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Replacement of fish meal by a mixture of vegetal proteins and vegetal proteins combined with prebiotics in Gilt-head sea bream diets *(Sparus aurata)*

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization of the United Nations
NRC	National Research Council
FM	Fish meal
PP	Plant protein
PUFAs	Polyunsaturated Fatty Acids
HUFAs	Highly Unsaturated Fatty Acids
EPA	Eicosapentaenoic Acid
DHA	Docosahexaenoic acid
CP	Crude protein
CL	Crude lipid
AA	Amino acid
EE	Ether extract
DM	Dry matter
Phos.	Phosphorus
MET	Methionine
LIS	Lysine
ARG	Arginine
VIT	Vitamin
EMFN	Extracted matter free of nitrogen
MOS	Mannoligosaccharides
CF	Condition factor
VSI	Viscerosomatic index
HSI	Hepatosomatic index
MFI	Mesenteric fat index
HI	Headless index
MI	Headless index
NEPI	Non-edible parts index
SGR	Specific growth rate
DFR	Daily feeding rate
FCR	Feed conversion rate
PER	Protein efficiency ratio
SL	Serous layer
ML	Muscular layer
SML	Submucous layer
VL	Villi length
VT	Villi thickness
LP	Lamina propria
GC	Goblet cells
PFI	Peripancreatic fat infiltration
ND	Nuclear displacement
CV	Cytoplasmic vacuolization

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ABSTRACT:

Three diets have been formulated to evaluate the effect of prebiotec (dry hydrolyzed intestinal mucosa) by supplementation in plant protein diets of gilt-head sea bream of 273g (average initial weight) for 60 days. The diets manufactured to be isonitrogenous (47% crude Protein, CP) and isolipidic (17% crude Lipid, CL) , two diets of them with total replacement of fish meal by a mixture of vegetable proteins, with and without prebiotec 0% Fish meal FM0 & 0% fish meal plus prebiotec FM0 + P. The third diet as the unique protein source 100% fish meal FM100.

The growth, nutritional, biometric and histological parameters have been determined at the end of trial, fish reached weights 284, 303 and 407g in the FM0, FM0+P, and FM100 respectively. A statistical differences were observed between FM100 and other two (FM0 & FM0+P) diets in specific growth rate (SGR), Daily feeding rate (DFR), feed conversion ratio (FCR) and protein efficiency ratio (PER).

Biometric indices show significant differences between FM100 and other two (FM0 & FM0+P) in condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI) and meat index (MI) as they were significantly decreasing by increasing FM replacement percent, also there were significant differences between FM100 and FM0 in Headless index (HI). The index of the non-edible parts (NEPI) was significantly higher in (FM0 & FM100) than FM100 while there were no significant differences between all diets in mesenteric fat index (MFI).

Moreover, body composition was affected by the substitution with vegetable proteins, there was a decrease in water, protein and ash content and an increase of lipid and energy content from the start to the end of the trial. At the end of trial fat content was higher in the gilt-head sea bream fed FM100 diet followed by FM0+P and the least amount of fat was in gilt-head sea bream fed with feed FM0 when there were no significant differences in energy content in all groups.

Protein and ash content also presents significant differences between the gilt-head sea bream that fed with feed FM0 and the gilt-head sea bream that fed other diets (FM0+P, FM100) where they were higher in FM0.

The histological parameters of gut and liver not affected by different experimental diets. The morphological evaluation showed all fish in intermediate case except in FM0+P there was a severe fat accumulation within the hepatocytes. .

The supplementation of prebiotec (dry hydrolyzed intestinal mucosa) was not useful contrary to the results of Tortosa, (2004) may be result to the parasite infections during the trial. The results of the current study showed that the immune role of the prebiotec was not enough to face the parasite infection and it is not recommended to use as a supplementation in vegetable meals in case of presence of parasites risk.

Key words: sea bream, vegetable mixture, fish meal, plant protein, prebiotics.

RESUMEN:

Tres dietas han sido formuladas para evaluar el efecto de prebiótico (mucosa intestinal hidrolizada seca) en la suplementación de las dietas con proteínas vegetales de dorada de 273g (peso inicial) durante 60 días. Las dietas fueron fabricadas para ser isonitrogenadas (47% de proteína bruta, PB) y isolipidicas (17% grasa bruta, GB), dos dietas con total reemplazo de la harina de pescado por una mezcla de proteínas vegetales, con y sin prebiótico, 0% harina de pescado FM0 & 0% harina de pescado más prebiótico FM0 + P. La tercera dieta como la única fuente de proteína 100% harina de pescado FM100.

Los parámetros del crecimiento, nutricionales, biométricos e histológicos se han determinado al final del experimento, los peces alcanzaron pesos 284, 303 y 407 g en FM0, FM0+P y FM100 respectivamente. Se observaron diferencias estadísticas entre FM100 y otras dos dietas (FM0 & FM0+P) en la tasa de crecimiento (TCI), tasa de alimentación diaria (TAD), índice de conversión de alimento (ICA) y coeficiente de eficiencia proteica (CEP).

Los índices biométricos muestran diferencias significativas entre FM100 y otros dos (FM0 & FM0 + P) en el factor de condición (FC), índice de viscerosomatico (IVS), índice hepatosomático (IHS) y índice de carne (ICAR) disminuyendo significativamente con el creciente porcentaje de reemplazo de FM, también allí hubo diferencias significativas entre FM100 y FM0 en el índice descabezado (ID). El índice de las partes no comestibles (IPNC) fue significativamente mayor en (FM0 & FM100) de FM100 mientras que no hubo diferencias significativas entre todas las dietas de índice de grasa visceral (IGV).

Además, la composición corporal fue afectada por la sustitución con proteínas vegetales, hubo una disminución en el agua, contenido de proteína y ceniza y un aumento del contenido de lípidos y energía desde el principio hasta el final de la prueba. Al final del experimento la grasa fue mayor en la dorada alimentada FM100 seguido FM0 + P y la menor cantidad de grasa fue de la dorada alimentada con alimentación FM0 cuando hubo no diferencias significativas en el contenido de energía en todos los grupos.

El contenido de proteína y cenizas también presenta diferencias significativas entre la dorada que alimentada con piensos FM0 y la dorada que alimentada con otras dietas (FM0 + P, FM100) donde ellos fueron superiores en FM0.

Los parámetros histológicos de intestino y el hígado no estuvieron afectados por las diferentes dietas experimentales. La evaluación morfológica demostró todos los peces en caso intermedio excepto en FM0 + P que hubo una grave acumulación de grasa en los hepatocitos.

La suplementación de prebiótico (mucosa intestinal hidrolizada seca) no fue útil, contrariamente a los resultados de Tortosa, (2004) puede ser resultado de las infecciones de parásitos durante la prueba. Los resultados del actual estudio demostraron que la función inmune del prebiótico no fue suficiente para hacer frente a la infección del parásito.

Palabras clave: dorada, mezcla vegetal, harina de, proteínas vegetales, prebióticos.

Résumé :

Trois régimes alimentaires ont été formulés pour évaluer l'effet des probiotiques (muqueuse intestinale sécher hydrolysée) dans la supplémentation du régime alimentaire avec des protéines végétales de dorade de 273 g (poids initial) pendant 60 jours. Ces régimes ont été fabriqués pour être iso-azotés (47% de protéines brutes, PB) et isolipidicas (17% de matières grasses brutes, GB), deux régimes avec le remplacement total de la farine de poisson par un mélange de protéines végétales, avec et sans prébiotique 0% farine de poissons FM0 & 0 % farine de poisson plus prébiotique FM0 + P. Le troisième régime est la seule source de protéine 100% de farine de poisson FM100.

Les paramètres de croissance nutritionnels, biométriques et histologique ont été déterminés à la fin de l'expérience, les poids de poisson enregistrés sont 284, 303 et 407 g pour FM0, FM0+ P et FM100 respectivement. Des différences statistiques entre FM100 et les deux autres régimes (FM0 & FM0 + P) pour le taux de croissance (TCI), le taux d'alimentation journalière (TAD), l'indice de conversion alimentaire (ICA) et le coefficient d'efficacité protéique (CEP) ont été enregistrées.

Les indices biométriques montrent des différences significatives entre FM100 et les deux autres régimes (FM0 & FM0 + P) pour le facteur de condition (FC), l'indice viscérosomatique (SVI), l'indice hépatosomatique (IHS) et l'indice de la viande (ICAR) diminuant de façon significative avec l'augmentation du pourcentage de remplacement FM ; il y a aussi une différence significative entre FM100 et FM0 pour l'indice du corps sans tête (ID). L'indice des parties non comestibles (IPNC) était significativement plus élevé pour (FM0 & FM100) par rapport FM100 alors qu'il n'y avait pas de différences significatives entre tous régimes pour l'indice de graisse viscérale (TVA).

En outre, la composition corporelle a été affectée par la substitution de protéine végétale, il y avait une diminution de la teneur en eau, en protéines et en cendres et une augmentation des lipides et de l'énergie à partir du début jusqu'à la fin de l'essai. A la fin de notre expérimentation, la matière grasse était plus élevée dans la dorade alimentée par FM100 suivi pour celle alimentée avec FM0 + P et la faible matière grasses a été enregistrée pour la dorade alimentée avec FM0 cependant il n'y avait pas de différence significative dans le contenu de l'énergie pour tous les groupes.

Le contenu de protéines et des cendres présente aussi des différences significatives entre la dorade nourrie avec FM0 et celles alimentés par les deux autres régimes (FM0 + P, FM100) où ils étaient plus élevés dans FM0.

Les paramètres histologiques des intestins et du foie ne sont pas affectés par les différents régimes expérimentaux. L'évaluation morphologique a montré tous les poissons dans le cas intermédiaire, sauf pour le FM0 + P qu'il y avait une grave accumulation de graisse dans les hépatocytes.

La supplémentation prébiotique (muqueuse intestinale hydrolyse seche) n'a pas été utile, contrairement aux résultats de Tortosa, (2004) qui peut résulter d'infections parasitaires pendant l'essai. Les résultats de cette étude ont montré que la fonction immunitaire de la prébiotique ne suffisait pas de faire face à l'infection par le parasite.

Mots-clés: la dorade, mélange végétal, farine de poisson, protéines végétales, prébiotiques

1. INTRODUCTION:

Meat production by aquacultured fish is growing rapidly and fish species are increasingly interesting for human nutrition. Fish is considered a great source of perfect animal protein; the muscles of fish have almost 15 - 20% protein content of its wet weight which has a balanced composition of amino acids. The type of lipid varies according to many factors such as fish species, size and nutrition, furthermore high concentrations of essential fatty acids such as ω-3 and ω-6 series; which are very important to prevent many diseases. Fish is also a good source to cover human's requirements of minerals such as potassium, phosphorus and iron as well as many vitamins. So, fish can be recommended as a very healthy food (FAO 1997).

1.1 AQUACULTURE PRODUCTION.

Aquaculture has been growing more rapidly than any other animal food-producing sector in the world. Worldwide, more than 1 billion people rely on fish as an important source of animal protein, healthy lipids, and essential micronutrients (Asian Development Bank, 2005). Global fish production from capture and aquaculture supplied about 158 million tons in 2012, with 66.6 million tons from aquaculture (41.9 from inland and 24.7 million tons from marine aquaculture), providing an apparent per capita supply of 19.2 kg (FAO, 2014).

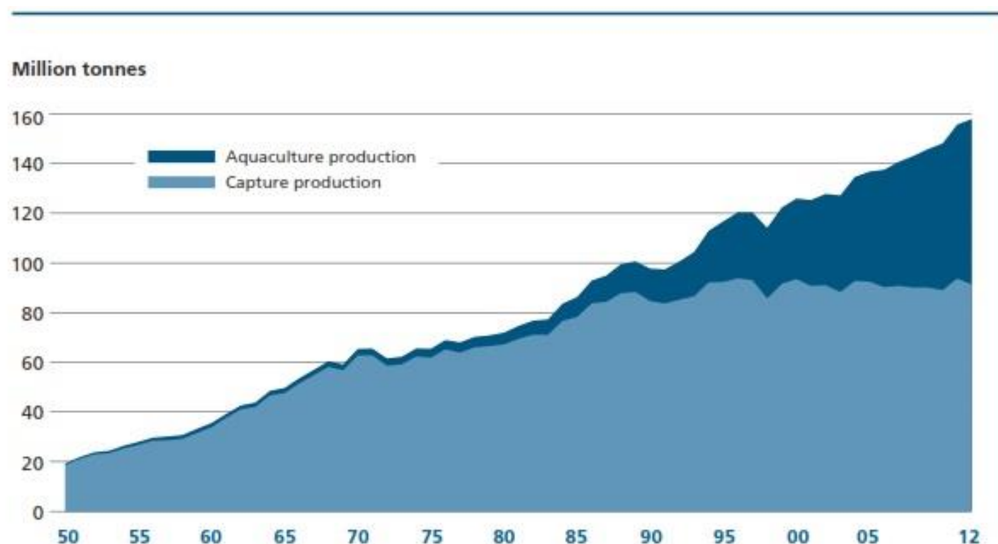

Figure 1: World capture fisheries and aquaculture production (FAO, 2014).

FAO aquaculture species increased to 567 species and species groups in 2012 (FAO, 2014). The composition of world aquaculture production was: finfish (66.3%, 44.151 million tons), molluscs (22.8%, 15.171 million tons), crustaceans (9.7%, 6.477 million tons), other species (1.3%, 0.865 million tons) (FAO, 2014).

1.1 .1 WORLD PRODUCTION OF GILT-HEAD SEA BREAM.

Gilt-head sea bream (Sparus aurata) total aquaculture production in Europe and the rest of the world in 2013 has reached 179.924 tons, according to statistics of APROMAR (Business Association of Marine Aquaculture Producers) and FEAP (Federation of European Aquaculture Producers). This value is higher than the 2012 with 11.42% (166.639 t).

