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Benefits of a commercial solid-state fermentation (SSF) product on growth performance, feed efficiency and gut morphology of juvenile Nile tilapia (*Oreochromis niloticus*) fed different UK lupin meal cultivars

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ABSTRACT

A nutritional investigation was conducted to determine whether growth performance, feed efficiency and midgut morphology of Nile tilapia is affected by United Kingdom cultivars of Yellow Lupin (Control, Y) (YLC) or Blue Lupin (Control, B) (BLC) inclusions compared to the addition of a commercial solid-state fermentation product (SSF) in separate dietary treatments for both lupin cultivars (YLS and BLS). After 49 days of feeding, tilapia receiving SSF supplemented diets (YLS & BLS) exhibited significantly greater weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and condition factor (K) compared to YLC or BLC without solid state fermentation. SSF inclusion exhibited significantly improving of enterocyte height within both the first and second half of the experiment ($P < 0.05$). The surface area of the midgut was not affected by lupin type or SSF ($P > 0.05$); although a potential response over time was apparent in the YLS group. Enterocyte microvilli were significantly wider in fish fed YLC diets ($P < 0.05$), whilst they were significantly longer in the BLC control compared to the YLC control ($P < 0.05$). Microvilli lengths of fish fed YLS were comparable to those receiving BLC diets and close to being significantly longer than those fed YLC alone ($P = 0.06$). Overall, BLC appeared to perform superiorly to YLC; whilst SSF inclusion promoted some desirable production parameters, seemingly most effectively in the YLC diet.

Keywords: Nile Tilapia; Growth; Feed utilisation efficiency; UK lupins; Solid State Fermentation; Gut morphology; Enterocyte microvilli integrity

INTRODUCTION

In response to the growing necessity to reduce fish meal use, plant proteins have been extensively adopted as the main solution for ensuring protein security within the diets of many aquatic species and poultry, with great successes achieved in the past two decades (Michael et al., 2016; Daniel, 2018). Meanwhile, the volumetric majority of commercially cultured finfish, including tilapias and carps, do not necessarily require marine-derived proteins in their diet (FAO, 2016; Daniel, 2017). However, their increasing production levels will continue to demand a simultaneous increase in the availability of high-quality but cost-effective dietary protein sources. Ingredient selection must be scrutinised to promote a sustainable development of the aquaculture industry and plant proteins should not be exempt from this consideration. In light of high crop importations, particularly of soya, calls are being made within Europe for diversification of protein-sources, for sustenance of the livestock sector (Bowyer et al 2019; Goda et al., 2019; Mercedes et al., 2015; El-Husseiny et al., 2018; Davies et al., 2019, Hassan et al., 2019). Importation to a level of dependency does not ensure protein security and the extended supply chain raises socioeconomic and environmental sustainability concerns such as financial cost, out-sourcing of industrial efforts and employment, little to no control over agricultural practices and the 'carbon-footprint' of transportation. At present, alternative proteins in aquafeeds should begin to no longer be viewed, or categorised, 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.

Lupins are seen to be a great potential competitor to soya bean (Bartkiene et al., 2015; 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 tilapia production in RAS (Recirculation Aquaculture Systems) and bespoke feed development. Consequently, there is much focus on lupins as a feed crop in the UK for the home and export market for pigs, poultry and fish, including the farming of tilapia.

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). With regards to lupins, their non-starch polysaccharide (NSP) content is of particular concern; even after de-hulling, this can remain higher than 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., 2003).

Nutritional inferiority of both hulled and de-hulled narrow-leaf lupin (*Lupinus angustifolius*), compared to soya bean meal, has been previously indicated in Nile tilapia (*Oreochromis niloticus*), with observations of depreciated growth following increasing inclusions of the lupin products (Chien and Chiu, 2003). Some have highlighted a requirement for breeding programmes and processing approaches to address anti-nutritional factors (ANFs) in lupins (Mercedes et al. 2015) but *in vivo* biological strategies aimed at degrading these compounds may present a complementary or alternative option.

