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Assessment of the long-term impact of high plant protein diets on the intestinal status of the on-growing gilthead seabream (*Sparus aurata*, L.)

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Doctoral Thesis in Science and Technology of Animal Production

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Directors:
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NOTA PRÈVIA

L'autor d'aquesta tesi doctoral va comptar inicialment amb el suport econòmic d'un contracte pre-doctoral per a la Formació del Personal Investigador de la Universitat Politècnica de València, que va entrar en vigor l'1 de març de 2014, al qual va renunciar posteriorment per incompatibilitat amb una nova ajuda de la que fou beneficiari. Des del 15 de setembre de 2014, l'estudiant ha comptat amb un contracte pre-doctoral per a la Formació de Professorat Universitari, del Subprograma de Formació i Mobilitat, dins del Programa Estatal de Promoción del Talento y su Empleabilidad del Ministeri d'Educació (FPU13/01278).

El centre d'adscripció de l'estudiant durant la realització de la tesi ha estat la Universitat Politècnica de València, on ha desenvolupat la seua tasca investigadora en el Grup d'Acuicultura i Biodiversitat de l'Institut de Ciència i Tecnologia Animal. No obstant, durant la realització de la tesi, s'ha col·laborat de activament amb el Laboratori de Bactèries Làctiques de l'Institut d'Agroquímica i Tecnologia dels Aliments. Tanmateix, l'estudiant va realitzar una estança d'investigació a l'institut Bindley Center de la Universitat de Purdue (West Lafayette, Indiana, Estats Units) durant tres mesos.

La investigació portada a terme ha estat finançada parcialment pel Vicerrectorat d'Investigació, Innovació i Transferència de la Universitat Politècnica de València, dins del projecte 'Pinsos per a aqüicultura sense farina de peix' (SP20120603).

Aquest treball ha estat redactat en anglés, ja que es tracta de l'idioma habitual per a la comunicació científica en el camp d'investigació, comptant amb l'informe favorable dels directors de la tesi i la conformitat de la comissió acadèmica del programa de doctorat en Ciència i Tecnologia de la Producció Animal.

L'estudiant de doctorat opta a la menció de Doctor Internacional, ja que compleix les següents circumstàncies: ha realitzat una estança d'investigació fora d'Espanya en un centre o institució de prestigi, el document de tesi ha estat redactat íntegrament en anglés (els resums també apareixen en valencià i castellà), la tesi ha estat prèviament avaluada per dos doctors experts pertanyents a institucions no espanyoles i un membre del tribunal avaluador es membre d'una institució d'educació superior o centre d'investigació no espanyol.

La tesi està estructurada amb una introducció general, un compendi d'articles i una discussió general amb les conclusions. Tots els coautors no doctors han expressat la seua acceptació per a la inclusió dels articles científics en aquesta tesi i la seua renuncia a presentar-los com a part d'altra tesi doctoral.

Llista d'articles publicats:

Estruch G, Collado MC, Peñaranda DS, Tomás Vidal A, Jover Cerdá M, Pérez Martínez G, Martínez-Llorens, S (2015) Impact of Fishmeal Replacement in Diets for Gilthead Sea Bream (*Sparus aurata*) on the Gastrointestinal Microbiota Determined by Pyrosequencing the 16S rRNA Gene. PLoS ONE 10(8): e0136389. doi: 10.1371/journal.pone.0136389

Estruch G, Tomás-Vidal A, El Nokrashy AM, Monge-Ortiz R, Godoy-Olmos S, Jover Cerdá M, Martínez-Llorens S (2018) Inclusion of alternative marine by-products in aquafeeds with different levels of plant-based sources for on-growing gilthead sea bream (*Sparus aurata*, L.): effects on digestibility, amino acid retention, ammonia excretion and enzyme activity. Archives of Animal Nutrition 2018. doi: 10.1080/1745039X.2018.1472408

Llista d'articles enviats a revistes indexades:

Estruch G, Collado MC, Monge-Ortíz R, Tomás Vidal A, Jover Cerdá M, Peñaranda DS, Pérez Martínez G, Martínez-Llorens S. (2018) Long term feeding with high plant protein based diets in gilthead seabream (*Sparus aurata*, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. En revisió a *BMC Veterinary Research*

Estruch G, Martínez-Llorens S, Tomás Vidal A, Monge-Ortíz R, Jover Cerdá M, Brown PB, Peñaranda DS (2018) Impact of total fishmeal replacement by plant sources in aqua feeds for gilthead seabream (*Sparus aurata*, L.) on the gut mucosa proteome. En revisió a *BMC Genomics*

Estruch G, Bäuerl C, Tomás-Vidal A, Jover-Cerdá M, Peñaranda DS, Pérez Martínez G, Martínez-Llorens S. Implementation of gilthead seabream (*Sparus aurata*, L.) intestine explant culture assay to determine mucosal pro-inflammatory responses to bacterial pathogens and impact of plant protein in feed. En revisió a *PLoS ONE*

D'altra banda, el treball realitzat també s'ha difós a través de les següents comunicacions a congressos:

Vegetable diets change the intestinal microbiota of gilthead seabream. Impact on productivity. S. Martinez-Llorens[2], G. Estruch[2], M.C. Collado[1], D. S. Peñaranda[2], A. Tomás Vidal[2], G. Pérez Martínez[1], M. Jover Cerdá[2]
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Aquaculture Europe Congress. Donostia – San Sebastián (Spain). October 14-17, 2014.
Publicació: Aquaculture Europe 2014 Abstracts, pages 782-783.

Impact of fishmeal replacement in diets for gilthead seabream (*Sparus aurata*) on gastrointestinal microbiota by pyrosequencing the 16S rRNA gene. G. Estruch[2], M.C. Collado[1], D. S. Peñaranda[2], A. Tomás Vidal[2], M. Jover Cerdá[2], S. Martinez-Llorens[2] and G. Pérez Martínez[1]
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VI Workshop Probióticos, Prebióticos y Salud: Evidencia científica. Oviedo (España). 5 y 6 de febrero de 2015.
Publicació: Nutrición Hospitalaria 2015 (Suplemento 1); 31:1-145. ISSN (papel): 0212-1611. ISSN (electrónico): 1699-5198.

¿Es posible y eficiente la sustitución total de la harina de pescado por harinas vegetales en dietas para dorada? G. Estruch[1], M.C. Collado[2], R. Monge[1], A. I. Navarro-Ramirez[1], S. Godoy[1], A. Tomas-Vidal[1], M. Jover Cerda[1], S. Martinez-Llorens[1] y G. Perez[2]
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[2] Instituto de Agroquímica y Tecnología de los Alimentos – Centro Superior de Investigaciones Científicas. Av. Agustin Escardino, 7. 46980. Paterna (Valencia)
XV Congreso Nacional de Acuicultura. Huelva (España). 13 al 16 de octubre de 2015.
Publicació: Actas del XV Congreso Nacional y I Congreso Ibérico de Acuicultura, páginas 30-31.

Impacto del uso de harinas vegetales y de subproductos marinos en dietas para dorada (*Sparus aurata*, L.) sobre el proteoma de la mucosa intestinal.

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XVI Congreso Nacional de Acuicultura. Zaragoza (España). 3 al 5 de octubre de 2017.

Publicació: Actas del XVI Congreso Nacional de Acuicultura, páginas 259-260.

Impact of fishmeal repacement by vegetable ingredients and marine origin by-products on the gut mucosa proteome of the gilthead seabream (*Sparus aurata*, L.)

G. Estruch[a], P. Brown[b], R. Monge-Ortíz[a], S. Godoy[a], D. Sánchez-Peñaranda[a], M. Jover-Cerdá[a], A. Tomás-Vidal[a], S. Martínez-Llorens[a]

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Aquaculture Europe Congress 2017. Dubrovnik (Croatia). October 17-20, 2017.

Publicació: Aquaculture Europe 17 Abstracts, pages 342-343.

“Potser no he arribat allà on planejava anar, però crec que he acabat al lloc on havia d’estar”

Douglas Adams

AGRAÏMENTS

Al començar a escriure aquestes línies gire la mirada enrere i no puc deixar d'advertir que ben poc queda d'aquell estudiant que es decidia a continuar els seus estudis de Biotecnologia i Aqüicultura amb la tesi doctoral.

Si entenem la vida com un compendi de projectes personals, la realització d'aquesta tesi ha estat sense cap dubte un dels més fructífers. Com en tota empresa, les dificultats han fet acte de presencia, però eren la condició indispensable per aconseguir un creixement personal. La paciència, l'esforç, la confiança en un mateix i l'estima i el suport de la gent que m'envolta han estat la millor recepta per superar tots els obstacles.

Durant aquests cinc anys, he tingut el privilegi de poder formar part d'un equip d'investigació i d'aprendre moltíssimes tècniques i nous coneixements. De participar de la comunitat universitària i iniciar-me en la docència. De conèixer altres estructures d'investigació, altres plantejaments i d'advertir de la importància dels recursos econòmics i el recolzament governamental, però especialment del compromís personal, a l'hora d'assolir els objectius d'investigació. De col·laborar amb equips d'altres països, intercanviar coneixements i viatjar a llocs que mai hagués imaginat.

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ABSTRACT

Although the inclusion of plant protein sources at high levels in aquafeeds for on-growing gilthead seabream has been successfully achieved on gilthead seabream in terms of growth, these diets are still associated to detrimental effects in feed efficiency and immune capacity. The intestine is the organ where takes place the first interaction of the host with dietary antigen or environmental bacteria, and plays a major role in the digestion of nutrients and the inflammatory and the immune response. The present PhD thesis focus on the impact of classical formulated high plant protein diets on fish performance, but especially, on evaluation of the intestinal status in on-growing fish long-term fed with high levels of fishmeal replacement.

Changes at intestinal level were characterized by using different approaches, including protein and amino acid digestibility and retention and ammonia excretion, digestive enzyme activity, histology, expression of genes related with inflammation, immunity, structure and digestion, but also using whole tissue-level techniques for the analysis of the impact on proteome and gut microbiota. Different levels of fishmeal replacement were assayed, although the impact of diets with total replacement, complemented by inclusion of alternative marine by-products or supplemented by free amino acids, received greater attention.

Total fish replacement produced a negative but minor impact on the growth and nutritive and digestive performance of on-growing gilthead seabream. Nevertheless, when fish were fed from juvenile stage with plant protein based diets, a higher negative impact in growth terms was noticed. Digestibility and metabolic use of amino acids was altered, but no differences were observed in the digestive enzyme activities. Nonetheless, feeding fish with total dietary fishmeal replacement by plant protein without any marine protein source was especially critical for survival rate. In these fish, gut histological assessment only revealed minor alterations related with an inflammatory response, but gene expression assay showed a down-regulation of several genes involved in the inflammatory and immune response. Moreover, a drastic change in the microbiota composition was observed, especially at the hindgut, revealing a possible lack of capacity to regulate a defensive response and to face with pathogen colonisation after a long-term coupling with these diet. Likewise, gut mucosa proteome analysis also suggests an

impact on biological processes related with the maintenance of gut homeostasis and the epithelial integrity. In contrast, total fishmeal replacement did not induce alterations at transcript or proteomic level when diet was complemented with marine ingredients, although some minor inflammatory signs were reported.

On the other hand, an *ex vivo* system to study the inflammatory and immune response of the gut mucosa to the presence of different bacteria was developed, and a preliminary assay evaluating the impact of the diet on this response was performed.

To sum up, present works represents a wide assessment at intestinal level of the effects of including plant protein sources at high levels in aqua feeds for on-growing gilthead seabream. Results indicate that alterations in the immune capacity, the gut homeostasis and the microbiota were observed when protein was exclusively provided by plant sources, and could explain the higher mortality reported with this diet.

Keywords: gilthead sea bream, protein sources, intestine, digestive capacity; immune system, microbiota, proteome, *ex vivo* assay

RESUM

Malgrat que la utilització d'alts nivells de proteïna vegetal en pinsos per a dorades en la fase d'engreixament s'ha aconseguit amb èxit en quan al creixement, aquestes dietes encara s'associen amb freqüència amb efectes negatius en l'eficiència nutricional i la capacitat immunitària. L'intestí és l'òrgan on es produeix la primera interacció entre el peix, els nutrients de la dieta i les bactèries de l'ambient, i juga un paper fonamental en la digestió dels nutrients i en la resposta inflamatòria i immune. Aquesta tesi doctoral es centra en l'impacte de diferents dietes experimentals amb un alt nivell de proteïna vegetal, i especialment, en l'avaluació de l'estat de l'intestí de les dorades d'engreixament alimentades durant un llarg període amb alts nivells de substitució de farina de peix.

Els distints canvis observats a nivell intestinal es van descriure mitjançant l'ús de distintes estratègies, com l'anàlisi de la digestibilitat i la retenció dels aminoàcids, de l'excreció d'amoni i de l'activitat enzimàtica, dels canvis histològic o de l'expressió de gens relacionats amb la funció i el manteniment de l'estructura intestinal, així com tècniques òmiques per a l'anàlisi del proteoma i de la microbiota intestinal. Es van assatjar diferents nivells de substitució de farina de peix, però l'impacte de les dietes amb substitució completa, bé complementada amb subproductes d'origen marí o suplementada amb aminoàcids lliures sintètics, va rebre major atenció.

La substitució completa de la farina de peix va tenir un efecte lleugerament negatiu sobre el creixement i l'eficiència digestiva i nutritiva de la dorada d'engreixament, encara que l'impacte era major quan els peixos eren alimentats des de la fase de juvenils amb aquesta dieta. La digestibilitat i el nivell de síntesis de proteïna es va veure alterada, encara que no s'observaren diferències significatives en l'activitat dels enzims digestius. No obstant, l'impacte de les fonts vegetals quan s'eliminaven per complet les fonts de proteïna marina de la dieta era especialment crític en la supervivència dels peixos. En l'intestí d'aquests peixos sols s'observaren xicotets indicis d'inflamació a nivell histològic, però també es va observar una disminució l'expressió de gens involucrats amb el procés inflamatori i la resposta immune. L'estudi de la microbiota intestinal va revelar canvis significatius en la composició, especialment a l'intestí posterior, suggerint una possible falta de capacitat de regular la resposta

immunitària i de modular la colonització per part de patògens després d'un llarg període d'alimentació amb aquesta dieta. D'altra banda, l'anàlisi del proteoma de la mucosa intestinal també va mostrar un impacte clar sobre diferents processos biològics relacionats amb el manteniment de l'homeòstasi intestinal i de la integritat de l'epiteli. Per contra, no es van observar un impacte de la substitució de la farina de peix a nivell d'expressió gènica o proteoma quan s'incloïa a la dieta una font complementària de proteïna d'origen marí, encara que sí que s'observaven alguns signes d'inflamació.

Per últim, es va desenvolupar un sistema *ex vivo* per avaluar la resposta inflamatòria i immune de la mucosa intestinal davant la presència de diferents bactèries, i es va realitzar un assaig preliminar per determinar l'efecte de la dieta sobre aquesta resposta.

En resum, en aquest treball s'ha realitzat una avaluació extensa i detallada dels efectes a nivell intestinal de la inclusió d'alts nivells de fonts de proteïna vegetal a les dieta per a les dorades d'engreixament. Els resultats indiquen que les alteracions en la capacitat immunitària, l'homeòstasi i la microbiota intestinal eren observades solament quan la proteïna era exclusivament obtinguda de fonts vegetals, i podrien explicar la major mortalitat observada amb aquestes dieta.

Paraules clau: dorada, proteïna vegetal, intestí, capacitat digestiva, sistema immunitari, microbiota, proteoma, assaig *ex vivo*

RESUMEN

Aunque el uso de altos niveles de fuentes de proteína vegetal en piensos para doradas de engorde se ha alcanzado con éxito en cuanto al crecimiento, estas dietas todavía están asociadas a efectos negativos en la eficiencia nutricional y en la capacidad inmunitaria. El intestino es el órgano donde se produce la primera interacción entre el pez, los nutrientes y las bacterias del medio, y desarrolla un papel crucial en la digestión de los nutrientes y la respuesta infamatoria e inmune. Esta tesis doctoral se centra en el impacto de distintas dietas con altos niveles de proteína vegetal, y especialmente, en la evaluación del estatus intestinal de las doradas de engorde alimentadas con altos niveles de sustitución de la harina de pescado durante un periodo largo de tiempo.

Los cambios observados en el intestino se caracterizaron mediante el uso de distintas estrategias, como el análisis de la digestibilidad y la retención de amino ácidos, de la excreción de amonio, de la actividad de enzimas digestivos, de los cambios histológico o de la expresión de genes relacionados con la función y el mantenimiento de la arquitectura intestinal, así como técnicas ómicas para el análisis del proteoma y de la microbiota intestinal. Se ensayaron distintos niveles de sustitución de harina de pescado, pero el impacto de las dietas con una sustitución completa, bien complementada con subproductos de origen marino o suplementada con aminoácidos libres sintéticos, recibió mayor atención.

La sustitución completa de la harina de pescado provocó una reducción, aunque ligera, del crecimiento y de la eficiencia digestiva y nutritiva de la dorada de engorde, aunque el impacto sobre el crecimiento era mayor cuando los peces eran alimentados desde la época de juveniles con estas dietas. La digestibilidad y el nivel de síntesis de proteína se vio alterada, aunque no se observaron diferencias significativas en la actividad enzimática digestiva. No obstante, el impacto de las fuentes vegetales cuando no había fuentes de proteína marina en la dieta era especialmente crítico para la supervivencia de los peces. En el intestino de estos peces solo se observaron diferencias menores relacionadas con la inflamación a nivel histológico, pero también se observó una disminución en la expresión génica de genes involucrados en la inflamación y la respuesta inmune. El análisis de la microbiota intestinal reveló cambios significativos en la composición de su composición, especialmente en el intestino posterior, sugiriendo una

posible falta de capacidad de regular la respuesta inmune y de modular la colonización de bacterias patógenas tras un largo periodo de alimentación con esta dieta. Por otro lado, el análisis del proteoma de la mucosa intestinal también mostró un claro impacto sobre distintos procesos biológicos relacionados con el mantenimiento del homeostasis intestinal y de la integridad epitelial. Por el contrario, no se observó un impacto de la sustitución de la harina de pescado a nivel de expresión génica o del proteoma cuando se incorporaba a la dieta una fuente de proteína marina complementaria, aunque sí que se observaron algunos signos menores de inflamación.

Por último, se desarrolló un sistema ex vivo para estudiar la respuesta inflamatoria e inmune de la mucosa intestinal a la presencia de distintas bacterias, y se realizó un ensayo preliminar en dorada para evaluar el efecto de la dieta sobre esta respuesta.

En resumen, en este trabajo se ha realizado una evaluación extensa y detallada de los efectos a nivel intestinal de la inclusión de altos niveles de proteína vegetal en la dieta para doradas de engorde. Los resultados indican que las alteraciones en la capacidad inmune, la homeostasis y la microbiota intestinal aparecían solo cuando la proteína procedía exclusivamente de fuentes vegetales, y podrían explicar la mayor mortalidad registrada con esta dieta.

Palabras clave: dorada, proteína vegetal, intestino, capacidad digestiva, sistema inmune, microbiota, proteoma, ensayo *ex vivo*

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AA - amino acid

ANF - anti-nutritional factors

Arg – arginine

ATP – adenosine triphosphate

Av. P – available phosphorous

Ca - calcium

CF - crude fiber

Cl - chlorine

CP - crude protein

CSF-1R - colony stimulant factor 1 receptor

Cys - cysteine

FAO – Food and Agriculture Organization

FEDNA – Fundación Española para el Desarrollo de la Nutrición Animal

GALT – gut associated lymphoid tissue

HSP70 – heat shock protein 70

Ig - immunoglobulin

IL – interleukine

Ile - isoleucine

K - potassium

Lys - lysine

Met - methionine

MHCIIα – major histocompatibility complex Iiα

mRNA – messenger ribonucleic acid

Na – sodium

NCCRP-1 – nonspecific cytotoxic cell receptor protein 1

NSP - non-starch polysaccharides

pIgR – polymeric immunoglobulin receptor

PPS plant protein sources

SGLT1 – sodium glucose linked transporter 1

TNF-α tumor necrosis factor α

TGF-β1 – tumor growth factor β1

Thre - threonine

Trp - tryptophan

Val – valine

General Introduction

1. Challenges in the gilthead seabream production. Fishmeal replacement

1.1 The gilthead seabream in the aquaculture sector

The gilthead seabream (*Sparus aurata*, Linnaeus 1758) is a fish of the genus *Sparus*, included within the *Perciformes* order, the widest order of the *Teleostei* subclass. Commonly, it reaches around 35 cm in length, although it can reach a length up to 1 meter and 5-7 kg weight when it is adult.

This marine species can be found in seagrass beds and sandy bottoms in subtropical areas, specifically in the eastern coastal regions of the North Atlantic Ocean, the African coast from Cape Verde to the strait of Gibraltar and the Canary Islands, and in the Mediterranean Sea, although individuals have been also reported in the Black Sea. It is mainly carnivorous, eating small molluscs as mussels and oysters, crustaceans and small fish, but it also feeds on plant sources in an accessory way (FAO. Fisheries & Aquaculture, 2017; FishBase, 2017).

The gilthead seabream is the most produced specie in the Mediterranean area. Total aquatic production is around 200,000 tonnes, and Spain is the fourth country in the world in terms of production after Turkey, Greek and Egypt. Specifically, the Valencian community reported 5,619 tonnes in 2016. Nevertheless, production in our country has decreased in the last years (15.3% from 2015), being very far from the 23,930 tonnes in 2008, and gilthead seabream has lost the leadership in the list of most produced species in favour of the sea bass. On this sense, most of the gilthead seabream production has moved to Turkey and Greece (Apromar, 2017).

Considering gilthead seabream production from extractive fishing has stabilized in the last years, ranging from the 6,000 to 8,500 tones, the aquaculture sector currently provides more than the 95% of the total gilthead seabream offer in the market (Figure 1). Hence, consumption has a high dependence of the aquaculture activity and in this regard, the gilthead seabream is the third most valuable fish aquaculture specie in the European Community, also in terms of amount of production, after the Atlantic salmon and the rainbow trout. (Apromar, 2017).

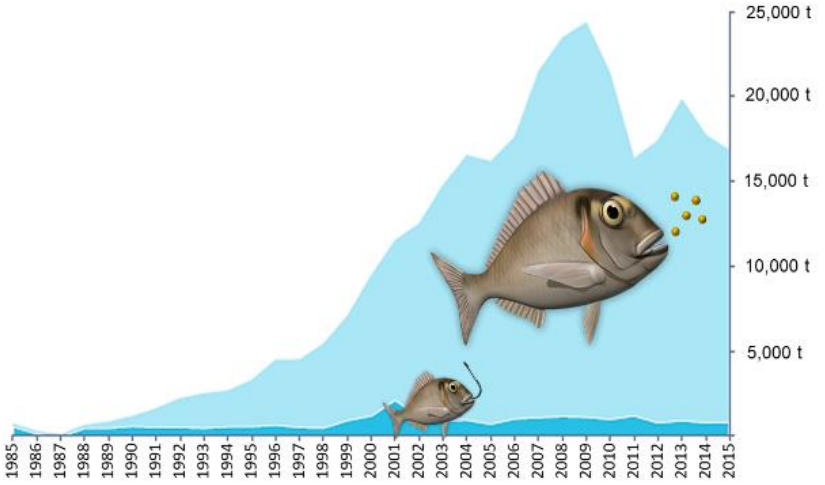

Figure 1. Evolution of gilthead seabream production sources: aquaculture, and extractive fishing, from 1985 to 2015 (own elaboration)

The intensive systems are the most extended way of production, especially using water cages. This system is used during the pre-growing and on-growing periods subsequently to other stages of production: reproduction, larval culture and juvenile growing. Water-cages production has several advantages in comparison to in-land production: is not necessary to pump water, oxygenate or treat the water, making this activity simpler and more economic. Nevertheless, it is not possible to manage temperature, so the fish need more time to reach a commercial weight. First commercial weight (350 g) is reached after 16 months, approximately. Commercial size ranges from 350 g to 1500 g.

Over the last 15 years, the main performance indicators of sea bream on-growing sector (growth, mortality and feed efficiency), which are decisive for the sustainable development of the activity, have not progressed significantly (Performance of the sea bass and sea bream sector in the Mediterranean, 2014). In order to improve the sustainability of the activity, it is necessary to accomplish new approaches to improve knowledge about the impact of the quality of juveniles and veterinary treatments on growth performance, susceptibility to disease and survival, and also about the nutritional requirements of the specie.

An increasing interest in genetics has been reported (Fernandes et al., 2017), since selective breeding programme could play an important role in the improvement of fish performance (Flynn et al., 1999; Antonello et al., 2009; Janssen et al., 2017). On the other hand, research on enhancing immune status of fish developing new preventive strategies has been an important research field until today (Reyes-Becerril et al., 2017; Bakopoulos et al., 2018).

Nutritive requirements for the gilthead seabream growing have been also widely studied, including dietary protein (45-46% in juveniles) (Santinha et al., 1996; Vergara et al., 1996), amino acid (AA) requirement (Kaushik, 1998; Lupatsch et al., 1998; Peres and Oliva-Teles, 2009; Santinha et al., 1996; Vergara et al., 1996), which are showed in Table 1, and dietary lipid level (21-22%) and essential fatty acid requirements (Benedito-Palos et al., 2009; Kalogeropoulos et al., 1992; Mongile et al., 2014; Vergara et al., 1999). A level of carbohydrates up to 20% is also desirable, since higher level can lead to detrimental effects on fish growth and feed efficiency (Enes et al., 2011). Requirements for vitamin B1 (Morris and Davies, 1995), vitamin B6 (Kissil et al., 1981), phosphorous (Pimentel-Rodrigues and Oliva-Teles, 2001), zinc and iron (Antony Jesu Prabhu et al., 2016) have been also assessed. However, information about nutritional requirements of the gilthead seabream and other farmed fish is far from complete (Jobling, 2016), especially concerning vitamin and minerals (Oliva-teles, 2000).

Table 1. AA requirements of juveniles of gilthead seabream

Essential AA	Ideal protein (g/100 g protein)
Arginine	5.55
Histidine	1.89
Isoleucine	2.55
Leucine	4.75
Lysine	5.13
Methionine + Cystein	2.60
Phenylalanine + Tyrosine	5.76
Threonine	2.98
Tryptophan	0.75
Valine	3.21

Adapted from Kaushik et al., 1998; Lupatsch et al., 1998; Peres and Oliva-Teles, 2009

1.2 Towards a sustainable production: fishmeal replacement

The high fishmeal and fish oil dietary levels in aquafeeds gives rise aquaculture would be a net consumer of fish, leading to the depletion of the natural fish grounds and to the unsustainability of the sector (Hardy, 2010). Moreover, global consumption of aquaculture products is increasing every year, so the fishmeal and fish oil necessary to satisfy the demand would also increase (Tacon and Metian, 2008).

Until 2005, fishmeal was the most cost-effective protein source, but in 2006, its price increased significantly, driving definitively to a change in aqua feeds formulation (Hardy, 2010; Tacon and Metian, 2008). In gilthead seabream farming, the cost of feed ranges from 30% to 50% of the total production cost due to high Feed Conversion Ratios (Merinero et al., 2005). Hence, this increase was especially dramatic for the production of this species and exerted a big pressure on feed manufacturers to dietary reduction of fishmeal and fish oil, leading to increase efforts on formulation of feeds containing alternative ingredients.

On this sense, composition of diets has evolved during the last years in order to securing the sustainability of the activity. However, since 'natural feed' of gilthead sea bream is being replaced by other nutrient sources, detailed knowledge on the nutritive demands of fish and the detrimental effects of alternative ingredients on fish physiology and fish performance, as well as their nutrient deficiencies (Ballester-Lozano et al., 2015) becomes even more important. Concerning fishmeal, a huge research effort on feasible replacements with alternative ingredient has been done. In fact, necessary percentages of fishmeal in feed for carnivorous species have been reduced approximately to the half (Hardy, 2010), leading to formulation of commercial feeds that contain around a 15% (Figure 2).

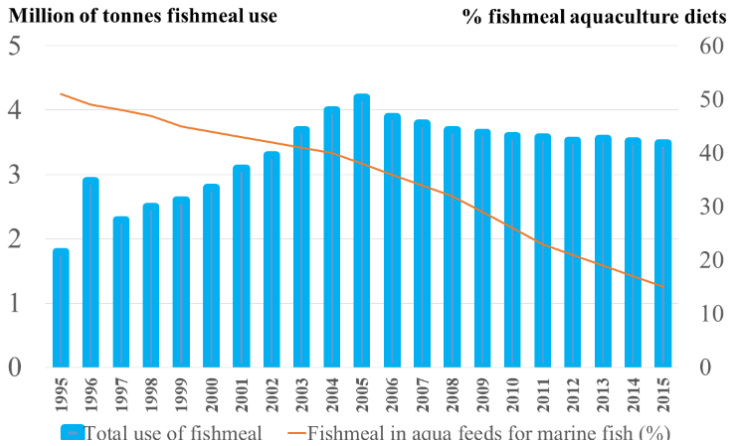

Figure 2. Evolution of total fishmeal use in aqua feeds and level of fishmeal in aqua feeds for marine fish from 1995 to 2015.
Adapted from: Tacon et al., 2011

In gilthead seabream production, several alternative sources have been assayed as replacements of fishmeal, including different vegetable sources as wheat and corn gluten (Pereira and Oliva-Teles, 2003), lupine meal (Pereira and Oliva-Teles, 2004), pea meal (Pereira and Oliva-Teles, 2002), rapeseed meal (Gómez-Requeni et al., 2004), soybean meal (Martínez-Llorens et al., 2007), sunflower meal (Sánchez-Lozano et al., 2007) or carob seed (Martínez-Llorens et al., 2012). Not only plant sources, but also algae microalgae (Shields and Lupatsch, 2012; Vizcaíno et al., 2014), processed animal proteins as blood meal and haemoglobin meal (Martínez-Llorens et al., 2008), poultry meal (Nengas et al., 1999), meat and bone meal (Moutinho et al., 2017a) or insect-derived meals (Piccolo et al., 2017) have been tested in different growing trials.

Nevertheless, although several alternative protein sources have provided positive results and look promising, cost of feed remains the main problem to be faced by the aquaculture industry. Feed companies are still trying to develop less expensive formulations by lowering fishmeal and fish oil levels but without affecting growth, feed efficiency, survival and other key productive parameters (Hardy, 2010). Partial fishmeal replacements were achieved relatively easily. However, complete or very high replacement seemed to cause several problems, including less growth, altered metabolism and negative impact on health status (Bell and Waagbø, 2008; Kaushik et al., 2004; Montero and Izquierdo, 2010).

2. Exploring new aqua feeds: plant protein sources

2.1 Potential alternative ingredients

The inclusion of alternative ingredients to fishmeal in aqua feeds formulation for aquatic species becomes crucial for the aquaculture development (Gatlin III et al., 2007). Potential alternative ingredients should possess certain characteristics:

- From a technical perspective, wide availability, competitive prices, and an easy handling, including procurement, shipping, storage and processing, are required.
- From the nutritional quality aspect, the characteristics that led fishmeal to be the main protein source for aqua feeds are the most desirable: high protein content, balanced AA profile (closed to fish requirements) high nutrient digestibility, lack of anti-nutritional factors (ANF), low levels of fibre and starch and good palatability (Gatlin III et al., 2007).

Plant protein sources (PPS) meet technical requirements, even above fishmeal, in terms of availability and price, and successfully partial replacements were achieved many years ago, even for carnivorous species, using different sources (Nengas et al., 1996; Robaina et al., 1995). The use of processed animal proteins, that also looked promising (Nengas et al., 1999), and present higher protein content than vegetable sources, a more suitable essential AA profile, and are free from ANF (Moutinho et al., 2017a) was prohibited in the European Union from 1999 to 2013, due to the arising of the bovine spongiform encephalopathy. It led scientists definitively to focus on vegetable sources as the main alternative to fishmeal. Indeed, despite the re-authorization of their use and their competitive prices, today represent only the 1% of total ingredients use in global aqua feed production (Coutinho, 2017).

2.2 Plant protein sources: drawbacks and limitations

Although PPS are potential alternative ingredients to fishmeal, they have some characteristics that limit their use in aquaculture, specially, the use in aqua feeds for carnivorous fish when they are included in high levels (Barrows et al., 2008; Gatlin III et al., 2007; Hardy, 2010; Oliva-Teles et al., 2015). These limitations, which have more or less importance depending on the source, represent challenges that are being addressed by research.

Vegetable meals present nutritional limitations (Table 2). A relatively low protein content is observed in many PPS, restricting its use in high-energy diets. Moreover, dietary essential AA profile required by fish is similar to the essential AA composition of fish (Akiyama et al., 1997), and vegetable sources display a very different profile with a limited content in some AA (Hardy, 2010). AA profile has importance not only on fish growth but also on health status, antioxidant defence and overall metabolism (Kiron, 2012; Li et al., 2009). On the other hand, non-soluble carbohydrates have a limited nutritional value for fish (Hardy, 2010; Stone, 2003).

Concerning micronutrients, fishmeal contains several vitamins, trace minerals and biologically active compounds that are also necessary to meet nutritional fish requirements, whilst vegetable meals are poor in trace elements (Hardy, 2010). Optimum levels of minerals for the different species of fish are less studied than macronutrient levels, and it is possible that some key trace and ultra-trace elements in fishmeal with an important role in fish physiology have not been identified. Not only content but also bioavailability of AAs and trace elements (Moyano López et al., 1999; Sugiura et al., 1999), that depends on the different sources (Santigosa et al., 2011), should be take into account.

PPS also show a high content in anti-nutritional factors (ANF) (Francis et al., 2001), including non-starch polysaccharides (NSP), which can disrupt the intestinal homeostasis, producing histopathological alterations and triggering an inflammatory response (Krogdahl et al., 2003). Many of them are destroyed or inactivated during manufacture or cooking extrusion pelleting of the aqua feeds (Hardy, 2010), including protease inhibitors, alkaloids, bitter-tasting compounds and lectins, which may interfere with nutrient digestion and absorption and cause disruption on the intestinal epithelial integrity. Nevertheless, immunological intolerance can be developed only with their physical presence (Gatlin III et al., 2007).

Table 2. Crude protein, crude fibre, NSP, starch, minerals and essential AA (%) in different PPS compared to fishmeal

	Moisture	CP	CF	NSP	Starch	Ca	Av. P	Na	Cl	Arg	Cys	Ile	Lys	Met	Thre	Trp	Val
Spanish corn	13.6	7.3	2.1	9.0	63.8	0.03	0.05	0.01	0.05	0.33	0.16	0.26	0.22	0.15	0.27	0.06	0.36
Soft wheat	10.3	12.9	2.4	10.8	58.4	0.05	0.15	0.02	0.08	0.62	0.28	0.45	0.36	0.21	0.37	0.15	0.55
Corn gluten	10.4	60.0	1.7	6.1	17.0	0.03	0.11	0.02	0.05	1.89	1.08	2.43	1.02	1.50	2.10	0.34	2.76
Sunflower meal	9.6	36.0	18.2	31.4	1.8	0.40	0.20	0.03	0.14	2.92	0.63	1.45	1.29	0.81	1.30	0.47	1.76
Pea	10.7	21.5	6.0	12.1	42.5	0.08	0.15	0.02	0.07	1.83	0.29	0.89	1.53	0.22	0.81	0.20	1.01
Horse bean	11.7	25.1	9.0	13.3	36.9	0.14	0.20	0.01	0.07	2.26	0.33	1.02	1.56	0.17	0.86	0.20	1.14
Soy bean meal	10.1	36.8	6.1	11.3	0.0	0.30	0.18	0.01	0.03	2.72	0.54	1.67	2.25	0.53	1.46	0.49	1.77
Soy meal	11.8	48.5	3.9	8.2	0.5	0.33	0.24	0.03	0.05	3.54	0.76	2.21	2.96	0.68	1.89	0.65	2.38
Fishmeal	7.0	70.0	0.4	0.8	0.0	2.55	2.00	0.90	1.55	4.13	0.63	2.87	5.25	1.96	2.87	0.74	3.43

Adapted from FEDNA tables, 2017
Crude protein, CP; Crude fiber, CF; Non-starch polysaccharides, NSP; Available phosphorous, Av. P

However, other ANF are not destroyed by the different processing steps (Hardy, 2010). Phytic acid can reduce the bioavailability of phosphorous and other essential minerals, especially zinc, as well as the apparent digestibility of the protein (Gatlin III et al., 2007). Hydrolysis products of glucosinolates, as goitrin and thiocyanate, inhibit the organic binding and uptake of iodin, interfering with the thyroid function (Gatlin III et al., 2007). Saponins have been related with lower feed intake (Bureau et al., 1998), interferences with cholesterol and bile salts metabolism and detrimental effects on the immune system and the gut function (Couto et al., 2014; Francis et al., 2002). Gossypol and phytoestrogens have been associated to reproductive problems in livestock and fish (Hendricks, 2002; Inudo et al., 2004). Erucic acid, xanthophylls and quercitin have also reported a negative impact on fish performance (Gatlin III et al., 2007).

Regarding NSP, although they are not toxic for fish, they are poorly digested, increasing the viscosity of the digestive content, impairing diffusion of nutrients and enzymes and binding or capturing steroids as bile acids. Hence, they can easily interfere with the uptake of dietary protein and lipids and reduce feed efficiency (Gatlin III et al., 2007; Hardy, 2010). Oligosaccharides have reported similar effects, particularly affecting the uptake of fat and minerals (Refstie et al., 1998). Beta-glucans may be also detrimental at long-term for fish (Gatlin III et al., 2007).

Finally, since fish taste preferences show low plasticity to the diet (Kasumyan and Döving, 2003), palatability of plant feedstuff could be another constraint for the use of high levels of vegetable sources in aqua feeds (Papatryphon and Soares Jr, 2001) and must be take into account during aqua feed design (Gatlin III et al., 2007). Palatability is affected by many dietary characteristics, including nutritional quality. Moreover, small soluble molecules, including certain AA (taurine, glycine, arginine, glutamic acid, alanine), betaine, nucleotides and organic acids have been recognized as feeding stimulants (Grey et al., 2009) and are rich in marine organisms like fish, squid, shrimp or krill, but less abundant in PPS, leading to a decreased palatability (Kader et al., 2010).

2.3 Classical strategies on the design of vegetable-based feeds

Different approaches have been applied to aqua feed design in order to remove or reduce the limitations of the PPS.

Using of plant high-protein concentrates, obtained by fermentation or mechanical pre-processing, partially solves the low protein and essential AA level of different vegetable raw meals and the high level of un-digestible carbohydrates such as fibre. However, prices of these concentrates are currently relatively expensive and may exceed fishmeal price. On this sense, the use of mixtures of different PPS, based on the achieve of an optimum AA profile, together with a high protein source such as corn or wheat gluten seem to be more suitable approach (Espe et al., 2006; Oliva-Teles et al., 2015).

Although it can be limited by the combination of different vegetable sources (Oliva-Teles et al., 2015), supplementation with free essential AA, especially lysine and methionine but also arginine and threonine, is sometimes needed in order to achieve the minimum essential AA requirements of the specie (Fournier et al., 2004). Inclusion of mineral supplements, as di-calcium phosphate, taurine supplementation, or the increase in the amount of vitamin and mineral premix on feeds when fishmeal is replaced, is also necessary (Gatlin III et al., 2007). Nevertheless, AA and mineral supplementation is also expensive, and the final cost of the product should be assessed in order to determine the potential commercial application of the new feed (Kissil and Lupatsch, 2004).

Impact of ANF can sometimes be also mitigated by supplementation. Phosphorous, zinc and also exogenous enzymes as phytase can be added in order to overcome the effect of phytic acid (Krogdahl et al., 2010), and triiodothyronine supplementation has proved to allow to overcome effect of glucosinolates (Hardy, 2010). Exogenous enzymes can be added to reduce negative effects of NSP, but the derived products (galaxies and xylems) are poorly tolerated by fish and may be even more problematic (Stone, 2003). Natural (heating, during the cooking extrusion process), chemical (solvent extraction) or biological approaches (addition of microorganisms) may be also used.

Use of probiotics and prebiotics can also improve the response of fish to fishmeal replacement. Gastrointestinal microbiota can be altered by dietary factors (Kim and Kim, 2013), and plays a key role on the gut

function. Therefore, inducing changes on the bacterial profile by the inclusion in vegetable-based diets of profitable bacteria (probiotics) or non-digestible food ingredient that stimulates the growth or the activity of growth- or health-promoting bacteria (prebiotics), or a combination of both (simbiotics), can lead to reduce negative impact on growth, digestion, immunity or disease resistance of the fish (Nayak, 2010). Characteristics of gut microbiota and its modulation by the diet will be discussed widely in the next section.

Nutritional value and digestibility of plant sources can also be increased by genetic engineering. New crops using genetic manipulated plants with desired characteristics, as plants with low phytic acid content (Guttieri et al., 2004) or with high lysine level (Stepansky et al., 2005)) have been tested. Production of specific selected strains of fish with a suitable genotype to adapt to fishmeal replacement could be an alternative pathway, although more studies about effects of genotype on nutrient utilization are needed.

Lastly, different feeding stimulants, such as mixtures of free AA, particularly lysine and methionine (Dias et al., 1997; Morimoto Kofuji et al., 2006), but also tissue extracts of marine organisms such as fish (Hidaka et al., 2000), shrimp (Mearns et al., 1987), mussel (Tandler et al., 1982), marine worms (Fuke et al., 1981), squid and krill (Morimoto Kofuji et al., 2006) have been used to improve palatability of formulated diets and increase feed intake. In addition, these supplementations may also help to combat nutritional deficiencies in alternative high plant protein-based diets (Kader et al., 2012), since marine by-products show a balanced AA profile and high amount of free essential AA, especially lysine and methionine, which are deficient in some of the main plant sources (Gatlin III et al., 2007).

2.4 Assessing the effects induced by plant protein sources

Enormous progresses have been achieved in the design of low-fish meal diets based on vegetable sources for gilthead sea bream and other carnivorous species. Indeed, it is feasible to formulate diets with higher levels of plant protein without impairing growth performance (Oliva-Teles et al., 2015), as has been observed in several assays using mixtures of different PPS (Benedito-Palos et al., 2016; De Francesco et al., 2007; Gómez-Requeni et al., 2004; Sitjá-Bobadilla et al., 2005). Successful total fishmeal replacements have even been reported in terms of growth, but with a high level of synthetic AA supplementation,

leading to mixtures more expensive than the fishmeal-based diets (Kissil and Lupatsch, 2004; Monge-Ortíz et al., 2016).

Nevertheless, despite the big effort on developing high plant proteins diets, these aqua feeds are still often associated to a reduced growth performance, feed intake, and high susceptibility to infection and disease (Krogdahl et al., 2010; Oliva-Teles, 2012), parameters which have a high impact on productivity in gilthead seabream farms. In order to better understand these detrimental effects of plant sources, different tissues and organs have been studied, including the liver, the head kidney, the muscle or the blood (Figure 3).

However, most of the research has focus on the intestinal status. The gastrointestinal tract is the first organ exposed to the different ingested ingredients and has a direct interaction with the environment, and research on this field suggest that the impact of dietary changes on fish performance can be explained by the disruption of the gut homeostasis (Baeza-Ariño et al., 2014), since the intestine plays a key role on digestion, absorption and metabolism of dietary nutrients, osmoregulation and immune defence (Minghetti et al., 2017). Thus, physiology of the gut and its response to dietary changes, particularly high levels of plant protein inclusion is often the main point of interest when new aqua feeds are being assayed.

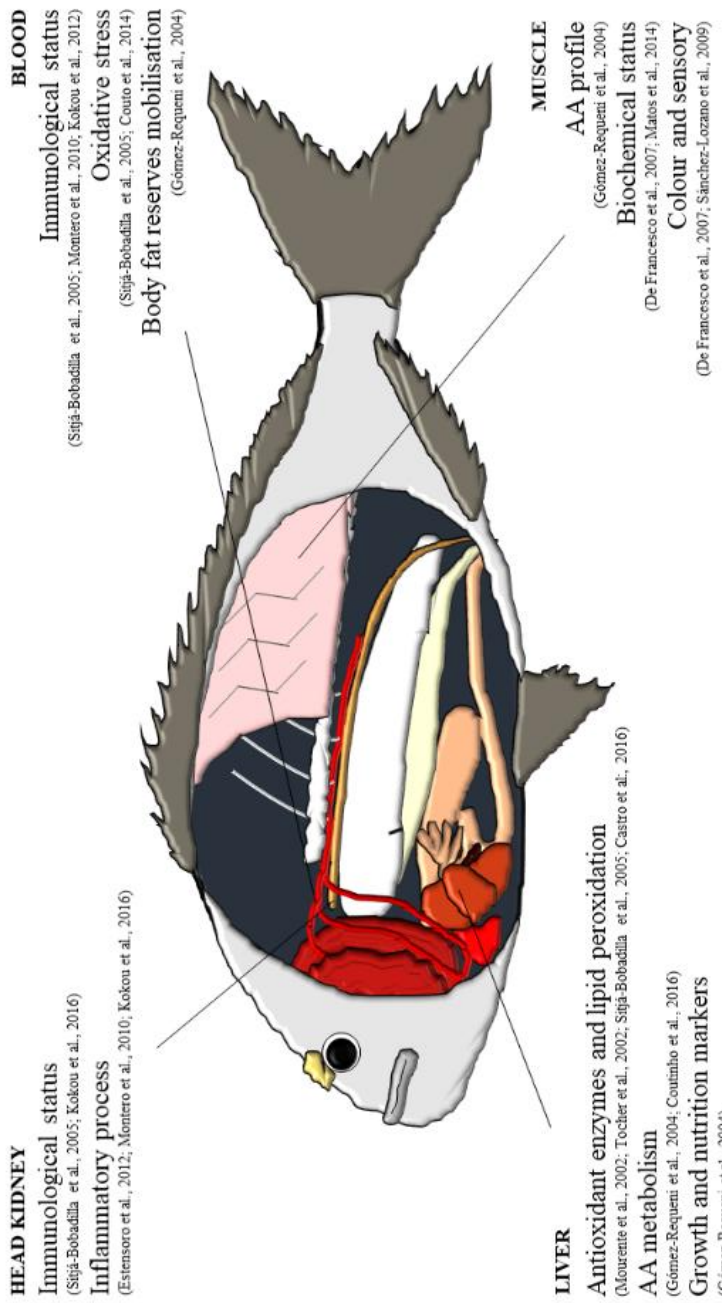

Figure 3. Previous studies evaluating the impact on different tissue and organs (except the intestine) in the gilthead seabream (own elaboration)

3. The gilthead seabream gut

3.1 The digestive tract

The anatomy, morphology and histology of the digestive tract of the gilthead seabream (Cataldi, 1987) has been previously studied in detail.

As a typical teleost, the gilthead seabream digestive canal shows a short oesophagus, which enters into the Y-shaped stomach with a very extensible bottom. The gastrointestinal pattern is typically carnivorous, with a well-defined stomach followed by a short intestine, with a relative length of 0.5-0.6. Digestion of food starts at the stomach and is completed in the intestine, the main organ for nutrient absorption. Four short finger-like appendages, the pyloric caeca, are located just at the beginning of the intestine, at the point where the *ductus hepaticus* and the *ductus pancreaticus* discharge. This specialization seems to have the function of extending the intestinal surface area in many fish (Clements and Raubenheimer, 2006). Finally, the gut forms two bends, and after the second bend, a narrowing, corresponding to a valve, is observed.

3.2 Intestinal structure and environment

3.2.1 Intestinal layers and cell types

Basic organization of the gut wall is similar to other fish or vertebrates (Banan Khojasteh et al., 2009). Four different layers can be distinguished along the gut from the inside out, with some modifications depending on the region: mucosa, submucosa, muscular and serose (Figure 4).

The mucosa is the innermost layer, and consists of the epithelium and the lamina propria. This layer is typically folded (Cataldi, 1987), in order to increase its functional surface. Intestinal villi and crypts as described in mammals are not found in fish (Aghaallaei et al., 2016), but many scientific works refer to the folds (or projections), typically villiform in the gut of different teleost, as villi (Baeza-Ariño et al., 2014; Banan Khojasteh et al., 2009; Cerezuela et al., 2013a; Fleurance et al., 2008). In fact, mucosal folds and furrows between folds can be compared with villi and crypts in the mammalian small intestine (Stroband and Debets, 1978). Other works have even distinguished between short and long villi (Omnes et al., 2015).

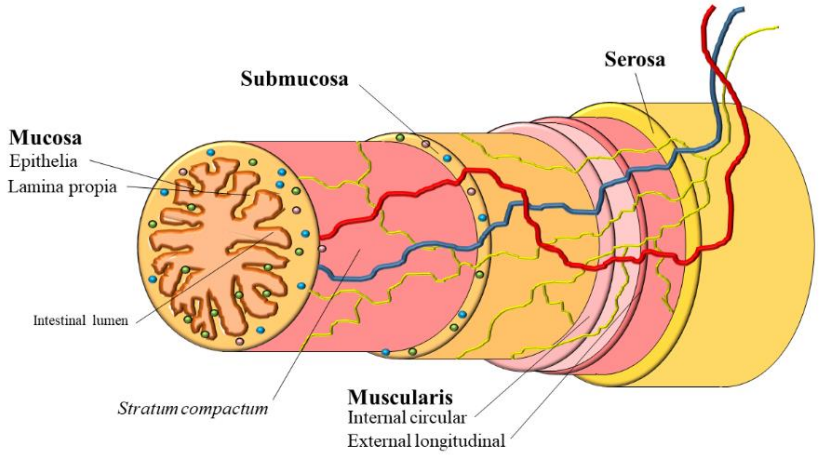

Figure 4. Layers in the intestine of the gilthead seabream (own elaboration)

The epithelium is coating the mucosa in all its surface and lining with the intestinal lumen. It is formed by a single layer of columnar epithelial cells, the enterocytes, and mucus-secreting cells, the goblet cells (Figure 5). Moreover, some infiltrate leucocytes, mainly T-lymphocytes, and a few B cells, can be also observed in the epithelium (Salinas et al., 2011), on the base or between the epithelial cells, as well as different endocrine cells (Elbal and Agulleiro, 1986).

The enterocytes are the main cells, and they principal function is the absorption of the nutrients. They are polarized structure, exhibiting pronouncedly differences between the apical and the basal part (Korneva and Bednyakov, 2011). In the apical side, cell membrane specializations called microvilli increase the apical cellular surface, forming the brush border membrane. It is covered by the glycocalyx, a filamentous structure with high levels of transmembrane mucin glycoproteins which protects the enterocyte and contributes to digestion of nutrients. However, it has not received too much attention in fish (Løkka and Koppang, 2016). The nuclei are located in the mid part towards the basal part. In the cytoplasm, mitochondria are highly abundant and the presence of vacuoles, related with the lipids uptake and digestion, and secondary lysosomes, is also normal in the apical part (Cerezuela et al., 2013a).

Different multiprotein structures intervene in the enterocytes junctions. From the apical to the basal side, tight junctions, adherens junctions and

desmosomes have been identified and described in different fish species (Banan Khojasteh et al., 2009; Hu et al., 2016). Tight junctions are composed by different transmembrane proteins, including occludins, tricellulins junctional adhesion molecule proteins and claudins, which interact with intracellular plaque proteins, mainly zona ocludens family and cingulin, in contact with the enterocytes cytoskeleton (Hu et al., 2016; Wells et al., 2017). Adherens junctions are composed by cadherin-catenin interactions, and together with tight junctions, regulate the function of the epithelial barrier (Sundell and Sundh, 2012).

