Feeding European sea bass with increasing dietary fibre levels: Impact on growth, blood biochemistry, gut histology, gut evacuation

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Abstract

Changing trends in fish feed formulation, with progressively higher inclusion levels of plant ingredients, are invariably introducing more fibre despite the fact that this component cannot be utilized by most fish. The effects of increasing insoluble dietary fibre level on growth, nutrient utilization, blood parameters and gut health in European sea bass (*Dicentrarchus labrax* L.) were studied over a period of 117 days. Moreover, investigation on digesta transit time through gastrointestinal evacuation pattern and digesta characteristics (moisture of digesta) were studied. Five iso-proteic diets were formulated to contain increasing insoluble fibre levels, neutral detergent fibre, NDF (7.2, 8.9, 11.5, 13.1 and 15.5%) derived by the inclusion of sunflower hulls and soybean hulls. No significant differences due to fibre inclusion levels were observed in final body weight, specific growth rate, feed intake, feed conversion rate, protein and lipid efficiency. No significant differences in serum total protein, glucose, triglycerides, alkaline phosphatase and inorganic phosphorous were found. All the histological sections showed normal intestinal architecture, and inflammatory and/or degenerative changes were not present in any histological section from all subjects examined. The investigation into gastrointestinal evacuation pattern revealed no significant differences between treatments, however higher dietary fibre levels seem to increase the time required to empty the stomach while the time required to empty 90% of the hindgut content was similar in all the treatments: around 46-47 h. No differences were found between diets in the moisture content of digesta along the digestive tracts. We can conclude that the different insoluble fibre levels tested in this trial have no effects on overall performances and feed efficiency in European sea bass. Results from blood biochemistry profile and

histology confirm good nutritional and health status of fish under all feeding treatments. The inclusion of fibre had no influence on digesta transit time. In formulation of feed for the on-growing of European sea bass insoluble fibre derived from sunflower hulls and soybean hulls can be included at a level of up to 15.5 %.

Keywords: European sea bass, fibre, growth, blood biochemistry, gut histology, gut evacuation

1.Introduction

Changing trends in fish feed formulation, with progressively higher inclusion levels of plant ingredients, are invariably introducing more fibre in aquafeeds. Cellulose and other fibrous carbohydrates are found in the structural components of plants and are indigestible to monogastric (simple-stomach) animals, such as fish. Fibre can be divided into soluble and insoluble (according to their extractability in a neutral buffer solution) that may have different antinutritive effects in endothermic animals (Dalsgaard et al., 2016). Soluble fibres in mammals, poultry and some fish species tend to increase digesta viscosity and retard absorption of nutrients (Krogdahl et al., 2005; Amirkolaie et al., 2005), while insoluble fibres appear to act largely as physical bulking agents (Bach Knudsen 2001) and tend to increase digesta transit, resulting in reduced absorption time (Krogdahl et al., 2005). Regarding insoluble dietary fibres, cellulose seems to be a relatively inert dietary component that may reduce the dry matter digestibility in Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua* L.), Nile tilapia (*Oreochromis niloticus* L.) and rainbow trout (*Oncorhynchus mykiss*) (Amirkolaie et al. 2005; Hansen & Storebakken 2007;

Kraugerud et al. 2007; Glencross 2009; Glencross et al. 2012; Lekva et al. 2010). Increasing dietary levels of cellulose have also negatively affected lipid digestibility in cod (Lekva et al. 2010) and energy and protein digestibility in rainbow trout (Glencross 2009; Glencross et al. 2012). Moreover, as reported by Altan and Korkut (2011), low dietary concentrations of dietary fibre (3–5 %) may have a beneficial effect on fish growth, but high dietary fibre (>8 %), on the contrary, may decrease dry matter digestibility of the diet and reduce the availability of other nutrients. Some discrepancies between values of maximum dietary fibre level have been reported for fish: less than 7 % (Altan and Korkut, 2011); less than 8 % (Eusebio et al., 2004); as low as possible and not exceeding 10 % (NRC, 2011). Information about the optimal percentage of fibre inclusion in practical feed formulation for the on-growing of the European sea bass (*Dicentrarchus labrax* L.) is scarce but Kousoulaki et al. (2015) reported that the fibre content in commercial feeds for this species can range between 1.5-3.2 %. The aim of the present study was to assess the effects of increasing dietary insoluble fibre level derived from sunflower hulls and soybean hulls on growth, nutrient utilisation, blood parameters, gut health, gastrointestinal evacuation pattern and digesta characteristics in European sea bass over 117 days, in order to identify the maximum amount of dietary fibre inclusion without negatively affecting zootechnical performance and health.

2. Materials and methods

2.1 Experimental diets

Five iso-proteic diets were formulated to contain increasing insoluble fibre levels (Neutral detergent fibre, NDF 7.2, 8.9, 11.5, 13.1 and 15.5%; F7.2, F8.9, F11.5, F13.1.

and F15.5, respectively). Diets were formulated with fishmeal and with a mixture of vegetable ingredients currently used in aquafeed (Parma et al., 2016; Bonvini et al., 2017). The fibre content was increased by increasing levels of a combination of sunflower hulls and soybean hulls to provide same proportion of fibre from each ingredient. Sunflower hulls and soybean hulls were chosen because they are one of the most used and available raw material for feed production. Lipid levels were slightly increased at increasing fibre content in order to compensate for the loss of available energy due to the higher fibre content. The diets were produced by extrusion process by Skretting Aquaculture Research Centre, Stavanger, Norway. The diameter of the pellet was 4 mm. Ingredients and proximate composition of the experimental diets are presented in Table 1.

