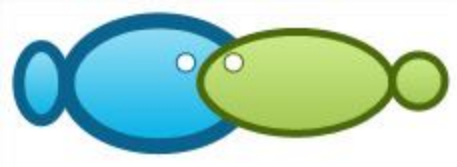

Partial and total replacement of fishmeal by cheaper plant and animal proteins with NucleoforceFish™ supplementation in diets for *Sparus aurata* influence fish performance, whole-body composition, and amino acid profile

[1]Asmaa M. El-Nokrashy, [2]Ramadan A. El-Banna, [2]Bahgat M. Edris, [3]Mohamed M. Abdel-Rahim, [4]Miguel Jover Cerdá, [4]AnaTomás Vidal, [1]Radi A. Mohamed, [3]Ashraf M. A. S. Goda

[1] Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafr Elsheikh, Egypt; [2] Department of Nutrition, Faculty of Veterinary Medicine, Cairo University, Egypt; [3] Aquaculture Division, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt; [4] Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology, Universitat Politècnica de València, València, Spain. Corresponding author: M. M. Abdel-Rahim, mohamed_m_ar@yahoo.com

Abstract. The current study evaluated the effects of elimination of dietary fishmeal (FM) with supplementation of Nucleoforce Fish™ as the source of nucleotides on growth, feed utilization, biometric indices, whole-body composition, and body amino acid profile of juvenile gilthead seabream, *Sparus aurata*. Fish fed six experimental diets (a 2x3 factorial design) with three levels of nucleotides at 0, 250, or 500 mg kg^{-1} and two levels of FM at 0, and 25%. Diets contained 45% crude protein, 18% lipid, 21 MJ kg^{-1} gross energy. Five hundred and forty fry gilthead seabream with an average initial body weight of 0.358±0.002 g $fish^{-1}$, were used. Fish were stocked into six cement ponds (each with 18 m^3); each was installed with three equal net-enclosures (each of 0.5 m^3) and stocked with 30 fish in triplicate. The trial was performed in 2017 and continued for 150 days. The results showed that dietary nucleotide supplementations at 250 mg kg^{-1} and 500 mg kg^{-1} significantly enhanced final body weight, weight gain, average daily gain, survival, condition factor, protein efficiency ratio, hepatosomatic index with decreased viscerosomatic index, especially in fish fed 500 mg kg^{-1} diet ($p < 0.05$) irrespective of FM levels. At 25% dietary FM level, the previously tested parameters were significantly improved compared with 0% fish meal diets irrespective of nucleotide levels. The content of protein, ether extract, and carcass energy in fish carcass increased in fish fed nucleotide-supplemented diets at 250 and 500 mg kg^{-1} in comparison with diets free of nucleotides irrespective of FM levels. Fish fed diets with 25% fish meal exhibited higher content of protein and lower content of both ether extract and energy irrespective of nucleotide levels. Amino acids (AA) of fish flesh fed nucleotide-supplemented diets increased over the control, especially at 250 mg kg^{-1} irrespective of FM levels, while AA was lower at 25% fishmeal diets as compared to 0% FM diets irrespective of nucleotide levels. In conclusion, nucleotides administered as feed additive at 500 mg kg^{-1} in diets contented 25% FM enhances the fish performance and increases the flesh quality in seabream.

Key Words: *Sparus aurata*, growth, survival, feed utilization, VSI, HSI, body chemical composition, amino acids (AA).

Introduction. Aquaculture production of marine fish expects to continue to increase to meet the growing global demand for seafood. Different commercial marine fish species use composite diets that contain high levels of protein, which are often provided by a fish meal from wild fisheries or by-products of the animal processing industries. Fish meal (FM) is an optimal protein source for fish feeds because of its nutritional value and high palatability to fish. Reduction or elimination of the contribution of FM in marine fish diets was the gateway for many nutritionists to provide economic and environmental benefits

by reducing the cost of feed for fish farmers while reducing the pressure of on species harvested for FM production and also serve as essential resources in the marine food web (FAO 2018).

The gilthead seabream, *Sparus aurata*, is being produced in considerable amounts in Europe (FAO 2018). As a carnivorous fish, it requires a high level of FM in its diets to provide an ideal amino acid profile and improve fish digestibility and growth. In the last decade, the increasing demand, price, and world supply fluctuations of FM have emphasized the need to search for alternative protein sources in marine aquafeeds. Substitution of FM by vegetable proteins was the solution proposed to escape for the sustainability and environmental effects dilemma, variable vegetable or other animal protein sources raw materials were used in substitution in different fish species with varying rates of inclusion (Deng et al 2011; Santigosa et al 2011; Wang et al 2012; Estruch et al 2018).

An obstacle faced by nutritional researchers regarding the substitution succession was the immune status of marine fish (Sitjà-Bobadilla et al 2005). Not only the immune status is affected by the FM replacement, but also the intestinal absorption is affected by this replacement as discussed by Santigosa et al (2011). According to Sitjà-Bobadilla et al (2005), the total alternative of FM protein with vegetable protein sources won't be feasible as the adverse effect on growth and general immune status won't compensate the reduction in cost due to the total replacement. Consequently, aid solutions were urged to solve this equation by using several feed additives such as nucleotides (Russo et al 2006; Ringø et al 2012; Reda et al 2018).