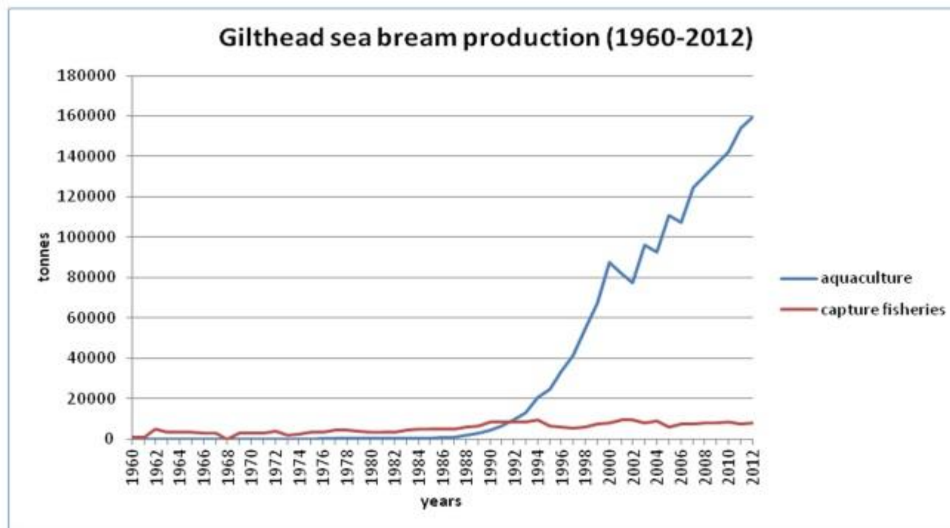

Figure 2: Global production of gilt-head sea bream 1950-2012 (FAO, 2014).

Greece is the major producer of gilt-head sea bream (Sparus aurata); Turkey is the second producer, while Spain is the third producer of the same fish followed by Egypt (FAO, 2014).

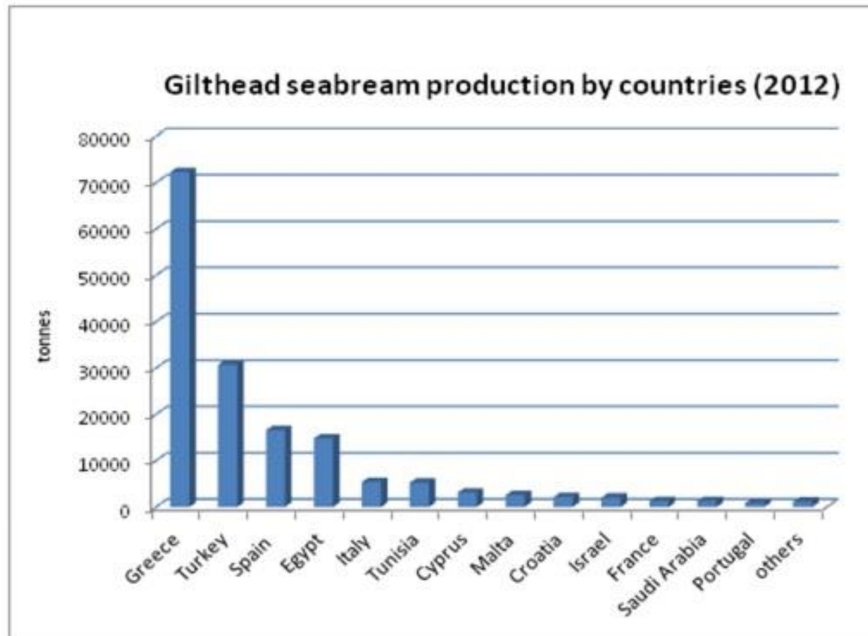

Figure 3. Global production of gilt-head sea bream from capture fisheries and aquaculture by country (FAO, 2014).

1.1.2 PRODUCTION OF GILT-HEAD SEA BREAM IN SPAIN.

The production of gilthead sea bream in Spain in 2013 has been 16.795 tons, with a decrease 13.6% less than 2012, while the maximum production of gilthead sea bream in Spain was in 2009 with 23.930 tons (APROMAR 2014).

In 2013, Valencia has led the gilt-head sea bream production from aquaculture in Spain with 6 974 t (42% of the total), followed by Murcia (3 730 t, 22%), Canarias (3 016 t, 18%), Andalucía (1 786 t, 11%) and Catalonia (1 292 t, 8%). (Figure 4)

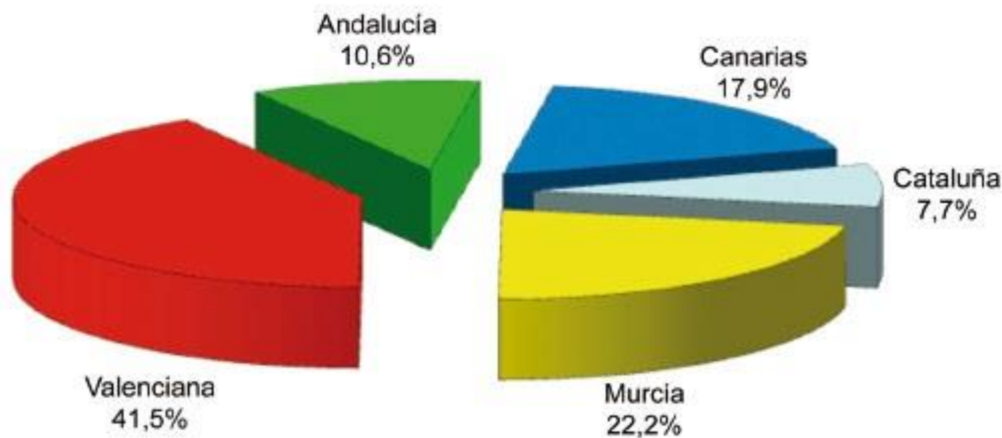

Figure 4: Distribution of gilt-head sea bream production in Spain in 2013 (APROMAR 2014).

1.2 OVERVIEW OF GILT-HEAD SEA BREAM.

1.2.1 TAXONOMIC DESCRIPTION.

The gilthead sea bream (Sparus aurata) is a carnivorous fish of the bream family Sparidae The gilt-head sea bream has many names depending on the country and regions in the same country but its name according to the Latin scientific classification is Sparus aurata (Linnaeus, 1758). Its taxonomic classification is the following (Froese et al., FishBase2010):

- Kingdom: Animalia
- Phylum: Chordata
- Class: Actinopterygii
- Order: Perciformes
- Family: Sparidae
- Genus: Sparus
- Species: *S. aurata*

1.2.2 ANATOMIC DESCRIPTION.

Gilt-head sea bream has a deep oval and compressed body. Its head has a regularly

curved profile and characterized by small eye, low mouth with small slightly oblique and thick lips as shown in figure 5.

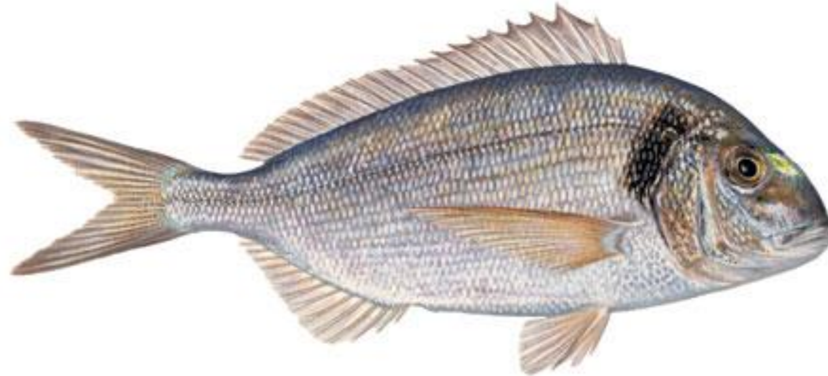

Figure 5: Model of gilt-head sea bream.

Gilt-head sea bream mouth is characteristic with mandible shorter than the maxilla. Each jaw has 4-6 canines and teeth have a molar shape distributed as 2-4 series in the higher jaw and 3-4 series in the lower jaw which has 1-2 teeth bigger. In addition, it has short gill rakers, the first branchial arch has 11-13 of them and there are 7-8 on the lower part. Its lateral line include almost 75-85 scales. Fish as well has single dorsal fin contain 13 soft and 11 hard rays, the anal fin presents 3 hard and 11-12 soft rays. The pectoral fins are long and pointed, while the ventral ones are shorter. The caudal fin has pointed lobes.

The fish is characterized by is silver-grey color and there is a big dark spot at the first part of the lateral line that covers also the upper part of the opercular bone. Also has gold and a black band between the eyes, the golden one is narrow in the middle part. The dorsal fin is blue-grey with a central black line while the color of caudal fin is grey-greenish white with black ends. (A. Moretti *et al.,* FAO 1999).

1.2.3 GEOGRAPHICAL DISTRIBUTION.

Gilt-head sea bream ranges from the Mediterranean and Black Sea to the eastern Atlantic Ocean from Senegal to the UK as shown with yellow parts in figure 6 (Kissil *et al.,* 2000).

Figure 6: Distribution of gilt-head sea bream (indicated by yellow color).

1.2.4 **HABITAT**.

Gilt-head sea bream is a coastal species and has a demersal behavior, inhabiting seagrass beds, rocky and sandy bottoms as well as in the surf zone commonly to depth of about 30 m, but the adults may occur to 150 m depth. It is a sedentary fish, solitary or forming small aggregations (A. Moretti et al., FAO 1999). It is an euryaline species and moves It is an euryaline species and moves in the beginning of spring towards safe coastal waters looking for plentiful food and moderate temperatures. In the end of autumn it returns again to the open sea for reproduction (L. Sola *et al.,* 2007).

1.2.5 BIOLOGY AND REPRODUCTION.

Gilt-head sea bream is a protandrous hermaphrodite: it is a functional male in the first two years and at over 30 cm in length becomes female. During the male phase, the bisexual gonad has functional testicular, with asynchronous spermatogenesis, and non functional ovarian areas (3, 4). Ovarian development is also asynchronous, and females are batch spawners that can lay

20 000-80 000 eggs per day for a period of up to 3 months. In the Mediterranean, they reproduce between October and December. The eggs are spherical and pelagic, with a diameter slightly lower than 1 mm and a single large oil droplet. The planktonic larval stage lasts about 50 days at 17-18° C (L. Sola *et al.,* 2007).

1.2.6 ENVIRONMENTAL CONDITIONS.

The optimal temperature range for the gilt-head sea bream is between 20-24 ° C, and the tolerable range of temperature is between 10 - 32 ° C. Gilt-head sea bream is euryhaline, it can tolerate a wide range of salinities, and it can survive in waters with only 5‰ salinity.
The minimum level of oxygen in the water for the gilt-head sea bream is around a 5 - 6 mg/l. With levels below 3 - 4 mg/l, the fish stop feeding; continuation of this stress may lead to death.

The optimal pH of gilt-head sea bream is approximately 8.3, since it is a marine fish. Alkalinity has no direct effect on fish. Should be given greater control in closed circuits, due to the continuous production of CO2 by nitrifying bacteria, so it is necessary to replace artificially the consumed alkalinity.

The main forms of nitrogen in the water are: N2, NH3, $NH4^{+}$, $NO2^{-}$, $NO3^{-}$, which are interrelated constitute the "nitrogen cycle". The presence of nitrogen compounds in aquaculture systems can become a limiting factor of production due to the toxic nature that have some of them.

The N2 is harmless for aquaculture up to levels of supersaturation in which may lead to "bubble disease". The solubility is low and is influenced by temperature and salinity.

Ammonia (NH3) may appear in small quantities in natural waters, but their presence is mainly due to be the main product of excretion of protein metabolism of aquatic organisms, although it can also come from the decomposition of organic matter (feces and uneaten feed).

Nitrites are also toxic, although its conversion into nitrates, which is a harmless product, is faster. The toxicity of nitrite is because it passes through the gills of fish, they pass into the blood and they oxidize ferrous iron from hemoglobin to ferric iron, creating a compound called ferrihemoglobin, so it is recommended to keep the levels below 0.01 mg/l.

Nitrates are practically harmless to fish because the toxicity values are very high, 1000 - 3000 mg/l $NO3^{-}$ (M. Pavlidis *et al.,* 2011).

1.3 NUTRITIONAL REQUIREMENTS.

The diet should be covering the following requirements:

a) **Proteins and amino acids:**

Requirements of protein I fish are very high, between 350 and 550 g / kg of feed, especially when compared with other farm animals, which are between 140-220 g / kg. These levels vary according to many factors, such as fish species, life stage, water temperature, feed intake, daily feed intake, frequency of feeding, protein quality, the composition of Amino acid and non-protein energy quality.

The importance of protein in the diets of fish is that it is the most expensive in the macronutrient composition of the feed, so a good understanding of the needs of each species can optimize the economic management of intensive farms.

In the carnivorous marine species, protein needs are around 45-55% of the diet. This is because their metabolic systems are evolutionarily adapted to digest proteins, which in turn implies a high excretion of ammonia, with an increase of energy expenditure.

The fact that the high protein feed leading to increased nitrogen excretion shows that a part of protein is not used for growth but for energy, and therefore may be partly replaced by energy nutrients, thereby reducing the high protein contents of the feed, which will make the feed cheaper.

Currently, intends to reduce the excretion of ammonia to the environment and increase retention of proteins, by controlling the relationship between protein energy and total energy in diets (Ep/Et). This relationship is very important because with a low Ep / Et, ingestion may stop before covering the minimum needs for growth, while a too high Ep / Et ratio means an undesirable waste of protein.

Moreover, the high price of the protein sources that have been used so far, such as fish

meal, make the search for maximum protein utilization is primary objective of investigations based on the nutrition of fish

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The maintenance requirement for digestible protein DP was independent of temperature and equaled 0.62g DP $kg^{-0.70}$. Efficiency of protein utilization for growth varied between 0.33 and 0.80 depending on the DP/DE ratio in the diet. The optimal protein utilization for protein deposition was estimated at K_{DPg} = 0.47. Using these values allows optimization of feeding for sea bream growth (Lupatsch & Kissil, 2003).

