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APPLIED STUDIES

The effect of feeding a novel multistrain yeast fraction on European seabass (*Dicentrachus labrax*) intestinal health and growth performance

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Fish were fed a single-strain yeast fraction (SsYF; 2 g/kg) or a multi-strain yeast fraction (MsYF; 0.8 g/kg) for 10 weeks. The results demonstrated significant ($p \leq 0.03$) elevations in weight gain, specific growth rate, protein efficiency ratio, and feed conversion ratio in fish fed the yeast fraction-supplemented diets. In the distal intestine, a significant elevation in microvilli density was observed after 5 and 10 weeks of dietary supplementation with MsYF and SsYF, respectively, compared to control fed fish ($p < 0.001$). A significant elevation ($p = 0.02$) in the perimeter ratio was observed in fish fed diets supplemented with the yeast fractions. After 10 weeks of feeding on the experimental diets, Rt-qPCR demonstrated a significant downregulation ($p < 0.05$) in the stress response genes, heat-shock protein 70 (*hsp70*) and proliferating cell nuclear antigen (*pcna*), in fish fed diets supplemented with the yeast fractions. Significant ($p < 0.05$) elevations in interleukin 1-beta (*il1β*) and interleukin-10 (*il10*) gene expression were observed in fish fed diets supplemented with the MsYF compared to the other dietary groups. These findings suggest that feeding an MsYF specifically at a lower incorporation rate < 1 g/kg, compared to a commercial SsYF at 2 g/kg, is effective in improving the intestinal health status and growth performance of European seabass.

KEYWORDS

growth performance, intestinal mucosal health, multistrain yeast fraction, seabass

Since the turn of the century, the use of prebiotics or functional ingredients with immune modulatory properties has become more prominent across animal feed industry. A key driving force has been the restrictions or ban on the use of prophylactic antibiotic growth promoters in various countries. In a wide range of species, both terrestrial and aquatic, a substantial body of literature exists demonstrating the advantageous effects of feeding functional prebiotics or immune modulatory substances (Kiron, 2012: Ganguly, Dora, Sarkar, & Chowdhury, 2013; Merrifield & Ringø, 2014; Song et al., 2014; Pourabedin & Zhao, 2015; Ringø et al., 2016; Dawood, Koshio, Abdel-Daim, & Doan, 2018; Dawood, Koshio, & Esteban, 2018). Beyond the scientific interest of assessing such feeding strategies, the use of a so-called "functional diet" has gained momentum in modern aquaculture, particularly in the European aquaculture sector (Encarnação, 2016). The present study is therefore in line with the ongoing strategy in fish farming, which is the improvement of health and welfare through dietary manipulation, especially with the use of health-promoting additives.

Investigations into the effects of functional feed ingredients from yeasts in animal feeds have largely been directed toward the identification of the functional effects of mannan-oligosaccharide (MOS). Many reports have concluded that the dietary inclusion of MOS can positively influence the health and performance of poultry, swine, and fish (Carbone & Faggio, 2016; Ferket, Parks, & Grimes, 2002; Kogan & Kocher, 2007; Torrecillas, Montero, & Izquierdo, 2014). Several recent reports have demonstrated the effectiveness of MOS in enhancing the health and growth performance of fish, including juvenile striped catfish, *Pangasiandon hypophthalmus* (Akter, Sutriana, Talpur, & Hashim, 2016) and rainbow trout, *Oncorhynchus mykiss* (Mínguez, Webster, & Villa, 2016); improving the gut morphology of starry flounder, *Platichthys stellatus* (Schmidt et al., 2017) and gilthead seabream, *Sparus aurata* (Eryalçin et al., 2017); and modulating the skin mucous barrier of Atlantic salmon, *Salmo salar* (Micallef et al., 2017). Although these studies are not directly comparable in terms of dietary regimes, rearing conditions, MOS products, and doses, it is likely that the molecular structure of MOS plays a significant role in the functional effects on the host's intestinal barrier response and physiology (Schiavone et al., 2014). Recent research concerning the yeast cell structure of *Saccharomyces cerevisiae* led to the discovery of complex strain-specific compositions and architectural characteristics of the cell wall (Schiavone et al., 2014; Schiavone, Sieczkowski, Castex, Dague, & Marie François, 2015). Among many distinctive traits, yeast strains can differ by the composition of their cell wall with, particularly certain strains differing by the architecture of the polysaccharide network at their surface. MOS derived from the cell wall of *S. cerevisiae* consist of linear chains of α-1,6-mannosyl units with short α-1,2 and α-1,3-linked mannose residues (Schiavone et al., 2015). The most recognized mode of action associated with MOS is in its ability to bind to enteropathogenic bacteria, preventing host colonization. Studies on pathogen adhesion recognition have shown that branched α-mannosides of yeast mannans bind with mannose-specific lectin-type receptors (Type 1 fimbriae) of enteropathogenic bacteria, such as *Escherichia coli* and *Salmonella* spp., preventing adhesion to the surface glycoproteins of intestinal villi (Firon, Ofek, & Sharon, 1983). However, a clear correlation between mannan concentration and pathogen immobilization is not always apparent, indicating the involvement of other binding factors. Critically, it appears that the structures of the mannosyls are important determinants of the binding capacity of yeasts (Ganner, Stoiber, Uhlik, Dohnal, & Schatzmayr, 2013). Detailed biochemical analysis and genetic studies have shown that mannoproteins in *S. cerevisiae* are highly branched *N*-glycosylated proteins, while *O*-mannosylated proteins have short mannose chains (Ballou, 1990). This may partly explain the different abilities of various yeast strains to bind specific bacteria.

Although much research has focused on the immunomodulatory effect of yeast β-1,3/1,6-glucans, MOS are likely to have a direct role in mobilizing the immune response once ingested through the activation of different microbial recognition receptors (MRRs). However, to date, the impact of the arrangement of the polysaccharides network at the surface of the yeast cell wall on the recognition by specific MRRs remains largely unknown.