Nucleotides are the main building blocks of nucleic acids of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), their general chemical structure is composed of a ribose sugar, one or more phosphate group and either pyrimidine or purine base (Gil 2002; Reda et al 2018). Nucleotides have essential physiological and biochemical functions, including encoding and deciphering genetic information, mediating energy metabolism, and cell signaling besides serving as components of coenzymes, allosteric effectors, and cellular agonists in terrestrial animals (Carver 1994). Nucleotide as a functional nutrient was researched by various researchers in many species, especially mono-gastric animals such as aquaculture and poultry, considering it a solution to enhance the general status of the body (Burrells et al 2001a; Jung & Batal 2012).

However, animals can obtain their required amount of nucleotides via exogenously or endogenously (Reda et al 2018). Because of the active de novo synthesis of nucleotides mainly in the liver, most animals appear to be almost independent of exogenous NT. Formation of nucleotides endogenously through salvage pathway, direct processing from amino acids or within the diet are not sufficient to fulfill the body need in addition to their high energy requirement for their internal production (Quan et al 1990; Hossain et al 2016). However, the requirements for exogenous nucleotides may increase under certain conditions, e.g., tissue injury, disfunction of liver, under-disease or stress, or in fast-growth life stage. For aquatic animals, dietary nucleotide supplementation has been demonstrated to enhance growth, lipid metabolism, disease resistance and development of small intestine (Lin et al 2009; Abtahi et al 2013; Liu 2016), disease resistance and immune responses (Burrells et al 2001a, b; Lin et al 2009). A positive correlation between addition of nucleotides and growth performance was also witnessed in many aquatic species such as gilthead seabream, European seabass *Dicentrarchus labrax*, hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus,* and Pacific white shrimp, *Litopenaeus vannamei* (Oliva-Teles et al 2006; Xu et al 2015; Jin et al 2018). It is also considered that under conditions of limited nucleotide intake or rapid growth, dietary nucleic acids may have a protein-sparing effect, as it limits the de novo synthesis of these molecules from its amino acid precursors (Carver 1999). Burrells et al (2001b) reported that under conditions of management and environmental stressors (vaccination, grading, saltwater transfer, etc.) associated with farming, a beneficial effect of dietary nucleotide supplementation on growth rate was observed in Atlantic salmon, *Salmo salar*.

The information on the effect of nucleotides on fish performance, and amino acid (AA) profile for sea bream is scarce. Thus, this study aims to evaluate the effects of elimination of dietary FM with supplementation of NucleoforceFish™ as a source of

nucleotides on survival, growth, feed utilization, biometric indices such as viscerosomatic index (VSI), hepatosomatic index (HSI), whole-body composition, and AA profile of juvenile gilthead seabream, *Sparus aurata*.

Material and Method

Experimental diets and feeding regime. Five hundred and forty fry gilthead seabream with an average initial body weight of 0.358±0.002 g fish^{-1} obtained from a private commercial fish farm, Egypt, were used. The fry were stocked into six cement ponds (each with 18 m^3) at El-Max Research Station, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. Each cement pond was installed with three net-enclosures (experimental net cage: 1*1*1 m) each of 0.5 m^3 water volume, and each net-enclosure was stocked with 30 fish. The cement ponds were supplied with underground saltwater (with a salinity of 32 ppt). Water temperature, dissolved oxygen, pH, and ammonia were monitored during the trial, to maintain water quality at the optimum range for seabream. Water temperature ranged from 24 to 28°C, salinity at 31±1 ppt, pH at 7.9±0.1, dissolved oxygen at > 5.5 ppm. The daily water exchange rate was 20% pond^{-1} day^{-1}, and fish were held under natural light (~12:12 h light:dark schedule). The trail was performed in 2017 during the period from 15 May to 15 October.

Experimental diets and feeding regime. Six isonitrogenous/isocaloric experimental diets were formulated to be 45% crude protein (CP) and 20 MJ kg^{-1} gross energy (GE). Two levels of fishmeal (0 and 25%) and three levels of NucleoforceFish™ (0, 250, and 500 mg kg^{-1} diet were evaluated. Diets 1-3 were abbreviated to FM0, FM0/250, and FM0/500 to provide 0, 250, and 500 mg NucleoforceFish™ (NF) kg^{-1} diet; while diets 4-6 were abbreviated to FM25, FM25/250, and FM25/500 to provide 0, 250, and 500 mg NucleoforceFish™ (NF), respectively. The proximate chemical composition of the experimental diets is presented in Table 1. The fish were fed on the experimental diets for 150 days, two meals a day (9:30 and 13:30 h), six days a week at ratio started from 10% of fish body weight (BW) at the beginning of the study, and finished with 5% at the end.

NucleoforceFish™ was obtained from Bioiberica® Spain; it contains 34% of free NT from inactivated yeast 92 extract, 80% pyrimidine, and 20% purine. The dry ingredients of the experimental diets were ground and mixed by blending machine into a homogenous mixture for 10 minutes. Oil source and hot water were added, and then diets were processed in an electric kitchen meat grinder (Moulinex ME605131 - HV8 - 1600 W, France) using cold-pressed. All diets were air-dried using an electrical fan for four hrs (moisture content of about 10%) after that dried at 45°C for 12 hrs. Dried diets were sieved using different feed sievers in three diameters: 1.5, 2, and 2.5 mm depending on growing fish size. The diets were packed in cellophane bags and cooled at -20°C before use. Fifty percent of the content of oil was added to each diet during the process of pelletizing, while the rest of the oil was added two days before feeding for increasing fish attractability.

