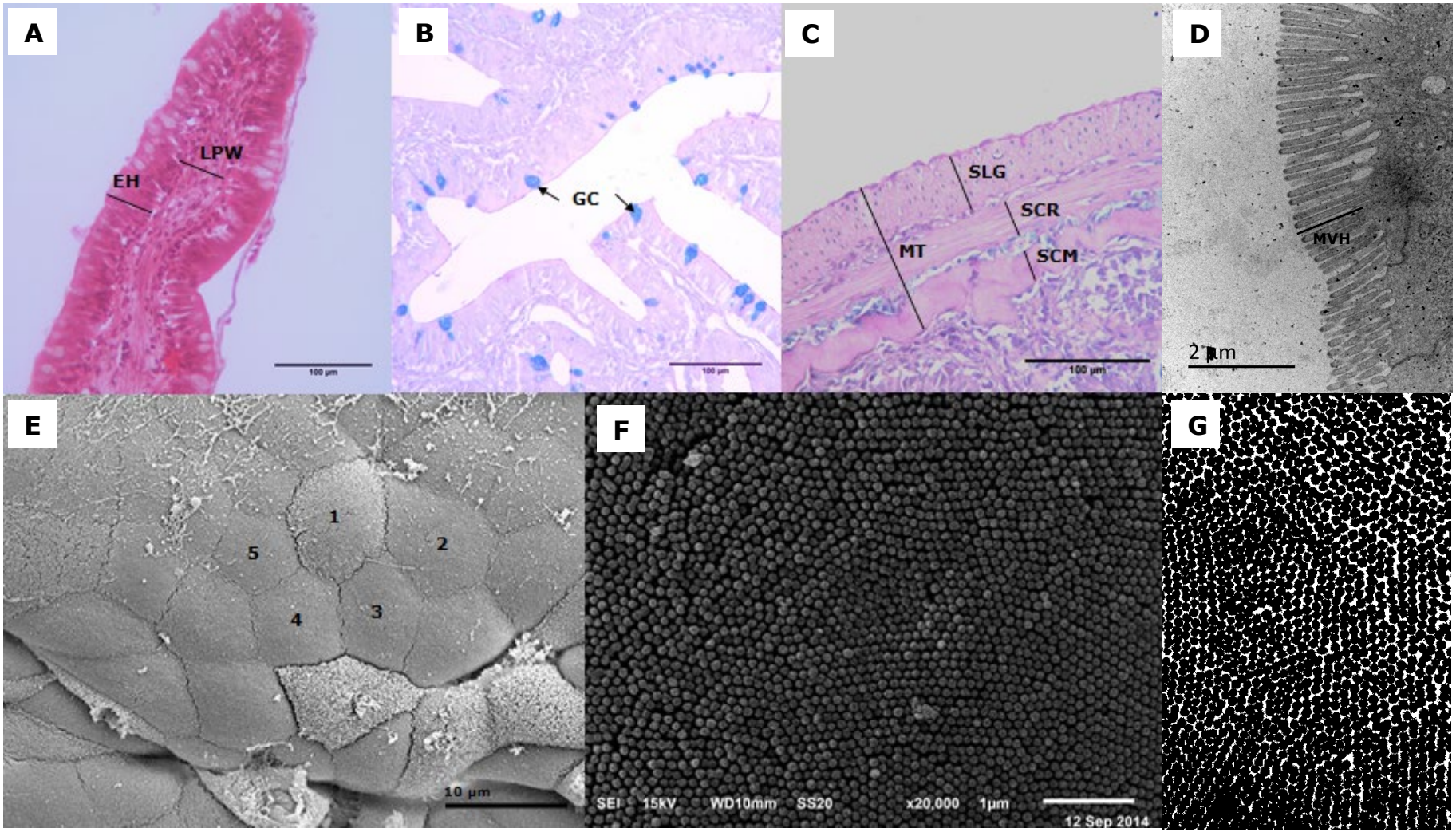

Fig. 1. Light and electron micrographs of rainbow trout posterior intestine displaying appraisal methods

