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Evaluation of different protein sources in fingerling grey mullet *Mugil cephalus* practical diets

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Abstract. An eight-week experiment was carried out to evaluate the effects of different protein sources (fish and haemoglobin meal, soybean meal and torula yeast), in practical diets, on growth, body composition and gut morphology of fingerling grey mullet (*Mugil cephalus*). Weight gain (%), SGR, FCR, N retention, PER, PGR, FDR and carcass composition of fish were not significantly affected by the dietary protein source. Fish fed the torula yeast based diet showed reduction in growth performance. Histological examinations performed on the alimentary tract of the fish showed a normal structural pattern in the experimental groups, as fundamental histological and histochemical aspects were similar if compared to the control group. The lower growth performance observed in fish fed a torula yeast based diet may be tentatively correlated with the presence of some detrimental morpho-functional aspects in the gut of these fish if compared to fish fed the other diets. Further studies are necessary to confirm this hypothesis.

Introduction

Mullet are an important species in traditional, coastal aquaculture in Italy, with total production averaging 3000 Mt per year. Farming of omnivorous fish, such as mullet, in brackish water under semi-intensive conditions, should allow under-utilized trophic resources to be fully exploited for animal production (Ravagnan 1990, 1992). Practical diets used in Italy still rely on a significant inclusion of expensive and limited marine raw materials such as fish meal. In Italian semi-intensive farming systems, mullet are intensively reared during the first year and later released into open ponds (Ravagnan 1990, 1992). The size of the fish and the level of energy stores at the time of release are likely to exert significant influence on the success of later growing stages. Therefore, inclusion of a 'safety' level of marine raw materials in the diet is still conservatively regarded as desirable in order to allow rapid growth of juveniles and significant energy storage in body tissues. Unfortunately, there is lack of

information on nutrient requirements of mullet, and different species (*Mugil cephalus*, *Chelon labrosus*, *Liza aurata*, *Liza ramada*, *Liza saliens*) could have different nutritional requirements according to their feeding habits (Yashouv and Ben-Sachar 1967; Albertini-Berhaut and Vallet 1971; Papaparaskeva-Papoutoglu and Alexis 1986; Benetti and Fagundes Netto 1991; Argyropoulou et al. 1992; Ojaveer et al. 1996).

Comparatively high growth rates are reported for mullet grown under natural or polyculture conditions, where juveniles feed mainly on zooplankton and possibly on microbial communities present in growing ponds (Ravagnan 1992). According to a number of authors, such results would suggest a potential for the use of yeast cells in diets (Papaparaskeva-Papoutsoglou and Alexis 1986; Pisarevskaya and Aksenova, 1991; Cardona and Castello 1994; Bhuiyan et al. 1999; Torras et al. 2000). Soybean protein has been extensively used as an alternative protein source for a number of fish species, allowing to significantly reduce fish meal inclusion in artificial diets. When included at high percentages in the diet, pathological changes in the gut of fish have been described (Pongmaneerat and Watanabe 1993; Hardy 1996; Burrells et al. 1999). Both of these protein sources, therefore, show some potential for use in juvenile mullet diets, targeting high growth rate and levels of body energy stores together with a reduction in feeding costs.

The objective of this study is to evaluate the effects of the use of soybean meal and torula yeast as protein sources in practical diets, on growth, body composition and gut morphology in fingerling grey mullet (*Mugil cephalus*).