Goblet cells display an enlarged apical cytoplasm, full of mucus-secretion granules, and narrow basal side. They are located between the enterocytes, although are less frequent in the apical part of the villi (Baeza-Ariño et al., 2014). They can contain acid or neutral content, although most of them have a mixture of both secretions (Elbal and Agulleiro, 1986). The subtypes and goblet cells distribution varies spatially along the gut (Baeza-Ariño et al., 2014; Deplancke and Gaskins, 2001) and can be an indicator of the physiological state of the fish (Cerezuela et al., 2013a).

Enterondocrine cells can be found isolated and scattered between the enterocytes, principally in the pyloric caeca and in the first part of the anterior intestine. They secret different hormones and regulatory peptides with a role in the digestive physiology, including pancreatic secretion, intestinal motility and the gallbladder contraction. Gastrin, substance P, pancreatic polypeptide, cholecystokin and met-enkephalin immunoreactive cells were identified in the intestine of the gilthead sea bream (Elbal and Agulleiro, 1986).

Finally, intestinal stem cells have been described in the furrows at the base of the intestinal folds in teleost species (Aghaallaei et al., 2016). Renewal of intestinal epithelium is necessary every certain period of time: cell proliferation and differentiation takes place at the base of the folds (Faro et al., 2009; Rombout et al., 1984) and then differentiated cells are pushed and migrate towards the fold tip (Aghaallaei et al., 2016), where they are shed, evidencing a dynamic system in a continuous transition state.

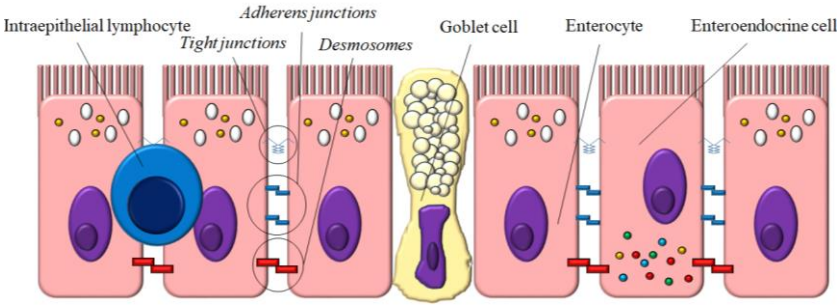

Figure 5. Cellular types in the epithelial layer. Enterocyte junctions (own elaboration)

The underlying lamina propria provides support and nutrition to the epithelia. It consists of a connective tissue net, nourish and oxygenate by lymphatic and blood capillaries, which contains a set of different immune-related cells, including but not limited to lymphoide cells, macrophages and eosinophilic and neutrophilic granulocytes (Rombout et al., 2011; Salinas et al., 2011).

The submucosa layer, as the lamina propria, is formed by a dense loose connective tissue and is crossed by blood and lymphatic capillaries and vessels which also support the mucosa and muscular layers (Groman, 1982). In many vertebrates, a muscular layer (*muscularis mucosae*) is observed between the tunica mucosa and the submucosa, but in several teleost fish, the *muscularis mucosae* is not present and a more or less distinct thick layer of densely packed connective tissue fibres named *stratum compactum* is observed (Banan Khojasteh et al., 2009; Carrassón et al., 2006). Specifically, in gilthead seabream, the *stratum compactum* is not clearly observed with light microscopy, and the lamina propria and the submucosa seem to form a continuous entity containing different immune cells. Indeed, the submucosa is often not defined as a separate layer in teleost (Løkka and Koppang, 2016).

Regarding the tunica muscularis, two different smooth muscular fibre layers, the internal circular and external longitudinal can be observed, separated by the myoenteric nerve plexus within a connective tissue with elastic fibres. Finally, the serosa consists on a loose connective tissue layer covered by a mesothelium and is attached to the mesenteric tissue, providing nutrients to the epithelium through blood vessels (Al Hussaini, 1949).

There is not a definitive consensus about considering two or three different sections in the intestine of the seabream. From an anatomic point of view, three sections can be considered: the section between the stomach's pyloric sphincter and the first bend of the gut (foregut, or anterior intestine), the section from the first bend to the ring-shaped narrowing after the second bend (midgut or mid intestine) and the section after the valve until the anus (hindgut or posterior intestine) (Venou et al., 2006). However, some research works have stablished other limits (Omnes et al., 2015; Pérez-Sánchez et al., 2013), also considering two sections divided by the second bend and calling foregut to the part of the alimentary canal from the mouth to the stomach (Calzada et al., 1998). From a histological perspective, although high differences along the gut are not observed, two different parts could be considered: the anterior intestine, which corresponds approximately to anterior and mid intestine, and the lower intestine or hindgut, which displays a higher number of goblet cells (Cataldi, 1987; Omnes et al., 2015). Differences in the direction of the folds (Calzada et al., 1998) and in the type and number of enteroendocrine cells (Elbal and Agulleiro, 1986) have also been reported between sections.

3.2.2 The gut mucus

The gastrointestinal mucus rests on the glycocalyx of the enterocytes and covers the single layer intestinal epithelium. It acts as a medium of protection, lubrication and transport between the luminal contents and the epithelium, and provides an ideal environment for the adhesion and growth of bacteria. It is composed of water (95%) and includes salts, lipids, phospholipids, cholesterol and a wide set of biologically active components, including antibodies, lysozymes, complement proteins, antibacterial peptides, lectins and pentraxins (Gómez and Balcázar, 2008). However, mucins are the major components of the mucus layer and the responsible of its properties of viscosity and elasticity (Bansil and Turner, 2006). They are extracellular high molecular weight glycosylated proteins synthetized and secreted by goblet cells (Johansson et al., 2013). that provides a big array of potential binding sites to bacteria. They tend to aggregate and form gels, exhibiting liquid crystalline order, and have particular adhesive and diffusion-through properties (Bansil and Turner, 2006). Hence, composition affects adhesiveness, viscoelasticity, transport and protective capacity of the mucus (Gomez et al., 2013), although the interaction with bacteria needs to be investigated in more detail (Løkka and Koppang, 2016).

Intestinal mucins have been characterized in gilthead seabream (Pérez-Sánchez et al., 2013). Structurally, there are two different families of mucins: the secreted gel mucins, including Mucin 2, Mucin 2-like and Mucin 19, and the membrane-bound mucins, which form the epithelial glycocalyx, such as Intestinal-Mucin, Mucin 13 and Mucin 18. From a chemical perspective, mucins are classified into neutral and acidic mucins (Deplancke and Gaskins, 2001): acidic mucins seem to protect against bacterial translocation (Deplancke and Gaskins, 2001), whilst neutrals mucins are related to digestion processes (Grau et al., 1992). Moreover, recent studies suggest that some mucins are also involved in signalling pathways related with cell proliferation, differentiation, adhesion, immune response, apoptosis, bacterial adhesion or inhibition and secretion of cellular products (Pérez-Sánchez et al., 2013), and can be used as biomarkers of the physiological status.

3.2.3 The gut microbiota

The intestinal microbiota is characterized by a high population density, a wide diversity and the high level of interaction with the surrounding environment (Denev et al., 2009), whose high load of organic material supports microbial growth (De Schryver and Vadstein, 2014). Its involved in a variety of gut functions, including metabolic, development and protective processes, contributing to the maintenance or disturbance of the normal nutritional, physiological and immunological status of the host (Nayak, 2010).

From a structural perspective, two different groups: the adherent or autochthonous microbiota, which colonize successfully the epithelia or the mucus, and is a part of the intestinal environment, and the transient or allochthonous microbiota, which is present transiently and temporarily in the intestinal tract, since it is not able to colonize the mucus layer or the epithelium (Denev et al., 2009; Nayak, 2010)

The composition and diversity of the gut microbiota of fish is the result of a complex process that involves the colonization, establishment and competition among different groups of bacteria, Several exogenous and endogenous factors are implicated, including host factors, as the developmental stage (Refstie et al., 2006), the gut structure (Sugita et al., 1985), the health status (Denev et al., 2009; Nayak, 2010) and the genetic background (Navarrete et al., 2012), nutrition factors, as the dietary form, protein source, dietary lipids and use of feed additives

(Romero et al., 2014), and environmental factors, including geographical location (Roeselers et al., 2011), water temperature (Sugita et al., 1989), season (Hagi et al., 2004) rearing and farming conditions (Ringo and Strom, 1994), salinity and trophic level (Wong and Rawls, 2012) and the existence of stressors (Lesel and Sechet, 1982). However, studies in zebrafish suggest that fish could present a core microbiota (Rombout et al., 2011), evidencing that there is a co-evolution of fish and the gastrointestinal microbiota and environment has little influence on microbiota composition. Equilibrium between species of resident bacteria provides stability in the microbial population (Denev et al., 2009), and diversity could be important in order to improve the response against different factors. On the other hand, differences among the different intestinal regions have been also described (Molinari et al., 2003).

Although several groups of microbes are represented, bacteria are the main constituent of the gut microbiota. Proteobacteria, Firmicutes and Actinobacteria seem to be the main phyla in fish gut microbiota (Navarrete et al., 2012, 2010; Wu et al., 2010). Acinetobacter, Achromobacter, Aeoromonas, Alteromonas, Bacteroides, Corynebacterium, Cytophaga, Flavobacterium, Micrococcus, Moraxella, Pseudomnas, Proteobacterium and Vibrio are the predominant genus in the gastrointestinal tract of the marine fish (Blanch et al., 1997; Brunvold et al., 2007; Cahill, 1990; Onarheim et al., 1994; Ringø et al., 2006; Zhou et al., 2009). Moreover, different lactic acid bacteria, including genus Lactobacillus, Lactococcus, Streptococcus, Leuconoc and Carnobacterium (Ringø and Gatesoupe, 1998) are also observed. Yeast have been also isolated in the fish intestine (Denev et al., 2009). The gastrointestinal microbiota of the gilthead seabream has been previously characterized by different techniques (Kormas et al., 2014; Parma et al., 2016; Savas et al., 2005; Silva et al., 2011), and most of the bacterial groups observed belonged to the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. However, individual fluctuations should be also take into account (Silva et al., 2011).

3.3 Intestinal function

The intestine of fish is a complex multifunctional organ which interacts directly with the surrounding environment. In conjunction with the associated organs (liver, glad bladder and pancreas) and with the

commensal microbiota that harbours, the gut mucosa carries out several functions, including digestion, absorption and metabolism of dietary nutrients, balance of water and electrolytes, immunity response of the host against pathogen and other aggressions from the external environment, elimination of contaminants and toxic metabolites from the environment, and the hormonal regulation of these processes (Buddington and Krogdahl, 2004; Denev et al., 2009; Minghetti et al., 2017).

3.3.1 Digestion and absorption of nutrients

After digestion in the stomach, chime containing non-digested proteins, peptides, lipids and carbohydrates enters to the intestine, whose role includes the secretion of digestive enzymes and hormones, harbouring the digestion of the nutrients and absorb and metabolize different types of molecules generated by the digestion process. A schematic view of the digestion process is showed in Figure 6.

Protein digestion in the intestinal lumen is carried out by different alkaline pancreatic-secreted proteolytic enzymes, including trypsin, chymotrypsin (endopeptidases) and carboxypeptidases (exopeptidases), which are secreted as inactive zymogens and then activated by active trypsin (endopeptidase). Resultant peptides can be hydrolysed to AAs by exopeptidases in the intestinal lumen, by brush border enzymes as aminopeptidases (exopeptidase) or by intracellular peptidases after transport across the enterocytes membranes (Santos Couto, 2013).

Lipids, including triglycerides, phospholipids, waxes and free fatty acids (Leaver et al., 2008) are hydrolysed by lipases, secreted by the pancreas, in presence of bile acid released by the gall bladder. Bile acid emulsify lipids and fat-soluble vitamins, favouring the action of lipases and the formation of lipid micelles. Carboxyl ester lipase seems to be the main lipase in marine fish (Kurtovic et al., 2009), although other lipase-type enzymes, as pancreatic lipase-colipase (Leger et al., 1977) and lipases from enterocyte including phospholipase (Tocher, 2003; Tocher et al., 2008) have been reported. Regarding carbohydrates, pancreatic α-amylase breaks α-(1-4)-glucoside bonds in starch polysaccharides, releasing di- and oligo-saccharides (Kokou et al., 2016) which are subsequently hydrolysed into monosaccharides by different disaccharidases in the brush border membrane. Maltase,

isomaltase, trehalase, sucrose and lactase have been identified in fish (Clements and Raubenheimer, 2006).

On the other hand, another brush border enzyme secreted by the enterocytes as the alkaline phosphatase is capable to dephosphorylate different molecules, including nucleotides, proteins and lipids. Its importance in fish digestion is completely understood, but seems to have more significance as a mucosal defence factor, dephosphorylating bacterial lipopolysaccharides (Chen et al., 2011). Nevertheless, it has been suggested as a marker of lipid absorption and enterocyte maturation (Bates et al., 2007).

General mechanisms of nutrient absorption across the brush border membrane of the enterocyte seem to be more or less conserved compared to mammals (Collie and Ferraris, 1995). Different absorption pathways have been described: nutrients can enter to the enterocytes by simple diffusion following a concentration gradient or by specialized membrane transporters (transcellular pathway), by facilitated or active transport. Another set of transporters in the basolateral membrane is responsible of the transport of not-metabolized nutrients from the enterocytes to the blood vessels in the mucosa layer, although diffusion processes are also described. Other molecules directly follow the paracellular pathway, diffusing between the enterocytes.

Di- and tripeptides are absorbed by the mediation of a brush border membrane transporter, the Peptide transporter 1 (Verri et al., 2010), and then hydrolysed by intracellular peptidases. Most of the luminal AAs are absorbed by active transport (Krogdahl et al., 2005), and present overlapping specificity to common specific transporters (Collie and Ferraris, 1995).

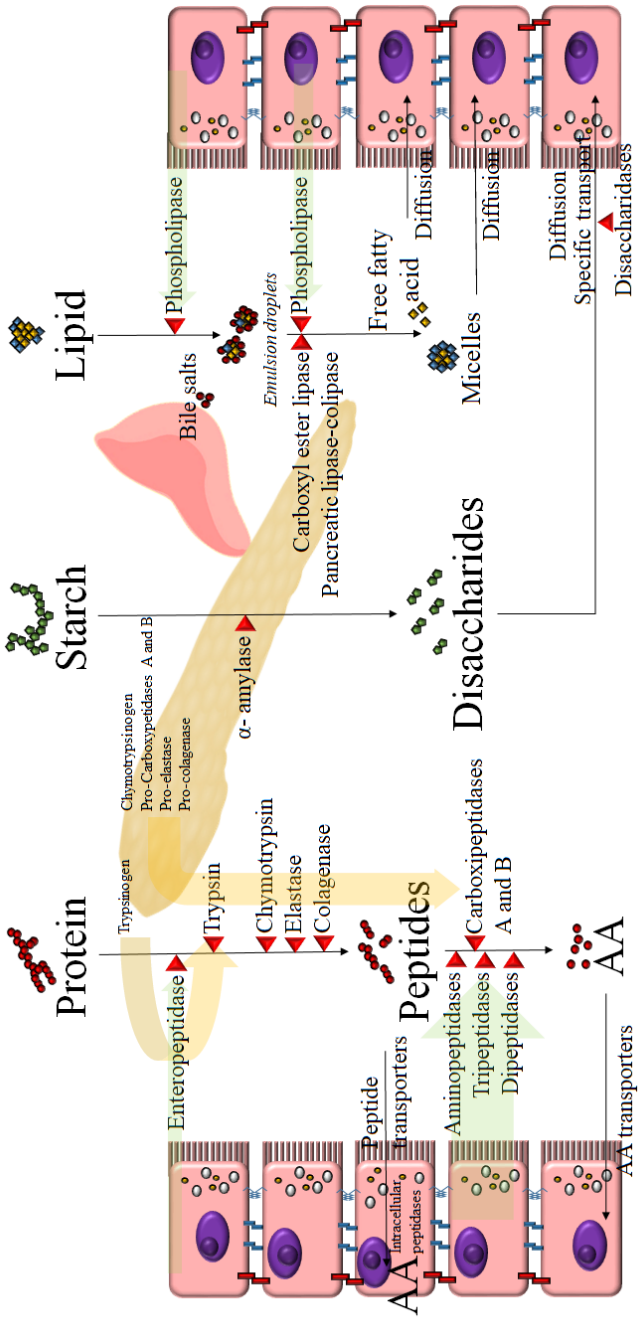

Figure 6. Schematic view of the digestive process of protein, starch and lipids in the intestine (own elaboration)

Molecules derived from lipid digestion are absorbed mainly by passive diffusion, although some membrane transporters participate in the absorption of some fatty acids and cholesterol (Bakke et al., 2010; Tocher, 2003). Then, lipid droplets are accumulated in the apical region of the enterocytes, metabolised and packed into lipoprotein particles that exit the enterocytes by exocytosis and reach the circulatory system (Caballero et al., 2003; Hernandez-Blazquez et al., 2006). Glucose and monosaccharides also enter the enterocytes by diffusion of by specific transporter mediation in the brush border membrane (SGLT1).

The proximal region, together with the pyloric caeca, seem to be the most active section in the digestion process and nutrient uptake (Nordrum et al., 2000) of peptides (Terova et al., 2013), carbohydrates (Krogdahl et al., 2005) and lipids (Hernandez-Blazquez et al., 2006; Nordrum et al., 2000; Tocher, 2003). However, the posterior region has proved to participate actively in the nutrients absorption process. AA transporters are present along the entire intestine with different rates of nutrient uptake (Santigosa et al., 2011), and zymogen granules secreted by goblet cells, which are more abundant in the distal part, are also involved in protein digestion (Al Hussaini, 1949). The high number of supranuclear absorptive vacuoles in distal enterocytes evidences that the uptake of intact macromolecules, such as proteins, takes place (McLean and Ash, 1987), although this could be more related with processes of antigen presentation for mucosal immunity than with nutrition processes (Quentel et al., 2007). Finally, the hindgut also plays a major role in lipid digestion in some species (Izquierdo and Henderson, 1998) and shows higher permeability to water-soluble molecules (Bakke-McKellep et al., 2000).

Intestinal digestive function is regulated by a set of hormones, regulatory peptides and neurotransmitters that can act in a paracrine (local signalling), endocrine (between the gut and the associated organs, liver, gallbladder and pancreas) or inter-organ way (between the gut and other organ systems) (Buddington and Krogdahl, 2004). The gastrointestinal tract is the large endocrine organ in vertebrates (Holst et al., 1996) and a wide number of different endocrine cells is observed (Elbal and Agulleiro, 1986) Although the specific cell types are species-dependent (Youson and Al-Mahrouki, 1999), signalling molecules secreted in response to chime entrance and pH reduction seem to be similar to mammals, including gastric inhibitory peptide, cholecystokinin, somatostatin, secretin, glucagon or peptide YY (Buddington and Krogdahl, 2004).

3.3.1.1 Role of intestinal microbiota

Gastrointestinal microbiota has also a role in the digestion and absorption of nutrients. Different phyla, including Proteobacteria, Bacteroidetes, Firmicutes and Fusobacteria are able to produce a set of different enzymes, including proteases, amylases, cellulases, lipases, chitinases, phosphatases and esterases (Bairagi et al., 2002; Gutowska et al., 2004; Ray et al., 2012) that can contribute to digestion. Different vitamins, AAs and metabolites can also be produced by gastrointestinal bacteria (Nayak, 2010). For example, anaerobic bacteria can supply volatile fatty acids from fermentation (Clements, 1997), release butyrate (Kapatral et al., 2003) and produce vitamins (Roeselers et al., 2011), including B12 (Sugita et al., 1994), while Actinobacteria can produce extracellular enzymes and a wide range of different secondary metabolites (Ventura et al., 2007). A role in the metabolism and trafficking of cholesterol, in the uptake of protein macromolecules (Bates et al., 2006) and in the host protein metabolism (Clements et al., 2009) in the hindgut has been suggested. However, is difficult to characterize the exact contribution of the gut microbiota, since many species are observed in a dynamic and complex environment as the gut (Romero et al., 2014).

3.3.2 Innate and adaptive immunity

The fish immune system involves different organs and comprises a wide set of innate and adaptive molecules and cells that allow the colonization of mucosal surfaces by commensal microbial communities and, at the same time, protect the host against pathogenic invasions (Gomez et al., 2013), in order to maintain a healthy status. Gut, skin and gills represent the major mucosal surfaces and immune barriers in teleost fish where the intestinal microbiota and antigen from the diet or the environment cross-talk with fish and the major part of the immune response is developed (Gomez et al., 2013).

The gut associated lymphoid tissue (GALT) in fish (Figure 7) shows similar characteristics to mammalian type 1 mucosal surfaces and exert similar physiological functions (Iwasaki, 2007). However, intestinal immunity in mammals is more complex, and some elements, lymph nodes, Peyer's patchs, M cells, Ig A and J chain are missed or well stablished in teleost fish (Rombout et al., 2011), which display a more diffusely organisation. Hence, two different compartments can be

differentiated: the lamina propria, containing a big set of lymphoid cells, macrophages, eosinophil cells, neutrophilic granulocytes and dendritic cells, and the epithelial layer, containing intraepithelial lymphocytes (Rombout et al., 2011; Salinas et al., 2011).

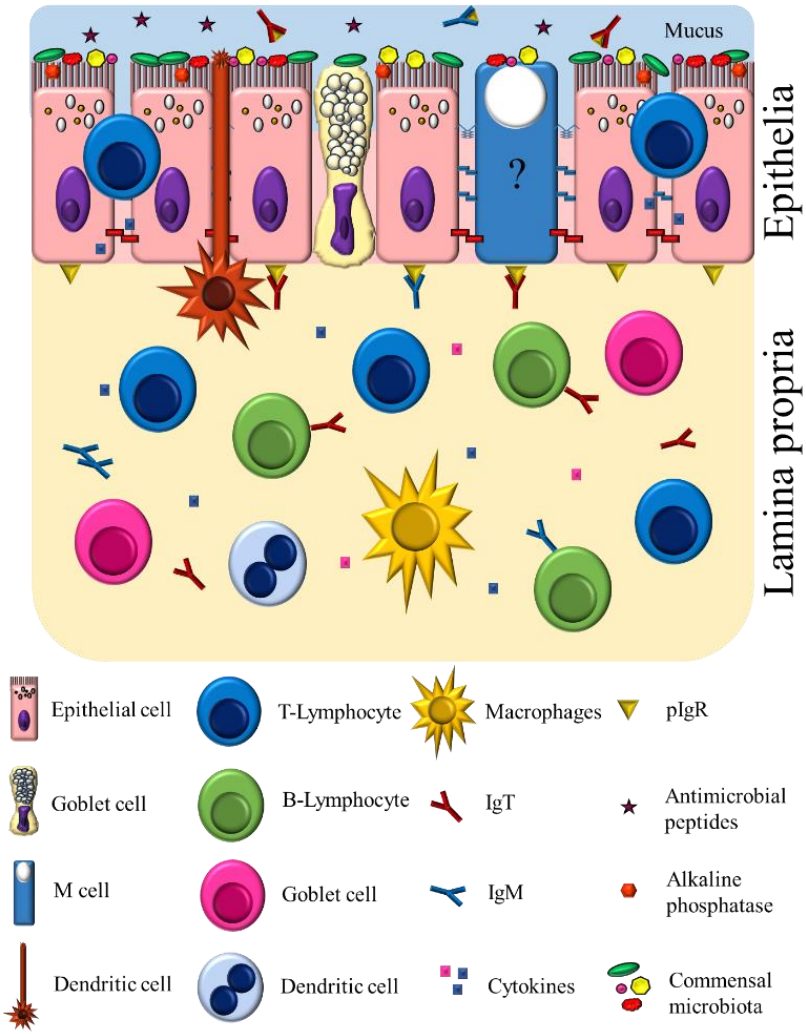

Figure 7. Schematic view of the gut mucosa surface and the elements in the GALT in teleost (own elaboration)

Additionally, the mucus layer covering the intestinal mucosa, the gastrointestinal microbiota and the acids, bile salts and enzymes secreted to intestinal lumen also contribute to the innate immune defence creating a hostile environment for the development of fish

pathogen (Gómez and Balcázar, 2008), and a balanced and dynamic interaction among all them is crucial to the maintenance of the intestinal homeostasis and the prevention of inflammatory or intestinal injury (Cerezuela et al., 2012).

Two different elements should be considered: the innate or nonspecific immune system, and the adaptive, acquired or specific immune system (Gómez and Balcázar, 2008), both involving a humoral and a cellular response.

3.3.2.1 Innate immunity

The innate system is crucial in fish since the production of antibodies is slower than in other vertebrates and is highly dependent of temperature (Ellis, 2001). It main function is the non-specific defence, the antigen presentation and the regulation of the immune response mediated by cytokine and chemokines (Gómez and Balcázar, 2008), providing a primary response against microbes including phagocytosis and the induction of the inflammation (Montalban-Arques et al., 2015). Specifically, the posterior intestine has been associated with important immune-regulatory functions (Løkka and Koppang, 2016) and showed a stronger antigen uptake capacity (Dimitroglou et al., 2010).

Mucus is involved in bacteria adhesion and antigen transport and provides the substrate in which the antibacterial mechanisms can take place (Gomez et al., 2013; Gómez and Balcázar, 2008). The level of mucins can modulate the effectiveness of the mucus layer, altering bacterial adhesion to the underline epithelium and intestinal permeability (Kim and Ho, 2010) and therefore the abundance and distribution of different bacteria (Asselin and Gendron, 2014). Additionally, it provides the substrate for a big set of biologically active components, including complement proteins, lysozyme, antimicrobial peptides, lectins and natural antibodies (Ellis, 1999; Gomez et al., 2013), which are part of the mucosal secretion and are involved in the innate immune responses against pathogen but also in the tolerance mechanisms to commensal microbes (Gomez et al., 2013; Nussbaum and Locksley, 2012). Their importance in defence mechanisms seems to differ between different species, although functions are commonly conserved in vertebrates (Gomez et al., 2013). Different elements identified in the humoral innate immunity in fish (except cytokines, which will be addressed later), and their functions, are listed in Table 3.

Table 3. Function of different molecules involved in the innate immunity of fish

Element	Function	References
Complement	Lysate of pathogen and apoptotic and necrotic cells. Initiation of inflammatory response. Modulation of adaptive immune response	Lange et al., 2004; Løvoll et al., 2006; Shen et al., 2011
Anti-microbial peptides	Anti-bacterial, anti-viral and anti-fungal activities	Paulsen et al., 2001; Silphaduang and Noga, 2001; Sveinbjornsson et al., 1996
Transferrin	Immune regulation. Antimicrobial and antioxidant activity. Macrophages activation	Bayne and Gerwick, 2001; Stafford and Belosevic, 2003
Pentraxins	Recruitment of complement and phagocytes to pathogen and damaged cells	Kodama et al., 1997
Lectins	Opsonisation. Activation of complement	Beck et al., 2012; Vaishnava, 2011
Antiproteases	Inhibition of bacteria proteinases	Alexander and Ingram, 1992; Hjelmeland, 1983
Natural antibodies	Identification of foreign conserved structures (nucleic acids, heat shock proteins, carbohydrates and phospholipids)	Sinyakov et al., 2002

Adapted from Gomez et al., 2013; Gómez and Balcázar, 2008

Regarding the cellular response, intrinsic immune cells but also enterocytes carry out immune functions. Enterocytes have a key role in the regulation of gut homeostasis, commensal colonization and immune response (Hill and Artis, 2010), since they interact directly with the microbiota through different pattern recognition receptors, including lectins, nod-like receptors and toll-like receptors, that recognize microbe-associated molecular patterns (Gomez et al., 2013). They produced intestinal alkaline phosphatase, preventing inflammation in response to commensal microbiota (Bates et al., 2007), and different antimicrobial peptides such as the β-defensin (Montalban-Arques et al., 2015). Moreover, enterocytes are responsible of antigen uptake in teleost, which takes place mainly in the mid or posterior intestine (Løkka and Koppang, 2016), and may induce tolerance, lead to an inflammatory and immune response by the release of cytokines and/or the activation of the adaptive primary and secondary response (Løkka and Koppang, 2016; Martin and Król, 2017). In contrast, some authors have suggested the presence of M-like cells as the responsible of

luminal antigen sampling (Fuglem et al., 2010), and a role of goblet cells and leukocytes have also been suggested (Løkka and Koppang, 2016). On the other hand, enterocytes participate actively in the immunoglobulin (Ig) transport from the lamina propria to the intestinal lumen (Rombout et al., 2011). Finally, the high rate of epithelial renewal could be an effective tool to remove pathogen (Gomez et al., 2013).

Innate immunity cells can be activated by recognition of different pathogen associated molecular patterns, including lipopolysaccharides, lipoproteins, flagellin, peptidoglycan, or bacterial nucleic acid structures mediated by pattern-recognition receptors (Montalban-Arques et al., 2015), or by cytokines secreted by other immune cells (Gómez and Balcázar, 2008). As a response, these cells initiate a response based on the production of antimicrobial molecules, including inflammatory cytokines and interferon (Le Page et al., 2000).

Eosinophilic granule cells, similar to mast cells in humans, are also abundant in the gut (Reite and Evensen, 2006) and seem to be recruited to inflammation sites in teleost. They contain different antimicrobial peptides, as lysozyme or piscidine (Silphaduang and Noga, 2001; Sveinbjornsson et al., 1996), but not much is known about they function. Phagocytic cells play an important role in the protection of the host. Macrophages and neutrophilic granulocytes are observed in teleost, even as the main antigen presenting cells (Rombout et al., 2011). Nevertheless, certain studies defend that macrophages are absent (Mulero et al., 2008) or in a low level (Salinas et al., 2008) in the gut of the gilthead seabream. Neutrophils are abundant in serum and are recruit to inflammation sites, and a low number in relation to eosinophils has been related to a healthy gut status (Rombout et al., 2011). Mucosal dendritic cells are not well stablished in the gut mucosa of fish, and although they have been characterized in zebrafish and rainbow trout (Bassity and Clark, 2012; Lugo-Villarino et al., 2010), seem to be scarce within the gut (Wittamer et al., 2011). Finally, nonspecific cytotoxic cells have been identified in different species, and a similar function to mammalian natural killer cells has been suggested (Evans and Jaso-Friedmann, 1992; Greenle et al., 1991), being rare in blood and common in lymphoid tissues.

Communication between different innate cells can be mediated by specific receptors in direct cell-to-cell contact or by the production of chemical messengers, the cytokines. These are secreted by host immune

cells in response to microbial antigens or molecules released by host damaged cells, and are involved in cell communication processes, regulating inflammatory mechanisms, including mucin synthesis (Sitja-Bobadilla et al., 2008). As in mammals, pathogenic and commensal bacteria could trigger the expression of pro-inflammatory and anti-inflammatory cytokines, respectively (Gomez et al., 2013). Among principal cytokines, the TNF-α and IL-1β are important pro-inflammatory cytokine in response to gram negative bacteria and are involved in the regulation of cellular activation and proliferation, cytotoxicity and apopotosis (Gómez and Balcázar, 2008). IL-6 is also related with the pro-inflammatory cascade response (Gómez and Balcázar, 2008; Savan and Sakai, 2006), but have reported anti-inflammatory effects (Scheller et al., 2011), as IL-10 and transforming growth factor β (Gomez et al., 2013; Moore et al., 2001). On the other hand, Interferon can interfere in the viral replication.

3.3.2.2 Acquired immunity

Activation of adaptive response can take place by cell to cell contact events mediated by adhesion molecules or by the secretion of cytokines. Different cytokines are involved in the communication between the innate and the acquired immune system and in the activation and proliferation of different cell subpopulations (Fischer et al., 2013; Uribe et al., 2011).

Lymphocytes carry out the cellular adaptive immunity response. T-lymphocytes interact with the bacteria present in the mucosal surface, developing tolerance or immunity against bacteria (Nutsch and Hsieh, 2012). They are abundantly present in the lamina propria but also in the intraepithelial area (Rombout et al., 2011; Salinas et al., 2011). This cell group includes, in most vertebrates, cytotoxic T lymphocytes, T helper cells and T regulatory cells (Fischer et al., 2013), that have a different role in the adaptive immune response, also in teleost. T- cell activation and induced proliferation leads to a T-cell mediated response executed by cytotoxic lymphocytes in the gut mucosa, including intraepithelial lymphocytes (Galindo-Villegas et al., 2013).

The function of B-lymphocytes consists on recognizing antigen and producing Igs against those antigens. However, B lymphocytes also secrete antibodies prior antigen activation (Gómez and Balcázar, 2008): these natural antibodies may be very important in fish since affinity maturation or class switch processes are not appreciable in fish

antibodies (Magor and Magor, 2001). Moreover, in some teleost species, a phagocytic and bactericidal activity has been identified in B cells in the systemic lymphoid organs (Zhang et al., 2010), that could also exist in mucosal B-lymphocytes. They are abundant in the lamina propria of both anterior and posterior intestine, especially in the distal part (Abelli et al., 1997); although can also be present in the epithelial region in a lower degree. Development of memory by the maintain of a B-cell profile against pathogens is much less developed than in mammals (Montalban-Arques et al., 2015).

Igs are the main components of the humoral adaptive immunity. In teleost, three different isotypes have been described: IgM, IgD and IgT. IgM is the most abundant Ig in the plasma of teleost and the main responsible of systemic humoral adaptive response, but is also observed in the response against pathogen in the different mucosal tissues, including the intestine (Salinas et al., 2011). IgD seems to be expressed in all immune tissues in teleost (Flajnik, 2002), but has not been correlated to mucosal immunity, whilst IgT isotype is associated to gut mucosal function, showing there a higher concentration than in serum, which suggests that it plays a key role in gut immunity (Gomez et al., 2013; Zhang et al., 2010). IgM, IgT and their response to infection and dietary challenges have been widely described in gilthead seabream (Piazzon et al., 2016). Polymeric immunoglobulin receptors (pIgR) in enterocytes bind and transport the secreted Igs across the gut mucosal epithelium (Rombout et al., 2011), and are probably involved in immune exclusion of commensal bacteria. The extracellular part of this receptor is co-secreted with the Igs, having a protecting role (Brandtzaeg et al., 2008). In addition to phagocytosis and pathogen neutralization, immune exclusion of commensal microbiota seems to be mediated by specific secreted antibodies that coats the resident bacteria surface (Zhang et al., 2010).

As the nutritional function, the immune function is regulated, besides from by cytokines, by an inter-organ signalling network and central nervous system nervous inputs (Buddington and Krogdahl, 2004). Thus, stress and other exogenous and endogenous factors can influence the immune response by hormonal regulation (Yada and Nakanishi, 2002).

3.3.2.3 Role of intestinal microbiota

The gut microbiota is crucial in the development and maturation of the gut immune system (Nayak, 2010). Early colonization and the subsequent interaction between bacteria and the gut, by the stimulation of the innate immunity (Leadbetter et al., 2002) or by the antigen uptake and presentation (Olafsen and Hansen, 1992), generates an immuno-physiologic regulation, leading to epithelial proliferation and maturation and to development, stimulation and maintenance of the immune system (Nayak, 2010).

Pathogenic, non-pathogenic and commensal bacteria coexist in the gut environment. Resident microbes have a significant role in intestinal immune responses, coexisting with the host in a dynamic equilibrium through continued and active signalling (Falkow, 2006). They have proved to enhance stability of the epithelial barrier and enterocyte proliferation (Cheesman et al., 2011) and to modulate the mucosal immune response (Rombout et al., 2011), Moreover, as a part of the natural resistance of the fish, they can exclude potential invaders by different mechanisms: by competitive exclusions for adhesion sites and nutrients (Gómez and Balcázar, 2008; Nayak, 2010; Rombout et al., 2011), determining the colonization capacity of other bacteria and limiting the interaction between fish pathogenic bacteria and the gut mucosa (Denev et al., 2009), by the production of organic acids, hydrogen peroxide, antimicrobials as bacteriocins, siderophores, lysozyme and antiviral substances (Gomez et al., 2013; Nayak, 2010; Rombout et al., 2011) and by modulation of the gut immune response against other bacteria (Denev et al., 2009; Rawls et al., 2004; Rombout et al., 2011).

Nevertheless, if the equilibrium between the commensal microbiota and the host is disturbed, present pathogen can growth and lead to a pathogenic status (Sekirov and Finlay, 2009). An imbalance of the microbiota ecology in favour of pathogenic groups or the reduction of the bacterial diversity (dysbiosis) could potentially damage some specific bacterial populations, lead to immune-related disadvantages and at last, to the occurrence of diseases (Montalban-Arques et al., 2015; Rombout et al., 2011).

3.3.3 Other functions

The gut is also involved in detoxification processes in fish, eliminating toxic metabolic wastes and lipophilic xenobiotic, which are transformed by cytochrome P-450 enzymes and eliminated by solute transporters called multidrug resistance proteins or P-glycoproteins (Cai et al., 2003; Doi et al., 2001). On the other hand, the gastrointestinal tract, together with the gills, kidneys and tegument, has an essential role in regulating the interchange of water and electrolytes with the environment (Bodinier et al., 2010), although the intestine has a minor role compared to oesophagus. As a euryhaline teleost, the gilthead sea bream needs to compensate the osmotic loss of water and passive gain of ions by the ion and water regulation (Kültz, 2015; McCormick, 2001). Water is absorbed following the paracellular pathway (Bischoff et al., 2014), whilst salts are absorbed (Na^+, K^+ and Cl^-) or secreted by different transcellular mechanisms, with an important role of the enzyme Na^+/K^+-ATPase (Bodinier et al., 2010; Foskett et al., 1983; Kültz, 2015).

4. Impact of plant sources on intestinal status: previous studies in gilthead seabream

A clearly impact on the gut structure and physiology has been described when high levels of fishmeal on aqua feeds are replaced by alternative PPS, affecting, as a consequence, the fish performance during the on growing periods. PPS inclusion in diets has been related with inflammatory processes and lack of capacity to regulate the mucosal integrity (Krogdahl et al., 2010), epithelial cells turnover and sloughing (Chikwati et al., 2013a), reduction in the activity of enzymes and nutrient transport in the brush border (Venold et al., 2013), deregulation of pancreatic proteases activity (Chikwati et al., 2013b) and reduction in the reabsorption of cholesterol and bile salts (Kortner et al., 2012). I.e., vegetable sources may affect all the functions in which the intestine is involved, and several research works have been performed in order to assess this impact in the intestine of the gilthead sea bream.

4.1 Effect on the intestinal structure and morphology

Different grades of histomorphological alterations have been described in fish at intestinal level in response to vegetable based diets (Krogdahl et al., 2010). Capacity to digest efficiently vegetables sources varies

among species, and the tolerance to plant ANF, which could be the cause of the gut morphology disruptions, seems to depend on the specie (Bonaldo et al., 2008) but also on the intestinal section (Baeza-Ariño et al., 2014), the age of the fish (Martínez-Llorens et al., 2007), the plant protein (Overland et al., 2009) and the level of substitution (Martínez-Llorens et al., 2012). Moreover, morphological changes can also reflect the digestive system adaptation to the vegetable sources: in the case of the gilthead seabream an effective trophic adaptation to plant based diets has been observed (Omnes et al., 2015), e.g, the compensatory increase in the relative length of the intestine (Santigosa et al., 2008) or in the villi length (Aslaksen et al., 2007; Omnes et al., 2015), allowing carnivorous fish to increase the functional surface for the digestion (Buddington et al., 1987) and compensate a lower activity of the brush-border membrane enzymes.

In salmonids, which have received greater attention, histopathological changes and inflammation have been reported commonly and more intensely in the distal intestine (Baeverfjord and Krogdahl, 1996), leading to enteritis, especially when soybean meal is included (Krogdahl et al., 2010).

4.1.1 Previous studies in gilthead seabream

Although the posterior intestine is more sensitive to fishmeal replacement than the anterior section in the gilthead seabream (Baeza-Ariño et al., 2014), it seems that gilthead seabream can tolerate high levels of plant sources in diets (Kokou et al., 2012; Sitjá-Bobadilla et al., 2005), and only moderate changes are observed in the distal intestine in most of the research works (Figure 8), without damage in the gut epithelia, even no histopathological changes in the proximal and distal intestine of on-growing gilthead seabream supporting the idea they can better tolerate high levels of vegetable sources inclusion in comparison to juveniles (Martínez-Llorens et al., 2007).

Classical morphological alterations used to include changes on the thick, shape and coalescence of the different gut folds, the expansion of the lamina propria, the increase on the infiltration of the submucosa, the lamina propria and the epithelial layer by different inflammatory cells, changes on the number and type of goblet cells and on the length and width of villi and microvilli. Additionally, the increased presence of fat vacuoles and supranuclear protein droplets in the enterocytes in the hindgut (Bonaldo et al., 2008; Kokou et al., 2017, 2015, 2012; Monge-

Ortíz et al., 2016; Santigosa et al., 2008; Sitjá-Bobadilla et al., 2005), which suggests a decreased proteolytic activity that lead to high absorption and accumulation in the distal part (Couto et al., 2016), were observed. Histological alterations were only found with a high or total fishmeal substitution (Santigosa et al., 2008; Sitjá-Bobadilla et al., 2005), but far from enteritis reported in other species (Heikkinen et al., 2006; Krogdahl et al., 2003; Refstie et al., 2000).

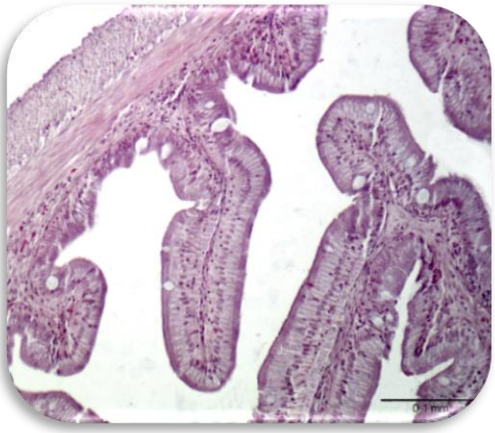

Figure 8. Detailed of villi in the foregut of gilthead seabream fed with high plant protein levels after haematoxylin eosin stain (20x). No significant morphological changes were observed

However, some aspects remain unclear. Martínez-Llorens et al., (2012) reported the reduction in villi length and thickness, the lamina propria, the serous layer thickness and the number of goblet cells, suggesting a reduction of the absorptive area and a lower mucus production. In contrast, Baeza-Ariño et al., (2014) also observed a significant reduction in the thickness of the serous layer, the villi and the lamina propria in the distal intestine with increasing levels of plant protein inclusion, but villi length and the number of goblet cells was increased. A link between the decrease of villi thickness and increase of goblet was suggested, based on a compensatory response to an unsatisfactory protein digestion, but it is not always observed (Monge-Ortíz et al., 2016). On the other hand, the reduction of the number of goblet cells (Martínez-Llorens et al., 2012; Santigosa et al., 2008) has been linked with the reduction of mucus production and lower intestinal protection. Finally, a relation between the reduction of villi length and the increase of dietary crude fibre was also suggested (Baeza-Ariño et al., 2014), but opposite observations have been also reported (Santigosa et al., 2008)(Nogales-Mérida et al., 2010).

4.2 Effect on the mucus and gut microbiota

The number of goblet cells, which produce and secrete mucin polymers (Estensoro et al., 2013) can be increased or decreased in response to different dietary changes. Although not many studies have been carried out focusing specifically on the impact of PPS on the gut mucus composition, quantitative and qualitative changes in the fish mucin secretion pattern (Estensoro et al., 2013; Løkka and Koppang, 2016) in response to pathogenic invasions (Álvarez-Pellitero, 2011), immunostimulants and probiotics (Cerezuela et al., 2012) or nutritional and pathological challenges (Pérez-Sánchez et al., 2013) have been reported.

Mucin synthesis and secretion is linked to innate immunity and to inflammatory processes taking place in the intestine (Álvarez-Pellitero, 2011; Sitjá-Bobadilla et al., 2005), and mucus is intimately associated with the commensal microbiota and changes in its composition can modulate the colonization of the intestinal tract by different bacterial groups (Estensoro et al., 2013). Hence, since fishmeal replacement can lead to immune dysfunctions (Sitjá-Bobadilla et al., 2005) or changes in the gut microbiota (Silva et al., 2011), it can also lead to qualitative or quantitative alterations of the mucus secretion (Álvarez-Pellitero, 2011).

On the other hand, the composition and diversity of the gut microbiota of fish is highly influenced by dietary factors nutrition factors (Romero and Ringo, 2014), and these impact has been assessed during the last decade using different techniques (Cerezuela et al., 2013a; Cordero et al., 2015; Dimitroglou et al., 2010; Guerreiro et al., 2016; Moutinho et al., 2017b; Savas et al., 2005).

4.2.1 Previous studies in gilthead seabream

The impact of PPS in the gilthead seabream intestinal microbiota has been studied. Silva et al., (2011) suggested a decrease of equitability and evenness in the intestinal microbiota in response to partial replacement. Kormas et al., (2014) observed a reduction of diversity from the reared individuals to the conventionally reared fish, although also suggested the existence of a core community in the gilthead seabream gut, similar to observed in zebrafish (Roeselers et al., 2011). Parma et al. (2016) did not observe significant differences in response to PPS, although some increasing trends with the level of replacement

were observed for *Cyanobacteria* and *Lactobacillaceae*. However, research works addressing this topic are scarce. Moreover, the technique used in the microbial analysis (Carda-Diéguez et al., 2013) and the type of sample collected (faeces, intestinal contents or tissues) will highly determine the microbial fraction characterized (allocthonous, authocthonous, or both), and then it's difficult to determine the real contribution of different species to the intestinal status, which would be of great interest for probiotics industry.

4.3 Effect on the digestion and the absorption of nutrients

The use of PPS leads to interferences with the performance of digestive enzymes and nutrient transporters (Alarcón et al., 1999; Fountoulaki et al., 2005; Santigosa et al., 2011), which determine the capacity of fish to digest and absorb dietary nutrients (Santigosa et al., 2011)(Vizcaíno et al., 2014). The activity of different enzymes has proven to be altered when they are included at high levels (Santigosa et al., 2008), maybe explained by ANF and enzyme inhibitors (Francis et al., 2001; Gatlin III et al., 2007) fibre content and the specific physicochemical properties of vegetable proteins (Hardy, 2010). Variations in the activity of brush border enzymes, related to absorptive capacity, in response to fishmeal replacement have been also reported in different species (Bakke-McKellep et al., 2000; Kokou et al., 2016). Both alterations can lead to modifications in the availability of dietary nutrients in the gut lumen.

AAs from different ingredients (and free AA) may have different bioavailability, so the utilisation of AAs of plant proteins mixture based diets is asynchronous (Gatlin III et al., 2007; Santigosa et al., 2011). Furthermore, intestinal transport system in fish shows overlapping specificity, with different AAs sharing the same transporter with different affinity: therefore, luminal abundance of each one can affect the rate of absorption of others, reducing their bioavailability (Berge et al., 2004). These interactions between AAs in alternative vegetable-based aqua feeds needs to be considered.

Histological modifications in response to PPS could also influence the effectiveness of nutrient transporters, since diffusion and absorption efficiency may be affected by the state of the enterocyte cell brush border membrane (Houpe et al., 1997). Moreover, a compensatory mechanism consisting on the up-regulation of transport capacities, by increasing transporters density or altering tissue mass (Collie and

Ferraris, 1995)(Krogdahl et al., 2005) has been suggested in fish to compensate eventual nutritional deficits such as can be caused by plant protein diets (Gómez-Requeni et al., 2004; Santigosa et al., 2011). Nevertheless, this mechanism has not proved to be efficient in gilthead seabream (Gómez-Requeni et al., 2004; Santigosa et al., 2011).

4.3.1 Previous studies in gilthead seabream

In gilthead seabream, a negative impact of PPS on the activity of digestive proteases and other enzymes located in the intestinal brush border of the gilthead seabream has been reported (Kokou et al., 2016; Santigosa et al., 2008; Silva et al., 2010), but not always leading to differences in terms of growth (Monge-Ortíz et al., 2016). This impact on digestive capacity is in concordance with the lower AA digestibility coefficients observed in response to higher levels of fishmeal replacement by vegetable ingredients can lead to lower AA digestibility coefficients (Dias et al., 2009), but greater retention coefficients, since the AA composition of fish is relatively stable and diets with high levels of fishmeal substitution use to contain lower amounts of essential AAs (Martínez-Llorens et al., 2012; Sánchez-Lozano et al., 2009). Besides, increases in ammonia production with high plant protein levels in diets (Bonaldo et al., 2011; Robaina et al., 1995) supports an excess or imbalance of dietary AAs and a decrease in the level of protein synthesis (Lied and Braaten, 1984; McGoogan and Gatlin III, 1999; Velazco-Vargas et al., 2014). Finally, protein source proved to modulate the expression of nutrient transporters (Terova et al., 2013), which could explain the observed decrease in the uptake capacity for different essential and non-essential AA in response to high levels of vegetable protein (Santigosa et al., 2011).

4.4 Effects on the inflammatory and the immune response

The inclusion of high levels of PPS in aqua feeds for carnivorous species can be considered as a stress factor, and as such, it leads to an immune response by the host (Tort, 2011). Detrimental effects on the immune system can be mediated by disturbances or morphological damage in the intestinal epithelium, changes in the microbiota composition or disruption in the functionality of mucus and of the epithelial barrier, triggering also an initially inflammatory response (Baeza-Ariño et al., 2014). The grade of impact depends on the specie (Santigosa et al., 2011), the level of plant ingredients (Sitjá-Bobadilla et al., 2005) and the duration of the dietary treatment (Tort, 2011).

Generally, immune system is less affected at short term, but after long periods facing the stressor, immune mechanisms that demand a continuous energy supply may collapse, leading to depressive or suppressive effects (Tort, 2011) and ultimately to fish disease (Martin and Król, 2017). Moreover, a sustained immune response also contributes to changes in the animal performance, redirecting energy and other resources from different physiological processes in order to face the stressor (Martin and Król, 2017). In rainbow trout, the suppression of innate immune capacity has been reported (Burrells et al., 1999), but inflammatory processes or immune stimulation has also been observed in other trials (Krogdahl et al., 2000; Rumsey et al., 1994). In the gilthead seabream, Sitjá-Bobadilla et al., 2005 reported that fishmeal substitution by a mixture of vegetable sources causes an anti-oxidative effect above 75% level of replacement and increased complement activity at 50% level, but decreased above 75%, suggesting a possible immunosuppression, whilst growth was only affected by total replacement.

Histological assessment of the intestine morphology in fish fed with high plant protein has revealed some morphological alterations that have been related with an inflammatory status, although generally no histopathological signs have been observed in the gilthead seabream. However, the evaluation of the immune impact, and especially, of the mechanism that mediates the response of the host require more than morphological observation.

In teleost, changes in inflammatory and immune markers have been registered in the intestine when they are fed with low fishmeal diets, and the triggering of a pro-inflammatory status was observed as a common response. In sea bass, the up-regulation of different pro-inflammatory cytokines in the distal gut of the sea bass was observed, as well as the enlargement of the submucosa layer and the negative impact on the GALT capacity against bacterial infection (Torrecillas et al., 2017). Salmonids developing enteritis reported a short-term up-regulation of several immune-related genes, but also genes involved in the proteolysis, transport, metabolism and detoxification (Sahlmann et al., 2013), and authors suggested that the maintenance of an inflammatory status during some days could explain the subsequent downregulation of genes related with endocytosis, exocytosis, detoxification, transporters and metabolic processes, leading the intestine to a lack of digestive, protective and metabolic capacity. Even in omnivorous species such as the common carp (*Cyprinus carpio*)

partial replacement has reported high gene expression of various inflammatory markers, morphological alterations, cell infiltration and decreased uptake capacity of the enterocytes (Urán et al., 2008). It suggests that inflammatory and immune response depend on the specie, but plant sources are, in any event potential inflammatory agents (Francis et al., 2001; Krogdahl et al., 2003).

