

Article

Study of Fishmeal Substitution on Growth Performance and Shelf-Life of Giltheadsea Bream (*Sparusaurata*)

Patricia Zaragoza¹, Silvia Martínez-Llorens², Isabel Fernández-Segovia³, José-Luis Vivanco⁴, Ana Tomas-Vidal^{2,*}, Ana Fuentes³, José Vicente Ros-Lis⁵ , Ramón Martínez-Máñez^{1,6}  and José Manuel Barat³

¹ Centro de Reconocimiento Molecular y Desarrollo Tecnológico, Unidad Mixta Universitat Politècnica de València-Universitat de València, 46022 Valencia, Spain; pzt100@gmail.com (P.Z.); rmaez@qim.upv.es (R.M.-M.)

² Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal (ICTA), Universitat Politècnica de València, 46022 Valencia, Spain; silmarll@dca.upv.es

³ Departamento de Tecnología de Alimentos, Universitat Politècnica de València, 46022 Valencia, Spain; isferse1@tal.upv.es (I.F.-S.); anfuelo@upvnet.upv.es (A.F.); jmbarat@tal.upv.es (J.M.B.)

⁴ Departamento de Proyectos de Ingeniería, Universitat Politècnica de València, 46022 Valencia, Spain; jvivanco@dpi.upv.es

⁵ Departamento de Química Inorgánica, Universitat de València, Burjassot, 46022 Valencia, Spain; J.Vicente.Ros@uv.es

⁶ CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), 46022 Valencia, Spain

* Correspondence: atomasv@dca.upv.es

Received: 1 April 2020; Accepted: 9 May 2020; Published: 15 May 2020



Abstract: In this work the effect of partial or total replacement of fishmeal by plant protein sources and krill and squid meal on growth performance and shelf-life of gilthead sea bream was evaluated. Plant protein diets with 50 g kg⁻¹ of krill and 100 g kg⁻¹ of squid were supplemented with synthetic amino acids and at the end of the growing period weight showed no significant differences. The spoilage process of the fish was followed by physicochemical and microbiological measurements together with a colorimetric sensor array (CSA) specially designed for that purpose. The changes in the physicochemical parameters and microbial growth showed that shelf-life of samples were in all cases lower than nine days. The CSA was not able to show significant differences between both diets, confirming the physicochemical and microbiological results. The fact that the type of feed had no effect on the freshness parameters studied demonstrates that total fishmeal replacement with plant protein blends in the proportions used in this work could be an excellent alternative for feed formulation in aquaculture.

Keywords: *Sparusaurata*; giltheadsea bream; fishmeal; plantprotein; shelf-life; colorimetric sensor array

1. Introduction

In aquaculture production, diet is one of the most important factors because of its influence on metabolic aspects of the fish and its role in the chemical composition of muscle and flesh quality. Intensive farming depends on the diet composition of the external feed supply, and these can affect the nutritional quality of aquaculture products. An artificial diet can not include the typical components of a natural diet, so a feed composition is capable of causing differences in the appearance and the quality of these products [1].

Fishmeal and fish oil provide the ideal amino acid profile and the essential fatty acids for proper fish development. However, the continuous increase in the demand for fishmeal and fish oil combined

with their high price has led to a search for new proteins and energy-rich ingredients to avoid dependence on expensive marine raw materials for fish feeding.

Currently, gilthead sea bream is one of the most important species in Mediterranean aquaculture [2]. Gilthead sea bream commercial diets use plant proteins to substitute fishmeal, but their optimum dietary level for growth and nutrient efficiency depends on the raw material [3–5] or the protein blend [6,7]. Overall, results indicated that up to 40% inclusion of different protein sources, such as single ingredient, reported good growth rates, but higher inclusions can cause growth problems, due to the deficiency of essential amino acids and to the presence of anti-nutritional factors in many plant raw materials, that produce intestinal diseases in fish. However, when a mixture of protein sources is used, fishmeal can be totally replaced, as has been shown in previous studies [8], without having any negative effects on fish growth. Besides the zootechnical parameters, several other effects of including plant proteins have been evaluated in gilthead sea bream feeding, such as histological alterations on the intestine or liver [9], effects on immune response [10,11] and even on sensory attributes [12]. In summary, fishmeal can be replaced at 75% for plant protein mixture without having any negative effects. Previous trials have demonstrated that high fishmeal substitution for other proteins had slight effects on the quality traits of commercial-size gilthead sea bream [3,4,8]. However, the spoilage of samples has been rarely compared in this type of studies [13,14].

Despite gilthead sea bream being one of the most consumed fish in the Mediterranean, the study of the post mortem processes and the effect of diet on flesh quality has not been studied in depth. The effect of diet (specifically the inclusion of vegetal oils) on deterioration has only been described by Álvarez-Trujillo [15] who concluded that using vegetable oil to replace fish oil had no significant effects on the shelf-life of gilthead sea bream in cold storage. Matos et al. [14] reported that high fishmeal and/or fish oil replacement by plant ingredients in diets for gilthead sea bream feeding has a substantial effect on early post mortem metabolic processes and proteolytic potential on muscle. Nevertheless, to the best of our knowledge, there is no available information about the effect of dietary fishmeal substitution by plant proteins on fish muscle shelf-life.

Food quality has been traditionally monitored by sensory analysis [16], chemical and microbial analysis and mechanical methods [17]. However, some of these methods are tedious, expensive, time-consuming and require skilled personnel [18]. As an alternative, the development of rapid non-destructive quality control techniques that can be applied at any stage of the supply chain, is of much importance [19–21]. In this sense, different authors have been working on the development of fast and reliable methods to monitor food freshness based on colorimetric sensor arrays (CSA) in recent years. CSA technology has been furthered by the major progress and cost reduction in recent years in camera technology and the processing power of computer hardware [20]. Applications using CSA for detection and identification of volatile organic compounds have been developed in the field of food quality and safety assessment [10,22–24]. Chromogenic sensor arrays are a powerful tool for monitoring *Sparus aurata* freshness [18]. The color changes of this kind of arrays allows fish freshness monitoring by the combination of the unspecific response of the diverse sensors and statistical analysis tools. However, as a result of matrix inversion problems, for classification purposes, the high number of available labels, which in this case were the responses of the six sensing materials used in CSA needs to be simplified. Principal components analysis (PCA) is a widely used technique in order to reduce the dimensionality of the data.

In this paper, the objective was to study the effect of partial or total replacement of fishmeal (FM) by blends of plant protein sources, on growth performance and shelf-life of gilthead sea bream (*Sparus aurata*).

2. Materials and Methods

2.1. Growth Trial

The trial was conducted in 9 cylindrical fibreglass tanks (1750 L, three per treatment) within a saltwater recirculation system (65 m³ capacity) with a rotary mechanical filter and a gravity biofilter (approximately 6 m³). All tanks were fitted with aeration equipment. The water was heated by a heat pump installed in the system, and the water removal per tank and hour was 2.5 times/hour.

During the experiment, the temperature was maintained at 23 ± 1 °C, dissolved oxygen was 6 mg L⁻¹, salinity was 37–38 g L⁻¹, pH was 7.5 and ammonium value was 0.0 mg L⁻¹. The photoperiod was natural throughout the experimental period, and all tanks had similar lighting conditions.

