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Biotechnological treatment of microalgae enhances growth performance, hepatic carbohydrate metabolism and intestinal physiology in gilthead seabream (*Sparus aurata*) juveniles close to commercial size

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ABSTRACT

The aim of this work was to evaluate the effects on growth performance, intermediary metabolism and welfare of the inclusion of two commercial microalgae-based ingredients called LB-ChromaBream (LB-CB) and LB-ChromaBream-plus (LB-CBplus) in the diet of gilthead seabream (*Sparus aurata*) close to commercial size. For this purpose, fish of ~182 g of initial body mass were fed to satiety (ad libitum) for 41 days with three different diets: (i) CONTROL diet (CTRL), with a commercial-like formulation; (ii) LB-CB diet, with a 10 % inclusion of microalgal product; (iii) LB-CBplus diet, with a 10 % inclusion of the same product but enzymatically hydrolysed to increase the bioavailability of the nutrients. The results obtained show that the use of these microalgal products leads an overall improvement in productive parameters in terms of growth (15 % in SGR) and feed efficiency (11 %), as well as a significant reduction in circulating cortisol with the LB-CBplus diet. Observations on plasma and liver metabolites, and particularly on hepatic metabolic enzymes, collectively indicate that microalgae supplementation of feed lead to a better use of carbohydrates as a source of energy in the liver and other tissues, potentially sparing triglycerides within this tissue, and a channelling hepatic triglycerides to fuels growth. Finally, the specimens fed the supplemented diets experienced a substantial improvement in intestinal health, achieved by longer intestines, a higher transepithelial resistance and better apparent permeability measured by electrophysiological methods, especially those fed LB-CBplus, which could explain the increase in productive performance by improving nutrient assimilation. In conclusion, this study shows that the experimental feeds, especially the one containing biotechnologically treated microalgae, are suitable for improving some important indicators of growth performance and physiological condition of gilthead seabream, thus revealing the potential for their inclusion in new functional feeds for this species at an advanced stage of the production cycle.

1. Introduction

It is estimated that the world's population would be around 11 billion people in the year 2100 (UN, 2017). If we add to this a continuous increase in per capita fish consumption (e.g. from 9.0 kg in 1961 to 20.5 kg in 2017) (FAO, 2020), we obtain a huge demand for food, and fish in particular. In 2018, nearly 50 % of fish production came from aquaculture (FAO, 2020), highlighting the great importance of this activity. However, feeds used in aquaculture have traditionally been composed of fishmeal (FM) and fish oil (FO), especially for carnivorous species, making them incompatible with environmental and economic sustainability. Therefore, it is essential to look for feedstuff alternatives to

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achieve a feed production less dependent on extractive fishing, while maintaining a correct nutritional profile of farmed species to assure their growth and welfare.

Microalgae are considered suitable for aquaculture nutrition, as they are the basis of the feed of some cultured organisms such as bivalves, and necessary for larval and juvenile stages of some fish species (Brown et al., 1997; Muller-Feuga, 2000). These microorganisms are characterised by a relatively high content of both protein and polyunsaturated fatty acids (Renaud et al., 1994; Huerlimann et al., 2010), so they can be added to the diet producing positive results, such as improved feed efficiency and animal welfare (Perera et al., 2020), or protection of the intestinal mucosa, improving functions such as nutrient absorption and assimilation (Vizcaíno et al., 2018). A possible disadvantage in the use of microalgae is their thick cell wall, which can cause digestibility problems, but the use of biotechnological treatments such as enzymatic hydrolysis is promising to overcome this limitation (Agboola et al., 2019). Following this procedure, even low inclusion level of enzyme-hydrolysed microalgae might improve the physiological condition in fish in a manner similar to including higher amounts of raw microalgae in aquafeeds (Galafat et al., 2020, 2022). However, the economic viability of enzymatic hydrolysis still limited for facilitating its industrial up-scaling (Camacho et al., 2019), especially for raw ingredients included at high rate in feeds.

Yet, there is a growing interest in their use as functional feed additives. This alternative is supported by numerous studies showing that the use of microalgae not only does not produce detrimental effects on farmed fish in terms of welfare, but also that could be beneficial in several aspects such as feed efficiency (Perera et al., 2020), gut health (Jorge et al., 2019) or pigmentation (Galafat et al., 2020), which is particularly important for the consumer (Ribeiro et al., 2017). Another aspect of microalgae inclusion in fish feeds recently studied is its effect on feed quality, with both feed durability and pellet fat loss, being found to be improved in those fish including microalgae-derived products in their diets (Alcaraz et al., 2021).

Most of the studies in which the inclusion of microalgae has been tested have been performed in juveniles during the fattening stage. However, as the production of algal biomass on a large scale is still not highly profitable, its inclusion would significantly increase overall feed costs during this stage, which is extended for most species. Nevertheless, few studies have been carried out to test if juvenile specimens, close to commercial size as a proof of concept in a shot-feeding period, can obtain benefits from the use of microalgae in their feed during the last, and shorter, step of the farming cycle.

Particularly, *Arthrospira* sp. (Cyanobacteria) outstands for its high protein content, up to 70 % on dry matter basis, with amino acid profile comparable to those found in some reference feed proteins (Galafat et al., 2020). Thus, the dietary inclusion of *Arthrospira* was evaluated in gilthead seabream fry and juveniles with no negative effects on growth performance or nutrient utilisation, but even favourable impacts on fish physiology were described (Galafat et al., 2022). *Nannochloropsis* sp. is a marine microalga characterised by its richness in eicosapentanoic acid (EPA, C20:5n-3), pigments and other natural antioxidants, and its availability at industrial or semi-industrial scale makes this microalga a promising candidate as a commercial additive in gilthead seabream aquafeeds (Sales et al., 2021). This study builds on our recent microalgae aquafeeds research, where Galafat et al. (2020) included up to 4 % of *Asthrospira* sp. biomass, and Sáez et al. (2022) up to 5 % of *Nannochloropsis gaditana* in diets for gilthead seabream juveniles that achieved final weight, weight gain, percent weight gain and specific growth rate comparable to the reference diet containing fishmeal and fish oil. Owing to the reported effects produced by those microalgae, this piece of research goes a step beyond to evaluate the potential synergistic effects of a new aquafeed formula combining the protein-rich (>50 %) microalgal biomass (*Arthrospira* sp.) with another EPA-rich (up to 30 % of total fatty acids) marine microalga (*Nannochloropsis* sp.), raw and enzymatically hydrolysed, during a short dietary pulse in fish specimens with a body size close to the commercial standard.