The scientific literature about the assessment of dietary non-Saccharomyces yeast species as probiotic, prebiotic, or immunostimulant in aquaculture is scarce as highlighted by recent reviews (Gatesoupe, 2007; Navarrete & Tovar-Ramírez, 2014; Øverland & Skrede, 2017). We identified fewer than 15 published scientific studies dealing with the assessment of dietary non-Saccharomyces yeast species in aquaculture fish, most of them published after

2010 (Grammes et al., 2013; Opazo, Fuenzalida, Plaza-Parrochia, & Romero, 2017; Øverland, Karlsson, Mydland, Romarheim, & Skrede, 2013; Reyes-Becerril et al., 2011; Reyes-Becerril et al., 2012; Sharma et al., 2018; Tapia-Paniagua et al., 2011; Tovar-Ramírez et al., 2010). Specific investigations on the effect of feeding different yeast cell wall fractions from different yeast species or strains is an emerging field in teleost fish (Grammes et al., 2013; Marques, Dhont, Sorgeloos, & Bossier, 2006; Øverland et al., 2013; Soltanian, Dhont, Sorgeloos, & Bossier, 2007).

We hypothesize that there could be a benefit to localized immune responsiveness and intestinal integrity of combining different yeast fractions obtained from distinct yeast strains specifically selected for their contrasting cell wall architecture and molecular structure. The present study aimed at investigating the effects of feeding a novel multistrain yeast fraction (MsYF) consisting of two strains of *S. cerevisiae* and one strain of *Cyberlindnera jadinii* and comparing this to a commercially available single-strain yeast fraction (SsYF) on intestinal health and growth performance of one of the most developed aquaculture species in the Mediterranean, the European seabass (*Dicentrachus labrax*) (Food and Agriculture Organization, 2008). This study was designed as a preliminary assessment of an MsYF concept combining Saccharomyces and non-Saccharomyces yeast strains prior to further mechanistic studies.

1 | MATERIALS AND METHODS

1.1 | Fish and experimental protocol

A 10-week feeding trial was conducted at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth (Plymouth, United Kingdom). European seabass fingerlings were obtained from Aquanatal (Anglesey, United Kingdom). After 4 weeks of acclimation, 225 seabass (15.5 ± 0.1 g) were randomly distributed into 9 × 110 L fiberglass tanks (25 fish/tank). The seabass were fed at a fixed rate of 2.0% of biomass per day, with three feeding times: 9:00 a.m., 1:00 p.m., and 4:30 p.m., and they were batch-weighed weekly to adjust daily feed input. Fish were maintained under a constant 12:12 hr light:dark photoperiod; and water quality parameters were maintained within a suitable range for the European seabass (Person-Le Ruyet, Mahe, Le Bayon, & Le Delliou, 2004). Water temperature and salinity averaged 24.1 ± 0.8°C and 26.6 ± 4.3 ppt, respectively; dissolved oxygen was maintained between 7.5 and 8 mg/L; pH between 6.8 and 7.5; ammonium between 0.04 and 0.08 mg/L; nitrite between 0.02 and 0.06 mg/L; and nitrate between 54 and 58 mg/L.

1.2 | Feed formulation and diets

A basal diet was formulated to meet the known nutritional requirements of juvenile European seabass (NRC, 2011) (Table 1). The SsYF product was obtained by extracting the yeast cell wall of a primary grown strain of *S. cerevisiae* and the MsYF product by combining yeast fractions from three specific strains from the Lallemand Yeast Culture Collection (two strains of *S. cerevisiae* and one strain of *C. jadinii*). Figure 1 presents the distinct biochemical, biophysical, and selected immune properties of these products. Briefly, total carbohydrates were determined by sulfuric acid hydrolysis (Francois, 2006), and quantitation of total protein was measured by Kjeldahl. Immune assays were performed with mononuclear peripheral blood cells (PBMCs) from healthy volunteers provided by the French Blood Establishment, and monocytes were isolated by Ficoll gradient isolation from a cytapheresis residue obtained from a healthy donor. The PBMCs were cultured for 2 hr (5% CO_2—37°C) on plastic in an macrophage- serum free media medium and were seeded at 1,000,000/well of a 96-well plate to obtain 100,000 monocytes/well after adhesion. After washing with buffer (Hanks balanced salt solution without calcium or magnesium), the remaining cells were monocytes used for experiments on TNF-α secretion and superoxide anion species (RLO). Quantification of TNF-α was performed on supernatants according to Cisbio (30200 Codolet, France) TNF-α Assay kit using the Homogeneous Time Resolved Fluorescence detection technique. The induction of RLO by monocytes was measured after stimulation by Zymosan or yeasts

TABLE 1 Dietary formulation (%) and proximate composition of experimental diets

	Control	SsYF	MsYF
Dietary formulation (%)			
Fishmeal LT94[a]	15.0	15.0	15.0
Soybean protein concentrate[b]	38.9	38.9	38.9
Corn gluten meal	16.0	16.0	16.0
Fish oil[c]	11.9	11.9	11.9
Corn starch[d]	16.6	16.6	16.6
Vitamin + mineral mix[e]	1.0	1.0	1.0
CMC-binder	0.5	0.5	0.5
SsYF product[f]	—	0.2	—
MsYF product[g]	—	—	0.08
Proximate composition			
Dry matter (DM; %)	94.5	94.8	93.5
Crude protein (%DM)	45.0	44.7	44.9
Crude lipid (%DM)	15.6	15.5	15.4
Ash (%DM)	5.6	5.2	5.9
Gross energy (MJ/kg)	21.72	21.30	20.21

Note. MsYF: multistrain yeast fraction; SsYF: single-strain yeast fraction.
[a] United fish products (Aberdeen, United Kingdom).
[b] HP-110, Hamlet Protein, Saturnvej 5, DK-8700 Horsens, Denmark (crude protein 57.5%; ash 6.8%; moisture 6.5%; lipid 2.5%).
[c] Epanoil (Seven Seas Ltd, Feltham, Middlesex, United Kingdom).
[d] Sigma-Aldrich (Poole, United Kingdom).
[e] Premier nutrition vitamin premix; each 1 kg of premix contains: 12.1% calcium, ash 78.7%, vit A 1.000 μg/kg, vit D3 0.100 μg/kg, vit E (as alpha tocopherol acetate) 7,000.0 mg/kg, copper (as cupric sulphate) 250.000 mg/kg, magnesium 1.56%, phosphorous 0.52%.
[f] Single-strain yeast fraction product (AgriMOS; Lallemand SAS, Blagnac, France).
[g] Multistrain yeast fraction product (Lallemand SAS).

