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Potential of processed animal protein versus soybean meal to replace fish meal in practical diets for European catfish (*Silurus glanis*): growth response and hepatic gene expression

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Running Head: Fish meal replacemnt for European catfish, *Silurus glanis*.

Abstract

A 9-week study was conducted to evaluate the potential of processed animal protein (PAP) in comparison of soybean meal (SM) to replace fish meal (FM) in practical diets for European catfish, *Silurus glanis* on growth performance, liver transaminase activities and expression of growth related gene. Seven isonitrogenous (440 g kg^{-1} crude protein) and isoenergetic (16.70 MJ kg^{-1}) practical diets were formulated by replacing 0 (control), 30, 60 and 100% FM with either SM or PAP. Each diet was randomly assigned to triplicate groups of 25 fish per cages fixed in the pond. Fish fed the diet substituted 100% FM from SM or PAP had lower ($P < 0.05$) growth performance, feed efficiency and protein retention compared to control and other groups. The decreased growth performance was concomitant with decline in the expression level of hepatic GHR/IGF-I axis genes. The gene expression and activity of hepatic protein metabolism enzymes were also positively correlated with growth performance. Findings of the present study indicated that both SM as well as PAP was equally effective in replacing FM up to 60% in the practical diet of *Silurus glanis*. Total replacement of FM either with SM or PAP induced negative influences on growth and feed utilization.

Key words: Processed animal protein; soybean meal; growth; protein metabolism; gene expression; *Silurus glanis*.

Introduction

Quality of dietary protein ingredients plays an important role in feed utilization and regulating growth of commercially farmed fish. Fish meal (FM) is the most important and widely used protein ingredient in aquaculture, especially for the production of carnivorous fish species (Hertrampf & Piedad-Pascual 2000; Glencross et al. 2007). However, increasing demand, uncertain availability and high price with the expansion of aquaculture made it necessary to search alternate protein sources. Plant proteins currently represent the only economic and sustainable protein alternative to fishmeal and are increasingly being used in commercial fish feed, with the most common being soybean meal (SM), which has a high protein content. However, the use of plant proteins in the diet of carnivorous species remains a challenge due to their high carbohydrate content and imbalanced amino acid composition (e.g. deficiency of methionine and lysine) (Lunger et al. 2007). The use of plant protein, specially soybean, also competes with human food production. Hence, there is an urgent need to identify protein rich resources other than plant protein that could be implemented in aquatic diets. Compared with plant proteins, terrestrial animal by-products are rich in protein with optimal amino acid profiles. In addition, they contain less carbohydrate and anti-nutritional factors (Tacon 1993; Gomes et al. 1995). Similar digestibility coefficients of protein and energy for animal by-product and fish meal reported in some aquatic species (Bureau et al. 1999; Zhou et al. 2004) emphasise the possibility of fish meal replacement by animal by-product ingredients.

Depending on the ratio of bone to soft tissue used in processing, the finished terrestrial animal by-product is designated as meat meal (containing crude protein >550 g kg^{-1} and ash <200 g kg^{-1}) or meat and bone meal (MBM) (containing crude protein <450 g kg^{-1} and ash >350 g kg^{-1}). High ash content limits the inclusion level of MBM in fish feed compared to meat meal. Previous studies with meat meal products (Lovell 1992; Shimeno et al. 1993a,b & 1996; Tacon 1994) documented convincing result with respect to FM replacement for fish feed. On a cost per unit protein basis, meat meal or processed animal protein (PAP) is an appealing protein source for most farmed finfish. In addition, PAP is also able to contribute to the nutritional needs for calcium, phosphorous and vitamin B_{12}. PAP is therefore a high-value resource which is as safe as any other protein and should not be regarded as a waste product by any means. Since 2001, the European Commission has banned the use of animal protein in animal feeds because of the spread of mad cow disease in the cattle, caused due to feeding the ruminants with animal protein feed that contained a

protein (prion) causing Bovine Spongiform Encephalopathy (BSE) or mad cow disease. The European Commission has uplifted this ban in 2013 and permitted to use animal protein from non-ruminant animals in fish feed in EU countries (Commission Regulation EU No. 56/2013).

Now, new advancements in nutrition research have allowed for the integration of nutrition and genomics analysis through the nutrigenomics approach, which has added to the understanding of the effect of diet on gene expression (Mutch et al. 2005). Nutrigenomics studies in farmed fish have addressed the replacement of different percentages of FM with plant protein in the diet. It has been reported that fish growth rates are mediated by the growth hormone (GH) / insulin like growth factor (IGF) axis (Company et al. 2001; Perez-Sanchez et al. 2002) and dietary protein sources may affect expression of GH and IGF-1 encoding genes (Kumar et al. 2011). However, changes in the expression of growth related genes due to replacement of FM with PAP in *Silurus glanis* has not been studied before.

European catfish (*Silurus glanis*) has been cultivated extensively in ponds in Central and Eastern Europe for 100 years. This is one of the candidate carnivorous species for culture in Europe because of fast growth, hardiness to stressful environments and high market value. However, only few nutritional studies on *Silurus glanis* have been reported (Bekcan et al. 2006; Slawski et al. 2011). The available commercial diet for European catfish contains about 45 g kg^{-1} protein and 10 g kg^{-1} lipid. Most of the protein is provided by fish meal (above 60%), which raises the manufacture price for European catfish feed. Studies on other carnivorous fish showed that animal protein can partially replace dietary FM without compromising growth performance and feed efficiency (Bureau et al. 2000; Webster et al. 2000; Millamena 2002; Wang et al. 2006). Hence, replacement of FM by alternative low cost protein such as PAP should be promoted for practical production of diet for European catfish. To our knowledge, no information is available on the study of replacement of FM with PAP in this fish. Accordingly, the aim of this study was to assess the potential of using PAP versus SM as FM substitute protein in practical diets of *Silurus glanis* and to evaluate possible effects on growth performance and expression of growth and protein metabolism related candidate genes in liver of European catfish (*Silurus glanis*).

Material and methods

Diets

Seven isonitrogenous (44 g kg^{-1} crude protein) and isoenergetic (16.70 MJ kg^{-1}) experimental diet were formulated. The control diet (FM) was formulated with 49% FM as protein ingredient. The other six experimental diets were formulated based on the replacement of 30%, 60%, and 100% FM mainly by either soybean meal (SM) or PAP. Corn gluten was used to maintain protein level in diets. Different experimental diets are as: Control (FM), SM_{30}, SM_{60}, SM_{100}, PAP_{30}, PAP_{60}, and PAP_{100}. The composition of the experimental diets is given in Table 1. All experimental diets were produced as 4 mm slow sinking pellets by HALTAP, Szarvas.