Therefore, the aim of this work was to evaluate the effects of the inclusion of two commercial products composed by that blend of freshwater and marine microalgae, one of them obtained after a biotechnological treatment for increasing nutrients bioavailability in gilthead seabream (*Sparus aurata*) juveniles close to commercial size. Specifically, we studied the effects of a 10 % dietary inclusion of those microalgal blends on growth performance, nutrient utilisation and welfare after a 41-day feeding trial in this species since *S. aurata* is one of the main fish species farmed in Europe, especially in the Mediterranean region, where its production exceeded 250 thousand tonnes in 2019 (APROMAR, 2020).

2. Material and methods

2.1. Animal maintenance and Ethics

Gilthead seabream (*S. aurata*) juveniles were provided by a commercial source (CUPIBAR, Chiclana de la Frontera, Cádiz) and acclimated to the indoor experimental facilities at the *Servicios Centrales de Investigacion en Cultivos Marinos* (SCI-CM, CASEM, University of Cadiz, Puerto Real, Cadiz, Spain; Spanish Operational Code REGA ES11028000312) with seawater in controlled conditions of salinity (36 ppt), temperature (19 °C), and under natural photoperiod at our latitude (36°31′45″ N, 6°11′31″ W, from January to May 2020). All experimental procedures were done following the guidelines for experimental procedures in animal research of the Ethics and Animal Welfare Committee of the University of Cadiz, according to the principles published in the European Animal Directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) for the protection of animals used in scientific experiments. In addition, the Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 16/16/2019/170).

2.2. Experimental diets

Three isonitrogenous and isolipidic experimental diets were formulated with a composition mimicking that of commercial aquafeeds for gilthead seabream at the CEIA$_3$-Universidad de Almería facilities (*Servicio de Piensos Experimentales*, http://www.ual.es/stecnicos_spe) (Table 1). This formulation constituted the control diet (CTRL). In addition, two commercial microalgae-based products were added at 10 %, constituting two supplemented diets named as LB-ChromaBream (LB-CB) and LB-ChromaBream-plus (LB-CBplus). These compounds were provided by *LifeBioencapsulation* S.L. (Almería, Spain) being freeze-dried concentrated products containing 800 g kg^{-1} of a blend of microalgae (LB-CB) or the same blend of microalgae but enzymatically hydrolysed (LB-CBplus). The microalgal blend was composed by 70 % *Arthrospira* sp. and 30 % *Nannochloropsis* sp. biomass. LB-CBplus was produced by enzymatic hydrolysis with a mixture of commercial proteases (Alcalase 2.4 L® and Flavourzyme 1000 L® from Novozymes A/S, Bagsvaerd, Denmark) under controlled conditions until free amino acids concentration reached a final value higher than 25 g L-leucine equivalents per 100 g protein. Following hydrolysis, the hydrolysate was heated at 80 °C for 15 min for inactivating the proteolytic enzymes. Chemical composition of both products was similar (47.5 % crude protein and 12.4 % crude lipid), but free amino acid content was lower in LB-CB (32.3 ± 1.9 g leucine equivalents 100 g^{-1} protein) compared to LB-CBplus (82.6 ± 2.3 g leucine equivalents 100 g^{-1}). Free glucose equivalents in both products never reached values higher than 5 g 100 g^{-1} biomass. Briefly, feed ingredients and microalgal additives were mixed together, and then water was added to the mixture (up to 300 g kg^{-1}) to make up homogeneous dough in a vertical helix ribbon mixer (Sammic BM-10, Sammic, Azpeitia, Spain). The dough was passed through a two screw laboratory extruder (Evolum 25 Clextral, France) to obtain 5 mm-diameter pellets. The extruder barrel consisted of four sections and the

Table 1
Ingredient composition and proximate composition (% on dry matter basis) of the experimental diets. CTRL: control; LB-CB: LB-ChromaBream; LB-CBplus: LB-ChromaBream plus.

Ingredients (% dry matter, DM)	CTRL	LB-CB	LB-CBplus
Fishmeal LT94[1]	15.0	15.0	15.0
Lysine[2]	1.2	1.2	1.2
Methionine[2]	0.5	0.5	0.5
Squid meal[3]	1.0	1.0	1.0
Fishmeal hydrolysate[4]	0.5	0.5	0.5
Microalgal product[5]		10.0	10.0
Wheat gluten[6]	15.0	13.0	13.0
Soybean protein concentrate[7]	35.0	33.0	33.0
Fish oil[8]	5.0	4.5	4.5
Soybean oil[9]	8.0	7.2	7.2
Soybean lecithin[10]	1.0	1.0	1.0
Wheat meal[11]	12.7	8.0	8.0
Choline chloride[12]	0.5	0.5	0.5
Betain[13]	0.5	0.5	0.5
Vitamin and mineral premix[14]	2.0	2.0	2.0
Vitamin C[15]	0.1	0.1	0.1
Guar gum[16]	2.0	2.0	2.0
Proximate composition (% DM)			
Crude protein	44.3	44.6	44.0
Crude lipid	14.3	14.6	13.9
Ash	6.0	5.8	5.9

[1](protein: 69.4 %; lipid: 12.3 %), Norsildemel (Bergen, Norway); [2]Suysegala (Sevilla, Spain); [3]Bacarel (UK); [4]CPSP (Sopropeche, France); [5]Life-bioencapsulation SL (Almería, Spain); [6](protein: 76.0 %; lipid: 1.9 %), Lorca Nutricion Animal (Murcia, Spain); [7](protein: 50.0 %; lipid: 1.0 %) Lorca Nutricion Animal (Murcia, Spain); [8]AFAMPES 117DHA (AFAMSA, Spain); [9]Aceites el Niño (Málaga, Spain); [10]Lecico P700 (Lecico GmbH, DE); [11](protein: 12.0 %; lipid: 2.0 %), local provider; [12,13]Sigma-Aldrich (Madrid, Spain); [14,15]Vitamin & Mineral Premix: Vitamins (IU or mg kg^{-1} premix): vitamin A (retinyl acetate), 2000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E, 10,000 mg; vitamin K_3 (menadione sodium bisulphite), 2500 mg; vitamin B1(thiamine hydrochloride), 3000 mg; vitamin B_2 (riboflavin), 3000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2000 mg; vitamin B9 (folic acid), 1500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (LifeBioencapsulation premix); [16]EPSA (Sevilla, Spain).

temperature profile in each section (from inlet to outlet) was 90, 92, 95, and 105 °C, respectively. The feeds were dried in a 12 m^3 drying chamber with forced-air circulation (Airfrio, Almería, Spain) at 30 ºC for 24 h, and stored at −20 ºC until use. Proximate analysis (dry matter, ash, and crude protein, N × 6.25) of feeds were determined according to official procedures (AOAC, 2002). Lipids were extracted following (Folch et al., 1957) methodology using chloroform/methanol (2:1 v/v) as solvent, and total lipid content was calculated gravimetrically.