products through chemiluminescence. Differences in the cell wall glycosylation of a specific yeast species was studied by measuring the extension of glycoproteins at the yeast cell surface. To do so, the cell surfaces of each strain were probed with Concanavalin A using an Atomic Force Microscope (Nanowizard III, JPK-Bruker, Haus 13, Eingand B, Berlin, Germany) according to Schiavone et al. (2017). The presence of the specific yeast species composing MsYF is shown by identification using ITS-PCR followed by DNA digestion using restriction enzymes *Cfo*I, *Hae*III, and *Hinf*I. DNA extraction was performed directly on the sample dry biomass after Schiavone et al. (2017).

Three experimental diets were tested in triplicate: the basal diet (Control), the basal diet supplemented with a commercial SsYF product at 2 g/kg of feed (SsYF; AgriMos, Lallemand SAS, Blagnac, France), and an MsYF product at 0.8 g/kg of feed (MsYF; Lallemand SAS). In-feed incorporation rates were selected based on the manufacturer's instructions. Each diet was produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food Equipment, Orton Southgate, Peterborough, United Kingdom, model no: HL1400—10STDA mixer). Warm water was added to reach a consistency suitable for cold press to form 1-mm pellets (PTM Extruder system, model P6, Plymouth, United Kingdom). The nutritional profile for each diet was determined according to AOAC (2007) official protocols (Table 2).

1.3 | Sampling schedule

After 5 and 10 weeks of feeding, a total number of six and nine fish per experimental group, respectively, were randomly netted and euthanized following Home Office Schedule 1 procedures (United Kingdom). Fish were individually measured for body weight (BW; ±0.1 g); fork length (FL; ±1 mm) and the posterior intestine (PI) were sampled using

(a)

Products	Total carbohydrates	Protein
SsYF	63 %	22 %
MsYF	56 %	30 %

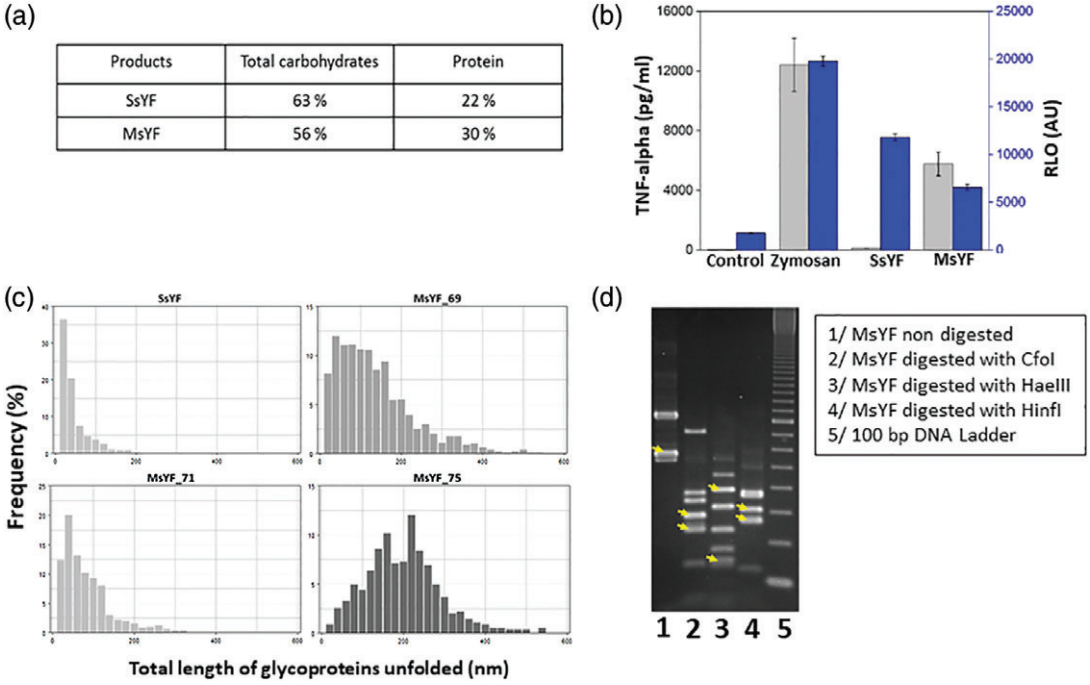

FIGURE 1 (a) Biochemical composition of the test products determined by the sulfuric acid hydrolysis method for total carbohydrates and Kejdahl method for protein. Total carbohydrates were calculated by adding α-glucans, β-glucans, mannans, and chitin; (b) secretion of reactive oxygen species (RLO) and TNF-α by human monocytes incubated with media (control), Zymosan, SsYF, or MsYF; (c) total length of glycoproteins fully unfolded at the surface of the single strain of *Saccharomyces cerevisiae* (SsYF) and of each strain of the multistrain product (MsYF strain 1, MsYF strain 2, and MsYF strain 3); (d) PCR (ITS) with primers ITS1 and ITS4, followed by DNA digestion by *Cfo*I, *Hae*III, or *Hin*fI. The presence of *C. Jadinii* was confirmed by the presence of its restriction profile (shown by yellow arrows). The other bands match the restriction profile of *S. cerevisiae*. ITS: internal transcribed spacer; MsYF: multistrain yeast fraction; PCR: polymerase chain reaction; RLO: superoxide anion species; SsYF: single-strain yeast fraction; TNF: tumor necrosis factor

TABLE 2 Primer pair sequences, gene name abbreviations, annealing temperature (anneal tm in °C), amplicon size (bp), and primer efficiency (eff) for genes used for real-time PCR