The studies conducted about protein requirements to be provided by a diet vary widely. A first approximation reports that dietary protein level of 40-50% provide good growth in *Sparus aurata* species. (Kaushik, 1997; Luquet y Sabaut, 1974; Sabaut y Luquet, 1973; Vergara *et al.,* 1996b). While a level of 55% in the diet is adequate to ensure good growth and good efficiency of protein utilization of gilt-head sea bream larvae (Vergara et al., 1996a).

Protein ingested by fish is hydrolyzed into amino acids which are absorbed in the intestinal tract and that are finally used for the synthesis of new proteins. Various experiments with different species of fish, have led to the conclusion that there are 10 essential amino acids. These are: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, and tryptophan. Although some species, as in the case of the gilt-head sea bream, may need other amino acids such as cysteine (Kaushik, 1997).

Although the quantitative determination of amino acid requirements is complex because there are interactions between them (Kaushik, 1989), It is known that an excess or deficiency in the ration causes an imbalance which reduces protein efficiency. Table 1 shows some of the amino acids required by gilt-head sea bream, according to recent studies.

Table 1. Requirements of some amino acids (% protein) in Gilt-head sea bream. (Tibaldi and Kaushik, 2002).

species	Arg	His	Ile	Leu	Lys	Me +Cys	Phe	Thr	Trp	Val
Gilt-head sea bream	5.4	1.7	2.6	4.5	5.0	2.4	2.9	2.8	0.6	3.0

Comparing the requirements of fish essential amino acids in general, expressed in % of the diet, with other farm animals you can observe that they are too high in fish, which requires the use of raw materials with high content in such amino acids, mainly fish and meat meal.

b) Energy.

The digestible energy (DE) content of the diet is the main factor determining voluntary consumption feeding in gilt-head sea bream and other fish species (Jobling and Wandsvik, 1983; Kentouri et al., 1995; Paspatis and Boujard,1996; Lupatsch et al., 2001).

The efficiency of utilization (above maintenance) of daily DE in sea bream reported to be constant at 0.50, regardless of energy intake. However, the efficiency of utilization of digestible protein (DP) varied between 0.33 to 0.60, with an optimum value of 0.47 (Lupatsch et al., 2001).

The relationship between DE intake and energy gain was linear, constant at KDEg= 0.67 and independent of feed intake and temperature. The daily requirement of DE for maintenance was dependent on temperature and determined as (16.6kJ x $exp^{0.055T}$)/BW in $kg^{0.82}$ (Lupatsch & Kissil, 2003).

Table 2 shows energy and protein requirements for gilt-head sea bream, according to

the growth potential for a specific weight and water temperature, and these are presented as a practical feeding calculated by Lupatsch et al. (2001).

Table 2. Recommended dietary energy and protein supply for growing Sparus aurata for different body weights (Lupatsch et al., 2001).

Body weight (g per fish)	10	100		250	
Weight gain (g per fish day^{-1})*	0.2	1.00		1.82	
DE_m (kJ per fish day^{-1})†	1.2	8.25		17.66	
DE_g (kJ per fish day^{-1})‡	3.3	17.36		35.19	
DE_{m+g} (kJ per fish day^{-1})§	4.5	25.61		52.85	
DP_m (g per fish day^{-1})‖	0.034	0.172		0.326	
DP_g (g per fish day^{-1})¶	0.096	0.398		0.694	
DP_{m+g} (g per fish day^{-1})§	0.130	0.570		1.019	
Food formulation at two DE levels					
DE level of diet (MJ kg^{-1})	16	16	20	16	20
Diet intake (g per fish day^{-1})	0.284	1.60	1.28	3.30	2.69
DP content (g kg^{-1})	455	345	432	309	387
FCR	1.14	1.60	1.28	1.80	1.44
DP : DE (g MJ^{-1})	28.5	21.6	21.6	19.3	19.3

* Predicted growth for Sparus aurata.
† Digestible energy required for maintenance = 55.8 kJ $kg^{-0.83}$ day^{-1} (Lupatsch et al., 1998).
‡ Digestible energy required for growth using energy efficiency of 0.50.
§ Digestible protein required for maintenance and growth.
‖ Digestible protein required for maintenance = 0.86 g BW $kg^{-0.70}$ day^{-1} (Lupatsch et al., 1998).
¶ Digestible protein required for growth using protein efficiency of 0.47. FCR, feed conversion ratio.

c) Vitamins and minerals.

The vitamins have be satisfied mainly in gilt-head sea bream diet since minerals in formulated diets are generally inexpensive. Absence of the B vitamins, including thiamine, riboflavin, pyridoxine, niacin and pantothenic acid lead to many pathologies in gilt-head sea-bream juveniles (Morris *et al*., 1995).

Covering the requirement of vitamins is very important for enhancing immune responses,

disease and stressing tolerance in gilt-head sea bream as shown in Table 3.

The mineral and vitamins premix should be supply the following requirements: minerals (g kg^{-1} of diet) and vitamins (IU or mg kg^{-1} of diet): CuSO4_5H2O, 2.0 g; FeSO4_7H2O, 25 g; ZnSO4_7H2O, 22 g; MnSO4_4H2O, 7 g; Na2SeO3, 0.04 g; KI, 0.026 g; CoCl2_6H2O, 0.1 g; Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4,500 mg; Vitamin K3, 220 mg; Vitamin B1, 320 mg; Vitamin B2, 1,090 mg; Vitamin B5, 2,000 mg; Vitamin B6, 500 mg; Vitamin B12, 1.6 mg; Vitamin C, 5,000 mg; Pantothenate, 1,000 mg; Folic acid, 165 mg; Choline, 60,000 mg. El- Husseiny et al. (2013).

Table 3. Reported levels of some vitamins and their effects on Gilt-head sea bream (H. Nakagawa *et al.,* 2007)

Vitamin	Dietary content (mg/kg diet)	Effective trial duration (days)	Improved parameters
Vitamin A	150 and 300 (retinol acetate)	7 or 14	Respiratory burst
	300	14 or 28	Leucocyte myeloperoxidase
	50 or 150	28 or 42	Leucocyte myeloperoxidase
Vitamin C	3000	14	Phagocytic activity
		42	Haemolytic complement activity
		56	Respiratory burst
Vitamin E	1200	30 to 45	Haemolytic activity, phagocytosis
	600	28	Cytotoxic activity of leucocytes
	1800	14	Cytotoxic activity of leucocytes
Combined vitamins C and E	3000 and 1200	30	Respiratory burst of phagocytes
		14	Blood glucose level after physical disturbance, crowding with anaesthesia, air exposure

The evaluation of mineral requirements in fish is complicated because fish may absorb some minerals both from the water and from the food (N.R.C. 1993). Minerals and trace elements requirements are shown to be limited to that for phosphorus. Pimentel-Rodrigues and Oliva-Teles (2001) estimated the requirements of phosphorus in gilthead seabream juveniles around 0.75 percent of the diet while Güthler (2005), determined the requirements of phosphorus to be about 0.65 percent of the diet. The mineral premix is usually used in formulation of diets.

1.4 FISH MEAL REPLACEMENT IN GILT-HEAD SEA BREAM DIET

Fish meal is usually the main source of protein in the diet of carnivorous fish, as it has a high protein content and has a balanced amino acid profile that resembles fish muscle. Furthermore, it is very digestible, is also very digestible and is a good source of essential fatty acids and minerals (Sargent and Tacon, 1999).

An increase in global demand for fishmeal due to increased aquaculture production, which is associated with stabilization of production of fishmeal, has led to decreased availability and increased prices for this commodity . Therefore, for the sustainable development of production of carnivorous fish is important to reduce dependence on fishmeal in feed formulation.

The protein content of the fish meal is between 60-75% and, as an animal protein source has a high proportion of the essential amino acids in a highly digestible form , mainly: methionine, cysteine, lysine, threonine and tryptophan as shown in table 4 (NRC 1983; M. L. WINDSOR, FAO 2011).

This raw material, rich in polyunsaturated fatty acids (PUFAs and HUFAs), especially in Eicosapentaenoic Acid (EPA) and Docosahexaenoic acid (DHA), improves overall health of the animal. Fatty acids also accumulate in the fish, passes finally to the consumer (C. Regost *et al.,* 2001).

Fish Meal energy content is much higher than in other protein sources, contain between 70-80% of the product in form of protein and digestible fat (NRC 1983; R. D. Miles and F. A. Chapman, 2015).

Fish meal has a relatively high content of phosphorus, available for the animal (assimilable phosphates and phosphoric acid). Vitamins that are presented at high levels are those of group B, including choline and vitamin B12, as well as vitamins A and D (R. D. Miles and F. A. Chapman, 2015).

Table 4. Profile of essential amino acids (%) of various protein raw materials (National Research Council NRC, 1983).

Amino acid	Fish meal	Blood meal	Meat meal	Corn gluten meal	Soybean meal	Wheat
Arginine	5.02	3.88	3.75	1.53	3.38	0.73
Histidine	1.80	5.59	1.04	1.06	1.19	0.36
Isoleucine	3.41	0.98	1.76	2.46	2.27	0.51
Leucine	5.64	11.86	3.29	7.92	3.65	1.02
Lysine	5.83	8.04	3.11	0.87	2.99	0.41
Methionine	2.27	0.95	0.70	1.14	0.58	0.24
Phenylalanine	2.94	6.36	1.83	3.05	2.36	0.72
Threonine	3.16	3.39	1.77	1.56	1.85	0.44
Tryptophan	0.83	1.13	0.32	0.23	0.71	0.30
Valine	4.68	8.13	2.63	2.40	2.25	0.65
C.P.	78.3	93.0	54.1	46.8	49.9	13.0
Total AA.E.	35.6	50.8	20.2	22.2	21.2	5.4
% AA.E./C.P.	45.4	54.8	37.3	47.5	42.5	41.4

Among the alternative sources of fishmeal are:

- Raw materials of animal origin: meat meal, feathers, bones and blood, etc. Today many of them are partially banned due to the possible occurrence of diseases that can affect humans such as encephalopathies.
- Raw materials of plant origin: among them seeds meals, leaves and derivatives.
- Raw materials of marine origin: including krill meal, squid meal, fishery residues, etc.
- Raw materials of industrial origin: such as single cell proteins or synthetic amino acids.

Robaina et *al.* (1995) reported that there is no significant effect on diet intake when they replaced sardine fish meal partially with 10, 20 and 30% of soybean meal (SBM) but they found a marked decrease in the activity of trypsin and protein digestibility with increasing SBM levels in diet may due to the presence of dietary phytic acid.

The partial replacement (20, 30 and 40%) of fish meal with corn gluten meal (CGM) or meat and bone meal (MBM) has no significant effect on growth, feed efficiency, protein efficiency ratio and protein production values. However, an increase of nitrogen excretion have been observed in fish fed the CGM and MBM diets leading to an increase in deamination activity and the released amount of ammonia into the water (Robaina *et al* ., 1997).

Kissil et al. (2000) tested rapeseed protein concentrate, with substitution levels of fishmeal 30, 60 and 100% in gilt-head sea bream with 12 g of average initial weight. To determine nutritional parameters, the gilt-head sea bream was fed to apparent satiation for 56 days.
They found that feeding rates were significantly lower at higher levels of substitution. The fish were fed without fishmeal (100%) obtained the worst conversion rate and the best conversion rates were obtained in fish which fed with 30% replacement of fishmeal by rapeseed. In addition, a decrease in the specific growth rate (SGR) with increasing the substitution level was also observed.

In an experiment conducted by Pereira *et al*. (2003), with juvenile gilt-head sea bream with 8 g of initial average weight, five experimental diets were tested with partial replacement of fish meal by corn gluten 0, 20, 40, 60 and 80. Gilt-head sea bream was fed to satiation twice daily for 84 days. They observed significant differences in growth only in the diet with 80% of substitution in which the final weight was lower. The protein and energy retention improved with increasing cornmeal except in the diet with 80% replacement in which the worst results were observed.

Gomez-Requeni *et al*. (2004) studied the replacement of 0, 50, 75 and 100% of fishmeal by a mixture of plant proteins of corn, wheat, pea, rapeseed and lupine, in gilt-head sea bream with initial average weight of 17g. The experiment was conducted for 81 days, fish were fed twice daily to satiety. The final weight, the growth rate and the daily feeding rate decreased

progressively and significantly with increasing protein content of plant origin. However, they did better conversion rates in diets with vegetable proteins.

Kissil & Lupatsch (2004), tested the substitution of fish meal by a mixture of plant protein (soy protein concentrate, wheat gluten, and corn gluten meal) at 4 levels 25, 50, 75 and 100. At the end of trial they showed the use of soy protein or corn gluten as the sole protein source in diets for gilt-head sea bream is not recommended but their use in combination with wheat gluten can provide a partial or complete alternative to fish meal. However, the cost of supplemental arginine made replacement economic at only the lowest replacement level (25%).

Replacement of fish meal by crude lupine meal or extruded was studied by Pereira *et al.* (2004) in juvenile gilt-head sea bream of average initial weight 42 g. The tested levels were 0, 10, 20% substitution of crude lupine meal, and 0, 10 and 20% of the extruded. No significant differences in nutritional parameters were found although the final weight of the gilt-head sea bream which fed 10% extruded lupine was significantly higher.

Sitjà-Bobadilla et al. (2005) tested the effect of fish meal replacement by a mixture of plant protein (PP) sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) balanced with indispensable amino acids in juvenile gilt-head sea bream for 6-month, they used three levels of replacement 50%, 75% and 100%. They found final body weight was progressively decreased with PP inclusion, but in PP50 and PP75-fed fish, feed efficiency (FE) was significantly improved and specific growth rates slightly reduced in comparison to fish fed the fish meal diet. In fish fed PP100 diet, FE remained unchanged and feed intake and growth decreased dramatically. In this group of fish, liver fat deposition was also largely increased, enterocytes showed an increased number of lipidic vacuoles and deposition of protein droplets, and the submucosa of intestine was dilated and infiltrated with eosinophilic granular cells.