2.3. Feeding protocol and sampling procedures

Three different treatments, corresponding to the three experimental diets, were applied over a period of 41 days, using a total of 108 specimens with an initial mean body mass of 182.5 ± 1.8 g. The animals were individually weighed and randomly distributed in 9 tanks of 400 L capacity (n = 12 fish/tank; initial stocking density 5.47 ± 0.06 kg/m^3) in the SCI-CM and were kept during the whole experimental period (May 28th to July 8th, 2020) in an open circulatory system as described above. Prior to offer the experimental diets, fish were acclimated to the experimental units for 10 days (May 17th to May 27th), and then feeding was supplied in 1 daily dose (10:30 a.m.) until apparent satiety (ad libitum), ensuring that the amount offered in each experimental unit was fully ingested. The feeding test was carried out blindly, in such a way that the three aquafeeds were labelled with different colours but with no reference to its composition, eliminating any source of subjectivity when feeding the animals. No mortality was recorded in any experimental group.

At the end of the 41-days feeding trial, a final sampling was done, in which 12 overnight fasted specimens from each experimental diet (4 fish/tank) were randomly selected, deeply anaesthetised with a lethal dose of 2-phenoxyethanol, and then individually weighed and measured. Blood was drawn from the caudal vessels with heparinised syringes and centrifuged at 13,000 × *g* for 20 min at 4 ºC to obtain plasma samples. Fish were then cervically sectioned in order to obtain biopsies of different tissues. Complete livers, as well as perivisceral fat, were also removed and weighed from each specimen. The entire intestine was removed for length measurement from the pyloric caeca to the rectum. Both plasma samples and liver biopsies were then snap-frozen in liquid nitrogen and stored at −80 °C until further biochemical analysis. For electrophysiological analyses, four additional fish from each experimental tank (12 fish/diet) were sampled to obtain intestine biopsies and be mounted as described below. Finally, the remaining four fish of each experimental unit were also weighted and measured to obtain the growth performance and biometric parameters described below for the total of animals assayed.

2.4. Growth performance and biometric parameters

The following growth parameters were evaluated: (i) specific growth rate (SGR) = (100 × (ln final body weight - ln initial body weight)/days; (ii) weight gain (WG) = (100 × (body weigh increase)/initial body weight; (iii) feed efficiency (FE) = weight gain/total feed intake; and (iv) condition factor (K) = (100 × body weight)/fork length^3.

Organosomatic indexes calculated as the ratio of tissue to body weight or fork length were determined for liver, perivisceral fat and intestine. They were estimated in accordance with the following equations: (i) Hepatosomatic index (HSI) = (100 × liver weight)/fish weight; and (ii) Intestine length index (ILI) = (100 × L_i)/L_b, where L_i and L_b are the intestine and fork body length, respectively; and (iii) Mesenteric index (MSI) = (100 × perivisceral fat weight)/fish weight.

2.5. Biochemical parameters of the plasma

Using commercial kits (SpinReact SA, St. Esteve d'en Bas, Girona, Spain), with reactions adapted to 96-well microplates, the metabolic parameters were spectrophotometrically analysed in each plasma sample, including the levels of glucose (Glucose-HK Ref. 13 1001200), lactate (Lactate Ref. 1001330), cholesterol (Cholesterol-LQ Ref. 41021) and triglycerides (TAG Ref. 1001311). Plasma total protein concentration was determined with the bicinchoninic acid method using the commercial BCA kit (BCA™ Protein assay kit, Pierce, Rockford, USA). Plasma cortisol levels were measured with the commercial Cortisol Enzyme Immunoassay Kit (Arbor Assays, K003-H1W) according to the manufacturer's indications. In short, plasma samples were 100-times diluted in the specific assay buffer after their reactions with the dissociation reagent. Then samples and standards were plated, in duplicate, with the specific monoclonal antibody and cortisol-peroxidase conjugate to produce the binding reaction. After 1 h of incubation, the plate was washed and substrate added to reacts with the bound cortisol-peroxidase conjugate, and then incubated and read at 450 nm. The limit of detection of the assay was 0.26 ng/mL with intra- and inter-assay coefficients of variation lower than 2 % and 3 %. All assays were performed with a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA), controlled by KCjunior Software for Microsoft® Windows.

2.6. Biochemical parameters of the liver

Frozen biopsies used for the assay of metabolites were mechanically

homogenised (blender) in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralised using 1 M KCO_3, centrifuged (30 min, 3220g and 4ºC), and then supernatants isolated to determine tissue metabolites. Tissue triglycerides levels were determined spectrophotometrically with a commercial kit (SpinReact, see above). Tissue glycogen concentration was quantified using the method described from (Keppler and Decker, 1974), where glucose obtained after glycogen breakdown with amyloglucosidase (Sigma-Aldrich, Ref. A7420) was determined with a commercial kit (SpinReact) as described before.

2.7. Activity of metabolic enzymes in liver

Frozen tissues for enzyme activity assays were homogenised by ultrasonic disruption in 10 volumes of ice-cold homogenisation buffer (50 mM imidazole, 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM sucrose; pH 7.5). Homogenates were centrifuged for 30 min at 3220 × *g* and 4ºC, and supernatants stored at −80 ºC for further analysis. The assays for the activity of several enzymes involved in glycogenolysis (GPase: glycogen phosphorylase, EC 2.4.1.1), glycolysis (HK: hexokinase, EC 2.7.1.1; PK: pyruvate kinase, EC 2.7.1.40), gluconeogenesis (LDH: lactate dehydrogenase, EC 1.1.1.27; FBP: fructose 1,6-bisphosphatase, EC 3.1.3.11), and lipid metabolism (HOAD: 3-hydroxyacyl-CoA dehydrogenase, EC 1.1.1.35) were performed as previously described for *S. aurata* tissues (Sangiao-Alvarellos et al., 2005, 2006; Vargas-Chacoff et al., 2016). Enzyme activities were determined using a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA), controlled by KCjunior Software for Microsoft® Windows. Activities were expressed as specific activities per mg of protein in the homogenate (U·mg $prot^{-1}$). Proteins were assayed in duplicate, as described above for plasma samples.

2.8. Voltage clamp in Ussing chambers

The anterior intestine was isolated and mounted as previously described (Estensoro et al., 2016) on a tissue holder of 0.25 cm^2, and positioned between two half-chambers containing 2 mL of physiological saline (160 mmol L^{-1} NaCl, 1 mmol L^{-1} $MgSO_4$, 2 mmol L^{-1} NaH_2PO_4, 1.5 mmol L^{-1} $CaCl_2$, 5 mmol L^{-1} $NaHCO_3$, 3 mmol L^{-1}KCl, 5.5 mmol L^{-1} glucose and 5 mmol L^{-1}Hepes, pH 7.800). During the experiments the tissue was bilaterally gassed with 0.3 % CO_2 + 99.7 % O_2 and the temperature was maintained at 19 °C to mimic stocking conditions. Short-circuit current (Isc, μAcm^{-2}) was monitored by clamping the epithelia to 0 mV. Voltage clamping and current injections were performed by means of a VCC MC8 voltage clamp amplifier (Physiologic Instruments, San Diego, USA). Bioelectrical parameters for each tissue were recorded continuously onto Labscribe3 running in a MacIntosh computer using an IWorx188 data acquisition system, from the time of mounting for a period of 90 min. Epithelial resistance (Rt, Ω.cm^2) was manually calculated (Ohm's law) using the current deflections induced by a bilateral ± 1 mV pulse of 3 s every minute. The apical side of the preparation was considered as the ground. Therefore, negative currents are absorptive, while secretory currents are positive.