Gene name	Primer name	Accession number	Primer sequence (5′–3′)	Aneal tm/amplicon/eff
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*—fwd	AY863148	TGCTGCTTTCACCTCCAAGAA	60/75/1.9
	gapdh—rev		CCATGTACTCCAGGTCGATGAA	
β-Actin	*βactin*—fwd	AJ493428	ATCCACGAGACCACCTACAA	60/79/2.0
	βactin—rev		ACAGCACAGTGTTGGCATAC	
Interleukin-1β	*il1β*—fwd	AJ269472	TTACCCACCACCCACTGACA	60/70/2.0
	il1β—rev		AAGCCCTTCAGTCTCTCCAT	
Interleukin-10	*il10*—fwd	AM268529	GCTGGGTCTGCTGTTCAACTA	60/66/2.1
	il10—rev		GCTGCATGGTTTCTGTGTTGTT	
Proliferating cell nuclear antigen	*pcna*—fwd	JQ755266	TGAAGTGTGCAGGAAACGAAGA	60/65/1.9
	pcna—rev		GGCGAGTGTGTCTGCATTGT	
Heat shock protein 70	*hsp70*—fwd	AY423555	TCCAGCGTGACAAGGTATCTG	60/64/1.9
	hsp70—rev		TCTTACCGGCAAGCCTTTCA	

scanning electron microscopy (*SEM*), histology, and gene expression analysis. For histological analysis, PI samples were removed and fixed in 10% marine formalin and maintained at 4°C for 48 hr. Samples were then transferred to 70% ethanol for long-term storage. For *SEM*, PI samples were washed in 1% S-carboxymethyl-L-cysteine buffer (pH 7.2) before being placed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (1:1 vol/vol, pH 7.2). For gene expression analysis, PI samples (<100 mg) were placed in a 1 mL RNA-later solution (Applied Biosystems, Paisley, United Kingdom) and stored at 4°C for 24 hr and then at −80°C until RNA extraction.

1.4 | Growth performance calculations

For analysis of growth performance, 25 fish/tank were bulk-weighed on a weekly basis. Growth and feed performance were assessed based on specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and Fulton's condition factor (K). Calculations were made using the following formulae: SGR (%BW/day) = 100 [(lnBW_f − lnBW_i)/T]; FCR = FI/WG; PER = WG/PI; K = BW/FL^3, where BW_f = final BW (g), BW_i = initial BW (g), T = duration of the trial (day), WG = weight gain (g), FI = estimated feed intake (g), PI = estimated protein intake (g), and FL = fork length (cm).

1.5 | Morphometric and *SEM*

Formalin-fixed PI samples were dehydrated (Leica TP1020), embedded in paraffin wax, sectioned at 5 μm thickness (Leica RM2235 microtome), and dried in an oven overnight. For each specimen, multiple sections were stained with hematoxylin and eosin (H&E) and Alcian Blue-PAS to assess the intestinal perimeter ratio (PR), intraepithelial leukocytes (IEL), and goblet cell abundance in the epithelium after Dimitroglou et al. (2009). Image analysis was conducted using Image J 1.47v software (National Institutes of Health, Maryland).

Samples for *SEM* were dehydrated in a graded ethanol series (30% alcohol, 50, 70, 90, and 100% twice) for 15 min at each step and critically point dried (K850 Emitech, South Stour, Ashford Avenue, Ashford, Kent, United Kingdom) with ethanol as the intermediate fluid and CO_2 as the transition fluid. The samples were then sputter-coated (K550 Emitech) with gold and viewed with a JSM 6610 LV scanning electron microscope. For each sample, multiple images were captured at ×500–×20,000 magnifications to assess general intestinal integrity. Microvilli density measurements were conducted in Image J using images at ×20,000 magnification according to Dimitroglou et al. (2009). Briefly, micrographs were converted to 8-bit, and the ratio of white/black (i.e., foreground/background) was calculated to give a microvilli density measure (arbitrary units [a.u.]).

1.6 | RNA extraction and cDNA synthesis

Total RNA was extracted using a TRI reagent (Ambion, Life technologies, Paisley, United Kingdom) according to the manufacturer's instructions, with some modifications. Briefly, 100 mg intestinal samples were removed from the RNA-later solution, and excess solution was removed by pressing the sample between sterile tissues. The samples were transferred into a tube containing 1 mL TRI reagent and homogenized for 10 min. Following this, 200 μL of chloroform was added, and after mixing, samples were centrifuged at 12,000*g* for 15 min. The upper aqueous phase was transferred into a tube containing an equal volume of isopropanol. Mixtures were vortexed and centrifuged at 14,000*g* for 15 min. Supernatants were discarded, and the precipitated RNA pellets were washed using 1 mL of 75% ethanol. Total RNA was dissolved in diethylpyrocarbonate water and, to remove any contaminating genomic DNA, was purified using RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Manchester, United Kingdom). The concentration and quality of RNA in each sample were determined by measuring 260/280 nm and 260/230 absorbance ratios (NanoDrop Technologies, Wilmigton, DE). The integrity of RNA was confirmed on a 1% Agarose gel. Samples were stored at −80°C. A 20-μL reaction volume containing of 1 μg of total RNA was used for cDNA synthesis, using the iScript cDNA synthesis kit (Bio-Rad, Watford, Herefordshire, United Kingdom). The

reaction was maintained at 25°C for 5 min and then 42°C for 30 min and was inactivated at 85°C for 5 min. The iScript cDNA synthesis kit contains a combination of oligo dTs and random hexamers to work with a wide variety of targets.

1.7 | Real-time PCR assay

PCR reactions were performed with the SYBR green method using a Quantstudio 12k Flex Real-time PCR thermal cycler (Applied Biosystems). Duplicate PCR reactions were carried out for each sample analyzed. Each PCR reaction was set on a 384-well plate by mixing 2 μL of diluted (1/10) cDNA with 5.5 μL 2× concentrated iQ SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM forward primer, and 0.3 μM reverse primer. The primers used and their sequences are presented in Table 3. The thermal profile for all reactions was 10 min at 95°C and then 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and displayed in all cases for one single peak. *β-actin* and *gapdh* were used as reference genes in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality (Bustin et al., 2009). The stability and suitability of *gapdh* and *β-actin* as reference genes were confirmed by generating an expression stability measure "*M*" for each reference gene using the calculations outlined in Vandesompele et al. (2002). No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. Modification of gene expression was represented with respect to the control group being sampled at the same time as the treatment group.