Solid-state fermentation (SSF) 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

104 bioactive components theoretically capable of degrading previously indigestible dietary
105 fractions. Enhanced animal performance has previously been observed following inclusion of
106 solid-state fermentation products in more contemporary formulations for poultry (Hooge et al.,
107 2010) and Nile tilapia *Oreochromis niloticus* (Hassaan et al., 2017). Diógenes et al (2018)
108 investigated commercial exogenous enzymes and the SSF product Synergen^{TM} produced by
109 Alltech, USA with very promising results on the performance of turbot (*Scophthalmus*
110 *maximus*) juveniles fed distillers' dried grains with solubles (DDGS) based diets.
111 In terms of enhancing the nutritional value of lupins, a recent study, investigating the pre-
112 treatment of lupin with Lactobacilli for inclusion in barramundi (*Lates calcarifer*) diets,
113 reported that nutritional value of the ingredient could be significantly improved through such
114 fermentation methods (Binh Van et al., 2015). To the authors' knowledge, no studies have yet
115 examined lupins in conjunction with solid-state fermentation products in finfish diets.
116 Furthermore, both lupins and exogenous bioactive supplements (asides from probiotics) have
117 sparsely been investigated with regards to their effects upon gut morphology, despite the
118 organ's integral function in assimilating nutrients and pathogen defence; hence the reasoning
119 behind this study. The midgut contains enzymes from the pancreas, intestinal wall and liver.
120 These enzymes attack proteins, lipids, and carbohydrates which obviously affect feed
121 efficiency utilization and consequently growth performance of fish, thus midgut
122 histomorphology was appraised at the start-, mid- and end-point of the trial to identify any
123 temporal responses to the experimental ingredients. Furthermore, the ultrastructure of the
124 midgut brush border was examined at the end of the study to identify any adverse effects upon
125 the dietetically and nutritionally sensitive microvilli. Carcass composition, along with
126 haematological and serological indices of health and immune status were also examined for
127 tilapia. Thus, the present investigation was conducted to evaluate the performance and midgut
128 morphology of Nile tilapia fed diets containing either yellow lupin (*Lupinus luteus*) or blue

lupin (*Lupinus angustifolius*) meals with and without inclusion of a solid-state fermentation product (Synergen™; Alltech Inc., KY, USA).

MATERIALS AND METHODS

Experimental system and animals

The nutritional trial was conducted within a Recirculating Aquaculture System *(*RAS*)* facility designed for nutrition trials with replicate tanks. For the study, YY super-male, black Nile tilapia (*Oreochromis niloticus*) was obtained from North Moore Tilapia (Lincolnshire, UK). The fish were stocked into 80 L tanks within a ~2200 L recirculating system which supplied a flow rate of ~600 L/hr. Acceptable water quality was maintained by biological and mechanical filtration, temperature was maintained at 26.0 °C ± 1, dissolved oxygen was maintained above 76.0 % and a 12 hrs light photoperiod was implemented with fluorescent lights and timers. During a 4 week adaptation period, the fish were fed a BioMar Efico Enviro (BioMar, DK) diet at ~2 % body weight (BW) per day. Following adaptation and grading, selected individuals were restocked into replicate groups of 50 individuals (n = 3), with resulting average initial fish weight of 36.22 g ± 0.16; corresponding to a stocking density of 20.12 kg/m^3 ± 0.13.

Experimental ingredients and diets

De-hulled kernel meal from narrow-leaf blue lupin (*Lupinus angustifolius* cv. Sanabor) and yellow lupin (*Lupinus luteus* cv. Pootalong) were supplied by Soya UK (Hampshire, UK), after de-hulling and milling by Alvan Blanch (Wiltshire, UK). These crops were cultivated in the U.K. as part of the Lupins in UK Agriculture and Aquaculture (LUKAA) initiative (Innovate UK). The solid-state fermentation product, Synergen™ (SSF), was kindly supplied by Alltech Inc. (KY, USA). Four experimental diets were formulated to satisfy all known nutrient requirements of Nile tilapia (NRC, 2011) and be isonitrogenous (N*6.25), isolipidic and isocaloric. These were two basal diets, with 15 % yellow or narrow-leaf blue lupin

inclusion to form reference (control) diets at a lupin level compliant with successful previous inclusions for warm water fish species). Synergen™- (Alltech®) supplemented diets were manufactured by including the SSF product at 0.1 % at the expense of corn starch (Table 1). The diets were formulated using feed formulation software (FeedSoft ™ FeedSoft corp., Dallas TX, USA) with inputted data from analysed ingredients actually employed.