2.2. Diets and Experimental Design

Three isonitrogenous (45% crude protein) and isolipidic diets (20% crude lipid) were formulated using commercial raw ingredients (Table 1, nutrient levels were similar to a commercial diet). The fishmeal substitution for a plant protein sources mix (wheat and wheat gluten, faba bean meal, soybean meal, pea meal, sunflower meal) was used at percentages of 0%, 75%, and 100% (diet FM100 or control diet, diet FM25, and diet FM0 without fishmeal, respectively). These diets were selected to have a control diet (FM100), a similar commercial diet (FM25) and an extreme diet (FM0). The plant protein mix was chosen due to the high protein content of the ingredients and supplemented with a crystalline amino acid to cover the amino acid requirement of the gilthead sea bream, previously described by Peres and Oliva-Teles [25]. All diets, except the control diet (FM100), were supplemented with taurine and calcium phosphate because fishmeal contains taurine and calcium that are not in plant protein meals and can be detrimental in the FM25 and FM0 diets. Additionally, soybean oil was included at 66 g kg⁻¹ in all diets following results obtained in a previous study by Martínez-Llorens et al. [26]. In diets FM25 and FM0, krill and squid meal were added (Table 1).

Table 1. Formulation and proximate composition of the experimental diets.

| | Diets | | |
|--|-----------------------------------|------|-----|
| | Ingredients (g kg ⁻¹) | | |
| | FM100 | FM25 | FM0 |
| Fish meal herring | 589 | 150 | |
| Wheat | 260 | 60 | |
| Wheat gluten | | 105 | 202 |
| Fababean meal | | 25 | 40 |
| Soybeanmeal | | 132 | 160 |
| Krill meal | | 50 | 50 |
| Pea meal | | 25 | 25 |
| Sunflowermeal | | 132 | 160 |
| Fish oil | 65 | 78 | 90 |
| Soybean oil | 66 | 66 | 65 |
| Squid meal | | 100 | 100 |
| Mono calcium phosphate | | 27 | 38 |
| Soybean lecithin | 10 | 10 | 10 |
| Taurine | | 20 | 20 |
| Methionine ^a | | 5 | 5 |
| Lysine ^a | | 5 | 10 |
| Multivitamin and minerals mix ^b | 10 | 10 | 10 |
| Proximate composition (g kg⁻¹ dry weight matter) | | | |
| Dry matter DM | 881 | 902 | 928 |
| Crude protein CP | 442 | 445 | 446 |
| Crude lipid CL | 185 | 201 | 200 |
| Ash | 101 | 101 | 88 |
| Carbohydrates ^c | 271 | 252 | 265 |

^a 7 L-Methionine and L-Lysine Clh: Guinama S.L.U. ^b Vitamin and mineral mix (values are g kg⁻¹ except those in parenthesis): Premix: 25; choline, 10; DL-a-tocopherol, 5; ascorbic acid, 5; (PO₄)₂Ca₃, 5. Premix composition: retinol acetate, 1,000,000 IU kg⁻¹; calciferol, 500 IU kg⁻¹; DL-a-tocopherol, 10; menadione sodium bisulphite, 0.8; thiamine hydrochloride, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides 12. ^c Carbohydrates, CHO (%) = 100-%CP-%CL-%Ash.

Diets were prepared using a semi-industrial twin-screw extruder (CLEXTRAL BC 45, St. Etienne, France). The processing conditions were as follows: screw speed of 100 rpm, a temperature of 110 °C, and pressure of 40–50 atm.

Composition of the diets were analysed following AOAC (Association of Official Agricultural Chemists) [27] procedures: dry matter (105 °C to constant weight), ash (combusted at 550 °C to constant weight), crude protein (N × 6.25) by the Kjeldahl method after acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganäs, Sweden) and crude lipid extracted with dichloromethane-methanol (Soxtec 1043 extraction unit, Tecator AB, Sweden). All analyses were performed in triplicate.

2.3. Fish and Feeding

The gilthead sea bream (*Sparusaurata*) were brought from a local marine fish farm (Piscimar S.A., Burriana. Castellón, Spain), transported alive to the Fish Nutrition Laboratory of the Universitat Politècnica de València (Spain) and randomized into experimental tanks (20 per tank). All fish were weighed every 30 days. Previously, fish were anesthetized with 30 mg L⁻¹ clove oil, containing 87% eugenol (Guinama[®], Valencia, Spain).

The test lasted 154 days (from November to April). The experimental diets were assayed in triplicate groups. The fish were fed by hand twice a day (09.00 h and 16.00 h) until apparent satiation. The pellets were distributed slowly to allow all fish to eat.

The experiment ended when the fish reached market weight (between 350 and 400 g). At the end of the growth trial, all fish were individually weighed, sacrificed by immersion in ice-cold water and stored at 4 °C, and fillets from 54 fish (6 per tank, 18 per treatment) were extracted for shelf-life evaluation. This experimental procedure was repeated three times at 15 day intervals.

2.4. Shelf-Life Study

2.4.1. Sample Preparation

The two fillets from one single fish were individually placed in plastic trays. A colorimetric sensor array (CSA) (made of six sensing materials filling a microplate) was placed inside the tray avoiding contact with the fish samples. In addition, three trays containing CSA were also packed in the absence of samples and used as controls for each experiment. Petri dishes filled with water were placed in the control trays to reproduce moisture conditions. All the trays were placed inside plastic bags and thermo-sealed. Finally, trays were stored at 4 °C for 11 days. The physicochemical and microbiological analyses were performed on days 0, 2, 4, 7, 9, and 11. Photographs of the CSA were also taken on the same days. This experimental procedure was repeated three times at 15 day intervals.

2.4.2. Chromogenic Array Preparation

Thymol blue, bromothymol blue, bromocresol green, aluminium oxide, and silica gel were purchased from Sigma–Aldrich (St. Louis, MO, USA). Analytical-grade solvents were acquired from Scharlab (Barcelona, Spain). All the reagents were used as received with no further purification. The binuclear rhodium complex with formula $[\text{Rh}_2(\text{C}_6\text{H}_4\text{PPh}_2)_2(\text{O}_2\text{CCH}_3)_2](\text{HO}_2\text{CCH}_3)_2$ was synthesized according to reported procedures [28]. Solutions were prepared according to the procedure described by Zaragoza et al. [18,29] and six final sensing materials were obtained:

- Preparation of probe 1, dye: thymol blue (40 mg), solvent: ethanol (8 mL), support: aluminum oxide.
- Preparation of probe 2, dye: bromothymol blue sodium salt (40 mg), solvent: ethanol (7 mL), support: silica gel.
- Preparation of probe 3, dye: bromocresol green (40 mg), solvent: ethanol (7 mL), support: silica gel.
- Preparation of probe 4, dye: dinuclear rhodium complex (40 mg), solvent: dichloromethane (6 mL), support: silica gel.