De Francesco et al. (2007) tested high-level fish meal replacement by plant proteins in gilthead sea bream Juveniles of 100 g initial body weight. Feed intake was higher in sea bream fed fish meal

diet, while feed efficiency and protein efficiency ratio were significantly higher in sea bream fed plant protein, also fish fed fish meal diet had a lower hepatosomatic index and a higher fillet yield.

Sanchez-Lozano, N. *et al*. (2007) experienced in gilt-head sea bream levels of 0, 10, 20, and 30% of replacement of fish meal by sunflower cake, experiment has been conducted in two phases. Fish started the first phase with an average weight of 44g were obtained significant differences in growth during the first 90 days of the experiment, the best replacement level was 10% and the worst was 30%.. However, in the second phase in which the fish were fed to satiation for 157 days, there were no differences in growth. Although the feed rate and conversion rate were higher in the diet with 30% replacement.

Martínez-Llorens *et al*. (2007) tested soybean meal in gilt-head sea bream with replacement levels of fish meal 20, 30, 40 and 50%. The experiment was conducted in two phases. In the first phase, the fish began the experiment with an average weight of 17 g, significant differences were found in growth parameters at the end of that phase. The fish were fed with replacement level of 50% fish reached the worst growth outcomes. However, in the second phase in which gilt-head sea bream left with an average weight of 80 g, no significant differences were found and therefore the fish growth was similar for all diets.

Bonaldo, A. et al. (2008) studied the effect of replacement of fish meal by soybean meal on the performance and gut histology of gilthead sea bream at 18 g body weight for 80 days, they used three levels of soybean meal (0, 180 & 300 g kg^{-1}) and the found d no significant effects on the specific growth rate, feed intake and feed conversion rate. They only found a moderately and diffusely expands in distal intestine and lamina propria of some fish fed 300 SBM due to an increase in cell infiltration represented by mononuclear cells.

Sanchez-Lozano, N. *et al*., 2009 carried out a Partial replacement of fish meal with 0, 30, 60 and 90% of vegetable mixture (Pea Protein Concentrate (PPC) and Rice Protein Concentrate (RPC)) in gilt-head sea bream diet, supplemented with Methionine and Lysine. They reported that there is no significant differences in feed intake ratio, protein efficiency ratio and feed conversion ratio.

No statistical differences were found in biometric parameters. No differences were observed in fish survival except for fish fed the 90% of diet.

Martínez-Llorens *et al*. (2009) tested soybean meal in gilt-head sea bream with replacement levels of fish meal of 0, 15, 30, 45, 60 and 75%. The fish began the experiment with an average weight of 242 g. Fish were fed with replacement level of 60 and 75% achieved the worst results of growth. Significant differences were found in protein efficiency in fish which fed the diets with replacement of 45, 60 and 75%. The best results of feed conversion ratio (FCR) occurred with 45% substitution of fishmeal by soybean cake.

Martínez-Llorens et al. (2011) carried out a study to determine the optimum fish meal and vegetable meals in diets for maximizing the economic profitability of Gilthead Sea bream, the recommended that the optimal fish meal levels for minimizing the economic conversion ratio were 33% (when soybean meal was included) and 14% (in the case of pea-rice concentrate mixture) and for maximizing the economic profit index, optimal fish meal levels were 31% and 28.8%, respectively. They concluded when fish meal prices increase, the highest beneficial level of the inclusion fish meal and the highest economic profit would be achieved with pea-rice mixture.

Sanchez-Lozano et al. (2011) studied the substitution of fish meal by pea protein concentrate in gilt-head sea bream at 51 g average initial weight, the use four levels of replacement 0, 162, 325 and 487 g kg^{-1} and found a decrease in final body weight by the increase of substitution level. The lowest specific growth rate (SGR) reached at the highest substitution level but other levels did not present statistical differences. Feed conversion ratio (FCR), protein efficiency ratio (PER) and body composition were not affected by the diets.

Martínez-Llorens et al. (2012) carried out a work to study the partial substitution of fish meal by carob seed germ meal (0, 17, 34 and 52%) in gilthead sea bream fingerlings of 10 g average weight for 83 days. They found a decrease in final body weight by the increase of level of substitution. The lowest specific growth rate (SGR) was found in fish fed the diet with 52% replacement, also this diet produced the least satisfactory results for feed intake (FI) and the food conversion ratio (FCR). On the other hand they found histological alterations in the fish fed 34% and 52% carob

seed germ meal, especially at 52% substitution level in the mucosal fold which observed shorter and thinner and exhibited a smaller number of goblet cells.

Vizcaíno, A.J. et al. (2014) tested the using of the microalga *Scenedesmus* almeriensis with different inclusion levels (0%, 12%, 20%, 25% and 39%) as fish meal alternative in diets of gilt-head sea bream juveniles in 45 days trial. They reported that growth rate and protein utilization in fish fed on a diet with 20% Scenedesmus meal tended to be higher, although not significantly different from those fed on control diet. None of the treatments caused adverse effects on body composition. This study confirmed the usefulness of S. almeriensis to partially replace fish meal in practical diets for gilthead sea bream juveniles.

Baeza-Ariño, R. et al. (2014) studied liver and gut alterations in gilt-head sea bream fed a mixture of vegetable protein concentrates (pea and rice mixture) at (30%, 60% and 90%) of fish meal substitution and compared with a control diet (0%). They found a significant changes especially in the case of the 90% substitution in parameters such as thickness of the layers, goblet cells and villi measurements. Structural integrity of the gut would not be significantly affected by a diet of up to 60% substitution. The alterations observed in the liver could not only be attributed to diet but also to possible individual variations.

Kokou et al. (2015) tested the replacement of fish meal by soybean meal with different levels of substitution 20, 40, and 60% in gilt-head sea bream of 16 g initial weigh, the found that growth and feed efficiency were affected negatively from 40% level.

Liver structure did not show any histological alteration from control at 20 and 40% levels. However, in the diet with 60% replacement lipid accumulation was observed within the hepatocytes. In the intestine the structure of the submucosa was dilated and leukocyte infiltration was also observed in some cases, which was more obvious in fish fed 60 % diet in relation to the fish fed fish meal.

1.5 PROBIOTICS:

Probiotic is an additive that contains live micro-organisms or mixtures of them incorporated into the diet to provide benefits to the host. These benefits may be due to different mechanisms of action: regulation of the intestinal microbial homeostasis, stabilization of the gastrointestinal barrier (Salminen, 1996), expression of bacteriocins (Mazmanian et al., 2008), production of enzymes that induce the absorption and improve nutrition (Hooper et al., 2002; Timmerman et al., 2005), immunomodulatory effects (Salzman et al., 2003), enzyme inhibition carcinogenic effects and interference with the ability of pathogens to colonize and infect the mucous membrane (Gill, 2003).

The use of probiotics became increasingly popular in aquaculture not only for disease prevention and improved nutrition but also because of an increasing demand for environment-friendly aquaculture. Dozens of scientific papers are published since late 1980s (Qi, 2008). The studies of prebiotics in fish and shellfish showed that they had effect on growth, feed conversion, gut microbiota, cell damage/morphology, resistance against pathogenic bacteria and innate immune parameters such as alternative complement activity (ACH50), lysozyme activity, natural haemagglutination activity, respiratory burst, superoxide dismutase activity and phagocytic activity (E. Ringo et al., 2010). Most microorganisms proposed as biological control agents in aquaculture cover the authorized list of microorganisms of EU and FDA as probiotics in feedings stuffs, which include the lactic acid bacteria, *Bacillus*, yeast.

A wide range of bacteria , both gram positive and gram negative bacteria, and yeasts have been investigated as probiotics with good results (Merrifield et al., 2010; Nayak, 2010; Dimitroglou et al., 2011; Rombout et al., 2011). They are also commercially available for use in aquaculture some probiotic supplements as one or more species of microorganisms. (Gatesoupe, 1999; Decamp y Moriarty, 2006).

Probiotics improve feed conversion efficiency and live weight gain and confer protection against pathogens through competition for the sites of adhesion, the production of organic acids (formic, acetic, lactic), hydrogen peroxide and other compounds such as antibiotics, siderophores, bacteriocins, lysozyme and modulate physiological and immunological responses in fish (Nayak, 2010). Ariğ et al., 2013 observed an improvement in growth of gilt-head sea bream larvae by Bacillus sp. Bacteria.

1.6 **PREBIOTICS**:

Prebiotic is an additive that selectively stimulates the growth and/or activity of one or a limited number of bacteria in the intestine (Gibson and Roberfroid, 1995). To classify a substance as a prebiotic should meet at least these three criteria: prebiotic must not be hydrolyzed or absorbed in the stomach or intestine, it should be selective to beneficial commensal bacteria furthermore its fermentation should induce local or systemic beneficial effects on the host (Manning y Gibson, 2004).

Prebiotics began to be studied in fish in 1995 (Hanley et al., 1995) and the beneficial effects described include the increase in the rate of growth, the stimulation of the immune system, changes in the intestinal microflora and alterations in the morphology of the gut (Olsen et al., 2001; Burr et al., 2005; Bakke-McKellep et al., 2007; Torrecillas et al., 2007; Ringo et al., 2010). Prebiotics studied in fish include oligosaccharides and other dietary fibers.

Li,P. & Gatlin D.M.III (2006) studied the use of dietary nucleotides in fish nutrition and concluded that dietary nucleotides according to many researches results improves growth in early stages of development, enhance larval quality via broodstock fortification, alter intestinal structure, increase stress tolerance as well as modulate innate and adaptive immune responses. Fishes fed nucleotide-supplemented diets generally have shown enhanced resistance to viral, bacterial and parasitic infection.

Gültepe, N. et al. (2011) studied the effect of supplementation with Mannanoligosaccharides (MOS) on growth parameters of gilt-head sea bream, the used two levels of MOS in diet 2 and 4 g Kg^{-1} added to fish meal based control diet for gilt-head sea bream 1-year-old. After 12 weeks they found no differences in survival rate among fish in all experimental diets but they observed a

significant improvability existed for both growth and feed utilization in fish fed diets supplemented with Bio-Mos. They recommended that dietary supplementation with 2 g kg^{-1} BIO-MOS seem to be most positive for gilt-head sea bream production.

1.7 JUSTIFICATION AND OBJECTIVES:

Gilt-head sea bream is the species most widely produced in the Mediterranean (APROMAR, 2014), but its production is decreasing by the decrease in sales price and is ceasing to be a profitable production. On the other hand, feed is one of the biggest costs in the production of aquaculture species, because feed is including raw materials very costly such as the fishmeal and fish oil. For this reason, it is interesting to study sources cheaper of alternative proteins, both animal and vegetable. However, the inclusion of these vegetable meals at high rates cause a reduction in growth and a lower nutritive efficiency (Martinez-Llorens *et al*., 2012), as well as immunosuppression (Sitja-Bobadilla *et al*., 2005), therefore, current research aims in part to the improvement of the feed through supplementation with additives, pre and probiotics, and other immuno-stimulants that outweigh the negative effects. In this sense a hydrolyzate of intestinal mucosa has provided good results in previous tests of pre-fattening gilt-head sea bream carried out in the GAB-UPV, but no results in the fattening phase.

The main objective of this study was to evaluate the inclusion of mixtures of vegetable protein concentrates in place of fishmeal, supplemented with hydrolyzate of intestinal mucosa in the diet to feed gilt-head sea bream *Sparus aurata*, and studying growth, nutritional parameters and intestinal health.

2. MATERIAL AND METHODS.

2.1 GENERAL DESCRIPTION OF THE INSTALLATION.

The experimental phase of the present study was conducted in the laboratory of aquaculture of the Department of Animal Science, Polytechnic University of Valencia. The laboratory is composed of different lines of experimentation, which have independent recirculation of water closed circuits that allow to perform various experiments with both freshwater and saltwater species.

This work was carried out on line 2 of the laboratory consisting of a closed water circuit seawater, which, after a correct cleansing, allows reuse, avoiding the excessive spending of water. This line consists of a network of channels that collect water from the tanks and lead them to a rotary filter, where solids are removed. Then water passes to a general well, and then to a biofilter that removes excess ammonium. This filter, water passes to another tank from which it is sent to tanks through drive pumps.

The installation also has a heat/cool pump to maintain constant temperature throughout the year and the supply of oxygen is carried out through a system of aeration pumps electro blowers, which take the outdoor air, filtered it and introduce it into the tanks through porous diffusers.

2.2 ELEMENTS OF THE SYSTEM.

2.2.1 TANKS.

Line 2 of the laboratory is made up of 18 cylindrical tanks of fiberglass, with a capacity of 1750 liters each (Figure 7). The tanks are distributed in a double row, with stands for the network of water, drainage, aeration and oxygenation. Nine of these tanks were used to carry out the experimental phase of the work

Figure 7: The fiberglass tanks of line 1 of the laboratory of aquaculture.

2.2.2 PUMPING SYSTEM.

Line 2 system presents a hydraulic circuit of large dimensions, so it is necessary to install a series of pumps to push water from the tank, which has a capacity of 17 m3 for salt water and 8 m3 for fresh water. Characteristics according to the manufacturer are: flow rate: 48-114 m3/h power: 5.5 kW, pump 1 is in charge of propel water to different installation tanks. The water that enters the tanks through vertical perforated PVC (poly vinyl chloride) pipe so that there is a tangential recirculation to the tank, controlled by keys of this material. The water drains through pipes to different channels where it is driven to a mechanical filter (removes material in suspension).

Pump 2 draws the water from the tank and leads to the biofilter. On the other hand is the heat pump that regulates the temperature of the water of the installation. It works with a power supply and consists of two compressors that perform the function of heat or cool water through the passage by two different heat exchangers.