Diet preparation

The ingredients were thoroughly combined in a Hobart Legacy mixer (Hobart Food Equipment, HL1400-10STDA; AU), for 30 min. Oil was subsequently added to the ingredient mix and left to combine homogenously for 30 min, followed by the addition of tepid water to achieve an appropriate consistency for pellet manufacture. Once an appropriate consistency was achieved, the dietary mix was immediately cold-press extruded (PTM Extruder System, P6; Devon, UK) and manually cut to form 2 mm diameter pellets. The moist pellets were oven-dried at 35 °C (Genlab, MINO 200 F; Cheshire, UK). The diets were packaged in sterilised containers and kept at room temperature (~15 °C) until used.

Chemical composition

Proximate compositional analysis was conducted according to standard AOAC (2016) procedures: moisture by oven-drying at 105 °C; crude protein via Kjeldahl method with a Gerhardt Kjeldatherm 40 digestion block and automated Vapodest 40 distillation unit (Gerhardt Laboratory Instruments; DE) (N x 6.25); crude lipid by hot-solvent extraction with a 6-place Soxtherm and Multistat control system (Gerhardt Laboratory Instruments; DE); ash via incineration at 550 °C for 12 hrs. The sum of moisture, crude protein, crude lipid and ash (%) was subtracted from 100 to calculate nitrogen-free extract (NFE).

Feeding and biomass sampling

The trial lasted a total period of 49 days. Throughout the trial, the animals were fed fixed rations between 2.0 and 3.5 % BW per day, which were incrementally reduced as the fish grew. The fish were weighed in bulk, by tank, on a weekly basis. This was performed, to an accuracy of 1 gram. Feeding rations were subsequently calculated for the following 6 days.

Performance evaluation

Weight gain (g) specific growth rate (g/d), protein efficiency ratio and condition factor were recorded for all fish from each aquarium at the initiation and the termination of the experiment was calculated using the subsequent formulae:

Weight gain (WG) = final body weight (g) – initial body weight (g).

Specific growth rate (SGR) =100× ((Ln (W2)-Ln (W1)) / T), Where: Ln = the natural log; W1 = Initial body weight; W2 = Final body weight and T= period of study (60 days).

Protein Efficiency Ratio (PER) = WG (g)/Protein intake (g).

Condition Factor = K value = (body weight, g) / (body length, cm^3) x 100)

Carcass composition

At day 49, sampling for whole carcass was performed by sacrificing and pooling ≥ 200 g of wet fish per tank (n = 3). The proximate composition of the carcasses was determined according to AOAC (2016)

Haematological parameters

At day 49, three fish per tank (n = 9) were sedated in buffered tricaine methanesulfonate (MS222) and blood was collected from the caudal vein. Haemoglobin levels (g/dl) were determined utilising Drabkin's cyanide-ferricyanide solution (Sigma) as described by Rawling et al. (2012). Haematocrit (% PCV) was determined using the microhaematocrit method described by Brown (1988). Serum was prepared in accordance with Rawling et al. (2012). Serum glucose (mg/dl) was measured by the Trinder method, a turbidimetric coupled-enzyme assay. In brief, phosphate buffer (100 mM, pH 7.0) containing 0.016 % (w/v) 4-

Aminoantipyrine, 2 x 10^{-4} % (w/v) peroxidase, 0.105 % (w/v) phenol and 0.1 % (v/v) Tween-20 was created as a colour reagent (all reagents Sigma). Three ml of colour reagent and 50 µl of serum were mixed and incubated for 15 min at 28 °C, rapidly cooled, then read at 550 nm. Serum lysozyme activity (U/ml) was analysed in accordance with methods described by Demers and Bayne (1997), which involved the lysis of Gram-positive *Micrococcus lysodeikticus* and measured by a turbidimetric assay.