- Preparation of probe 5, dye: dinuclear rhodium complex (80 mg), solvent: dichloromethane (6 mL), support: silica gel.
- Preparation of probe 6, dye: dinuclear rhodium complex (140 mg), solvent: dichloromethane (6 mL), support: silica gel.

2.4.3. Analytical Determinations

On each sampling day, three trays (one per diet) were randomly selected, and physicochemical and microbial analyses were carried out. All the analyses were performed in triplicate.

Headspace gas measurement. The gas composition of tray headspace was measured using an O₂ and CO₂-meter (Checkmate 9900, PBI Dansensor A/S, Ringsted, Denmark). Gas sampling was carried out with a needle connected to the gas analyzer.

pH, total volatile basic nitrogen (TVB-N) and thiobarbituric acid (TBA) index. pH measurements were carried out using a digital pH-meter micro pH 2001 (Crison Instruments, S.A., Barcelona, Spain) with a puncture electrode (Crison 5231). The measurements were taken in five different locations of each fillet along the dorsal muscle, approximately 1–2 cm apart. The rest of physicochemical analyses were performed on the minced fillets. The contents of TVB-N were determined by steam distillation according to the method described by Malleand Tao [29] and expressed as mg N/100 g of muscle. The TBA index was used to evaluate the extent of lipid oxidation. This parameter was determined using a spectrophotometric method [30] and the results were expressed as mg malondialdehyde (MDA)/kg of muscle.

ATP-related compounds and K₁-value. The ATP-related compounds, consisting of inosine-5'-monophosphate (IMP), inosine (Ino), and hypoxanthine (Hx), were assayed by HPLC following the method described by Barat et al. [31] with minor modifications [19]. K₁-values were calculated as described elsewhere [32].

All the chemical reagents were provided by Sigma–Aldrich (St. Louis, MO, USA).

Microbial analyses. Mesophilic counts were performed according to the method provided in the ISO 4833:2003 standard [33]. All the culture media were provided by Scharlau Chemie S.A. (Barcelona, Spain). The results are expressed as log cfu g⁻¹.

2.5. Digital Imaging and Data Collection

Photographs of the colorimetric arrays were also taken on days 0, 2, 4, 7, 9, and 11. Images of the optical sensing array samples were captured using a polyurethane lightbox, with constant lighting conditions. Colorimetric array data were extracted from the images using Photoshop Pro 5 software to isolate CIELab coordinates. L* is lightness, a* deviation towards red (positive values) or green (negative values) and b* deviation towards yellow (positive values) or blue (negative values).

2.6. Statistical Analysis

The data obtained from the physicochemical and microbial analyses were analyzed with a multifactor ANOVA, considering the interactions among factors (diet and time of storage), with the variables being the parameters. The method used for multiple comparisons was the LSD test (least significant difference) with a significance level of $\alpha = 0.05$. Statistical data processing was performed with the Statgraphics Centurion software (Statpoint Technologies, Inc., Warrenton, VA, USA).

Color data were analyzed using a principal component analysis (PCA). The main feature of a PCA is the coordination of the data in the new base (scores plot) and the contribution to each component of the sensors (loads plot). PCA decomposes the primary data matrix by projecting the multi-dimensional data set onto a new coordinate base formed by the orthogonal directions with maximum data variance. The eigenvectors of the data matrix are called principal components (PCs). PCs are ordered based on the amount of variance (PC1, PC2, etc.).

CIELabcolor data were used to perform the PCA analysis using MATLAB R2017a (The Mathworks). The original variables, in this case, were the responses of the six sensing materials used in CSA. The mean

centering pre-processing technique was applied to a dataset of 18 measurements (color features i.e., six L*, a* and b* coordinates) on seven different days, in the three experiments (i.e., 9 initial samples \times 3 types of diet \times 3 experiments).

3. Results and Discussion

3.1. Effect of Fishmeal on Fish Growth

The diets formulation was made from a variety of feed ingredients to satisfy the nutrient requirements of gilthead sea bream. For this study, a protein sources mixture, supplemented with synthetic amino acids [25], as a substitute for fishmeal in gilthead sea bream diets, was selected. The most common alternatives for FM as a protein source are different kinds of plant protein meals that vary in content of available nutrients, but by using a mixture of different ingredients [34] and supplementing the diet with indispensable amino acids [34] the formulation of nutritionally complete diets with an inclusion level of FM as low as 10% can be obtained.

Survival rates were 86%–88% and did not differ significantly among treatment groups. At the end of the growth period, weight, and specific growth rate (SGR) showed no significant differences (Table 2).

Table 2. Survival and growth of gilthead sea bream fed experimental diets.

| | FM100 | FM25 | FM0 | SEM |
|---|-------|-------|-------|-------|
| Initial weight (g) | 129.5 | 130 | 125.5 | 3.54 |
| Final weight (g) | 393.7 | 423.7 | 383 | 10.86 |
| Survival (%) | 88.33 | 88.33 | 86.66 | 5.53 |
| SGR (% day ⁻¹) ^a | 0.73 | 0.77 | 0.71 | 0.02 |

Means of triplicate groups. SEM: pooled standard error of the mean. Initial weight was considered as covariable for final weight and SGR. Newman–Keuls test. ^a Specific growth rate (% day⁻¹) SGR = $100 \times \ln(\text{final weight}/\text{initial weight})/\text{days}$.

In the present study, gilthead sea bream fed a diet in which fishmeal was totally replaced by a plant protein sources mix grew the same as fish fed on a fishmeal control diet, without any effects on growth performance and fish survival. The success of the diets was not only attributed to its adequate amino acid profile, but also to the addition of krill and squid meal, with a high protein quality, leading to good acceptance of the FM25 and FM0 diets.

3.2. Shelf-Life Studies

3.2.1. Physicochemical Study

Regarding the shelf-life of gilthead sea bream, different physicochemical and microbial parameters were studied.

Headspace gas composition. Changes in CO₂ and O₂ contents in the headspace of the trays during cold storage of gilthead sea bream are shown in Figure 1a. The initial CO₂ content was about 1% in the three formulations, increasing the concentration throughout storage until a level of 6.9% on day 11 of study in the feed FM25. On the contrary, the initial content of O₂ was about 21% although this decreased during storage, especially in gilthead sea bream fed with FM25 (13% of O₂). These results are mainly due to the aerobic microbial growth. Significant differences were found in both gases over time (Table 3). With regards to the feed type, a higher value of CO₂ was only observed for the FM25 diet on the last day of study.