The threshold cycle (Ct), defined as the point at which the fluorescence rises appreciably above the background fluorescence, was determined manually for each run. PCR efficiencies for each set of primers were determined using serial dilutions of cDNA ($n = 3$) and the resulting plots of Ct versus the logarithmic cDNA input using the Equation E: (PCR efficiency) = 10(−1/slope); see Table 2 (Rasmussen, 2001). The normalized expression level (NEL) of target genes were calculated on the basis of Ct deviation (ΔCt) of the unknown sample versus a control sample and expressed in comparison to the reference genes *gapdh* and *β-actin* according to calculations outlined by geNorm manual (http://medgen.ugent.be/~jvdesomp/genorm/) and Vandesompele et al. (2002).

1.8 | Statistical analysis

All statistical analyses for realtime-quantitative polymerase chain reaction (Rt-qPCR) data were carried out using the permutation test in R following Ohmel (1996). All other statistical differences (Intestinal morphometry, scanning electron microscopy *(SEM)*, growth performance data) were assessed by one-way ANOVA tests (SPSS version 22, SPSS Inc., IL), with Tukey high significant difference post-hoc test where differences in experimental groups occurred. Significance was accepted at $p < 0.05$. Data are presented as mean ± *SD*.

TABLE 3 Body size, growth, and feed performance of European seabass over the 10-week trial's duration. Mean ± *SD* ($n = 3$)

	Control	SsYF	MsYF	*p*-Value
Initial body weight (g)	15.5 ± 0.2	15.5 ± 0.2	15.4 ± 0.2	0.987
Final body weight (g)	33.9 ± 0.9^{a}	36.1 ± 0.6^{b}	37.1 ± 1.2^{b}	0.016
Final condition factor (K)	1.01 ± 0.03	1.11 ± 0.03	1.12 ± 0.12	>0.05
Specific growth rate (%/day)	1.32 ± 0.02^{a}	1.43 ± 0.03^{b}	1.42 ± 0.03^{b}	0.003
Feed conversion ratio	1.82 ± 0.02^{a}	1.64 ± 0.02^{b}	1.53 ± 0.03^{b}	<0.001
Protein efficiency ratio	0.64 ± 0.04^{a}	0.71 ± 0.02^{b}	0.84 ± 0.04^{b}	0.027
Survival (%)	88.0 ± 3.3^{a}	94.7 ± 3.3^{ab}	98.7 ± 1.9^{b}	0.014

Note. Different superscript letters indicate significant differences between treatments. MsYF: multistrain yeast fraction; SsYF: single-strain yeast fraction.

2 | RESULTS

2.1 | Growth performance

Growth performance data are presented in Table 3. A significant improvement of SGR was observed in fish fed the SsYF (1.43 ± 0.03%BW/day; $p = 0.016$) and MsYF (1.42 ± 0.03%BW/day; $p = 0.003$) diets compared to fish fed the control diet (1.32 ± 0.02%BW/day). In accordance with SGR, there was a significant positive effect ($p = 0.016$) in final BW observed in fish fed SsYF (36.1 ± 0.6 g) and MsYF (37.1 ± 1.2 g) compared to the control group (33.9 ± 0.92 g). Compared to the control group (1.76 ± 0.02), FCR was significantly lower in fish fed MsYF (1.53 ± 0.03; $p < 0.001$) and SsYF (1.60 ± 0.02; $p = 0.002$). Likewise, significant elevations in PER were observed in fish fed MsYF and SsYF compared to control fed fish ($p < 0.03$). After 10 weeks, percentage survival was significantly higher in MsYF (98.7 ± 1.9%; $p = 0.012$) compared to fish fed the control diet (88.0 ± 3.3%) and the SsYF-supplemented diet (94.7 ± 3.3%).

2.2 | Intestinal morphometry and *SEM* analysis

European seabass from all groups presented an intact intestinal epithelial barrier with extensive mucosal folds, abundant IEL, and numerous goblet cells. Epithelial surfaces appeared healthy with uniform enterocyte formations and densely packed microvilli with no signs of cellular or microvilli disruption or necrosis. Histological morphometric analysis demonstrated that the PI PR was significantly elevated in fish fed SsYF after 5 and 10 weeks (3.61 ± 0.44 and 4.32 ± 0.34, respectively) compared to control fed fish (3.01 ± 0.42 and 3.21 ± 0.41, respectively; Table 4.). In comparison, fish fed the MsYF-supplemented diet exhibited a significant elevation in PR only after 10 weeks (4.22 ± 0.02) compared to the control group (3.22 ± 0.11). There were no statistical differences in PR values between the SsYF and MsYF experimental groups throughout the experiment. After 5 weeks, PI microvilli density was significantly elevated in fish fed SsYF (2.90 ± 1.02 a.u.; $p = 0.02$) and MsYF (4.80 ± 1.03 a.u.; $p = 0.001$) compared to the control group (1.62 ± 0.51 a.u.), with a significant elevation in the MsYF compared to SsYF group ($p = 0.006$) (Figure 2a). Likewise, after 10 weeks, the SsYF- (3.51 ± 0.43 a.u.) or MsYF (3.54 ± 0.71 a.u.)-fed fish exhibited a significant elevation ($p < 0.001$) in PI microvilli density compared to fish fed the control diet (1.71 ± 0.23 a.u.) (Figure 2b).