Midgut histology

Specimens for mid gut evaluation were sampled from the initial stock at day 0 (n = 9), followed by sampling of 3 fish per tank at days 26 and 49 (n = 9), for light microscopy (LM). Specimens for transmission electron microscopy (TEM) were also obtained from the fish sampled at 49 days.

For light microscopy preparation (LM), the specimens were fixed in 10 % formalin which was replaced by 70 % ethanol after 24 hrs. 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. Functional surface area was evaluated by measuring the outer mucosal (OP) and inner epithelial (IP) perimeters of cross-sections, to calculate perimeter ratio (PR) (arbitrary unit: AU) as follows:

Perimeter ratio (PR) = OP / IP (As described by Bowyer et al., 2019)

For Transmission Electron Microscopy (TEM) appraisal, the samples were processed in accordance with methods detailed by Dimitroglou et al. (2009). In brief, specimens were fixed in sodium cacodylate-buffered 2.5 % glutaraldehyde then post-fixed in sodium cacodylate-buffered 1 % osmium tetroxide (OsO_4). Dehydration was conducted in graded levels of ethanol;

thereafter infiltration, curing and polymerisation 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. Micrographs providing an aerial view of the brush border were used to measure microvilli counts (MVCT) (no. /μm^2) using 1 μm^2 quadrants placed upon standardised co-ordinates. Estimated total absorption surface area (TAS), expressed as μm^2 per 1 μm^2 foot-print, was calculated using the previous parameters as follows:

TAS (μm^2 / 1μm^2) = ((2π x ½MVD x MVL) + (2π x ½MVD^2)) x MVCT

Economical evaluation

The economic value of the diets was determined according to (Abdel Rahman et al. 2010 and Salama et al. 2010) using the following equations:

Feed Conversion Ratio (FCR) = Feed intake (FI) (g)/WG (g).

Relative feed cost/kg fresh fish = Values of feed cost/kg fresh fish / the minimum value of the same parameter.

Feed cost/ 1 kg gain ($) = Feed intake per kg gain (FCR) × cost/kg diet ($).

Economic conversion rate (ECR) = Cost of diet ($ kg^{-1}) x Feed conversion ratio (FCR).

Statistical analyses

All statistical analyses were performed using Sigma Plot 13.0 (SyStat Software Inc.; IL, USA). All values expressed as percentages herein were arcsine-transformed prior to

253 statistical analysis. Fish and feed performance, carcass composition, haemato-serological and
254 midgut ultrastructural parameters were analysed via Two-Way Analysis of Variance (ANOVA)
255 with Fisher's LSD Method for pair wise multiple comparisons. Variables tested were lupin
256 type (YL and BL) and SSF presence (0 and 0.1 % SSF, i.e. control and SSF inclusion), with a
257 test of interaction between these two variables. Enterocyte height (EH) and absorptive surface
258 area (PR) were analysed via Three-Way ANOVA with Fisher's LSD Method. Variables tested
259 were lupin type, SSF presence and time (day 0, 26 and 49), with interactions assessed between
260 the three variables. Significant effects of time alone upon all treatments collectively are not
261 presented in figures herein. Analyses were performed upon ranked means, where data
262 expressed non-normal distribution and are referred to as such within. Statistical significance
263 was considered as $P \leq 0.05$ throughout.

264 Fish protocols were approved by the Institutional Animal Care and Welfare Committee
265 and conforming to European Union statutory regulations for Animal Scientific Procedures
266 (APC's).

267 **RESULTS**

268 Growth performance, feed efficiency and fish condition results are displayed in Table
269 2. Average final fish weight (FW), weight gain (WG) and specific growth rate (SGR) were all
270 significantly increased by SSF (Synergen^{TM}) inclusion (P = 0.02, P = 0.02, P = 0.03,
271 respectively); no significant effects of lupin type, or lupin type–SSF interaction were observed.
272 Significant effect was also detected upon the improvement of FCR with the SSF product
273 inclusion against the corresponding control lupin diets fed to tilapia. Furthermore, no
274 significant effects of SSF, lupin type or an interaction between the two dietary variables upon
275 PER (P = 0.07) No significant effects upon K-factor (K-F) were observed.