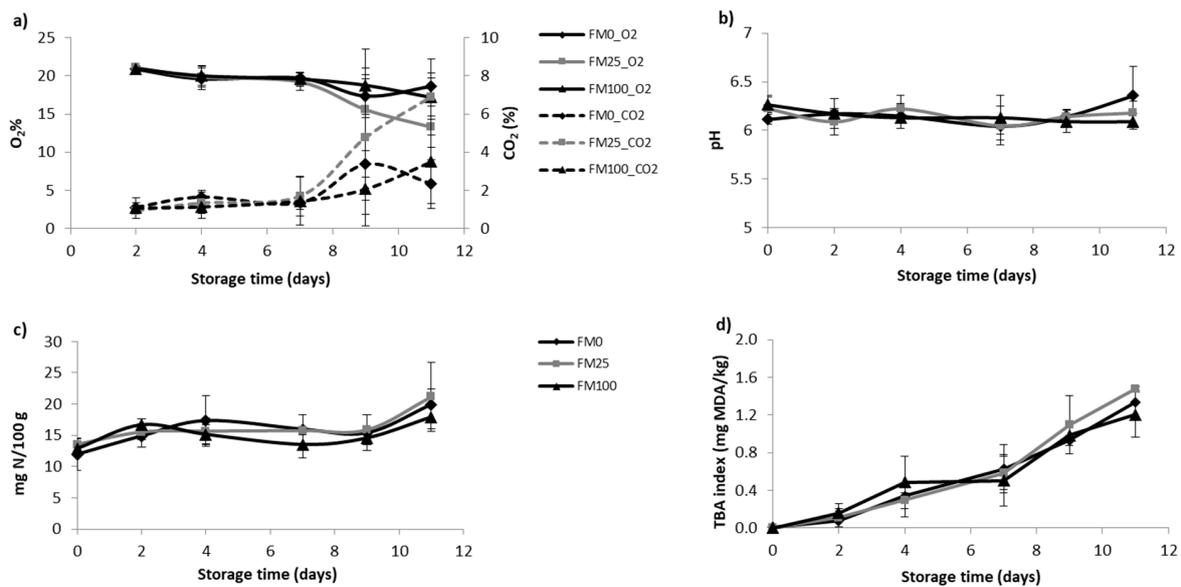


Figure 1. Changes in O₂ and CO₂ contents (a), pH (b), TVB-N (c), and TBA index (d) in samples of gilthead sea bream during 11 days of storage at 4 °C. (Means and standard deviations, $n = 3$). (FM0, FM25 and FM100: diets detailed in Table 1). MDA: malonaldehyde.

Table 3. Results of the study of the effect of diets, storage time and their interaction on the physicochemical and microbial parameters.

| | Diets | | Storage Time | | DietsxStorage Time | |
|-----------------------|---------|----|--------------|-----|--------------------|----|
| | F-Ratio | | F-Ratio | | F-Ratio | |
| O ₂ | 1.39 | ns | 12.97 | *** | 1.14 | ns |
| CO ₂ | 1.97 | ns | 12.10 | *** | 1.00 | ns |
| pH | 0.04 | ns | 1.81 | ns | 1.30 | ns |
| TVB-N | 1.09 | ns | 7.85 | *** | 0.60 | ns |
| TBA index | 0.30 | ns | 77.40 | *** | 0.73 | ns |
| K ₁ -value | 0.87 | ns | 33.26 | *** | 0.45 | ns |
| Mesophilic | 1.63 | ns | 26.12 | *** | 0.44 | ns |

ns: non significant; *** $p < 0.001$.

pH, total volatile basic nitrogen (TVB-N) and thiobarbituric acid (TBA) index. The evolution of pH, TVB-N and TBA index is shown in Figure 1b–d, respectively. The obtained pH values (between 6.04 and 6.26) agree with other studies on the same fish species [31,35]. This parameter did not significantly change during the storage time. It is important to note that the main changes in pH take place in the first hours after slaughter [36]. However, in this study, the changes in pH during the *rigor mortis* were not monitored. The type of feed did not affect the behaviour of pH.

The TVB-N values did not show an important variation during storage, only a slight increase was observed between days 0 and 2, and also at the end of the study (day 11) (Figure 1c). Regarding the effect of feed, no differences were observed ($p > 0.05$) (Table 3). The TVB-N contents ranged between 12–20 mg N/100 g for gilthead sea bream fed with FM0, 13–21 mg N/100 g for FM25 and 13–18 mg N/100 g for FM100. The evolution reported for this parameter was similar to that found in other studies on gilthead sea bream [35,37]. According to Dalgaard [38] acceptability limits of 25, 30 and 35 mg N/100 g have been established for different fish species. In this study, the highest value reached at the end of the storage was 21 mg N/100 g, which is lower than the limits mentioned above.

The values of TBA were low in the first days of study (ranged from 0 mg malonaldehyde/kg at the beginning of the study to 0.5 mg malonaldehyde/kg at day 7), which show that no lipid oxidation took place at the beginning of storage (Figure 1d). Although this parameter progressively increased,

the values at the end of the study did not reach 1.5 mg malonaldehyde/kg. According to Connell [39], fish with values higher than 2 mg malonaldehyde/kg would be rejected due to unpleasant flavors. In this study the TBA index stood below this value in all samples, which means that lipid oxidation was limited during the study period. No significant differences were found in this parameter between the different feeds. The soybean oil and protein sources used in the three formulations contain large amounts of tocopherols [40] and phenolic compounds [41,42], which are antioxidants. Soybean oil is reportedly stable and also rich in natural antioxidants. In the present study, muscle lipid oxidation was not significantly altered by partial or total fishmeal replacement for vegetable ingredients. This is according to the results obtained in other studies, which have reported that plant meal or plant oil dietary inclusion showed lower TBARS values in fish muscle [43,44]. Different studies have shown that the presence of tocopherols from vegetal oils in diets could decrease lipid oxidation in the fish muscle [45–47]. Therefore, in this study, the use of soybean oil as a partial lipidic source in the feeds may perhaps enhance the oxidative stability of fish.

ATP-related compounds and K_1 -value. Figure 2 shows the changes in IMP, Ino, and Hx, in the samples. IMP values decreased throughout the storage period, while Ino and Hx contents increased slightly.

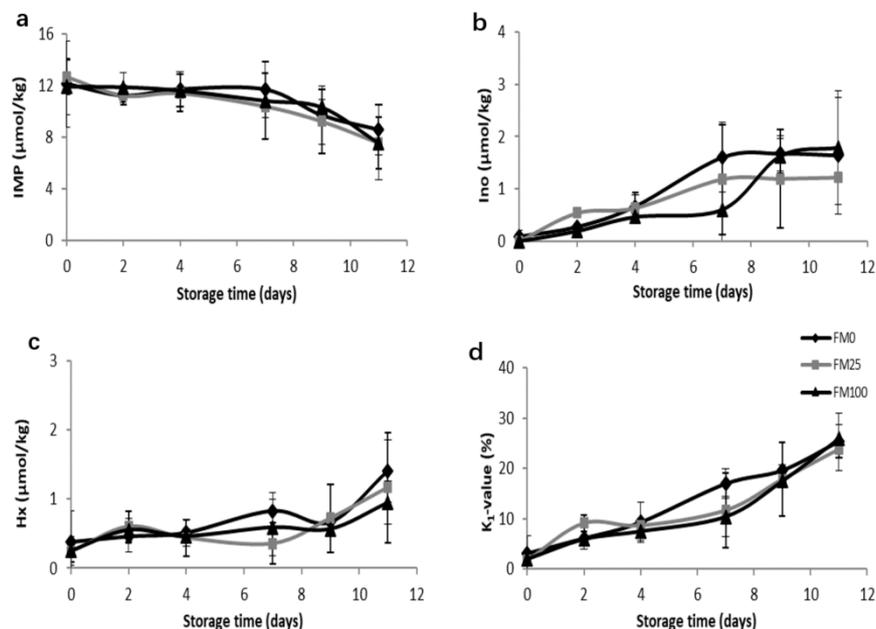


Figure 2. Changes in inosine-5'-monophosphate (IMP) (a), inosine (Ino), (b), hypoxanthine (Hx), (c), and K_1 -value (d) in samples of gilthead sea bream during 11 days of storage at 4 °C. (Means and standard deviations, $n = 3$). (FM0, FM25, and FM100: diets detailed in Table 1).