Materials and methods

Three different diets were formulated: a practical control diet (CD) based on a commercial formulation (A.S.A. s.r.l. – Agridea, San Martino Buon Albergo, VR, Italy), a soybean-based diet (SB) (25% of fish meal and haemoglobin meal protein were replaced with soybean protein), and a torula yeast-based diet (TY) (25% of fish meal and haemoglobin meal protein were replaced with torula yeast protein). Protein content was designed to be in accordance with Argyropoulou et al. (1992). Composition and proximate analysis of the diets are reported in Table 1. Wild fingerling grey mullet (*Mugil cephalus*) weighing approx 1.5 g were obtained from a commercial fish farm (Azienda Agricola Ravagnan, Valle Ca' Pisani, Porto Viro, RO, Italy), then transferred to the experimental station "Consorzio per l'ecologia e l'acquacoltura costiera" (Porto Viro, RO, Italy) and maintained as a group for 2 weeks. Fish were randomly divided into six 50-l flow-through aquaria (50 fish/aquarium) for a further 2-week adaptation period after sampling of 30 individuals for whole body proximate analysis. During this period the fish were fed a CD diet and closely monitored to quantify a daily ration which would be completely consumed within 30 min at the latest. Water temperature was kept at 26 ± 1.0 °C throughout the trial, while DO varied between 5.0 and 6.0 mg/l. Salinity was constant at 38‰.

Table 1. Composition (g/kg) and proximate analysis (% of dry matter) of the practical control diet (CD), the soybean based diet (SB), and the torula yeast based diet (TY).

Composition	Diets		
	CD	SB	TY
Fish meal	133.0	78.0	88.0
Spray-dried blood meal	70.0	67.0	59.0
Soybean meal	200.0	280.0	200.0
Torula yeast	–	–	80.0
Wheat flour	275.0	253.0	251.0
Wheat middlings	120.0	120.0	120.0
Wheat bran	100.0	100.0	100.0
Fish oil	89.0	89.0	89.0
Calcium carbonate	10.0	10.0	10.0
Vitamin/mineral mix[a]	2.0	2.0	2.0
Vitamin C[b]	1.0	1.0	1.0
Proximate analysis			
Crude protein[c]	31.2	30.0	30.2
Crude fat	11.9	11.3	11.5
NFE[d]	51.2	53.3	52.7
Ash	5.7	5.4	5.6

[a]Providing, per kg diet: vitamin A acetate 2500 UI, vitamin D_3 (colecalciferol) 1000 UI, vitamin E 120 UI, menadione (as sodium bisulfite) 8 mg, thiamin 8 mg, riboflavin 16 mg, pyridoxine (as pyridoxine hydrochloride) 8 mg, niacin 80 mg, folic acid 4 mg, vitamin B_{12} (cyanocobalamin) 0.02 mg, D-biotin 0.45 mg, calcium pantothenate 35 mg, Fe (as citrate) 16 mg, Mg (as sulfate) 15 mg, Zn (as $ZnSO_4$) 21 mg, Cu (as $CuSO_4$) 2.1 mg, I (as $KIO_3 \cdot C_2H_6N_2 \cdot 2HI$) 1.6 mg, Se (as sodium selenite) 0.07 mg.
[b]Polyphosphate.
[c]N $\times$6.25.
[d]Nitrogen-free extract (calculated by difference).

From the beginning of the trial, each experimental diet was hand-fed to duplicate groups of mullet, twice a day, as a paste, at a rate of 4% (as dry matter) of the biomass in each aquarium. Diet rations were chosen on the basis of the observations gathered during the 2 weeks. The fish were bulk weighed every 14 days following a 24-h fast and rations were adjusted accordingly. The total trial duration was 8 weeks.

At the end of the feeding trial the fish remaining in each aquarium were killed using tricaine methansulfonate following a 24-h fast. They were then bulk weighed, counted and analyzed for whole body composition. Carcass proximate analysis was expressed on a wet weight basis as recommended by Shearer (1994). All the chemical analyses were carried out in duplicate. Moisture, protein, lipid and ash content of the diets and fish were carried out following AOAC (1996).

Histological analysis of the alimentary canal was performed on five fish per dietary treatment (sampled fish, total $n = 15$). The gut was promptly excised from the abdominal cavity of each mullet. Subsequently, small samples of the oesophagus, stomach (both glandular and gizzard-like zones) and intestine

(distinguished by the pyloric caeca, long coiled mid tract, and short distal tract) were collected from each sampled fish (total number of examined gut samples, $6 \times 15 = 90$). The samples were fixed for 24 h at 4 °C in 4% paraformaldehyde in phosphate buffered saline (PBS), and paraffin-embedded after a treatment in a graded ethanol series. Dewaxed sections (4 μm-thick) were stained with haematoxylin–eosin (HE) in order to identify histological details, and Alcian blue (AB) pH 2.5 and periodic acid-Schiff (PAS) reactions to identify acidic and neutral glycoconjugates (Domeneghini et al. 1998, 1999). The latter histochemical reactions were performed in order to describe the epithelial mucous cells which secrete the glycoconjugates entering the gut mucous secretions. Observations were conducted using a BX51 Photomicroscope (Olympus, Italy).