276 ***Carcass composition*** appeared not to be affected by lupin type, SSF inclusion or interaction
277 between the two dietary variables; the results are displayed in Table 3.

278 ***Haematological*** analysis of haematocrit (% PCV), haemoglobin (g/dl), serum glucose (mg/dl)
279 and serum lysozyme (U/ml) did not appear to show any significant responses to the dietary
280 variables; the results of which are displayed in Fig. 1.
281 ***Enterocyte Height*** (EH) results are displayed in Fig. 2. No significant effect of lupin type or
282 SSF inclusion alone was identified upon ranked EH. However, time was observed to
283 significantly affect EH ($P = 0.02$), with the overall ranks at day 26 significantly differing from
284 day 0 ($P = 0.01$) and likewise, ranks at day 49 differing from day 0 ($P = 0.01$). Within the
285 controls, EH was observed to not be significantly different between days 0 and 26, however
286 the values were indicated to be significantly different between days 26 and 49 ($P = 0.03$) and
287 days 0 and 49 ($P = 0.01$). With the SSF diets, a significant difference in EH was observed
288 between days 0 and 26 ($P < 0.01$) and days 26 and 49 ($P = 0.02$), however no difference was
289 observed between days 0 and 49. Consequently, a significant interaction between SSF inclusion
290 and time was observed upon EH ($P = 0.01$). The controls displayed significantly lower EH than
291 the SSF treatments at day 26 ($P = 0.02$), whilst the trend was significantly the inverse at day
292 49 ($P = 0.04$).
293 ***Perimeter Ratio*** (PR) results are displayed in Fig. 2. Time was observed to significantly affect
294 grouped PR alone between days 0 and 26 ($P < 0.01$) and consequently days 0 and 49 ($P < 0.01$)
295 but no difference was detected between the overall values recorded between days 26 and 49.
296 Significant effects of time upon PR were independent of lupin type and SSF. However of note,
297 a near significant interaction between lupin type, SSF inclusion and time was detected ($P =$
298 0.06), as a consequence of a numerical decrease in PR within the YLS group between day 26
299 and 49, indicating an increase in relative surface area.
300 **Midgut ultrastructure** results are displayed in Table 4 and micrographs are presented in Fig.
301 3. No significant effects of any dietary variables were observed upon microvilli counts (MVCT)
302 ($P > 0.05$). However counts were numerically higher in the BL-fed fish, attributable to

303 microvilli diameter (MVD) being significantly lower in this group ($P = 0.03$). No effect of SSF
304 inclusion or interaction between the dietary variables was detected upon MVD. No significant
305 effect of lupin type or SSF inclusion alone was observed upon microvilli height (MVH).
306 However, a significant interaction between lupin type and SSF inclusion was detected ($P =$
307 0.03). The difference was detected to lie between the control diets, in the absence of SSF, with
308 significantly lower MVH in YLC than BLC ($P = 0.01$). However, there was no significant
309 difference between YLS and BLC or BLS, thus a near significant difference was apparent
310 between the YLC and YLS groups ($P = 0.06$). However visually, the YLS microvilli surface
311 of the gut enterocytes appeared more densely arranged than for the YLC group that were more
312 fragmented in appearance in images J and I (Fig. 3), respectively. This was also observed for
313 lateral images of the microvilli brush border; A & B (YLC) and C & D (YLS) respectively.
314 No significant effect of lupin type, SSF presence or an interaction between the two dietary
315 variables was observed upon estimated total absorption surface area (TAS), at the
316 ultrastructural level. However the TAS of the YLC group appeared somewhat lower than the
317 other dietary groups

318 The relative feed cost/kg of fish fed diets containing a commercial SSF product
319 (Synergen™ SSF) was lower than the feed used as the control (88, 92 % vs. Control 100%)
320 (Table 1). The data also indicate that Nile tilapia fed diets containing the SSF product
321 (Synergen™ SSF) recorded the best FCR and ECR values. The YLS diet had the lowest in total
322 feed cost and the best relative feed cost/kg gain, while fish fed the control diet recorded the
323 highest in total feed cost/kg fish gain.