2.9. Permeability assay

After 20 min of tissue stabilisation, the saline was replaced for fresh well gassed solution to a final volume of 2 mL per chamber. Enough FITC-dextran (average mol wt. 4000, Sigma, Madrid) prepared as a concentrated stock of 1 mg/mL was added to final concentrations of 0.5 mg/mL to the apical chamber and a sample (0.1 mL) collected from either the apical or basolateral compartments after 5 min of mixing to establish time zero. After exactly 1-hour, new samples from both the donor and receiver compartments were collected to fresh vials. Fluoresce measurements were performed using a Multi-Mode Microplate Reader BioTekSynergyTM 4 (BioTek® Instruments, Winooski, VT, USA) set for excitation wavelength at 492 nm and emission wavelength at 520 nm. The apparent permeability (Papp) was estimated using the equation: Papp= (V*dC)/(A*C0*dT), where Papp is the permeability in centimetres per second, V is the volume of the receiver chamber, A is the surface area of the tissue in square centimetres, C0 is the starting concentration in the donor compartment (apical) and dC/dT is the rate of concentration change (ng/sec) of FITC in the receiving chamber (basolateral).

2.10. Statistical analyses

All results are expressed as the mean ± SEM (standard error of the mean). Data on feed intake and growth indexes are the mean ± SEM of triplicate tanks, whereas data on somatic indexes are the mean ± SEM of 12 fish, whereas data on body mass are represented by the mean ± SEM of 36 fish. All data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests, respectively, with $p < 0.05$. Differences among treatments (CTRL, LB-CB, LB-CBplus) in all parameters were analysed by one-way analysis of variance (ANOVA, $p < 0.05$). In those parameters where statistically significant differences were detected between groups (level of significance at $p < 0.05$), the Tukey's test was carried out. The software package GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, US) was used for all tests performed and generated figures.

3. Results

3.1. Growth performance and biometric parameters

No mortality occurred during the experiment. The starting weight of the specimens was the same for the three experimental groups, growing allometrically ($1.5 < K < 2.5$) up to 233.0 g on the control diet, 240.8 g on the LB-CB diet and 247.7 g on the LB-CBplus diet (Table 2), with

Table 2
Growth performance and somatic indexes of gilthead seabream juveniles close to commercial size fed to visual satiety from May 28th to July 8th, 2020, with a control diet and two supplemented diets with 10 % of the LB-CB and LB-CBplus microalgae products. Data on feed intake and growth indexes are the mean ± SEM of triplicate tanks. Data on somatic indexes are the mean ± SEM of 12 fish, whereas data on initial body mass are the mean ± SEM of 36 fish. Different superscript letters in each row indicate significant differences among dietary treatments based on one-way ANOVA and Tukey's test ($p < 0.05$). CTRL: control; LB-CB: LB-ChromaBream; LB-CBplus: LB-ChromaBream-plus.

	CTRL	LB-CB	LB-CBplus	*p*[a]
Initial body mass (g)	182.50 ± 0.16	182.60 ± 0.30	182.60 ± 0.17	0.956
Final body mass (g)	233.0 ± 0.66^{a}	240.8 ± 3.20^{ab}	247.7 ± 2.42^{b}	0.013
Kb	2.06 ± 0.03	2.00 ± 0.02	2.01 ± 0.03	0.169
WG (%)c	27.65 ± 0.47^{a}	31.89 ± 1.54^{ab}	35.63 ± 1.20^{b}	0.008
SGR (%)d	0.59 ± 0.01^{a}	0.67 ± 0.03^{ab}	0.74 ± 0.02^{b}	0.008
FIe	78.65 ± 0.89^{a}	80.48 ± 1.44^{a}	87.06 ± 0.52^{b}	0.003
FEf	0.64 ± 0.02^{a}	0.72 ± 0.03^{ab}	0.75 ± 0.02^{b}	0.047
HSI (%)g	1.32 ± 0.05	1.21 ± 0.04	1.25 ± 0.08	0.445
MSI (%)h	1.15 ± 0.17	1.57 ± 0.22	1.24 ± 0.11	0.216
ILI (%)i	74.07 ± 3.15^{a}	88.44 ± 3.18^{b}	85.70 ± 2.35^{b}	0.003

[a] Values resulting from one-way analysis of variance.
[b] Condition factor= (100 ×body weight)/fork length^3.
[c] Weight gain (%)= (100 ×(body weigh increase)/initial body weight.
[d] Specific growth rate= 100 × (ln final body weight − ln initial body weight)/days.
[e] Feed Intake= (grams of aquafeed consumed/tank)/week.
[f] Feed Efficiency=weight gain/total feed intake.
[g] Hepatosomatic index= (100 ×liver weight)/fish weight.
[h] Mesenteric index= (100 ×perivisceral fat weight)/fish weight.
[i] Intestine length index= (100 ×intestine length)/fork length.

significantly higher weight gain (WG) of specimens fed the LB-CBplus diet when compared to those fed the control diet. Animals with the LB-CB diet obtained intermediate weight gains to both groups. In addition, specific growth rates (SGR) were statistically higher in fish fed the LB-CBplus diet compared to the CTRL group, with the LB-CB diet having values between the others fish. The same pattern was also produced in Feed Intake and Feed Efficiency (FE) (Table 2). No significant differences were found in the hepatosomatic (HSI) and mesenteric (MSI) indices; however, there was a significant increase in the intestine length index (ILI) in fish fed the diets supplemented with microalgae, being higher in both supplemented diets (LB-CB and LB-CBplus) respect to the CTRL diet (Table 2).

3.2. Blood and tissue biochemistry

Results on plasma and hepatic parameters are shown in Table 3. Plasma samples revealed no significant differences in circulating protein and plasma glucose levels. However, a significant increase in plasma lactate levels, concomitantly found with a significant decrease in triglyceride (TAG) and cholesterol levels were found in fish fed the LB-CB diet compared to those fed the control and the LB-CBplus diet. In addition, significantly higher cortisol levels were observed in the LB-CB group compared to LB-CBplus fish.