2.2 Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. European sea bass juveniles were obtained from Maricoltura Mattinatese (Mattinata, Foggia, Italy). At the beginning of the trial, 60 fish (initial average weight: 69.4 ± 2.3 g) per tank were randomly distributed into fifteen 900 L square tanks with a conical base. Each diet was administered to triplicate groups, assigned in a completely random manner, over 117 days. Tanks were provided with natural seawater and connected to a closed recirculating system (overall water volume: 18 m^3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25mJ/cm^2: 32m^3 h^{-1}, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astralpool, Barcelona, Spain). The water exchange rate within each tank was 100% every hour, while the overall water

renewal amount in the system was 5 % daily. During the trial, the temperature was kept constant at 22 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant (8.0 ± 1.0 mg L^{-1}) by a liquid oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}), nitrite (≤ 0.2 mg L^{-1}) and salinity (25 g L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added on a daily basis to keep pH constant at 7.8–8.0. Feed was provided to apparent satiation by oversupplying the feed by automatic feeders by approximately 10% of the daily ration, twice a day for six days a week, while one meal was supplied on Sundays, as reported by Mongile et al. (2014). Each meal lasted 1 hour, after which the uneaten pellets of each tank were gathered, dried overnight at 105°C, and their weight was deducted for overall calculation.

2.3 Sampling

At the beginning and at the end of the experiment, all the fish in each tank were anaesthetised by 2-phenoxyethanol at 300 mg L^{-1} and individually weighed. Specific growth rate (SGR), voluntary feed intake (VFI) and feed conversion rate (FCR) were calculated. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and on a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER), gross protein efficiency (GPE) and gross lipid efficiency (GLE) were calculated. Furthermore, at the end of the trial, wet weight, viscera, liver and mesenteric fat weight were individually recorded for 10 fish per tank to determine viscerosomatic index (VSI), hepatosomatic index (HSI) and mesenteric fat

index (MFI). At the end of the trial, five fish per tank (15 fish per dietary treatment) were sampled for intestine histology examination. At the end of the trial, the fish left were kept in the same rearing and feeding conditions for three more days and then were sampled to perform blood analyses of serum total protein (TP), triglycerides (TRIG), glucose (GLU), alkaline phosphatase (ALP) and inorganic phosphorus (P). Blood from 4 fish per tank was collected 5 h postprandial from the caudal vein. Samples were then centrifuged (3000 g for 10 min at 4°C), serum aliquots were stored at 4°C and analysed during the same day according to Bonvini et al. (2017). All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.4 Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Voluntary Feed Intake (VFI) (g feed $fish^{-1}$) = g feed ingested / fish number. Feed conversion ratio (FCR) = feed intake / weight gain. Viscerosomatic index (VSI) (%) = 100 * (viscera weight / body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight / body weight). Mesenteric fat index (MFI) (%) = 100 * (mesenteric fat weight / body weight). Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Gross

lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish.

2.5 Histology

After euthanasia of 5 fish per tank, the gut was removed and the intestine was divided into two segments (midgut and hindgut). From each segment, a 5 mm-long piece was sectioned and fixed in 10% buffered formalin. Samples were processed for routine histology to obtain a transversal section, which was stained with haematoxylin and eosin (H&E). Sections were evaluated blind under a light microscope (Nikon Eclipse 80i, Nikon Corporation, Japan) to verify the preservation of the normal intestinal architecture. In particular, the histological investigation was focused on the main cell constituents of the mucosal layer (goblet cells, supranuclear absorption vacuoles in the enterocytes), capillary within the intestinal folds, lymphoplasmacytic cells within lamina propria (GALT-like tissue). Moreover, any possible degenerative and diet adaptive induced changes were taken into consideration. Photographs of the sections were made with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) equipped with a Nikon Digital Sight SD-MS camera and the Nikon software NIS-Elements. Adobe Photoshop CS3 Extended was used for the final photographic preparation without altering the original integrity of the pictures.

2.6 Gastrointestinal evacuation, time and digesta characteristics

Following the feeding trial, sampling for gastrointestinal evacuation pattern and digesta characteristics was conducted. We adopted the approach used in the studies by Adamidou et al. (2009) and Nikolopoulou et al. (2011). Fish were kept fasting for 72 h before being fed to make sure that the gastrointestinal tract was empty. Four fish per tank were sacrificed at 1, 4, 10, 16, 28 and 48 h after feeding a single meal to satiation. Each sampled fish was weighed, then the abdominal cavity was opened and the digestive tract carefully removed and separated into three parts: stomach, midgut and hindgut. Midgut was defined as the section from the pyloric sphincter to the ileorectal valve and hindgut from the ileorectal valve to the anus. Stomach and intestinal contents from the above sections were collected in pre-weighed dishes, weighed for each fish separately, dried and reweighed. The measured weights were used for the calculations described below. The geometric means of stomach and intestinal dry digesta content divided by the fish weight were regressed against time separately for each diet, in order to fit to a model for calculating gastric evacuation rate (GER) and gastric evacuation time (GET) for the stomach, midgut and hindgut filling time (MFT, HFT) and midgut and hindgut evacuation time (MET, HET) for midgut and hindgut.

2.7 Stomach evacuation pattern calculation

In the case of stomach, GER was calculated according to the formula described by Elliott (1972) and adapted by Nikolopoulou et al. (2011) The geometric mean of the stomach dry digesta content divided by the fish weight was regressed against time separately for each diet. GER is estimated as the value of r of the regression model:

$W_t = A\, e^{-rt}$

239 which is equivalent to the semi-logarithmic model:

240 $lnW_t = lnA - rt$

241 where Wt is the geometric mean weight of stomach dry matter digesta at time t, A is an
242 intercept estimated from the model regression and r is the rate of gastric evacuation. GET
243 50%, GET 75% and GET 90% is the evacuation time (expressed in hours) required to
244 empty 50%, 75% and 90% of the stomach. It was computed through:

245 $GET\ p\% = [\ln 100 - \ln(100-p)]/r$

246 where *p* is the digestible organic matter to be evacuated from the stomach.