Table 1

Formulation and proximate composition of the experimental diets (g kg^{-1})

Formulation and proximate analysis	*Experimental diets**					
	FM0			*FM25*		
	N0	*N250*	*N500*	*N0*	*N250*	*N500*
	D1	*D2*	*D3*	*D4*	*D5*	*D6*
Fish meal	0	0	0	185	185	185
Poultry by-product	294	294	294	220	220	220
Soybean meal	234	234	234	175	175	175
Corn gluten	134	134	134	100	100	100
Wheat bran	76	75.75	75.50	89	88.75	88.50
Squid	134	134	134	100	100	100
Fish oil	118	118	118	121	121	121
Vitamins[1]	10	10	10	10	10	10
Nucleotides[2]	0	0.25	0.5	0	0.25	0.5
Proximate composition (%)						
DM, %	93.9	93.6	92.9	93.3	93.5	93.8
Protein, %	45.50	45.90	46.60	46.10	45.75	46.00
Lipid, %	18.00	17.80	17.60	18.10	18.20	17.70
Ash, %	15.35	15.70	15.10	15.40	15.65	15.85
Fiber, %	2.93	2.53	2.68	2.55	2.92	3.30
NFE[3], %	18.22	18.08	18.02	17.85	17.48	17.16
Gross energy (MJ/kg)[4]	20.97	20.96	21.04	21.09	20.98	20.79
P/E ratio (mg CP:kJ)	90.74	91.58	92.63	91.43	91.19	92.55
Amino acid profile (g kg^{-1})						
Aspartic (ASP)	3.78	3.62	3.72	3.62	3.78	3.74
Threonine (THR)	1.75	1.66	1.73	1.66	1.77	1.72
Serine (SER)	2.03	2.28	2.33	2.28	2.18	2.09
Glutamic (GLU)	7.47	6.99	7.28	6.99	6.96	7.38
Glycine (GLY)	2.54	2.15	2.21	2.15	2.66	2.46
Alanine (ALA)	3.28	2.82	2.98	2.82	3.17	3.05
Valine (VAL)	2.25	2.45	2.56	2.45	2.54	2.34
Isoleucine (ILE)	1.69	1.77	1.82	1.77	1.82	1.67
Leucine (LEU)	3.61	3.79	3.95	3.79	3.68	3.61
Tyrosine (TYR)	1.57	1.6	1.62	1.6	1.61	1.59
Phenylalanine (PHE)	2.01	2.09	2.13	2.09	2.04	2.02
Histidine (HIS)	0.99	0.79	0.83	0.79	0.84	0.92
Lysine (LYS)	2.42	1.8	1.88	1.8	2.21	2.15
Arginine (ARG)	2.7	2.44	2.49	2.44	2.56	2.64
Proline (PRO)	2.84	2.67	2.98	2.67	2.71	2.85
Cysteine (CYS)	0.58	0.83	0.87	0.83	0.78	0.65
Methionine (MET)	1.08	0.97	0.98	0.97	1.16	1.06
Price (US$/kg feed)	0.844	0.858	0.872	0.961	0.975	0.989

FM_{25} (25% fish meal), FM_0 (0% fish meal), N0 (0 mg nucleotide), N_{250} (250 mg nucleotide), N_{500} (500 mg nucleotide); [1] Vitamin and mineral mixture each: 1 kg of a mixture contains 4800 I.U. Vit. A, 2400 IU cholecalciferol (Vit. D), 40 g Vit. E, 8 g Vit. K, 4.0 g Vit. B_{12}, 4.0 g Vit. B2, 6 g Vit. B6, 4.0 g pantothenic acid, 8.0 g nicotinic acid, 400 mg folic acid, 20 mg biotin, 200 gm choline, 4 g copper, 0.4 g iodine, 12 g iron, 22 g manganese, 22 g zinc, 0.04 g selenium, 1.2 mg niacin, 12 mg D-calcium pantothenate, 26 mg pyridoxine. HCl, 6 mg riboflavin, 7.2 mg thiamin. HCl, 1.2 mg sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg ferrous sulfate ($FeSO_4.7H_2O$, 20% Fe), 65 mg manganese sulfate ($MnSO_4$, 36% Mn), 89 mg zinc sulfate ($ZnSO_4.7H_2O$, 40% Zn), 150 mg copper sulfate ($CuSO_4.5H_2O$, 25% Cu), 28 mg potassium iodide (KI, 24% K, 76% I), 1000 mg celite AW521 (acid-washed diatomaceous earth silica); [2] Nucleoforce Fish™ is concentrated balanced free nucleotides and active precursors, obtained from yeast produced by the bioiberica company, Spain. https://www.bioiberica.com/animal-health/animal-nutrition/fish/nucleoforce-fish-1/#sthash.oj8NWjSA.dpbs; [3] By differences; [4] Calculated using gross caloric values of 23.62, 39.52, and 17.15 kJ/g for protein, fat, and carbohydrate, respectively, according to Brett (1973).

Analytical methods. At the beginning of the trial, a random pooled sample of 100 fish was collected, anesthetized with t-amyl alcohol, and sacrificed for determination of initial whole-body proximate composition. At the termination of the trial, five fish were randomly selected from each net-enclosure and anesthetized with t-amyl alcohol, sacrificed, and homogenized in a blender, to determine the final whole-body proximate

composition. The fish were pooled for each net-enclosure, oven-dried, ground, and stored at -20°C for subsequent analysis. The chemical composition of fish and diet samples were analyzed according to the procedures of AOAC (2000). Dry matter was determined after drying the samples in an oven (105°C) for 24 h. Ash by incineration at 550°C for 12 h. Crude protein was determined by micro-Kjeldahl method, N% × 6.25 (using Kjeltech autoanalyzer, Model 1030, Tecator, Höganäs, Sweden) and crude fat by soxhlet extraction with diethyl ether (40-60°C). Fish were weighed individually every four weeks, and the feeding rates were re-adjusted depending on the actual weight of the fish sample and assess fish health status.