A = H&E-stained fold (20 X mag.): *EH* = enterocyte height, *LPW* = lamina propria width, scale bar = 100 µm; **B** = PAS-stained fold (20 X mag.): *GC* = goblet cell; **C** = PAS-stained muscularis (10 X mag.), MT = muscularis thickness, SLG = stratum longitudinale, SCR = stratum circulae, SCM = stratum compactum, scale bar = 100 µm: **D** = Brush border Epithelial (20,000 X mag. TEM), MVH = microvilli height, scale bar = 2 µm: **E** = Epithelial surface (2,500 X mag. SEM), *numbers* indicate individual enterocytes, scale bar = 10 µm. **F** = Epithelial surface (20, 000 X mag. SEM), scale bar = 1 µm. **G** = threshold-reversed epithelial layer displaying microvilli in *black* (20,000 X mag. SEM).

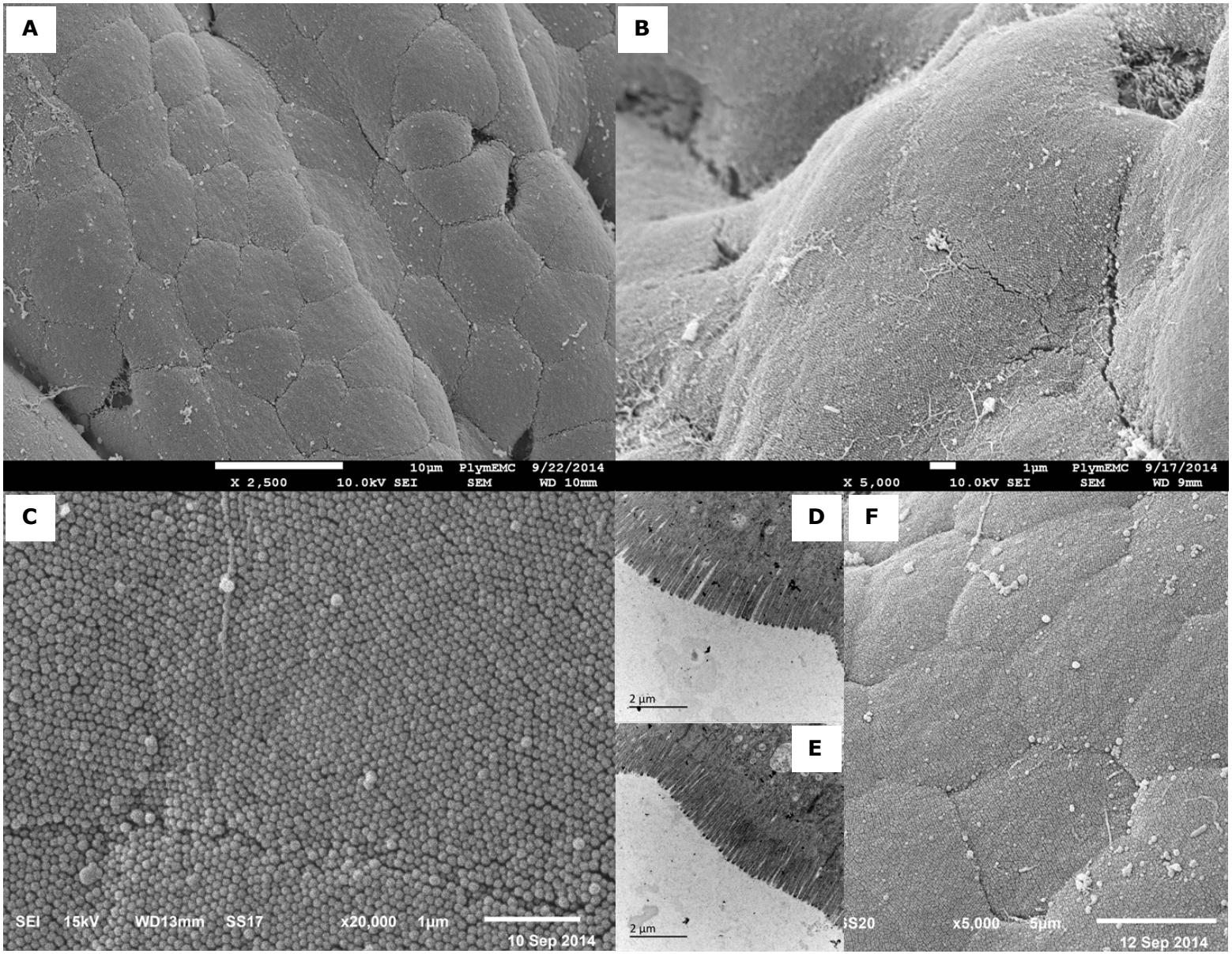

Fig. 2. Intestinal ultrastructure of fish fed the basal lupin diet (LC) after 10 weeks.
A = 2,500 X mag. SEM, **B** = 5,000 X mag. SEM, **C** = 20,000 X mag. SEM, **D** & **E** = 20,000 X mag. TEM, **F** = 5,000 X mag. SEM