Data were subjected to a one-way ANOVA to evaluate the effects of protein source. Student–Newman–Keuls was used as a *post hoc* test for comparison of the means among different samples, using the Prism 3.0 statistical package (GraphPad Software, San Diego, CA, USA).

Results

No significant differences in any of the growth and feed efficiency parameters were found, although fish fed a TY diet showed a tendency towards lower growth, feed efficiency and energy storage rate, the latter expressed as fat deposition rate FDR [(L_n final body lipid $-$ L_n initial body lipid) $\times 100$/number of feeding days] (Table 2). On the other hand, fish fed CD showed a tendency towards better protein efficiency as expressed by a protein efficiency ratio PER [(weight gain/apparent protein intake) $\times 100$] (Table 2).

Carcass composition at the end of the trial was not significantly different between fish fed different experimental diets (Table 3).

Some common structural features of the alimentary canal were evident in fish fed the experimental diets. The oesophagus (Figure 1) showed a tunica mucosa which was lined by both a stratified and simple columnar epithelium in fish fed CD and SB diets. The surface epithelium contained several mucous cells which were intensely AB pH 2.5- and PAS-reactive, thereby indicating that oesophageal mucous cells synthesized both acidic and neutral glycoconjugates. In TY diet-fed fish, the oesophageal mucous cells contained a larger quantity of acidic glycoconjugates, as the AB pH 2.5-reactivity was more intense than the PAS-reactivity. The glandular stomach mucosa (Figure 2) was lined by a simple columnar epithelium which was intensely PAS-reactive (and AB-unreactive) independently from the diet (indicating synthesis of neutral glycoconjugates only). The tubular gastric glands were histochemically unreactive in all cases.

Differences between fish fed different experimental diets were also evident. The mucosa of the gizzard-like zone of the stomach, as well as the inner koilin-like material, showed a strong PAS-reactivity in fish fed CD (Figure 2b) and SB diets. On the other hand, a lower PAS-reactivity was present in TY

Table 2. Growth and feed efficiency of grey mullet fed diets containing different protein sources.[a]

	CD	SB	TY	Pooled SEM[b]
Initial weight (g)	1.5	1.4	1.7	0.12
Final weight (g)	4.7	4.7	4.3	0.28
Gain (%)[c]	213.5	227.5	154.0	15.0
SGR[d]	2.04	2.11	1.67	0.08
Feed intake (g)	14.0	15.8	11.7	1.4
FCR[e]	4.3	4.9	4.5	0.22
N retention (%)[f]	13.7	11.5	13.1	0.92
PER[g]	0.70	0.60	0.61	0.03
PGR[h]	2.48	2.54	2.16	0.12
FDR[i]	1.48	1.58	1.02	0.26

[a]Means of two aquaria (50 fish each) after an eight-week period. Means were not significantly different ($p < 0.05$).
[b]Pooled standard error of the means.
[c]Percentage of initial weight.
[d]SGR: specific growth rate = (L_n final weight − L_n initial weight) × 100/number of feeding days.
[e]FCR: feed conversion ratio = apparent dry feed intake/weight gain.
[f]Nitrogen retention = (nitrogen deposition/apparent nitrogen intake) × 100.
[g]PER: protein efficiency ratio = (weight gain/apparent protein intake) × 100.
[h]PGR: protein growth rate = (L_n final body nitrogen − L_n initial body nitrogen) × 100/number of feeding days.
[i]FDR: fat deposition rate = (L_n final body lipid − L_n initial body lipid) × 100/number of feeding days.