Growth performance and feed utilization indices. Mean final body weight (FBW) of each experimental treatment was determined by dividing total fish weight in each net-enclosure by number of fish. Weight gain (WG), average daily gain (ADG), protein efficiency ratio (PER), survival (%, S) and condition factor (K) were calculated using the following equations:

WG = final body weight (g) - initial body weight (g)

ADG = average daily gain = (FBW - IBW)/t

where: FBW is final body weight (g); IBW is initial body weight (g); ln = natural logarithmic; t = time in days;

Protein efficiency ratio (PER) = fish weight gain (g)/protein intake (g)

Survival (%) (S) = 100 (final number of fish/initial number of fish)

Condition factor (K) = $100 \times (BW/L^3)$

where: BW is fish body weight (gram); L is total fish length (cm).

Biometric indices. At the end of this experiment, four fish from each net-enclosure were sacrificed to obtain their final biological records including liver and viscera weights to determine hepatosomatic (HSI) and viscerosomatic (VSI) indices, as follows:

Hepatosomatic index, HSI = 100 * [liver weight (g)/total body weight (g)] according to Schreck & Moyle (1990);

Viscerosomatic index, (VSI) = 100 * [total weigh of all viscera (g)/total body weight of the fish before removal of the viscera (g)]

Protein and amino acids profile. At the end of this experiment, sub-samples (5-10 mg) of the freeze-dried samples were extracted in Eppendorf tubes in 1 mL 6% tri-chloro-acetic acid (TCA) under rotation for 24 h at 4°C. After centrifugation (15000 x g, 10 min, 4ºC), the supernatant was used for AA analysis after appropriate dilution in borate buffer (100 mM, pH 10.4).

The precipitate was washed once in 6% TCA, re-centrifuged, and transferred to a 10 mL tube and dissolved in 4 mL of 1 M NaOH by rotation for 48 h at room temperature for analysis of total protein and AA.

After centrifugation (15000 x g, 10 min, 20°C), the supernatant was collected and appropriately diluted to 0.5 M NaOH with distilled water, and used for determination of total protein by the method of Lowry et al (1951), using bovine serum albumin (BSA, Sigma A-7638) in 0.5 M NaOH as standard and 0.5M NaOH as blank. The color was allowed to develop in darkness for 30 min and, after an additional mixing, the sample absorbance was read on a spectrophotometer at 750 nm. All reagents used in the analyses were prepared from the glass-distilled, ion-exchanged amino acid analysis was performed by reversed-phase chromatography using high-performance liquid chromatography (HPLC) with fluorimetric detection.

The analytical reproducibility based on repetitive analyses of standards was < 1% for all amino acids except proline (4%). The applied HPLC procedure did not separate phosphoserine and aspartic acid. The AA values (µmoles mg^{-1} dry weight (DW) of analyzed material) were converted to the equivalent protein content and expressed both

in molar terms of the various amino acids (μmoles mg^{-1} DW), and in weight-specific terms as an equivalent to protein content (μg mg^{-1} DW) (Urschel et al 2007).

Statistical analysis. Data were statistically analyzed with a one-way and two-way analysis of variance (ANOVA) using the software SPSS (Standard Version 22.0 SPSS Inc. Chicago, Illinois). Duncan's multiple range test was used to compare differences between treatment means when significant F values were observed (Duncan 1955), at $p \leq 0.05$ level. All percentage data were arc-sin transformed before analysis (Zar 1984). However, data are presented untransformed to facilitate comparisons.

Results

Growth performance, feed utilization, and biometric indices. The growth performance parameters of seabream are presented in Tables 2 and 3. The obtained data showed that the FBW, WG, and ADG were significantly ($p \leq 0.05$) affected either by the addition of nucleotides and/or the dietary level of FM. The highest growth performance was recorded for fish fed diet D6 (FM25/N500) without significant differences with D5 (FM25/N250), while the lowest values were recorded for fish fed D1 (FM0/N0). The main effects of dietary FM levels irrespective nucleotide levels or dietary nucleotide levels irrespective FM levels showed a significant moral improvement on growth performance index in favor of FM25 and nucleotide supplemented diets especially N500 (Table 3). The same trend was observed for protein efficiency ratio (PER), survival (%), condition factor, and biometric indices (VSI, and HSI). The improvement ratio of fish survival was 7 and 14%, with the addition of nucleotide at 250 and 500 mg kg^{-1} feed, respectively. Condition factor (K) improved in seabream fed diets contained FM25. A reduction with 14.5% in the values of VSI in the FM25 group compared with the FM0 group was detected. Increasing the nucleotides level from 0 to 500 mg kg^{-1} decreased the value of VSI with 9% and increased HSI with 15%.

Whole-body proximate composition. The whole-body chemical composition of seabream is presented in Tables 4 and 5. The results showed that, with increasing dietary nucleotide level (irrespective FM levels), the fish body exhibited higher significant ($p \leq 0.5$) content of protein, lipids, and carcass energy without substantial differences between N250 and N500 groups. Nucleotides-enriched diets increased significantly ($p \leq 0.5$), the fish body content of lipids with 16.3 and 12.8% for the N250 group, and N500 group, respectively, compared with the control (N0). Also, carcass energy increased with 6.8 and 5.2% for the N250 group, and N500 group, respectively, compared with the control (N0). However, a significant decrease in the fish body content of ash with percent 11.1 and 4.5% were detected for the N250 group, and N500 group, respectively, compared with the control (N0). Seabream fed FM25 diets (irrespective nucleotide levels) exhibited significantly higher content of protein (3.8%), and ash (9.1%), and lower content of lipids (17.3%) and carcass energy (3.8%) compared with FM0 groups.