247

248 *2.8 Midgut and Hindgut Filling/Evacuation pattern calculations*

249

250 In the case of midgut and hindgut the best model is the quadratic regression,
251 represented as a parabola. It was not possible to calculate GER, because the trend of
252 digesta in midgut and hindgut was not linear. Points from each curve were estimated to
253 determine the time of maximum midgut and hindgut filling and evacuation time. The
254 MFT and HFT, (maximum filling time) was calculated as the vertex of the parabola. In
255 these cases, the equations used were:

256 $W_t = A + r_1 t + r_2 t^2$

257 where r_1 is the coefficient of the linear part of the model and r_2 is the parameter of the
258 quadratic part of the model.

259 The vertex of the parabola:

260 $[-(r_1/2r_2), (4Ar_2 - r_1^2)/4r_2]$

261 and MFT and HFT:

262 $-(r_1/2r_2)$

MET and HET 50%, 75% and 90% is the evacuation time (expressed in hours) required to empty 50%, 75% and 90% of midgut and hindgut and was computed through: $(1-p)*[(4Ar_2 - r_1^2)/4r_2]=A+ r_1t+r_2t^2$.

MET and HET 50% , 75% and 90% is the solution with respect to t of the equation: $A-(1-p)*[(4Ar_2 - r_1^2)/4r_2]+ r_1t+r_2t^2=0$ by Newton Raphson optimization where p=0.50, 0.75, 0.90.

2.9 Digesta moisture determination

The weights of stomach and intestine contents, at each sampling time, were used to calculate the moisture of digesta as percentage of digesta weight in each gastrointestinal segment, according to Nikolopoulou et al. (2011).

2.10 Analytical methods

Diets and whole body were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450 °C. NDF, acid-detergent fibre (ADF) and lignin were determined according to Van Soest and Robertson, (1981). Cellulose content was determined based on the ADF–lignin. Hemicellulose content was determined based on NDF–ADF content. Gross energy was determined by a calorimetric bomb (Adiabatic

Calorimetric Bomb Parr 1261; PARR Instrument, IL, U.S.A.). Serum glucose, alkaline phosphatase, inorganic phosphorus, triglycerides and total protein were measured using colorimetric methods (Total protein OSR6232, Triglyceride OSR61118, Glucose OSR6121, Alkaline phosphatase OSR6004, Inorganic phosphorus OSR6122; Beckman Coulter, Brea, CA, USA) on an automated analyser (AU 400, Beckman Coulter, Brea CA, USA).

2.11 Statistical analysis

All data are presented as mean ± standard deviation (SD). Tank was used as the experimental unit for analyzing growth and performance, a pool of five sampled fish was considered the experimental unit for analyzing carcass composition, whereas individual fish was used for analyzing VSI, HSI, MFI, and blood parameters. Data of growth performance, VSI, HSI, MFI, nutritional indices and blood parameters were analysed by a one-way ANOVA and in case of significance ($P \leq 0.05$) Tukey's post hoc test was performed. The normality and/or homogeneity of variance assumptions were validated for all data preceding ANOVA. Moisture of digesta was analysed by two-way ANOVA using diet and time as independent factors. Statistical analysis was performed using the software R version 3.1.0 (Revolution analytics, Palo Alto, CA, USA). The differences among treatments were considered significant at $P \leq 0.05$.

3. Results

3.1 Growth and blood biochemistry

311

312 Growth performances are summarised in Table 2. No significant differences due to
313 fibre inclusion levels were observed after the 117 days in terms of growth performance
314 (final body weight and SGR), feed intake (VFI) and feed utilisation (FCR). Data on
315 biometric indices, body composition and nutritional indices are shown in Table 3. VSI
316 and HSI values significantly decreased with increasing fibre dietary levels, while no
317 significant differences were found in MFI. Regarding whole body protein content, fish
318 fed diet F7.2 showed higher value in comparison to those fed F13.1, while no significant
319 differences were found among the lipid, ash and moisture content of fish. No significant
320 differences among treatments were found in PER, GPE and GLE. Serum TP, GLU, TRIG,
321 ALP and P levels are shown in Table 4. No significant differences among treatments were
322 found. Data on P were significant for ANOVA (P = 0.0469) but no differences among
323 treatments were detected by the multiple comparison test.

324

325 *3.2 Histology*

326

327 All the histological sections showed normal intestinal architecture. No differences
328 were found in the mucosal layer (goblet cells, supranuclear absorption vacuoles in the
329 enterocytes), capillarity network within the intestinal folds, lymphoplasmacytic cells
330 within the *lamina propria*. Moreover, inflammatory and/or degenerative changes were
331 not present in the histological sections taken from the examined subjects (Fig. 1).

332

333 *3.3 Gut evacuation rate and time*

334

Data on GER, GET, MFT, HFT, MET and HET are presented in Table 5. Dry weight of the stomach content decreased with time as shown in Fig. 2. No significant differences were found among treatments for stomach, midgut and hindgut however diet F7.2 required 22 h to evacuate 90% of the initial digesta content, while diet F13.1 and F15.5 required 38 h and 35 h respectively. The data extracted for the midgut and for the hindgut evacuation fitted a quadratic model (Fig. 3 a, b). According to the equations, the time required to empty the whole gastrointestinal tract was estimated to be around 46-47 h for fish fed under all treatments. Data on moisture of digesta for each gastrointestinal tracts at 1, 4, 10, 16, 28, and 48 h post prandial are shown in Table 6. No differences were found between treatments, but moisture in the stomach changed with time increasing from 1 to 4 h postprandial and then remained constant until 28 h postprandial.