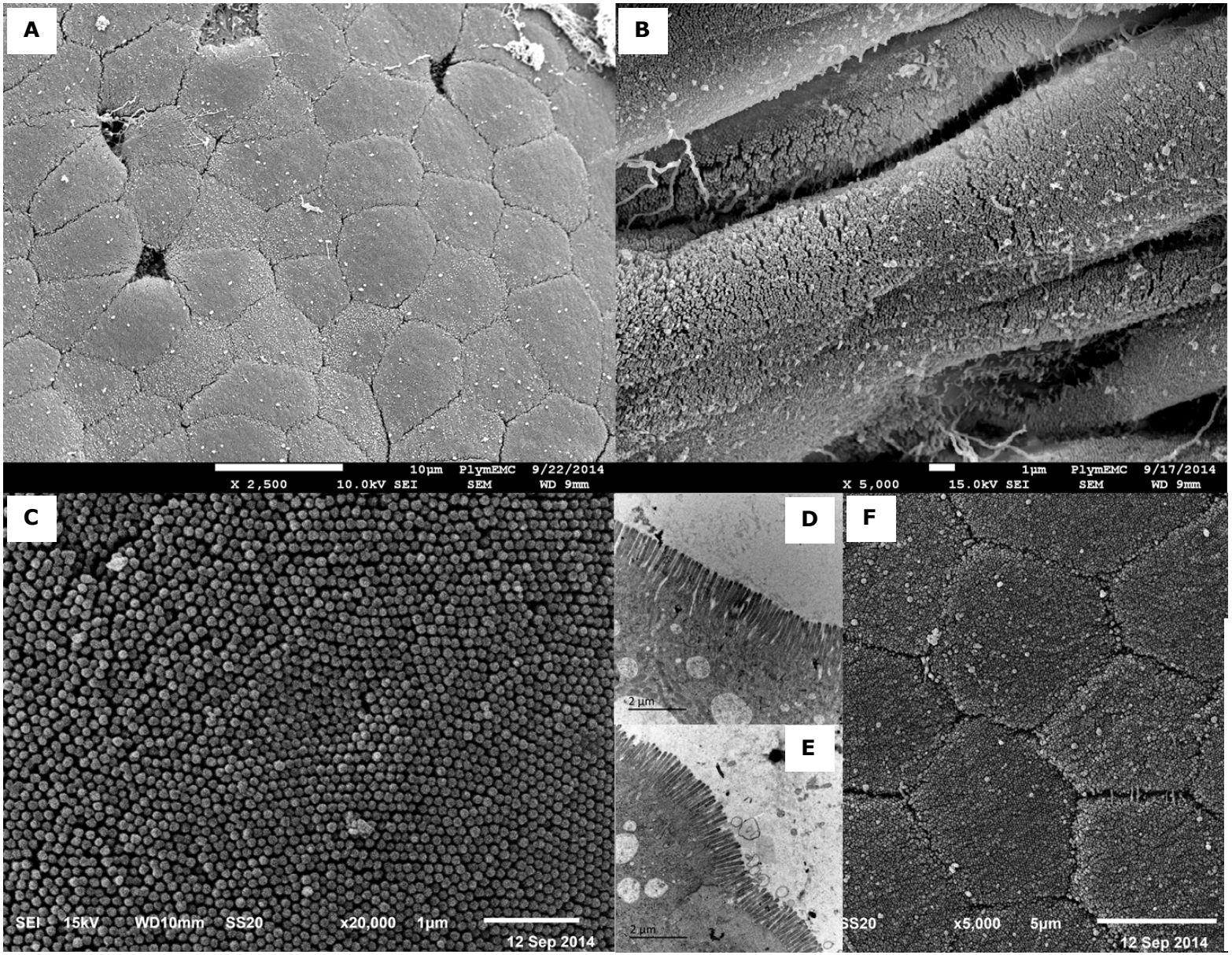

Fig. 3. Intestinal ultrastructure of fish fed a 0.1 % SYN inclusion (LS0.1) after 10 weeks.
A = 2,500 X mag. SEM, **B** = 5,000 X mag. SEM, **C** = 20,000 X mag. SEM, **D** & **E** = 20,000 X mag. TEM, **F** = 5,000 X mag. SEM