Fish and experimental conditions

The experiment was carried out at Research Institute for Fisheries and Aquaculture (HAKI), Szarvas, Hungary. At experimental setup, 525 European catfish (*Silurus glanis*) (350.94 ± 5.24 g, mean ± SE, n=21 cages) were randomly distributed into 21 cages of 3 X 3 X 3 m fixed in the pond. The trial lasted for nine weeks. Continuous aeration was provided from a paddlewheel aerator installed in the pond to ensure adequate oxygen level during the whole experimental period. During this period fish in each cage were fed with their corresponding experimental diets twice a day (08.00 and 16.00 h) with daily feeding rate 2.5% of total body weight. Fish were weighed individually at the beginning of the experiment and at every other week interval during the experimental period to adjust the feeding level for subsequent week. On the designated weigh days, fish were weighed in the morning, not being fed for 16 hours prior. Water quality was monitored throughout the experiment. All the water parameters were in optimum range (temperature 17 – 20 ^{0}C, pH 7.0 – 8.0, dissolve oxygen 6.4 – 8.1 mg l^{-1}).

Sampling

All fish were weighed (g) individually at the end of experiment to assess weight gain. Growth performance of fish such as percentage weight gain, body mass gain (%), metabolic growth rate (MGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and protein productive value (PPV) was calculated based on the following standard formulae: Body mass gain (%) = [(Final weight –Initial weight) / Initial weight] x 100; Metabolic growth rate (MGR, g $kg^{0.8}$ day^{-1}) = (Body mass gain in g) / [{(initial body mass in g / 1000)^$^{0.8}$ + (final body mass in g / 1000) ^$^{0.8}$}/2] / number of trial days; Apparent feed conversion ratio (AFCR)= Total dry feed intake (g)/ wet weight gain (g); Apparent protein efficiency ratio

(APER) = Net weight gain (wet weight) / Protein fed; Protein productive value (PPV) = [(final fish body protein in g – initial fish body protein in g) / total protein consumed in g] x 100.

Nine fish from the start population and three fish from each cage at the end of experiment were randomly collected for analyses of whole body proximate composition. In addition, another four fish from each cage were anaesthetized, killed with a blow to the head and then dissected to collect the targeted organ (liver) at the end of experiment. Four liver samples were taken from the same area of liver on each fish. Liver samples (3-5 g) from two fishes were immediately frozen in liquid nitrogen, and stored in at – 80 ^{0}C until analyses of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Similarly, liver samples from another two fishes were immediately stored in an RNAlater at –80 ^{0}C until RNA extraction for gene expression analysis.

Analysis

Proximate analysis

Proximate composition of the feed and fish was analysed by standard methods of AOAC (1995). Dry matter was determined gravimetrically after drying at 105 ^{0}C for 4 h. Total nitrogen was determined by Kjeldahl-method using a digestion block (KJELDATHERM, Gerhardt, Germany) and distillation method (VAPODEST 30, Gerhardt, Germany), and crude protein calculated as N X 6.25. Fat was determined by Soxhlet method using a semi-automatic system (SOXTHERM 2000, Gerhardt, Germany) and diethyl ether (boiling point, 40 – 60 ^{0}C) as a solvent and ash content was estimated by after combustion at 550 ^{0}C for 4 h. Crude fibre content was determined in fat extracted feed sample by digestion with sulphuric acid (0.51 mole l^{-1}) and potassium hydroxide 0.89 mole l^{-1}) in a GERHARDT Crude Fibre apparatus (Gerhardt, Germany). Total carbohydrate was calculated by differences as, 1000 – (crude protein + fat + ash + fibre). The gross energy of experimental diets was calculated as described by Halver (1976). The amino acid contents in diets were analysed by the accredited laboratory of the Hungarian Food Chain Safety Office (www.nebih.gov.hu) following the ISO 13903:2005 Official Method. The essential amino acid compositions of the experimental diets are given in Table 2.

Protein metabolism enzymes

SIGMA-ALDRICH® Alanine Aminotransferase (ALT) Activity assay kit (Catalog # MAK052, SIGMA-ALDRICH, USA) and Aspartate Aminotransferase (AST) Activity assay kit (Catalog # MAK055, SIGMA-ALDRICH, USA) were used to determine ALT and AST activity in liver of fish. The principle for ALT activity measurement is transfer of an amino group from alanine to α-ketoglutarate resulting in the generation of pyruvate, and in the production of a colorimetric (570 nm) product proportional to the ALT enzyme activity present. One unit of ALT is defined as the amount of enzyme that generates 1.0 µmole of pyruvate per min at 37 ^{0}C. Similarly, measurement of AST activity was related to transfer of an amino group from aspartate to α-ketoglutarate resulting in the generation of glutamate, and in the production of a colorimetric (450 nm) product proportional to the AST enzyme activity present. One unit of ALT is the amount of enzyme that generates 1.0 µmole of glutamate per min at pH 8.0 at 37 ^{0}C.

Gene expression analysis

RNA extraction

Total RNA was isolated from liver samples using a Promega RNA isolation Kit (Cat No. Z3100, USA) according to the manufacturer's instructions. The quantity of the RNA was assessed using a Nano-Drop spectrophotometer (NANODROP 2000, Thermo Scientific, Wilmington, Delaware, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and the purity by measuring the OD260/OD280 absorption ratio (>1.95).

First strand cDNA synthesis

cDNA was generated from 1µg of total RNA using the Omniscript Reverse Transcriptase cDNA synthesis kit (Qiagen, Germany) for reverse transcriptase polymerase chain reaction (RT-PCR) following the manufacturer's protocol. The product of the first strand cDNA synthesis was stored at – 80 ^{0}C until the quantative RT-PCR (qRT-PCR) runs.