The IMP levels were higher than 10 $\mu\text{mol/g}$ at the beginning of the study (Figure 2a). These high levels could be due to the fact that the conversion of ATP to IMP by partial dephosphorylation and deamination, is usually completed in one day and it might be totally autolytic. IMP levels of this magnitude have been found in other studies on gilthead sea bream after one day of slaughter [48]. The concentration of this metabolite progressively decreased throughout storage, although at the end of the study (day 11) the levels were still higher than 5 $\mu\text{mol/g}$. This agrees with other studies, where the final contents of IMP at the end of the shelf-life were similar to the levels found in the present work [48]. The decrease of IMP is due to the degradation of this metabolite, which is converted to Ino and Hx due to autolytic and microbial enzymes [49].

The initial contents of Ino were very low, although they increased during the storage period, reaching maximum levels of 1.79 $\mu\text{mol/g}$ (feed FM100 on day 11 of the study) (Figure 2b). The Hx level was low during the study period (Figure 2c). This means that Ino degradation to Hx was very

slow, with Ino being the main metabolite of IMP degradation at the end of the storage time. A similar pattern was observed in other studies on gilthead sea bream [48].

These results show that the degradative changes (autolytic and microbiological) that took place in the samples during the storage period were limited because IMP turned into Ino, but only a slight degradation of Ino to Hx was observed at the end of the study.

The initial K_1 -values were lower than 5% for the three types of feeds (Figure 2d). Gilthead sea bream with a high degree of freshness has been reported to have K_1 -values lower than 10% [50]. This parameter significantly increased during storage to values around 25% on day 11. These results agree with other studies on fresh gilthead sea bream [38]. No significant differences between the different feeds were found.

3.2.2. Microbial Analyses

Mesophilic growth of samples in cold storage is shown in Figure 3. The microorganism growth profile was similar for the three types of samples. The initial mesophilic counts were low in all cases, gradually increasing from day 4 of storage until reaching values higher than 8 log cycles at the end of the study. These results demonstrate that there was not a good co-relation between the TVB-N and the microbial spoilage, as in other studies with this fish species [36]. Samples exceeded the acceptability limits of 10^6 – 10^7 cfu/g as proposed by several authors [51,52] on day 9 of storage. These results demonstrate that there was not a good co-relation between the TVB-N and the microbial spoilage, as in other studies with this fish species [36]. Taking into account the microbial quality, a shelf-life shorter than 9 days should be established for gilthead sea bream, regardless of the type of feed, in the storage conditions applied in this study.

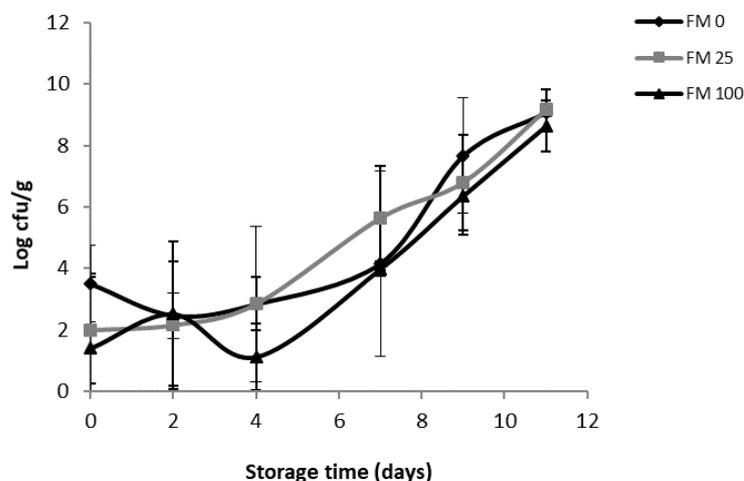


Figure 3. Changes in mesophilic bacteria in samples of gilthead sea bream during 11 days of storage at 4°C. (Means and standard deviations, $n = 3$). (FM0, FM25, and FM100: diets detailed in Table 1).

3.2.3. Study of the Effect of Diets, Storage Time and Their Interaction on the Physicochemical and Microbial Parameters

The multifactor ANOVA was carried out taking into consideration the two factors (type of diet and storage time), as well as the interactions between them. Table 3 shows the F-ratio obtained in this analysis for each parameter. The F-ratio represents the quotient between variability due to the considered effect and the residual variance. The effect of feed type was not significant for any of the parameters studied. The storage time had a significant effect on the parameter changes, except for pH. The interaction between the two factors was non-significant ($p < 0.05$) in either case, which demonstrates that the evolution of the parameters throughout the storage time was not affected by the type of diet, as mentioned above.

3.2.4. Changes in the Colorimetric Array

The chromogenic array used in this study consisted of six sensing materials (based on aluminum oxide and silica gel) containing pH indicators and Lewis acids. In this case, the responses of the six sensing materials used in CSA (18 color features corresponding to six L^* , a^* , and b^* coordinates), need to be simplified. Principal components analysis (PCA) has been used for this purpose, and the mean centering pre-processing technique was applied.

PCA decomposes the primary data matrix by projecting the multi-dimensional data set onto a new coordinate base formed by the orthogonal directions with maximum data variance. The eigenvectors of the data matrix are called principal components (PCs). PCs are ordered based on the amount of variance (PC1, PC2, etc.). Three PCAs were done for each formulation. Figure 4 shows the percent variability explained by each principal component for each formulation. The number of PCs needed to account for 95% of variance were 5, 4, and 4 for FM0, FM25, and FM100, respectively.

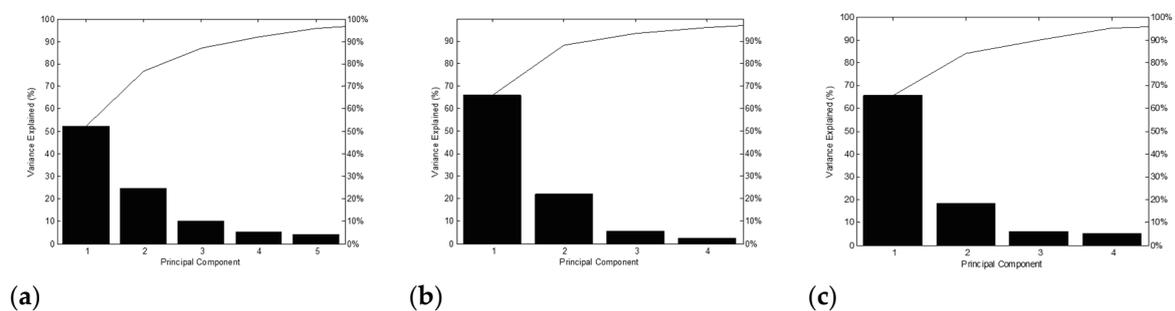


Figure 4. The plot of the percent variability explained by each principal component for (a) FM0, (b) FM25, and (c) FM100. (FM0, FM25, and FM100: diets detailed in Table 1).