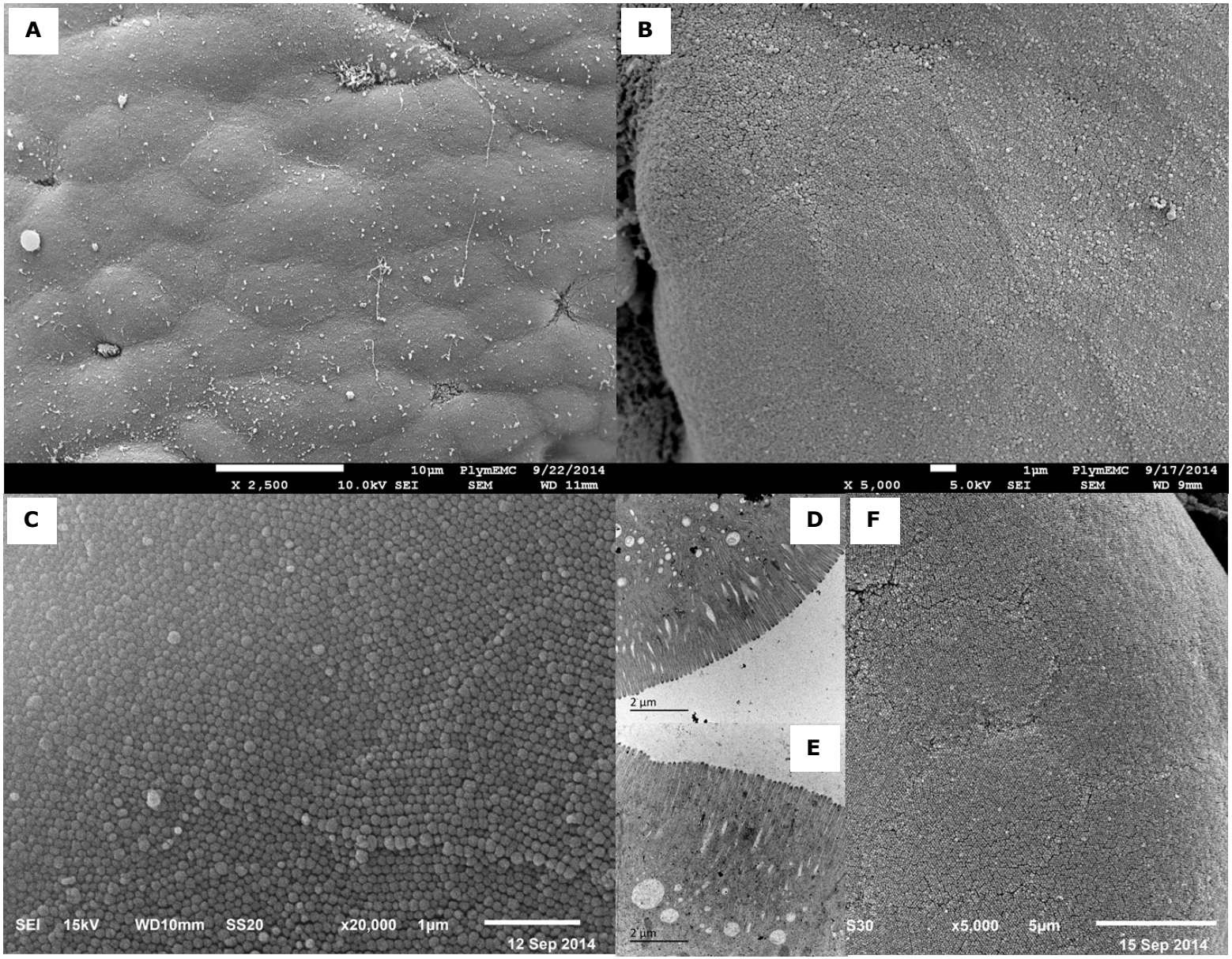

Fig. 4. Intestinal ultrastructure of fish fed a 0.5 % SYN inclusion (LS0.5) after 10 weeks.
A = 2,500 X mag. SEM, **B** = 5,000 X mag. SEM, **C** = 20,000 X mag. SEM, **D** & **E** = 20,000 X mag. TEM, **F** = 5,000 X mag. SEM