Real-time quantative RT-PCR

Highly purified salt-free 'OliGold' primers (Eurogentec, Seraing, Belgium) for the quantification of the target genes were designed using the Lightcycler probe design software, version 1.0 (Roche Diagnostics, Vilvoorde, Belgium). The primer sequences and calculated efficiency is enlisted in Table 3. qPCR analyses were performed on an Mx3000P QPCR

System (Agilent Technologies, Belgium). Reactions containing 5µL of 5×diluted cDNA, 10pmol each of forward and reverse primers, 0.4µL ROX dye (1:500 dilution) and 10µl Brilliant II SYBR Green qPCR (Agilent) were performed in a four-step experimental run protocol: a denaturation program (10 min at 95°C); an amplification and quantification program repeated 40 times (30 s at 95 ^{0}C, 50 s at 55 ^{0}C, 40 s at 72 ^{0}C); a melting curve program (55–95 ^{0}C with a heating rate of 0.10 ^{0}C/s and a continuous fluorescence measurement) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, 'no-template' controls (i.e. with water sample) for each set of genes were also run to ensure no contamination of reagents, no primer–dimer formation, etc. β-actin has been reported as the most suitable reference gene in *Silurus* species (Wu et al. 2009). Moreover, the calculated value of coefficient of variation (%) (from CT value of 30 random samples) for β-actin was 4.40, suggesting its stable expression level in these test conditions. Therefore, it was used as endogenous standard to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

Statistical analysis

All data were subjected to analysis of variance using SPSS 22 for Windows. Differences among the means were tested by Duncan multiple range test. The level of significance chosen was $P < 0.05$.

Results

Proximate composition of whole body

The effect of different inclusion levels of SM and PAP on the body composition of European catfish is presented in Table 4. Whole body composition analysis showed that replacement of dietary FM with either SM or PAP significantly increased ($P < 0.05$) whole body moisture content and decreased ($P < 0.05$) whole body crude protein content. Fish fed the SM_{30} and SM_{60} diets had higher ($P < 0.05$) whole body moisture and crude protein content than that of fish fed the SM_{100} diet. Similarly, fish fed PAP_{30} and PAP_{60} diets had higher ($P < 0.05$) whole body crude protein content than that of fish fed the PAP_{100} diet. Replacement of dietary FM with SM significantly ($P < 0.05$) decreased whole body crude lipid up to 60% replacement level, however it increased ($P < 0.05$) at 100% replacement level. Whereas similar whole body crude lipid content was found in fish fed the FM and

236 PAP_{60} diet, it was lower ($P < 0.05$) than that of fish fed the $PAP_{30\ and}$ PAP_{100} diet. No
237 significant differences were found in whole body ash content among different diet treatments.
238 **Growth performance and nutrient utilization**
239 No mortality was registered in any of the groups throughout the feeding experiment.
240 All groups had approximately the same initial weight of 350.94 ± 5.24 g (mean ± SE, n=21
241 cages), with no significant differences in start weight between cages. The dietary SM or PAP
242 level significantly ($P < 0.05$) affected the growth performance in the present study. There
243 were no significant differences in body mass gain (%) and MGR among fish fed the diets
244 with 0, 30 and 60% replacement of FM from either SM or PAP (Table 5). However, body
245 mass gain (%) and MGR in fish fed the diet with 100% replacement of FM from either SM or
246 PAP were similar ($P > 0.05$) and significantly ($P < 0.05$) lower than other groups.
247 When either SM or PAP replaced 100% FM, AFCR and APER were lower ($P < 0.05$)
248 and higher ($P < 0.05$) respectively compared with the other groups, however no significant
249 differences were observed at or up to 60% substitution level (Table 5). As the replacement
250 level was increased, the PPV decreased ($P < 0.05$) and lowest PPV was found in 100%
251 substitution level (Figure 1).
252
253 **Expression of growth related genes**
254 Hepatic IGF-I mRNA expression level was significantly downregulated in fish fed
255 with SM_{100} and PAP_{100} compared to the control (FM) and to the other experimental groups
256 (Figure 2A). Relative to the control, a 3.31 ($P < 0.05$) and 3.67 ($P < 0.05$) fold reduction in
257 IGF-I transcript level was observed respectively in SM_{100} and PAP_{100}.
258 Significant reduction in GHR mRNA level was also seen for fish fed the diets with
259 SM_{100} and PAP_{100} with 3.87 and 3.44 fold lower expression than in the control (Figure 2B).
260 The drops in the GHR transcript level in these two groups (SM_{100} and PAP_{100}) were also
261 significantly lower than other dietary treatments. Likewise, in SM_{30} and PAP_{60} GHR
262 expression level was also significantly downregulated compared to the control, nevertheless,
263 these reductions remained significantly higher to both SM_{100} and PAP_{100}.
264 The expression pattern of the GH gene among dietary treatments was almost opposite
265 to the IGF and GHR (Figure 2C). Contrary to IGF and GHR, the mRNA transcript level of
266 GH in fish group subjected to SM_{100} and PAP_{100} elevated significantly, and prevailed the
267 control by 2.12 and 2.03 fold respectively. Similarly, a significant increment in GH transcript
268 level compared to the control was also documented for PAP_{60}.

Expression of protein metabolic enzyme (ALT and AST) genes

Under the different experimental conditions, mRNA expression level of ALT and AST followed the same pattern as the IGF-I expression profile; the level dropped significantly in SM_{100} and PAP_{100} relative to all treatments including the control (Figure 3). Compared to the control level, relative declines in ALT expression in SM_{100} and PAP_{100} were 1.73 fold ($P < 0.01$) and 1.65 fold ($P < 0.05$) respectively. Likewise, SM_{100} and PAP_{100} displayed a 3.59 fold ($P < 0.05$) and 2.60 fold ($P < 0.05$) decline in AST expression comparative to the control expression level.

Protein metabolism enzyme activity

In general, ALT and AST activity responses (Figure 4) paralleled mRNA expression data (Figure 4) quite well for these two enzymes. Dietary inclusion of SM or PAP significantly ($P < 0.05$) changed the activities of protein metabolic enzymes (ALT and AST) in liver of European catfish (Figure 2). The activities of ALT and AST in liver decreased with increasing dietary SM or PAP level. When the substitution level was 30%, no significant differences were observed between this group (SM_{30} or PAP_{30}) and the control group. However, when the substitution level was 60% or higher, activities of ALT and AST in liver were lower ($P < 0.05$) than in the control group.

Discussion

Feeding the minimum fish meal diets is practically important in commercial fish farming. Soybean meal is widely used as the most cost effective alternative for FM in feeds for many aquaculture species (Storebakken et al. 2000). However, recent studies showed that processed animal protein ingredients are highly digestible and have good nutritive values for fish, suggesting PAP as one of the potential ingredient to replace fish meal (Bureau et al. 1999, 2000). In this point of view, we compared the capacity of FM replacement by PAP and SM in the diet of European catfish *Silurus glanis*.