2.2.3 WATER TREATMENT SYSTEMS.

2.2.3.1 Mechanical filter.

The mechanical filter or rotary drum filter, works retaining solid particles that are suspended in the water, making them go through a mesh of 70 microns in step. Particles trapped in the mesh are eliminated through a cleaning device comprising a Jet spray of fresh water.

2.2.3.2. Biofilter.

The biofilter of the laboratory is in a tank of 24 m3 (6 x 2 x 2 m). It is an essential element in closed-circuit installations since its mission is to reduce the concentration of ammonia in the water, coming from the metabolic excretions of fish and decomposition of organic nitrogen of fecal origin and not ingested feed particles.

A biofilter of the so-called "rain or dripping", consisting of a tank full of plastic material (biobolas) it is not submerged, which supports populations of bacteria that are responsible for the biological purification of the water. These colonies of bacteria oxidize the nitrogenous compounds in the water.

The bacteria that oxidize ammonium and transform it into nitrite (toxic), belong to the genus Nitrosomonas sp. Which they carry out the second part of the process, to oxidize the nitrites to nitrates they belong to the genus Nitrobacter sp. The amount of bacteria in the biofilter is proportional to the surface that have to be fixed.

2.2.4 AERATION SYSTEM.

This system is composed of two pumps electrosoplantes, situated outside the main ship and protected by a deckhouse. The power of each one of them is 1.5 kW. Air pumps generating flows through PVC pipes that distributed it to each of the tanks of the installation. Each tank is equipped with a system consisting of tubing, shutoff valve and porous rubber, which allows us to control the air inlet.

Diffusion in water is achieved through a porous rubber to micronize air bubbles. Gums are placed at the bottom of the tanks to facilitate the transfer of oxygen to the water.

2.2.5 WATER CHANNELING SYSTEM.

2.2.5.1 Canals.

Gutters network that runs the installation is designed to drive the water that is poured from different tanks to the Rotary filter. Gutters are concrete and are located at ground level and protected by grilles.

Due to the process of nitrification, the water from the system tends to acidify it. It is for this reason that time is needed to decreasing the pH by the addition of certain products (bicarbonate or calcium carbonate $CaCO_3$) that are added to the water by means of the network of gutters.

In addition, the salinity of the water is controlled also from gutters, adding fresh water when this parameter increases.

2.2.5.2 Pipes.

Different lines that we find in the installation are of varied diameters depending on its function. They can be manufactured in various materials, mainly PVC, polyethylene or polypropylene.

2.2.6 EMERGENCY SYSTEM.

The emergency system is of vital importance in the laboratory. In the case of detecting any failure in the installation, it could be corrected in time without causing great losses.

The system consists of the following elements:

a) Dialler: connected to different numbers that communicate with the technician responsible for the installation, in case of emergency.
b) Electrical generator: is responsible for supplying power to the different system pumps or pumps in reserve, in case of power failure.
c) Group of solenoid valves of oxygen: allow the passage of oxygen to the different tanks, when the main ventilation system fails.
d) Oxygen bottles: outside the main ship, in a protected area.
e) Reserve electrosoplantes game.

The elements that make up the system of emergency are established automatically in case of a cut in the power supply of the installation.

2.3 EXPERIMENTAL DESIGN.

2.3.1 FISH.

For the study of this work 135 animals from (MAREMAR) Sagunto, Valencia. Fish with an average initial weight of 273g were used. The gilt-head sea bream were acclimated and fed with a diet control for at least one week. The fish were weighed and distributed in tanks.

The diets were tested in triplicate. Fish were fed to satiation, twice a day (morning and afternoon), six days a week, but Saturdays is fed via a single morning socket. Bottles of feed were weighed at the end of the day for the daily intake of each tank.

The study lasted for 13 weeks until the weight of the gilt-head sea bream is a commercial weight. The growth is controlled through monthly samplings. To speed up sampling and facilitate the handling of animals used essence of oil as anesthetic.

At the beginning and end of the test, 45 fish were randomly reserved, five of each tank, that served as the final and initial samples of the experiment and were slaughtered for later analysis.

2.3.2 EXPERIMENTAL DIETS.

All the experimental diets used in this work were developed by extrusion cooking process in the feed factory of the Department of Animal Science, Polytechnic University of Valencia. For this a semi-industrial extruder of Clextral model BC45 it was used (Figure 8).

Three diets were designed, developed and manufactured all to be isonitrogenous and isoenergetic, two diets of them with total replacement of fish meal by a mixture of vegetable proteins, with and without prebiotic (Dry hydrolyzed mucosa) 0% Fish meal FM0 & 0% fish meal plus prebiotic FM0 + P. The third feed as the unique protein source 100% fish meal FM100.

The feeds were manufactured by extrusion in the feed mill of the group of aquaculture and biodiversity GAB (UPV), the ingredients and the composition of experimental diets are shown in tables 5&6

Table 5. Ingredients of experimental diets.

Ingredients (g kg -1)	FM100	FM0	FM0+P
Fish Meal	616	0	0
Wheat	285	34	34
Wheat Gluten	0	288	288
Soy bean	0	477	477
Soy bean oil	97	87	87
Fish Oil	46	100	100
Phos.	0	38	38
Taurine	0	20	20
MET	0	6	6
LIS	0	6	6
ARG	0	3	3
VIT	20	20	20

Table 6. Composition of experimental diets.

Composition (%D.M.)	FM100	FM0	FM0+P
D.M.	86.56	84.51	83.89
C.P.	46.35	47.99	48.53
E.E.	16.86	17.38	16.90
Ash	9.15	6.05	6.32
EMFN	27.65	28.59	28.24
Energy KJ/Kg	21623	21701	21055

Note: FM0+P is the same mixture of FM0 but with the addition of prebiotic as following: for 1Kg of diet FM0+P = 990 g FM0 + 10 g prebiotic

For the manufacture of diets each of the dry ingredients were weighed individually and mixed, with the exception of the vitamins that were added later to avoid losses and oils, to avoid the formation of lumps. Then proceeded to grind with a hammer mill and after this, all the ingredients were introduced in the mixer, where the vitamins were added. Fish oil was introduced when the

rest of the ingredients were mixed properly. Finally, the feed was extruded to certain speed, pressure and temperature conditions.

After the grinding was the mixing of raw materials, which took place in a mixer for a period of time of 20 min. Vitamins and oils were introduced in the mixer when the other raw materials took minutes mixing.

Finally, the processing of feed was in the semi-industrial extruder, whose speed, pressure and temperature conditions were 100 r.p.m, 40-50 atm and 100 - 110 ° C respectively and 3 - 4mm sizes of the pellet (Figure 8).

Figure 8. Extruder semi-industrial Clextral BC45.

2.4 WORK ROUTINE.

The purpose of the daily routine of work was to ensure the correct operation of the system at all times. Tasks and actions listed below, sought the welfare of animals and the adequate water quality, as well as control of all the elements that made up the system.

2.4.1 GENERAL INSTALLATION REVIEW.

Daily found the normal functioning of all the equipment and elements of the laboratory. The water level of the main pipe and the tank, the correct operation of the Rotary filter, the system of pumping and ventilation system, as well as the inputs and outputs of water tanks were reviewed.

2.4.2 CONTROL OF WATER QUALITY, PHYSICAL AND CHEMICAL PARAMETERS.

Three times a week the water tanks were sampled to ensure quality conditions are adapted to the needs of the fish.

The physical-chemical parameters that were checked and the necessary instruments to measure were as follows:

Temperature and dissolved oxygen: measured with probe or portable Oximeter (OxiGuard Handy Beta). The device displayed directly on the display temperature (° C) water and the concentration of oxygen dissolved (in mg/l or % of saturation), pH: was measured by pH meter. Nitrites and ammonium: controlled from the result of the test colorimeter. Salinity: salinometer or Refractometer (Hanna Instruments).

The following table shows the mean values of the parameters controlled during the experimental phase of the study.

Table 7. Values of physical-chemical parameters of the water during the experiment.

Ammonium (mg/l)	0.25
Nitrites (mg/l)	0.3 ± 0.2
Nitrates (mg/l)	100 ± 25
Salinity (‰)	30-33
Oxygen (mg/l)	6 ± 0.5
Temperature (ºC)	23±1ºC
pH	7±0.5

2.4.3 CONTROL OF GROWTH

Monthly control of weight (sampling) was to learn about the evolution of the growth of the animals. For this reason, fish fasted 24 hours prior to the check. We extracted all the fish for a

same tank (taking advantage of the vacuum to clean them). They were then placed in tanks filled with water that was added essence of oil (anesthetic), which facilitated the handling of animals. During the surveys at the beginning and end of the experiment were weighed individually while, elsewhere, they weighed between 2-5 fish, depending on the size. Once heavy fish were returned to their respective tanks.

Growth, nutrient efficiency parameters and energy were obtained by the following expressions:

- **Specific growth rate (%/day):**

  SGR = [(Ln (final weight mean) − Ln (initial weight mean))/time] *100

- **Daily feeding rate (%/day):**

  DFR = [total intake / ((final biomass+ initial biomass)/2)]*100

- **Feed conversion rate:**

  FCR= [total intake / (final biomass – initial biomass)]

- **Protein efficiency ratio:**

  PER= Weight gain/protein intake.

- **Energy:**

  E = 51.78 * C (Carbon) – 19.387 * N (Nitrogen)

2.4.4 FINAL CONTROLS. BIOMETRICS AND BIOMETRIC INDICES.

At the beginning and end of the experiment, five fish in each tank were taken randomly. These were reserved for biometric analysis (Figure 9) subsequent to that they would determine the physiological characteristics and body parameters of the gilt-head sea bream, both at the beginning and at the end of the trial.

The parameters that were measured during the biometric analysis of fish are detailed below:

-Total length (cm): measured from the end of the jaw to the end of the caudal fin rays.

-Total weight (g): individual weight of each whole animal after slaughter.

-Weight of the carcass (g): individual weight of each animal after having extracted all the visceral content.

-Visceral weight (g): weight of the visceral contents of the animal (liver, digestive, visceral fat, heart, gonads, and spleen).

-Weight of the visceral fat (g): weight of mesenteric fat in the abdominal cavity of the individual.

-Head weight (g): weight of the head of the fish from the end of the jaw to the operculum.

- Muscles weight: the weight of muscles fillet after removing the spine.

Figure 9. Detail of final biometrics of the experiment.

After we made the biometrics and known all the above parameters, we proceeded to the calculation of the biometric indices, obtained by the following expressions:

- Condition factor:

$CF = \text{total weight (g)} / \text{total length}^3 \text{ (cm)}$

- Viscerosomatic index:

$VSI = (\text{total viscera weight (g)} / \text{total weight (g)}) * 100$

- Hepatosomatic index:

HSI = (liver weight (g) / total weight (g)) * 100

- Mesenteric fat index:

MFI= (visceral fat weight (g) / total weight (g)) *100

- Headless index:

HI = [(total weight (g) - (head weight (g) + viscera weight (g))) / total weight (g)] * 100

- Meat index:

MI = (Meat weight (g) / total weight (g)) * 100

- Non-edible parts index:

NEPI = [(viscera weight (g) + head weight (g) +fins weight (g) + gills weight (g)) / total weight (g)] *100

2.5 ANALYTICAL METHODS.

The main macronutrients of fish, feed and raw materials employed in the elaboration of the same, were analyzed in the laboratory of the nutrition unit of the Department of Animal Science, Polytechnic University of Valencia.

The fish of a same tank, after analysis, were crushed completely (including viscera) and frozen in tubs properly tagged. Feedingstuffs and raw materials (flour and carbohydrates) were ground and stored in refrigerator.

2.5.1 DETERMINATION OF THE DRY MATTER.

2.5.1.1 Materials.

- Drying stove
- Calcium chloride desiccator
- Porcelain crucibles
- Metal tongs
- Balance

2.5.1.2 Methodology.

Using clamps, crucibles (numbered) of the stove where a few hours have been extracted to remove moisture (Figure 10) and are introduced in a desiccator until they have cooled down completely. Then in the Crucible previously moronic, they weigh between 2.5-3 g of the sample to be analyzed and are introduced again in the drying oven at 103-105 ° C for 24 hours, so the sample is completely free of moisture. Finally, crucibles are removed and left in a desiccator for half an hour, approximately. Once cooled they weighed.

2.5.1.3 Calculations.

A = weight of the Crucible

B = weight of the crucible with the sample

C = weight of the crucible with the dried sample

DM = dry matter.

- $\%DM = (C - A) / (B - A) * 100$

Figure 10: Drying oven with crucibles.

2.5.2 DETERMINATION OF ASH.

The ashes are the residue obtained after burnt dry matter sample to constant weight. They are composed of carbonates, phosphates, oxides or sulphates mineral.

2.5.2.1 Materials.

- Porcelain crucibles
- Calcium chloride desiccator
- Muffle of incineration
- Drying stove
- Metal tongs
- Balance

2.5.2.2 Methodology.

Once determined the matter dry, is percaline the sample until it stops emitting smoke and crucibles are introduced into a flask of incineration at 550 ° C for 5 hours (Figure 11). After that time, turns off the appliance and samples are left inside for at least one hour, in order to not burn the heat given off by the product. When we open the flask without danger, we introduce crucibles in drying stove for a while, so that the temperature change is not so abrupt. The samples we finally went to a desiccator. Once cooled, they weigh.

Figure 11: muffle

2.5.2.3.-calculations

A = weight of the Crucible.

B = weight of the crucible with the sample.

C = weight of the crucible with the ash.

- %Ash = $(C - A) / (B - A) * 100$

2.5.3 DETERMINATION OF THE CRUDE FAT.

The method is based on the solubility of. Lipid in organic solvent, by what is called crude fat or Ethereal extract at the fraction of the sample which is soluble in ether with a boiling point of 40 to 60° C.