4.4.1 Previous studies in gilthead seabream

In gilthead seabream, dietary modulation of several molecules involved in intestinal immunity, such as cytokines and other inflammation and immune-related proteins, Igs, cytoskeletal proteins, tight and gap junction proteins, proteins related with cell differentiation and proliferation, pattern recognition receptors, antimicrobial peptides, the alkaline phosphatase or the transferrin, has received great attention (Calduch-Giner et al., 2012; Cerezuela et al., 2013b; Estensoro et al., 2012; Guzmán-Villanueva et al., 2014; Montero and Izquierdo, 2010; Pérez-Sánchez et al., 2015; Piazzon et al., 2016; Reyes-Becerril et al., 2013, 2011, 2008; Santos Couto, 2013). However, specific studies addressing the long-term effects of high plant protein diets in adult gilthead seabream are scarce (Table 4).

Table 4. Impact of different fishmeal replacement formulations on the intestinal immune status of gilthead seabream reported in previous studies

Diet	Effect	Reference
60% bioprocessed soy	Greater number of leukocytes Upregulation of CSF-1R Stability in HSP70, MHCIIα, NCCRP-1	Kokou et al., 2015
>20% soy protein concentration	Higher expression of HSP70, MHCIIα, NCCRP-1 Stability in CSF-1R, TGF-β1 and β-2-microglobulin	Kokou et al., 2017
5% level of fishmeal	Up-regulation of pro-inflammatory markers and higher grade of cell infiltration in submucosa in the foregut Changes in antioxidant defences, cell differentiation and proliferation, epithelial architecture and permeability, immunity and mucus production (upregulation in foregut, downregulation in hindgut)	Estensoro et al., 2016

Among the effects noticed, it seems that a common response to different high plant protein formulation is not observed. For instance, CSF-1R, which has been linked with macrophage activation (Chen et al., 2004; Roca et al., 2006), inflammation or infection (Chitu and Stanley, 2006), and downregulated in response to probiotics (Reyes-Becerril et al., 2008), but also different heat-shock proteins, related with the immune response, and markers of nonspecific cytotoxic cells and antigen presenting cells, respectively, were differently expressed in response to different dietary treatments, although intestinal inflammation was observed in both cases (Kokou et al., 2017, 2015). On the other hand, previous studies also suggest that different immune and homeostatic mechanisms in gilthead seabream can be altered in a different way along the gut by PPS (Estensoro et al., 2016).

5. Assessing the gut status: traditional and novel techniques

Initially, studies regarding fishmeal replacement focused on suitability of potential new alternative ingredients according to dietary requirements and on the levels of inclusion. On this sense, fish growth, growth and nutritive parameters, fish body composition, macronutrient and micronutrient digestibility were traditionally assessed. However, the necessity of formulation of diets with very low fishmeal level demands a broad knowledge about the interactions between diet and fish physiology and the contribution of different tissues and organs (Rodrigues et al., 2012). Hence, the knowledge provided by different fields such as chemistry, biochemistry, physiology, microbiology, microscopy, immunology and molecular biology is crucial in order to obtain a global comprehension (Jobling, 2016).

The study of the dietary effects on the intestinal status needs the integration of the research about the ingredients, the feed intake and the physiological mechanisms involved in its regulation, nutrient requirements and utilisation, metabolic pathways, fish growth, immune status and stress resistance (Jobling, 2016). Commonly, approaches as the histology assessment, determination of digestive enzyme activities, nutrient uptake and retention studies, microbiological studies, gene expression assays or epithelial resistance assays have been carried out.

Nevertheless, in the last decades, -omics technologies have been widely implemented in farm animal field, including aquaculture (Lokman and

Symonds, 2014; Rodrigues et al., 2012). These high-throughput tecniques allow to perform large-scale studies of the whole set of transcripts (transcriptomics), proteins (proteomics), metabolites (metabolomics) or microbial genomes (metagenomics) in a particular tissue, moment and conditions, since they are dynamic from the temporal and spatial point of view and response to different intrinsic and extrinsic factors. These studies contribute to obtain a holistic view of the molecular physiology and to better understand the molecular mechanisms involved responses (Martin et al., 2016). Thus, they can provide more information than techniques that only focus in individual and/or predetermined elements (Lokman and Symonds, 2014) and to identify gene networks and metabolic pathways in which common research is not usually focusing (Martin and Król, 2017). In the case of the study of fishmeal and fish oil replacement impact in the gut, dietary nutrients may influence gene activation, protein expression, enzyme activity, metabolism and the microbiota in the intestine (Jobling, 2016)(Martin and Król, 2017), and - omics have been used to determine changes in the gut transcriptome (Calduch-Giner et al., 2012) and microbiome (Kormas et al., 2014; Parma et al., 2016). To the best of our knowledge, proteomics and metabolomics have not been used in the assessment of dietary impact in the intestine up to today.

On the other hand, most of research about dietary impact on the intestinal status has been performed following through *in vivo* experiments, in which different variables are assessed or analysed after a feeding period, using different experimental diets and with variable conditions, including bacterial challenges. However, some effects may be difficult to evaluate by *in vivo* approaches, especially when specific and controlled experimental conditions are required. Therefore, *in vitro* studies based on isolated cells (Boltaña et al., 2014; Sepulcre et al., 2007) or established cell lines (Minghetti et al., 2017) culture, but also ex vivo studies, which allow to maintain the histological features and interactions between cells (Resau et al., 1991), can complement knowledge provided by in vivo approaches. In fish, the intestinal sack *ex vivo* method has been used in order to evaluate changes in the morphology or the associated microbiota after exposure to bacteria (Hartviksen et al., 2015; Nematollahi et al., 2005), allowing to evaluate the interactions between specific bacterial strains and the intestine. Moreover, this type of approaches may contribute to reduce the number of fish in research, but they are limited by the short term viability of the intestinal tissue in *ex vivo* conditions.

Justification and objectives

Aquaculture faces with the challenge of developing new alternative and sustainable aquafeeds for the production of different species, including gilthead seabream. These new feeds need to be based on alternative sources, with lower cost in comparison to fishmeal and fish oil based diets but no detrimental effects on the fish performance in terms of productivity. Plant sources meet many of the necessary characteristics in terms of availability and price for being used as a replacement to fishmeal. In this regard, promising results have been achieved after partial substitution of fishmeal by PPS, compensating nutritional limitations by different strategies in the design of diets. Nevertheless, high plant proteins are still associated to reduce growth, feed intake and immune capacity.

Fish performance is a complex process which depends on the physiology of different body tissue and organs. Therefore, in the field of aquaculture nutrition research, the gut physiological responses become one of the main point of interest, since the intestine represents a complex biological system that interacts directly with dietary ingredients and environment, plays a major role in the nutrients digestion and absorption and in the immunity.

Both stablished and novel whole-tissue level techniques can contribute to shed light on the physiological response of the intestine to high levels of plant sources and in the interactions between nutrition, intestinal microbiome and immune system. This new knowledge will contribute to improve in the design of new feed strategies.

The present thesis started on the confirmed hypothesis that the inclusion of vegetable sources at high levels in aquafeeds for on-growing gilthead seabream has negative effects on gilthead seabream performance than can ultimately lead to high impact on productivity.

The overall goal was to characterise the long-term changes happened at intestinal level in response to plant protein by using different approaches in order to obtain further knowledge on the interactions between diet, nutrition and immunity that take place in this tissue. In this regard, the following aspects were given particular attention: fish growth and nutritive performance, protein and AA digestibility and retention and ammonia excretion, and on intestinal enzyme activity, histology, expression of genes related with inflammation, immunity, structure and digestion, gut mucosa proteomic profile and metagenome have been assessed along the present work.

Specific objectives of this thesis were:

- To assess the effect of high plant protein-based experimental diets on growth and digestive performance in the on-growing gilthead seabream
- To characterize, for the first time in gilthead seabream, the impact of total fishmeal replacement:
    - on the expression of inflammatory, integrity and immune-related genes
    - on the gut microbiota composition
    - on the gut mucosa proteome
- To integrate the results of classical and novel approaches in the assessment af the intestinal status
- Developing a new method to study the dietary impact on the interactions between immune status and intestinal bacteria by gene expression determination.

Considering three different dietary levels of fishmeal replacement (50%, 75% and 100%) by a mixture of different PPS, and the complementation (or not) with 15% level of squid and krill meal in the case of the high partial (75%) or total replacement (100%), in order to improve AA profile, six different experimental diets were initially formulated (Table 1):

Table 1. Experimental diets assayed

Diet	Fishmeal replacement level (%)	Marine by-products inclusion (%)	Essential AA supplementation (g/kg)
FM100 / FM / CTR	0%		
FM50	50%		Met(5)
FM25+	75%	15%	Met(5), Lys(5)
FM25	75%		Met(5), Lys(5)
FM0+ / VM+	100%	15%	Met(5), Lys(10)
FM0 / VM / AA0 / PP	100%		Met(7), Lys(10), Arg (5), Thr(3)

Analyses in different Chapters are summarized in Figure 1. In **Chapter 1**, the long-term impact of the different experimental diets on growth and nutritive parameters, biometrics, whole-body composition, AA and protein digestibility, ammonia excretion and digestive enzyme activity, in order to determine the maximum feasible level of replacement without affecting the growth and digestive performance of the on-growing gilthead seabream.

The following chapters focused on the intestinal response of gilthead seabream to total fishmeal replacement, complemented or not with alternative marine ingredients (VM+ and VM, respectively), compared to response to the fishmeal based diet (FM). **Chapter 2** evaluated the changes at histological level and in the expression of genes related with inflammatory events, the immune system, the maintenance of the integrity of the gut epithelia and nutrient digestion. **Chapter3** evaluate the composition and diversity of the intestinal prokaryotic community in the groups FM100 and AA0. The impact on the proteomic profile of the gilthead seabream intestinal mucosa was also evaluated on **Chapter 4**.

Finally, **Chapter 5** described an *ex vivo* procedure to assess the short-term changes in the expression of genes related with the intestinal defence performance in response to presence of different pathogen and its modulation by dietary protein source.

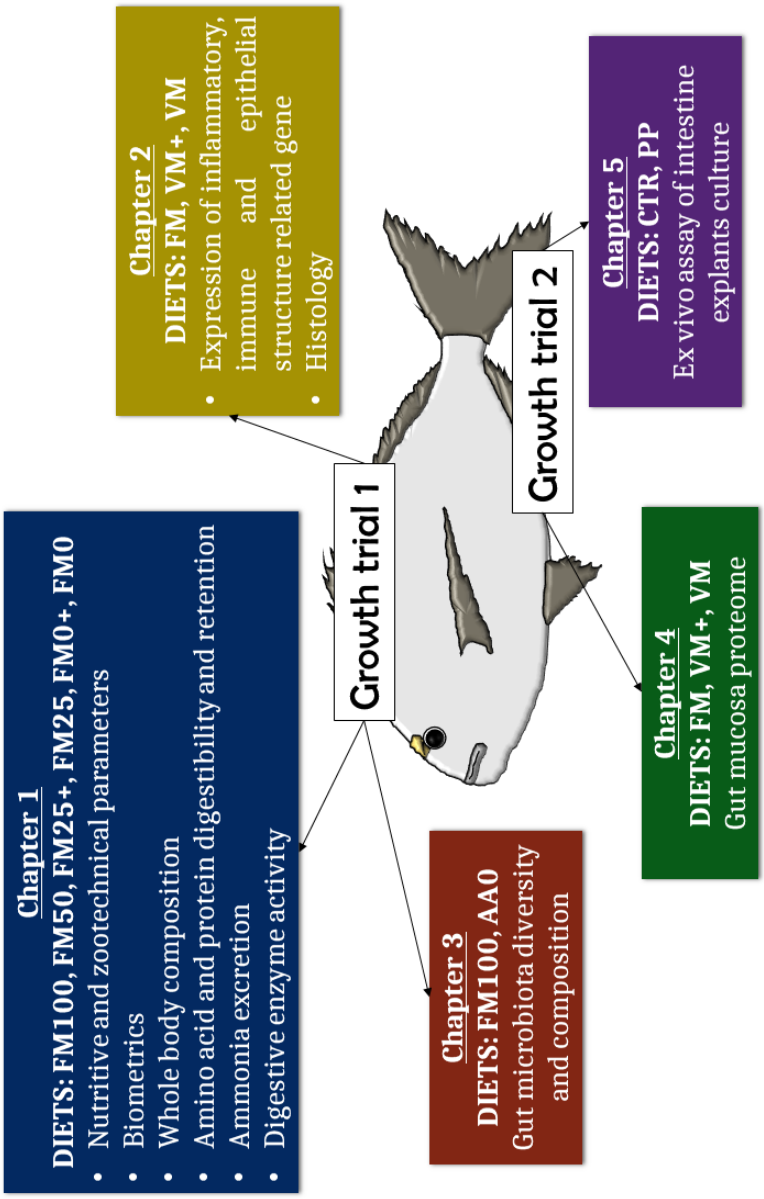

Figure 1. Different analysis carried out during the PhD Thesis performance (own elaboration)

Chapter 1.

Inclusion of alternative marine by-products in aquafeeds with different levels of plant-based sources for on-growing gilthead seabream (*Sparus aurata*, L.): effects on digestibility, amino acid retention, ammonia excretion and enzyme activity

Inclusion of alternative marine by-products in aquafeeds with different levels of plant-based sources for on-growing gilthead seabream (*Sparus aurata*, L.): effects on digestibility, amino acid retention, ammonia excretion and enzyme activity

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Abstract

The search for new sustainable aquafeeds for the species with greater economic importance, such as the gilthead seabream in Europe, is one of the main challenges in the aquaculture sector. The present work tested fishmeal replacement by a mixture of plant meals at different levels, as well as the use of marine by-products with attractant properties and high-quality protein in high plant protein diets. In order to do that, effects on growth and biometric parameters, digestibility, amino acid retention, excreted ammonia and proteases and amylase activity were assessed, using six different diets: FM100 (100% of protein provided by fishmeal), FM50 (50% of replacement), FM25 (75% of replacement) and FM0 (100% of replacement), but also FM25+ (75% of replacement and 15% of squid and krill meal inclusion), and FM0+ (100% of replacement and 15% of squid and krill meal inclusion). In group FM0, a clear impact of dietary changes was observed on growth, survival and ammonia excretion. Amino acid retention in group FM0+ was also significantly affected, which can be explained by the limited content of certain amino acids in this diet. On the other hand, no significant differences were observed in most biometric parameters or in enzyme activity. In conclusion, complete fishmeal replacement can be achieved by using a mixture of plant-based sources, but supplementation with complementary marine ingredients can prevent detrimental effects on growth, survival, nutritional parameters and protein metabolism.

Keywords: *gilthead seabream, vegetable protein, krill meal, ammonia nitrogen, digestibility, enzyme activity*

1. Introduction

Fishmeal has traditionally been used as the main ingredient in diets for carnivorous fish, due to its high content in high-quality protein and its digestibility and palatability. Nevertheless, the reduced availability of this product and the increase of its price demand finding alternative protein sources. As a carnivorous species, the gilthead seabream (*Sparus aurata*, L.) needs a high level of protein in aquafeeds. Plant-based sources, such as oilseed and cereals and their by-products, have a stable nutritional composition and high market availability. Wheat and corn gluten (Pereira and Oliva-Teles 2003), lupin meal (Pereira and Oliva-Teles, 2004), pea meal (Pereira and Oliva-Teles 2002), rapeseed meal (Gómez-Requeni et al. 2004), soybean meal (Martínez-Llorens et al. 2007), among other single ingredients, have been studied as fishmeal replacements in gilthead sea bream. However, the use of plant ingredients has some drawbacks, such as low digestibility, imbalances in essential amino acids (EAA) and low palatability.

On the one hand, the high content in non-starch polysaccharides (NSP), observed in plant meals (Francis et al. 2001), can alter the activity of the different digestive enzymes, which affects the digestion and absorption of nutrients (Fountoulaki et al. 2005) and, as a consequence, the growth performance of fish. Carnivorous fish are not able to efficiently digest carbohydrates, but use them as a source of energy when found in high proportions in diets, which leads to impaired growth parameters (Bowyer et al. 2013). Different effects of different plant-based sources on the activity of various digestive enzymes have been reported in different species (Bowyer et al. 2013; Hartviksen et al. 2014), including gilthead sea bream (Santigosa et al. 2008).

On the other hand, insufficient levels of EAA can be partially solved by using diverse plant-based sources in feeds, as a consequence of the complementation between different amino acids profiles present in the various plant sources. In fact, best results in sea bream growth assays have been achieved with partial substitutions of fish meal with mixtures of different plant ingredients (De Francesco et al. 2007; Dias et al. 2009; Sánchez-Lozano et al. 2009). Moreover, certain studies (Kissil and Lupatsch 2004; Monge-Ortiz et al. 2016) have been able to formulate a feed with total substitution of the fishmeal by vegetable ingredients. Nevertheless, the required amino acid profile for on-growing sea bream fed only plant protein sources can just be achieved by supplementing free amino acids (Monge-Ortiz et al. 2016) or by including

complementary ingredients, in order to combat the nutritional deficiencies of these diets (Kader et al. 2012).

Lastly, the use of attractants in fish feed is necessary to minimise the negative impact of plant meals in growth rate (Gomes et al. 1995; Venou et al. 2003) and improve palatability and feed intake. Different kinds of attractants, like chemical attractants or extracts of marine organisms such as krill meal (Torstensen et al. 2008), have been tested. Moreover, marine by-products also show a balanced amino acid profile closer to fishmeal and provide free amino acids (Kader et al. 2012), which improves the amino acid profile of high plant protein diets by complementing some of the deficiencies of plant-based diets (Kolkovski et al. 2000; Mai et al. 2006) and reducing the level of supplementation with crystalline amino acids.

The inclusion of squid and krill meal in diets for gilthead sea bream has been previously assessed. Nevertheless, most of the studies focus on larvae (Kolkovski et al., 2000; Cahu and Zambonino Infante, 2001) or small fish (Kader et al., 2010, 2012). Moreover, research works with larger fish in which these alternative ingredients have been used in diets with high levels of plant protein also include high levels of free amino acids, leading to mixtures more expensive than fishmeal-based diets (Monge-Ortíz et al., 2016). Therefore, the main goal of the present research work was to assess the impact of different levels of inclusion of a plant protein mixture in aquafeeds on the growth and digestive performance of on-growing gilthead seabream. To achieve the minimum requirements of EAA for the on-growing gilthead seabream, diets were supplemented with crystalline amino acids and, in the case of high partial or total replacement, the inclusion of squid and krill meal as complementary ingredients was also tested in order to improve the essential amino acid profile of the experimental diets. The impact of the different diets on biometrics, biochemical composition, digestion and retention of essential and non-EAA, ammonia excretion and digestive enzyme activities was also evaluated.

2. Materials and methods

2.1 Experimental setup

The growth assay was conducted in 18 cylindrical fiberglass tanks (1750 l) within a marine water recirculating system (75 m^3 capacity) with a rotary mechanical filter and a gravity biofilter (6 m^3 capacity) in the Universitat Politècnica de València (UPV). All tanks were equipped with aeration and the water was heated with a heat pump installed in the system. During the experiment, water conditions were as follows: 23±1.5º C, 30±1.7 gL^{-1} salinity, 6±0.5 mg O_2L^{-1} and pH 7.5. All tanks had similar lighting conditions with a natural photoperiod (from November-March, with an average of 11h of light).

2.2 Fish

Sea bream were obtained from the fish farm PISCIMAR in Burriana (Valencia, Spain). Fish acclimated to laboratory conditions for 2 weeks, feeding a standard commercial diet with a proximal composition of 48% crude protein (CP), 23% ether extract (EE), 11% crude ash (CA), 2% crude fibre (CF) and 14% nitrogen free extract (NFE). Then, the 360 fish were randomly distributed in the 18 tanks, in groups of 20 in each one. The experiment was initiated with fish weighing 128±5.3 g.

2.3 Ethics statements

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the UPV, following the Spanish Royal Decree 53/2013 on protection of animals used for scientific purposes (Boletín Oficial del Estado, 2013).

2.4 Diets

Six isonitrogenous and isoenergetic diets were formulated (FM100, FM50, FM25+, FM25, FM0+ and FM0). They differed in the level of fishmeal, the inclusion of krill and squid meal as attractants and the addition of different synthetic crystalline amino acids. Various levels of fishmeal replacement were tested: 0% (FM100), 50% (FM50), 75% (FM25+ and FM25) and 100% (FM0+ and FM0). Diets FM25+ and FM0+ included alternative marine by-products (15%) from different companies: squid meal (Max Nollert, Utrecht, Netherlands), at 10%

level, and squid meal (Ludan Renewable Energy, Valencia, Spain), at 5%. All diets, except for FM100, were supplemented with free amino acids in order to meet the optimum amino acid requirements for gilthead seabream (Peres & Oliva-Teles 2009). The formulation of experimental diets, essential amino acid dietary content and amino acid optimum requirements for the on-growing gilthead seabream are shown in Table 1.

Table 1. Formulation and proximate composition of experimental diets

	FM100	FM50	FM25+	FM25	FM0+	FM0	Optimum[1]
Ingredients [g·kg^{-1}]							
Fish meal	589	295	150	150			
Wheat meal	260	66	60				
Wheat gluten		130	125	220	222	295	
Soybean meal		130	132	160	160	182	
Bean meal		25	25	42	40	41	
Pea meal		25	25	42	40	41	
Sunflower meal		130	132	160	160	158	
Squid meal			100		100		
Krill meal			50		50		
Fish oil	38	64	78	77	90	90	
Soybean oil	93	91	66	91	65	90	
Soy Lecithin	10	10	10	10	10	10	
Vitamin-mineral mix*	10	10	10	10	10	10	
$Ca(H_2PO_4)_2$		19	27	28	38	38	
Taurine						20	
DL-Methionine		5	5	5	5	7	
L-Lysine-HCl			5	5	10	10	
L-Arginine						5	
L-Threonine						3	
Proximate composition [g·kg dry matter^{-1}]^{T}							
Dry matter	881	914	902	928	928	939	
Crude protein	442	447	445	450	446	451	
Ether extract	185	193	201	210	200	198	
Crude ash	101	98	101	90	88	75	
Crude fibre	10	35	39	42	46	42	
Nitrogen Free Extract$^{\mu}$	260	219	214	213	209	222	
Non-starch Polysaccharides	109	175	178	197	199	206	
Essential amino acids [g·100g dry matter^{-1}]							
Arginine	3.39	3.87	3.86	3.16	3.58	3.30	2.50
Histidine	1.00	1.11	0.81	0.90	0.81	0.82	0.85
Isoleucine	1.47	1.30	1.24	1.26	1.08	1.17	1.15
Leucine	3.24	2.84	3.11	3.03	2.45	2.98	2.24
Lysine	3.68	2.60	2.78	2.12	2.38	2.26	2.31
Methionine	1.16	1.14	1.06	1.09	1.05	1.06	1.17
Phenylalanine	1.8	1.75	1.78	1.67	1.76	1.87	
Threonine	1.98	1.66	1.50	1.45	1.28	1.44	1.34
Valine	2.01	1.67	1.60	1.57	1.32	1.47	1.44

*Vitamin and mineral mix (values are [g·kg complete diet^{-1}]): Premix: 5; Choline, 2; DL-a-tocopherol, 1; ascorbic acid, 1; $(PO_4)_2Ca_3$, 1. Premix composition: retinol acetate, 10000[IU·kg complete diet^{-1}]; calcipherol, 5 [IU·kg complete diet^{-1}]; DL-a-tocopherol, 0.1; menadione sodium bisulphite, 0.008; thiamine hydrochloride, 0.023; riboflavin, 0.023; pyridoxine hydrochloride, 0.15; cyanocobalamine, 0.25; nicotinamide, 0.15; pantothenic acid, 0.06; folic acid, 0.0065; biotin, 0.0007; ascorbic acid, 0.75; inositol, 0.15; betaine, 1; polypeptides 0.12.
TDry matter [g kg^{-1} wet matter]: FM100 = 881; FM50 = 914; FM25+ = 902; FM25 = 928; FM0+ = 928; FM0 = 939.
HNitrogen Free Extract (NFE) = 1000 – Crude protein – Ether extract – Crude ash – Crude fibre
IOptimum essential amino acid profile according to Peres and Oliva-Teles (2009)

All diets were prepared as pellets by cooking-extrusion using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France) located at the UPV. The processing conditions were as follows: 100 rpm screw speed, 110º C, 40 atm pressure and 3-5 mm diameter pellets.

2.5 Biochemical analyses

The diets, their ingredients, fish and faeces were analysed according to the Association of Official Agricultural Chemists (AOAC 1995) procedures: dry matter (DM) by heating at 105° C to constant weight (2001.12), CA by incineration at 550° C to constant weight (942.05), CP, N × 6.25, by the Kjeldahl method after an acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden; 954.01), EE by methyl-ether extraction (Soxtec 1043 extraction unit, Tecator; 920.39) and CF by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator; 989.03). All analyses were performed in triplicate except for faeces. Chemical analyses of ingredients were determined prior to diet formulation. Proximate composition of experimental diets is shown in Table 1.

2.5.1 Amino acid analysis

Amino acids of raw materials, experimental diets, fish (five per tank) and faeces were analysed prior to diet formulation through a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature control module, following the method described by Bosch et al. (2006). Aminobutyric acid was added as an internal standard pattern before hydrolysation. The amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and

cysteic acid after oxidation with performic acid. Amino acids were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm) and then transformed to methionine and cysteine. Essential and non-essential amino acid content of different ingredients are shown in Supplemental material (S1). The amino acid content of fish is shown in Supplemental material (S2). Retention efficiencies of ingested amino acid (AAIRE) were calculated for each experimental group.

2.6 Growth assay

The trial lasted 154 d. Fish were observed daily in the tanks and were weighed individually every 4 weeks, using clove oil containing 87% eugenol (Guinama ®, Valencia, Spain) as an anaesthetic (1 mg·100ml of water^{-1}) to minimise their suffering, in order to evaluate fish growth along the assay, determine growth parameters and asses their health status. At the end of the experiment, fish were sacrificed by cold shock after anaesthesia.

Each of the six experimental diets was randomly assigned to three tanks (triplicate groups). Fish were fed by hand twice a day (9:00 h and 17:00 h) to apparent satiation, distributing the pellets slowly, allowing all fish to eat and making sure no feed remained at the bottom of the tanks, in a weekly regime of 6 d of feeding and one of fasting.

The growth and nutrient efficiency and utilisation indices considered were: feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER) and protein productive value (PPV). Survival rate (S) was also determined.

$$\mathrm{FI} = 100 \cdot \frac{\text{feed consumption [g]}}{\text{average biomass [g]} \cdot \text{days}}$$

$$\mathrm{FCR} = 100 \cdot \frac{\text{feed offered [g]}}{\text{weight gain[g]}}$$

$$\mathrm{PER} = 100 \cdot \frac{\text{weight gain [g]}}{\text{protein intake [g]}}$$

$$\mathrm{PPV} = 100 \cdot \frac{\text{protein gain [g]}}{\text{protein intake [g]}}$$

$$\mathrm{S} = 100 \cdot \frac{\text{final number of fish}}{\text{initial number of fish}}$$

2.7 Biometric indices

Condition factor (K), viscerosomatic index (VSI), hepatosomatic index (HSI) and visceral fat index (VFI) were calculated at the end of the growth assay, using five fish per tank randomly selected.

$$K = 100 \cdot \frac{\text{total weight [g]}}{\text{total length [cm}^3\text{]}}$$

$$VSI = 100 \cdot \frac{\text{visceral weight [g]}}{\text{empty fish weight [g]}}$$

$$HSI = 100 \cdot \frac{\text{liver weight [g]}}{\text{fish weight [g]}}$$

$$VFI = 100 \cdot \frac{\text{visceral fat weight [g]}}{\text{empty fish weight [g]}}$$

2.8 Digestibility assay

A digestibility experiment was performed after the feeding trial in six digestibility tanks of 250 L, following the Guelph System protocol (Cho et al. 1982), using five fish per experimental group randomly selected. Before feeding, fish were fasted for 2 d. During a period between 7 to 14 d, until enough wet faeces for the analysis were collected, fish were fed to satiation once a day in the morning (9:00) with the same six experimental diets, but chromium oxide (50 $g \cdot kg^{-1}$) was added as an inert marker. Uneaten feed was removed from the columns (15:00). Samples of wet faeces from each tank were collected from decantation columns, just before the morning feeding, and dried at 60ºC for 48h prior to analysis.

Chromium oxide was determined in the diets and faeces using an atomic absorption spectrometer (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA) after acid digestion. Analysis was performed in duplicate. CP and amino acids content in diets and faeces were used to determine apparent digestibility coefficients (ADC, equation 1).

(1) $$ADC_N(\%) = 100 \cdot \left[1 - \left(\frac{\%\ marker\ in\ diet}{\%\ marker\ in\ faeces}\right) x \left(\frac{\%N\ in\ faeces}{\%\ N\ in\ diet}\right)\right],$$

where N is the nutrient (CP or respective amino acid)

Retention efficiencies of the digested crude protein (PDRE, %) and of the digested EAA (AADRE, %) were also calculated.

2.9 Ammonia excretion

The ammonia excretion was established for the different experimental groups. Analyses were carried out following the method detailed by McGoogan and Gatlin (1999). The pump was turned off during the entire ammonia sampling period, altering the recirculating nature of the system in order to estimate the ammonia increase due to excretion, but aeration was continually provided to each tank.

Water samples were taken before feeding and then at intervals of two h after feeding for a 24-h period (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h). Total ammonia nitrogen (TAN) concentration was measured in all water samples using the Orion® 4-Star Plus probe (ThermoScientific®, Waltham, Massachussets, USA) with an ammonia specific electrode. Measurements were performed following the procedures detailed by García García et al. (2011), using hydrochloric acid (J.T. Baker®, Avantor™, Central Valley, USA) and sodium hydroxide (Scharlau, Scharlab, Barcelona, Spain).

Each diet was tested three different days in three different tanks (n=9). Average ammonia concentrations were adjusted to fish weight and feed ingestion in the different tanks, expressing accumulated ammonia excretion and ammonia excretion per h in mg·kg fish^{-1}·kg feed^{-1}.

2.10 Enzymatic activity

Digestive tracts of two fish per tank randomly selected were sampled at the end of the assay. Fish were dissected in order to obtain the digestive tract. Stomach (S) and gut (G) were stored separately at -20º C until enzymatic extraction.

Enzyme extracts for protease analysis were obtained by manual disaggregation, dilution in distilled water (1 g of sample : 3 ml of distilled water) followed by homogenisation by T25 – Digital ULTRATURRAX®, maintaining tubes on ice, and centrifugation at 12000 rpm and 4º C for 15 min. Gut contents (GC) were removed from the tissue and diluted in distilled water (1:3) before centrifugation at 16000 g and 4º C for 15 min. Supernatants were stored at 20º C until enzyme analysis.

Pepsin assays were performed on S samples and total alkaline protease assays on G samples, while trypsin, chymotrypsin and α-amylase assays

were performed on G and GC samples. Total protein concentration was assayed according the Bradford procedure (Bradford, 1976), using bovine serum albumin (2 mg·ml^{-1}) as a standard. Enzyme activities were expressed in U·mg of total protein^{-1} for the S and G samples (equation 2) and in U·mg of content^{-1} for the GC samples (equation 3).

$$(2)\ \frac{U}{mg\ total\ protein} = \frac{\Delta Abs\ x\ Vtotal}{\varepsilon\ x\ Vsample\ x\ d} \cdot \frac{ml}{mg\ total\ protein}$$

$$(3)\ \frac{U}{mg\ content} = \frac{\Delta Abs\ x\ Vtotal}{\varepsilon\ x\ Vsample\ x\ d} \cdot \frac{ml}{mg\ content}$$

2.10.1 Pepsin activity

Total acid protease activity was evaluated using 0.5% haemoglobin p/v as substrate in 100 mM glycine – HCl buffer, pH 2.5, at 280 nm, following the method detailed by Anson (1938) and modified by Díaz-López et al. (1998). One unit of activity was defined as 1 μg of tyrosine released per min (Extinction coefficient = 0.0071 ml·μg^{-1}·cm^{-1}).

2.10.2 Total alkaline protease activity

Total alkaline protease activity was tested using 1% casein p/v as substrate in 100 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 7.5, at 280 nm, following the method detailed by Kunitz (1947) and modified by Walter (1984). One unit of activity was defined as 1 μg of tyrosine released per min (Extinction coefficient = 0.0071 ml·μg^{-1}·cm^{-1}).

2.10.3 Trypsin activity

Trypsin activity was obtained by a kinetic assay using Nα-Benzoyl-D,L-arginine p-nitroanilide 0.5 mM as a substrate in 50 mM Tris-HCl buffer containing 20 mM $CaCl_2$, pH 8.2, following the method developed by Erlanger et al. (1961). The increase in absorbance at 405 nm was measured every 30 s for 5 min. One unit of activity was defined as 1 μg of p-nitroanilide released per min (Extinction coefficient = 0.0637 ml·μg^{-1}·cm^{-1}).

2.10.4 Chymotrypsin activity

Chymotrypsin activity was obtained by a kinetic assay using N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide 0.5 mM as a substrate in 200

mM Tris-HCl buffer containing 50 mM $CaCl_2$, pH 7.6, following the method developed by Erlanger et al. (1961). The increase in absorbance at 405 nm was measured every 30 s for 5 min. One unit of activity was defined as 1 μg of p-nitroanilide released per min (Extinction coefficient = 0.0637 $ml \cdot \mu g^{-1} \cdot cm^{-1}$).

2.10.5 α-amylase activity

α-amylase activity was determined by a kinetic assay using a commercial kit (Amylase MR, Cromatest, Linear Chemicals S.L., Barcelona, Spain), following manufacturer's instructions. The increase in absorbance at 405 nm was measured every 30 s for 5 min, after an incubation period of 1 min. One unit of activity was defined as 1 μg of 2-chloro-p-nitrophenol released per min during the enzymatic reaction at 37º C (Extinction coefficient = 0.0818 $ml \cdot \mu g^{-1} \cdot cm^{-1}$).

2.11 Statistical analysis

Different growth and nutrient indices, biochemical parameters, biometric indices, ADC, AAIRE, AADRE, ammonia excretion indices and specific enzyme activities were analysed through an analysis of variance using the statistical package Statgraphics ® Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA), with a Newman-Keuls test for the comparison of the means. All percentage data were arcsine transformed prior the analysis. Initial weight was used as a covariate in the analysis of growth indices. The results are shown as the mean ± standard error of the mean (SEM). The level of significance was set at $p<0.05$.

3. Results

3.1 Fish growth and nutritive efficiency

Growth and nutritive efficiency indices of the growth experiment are shown in the Table 2. Statistical differences were determined in the final weight (FW), FCR, PER and PPV between groups FM25 and FM0. There were also significant differences between group FM25+ and groups FM50, FM25 and FM0 in FI. Besides, survival rate was significantly lower in group FM0.

Table 2. Growth, nutritive and biometric indices of gilthead sea bream fed the different experimental diets

	FM100	FM50	FM25+	FM25	FM0+	FM0	SEM
IW [g]	131.2	125.9	130.2	126.1	129.6	127.2	2.16
FW [g]	393.1^{ab}	401.9^{ab}	422.7^{a}	390.2^{ab}	384.6^{ab}	360.4^{b}	12.53
FI [g·100g fish^{-1} day $^{-1}$]	1.3^{ab}	1.4^{a}	1.3^{b}	1.4^{a}	1.3^{ab}	1.4^{a}	0.03
FCR	2.1^{ab}	2.1^{ab}	1.9^{b}	2.3^{ab}	2.18^{ab}	2.4^{a}	0.09
PER	1.1^{ab}	1.1^{ab}	1.2^{a}	1.0^{ab}	1.1^{ab}	0.9^{b}	0.05
PPV [%]	18.8^{ab}	18.5^{ab}	21.2^{a}	17.4^{ab}	20.1^{ab}	16.7^{b}	0.90
S [%]	88.3^{a}	85.0^{a}	88.3^{a}	78.3^{a}	86.7^{a}	60.0^{b}	5.44
K [g·cm^{-3}]	1.9	1.9	1.9	1.7	1.8	1.7	0.08
VSI [%]	9.2	9.95	10.3	8.9	9.6	8.6	0.80
HSI [%]	1.6^{a}	1.4^{ab}	1.3^{ab}	1.3^{ab}	1.3^{ab}	1.2^{b}	0.06
VFI [%]	1.5	1.3	1.2	1.3	1.8	1.4	0.18

IW, initial weight; FW, final weight; FI, feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; PPV, protein productive value; K, condition factor; VSI, viscerosomatic index; HIS, hepatosomatic index; VFI, visceral fat index.
Means of triplicate groups; data in the same row with different superscripts differ at $p<0.05$; SEM: pooled standard error of the mean; Newman-Keuls test was applied for the comparison of the means.
IW was considered as covariable for final weight

3.2 Biometric and body composition

Regarding biometric parameters (Table 2), significant differences were only observed in the HSI, which was higher for group FM100 compared to FM0. No differences were detected in the proximate composition of the fish, as shown in the Supplemental material (S2).

3.3 Digestibility and protein and amino acid retention efficiency

No differences were observed in the amino acid composition of whole body fish between experimental groups (Supplemental material, S2).

The ADC_{CP}, ADC_{aa} and ADC_{CL} are shown in Table 3. Lower values were observed for all EAA in groups FM25 and FM0, while higher values were perceived for groups FM100 and FM25+. The biggest differences were detected for arginine, threonine and valine, but no statistical analysis was performed. Differences in the digestibility of CP and EE were minor (ranging from 96.5 to 92.9 for CP and from 98.9 to 97.3 for EE), although a slight negative effect of fishmeal replacement and a slight positive effect of complementary marine ingredients could be noticed.

Table 3. ADC of crude protein (CP) and amino acids in the gilthead sea bream fed different experimental diets

	FM100	FM50	FM25+	FM25	FM0+	FM0
ADC_{CP} [%]	96.5	94.1	95.2	94.2	94.1	92.9
ADC_{EAA} [%]						
Arginine	96.2	93.5	94.8	90.3	93.1	89.2
Histidine	95.7	94.9	96.9	94.1	94.5	93.4
Isoleucine	96.4	94.2	97.1	93.5	95.7	92.2
Leucine	96.4	95.1	96.8	94.8	96.2	93.0
Lysine	97.8	96.7	97.0	94.8	97.5	93.9
Methionine	97.2	96.3	97.1	96.1	97.3	96.3
Phenylalanine	96.9	96.4	97.2	95.5	96.7	94.6
Threonine	96.0	95.2	95.8	92.1	93.8	91.4
Valine	96.1	94.8	96.3	93.6	94.9	91.2
ADC_{NEAA} [%]						
Alanine	96.0	96.3	97.0	94.8	95.2	90.6
Aspartate	92.3	90.3	91.0	89.5	92.0	87.9
Cysteine	91.6	90.9	91.6	90.1	90.5	89.0
Glutamine	97.0	96.5	98.2	96.8	97.8	95.6
Glycine	92.7	92.3	91.1	91.6	92.0	87.9
Proline	96.4	94.8	96.8	95.1	97.0	94.9
Serine	95.5	95.2	96.1	94.3	95.6	93.2
Tyrosine	97.6	96.1	96.2	95.7	95.9	94.9

EAA, essential amino acids; NEAA, non-essential amino acids

The PDRE and AADREs are shown in Table 4. Higher AADREs were generally obtained in group FM0+, while lower AADREs were observed in group FM100 for isoleucine, lysine and valine, in groups FM100 and FM50 for histidine and in group FM0 for phenylalanine.

Table 4. Retention efficiencies of digested protein and digested essential amino acids in gilthead sea bream fed different experimental diets

	FM100	FM50	FM25+	FM25	FM0+	FM0	SEM
PDRE	19.44	19.67	22.30	18.43	21.32	18.02	0.955
AADRE (%)							
Arginine	20.12	15.97	22.91	23.85	25.68	21.38	2.140
Histidine	20.09^{c}	18.21^{c}	29.81^{ab}	21.95^{bc}	31.25^{a}	21.61^{bc}	2.245
Isoleucine	24.07^{c}	26.62^{bc}	33.43^{ab}	27.26^{bc}	38.13^{a}	29.70^{bc}	1.999
Leucine	17.03^{b}	19.21^{b}	20.71^{b}	17.04^{b}	25.95^{a}	17.06^{b}	1.347
Lysine	19.45^{b}	25.44^{a}	28.17^{a}	28.54^{a}	29.17^{a}	28.39^{a}	1.884
Methionine	21.14	21.67	25.95	20.16	24.79	18.67	2.034
Phenylalanine	15.40^{ab}	15.86^{ab}	19.62^{a}	15.69^{ab}	19.84^{a}	12.34^{b}	1.320
Threonine	15.60	24.86	25.03	20.74	29.75	19.30	3.356
Valine	21.58^{c}	25.82^{bc}	32.00^{b}	26.87^{bc}	38.38^{a}	28.80^{b}	1.710

PDRE, Protein Digestion Retention Efficiencies; ADRE, Amino acid Digestion Retention Efficiencies

PDRE [%] = 100 · (protein gain [g]/(($ADC_{protein}$/100) · protein ingested [g])); AADRE [%] = 100 · (amino acid gain [g]/(($ADC_{amino\ acid}$/100) · amino acid ingested [g]))

Means of triplicate groups (n=3); data in the same row with different superscripts differ at p<0.05; SEM: pooled standard error of the mean; Newman-Keuls test was applied to compare the means.

3.4 Ammonia excretion

Accumulated ammonia excretion and ammonia excretion per h are shown in Figure 1. Accumulated ammonia excretion was significantly higher for diet FM0 from the first sampling point (2 h after feeding) to the end of the assay, which was mainly due to higher ammonia excretion during the first half of the experiment. There were significant differences in ammonia production in the first five two-h intervals after feeding, especially between groups FM0 and FM100, FM50 and FM25+. Statistical differences in ammonia production were also determined between the 16th and the 18th hour after feeding. Maximum ammonia excretion values were determined at the 4th (FM50, FM25+, FM25 and FM0) and 8th h (FM100 and FM0+).

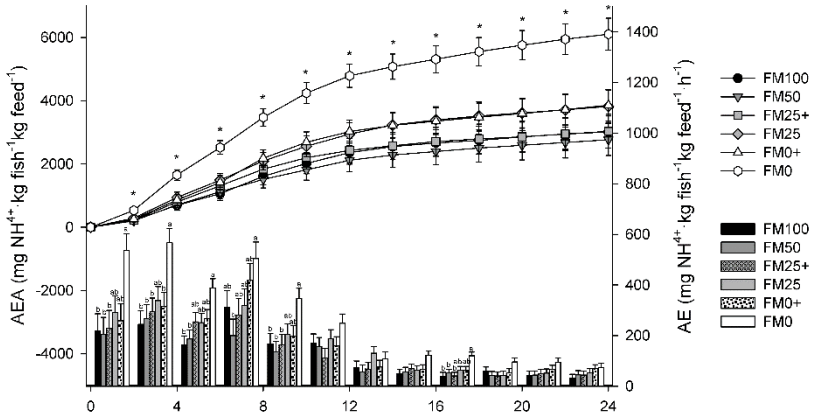

Figure 1. Ammonia excretion accumulation (AEA) and ammonia excretion (AE) per h in the different experimental groups

Means of triplicate groups and standard error of the mean; asterisks indicate significant differences of the group FM0 in the AEA with the other groups, at $p<0.05$; different superscripts indicate differences between groups in the AE, at $p<0.05$; Newman-Keuls test was applied to compare the means.

3.5 Enzyme activity

3.5.1 Protease activity

No significant differences were observed in pepsin activity in the stomachs of fish fed the different experimental diets (Figure 2(a)). A higher average value was obtained in group FM25+, followed by FM100, while the average activity registered in the other groups was

very close. A similar pattern was observed in proteases activity in the gut tissue (Figure 2(b)). In this case, lower average activities of total alkaline protease and trypsin were observed in group FM0. Chymotrypsin activity was also determined in gut tissue, but the results are not shown since it was a very low or non-existent level.

No statistical differences were observed on proteases activity in GC samples (Figure 2(c)). The same pattern could be observed in the total alkaline protease, trypsin and chymotrypsin activity registered in the different experimental groups. The highest values were obtained, in this case, in group FM100. The ratio trypsin / chymotrypsin was similar for all experimental groups, although it was slightly lower for group FM0+.

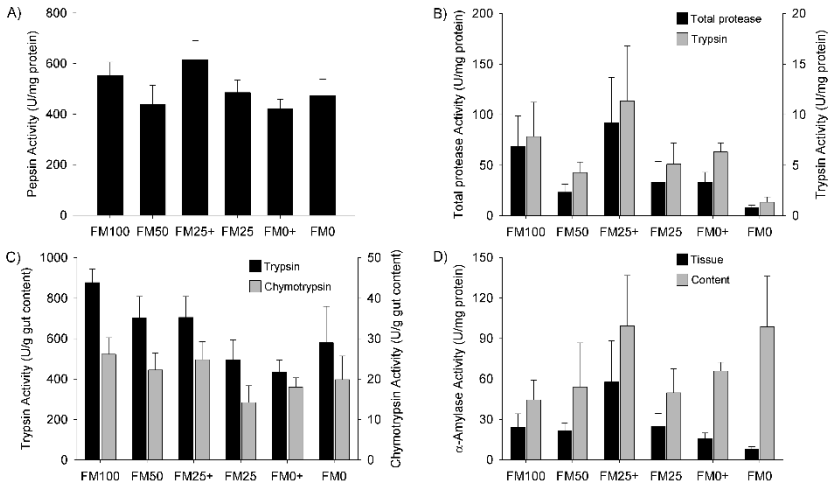

Figure 2. Enzymatic activity determined in the gastrointestinal tissue and contents of fish fed the different experimental diets.

A) Pepsin activity in stomach tissue; B) Total alkaline protease (black bars) and trypsin (grey bars) activity in gut tissue; C) Trypsin (black bars) and chymotrypsin (grey bars) activity in gut contents; D) α-amylase activity in gut tissue (black bars) and the gut contents (grey bars)

Means of six fish per treatment (n=6), in U/mg protein or U/g gut content, and standard error of the mean; different superscripts indicated differences, at p<0.05

3.5.2 α-Amylase activity

There were no significant differences observed between experimental groups when α-amylase activity was determined in gut tissue or gut content (Figure 2(d)). Highest average values were registered in group FM25+.

4. Discussion

The inclusion of krill and squid meal in diets does not prove to have a positive attractant effect on fish on the present research work, in contrast to previous reports with squid and krill meal (Kader et al. 2012). In fact, higher values of FI were observed for the groups of fish fed with diets containing a partial or complete fishmeal substitution without squid and krill meal inclusion (FM50, FM25 and FM0). However, fish were fed to satiation and intake is regulated by fish according to the energy level of the feeds (Sánchez-Lozano et al. 2007). Thus, plant-based diets without marine-ingredient complementation could be deficient from an energetic point of view and it could dissemble a possible attractant effect. In this sense, although the different feeds assayed in this experiment were formulated as isoenergetic, digestive crude energy should be considered in further studies.

Despite fish in group FM25+ showed the lower FI value, this group reached the highest FW, even over group FM100. There were no statistical differences between groups FM100, FM50, FM25+, FM25 and FM0+, which suggests that high partial substitution of marine-origin ingredients does not have a negative impact on fish growth (Benedito-Palos et al. 2007; De Francesco et al. 2007; Dias et al. 2009), while a lower FW in group FM0 did confirm a negative effect on growth when diets with total replacement are used (Gómez-Requeni et al. 2004). Although there are reports of successful total substitution of fishmeal in terms of growth performance, the diets used contained a higher level of free amino acid supplementation (Kissil and Lupatsch 2004) or included small amounts of marine-origin ingredients (Monge-Ortíz et al. 2016). Thus, squid and krill meal inclusion has a positive effect on growth at the same level of fishmeal substitution, reducing the impact of fishmeal replacement.

As a consequence of higher FW and lower FI, group FM25+ showed a minor FCR and higher PER and PPV. As in other species (Torstensen et al. 2008), high plant protein inclusion does not seem to affect protein utilisation in gilthead sea bream and, in fact, it seems to be the most efficient food strategy for gilthead seabream during the on-growing period. In contrast, total replacement seems to have a negative effect on protein utilisation, which will be discussed later.

It is important to point out that the group FM0 had a low survival rate. The number of casualties was particularly high in the last third of the trial, but no obvious signs of disease were observed in the dead fish. Although the causes of the increase in mortality in all groups remain unclear to this day, water was partially changed in the marine water recirculating system and consequently some bacteria could have been introduced into the system and have led to the death of weaker fish. Besides, high fishmeal replacement has been related with immune disorders (Sitjá-Bobadilla et al. 2005) and could explain the higher mortality in group FM0.

A significant impact of fishmeal replacement or inclusion of squid and krill meal was not observed in the biometric parameters or in the chemical composition of the fish. Only differences in the HSI were registered between fish fed the FM100 and FM0 diets, so the decrease of liver weight could be related to feed with high plant protein levels (Sánchez-Lozano et al. 2009; Martínez-Llorens et al. 2012), although this relation has not been observed in some previous studies (Linn et al. 2014; Monge-Ortiz et al. 2016).

Digestibility of dietary protein and EAA and their retention efficiency indices should be taken into account in the design of new diets in order to improve amino acids profile to better suit fish requirements. Differences in crude dietary protein digestibility are influenced by the content of antinutritional factors, the physicochemical properties of proteins or fibre level, among others (Martínez-Llorens et al. 2012), which affect amino acid availability (Francis et al. 2001). Moreover, since different amino acids are absorbed by the brush border membrane of the enterocytes through the same specific transporters with different affinities, differences on amino acid profile can compromise their availability and absorption (Berge et al. 2004), leading to variations in their digestibility and retention coefficients.

Finally, the method for faeces collection should be considered, since the column or decantation method, used in the present work, normally gives higher ADC than other methods, such as the stripping method or the use of faeces collection devices (Vandenberg and De la Noüe 2001), due to nutrient leaching during the time between the release and the collection of the faeces (Spyridakis et al. 1989; Vandenberg and De la Noüe 2001). On the other hand, the stripping method seems to underestimate the digestibility coefficients, leading to stress events, possible disruptions of nutrients absorption and therefore to the

obtention of samples in which the absorption process has not been completed (Clements and Raubenheimer 2006).

A slight decreasing trend in digestibility coefficients with higher levels of replacement has been observed in the present work, similarly to previous studies using the same faecal collection method (Dias et al. 2009), although higher differences were expected due to the higher fibre and NSP levels in diets with plant meals. Digestibility of EAA only differs slightly in the different experimental groups, except in the case of FM25 and FM0 and particularly for Arg, Ile, Lys, Thr and Val. Except for the FM0 group, the effects on fish growth, PER and PPV were minor, suggesting the other diets supply enough digestible EAA to meet nutritional requirements. On the other hand, a possible EAA imbalance caused by the different bioavailability of EAA which depends on the source (Santigosa et al. 2011), could be the reason of the poorer digestibility performance in the case of FM0. A positive effect of marine-complementary ingredients is observed in groups FM25+ and FM0+ in comparison to groups with equal dietary fishmeal. As aforementioned, these sources have proved to reduce deficits in aqua feeds with high levels of plant protein (Kolkovski et al. 2000), showing a more balanced amino acid profile and a high amount of free amino acids (Kader et al. 2012).

Since the EAA composition in fish bodies was the same for all dietary groups, AADREs were a reflection of the amino acid composition of the diets and they were greater in the dietary groups with a limited content of the different amino acids, particularly noticeable for group FM0+ and especially lower for group FM100, which showed higher level of dietary EAA (Sánchez-Lozano et al. 2009; Martínez-Llorens et al. 2012). In this sense, higher EAA retention has been reported in diets with higher levels of fishmeal substitution in similar previous studies (Gómez-Requeni et al. 2004; Sánchez-Lozano et al. 2009).