Dietary SM or PAP levels significantly affected the growth response of European catfish. With increasing dietary SM or PAP, growth and nutrient utilization significantly decreased. Especially, when replacing FM more than 60%, the body mass gain (%), feed efficiency and protein efficiency were significantly lowered than those of control and other groups. However, no significant differences in body mass gain (%), MGR, AFCR and APER were observed among fish fed the diets with less than 60% protein from either SM or PAP.

These results indicate that 60% of FM could be replaced by either SM or PAP without significantly reducing growth and nutrient utilization under the specific formulated diets while higher substitution levels reduced the growth. This corroborates well with the results of some previous studies. Lin & Luo (2011) found that when SM replaced 100% protein from FM in the diet of tilapia, weight, relative weight gain ratio and specific growth rate were lower while no differences were observed at or less 75% substitution level. Gallagher (1994) also found 75% FM protein could be replaced by SM protein in the diets for hybrid striped bass *Morone chrysops* x *M. sasatilis*. Similar results have also been documented in some freshwater fish, such as carp (Viola et al. 1982), blue catfish (Webster et al. 1992), tilapia (*Oreochromis mossambicus*) (EI-Sayed 1999) and Nile tilapia (*O. niloticus*) (EI-Saidy & Gaber 2002). Regarding replacement of FM with PAP, Nengas et al (1999) were able to replace 50% of the protein provided by fish meal with poultry by-product meal (PBM) in diets for gilthead seabream; however, reductions in growth performance were seen at the 75% level of FM replacement. Similarly, negative effects on growth performance and nutrient utilization were observed when more than 50% of FM was replaced with PBM in black sea turbot (Yigit et al. 2006), European eel (Gallagher & Degani 1998) and chinook salmon (Fowler 1991).

Essential amino acid deficiency is one of the major factors limiting the utilization of alternate protein sources as fish meal substitutes (Glencross et al. 2007). The dietary amino acid analysis showed that the ΣEAA decreased with increasing SM or PAP, which was positively correlated with growth response. Substitution of 100% dietary FM with SM or PAP results in a significant decrease of ΣEAA in the diet. The dietary lysine and threonine content decreased with the increasing dietary SM or PAP, whereas the dietary methionine level was decreased only due to inclusion of SM in place of FM. This suggests that the reduced growth was related to essential amino acids deficiencies, especially lysine and threonine at more than 60% substitution level. Since the quantitative requirement for most of essential amino acids have not been determined for European catfish, the amino acid pattern of its relative species, *Ictalurus punctatus* was taken as the index of requirement (Li et al. 2004). It appears that lysine, methionine and threonine content in the SM_{100} diet and lysine and threonine content in PAP_{100} diet could not satisfy the EAA requirement, although histidine, valine and phenylalanine were relatively higher (NRC 2011). This confirmed that imbalance of essential amino acid accounted for reduced growth at high substitution level of SM or PAP. The supplementation of crystalline amino acids and/or other ingredients could

335 improve the EAA composition of the diet, and subsequently probably improve the growth of
336 European catfish, which needs to be examined in further studies. Another possible reason for
337 depressed growth and nutrient utilization could be high undigested fibre in SM_{100} and PAP_{100}
338 diets. Undigested fibre reduces digestible energy on the higher inclusion level of alternate
339 protein ingredients and less digestible energy resulted in more amino acid oxidation and /or
340 conversion of amino acid to glucose and /or fat (Francis et al. 2001; Krogdahl et al. 2005).
341 Efficient protein synthesis requires sufficient availability of all essential amino acids
342 (Dabrowski & Guderly 2002). Unbalanced amino acid concentrations in a diet results in
343 increased protein degradation (Langar et al. 1993) and thereby increased protein turnover
344 (Martin et al. 2003). High inclusion of alternate protein ingredients lower nitrogen retention
345 in salmon and trout due to less available digestible energy and an unbalanced amino acid
346 profile (Cheng et al. 2003; Refstie et al. 2000). Similarly, in the present study, PPV of fish
347 decreased with increasing dietary SM or PAP levels. There was no significant difference in
348 PPV among fish fed diets with 0%, 30% and 60% replacement of FM from SM or PAP, but
349 when FM substitution level was more than 60%, PPV was significantly lower than in the
350 control. EAA imbalance could be one reason for declining PPV. Also, whole body crude
351 protein decreased with increasing dietary SM or PAP level and fish fed the diet with 100%
352 substitution of FM from SM or PAP had significantly lower crude protein content. This
353 indicates that 60% of FM replacement either with SM or PAP provides optimum digestible
354 energy and a balanced amino acid profile for European catfish growth.
355 The GH/IGF-I axis provides an integral signal for growth and nutrient partitioning
356 (Beckman & Dickhoff 1998), and is also involved in tissue differentiation, metabolism,
357 reproduction, behaviour and immunity. In the present study, the gene expression patterns of
358 growth regulating hormones and receptors e.g GH, IGF-I, GHR were investigated. In general,
359 GH and IGF-I have metabolic functions, so it can be well anticipated that dietary
360 modifications are the principal regulator of GH/IGF-I axis in fish. Gómez-Requeni et al
361 (2003) have demonstrated that in conjunction with reduced growth rates, the changes in the
362 dietary EAA content could induce some sort of liver GH resistance. Similarly, the results
363 presented herein indicate that the expression of the GH-liver axis was affected by dietary
364 treatment. Besides dietary EAA imbalance discussed earlier explicitly for SM_{100} and PAP_{100},
365 the cessation in growth rate in these two treatments is also attributed to the decline in the
366 binding capacity of GH to the hepatic GH receptors concomitant with the decrease in GHR
367 mRNA level. This leads to a reduction of IGF-I synthesis (or transcript) since there is a

368 reduced signal from the GHR. As a consequence, IGF-I can no longer perform its negative
369 feedback on GH secretion; consequently it triggers GH levels to rise as was seen in SM_{100}
370 and PAP_{100} with an elevated GH transcript level. Apparently, the elevated mRNA transcript
371 level of GHR/IGF-I axis genes in FM (control) diet fed fish might be responsible for the
372 higher growth rate. The opposing response of GH and IGF-I mRNAs in the present study is
373 consistent with the observation in rabbit fish (*Siganus guttatus*), groupers (*Epinephelus*
374 *coioides*) and common carp (*Cyprinus carpio*) under dietary modifications (Ayson et al.
375 2007; Pedroso et al. 2006; Sinha et al. 2012). Furthermore, IGF binding proteins (IGFBP)
376 also form an integral part of GH/IGF-I axis as IGF-1 can act on these target sites to stimulate
377 cell proliferation, differentiation and ultimately body growth. Therefore, the investigation of
378 the expression profile of IGFBP and their regulatory role for functioning of IGF-I will be
379 crucial for future research. Nevertheless, in the present study, GH/IGF axis was investigated
380 only at the mRNA level. Changes in gene expression do not always translate into comparable
381 changes in protein function, although there was a fairly good correlation in the present study
382 with the growth rate. In future studies, investigation of these hormones/proteins at the
383 translational level will be crucial.