The PCA scores were used for the multivariate ANOVA analysis. Figure 5 shows the plot of canonical variables $cv1$ versus $cv2$. As can be observed at the beginning of the experiment some biochemical changes occur that influence the array response, then during days 2 and 4 no significant changes are observed, but for day 7 and especially on day 11 a strong color modulation was produced. This behavior can be assigned to the generation of reactive metabolites since this kind of array are not responsive to the concentration of carbon dioxide in the tray headspace.

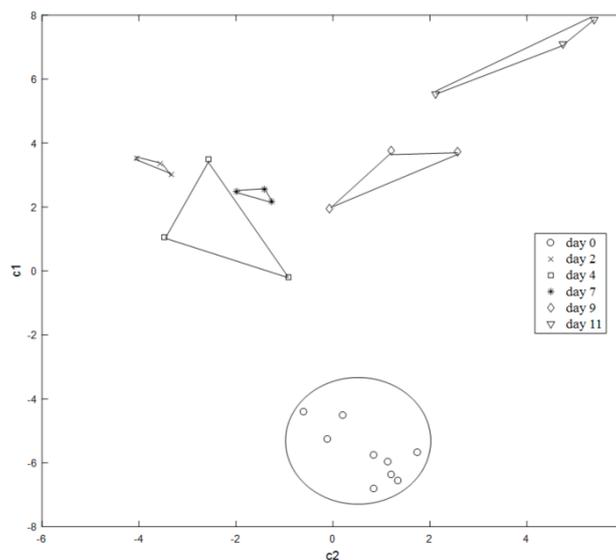


Figure 5. The plot of principal components of $cv1$ vs. $cv2$, using PCA scores for FM25. (FM25 diet detailed in Table 1).

Most importantly, for the purpose of this work is that no significant differences in the response of the colorimetric sensing array were observed in the evolution of the fish samples regardless of the diet used.

4. Conclusions

Diets for gilthead sea bream in the on-growing phase containing different plant proteins sources mixture supplemented with synthetic EAA can replace 100% of FM without negatively affecting gilthead sea bream performance. Likewise, these results clearly indicate that the use of synthetic amino acids to balance diet with high or total FM replacement is a successful approach that has no effect on growth performance.

The use of alternative diets based on plant proteins sources does not have a significant effect on the physicochemical parameters of pH, TVB-N, TBA index or K1-value, nor on the growth of mesophilic bacteria, compared to diets based on fishmeal. The evolution of these parameters throughout the storage time indicates that the loss of quality of gilthead sea bream follows a similar pattern in fish fed with diets of 100% fishmeal or in those in which all the fishmeal has been replaced by plant proteins sources. The use of one diet or another does not affect the shelf life of the product. The CSA designed does not discriminate between the different feeds. It could be stated that there is no limitation in the percentage of reduction of fishmeal according to these parameters. Therefore, from this point of view, plant protein meals could be an excellent alternative to fishmeal in aquaculture.

Author Contributions: Conceptualization, A.T.-V., S.M.-L. and I.F.-S.; methodology, A.T.-V., I.F.-S. and A.F.; software, J.-L.V.; investigation, P.Z. and A.F.; data curation, J.-L.V.; writing—original draft preparation, J.-L.V.; writing—review and editing, J.V.R.-L., J.-L.V., R.M.-M., I.F.-S. and A.T.-V.; visualization, J.-L.V.; supervision, A.T.-V., S.M.-L., I.F.-S. and A.F.; funding acquisition, R.M.-M. and J.M.B. All authors have read and agreed to the published version of the manuscript.

Compliance with Ethical Standards: The *Sparusaurata* study complied with European Union Council Directive 2010/63/UE which lays down minimum standards for the protection of animals, and also in accordance with the Spanish national regulations (Spanish Royal Decree 53/2013) that protect animals used in experimentation and for other scientific purposes. The protocol was approved by the Universitat Politècnica de València (UPV) Ethics Committee. Animals were euthanized by an immersion in ice water and then dissected.

Funding: This research received no external funding.

Acknowledgments: We thank to the financial support of the Universitat Politècnica de València, Spain.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Grigorakis, K. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparusaurata*) and sea bass (*Dicentrarchuslabrax*) and factors affecting it: A review. *Aquaculture* **2007**, *272*, 55–75. [[CrossRef](#)]
2. Fezzardi, D.; Massa, F.; Àvila-Zaragoza, P.; Rad, F.; Yücel-Gier, G.; Deniz, H.; Salem, M.H.A.; Hamza, H.A.; Salem, S. Ben Indicators for sustainable aquaculture in Mediterranean and Black Sea countries: Guide for the use of indicators to monitor sustainable development of aquaculture. *Gen. Fish. Comm. Mediterr. Stud. Rev.* **2013**, *93*, 1–60.
3. Martínez-Llorens, S.; Moñino, A.V.; Vidal, A.T.; Salvador, V.J.M.; Pla Torres, M.; JoverCerdá, M. Soybean meal as a protein source in gilthead sea bream (*Sparusaurata* L.) diets: Effects on growth and nutrient utilization. *Aquac. Res.* **2007**, *38*, 82–90. [[CrossRef](#)]
4. Sánchez Lozano, N.B.; Tomás Vidal, A.; Martínez-Llorens, S.; Nogales Mérida, S.; Blanco, J.E.; Moñino López, A.; Pla Torres, M.; Cerdá, M.J. Growth and economic profit of gilthead sea bream (*Sparusaurata*, L.) fed sunflower meal. *Aquaculture* **2007**, *272*, 528–534. [[CrossRef](#)]
5. Martínez-Llorens, S.; Baeza-Ariño, R.; Nogales-Mérida, S.; Jover-Cerdá, M.; Tomás-Vidal, A. Carob seed germ meal as a partial substitute in gilthead sea bream (*Sparusaurata*) diets: Amino acid retention, digestibility, gut and liver histology. *Aquaculture* **2012**, *338*, 124–133. [[CrossRef](#)]