Ammonia excretion is a useful indirect tool to assess the metabolic use of protein in the diets (Velazco-Vargas et al. 2014) and, as a potentially toxic factor in aquaculture, its control is of great importance. Excess of dietary amino acids may be either not absorbed or metabolically derived, which leads to an increase of ammonia production (McGoogan and Gatlin III 1999). Moreover, an increased excretion can be expected with a lower-level protein synthesis (Lied and Braaten 1984), also expressed as lower growth and protein retention (Bonaldo et al. 2011). Previous studies have reported an increase of ammonia production in

response to high levels of vegetable meal inclusion in sea bream diets (Robaina et al. 1995; Bonaldo et al. 2011). In this sense, a higher ammonia excretion and lower growth and protein retention are observed in the diet with the highest inclusion of plant-based sources. Inasmuch as the protein dietary level is very similar in all experimental groups, the higher ammonia production in group FM0 in comparison to the other experimental groups can be explained by the lower digestibility of some EAA in this diet, which leads to an imbalance of ingested amino acids, a higher catabolism level of amino acids exceeding the required profile and, ultimately, a lower growth.

The ability of fish to digest and use nutrients depends on an appropriate performance of the different digestive enzymes (Vizcaíno et al. 2014). The source, quality and concentration of dietary nutrients can modulate the intestinal enzymatic profile (Santigosa et al. 2008), while the activity of these enzymes in the digestive tract can be used as an indicator of digestive capacity and nutritional status of the fish (Engrola et al. 2007). The use of plant-based alternative ingredients can lead to interferences with nutrient digestion and utilisation (Alarcón et al. 1999) due to antinutritional factors or enzyme inhibitors.

Previous studies have reported negative effects of plant protein sources on pepsin, alkaline protease, trypsin and chymotrypsin activity on seabream (Santigosa et al. 2008; Silva et al. 2010), although impact at enzyme level did not always lead to differences in growth and feed utilisation (Monge-Ortiz et al. 2016). The results obtained in this research suggest that there is a certain effect of plant meal inclusion on the different protease activity in the gastrointestinal tissue and digestive contents, although there were no statistical differences observed between groups. As with growth and nutritive parameters, values obtained for group FM25+ in the intestinal tissue are the highest, showing the high digestive capacity of fish in this group. Nevertheless, differences in protease activity between different gut sections —not considered in the present work— have been reported in different species, being a more decisive factor than the diet itself (Deguara et al. 2003; Sørensen et al. 2011; Hartviksen et al. 2014). Moreover, digestive capacity depends not only on enzyme level, but also on digestion time (i.e. the time during which the enzymes act on the nutrients) (Fountoulaki et al. 2005), which varies depending on protein source, since intestine length increases when high plant protein diets are used (Santigosa et al. 2008). calculating the cumulative enzyme activity, from feeding time to 24 h after, can avoid variations due to sampling

time and digestion patterns (Fountoulaki et al. 2005) and therefore should be taken into account in upcoming research, as well as sampling time after feeding (Venou et al. 2003; Yúfera et al. 2012), pH and temperature (Hidalgo et al. 1999; Deguara et al. 2003; Nikolopoulou et al. 2011).

Regarding amylase activity, previous studies show that the amylase activity can be positively influenced by the dietary carbohydrate level (Kuz'mina 1996), but negatively affected by the level of dietary starch and plant protein inclusion (Kokou et al. 2016). In the present study, diets with the highest level of plant meal showed a lower content of dietary starch (the level of carbohydrates is similar in all experimental diets) and the effects of both factors remain unclear, since no differences between groups were determined.

To conclude, in this article we have reported that a complete fishmeal substitution in gilthead seabream diets during the on-growing period can be achieved without a high impact on biometric and biochemical parameters, protein and amino acid digestibility and enzyme activity. Nevertheless, the inclusion of marine-complementary ingredients such as squid and krill meal is necessary in lower percentages to avoid adverse effects on growth and nutritional efficiency parameters when a complete fishmeal replacement is performed.

Supplementary material

Supplementary material (S1 and S2) is included in ANNEX I

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Chapter 2.

Long-term feeding with high plant protein based diets in gilthead seabream (*Sparus aurata,* L.) leads to changes in the inflammatory and immune related gene expression at intestinal level

Long-term feeding with high plant protein based diets in gilthead seabream (*Sparus aurata*, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level

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Abstract

Background

In order to ensure sustainability of aquaculture production of carnivourous fish species such as the gilthead seabream (*Sparus aurata*, L.), the impact of the inclusion of alternative protein sources to fishmeal, including plants, has been widely assessed. With the aim of evaluating long-term effects of vegetable diets on growth and intestinal status of the on-growing gilthead seabream (initial weight=129g), three experimental diets were tested in the present work: a strict plant protein-based diet (VM), a control fishmeal based diet (FM) and a plant protein-based diet with 15% of marine ingredients (squid and krill meal) alternative to fish meal (VM+). Fish intestines were sampled after 154 days. Besides studying growth parameters and survival, the gene expression related to inflammatory response, immune system, epithelia integrity and digestive process was analysed in the foregut and hindgut sections, as well as different histological parameters in the foregut.

Results

There were no differences in growth performance and feed utilization, although a higher fish mortality was recorded in the VM group. In addition, this group reported lower expression in genes related to pro-inflammatory response, as Interleukine-1β (*il1β*), Interleukine-6 (*il6*) and cyclooxigenase-2 (*cox2*), immune-related genes as immunoglobulin M (*igm*) or bacterial defence genes as alkaline phosphatase (*alp*). In contrast, the VM+ group yielded similar survival rate to FM and the gene expression patterns indicated a higher induction of the inflammatory and immune markers (*il1β*, *cox2* and *igm*). However, major histological changes in gut could not be detected.

Conclusions

Using plants as the unique source of protein at long term may have been the reason of a decrease in the level of certain pro-inflammatory mediators or immune-related molecules. This could reflect a possible lack of local immune response at the intestinal mucosa, explaining the higher mortality observed. The inclusion of krill and squid meal in vegetable diets, even at low concentrations, provided an improvement in both, nutrition and survival parameters, which could be explained by the maintenance of an effective immune response throughout the assay.

Besides, from an economic point of view, the addition of krill by-products and squid meal appears as a good alternative for seabream feeding.

Keywords
gilthead seabream, vegetable meal, squid meal, krill meal, histology, gene expression

Background

Fishmeal replacement in feeds is one of the main challenges in aquaculture farming in order to ensure the sustainability of the production of aquaculture species, especially in carnivorous species [1]. Plant sources have been used as substitutes in order to reduce the use of fishmeal [2] and to develop more economic and environmentally sustainable feeds compared to fishmeal based diets [1,3].

Tolerance to vegetable products depends on species [4]. In the case of gilthead seabream, although high or total replacements of fishmeal by vegetable meal have been successfully achieved in terms of growth [5, 6], detrimental effects on nutrient digestibility and absorption [7,8] have also been reported. Moreover, histomorphological gut and liver alterations [4, 9-11], immune status disorders [9] or gut microbial imbalances [12] have been described. Thus, the use of certain agricultural by-products seems to ultimately lead to a lower nutritive efficiency and an increase in both the susceptibility against diseases and bacterial and parasitic infections [13], which may be induced by an immune deficiency status or disruptions on the inflammatory response.

Hence, dietary and nutritional factors have a great influence on growth and immune response of fish [14]. Among other physiological processes, fish gut particularly plays a key role in the digestion and absorption of nutrients, in the immune response to potential pathogenic invasion and in the protection against environmental stressors [15]. The intestinal status in response to dietary changes has been widely assessed in fish, including gilthead seabream [16-21]. In particular, the impact of low fishmeal diets on the intestinal physiology of different species has been assessed in different stages of the growing phase [22,23].

A wide set of physiological parameters can be evaluated by using different techniques. Gene expression approaches allow to analyse different genes involved in different processes [24] including digestion (digestive enzymes, nutrient transporters), epithelial structure, inflammatory processes (cytokines and other proinflammatory mediators), and innate and adaptive immune response (mucins, genes codifying for antibodies), obtaining a snapshot of the whole response that can indeed provide hints and new insights to dietary impact on the intestinal status. On the other hand, histological assessment of the different gut layers can provide some valuable information on

inflammatory reactions, as well as morphological adaptations to face with the dietary modifications [25].

In addition to detrimental effects associated to anti-nutritional factors [26, 27], whose impact depends on the tolerance of different species, high fishmeal substitutions by vegetable meals in fish diet could result in amino acid imbalances and palatability problems [1, 27], which could have an influence in the feed intake and negatively affect the fish performance [28]. In order to achieve the minimum requirements, diets with high fishmeal substitution usually need a supplementation with synthetic amino acids, that increases the price of the diet and could have different adverse effects in nutrient utilisation [29]. Nevertheless, the addition of complementary ingredients, as opposed or in combination with the amino acid supplementation, seems to be more effective to achieve an ideal amino acid profile when alternative vegetable-based diets are used [28]. Indeed, marine by-products, including squid meal or krill meal, are regarded as a high quality protein source, since they show a balanced amino acid profile and contain a considerable amount of free amino acids [28]. Furthermore, these marine ingredients yield several profits, such as acting as feed-attractant that improves feed intake or offsetting some of the deficiencies observed with high plant protein diets for marine carnivorous fish [28, 30, 31].

This work focuses on the impact of a complete replacement of fishmeal during the on-growing period on the intestine of gilthead seabream through the gene expression study of a broad set of genes related to inflammatory response, immune system, gut epithelia integrity, digestive enzymes and peptide transporters. In addition, the effect of the inclusion of marine byproducts (squid and krill meal) in sea bream plant based diets as a source of marine protein was also assessed in terms of growth parameters and gene expression. The study was supplemented with histological analysis of the foregut, aiming to understand the possible effects in relation to nutrient absorption and inflammatory processes at the morphological level.

Methods

Ethics statements

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica

de València (UPV), following the Spanish Royal Decree 53/2013 and the European Directive 2010/63/UE on the protection of animals used for scientific purposes.

Fish were weighed individually every month during the feed assay, using clove oil with an 87% of eugenol (Guinama ®, Valencia, Spain) as an anaesthetic (1 mg / 100 mL of water) to minimize their suffering.

At the end of the growth assay, fish were euthanized by decapitation, after fish were anesthetized with clove oil dissolved in water (1 mg / 100 mL of water), thus minimizing their suffering.

Design of the experiment

Rearing system, fish and growth assay

The experiment was conducted at the Universitat Politècnica de València (UPV) in a recirculating saltwater system (75m3 capacity) with a rotary mechanical filter and a 6 m^3 capacity gravity biofilter. Nine cylindrical fiberglass tanks with a capacity of 1750 L were used, and water temperature, salinity, dissolved oxygen and pH were as follows: 22.0±0.52º C, 30±1.7 g/L, 6.5±0.49 mg/L, 7.5-8.5. Water parameters were daily measured. All tanks had similar lighting conditions, with a natural photoperiod (from November to March, average of hours of light: 11h).

The seabreams were provided by the fish farm PISCIMAR, in Burriana (Castelló, Spain). The feed was given by hand twice a day (at 9:00 and 17:00 hours) up to apparent satiation with a standard commercial (48% crude protein, 23% ether extract, 11% crude ash, 2% crude fibre and 14% nitrogen free extract) diet during the two-month acclimation period to laboratory conditions. The weekly feeding regimen consisted of six days of feeding and one day of fasting. Growth assay started with fish with an average weight of 129±19 g.

Seabream were randomly distributed into 9 fiberglass tanks (twenty fish per tank), and three different experimental diets (a vegetable diet, VM; a fishmeal-based diet, FM and a vegetable diet with marine ingredients, VM+) were randomly assigned to three of them (n=3). Feeding parameters remained the same as during the acclimatation period. The experiment finished when the fish achieved a commercial size, (average

weight ~350g), and fish were sacrificed afterwards, 154 days after the beginning of the assay.
Fish weight (g) and survival rate (%) were assessed monthly. Final weight (g) (FW), specific growth rate (%/day) (SGR), feed intake (g/ 100 g fish · day) (FI), feed conversion ratio (FCR), and survival (%) (S) were determined when the experiment was completed. The SGR, the FI and the FCR were obtained taking into account the reported monthly biomass of dead fish.

Diets

Diets were prepared as pellets by cooking-extrusion with a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France); located at UPV. The processing conditions were as follows: 0.63 g screw speed, 110º C and 30-40 atm.

Three isonitrogenous and isoenergetic diets were formulated using commercial ingredients, whose proximal composition was previously analysed according to AOAC (Association of Official Agricultural Chemists) procedures: VM diet was based on a mixture of vegetable meals as a protein source and included synthetic amino acids in order to accomplish the minimum requirements of essential amino acids [32]. In FM the protein was provided by fishmeal, although wheat meal was incorporated as a source of carbohydrates. Synthetic amino acids were not included. Finally, VM+ contained a mixture of vegetable meals similar to the VM diet one, but squid meal and krill meal were added to the feed at 10 and 5% level, respectively, reducing the concentration of free amino acid supplementation. These meals were obtained from different companies as by-products: squid meal was provided by Max Nollert (Utrecht, Netherlands) and krill meal by Ludan Renewable Energy (Valencia, Spain).

Amino acids of raw materials and experimental diets were analysed, prior to diet formulation, through a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature control module. Aminobutyric acid was added as an internal standard pattern before hydrolysation. The amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after

oxidation with performic acid. Amino acids were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm).

Table 1. Proximal composition and essential amino acid profile of the different aqua feed ingredients

	FM	WM	WG	BBM	SBM	PM	SFM	SM	KM
Proximate composition (% dry weight)									
DM	90.3	87.8	93.3	89.0	88.1	86.6	89.6	88.0	88.8
A	16.8	1.6	0.9	3.0	7.1	3.4	6.7	9.1	10.4
CL	9.3	1.8	0.9	1.1	2.2	0.8	1.5	15.1	22.5
F	0.1	2.8	0.4	9.1	3.6	6.2	18.7	0.9	4.0*
NFE**	2.5	82.4	16.8	65.7	37.2	70.9	37.4	3.8	7.0
NSP	2.6	23.8	17.2	33.3	32.4	29.9	50.1	4.7	11.0
CP	71.3	11.4	81.0	21.1	49.9	18.7	35.7	71.1	56.1
Essential aminoacids (g 100 g^{-1} dry matter)									
Arginine	5.86	0.38	2.57	1.99	3.66	1.76	3.33	5.90	4.14
Histidine	2.54	0.26	1.45	0.74	1.42	0.58	1.14	1.85	1.26
Isoleucine	3.40	0.36	3.01	1.03	2.33	0.98	1.56	2.28	3.19
Leucine	6.55	0.80	5.79	2.04	4.22	1.78	2.48	4.16	4.67
Lysine	6.01	0.37	1.21	1.92	3.45	1.92	1.39	3.85	3.77
Methionine	2.30	0.22	0.88	0.31	0.92	0.36	1.00	1.76	1.66
Phenylalanine	3.73	0.49	4.31	1.10	2.60	1.11	1.86	2.14	2.97
Threonine	3.55	0.30	1.95	0.94	1.98	0.86	1.52	2.19	2.74
Valine	3.88	0.47	3.26	1.13	2.30	1.06	1.73	2.70	3.12

*4% of chitin

**NFE = 100—CP—CL—F

Origin and price of the different ingredients (ingredient, origin, price in € kg ingredient^{-1}): FM, Vicens i Batllori S. L. (Girona, Spain), 1.51; WM, Desco S. L. (Museros, Spain), 0.15; WG, Ercros S. A. (Barcelona, Spain), 0.76; BBM, Desco S. L. (Museros, Spain), 0.27; SBM, Desco S. L. (Museros, Spain), 0.31; PM, Desco S. L. (Museros, Spain), 0.23; SFM, Desco S. L. (Museros, Spain), 0.17; SM, Max Nollert (Utrecht, Netherlands), 4.28; KM, Ludan Renewable Energy (Valencia, Spain), 0.25.

FM, fishmeal; WM, wheat meal; WG, wheat gluten; BBM, broad bean meal; SBM, soybean meal; PM, pea meal; SFM, sunflower meal; SM, squid meal; KM, krill meal; DM, dry matter; A, ashes; CL, crude lipid; F, fiber; NFE, nitrogen free extract; NSP, non-starch polysaccharides; CP, crude protein

Proximate composition and essential amino acids of different ingredients are shown in Table 1. The ingredients used, the proximate composition and the essential amino acids of the experimental feeds are included in Table 2.

Table 2. Price, ingredients, proximal composition and essential aminoacid profile of diets tested in the growth assay

	VM	FM	VM+
Price (€ kg^{-1})*	0.79	1.09	1.05
Ingredients (g kg^{-1})			
Fishmeal		589	
Wheat meal		260	
Wheat gluten	295		222
Broad bean meal	41		40
Soybean meal	182		160
Pea meal	41		40
Sunflower meal	158		160
Krill meal			50
Squid meal			100
Fish oil	90	38.1	77.5
Soybean oil	90	92.9	77.5
Soy Lecithin	10	10	10
Vitamin-mineral mix**	10	10	10
Calcium phosphate	38		38
Arginine	5		
Lysine	10		10
Methionine	7		5
Taurine	20		
Threonine	3		
Proximate composition (% dry weight)			
DM	93.9	88.1	92.83
A	7.4	10.1	8.8
CL	19.8	18.5	20
F	4.3	0.8	4.6
NFE***	23.2	22.8	21.9
NSP	21.5	7.7	20.6
CP	45.0	44.2	44.6
DP******	41.8	42.7	42.0
Essential amino acids (g 100 g^{-1})			
Arginine	3.30	3.39	3.58
Histidine	0.82	1.00	0.81
Isoleucine	1.17	1.47	1.08
Leucine	2.98	3.24	2.45
Lysine	2.26	3.68	2.38
Methionine	1.06	1.16	1.05
Phenylalanine	1.87	1.80	1.76
Threonine	1.44	1.98	1.28
Valine	1.47	2.01	1.32

* The price of the diets was obtained from the individual prices of the different marine and vegetable meals (included in Table 1) and the other ingredients (price in € kg^{-1} ingredient): soybean oil, 0.63; soy lecithin, 1.15; vitamin-mineral mix, 2.75; calcium phosphate, 2.07; arginine, 7.64; lysine, 1.68; methionine, 3.52; taurine, 2.20; threonine, 1.30.

**Vitamin and mineral mix (values are g kg^{-1} except those in parenthesis): Premix, 25; choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. The Premix is composed of: retinol acetate, 1 000 000 (IU kg^{-1}); calcipherol, 500 (IU kg^{-1}); DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides, 12.

***NFE = 100—CP—CL—F
****DP = CP x ADC_{CP}; ADC_{CP} = Apparent Digestibility Coefficient of Protein: ADC_{CP}(VM) = 0,93; ADC_{CP}(FM)= 0,97; ADC_{CP}(VM+) = 0,94
DM, dry matter; A, ashes; CL, crude lipid; F, fiber; NFE, nitrogen free extract; NSP, non-starch polysaccharides; CP, crude protein; DP, digestible protein

A digestibility experiment was performed after the growth assay, using five randomly selected fish per experimental group and digestibility tanks of 250 liters of capacity (one per experimental group). Apparent digestibility coefficient of the crude protein was obtained, according to the Guelph System Protocol [33], by the Chromium Oxide determination method. After two days of fasting, digestibility assay started and lasted 14 days. Fish were fed to satiation once a day (9:00 hour) with the same experimental diets containing chromium oxide (50g kg-1) as an innert marker and uneaten food was then removed from the columns (15:00). Wet faeces were collected from decantation columns just before the next morning feeding and then dried at 60ºC for 48 h prior to analysis. After acid digestion, an atomic absorption spectrometer (Perkin Elmer 3300, Perkin Elmer, Boston, MA, USA) was used for Chromium oxide determination in duplicate in diets and faeces. Apparent digestibility coefficient of the crude protein (CP) was calculated as follows (Equation 1):

$$(1)\quad ADC_N\ (\%) = 100 \cdot \left(1 - \left(\frac{\%\ marker\ in\ diet \cdot \%\ CP\ in\ faeces}{\%\ marker\ in\ faeces \cdot \%\ CP\ in\ diet}\right)\right)$$

Economic assessment

The Economic Conversion Rate (ECR) and the Economical Profit Index (EPI) [2] were calculated for each experimental group using Equations (2) and (3), respectively. The currency type for economic evaluations was the euro (€). The price of the diets was obtained from the individual prices of the different ingredients. Gilthead seabream sale price was 5.37 € Kg fish-1, based on prices of the Spanish Wholesale market on January 2017. With the aim of showing the impact of the fish mortality on economic profit in on-growing phase, biomass of dead fish was considered and therefore was not included in the total final biomass, and the initial number of fish was used to standarize when the EPI was determined.

$$(2)\quad \text{ECR}\ (€ \cdot \text{kg fish}^{-1}) = \text{FCR}\ (\text{kg diet} \cdot \text{kg fish}^{-1}) \cdot \text{Price of diet}\ (€ \cdot \text{kg diet}^{-1})$$

$$(3)\quad \text{EPI}\ (€ \cdot \text{fish}^{-1}) = \frac{\text{Final biomass(kg fish)} \cdot \text{Sale price}\ (€ \cdot \text{kg fish}^{-1}) - \Delta\text{biomass (kg fish)} \cdot \text{ECR}\ (€ \cdot \text{kg fish}^{-1})}{\text{Initial number of fish}}$$

Sampling

In order to assess gene expression and histological changes throughout the intestinal tract, intestinal samples from three fish per tank were sampled at the end of the growth assay after one day of fasting (40 hours after the last feed). Based on the separation on sections proposed in previous researches [34], three different sections were considered but only pieces of foregut (FG) and hindgut (HG) were collected and stored in RNA later (Ambion Inc., Huntington, UK) at 4ºC overnight and then at -20ºC until RNA extraction. Pieces of FG section (two fish per tank, n=6) were stored in phosphate buffered formalin (4%, pH 7.4) for the histological assessment.

Gene expression

RNA extraction and cDNA step

Total RNA was extracted from FG and HG tissues by traditional phenol/chloroform extraction, using TRIzol Reagent (Invitrogen, Spain), and then purified and treated with DNase I using NucleoSpin® RNA Clean-up XS kit (Macherey-Nagel, Düren, Germany), according to guide instructions. Total RNA concentration, quality and integrity were evaluated using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain) and samples were stored at -80ºC until complementary DNA (cDNA) synthesis.

cDNAwas synthetized from 1 µg of total RNA input using the qScript cDNA Synthesis Kit (Quanta BioScience), according to the manufacturer's instructions, using the Applied Biosystems 2720 Thermal Cycler. The cycling conditions were 22 ºC for 5 min, 42 ºC for 30 min, and 85 ºC for 5 min. Total RNA samples were stored at -80 ºC until gene expression was analysed.

Measurement of gene expression by SYBR Green Assay Real Time quantitative RT-PCR (qPCR)

Reference and target genes

Four candidate reference genes (*ef1α*, *gapdh*, *rps18*, *βact*; Table 3) were tested to be used as housekeeping genes in the gene expression assay. The stability of these genes was determined using six cDNA pooled

samples, obtained each one from combine equally volumes of cDNA samples from the same section in a given experimental group. Ribosomal protein s18 (*rps18*) and β-actin (*βact*) were selected as reference genes for the normalization of gene expression based on the stability of its expression in the cDNA pools and the cDNA specificity in the amplification, confirmed by melting curve analysis [see Additional file 1].

Expression stability of reference genes in individualized samples was determined using the BestKeeper program [35], which reports a standard deviation (SD[±Cq]) lower than 1 for both genes (0.54 for *rps18* and 0.68 for *βact*, $p<0.05$) and Cq arithmetic means of 20.19±1.46 and 17.96±1.6 for *rps18* and *βact*, respectively. The BestKeeper's calculated variations in the reference genes are based on the arithmetic mean of the Cq values.

On the other hand, eighteen candidate target genes (Table 3) were previously tested by RT-qPCR. The proinflammatory cytokines genes *il1β*, *il6* and *il8*, and other proinflammatory molecules, as *tnfα*, *casp1* and *cox2* were included due to their relevance as inflammation markers [16,20]. Genes encoding different mucins (*imuc*, *muc2*, *muc2L*, *muc13* and *muc19*), which contribute to protect the intestine epithelium against a broad spectrum of damages [19], and specific antibodies (*igm*) were also chosen to assess the response of the innate and adaptive immunity, respectively. A tight junction protein, such as *ocl*, and an essential component of microtubules such as *tub* [16] were included in the expression pretesting due to their involvement in the maintenance of the epithelial gut integrity. Regarding the selected genes encoding digestive enzymes and nutrient transporters, *αamy* and *tryp* are digestive enzymes responsible for hydrolysis of carbohydrates and proteins, respectively, and *pept1* is a peptide transporter at the brush border membrane of the enterocytes with an important role in the intestinal absorption [36]. Finally, the gene expression of the *alp*, responsible of removing the phosphate groups of many different molecules [37], was also determined.

This preliminary gene expression test was performed using the cDNA pooled samples used in the reference gene evaluation [see Additional file 2]. Target genes for the further individualized assesment were selected based on their function, potential fold-change differences between diets and intestine segments (significant differences cannot be

determined by a statistical analysis since n=1), gene expression level and nonspecific amplifications. Later on, relative gene expression of the nine selected genes (*il1β*, *il6*, *cox2*, *igm*, *imuc*, *ocl*, *pept1*, *tryp*, *alp*) was determined at the FG and at the HG in nine fish per dietary treatment.

Table 3. Primer sequences of candidate genes (reference and target genes) in the RT-qPCR assay

Gene	Abbreviation	GeneBank ID	Primer Forward	Primer Reverse	Lenght	Reference
REFERENCE GENES						
Elongation Factor 1α	EF-1α	AF184170	CTGTCAAGGAAATCCGTCGT	TGACCTGAGCGTTGAAGTTG	87	[13, 17]
Glyceraldehide 3-phosphate dehydrogenase	GAPDH	DQ641630	CCAACGTGTCAGTGGTTGAC	AGCCTTGACGACCTTCTTGA	80	[14]
Ribosomal Protein S18	Rps18	AM490061	AGGGTGTTGGCAGACGTTAC	CGCTCAACCTCCTCATCAGT	97	[14]
β-Actin	β-Act	X89920	TCTGTCTGGATCGGAGGCTC	AAGCATTTGCGGTGGACG	113	[16]
TARGET GENES						
Interleukin 1β	IL-1β	AJ277166	GCGACCTACCTGCCACCTACACC	TCGTCCACCGCCTCCAGATGC	131	[14]
Interleukin 6	IL-6	AM749958	AGGCAGGAGTTTGAAGCTGA	ATGCTGAAGTTGGTGGAAGG	101	[13]
Interleukin 8	IL-8	DQ976619	GCCACTCTGAAGAGGACAGG	TTTGGTTGTCTTTGGTCGAA	164	[13, 17]
Tumor Necrosis Factor α	TNF-α	AJ413189	CAGGCGTCGTTCAGAGTCTC	GAGATCCTGTGGCTGAGAGG	83	[14]
Cyclooxygenase 2	COX-2	AM296029	GAGTACTGGAAGCCGAGCAC	GATATCACTGCCGCCTGAGT	192	[13, 17]
Caspase 1	CASP-1	AM490060	ACGAGGTGGTGAAACACACA	GTCCGTCTCTTCGAGTTTCG	92	[13]
Intestinal Mucin	I-Muc	JQ277712	GTGTGACCTCTTCCGTTA	GCAATGACAGCAATGACA	102	[16]
Mucin 2	Muc-2	JQ277710	ACGCTTCAGCAATCGCACCAT	CCACAACCACACTCCTCCACAT	90	[16]
Mucin 2-like	Muc-2L	JQ277711	GTGTGTGGCTGTGTTCCTTGCTTTGT	GCGAACCAGTCTGGCTTGGACATCA	67	[16]
Mucin 13	Muc-13	JQ277713	TTCAAACCCGTGTGGTCCAG	GCACAAGCAGACATAGTTCGGATAT	67	[16]
Mucin 19	Muc-19	JQ277715	TGCTTGCTGATGACACAT	TTCACATAGGTCCAGATATTGA	128	[16]
Immunoglobulin M	IgM	JQ811851	TCAGCGTCCTTCAGTGTTTATGATGCC	CAGCGTCGTCGTCAACAAGCCAAGC	131	[15]
Occludin	Ocl	JK692876	GTGCGCTCAGTACCAGCAG	TGAGGCTCCACCACACAGTA	81	[13, 17]
Tubulin	Tub	AY326430	AAGATGTGAACTCCGCCATC	CTGGTAGTTGATGCCCACCT	98	[13]
α-Amylase	α-Amy	AF316854	TGGTGGGACAATCAGAGTCA	GTCCAGGTTCCAGTCGTCAT	85	[13, 17]
Alkaline phosphatase	AlP	AY266359	TTACTGGGCCTGTTTGAACC	GATCTTGATGGCCACTTCCAC	102	[13, 17]
Trypsin	Tryp	AF316852	GGTCTGCATCTTCACCGACT	AAAGGCAGCAGAGTGATGGT	85	[17]
Peptide transporter 1	Pep T-1	GU733710	TTGAACATAACGTCGGGTGA	AATTTTGCATTTCCCTGTGG	92	[13]

RT-PCR assay conditions

All qPCR assays and expression analyses were performed using the Applied Biosystems 7500 Real-Time PCR with SYBR® Green PCR Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA). The total volume for every PCR reaction was 10 µL, performed from diluted (1:50) cDNA template (1 µL), forward and reverse primers (10 µM, 1 µL), SYBR® Green PCR Master Mix (5 µL) and nuclease-free water up to 10 µL.

After an initial Taq activation of polymerase at 95 ºC for 10 min, 42 cycles of PCR were performed with the following cycling conditions: 95 ºC for 10 s and 60 ºC for 20 s in all genes, except for alp (with annealing and extension step at 55 ºC). In order to evaluate assay specificity, a melting curve analysis was directly performed after PCR by slowly increasing the temperature (1º C / min) from 60 to 95 ºC, with a continuous registration of changes in fluorescent emission intensity.

The analysis of the results was carried out using the $2^{-\Delta\Delta Ct}$ method [24]. The target gene expression quantification was expressed relative to the expression of the two reference genes (*rps18* and *βact*). A cDNA pool from all the samples was included in each run and acted as a calibrator, and a non-template control for each primer pair, in which cDNA was replaced by water, was run on all plates. Reference and target genes in all samples were run in duplicate PCR reactions.

Histological analysis

Fragments of FG fixed in formalin were routinely dehydrated in ethanol, equilibrated in UltraClear (Bio-Optica Milano s. p. a., Milan, Italy), and embedded in paraffin according to standard histological techniques. Transverse sections were cut with a thickness of 5 µm with a microtome Shandom Hypercut (four sections per paraffin block were obtained) and dyed with the haematoxylin-eosine staining method. A total of 72 FG sections, obtained from 18 different paraffin blocks (n=6), were analysed under the light microscope (Nikon, Phase Contrast Dry JAPAN 0.90), focusing on possible inflammatory changes and other disorders.

A combination of different criteria reported by several authors [7,9, 38-40] was used to measure the following parameters at FG sections: serous layer (SL), muscular layer (ML), submucosa layer (SML), villi

length (VL), villi thickness (VT) and lamina propria thickness (LP), and number of goblet cells per villus (GC). The longitudinal external muscularis layer was not clearly distinguished and was included in the measurement of the serous layer (SL); therefore, the muscular layer (ML) only included the internal circular muscular layer. Six measurements per section in each parameter were performed and average means were obtained for each sample (n=6). Moreover, a continuous scoring system (Figure 1), ranging from 1 to 4, was used to assess the supranuclear vacuolization on the epithelia (V), the position of the nuclei of the enterocytes (EN) and the lymphocytic infiltration of the epithelial layer (EI), the lamina propria (LPI) and the submucosa (SMI) in each sample (n=6).

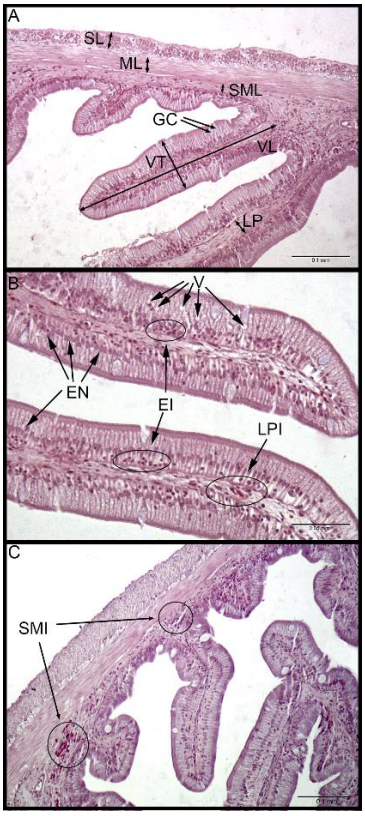

Figure 1. Evaluation and scoring system used to assess histological parameters of gilthead seabream foregut

A) Measurements performed in a foregut histological section (20x).
B) Detail of villi with a certain grade of infiltration of the lamina propia and the epithelia. Enterocytes nuclei were displaced in some cases. Epithelial vacuolization can also be observed in a normal grade (40x). C) Enterocytes showed aligned nuclei in a basal position. Villi presented a low grade of infiltration of their lamina propia and of the epithelia, and low vacuolization. A certain grade of infiltration in the submucosa layer can be observed (20x).
SL, ML, SML, VL, VT and number of GC were measured six times per section, and averages were obtained for each section (six sections per group, n=6). V, EN, EI, LPI and SMI were assessed in each section (n=6) using the following scoring system: V, normal (1) to hypervacuolated (4); EN, basal (1) to apical (4); EI, low (1) to markedly increased (4); LPI, low (1) to markedly increased (4); SMI, low (1) to markedly increased (4).
SL, serous layer; ML, muscular layer; SML, submucosa layer; VL, villi length; VT, villi thickness; LP, lamina propria; GC, goblet cells; V, supranuclear absorptive vacuoles; EN, enterocytes nuclei; EI, epithelial infiltration; LPI, lamina propria infiltration; SMI, submucosa infiltration

Statistics

Statistical data analyses were carried out with Statgraphics © Centurion XVI software (Statistical Graphics Corp., Rockville, MO, USA). All percentage data were arcsine transformed prior the analysis.

Differences in fish weight and survival between dietary groups were monthly evaluated by simple analysis of variance, considering the tank as the experimental unit. At the end of the growth trial, economic indeces (ECR and EPI) and livestock data (FW, SGR, FCR, FI and S) were subjected to simple variance analysis. Each group in the calculation represented the combined group of fish per single tank (triplicate tanks per treatment). Student Newman-Keuls test was used to assess specific differences among diets at the 0.05 significance level. Descriptive statistics are shown as the mean ± pooled standard error of the mean (SEM).

Relative gene expression data was statistically analysed by two-way analysis of variance using Newman-Keuls test. Differences in expression were considered statistically significant when $p<0.05$. Data was expressed with the mean and the standard error for each gut section and experimental group. Differences in the gene expression between sections within each group, between experimental groups, and between same sections in different dietary groups were determined.

Finally, histological measurements in foregut were showed as the mean ± standard error of the mean and it was analysed through an ANOVA, with a Newman-Keuls test for the comparison of the means and a level of significance set at $p<0.05$. PCA was used to analyse the histological scored parameters of gut (V, EN, EI, LPI and SMI). Statistical differences between experimental groups were estimated by an analysis of variance (ANOVA) using the first and second Principal Components of the PCA, with a Newman-Keuls test ($p<0.05$).

Results

Economic indices

Statistically differences were determined in the ECR between the groups FM and VM, whilst the EPI was significantly higher in the groups FM and VM+ (Table 4). Differences in the ECR can be

explained by the higher cost of the FM feed, while the lower number of fish at the end of the growth assay in the different tanks assigned to the VM treatment led to a significant lower EPI in this dietary group.

Table 4. Growth and economic indices of seabream fed experimental diets at the end of the experiment

	VM	FM	VM+	SEM
IW (g)	127.2	131.2	129.6	4.1
FW (g)	360.4	393.1	384.6	13.2
SGR (% day^{-1})	0.69	0.72	0.73	0.03
FI (g 100 g fish^{-1} day^{-1})	1.38	1.35	1.33	0.02
FCR	2.40	2.14	2.08	0.10
S (%)	60.0^{b}	88.3^{a}	86.7^{a}	5.18
ECR (€ kg fish^{-1})*	1.90^{b}	2.35^{a}	2.24^{ab}	0.10
EPI (€ fish^{-1})*	0.99^{b}	1.36^{a}	1.33^{a}	0.07

*Price of diets: VM = 0.79 €; FM = 1.09 €; VM+ = 1.05 €. Sale price of gilthead seabream = 5.37 € kg fish^{-1}.
Means of triplicate groups (n=3). Data in the same row with different superscript letters differ at $p<0.05$.
IW, initial weight (g); FW, final weight (g); SGR, specific growth rate (% day^{-1}) = 100 •·ln (final weight (g)/ initial weight (g)) / days; FI, feed intake (g 100 g fish^{-1} day^{-1}) = 100 • feed consumption (g) / (average biomass (g)· days); FCR, feed conversion ratio = feed offered (g) / weight gain (g); S, survival (%) = 100 • (final number of fish / initial number of fish); ECR, Economic Conversion Rate (€ kg fish^{-1}); EPI, Economical Profit Index (€); SEM, standard error of the mean

Growth assay and growth indices

Statistically significant differences were determined in the average weight of fish after 112 days from the beginning of the growth assay, registering higher weight in those fish fed FM and VM+ than in fish fed VM (Figure 2), although no significant differences were observed in subsequent sampling points and at the end of the feeding trial. On the other hand, survival rate of fish fed VM began to decrease after 112 days of the experiment in comparison to the rates observed in the other two groups (FM and VM+). Survival rate continued decreasing at VM group as the growth trial progressed, but no disease signs were reported in dead fish. No differences were observed in the growth parameters, which are shown in Table 4.

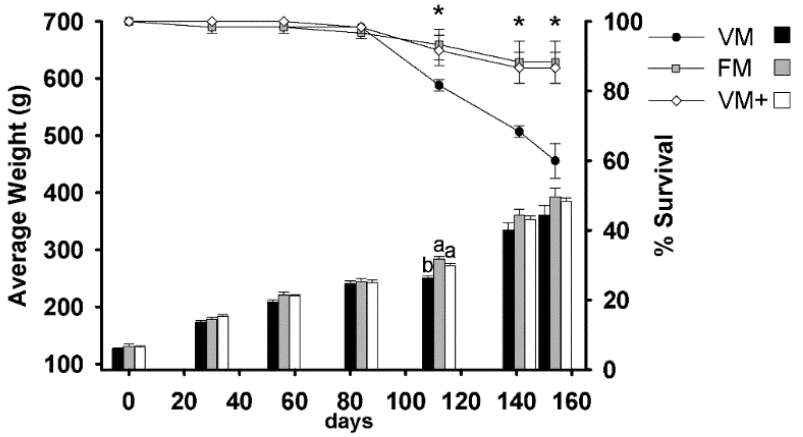

Figure 2. Average weight (g) and survival rate (%) evolution of gilthead seabream along the assay period

Average weight mean and standard error (bars) and survival rate (line) of each experimental group were displayed in different colours (Black: VM; Grey: FM; White: VM+). Different superscripts on the bars indicate significant statistical differences in the average weight during the growth trial ($p<0.05$). Data are means of triplicate groups (n=20). Asterisks indicate the existence of significant differences in the survival rate along the assay at $p<0.05$.

Gene expression

Inflammation and immune system genes

The diet was determined as a significant factor affecting the expression of *il1β*, *il6*, *cox2* and *igm* (Table 5). Fish fed VM and FM reported lower expression levels of *il1β* (Figure 3A), *cox2* (Figure 3C) and *igm* (Figure 3D) in comparison to VM+ group, and a lower expression of il6 (Figure 3B) was observed in the VM group. *Igm* and *i-muc* relative expression were affected by the section (Table 5): *igm* (Figure 3D) had a higher expression in FG than in HG, specially in the group VM+, and *i-muc* (Figure 3E) reported a remarkably higher expression in the HG.

Table 5. p-values* determined for diet, intestinal section and the interaction between both factors on the gene expression assay

Gene	Diet	Section	Diet x section
il1β	<0.05	ns	ns
il6	<0.05	ns	ns
cox2	<0.01	ns	ns
igm	<0.0001	<0.05	<0.05
imuc	ns	<0.01	ns
ocl	<0.01	ns	ns
alp	<0.01	ns	ns
tryp	ns	ns	ns
pept1	<0.05	<0.0001	ns

*p-values were obtained using the Student Newman-Keuls test in a two-way analysis of variance

il1β, Interleukine-1β; *il6*, Interleukin-6; *il8*, Interleukine-8; *cox2*, Cyclooxigenase-2; *imuc*, Intestinal Mucin; *igm*, Immunoglobulin M; *ocl*, Occludin; *alp*, Alkaline Phosphatase; *tryp*, Trypsin; *pept1*, Peptide Transporter 1; ns, non significant

Structural, enzyme and nutrient transport genes

Expression of *ocl*, *alp* and *pept1* was significantly influenced by the diet (Table 5). VM showed a lower expression of *ocl* (Figure 3F) and alp (Figure 3G) in comparison to the other two groups, and a lower expression of *pept1* (Figure 3I) in comparison to FM, but higher in comparison to VM+. *tryp* relative expression (Figure 3H) showed a large individual variation and no significant differences were found at diet or section level. Finally, the expression of *pept1* was also affected by the section (Table 5), being overexpressed in the FG compared to HG in all experimental groups (Figure 3I).

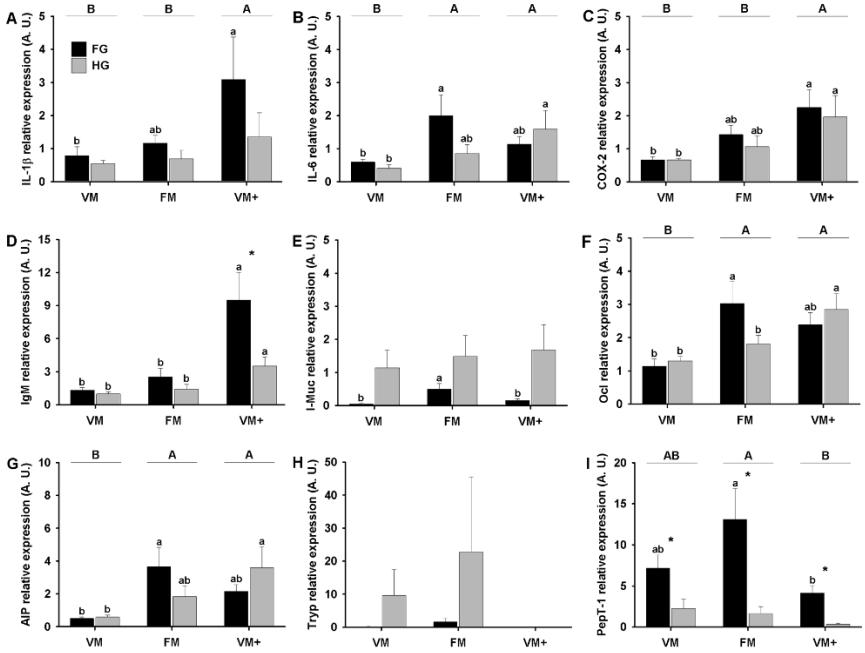

Figure 3. Relative gene expression in the intestine of gilthead seabream fed different experimental diets

A) Interleukine-1β (IL-1β); B) Interleukine-6 (IL-6); C) Cyclooxigenase-2 (COX-2); D) Intestinal Mucin (i-MUC); E) Immunoglobulin M (IgM). F) Occludin (Ocl); G) Alkaline Phosphatase (AlP); H) Trypsin (Tryp); I) Peptide Transporter 1 (PepT-1). Bars represent relative gene expression (mean + standard error, n=9), for each group, in the foregut (FG, black bars) and the hindgut (HG, grey bars). Superscript letters on the bars indicate differences between experimental groups in each section, at $p<0.05$. Asterisks indicate differences between intestinal sections in each experimental group, at $p<0.05$. Capital letters at the top of the graph indicate differences between experimental groups, regardless the intestinal section (n=18, $p<0.05$), when interaction between factors (diet and section) is not significative (Table 5).

Histological analysis

Light microscope observations revealed minor histological alterations in the FG. Fish fed VM exhibited thinner villi than the fish fed the FM diet (Table 6). No differences were determined in the thickness of the three layers of the intestinal wall, nor in the length of the villi and the thickness of the lamina propria. GC were increased in many of the fish fed the vegetable diets, especially for the fish fed VM+, although no significant differences were determined between dietary groups.

On the other hand, assessment by scoring of different parameters of the gut (Figure 4) revealed differences on the number of supranuclear absorptive vacuoles in the epithelial layer (V), the displacement of the enterocytes nuclei to apical positions (EN), and the degree of inflammatory cells infiltration in the submucosa layer (SMI), higher in the foregut sections belonging to VM and VM+ groups in the three cases. PCA dispersion graph (Figure 4) based on this assessment showed a clear separation among the FM group and the groups fed with plant-based diets. First Component (PC1) explained the 53,7% of the variability and was related with higher inflamation. In this sense, ANOVA based on the PC1 confirmed the existence of significant differences between FM sections and sections of the groups of fish fed vegetable diets (VM and VM+).

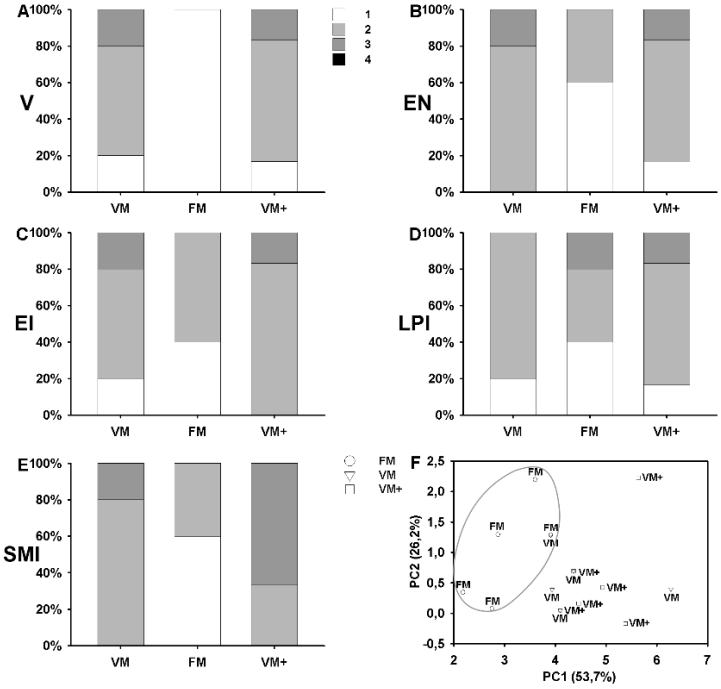

Figure 4. Histological assessment of foregut sections of gilthead seabream fed different experimental diets, according to Figure 1

Frequency bar charts showing differences in (A) supranuclear absorptive vacuolization (V), (B) enterocytes nuclei (EN), (C) enterocytes infiltration (EI), (D) lamina propria infiltration (LPI) and (E) submucosa infiltration (SMI). (F) Dispersion graph representing values of the first and second components for each foregut section assessed, obtained from the Principal component analysis of histological foregut scores according to diet. Only sections evaluated in all parameters were included in the Principal component analysis (n=5 for VM and FM, n=6 for VM+).

Table 6. Dietary effect on the histomorphology of the foregut a of gilthead seabream

	VM	FM	VM+	SEM
SL (µm)	52.9	50.3	61.7	5.2
ML (µm)	56.6	58.4	55.8	5.4
SML (µm)	40.4	44.3	43.0	4.5
VL (µm)	512.2	621.7	568.7	57.4
VT (µm)	85.2^{b}	101.1^{a}	93.4^{ab}	4.1
LP (µm)	9.9	14.2	14.0	1.3
GC	4.4	3.2	4.9	1.1

Means for SL, ML, SML, VL, VT, LP and GC were calculated from the average mean of each sample (n=6). Data in the same row with different superscript letters differ at $p<0.05$.
SL, serous layer; ML, muscular layer; SML, submucosa layer; VL, villi length; VT, villi thickness; LP, lamina propria; GC, globet cells; SEM, standard error of the mean

Table 7 shows a summary with all the effects observed on growth, gene expression and gut histology, taking the FM group as a control.

Table 7. Summary of dietary effects on growth performance and intestinal gene expression and histology

	VM	VM+
Growth performance	Higher mortality	
Gene expression	Lower expression of *il6*, *ocl*, *alp* and *pept1*	Higher expression of *il1β*, *cox2* and *igm* Lower expression of *pept1*
Foregut histology	Thinner villi More apical enterocytes nuclei Higher V and SMI	Thinner villi More apical enterocytes nuclei Higher V and SMI

il1β, Interleukine-1β; *il6*, Interleukine-6; *cox2*, Cyclooxigenase-2; *igm*, Immunoglobuline M; *alp*, Alkaline phosphatase; *pept1*, Peptide Transporter-1; V, vacuolization; SMI, submucosa infiltration; PFI, peripancreatic fat infiltration

Discussion

Zootecnical and economical parameters

Based on the evolution of mean weight and survival rates, the impact of the different feeds on the growth and survival can be observed from

112 days of the growth assay. However, although survival rates of fish fed the VM diet was significantly decreasing from this time until the end of the trial, no significant differences in terms of mean weight were registered at the 140 and 154 days. The size of the dead fish found in the VM tanks in the final stage of the assay was smaller than the average size of the fish in these tanks, which could explain the disappearance of significant differences in the mean weight at the end of the trial. Variability in the different experimental groups prevents signficant differences in growth indices, although results show certain negative impact of the VM diet on the SGR and specially on the FCR. On the other hand, the less growth performance and higher mortality reported in the VM group are manifested in the economic indices. In this sense, FM and VM+ diets showed a similar efficiency under an economical point of view.

Intestinal status

Fish perfomance, including growth and survival, could be compromised by alterations in the intestinal homeostasis [11]. In order to learn about the long term dietary impact on the intestinal tract, the expression of different genes related to the inflammatory, the innate and adaptive immune system, the epithelial integrity and nutrient digestion and transport was determined, and the gut morphology was assessed.

Fishmeal replacement by different vegetable sources has been associated with occurrence of gut inflammation in different species [41,42]. Previous research has reported the up-regulation of the expression of different inflammatory markers [22, 23, 43, 44], higher grade of cell infiltration in the submucosa and changes in the expression of genes related with several processes, including antioxidant defences, cell differentiation, epithelial permeability, immunity and mucus production [22, 43] in response to moderate and high levels of plant protein sources inclusion.

In the present work, the group VM+, in which fishmeal was totally replaced by plant sources and squid and krill meal were included at 15% level, reported the up-regulation of pro-inflammatory markers (*il1β* and *cox2*) and *igm* compared to control group (FM). The increase of gene expression in relation to inflammatory mediators has been linked to the regulation of the inflammation [20] and the activation of the innate immunity in response to infection [45], and it has been observed as a common response against low fishmeal based diets in several species

[23]. On the other hand, although Immunoglobulin T has been recently suggested as the main inmunoglobulin in the mucosal responses in gilthead seabream [46], IgM plays a key role in the gut mucosal immune reactions against pathogens or environmental stress, and also in the triggering of the humoral response [18, 47]. Additionally, high levels in fish fed with plant sources based diets have been reported [48]. Thus, the up-regulation of these genes could reflect that fish fed VM+ were developing an inflammatory process at the intestinal mucosa level, and are able to maintain an active local immune system after the growth trial.