324 **DISCUSSION**

325 The potential of Solid State Fermentation (SSF) products to improve aquaculture feeds
326 by releasing plant nutrients that are unavailable to fish and reducing Anti-Nutritional Factors
327 (ANFs) within specific plant feed ingredients requires more study to validate their efficiency

328 in fish diets. The present trial was conducted to establish and document the effects of a
329 nutritional solution produced by a commercial SSF product (Synergen™ SSF) on Nile tilapia
330 production and health when supplemented to diets containing blue and yellow lupin as a UK
331 initiative to produce home grown plant crops for animal production and aquaculture in Europe.
332 We included both British grown lupin meal varieties (Blue and Yellow) at realistic inclusions
333 of 15%. This was based on previously tested levels with success for several warm water fish
334 species. Of particular relevance to this study, it was confirmed by Abdel-Moneim and Yones
335 (2010) that up to 50% of lupin kernel meal (LKM) can be included by replacing the fish meal
336 component in diets of juvenile red hybrid tilapia (Oreochromis niloticus x O. mossambicus)
337 with positive effects on growth performance and nutrient utilization. Our Lupin (SSF-free)
338 based diets all gave comparable results to Abdel-Moneim and Yones (2010) that was evident
339 of lupins being as good a plant protein concentrate for conventional soybean for tilapia. It is
340 recognised too that it might also be desirable to run experimental diets against a range of
341 practical commercial tilapia diets for comparisons in practical conditions.
342 In the present study, comparisons between the lupin basal diets seems to suggest that there was
343 no significant effect of lupin kernel meal (LKM) type on performance. This appears to be in
344 agreement with findings by Glencross et al. (2006), whom did not observe any difference in
345 performance between rainbow trout fed yellow (cv.Wodjil) or narrow-leaf (cv. Gungarru) lupin
346 protein concentrates. Furthermore, a later study by Glencross et al. (2011), did not detect any
347 significant difference in performance between rainbow trout fed between yellow and narrow-
348 leaf (blue lupin) varieties. On the contrary, Salini and Adams (2014) reported that the FCR of
349 fish fed yellow lupin cultivar (Pootalong) was higher than all other dietary treatments and
350 significantly greater than the narrow-leaf lupin (cv. Jindalee); indicating that Pootalong (YL)
351 was of lower nutritional quality. Furthermore, Sharawy et al (2016) observed that fish meal
352 (FM) could be substituted by solid state fermented soybean meal (SSF-SBM) with yeast, The

353 results of this latter work indicate that SSF-SBM with yeast, *S. cerevisiae*, is an acceptable
354 alternative plant protein source that can replace up to 50% of dietary FM protein in diets of
355 Indian white shrimps yields comparable growth and production, and is as cost effective, as the
356 control diet. The present results with tilapia indicated that lupin meals treated by the SSF
357 containing natural exogenous enzymes likely improved the digestibility of NSP, overall feed
358 efficiency and reduce the levels of toxic alkaloids, a factor which could limit the acceptance of
359 lupin seeds (Glencross and Hawkins, 2004). It should be noted that due to the difficulty in
360 obtaining faeces from tilapia under the conditions defined, it was not possible to report
361 coefficients of digestibility in the study but this would be considered in future experiments with
362 lupin incorporation together with SSF or other exogenous feed enzyme supplementation and
363 using a suitable inert marker such as yttrium oxide.

364 The YLC diet in the present study resulted in numerically inferior SGR, FCR and PER
365 values compared to the BLC diet, and these findings are consistent with those of Salini and
366 Adams (2014). Following inclusion of Synergen™ (SSF), the fish showed significant
367 improvements to their overall weight gain, final weight and SGR and FCR in both lupin
368 varieties tested. Ilham et al (2018) also noted that the growth of barramundi (*Lates calcarifer*)
369 fed fermented lupin meal (FLM) attained similar growth to fish fed a fish meal based diet
370 indicating that fermented lupin meal had a potential to substantially replace 75% FM protein
371 in the diets of barramundi.