4. Discussion

To our knowledge, this is the first study designed to specifically evaluate the effects of graded levels of dietary insoluble fibre derived from sunflower hulls and soybean hulls on performance, health and digesta transit time of European sea bass using formulations at practical level. Crude fibre which is basically cellulose, insoluble hemicellulose and lignin, does not have any value in the nutrition of carnivorous fish and according to Altan and Korkut (2011) should be restricted to less than 7 % in fish diet. The results of our study demonstrated that neutral detergent fibre (hemicellulose, cellulose and lignin) up to level of 15.5 % had no effects on growth performance, feed intake and protein/lipid utilisation. The performances registered in this trial are similar to those found in other studies on European sea bass (Tulli et al., 2010; Guerreiro et al., 2015; Bonvini et al.,

359 2018). Hansen and Hemre (2013) reported that plant protein can contain soluble fibres
360 and antinutrients which can interfere with nutrient digestibility. Soluble fibres increase
361 the viscosity of gut content, which potentially can reduce digestible enzyme activities and
362 negatively affect nutrient digestion and absorption (Leenhouwers et al., 2006). Recently,
363 Adamidou et al. (2011) reported that an inclusion of fibre up to a level of 5 g 100 g^{-1} did
364 not affect growth performance or nutrient digestibility in sharpsnout seabream (*Diplodus*
365 *puntazzo*). Bou et al. (2014) have demonstrated that using diets with the inclusion of fibre
366 from cellulose up to 18 % at the expense of carbohydrate did not affect growth
367 performance in gilthead sea bream (*Sparus aurata*). Both sharpsnout and gilthead sea
368 bream belong to the family of Sparidae, which are considered omnivorous fish and more
369 predisposed to digest plant-based ingredients then carnivorous species, such as European
370 sea bass (Stone, 2003). In general, dietary fibre did not affect whole body composition
371 except for the protein content which was slightly lower in F13.1 compared to F7.2 but
372 without resulting in lower protein efficiency.
373 VSI and HSI values increased in diets with the lowest fibre inclusion. No evidence is
374 reported in the literature about any influence of fibre levels on somatic indices. One
375 possible explanation of the trend in HSI, found in our study, can be related to the decrease
376 in level of wheat in diets at the increasing of fibre. Previous studies on European sea bass
377 and other species reported that increasing NFE, wheat and starch level in the diets can
378 increase HSI values probably due to an enhancement in glycogen liver deposition
379 (Bonaldo et al., 2008, 2010; Bonvini et al., 2017; Bou et al., 2014).
380 Information on the nutritional status and health in fish species can be achieved through
381 the study of blood metabolites (Bonaldo et al., 2015; Bonvini et al., 2015). To our
382 knowledge, no studies have assessed blood parameters in response to fibre inclusion in

383 diets in marine species. Among the blood parameters, TP, TRIG, GLU, ALP and P seem
384 to have potential as predicative diagnostic tools for evaluation of European sea bass
385 nutritional status (Peres et al., 2014). In this trial, no differences were found among
386 treatments in all parameters analysed. TP level is usually very stable in well-nourished
387 animals but decreases under fasting conditions (Peres et al., 2014). In this study, plasma
388 TP averaged between 5.6 and 5.1 g dl^{-1} and these values are within the ranges reported
389 for European sea bass under good nutritional status (Peres et al., 2014). TRIG levels are
390 in agreement with previously reported values for this species (Bonvini et al., 2017), but
391 higher than those reported by Peres et al. (2014), a difference which can be related to the
392 different lipid level and formulation of the diet used, in comparison to our study. GLU
393 levels are in agreement with previously reported values for this species (Peres et al.,
394 2014). Adamidou et al. (2009), reported that GLU levels in European sea bass serum were
395 also affected by the type of carbohydrate ingested, with wheat starch showing the most
396 rapid increase and decrease in serum glucose compared to fish fed pea and chickpea diets,
397 while faba bean starch resulted in a delay in serum glucose peak and a lower range of
398 glucose values. ALP levels are higher in comparison to Peres et al. (2014). Alkaline
399 phosphatase is involved in the absorption and transport of lipid and carbohydrates from
400 the intestine, and intestinal activities are positively correlated with food ingestion and
401 growth rate (Lemieux et al., 1999). Finally, inorganic phosphorus levels are in agreement
402 with previously reported values for this species (Peres et al., 2014). Plasma electrolytes
403 (univalent and bivalent) are considered to be valuable indicators of secondary stress and
404 osmoregulation ability in fish (Roque et al., 2010). Plasma phosphorus has been identified
405 as a good indicator of stress (i.e. starvation and stocking density) and pathological
406 situations (Roque et al., 2010; Peres et al., 2014).

Besides the study of blood metabolites, histological analysis of the digestive system is considered a valid tool for evaluating the gut health status. No inflammatory and/or degenerative changes were recorded in any of the histological sections of the gut. The examination revealed no alteration in the mucosal layer (goblet cells and supranuclear absorption vacuoles in the enterocytes), no vascular changes within the intestinal folds and no lymphoplasmacytic infiltration within the *lamina propria*. Few references are available in literature on the effects of fibre on fish intestine. For example, Olsen et al. (2007) reported in Atlantic cod an increase in cellularity of the *lamina propria* and a modest goblet cell hypertrophy and hyperplasia especially in fish fed a diet with 100% of plant protein mixture. These results were ascribed to a significant amount of fibres in plant-based diets. In our study these morphological changes were not observed in any of the diet treatments confirming a good tolerance of European sea bass intestine to a fibre content up to 15.5%. Leigh et al. (2017) reported in zebrafish (*Danio rerio*) changes in the epithelial surface area and overall gut length as a response to fibre intake level but without any inflammatory or degenerative effects.

Dietary fibre can affect gastrointestinal transit time of feed (Zhou et al., 2004). The investigation on gastrointestinal evacuation rate/time in this study reported no significant differences between treatments, however higher fibre dietary levels led to an increased time required to empty the stomach. A marked difference in term of mean values was infact observed between diets F7.2, F13.1, F15.5 which required 22, 38 and 35 h, respectively to empty 90% of the stomach content. In addition slower evacuation was also observed in F13.1 and F15.5 compared to F7.2 to empty 50% and 75% of the stomach content. In the present trial if we consider that the inclusion of high dietary fibre level did not exert a negative effect on performance and feed utilization, a possible explanation

may be related to a reduced gastric evacuation at high fibre level which might have improved feed digestion and absorption.