6. Sánchez-Lozano, N.B.; Martínez-Llorens, S.; Tomás-Vidal, A.; Cerdá, M.J. Effect of high-level fish meal replacement by pea and rice concentrate protein on growth, nutrient utilization and fillet quality in gilthead seabream (*Sparusaurata*, L.). *Aquaculture* **2009**, *298*, 83–89. [[CrossRef](#)]
7. Estruch, G.; Tomás-Vidal, A.; El Nokrashy, A.M.; Monge-Ortiz, R.; Godoy-Olmos, S.; JoverCerdá, M.; Martínez-Llorens, S. Inclusion of alternative marine by-products in aquafeeds with different levels of plant-based sources for on-growing gilthead sea bream (*Sparusaurata*, L.): Effects on digestibility, amino acid retention, ammonia excretion and enzyme activity. *Arch. Anim. Nutr.* **2018**, *72*, 1–19. [[CrossRef](#)]
8. Monge-Ortiz, R.; Llorens, S.M.; Márquez, L.; Moyano, F.J.; Jover-Cerdá, M.; Vidal, A.T. Potential use of high levels of vegetal proteins in diets for market-sized gilthead sea bream (*Sparusaurata*). *Arch. Anim. Nutr.* **2016**, *70*, 155–172. [[CrossRef](#)]
9. Baeza-Ariño, R.; Martínez-Llorens, S.; Nogales-Mérida, S.; Jover-Cerda, M.; Tomás-Vidal, A. Study of liver and gut alterations in sea bream, *Sparusaurata* L., fed a mixture of vegetable protein concentrates. *Aquac. Res.* **2016**, *47*, 460–471. [[CrossRef](#)]
10. Sitjá-Bobadilla, A.; Peña-Llopis, S.; Gómez-Requeni, P.; Médale, F.; Kaushik, S.; Pérez-Sánchez, J. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparusaurata*). *Aquaculture* **2005**, *249*, 387–400. [[CrossRef](#)]
11. Estruch, G.; Collado, M.C.; Monge-Ortiz, R.; Tomás-Vidal, A.; Jover-Cerdá, M.; Peñaranda, D.S.; Pérez Martínez, G.; Martínez-Llorens, S. Long-term feeding with high plant protein based diets in gilthead seabream (*Sparusaurata*, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. *BMC Vet. Res.* **2018**, *14*, 302. [[CrossRef](#)] [[PubMed](#)]
12. De Francesco, M.; Parisi, G.; Pérez-Sanchez, J.; Gomez-Réqueni, P.; Médale, F.; Kaushik, S.J.; Mecatti, M.; Poli, B.M. Effect of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparusaurata*) on growth and body/fillet quality traits. *Aquac. Nutr.* **2007**, *13*, 361–372. [[CrossRef](#)]
13. Hernández, M.D.; López, M.B.; Álvarez, A.; Ferrandini, E.; García, B.G.; Garrido, M.D. Sensory, physical, chemical and microbiological changes in aquacultured meagre (*Argyrosomusregius*) fillets during ice storage. *Food Chem.* **2009**, *114*, 237–245. [[CrossRef](#)]
14. Matos, E.; Silva, T.S.; Colen, R.; Dinis, M.T.; Dias, J. Plant protein and vegetable oil-based diets modulate gilthead seabream (*Sparusaurata*) muscle biochemical status and proteolytic enzymes at an early postmortem stage. *Aquac. Nutr.* **2014**, *20*, 153–162. [[CrossRef](#)]
15. Álvarez Trujillo, A.M. Vida útil de la Dorada Almacenada en Refrigeración con Hielo: Influencia de Distintos Factores del Cultivo. Ph.D. Thesis, Universidad de Murcia, Murcia, Spain, 2012.
16. Goulas, A.E.; Kontominas, M.G. Effect of salting and smoking-method on the keeping quality of chub mackerel (*Scomberjaponicus*): Biochemical and sensory attributes. *Food Chem.* **2005**, *93*, 511–520. [[CrossRef](#)]
17. Olafsdottir, G.; Nesvadba, P.; Di Natale, C.; Careche, M.; Oehlenschläger, J.; Tryggvadottir, S.V.; Schubring, R.; Kroeger, M.; Heia, K.; Esaiassen, M. Multisensor for fish quality determination. *Trends Food Sci. Technol.* **2004**, *15*, 86–93. [[CrossRef](#)]
18. Zaragoza, P.; Fuentes, A.; Fernández-Segovia, I.; Vivancos, J.-L.; Rizo, A.; Ros-Lis, J.V.; Barat, J.M.; Martínez-Mañez, R. Evaluation of sea bream (*Sparusaurata*) shelf life using an optoelectronic nose. *Food Chem.* **2013**, *138*, 1374–1380. [[CrossRef](#)]
19. Gribbestad, I.S.; Aursand, M.; Martinez, I. High-resolution 1H magnetic resonance spectroscopy of whole fish, fillets and extracts of farmed Atlantic salmon (*Salmo salar*) for quality assessment and compositional analyses. *Aquaculture* **2005**, *250*, 445–457. [[CrossRef](#)]
20. Folkestad, A.; Wold, J.P.; Rørvik, K.-A.; Tschudi, J.; Haugholt, K.H.; Kolstad, K.; Mørkøre, T. Rapid and non-invasive measurements of fat and pigment concentrations in live and slaughtered Atlantic salmon (*Salmo salar* L.). *Aquaculture* **2008**, *280*, 129–135. [[CrossRef](#)]
21. Olsen, S.H.; Sørensen, N.K.; Larsen, R.; Elvevoll, E.O.; Nilsen, H. Impact of pre-slaughter stress on residual blood in fillet portions of farmed Atlantic cod (*Gadusmorhua*)—Measured chemically and by visible and near-infrared spectroscopy. *Aquaculture* **2008**, *284*, 90–97. [[CrossRef](#)]
22. Chen, Q.; Zhang, C.; Zhao, J.; Ouyang, Q. Recent advances in emerging imaging techniques for non-destructive detection of food quality and safety. *TrAC Trends Anal. Chem.* **2013**, *52*, 261–274. [[CrossRef](#)]
23. Salinas, Y.; Ros-Lis, J.V.; Vivancos, J.-L.; Martínez-Mañez, R.; Marcos, M.D.; Aucejo, S.; Herranz, N.; Lorente, I.; Garcia, E. A novel colorimetric sensor array for monitoring fresh pork sausages spoilage. *Food Control* **2014**, *35*, 166–176. [[CrossRef](#)]