Amino acids (AA) profile. Biochemical analyses of fish extraction, total protein (% of dry weight basis: DM) and (AA) (g/100 g DW protein) in the fish flesh of juvenile seabream fed six experimental diets with different levels of nucleotides and FM are presented in Table 6 and Figure 1. The results showed that, with increasing dietary nucleotide level (irrespective FM levels), the fish body exhibited higher significant ($p \leq 0.5$) content of protein, total AA, and 15 from 19 specific amino acids that were analyzed compared with N0 group. An improvement with 6.8, 8.2, and 43.6% in fish extraction content of protein, AA, and total AA at N250 group compared with N0, while there are no significant differences between N250 group and N500 group. Seabream fed FM25 diets (irrespective nucleotide levels) exhibited lower non-significant content of protein, and AA, and lower significant content of 18 from 19 amino acids that were analyzed compared with the N0 group. A reduction in the percentage of total AA with 43.2% was detected at the FM25 group compared with the FM0 group.

Table 2

Growth performance, feed utilization, and biometric indices of juvenile gilthead seabream, *Sparus aurata* fed different experimental diets

Measured parameters	*FM_0*			*FM_{25}*			*P-value*
	N_0	*N_{250}*	*N_{500}*	*N_0*	*N_{250}*	*N_{500}*	
	D1	*D2*	*D3*	*D4*	*D5*	*D6*	
IBW (g $fish^{-1}$)	0.35±0.01	0.36±0.01	0.36±0.01	0.35±0.01	0.36±0.01	0.36±0.01	0.921
FBW (g $fish^{-1}$)	$11.76±0.27^{d}$	$12.38±0.23^{cd}$	$13.14±0.19^{bc}$	$12.60±0.36^{c}$	$13.63±0.16^{ab}$	$14.25±0.24^{a}$	0.714
WG (g $fish^{-1}$)	$11.40±0.26^{d}$	$12.02±0.23^{cd}$	$12.78±0.19^{bc}$	$12.25±0.36^{c}$	$13.27±0.16^{ab}$	$13.89±0.24^{a}$	0.713
ADG (g $fish^{-1}$ day^{-1})	0.076^{d}	0.080^{cd}	0.085^{bc}	$0. 082^{c}$	0.089^{ab}	0.093^{a}	0.709
PER (%)	$0.90±0.01^{d}$	$0.96±0.02^{c}$	$1.02±0.01^{b}$	$0.92±0.010^{cd}$	$1.07±0.03^{b}$	$1.14±0.01^{a}$	0.020
Survival (%)	66.67^{b}	73.33^{ab}	76.67^{ab}	73.33^{ab}	76.67^{ab}	83.33^{a}	0.848
K	$1.29±0.03^{b}$	$1.33±0.03^{b}$	$1.36±0.03^{ab}$	$1.30±0.03^{b}$	$1.36±0.01^{ab}$	$1.42±0.02^{a}$	0.761
VSI (%)	$10.40±0.25^{a}$	$10.20±0.16^{a}$	$9.76±0.21^{a}$	$9.09±0.29^{b}$	$8.58±0.11^{bc}$	$8.29±0.13^{c}$	0.757
HSI (%)	$1.20±0.02^{c}$	$1.33±0.03^{b}$	$1.42±0.02^{a}$	$1.22±0.01^{c}$	$1.25±0.01^{c}$	$1.31±0.02^{b}$	0.010

* Values are means±SEM, N = 3 per treatment group; ** Means in a row without a common superscript letter are significantly different ($p < 0.05$); IBM = initial body weight, FBW = final body weight, WG = weight gain, ADG = average daily gain, PER = protein efficiency ratio, k = condition factor, VSI = viscerosomatic index, HSI = hepatosomatic index; FM = fishmeal; N = nucleotides, FM_0 = 0% fish meal, FM_{25} = 25% fish meal, N_0 = no nucleotide, N_{250} = 250 mg nucleotide, N_{500} = 500 mg nucleotide; D1 = FM0/N0; D2 = FM0/N250; D3 = FM0/N500; D4 = FM25/N0; D5 = FM25/N250; D6 = FM25/N500.

Table 3

The main effects of dietary FM levels irrespective N levels or dietary N levels irrespective FM levels on growth performance, feed utilization, and biometric indices of juvenile gilthead seabream, *Sparus aurata* fed different experimental diets