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Table 1. Feed formulations, proximate compositions and element concentrations of the experimental rainbow trout diets.

Ingredient (g / kg)	Diets			
	LC	LS1	LS5	FMC
Yellow Lupin	300.00	300.00	300.00	-
Herring Meal [1]	250.00	250.00	250.00	638.63
Soyabean meal [2]	180.71	180.71	180.71	-
Fish Oil [3]	137.15	137.15	137.15	119.97
Corn Starch [4]	70.14	69.14	65.14	229.40
Soya Protein Concentrate [5]	50.00	50.00	50.00	-
Carboxyl-methyl-cellulose [6]	5.00	5.00	5.00	5.00
Vitamin/Mineral Premix [7]	5.00	5.00	5.00	5.00
Ascorbyl-Phosphate [8]	1.00	1.00	1.00	1.00
Yttrium Oxide [9]	1.00	1.00	1.00	1.00
Synergen™	-	1.00	5.00	-
Proximate composition (g/kg)				
Crude protein	432.3	434.9	440.1	4307
Crude lipid	208.1	206.9	198	2047
Ash	66.8	70.5	67	66
NFE	370.3	376.2	414.5	417.1
Gross energy (MJ/kg)	21.66	21.68	21.63	23.21

[1] LT94 herring meal (CC Moore, UK); [2] HP100 (Hamlet, DK); [3] Epanoil (Seven Seas, UK); [4] (Sigma Aldrich, UK); [5] SPC 60 (BioMar, DK); [6] (Sigma Aldrich, UK); [7] PNP Fish: Ash 78.7 %, Ca 12.1 %, Mg 1.56 %, P 0.52 %, Cu 0.25 g/kg, Vit. A 1.0 µg/kg, Vit D3 0.1 µg/kg, Vit. E 7 g/kg (Premier Nutrition, UK); [8] Rovimix (DSM, UK); [9] (Sigma Aldrich, UK).

Table 2. Fish and feed performance values of the dietary treatments.

	Diet			
	LC	LS1	LS5	FMC
IW (g)	44.04 ± 0.44	43.26 ± 0.18	43.65 ± 0.34	43.38 ± 0.17
FW (g)	140.42 ± 5.71[c]	135.44 ± 3.56 [c]	146.54 ± 2.33 [b]	159.56 ± 4.76 [a]
WG (g)	97.07 ± 4.63 [c]	93.33 ± 1.67 [c]	104.29 ± 1.14 [b]	117.02 ± 4.39 [a]
FCR	1.25 ± 0.47 [c]	1.22 ± 0.34 [c]	1.08 ± 0.20 [b]	0.99 ± 0.16 [a]
SGR	1.64 ± 0.76 [c]	1.65 ± 0.70 [c]	1.76 ± 0.58 [b]	1.87 ± 0.56 [a]
PER	1.73 ± 0.07 [c]	1.70 ± 0.03 [c]	1.86 ± 0.03 [b]	1.97± 0.06 [a]
Survival (%)	100	100	100	100
Somatic indices				
K-F	1.79 ± 0.11	1.75 ± 0.09	1.75 ± 0.04	1.85 ± 0.04
HIS	1.05 ± 0.06	0.99 ± 0.09	1.02 ± 0.05	0.94 ± 0.03

Abbreviations: LC = yellow lupin control diet; LS0.1 = yellow lupin basal + Synergen™ (0.1%); LS0.5 = yellow lupin basal + Synergen™ (0.5%); FMC = fishmeal reference/control diet; IW = initial fish weight; FW = final fish weight; WG = weight gain (g); FCR = feed conversion ratio; SGR = specific growth rate; PER = protein efficiency ratio; K-F = k-factor condition index; HIS = hepatosomatic index

[a, b, c] Diets possessing the same superscript in the same row are not significantly different ($P \leq 0.05$), no superscripts indicate no significant difference between any diets.