On the other hand, the evacuation time for the hindgut was very similar in all the treatments, around 46-47 h to empty 90% of hindgut content. There is little published information regarding the effects of fibre on gastric evacuation in marine carnivorous species. When plant-based ingredients are included in the diet, gastric evacuation time is higher, increasing more with legumes than with cereals (Adamidou et al., 2009). Moreover, Venou et al. (2003) reported that differences in ingredient processing can also modify gastric evacuation time, which is higher for extruded cereals than for raw cereals in gilthead sea bream. In the same species, García-Meilán et al. (2014) found that differences in diet composition, such as high lipid levels or high starch content, may be involved in the differential transit rate and Fountoulaki et al. (2005) found that a low transit rate was related to a high lipid content. Since in the present work diets were designed at a practical level in order to identify the maximum amount of dietary fibre inclusion without negatively affecting zootechnical performance and health, we cannot totally exclude the effects that the differences in the lipid content could have exerted on the gastric evacuation pattern. Moisture of digesta in the gastrointestinal tract showed no differences between treatments. Moisture of stomach increased with time from 1 to 4 h after feeding and remained unchanged until 28 h post prandial. The water required for feed moisturisation originates from feed water, initial water absorption of pellets, drinking and stomach secretions (Kristiansen and Rankin, 2001). In this context, dietary fibre behaves within the gastrointestinal tract as a polymer matrix with variable physicochemical properties including water-holding capacity (Kay, 1982).

5. Conclusion

In conclusion, the different fibre levels derived from sunflower hulls and soybean hulls tested in this trial had no effects on overall performances and feed efficiency in European sea bass. Results from blood biochemistry profile and gut histology confirm a good nutritional and health status of fish under all feeding treatments. The inclusion of fibres derived from sunflower and soybean hulls had no influence also on digesta transit time and digesta moisture even if higher dietary fibre levels seem to increase the time required to empty the stomach. According to the results it seems feasible to include insoluble fibre up to 15.5% in feed formulation for the on-growing of European sea bass without negatively affecting zootechnical performance and health.

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Table 1. Ingredients and proximate composition of the experimental diets

	F7.2	F8.9	F11.5	F13.1	F15.5
Ingredient, % of the diet					
Sunflower Hulls	0.0	1.5	3.2	4.8	6.4
Soybean Hulls	0.0	2	4.2	6.4	8.6
Fish meal	20	20	20	20	20
Soybean concentrate	13	13	13	13	13
Wheat	28.4	23.3	17.9	12.4	6.9
Corn gluten	8	8	8	8	8
Wheat gluten	15.2	15.6	16.1	16.6	17
Fish oil	7.5	8	8.6	9.2	9.8
Rapeseed oil	7.5	8	8.6	9.2	9.8
Vit/Min premix[1]	0.5	0.5	0.5	0.5	0.5
Proximate composition, % on a wet weight basis					
Moisture	7.28	7.77	7.71	7.49	7.74
Protein	43.6	43.2	43.5	43.8	43.7
Lipid	22.5	24.3	24.8	25.4	27.1
Ash	4.42	4.47	4.53	4.63	4.73
NDF[2]	7.20	8.93	11.46	13.14	15.46
ADF[3]	2.79	4.45	5.99	7.11	9.26
Hemicellulose	4.41	4.48	5.48	6.03	6.20
Cellulose	2.33	3.37	4.73	5.73	7.07
Lignin	0.46	1.08	1.26	1.38	2.20
Gross energy MJ kg^{-1}	21.5	22.1	22.1	22.5	23.0

[1] Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011)
[2]Neutral detergent fibre
[3]Acid detergent fibre

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Table 2. Growth performance and feed intake of European sea bass fed experimental diets over 117 days.

	Experimental diets					
	F7.2	F8.9	F11.5	F13.1	F15.5	*P* value
IBW (g)	69.5 ± 2.1	68.1 ± 4.0	68.7 ± 1.1	69.7 ± 1.3	71.2 ± 2.5	0.5944
FBW (g)	215 ± 11.7	204 ± 14.9	215 ± 15.9	223 ± 6.3	219 ± 15.8	0.5369
SGR (% day^{-1})	0.97 ± 0.03	0.94 ± 0.02	0.97 ± 0.07	0.99 ± 0.01	0.96 ± 0.04	0.5136
VFI (g feed $fish^{-1}$)	237 ± 7.7	219 ± 7.7	230 ± 11.6	232 ± 8.1	237 ± 13.4	0.2759
FCR	1.63 ± 0.06	1.43 ± 0.24	1.58 ± 0.11	1.51 ± 0.03	1.60 ± 0.06	0.3773
Survival %	100 ± 0.0	100 ± 0.0	99.4 ± 1.0	100 ± 0.0	100 ± 0.0	0.4516

Data are given as the mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 * (ln FBW- ln IBW) / days.
VFI = Voluntary Feed Intake (g feed/fish) = g feed ingested / number of fish
FCR = Feed conversion rate = feed intake / weight gain.

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Table 3. Biometric indices, body composition and nutritional indices of European sea bass fed the experimental diets

	Experimental diets					
	F7.2	F8.9	F11.5	F13.1	F15.5	*P* value
Biometric indices						
VSI	$12.1^{b} \pm 1.5$	$11.4^{ab} \pm 1.8$	$11.3^{ab} \pm 2.5$	$11.1^{ab} \pm 1.4$	$10.5^{a} \pm 1.9$	0.0303
HSI	$3.2^{c} \pm 0.5$	$3.1^{c} \pm 0.5$	$2.7^{b} \pm 0.6$	$2.6^{ab} \pm 0.5$	$2.3^{a} \pm 0.5$	<0.0001
MFI	6.7 ± 1.5	6.1 ± 1.8	6.4 ± 2.5	6.3 ± 1.5	5.8 ± 1.6	0.4604
Whole body composition, %						
Protein	$17.1^{b} \pm 0.1$	$16.4^{ab} \pm 0.3$	$16.6^{ab} \pm 0.3$	$16.2^{a} \pm 0.5$	$16.3^{ab} \pm 0.3$	0.0465
Lipid	17.6 ± 1.3	18.6 ± 1.4	17.6 ± 2.3	17.8 ± 1.9	19.0 ± 1.1	0.7498
Ash	2.8 ± 0.21	2.7 ± 0.03	2.7 ± 0.07	2.7 ± 0.04	2.8 ± 0.15	0.7554
Moisture	61.6 ± 0.8	61.2 ± 0.4	60.9 ± 0.5	60.3 ± 0.5	60.0 ± 1.9	0.3485
Nutritional indices						
PER	1.41 ± 0.05	1.43 ± 0.08	1.46 ± 0.11	1.51 ± 0.03	1.43 ± 0.05	0.4484
GPE	24.4 ± 0.97	23.5 ± 1.78	24.2 ± 1.24	24.2 ± 0.62	23.2 ± 0.65	0.6601
GLE	56.4 ± 7.1	57.2 ± 8.2	52.9 ± 11.7	54.2 ± 6.3	52.7 ± 4.1	0.9343