TABLE 4 Cytoarchitecture of the distal intestine of European seabass after 5 and 10 weeks of feeding experimental diets. Mean ± *SD* ($n = 3$)

	Control	SsYF	MsYF	*p*-Value
Week 5				
Perimeter ratio	3.01 ± 0.42^{a}	3.61 ± 0.44^{b}	3.21 ± 0.41^{ab}	0.04
Mucosal fold length (μm)	325 ± 106	384 ± 147	335 ± 99	0.55
Goblet cells density (*n*/100 μm)	17.24 ± 6.81	9.23 ± 1.52	11.24 ± 3.41	0.12
IEL density (*n*/100 μm)	4.50 ± 1.01	8.31 ± 3.44	9.33 ± 4.22	0.16
Week 10				
Perimeter ratio	3.22 ± 0.11^{a}	4.32 ± 0.34^{b}	4.22 ± 0.41^{b}	0.02
Mucosal fold length (μm)	318 ± 74^{a}	391 ± 77^{b}	337 ± 96^{ab}	0.02
Goblet cells density (*n*/100 μm)	10.34 ± 3.41	14.33 ± 6.94	13.01 ± 3.84	0.65
IEL (*n*/100 μm)	8.81 ± 4.93	12.54 ± 4.02	9.72 ± 3.51	0.20

Note. Different superscript letters indicate significant differences between treatments. IEL: intra epithelial leukocytes; MsYF: multistrain yeast fraction; SsYF: single-strain yeast fraction.

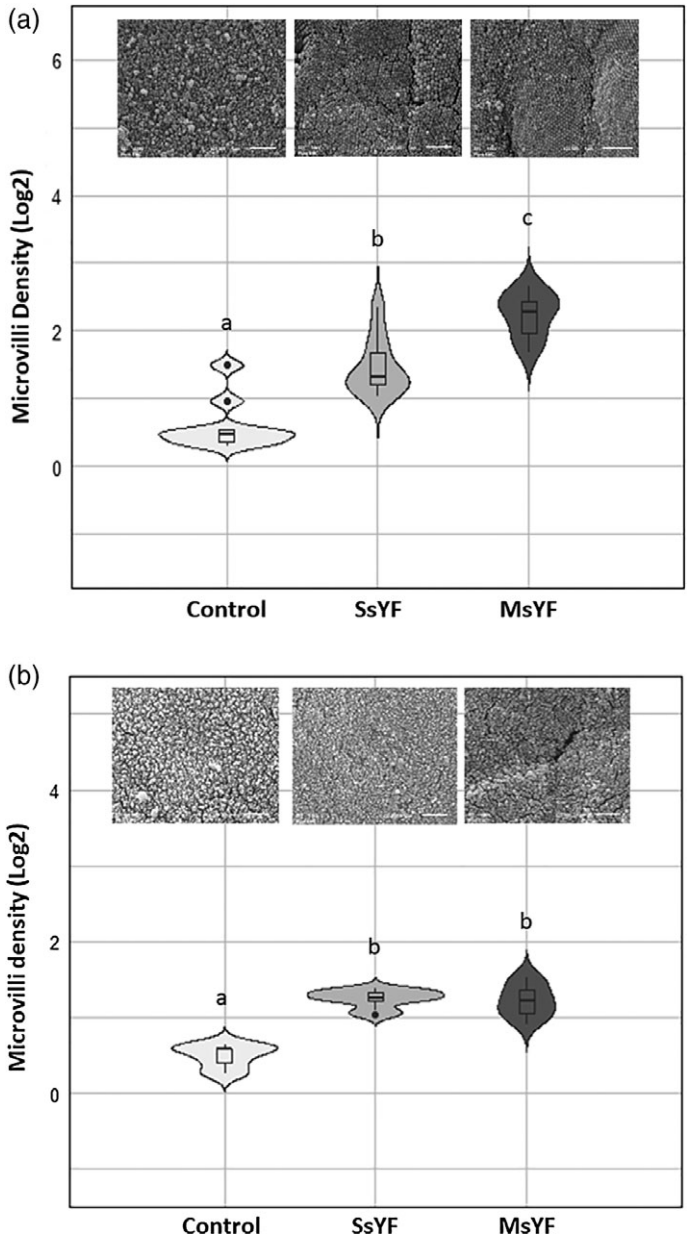

FIGURE 2 Microvilli density (arbitrary units; a.u.) of the distal intestine of European seabass after (a) 5 weeks and (b) 10 weeks of feeding experimental diets. Violin plots show representative *SEM* images of intestinal samples taken to perform microvilli density analysis (10 samples/treatment). Different superscript letters indicate significant differences between treatments (scale bar = 1 μm). MsYF: multistrain yeast fraction; SsYF: single-strain yeast fraction; *SEM*: scanning electron microscopy

2.3 | Intestinal Rt-qPCR

Gene expression analysis demonstrated a significant downregulation ($p < 0.03$) in *hsp70* and *pcna* expression in both the SsYF (0.17 ± 0.06 NEL) and MsYF groups (0.11 ± 0.07 NEL) compared to the control (0.48 ± 0.15 NEL), with no differences between SsYF and MsYF. In contrast, fish fed the MsYF, but not the SsYF, exhibited a significant