Values expressed as mean ± S.D. (n = 4). Statistical test: ANOVA + Fisher's LSD

Weight gain (g) = $FW_f - FW_i$; Whereby; FW_f = final fish weight (g) and FW_i = initial fish weight (g); *Specific growth rate (SGR) = 100 x ((Ln* W_f *– Ln* W_i*) / DF);* Whereby; Ln = natural log, W_f = final tank biomass (g), W_i = initial tank biomass (g) and DF = days fed. *Feed conversion ratio (FCR) =* $(W_f - W_i)$ */ FI;* Whereby; W_f = final tank biomass (g), W_i = initial tank biomass (g) and FI = feed intake (g). *K-factor (AU) = 100 x (FW /* FL^3*);* Whereby; FW = fish weight (g) and FL = fork length (cm). *Hepatosomatic index (HSI) = 100 x (LW / FW);* Whereby; LW = whole liver weight (g) and FW = fish weight (inc. liver) (g).

Table 3. Apparent macronutrient digestibility coefficients (ADC) (%) of the experimental lupin-based and fishmeal-based rainbow trout diets.

	Diet			
	LC	LS1	LS5	FMC
ADC (%)				
DM	96.97 ± 0.43	96.71 ± 0.21	97.08 ± 0.69	96.12± 0.65
CP	83.65 ± 0.02^{c}	85.26 ± 0.03 ^{b}	85.55 ± 0.03^{a}	85.12± 0.02^{b}
CL	89.11 ± 0.77	88.93 ± 0.10	90.43 ± 0.79	89.12± 11
CF	33.99 ± 1.05^{c}	35.52 ± 1.60 ^{b}	44.69 ± 2.62^{a}	44± 0.69^{a}
GE	66.29 ± 0.62^{c}	68.41 ± 0.83 ^{b}	71.76 ± 0.15 ^{a}	69.42±0.11^{b}

Abbreviations: DM = dry matter; CP = crude protein; CL = crude lipid; CF = crude fibre; GE = gross energy

Table 4. Whole-body proximate composition (g/kg) of the fish fed the experiment diets.

	Diet			
	LC	LS1	LS5	FMC
Dry matter	297.8± 0.01	302.7± 0.01	302.1 ± 0.00	297.6± 0.01
CP	152.4 ± 0.18 [b]	154.7 ± 0.24 [b]	163.4 ± 0.02 [a]	162.9 ± 0.11 [a]
CL	110.6 ± 0.27	112.8 ± 0.48	114.1 ± 0.42	109.1 ± 0.53
Ash	20.0 ± 0.01	21.0 ± 0.17	20.8 ± 0.17	20.1±0.12
GE (MJ/kg)	78.7 ± 0.04 [b]	80.1 ± 0.00 [a]	79.8 ± 0.03 [a]	79.9 ± 0.03 [a]

Abbreviations: CP = crude protein; CL = crude lipid; GE = gross energy. Values expressed as mean ± S.D. (n=3) of whole carcass (on wet basis). Statistical tests: ANOVA + Fisher's LSD.

[a,. b, c] Diets possessing the same superscript in the same row are not significantly different (P≤0.05), no superscripts indicate no significant difference between any diets

Table 5. Haematological and serological parameters of rainbow trout fed the experimental diets.

	Diet			
	LC	LS1	LS5	FMC
Hb (g/dl)	0.18 ± 0.03	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.01
Hct (% PCV)	36.50 ± 4.75 [a]	40.50 ± 2.78 [b]	36.00 ± 3.02 [ac]	33.14 ± 1.68 [c]
Glc (mg/dl)	44.19 ± 8.47 [a]	47.26 ± 7.25 [a]	65.65 ± 7.38 [b]	72.90 ± 12.45 [b]
Lyz (U/ml)	1156.46 ± 116.03 [a]	1427.98 ± 279.75 [b]	1450.71 ± 235.75[b]	1167.31 ± 109.42 [a]

Abbreviations: Hb = haemoglobin; Hct = haematocrit; Glc = serum glucose; LYZ = serum lysozyme; PCV = packed cell volume

Values expressed as mean ± S.D. Statistical test: ANOVA + Fisher's LSD. Hb n=8; Hct n=8; Glc n=8 (LC, FMC) and n=7 (LS0.1, LS0.5); LYZ n=8.