In contrast, this up-regulation is not observed in the VM group, which showed a lower expression of different pro-inflammatory markers and other genes related with the immune defence (*igm*, *alp*) and the regulation of epithelial permeability (*ocl*), even lower compared to the FM group. Occludin has been suggested as a key protein in the epithelial integrity maintenance and in the regulation of permeability and other properties of the epithelial barrier [49], being a marker of integrity of the tight junction between the enterocytes, and its underexpression could suggest deficiencies in the regulation of the gut inflammatory response [16, 20]. Importance and physiologic function of alkaline phosphatase in digestion and a possible dietary regulation of its expression remain unclear, but it has been described as a gut mucosal defense factor, which seems to be implicated in the mucosal defence through the dephosphorylation of the lipopolysaccharides (LPS) from the endotoxins of gram-negative bacteria [50]. Microbial LPS upregulates alp and its activity reduces toxicity of LPS [37], preventing from excessive inflammation in response to commensal microbes and helping to maintain the balance and integrity of the intestinal epithelial barrier [51].

The down-regulation of the expression of the genes could reflect that fish fed the VM diet were not triggering an inflammatory response at the end of the growth trial, as well as certain grade of immune mechanism suppression at local level, maybe evidencing a stress response. This depressed status could explain the higher mortality reported in this group and it could be linked with microbial imbalances that have been described in response to total fishmeal replacement in gilthead seabream [12].

In this sense, inclusion of high levels of plant protein sources in aqua feeds for carnivorous species can be considered as a chronic stress

factor, triggering a reponse by the host [52], which redirects more energy and resources to face with the stressor [53]. After long periods, immune mechanisms and other pathways that demand a continuous energy supply can be affected, leading to depressive or suppresive effects [52], leading to a chronic stress status. The suppresion of inflammatory and immune mechanisms in response to long term feeding high plant protein diets has been observed in previous research in different species [54,55], including the gilthead seabream [9], and a differential response was also observed in different intestinal sections [22].

Exposition to antinutrients included into the vegetable-based diets (VM+, and specially, in VM) throughout the growth assay could initially determine a prolongued inflammatory reaction in both experimental groups, demanding an additional energy expenditure that fish fed VM are not able tot sustain. Therefore, differences in the inflammatory and immune status of the gut between the VM and VM+ group at the end of the growth assay might be explained by dietary composition.The VM diet only includes vegetable meals, and synthetic amino acids were added in order to comply minimum amino acid requirements [32], while in the VM+ diet squid and krill meal —which have higher quality protein than vegetable meals and could improve essential amino acid profile in terms of bioavailability— were included at 10% and 5% levels, respectively, and the amount of synthetic amino acids was lower. This inclusion of marine by-products at 15% level could favour the maintenance of an active gut proinflammatory response along the experiment, while the VM diet could be a deficient diet from a nutritional point of view and fish could be unable to meet the energy requirements to sustain the inflammatory response during all the growth trial. On the other hand, chitin, which is present in the krill meal at 4%, could increase the activity of the seabream immune system [56]. Composition in fibre, non-starch polysaccharides and fatty acids was very similar in both experimental diets and did not seem to be the reason of the observed differences.

On the other hand, the higher expression of *pept1* at the FG of fish confirms that this is the main production site and the intestinal section in which most of the absorption of small peptides takes place in gilthead seabream [36]. The downregulation of the peptide transporter in the anterior intestine of fish fed VM, and especially of fish fed VM+, could be related to a greater presence of non-starch polysaccharides, saponins or other antinutrients in the vegetable based diets, which could alter the

gut integrity and reduce the gastrointestinal passage of the food [57], and also to a lower digestibility of vegetable protein, which possibly contributes to a lower small peptide transport.

Finally, some possible minor inflammatory signs were observed at histological level in the present work in fish fed with both plant protein-based diets (VM and VM+), which could suggest that fish fed VM could develop an inflammatory reaction at certain point of the growth assay, before a possible suppression of inflammatory and immune mechanisms. Modifications include a higher grade of vacuolization in the epithelia and an increase of cell infiltration in the submucosa layer. Presence of supranuclear absorptive vacuoles in the epithelial layer is normal, but their excessive accumulation could be related to changes in the function of enterocytes [58], and it is often accompanied with evident signs of inflammation, as immune cell infiltration, as it has been observed in previous studies in response to different experimental diets in different species and in different segments of the gut [4, 7, 59-62]. Moreover, villi with a higher content of GC were observed in the gut of fish fed diets containing vegetable meals, especially on the VM+ group, compared to the FM group, although no statistical differences were determined because a reduced number of GC in some villi in all experimental groups was observed. The increase in the number of GC has been noticed in rainbow trout [59], likewise in seabream fed with vegetable-based diet [6, 11], suggesting a possible alteration of secretory processes. GC secreted a mucus gel that covered the epithelium of the intestinal tract [63], so that the thicker mucus layer observed in fish fed vegetable based diets during the sampling process is consistent with these findings, although no differences were reported between experimental groups in the imuc expression in the HG, were it is constitutively expressed accordin to previous research [19]. However, no enteritis features in the FG were found, which is in accordance to previous studies [7]. In this sense, tolerance to antinutrients, which may be the cause of enteritis [59], seems to depend on species [4], and gilthead seabream seems to tolerate high levels of plant sources in diets without intestinal structural damage [9, 64], and only moderate changes, without pathological signs, have been observed in most research works [4, 7, 9, 25, 43, 44, 64]. In this sense, a higher degree of cellularity and the widening of the lamina propria -described as signs of inflammation- of fish fed vegetable diets, were not noticed in the present experiment, but similar observations were also made [6,10,11] in feeding trials with high levels of fishmeal replacement, so this point must be clarified.

On the other hand, thinner villi observed in the FG of fish fed with VM could be affecting the nutrients absorption capacity, although impact on growth could be more related with the allocation of energy to face with an prolongued inflammatory status than with histomoprhological changes. However, a similar effect with high levels of fishmeal replacement has been observed [10] and further investigation should be also performed on this issue to explain that response.

Conclusion

Total replacement of fishmeal by vegetable protein sources in diets for the on-growing of gilthead seabream had a negative impact on long-term fish survival under the experimental conditions, maybe caused by a lack of gut mucosal immune response derived from a lingering poor nutritional status. The inclusion of squid and krill meal in vegetable-based diets seemed to produce a long-term inflammation response in the gut along the growth assay, but no negative effects on fish survival were reported. However, development of vegetable-based diets that do not cause gut inflammatory reactions is needed in order to ensure, not only growth and survival, but also health status and welfare of fish.

Abbreviations

UPV: Universitat Politècnica de València; **VM**: Vegetable diet; **FM**: Fishmeal-based diet; **VM+**: Vegetable diet with marine ingredients; **FW**: Final weight; **SGR**: Specific growht rate; **FI**: Feed intake; **FCR**: Feed conversion ratio; **S**: Survival; **CP**: Crude protein; **FG**: Foregut; **HG**: Hindgut; **ECR**: Economic Conversion Rate; **EPI**: Economical Profit Index; ***ef1α***: Elongation Factor 1α; ***gapdh***: Glyceraldehide 3-phosphate dehydrogenase; ***rps18***: Ribosomal protein S18; ***βact***: β-Actin; ***il1β***: Interleukin 1β; ***il6***: Interleukin 6; ***il8***: Interleukin 8; ***tnfα***: Tumor Necrosis Factor α; ***cox2***: Cyclooxygenase 2; ***casp1***: Caspase 1; ***imuc***: Intestinal Mucin; ***muc2***: Mucin 2; ***muc2L***: Mucin 2-like; ***muc13***: Mucin 13; ***muc19***: Mucin 19; ***igm***: Immunoglobulin M; ***ocl***: Occludin; ***tub***: Tubulin; ***αamy***: α-Amylase; ***alp***: Alkaline Phosphatase; ***tryp***: Trypsin; ***pept1***: Peptide Transporter 1; **SL**: Serous layer; **ML**: Muscular layer; **SML**: Submucosa layer; **VL**: Villi length; **VT**: Villi thickness; **LP**: Lamina propria thickness; **GC**: Goblet cells; **V**: Supranuclear vacuolization; **N**: Position of the nuclei of the enterocytes; **EI**: Lymphocytic infiltration of the epithelilal layer; **LPI**: Lymphocytic infiltration of the lamina propria; **SMI**: Lymphocytic infiltration of the

submucosa; **SEM**: Standard error of the mean; **ANOVA**: Analysis of variance

Supplementary material

Supplementary material (Additional file 1 and Additional file 2) is included in ANNEX II

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Chapter 3.

Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene

Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene

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Abstract

Recent studies have demonstrated the impact of diet on microbiota composition, but the essential need for the optimization of production rates and costs forces farms and aquaculture production to carry out continuous dietary tests. In order to understand the effect of total fishmeal replacement by vegetable-based feed in the sea bream (*Sparus aurata*), the microbial composition of the stomach, foregut, midgut and hindgut was analysed using high-throughput 16S rDNA sequencing, also considering parameters of growth, survival and nutrient utilisation indices.

A total of 91,539 16S rRNA filtered-sequences were analysed, with an average number of 3661.56 taxonomically assigned, high-quality sequences per sample. The dominant phyla throughout the whole gastrointestinal tract were *Actinobacteria*, *Protebacteria* and *Firmicutes*. A lower diversity in the stomach in comparison to the other intestinal sections was observed. The microbial composition of the Recirculating Aquaculture System was totally different to that of the sea bream gastrointestinal tract. Total fishmeal replacement had an important impact on microbial profiles but not on diversity. *Streptococcus (p-value: 0.043) and Photobacterium (p-value: 0.025)* were highly represented in fish fed with fishmeal and vegetable-meal diets, respectively. In the stomach samples with the vegetable diet, reads of chloroplasts and mitochondria from vegetable dietary ingredients were rather abundant. Principal Coordinate Analysis showed a clear differentiation between diets in the microbiota present in the gut, supporting the presence of specific bacterial consortia associated with the diet.

Although differences in growth and nutritive parameters were not observed, a negative effect of the vegetable diet on the survival rate was determined. Further studies are required to shed more light on the relationship between the immune system and sea bream gastrointestinal tract microbiota and should consider the modulation of the microbiota to improve the survival rate and nutritive efficacy when using plant-based diets.

Introduction

The gilthead sea bream (*Sparus aurata*) is a species of the family Sparidae being produced in large amounts in Europe. As a carnivorous fish, it requires a high level of fishmeal in its diets to provide an ideal amino acid profile and reach high digestibility and growth. Despite this, fishmeal substitution by plant protein sources in sea bream diets is necessary to maintain the profitability of the farms. Therefore, in recent years, a large research effort has been made in this field to reduce fishmeal and/or fish oil in aquafeeds by plant sources [1, 2]. However, plant protein sources contain certain undigestible components (non-starch polysaccharides) [3] and antinutritional factors (protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, allergens) [4]. These compounds can affect nutrient digestibility and absorption [5], as well as gut integrity [6,7], promoting bacteria ingress and, therefore, change the gut microbiota in terms of microbial abundance and species richness.

Despite these problems associated to vegetable proteins, a successful replacement of total fishmeal by a vegetable protein concentrate mixture has been reported [8]. However, alterations in the gut histology of sea bream have been observed with fishmeal replacement above a 60% level [9], as well as immunosuppression above 75% of fishmeal substitution [6]. An imbalanced microbiota may provoke an alteration of the immune regulatory functions of the gut and contribute to the development of diseases [10].

Many exogenous and endogenous factors, such as species, age and developmental stage, bacterial colonisation during the larval stage, geographic location, seasonality and other environmental factors, especially temperature, antibiotic use during fish growth, or the individual genetics of each fish can alter the gut microbiota composition [11]. However, food is one of the main factors putting selective pressure on the gastrointestinal microbial composition [12]. Differences in the amount of the microbiota population between fish fed live food or artificial feed have been observed [13]. Also, diets with plant meals have an impact on the microbiota composition [14], affecting the gastrointestinal tract (GIT) morphology and increasing the damage of the absorptive area [15], although differences in microbiota composition were not observed in herbivorous fish species such as the Crucian Carp (*Carassius auratus gibelio* x *Cyprinus carpio*) [16].

It is frequently considered that fish gut is usually divided into three sections [17, 18]: the first segment or foregut (FG) is generally the longest part and has mainly an absorptive function, the second segment or midgut (MG) contains enterocytes having a high pinocytotic activity for macromolecule transport, and the third segment or hindgut (HG) is the shortest of them, for which different functions have been proposed. Different digestive functions may also be related to different microbial content as occurs in mammals [19]. Furthermore, although the gut associated lymphoid tissue (GALT) [10] in fish does not reach the level of organisation shown in mammals, abundant lymphocytes are found in the lamina propria. The midgut has been proposed as the segment that has a clearer function of antigen capture and immune stimulation [20], and also a high presence of immunoreactive cells has been associated to the posterior intestine [21].

Fish microbiota has traditionally been studied by culture methods and subsequent identification based on biochemical and phenotypic characteristics of bacteria [22]. The development of PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) [23] and other molecular methods in recent years has allowed the characterisation of total microbiota in fish, both marine fish and freshwater, such as *Oncorhynchus mykiss* [24], *Gadus morhua* [14], *Salmo salar* [25], *Paralichthys olivaceus* [12] and many other species, including *Sparus aurata*[7,15,26]. However, new modern sequencing techniques such as 454 pyrosequencing (Roche, Basel, Switzerland) have been applied to study the microbiota of zebrafish (*Danio rerio*) [27], and also economically important species such as *Cyprinus carpio* [28], *Oncorhynchus mykiss* [29], *Dicentrarchus labrax* [30] or *Sparus aurata*[31].

The aim of this study was to assess the impact of a total vegetable diet during the fattening period of sea bream on zootechnical parameters, but also on an increasingly relevant biological aspect, the gut microbiota composition, that may in turn have a number of physiological consequences ranging from feed component utilisation to immune competence. Furthermore, to the best of our knowledge, this study represents the first report of microbiota composition along the GIT in sparids fed only with vegetable meals as a source of protein using high-throughput techniques.

Materials and methods

Rearing system

The trial lasted 154 days (from December 2012 to May 2013) and was conducted in six cylindrical fibre glass tanks (1750 L) as part of a recirculating saltwater system (75 m^3 capacity) with a rotary mechanical filter and a 6 m^3 capacity gravity biofilter. All tanks were equipped with aeration, and the water was heated with a heat pump installed in the system. The water temperature was 22.0±0.52 ºC, salinity was 30±1.7 g L^{-1}, dissolved oxygen was 6.5 ± 0.49 mg L^{-1}, and pH ranged from 7.5 to 8.5. The photoperiod was natural and all tanks had similar lighting conditions.

Fish

Sea bream were obtained from the fish farm PISCIMAR in Burriana (Valencia, Spain) and after two months of acclimation to laboratory conditions, feeding a standard commercial diet, were distributed in the six tanks in groups of 20 in each tank. The experiment was initiated with fish weighing 130 ± 19 g, however, with slight differences between the tanks.

Ethics statements

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes [32].

Diets and feeding

Diets were prepared as pellets by cooking-extrusion with a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France); located at UPV. The processing conditions were as follows: 0.63 g screw speed, 110 ºC and 30-40 atm. Proximate analyses of diet ingredients, diets and faeces were based on AOAC procedures [33].

Two isonitrogenous and isoenergetic diets (FM100 and AA0) were formulated using commercial ingredients (Table 1). FM100 contained fishmeal as the main protein source, wheat meal, fish and soy oil and a vitamin-mineral mix. In the AA0 diet, fishmeal and wheat meal were replaced by a mixture of vegetable meals, and synthetic aminoacids were added in order to balance the aminoacid composition. Proximate composition, including digestible protein (DP), is also shown in Table 1. Apparent digestibility of the protein of feeds was determined using the method detailed by Sánchez Lozano et al. [34].

Table 1. Ingredient content and proximate composition of experimental diets

	FM100	AA0
Ingredients (g kg^{-1})		
Fishmeal	589	
Wheat meal	260	
Wheat gluten		295
Bean meal		41
Soybean meal		182
Pea meal		41
Sunflower meal		158
Fish oil	38,1	90
Soybean oil	92,9	90
Soy Lecithin	10	10
Vitamin-mineral mix*	10	10
Calcium phosphate		38
Taurine		20
Methionine		7
Lysine		10
Arginine		5
Threonine		3
Proximate composition (% dry weight)		
Dry matter	88	94
Ash	10,1	7,4
Crude lipid (CL)	18,5	19,8
Fibre	1	4,2
NFE**	26	22,2
Non-starch polysaccharides	10,9	20,6
Protein (CP)	44.2	45.0
Digestible Protein (DP)	42,4	41,4

*Vitamin and mineral mix (values are g kg^{-1} except those in parenthesis): Premix: 25; Choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition: retinol acetate, 1 000 000 IU kg^{-1}; calcipherol, 500 IU kg^{-1}; DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12.

**Nitrogen free extract (NFE, %) = 100 - %CP - %CL - %Fibre

Growth assay

Each experimental diet was assayed along 154 days in three tanks, randomly assigned. Fish were handfed twice a day (09:00 and 17:00 hours) to apparent satiation in a weekly feeding regimen of six days and one of fasting. Pellets were distributed slowly, permitting all fish to eat. Fish were observed daily in tanks and were weighed individually every four weeks, using clove oil containing 87% eugenol (Guinama ®, Valencia, Spain) as an anaesthetic (1 mg / 100 mL of water) to minimize their suffering, in order to evaluate fish growth along the assay, determine growth parameters and assess their health status. Growth and nutrient utilisation indices considered were as follows:

Specific growth rate (% day^{-1}) (SGR) = 100·ln (final weight / initial weight) / days
Feed intake (g 100 g fish^{-1} day^{-1}) (FI) = 100 · feed consumption (g) / (average biomass (g)· days)
Feed conversion ratio (FCR) = feed offered (g) / weight gain (g)
Protein efficiency ratio (PER) = weight gain (g) / protein offered (g)
Survival (%) (S) = 100 · (final number of fish / initial number of fish)

Sampling of gastrointestinal contents

Gastrointestinal contents of three fish per tank were sampled at the end of the assay in the laboratory, 154 days after initiation of the experiment. The fish were slaughtered to obtain samples of the gastrointestinal content in the different sections of the GIT.

The criterion used to determine when the animals should be humanely sacrificed was their commercial size (over 300 g). To ensure the presence of content along the whole digestive tract, fish were fed at 20:30 on the day before and at 8:30 on the sampling day, 30 minutes before initiation of sampling.

Fish were anesthetized using clove oil dissolved in water (1 mg / 100 mL of water), in order to minimize suffering of the animals, sacrificed by decapitation, and then dissected in order to obtain the digestive tract. Four different sections were considered: stomach (ST), foregut (FG), midgut (MG) and hindgut (HG). Gastrointestinal content was obtained by scrapping the gastric/intestinal mucosa with a spatula. Thus, a total of four gastrointestinal content samples were obtained per fish, placed in Eppendorff tubes and immediately frozen in liquid nitrogen. Later, they were stored at -80 ºC until DNA extraction. Moreover, a 500 mL

water sample was obtained from the recirculating saltwater system and stored at -20 ºC.

72 samples of gastrointestinal content were obtained in the sampling. Nevertheless, samples were pooled after 16S ribosomal RNA gene PCR amplification to simplify the pyrosequencing assay and subsequent microbiota analysis. Each pool was made up of 3 samples from the same gastrointestinal section, proceeding from the same tank. Hence, each pool represents a particular digestive section of a single tank, having a total of 24 pools. The water sample was assayed simultaneously; i. e. 25 different sets of sequences were obtained from the raw pyrosequencing data.

DNA extraction

Total DNA was isolated from the gastrointestinal content samples by using the Genomic DNA from tissue Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions.

PCR amplification and pyrosequencing

A barcoded primer set based on universal primers 27F and 533R was used to amplify 500 bps of the 16S rRNA genes covering the V1 to V3 regions. PCR was carried out using a high-fidelity KAPA-HiFi polymerase (Kappa Biosystems, US) with an annealing temperature of 52 °C and 30 cycles to minimise PCR biases. All samples assigned to the same pool shared a common barcode. The final DNA per sample was measured using the Agilent High Sensitivity DNA assay in the Agilent 2100 Expert.

Purified PCR products were pooled in equimolar amounts, as described in the 454 Roche protocol, and submitted for pyrosequencing, using the Genome Sequencer GS Junior Series (454 Life Science, Branford, USA). PCR products were pyrosequenced from the forward primer end only at Servei Central de Suport a la Investigació Experimental (SCSIE) of the Universitat de València (Valencia, Spain).

Livestock data statistical analysis

Statistical data analyses were carried out with Statgraphics © Centurion XVI [35]. SGR, FCR, FI, PER and S data were subjected to multifactor

variance analysis, introducing the initial live weight as a covariate in growth data. All percentage data were arcsine transformed prior the analysis. Each group in the calculation represented the combined group of fish per single tank (triplicate tanks per treatment). The Newman – Keuls test was used to assess specific differences among diets at the 0.05 significance level. Descriptive statistics are mean ± SE unless otherwise noted.

Sequence data analysis

From the resulting raw data set, provided by pyrosequencing, low quality sequences were filtered out to remove sequences having a length shorter than 150 nucleotides. A dereplicate request on the QIIME pipeline was used to identify representative sequences for each operational taxonomic unit (OTU) generated from complete linkage clustering with a 97% similarity, and chimeric sequences were removed using UCHIME software [35]. Reads of chloroplast, mitochondria or eukaryotic origin were also excluded by filtering sequences. Alpha diversity indices were determined from rarefied tables using the Shannon-Wiener index for diversity and the Chao1 index for species richness; Observed Species (number of unique OTUs) and Phylogenetic Distance (PD_whole) were also determined. A beta diversity distance matrix was computed from the previously constructed OTU table using UniFrac analysis. Unweighted (presence/absence matrix) and weighted (presence/absence/abundance matrix) UniFrac distances were used to construct two- and three-dimensional Principal Coordinate Analysis (PCoA) plots. Biplots were generated as part of the beta diversity analysis in QIIME, using genus level OTU tables showing principle coordinate sample clustering alongside weighted taxonomic group data. Data on assigned sequences at genus level shared between samples were used to generate a Venn diagram.

Relative frequencies of different taxonomic categories were calculated using the Statistical Analysis of Metagenomic Profiles program (STAMP v.2.0.0). Statistical differences between experimental fish samples were estimated by ANOVA analysis with the Games-Howell post-hoc test and the multiple test correction of Benjamini-Hochberg and differences between diets were calculated by T-test as implemented in STAMP.

DNA sequences were deposited in the MG-RAST server database (http://metagenomics.anl.gov/, with access numbers 4548816.3 to

4548840.3), under the project name "Seabream Gastrointestinal Microbiota."

Results

Performance factors of gilthead sea bream

No differences were found in growth and nutritive parameters (Table 2) between fish fed two diets during the on-growing phase. However, fish survival showed significant differences, and the FM100 diet presented a higher survival rate (88%) than the AA0 diet (60%).

Table 2. Main performance of gilthead sea bream fed diet FM100 or AA0

	FM100	AA0	SEM
Final weight (g)	393	360	15.7
Survival (%)	88[a]	60[b]	5.5
SGR (% day^{-1})	0.72	0.69	0.040
FI (g 100 g fish^{-1}day^{-1})	1.35	1.38	0.019
FCR	2.14	2.40	0.122
PER	1.06	0.96	0.063

Means of triplicate groups. Data in the same row with different superscripts differ at $P<0.05$. SME: pooled standard error of the mean.
Specific growth rate (%day−1), SGR=100×ln(final weight/initial weight)/days.
Feed Intake ratio (g 100 g fish−1day−1), FI=100×feed consumption (g)/average biomass (g)×days.
Feed Conversion Ratio, FCR = feed offered (g)/ weight gain (g).
Protein Efficiency Ratio, PER = Weight gain (g)/Protein intake (g)
Initial average weight: FM100; Tank 1: 133±18.9, Tank 2: 136±23.7, Tank 3: 125±16.0. AA0; Tank 1: 129±21.2, Tank 2: 127±15.1, Tank 3: 126±17.0.

Gut microbiota composition of gilthead sea bream

After quality filtering and length trimming, 91,539 16S rDNA sequences were analysed, with an average number of 3661.56 taxonomically assigned, high-quality sequences per sample. The microbiota throughout the GIT of gilthead sea bream was analysed and sequences annotated in OTUs with the QIIME pipeline using the GreenGenes database. A total of 2,813 de novo OTUs at 97% identity were identified in gilthead seabream GIT. A total of 43,177 sequences (4,793 hits, with an average percentage of identity of 99.16) could be identified at species level, 56,400 (7,026 hits) at genera level and 70,721 (7388 hits) at family level. Families grouped schematically as shown in Fig. 1 using a minimum identity cut-off of 80%.

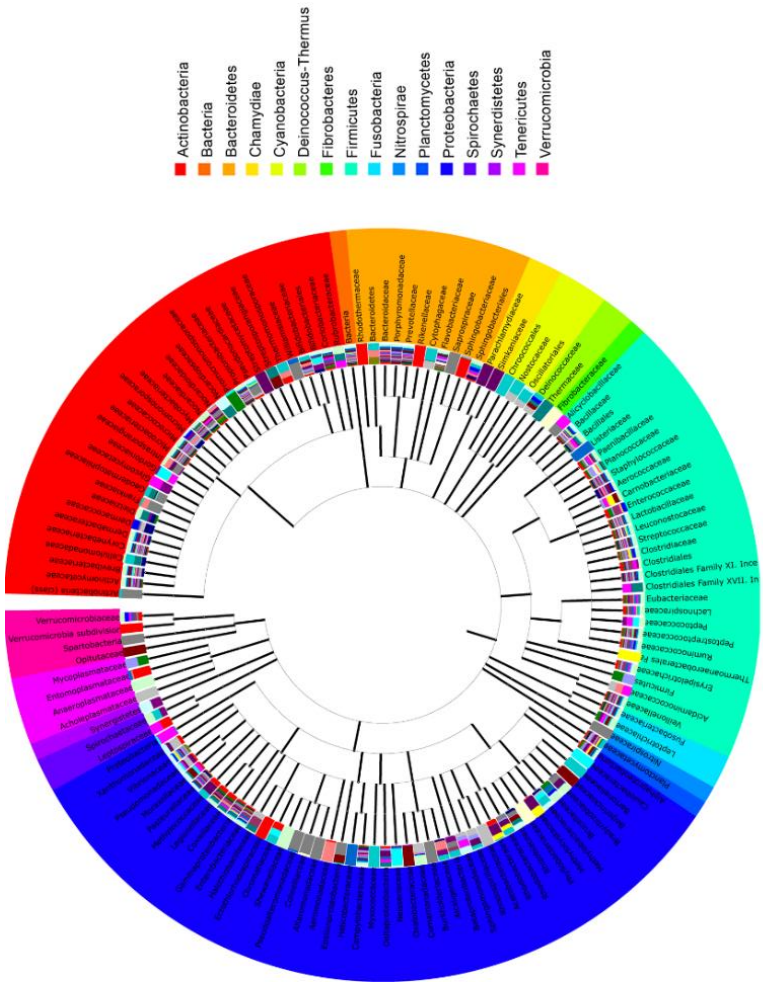

Fig. 1. Circular tree representation of microbiota associated to the GIT of the gilthead sea bream, at family taxonomic level.

Sequences were assigned to different families using GreenGenes database in MG-RAST, with a minimum percentage of identity cutoff of 80% and a minimum alignment length cutoff of 15. Different colours were assigned by phyla. Likely chloroplast and mitochondria sequences were omitted. Amount of different colours in the bars are representative of the number of different MIDs in which each taxon was found. Phylogenetic relations between different taxa are shown.

When analysing separately sequences from different segments of the GIT from all the animals analysed, a lower number of sequences was found for the ST in comparison with the gut sections. No significant differences in species richness (Chao1 index) among different gut

sections (FG, MG, HG) could be found when a rarefaction analysis was performed (Fig. 2).

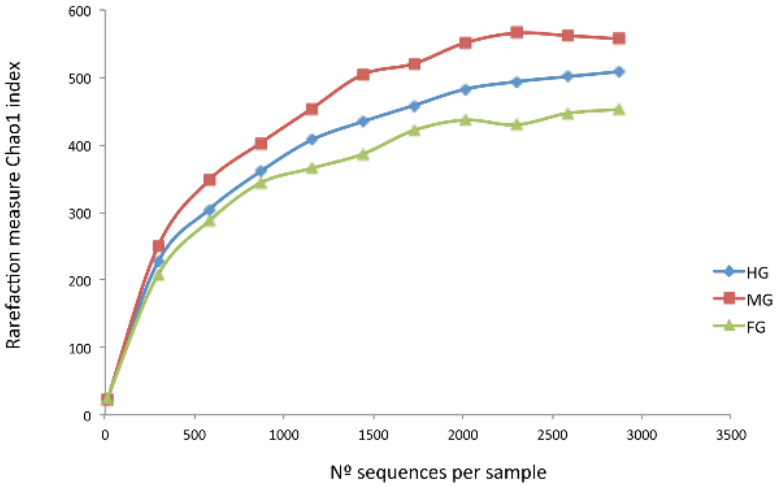

Fig. 2. Rarefaction curves (Chao1 index) showing the microbial community complexes in the different gut sections of the gilthead sea bream.

Orange = FG; Blue = MG; Red = HG.

The dominant phyla in ST were Firmicutes (29.1%), *Proteobacteria* (26.0%), and *Actinobacteria* (24.8%). In the FG, the predominant phyla were *Actinobacteria* (34.8%) and *Firmicutes* (33.0%), followed by *Proteobacteria* (24.8%). *Firmicutes* was the dominant phylum in the MG (33.7%) and *Actinobacteria* and *Proteobacteria* exhibited similar percentages (27.6% and 25.6%, respectively). Finally, Actinobacteria was the most represented phylum in the HG (36.5%), followed by *Proteobacteria* (31.7%), while *Firmicutes* was less abundant (23.7%) in comparison with other sections of the GIT. Another 21 phyla were found along the whole digestive tract, including *Bacteroidetes* (from 3.6% to 6.7%). A large percentage of unassigned bacteria was also found ranging from 2.8% (in the FG and the MG) to 13.0% (in the ST).

Although all the *Cyanobacteria* sequences, including sequences assigned to the *Chloroplast class*, were removed from the analysis, some non-plant derived sequences belonging to the*4C0d*-2; *ML635J-21*; *Nostocophycideae* and *Oscillatoriophycideae* classes, (of the families *Xenococcaceae* and *Phormidiaceae*), related to marine algae, were also found.

Performing the analysis at genus taxonomic level, *Streptococcus* (7.8%) and *Clostridium* (7.2%) were the most abundant genera among *Firmicutes*. Regarding *Actinobacteria*, the genus *Corynebacterium* was predominant along the whole digestive tract, including the ST (11.5%), followed by the genus *Propionibacterium* (4.5%). Families of the phylum Proteobacteria highly observed in the stomach were *Vibrionaceae* (11.3%), mainly from the genus *Photobacterium* (8.4%), and *Enterobacteriaceae* (3.6%). The percentage of unassigned sequences in this section was remarkable (13.7%).

Different genera of *Proteobacteria* were found in the FG in higher proportions, such as *Photobacterium* (4.4%), *Enhydrobacter* (3.7%), an unassigned genus (UG) of the family *Enterobacteriaceae* (3.7%) and *Sphingomonas* (2.7%). In relation to *Actinobacteria*, genera *Corynebacterium* and *Propionibacterium* were found in higher amounts than in the ST (20.3% vs 11.5%, p-value=0.243, and 8.4% vs 4.5%, p-value=0.085, respectively). Among *Firmicutes*, *Streptococcus* was the most abundant genus (14.1%), followed by *Staphylococcus* (2.7%), *Finegoldia* (2.4%) and *Lactobacillus* (1.9%), while the genus *Clostridium* decreased in the FG when compared to the ST (0.1% vs 7.2%, p-value=0.090).

In the MG, *Firmicutes* was the most abundant phylum, dominated by *Lactobacillus* (7.1%), *Streptococcus* (6.6%), *Proteiniclasticum* (3.8%), *Megamonas* (2.8%), *Staphylococcus* (2.2%) and *Finegoldia* (1.6%). The phylum *Actinobacteria* was represented mainly by the genera *Corynebacterium* (13.4%) and *Propionibacterium* (11.5%). Regarding *Proteobacteria*, major changes with respect to the FG were not observed. *Photobacterium* (4.4%) and an UG of the family *Enterobacteriaceae* (3.8%) were observed, as well as *Pseudomonas* (3.6%) and other unassigned genera at this level belonging to the families *Legionellaceae* (2.3%) and *Rhodobacteraceae* (2.1%). The genus *Bacteroides* was relatively abundant in this section (4.5%).

Finally, an UG of the family *Micrococcaceae* was well-represented (4.5%) among genera of *Actinobacteria*, but *Corynebacterium* (17.1%) and *Propionibacterium* (10.4%) were still observed as the most abundant genera belonging to this phylum. The genus *Photobacterium* was highly represented in this section (11.5%), and other observed genera were the above-mentioned UG of families *Rhodobacteraceae* (4.1%), and *Legionellaceae* (4.1%). Regarding *Firmicutes*,

Streptococcus exhibited a similar percentage when compared to the MG (8.2%), and the same genera identified in previous sections were found, such as *Lactobacillus* (2.0%), *Staphylococcus* (1.7%) and *Finegoldia* (1.4%).

Recirculating Saltwater System Microbiota

In order to evaluate the mutual impact of the recirculating saltwater system and sea bream GIT, the microbiota present in the water was also analysed. *Bacteroidetes* (54.5%) was the predominant phylum, with *Flavobacteriaceae* (42.4%) as the most common family, and *Sediminicola* being the most represented genus (25.0%). *Saprospiraceae* were also identified (9.4%). The other predominant phyla were *Proteobacteria* (31.1%), including the family *Rhodobacteraceae* (14.2%), and *Planctomycetes* (3.08 %), while the percentage of the phyla *Actinobacteria* and *Firmicutes* were lower than 1.5%, in contrast with those levels observed in sea bream GIT. Another 13 phyla were observed. Hence, dominant families observed in the gastrointestinal microbiota were significantly different from those observed in the water (Fig. 3).

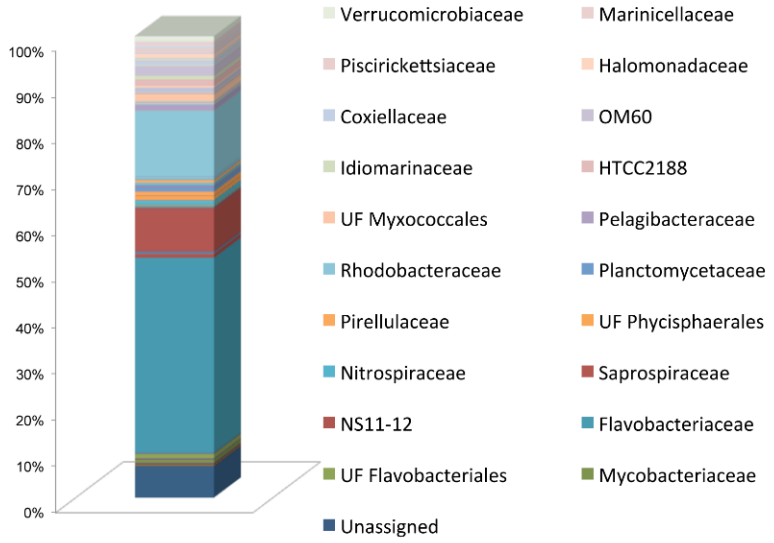

Fig. 3. Relative abundance (%) of main taxa present in the water of the RAS, at family taxonomic level.
Families with abundance lower than 0,5% in all samples were not shown.

Impact of fishmeal replacement on gut microbiota composition of gilthead sea bream

Alpha diversity metrics (Chao1 and Shannon-Wiener indices) did not show differences between diets throughout the GIT. No significant differences were observed in Phylogenetic Distance or the Number of Species when fish fed the fishmeal diet were compared to fish fed the vegetable mixture diet (Fig. 4). Rarefaction curves were shown in S1 Fig.

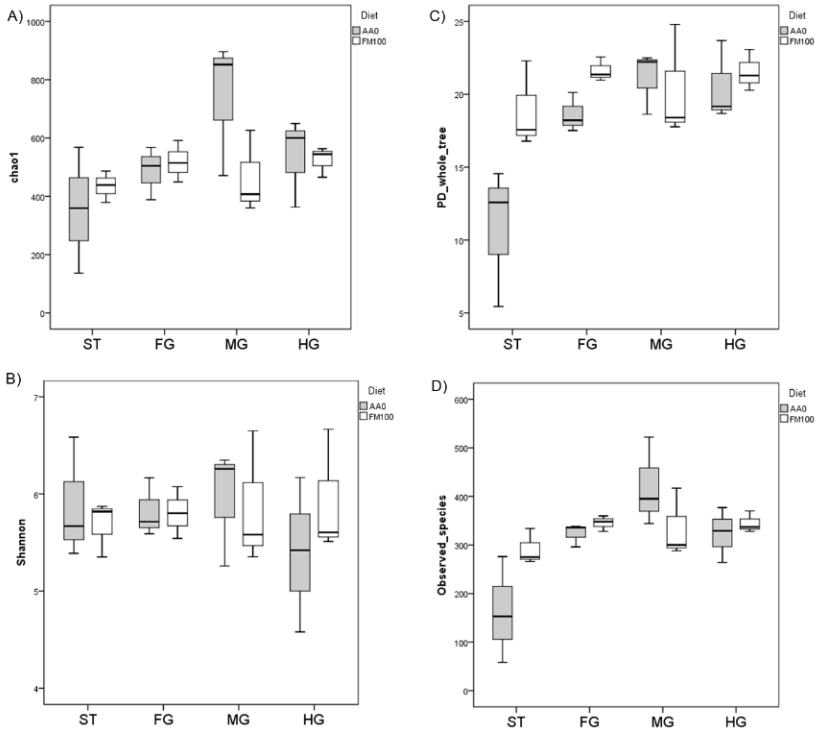

Fig. 4. Alpha diversity metrics, Phylogenetic Distance and Observed Species throughout the GIT of the gilthead sea bream.
A) Chao1 index (chao1); B) Shannon-Wiener index (Shannon); C) Phylogenetic distances (PD_whole_tree); D) Observed species (Observed_species). Different indices were represented by Box-Whisker diagrams for the two groups of fish and significant differences are indicated with an *.

The Venn diagram showed a broader perspective (Fig. 5). A core of 10, 11 and 19 bacterial families was shared by the two groups of fish in the FG, MG and HG, respectively. A greater number of families

specifically associated with the diet FM100 was found in the MG (7 vs. 4 linked to the AA0 group), while fishmeal replacement slightly increased the number of specific genera in the FG (5 vs 6) and HG (5 vs 7). In the FG, Actinomycetaceae, Carnobacteriaceae, Micrococcaceae, Neisseriaceae and Pasteurellaceae families were also exclusive of fish fed FM100, while Methylobacteriaceae, Moraxellaceae, Porphyromonadaceae, Pseudomonadaceae, Shingomonoadaceae and Vibrionaceae were only observed in fish fed AA0. In the MG, the unique families in the FM100 group were *Bacteroidaceae*, *Coxiellaceae*, *Enterobacteriaceae*; *Lachnospiraceae*; *Legionellaceae*, *Pasteurellaceae* and *Ruminococcaceae*, whilst *Acetobacteraceae*, *Clostridiaceae*, *Leuconostocaceae* and Vibrionaceae were only found in the AA0 group. Finally, in the HG, *Actinomycetaceae*, *Coxiellaceae*, *Micrococcaceae*, *Pasteurellaceae* and *Rhizobiaceae* families were exclusively present in fish fed FM100; in contrast, *Clostridiaceae*, *Comamonadaceae*, *Enterococcaceae*, *Moraxellaceae*, *Nocardiopsaceae*, *Pseudomonadaceae* and *Vibrionaceae* were solely observed when fish were fed AA0.

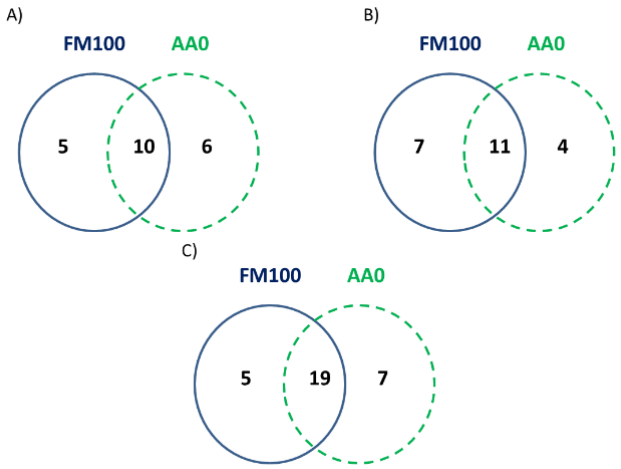

Fig. 5. Venn diagrams for the different gut sections, at family taxonomic level.

OTUs included were present in percentages above 1**%.** Common bacterial families are displayed in the middle regions and specific bacterial families of fish fed the AA0 and the FM100 diet are displayed in green and blue line colourd, respectively. (A) FG; (B) MG; (C) HG.

Significant differences at genus level were found among diets when all the sections were considered (Fig. 6), highlighting *Photobacterium* (p-

value: 0.025) and *Streptococcus* (p-value: 0.043), which were highly represented in the AA0 and FM100 diets, respectively. Moreover, the AA0 diet exhibited higher relative percentages of unassigned sequences (p-value: 0.011).

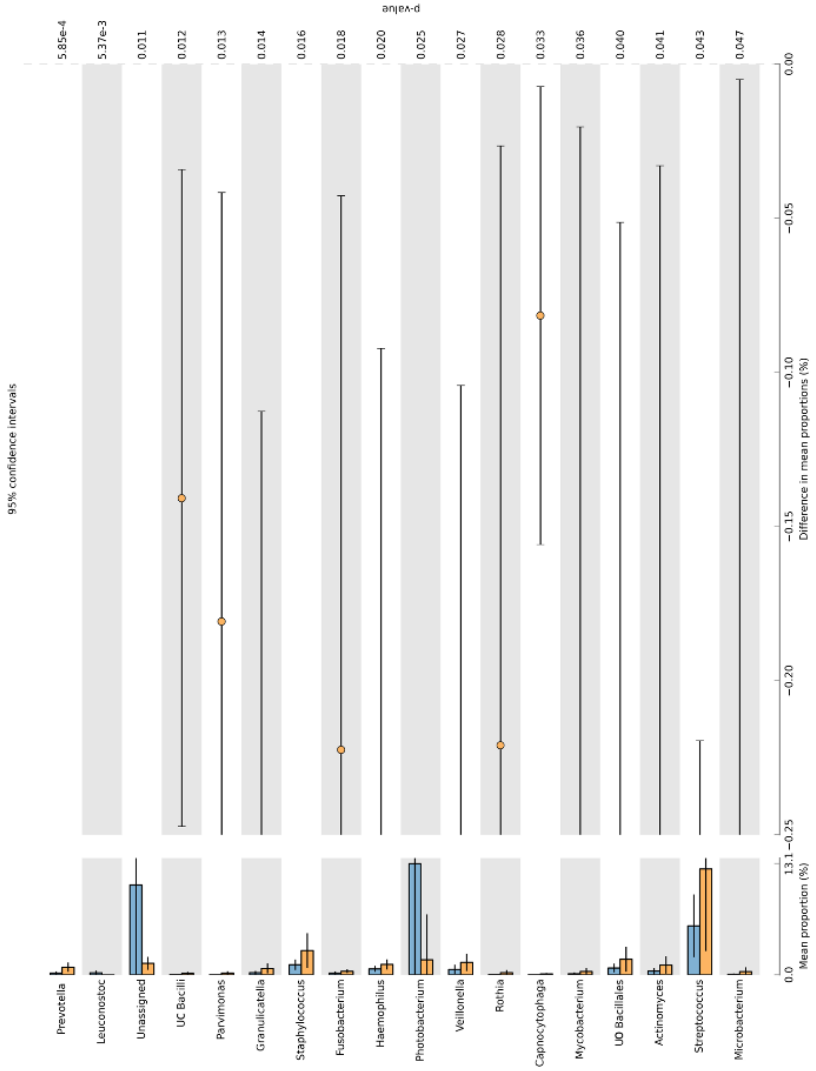

Fig. 6. Significant differences between diets at genus level, independent of the gut section.

Mean proportions, 95% confidence intervals and p-values are represented for each taxon for the two groups of fish. T-tests were used when comparing the relative abundances of individual taxa between AA0 and FM100.

Fig. 7 represents the evolution of the main taxa along the intestinal tract according to the diet, at phylum and genus level. Comparisons in the ST are omitted due to the higher percentages of plant-derived sequences, especially in fish fed AA0 (Table 3).

Table 3. Percentages of Chloroplast, Algae, Mitochondria and Bacterial Sequences in all the pools of different sections and tanks

	N. of sequences	% Chloroplast sequences	% Algae sequences	% Mitochondria sequences	% Bacterial sequences	N. of bacterial sequences
FM100						
ST1	3727	32.28	0.00	3.17	64.56	2406
ST2	3670	6.29	0.00	5.61	88.09	3233
ST3	2646	0.04	0.00	0.00	99.96	2645
ST		12.87	0.00	2.93	84.20	
FG 1	3568	3.00	0.00	0.95	96.05	3427
FG 2	7338	5.04	0.00	11.37	83.59	6134
FG 3	4689	10.11	0.13	20.54	69.23	3246
FG		6.05	0.04	10.95	82.96	
MG 1	3721	0.56	0.00	0.13	99.30	3695
MG 2	4334	1.15	0.00	0.48	98.36	4263
MG 3	4065	10.11	0.00	10.75	79.14	3217
MG		3.94	0.00	3.79	92.27	
HG 1	3926	0.36	0.00	0.03	99.62	3911
HG 2	3661	12.48	0.00	9.31	78.20	2863
HG 3	3499	5.06	0.00	6.23	88.71	3104
HG		5.97	0.00	5.19	88.84	
AA0						
ST1	2633	48.20	0.00	5.32	46.49	1224
ST2	4519	84.42	0.00	8.48	7.10	321
ST3	3995	88.39	0.00	9.01	2.60	104
ST		73.67	0.00	7.60	18.73	
FG 1	2573	17.61	0.04	1.44	80.92	2082
FG 2	3579	18.75	0.00	1.45	79.80	2856
FG 3	3810	4.17	0.05	0.16	95.62	3643
FG		13.51	0.03	1.02	85.44	
MG 1	3386	2.84	13.94	0.74	82.49	2793
MG 2	2693	10.36	0.00	0.93	88.71	2389
MG 3	3633	5.53	1.40	0.41	92.65	3366
MG		6.24	5.11	0.69	87.95	
HG 1	3015	3.78	0.10	0.17	95.95	2893
HG 2	2890	9.03	0.07	0.87	90.03	2602
HG 3	2688	6.96	0.00	0.45	92.60	2489
HG		6.59	0.06	0.49	92.86	
WATER	3281	0.00	0.24	0.00	99.76	3273

ST: Stomach, FG: Foregut, MG: Midgut, HG: Hindgut

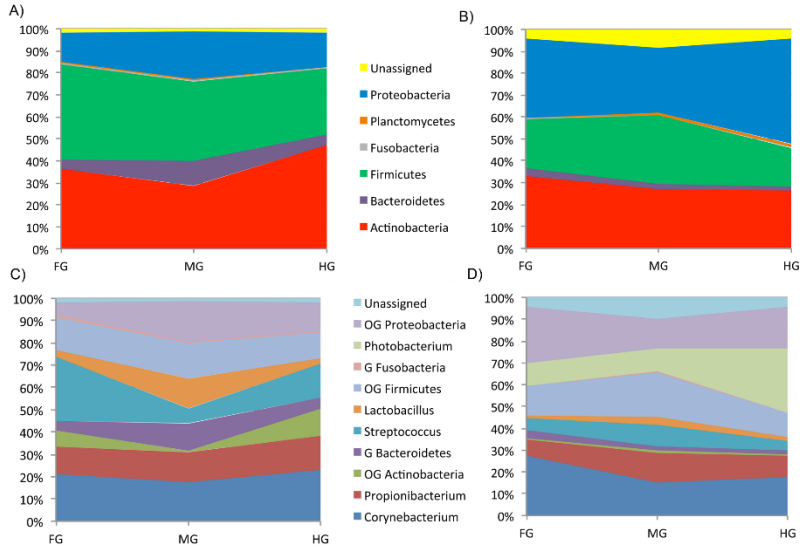

Fig. 7. Relative abundance (%) of the main taxa present throughout the gut of the gilthead sea bream, according to the diet, at phylum and genus taxonomic level.

Different sections (FG, MG, HG) are displayed in the X axis; relative abundance of different taxons are represented in the Y axis. Only phyla or genera with abundance higher than 0,5% in any gut section were shown. Only genera with abundance higher than 10% in any gut section were shown separately (G=genera; OG=other genera)* A) Microbial community of fish fed FM 100, at phylum taxonomic level B) Microbial community of fish fed AA0, at phylum taxonomic level C) Microbial community of fish fed FM100, at genus taxonomic level D) Microbial community of fish fed AA0, at genus taxonomic level

* **OG Proteobaceria**: Enhydrobacter, UG Enterobacteriaceae, Haemophilus, UG Legionellaceae, Neisseria, Pseudomonas, UG Rhodobacteraceae and Shingomonas; **OG Firmicutes**: Delftia, Finegoldia, Granulicatella, Megamonas, Peptoniphilus, Proteiniclasticum, Staphylococcus and Veillonella; **G Bacteroidetes**: Bacteroides, Paraprevotella, Porphyromonas and Prevotella; **OG Actinobacteria**: Actinomyces, Microbacterium and UG Micrococcaceae.

In the FG, the presence of *Proteobacteria* was more relevant in AA0 samples than in FM100 (36.2 % vs 13.4%, p-value=0.222), while *Firmicutes* were more abundant in FM100 than in AA0 (43.1% vs 22.8%, p-value=0.090). *Streptococcus* genus abundance was higher in the FM100 group than in AA0 (23.4% vs 4.7%, p-value=0.097), and fishmeal replacement also affected negatively the genera *Staphylococcus* and *Lactobacillus* throughout the gut, including the FG (3.3% vs 2.1%, p-value=0.422 and 2.7% vs 1.1%, p-value= 0.099, respectively). In contrast, the genus Finegoldia was present in lower proportions in FM100 than in AA0 (1.3% vs 3.6%, p-value=0.300). The

genera *Photobacterium* (8.7% vs 0.1%, p-value=0.364), *Enhydrobacter* (7.0% vs 0.4%, p-value=0.339), *Sphingomonas* (5.1% vs 0.3%, p-value=0.242) and an UG of the family Enterobacteriaceae (6.9% vs 0.6%, p-value=0.331) were more highly represented in the FG of fish fed the plant-based diet than in FM100.

At phylum level, differences between the FM100 group and the AA0 group, in abundance of Firmicutes (36.2% vs 31.2%, p-value=0.361) and Proteobacteria (21.90% vs 29.2%, p-value=0.501), decreased in the MG, although Bacteroidetes were more abundant in fish fed the fishmeal diet than in fish fed the vegetal diet (11.0% vs 2.4%, respectively, p-value=0.279). At genus level, *Bacteroides* were highly represented in the MG of fish fed FM100 than in AA0 (8.5% vs 0.5%, p-value=0.268), such as *Lactobacillus* (11.0% vs 3.2%, p-value=0.251), *Megamonas* (3.7% vs 1.9%, p-value=0.436) and *Staphylococcus* (3.4% vs 0.9%, p-value=0.263). The most abundant genera of the phylum Firmicutes in animals receiving the vegetable diet were *Streptococcus* (7.8%, vs 5.5% in fish fed FM100, p-value=0.363) and *Proteiniclasticum* (7.6%), observed exclusively in this group of fish (p-value=0.372). Among Proteobacteria, Enterobacteriaceae (7.0%), and Legionellaceae (3.8%) were the most represented families in this section in fish fed FM100, while in AA0 samples of the MG the most common genus of Proteobacteria was *Photobacterium* (8.8%), followed by *Pseudomonas* (5.3%) and an UG of Rhodobacteraceae (3.0%).