In the liver, no effect of dietary supplementation on glucose content was found. However, statistically differences were obtained for glycogen and TAG, both showing a decrease in their storage levels in fish fed with the LB-CB microalgae compared to those fed with the CTRL diet, whereas animals fed with the LB-CBplus supplemented diet showed intermediate values.

3.3. Metabolic enzymes

The effects of dietary supplementation on the activity of several metabolic enzymes related to glycogenolysis, glycolysis, gluconeogenesis and lipid metabolism was also evaluated (Fig. 1). No significant differences were found in the liver activity of HOAD enzyme, although a slight decrease in this activity was observed in fish fed both microalgae-supplemented diets. GPase activity showed a significant increase with the ingestion of the LB-CB diet compared to the CTRL group, with intermediate levels of this enzyme activity in fish ingesting the LB-CBplus diet. For the gluconeogenic enzyme FBP, the opposite pattern was observed, with the lowest values detected in the LB-CB group. Hepatic HK and PK activities were significantly increased in fish fed with both microalgae-supplemented diets compared to the CTRL fish. LDH enzyme activity showed a significant increase in the LB-CB diet compared to both the CTRL and the LB-CBplus diets.

Table 3
Blood and liver biochemistry of gilthead seabream juveniles close to commercial size fed to visual satiety from May 28th to July 8th, 2020, with a control diet and two supplemented diets with 10 % of the LB-CB and LB-CBplus microalgae products. Data are the mean ± SEM of 12 fish. Further information, as described in legend of Table 1.

	CTRL	LB-CB	LB-CBplus	p^1
Plasma glucose (mM)	4.59 ± 0.06	4.55 ± 0.09	4.54 ± 0.12	0.908
Plasma lactate (mM)	3.34 ± 0.09^{a}	3.76 ± 0.07^{b}	3.42 ± 0.08^{a}	0.001
Plasma triglycerides (mM)	4.62 ± 0.19^{b}	3.73 ± 0.13^{a}	4.42 ± 0.09^{b}	0.001
Plasma proteins (mg mL^{-1})	77.18 ± 3.15	78.28 ± 3.73	80.43 ± 5.02	0.846
Plasma cholesterol (mg dL^{-1})	346.6 ± 14.6^{b}	294.8 ± 8.4^{a}	388.1 ± 7.7^{b}	0.004
Plasma cortisol (ng mL^{-1})	18.44 ± 3.34^{ab}	27.07 ± 2.89^{b}	16.74 ± 2.06^{a}	0.031
Hepatic glucose (mg gww^{-1})	67.60 ± 1.58	66.90 ± 1.44	65.01 ± 1.25	0.423
Hepatic glycogen (mg gww^{-1})	192.4 ± 7.1^{a}	166.2 ± 4.8^{b}	172.9 ± 6.7^{ab}	0.016
Hepatic triglycerides (mg gww^{-1})	33.81 ± 1.08^{a}	29.41 ± 0.98^{b}	31.7 ± 1.35^{ab}	0.036

3.4. Voltage clamp in Ussing chambers

Electrophysiological analysis of the intestine showed no significant differences in the short-circuit current (Isc) for any of the experimental groups, with Isc values < 0 (Fig. 2A). However, there were significant differences in the epithelial resistance (Rt) among the three experimental groups, being the LB-CBplus group the one with the highest values and the CTRL group the one with the lowest values (Fig. 2B).

3.5. Permeability assay

Intestinal permeability assays showed significant differences in the apparent intestinal permeability (Papp), with the group supplemented with LB-CBplus showing a significant decrease in Papp compared to the CTRL group, and the LB-CB fed fish having intermediate Papp values compared to the other treatments (Fig. 2C).

4. Discussion

As shown in previous studies, the use of microalgae in fish feeds is beneficial even with at low or very low inclusion levels, and the results obtained by Perera et al. (2020) provide evidence for *Sparus aurata* at the juvenile stage. For example, it was found that the incorporation of microalgae in functional feeds for juvenile *S. aurata* produces an increase in feed efficiency from 7.4 % to 13.5 % and reduces the amount of feed used by 148 kg per tonne of farmed fish, which translates into a positive balance for cost saving during the production cycle (Perera et al., 2020). In the present study, we tested whether the inclusion of microalgae at the last stage of cultivation, closer to the commercial size for this fish species, could be also considered to obtain the well-known benefits of these microorganisms in terms of growth performance, intermediary metabolism and welfare, with a moderate increase in feed cost (Table 4). The use of LB-CB and LB-CBplus would reduce 174 kg and 230 kg, respectively, the amount of feed requested for increasing one ton of fish biomass, but the feed cost increases up to 0.50 €/kg during the supplementation period. However, the positive effects observed on metabolic and physiological status of fish could justify the dietary use of those products during a short pulse administration (up to 40 days).

In terms of growth performance, this study shows that the microalgae inclusion, especially with those cells previously treated by biotechnological processes (LB-CBplus) significantly promoted weight gain (WG) of the animals, which is also supported by the improved specific growth rate (SGR) and feed efficiency (FE) obtained. Moreover, FE increased even when a slight enhance in the feed intake was observed in fish from the LB-CBplus group. This corroborates that the higher growth of these fish is not only due to a higher feed intake, but also to the fact that the nutrients contained in the aquafeed are more easily assimilated when biotechnologically treated. The increase in feed intake of LB-CBplus supplemented diet may also indicate some difference in the palatability of the feed, or in other appetite-related issues that will require further investigations. These results are in accordance with a previous study, where gilthead seabream juveniles also obtained higher WG, SGR and final mean body mass when fed with a diet including a 20 % of *Scenedesmus almeriensis* compared to a control diet with a commercial composition (Vizcaíno et al., 2014), and in juvenile fish of the same species fed with low (1 %) or very low (0.5 %) doses of different microalgae extracts, where a clear improvement of FE was also observed (Perera et al., 2020). These observations indicate that *S. aurata,* at different size/body mass, may benefit from diets including microalgae at different proportions in their formulation. Moreover, different treatments to weaken the cell wall of microalgae such as *Nannochloropsis*