Table 3. Proximate carcass composition (% wet weight) of grey mullet fed diets containing different protein sources.[a]

	CD	SB	TY	Pooled SEM[b]
Moisture	68.8	70.3	70.2	0.83
Crude protein	17.6	17.5	18.2	0.37
Crude lipid	10.6	10.7	10.1	1.10
Ash	1.9	1.3	1.3	0.30

[a]Means of pooled samples from two aquaria (50 fish each) after a 8-week period. Means were not significantly different ($p < 0.05$).
[b]Pooled standard error of the means.

diet-fed fish, their koilin-like material being less heavily stained (Figure 2c). The intestinal mucosa of fish fed a CD diet contained mucous cells, together with enterocytes. Mucous cells were frequent in the pyloric caeca (Figure 3a), in the middle intestine (Figure 3b) (where their frequency was very high at the basis of intestinal folds), and in the distal intestine (Figure 3c). These mucous cells were AB- and PAS-reactive, with a higher AB-reactivity towards the distal intestine (Figure 3c), indicating that the mucous cells of this tract were more rich in acidic glycoconjugates than the mucous cells of the pyloric caeca and mid intestine. SB diet-fed fish exhibited a lower number of epithelial mucous cells in the middle intestine and a higher number of more intensely stained mucous cells in the distal intestine compared to fish fed a CD diet