In most feed and raw materials gross fat fraction will be constituted basically by fat (triesters of glycerol with acid grease high number of carbon atoms), that they are the most abundant lipids, but it can also contain other different chemical natures lipid and non-lipid compounds soluble in ether (pigments, fat-soluble vitamins,...). Phospholipids, which are not completely soluble in ether, are not included in a quantitative way.

When the sample to be analyzed may have lipids linked to other substances (animal products, gluten, dried pulp, dairy products, distilleries, feed enriched with fat,...) or in the form of soaps (Lee,...), is necessary to make a prior hydrolysis with hydrochloric acid prior to extraction with organic solvent so that they can be removed completely.

2.4.3.1 Materials.

- Analytical balance.
- Stove 105 ° C.
- XT10 Ankom extraction equipment (Figure 12).
- Bags filter lace Ankom.
- (Ankom 1915) heat sealer.
- Desiccator.

REAGENTS:

Light petroleum (BP 40-60 ° c, attention: volatile and flammable liquid)

Figure12: XT10 Ankom extraction equipment

2.5.3.2 Methodology.

Hydrolysis:

The analysis will take place in duplicate.

-Numbered in pencil lace bag, record the weight of the bag and tare.

-Add 0.25 g of celite, tare.

- Add approximately 0.3 g of sample and seal the bag.

- Despite two bags with celite which will be white.

- Place the bags in the holder and insert in the extraction equipment.

- Add 200 ml of petroleum ether at the bottom of body of extraction and 150 ml of petroleum ether in the upper part of the body of extraction (to cover the bags) and deposit the body of the extraction in the equipment.

- Open the water supply

- 60 min removal time, Enter

- Temperature of 90° C, Enter extraction

- wash 8 min, Enter time

- Make sure the water is connected, Start

The ANKOM HCI automatically heat and maintain the set temperature. Since hydrolysis is complete, samples are automatically washed with cold water. The instrument will warn when the process is complete.

- Insert in oven at 105 ° C for 24 hours.

- Remove from stove, put in a desiccator 10 min and weigh.

- Equipment must be cleaned the bottom of body pump where is the fat extracted with a blotting paper (with caution since it is hot, wait a few minutes so that was cool) and recover the deposit recycled ether extraction.

2.5.3.2 Calculation.

- A: bag weight.
- B: celite weight.
- C: bag weight + celite weight.
- D: Water weight.
- E: bag + celite + sample.
- F: bag + celite + sample without fat.
- G: Blank
- H: D+F

$$\%Cf = [\{(E-C*G)-(H-C*G)\}/ (E-C*G)] * 100$$

2.5.4. DETERMINATION OF CRUDE PROTEIN.

Protein have been determined according to AOAC Official Method 968.06, by a device can measure both nitrogen and carbon at the same time. Nitrogen is measured and converted to equivalent protein by a numerical factor.

2.5.4.1 Materials.

- Balance: Accurate to 0.01 mg.
- Specific aluminum paper for samples.
- Small spoon.
- Metal tong.
- Nitrogen & carbon analyze device (Figure13).

Figure13: Nitrogen and carbon analysis device.

2.5.4.2 Methodology.

The weight of 0.25 grams of samples put in aluminum papers specific to the analysis and then close it well and put it in capsules placed inside the device directly. After 5 minutes, you get both nitrogen and carbon.

2.5.4.3 Calculation.

The device calculates the protein automatically by equations based on numerical value of 6.25 to convert nitrogen to protein.

2.6 HISTOLOGY

For the histological analysis, the tissues have been prepared from Liver, anterior intestine and posterior intestine and kept in formaldehyde.

2.6.1 Materials.

- Plastic boxes.
- Gelatinized slides.
- Covers.
- Brushes.
- Metal tongs.
- Paraffin.
- Water at 30ºC.
- Glue.
- Histocentre 2. (Figure14).
- Microtome.

Figure 14: the paraffin equipment (Histocentre 2).

2.6.2 Methodology.

The steps of work have been done as the following:

- Keep "tissue" in formaldehyde until their preparation.

- Label the box with the sample code (by pencil)
- Place the sample in the box
- These boxes are put in formaldehyde in a big pot
- Put the samples in (*el revolver*) equipment (Citadel, 2000) figure15.
- Fill each bucket of the gun with your solution ((2º, 3º etc. - rotate to remove trays)
- Put samples of the pot in aluminum baskets (large or small)

Figure15: El revolver equipment (Citadel, 2000).

- Turn on the equipment and give to "start" program "C"
- Finished the process, take samples and put them in the paraffin in the equipment (Histocentre 2). Turn on 3 buttons (pool paraffin, paraffin Jet and the cold dish.)
- Take the transparent plastic box, put in the "hot plate" and fill the bottom with paraffin

- Take the sample and place it in the center of the transparent box, take the part of color and place on the transparent and fill with paraffin.
- Take the preparation and put in the "cold plate"
- When the preparations are cool remove the transparent part
- Carving the sample
- Once carved are cut with the microtome (Hypercut) Figure 16.

Figure 16: The microtome (Hypercut).

- Placed in the microtome, first cut is at 30 until you reach the sample, then at 10 until you can see that it is well and after that cut at 5.
- Cuts several times to make the sample size of blade
- Pick up it with a brush and end of another one.
- Quickly immerse in water $30^{o}C$
- Catch it with the objects slide (blade)
- Let it dry on free air.
- Put the reagents in the trays.
- Put the objects slides in the basket of slides and immerse following steps:

Step	Reagent	Time
1	Xylene	15 minutes
2	Ethanol100%	10 minutes
3	Ethanol 96%	5 minutes
4	Ethanol 70%	5 minutes
5	Hematoxylin	3 minutes
6	Wash	5 minutes
7	Eosin	8 minutes
8	Wash	4 minutes
9	Ethanol 70%	10 seconds
10	Ethanol 96%	30 seconds
11	Ethanol 100%	30 seconds
12	Ethanol 100%	1 minute
13	Xylene	3 minutes
14	Xylene	3 minutes

24. Dry
25. Take the objects covers.
26. Take the glue (Eukitt) with plastic pipettes. Always in Brooder.
27. Put small droplets out of the sample and put the objects covers and press slightly until there are no bubbles
28. Take photos.

2.7 STATISTICAL ANALYSIS.

All this work analyses were performed with the statistical package StatGraphics Plus XVII version 17.1.03 (Copyright 1982-2014, Statistical Graphics Corp.). To study the differences between the variable p of the treatments applied, was performed a multivariate variance analysis, using the Newman-Keuls test for the comparison of individual means.

For the analysis of the variables of growth (average final weight and overall Instantaneous growth rate IGR) and to correct differences in weight at the start of the trial, was used as a covariate average initial weight of the fish.

The data are expressed as mean ± SE (standard error of the mean), indicating also the number of observations of each analysis (n). The inference was made with a risk of first kind equal to 0.05, or what is the same, with a 95% confidence interval.

3. RESULTS AND DISCUSSION.

3.1 GROWTH AND SURVIVAL.

The evolution of the average body weight during the experiment along of trial in 60 days shown in Figure 17, which shows that the growth of fish which fed with feed FM100 (100% fish meal) reached the highest level of growth while the growth of fish which fed the FM0 and FM0 + P fish was very similar.

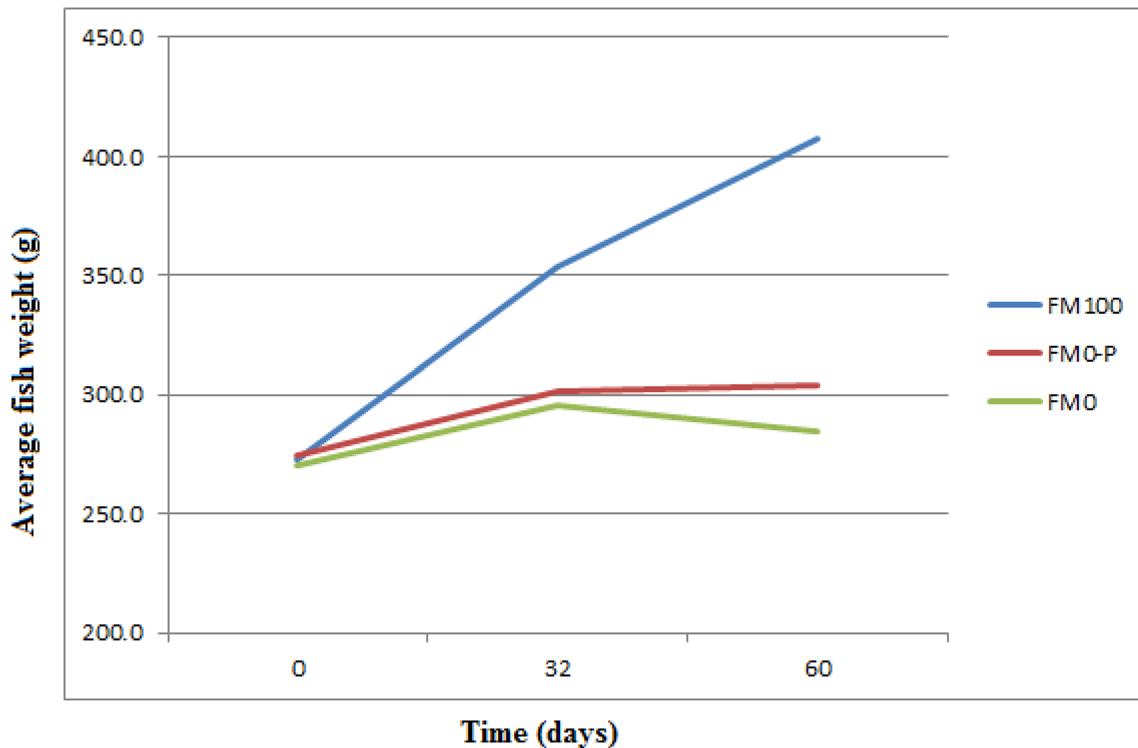

Figure 17: Evolution of average body weight (g) of gilt-head sea bream throughout the experiment.

The final survival of the experiment was 75.5% in fish which fed FM0 diet, 73.3% in FM0+P and 100% in FM100. The significant differences in survival between different types of diets not only because the effect of diet but also because there was a parasite *Sparicotyle chrysophrii* that could affect the survival percentage, growth and the final weight.

3.2 GLOBAL GROWTH AND FEED EFFICIENCY.

Table 8 shows the global results of growth and nutritional parameters with differences between these parameters.

Table 8: Parameters of growth and feed efficiency at the end of the experiment.

Treatment	FM0	FM0+P	FM100
Initial weight n=3	270 ±11.9	275 ±11.9	273 ±11.9
Final weight n=3	284^{b} ±12.9	303^{b} ±12.9	407^{a} ±12.9
SGR n=3	0.09^{b} ±0.02	0.17^{b} ±0.02	0.67^{a} ±0.02
DFR n=3	1.13^{b} ±0.05	1.12^{b} ±0.05	1.41^{a} ±0.05
FCR n=3	10.88^{a} ±0.49	7.37^{a} ±0.40	2.14^{b} ±0.40
PER n=3	0.17^{b} ±0.05	0.29^{b} ±0.05	1.01^{a} ±0.05

Note. Different letters indicate differences between means. Newman-Keuls Test ($p<0.05$).
SGR: Specific growth rate**, DFR:** Daily feeding rate**, FCR**: Feed conversion ratio, **PER**: Protein efficiency ratio.

There were significant differences between diet FM100 and other diets (FM0, FM0+P) in growth parameters: final weight, specific growth rate (SGR), daily feeding rate (DFR), feed conversion ratio (FCR) which were better in the diet FM100 and Protein efficiency ratio (PER) but no significant differences observed between diets FM0 and FM0+P in the same parameters.

The total of replacement fish meal was achieved the lowest final weight and specific growth rate (SGR) furthermore worst feed conversation ratio (FCR), which was consistent with other studies which obtained the worst results in total and high levels replacement of fish meal by plant protein sources such as (Robaina *et al.* (1995), Nengas *et al.* (1996), Kissil et *al.* (2000), Kissil & Lupatsch (2002), Ceulemans *et al.* (2003), Pereira T. G. & A Oliva-Teles (2003), Martínez-Llorens et *al.* (2007), Martinez-Llorens et *al.* (2009)). This negative effect by increasing plant protein levels probably due to low palatability, lack in essential amino acids and/or the existence of antinutritional factors Kissil *et al.* (2000). Nevertheless, Kissil & Lupatsch (2004) and Tomas et al. (2011), obtained good results with total replacement of fish meal by plant protein.

On the other hand, there were no significant differences between the diets FM0 which consist of only vegetable protein sources and the other diet FM0+P which consist of vegetable protein sources plus dry hydrolyzed mucosa in final weight, SGR, PER and FCR in contrast to the results of Tortosa, (2004) who obtained a high final weight, SGR, PER and better FCR in the case of using (dry hydrolyzed mucosa) in the diet of juvenile gilt-head sea bream.

The fish were fed FM100 kept high SGR during the experiment unlike which fed other diets FM0 & FM0+P, although the presence of the parasite may effect, because the increase in immune defense mechanism occurs with high level of fish meal and the opposite with high level of vegetable meal inclusion (Sitjà- Bobadilla et al., 2005).

3.3 BIOMETRIC PARAMETERS.

Table 9 shows the results of the effect of the diets (FM100, FM0 and FM0 + P) on biometric parameters of gilt-head sea bream at the end of the experiment.

At the end of the experiment, there were significant differences between the FM100 diet and other diets FM0, FM0-P in the condition Factor (CF), Viscerosomatic index (VSI), Hepatosomatic index (HSI) and meat index (MI), but there were no significant differences between diets FM0 and FM0+P in the same parameters, also there were no significant differences between all the diets in Mesenteric fat index (MFI).