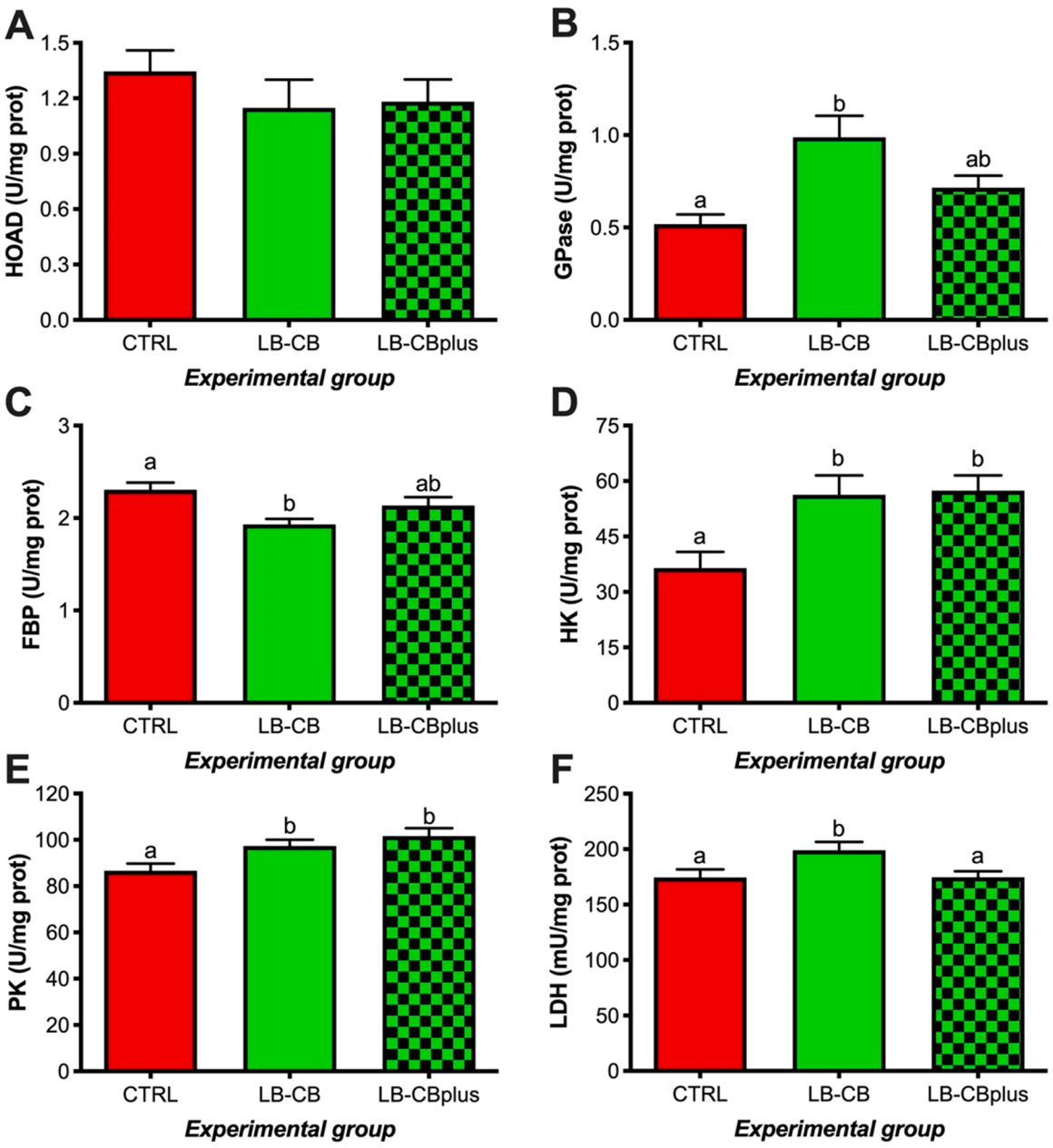

Fig. 1. Specific activity (U mg protein^{-1}) of metabolic enzimes in the liver of gilthead seabream juveniles fed to visual satiety from May 28th to July 8th, 2020 (41 days). Data are the mean ± SEM of 12 fish. HOAD: 3-hydroxyacyl-CoA dehydrogenase (A), GPase: glycogen phosphorylase (B), FBP: fructose 1,6-bisphosphatase (C), HK: hexokinase (D), PK: piruvate kinase (E), LDH: lactate dehydrogenase (F). Different letters in each panel mean statistical differences after one-way ANOVA and Tukey test ($p < 0.05$). CTRL: control; LB-CB: LB-ChromaBream; LB-CBplus: LB-ChromaBream-plus.

gaditana, allow to increase its bioavailability, showing better results in the growth performance of the African catfish (*Clarias gariepinus*) (Agboola et al., 2019), while others such as the diatom *Phaeodactylum tricornutum* did not produce benefits in terms of final body mass in *S. aurata*, although induced an immunostimulatory effect (Reis et al., 2021). Indeed, the main change observed in the present study after treating microalgae with commercial enzymes was a significant increase in the content of total free amino acids in the soluble protein fraction in LB-CBplus. This product contains almost three times higher level of free amino acids than that found in the raw microalgae-based product. This finding represents higher amino acid bioavailability in the hydrolysate, which probably explain the higher growth performance obtained in LB-CBplus-fed fish. As a whole, results indicate that biotechnological treatments of microalgae are good options for obtaining the maximum potential of the nutrients contained in them. In addition, neither LB-CB nor LB-CBplus negatively affected the allometric growth of fish, as the condition factor (K) was unaltered, with values of around 2 in all experimental groups. These values are within the normal range for sea bream (Ortega, 2008). Furthermore, the absence of significant differences in the MSI and HSI compared to the control diet indicates that there was no additional accumulation of hepatic or perivisceral fat, suggesting that final mean weight reached is associated with fillet yield and no with higher perivisceral fat accumulation. Moreover, both products increased the length of the intestine and presumably their absorptive capacity (Perera et al., 2020), which can be the reason for the increased feed efficiency observed. In this sense, the inclusion of vegetal components is often detrimental to the intestinal health, since carnivorous species, as the sea bream, will require to potentiate morphological or physiological adaptations for a better processing and assimilation of nutrients. However, here we show that the inclusion of microalgae