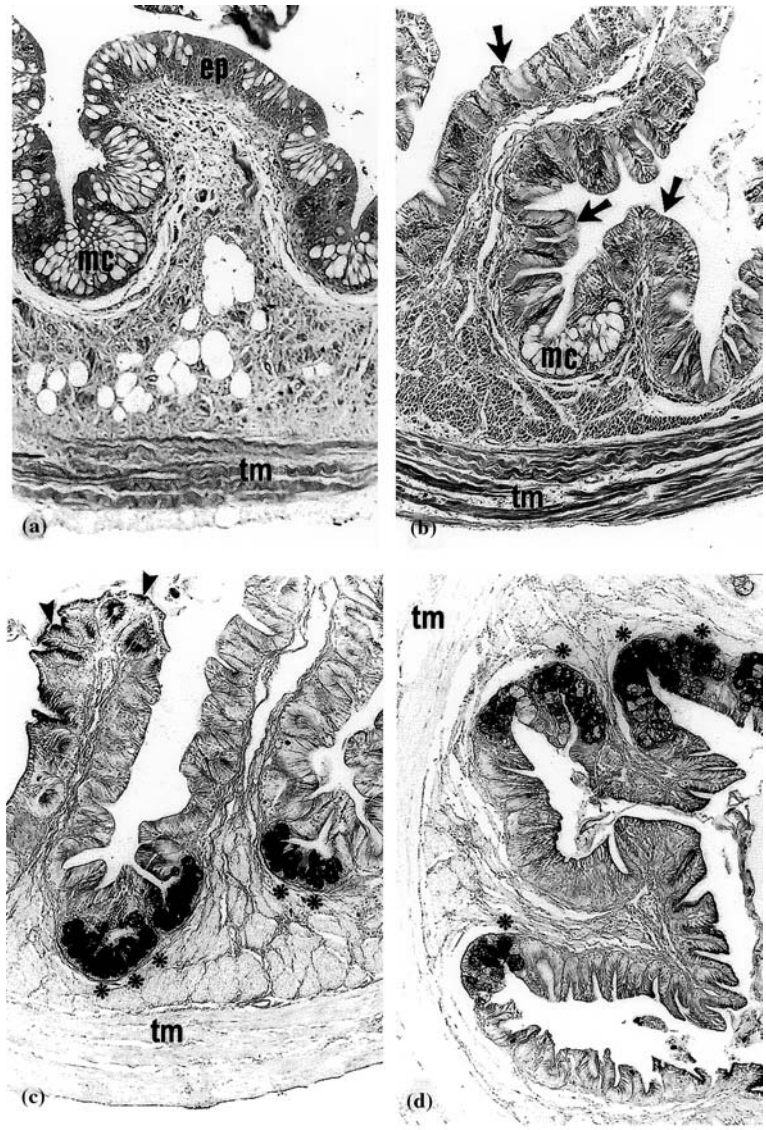

Figure 1. Histology of the oesophagus of *Mugil cephalus*. All figures, 150×: (a) CD, the surface epithelium (ep) is stratified and contains several grouped mucous cells (mc). HE stain. (b) SB diet, the surface epithelium (arrows) is simple columnar. A group of mucous cells (mc) is evident at the bottom of oesophageal folds. HE stain. (c) SB diet, the mucous cells are intensely reactive (asterisks). The apical zone of columnar surface epithelium is also positive (arrowheads). PAS reaction. (d) TY diet, the mucous cells are strongly reactive (asterisks). AB pH 2.5 reaction.

(Figures 4a, b). The number of mucous cells in the middle intestine of fish fed a TY diet was dramatically lower compared to fish fed the other diets (Figure 4c). Moreover, the mucosal folds of the mid intestinal tract of fish fed

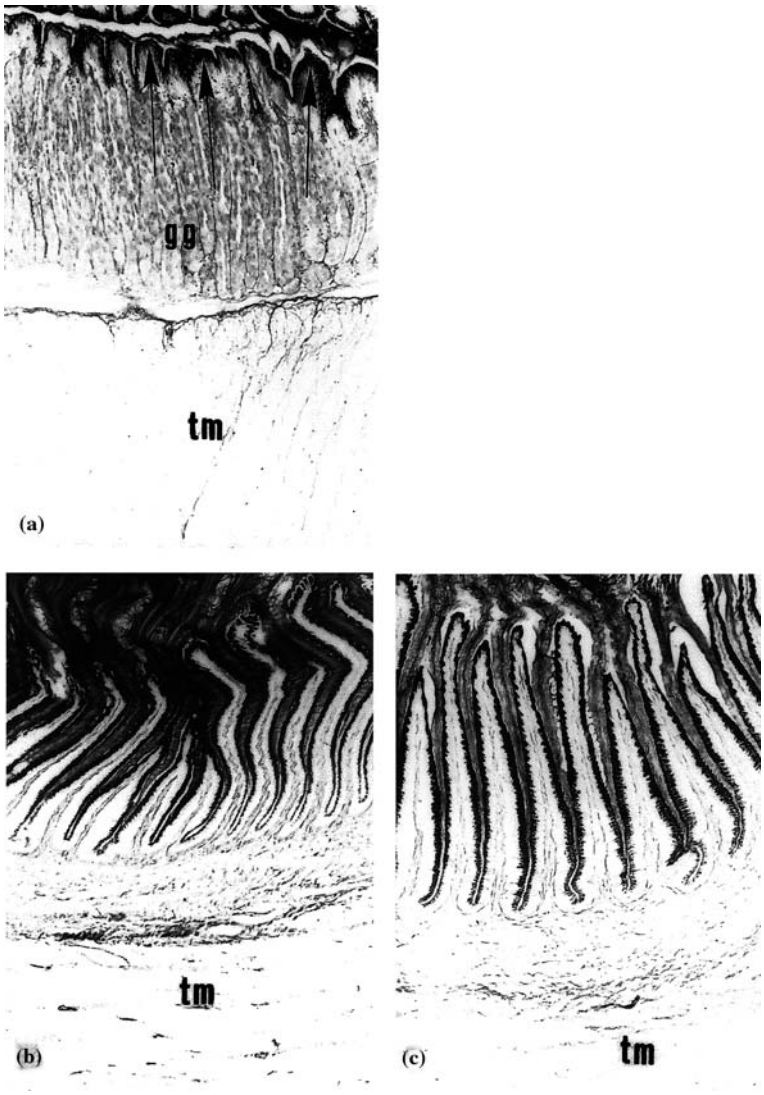

Figure 2. Histology of the stomach. All figures, PAS reaction, tm = tunica muscularis, 150×. (a) SB diet, the surface epithelium (arrows) of the glandular stomach is strongly positive. The gastric glands (gg) are histochemically unreactive. (b) CD diet, the koilin-like material (top of the picture) of the gizzard-like stomach is strongly positive. (c) TY diet, the koilin-like material is moderately reactive.

this latter diet showed signs of epithelial shedding (data not shown), and appeared irregular in shape, while the brush border of surface epithelium was evidently thicker (Figure 4c) than in fish fed either a CD or SB diet. The distal intestine of fish fed a TY diet showed a large number of mucous cells (Figure 4d).