Measured parameters	*Experimental treatments*						*P-value*
	Dietary FM levels irrespective N levels		*P-value*	*Dietary N levels irrespective FM levels*			
	FM_0	*FM_{25}*		*N_0*	*N_{250}*	*N_{500}*	
IBW (g $fish^{-1}$)	0.36±0.01	0.36±0.01	0.778	0.35±0.01	0.36±0.01	0.36±0.01	0.369
FBW (g $fish^{-1}$)	$12.43±0.22^{b}$	$13.49±0.25^{a}$	0.000	$12.18±0.30^{c}$	$13.01±0.22^{b}$	$13.70±0.14^{a}$	0.000
WG (g $fish^{-1}$)	$12.07±0.20^{b}$	$13.14±0.21^{a}$	0.000	$11.83±0.11^{c}$	$12.65±0.13^{b}$	$13.34±0.12^{a}$	0.000
ADG (g $fish^{-1}$ day^{-1})	0.081^{b}	0.088^{a}	0.008	0.079^{b}	0.084^{ab}	0.089^{a}	0.007
PER (%)	$0.96±0.02^{b}$	$1.04±0.04^{a}$	0.000	$0.91±0.04^{c}$	$1.02±0.02^{b}$	$1.08±0.01^{a}$	0.000
Survival (%)	72.22^{b}	77.78^{a}	0.064	70.00^{c}	75.00^{b}	80.00^{a}	0.035
(k)	$1.33±0.02^{b}$	$1.36±0.02^{a}$	0.162	$1.30±0.03^{b}$	$1.35±0.01^{b}$	$1.39±0.02^{a}$	0.009
VSI (%)	$10.12±0.17^{a}$	$8.65±0.16^{b}$	0.000	$9.75±0.17^{a}$	$9.39±0.13^{b}$	$9.03±0.16^{c}$	0.012
HSI (%)	$1.32±0.02^{a}$	$1.26±0.01^{b}$	0.003	$1.21±0.01^{c}$	$1.29±0.02^{b}$	$1.37±0.02^{a}$	0.000

* Values are means±SEM, N = 3 per treatment group; ** Means in a row without a common superscript letter are significantly different ($p < 0.05$); IBM = initial body weight, FBW = final body weight, WG = weight gain, ADG = average daily gain, PER = protein efficiency ratio, k = condition factor, VSI = viscerosomatic index, HSI = hepatosomatic index; FM = fishmeal; N = nucleotides, FM_0 = 0% fish meal, FM_{25} = 25% fish meal, N_0 = no nucleotide, N_{250} = 250 mg nucleotide, N_{500} = 500 mg nucleotide.

Table 4

Proximate body composition of juvenile gilthead seabream, *Sparus aurata* fed different experimental diets

Measured parameters	*FM_0*			*FM_{25}*			*P-value*
	N_0	*N_{250}*	*N_{500}*	*N_0*	*N_{250}*	*N_{500}*	
	D1	*D2*	*D3*	*D4*	*D5*	*D6*	
Dry matter (%)	28.81±0.80	29.51±0.46	29.34±1.66	29.06±0.80	29.10±1.36	30.48±1.99	0.754
Protein (%)	62.83±0.34^{e}	65.17±0.39^{c}	63.93±0.12^{d}	65.77±0.23^{bc}	66.47±0.12^{ab}	66.97±0.18^{a}	0.000
Ether extract (%)	21.34±0.46^{ab}	21.88±0.60^{a}	21.61±0.90^{ab}	14.66±0.22^{d}	19.97±0.21^{bc}	18.98±0.46^{c}	0.000
Ash (%)	16.61±0.13^{c}	15.33±0.12^{e}	16.01±0.23^{d}	18.64±0.23^{a}	16.03±0.24^{d}	17.65±0.13^{b}	0.000
Carcass energy (Kcal/100 g)	555.82±3.29^{b}	574.11±6.94^{a}	564.60±8.90^{ab}	509.34±1.56^{cd}	563.36±2.67^{ab}	556.86±3.37^{b}	0.000

* Values are means±SEM, N = 3 per treatment group; ** Means in a row without a common superscript letter are significantly different ($p < 0.05$); FM = fishmeal; N = nucleotides, FM25 (25% fish meal), FM_{25} (25% fish meal), FM_0 (0% fish meal), N0 (0 mg nucleotide), N_{250} (250 mg nucleotide), N_{500} (500 mg nucleotide); D1 = FM0/N0; D2 = FM0/N250; D3 = FM0/N500; D4 = FM25/N0; D5 = FM25/N250; D6 = FM25/N500; Initial sample (% dry matter basis): dry matter = 28.60±0.17%; crude protein = 67.30±0.26%; ether extract = 16.10±0.26%; ash = 10.87±0.23%; carcass energy = 531.56±2.26 Kcal/100 g.

Table 5

The main effects of dietary FM levels irrespective N levels or dietary N levels irrespective FM levels on proximate body composition of juvenile gilthead seabream, *Sparus aurata* fed different experimental diets

Measured parameters	*Experimental treatments*						
	Dietary FM levels irrespective N levels		*P-value*	*Dietary N levels irrespective FM levels*			*P-value*
	FM_0	*FM_{25}*		*N_0*	*N_{250}*	*N_{500}*	
Dry matter (%)	29.22±0.85	29.55±1.20	0.761	28.94±0.80	29.31±0.76	29.91±1.51	0.369
Protein (%)	63.98±0.24^{b}	66.40±0.16^{a}	0.000	64.30±0.28^{b}	65.82±0.24^{a}	65.45±0.13^{a}	0.000
Ether extract (%)	21.61±0.60^{a}	17.87±0.33^{b}	0.000	18.00±0.36^{b}	20.93±0.41^{a}	20.30±0.62^{a}	0.000
Ash (%)	15.98±0.16^{b}	17.44±0.18^{a}	0.000	17.63±0.17^{a}	15.68±0.20^{c}	16.83±0.19^{b}	0.000
Carcass energy (Kcal/100 g)	564.84±5.6^{a}	543.19±2.5^{b}	0.000	532.58±2.8^{b}	568.74±4.7^{a}	560.73±5.6^{a}	0.068

* Values are means±SEM, N = 3 per treatment group; ** Means in a row without a common superscript letter are significantly different ($p < 0.05$); FM = fishmeal; N = nucleotides, FM25 (25% fish meal); FM_{25} (25% fish meal), FM_0 (0% fish meal), N0 (0 mg nucleotide), N_{250} (250 mg nucleotide), N_{500} (500 mg nucleotide); Initial sample (% dry matter basis): dry matter = 28.60±0.17%; crude protein = 67.30±0.26%; ether extract = 16.10±0.26%; ash = 10.87±0.23%; carcass energy = 531.56±2.26 Kcal/100 g.