Data are given as the mean (n=3; n=30 for VSI, HSI, MFI) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
MFI = Mesenteric fat index (%) = 100*(mesenteric fat weight/FBW).
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
GLE = Gross lipid efficiency = 100*[(%final body lipid*FBW) - (%initial body lipid*IBW)]/total lipid intake fish.

Table 4. Blood biochemistry of European sea bass fed the experimental diets

	Experimental diets					
	F7.2	F8.9	F11.5	F13.1	F15.5	*P* value
TP (g dL^{-1})	5.5 ± 0.40	5.6 ± 0.98	5.3 ± 0.70	5.5 ± 0.63	5.1 ± 1.16	0.5586
TRIG (mg dL^{-1})	1884 ± 818	2023 ± 600	1769 ± 788	1790 ± 715	1797 ± 670	0.9064
GLU (mg dL^{-1})	98 ± 55.1	90 ± 30.1	77 ± 17.3	89 ± 26.0	111 ± 33.8	0.2544
ALP (U L^{-1})	235 ± 58.9	252 ± 59.1	227 ± 56.7	207 ± 49.7	210 ± 51.9	0.2300
P (mg dL^{-1})	8.4 ± 0.85	9.4 ± 1.21	8.5 ± 0.72	8.4 ± 1.01	8.9 ± 1.00	0.0469

Each value is mean from 12 samples ± SD. Data on P were significant for ANOVA ($P = 0.0469$) but no differences among treatments were detected by the multiple comparison test.
TP = total protein
TRIG = triglycerides
GLU = glucose
ALP = alkaline phosphatase
P = inorganic phosphorus

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Table 5. Gastric evacuation rate (GER), gastric evacuation time (GET, expressed in hours), midgut filling and evacuation time (MFT, MET, h) and hindgut filling and evacuation time (HFT, HET, h) of European sea bass fed the experimental diets

	Experimental diets				
	F7.2	F8.9	F11.5	F13.1	F15.5
Stomach					
GER	0.1031	0.0945	0.0899	0.0607	0.0657
GET 50% (h)	6.72	7.33	7.71	11.43	10.55
GET 75% (h)	13.44	14.66	15.41	22.86	21.09
GET 90% (h)	22.32	24.36	25.60	37.96	35.04
R^2	0.82	0.92	0.92	0.98	0.77
Midgut					
MFT (h)	12.99	21.01	12.52	19.45	19.99
MET 50% (h)	35.63	39.50	35.39	38.65	38.88
MET 75% (h)	40.72	43.65	35.39	42.97	43.12
MET 90% (h)	43.36	45.81	43.20	45.21	45.33
R^2	0.33	0.55	0.33	0.43	0.53
Hindgut					
HFT (h)	25.71	23.28	23.71	25.66	25.34
HET 50% (h)	41.60	41.00	40.07	41.54	41.20
HET 75% (h)	45.71	44.99	43.74	45.11	44.76
HET 90% (h)	47.03	47.06	45.66	46.97	46.62
R^2	0.71	0.42	0.65	0.70	0.60

No significant differences among treatments and within each gastrointestinal tract were detected $P \leq 0.05$

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Table 6. Moisture, % of digesta obtained for each gastrointestinal tract of European sea bass from 1 to 48 hours (T1, T48) after feeding

Stomach	T1	T4	T10	T16	T28	T48	Time	Diet	Time*Diet
F7.2	63.0 ± 2.5[a]	73.9 ±4.3 [ab]	77.9±5.0[b]	82.9±2.6[b]	77.1±1.2[b]				
F8.9	58.9±11.5[a]	72.9±3.4[b]	77.2±4.4[b]	80.4±1.4[b]	80.0±4.9[b]				
F11.5	60.4±6.8[a]	69.3±9.3[b]	79.8±5.0[b]	80.5±2.9[b]	80.8±6.1[b]		0.0001	0.43	0.115
F13.1	59.4±4.2[a]	72.3±3.0[b]	75.9±4.1[b]	77.1±5.1[b]	78.8±8.8[b]				
F15.5	59.6±3.0[a]	74.6±5.2[b]	80.5±2.2[b]	78.4±2.2[b]	75.1±6.3[b]				
Midgut									
F7.2		86.1±2.4	83.0±7.1	85.4±1.6	87.5±1.5				
F8.9		87.0±2.0	85.3±1.1	85.9±2.2	85.3±2.8				
F11.5		85.4±1.6	85.4±2.6	83.2±6.8	85.5±4.7		0.608	0.647	0.769
F13.1		84.5±5.7	85.5±1.4	84.2±3.9	84.2±6.1				
F15.5		85.1±2.5	85.1±2.0	85.1±3.8	84.9±2.6				
Hindgut									
F7.2		80.2±9.1	80.4±10.1	82.5±2.3	79.5±5.9	80.4±6.7			
F8.9		80.8±3.4	81.9±1.9	78.8±10.4	81.6±5.3	77.8±5.1			
F11.5		77.1±8.7	80.8±7.2	80.3±8.6	79.0±7.2	71.3±7.3	0.140	0.087	0.936
F13.1		84.0±5.0	83.0±2.2	79.7±11.9	82.1±4.5	80.6±3.4			
F15.5		81.2±5.8	83.0±2.4	81.3±6.0	81.6±5.7	75.9±11.1			

Values within a row with different superscripts differ significantly at $P < 0.05$. Statistical significance by two-way ANOVA using time and diet as independent factors.