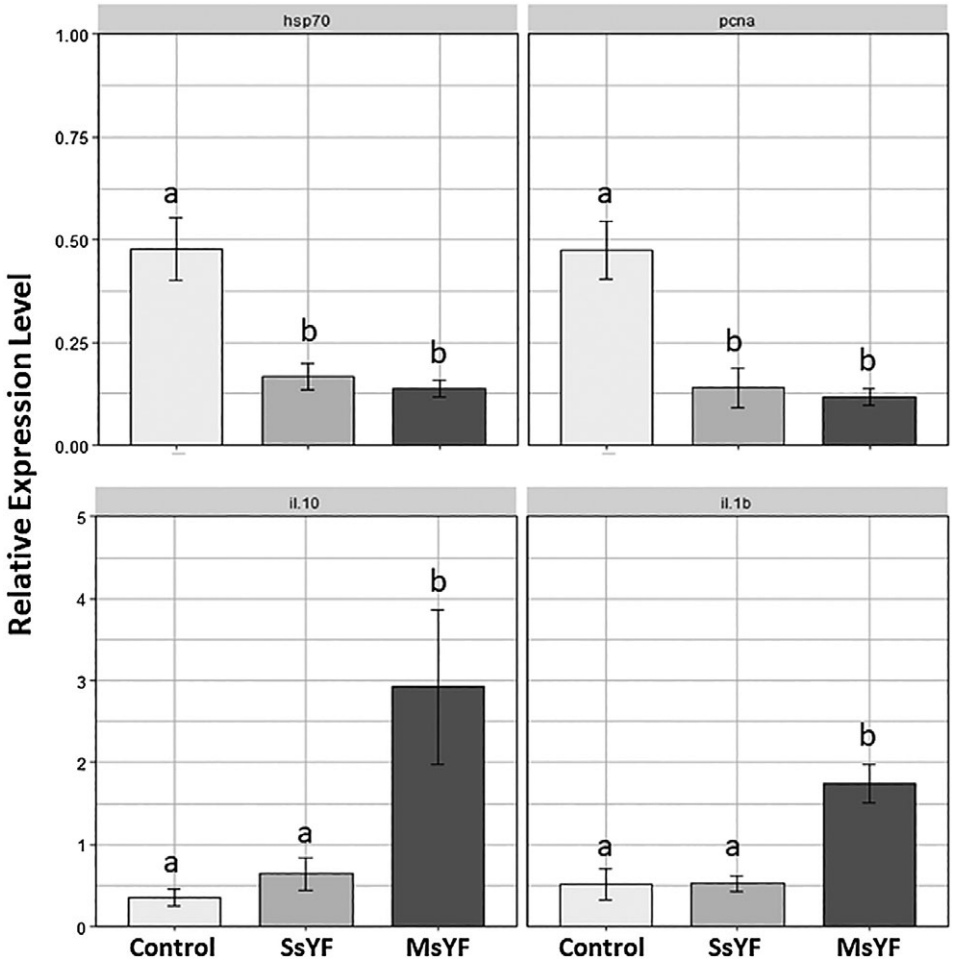

FIGURE 3 Gene expression data (relative expression level) for seabass posterior intestinal samples after 10 weeks of feeding control diet and diets supplemented with SsYF or MsYF (4 samples/treatment). Different superscript letters indicate significant differences for each pairwise comparison between treatments. MsYF: multistrain yeast fraction; SsYF: single-strain yeast fraction

upregulation ($p < 0.03$) in *il10* (2.92 ± 1.89 NEL) and *il1β* (1.75 ± 0.46 NEL) gene expression compared to fish fed the control diet (Figure 3).

3 | DISCUSSION

The current study is the first to report an effect of dietary supplementation with an MsYF product on seabass intestinal health and performance compared to a commercial SsYF product. The positive effects of feeding SsYF rich in MOS on fish growth performance have been well documented previously. Torrecillas et al. (2012) fed European seabass an SsYF rich in MOS at 4 g/kg for 60 days and reported significant elevations in BW and length, condition factor, and SGR compared to fish fed the control dietary regime. Similarly, in another study, Torrecillas et al. (2013) reported significant elevations in weight gain, total length, and SGR in seabass fed an SsYF diet compared to a control diet. In other fish species, Rodriguez-Estrada, Satoh, Haga, Fushimi, and Sweetman (2009) fed rainbow trout an SsYF diet at 4 g/kg for 12 weeks and reported higher weight gain and SGR compared to fish fed a control diet. The results

from the current investigation are in line with previous studies confirming that feeding diets containing an SsYF to seabass at a dose of 2 g/kg had a positive effect on growth performance. Interestingly, our results indicate that the dietary supplementation of the MsYF at a lower dose (<1 vs. 2 g/kg) achieved a similar level of efficacy in terms of benefits to growth and feed performance compared to seabass fed the SsYF-supplemented diet.

The supplementation of aquafeeds with SsYFs and other functional prebiotics has improved gut morphometry in fish by increasing intestinal absorptive area, villi structure complexity, microvilli density, and height (Torrecillas et al., 2014). Torrecillas et al. (2013) reported an improvement in the cytoarchitecture of the intestinal epithelial barrier of seabass fed diets containing an SsYF rich in MOS at 4 g/kg compared to fish fed the control regime. The authors concluded that the effect of the SsYF likely depends on the dose and duration of supplementation as well as on the structure of the SsYF. The current study reported similar effects of feeding an SsYF product rich in MOS at 2 g/kg and MsYF products at a lower dose of 0.8 g/kg on the intestinal integrity and growth performance of European seabass. Previously, Dimitroglou et al. (2009) reported a significant elevation in microvilli density in rainbow trout fed diets containing SsYF 2 g/kg for 16 weeks. Likewise, Dimitroglou et al. (2011) reported elevations in microvilli density as well as a higher gut absorptive surface in Atlantic salmon fed diets containing an SsYF at 4 g/kg for 14 weeks when compared to fish fed the control regime. In the current study, there was a significant elevation in microvilli density in European seabass fed the diets supplemented with the MsYF after 5 weeks compared to SsYF (Figure 2a). To the authors' knowledge, this study is the first to report a beneficial effect of feeding an MsYF product below an inclusion level of 1 g/kg on the intestinal morphology of a marine fish.