Table 6

The main effects of dietary FM levels irrespective N levels or dietary N levels irrespective FM levels on protein (% DM) and amino acids (AA) (g/100 g DM) of juvenile gilthead seabream, *Sparus aurata* fed different experimental diets

Amino acids	*Dietary FM levels irrespective N levels*		*P-value*	*Dietary N levels irrespective FM levels*			*P-value*
	FM_0	FM_{25}		N_0	N_{250}	N_{500}	
Protein[a] (% DM)	40.48±1.11	38.24±0.40	0.131	37.96±0.13[b]	40.54±0.51[a]	39.59±0.42[a]	0.002
AA[b] (g/100 g DM protein)	39.28±1.54	38.49±0.38	0.645	36.98±0.23[b]	40.03±0.38[a]	39.64±0.23[a]	0.000
Alanine	2.31±0.32[a]	1.22±0.19[b]	0.043	1.38±0.15[b]	2.25±0.22[b]	1.68±0.15[a]	0.014
Arginine	2.77±0.19[a]	1.57±0.32[b]	0.033	1.75±0.23[b]	2.64±0.16[a]	2.12±0.18[ab]	0.022
Aspartic acid	4.16±0.17[a]	2.62±0.46[b]	0.034	2.80±0.34[b]	3.86±0.19[a]	3.51±0.20 [ab]	0.034
Cysteine	0.75±0.10[a]	0.35±0.06[b]	0.023	0.41±0.06[b]	0.67±0.08[a]	0.58±0.06[ab]	0.042
Glutamic acid	2.53±0.20[a]	1.65±0.13[b]	0.022	1.85±0.13[b]	2.41±0.16[a]	2.01±0.12[ab]	0.041
Glycine	1.81±0.14[a]	1.07±0.11[b]	0.014	1.24±0.10[b]	1.67±0.12[a]	1.41±0.13[ab]	0.070
Histidine	0.008[a]	0.005[b]	0.016	0.01±0.0	0.01±0.0	0.01±0.0	----
Isoleucine	1.06±0.07[a]	0.73±0.06[b]	0.024	0.79±0.05[b]	1.01±0.06[a]	0.90±0.05[ab]	0.031
Leucine	2.15±0.10[a]	1.35±0.08[b]	0.004	1.59±0.12	1.90±0.13	1.76±0.13	0.252
Lysine	2.23±0.16[a]	1.45±0.16[b]	0.025	1.62±0.13[b]	2.15±0.12[a]	1.76±0.12[b]	0.029
Methionine	1.30±0.11[a]	0.42±0.10[b]	0.004	0.68±0.13	1.04±0.14	0.86±0.14	0.226
Phenylalanine	1.21±0.13[a]	0.70±0.10[b]	0.040	0.77±0.08[b]	1.17±0.09[a]	0.93±0.06[ab]	0.016
Phosphoserine	0.002	0.0013	0.519	0.0015[b]	0.001[c]	0.002[a]	0.000
Proline	1.78±0.23[a]	0.88±0.17[b]	0.033	1.01±0.11[b]	1.68±0.15[a]	1.31±0.16[ab]	0.021
Serine	1.41±0.16[a]	0.76±0.08[b]	0.023	0.91±0.09	1.32±0.13	1.03±0.09	0.044
Taurine	0.08±0.01[a]	0.05±0.005[b]	0.012	0.055±0.005[b]	0.075±0.008[a]	0.065±0.005[ab]	0.101
Threonine	1.54±0.17[a]	0.76±0.12[b]	0.022	0.88±0.11[b]	1.39±0.14[a]	1.19±0.12[ab]	0.039
Tyrosine	1.23±0.22[a]	0.55±0.08[b]	0.044	0.62±0.06[b]	1.14±0.14[a]	0.91±0.12[ab]	0.023
Valine	1.75±0.21[a]	0.96±0.07[b]	0.024	1.12±0.09[b]	1.60±0.17[a]	1.35±0.11[ab]	0.063
Total AA	30.08±2.66[a]	17.08±2.26[b]	0.020	19.46±2.0[b]	27.95±2.2[a]	23.34±2.0[ab]	0.040

[a] Protein determined with the bovine serum albumin method of Lowry et al (1951); [b] AA in weight is calculated as protein (i.e. from the amino acid mole weight subtracted by the mole weight of a water molecule, which resembles the AA before hydrolysis); P: protein (% DM); AA = amino acids (g/100 g DM); FM = fishmeal; N = nucleotides; FM_{25} (25% fish meal), FM_0 (0% fish meal), N0 (0 mg nucleotide), N_{250} (250 mg nucleotide), N_{500} (500 mg nucleotide).