A significantly higher presence of *Proteobacteria* (47.9% vs 15.5%, p-value=0.022) and lower abundances of *Firmicutes* (17.6% vs 29.9%, p-value=0.079) and *Actinobacteria* (26.2% vs 46.9%, p-value=0.092) were observed in the HG of fish fed the plant-based meal diet compared to FM100. An UG of the family Micrococcaceae was exclusively observed in fish fed FM100 (8.6%, p-value=0.269), although as stated above, *Corynebacterium* and *Propionibacterium* were the most abundant genera belonging to this phylum in fish fed both diets. *Streptococcus* was the major taxon among the *Firmicutes* genera in the FM100 group, being significantly overrepresented compared to the AA0 samples (12.7% vs 3.7%, p-value=0.009), followed by *Staphylococcus* (2.6% vs 0.8%, p-value=0.232) and *Lactobacillus* (2.2% vs 1.8%, p-value=0.723). *Proteiniclasticum* (2.4%) was only found in fish fed the vegetable diet, as occurred in the MG (p-value=0.365). The *photobacterium* was very abundant and exclusively observed in the HG of fish fed AA0 (25.4%, p-value=0.103), and the above-mentioned UG of the family *Rhodobacteraceae* was also

abundant in this group of fish (8.0%, vs 0.2% in the FM100 group, p-value=0.230). In fish fed FM100, an UG of the family *Legionellaceae* was the most observed genus belonging to the phylum Proteobacteria (6.3%, vs 2.0 in fish fed AA0, p-value=0.519).

Finally, unweighted and weighted PCoA showed a certain differentiation in the microbiota associated to the gut of fish fed FM100 and AA0 (Fig. 8**).** Unweighted PCoA (PC1 = 13%, PC2 = 10%, PC3 = 10%) grouped samples by diet. A higher separation between different sections of the gut of fish fed AA0 was observed in comparison with FM100 samples, although two outlier samples corresponding to FM100 were found. Separation among diets was clearer in the weighted PCoA (PC1 = 45%, PC2 = 19%, PC3 = 8%). First Component grouped different sections of the intestine of fish fed fishmeal, while gut sections of fish fed the vegetable diet appeared more separated from each other along the X axis. Second Component of the PCoA had the opposite effect on samples, grouping AA0 gut sections and separating FM100 ones.

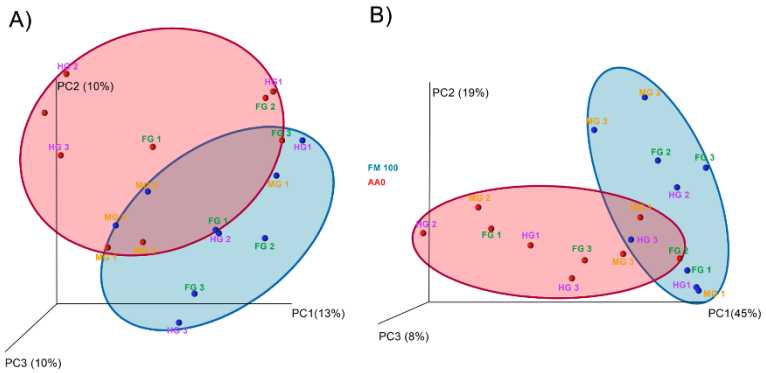

Fig. 8. Principal coordinates analysis (PCoA) of Unweighted (A) and Weighted (B) Unifrac distances of microbial communities associated to the gut, according to diet.

A beta diversity distance matrix was computed from the previously constructed OTU table using UniFrac analysis. Unweighted (presence/absence matrix) and weighted (presence/absence/abundance matrix) UniFrac distances were used to construct the PCoA plots. Circles in red and blue represent different gut section of fish fed AA0 and FM100, respectively.

Discussion

Gastrointestinal microbiota of gilthead sea bream

Although Microbiota composition seems to differ among fish species, in general terms, fish harbour a microbiota that is dominated mainly by the phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* [37] .All fish gut samples in this study and most fish gut samples in previous studies shared *Proteobacteria* and *Firmicutes* as the most dominant phyla [27,38,39]. *Actinobacteria* were found in our samples and also in grass carp [38] and rainbow trout [39]. Nevertheless, *Bacteroidetes* [30] and *Fusobacteria* [28] were the most representative phyla in seabass and carp, respectively. At genus level, *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Bacteroides*, *Corynebacterium*, *Clostridium*, *Cytophaga*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Photobacterium*, *Pseudomonas* and *Vibrio* have been described as the most common genera retrieved in marine fish, as well as different genera of lactic acid bacteria (*Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*), belonging to the phylum Firmicutes [10,22,25,27].

According to our own results, most of these genera were present in the GIT of gilthead sea bream. DNA extraction was performed on gastrointestinal content samples, obtained from the gut after scraping the mucosa, thus genera represented both, luminal (allochthonous) and mucosal communities (autochthonous).

Results were consistent with results obtained on the microbiota present in the stomach and the gut in sea bream with other methods [13,26]. However, in a previous study using tag pyrosequencing [31], Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the most abundant phyla observed in the gut, whilst Diaphorobacter (belonging to β-Proteobacteria) was the dominant genus in all fish examined. This finding could be explained by the fact that bacterial DNA was extracted from the gut tissue, whereas the majority of previous works had analysed the microbiota from the intestinal content [40].

In the present work, the analysis of the microbiota of the stomach was limited by chloroplast and mitochondria sequence contamination, which has been found in 16S rRNA gene analysis when using 454

pyrosequencing for the analysis of microbial communities in plants and folivorous arthropods [41]. This technical limitation, intrinsic to 16S rDNA sequencing, must be born in mind. Chloroplast and mitochondria sequences were ruled out for further quantitative analysis, although it also eliminated Cyanobacteria and *Mitochondria* (Rikettsiales), thus, introducing a population bias, mainly in the ST samples where aquatic bacteria (Cyanobacteria) could be highly represented, while Rickettsiales are pathogenic or endosymbionts not expected in the stomach. Relative abundance of *Chloroplasts and Mitochondria* was much higher in the stomach, especially in fish fed the AA0 diet, compared with gut sections (always below 20% and 10% for diets AA0 and FM100, respectively) and the differences between the diets disappeared when moving through the GIT, evidencing that they must predominantly correspond to vegetable components of the diet that are degraded during digestion. In addition, the lower microbial diversity found in the stomach could also be due to the restrictive environmental conditions found in the ST, as occurs in most vertebrates.

Recirculating saltwater system microbiota

In the present study, the microbiota of the Recirculating Aquaculture System (RAS) was dominated by different genera of the family *Flavobacteriaceae*, widely distributed in diverse habitats, including marine environments, in which they may be numerically dominant [42]. The family *Rhodobacteraceae*, which often occurs in aquatic habitats, was abundantly observed in other RAS [43], and *Saprospiraceae*, which was also well-represented, was found to be linked with activated sludge [44] and its presence could be due to the high content of organic matter in the system. Uptake of RAS water by marine fish takes place continuously, hence GIT microbiota is expected to be a mixture of autochthonous and allocthonous bacteria [45]. Nevertheless, in our study, RAS had a different microbiota composition and greater diversity of that observed in the gilthead sea bream GIT. *Flavobacteriaceae* and *Saprospiraceae* were underrepresented in all sections compared to water, while, in comparison, *Rhodobacteraceae* was found in higher percentages in the whole digestive tract, particularly in the HG of AA0, suggesting the diet could affect the colonization of GIT by bacteria present in the surrounding water. Further studies should be performed in order to clarify the origin of different bacterial groups and their capacity to colonize the GIT of fish.

The microbial community of Recirculating Aquaculture Systems (RAS) is influenced by several factors, such as feed type, feeding regime, management routines, variation in system design, water composition parameters [46] and the selective pressure of biofilters [47]. In addition, each fish species introduces its own microbiota of skin, gills and GIT [48]; make-up water also alters its original microbial composition, and fish feed, equipment used in and about the system and staff/visitors in contact [49] may also introduce different taxa of bacteria. Moreover, storage and processing of samples and PCR efficiency can affect the presence and relative abundance of different taxa [30, 50]. Hence, microbial diversity and composition in RAS water varies from one system to another, making comparisons difficult [51].

Impact of fishmeal replacement

Total fishmeal replacement by plant protein concentrate has been reported in sea bream juveniles [8] with positive growth and nutrient efficiency. The success of total fishmeal substitution was due to the high digestibility of the protein source concentrate and also to the balanced dietary amino acid profile.

In our study, no significant differences in terms of growth and nutritive parameters could be found between diets. However, significant differences were observed in the survival rate. Mortality did not seem to be associated with any specific pathology. The presence of high non starch polysaccharide and other antinutrients substances, as tannins, in the AA0 diet, may cause a decrease in the availability of nutrients, including amino acids, producing imbalances with the direct consequence on immune organs and responses [52].
In STs of fish fed FM100, the genera *Corynebacterium*, *Propionibacterium* and *Clostridium* were the most abundant, while the family *Enterobacteriaceae*, was most abundant in fish fed the vegetable diet. In fact, the latter organisms are regarded as efficient secretors of polysaccharide hydrolases [53,54], being compatible with the higher fibre content of AA0.

Fishmeal replacement did not induce significant changes in microbial richness throughout the gut, as no significant differences in Alpha diversity indices, Observed Species and Phylogenetic Distance were determined between the two groups of fish, which is in agreement with previous reports [26]. Other studies in rainbow trout reported higher bacterial richness [29], lower microbial diversity [55] or only minor

changes in the microbiota composition [56] with different levels of substitution, while contradictory effects of fishmeal replacement on the microbial diversity were observed in Atlantic salmon [57,58].

Fishmeal replacement had a negative effect on the relative abundance of *Firmicutes* throughout the gut, particularly on the genera *Streptococcus* and *Lactobacillus*, which are lactic acid bacteria. These are prevalent constituents of the intestinal microbiome of many fish species and are generally considered beneficial organisms associated with a healthy intestinal epithelium [59], and some strains of these taxa can inhibit adhesion of several fish pathogens, ensuring the maintenance of a balanced microbiota, which is crucial in the prevention of diseases, especially GIT infections [60]. On the other hand, fish fed the vegetable mixture diet exhibited a higher percentage of *Proteobacteria* along the whole digestive tract. The genus *Photobacterium* was highly represented in all sections of the gut of fish fed AA0, particularly in the HG. Some species of the genus *Photobacterium* are secondary pathogens of marine life and its great abundance in the GIT of this group of fish might suggest an alteration of gut immune mechanisms of gilthead sea bream. Nevertheless, differences in *Photobacterium* abundance could also be explained by differences in fibre and NSP between FM100 and AA0, as this genus has also been previously reported to degrade cellulose [53,54]. The genus *Pseudomonas*, which also has cellulolytic activity, was slightly more abundant throughout the GIT of AA0, especially in the MG.

It is likely that low or moderate fishmeal substitutions in sea bream feeds do not have a significant effect, or even have a positive effect, on growth [61] and also on microbiota composition and diversity [7], preventing the establishment of an evident dietary effect [26]. Nevertheless, high fishmeal replacement could produce alterations in the non-specific immune system in gilthead sea bream [6], which could be the main reason of the higher mortality in the group of fish fed the AA0 diet. Soybean protein has been reported to induce enteropathy in salmonids and others fish species [62], with a variety of intestinal disturbances including increased permeability [63] promoting inflammatory secretions that lead to greater immune sensitivity [64,65]. In our work, changes in the microbial patterns detected in the vegetable diet, particularly in the immune-competent segments of the hindgut, could also render fish prone to infection. An imbalanced microbiota could alter immune regulatory functions of the gut and contribute to the development of diseases [13], particularly if Proteobacteria are the

dominant clade, which includes potential pathogen genera (such as *Pseudomonas* and *Photobacterium*). This is in agreement with the observed susceptibility to infection of Atlantic salmon fed soybean, showing high levels of lysozyme and IgM in the mid- and distal-intestinal mucosa and an elevated gut inflammatory response [66].

A core microbiota has been suggested in different species [27,39,56], however, large individual variations within fish with a similar genetic background, fed the same diet and maintained under the same environmental conditions, have been described in previous reports [26,29,55] probably due to a strong host genotype influence on the bacterial composition [39,58]. In the present study, PCoA showed that gut content samples from fish that followed the same diet clustered together, although the AA0 diet showed greater dispersion. Hence, endogenous and exogenous factors but also the great variability of sources and proportions of ingredients used in feeds can modify the microbiome constitution. The link between diet and gut microbiota and the related changes in gut morphology and the immune system should be subject to further investigation, in order to understand the greater mortality observed as a consequence of the vegetable diet.

In conclusion, our study revealed that the total fishmeal replacement in diets for gilthead sea bream was nutritionally satisfactory and introduced no change in the total microbial diversity or richness, but altered the GIT microbiota profile at HG level , being a GIT section rich in immune cells. There was also an increase in the mortality rate. Further studies will determine if the adverse effect observed, possibly at immune level, was due to vegetable components of the diet or if it was the consequence of the microbial imbalance that they caused, or both. Development of new diets with new sources of ingredients, and possibly probiotics, will help in these investigations that constitute the keystone to the development of more efficient, economic and sustainable feeds in aquaculture.

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Supporting Information

Supporting information (S1 Fig.) is included in ANNEX III

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Chapter 4.

Impact of total fishmeal replacement by plant sources in aqua feeds for gilthead seabream (*Sparus aurata*, L.) on the gut mucosa proteome

Impact of total fishmeal replacement by plant sources in aqua feeds for gilthead seabream (*Sparus aurata*, L.) on the gut mucosa proteome

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Abstract

Background

The digestive tract, particularly the intestine, represents one of the main site of interactions with the environment, playing the gut mucosa a crucial role in the digestion and absorption of nutrients, and in the immune defence. Previous researches have proven that the fishmeal replacement by plant sources could have an impact on the intestinal status, compromising relevant productive parameters, such as feed efficiency, growth or survival. In order to evaluate the long-term impact of total fishmeal replacement on intestinal mucosa, the gut mucosa proteome was analysed in fish under the following experimental dietary treatments: FM (fishmeal based diet), VM (all the protein was supplied by plant sources) and VM+ (plant protein based diet including a 15% of alternative marine sources).

Results

Total fishmeal replacement, with (VM+) or without marine ingredients inclusion (VM), reported a negative impact on growth and biometrics, being more pronounced in VM group. On the other hand, at proteomic level, the VM group showed a different profile with a big set of underrepresented proteins. Functional analysis revealed several biological processes affected, such as intracellular transport, assembly of cellular macrocomplex, protein localization and protein catabolism, as well as several molecular functions, mainly related with binding to different molecules and the maintenance of the cytoskeleton structure.

Discussion

Downregulated proteins in VM group included molecules which have a crucial role in the maintenance of the normal function of the enterocytes, and therefore, of the epithelium, including permeability, immune and inflammatory response regulation and nutritional absorption. Nutritional deficiencies due to total plant protein based diets could explain the impact on the gut mucosa in the VM group, since long-term feeding with these diets may be considered as a stress factor and lead to a negative impact on growth and immune system mechanisms. The inclusion of 15% of alternative marine sources may

improve the intestinal Health at proteomic level, but was not enough to reach the growth rate of the FM group.

Keywords

gilthead seabream; intestine, plant sources, krill meal, squid meal, mucosa, proteome

Background

Reducing fishmeal and fish oil content, or their total replacement, in aquafeeds is becoming necessary for ensuring aquaculture sustainability, being particularly relevant in the production of carnivorous fish. As one of the most important Mediterranean aquaculture species, the gilthead sea bream (*Sparus aurata*, L.) has received great attention, and several studies have been focused on defining new feeds and alternative protein sources [1, 2, 3].

Vegetable meals are likely the most commonly protein source used as alternative to fishmeal and marine origin by-products, and its inclusion in aquafeeds has been successfully achieved, even with a total replacement [4, 5]. Nevertheless, previous studies have demonstrated that the use of vegetable sources could affect not only fish growth, but also digestive capacity or immune status [6, 7]. In this sense, the presence of anti-nutritional factors and the imbalances in essential amino acids, *inter alia*, may be responsible for the appearance of multiple changes at the gut level [8].

The gut mucosa, a layer which consist of the intestinal epithelium and the underlying connective tissue or lamina propria, plays an important role in digestion, absorption and metabolism of dietary nutrients, ion regulation and immune defence [9]. The gastrointestinal tract is continuously exposed to the presence of water, ions, dietary nutrients and different bacteria, being the main entrance of pathogens in fish [10]. Mucus covering the mucosa is the first line of defence, containing a set of biologically active components (antibacterial peptides, lysozymes, complement proteins, lectins and humoral antibodies) and preventing the colonization of pathogenic agents [11]. Nevertheless, gut epithelia, formed by intestinal epithelial cells, is the highly selective barrier against commensal and potentially pathogenic luminal microbes [12], being a protective barrier but also an interactive layer that regulates the fluxes of solutes, nutrients, antigens and immune-related molecules between the lumen and lamina propria [13]. In this regard, epithelial cells contribute to the innate and adaptive response through to the interaction with immune cells from lamina propria [14]. Hence, the effects of fishmeal replacement on intestinal mucosa function and structure could be a determinant parameter for the progression of fish performance.

Omics technologies have been used in aquaculture during the last decade [15]. Specifically, proteomics is considered a powerful tool to reach a deep understanding of fish biology, taking into account the post-transcriptional and post-translational regulation of the protein activity and providing relevant physiological information [16, 17], which can be missed by transcriptomics, since protein levels do not always correspond with levels of expressed mRNA [18]. Two-dimensional gel electrophoresis has been the most used technique in quantitative proteomics up to today. However, limitations on reproducibility, dynamic range and analytical throughput, together with cost-reduction of gel-free strategies have lead mass spectrometry (MS) approaches, and particularly, liquid chromatography (LC) coupled to MS (LC-MS), to become the most-widely used technology for high-throughput proteomic studies of biological tissues and other complex mixtures, allowing to analyse simultaneously a large number of protein [19, 20].

The study of the gut mucosa using omics could help for a better understanding of the relation between nutritional changes and fish performance, as well as of its role in intestinal health, contributing to the feasibility of incorporating high dietary levels of plant proteins in aquafeeds for carnivorous species. In this regard, proteomic studies have been carried out in numerous species, such as Atlantic salmon (*Salmo salar*) [21, 22], rainbow trout (*Oncorhynchus mykiss*) [23, 24, 25], zebrafish (*Danio rerio*) [26], Nile tilapia (*Oreochromis niloticus*) [27] and also in gilthead seabream [28]. However, most of these works focused on the response of the liver metabolism to dietary changes and few authors have studied the gut response to changes in dietary composition or feeding pattern. Along these lines, changes in the protein composition, including enzymes and serum albumin, in the pyloric caecea of rainbow trout in response to different alternative protein sources have been evaluated [29]. Impact of short-term starvation in the proteome of the gut epithelia in this species has been also assessed [30], reporting an increase of the lymphocytes cytotoxicity, a reduction of the permeability to macromolecules and a negative impact on the inhibition on serine protease-induced stress and bacterial infection. Furthermore, alterations in lipid and energy metabolic activity in the intestine of Atlantic salmon in response to fish oil replacement have been also observed in previous research [22].

Nevertheless, to the best of our knowledge, this research work has assessed, for the first time, using LC-MS/MS, the long-term differences in the gut mucosa of the gilthead seabream in response to complete

replacement of fishmeal by plant protein sources in aquafeeds for gilthead seabream.

Methods

Experimental setup

The growth assay was conducted in three octagonal concrete tanks (4000 L) using a marine water recirculating system (75 m^3 capacity), which includes a rotary mechanical filter and a gravity biofilter (6 m^3 capacity), at the Universitat Politècnica de València (UPV). All tanks were equipped with aeration, and the water was heated with a heat pump installed in the system. During the experiment, water parameters were as follows: 23±1.5 ºC, 30±1.7 g L^{-1} salinity, 6±0.5 mg O_2 L^{-1}, and 7.5 pH. All tanks had similar lighting conditions, with a natural photoperiod (from January to August).

Fish and acclimatation

Gilthead seabream came from the fish farm BERSOLAZ (Bersolaz Spain, S.L.U, Culmarex Group), located in Port de Sagunt (Valencia, Spain). After their arrival at the facilities at the UPV, fish were acclimated to laboratory conditions for two weeks, being fed daily by hand, to apparent satiation, three times per day (8:00, 13:00 and 18:00) with a standard commercial diet (proximate composition: 55.2% crude protein (CP), 17.3% crude lipid (CL), 11.6% carbohydrate, 9.4% ash (A) and 6.4% moisture) which was also provided by BERSOLAZ. Fish were weighed before starting the growth assay (initial weight = 9.6±1.9 g) and then randomly distributed into the three experimental tanks (80 fish per tank).

Diets

Experimental diets were manufactured as pellets by cooking-extrusion using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, Firminy, St Etienne, France) located at the UPV, with 100 rpm screw speed, 110 ºC, 40 atm pressure and 3-5 mm diameter pellets as processing conditions.
Three different diets were assayed: the FM diet, a fishmeal based control diet, in which fishmeal (59%) was the main source of protein; the VM diet, a vegetable-meal based diet in which the whole protein

content was of plant origin, and the VM+ diet, a vegetable-meal based diet including 10 % squid meal and 5% krill meal, which were obtained as by-products from the companies Max Nollert (Utrecht, Netherlands) and Ludan Renewable Energy (Valencia, Spain), respectively, thereby containing a 15% level of marine-origin protein, which is similar to gilthead seabream commercial diets. VM and VM+ were supplemented with different synthetic crystalline amino acids in order to achieve minimum amino acid requirements [31]. Ingredients and proximate composition of the experimental diets are shown in Table 1.

Table 1. Ingredients and proximal composition of diets tested in the growth assay

	FM	VM	VM+
Ingredients (g $100g^{-1}$)			
Fishmeal	58.9		
Wheat meal	26.0		
Wheat gluten		29.5	22.2
Bean meal		4.1	4.0
Soybean meal		18.2	16.0
Pea meal		4.1	4.0
Sunflower meal		15.8	16.0
Krill meal			5.0
Squid meal			10.0
Fish oil	3.81	9.0	7.75
Soybean oil	9.29	9.0	7.75
Soy Lecithin	1.0	1.0	1.0
Vitamin-mineral mix*	1.0	1.0	1.0
Calcium phosphate		3.8	3.8
Arginine		0.5	
Lysine		1.0	1.0
Methionine		0.7	0.5
Taurine		2.0	
Threonine		0.3	
Proximate composition (% dry weight)			
DM	89.5	88.4	89.5
CP	43.5	42.0	40.7
A	9.9	6.6	7.2
CL	14.3	18.3	18.8
CHO	20.8	21.4	22.8

*Vitamin and mineral mix (values are g kg^{-1} except those in parenthesis): 25; choline, 10; DL-atocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5; retinol acetate, 1 000 000 (IU kg^{-1}); calcipherol, 500 (IU kg^{-1}); DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12.
DM, dry matter; CP, crude protein; A, ashes; CL, crude lipid; CHO, carbohydrates

Macronutrients and amino acids analysis

Chemical analyses of ingredients were determined prior to diet formulation. Ingredients were analysed according to AOAC (1990) procedures: dry matter (DM) by heating at 105 °C to constant weight, ash (A) by incineration at 550 °C to constant weight), crude protein (CP), N × 6.25, by the Kjeldahl method after an acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden), crude lipid (CL) by methyl-ether extraction (Soxtec 1043 extraction unit, Tecator) and crude fibre (CF) by acid and basic digestion (Fibertec System M., 1020 Hot Extractor, Tecator). All analyses were performed in triplicate. Diets were also assayed using the same procedures. Proximate composition is also showed in Table 1.

Amino acids of raw materials and experimental diets were also analysed, through a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature control module. Aminobutyric acid was added as an internal standard pattern before hydrolysation. The amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were determined separately as methionine sulphone and cysteic acid after oxidation with performic acid. Amino acids were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm), and then transformed to methionine and cysteine. Essential and non-essential amino acids content of different diets were shown in the Supplementary Data (S1).

Growth assay

The trial lasted 221 days. Fish were observed daily in tanks in order to check their health status. At the end of the experiment, fish were sacrificed by cold shock after anaesthesia using clove oil (87% eugenol, Guinama ®, Valencia, Spain) dissolved in water (1 mg / 100 mL of saltwater), in order to minimize the suffering of fish, and were weighed individually, in order to evaluate fish growth and determine growth parameters. During the experiment, fish were fed by hand to apparent satiation, three times per day during the first 60 days (8:00, 13:00 and 18:00) and twice per day (9:00 and 14:00) from then up to the end of

the experiment. Food managers distributed the pellets slowly, allowing all fish to eat, in a weekly regime of six day of feeding and one of fasting. Temperature, pH, oxygen, ammonia, nitrite and nitrate concentrations were monitored throughout the growth assay.

Biometric analysis

Final weight (FW), condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI) and visceral fat index (VFI) were obtained at the beginning and at the end of the growth assay, using five fish per tank. The weight (GW) and the length of the gut tract (GL) were also measured.

Statistics

Biometric indices were analysed through an analysis of variance using the statistical package Statgraphics ® Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA), with a Newman-Keuls test for the comparison of the means and a level of significance of $p<0.05$. All percentage data were arcsine transformed prior the analysis.

Proteomics

Sampling

At the end of the growth trial, three fish per tank were slaughtered on ice after euthanizing with clove oil and dissected in order to obtain the gastrointestinal tract. Fish fasted 24 hours before sampling.

After discarding the stomach and pyloric caeca, the first intestinal third of the gut (foregut) was removed, sliced longitudinally and washed with phosphate buffered saline solution to remove digesta. Intestinal mucosa was scraped using sterilized large scalpel blades, stored in Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80º C.

Tissue extraction and protein precipitation

Gut mucosal scrapings were placed in 8M urea (Malinckrodt AR®, LabGuard®) in homogenization tubes (^{RT}Precellys® Ceramic Bead Tube, 1.4 mm / 0.5 mL tubes) and then ground using the homogenizer PrecellysTM Control Device (Bertin Technologies), with the following

conditions: 6500 m/s and 3 rounds of 20 s. Tubes were centrifuged (14000 rpm, 4º C, 15 min) and supernatants transferred to new Eppendorf tubes.

Tissue extracts were subjected to cold acetone precipitation: cold acetone (Acetone HPLC grade, Fisher Chemical) was added to samples in a proportion of 5:1 (5 ml cold acetone: 1 mL sample), tubes were incubated overnight at -20ºC and then centrifuged at 14000 rpm and 4ºC during 10 min. Supernatants were discarded and pellets were dried, resuspended in 8M urea, shaken for two hours at room temperature using a vortex and then centrifuged (14.000 rpm, room temperature, 5 min). Supernatants were collected, protein concentration was determined using a BCA assay kit (ThermoScientific, Meridian Rd., Rockford, IL, USA) and samples stored at -20ºC.

Denaturation, reduction, alkylation and digestion

A volume of sample with a protein amount of 50 µg was subjected to simultaneous denaturation and reduction, using DL-dithiothreitol (Sigma) in 25 mM ammonium bicarbonate (Sigma-Aldrich) Buffer (ABC), in a final concentration of 10 mM, and incubating at 60º C and 750 rpm agitation for 60 min. Iodoacetamide (Sigma) in 25 mM ABC Buffer was used for alkylation, in a final concentration of 20 mM, and vortexed at room temperature for 60 min in dark conditions. 25 mM ABC buffer was added to samples after alkylation to reduce the urea concentration, in a proportion of 5:1 (5 mL 25 mM ABC buffer: 1 mL sample). A trypsin/lysine-C enzyme mix (Trypsin/Lys-C mix mass spec grade, Promega) was used for the digestion. Enzyme pellets were resuspended with 25 mM ABC buffer and 3.2 µg of enzyme mix were added per sample. $CaCl_2$ solution was added to samples in a final concentration of 1 mM just before starting incubation at 37ºC and 500 rpm agitation for 3 hours. An additional 1.6 µg of enzyme mix was added to samples and these were incubated again in the same conditions overnight.

C18 column purification

Digested peptides were purified using C18 columns (MicroSpin Column 96/pk, C_{18} Silica, 5-200 µL loading, 5-60 µg capacity, The Nest Group, Inc.). Columns were previously conditioned with 200 µL 100% acetonitrile (ACN) twice and 200 µL 3% ACN 0.1% formic acid (FA) (Fluka Analytical) twice, by centrifuge at 2000 rpm at room

temperature for 2 min. Elutions were discarded. Samples were loaded onto columns and centrifuged with the same conditions, and the elution was reloaded and centrifuged again. Columns were washed four times with 200 µL 3% ACN 0.1% FA. Finally, columns were placed in new clean tubes and eluted twice with 100 µL 60% ACN 0.1 % FA. Samples were dried using a vacuum centrifuge and stored at -20 ºC.

LC-MS/MS load

Samples were resuspended in 50 µL 3% ACN 0.1% FA, vortexed for 30 min at room temperature and centrifuged (14000 rpm, room temperature, 10 min). Peptide concentration was determined using the BCA assay kit and 3% ACN 0.1% FA was added to each sample to achieve a final concentration of 0.1 µg/µL.

LC-MS/MS assay

The samples were analyzed using the Dionex UltiMate 3000 RSLC Nano System coupled to the Q Exactive™ HF Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Waltham, MA, USA). Peptides (10 µl) were loaded onto a trap column (20 µm x 350 mm) and washed using a flow rate of 5 µl/minute with 2% ACN 0.01% FA. The trap column was then switched in-line with the analytical column after 5 minutes. Peptides were separated using a reverse phase Acclaim PepMap RSLC C18 (75 µm x 15 cm) analytical column using a 120 min method at a flow rate of 300 nL/min. Mobile phase A consisted of 0.01% and a mobile phase B consisted of 80% ACN 0.01 % FA. The linear gradient started at 5% B and reached 30% B in 80 min, 45% B in 91 min, and 100% B in 93 min. The column was held at 100% B for the next 5 min before being brought back to 5% B and held for 20 minutes. Sample was injected into the QE-HF through the Nanospray Flex™ Ion Source fitted with an emission tip from Thermo Scientific. Data acquisition was performed monitoring the top 20 precursors at 120,000 resolution with an injection time of 100 millisec.

Data Analysis

The freely available MaxQuant software package (version 1.5.5.1, Max Planck Institute of Biochemistry) was used for the analysis of mass-spectrometric data set. Only 896 protein sequences are registered in the UniProt database for gilthead seabream species (updated to 03/10/2017), and 2695 if a higher taxon as ‘Sparidae’ was considered.

In order to perform an efficient protein identification, the UniProt database for the teleost fish zebrafish (*Danio rerio*; 59075 sequences, updated to 03/10/2017), which genome sequence is available [16], was used for the mass spec file analysis.

The search parameters were: first search peptide tolerance: 20 ppm, main search peptide tolerance: 4.5 ppm, other instrument group-specific parameters by default. The enzymes considered were trypsin and LysC, with 2 Max. missed cleavages. Oxidation of methionine residues (variable) and carbamidomethylation of cysteine residues (fixed) were included as modifications. Sequences and identification of global parameters were used by default, with a False Discovery Ratio of 0.01. Match between runs was considered, with a Match time window of 1 min and an Alignment Time Window of 20 min. Label-free quantification (LFQ) was used to obtain the normalized LFQ intensity. LFQ intensity values were obtained from the MaxLFQ algorithms, included in the MaxQuant software packwages. These algorithms were developed in order to achieve a highest accuracy of quantification in label-free LC-MS/MS assays without "house-hold proteins", extracting the maximum ratio information from peptide signals in a given number of samples [32].

Contaminants and reverse proteins were removed from the analysis. Only proteins with at least 2 MS/MS counts, and a minimum of two different peptides used for identification (with the UniProt database) were considered for quantitative analysis. InfernoRDN application (Pacific Northwest National Laboratory), which provides an easy-to-use to R (version 3.4.0) for proteomic data analysis, was used to analyse both intensity and LFQ intensity data sets, including analysis of variance (ANOVA), principal component analysis (PCA), cluster analysis and heatmap plot generation. Only proteins with values of intensity (or LFQ intensity) in all the samples were considered for the ANOVA.

After ANOVA analysis, proteins with a p-value<0.05 were subjected to 2-groups cross comparison. Proteins with an average fold change (FC)≥2 or ≤0.5, or with a t-test<0.05 (and a FC ≥1.5 or ≤0.75) were selected for the functional analysis of the different comparisons (FM vs VM, and FM vs VM+).

Reproducibility validation

Two samples were run per triplicate in the Q-Exactive in order to validate the reproducibility of the assay. Data was analysed using the same database and search parameters, but match between runs was not considered. Contaminants and reverse proteins were removed, and peptides and proteins only present in one or two runs were considered when MS/MS counts ≥ 6. Taking as basis the identified peptides, the variation coefficient of intensity values for each peptide was reported. If we consider the identified proteins, the variation coefficients of both intensity and LFQ intensity values were determined. In both cases, the median of each set of variation coefficients were determined.

Functional annotation

Only protein sets obtained from the intensity data analysis were analysed. This decision is further addressed later in the 'Discussion' section.

An Enrichment Analysis (two-tailed Fisher's Exact Test) was performed, using Blast2GO software (version 2.8.0), for each comparison [33]. The test list included the differentially expressed proteins after the fold-change and t-test filtering, and the reference list (or background) was obtained from the UniProt Danio rerio database, containing the different Gene Ontology™ terms (GO terms) associated to all the proteins of this database. Enriched GO terms were filtered according to p-value (<0.05) and FDR (<0.05) and classified in the three GO annotation domains: biological process, cell component and molecular function.

Gene Ontology IDs of the GO terms overrepresented in the test list were provided to ReViGO (Reduce Visualize Gene Ontology), which summarizes them removing redundant terms [34]. Danio rerio database was used, and SimRel was used as the semantic similarity measure, setting an 'Allowed Similarity' value of 0.5 (small) to reduce the length of the results. Treemaps from the ReViGO analysis were displayed using the package 'treemap' of R (version 3.4.0).

KEGG pathways

The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) was used for the KEGG annotation [35, 36]. The list of differentially expressed used as input in the Blast2GO analysis was also used for the DAVID functional annotation using the *Danio*

rerio sequences included in its repository. Pathways with a p-value<0.05 were considered as significantly affected.

Results

Biometric assessment

Fish weight (FW), condition factor (CF), hepatosomatic index (HSI), visceral fat index (VFI) and gut weight (GW) were affected by the dietary composition (Table 2). FM group reported mostly the highest values, while in the most of the cases VM group registered the lowest indices. On the other hand, VM+ group showed intermedia results between FM and VM, except for the CF.

Table 2. Biometric indices of fish before and after the growth period in the different experimental groups

	I	FM	VM	VM+	SEM
FW	9.6	180.9^{a}	103.6^{c}	142.7^{b}	10.35
CF	1.25	2.15^{a}	1.91^{ab}	1.73^{b}	0.085
VSI	0.110	0.072	0.071	0.079	0.004
HSI	1.15	1.42^{a}	0.91^{c}	1.13^{b}	0.063
VFI	0.65	1.79^{a}	0.95^{b}	1.23^{ab}	0.201
GW	0.21	3.62^{a}	2.65^{b}	3.67^{a}	0.271
GL	7.4	11.1	10.7	14.3	1.44

FW, fish weight; CF, condition factor; VSI, viscerosomatic index; HSI, hepatosomatic index; VFI, visceral fat index; GW, gut weight; GL, gut length; SEM, pooled standard error of the mean

FW (g); CF (g cm^{-3}) = 100 · total weight (g)/ total length (cm)^{3}; VSI (%) = 100 visceral weight (g) / total weight (g); VSI (%) = · 100 visceral weight (g) / total weight (g); HSI (%) = 100 · liver weight (g) / total weight (g); VFI (%) = 100 visceral fat weight (g) / total weight (g); GW (g); GL (cm).

Means of 10 fish (n=10); data in the same row with different superscripts differ at P<0.05. Newman-Keuls test was applied for the comparison of the means

Proteomic profile

LC-MS/MS assay

1355 proteins were identified after the MaxQuant assay. After removing contaminants and reverse sequences, the list reduced to 1328 proteins. 754 (56.78%) of them were found in all the samples. A summary of the proteins identified in the different groups and individual samples is shown in Table 3. Samples from the VM group,

especially VM2 (63.6%), reported lower percentages of identifications in comparison to the total amount, but a similar number of proteins was represented in all the groups.

Table 3. Number of proteins identified in the different runs and experimental groups

	FM			VM			VM+		
	FM1	**FM2**	**FM3**	**VM1**	**VM2**	**VM3**	**VM+1**	**VM+2**	**VM+3**
Identifications	1233 (92.8%)	1225 (92.2%)	1244 (93.7%)	1068 (80.4%)	845 (63.6%)	1219 (91.8%)	1247 (93.9%)	1229 (92.6%)	1257 (94.7%)
Represented in the group*		1291 (97.2%)			1279 (96.3%)			1299 (97.8%)	
Represented in all samples		1163 (87.6%)			776 (58.4%)			1174 (88.4%)	

*Proteins represented in the group were identified in at least one run of the group

Reproducibility validation

S2 (Supplementary Data) summarizes the reproducibility assessment of the LC-MS/MS assay. Percentage of peptides and proteins that display intensity values in the three runs of both sets of runs (R1 and R2) is around the 65 % and the 80%, respectively. However, these percentages increase to 100% (75% when we work with LFQ Intensity) after removing the low abundant peptides (or proteins) from the analysis, confirming a high reproducibility in qualitative terms.

After filtering, the percentage of peptides showing a variation coefficient in intensity below 20% was 78% for R1 and 79% for R2. Regarding the proteins, this percentage ranged from 66% to 69%, if the intensity values were considered, and from 72% to 75% when LFQ intensity data set was used. The medians of the variation coefficients for both sets of runs were around the 13% and the 8%, working with Intensity and LFQ Intensity, respectively. These values are common and highly acceptable for a LC-MS/MS assay.

Quantitative analysis

After filtering proteins that display a total MS/MS counts<2, 1270 protein IDs were retained. The set of identified proteins, including intensities and LFQ intensities, is available in the Supplementary Data (S3).

After PCA, considering intensity (Figure 1A) and LFQ intensity values (Figure 1B), the samples belonging to FM and VM+ groups grouped closer and separately to VM samples.

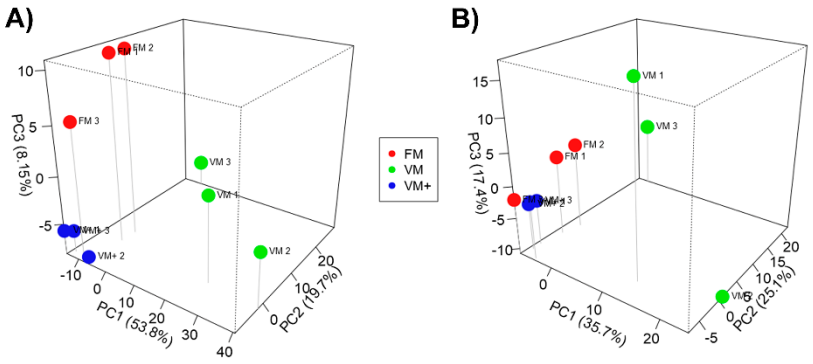

Figure 1: PCA three-dimensional plot, considering all the proteins identified in the MaxQuant assay

A) Taking Intensity values. B) Taking LFQ intensity data**.**
Percentages represent the variability of data sets which is explained by the different Principal Components

The number of total identifications and differentially expressed proteins among groups, considering the intensity and the LFQ intensity, is reported in Table 4. The number of differentially expressed proteins was lower when LFQ intensity was used, since the intensity is normalized globally across all the samples, and an under-expression of numerous proteins can be observed in the group VM in comparison to the other two groups.

Table 4. Number of proteins considered for quantitative analysis and differentially expressed between experimental groups

	Intensity	LFQ Intensity
Proteins with global intensity ≠ 0	1265	1208
Proteins differentially expressed with p<0.05	229	61
Proteins missing in one or two groups	13	17

If the PCA analysis is performed taking as basis the differential proteins (Figure 2A and 2B), each experimental group showed a differential distribution. The heatmap plot confirmed the variability observed in the PCA distribution, mainly for VM group, which was classified in a different hierarchical branch (Figure 2C and 2D), while the FM and VM+ samples showed a similar proteomic profile and cluster together. The set of differentially expressed proteins, including their individual and average intensity/LFQ intensity values, fold change and t-test values, for each comparison, is reported in the Supplementary Data

(S4). Comparing groups in pairs (Table 5), in the case of the intensity analysis, FM and VM+ groups showed a generalized over-expression of the whole-set of proteins compared to VM. In the case of the LFQ intensity analysis, some proteins were over-expressed in the VM group compared to other experimental groups, although the majority of the proteins with a differential expression were also underrepresented or absent in the VM group. Comparing the intensity and LFQ intensity approaches, 22 and 25 proteins overexpressed or only present in the FM and the VM+ groups, respectively, in comparison to the VM group, were common for both Intensity and LFQ Intensity data sets. The list including the significantly over- or under- represented proteins for each comparison, and the proteins exclusively found in specific group are shown in Supplementary Data (S5).

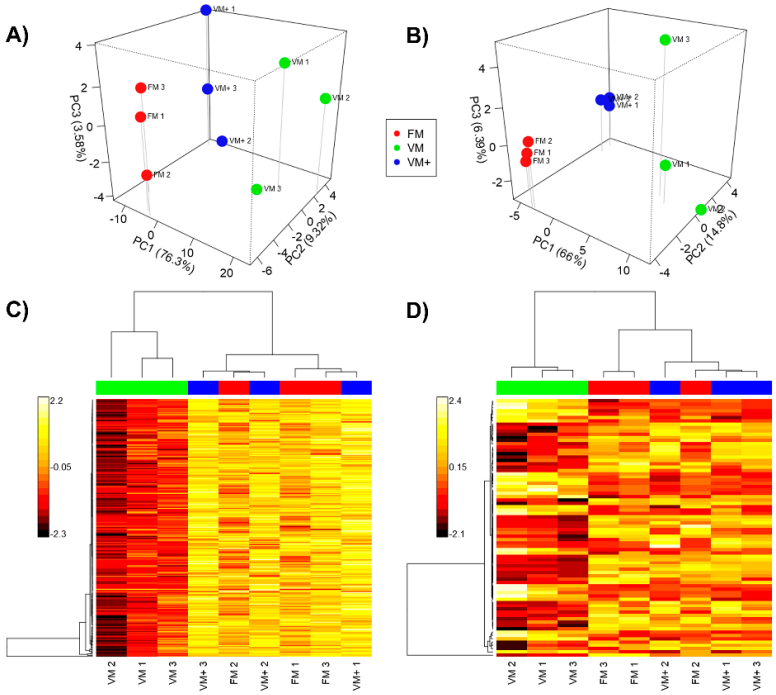

Figure 2 PCA three-dimensional plot and heatmap plot, including only proteins differentially expressed between groups

A) PCA plot considering intensity data. B) PCA plot considering LFQ intensity data. C) HeatMap plot considering intensity data. D) HeatMap plot considering LFQ intensity data.

Percentages in (A) and (B) represent the variability of data sets which is explained by the different Principal Components. HeatMap were constructed based on hierarchical clustering of samples using the Complete linkage as agglomeration method with Euclidean distances

Table 5. Comparisons between experimental groups after filtering based on the fold change and the t-test

	Intensity		LFQ Intensity	
	Overexpressed (Present only) in the first group	Overexpressed (Present only) in the second group	Overexpressed (Present only) in the first group	Overexpressed (Present only) in the second group
FM vs VM	193 (12)	0 (1)	20 (14)	14 (0)
FM vs VM+	9 (1)	33 (1)	6 (2)	1 (3)
VM+ vs VM	216 (11)	0 (0)	20 (15)	19 (0)

For each comparison, the proteins over-expressed or present only in the first group were under-expressed or absent in the second group, and vice versa.

In order to evaluate if differential expressed proteins were common or specific of each comparison, Venn diagrams were created for intensity and LFQ intensity data sets (Figure 3). Most of the differentially expressed proteins of the FM *vs* VM and VM+ *vs* VM comparisons were shared, even using LFQ intensities. As a consequence, the functional annotation and the KEGG Pathway analysis were performed for the FM vs VM comparison, taking intensity values. This decision is further considered in the Discussion section.

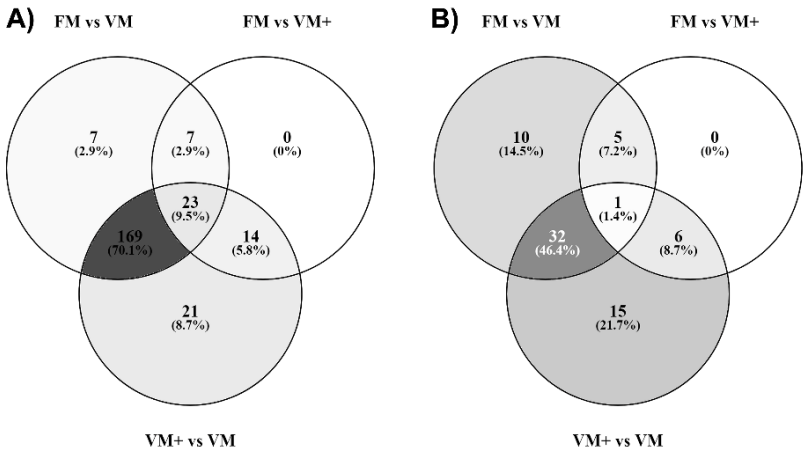

Figure 3: Venn diagrams comparing the differentially expressed protein sets in the three two-groups comparison

A) Considering intensity data. B) Considering LFQ intensity data. Percentages are referred to the total number of differentially expressed proteins in each approach

Functional annotation

A total of 199 protein IDs, from the set of differentially expressed proteins in the comparison FM *versus* VM (206 proteins), were recognized by Blast2GO and submitted to the Enrichment Analysis. All the enriched GO terms delivered by Blast2GO, and classified in three different domains of the Gene Ontology annotation (the biological processes in which the protein is involved, the molecular function of the protein, and its location in the cell) have been detailed in Table S6 (Supplementary Data). A graphical summary of these enriched GO terms for each domain, taking into account the significance of each term, is shown in Figure 4.

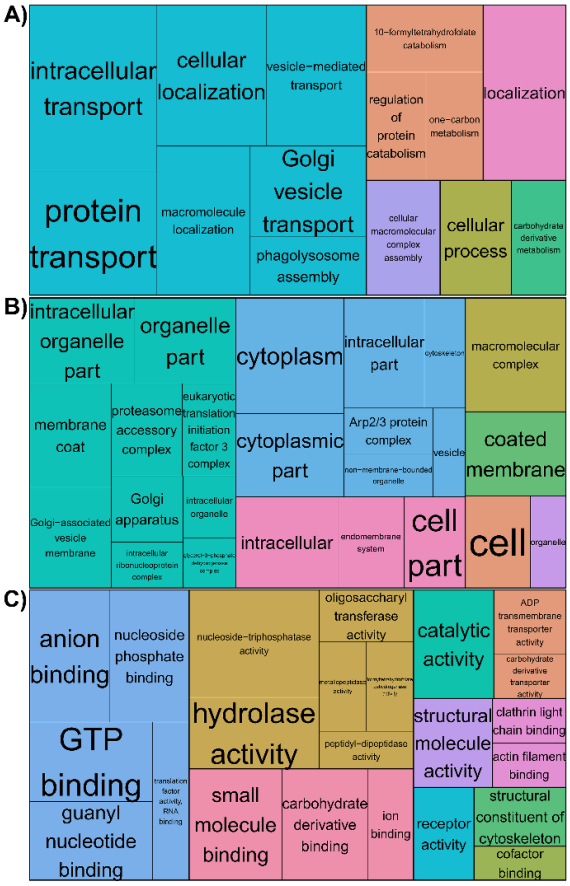

Figure 4: Graphical summarizes of the enriched Gene Ontology terms for the three categories

A) Biological processes. B) Cell components. C) Molecular functions

Among the biological processes affected, intracellular transport processes, mediated or not by vesicles, the assembly of cellular macrocomplex, as the phagolysosome, localization of protein and other macromolecules, protein catabolism and one carbon metabolic processes mediated by tetrahydrofolate were the most common. Regarding the cell components, membrane proteins, including Golgi and the endomembrane system, were most affected by dietary treatment, but several cytosolic proteins were also altered. Most of the proteins are constituents of the cytoskeleton, vesicles and different intracellular protein complexes as the proteasome, the Arp2/3 protein complex, ribonucleoprotein complexes, the eukaryotic translation initiation factor 3 complex or the glycerol-3-phosphate dehydrogenase complex. Finally, several molecular functions were enriched according to Blast2GO analysis: binding to nucleotides, small molecules, ions, carbohydrate derivatives, cofactors and cytoskeleton proteins, nucleoside-triphosphatase and hydrolase activity, catalytic activity, structural molecule activity, receptor activity and structural constituent of cytoskeleton, among others.

KEGG pathways

From the list of 206 differentially expressed proteins, 201 IDs (1 was redundant, and 4 were not recognised) were identified by DAVID. The 58.7% (118) presented KEGG annotation and 5 KEGG pathways were significantly affected (p-value<0.05) (Table 6).

Table 6. Affected KEGG pathways according to DAVID analysis, including the number of proteins altered in each pathway

KEGG Pathway	Nº of proteins	%*	PValue
Phagosome	14	11.9	0.00002
Proteasome	7	5.9	0.00107
Salmonella infection	8	6.8	0.00440
Regulation of actin cytoskeleton	13	11.0	0.01318
Amino sugar and nucleotide sugar metabolism	5	4.2	0.03177

* Percentage of proteins altered in the specific KEGG pathway from the total of proteins with KEGG annotation

Discussion

Biometric Parameters: Growth and Survival

The present assay reported significant differences in weight and other biometric indices, indicating an effect of total fishmeal replacement on growth performance of gilthead seabream. Diets were designed to cover

the minimum requirements for essential amino acids in this species [31]. In previous trials, total fishmeal replacement has been successfully achieved for gilthead seabream without affecting fish growth [5, 37]. This could be explained by the differences in initial fish weight (around 10 g in the present trial compared to more than 100 g in the previous assays), since lower levels of fishmeal replacement can be reached during the first stages of growth [1].

On the other hand, the inclusion of alternative marine sources at 15% level in a vegetable meal based diet had a positive effect on fish growth compared to the VM diet, although in contrast with other studies [38], fish growth did not reach the levels obtained with the fishmeal based diet.

Proteome Analysis

MaxLFQ algorithms were used in order to achieve the highest quantification accuracy in the proteomic assay. Nevertheless, their application requires the existence of a dominant population of proteins that change minimally between experimental conditions [32]. In the present work, a big set of proteins was observed to be under expressed in the VM group in comparison to FM and VM+ samples, and this could have affected obtaining of LFQ Intensity data and hide potential interesting differences between dietary groups. In this regard, the number of differentially expressed proteins was markedly reduced, and some proteins were found as over-expressed in the VM group, although most of proteins over-represented in the FM and VM+ groups were also observed using the Intensity analysis. Considering samples preparation was carried out in parallel in unique experimental condition, and samples were loaded randomly in the LC-MS/MS after peptide quantification, similar LC-MS/MS performances in quantitative terms were expected. Since reproducibility assessment showed also a good correlation when Intensity data was considered, functional annotation was only carried with the outputs of the Intensity data, although both sets of data were considered for quantitative analysis.