The fish's intestinal epithelium acts as the first line of defense against potentially harmful substances or pathogens and is continuously in contact with the resident microbiota, the external environment, and dietary compounds. The results of the current study demonstrated a clear difference in the gene expression profiles of selected stress and innate immune response genes in the PI of seabass fed diets supplemented with SsYF or MsYF for 10 weeks. The heat shock protein 70 (*hsp70*) family is the best studied group of heat shock proteins in aquatic organisms and serves as a stress biomarker, known to be induced during exposure to temperature or salinity, handling, toxins, or other stressors in aquatic organisms (Basu et al., 2002; Roberts, Agius, Saliba, Bossier, & Sung, 2010). In the current study, fish fed both the SsYF or MsYF exhibited a significant downregulation in gene expression of *hsp70* compared to the control diet. Similarly, rainbow trout fed a commercial SsYF rich in MOS and β-glucans at a dose of 2 g/kg exhibited a significant downregulation in gene expression of *hsp70* compared to the control regime (Ahmadi, Farahmand, Miandare, Mirvaghefi, & Hoseinifar, 2014). Likewise, in the present study, a significant downregulation was observed in the gene expression of proliferating cell nuclear antigen (*pcna*), which plays an important role in cellular and DNA replication (Paunesku et al., 2001), in fish fed the diets supplemented with the yeast fractions. Previously, Torrecillas et al. (2012) reported that, 4 hr postinoculation with *Vibrio anguillarum*, European seabass fed diets supplemented with a SsYF at 4 g/kg exhibited a significantly lower plasma cortisol level compared to fish fed the control regime. In comparison, the current investigation fed an SsYF or MsYF diet at a lower dose and still observed a decrease in gene expression of both *hsp70* and *pcna*. This suggests that both the SsYF and MsYF had an effect on the stress response of European seabass at the transcript level. Furthermore, the significant reduction in *pcna* expression infers that fish fed the yeast fractions may have a lower level of enterocyte turnover and thus more mature enterocytes that would allow for better nutrients absorption. Indeed, this notion is supported by the observed increase in microvilli density and enhanced feed utilization in fish fed diets containing the yeast fractions.

Feeding European seabass with MsYF triggered a specific immune response in the PI by increasing the expression of both *il1β* and *il10* compared to fish fed the control and SsYF regimes. As a pleitropic proinflammatory cytokine, *il1β* can affect nearly every cell type, often in concert with other proinflammatory cytokines such as TNFs (as reviewed by Dinarello, 1997; Bird et al., 2002; Huising, Stet, Savelkoul, & Verburg-van Kemenade, 2004; Savan & Sakai, 2006). In fish, *il1β* is one of the first cytokines released during inflammation and is an important effector cytokine of the inflammatory response (Engelsma et al., 2002). The current study demonstrated that *il1β* gene expression in European seabass fed the MsYF diet was significantly upregulated compared to fish fed the control diet. In contrast, fish fed the SsYF diet displayed no difference in *il1β* gene expression compared to control group, which is in

agreement with Torrecillas et al. (2015) using a similar SsYF product at an inclusion level of 1.6 g/kg. Furthermore, in this study, European seabass fed the MsYF diet for 10 weeks, but not the SsYF, exhibited a significant upregulation in the gene expression of the anti-inflammatory effector cytokine interleukin-10 (*il10*) compared to the control group. Similarly, Torrecillas et al. (2015) reported an upregulation in *il10* posterior intestinal gene expression in European seabass after 8 weeks of feeding diets containing a SsYF at 4 g/kg. Interleukin-10 is an important effector cytokine in the maintenance of the fine balance between a swift and potent immune response against invading pathogens and the control of detrimental pathological injury (Foey & Picchietti, 2014; Raida & Buchmann, 2008). The fact that fish fed MsYF exhibited an upregulation in the expression of both *il1β* and *il10* suggests that the dietary inclusion of the MsYF could be promoting a potent but balanced inflammatory response providing immunological protection and maintenance of the intestinal of the intestinal barrier. In summary, we hypothesize that the differences detected in the gene expression profiles of *il-1β* and *il-10* between the SsYF and MsYF groups could, in part, be explained by the different cell wall architectures of the yeast strains composing the products. The combination of the three yeast strains within the MsYF product could activate different pattern recognition receptors compared to SsYF, including toll-like receptors as suggested by the enhanced TNF-α production measured with incubation with human monocytes. This would, in turn, be expected to strengthen the innate immune response to both intra- and extracellular pathogens by activating macrophage and natural killer cell populations in the intestinal mucosal tissue. However, further mechanistic studies are required to investigate this notion.

Several limits inherent to our study and to a majority of similar studies in the field must, however, be highlighted. Even though our results showed positive effects on performance and intestinal morphology parameters, the restricted functional significance of transcriptomic makes inference on immune mechanisms based on immune genes expression difficult. However, the use of gene expression as biomarkers remains practical, cost-effective, and widely used allowing some cross-studies comparative assessment. Recently, several practical feeding studies with European marine fish species were conducted by our group to assess the potential benefit MsYF can bring under commercial farming conditions and confirmed the potential of MsYF as an immune modulation strategy to improve fish health (Rawling et al., 2017; Rawling et al., 2017; Rawling et al., 2018). Finally, it must be noted that, although this study did not investigate the effect of the two yeast products on the intestinal microbiota, it is not possible to exclude a contribution of the gut microbiota to the observed effects on the gene expression of both the stress and immune response markers. Indeed, yeast fraction products are often considered prebiotic, with effects being attributed to changes in the microbiota (Ringø et al., 2010). A previous study reported a modulation of the microbiota of seabass when fed an SsYF diet at 4 g/kg compared to fish fed a control regime (Torrecillas et al., 2013). Several other studies report similar effects on other fish species, such as gilthead seabream (*S. aurata*) or rainbow trout, fed inclusion rates as low as 2 g/kg (Dimitroglou et al., 2009; Dimitroglou et al., 2011).

In conclusion, the impetus of using functional feed additives in aquafeeds is to promote intestinal health and improve fish performance. The present study has demonstrated, for the first time, that dietary inclusion of a novel MsYF at a dose below 1 g/kg can enhance the growth performance and intestinal epithelial structure of European seabass by promoting SGR, FCR, and PER and microvilli density and increasing the intestinal surface area compared to fish fed the nonsupplemented diet. Dietary supplementation with the MsYF product also stimulated the innate immune response in the PI of European seabass in a specific fashion compared to fish fed the SsYF in terms of an elevation in the gene expression profiles of *il-1β* and *il-10*. This distinct (anti-) inflammatory signature, suggestive of a potent but balanced inflammatory response and achieved at a lower in-feed incorporation rate compared to the SsYF, could have a significant effect on preserving a healthy mucosal barrier, which may in turn enhance protection against enteric pathogens. This preliminary result merits further attention using a dose–response design, analysis of individual strain and strain combinations, and disease challenge studies.

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