384 Aspartate aminotransferase (ALT) and alanine aminotransferase (AST) activity is
385 highly correlated with the protein utilization and changes in their activities have been used to
386 provide an indication of disturbance by replacement of FM with alternate protein sources to
387 the metabolic function and nutrient utilization by fish (Krogdahl et al. 2003;Lin & Luo 2011).
388 In the present study, kinetics of protein metabolism enzymes (ALT and AST) activities in
389 hepatic tissue were accompanied with a concomitant response at the transcript level. mRNA
390 expression levels and activities of these two enzymes clearly reduced with increase in dietary
391 SM or PAP level and remarkably the lowest activities were found in the fish fed the diet
392 substituted with 100% FM from SM or PAP. This signifies that the utilization of dietary
393 protein decreased and the liver was damaged to a certain extent (Lin & Luo 2011). These
394 responses are in consistent with the growth, and positively correlated with the growth
395 performance and feed utilization. As hepatic ALT and AST gene expression were also
396 significantly altered in response to dietary increment of SM and PAP, it can be deduced that
397 the reduction in protein utilization from different dietary sources could also be attributed to a
398 transcriptional changes. In general, amino transferases, such as ALT and AST, catabolize
399 amino acids and transfer amino groups to alpha-keto acid (reversible catalysis). Interestingly,
400 we found that the level of essential amino acids is reduced particularly in SM_{100} and PAP_{100}

401 (Table 2) which corresponds to the reduced expression and activities of ALT and AST in
402 these two dietary treatments. It is therefore tempting to speculate that when the essential
403 amino acids are deficient, the keto acids may be reduced thereby tends to decline the activity
404 of ALT and AST.
405
406 **Conclusions**
407 The results demonstrated that the use of either SM or PAP as a dietary protein source
408 to completely replace FM negatively impacts the growth performance and efficiency with
409 which dietary protein is used. The decreased growth performance was associated with
410 significant decreases in the expression of hepatic GHR/IGF-I axis genes. The activity and
411 gene expression of hepatic protein metabolism enzymes (ALT and AST) were positively
412 correlated with growth performance and protein utilization efficiency. In conclusion, both
413 SM as well as PAP was equally effective in FM replacement up to 60% in the practical diet
414 of *Silurus glanis*. Imbalance in essential amino acid lysine and threonine and high fibre
415 content were effective to decrease growth performance of fish at 100% FM replacement.
416 Equal FM replacement capacity and low cost of PAP compare to SM, makes PAP as a more
417 suitable ingredient to replace FM in the diet of European catfish, *Silurus glanis*. This study is
418 the first to evaluate and provide the data on the substitution possibilities of FM by PAP in the
419 diet for European catfish, *Silurus glanis*, a commercially important fish species, particularly
420 in Central and Eastern Europe.
421
422 **Acknowledgement**
423 The financial support of this project (Harcsatech GOP-1.1.1-11-2011-0028) by
424 Ministry of Agriculture, Hungary is gratefully acknowledged. This experiment was also
425 partially supported by the AQUAREDPOT project funded by the European Commission
426 (FP7-316266).
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578

Figure 1: Protein productive value (PPV) of European catfish, *Silurus glanis* fed practical diets with different levels of substitution of FM by SM or PAP (mean ± SE). Mean values with different letters having significant difference ($P < 0.05$).

Figure 2: Relative expression of growth genes (A) insulin like growth factor–I (IGF-I), (B) growth hormone receptor (GHR) and (C) growth hormone (GH) in the liver of European catfish, *Silurus glanis* fed practical diets with different levels of substitution of FM by SM or PAP (mean ± SE). Mean values with different letters having significant difference ($P < 0.05$).

Figure 3: Relative expression of ALT and AST gene in liver of European catfish, *Silurus glanis* fed practical diets with different levels of substitution of FM by SM or PAP (mean ± SE). Mean values with different letters having significant difference ($P < 0.05$).

Figure 4: Protein metabolism enzymes (ALT and AST) activity in the liver of European catfish, *Silurus glanis* fed practical diets with different levels of substitution of FM by SM or PAP (mean ± SE). Mean values with different letters having significant difference ($P < 0.05$).

Table 1: Formulation (g kg^{-1}) and proximate composition (g kg^{-1}, dry matter) of the experimental diets used in the experiment.

Ingredient	**FM (Control)**	**SM_{30}**	**SM_{60}**	**SM_{100}**	**PAP_{30}**	**PAP_{60}**	**PAP_{100}**
Fishmeal	490	350	200	0	350	200	0
Extr. soya	0	200	300	400	0	0	0
PAP-55	0	0	0	0	150	300	450
W. wheat	280	200	180	160	280	270	285
Maize	58	68	48	33	60	62	43
Corn gluten	0	0	80	200	0	20	80
Blood meal	50	50	50	50	50	50	50
Yeast, f.g.	50	50	50	50	50	50	50
Vit-Min mix[1]	20	20	20	20	20	20	20
Lignin phosphate	7	7	7	7	7	7	7
Fish oil	5	15	25	40	5	5	5
Linseed oil	40	40	40	40	28	16	10
Proximate composition (Dry basis)							
Moisture	77.5	86.9	90.2	96.5	75.1	81.4	98.8
Dry Matter (%)	922.5	913.1	909.8	903.5	924.9	918.6	901.2
Crude Protein (CP)	427.0	428.2	433.4	428.7	427.9	448.4	451.5
Crude Fat	110.1	110.8	107.0	110.9	98.1	87.1	80.9
Crude fibre	17.7	25.3	27.5	29.5	23.6	27.5	31.5
Ash	135.0	113.7	91.3	51.5	130.4	121.4	105.2
Total Carbohydrate	310.2	322.0	340.8	379.4	320.0	315.6	330.9
Gross energy (MJ kg^{-1})	16.49	16.73	16.99	17.71	16.23	16.08	16.15