Taking account these considerations, a high impact of total fishmeal replacement was observed in gut mucosa proteome. Approximately 20% of identified proteins were significantly underrepresented in the VM gut mucosa in comparison to the FM and VM+ groups, representing nearly 80% of the complete set of differentially expressed proteins (almost the 50% considering the LFQ Intensity data).

Moreover, despite differences observed on growth between FM and VM+ groups at the end of the growth trial, intestinal status of fish fed VM+ diet did not seem to be affected, leading to focus on the functional differences between FM and VM.

The replacement of fishmeal by alternative protein sources can lead to an impact on a great variety of biological processes and metabolic pathways in the gut mucosa [39], which could affect fish performance [29]. Although most of the knowledge about protein function was obtained from human studies, thus its application to fish physiology has to be considered with caution [39], present results seem to point to long-term feeding with a complete vegetable diet may cause a loss of part of the mucosa functionality.

Most of the downregulated proteins identified in VM group were related with transport of molecules, cell communication, cell metabolism, structural functions and assembly of protein complexes as the phagosome or the proteasome, necessary for the normal function of the enterocytes and therefore, of the epithelium.

Effects on the enterocytes functionality

The homeostatic balance between epithelial cell proliferation and apoptosis is essential for the maintenance of the epithelial function, including regulation of epithelial permeability, the inflammatory response or the absorption of nutrients [40]. Apoptosis plays a central role in epithelial organization and cell turnover, and defects on apoptotic pathways in enterocytes have been related to villus atrophy, epithelial hyperplasia or loss of normal absorptive function [41]. The 26S proteasome, a key multiprotein complex in cell proteostatic mechanisms [42], and other proteasome subunits, were underexpressed in group VM, as well as the villin, a major actin-modifying protein which plays a key role on the assembly of the brush border [43]. Villin is involved in several functions in the epithelia, including antiapoptotic activity, regulation of epithelial cell morphology, differentiation, maturation and motility and actin reorganization [40]. Other proteins related with apoptotic regulation, the regulation of cell polarization, the migration of cells and the maintenance of a homeostatic state, such as the gelsolin [41], the Ap1m2 [44], the annexins A1 [45] and A2 [46] or the AP2-complex [47] were also downregulated in this group.

In rainbow trout liver, a downregulation effect on the proteasome pathway in response to starvation was reported [23] and pathways involved in cellular protein degradation seem to be sensitive to plant protein inclusion [24], while the partial replacement of fishmeal by soybean meal induced inflammation, cellular repair and apoptosis in the distal intestine of Atlantic salmon [48]. These results suggest that the proteasome pathway could play a protective role in the epithelial cells, and it can be regulated by the dietary composition and energy level. In this regard, the impact on the regulation of the apoptosis mechanisms reported in the present work might be explained by dietary factors.
Furthermore, several proteins involved in protein synthesis and metabolism, such as translation-related proteins and the mentioned apoptotic-related proteins, were also under-expressed in gut mucosa of fish fed VM diet. Therefore, an unbalanced or deficient protein turnover between protein synthesis and degradation into amino acids could take place, leading ultimately to not satisfy the necessary renewal of proteins in the cell. Indeed, higher rates of cell renewal have been related to inflammatory responses [49] in order to maintain the population of functional enterocytes, so a reduced cell recovery rate could be associated with the loss of the epithelium properties [48].

On the other hand, intracellular transport processes, especially protein transport and Golgi vesicle-mediated transport, were altered in the VM group. Golgi complex is the main organelle involved in protein transport and plays a crucial role in the maintenance of homeostasis in polarized cells such as the enterocytes [50]. In this regard, proteins such as clathryn, coatomer protein and adaptor-related protein complex, which are related to intracellular protein transport, were under-regulated in the VM group.

On the other hand, one-carbon metabolism, which supports amino acid metabolism, nucleotide biosynthesis and redox defence, among several physiological processes [51], and the carbohydrate derivatives metabolism, which is also related with many cellular functions, were also altered.

Effect on epithelial permeability, immune response and inflammatory activity

The under-expression of proteins such as actin cytoskeleton-related proteins and myosin light chain kinase could be related to a lack of capacity to regulate the permeability of the intestinal barrier, which is

necessary for an inflammatory and immune response against luminal environmental changes. The actin cytoskeleton dynamics seem to be regulated by the phosphorylation of the myosin light chain [52] and also by several different cytoskeletal, scaffolding, signalling and polarity proteins [53]. It is anchored to epithelial tight junctions between the enterocytes, which play an important role in the regulation of epithelial barrier permeability by luminal and tissue stimuli and in the selective exchange of molecules between the intestinal lumen and lamina propria [54, 55], being a crucial structure for the intestinal status. Thus, disruptions on this regulation mechanism can lead to inflammatory reactions and affected immune states [13, 56] and also to malabsorption of nutrients [57].

Since intestinal barrier is continuously exposed to commensal bacteria and dietary nutrients, these can have an influence in the pathways related to the expression and localization of tight junction proteins [58]. An increase of transepithelial uptake capacity, perhaps caused by an increased permeability, in response to saponins, which are present in soy, has been reported in different species, including fish [59]. Nevertheless, an impact on gene expression of tight junction proteins has been observed in fish fed high soy dietary levels [60], which suggested the tightening of the tight junctions, maybe in response to antinutrients, Therefore, further research is needed in order to go into the exact role in permeability regulation of the different proteins.

The intestinal epithelium is involved in modulation of the gastrointestinal microbiota through the activation of inflammatory responses [61, 62] or by immunotolerance development to luminal microbiota [63]. Bacterial translocation through the epithelial barrier can take place following the paracellular route, between adjacent epithelial cells [64] or through the enterocytes [63] by the formation of phagosomes [11]. Phagocytosis has been also described in macrophages/monocytes and neutrophils during the innate immune response [11]. Thus, besides the effect on epithelial permeability, the alterations in phagocytic processes observed in the present work could be also linked to a lack of capacity in the gut mucosa of initiate an inflammatory process, exert an effective innate immune response and develop an inmunotolerance to commensal bacteria. A remarkable impact on the gut microbiota composition of the gilthead seabream has been reported when fishmeal was completely replaced by vegetable sources [37], and the differences in the gut bacterial community could be explained by an immune dysregulation. In this regard, the

underexpression in the VM group of proteins related to the modulation of inflammatory and immune reactions, such as the leukotriene A-hydrolase [65], the annexins [45], Meprin A [66] or the angiotensin converting enzyme [67] may also be related.

Extensive research has been carried out regarding to the impact of including plant protein sources in the diet on the inflammatory and immune response of fish, also in gilthead seabream [6, 68, 69]. The level of fishmeal replacement, as well as the duration of the dietary treatment, seems to be decisive in the trigger of an immunostimulated or immunosuppressed status [6, 70]. In this respect, the suppression of innate immune capacity by high levels of inclusion of plant proteins has been previously observed in rainbow trout [71], but also in gilthead seabream. The long-term decrease in the plasma complement level after feeding with a fishmeal replacement above 75% level has been described [6], suggesting a possible immunosuppression. Moreover, a long-term immunosuppression at the gut mucosa level, based on gene expression, was suggested in fish fed using only plant protein sources [72]. On the other hand, a transcriptomic modulation induced by dietary decrease of fishmeal and fish oil was also observed [73], reporting an upregulated expression of inflammatory markers with higher grade of leucocyte infiltration in the submucosa, especially in the anterior intestine, and changes in other genes related with cell differentiation and proliferation, antioxidant defence, immunity, epithelial architecture and permeability and mucus production. At the histological level, the inclusion of vegetable ingredients has revealed changes potentially related to intestinal inflammation, based on the number of goblet cells, the level of infiltration of leukocytes, the grade of supranuclear vacuolization and the submucosa thickness [6, 7, 69, 72], although major histopathological signs were not reported.

Effects on nutritional absorption/secretion

The role of the gut mucosa on the absorption of nutrients could be also negatively affected by the total fishmeal replacement. The structural modifications in the gut epithelia described in seabream in response to plant protein inclusion in diets [6, 7, 74] could modulate nutrient transport, since transporters are immersed in the lipid membrane of the enterocytes. Moreover, the impact of vegetable sources on the digestive protease balance [7], the brush border enzyme activities [75] or the asynchronous utilization of amino acids from different origins [76] can lead to a lower luminal nutrient availability [77]. In fact, nutrient

absorption in gilthead seabream is affected by the use of high levels of vegetable sources [77]. In the present work, VM group showed a downregulation of several proteins related to the metabolism and transport of proteins and with the maintenance of the enterocyte structure, which could have had a consequence on the nutrient assimilation performance [57], decreasing fish growth.

Deficiencies of plant protein based diets

High plant protein diets deficiencies have been related with the presence of anti-nutrient factors, as non-starch polysaccharides [78], but differences in luminal amino acid bioavailability, which depends on the source, could lead to differences in its exploitation [77, 79] and to essential amino acid imbalances. Several amino acids are involved in different physiological process related with the maintenance of the gut integrity and in the regulation of different immune and metabolic pathways [8, 80, 81, 82, 83], so imbalances can compromise the normal intestinal function and lead to immune dysfunctions [6].

Therefore, long term feeding with nutritionally deficient diets could be considered as a chronic stress, involving a high energy and resource expenditure and a high metabolic activity [70] and affecting the immune status [84]. This impact depends on the intensity and the duration of the stressing factor, but long term stressors, such as the diet could be, normally shows immune suppressive or depressive effects [70]. Since immune mechanisms require a continuous energy availability, they will face a lack of resources at a long term, which can be ultimately lead to a higher mortality [72].Moreover, the allocation of most of the energy expenditure to the maintenance of an effective immune response during the trial could also explain differences in growth observed in the present trial.

The inclusion of marine sources at 15% level seems to prevent these detrimental effects, but further research is necessary in order to better understand how small dietary changes can have such high effects on the immune status of the fish, that manifest, ultimately, in fish growth, feed conversion ratio and survival, which are the most important parameters from the productivity point of view.

Conclusions

In the present study, a long term downregulation of proteins involved in epithelial permeability, inflammatory response and enterocyte homeostasis (including cell apoptosis, metabolism and protein transport) was observed in the gut mucosa of gilthead seabream with the complete replacement of fishmeal by vegetable ingredients, leading to poor growth and nutritive performance. This outcome suggests a possible suppression of the function of the gut epithelia over the long term.

Supplementary Material

Supplementary files (S1, S2) are included in ANNEX IV. S3, S4, S5 and S6 will be available after manuscript publication.

Ethics approval

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 and the European Directive 2010/63/UE on protection of animals used for scientific purposes

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Chapter 5.

Implementation of gilthead seabream (*Sparus aurata*, L.) intestine explant culture assay to determine mucosal pro-inflammatory responses to bacterial pathogens and impact of plant protein in feed

Implementation of gilthead seabream (*Sparus aurata*, L.) intestine explant culture assay to determine mucosal pro-inflammatory responses to bacterial pathogens and impact of plant protein in feed

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Abstract

The interactions between microbiota and fish immunity at gut level have been widely discussed during decades. However, in vivo approaches reported several limitations to evaluate these interactions. This work implemented an ex vivo assay of intestine explants culture to determine possible inflammatory and immune responses at gene expression level after exposure to bacterial pathogens. This system showed to be efficient to reduce the number of fish, increasing time of incubation and preserving the immune features of the intestinal mucosa. Tissue viability, RNA integrity, impact of the ex vivo culture on expression of target genes and data consistency between duplicated conditions was assessed to confirm the feasibility of the assay and to optimize conditions. Additionally, the effect of high plant protein inclusion in diets during a short or long period wastested in both intestinal sections (foregut and hindgut). Significant expression in pro-inflammatory cytokines IL-1B and IL-6 was elicited after 6 hours of exposure to *Vibrio Algynoliticus*, while COX-2 expression was significantly induced by *P. damselae* subsp. *pisicida*, although high correlation was observed in the expression of the pro-inflammatory markers. Finally, dietary groups did not show differences in expression when explants were not challenged with pathogen, but explants from fish fed the plant-based diet during a short term showed higher responses to bacteria exposure.

Introduction

The fish intestine is a complex biological system which represents a major barrier of defence against pathogens and plays a crucial role in the digestion and absorption of nutrients, osmoregulation and immune and inflammatory response [1]. Furthermore, the intestine participates in the modulation of gastrointestinal microbiota inducing inflammatory responses against pathogenic bacteria or developing immunotolerance to luminal bacteria [2].

The interactions between fish intestinal immunity, pathogen and commensal microbiota in the gut have been widely reviewed [3]. Bacterial challenges *in vivo* require specialized settings, expensive operating costs and a high number of fish, and is difficult to perform and achieve the desired experimental working conditions [4].

In this regard, *ex vivo* systems have been developed and proved to be useful tools to evaluate the possible effects of different bacterial strains on the intestine health providing very reliable information about the interactions between the bacteria and the host. Despite some restrictions related to tissue viability, the ex vivo intestinal sack method [5]has been used to assess the histological and microbial changes in fish in response to bacteria exposure [4, 6-9]. As drawbacks, this method is highly restricted by the tissue viability under experimental conditions [4, 5]. The development of new experimental models based on tissue explants culture have allowed maintaining the tissues lifespan, as well as immune and histological features [10-12]. These systems have been used to register responses to exposure to specific bacteria in human tissue explant cultures, also at gene expression level [12].

On the other hand, the necessity to replace fishmeal by alternative protein sources in aquafeeds has lead researchers to focus on the impact of the inclusion of new ingredients, such as plant protein sources, in the performance of produced fish. Although their use at high levels without impairing growth performance is feasible [13, 14], negative effects on growth performance, feed intake or immune capacity have been observed [15]. Focusing on fish intestine, plant sources have been related to morphological alterations, changes in the intestinal bacterial community, inflammatory events and lack of capacity to regulate the intestinal epithelia integrity [16], as it has been reported *in vivo* by the evaluation of changes in histology and in the gene expression pattern [17-19].

Research related to the impact of fishmeal replacement becomes even more relevant for carnivorous species, such as gilthead seabream. Several studies have been carried out in this species, assaying different levels of substitution and alternative ingredients [20-22], to evaluate, *in vivo*, immune parameters in response to fishmeal replacement [23-25] or bacterial infection [26-29].

The *ex vivo* response of the intestinal tissue to different protein sources after culture with different bacteria has been previously addressed in other species [4]. However, to the best of our knowledge, this is the first study involving gene expression determination in intestinal explants after *ex vivo* bacterial exposure in fish.

In the present work, an *ex vivo* method based on an intestine explant culture system has been developed to evaluate the inflammatory and immune response, at gene expression level, of the gilthead seabream intestine after exposure to different bacterial strains. Moreover, an exploratory assay was also performed to evaluate the impact of total fishmeal replacement by a plant mixture during a short and long-term period by measuring the inflammatory and immune responses.

Materials and methods

Ethic statement

The experiment was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes [30].

Bacterial strains and growth conditions

Pseudomonas anguilliseptica (CECT901), *Vibrio algynoliticus* (CECT521) and *Photobacterium damselae* subsp. *Piscicida* (CECT 7198) strains were obtained from the Colección Española de Cultivos Tipo (CECT; Paterna, Spain), and cultured with the recommended media under agitation (Tryptic Soy Broth for *P. anguilliseptica* and Marine Broth for *V. algynoliticus* and *P. damselae subsp. piscicida*) at 26º C for 2 days (*P. anguilliseptica* and *P. damselae* subsp. *Piscicida*) and at 30º C for 1 day *(V. algynoliticus).*

Bacterial cell counts were estimated by linear regression to a previously established standard curve for each strain correlating colony forming units (cfu)/mL to optical density (600 nm). In the particular case of *P. anguilliseptica*, its aggregant nature limited the good correlation between cfu/mL and optical density, so the standard curve registered for *P. damselae* subsp. *piscicida* was used to estimate the bacterial concentration (cfu/mL).

Fish, rearing system conditions, diets and feeding conditions

Juveniles of gilthead seabream were obtained from the fish farm BERSOLAZ (Bersolaz Spain, S.L.U, Culmarex Group) in Port de Sagunt (Valencia, Spain) and transported to the facilities at the Universitat Politècnica de València, where the growth trial was conducted. Fish were harboured in two octagonal concrete tanks (4000 L) within a marine water recirculating system (75 m^3). Features of the system and water parameters set were described in previous growth trials carried out on these facilities [31]. Lighting conditions were determined by the natural photoperiod. Temperature, pH, oxygen, ammonia, nitrite and nitrate concentrations were monitored along the experiment. The fish were daily fed by hand to apparent satiation two times per day (9:00h and 17:00h). The pellets were slowly distributed, allowing fish to eat, in a weekly regime of six day of feeding and one day of fasting.

Two experimental diets were designed and assigned to a different concrete tank. Diets were prepared by cooking extrusion process using a semi-industrial twin-screw extruder (CLEXTRAL BC-45, St. Etienne, France). A fishmeal based control diet (CTR), in which most of the protein was provided by fishmeal (59%), and a plant protein based diet (PP), in which all the fishmeal was replaced by plant sources and synthetic amino acid were added to meet the minimum amino acid requirement for gilthead seabream juveniles [32]. Ingredients and proximate composition are showed in Table 1. Prior to diet formulation, dry matter (DM), crude protein (CP), crude lipid (CL) and crude fibre (CF) of different sources and ingredients used were analysed according to AOAC procedures [33]. All analyses were performed in triplicate. Amino acids of raw materials were also analysed by reverse phase – high performance liquid chromatography [34]. Macronutrients and essential amino acids content were also determined in the experimental diets, and they are also shown in Table 1.

Table 1. Ingredients, proximal composition and essential amino acids profile of experimental diets

	CTR	PP
Ingredients (g kg^{-1})		
Fishmeal	589	
Wheat meal	260	
Wheat gluten		295
Broad bean meal		41
Soybean meal		182
Pea meal		41
Sunflower meal		158
Krill meal		
Squid meal		
Fish oil	38.1	90
Soybean oil	92.9	90
Soy Lecithin	10	10
Vitamin-mineral mix*	10	10
Calcium phosphate		38
Arginine		5
Lysine		10
Methionine		7
Taurine		20
Threonine		3
Proximate composition (% dry weight)		
DM	88.1	93.9
A	10.1	7.4
CL	18.5	19.8
F	0.8	4.3
NFE**	22.8	23.2
NSP	7.7	21.5
CP	44.2	45.0
Essential aminoacids (g 100 g^{-1})		
Arginine	3.39	3.30
Histidine	1.00	0.82
Isoleucine	1.47	1.17
Leucine	3.24	2.98
Lysine	3.68	2.26
Methionine	1.16	1.06
Phenylalanine	1.80	1.87
Threonine	1.98	1.44
Valine	2.01	1.47

*Vitamin and mineral mix (values are g kg^{-1} except those in parenthesis): Premix, 25; choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. The Premix is composed of: retinol acetate, 1 000 000 (IU kg^{-1}); calcipherol, 500 (IU kg^{-1}); DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamine, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides, 12.

**NFE = 100—CP—CL—CF

DM, dry matter; A, ashes; CL, crude lipid; F, fiber; NFE, nitrogen free extract; NSP, non-starch polysaccharides; CP, crude protein

Experimental design

Three different ex vivo experiments were conducted. The scheme of the experimental design is shown in Fig 1. *Experiment 0* was designed in order to estimate the maximum length of incubation for gilthead seabream intestine under ex vivo conditions by LDH activity assessment. Afterwards, in *experiment 1*, the best reference gene to determine the gene expression under *ex* vivo conditions was stablished. Furthermore, a preliminary assay was performed at different time of incubation in samples obtained from fish long-term fed the CTR diet and the PP diet (average weight=68±37.8g) and exposed to different bacteria strains. Finally, experiment 2 focused on gene expression data consistency using samples belonging to fish long-term fed the CTR and the PP diet, and short-term (15 days) fed the PP diet (average weight=252±70.1g). This last assay will allow us the evaluate the impact of short or long-term feeding with PP diet in the intestine before and after the ex vivo bacterial exposure.

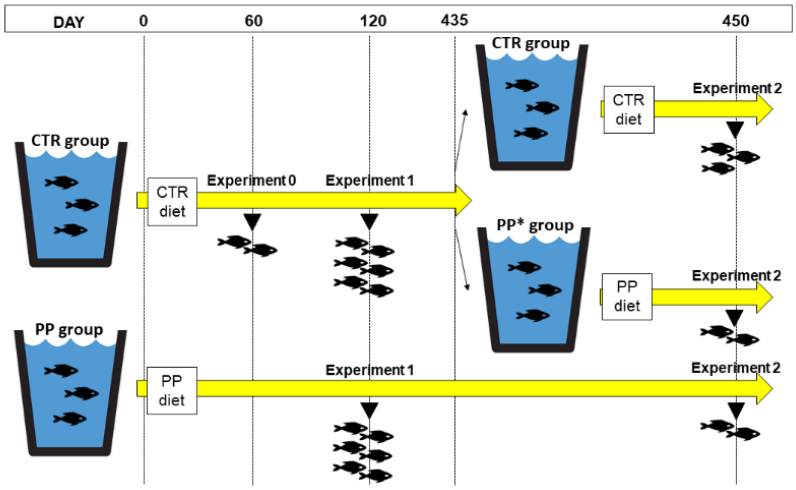

Fig 1. Summary of the experimental design.
The different *ex vivo* experiments were performed at days 60, 120 and 450. Initially, fish were distributed in two tanks and fed with CTR and PP diets. Fifteen day before the experiment 2, fish of the CTR group were divided in two groups, and one of them change its feed to the PP diet.

Ex vivo assay development

Bacterial stimuli preparation

At the day of the ex vivo explant culture experiment, optical density (600 nm) of the bacterial cultures was determined and bacterial cell number was estimated using the standard curves established for each strain. Then, bacterial cultures were centrifuged at 4.000 g for 20 min, washed once with PBS, and re-suspended in CO2-independent culture medium (Gibco, ThermoFisher) to a final concentration of $3 \cdot 10^7$ ufc/mL in the case of *V. algynoliticus*, and $1 \cdot 10^7$ ufc/mL of *P. damselae* subsp. *piscicida* and *P. anguilliseptica*.

Tissue collection and preparation

Fish were sacrificed by immersion in benzocaine (60 ppm) during 15 min. Then, they were dissected and the intestine was obtained and separated in two sections (foregut and hindgut). Each section was cut with a scalpel in small pieces (4 mm x mm), which were immediately placed in culture filter plates (15 mm diameter wells with 500 um bottom-mesh, Netwell culture systems, Costar, Cambridge, MA) with the epithelial surface uppermost, and filters were placed into wells containing 1 mL of the different bacterial solutions in CO2-independent culture medium. 100 µL of the corresponding bacterial solutions were finally added to epithelial surface to ensure that samples were completely submerged.

Ex vivo system conditions

Samples were incubated at 22º C in independent CO_2 atmosphere. The incubation time varied in the different experiments. At the end of the incubation time, samples were carefully collected from the culture filter plates and stored in 100 mM Tris-HCl at 4ºC or RNA later (Ambion Inc., Huntingdon, UK) at -80º C for LDH activity evaluation or RNA isolation, respectively. pH changes in the explant culture medium due to different bacterial treatments were monitored.

Experiments

Experiment 0

Explants of foregut (FG) and hindgut (HG) from two fish (CTR group) were incubated during 6 h and 24 h. No bacterial cultures were added to the explant culture medium. LDH enzymatic activity was determined at tissue level at 0, 6 and 24 h of ex vivo culture, whereas in the culture medium supernatant at 4, 6 and 24 h.

Experiment 1

Explants of FG and HG from fish from both dietary groups (CTR and PP, 120 days) were exposed to *V. algynoliticus*, *P. damselae* subsp. *piscicida* and *P. anguilliseptica* for 4 and 6 h. Adequate controls without bacteria were included and two fish per group were used per experimental point: initial samples (0 h), 4 h and 6 h of *ex vivo* exposure to bacteria. An electrophoresis gel with pooled RNA samples for each challenged group was run to confirm good RNA integrity (S1 Fig). Gene expression was determined in all samples.

Experiment 2

Following the experiment 1 design, FG and the HG explants were obtained from fish long-term fed with CTR and PP diets (450 days), and from fish fed with CTR diet during all their growth period except the last 15 days, when they were fed with PP (group PP*). Samples were incubated during 6 h in presence of *V. algynoliticus* and *P. damselae* subsp. *piscicida*. A control group without bacterial exposure was also included. Three fish from the CTR group and two from the PP and PP* were used to obtain initial samples (2 per fish and section) and pieces for the ex vivo assay. Every single condition (fish/section/stimuli) was assayed in duplicate in the *ex vivo* system. Tissue samples were stored in RNA later for the subsequent gene expression assay.

LDH activity assay

LDH activity was determined in the tissue (U/mg protein) and in the surrounding explant culture medium (U/L) at different times (0, 4, 6 and 24 h) of the incubation, using a commercial kit (BioSystems S. A.,

Barcelona, Spain), by measuring NADH absorbance et 340 nm. Tissue was weighed, homogenised in Tris-Hcl 100mM while maintaining the tubes on ice, centrifuged at 12.000 rpm and 4º C during 15 min and supernatant was collected for LDH assessment. Total protein in tissue extracts was determined using Bradford (Bradford, 1976).

Gene expression

RNA extraction and cDNA step

Total RNA was extracted from intestinal tissue samples using the phenol/chloroform method with TRIzol Reagent (Invitrogen, Spain) and treated with DNAse I (Roche) to remove DNases. Total RNA concentration, quality and integrity were assessed using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain).

1 µg of total RNA was used for cDNA synthesis reaction using the qScript cDNA synthesis kit (Quanta BioScience), according to the manufacturer's instructions. An Applied Biosystems 2720 Thermal Cycler was used with the following cycling conditions: 22 ºC for 5 min, 42 ºC for 30 min, and 85 ºC for 5 min. cDNA samples were stored at -20º C until gene expression was analysed.

Reference gene

Four candidate genes (**Table 2**) were tested to be used as reference genes, further assessing RNA integrity along the assay. The Cq of the four genes was determined in six pooled samples from Experiment 1 (two dietary groups: CTR and PP; three times: 0, 4 and 6 h).

Table 2. Primer sequences of candidate genes (reference and target genes) in the RT-qPCR assay

Gene	Abbreviation	GeneBank ID	Primer Forward	Primer Reverse	Lenght	Reference
REFERENCE GENES						
Elongation Factor 1α	EF-1α	AF184170	CTGTCAAGGAAATCCGTCGT	TGACCTGAGCGTTGAAGTTG	87	[35, 36]
Glyceraldehide 3-phcsphate dehydrogenase	GAPDH	DQ641630	CCAACGTGTCAGTGGTTGAC	AGCCTTGACGACCTTCTTGA	80	[37]
Ribosomal Protein S18	Rps18	AM490061	AGGGTGTTGGCAGACGTTAC	CGCTCAACCTCCTCATCAGT	97	[37]
β-Actin	β-Act	X89920	TCTGTCTGGATCGGAGGCTC	AAGCATTTGCGGTGGACG	113	[38]
TARGET GENES						
Interleukin 1β	IL-1β	AJ277166	GCGACCTACCTGCCACCTACACC	TCGTCCACCGCCTCCAGATGC	131	[37]
Interleukin 6	IL-6	AM749958	AGGCAGGAGTTTGAAGCTGA	ATGCTGAAGTTGGTGGAAGG	101	[35]
Cyclooxygenase 2	COX-2	AM296029	GAGTACTGGAAGCCGAGCAC	GATATCACTGCCGCCTGAGT	192	[35, 36]
Intestinal Mucin	I-Muc	JQ277712	GTGTGACCTCTTCCGTTA	GCAATGACAGCAATGACA	102	[38]
Immunoglobulin M	IgM	JQ811851	TCAGCGTCCTTCAGTGTTTATGATGCC	CAGCGTCGTCGTCAACAAGCCAAGC	131	[39]
Occludin	Ocl	JK692876	GTGCGCTCAGTACCAGCAG	TGAGGCTCCACCACACAGTA	81	[35, 36]

RT-PCR assay conditions

Relative gene expression of six genes (**Table 2**) was determined in the foregut and hindgut samples. Genes were related to the inflammatory process (IL-1beta, IL-6 and COX-2), the immune status (IgM and I-Muc) and the maintenance of the epithelial integrity (Ocl).

All qPCR assays and expression analyses were performed using the Applied Biosystems 7500 Real-Time PCR with SYBR® Green PCR Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA). After an initial Taq activation of polymerase at 95 ºC for 10 min, 42 cycles of PCR were performed with the following cycling conditions: 95 ºC for 10 s and 60 ºC for 30 s in all genes. In order to evaluate assay specificity, a melting curve analysis was directly performed after PCR cycles by slowly increasing the temperature (1º C/min) from 60 to 95 ºC, with a continuous registration of changes in fluorescent emission intensity. The total volume for every PCR reaction was 20 µl, performed from diluted (1:20) cDNA template (5 µl), forward and reverse primers (10 µM, 1 µL), SYBR® Green PCR Master Mix (10 µl), ROX (2 µL, 10 nM) and nuclease-free water up to 20 µl. The analysis of the results was carried out using the $2^{-\Delta\Delta Ct}$ method. The target gene expression quantification was expressed relative to the expression of the selected reference gene. A cDNA pool from all the samples was included in each run and acted as a calibrator, and a non-template control for each primer pair, in which cDNA was replaced by water, was run on all plates. Reference and target genes in all samples were run in duplicate PCR reactions.

Statistics

Statistical data analysis was performed with Statgraphics© Centurion XVI software (Statistical Graphics Corp., Rockville, MO, USA).

In *Experiment 0*, LDH enzymatic activity in the tissue and the supernatant was statistically analysed by one-way analysis of variance (ANOVA) using Newman-Keuls test to determine possible differences across the assay (0, 4, 6 and 24 hours) in FG and HG.

In *Experiment 1*, the expression stability of reference genes was assessed using the BestKeeper program, basing on the arithmetic means of the Cq values [40]. Lower deviation in the expression is related to better stability. Furthermore, the relative gene expression in the initial

(0 h) and control samples (4 and 6 h) was statistically analysed by ANOVA to determine the impact of experimental conditions on gene expression. Gene expression of cultured pieces was normalised with the expression of control samples at 4 and 6 hours. Multifactorial analysis was used to determine the significance ($p<0.05$) of different factors considered (dietary treatment: CTR/PP, intestinal section: FG/HG and bacterial stimuli*: P. damselae* subsp. *piscicida*/*P. anguilliseptica*/*V. algynoliticus*) at both times and to determine differences in normalised gene expression between dietary groups, sections and bacterial stimuli, using Newman-Keuls test. Data was expressed with the mean and the standard error of the normalised expression values, and differences were considered statistically significant when $p<0.05$.

In *Experiment 2*, a multifactorial analysis of variance (two factors: diet and section) of the average expression in the initial samples was initially performed. On the other hand, relative gene expression values reported in duplicate samples for the same conditions (diet, section and stimuli) were randomly assigned to different variables (x and y). Data consistency was evaluated for each gene by simple regression analysis using the model $y = ax$. 95% confidence intervals for a ($a \pm 1.96\sigma$) were obtained for each gene to validate the hypothesis $a=1$ ($y=x$). As in the experiment 1, impact of experimental conditions on the relative gene expression was statistically analysed by ANOVA. Average gene expression for initial (0 h) and control samples (6 h) were used for the analysis. A correlation analysis with the expression data set of all cultured samples was carried out and the Pearson product-moment coefficient was obtained for each pair of genes. Finally, gene expression in the stimulated intestinal pieces was normalised with the average expression of control samples and multifactorial analysis was used to evaluate the impact of dietary conditions, section and bacterial stimuli (dietary treatment: CTR/PP/PP*, intestinal section: FG/HG and bacterial stimuli*: P. damselae* subsp. *piscicida*/*V. algynoliticus*). Significant differences between dietary groups, sections or bacterial stimuli, as well as in the response in each dietary group to different bacteria assayed, were determined using the Newman-Keuls test. Expression was represented by mean and the standard error of the average normalised expression values and differences were considered statistically significant when $p<0.05$.

Results

Experiment 0

LDH activity in the tissue and in the surrounding media reported the minimum and maximum values in both intestinal section (FG and HG) at 24 h, respectively (Fig 2). Only slight differences were observed between 0, 4 and 6 hours in both media and tissue level. LDH has been described as a potential marker of cell damage (Legrand et al., 1992), and the release of this enzyme to surrounding media in eukaryotic cell cultures has been correlated with the number of dead cells in the tissue. Therefore, 6 hours was established as the maximum time of incubation.

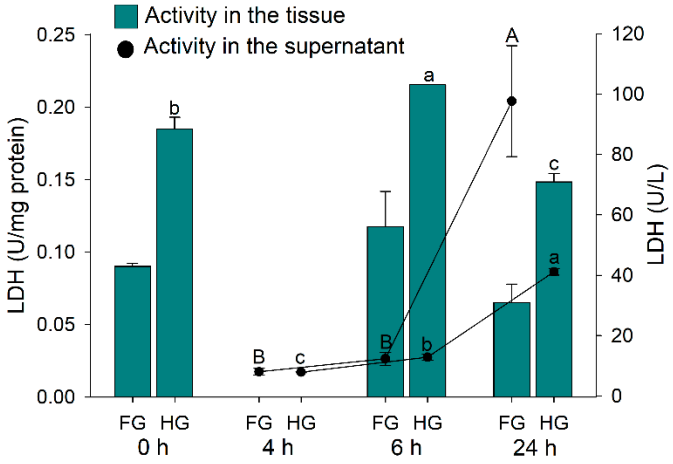

Fig 2. Assessment of the LDH activity.

LDH activity in the tissue (U/mg protein) and in the surrounding DMEM (U/L) is expressed by mean and standard error (n=2), for both intestinal sections (FG and HG) and four different times (0, 4, 6 and 24 hours). Different superscripts on the bars indicate significant differences between different times for each section ($p<0.05$).

Experiment 1

Housekeeping gene expression

EF-1α showed the maximum stability among the analysed genes and was selected as the housekeeping gene in the gene expression assay (Table 3). No significant differences were observed in Cq values reported at different times for all the candidate genes (Fig 3), suggesting a low grade of RNA degradation along the assay, which was supported by the integrity of 28S/18S in the gel electrophoresis (S1 Fig).

Table 3. BestKeeper assessment of the candidate housekeeping genes

	β-Actin	EF-1α	RPS18	GAPDH
Mean ± Std dev	17.42±0.54	19.97±0.43	20.33±0.59	21.28±0.66
Min	16.47	18.98	19.49	19.93
Max	18.40	20.63	21.30	22.43

Gene expression is represented by mean Cq and standard error (n=6)

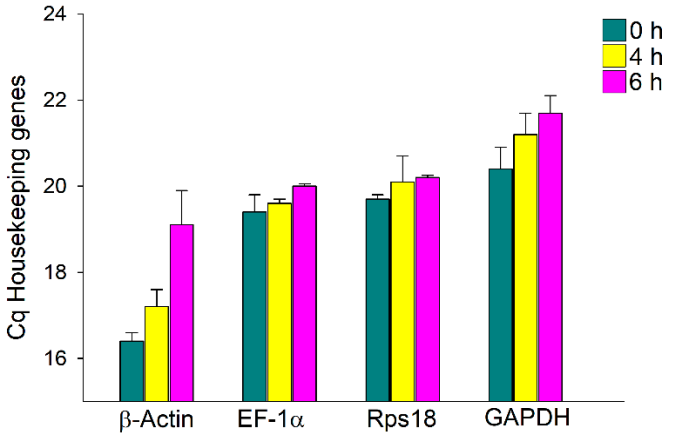

Fig 3. Evaluation of the candidate housekeeping gene stability along the assay.

Cq of the different genes in pooled samples at different times is expressed by mean and standard error (n=2). Different superscripts on the bars indicate significant differences between different times for each section ($p<0.05$)

Impact of the ex vivo system

Gene expression of all genes was altered by *ex vivo* experimental conditions when explants were cultured in CO_2 independent medium without bacteria (Fig 4). IL-1β, IL-6 and COX-2 showed an increased expression, although no significant differences were determined for IL-1β. A decrease in the expression of IgM was observed, while Ocl showed the maximum expression at 6 h. No differences were observed in the expression of I-Muc. Hence, expression results in samples cultured with the different bacteria were normalised with the expression of the control samples, for each fish and section. Additionally, the pH of different DMEM solutions remained stable during all the assay (Control: 7.37±0.05, V. *algynoliticus*: 6.92±0.09, P. *damselae* subsp. *piscicida*: 6.79±0.09, P. *anguilliseptica*: 7.55±0.05), discarding any effect of pH modulation on gene expression.

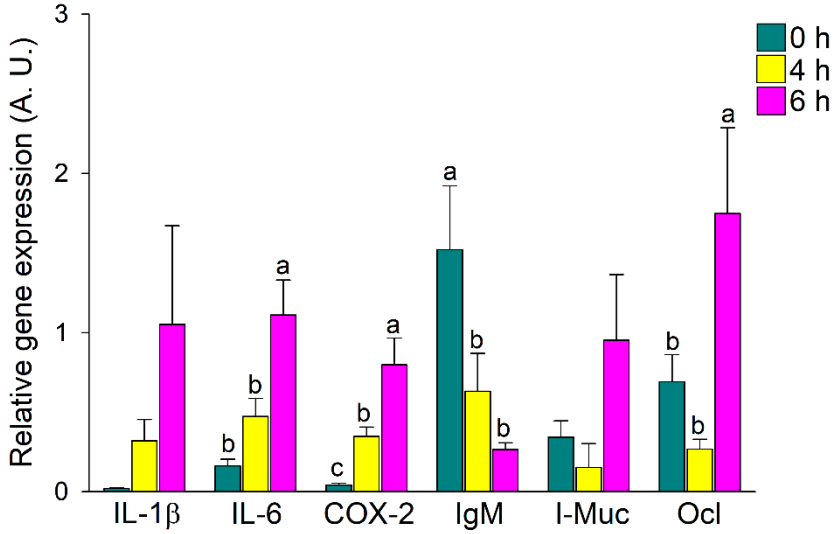

Fig 4. Impact of the *ex vivo* method on the expression of different genes in Experiment 1.

Relative gene expression (A. U.) of the different gene is expressed by mean and standard error (n=4) for the three different times (0, 4 and 6 hours). Different superscripts on the bars indicate significant differences between different times for each gene ($p<0.05$).

Effects of intestinal section, dietary treatment, bacteria stimuli and time of incubation

The effect of different factors was evaluated at 4 and 6 hours in the ex vivo explant culture system, and gene expression results were analysed by multivariate analysis of variance to determine significant differences between the different experimental groups (FG and HG; CTR and PP; Control, *P. anguilliseptica*, *P. damselae subsp. piscicida*, *V. algynoliticus*).

IL-6 and Ocl were significantly altered by the section and the diet at 4 hours of incubation, respectively (Table 4): IL-6 reported a higher expression in the foregut (Fig 5B), while Ocl showed higher expression in the CTR group (Fig 5A). However, no significant differences were reported between control samples and samples submitted to bacterial exposure (Fig 5C) at 4 hours.

Table 4. Effect of different factors on normalised gene expression values in Experiment 1

		IL-1β	IL-6	COX-2	IgM	I-Muc	Ocl
At 4 h	*Diet*	0.481	0.181	0.228	0.074	0.121	0.017*
	Section	0.078	0.005*	0.670	0.223	0.515	0.098
	Stimuli	0.145	0.116	0.523	0.771	0.940	0.680
At 6 h	*Diet*	0.074	0.011*	0.011*	0.510	0.006*	0.007*
	Section	0.391	0.336	0.133	0.516	0.004*	0.015*
	Stimuli	0.001*	0.083	0.024*	0.137	0.201	0.101

p-values obtained for each factor in the multifactorial analysis. Significant values are indicated by *

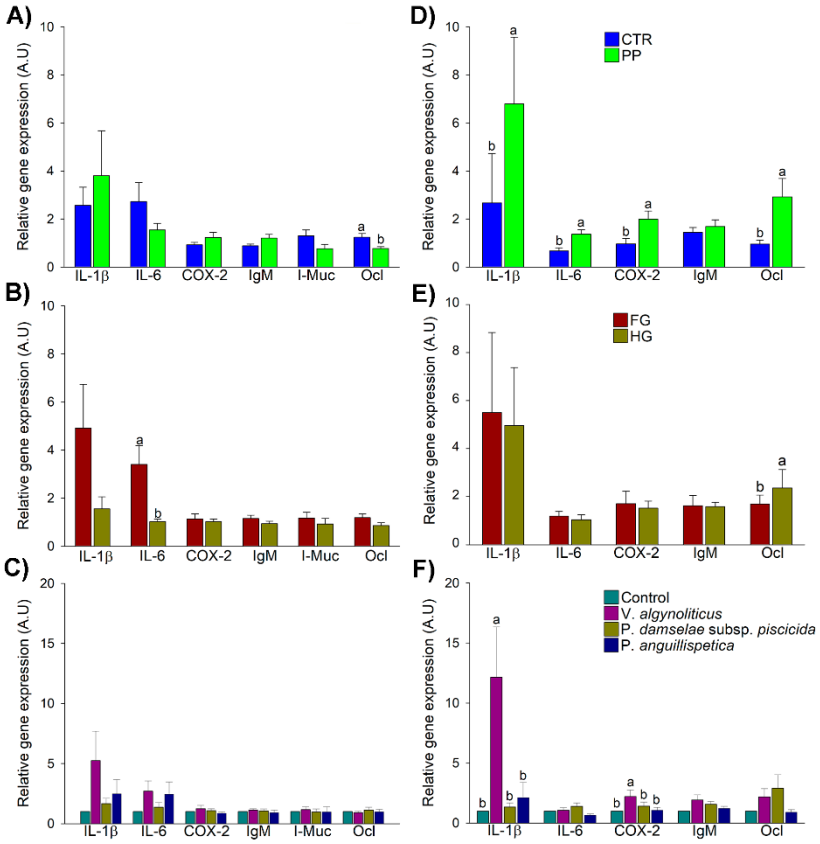

Fig 5. Impact of the diet, section and different stimuli in Experiment 1
Relative gene expression (A. U.) of the different gene is expressed by mean and standard error. Different superscripts on the bars indicate significant differences between different conditions (diet/section/stimuli) for each gene ($p<0.05$). A) Effect of dietary treatment (n=12) at 4 hours. B) Effect of intestinal section (n=12) at 4 hours. C) Effect of bacterial stimuli (n=8) at 4 hours. D) Effect of dietary treatment (n=12) at 6 hours. E) Effect of intestinal section (n=12) at 6 hours. F) Effect of bacterial stimuli (n=8) at 6 hours.

In contrast, a significant effect of the bacterial exposure was found in the expression of IL-1β and COX-2 at 6 hours (Table 4), being significantly higher after exposure to V. *algynoliticus* (Fig 5F). In intestinal explants from fish fed the PP diets, longer incubation times showed significantly higher expression values in IL-1B, IL-6, COX-2 and Ocl (Fig 5D**).** Regarding explants from different sections, a higher expression in Ocl was found in the HG (Fig 5E). Variability registered in the expression of I-Muc prevented to stablish any reliable effect of different factors at 6 h.

Experiment 2

Impact of fishmeal replacement in gene expression before ex vivo challenge

No significant effect of the different dietary treatments nor the intestinal section were observed after the growth period (Table 5). IL1β and COX-2 showed an average higher expression in the hindgut of fish fed in the PP* group, and I-Muc expression reported higher values in both intestinal sections of fish fed the CTR diet, but also high individual variation was registered (Fig 6).

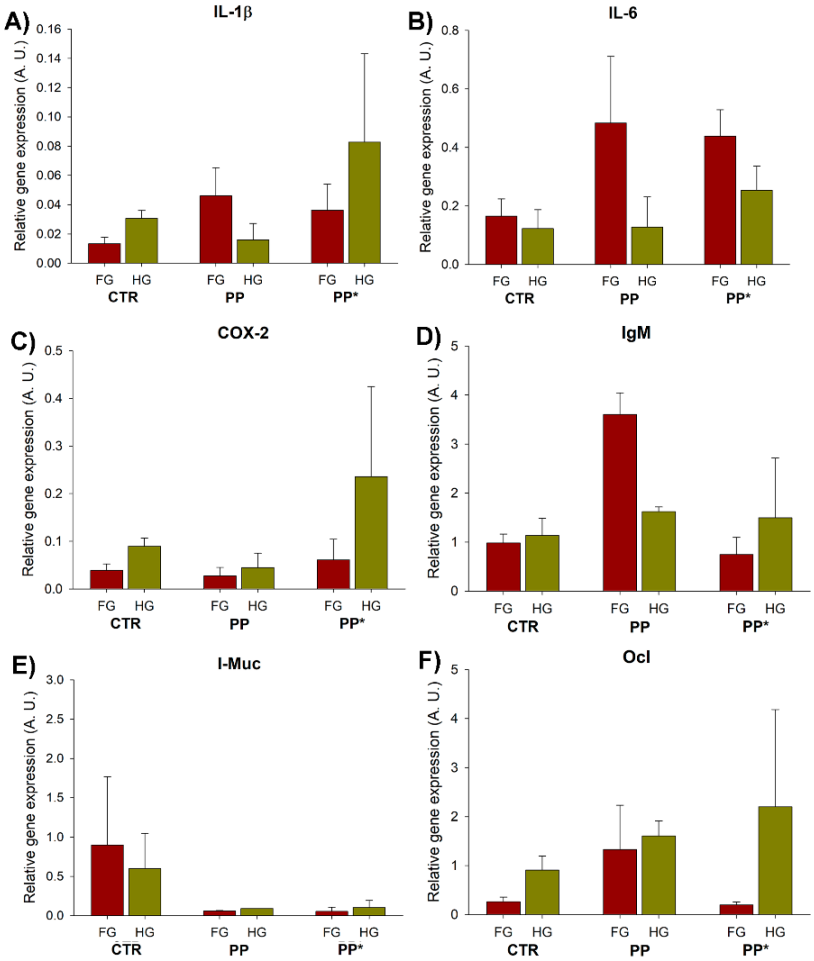

Fig 6. Inflammatory and immune gene expression in different dietary groups after the feeding period and prior to ex vivo challenge

Relative gene expression (A. U.) of the different gene is expressed by average mean and standard error (n=3 for CTR, n= 2 for PP and PP*) in foregut and hindgut samples. Different superscripts on the bars indicate significant differences for each gene ($p<0.05$).

Table 5. Effect of different factors on initial gene expression values in Experiment 2

	IL-1β	IL-6	COX-2	IgM	I-Muc	Ocl
Diet	0.415	0.201	0.273	0.059	0.360	0.452
Section	0.842	0.100	0.170	0.584	0.798	0.143

p-values obtained for each factor in the multifactorial analysis. Significant values are indicated by *

Impact of the ex vivo system

Confirming the previous results, the expression of pro-inflammatory genes was increased along the assay, although significant differences were only reported in the expression of IL-6 and COX-2 (Fig 7). Expression of IgM and Ocl was also increased, but expression in control samples was not significant higher than the observed before the *ex vivo* assay.

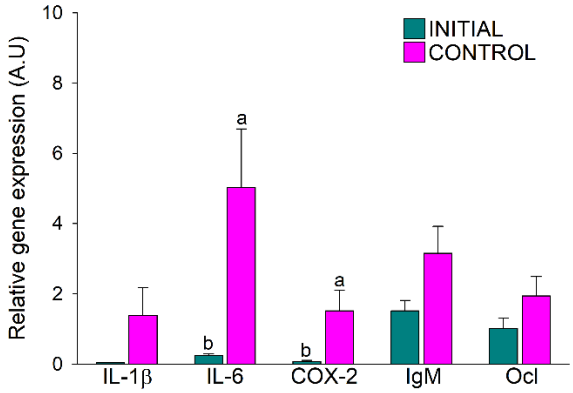

Fig 7. Impact of the *ex vivo* method on the expression of different genes in Experiment 2

Relative gene expression (A. U.) of the different gene is expressed by average mean and standard error (n=14) in initial and control samples. Different superscripts on the bars indicate significant differences for each gene ($p<0.05$).

Data consistency

The value a=1 is included in the 95% confidence interval for the genes IL-1β, IL-6, COX-2, IgM and Ocl (Fig 8), so the hypothesis $y=x$ cannot be rejected. The adjustment to the lineal model is particularly good for pro-inflammatory genes (IL1-β, IL-6, COX-2). I-muc expression reported a high variability between duplicates and no significant relation can be stablished between *x* and *y* data, and was not considered in further analyses.

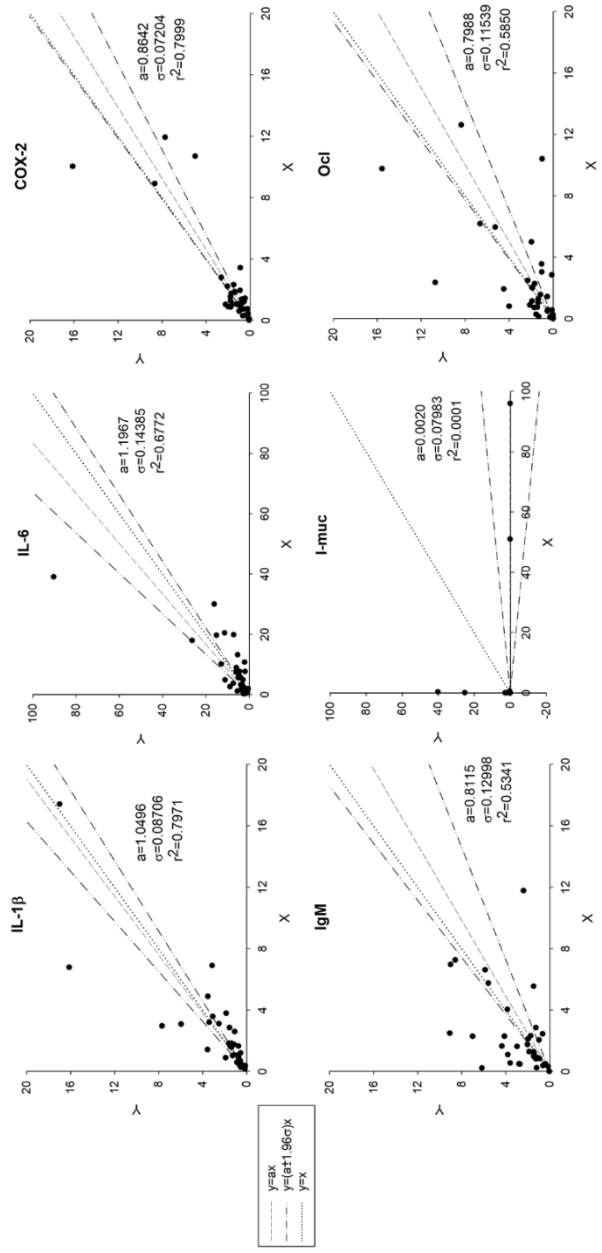

Fig 8. Reproducibility assessment of the *ex vivo* assay

Relative gene expression values of duplicate samples for the same conditions (diet, section and stimuli) were represented as 'x,y'. The linear models $y=ax$ and limits of the 95% confidence intervals were represented for each gene. Values for a, σ and r^2 are also included

Multivariate analysis

Correlation analysis reported that some all the genes were significantly-correlated, except COX-2 and IgM, although only high Pearson product-moment coefficients were obtained for the pairs IL-1β / IL-6 and IL-1β / COX-2 (Fig 9).

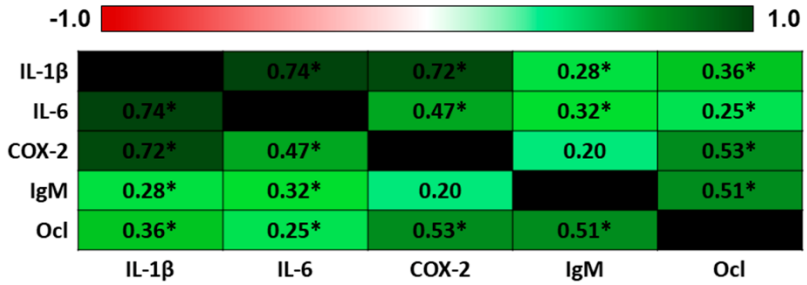

Fig 9. Correlation analysis of gene expression determined in samples after the ex vivo assay

Pearson product-moment coefficients between each pair of genes. Significant correlations are indicated with a *.