372 In the present study, no statistically significant differences in Protein Efficiency Ratio,
373 (PER) were found among the dietary treatments of Nile tilapia fed SSF inclusion but trends
374 appeared to display a numerical increase in PER of SSF-fed fish, particularly with regards to
375 the yellow lupin diet. Furthermore, Yadava et al. (2009) found that Allzyme®-SSF was
376 observed to improve protein efficiency in a practical broiler formulation, indicating that solid-
377 state fermentation processes and exogenous enzymes can impart residual bioactivity capable

of aiding in the animal's efficacy of converting protein to body mass. Our growth performance and feed utilisation data compared well as for tilapia fed lupins by Moneim and Yones (2010) indicating that our basal diet of 15% lupin was a satisfactory reference diet for SSF evaluation. It should be cautioned that there is no specific control or reference diet for tilapia available as ingredient formulations vary extensively. It was therefore deemed appropriate to compare two types of lupin meals at fixed levels with and without SSF addition.

Extensive reviews of carbohydrate utilisation by fish have been published by a number of authors, the general consensus affirming that complex carbohydrates are poorly digested outside of strictly herbivorous fish but lower molecular weight carbohydrates (i.e. mono- and disaccharides) tend to be readily available (Krogdahl et al., 2005). Carbohydrates which are not degraded may impart undesirable characteristics upon the GIT and its contents, such as increasing intestinal viscosity (Bedford and Partridge, 2010), thus classifying many of them as fibre type ANFs. However, if low molecular-weight carbohydrates become available to the animal then they may be utilised as metabolisable energy, reducing the metabolic burden of gluconeogenesis and subsequently leading to a carbohydrate induced protein-sparing effect. This has been widely acknowledged as effective in a vast number of finfish species, including Nile tilapia, as reviewed by Krogdahl et al. (2005). Glencross et al. (2003) examined the influence of lupin oligosaccharides on nutrient digestibility in rainbow trout, concluding that removal of these components by α-galactosidase significantly improves nitrogen digestibility and that monosaccharide derivatives of this process are readily absorbed by the animal. Galactosidase has been reported as secreted by *A. niger* during solid-state fermentation of wheat bran (Passos et al., 2015), thus there is a strong possibility of such occurrences during this study. In the present study with tilapia, the Synergen™ product used is a natural solid-state fermentation complex of Aspergillus niger that contains residual enzyme activity as described by Diógenes et al (2018). 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. Because all the enzymes have been produced together during the SSF process, they work in synergy resulting in a greater nutrient release than with individual enzymes or cocktails of different enzymes as demonstrated successfully for piglets (Taylor-Pickard, 2014). More nutrients released from the diet means for example, that more energy is released which can be used by the gastro-intestinal cells and stimulating localised ultra-structure development such as enterocyte micro-villi.

At the gastrointestinal level of the tilapia undergoing the various dietary treatments, distinct trends in epithelial Enterocyte Height (EH), relating to the presence or absence of SSF, were identified in the current study. Whilst both the control diets displayed no marked alteration to EH until the mid-point of the trial, significant heightening of enterocytes lining the gut was indicated thereafter; meanwhile, the opposite trend was the case of the SSF diets. Overall, the present study provides a quantitative appraisal of enterocyte height from a view of developmental progressions, rather than a final suggested change due to respective treatment effect. These fluctuations are proposed as an indication of dietary adaption; this could be due to nutrient provision and possibly establishment of equilibria within the gut microbiome although not tested in this study.

In terrestrial mono-gastric animals, some studies have indicated that exogenous enzyme sources can liberate sufficient bioavailable nutrients to trigger enhanced performance without effect upon epithelial parameters (Amerah et al., 2008; Owens et al., 2008). Conversely, the results of this study indicate that exogenous facilitators of fermentation within the gut can elicit a positive effect upon epithelial cells and they demonstrate effectively that time is a critical factor in confidently identifying whether additives of this kind modulate morphology.