[1]Vit-Min mix (Cargill Takarmany Zrt.) (quantity kg^{-1}): vitamin A – 1000000 IU; vitamin D_3 – 80000 IU; vitamin E – 5000 mg; vitamin K_3 – 334 mg; vitamin B_6 – 200 mg; vitamin C (ascorbic acid monophosphate) – 11300 mg; Ca – 114 g; P – 78 g; Na – 1 g; Fe – 670 mg; Zn – 1070 mg; Mn – 160 mg; Cu ($CuSO_4$*$5H_2O$) – 200 mg; Se – 20 mg; Lysine – 70 g; Methionine – 198 g

Table 2: Essential amino acid profile of the experimental diets (mg amino acid per g protein)

	FM (Control)	**SM_{30}**	**SM_{60}**	**SM_{100}**	**PAP_{30}**	**PAP_{60}**	**PAP_{100}**
Essential amino acids (EAA)							
Lysine	63.7	70.1	61.1	43.4	60.8	54.2	45.8
Methionine	19.4	16.3	18.5	14.0	18.9	25.0	20.8
Arginine	38.4	53.5	45.0	38.5	43.9	38.8	39.9
Histidine	51.3	40.6	36.9	36.4	56.1	65.3	50.9
Threonine	40.3	40.9	37.4	28.9	39.0	35.9	30.1
Valine	69.1	53.9	57.9	49.7	57.3	63.8	59.6
Leucine	77.5	77.3	89.1	74.4	67.3	76.9	70.2
Isoleucine	34.4	39.9	36.7	33.6	36.5	34.1	29.5
Phenylalanine	47.8	52.3	52.4	46.9	51.4	48.4	45.4
ΣEAA[a]	441.9	444.9	434.9	365.8	431.2	442.5	392.2

[a]ΣEAA, Σ essential amino acid

Table 3: PCR primer sequences, accession numbers, melting temperatures and calculated efficiency.

Gene	**Accession no.**	**Sequence of primer (5′ → 3′)**	**Efficiency**
Target gene			
Growth hormone	AY157496	F: GCTGCACACCTCCTACC R: GTGGAGCCAGAGAGTCG	99.6%
Insulin-like growth factor-I	X79077	F: CGTGGGGATGTCTAGCG R: CCCGGCACAAGGTATACG	106%
Growth hormone receptor	AY336104	F: TCCCTCTGTACTTCCGCC R: GGTGTCAGATACCCCACG	139.1%
GOT	AB905611	F: CTTTGCCAGTGGGGACA R: ACTCGCTTGGCCTCTTC	114%
GPT	AB911122	F: CTTGTGACCCGGACAAC R: CGCCCAGCTCAGCTAAT	96.2%
Reference gene			
Beta actin		F: GAGCACCCAGTCCTTCTTAC R:TGCCCATCTCCTGCTCAAAGT	102%

The accession number refers to the registered sequence used from Gene bank. F: forward, R: reverse.

Table 4: Proximate composition (g kg^{-1}, wet weight basis) in whole body of European catfish fed practical diets with different levels of replacement of FM by SM or PAP (mean ± SE; n=9).

Treatments	Moisture	Crude protein	Crude lipid	Ash
FM (Control)	729.3 ± 0.1^{c}	157.8 ± 1.5^{a}	53.4 ± 3.6^{b}	23.7 ± 0.4
SM_{30}	774.2 ± 1.5^{a}	143.9 ± 1.1^{a}	40.2 ± 1.7^{c}	20.4 ± 0.1
SM_{60}	782.7 ± 1.8^{a}	142.5 ± 5.1^{a}	32.9 ± 2.0^{c}	23.1 ± 0.4
SM_{100}	752.8 ± 1.7^{b}	137.6 ± 5.2^{b}	72.6 ± 9.1^{a}	19.6 ± 0.8
PAP_{30}	749.6 ± 0.6^{b}	141.7 ± 3.4^{a}	75.5 ± 0.3^{a}	20.6 ± 0.8
PAP_{60}	769.6 ± 0.6^{ab}	147.7 ± 3.2^{a}	55.8 ± 0.5^{b}	22.1 ± 0.3
PAP_{100}	764.3 ± 0.7^{ab}	137.0 ± 0.8^{b}	63.5 ± 0.9^{ab}	20.9 ± 0.2

Values within same column with different superscripts are statistically different at $P < 0.05$.

Table 5: Growth performance of European catfish fed practical diets with different levels of substitution of FM by SM or PAP (mean ± SE)

Treatments	Initial weight (g)	Final weight (g)	Weight gain (g)	Body mass gain (%)	Metabolic growth rate ($gkg^{0.8}day^{-1}$)	AFCR	APER
FM (Control)	351.03 ± 0.09	935.12 ± 7.10^{a}	584.08 ± 7.10^{a}	166.39 ± 2.02^{a}	6.50 ± 0.10^{a}	1.31 ± 0.04^{b}	1.78 ± 0.05^{a}
SM_{30}	350.88 ± 0.08	899.83 ± 10.88^{a}	548.93 ± 10.93^{a}	156.44 ± 3.14^{a}	5.98 ± 0.15^{a}	1.32 ± 0.10^{b}	1.77 ± 0.11^{a}
SM_{60}	351.31 ± 0.05	903.68 ± 18.61^{a}	552.37 ± 18.61^{a}	157.23 ± 8.14^{a}	6.04 ± 0.21^{a}	1.32 ± 0.11^{b}	1.75 ± 0.12^{a}
SM_{100}	351.24 ± 0.09	757.07 ± 9.65^{c}	405.83 ± 9.66^{c}	115.54 ± 2.75^{c}	4.03 ± 0.12^{c}	1.77 ± 0.07^{a}	1.32 ± 0.10^{b}
PAP_{30}	350.97 ± 0.11	882.64 ± 4.17^{a}	531.66 ± 4.17^{a}	151.48 ± 1.19^{a}	5.73 ± 0.06^{a}	1.36 ± 0.05^{b}	1.72 ± 0.06^{a}
PAP_{60}	350.82 ± 0.09	862.71 ± 15.26^{ab}	511.89 ± 15.69^{ab}	145.91 ± 7.29^{ab}	5.46 ± 0.16^{ab}	1.39 ± 0.09^{b}	1.61 ± 0.14^{a}
PAP_{100}	351.03 ± 0.13	749.37 ± 13.29^{c}	398.33 ± 13.06^{c}	113.47 ± 3.64^{c}	3.94 ± 0.16^{c}	1.86 ± 0.11^{a}	1.19 ± 0.08^{b}

Values within same column with different superscripts are statistically different at $P < 0.05$.