[a, b] Diets possessing the same superscript in the same row are not significantly different ($P \leq 0.05$), no superscripts indicate no significant difference between any diets

Table 6. Morphological parameters of the posterior intestine of the lupin-fed fish, with and without Synergen™ inclusion.

	Diet		
	LC	LS1	LS5
Macrostructure			
EH (μm)	38.55 ± 2.24 [a]	41.24 ± 1.04 [b]	41.36 ± 0.31 [b]
GC (no./mm)	224.06 ± 26.95 [a]	226.57 ± 18.45 [a]	182.63 ± 19.33 [b]
LPW (μm)	16.57 ± 3.53	16.44 ± 2.08	16.02 ± 2.31
Muscularis			
MTI	1.65 ± 0.13	1.49 ± 0.37	1.79 ± 0.29
SLON (%MT)	34.83 ± 5.63	34.84 ± 7.07	34.97 ± 3.80
SCR (%MT)	25.28 ± 2.55	26.09 ± 2.81	25.98 ± 5.36
SCM (%MT)	16.07 ± 1.56	17.84 ± 3.63	17.49 ± 3.10
Ultrastructure			
MVL (μm)	1.40 ± 0.34	1.22 ± 0.20	1.48 ± 0.19
MVD (μm)	0.13 ± 0.02	0.12 ± 0.00	0.12 ± 0.01
MVCT (/μm^2)	82.51 ± 9.40 [a]	69.90 ± 6.19 [b]	85.74 ± 7.32 [a]
EAA (μm^2)	41.22 ± 2.11	41.39 ± 2.35	41.29 ± 2.31
ETAS (μm^2)	1833.75 ± 400.06 [a]	1365.51 ± 216.40 [b]	1877.63 ± 396.65 [a]
MVCV (%)	89.92 ± 2.18 [a]	88.07 ± 1.65 [a]	92.04 ± 1.58 [b]

Abbreviations: EH = enterocyte height; GC = globlet cell counts; LPW = lamina propria width; MT = total muscularis thickness; MTI = muscularis thickness index; SLON = % stratum longitudinale of MT; SCR = % stratum circulae of MT; SCM = % stratum compactum of MT; MVL = microvilli length; MVD = microvilli diameter; MVCT = microvilli counts; EAA = enterocyte apical area; ETAS = estimated total absorptive surface area per enterocyte; MVCV = microvilli coverage.

Values expressed as mean ± S.D. Statistical tests: ANOVA + Fisher's LSD (LPW, MTI, SLON, SCR, SCM, MVL, MVD, MVCT, EAA, ETAS, MVCV); Kruskal-Wallis + Mann-Whitney U (EH). n = 8.

Table 7. Proteolytic enzyme activities in digesta and mucosa of fish fed the lupin based diet, with and without SYN inclusion.

	Diet		
	LC	LS1	LS5
Digesta			
TAP* (U/g)	17.50 ± 3.75	16.03 ± 1.26	17.71 ± 1.32
Trypsin (U/g)	68.18 ± 21.31	66.97 ± 14.02	65.03 ± 13.89
Chymotrypsin (U/g)	325.50 ± 129.00	290.29 ± 27.89	368.57 ± 98.38
ALP (U/g)	51.64 ± 8.94 [b]	78.92 ± 17.14 [a]	79.37 ± 20.56 [a]
LAP (U/g)	5.91 ± 1.27 [b]	7.61 ± 2.85 [a]	8.50 ± 1.06 [a]
Mucosa			
ALP (U/g)	216.73 ± 56.31	203.24 ± 57.98	169.73 ± 68.43
LAP (U/mg)	837.87 ± 225.36	737.07 ± 113.59	692.20 ± 102.33

Abbreviations: TAP = total alkaline protease; ALP = alkaline phosphatase; LAP = L-leucine aminopeptidase.

*10^{-3}.

Values expressed as mean ± S.D. Statistical tests: ANOVA + Fisher's LSD