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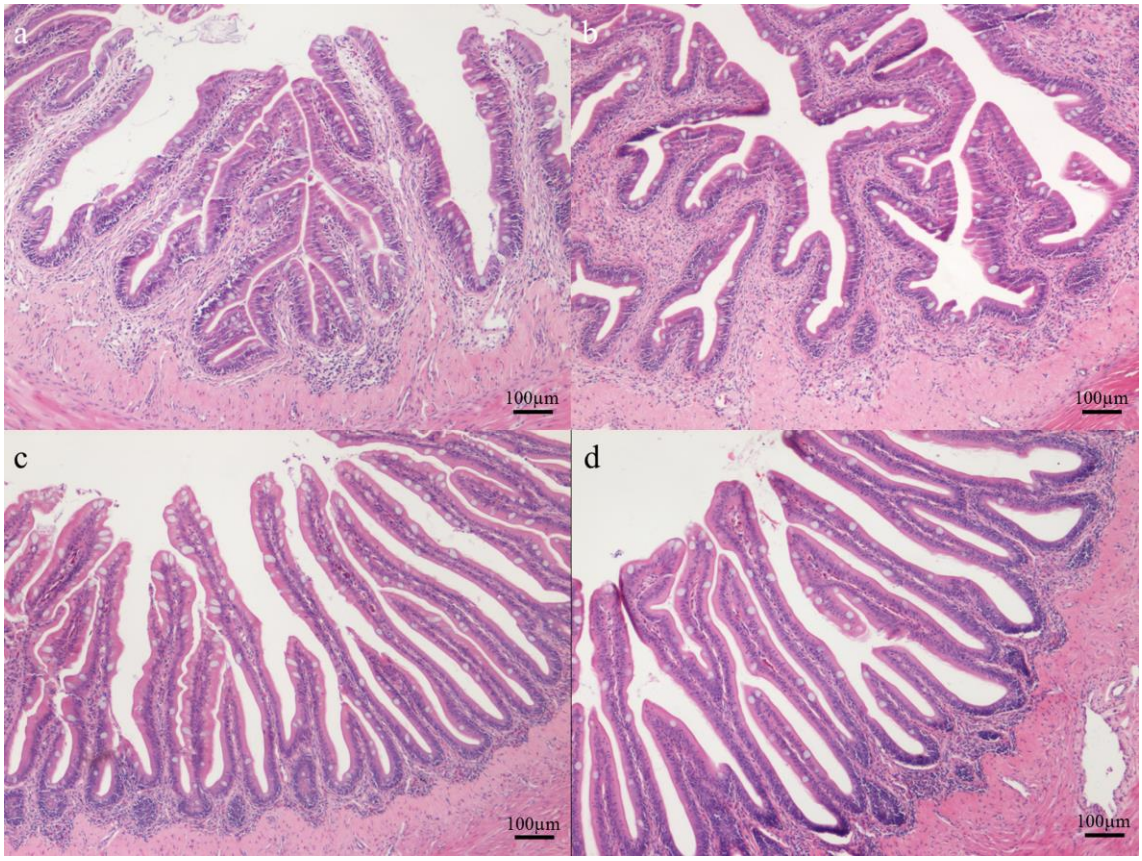
Figure captions

Figure 1. Histology of the midgut (a, b) and hindgut (c, d) of European sea bass fed diets F7.2 (a, c) and F15.5 (b, d). All the histological sections showed normal architecture of the mucosal layer (regular columnar epithelium with polarized and basally located nuclei), submucosal (loose connective tissue rich in capillary network) and muscular layer (H&E, bar=100 µm).

Figure 2. Exponential curves showing stomach evacuation of g digesta dry matter % body weight (DM %BW) over the 48 h sampling period of European sea bass fed the experimental diets. No significant differences among treatments were detected $P \leq 0.05$.

Figure 3. Midgut (a) and hindgut (b) quadratic curves describing the evacuation of g digesta dry matter % body weight (DM %BW) over the 48 h sampling period of European sea bass fed the experimental diets. No significant differences among treatments were detected $P \leq 0.05$.

668 Figure 1



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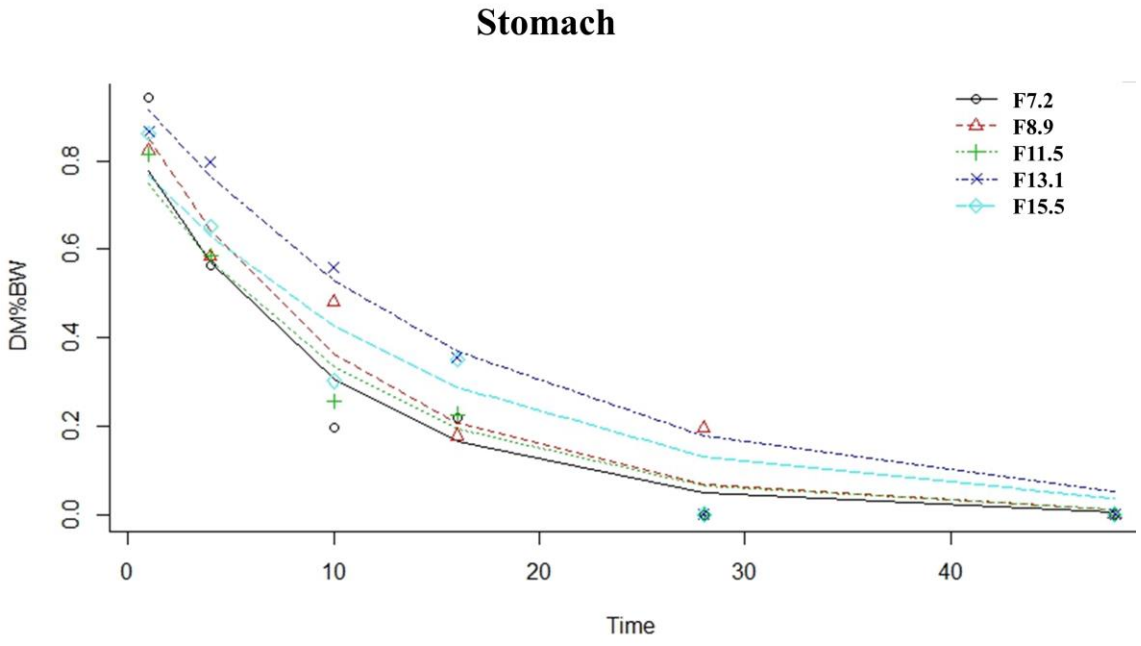