24. Salinas, Y.; Ros-Lis, J.V.; Vivancos, J.-L.; Martínez-Máñez, R.; Marcos, M.D.; Aucejo, S.; Herranz, N.; Lorente, I. Monitoring of chicken meat freshness by means of a colorimetric sensor array. *Analyst* **2012**, *137*, 3635–3643. [[CrossRef](#)]
25. Peres, H.; Oliva-Teles, A. The optimum dietary essential amino acid profile for 560 gilthead seabream (*Sparusaurata*) juveniles. *Aquaculture* **2009**, *296*, 81–86. [[CrossRef](#)]
26. Martínez-Llorens, S.; Vidal, A.T.; Moñino, A.V.; Torres, M.P.; JoverCerdá, M. Effects of dietary soybean oil concentration on growth, nutrient utilization and muscle fatty acid composition of gilthead sea bream (*Sparusaurata* L.). *Aquac. Res.* **2007**, *38*, 76–81. [[CrossRef](#)]
27. AOAC. *Official Methods of Analysis of the Association of Analytical Chemists International*; Association of Official Analytical Chemists: Arlington, VA, USA, 2005.
28. Moragues, M.E.; Esteban, J.; Ros-Lis, J.V.; Martínez-Máñez, R.; Marcos, M.D.; Martínez, M.; Soto, J.; Sancenón, F. Sensitive and selective chromogenic sensing of carbon monoxide via reversible axial CO coordination in binuclear rhodium complexes. *J. Am. Chem. Soc.* **2011**, *133*, 15762–15772. [[CrossRef](#)]
29. Zaragoza, P.; Fernández-Segovia, I.; Fuentes, A.; Vivancos, J.-L.; Ros-Lis, J.V.; Barat, J.M.; Martínez-Máñez, R. Monitorization of Atlantic salmon (*Salmo salar*) spoilage using an optoelectronic nose. *Sens. Actuators B Chem.* **2014**, *195*, 478–485. [[CrossRef](#)]
30. Vyncke, W. Direct determination of the thiobarbituric acid value in trichloroacetic acid extracts of fish as a measure of oxidative rancidity. *Fette Seifen Anstrichm.* **1970**, *72*, 1084–1087. [[CrossRef](#)]
31. Barat, J.M.; Gil, L.; García-Breijo, E.; Aristoy, M.-C.; Toldrá, F.; Martínez-Máñez, R.; Soto, J. Freshness monitoring of sea bream (*Sparusaurata*) with a potentiometric sensor. *Food Chem.* **2008**, *108*, 681–688. [[CrossRef](#)] [[PubMed](#)]
32. Fernández-Segovia, I.; Fuentes, A.; Aliño, M.; Masot, R.; Alcañiz, M.; Barat, J.M. Detection of frozen-thawed salmon (*Salmo salar*) by a rapid low-cost method. *J. Food Eng.* **2012**, *113*, 210–216. [[CrossRef](#)]
33. ISO. ISO 4833:2003—Microbiology of Food and Animal Feeding Stuffs—Horizontal Method for the Enumeration of Microorganisms—Colony-Count Technique at 30 Degrees C. Available online: <https://www.iso.org/standard/34524.html> (accessed on 26 March 2020).
34. Kaushik, S.J.; Coves, D.; Dutto, G.; Blanc, D. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchuslabrax*. *Aquaculture* **2004**, *230*, 391–404. [[CrossRef](#)]
35. Grigorakis, K.; Taylor, K.D.A.; Alexis, M.N. Seasonal patterns of spoilage of ice-stored cultured gilthead sea bream (*Sparusaurata*). *Food Chem.* **2003**, *81*, 263–268. [[CrossRef](#)]
36. Bagni, M.; Civitareale, C.; Priori, A.; Ballerini, A.; Finoia, M.; Brambilla, G.; Marino, G. Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (*Dicentrarchuslabrax*) and sea bream (*Sparusaurata*). *Aquaculture* **2007**, *263*, 52–60. [[CrossRef](#)]
37. Fuentes, A.; Masot, R.; Fernández-Segovia, I.; Ruiz-Rico, M.; Alcañiz, M.; Barat, J.M. Differentiation between fresh and frozen-thawed sea bream (*Sparusaurata*) using impedance spectroscopy techniques. *Innov. Food Sci. Emerg. Technol.* **2013**, *19*, 210–217. [[CrossRef](#)]
38. Dalgaard, P. *Freshness, Quality and Safety in Seafoods F-FE 380A/00 [mAY 2000]*; Danish Institute for Fisheries Research: Silkeborg, Denmark, 2000; ISBN 1841701106.
39. Connell, J.J. Method of assessing and selecting for quality. In *Controlfish Quality*, 4th ed.; Fishing News Books Ltd: Surrey, UK, 1975; pp. 157, 159–160.
40. Kamal-Eldin, A. Effect of fatty acids and tocopherols on the oxidative stability of vegetable oils. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 1051–1061. [[CrossRef](#)]
41. Adom, K.K.; Liu, R.H. Antioxidant activity of grains. *J. Agric. Food Chem.* **2002**, *50*, 6182–6187. [[CrossRef](#)] [[PubMed](#)]
42. Tyug, T.S.; Prasad, K.N.; Ismail, A. Antioxidant capacity, phenolics and isoflavones in soybean by-products. *Food Chem.* **2010**, *123*, 583–589. [[CrossRef](#)]
43. López-Bote, C.J.; Diez, A.; Corraze, G.; Arzel, J.; Alvarez, M.; Dias, J.; Kaushik, S.J.; Bautista, J.M. Dietary protein source affects the susceptibility to lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchuslabrax*) muscle. *Anim. Sci.* **2001**, *73*, 443–449. [[CrossRef](#)]
44. Hosseini, S.V.; Abedian-Kenari, A.; Rezaei, M.; Nazari, R.M.; Feás, X.; Rabbani, M. Influence of the in vivo addition of alpha-tocopheryl acetate with three lipid sources on the lipid oxidation and fatty acid composition of Beluga sturgeon, *Husohuso*, during frozen storage. *Food Chem.* **2010**, *118*, 341–348. [[CrossRef](#)]

45. Ng, W.-K.; Wang, Y.; Ketchimenin, P.; Yuen, K.-H. Replacement of dietary fish oil with palm fatty acid distillate elevates tocopherol and tocotrienol concentrations and increases oxidative stability in the muscle of African catfish, *Clarias gariepinus*. *Aquaculture* **2004**, *233*, 423–437. [[CrossRef](#)]
46. Regost, C.; Jakobsen, J.V.; Rørå, A.M.B. Flesh quality of raw and smoked fillets of Atlantic salmon as influenced by dietary oil sources and frozen storage. *Food Res. Int.* **2004**, *37*, 259–271. [[CrossRef](#)]
47. Wang, Y.; Yuen, K.-H.; Ng, W.-K. Deposition of tocotrienols and tocopherols in the tissues of red hybrid tilapia, *Oreochromis sp.*, fed a tocotrienol-rich fraction extracted from crude palm oil and its effect on lipid peroxidation. *Aquaculture* **2006**, *253*, 583–591. [[CrossRef](#)]
48. Alasalvar, C.; Taylor, K.D.A.; Öksüz, A.; Garthwaite, T.; Alexis, M.N.; Grigorakis, K. Freshness assessment of cultured sea bream (*Sparus aurata*) by chemical, physical and sensory methods. *Food Chem.* **2001**, *72*, 33–40. [[CrossRef](#)]
49. Surette, M.E.; Gill, T.A.; LeBlanc, P.J. Biochemical basis of postmortem nucleotide catabolism in cod (*Gadus morhua*) and its relationship to spoilage. *J. Agric. Food Chem.* **1988**, *36*, 19–22. [[CrossRef](#)]
50. Lougovois, V.P.; Kyranas, E.R.; Kyranas, V.R. Comparison of selected methods of assessing freshness quality and remaining storage life of iced gilthead sea bream (*Sparus aurata*). *Food Res. Int.* **2003**, *36*, 551–560. [[CrossRef](#)]
51. Anderson, M.D.R.P.; Vicente, C.Y.P. *Microbiología Alimentaria: Metodología Analítica para Alimentos y Bebidas*; Diaz de Santos: Madrid, Spain, 1999; ISBN 9788479784249.
52. Özyurt, G.; Kuley, E.; Özkütük, S.; Özogul, F. Sensory, microbiological and chemical assessment of the freshness of red mullet (*Mullus barbatus*) and goldband goatfish (*Upeneus moluccensis*) during storage in ice. *Food Chem.* **2009**, *114*, 505–510. [[CrossRef](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).