Effects of intestinal section, dietary treatment and bacteria stimuli

Multifactorial analysis reported the diet as a significant factor altering the expression of COX-2 and Ocl, while the expression of pro-inflammatory genes (IL-1β, IL-6 and COX-2) was affected by the different bacterial stimuli assayed (Table 6). No differences were reported between sections.

Table 6. Effect of different factors on normalised gene expression values in Experiment 2

	IL-1β	IL-6	COX-2	IgM	Ocl
Diet	0.533	0.601	0.025*	0.120	0.044*
Section	0.157	0.138	0.168	0.864	0.486
Stimuli	0.003*	0.036*	0.021*	0.218	0.163

p-values obtained for each factor in the multifactorial analysis. Significant values are indicated by *

Ex vivo exposure to V. *algynoliticus* and *P. damselae* subsp. piscicida increased the expression of IL-1β and IL-6, and COX-2, respectively, compared to control samples (Fig 10A). Regarding the dietary impact, highest expression values in response to exposure were registered in the

PP* group for all evaluated genes, although significant differences compared to groups CTR and PP were only determined in COX-2 and Ocl (Fig 10D and Fig 10E). On the other hand, significant differences in the response to the different bacteria were reported only for IL-1B, which was higher after exposure to *V. algynoliticus* compared to *P. damselae* subsp.*piscicida* in the three experimental groups (Fig 10B). However, no differences in the response to the same bacteria stimuli were determined between diets.

Discussion

Developing an *ex vivo* intestinal culture assay

In the current study, an *ex vivo* system using intestine explants has been developed, as an efficient alternative to in vivo experiments, to evaluate the impact of bacteria exposure in the expression of inflammatory and immune-related genes in the intestine.

Ex vivo approaches based on explants culture proved to be useful to analyse pro-inflammatory responses [12]. In fish, different attempts have been done to evaluate the interactions pathogen-host [41], especially by the intestinal sack method [4, 7-9, 42]. Nevertheless, this technique uses whole guts or entire sections to assay each single stimuli [5], making more difficult the comparison between different stimuli when high variability is registered. Moreover, the use of whole gut limits tissue viability and therefore the length of incubation, as prolonged incubations (>1 hour) lead to necrosis and degradation [4], while shorter times of incubation might not be enough to observe immune response. In fact, in the present work, significant differences in the expression of inflammatory markers were not reported at 4 hours. The *ex vivo* assay developed was able to maintain tissue integrity, based on LDH and reference gene results, up to 6 hours of incubation, being noticeably superior to the observed with the intestinal sack method. Additionally, the use of several explants from a single intestine increases experimental efficiency, reducing number of fish and therefore, individual variability.

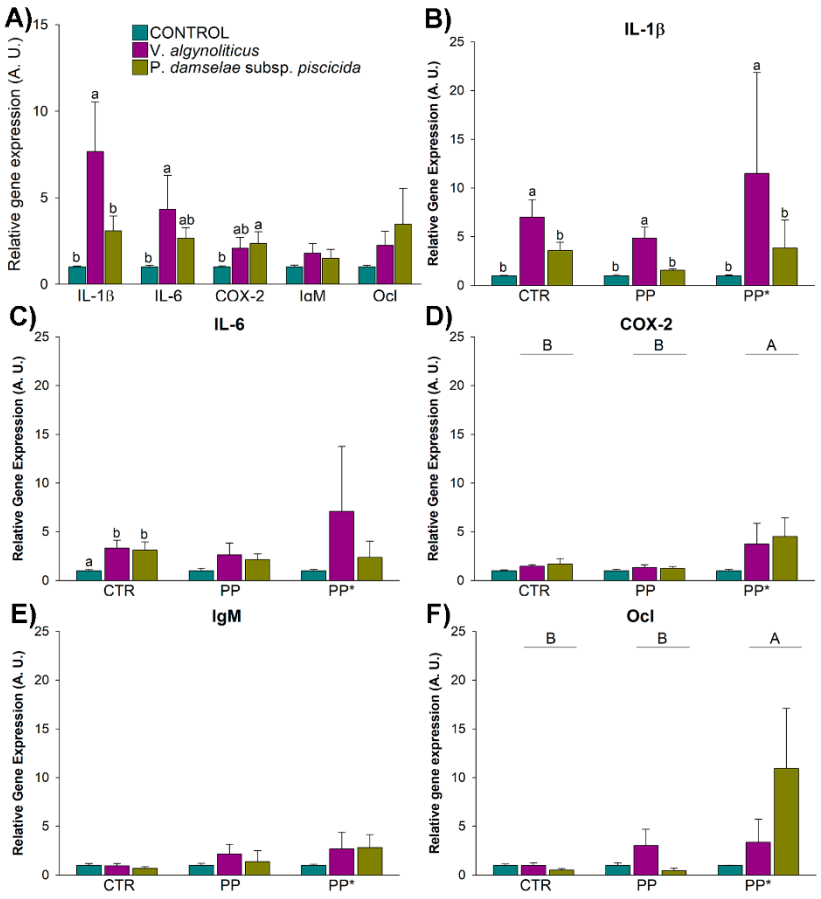

Fig 10. Impact of ex vivo exposure to different bacteria in the different dietary groups in Experiment 2

Relative gene expression (A. U.) of the different gene is expressed by mean and standard error. Different superscripts on the bars indicate significant differences between different ex vivo conditions (Control/*V.algynoliticus*/*P.damselae* subsp. *piscicida*) for each gene ($p<0.05$). Capital letters at the top of the graph indicate differences between dietary groups ($p<0.05$) A) Effect of bacterial stimuli on the expression of different genes (n=14). B) Effects on IL-1β expression in the three dietary groups. C) Effects on IL-6 expression in the three dietary groups. D) Effects on COX-2 expression in the three dietary groups. E) Effects on IgM expression in the three dietary groups. F) Effects on IgM expression in the three dietary groups (n=6 in CTR, n=4 in PP and PP*)

The expression of different target genes was significantly altered by *ex vivo* culture, even without exposure to bacteria. The increase of pro-inflammatory genes (IL1-β, IL-6, COX-2) is most probably due to tissue injury caused by dissection which leads to an inflammatory or

stress reaction [43, 44] and cytokine expression seem to be mainly modulated at gut local level [45].

The effect on the other three genes needs further studies. Although IgM registered a high basal expression at the gut [29], recent studies pointed out that IgT could be the main immunoglobulin in the mucosal responses [29, 46] and drastic changes in IgM could not be expected. Ocl was also related to inflammatory events [35], but its regulation depends on several cytoskeletal, scaffolding, signalling and polarity proteins [47]. High variability reported at 6 hours prevented obtaining valuable conclusions on the expression of I-Muc.

Finally, in *Experiment 2*, two explants obtained from the same section of each fish were cultured in the presence of the same bacteria to assess the consistency of data obtained after the *ex vivo* assay, since a similar response was expected from adjacent intestinal pieces, although they can't be considered technical replicates and the stochastic nature of transcription should be taken into account [48]. Regression analyses show a relatively good consistency in the expression data set of pro-inflammatory genes, as well as it may be acceptable for IgM and Ocl. I-Muc showed large differences in most of the replicates. High variability in I-Muc expression between fish in the same experimental conditions was previously reported [49], and I-Muc expression was not considered at 6 hours.

Impact of fish replacement on the response to bacterial exposure

The inclusion of alternative protein sources to quality fishmeal, such as plant protein sources, in aquafeeds for carnivorous fish can lead to amino acid imbalances and, hence, to immune dysfunctions [23,50], besides possible inflammatory effects caused by anti-nutrients [16, 51], leading ultimately to pathogenic invasion, disease and death.

In order to evaluate the intestinal immune status, three pro-inflammatory markers (IL1-β, IL-6, COX-2) were selected. These molecules are secreted by host cells during the inflammatory process in response to microbial antigen of pathogenic bacteria or other cytokines and chemokines [2, 52]. Additionally, IgM is considered as the most abundant immunoglobulin in plasma, high levels in fish fed with plant sources based diets have been reported [53] and its expression was induced in mucosal tissues as response to pathogen infection [54]. The

I-Muc, as a mucin, is involved in epithelia protection and bacteria adhesion and growth, and has been suggested as a resilience biomarker to inflammation in fish [38]. Finally, Ocl is as key protein in the regulation of tight junctions between enterocytes, and therefore in the permeability of the epithelial barrier [47]. The expression was assessed on the foregut and the hindgut, since a different performance in the immune processes has been suggested [55], but no significant differences were observed in the ex vivo response assay.

Up-regulation of several immune-related genes in response to fishmeal replacement has been previously observed and it seems to alter the intestinal response to *ex vivo* and *in vivo* infections [50]. Although some interesting trends are observed, no differences between dietary groups were reported before the *ex vivo* assay, maybe as a consequence of low number of fish. However, differences emerged after the ex vivo exposure to bacteria.

Three different pathogenic bacteria, previously identified in farmed gilthead seabream under disease, were used: *Photobacterium damselae* subsp. *piscicida* has been identified as the causal agent of pasteurellosis [56], *Pseudomonas anguilliseptica* is related to 'winter disease' [57], and *Vibrio algynoliticus* has been described as the causal agent of vibriosis [58], but also has been associated to other Vibrio species in high mortality outbreaks [59].

As expected, *ex vivo* exposure to pathogen bacteria leads to an increase in the expression of pro-inflammatory genes, especially in the case of IL-1β in response to *Vibrio algynoliticus*, but also IL-6 and COX-2. Differences in the responses to different stimuli could be due to a dose effect [60] or to differences in the immune mechanisms triggered by the exposure to different bacterial strains, but a dose-dependent study should be performed to shed light on this issue.

Interleukins are quickly released as part of the non-specific innate immune response [61]: IL-1β is one of the main pro-inflammatory cytokines secreted in response to gram negative bacteria [3] and its release is followed by IL-6 release in the pro-inflammatory cascade [52, 62]. Then, expression of COX-2 is rapidly induced by different inflammatory mediators [63, 64], including IL-1β [65]. A transcriptional modulation of IL-1β by infection has been suggested [28], indeed, increased expressions of IL-1β have been reported in the intestine of different species, including gilthead seabream [28], after

gram negative bacteria intraperitoneal injections. An increase of IL-1β and COX-2 expression in gilthead seabream immune cells was also observed after *in vitro* challenge with different bacteria or different commercial pathogen associated molecular pattern (PAMP) solutions [60, 64, 66, 67]. Additionally, multivariate analysis revealed a high correlation in pro-inflammatory genes response, and similar expression patterns are exhibited in the different conditions assayed, which has been previously observed in *in vitro* challenges in different teleost immune cells [60, 64]. These results suggest that intestinal explants are preserving the ability to trigger an inflammatory response, and interactions between different gut cells remain functional during the culture time after excision from host.

On the other hand, an increase of IgM expression has also been reported in response to *in vivo* parasite infection, but very late after the exposure [39], maybe because antibodies production in fish is slower than in other vertebrates [68]. Concerning Ocl, it has been related to inflammatory processes [35], and the increased expression of other key tight junction proteins, such as claudins, has been observed in fish under infection [69], but the chronology of this response remains unclear.

Two independent preliminary experiments testing the dietary impact on the intestinal response to ex vivo bacteria exposure were carried out. Both experiments cannot be combined or compared, since they were performed with fish with different size, then tolerance to plant sources [21]. In experiment 1, PP group registered at 6 hours the highest expression response for inflammatory genes, supporting the hypothesis that plant sources could alter the intestinal response to *ex vivo* infection [50]. Differences at histological level in response to *ex vivo* exposure to *Aeromonas salmonicida* between fish fed with different protein sources have been previously reported [4]. In contrast, in experiment 2 higher values were observed in the PP* group (fed with PP diet only during the 15 days prior to assay). In so far as deficient diets could be considered a stress factor, long term feeding could determine suppressive or depressive effects on the immune mechanisms [23, 49, 70], which could explain the obtained results. Lower values reported in CTR group could be related to a higher protection in host from bacterial adhesion and growth. However, results should be analysed with caution since a wide individual variation in the levels of expression of inflammatory and immune genes has been reported in other species [71], and level of expression before the ex vivo trial conditions the inflammatory and immune capacity registered after bacterial exposure

[50]. Further research should be performed to confirm the impact of diet on the ex vivo intestinal immune capacity. Finally, although the ex vivo assay allow to register changes in the expression of pro-inflammatory markers, as well as differences in the response between experimental groups, differences in the response to different stimuli between dietary groups were not observed.

In summary, a new experimental system based on fish intestinal explants culture to evaluate the short-term inflammatory and immune response to bacterial ex vivo exposure has been successfully implemented in gilthead sea bream, being easily adapted to other teleost species. Developing and optimisation of ex vivo methods, including tissue or cell culture, will contribute to better understand the interactions between immune system and microbiota and to reduce further the number of animals used in research.

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Supporting information

S1 Fig is included in ANNEX V

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General Discussion

1. Study sequence

Initially, growth and digestive performance in response to six experimental diets (based on different levels of fishmeal replacement with the inclusion or not of marine by-products) were evaluated (**Chapter 1**). Adverse effects on growth and nutritional efficiency parameters were observed when fishmeal was totally replaced by PPS (diet FM0 = VM). Furthermore, slight decrease in the AA and protein digestibility was observed with an increased level of plant protein, although no differences were determined in the enzymatic activity, suggesting not a high impact on digestive capacity. However, an increase of ammonia production was observed in the group VM, suggesting a possible impact on the metabolic use of AA. This effect on AA metabolism maybe triggered by imbalances in the AA bioavailability, which could explain the lower growth rate observed. Finally, the inclusion of alternative marine by-products in diets with total fishmeal replacement (diet FM0+ = VM+) reported a positive effect in terms of growth, nutritive and digestibility parameters compared to the VM diet.

Nevertheless, the highest impact of plant protein inclusion in the diets was observed at survival rate, which was dramatically lower in the VM group. Due to absence of specific signs of disease in death fish, the hypothesis raised was that the high mortality could be attributed to a poor health status induced by the long term feeding with a plant protein based diet. In order to obtain a better picture of the intestinal health status, the expression of different genes related to the inflammatory response, immune status, gut epithelia integrity and digestive process was determined in the intestine of fish fed with diets with total fishmeal replacement (VM and VM+) compared to fish fed with the fishmeal based diet (diet FM100 = FM). Additionally, the impact of total replacement on gut histology and intestinal microbiota composition were evaluated.

The lower expression of inflammatory, immune and epithelial integrity-related genes observed in fish fed long-term with the VM diet, in contrast to the observed in fish fed with the diet including squid and krill meal (VM+) (**Chapter 2**) suggests a possible chronic stress status of the intestinal tract in fish belong to VM group. This status, maybe induced by a deficient nutrition in the VM group, could be the responsible of the loss of the capacity to exhibit a mucosal inflammatory and immune response at the end of the growth trial,

leading to the higher mortality registered. On the other hand, the VM+ diet seemed to allow fish to maintain the inflammatory and immune activity at long-term at intestinal level. Histological assessment only revealed minor inflammatory signs, (**Chapter 2**) but a significant impact was not expected since gilthead seabream have not showed major histological changes in response to high plant protein diets (Baeza-Ariño et al., 2014; Bonaldo et al., 2008; Sitjá-Bobadilla et al., 2005). Additionally, the analysis of the gut microbiota profile in the FM (FM100) and VM (AA0) groups revealed relevant differences, particularly at hindgut section (**Chapter 3**). High population of *Streptoccocus* and Lactobacillus were registered in the intestines of fish fed with FM, while a significant increase of Proteobacteria population, especially *Photobacterium* species was observed in the VM group. This genus includes different bacteria related to primary or secondary infections in gilthead seabream, and their presence in the intestine of fish of the VM group, together with the absence of a local inflammatory and immune response could explain the higher mortality registered.

Proteomics is a powerful tool in physiological studies, providing relevant information that can be missed by approaches based on mRNA (Forné et al., 2010; Pandey and Mann, 2000; Rodrigues et al., 2012). Therefore, although possible changes in the gut were assessed at histological and transcripts level, the study of the gut mucosa proteome helps in order to deeper understand the long-term impact of the plant protein on the intestinal status.

With this aim, a new long-term feeding trial was conducted, using only the FM, VM and VM+ diets. The subsequent analysis suggested a loss of gut mucosa functionality in the VM group (**Chapter 4)**, based on the downregulation of a big set of proteins involved in epithelial permeability, inflammatory response and enterocytes homeostasis. These results were coherent with the suggested local immune depression registered in the first trial.

In terms of growth, differences were observed despite the inclusion of marine by-products, in contrast to the first feeding trial when no significant growth differences were reported between FM and VM+ (initial weight= 128±5.3g). It is necessary to point out that the second feeding trial started with an average initial fish weigh of 9.6±1.9 g, and differences in growth performance can be attributed to the strict nutrition requirements and the lower tolerance to plant ingredients during the first growth stages (Martínez-Llorens et al., 2007; Santos

Couto, 2013). In contrast to fish belonging to VM group, fish fed the VM+ diet showed a gut mucosa profile similar to the exhibited by fish in the FM group, supporting the hypothesis that the diet VM+ allows to maintain the gut functionality and the capacity to trigger an inflammatory response after a long period, despite the negative effects observed in growth.

Finally, the interactions between gut microbiota-immunity-nutrition have been poor studied in most fish species, including the gilthead sea bream. Indeed, the wide variety of nutrients, immune mechanisms and bacteria that are involved in the intestinal ecosystem of a single fish makes very difficult this kind of study by in vivo approaches. Hence, an *ex vivo* system was developed to evaluate the response of the gut mucosa to the exposure by different bacteria, and it was used in a preliminary assay to assess the impact of fishmeal replacement in the gilthead seabream intestine (**Chapter 5**).

2. Final disclosure

The main goal of this thesis was to shed light on the impact of high plant protein levels on the intestinal status of the on-growing gilthead seabream. Hence, according to the results, the first question arising is*: has plant protein altered the intestinal status on on-growing gilthead seabream in terms of digestive and immune capacity?*

2.1 Digestive performance

In the first trial, no differences were reported in digestive enzymatic activity, and histological assessment was not able to identify any change that could be related with a poor digestive performance (Chapter 2). Only a slight lower digestibility for dietary AA (Chapter 1), even after complementation with marine ingredients, being probably related to the plant protein dietary level. These minor differences leaded to a significant lower growth and nutritive performance in the VM group, but not in the VM+ group during the on-growing phase. Increased ammonia excretion observed in the VM group suggested a great impact on AA metabolism, which might be related to dietary AA bioavailability (Santigosa et al., 2011).

On the other hand, the second growth trial reported a higher impact on growth performance in response to fishmeal replacement by plant sources, evidencing nutritional deficiencies in diet VM suggested in

Chapter 1. However, although the inclusion of squid and krill meal (VM+) significantly improved growth compared to the plant protein based diet (VM), it did not allow to reach growth parameters registered in the FM group.

As it was stated, impact of PPS inclusion seems to depend of the fish size. Previous research has leaded to consider that minimum nutrient requirements may be most demanding in juveniles and during the first stages of growth (Santos Couto, 2013). Indeed, tolerance to plant proteins inclusion seem to be lower in younger fish (Martínez-Llorens et al., 2007). Although the digestive capacity was not directly assessed after the second feeding trial, the downregulation of several proteins involved in the gut homeostasis in the VM group (Chapter 4) could indicate a higher impact on the digestive performance. In this line, the downregulation of functional marker genes has been reported in sea bream juveniles in response to plant ANF (Santos Couto, 2013). In contrast, no impact was reported in the gut mucosa proteome of fish fed the VM+ diet, suggesting that squid and krill meal prevents from loss of functionality at long-term. The higher AA requirements of small fish and the likely maintenance of sustained inflammatory response to PPS which requires a continuous energy expenditure along the feeding trial (Tort, 2011) could explain differences in growth compared to the FM group.

Differences in growth are not necessary related to differences in the digestive capacity. Energy expenditure and resources designated to growth can be allocated to other processes, such as the inflammatory and immune responses (Tort, 2011). Additionally, since the enterocytes metabolism is largely dependent of the AA absorbed from the lumen (Reeds et al., 2000), a prolonged feeding with a deficient diet since the first stages of growth could have a significant impact on its function.

Higher retention indices for most essential AAs were observed for group VM+ (Chapter 1). It is well known that retention indices are highly dependent of the dietary AA profile, and higher indices have been observed typically in plant protein based diets with limited content on essential AA (Gómez-Requeni et al., 2004; Sánchez-Lozano et al., 2009). Nevertheless, although dietary profile explains the higher retentions reported for VM+, values obtained for the VM group were far from the expected according to dietary AA profile, suggesting they are not being absorbed or metabolized (Chapter 1). These findings, together with the higher ammonia excretion reported in this group in

the first growth trial, confirm nutritional deficiencies in the VM diet, probably based on luminal AA bioavailability, which leads to increased catabolism of AA exceeding the required profile. These deficiencies could be the responsible of lower growth, and probably, immune deficiencies at long term, as will be widely discussed later, and considering differences in the fish weight at the beginning of the feeding trials and the duration of each one, can explain the observed results in both trials.

In conclusion, although all experimental diets meet the minimum requirements in essential AAs for the gilthead seabream (Peres and Oliva-Teles, 2009), results seem to point out that supplementation with free AAs is less efficient achieving an optimum AA profile compared to the squid and krill meal inclusion, and complementing nutritional deficiencies related to PPS. The intestine digestive activity did not seem to be highly affected by total replacement of fishmeal by PPS during the on-growing phase. However, the impact seemed to be higher when fish were fed with plant based diets since their first stages of growth, evidencing nutritional limitations of the VM diet and leading to a loss of gut mucosa functionality which can explain increased differences in growth.

2.2 Inflammatory and immune status

Regarding the immune status, some considerations are need to be previously considered. The existence of a pro-inflammatory status at gut level commonly evidences an immune response against a dietary or environmental antigen, including bacteria. Although inflammation cannot be considered a positive feature, is an indicator of the functionality of the gut associated lymphoid tissue. In contrast, absence of a pro-inflammatory response means that the intestine has not response to any antigen, because the antigen was missed, but also because there is a lack of capacity to develop an inflammatory and immune response against the antigen, maybe due to energetic or resources exhaustion (Tort, 2011). This chronic status will lead fish to weakness, disease and dead (Martin and Król, 2017). The trigger and maintenance of a pro-inflammatory status demand a high energy and resources, since processes such as production of antibodies, synthesis of proteins related with the innate immunity or immune cell production, recruitment and differentiation demand a continuous availability. Moreover, this prolonged stress may involve the switch of energetic

metabolism, allocating most of the energy resources to face with them, and not in growth (Martin and Król, 2017; Tort, 2011)

Present results suggest that the exclusive use of plants as protein sources in aqua feeds for gilthead seabream has, at long term, a significant impact on the immune response of fish, which is reflected in an increased mortality.

In the first trial, the study of the expression of pro-inflammatory and immune-related genes at intestinal level in fish long-term fed the VM diet (Chapter 2) suggested an inability to develop an inflammatory response at local level compared to fish in VM+ group, which showed a response similar to the reported against low fishmeal based diets in several species in previous research (Torrecillas et al., 2017). On the other hand, changes observed in the gut microbiota composition in the of the VM group compared to FM group (Chapter 3), particularly in the hindgut, which has been related with the immune function (Løkka and Koppang, 2016), consisted especially on the increased presence of *Photobacterium* and the decreased in the lactic acid bacteria population, which have been associated to healthy intestinal status (Dimitroglou et al., 2009), also supported this idea. Both observations (local immune dysfunction and microbiota disruption) could explain the higher mortality reported in the VM group compared to groups FM and VM+. Additionally, the gut mucosa proteome analysis after the second feeding trial (Chapter 4) revealed a great impact of the VM diet on the epithelial permeability, inflammatory response, enterocytes homeostasis and gut mucosa functionality in general, while the VM+ diet allowed fish to maintain the gut mucosa proteomic profile observed in the FM group.

In the present work, the intestinal inflammatory status was not evaluated in response to complete fishmeal replacement during a short period. However, different aspects lead us to believe that this could be the most likely scenario for the VM and the VM+ groups, since:

- Previous research has reported inflammation in response to high plant protein diets (Torrecillas et al., 2017).
- Short stressing periods seem not to have a high impact on immune mechanisms (Tort, 2011).
- An inflammatory status is also observed at long term in the group VM+ (Chapter 2)
- Minor histological alterations observed in the VM and VM+ groups (Chapter 2) could be indicative of certain grade of

immune cell mobilisation in a previous pro-inflammatory status

- The stressor factor (the diet) has been in contact with the fish since the beginning of the feeding trial.

Hence, a prolonged inflammatory status could be expected during the growth experiment in the VM and VM+ groups, demanding the high energy expenditure aforementioned, which could also partially explain the reported differences in growth, particularly in the second feeding trial. Therefore, loss of inflammatory capacity and immune depression at gut level in the VM group at long term, as well as impact on intestinal homeostasis reported in Chapter 4, suggest that fish fed the VM diet, in contrast to fish fed the VM+ diet, could be unable to meet the energy and resources requirements to sustain an inflammatory response during all the growth assay due to nutritional dietary deficiencies, decreasing the efficiency of local immune mechanisms and leading ultimately to a chronic immune suppression (Tort, 2011), exhaustion, weakness, disease, and death (Figure 1). The dependence of enterocytes on luminal AA absorption (Reeds et al., 2000) could accentuate this effect. The loss of digestive, protective and metabolic capacity of the intestine after the maintenance of an inflammatory status during some days has been suggested for other species (Sahlmann et al., 2013). Moreover, alteration of the capacity of the host to response to bacteria adhesion and colonisation could explain the changes in the bacterial community observed (Chapter 3).

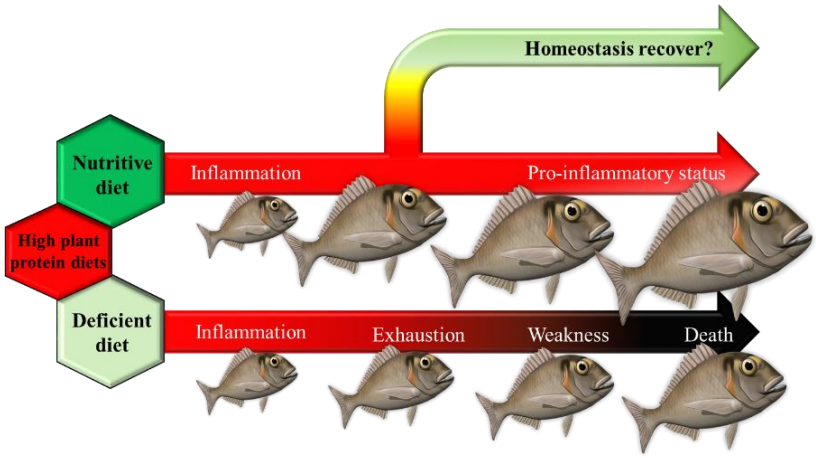

Figure 1. Graphic model of the response of gilthead seabream to high plant protein diets (own elaboration)

On the other hand, the exploratory assayed using intestine explants carried out in Chapter 5 suggested that feeding with the VM diet during a short period lead to increased expressions of pro-inflammatory makers after ex vivo exposure to different pathogenic bacteria, when it was compared to inflammatory response triggered in presence of cultured bacteria in fish fed long term the VM diet. However, this results should be only considered preliminary data due to low number of individuals analysed.

Therefore, the impact of the VM diet on the local immune and inflammatory status of on-growing gilthead seabream can be explained by the nutritional deficiencies suggested for this experimental diet. However, how this diet could lead to depressed local inflammatory and immune capacity while the inclusion of marine sources at 15% allows fish to sustain an efficient pro-inflammatory status at long-term and prevents from increased mortality?

The inclusion of marine sources, which show a balanced AA profile closed to fishmeal (Kader et al., 2012) and can complement some of the deficiencies of high plant protein diets (Kolkovski et al., 2000; Mai et al., 2006) could prevent from energy and resources exhaustion and death, allowing to maintain the pro-inflammatory status and the intestinal function, even recover the intestinal homeostasis (Figure 1), since an effective trophic adaptation to plant based diets has been observed in gilthead seabream (Omnes et al., 2015). In this line, different AAs, such as the glutamine, glutamate, arginine, histidine, methionine, cysteine, taurine, glycine, lysine or threonine seem to have different roles in different physiological process related with the maintenance of the gut integrity and the normal intestinal function (Iizuka and Konno, 2011; Kiron, 2012; Wang et al., 2009) and are also involved in the synthesis of an array of proteins such as antibodies and in the regulation of different immune and metabolic pathways (Jobgen et al., 2006; Meijer, 2003), playing an important role in immune mechanisms (Kiron, 2012; Li et al., 2007). Therefore, AA imbalances can lead ultimately to immune dysfunctions (Sitjá-Bobadilla et al., 2005).

Finally, and from an economical perspective, the VM diet, although was the most economic diet, can't be considered as an optimal diet, since the impact on fish survival has a significant impact on economical indices determined (Chapter 2). In contrast, the VM+ diet, which has a

similar cost compared to the FM diet, also displayed similar economical parameters to the fishmeal based diet.

In short, findings of this thesis supports the idea that PPS can induce pro-inflammatory status in the intestine of gilthead seabream when they are included in high levels in aqua feeds. Maintenance of immune mechanisms requires a continuous energy availability, and a nutritionally deficient diet could lead to lack of resources at a long term, then immune depression, weakness and ultimately to a higher mortality (Martin and Król, 2017; Tort, 2011). Moreover, the allocation of most of the energy expenditure to the maintenance of an effective immune response (Martin and Król, 2017) during the trial could also explain differences in growth, since digestive capacity did not seem to be highly altered in on-growing gilthead seabream.

3. Conclusions

This thesis comprises a deep evaluation of the impact of the high plant protein diets on intestinal performance in gilthead seabream, using cutting edge technologies, such as omics or intestinal explants culture. The overall work allowed the following conclusions:

- An experimental diet based on total fishmeal replacement by plant sources leaded to detrimental effects on key gilthead seabream zootechnical parameters such as growth, nutritive efficiency and survival, which could be related with nutritional deficiencies in high plant protein diets

- Long-term feeding with high plant protein diets can induce certain immunosuppression in the gut lymphoid tissue which could be related to the low survival rates observed, and ultimately to the loss of part of functionality of the gut mucosa.

- Fishmeal replacement had a long term high impact on the gut microbiota composition, especially at hindgut level. This disruption could be related to a depressed immune capacity and partially explain the mortality reported.

- Inclusion of marine by-products in the plant protein diet did not prevent from inflammatory signs, but proved to improve growth, nutritive efficiency, survival and immune capacity

compared to plant protein based diets and to maintain the proteomic profile observed in fish fed with fishmeal based diets

- Intestinal microbiota and the mucosa proteome in response to complete fishmeal replacement by plant sources was assessed for the first time in teleost by 16s rDNA pyrosequencing and LC-MS/MS, respectively, reporting an increase of potential damaging bacteria and a high impact on intestinal homeostasis

- Ex vivo approach based on intestinal explants culture represent a novel and promising technique to evaluate interactions between the intestinal bacteria and the immune performance of fish and the impact of different extrinsic factor such as the diet

4. Future perspectives

Although the present work has assessed deeply the impact of PPS on intestinal status, further studies in this topic are necessary. Alternative ingredients or single nutrients and their inclusion level need further approaches, as well as the wide variety on innate mechanisms based on humoral or cellular responses (Gomez et al., 2013), or the big range of bacteria that can interact with the fish through the gut mucosa (Kiron, 2012). Moreover, the high variability reported between individuals in terms of microbiota (Silva et al., 2011) and inflammatory response (Rojo et al., 2007) also represents a significant challenge. Future approaches based on the combination of *in vivo*, *ex vivo*, and *in vitro* approaches will help to shed light to the complex dynamic involving nutrition, immunity and microbiota.

Specifically, *ex vivo* approaches proved to be a promising tool to evaluate the interactions between bacteria and local immune system under controlled experimental conditions (Bäuerl et al., 2013), and how the diet can affect those interactions. The present thesis implemented for first time an ex vivo assay to register inflammatory responses by gene expression determination after bacteria exposure (Chapter 5), increasing the length of the incubation in comparison to previous *ex vivo* approaches (Nematollahi et al., 2005), and also allowing to assay the impact of several different bacteria in a single fish and therefore to reduce the number of fish in research, as EU recommends (Revision of the UE directive for the protection of animals used for scientific purposes [Directive 86/609/EEC]; 8th September 2010).

From a basic biologic perspective, research on impact of alternative ingredients to fishmeal should look on three important points:

- The role and importance of specific micronutrients of fishmeal (Coutinho, 2017) in the different physiological processes at systemic or organ level, as well as the detrimental effects of their deficiencies, which can contribute to understand how small dietary changes can have such high effects on fish performance.
- The intestinal physiology in teleost. Most of hypothesis raised are based on knowledge from mammals, and although most functions are conserved (Rauta et al., 2012), differences between fish have also been reported and intestine should be deeply described at both digestive and immune level in different species.
- The interaction between bacteria with the gut associated lymphoid tissue, as well as its possible contribution to digestion.

The knowledge provided by basic research could be used by aquaculture industry to improve productivity in fish farm by developing new alternative aqua feeds. Furthermore, the design and implementation of selective breeding programmes (Janssen et al., 2017; O'Flynn et al., 1999) based on the fish performance in response to plant sources, in terms of growth and immune resistance, could have a great impact on productivity. Addressing this challenge will require a vast knowledge of the relation between genotype and phenotype and the identification of reliable biomarkers of trophic adaptation or immune capacity.

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Annexes

ANNEX I. SUPPLEMENTARY MATERIAL OF CHAPTER 1

S1. Essential and non-essential amino acid profile of the different aqua feed ingredients [g·100g dry matter^{-1}]

	FM	WM	WG	BM	SBM	PM	SFM	SM	KM
EAA [g·100g dry matter^{-1}]									
Arginine	5.86	0.38	2.57	1.99	3.66	1.76	3.33	5.90	4.14
Histidine	2.54	0.26	1.45	0.74	1.42	0.58	1.14	1.85	1.26
Isoleucine	3.40	0.36	3.01	1.03	2.33	0.98	1.56	2.28	3.19
Leucine	6.55	0.80	5.79	2.04	4.22	1.78	2.48	4.16	4.67
Lysine	6.01	0.37	1.21	1.92	3.45	1.92	1.39	3.85	3.77
Methionine	2.30	0.22	0.88	0.31	0.92	0.36	1.00	1.76	1.66
Phenylalanine	3.73	0.49	4.31	1.10	2.60	1.11	1.86	2.14	2.97
Threonine	3.55	0.30	1.95	0.94	1.98	0.86	1.52	2.19	2.74
Valine	3.88	0.47	3.26	1.13	2.30	1.06	1.73	2.70	3.12
NEAA [g·100g dry matter^{-1}]									
Alanine	4.32	0.43	2.00	1.10	2.16	0.96	1.30	3.90	3.25
Aspartate	6.97	0.65	2.23	2.91	6.54	2.72	3.55	5.15	5.92
Cysteine	0.56	0.20	1.12	0.24	0.47	0.24	0.65	0.56	0.37
Glutamine	10.00	3.40	31.98	4.65	10.67	4.23	7.51	9.27	7.39
Glycine	4.26	0.48	2.45	1.15	2.11	0.97	2.49	7.98	2.73
Proline	2.86	1.09	10.82	1.00	2.46	0.85	1.6	4.17	2.26
Serine	3.41	0.53	3.67	1.36	2.74	1.11	1.85	2.86	2.43
Tyrosine	2.67	0.08	2.29	0.47	1.41	0.40	0.74	1.38	2.68
EAA/NEAA	1.08	0.53	0.43	0.87	0.80	0.84	0.91	0.76	1.02

FM, fishmeal; WM, wheat meal; WG, wheat gluten; BM, bean meal; SBM, soybean meal; PM, pea meal; SFM, sunflower meal; SM, squid meal; KM, krill meal; EAA, essential amino acids; NEAA, non-essential amino acids

S2. Biochemical and amino acid body composition of the initial gilthead seabream and those one fed experimental diets

	Initial	FM100	FM50	FM25+	FM25	FM0+	FM0	SEM
Biochemical (%)								
Moisture	64.2	62.3	64.4	64.0	64.2	63.1	64.4	0.72
CP	47.0	47.0	49.6	49.6	49.3	49.6	50.4	1.30
CL	44.9	42.8	41.6	41.4	40.7	40.3	39.7	1.25
CA	8.3	7.2	8.2	7.7	8.9	8.7	9.0	0.72
EAA (g 100 g wet weight^{-1})								
Arginine	1.61	1.47	1.37	1.59	1.56	1.72	1.54	0.099
Histidine	0.36	0.39	0.39	0.42	0.40	0.45	0.38	0.023
Isoleucine	0.50	0.65	0.63	0.68	0.64	0.71	0.66	0.033
Leucine	1.20	1.16	1.14	1.18	1.14	1.25	1.16	0.050
Lysine	1.27	1.42	1.33	1.38	1.29	1.36	1.38	0.072
Methionine	0.41	0.47	0.47	0.47	0.45	0.49	0.44	0.026
Phenylalanine	0.60	0.58	0.58	0.63	0.58	0.67	0.55	0.028
Threonine	0.72	0.66	0.79	0.69	0.66	0.73	0.65	0.066
Valine	0.67	0.81	0.80	0.85	0.81	0.89	0.82	0.033
NEAA (g 100 g wet weight^{-1})								
Alanine	1.07	1.07	1.10	1.11	1.06	1.13	1.08	0.033
Aspartate	1.57	1.61	1.58	1.61	1.51	1.62	1.60	0.073
Cysteine	0.07	0.13	0.16	0.13	0.13	0.15	0.11	0.019
Glutamine	2.33	2.41	2.34	2.45	2.34	2.53	2.43	0.091
Glycine	1.24	1.25	1.39	1.35	1.30	1.46	1.30	0.110
Proline	0.75	0.70	0.78	0.77	0.75	0.79	0.78	0.046
Serine	0.70	0.59	0.57	0.58	0.58	0.62	0.58	0.022
Tyrosine	0.44	0.42	0.41	0.45	0.44	0.49	0.40	0.021
EAA/NEAA	0.90	0.93	0.90	0.93	0.93	0.94	0.92	0.029

CP, crude protein; CL, crude lipids; CA, crude ash; EAA, essential amino acids; NEAA, non-essential amino acids

Moisture (%) = 1 – % Dry Matter; CP (% dry weight); CL (% dry weight); A (% dry weight)

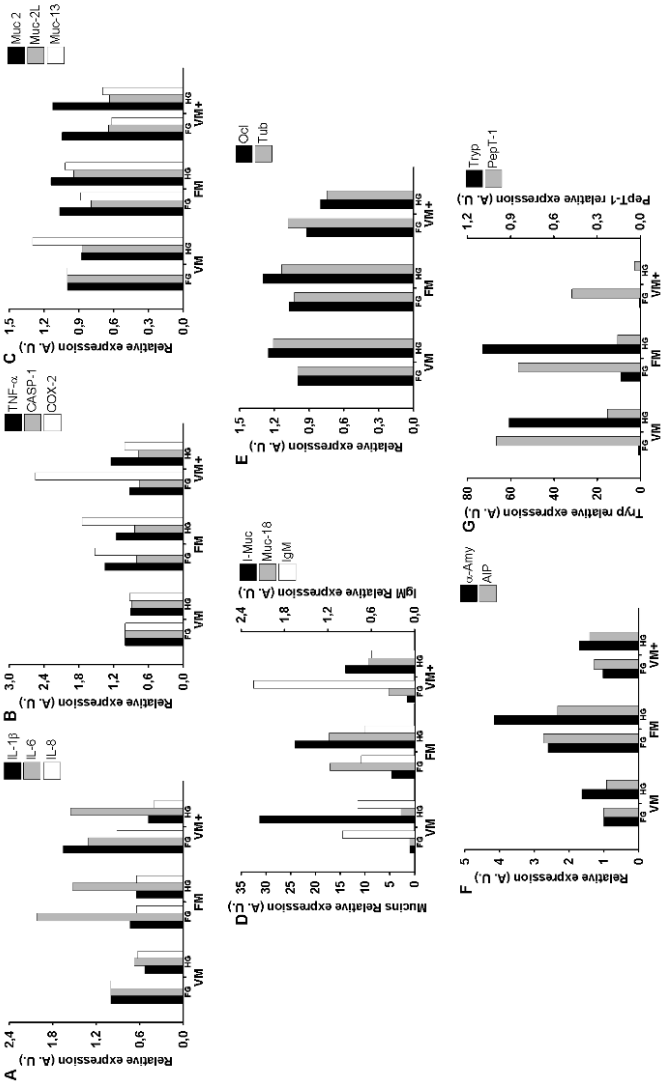
Means of triplicate groups (n=3). SEM: pooled standard error of the mean. Newman-Keuls test was applied for the comparison of the means.

ANNEX II. SUPPLEMENTARY MATERIAL OF CHAPTER 2

Additional file 1 (.xls). Cq values reported in cDNA pooled samples when evaluating candidate reference genes.

Pooled sample	ef1α	gapdh	rps18	βact
FM - FG	21.5	23.0	21.1	19.1
FM – HG	19.8	21.7	20.1	17.8
VM – FG	19.3	21.2	20.7	17.8
VM – HG	20.8	22.4	21.1	19.0
VM+ - FG	19.4	21.2	20.3	17.7
VM+ - HG	20.6	22.1	20.8	18.6
Average	20.3	22.0	20.7	18.3
Standard deviation	0.86	0.71	0.41	0.64

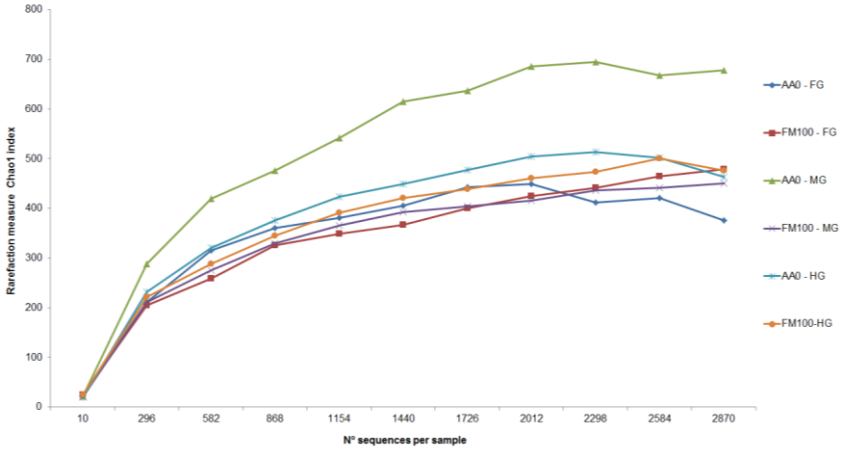
It Includes Cq determined for different candidate reference genes in six different cDNA pooled samples, and the average and standard desviation.



Additional file 2 (.tiff). Relative gene expression of candidate target genes in cDNA pooled samples.

A) Interleukine-1β (*il1β*), Interleukin-6 (*il6*) and Interleukine-8 (*il8*); B) Tumor Necrosis Factor–α (*tnfα*), Caspase 1 (*casp1*), Cyclooxigenase-2 (*cox2*); C) Mucin 2 (*muc2*), Mucin 2-like (*muc2L*), Mucin 13 (*muc13*); D) Intestinal Mucin (*imuc*), Mucin 18 (*muc18*), Immunoglobulin M (*igm*); E) Occludin (*ocl*) and Tubuline (*tub*); F) α-Amylase (*αamy*) and Alkaline Phosphatase (*alp*); G) Trypsin (*tryp*) and Peptide Transporter 1 (*pept1*). Different genes are represented with different colours. Bars represent relative gene expression of cDNA pools (one per section and treatment), in the foregut (FG) and the hindgut (HG). cDNA pool of the foregut of fish fed VM was used as a calibrator**.**

ANNEX III. SUPPORTING INFORMATION OF CHAPTER 3



S1 Fig. Rarefaction curves (Chao1 index) showing the microbial community complexes in the different gut sections of gilthead sea bream fed different experimental diets (AA0 and FM100).

ANNEX IV. SUPPLEMENTARY MATERIAL OF CHAPTER 4

S1. Essential and non-essential amino acids of the experimental diets

	FM	VM	VM+
EAA (g·100g-1)			
Arginine	3.10	4.49	5.16
Histidine	0.98	0.79	0.70
Isoleucine	2.09	1.60	1.53
Leucine	3.59	2.82	2.63
Lysine	3.16	1.92	1.99
Methionine	1.03	0.91	0.74
Phenilalanine	2.01	1.93	1.83
Threonine	1.89	1.41	1.19
Valine	2.55	1.84	1.74
NEAA (g·100g-1)			
Alanine	2.84	1.47	1.46
Aspartate	4.09	2.72	2.80
Cystein	0.35	0.50	0.58
Glicine	2.90	1.82	2.06
Glutamine	7.52	11.45	10.36
Proline	2.29	3.53	3.22
Serine	1.81	1.77	1.68
Tyrosine	1.31	1.07	0.99
EAA/NEAA	0.88	0.73	0.76

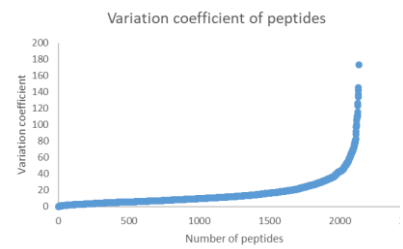
EAA, essential amino acids; NEAA, non-essential amino acids

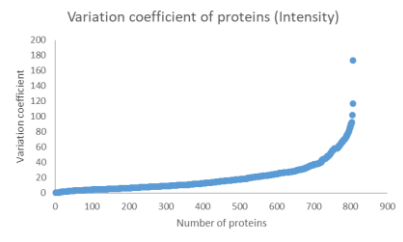
S2. Reproducibility assessment of the LC-MS/MS assay

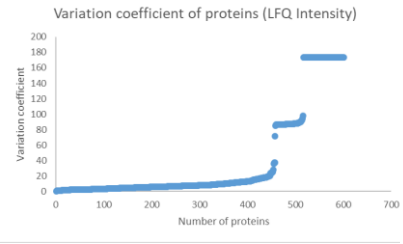
	R1	R2
Peptides Intensity		
Number of peptides	3413	3102
Peptides with Intensity ≠ 0 in the three runs	*2147 (63%)*	*2042 (66%)*
Number of peptides after filtering	2136	2028
Peptides with Intensity ≠ 0 in the three runs	*2130 (100%)*	*2019 (100%)*
Number of peptides with CV<20% (% of the total)	1656 (78%)	1600 (79%)
CV median	10.37%	10.48%
Protein Intensity		
Number of proteins	1070	990
Proteins with Intensity ≠ 0 in the three runs	*805 (75%)*	*804 (81%)*
Number of proteins after filtering	807	804
Proteins with Intensity ≠ 0 in the three runs	*801 (99%)*	*802 (100%)*
Number of proteins with CV<20% (% of the total)	530 (66%)	554(69%)
CV median	12.68%	12.89%
Protein LFQ Intensity		
Number of proteins	1070	990
Number of proteins after filtering	592	582
Proteins with LFQ Intensity ≠ 0 in the three runs	*459 (78%)*	*431 (74%)*
Number of proteins with CV<20% (% of the total)	445 (75%)	420 (72%)
CV median	7.53%	7.78%

Two different samples (R1 and R2) were run per triplicate and the coefficient of variation (CV) of the intensity of the identified peptides, and of the intensity and the LFQ intensity of the identified proteins, was determined after filtering. Filtering parameters mentioned in the Material and Methods section were used.

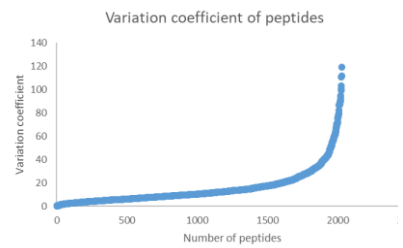
R1

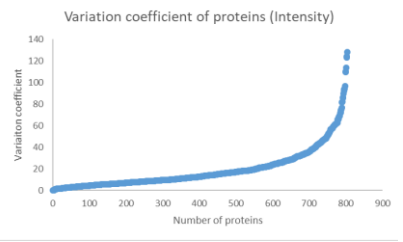

Variation coefficient of peptides
Variation coefficient
Number of peptides

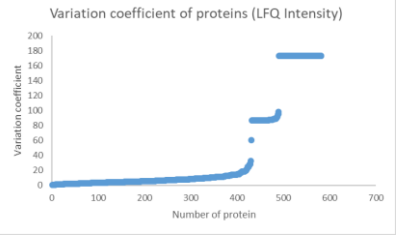

Variation coefficient of proteins (Intensity)
Variation coefficient
Number of proteins


Variation coefficient of proteins (LFQ Intensity)
Variation coefficient
Number of proteins

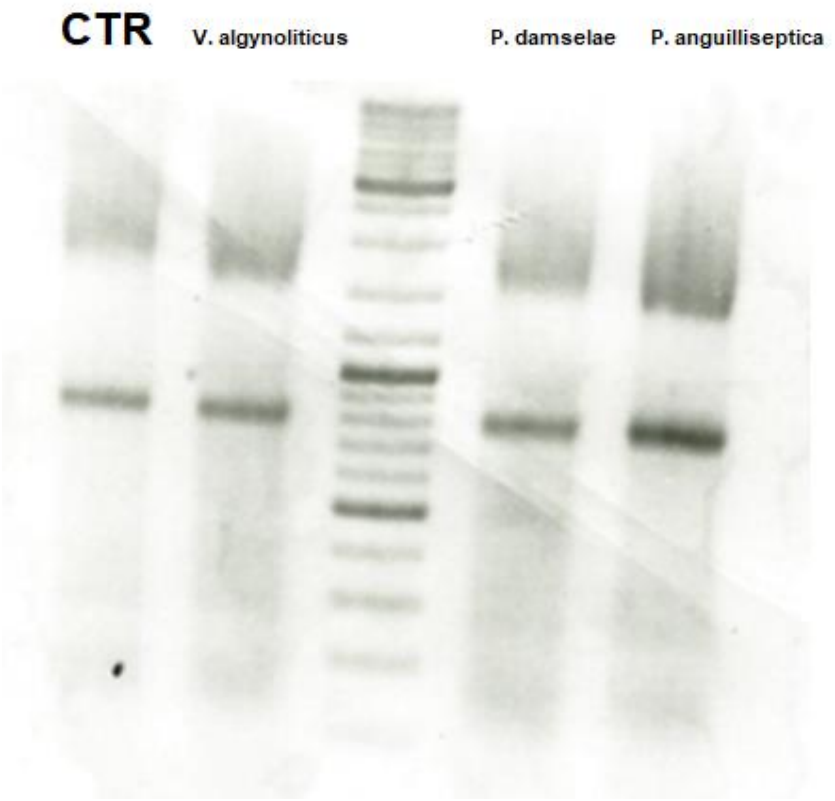
R2


Variation coefficient of peptides
Variation coefficient
Number of peptides


Variation coefficient of proteins (Intensity)
Variaiton coefficient
Number of proteins


Variation coefficient of proteins (LFQ Intensity)
Variation coefficient
Number of protein

ANNEX V. SUPPORTING INFORMATION OF CHAPTER 5



S1 Fig. Electrophoresis of pooled RNA samples from explants after 6 hours of incubation

Integrity of 28S and 16S bands suggests good RNA integrity after the *ex vivo* assay