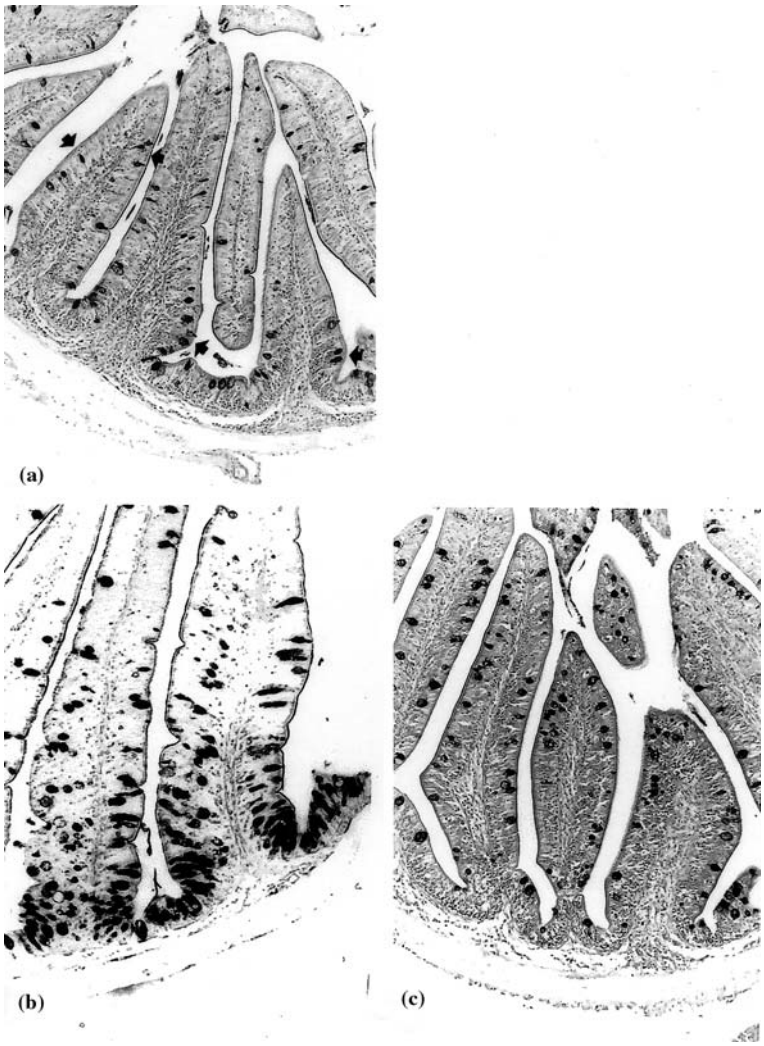

Figure 3. Histology of the intestine in CD diet-fed fish. (a) pyloric caeca, the intestinal folds contain frequent mucous cells (arrows). Combined AB pH 2.5/PAS reaction, 150×. (b) mid intestine, the intestinal folds show the presence of several mucous cells, specially frequent at their base. PAS reaction, 200×. (c) distal intestine, the mucous cells are frequent. Combined AB pH 2.5/ PAS reaction, 150×.

Discussion

Fish gained almost three times their initial weight at the end of the experiment, thereby indicating a good overall growth rate for all of the diets used. The good utilization of the experimental diets by the fish is also mirrored by the absence of significant differences in carcass composition at the end of the experiment.