FCR: feed conversion ratio

PER: protein efficiency ratio

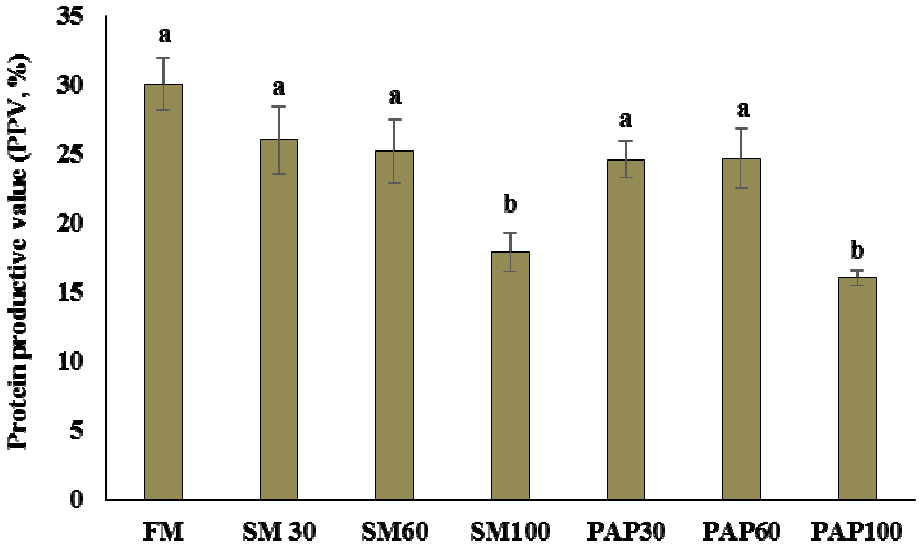
Protein productive value (PPV, %)
35
30
25
20
15
10
5
0
a
a
a
b
a
a
b
FM
SM 30
SM60
SM100
PAP30
PAP60
PAP100

Figure 1

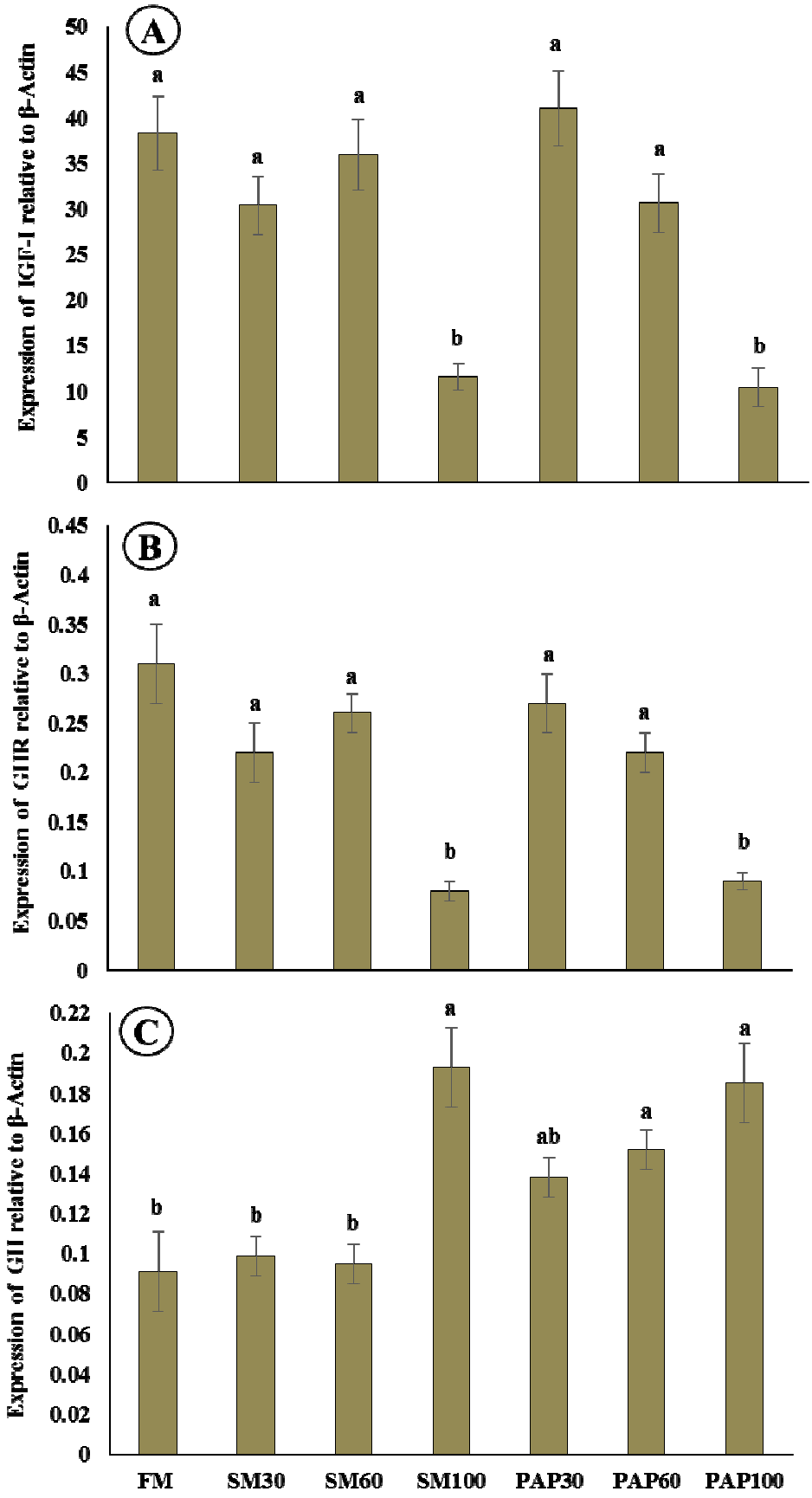
A
Expression of IGF-I relative to β-Actin
50
45
40
35
30
25
20
15
10
5
0
a
a
a
b
a
a
b
B
Expression of GHR relative to β-Actin
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
b
a
a
b
C
Expression of GH relative to β-Actin
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
b
b
b
a
ab
a
a
FM
SM30
SM60
SM100
PAP30
PAP60
PAP100