Perimeter ratios of the midgut (PR) were observed to change noticeably from the initial measurements in the first half of the investigation. This indicates that the functional border of

the midgut, in all treatments, increased in surface area after the feeding of the commercial diet was ceased and tilapia were fed diets containing lupins and SSF for the duration of the trial. Conversely, Smulikowska et al., (2014) reported that increasing levels of both yellow and narrow-leaf blue lupin caused reducing effects to the potential absorptive area of the intestine in broilers. The results of this study are of interest as greater surface area is routinely considered to be a beneficial characteristic but it appears it may not always necessarily be indicative of a higher-quality diet formulations. By day 26, all treatments were almost indistinguishable in their relative gut surface area, which was by and large maintained until day 49 in the YLC, BLC and BLS treatments. However the continuation to day 49 saw YLS-fed fish display a substantial increase in functional surface area compared to day 26, thus also a divergence away from the other treatments. This seems to correspond with findings by Olkowski et al. (2010), who detected significant elongation of villi in the intestine of broilers fed yellow lupin-based diets supplemented with non-starch polysaccharide degrading enzymes. Overall, the present results for tilapia appear to show a more pronounced effect of lupins upon gut macrostructure than has previously been reported in finfish (Omnes et al., 2015; Refstie et al., 2006; Glencross et al., 2004). To the author's knowledge, no known studies have examined tissue at an ultrastructural level following feeding of lupin diets to monogastric species, let alone in partnership with fermentation products. Therefore, cross-literary comparisons are difficult. Yellow lupin appeared to elicit poorer potential for functional surface area at an ultrastructural level, compared with narrow-leaf lupin. Thicker microvilli appeared to reduce potential for higher density, whilst the microvilli provided less surface area due to a reduced length. There was no apparent influence of improvement to these parameters when the yellow lupin diet was supplemented with SSF. However, the fact that YLC group had significantly shorter microvilli than the BL groups but the YLS average microvilli length was comparable to the BL groups suggests a positive effect may still have occurred. These results seemed to display trends

towards improved absorption surface area (TAS) in the YLS, with no noticeable difference with the narrow-leaf blue lupin fed tilapia. This suggests that negative effects of yellow lupin upon gut ultrastructure can be mitigated to an appreciable extent by SSF inclusion such as Synergen^{TM} and allowing greater surface area potential for assimilation of nutrients. Promotion of these kinds of ultrastructural characteristics has also been associated with low molecular-weight prebiotics (MOS) Dimitroglou et al. (2009), supporting the benefits of non-starch polysaccharide degradation by exogenous enzymes. Considering all of these patterns, it would be highly beneficial for future studies to quantify effects upon nutrient digestibility and modulation of the gut microbiota in tilapia and in other commercially important fish species fed various grains, plant protein concentrates and seed meals in combination with SSF products.

It was evident that the commercial SSF product evaluated in this study significantly improved several production parameters for tilapia and can play a positive role in contributing to cost effective and sustainable diet formulations for aquaculture.

CONCLUSION

To our best knowledge, this study is the first study of its kind investigating the inclusion of a solid-state fermentation product in finfish diets containing lupin. Without SSF-inclusion, the narrow-leaf lupin appears to elicit preferable production characteristics compared to yellow lupin. The result indicated that inclusion of the specific commercial solid- state fermentation, SSF product evaluated in this study can be an effective means of promoting better performance and to some extent, improving gut morphology, most probably due to a supply of exogenous bioactive components capable of degrading indigestible dietary fractions in a lower quality plant feed ingredient. This appeared most pronounced when SSF was added to a yellow lupin diet in tilapia. Clearly, there is scope for more extensive studies with high value carnivorous species such as salmonids (rainbow trout and salmon) as well as marine fish e.g. seabass, seabream, to improve the value of plant ingredients in diet formulations. SSF products such as

478 Synergen™ have promising capacity to improve fish growth and feed performance as well as
479 enhancing gut integrity and ultimately the health and welfare of farmed fish. They will also
480 likely impart the benefit of reducing environmental impact through more efficient digestion
481 and may provide an economic gain in fish production costs. Tilapia production in Europe is
482 being realised and there are several feed manufacturers engaged in specialised diets for this
483 species as well as for the export market particularly to Africa. The use of sustainable plant
484 ingredients like lupins can help reduce our soy bean import dependency and coupled with SSF
485 supplementation can be a positive asset to meet this agenda.

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