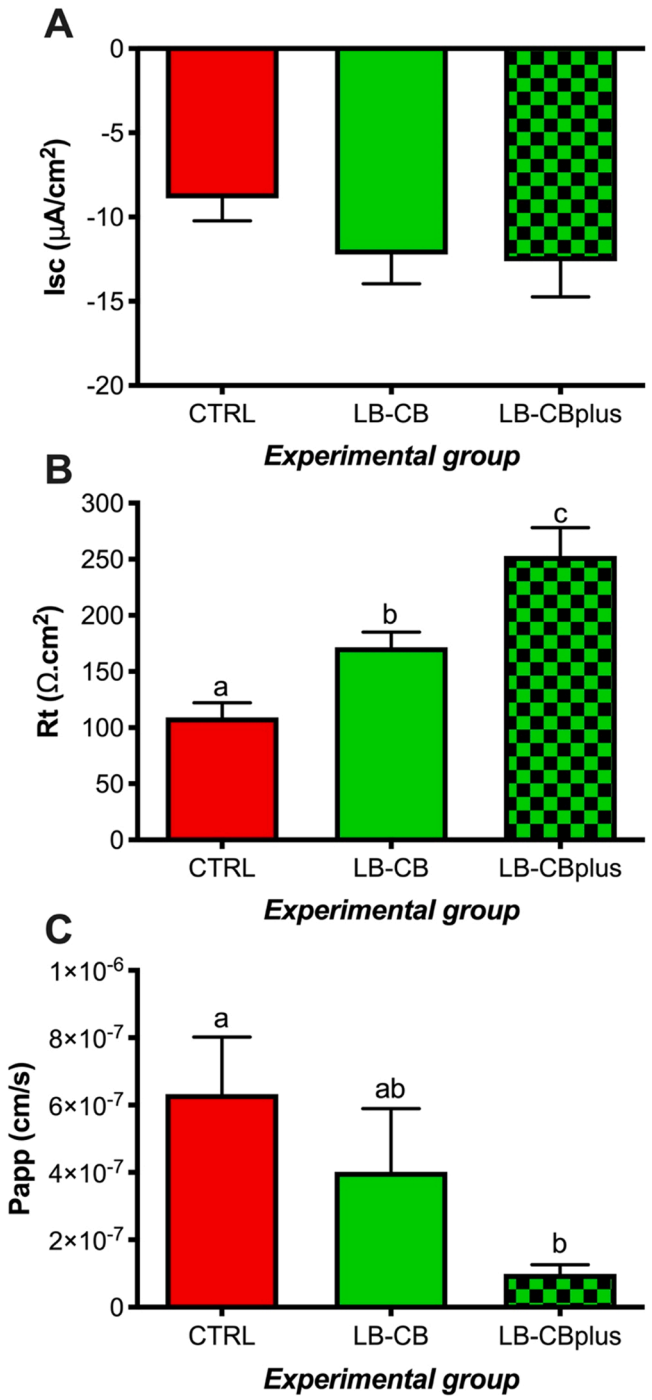

Fig. 2. Electrophysiological response and intestinal apparent permeability of juvenile gilthead sea breams fed to visual satiety from May 28th to July 8th, 2020 (41 days). Data are the mean ± SEM of 12 fish. Short-circuit current (Isc, A), Epithelial resistance (Rt, B), Apparent permeability (Papp, C). Further information, as described in legend of Fig. 1.

benefits intestinal health in sea bream, even when included as whole cells. Tissue resistance, the gold standard to test selectivity and integrity of epithelia, was increased due to the addition of both microalgae blends. Moreover, apparent tissue permeability, concomitantly with the elongation of intestine, responds accordingly and shows reduced values when compared to the control. The increased resistance and reduced permeability of the intestine are known factors of an improved intestinal physiology in fish (Estensoro et al., 2016; Sitjà-Bobadilla et al., 2019).

In terms of intermediary metabolism, proteins are used as a primary

Table 4
Estimation of using microalgae-based products on the feed saving and global balance of feed costs for producing one ton of farmed fish fed with a control diet and two supplemented diets with 10 % of the LB-CB and LB-CBplus microalgae products.

	CTRL	LB-CB	LB-CBplus
Feed efficiency (kg fish·kg feed^{-1})	0.64	0.72	0.75
Total feed (kg feed·ton fish^{-1})	1563	1389	1333
Feed saving (kg feed·ton fish^{-1})		174	230
Cost feed saving (€·ton fish^{-1})[1]		261	345
Product use (kg ton fish^{-1})		139	133
Product cost (€ ton fish^{-1})[2]		695	798
Balance (€·ton fish^{-1})[3]		556	665
Feed cost increase (€ kg feed^{-1})		0.40	0.50

[1]For the estimation it has been considered a value of 1.5€ kg feed^{-1}
[2]Estimated product cost: LB-CB (5 € kg^{-1}) and LB-CBplus (6 € kg^{-1})
[3]Estimated as *cost feed saving* (€ ton fish^{-1}) – *product cost* (€ ton fish^{-1}).

energy source (Mommsen and Walsh, 1992) and a source of amino acids for the formation of hormones and enzymes, as well as for tissue growth and turnover (De la Higuera, Cardenete, 1993). In spite of LB-CBplus-fed fish showed the highest mean value, no differences in plasma protein concentration were detected between experimental groups pointing to a homeostatic load at circulating levels, regardless of the experimental feed. In addition, plasma glucose did not differ among treatments, which also indicate that homeostatic mechanisms operate in the three groups to keep plasma glucose around normal values for the species (see Martos-Sitcha et al., 2013, 2014; Skrzynska et al., 2017, 2018). However, different results were obtained for plasma lactate and triglycerides (TAG), showing an inverse pattern. Lactate is synthesised by the anaerobic pathway in the white muscle of the fish (Palomares, 2009) and its formation is lowered when the organism has available metabolites that can be used aerobically by the mitochondria. Therefore, the increase of plasma lactate in fish fed diets supplemented with LB-CB strongly suggests that different effects are produced on the intermediary metabolism of animals depending on whether the microalgae are biotechnologically treated or not. The proteolysis of microalgal biomass can release inner bioactive compound with additional functional activities. For instance, Sáez et al. (2022) evidenced that enzymatically-treated *N. gaditana* was responsible for a trend towards increased in vivo antioxidant effects on muscle lipids compared to untreated microalgal biomass in gilthead seabream juveniles. These authors suggested that increased release and further bioavailability of some bioactive compounds contained in the cells might have occur, as was confirmed in that study for total phenolics compounds. Apparently, microalgae favour oxidative over anaerobic metabolism when they are enzymatically hydrolysed. This may be related to a higher bioavailability of fatty acid for oxidation, which merits further investigation. Other study in sea bream juveniles also report a decrease in plasma lactate when using diets supplemented with microalgae extracts (Perera et al., 2020). In the case of TAG, they are one of the main energy sources when sufficient oxygen is available for their aerobic use in the mitochondria (Tocher, 2003). The decrease of plasma triglycerides in fish fed with the diet supplemented with LB-CB may be related to a lower bioaccessibility (i.e., fatty acids contained in raw microalgae) of untreated microalgae in the intestine of fish. Also, the decrease in plasma TAG may be the result of a higher use of triglycerides as energy substrate in the LB-CB group, though this is unlikely to occur, at least in muscle, given the increase in plasma lactate observed with this diet. Our results for cholesterol are also consistent with the effects observed in zebrafish, where the higher concentration of microalgae meal tested in feed resulted in a significant decrease in plasma cholesterol (Carneiro et al., 2020), that would support the importance of microalgae pre-treatment to promote release, bioavailability and absorption of some key nutrients from microalgae into the bloodstream. Microalgae-supplemented diets, either raw or hydrolysed, enriched the same fatty acids the

experimental diets, but it is unclear if quantitative or qualitative changes in muscle lipid content may occur as consequence of the enzymatic pre-treatment. Further studies aimed at fully understanding the intrinsic mechanisms of lipid metabolism are needed.