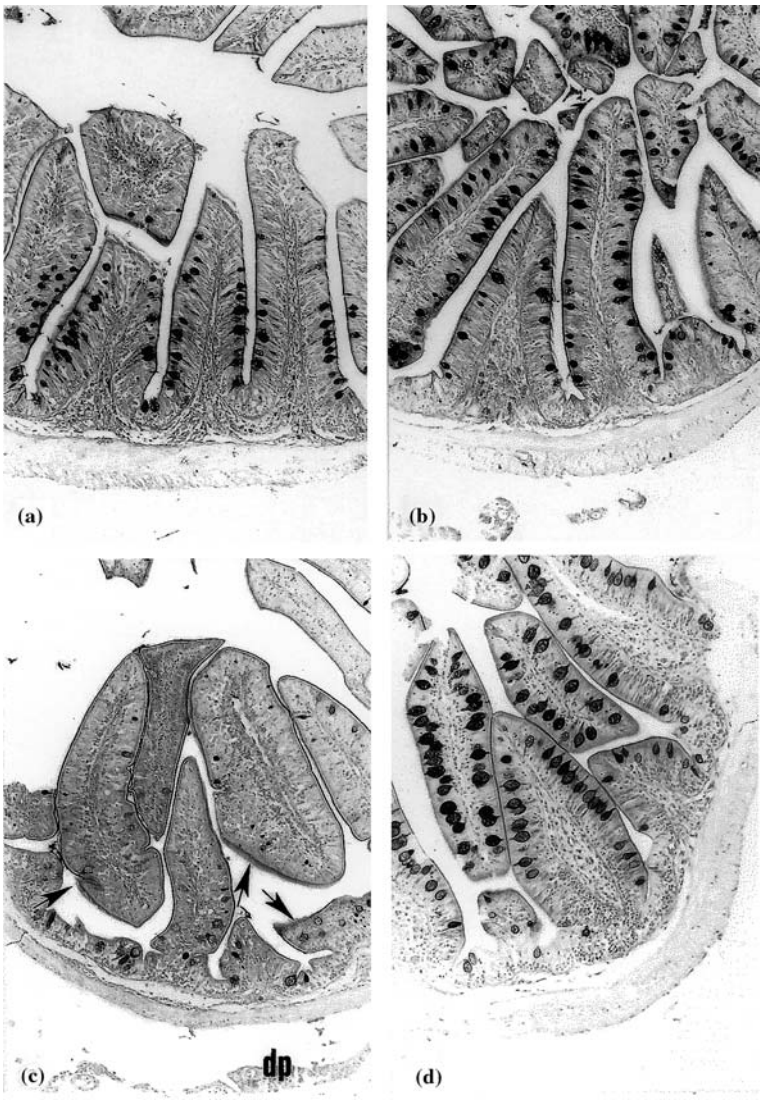

Figure 4. Histology of the intestine. All figures, combined AB pH 2.5/PAS reaction, 150×. (a) SB diet, within the mucosal folds of the mid intestine the mucous cells are less numerous in comparison with CD fish. (b) SB diet, the mucosal folds of the distal intestine contain more numerous mucous cells than the same tract of CD fish. (c) TY diet, the mucous cells of mid intestine are scarce and slightly reactive. The brush border appears very thick in some areas (arrows) (dp = diffuse pancreas). (d) TY diet, numerous mucous cells in the distal intestine.

Body lipid stores possibly reflected the fat content of the diets, which was identical in all the experimental diets tested, rather than the protein quality (Haard 1992; Rasmussen et al. 2000). FCRs were in line with values reported for grey mullet in the literature (Ravagnan 1992).

Grey mullet is reported to be planktivorous at the early stages of development (between 20 and 50 mm length), after which they change to a herbivorous feeding habit (Albertini-Berhaut 1973; Shehadeh et al. 1973; Tamaru et al. 1992). Fish used in the present study were well above this size, and were therefore in a development stage that could make good use of vegetable proteins. In addition, soybean may also be a source of valuable ω6 fatty acids which seems to be required for optimal growth by grey mullet (Argyropoulou et al. 1992; Tamaru et al. 1992). Growth rates were in fact not significantly different between SB and CD diet groups, even though the latter diet had a slightly higher protein to carbohydrate ratio, which is reported to promote higher growth rate in some mullet species such as thick-lipped grey mullet (*Chelon labrosus*) (Ojaveer et al. 1996). Fish fed a CD diet exhibited a tendency towards better protein utilization, when compared to fish fed on SB or TY diet. Different vegetable proteins seem to have different nutritional value for mullet and different mullet species may respond differently to the same vegetable protein. Davies et al. (1997) reported growth reduction in thick-lipped grey mullet (*Chelon labrosus*) fed diets in which 9 or 18% of fish meal was replaced by algae meal. On the other hand, practical growout diets based on vegetable protein sources are successfully used for mullet after the juvenile stage under commercial conditions (G.Wm. Kissil, personal communication). Different amino acid profiles of different vegetable proteins may at least partially explain such differences, given the fact that amino acid requirements of mullet are largely unknown.