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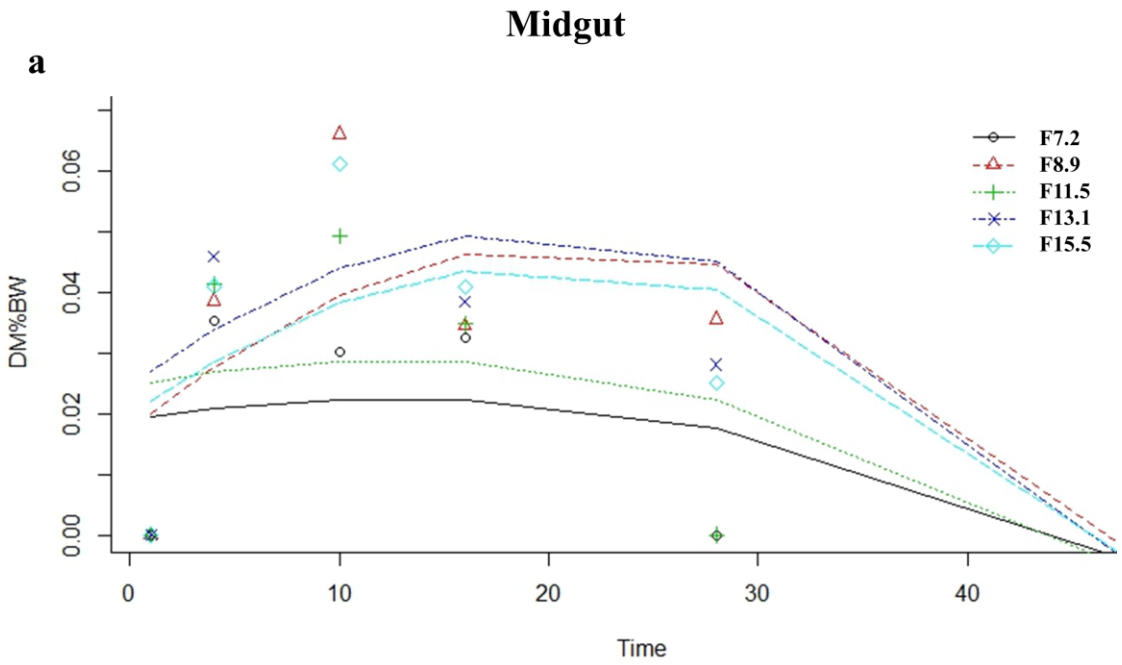
680 Figure 3



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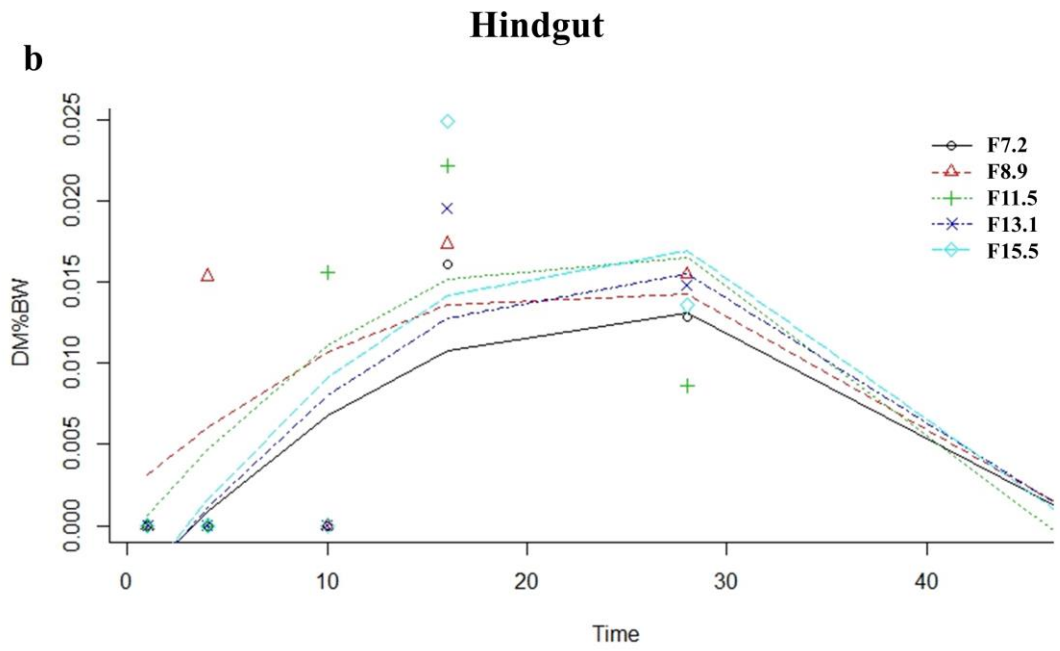
Hindgut
b
DM%BW
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0.010
0.015
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0.025
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10
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30
40
Time
F7.2
F8.9
F11.5
F13.1
F15.5

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