On the other hand, there were significant differences between FM0 diet and other diets (FM0+P, FM100) in the index of the non-edible parts (NEPI), when there was no significant difference between FM0+P and FM100 in this parameter. Also found significant differences between FM100 diet and FM0 diet in Headless index (HI), but there were no significant differences between FM0+P and two other diets.

The diet FM100 a highest condition factor, liver weight, viscera weight, meat content and carcass with very clear significant differences with other two diets FM0 and FM0+P.

Table 9: Effect of diet on biometric indices gilt-head sea bream at the end of the experiment.

		FM0	**FM0+P**	**FM100**
CF n=12	Mean	1.58[b]	1.68[b]	2.02[a]
	SEM	0.04	0.04	0.04
VSI n=12	Mean	4.42[b]	5.05[b]	6.70[a]
	SEM	0.30	0.30	0.30
HSI n=12	Mean	0.79[b]	0.85[b]	1.32[a]
	SEM	0.09	0.09	0.09
MFI n=12	Mean	0.47	0.51	0.64
	SEM	0.08	0.08	0.08
MI n=12	Mean	50.86[b]	51.82[b]	55.51[a]
	SEM	0.81	0.81	0.81
NEPI n=12	Mean	46.99[a]	44.99[a]	42.10[b]
	SEM	0.74	0.74	0.74
HI n=12	Mean	71.86[b]	73.46[ab]	73.71[a]
	SEM	0.60	0.60	0.60

Note: Measurement of 12 replicates per group (n = 12). Different letters indicate differences between means. Newman-Keuls Test ($p < 0.05$). CF: Condition Factor, VSI: Viscerosomatic index, HSI: Hepatosomatic index, Mesenteric fat index (MFI), MI: Meat index, NEPI: non-edible parts index, HI: Headless index

The Hepatosomatic index HIS was high in FM100 diet and low in FM0&FM0+P inconsistent with the results of (Robaina et al. (1995), Pereira & Oliva-Teles (2003) & Nogales S. et al. (2010)) who found no significant differences in HSI at high replacement levels. While (Deguara et al. (1999) & Nogales S. et al. (2011) observed the decrease of HIS by the increasing of plant protein (PP) level.

The Viscerosomatic index VSI is high in FM100 and less in FM0&FM0+P contradict the results of Pereira & Oliva-Teles (2003) & Nogales et al. (2011) which explain no significant differences in VSI at high replacement levels. Also Nogales S. et al. (2010) showed that there were significant differences in condition factor (CF), Mesenteric fat index (MFI) & Headless index (HI), this result is agree with my results only in MFI.

Tortosa, (2004) has found no significant differences between diets FM100, FM0 and FM0+P in the biometric indices CF, HIS, VSI and MFI. My work has the same result of his work in MFI for all diets and the same results of CF, HIS & VSI only between FM0 and FM0+P.

3.4 BODY COMPOSITION.

The experiment began with gilt-head sea bream of initial average weight 273g whose body composition is shown in Table 10 beside body composition at end of the experiment.

Table 10: Body Composition (% Dry Matter, MS) of gilt-head sea bream at the beginning and end of the experiment.

parameters	Start	FM0	FM0-P	FM100
Moisture **n=3**	72.1	66.0 ± 0.79	65.0 ± 0.79	63.4 ± 0.79
Crude fat **n=3**	24.24	35.95[b] ± 1.05	40.38[a] ± 1.05	42.68[a] ± 1.05
Crude protein **n=3**	64.04	53.23[a] ± 0.64	50.01[b] ± 0.64	49.27[b] ± 0.64
Ash **n=3**	10.13	11.38[a] ± 0.40	7.99[b] ± 0.34	6.98[b] ± 0.40
Energy (kJ g^{-1}) **n=3**	6	6.8 ±15	6.8 ±15	7.2 ±15

The body composition of the gilt-head sea bream at the end of the experiment was affected by the substitution with vegetable proteins (table 10), there was a decrease in water, protein and ash content and an increase of lipid and energy content from the start to the end of the trial correspond to the results of Pereira & Oliva-Teles (2003) except the result of protein which increased at the end of his trial, and discordant with the results with the results of Kissil & Lupatsch (2004) which there were no significant differences in body composition between the start and the end of trial also between all diets at different levels of substitution up to 100% PP, Also Martínez-Llorens et al. (2009) didn't found any significant differences in body composition between the start and the end of the trial and between all diets up to 75% replacement of fish meal by plant protein sources.

At the end of trial fat content was higher in the gilt-head sea bream fed FM100 diet followed by FM0+P and the least amount of fat was in gilt-head sea bream fed with feed FM0 when there were no significant differences in energy content in all groups.

The ash content also presents significant differences between the gilt-head sea bream that fed with feed FM0 and the gilt-head sea bream that fed other diets (FM0+P, FM100). From these results, the consequence will be slower growth of fish fed diets without fish meal, and thinner (greater % of ashes and less fat).

3.5 HISTOLOGY OF GUT AND LIVER.

The measurement of the gut parameters were shown in figure 18, the measurements were shown no statistical differences between all parameters of gut returns to the effect of diet in both proximal and distal intestine, also there were no significant differences in liver parameters (Hepatocyte diameter and Nucleus diameter) as shown in table 11.

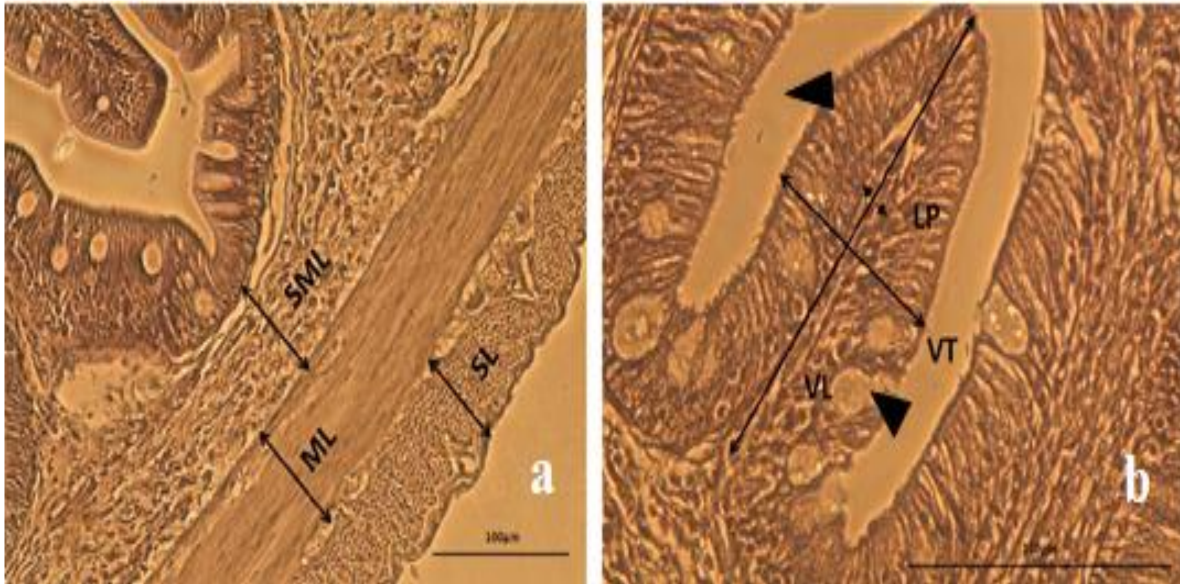

Figure 18: Detail of different measurements in the gut. (a) Detail of the intestine (20x) with measurements of the serous layer (SL), muscular layer (ML) and submucous layer (SML. (b) Detail of intestine villi (40x) with measurements of the villi length (VL), villi thickness (VT), lamina propia (LP) and (◀) Goblet cells (GC).

The morphology of both gut and liver were described and four parameters were observed in the gut: lamina propia infiltration, enterocytes infiltration, cells separation and nucleus misalignment (Figures 19&20). On the other hand three parameters were observed in the liver: cytoplasmic vacuolization (CV), peripancreatic fat infiltration (PFI) and nuclear displacement (ND). For estimations, a grading scale of 1–4 (1 = not observed, 2 = few, 3 = medium, 4 = severe) was used.

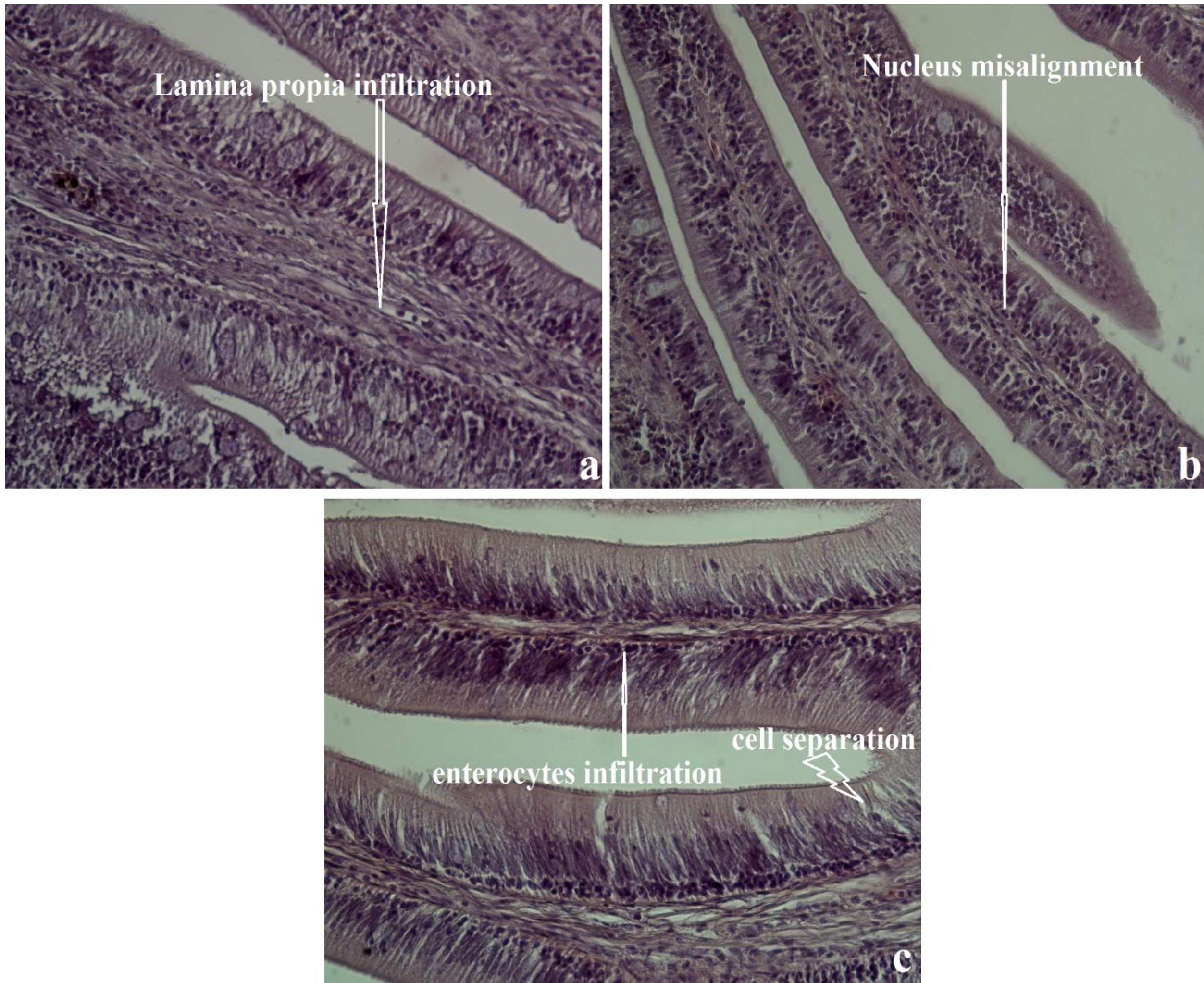

Figure 19: detail of morphological evaluation of proximal intestine PI; (a) FM0 diet (40x), (b) FM0+P diet (40x) & (b) FM0100 (40x).