The contrasting trends for plasma TAG and lactate indicate a shift in the energy substrate preferentially used, and thus an overall change in the relative contribution of anaerobic metabolism and TAG oxidation. An additional indication of this change comes from the levels of plasma cortisol, which increased in fish fed with LB-CB. This increase is known to result in higher energy expenditure and lower growth rate (Jerez-Cepa et al., 2019). In addition, dietary fatty acids are known to play an important role in regulating cortisol release in fish (Ganga et al., 2006). For example, in gilthead seabream, dietary deficiencies in n-3 HUFA raised the basal plasma cortisol levels and altered the pattern of cortisol release after stress (Montero et al., 1998). In our study, lower bioavailability of some essential fatty acids may have occurred in fish fed with non-biotechnologically treated microalgae, raising the basal levels of cortisol and promoting changes in intermediary metabolism. Conversely, low cortisol levels can stimulate protein synthesis, leading to better somatic growth (Van Der Boon et al., 1991). Thus, increasing the bioavailability of fatty acids contained in microalgae by the biotechnological treatment used could be useful to manage cortisol release. However, cortisol release to the plasma is modulated by many other external factors. Therefore, further studies are needed to test whether the effect of decreasing plasma cortisol in response to microalgae containing feeds are useful under different routine aquaculture situations such as increased stocking density, exposure to air during handling or sorting, among others potentially stressful conditions.

Regarding liver metabolites, no significant differences were observed in free glucose among the experimental groups, but hepatic glycogen and TAG levels showed a decrease in the microalgae-fed fish when compared to control diets, being more evident with the LB-CB diet. The liver is the main site for lipogenesis in fish and liver carbohydrate may control overall lipogenesis (Hemre et al., 2002). Both metabolites, i.e. glycogen and TAG, act as energy reserve, so our results suggest a greater energy investment in maintenance and growth rather than in storage, a result in line with the biometric outcomes obtained. Indeed, results with this diet (LB-CB) suggest that TAG, both from the liver and from plasma, are preferentially channelled towards growth. In this regard, enzyme activities at hepatic level also support this idea, where microalgae inclusion enhances carbohydrate use within the organ, but promote lipid utilisation in other tissues (see below).

Our results show a significant increase in GPase activity in fish fed the LB-CB diet compared to those fed the control diet. This enzyme catalyses the dephosphorylation of glucose-6-phosphate to glucose, using glycogen as substrate (Hallgren et al., 2003). Thus, higher activity of the enzyme is well correlated with the decrease in hepatic glycogen observed with LB-CB supplementation. HK and PK showed similar activity patterns, with a significant increase in liver of fish ingesting both diets supplemented with microalgae. While HK is the first step in glycolysis, phosphorylating glucose to be used by cells, PK catalyses the last step of glycolysis producing pyruvate and ATP (Perera et al., 2020). Therefore, our results support that diet supplementation with microalgae promotes the metabolic use of carbohydrate as energy source in the liver, both from plasma and from the own glycogen reserves. Interestingly, our previous study testing a low inclusion (1 %) of microalgae in diet for sea bream juveniles also showed an increase in hepatic HK activity, but not in PK activity, suggesting that supplementation promoted the hepatocyte capacity for glucose uptake and glycogen storage, but not its oxidation for energy (Perera et al., 2020). Thus, and although these studies were performed in two different life stages of sea bream, it seems that the inclusion of microalgae derived products in aquafeeds consistently promote glucose uptake by the hepatocytes, but its final fate (i.e. storage or oxidation) depends on the level of microalgae inclusion. In our current study, the use of glycogen-derived glucose for energy in the liver of fish fed with microalgae supplemented diets also agree with a significant decrease in FBP enzyme activity in LB-CB fish, as this enzyme is involved in the gluconeogenesis (Vargas-Chacoff et al., 2016). Liver FBP activity, also decreased in sea bream juveniles fed with low inclusion (1 %) of microalgae (Perera et al., 2020).

In addition, a significant increase in LDH activity was observed in fish fed with the LB-CB diet. Liver LDH has a gluconeogenic function (Furné et al., 2012) and may, in theory, be important to recycle lactate from plasma into glucose, which can be either used for storage as glycogen or for energy within the liver or elsewhere. The higher amount of lactate to be processed, reflected by the higher plasma lactate in fish fed microalgae supplemented diets, may be in line with the increased LDH activity observed in the liver. This is in agreement with a previous study in gilthead seabream supplemented with white tea, where an increase in liver LDH activity parallels an increase in plasma lactate (Pérez-Jiménez et al., 2012). However, the formation of pyruvate from lactate by LDH at hepatic level (i.e. Cori cycle), and its further conversion into glycogen, has not been clearly explained in fish, and an early study analysing lactate metabolism in a large number of fish species suggested that little blood lactate is taken up by the liver (Dando, 1969). More recent studies also suggested that using lactate as a precursor for liver glycogen is unlikely in fish (Omlin et al., 2014). Thus, the relationship between plasma lactate and liver LDH activity in fish remains obscure and deserves further assessments.

Finally, no significant differences in HOAD activity were observed among the three experimental groups, although a slight reduction in its activity was observed in response to the diets supplemented with both LB-CB and LB-CBplus. The HOAD enzyme is involved in the β-oxidation of fatty acids (Guerreiro et al., 2019), and its activity is directly related to the availability of lipids (Turner et al., 2007). Therefore, this observation may be related with the lower presence of triglycerides in the liver of fish fed with both experimental diets. Alternatively, it may result from the observed enhanced hepatic glucose oxidation for energy, which would spare triglycerides in the liver, making them more available to fuel muscle growth.

5. Conclusion

It is concluded that the microalgae-derived products evaluated, especially after a biotechnological treatment, are suitable for aquafeed supplementation for *S. aurata*, close to commercial size. This is supported by a clear improve of growth performance and hepatic carbohydrate metabolism, being also proved not to result in detrimental effects on intestinal physiology, but instead, to produce longer and more efficient intestines. However, further studies are required to test if the inclusion of these products at lower dietary inclusion levels allow fish to better handle stressful situations at this advanced stage of the farming cycle. Likewise, the effects of microalgae inclusion on marketable product quality such as texture, colour or fillet composition should be evaluated, as these are important features of fish near harvest.

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CRediT authorship contribution statement

Conceptualization: **M.I.S., S.T.T., J.A.M.-S.**; Data curation: **E.P., J. A.M.-S.**; Formal analysis: **L.M.-R., A.B., J.F., J.A.M.-S.**; Funding acquisition: **M.I.S., F.J.A., J.M.M, J.A.M.-S.**; Methodology: **L.M.-R., A. B., M.I.S., F.J.A., J.F., J.A.M.-S.**; Project administration and Supervision: **J.A.M.-S.**; Writing – original draft: **L.M-R.**; All authors reviewed, edited and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data that support the findings of this study are all presented in the figures and tables, as well as available from the authors upon reasonable request.

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