Figure 2

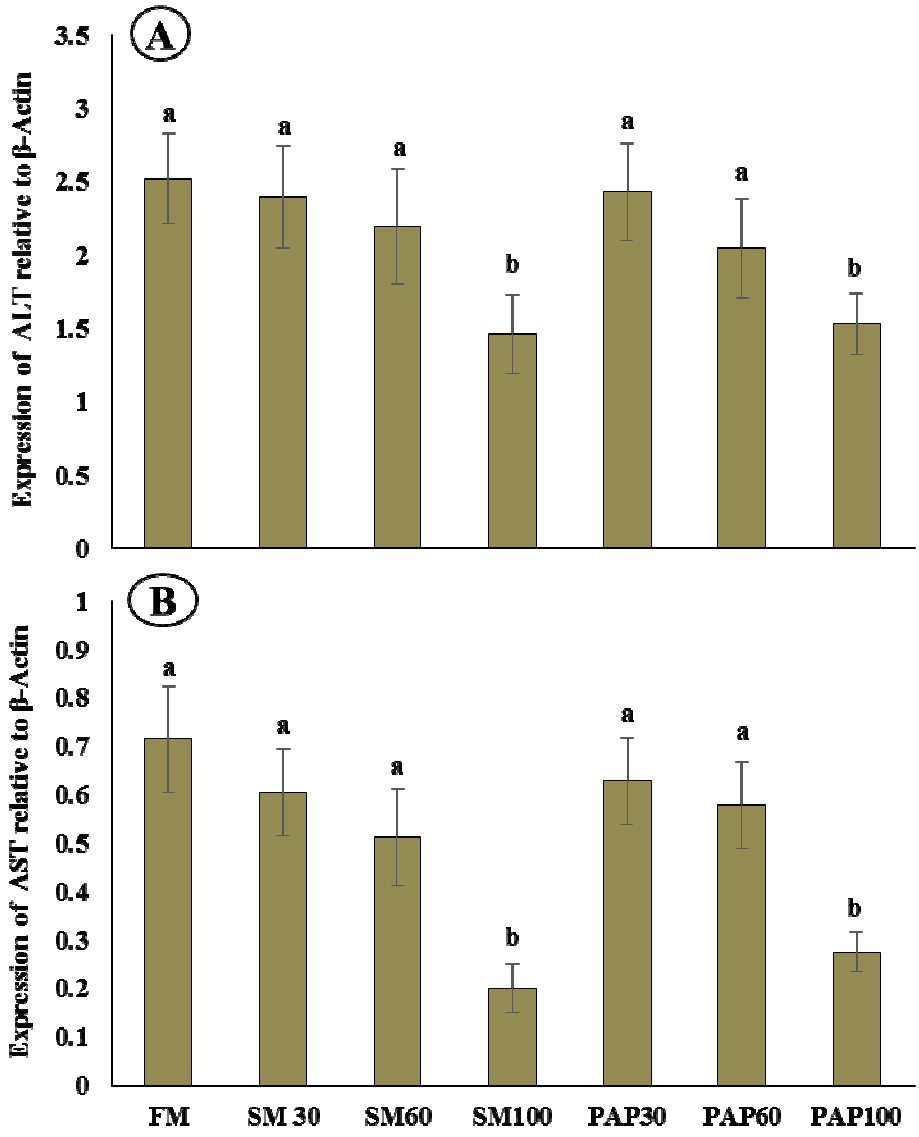
A
Expression of ALT relative to β-Actin
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
b
a
a
b
B
Expression of AST relative to β-Actin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
a
b
a
a
b
FM
SM 30
SM60
SM100
PAP30
PAP60
PAP100

Figure 3

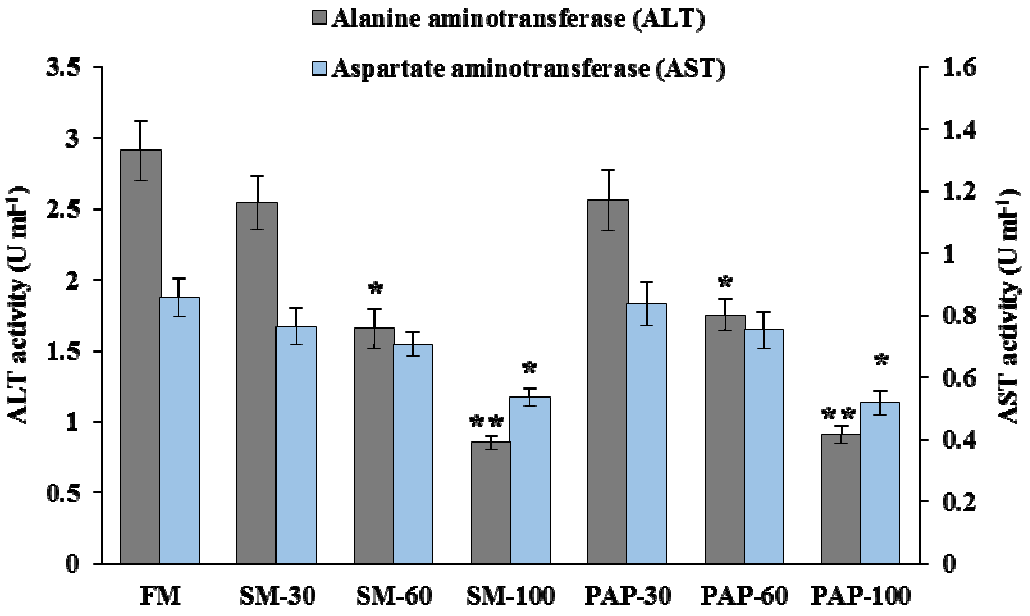
Alanine aminotransferase (ALT)
Aspartate aminotransferase (AST)
ALT activity (U ml-1)
AST activity (U ml-1)
3.5
3
2.5
2
1.5
1
0.5
0
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
FM
SM-30
SM-60
SM-100
PAP-30
PAP-60
PAP-100

Figure 4