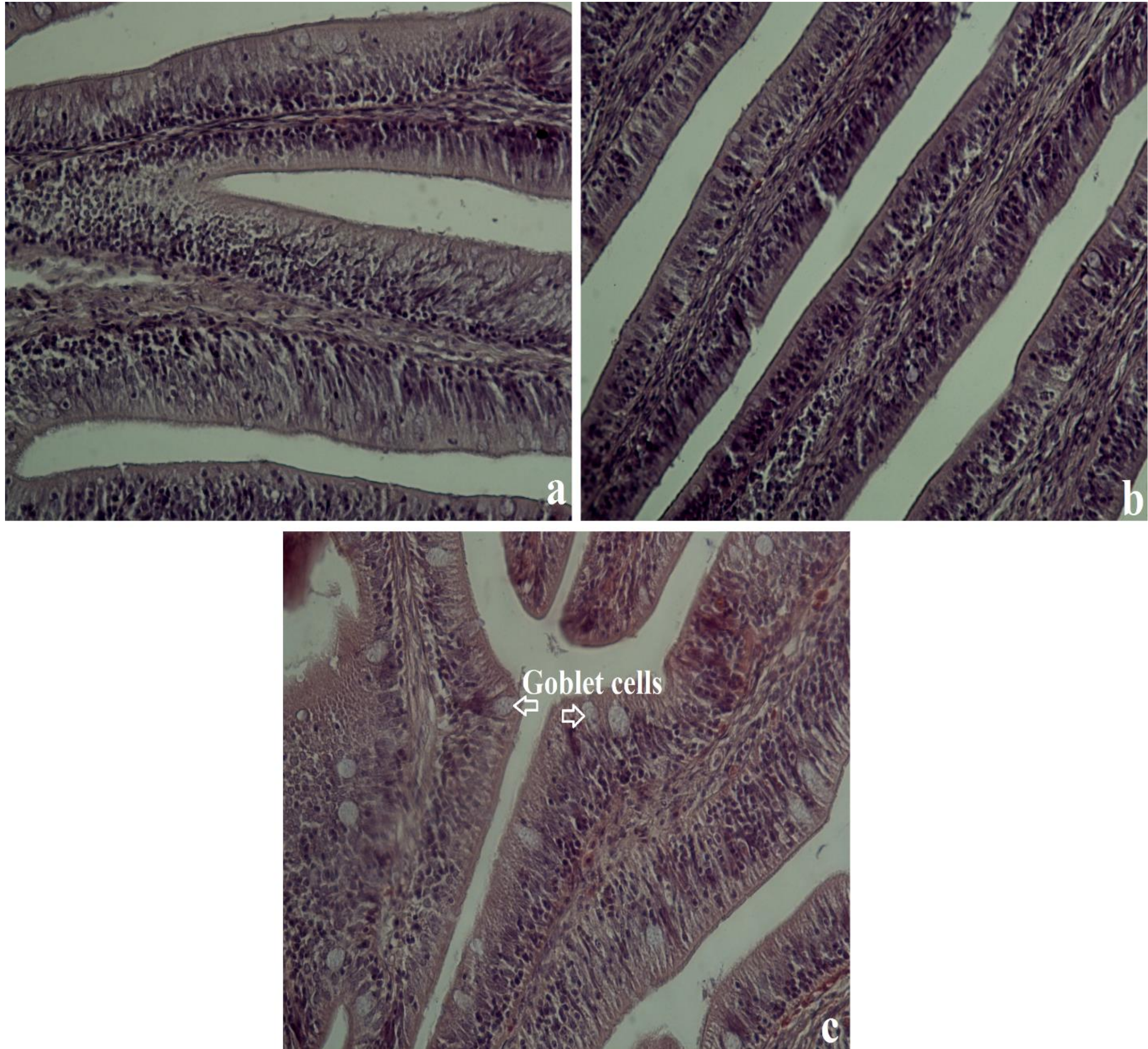

Figure 20: Distal intestine histology; (a) FM0 diet (40x), (b) FM0+P diet (40x) & (b) FM0100 (40x).

Table 11: Effect of diets on gut and liver parameters.

Proximal intestine (PI)			
parameters	FM0	FM0+P	FM100
SL (µm) **SEM***	41.70 ±16.68	56.05 ±16.68	72.81 ±16.68
ML (µm) **SEM**	64.90 ±22.37	58.76 ±22.37	61.22 ±22.37
SML (µm) **SEM**	41.64 ±13.13	37.38 ±13.13	43.14 ±13.13
VL (µm) **SEM**	381.45 ±47.25	485.04 47.25	362.85 47.25
VT (µm) **SEM**	58.00 ±3.13	53.51 ±3.13	59.00 ±3.13
LP (µm) **SEM**	25.73 ±3.88	19.54 ±3.88	18.63 ±3.88
GC **SEM**	4.92 ±2.22	5.72 ±2.22	6.47 ±2.22
Distal intestine (DI)			
parameters	FM0	FM0+P	FM100
SL (µm) **SEM**	49.18 ±11.32	79.99 ±11.32	51.43 ±11.32
ML (µm) **SEM**	72.30 ±17.31	62.46 ±17.31	51.89 ±17.31
SML (µm) **SEM**	37.91 ±7.84	41.72 ±7.84	33.11 ±7.84
VL (µm) **SEM**	293.08 ±40.87	322.43 ±40.87	389.44 ±40.87
VT (µm) **SEM**	54.78 ±6.07	53.75 ±6.07	56.90 ±6.07
LP (µm) **SEM**	14.73 ±2.82	10.89 ±2.82	14.27 ±2.82
GC **SEM**	5.69 ±1.70	6.43 ±1.70	6.46 ±1.70
Liver			
parameters	FM0	FM0+P	FM100
Hepatocyte diameter (µm) **SEM**	4.74 ±0.63	4.29 ±0.63	6.43 ±0.63
Nucleus diameter (µm) **SEM**	2.05 ±0.12	2.34 ±0.12	2.47 ±0.12

*Standard error of the mean, (SL; µm): Serous layer, (ML; µm): Muscular layer, (SML; µm): Submucous layer, (VL; µm): Villi length, (VT; µm): Villi thickness, (LP; µm): Lamina propria, (GC): Goblet cells.

The morphological evaluation of proximal intestine showed both FM100 and FM0+P diets had the same degree of lamina propia infiltration and nucleus misalignment while FM0 was higher in the same parameters, but all diets had the same enterocyte infiltration and cells separation.

At the level of distal intestine both FM100 and FM0+P diets had the same degree of lamina propia infiltration when FM0 was lower in these two parameter, also FM0 and FM0+P had the same cells separation while FM100 was higher in this parameter, but all diets had the same enterocyte infiltration and nucleus misalignment as shown in table 12.

Table 12: Morphological evaluation of gut and liver.

Proximal intestine (PI)				
Treatment	**Lamina propia infiltration**	**Enterocyte infiltration**	**Cells separation**	**Nucleus misalignment**
FM0	3-4	2-3	2-3	3
FM0+	1-2	2-3	2-3	2-3
FM100	1-2	2-3	2-3	2-3
Distal intestine (DI)				
Treatment	**Lamina propia infiltration**	**Enterocyte infiltration**	**Cells separation**	**Nucleus misalignment**
FM0	1-2	2-3	1-2	2-3
FM0+	2-3	2-3	1-2	2-3
FM100	2-3	2-3	2-3	2-3
Liver				
Treatment	**Peripancreatic fat infiltration (PFI)**	**Nuclear displacement (ND)**	**Cytoplasmic vacuolization (CV)**	
FM0	2-3	3-4	2-3	
FM0+	4	3-4	2-3	
FM100	2	3	2-3	

Liver morphological evaluation showed no differences between all diets in cytoplasmic vacuolization (CV) which reflect a moderate deformed hepatocytes (Figure 21) when peripancreatic fat infiltration (PFI) was severe in FM0+P, few to medium in FM0 and few in FM100 (Figure 22).

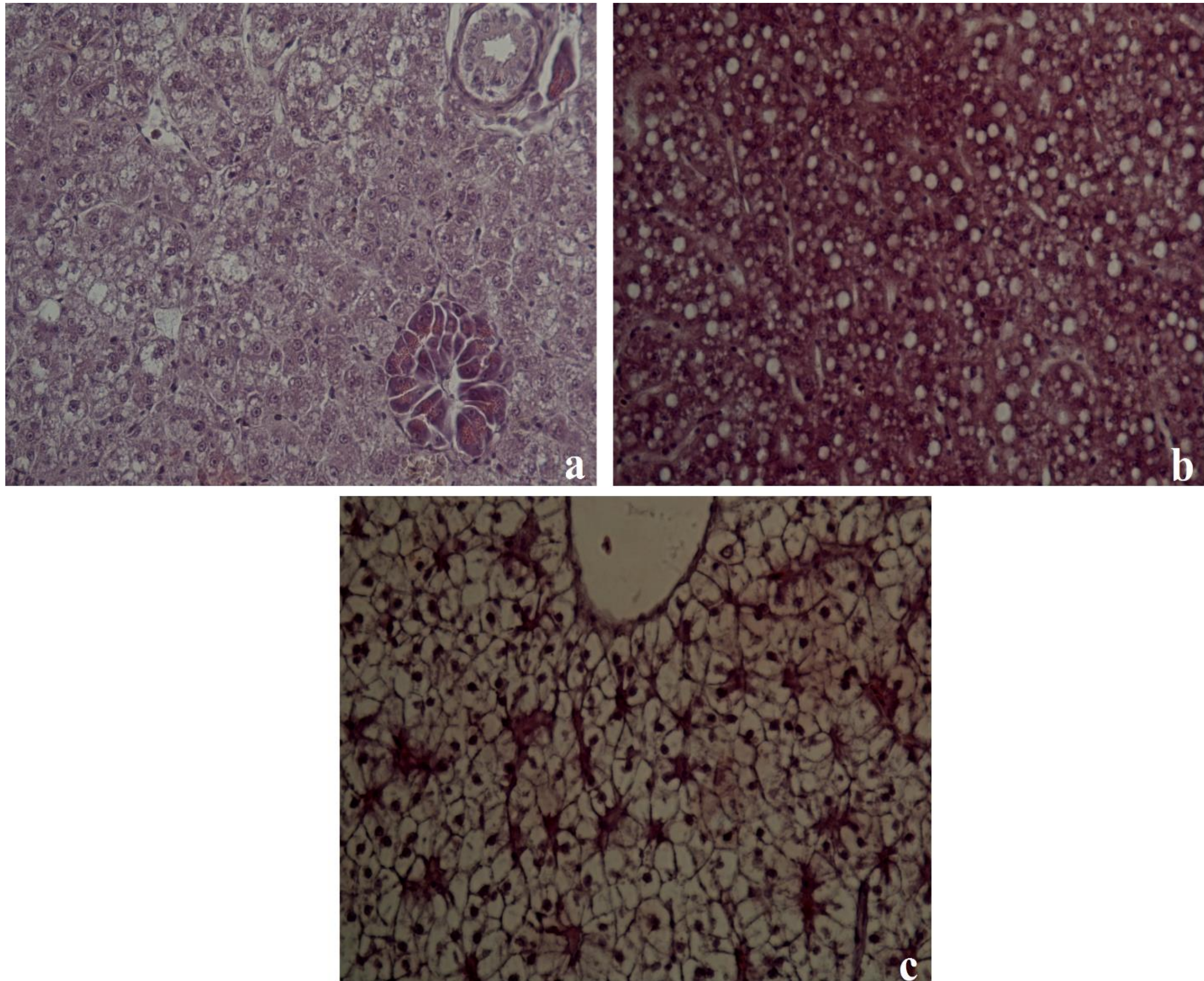

Figure 21: Effect of diets on cytoplasmic vacuolization (CV) & nuclear displacement (ND); (a) FM0 diet (40x), (b) FM0+P diet (40x), (c) FM100 diet (40x).

Nuclear displacement (ND) in both FM0 and FM0+P was ranged between medium and severe which mean the majority of nuclei was not in the central position of the hepatocyte while the same parameter was less in the case of FM100 diet (Table 12).

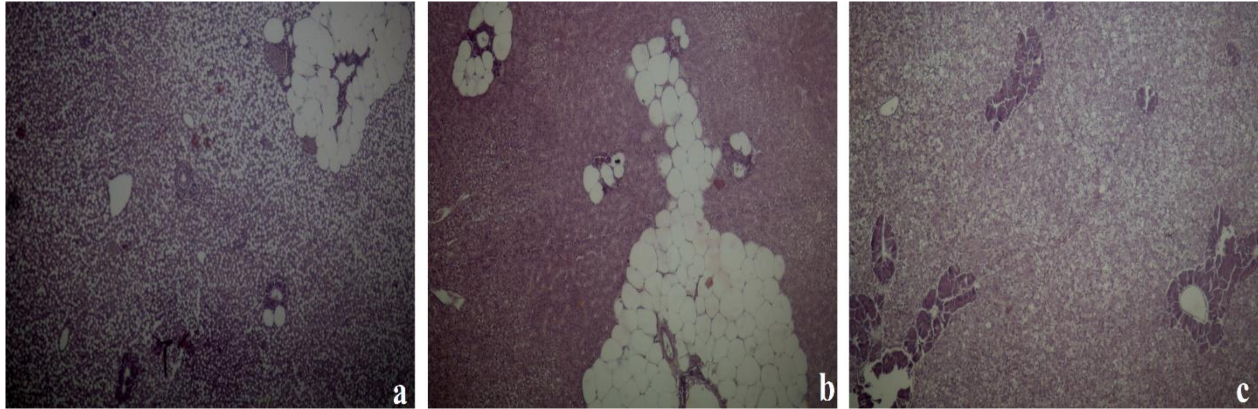

Figure 22: Peripancreatic fat infiltration (PFI) in liver; (a) FM0 diet (10x), (b) FM0+P diet (10x), (c) FM100 diet (10x).

Present results agree with Baeza-Ariño et al (2014) in of SL, VL&VT of the proximal intestine and ML of the distal intestine also in the SML of both proximal and distal intestine which there were no significant difference between diets FM100 and the diet with high level of replacement up to 90% PP. When these authors contradict with current results in SL, VL & VT of the distal intestine; they had smaller SL layer, longer and thinner villi at high level of replacement. Also they found a significant difference in the number of GC which was higher at high level of replacement as well as in LP thickness which was thinner at 90% substitution at the level of both proximal and distal intestine. They observed a severe nuclear displacement (ND) and cytoplasmic vacuolization (CV) at high level of substitution but it was few in the case of FM100 diet. On the other hand peripancreatic fat infiltration (PFI) was few at 90% PP diet and higher a little bit in FM100 diet contrary to my results.

The results of Martínez-Llorens et al. (2012) corresponds with the results of this work in ML, SML and LP which there were no significant differences between FM100 diet and the high level of substitution up to 52% PP when they had a significant differences between the two diets in other intestine parameters; smaller SL, shorter and thinner villi and less number of GC in the case of 52% PP diet. The liver was healthy at FM100 diet and in intermediate case at 52% PP replacement level. On the other hand Kokou et al. (2015) had a high lipid accumulation within the hepatocytes and high enterocyte infiltration at 60% replacement of fish meal by soybean protein.

Tortosa, 2014 found no significant differences in SL, ML, SML and VL between all his trial diets (FM100, FM0 and FM0+P) as obtained in present results but he observed a significant differences between FM0 and FM100 in VT which was thinner in FM0 also in GC which was fewer in FM100.

4. CONCLUSION:

The results of growth, nutritional, biometric and histological parameters at the end of trial and under this special case (presence of parasites) showed that FM100 diet was the best in all parameters with significant differences except in the body content of protein which was lower than other diets. So the total replacement with supplementation with dry hydrolyzed intestinal mucosa was not useful, because FM0+P not showed any immune defense when FM100 showed a good immune defense against the parasite.

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