The use of torula yeast resulted in a tendency towards a reduction in growth performance and feed utilization, although there was no significant differences between the TY diet and the other experimental diets. Again, the low statistical power of the experiment may partially have masked significant differences. The apparent lower performance of torula yeast as a protein source for mullet was quite unexpected, as practical yeast-based diets have been reported to be successfully used in Asia in mullet production (E. Grimaldi, personal communication). However, it should be noted that under the definition of yeasts we do include many different products obtained by different technologies (substrate used and further processing), which are likely to affect nutritional value of the product even within the same type of yeast (Hertrampf and Piedad-Pascual, 2000). As such, complete characterization of the yeast product used in different trials would be needed for proper comparison.

Histological observations resulted in agreement with growth and feed efficiency data for the grey mullet. None of the experimental diets induced severe damage to the gut, as demonstrated by the fact that the alimentary canals of fish fed either SB or TY diets were structurally similar to those of fish fed a CD diet. The oesophageal mucosa did not show differences between fish subjected to different dietary treatments, thereby indicating that the protein source of the diet did not affect the structure of this tract of the alimentary canal. The only difference between dietary treatments was a higher presence of acidic (Alcian blue pH 2.5-reactive) glycoconjugates in the oesophageal mucous cells of TY

diet-fed fish, most likely due to a local protective response towards this feed component. In many other fish species acidic components of the gut glycoconjugates are reputed to protect the gut mucosa from lumenal content (Domeneghini et al. 1998).

The surface epithelium of the gastric mucosa synthesized neutral (PAS-reactive) glycoconjugates irrespective of the diet, with a lower synthesis in the gizzard-like zone of the TY diet-fed fish. The koilin-like material of these fish also showed a lower density in comparison with fish fed either CD or SB diets.

The number of mucous cells in the mid intestinal tract was lower in fish fed SB and TY diets. These also showed irregularly shaped intestinal folds with thick brush border, as in starved fish (Hall and Bellwood 1995). This suggests that a TY diet was possibly less nutritive than the other two diets, resulting in detrimental effects mainly affecting the middle intestinal tract. This in turn may lower the growth of fish fed a TY diet. The number of mucous cells in the distal intestine was lower in the SB diet-fed fish and, to a lesser extent, in TY diet-fed fish compared with fish fed a CD diet. It is well known that the distal end of the fish alimentary canal can absorb and digest proteins via a pinocytotic pathway, and that gut glycoconjugates, above all the acidic glycoconjugates, are structures that regulate protein transfer from the lumen to the mucosa (Segner et al. 1994). The integrity of the distal intestine mucosa as well as the histochemical reactivity and the numbers of mucous cells can therefore indicate the secretory and absorptive efficiency of this intestinal tract.

Conclusions

On the basis of the results of the present study soybean meal may be regarded as a valuable protein source for juvenile grey mullet under intensive culture, as it can provide up to almost 50% of dietary protein without significant detrimental effects on growth, feed efficiency, body energy stores, and the structural pattern of the gut. Due to some detrimental effects of torula yeast-based diet upon gastric and intestinal tracts of the alimentary canals of the fish, no definitive conclusions can be drawn from the results of this experiment about the suitability of torula yeast as protein source in juvenile grey mullet diets. Moreover, the torula yeast diet showed a tendency towards a lower performance in fish if compared to the commercial control diet and to the soybean-based diet.

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