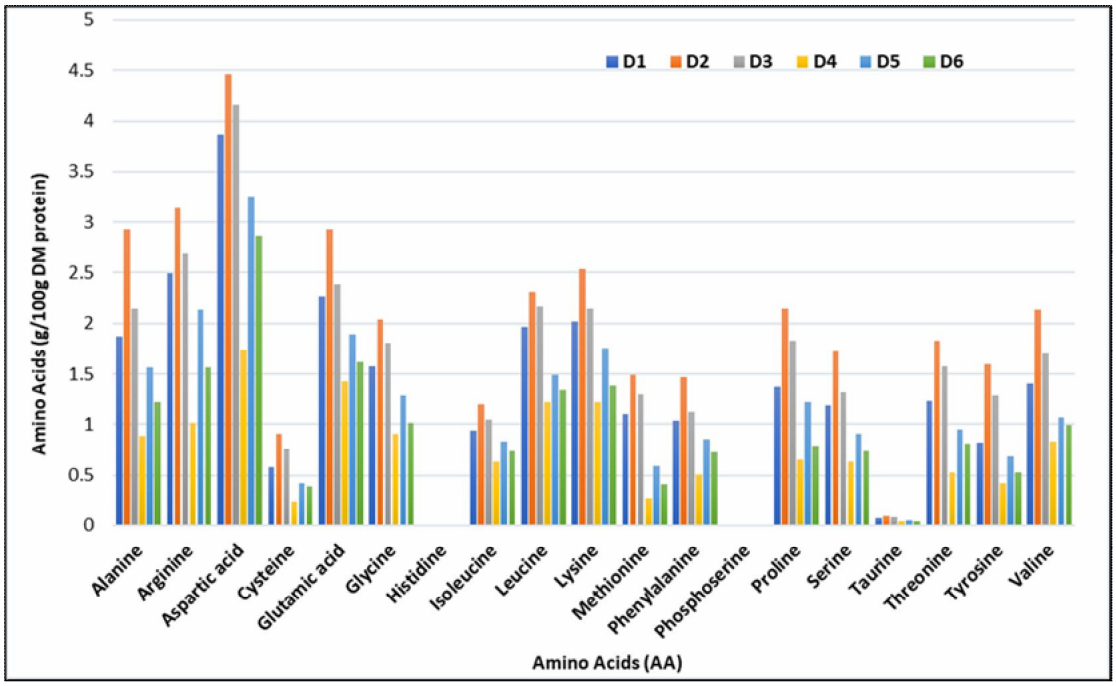

Figure 1. Biochemical analyses of fish extraction, and amino acids profile (AA) (g/100 g DW protein) of juvenile gilthead seabream, *Sparus aurata* flesh fed six experimental diets with different levels of nucleotides and fishmeal. Where: D1 = FM0/N0; D2 = FM0/N250; D3 = FM0/N500; D4 = FM25/N0; D5 = FM25/N250; D6 = FM25/N500.

Discussion. Nucleotides are the building blocks of DNA and RNA. They are a group of vital biochemical substances that play different physiological roles in every biological process that is indispensable to the support of life. For instance, the crucial cellular function, metabolism, biosynthetic pathways, biological regulators, activated intermediates of energy transport in cells and cell signaling (Li & Gatlin 2006; Krüger & van der Werf 2018). Nucleotides are synthesized de novo in most fish tissues, but with low quantity under certain conditions (Do Huu 2016). The dietary supplementation of nucleotides for fish may be of significant benefit, particularly to mitigate the adverse effects at the time of physiological stress (Hess & Greenberg 2012). FM is one of the most important natural sources of nucleotides, but unfortunately, it is the most expensive aqua-feed ingredient with limited resources and a continuous decrease in the available quantities of it due to the increase in demand. Therefore, researchers have been looking for other alternative feedstuffs containing high levels of nucleotides to address the growing shortage of FM (Do Huu et al 2012). The potential positive effect of nucleotides supplementation in low FM diet observed in many studies (Liu 2016) could be related to different mechanisms. Specific nucleotides have been detected to have notable attractant properties for fish diets.

Results of the current research work revealed a significant improvement in seabream growth indices (FW, WG, and ADG) for fish fed nucleotides-enriched diets compared with the nucleotide-free diets (D1, and D4) in both fish meal levels. This result is in accordance with Borda et al (2003), who reported that exogenous supply of nucleotides might promote the growth of seabream larvae to meet their high rate of cell replication. Also, the application of nucleotides demonstrated a positive influence on growth performance when added to formulated fish diets (Lin et al 2009; Tahmasebi-Kohyani et al 2012; Ringø et al 2012). Dietary uptake of exogenous nucleotides, isolated from yeast, may optimize cell proliferation to promote rapid growth. Özlüer-Hunt et al (2014) found that growth rates of rainbow trout juveniles, *Onchorchyncus mykiss* was positively affected associated with increasing gastric pepsin activity and intestinal trypsin and lipase activity when FM was replaced with nucleotides. The same positive effect of dietary nucleotides on fish growth was discovered in other fish species such as on beluga sturgeon (Abtahi et al 2013), *Argyrosmus regius* (Saenz de Rodriganez et al 2013), hybrid tilapia *Oreochromis niloticus* x *Oreochromis aureus* (Xu et al 2015), juvenile

turbot, *Scophthalmus maximus* (Peng et al 2013; Fuchs et al 2015), zebrafish (Guo et al 2017), grass carp *Ctenopharyngodon idellus* (Tie et al 2018), and black tiger shrimp *Penaeus monodon* (Do Huu et al 2012).

The effects of dietary FM level irrespective nucleotides levels showed a significant improvement on the growth performance index in favor of FM25. This result agrees with many authors such as Do Huu et al (2012), who stated that aqua-feed without FM contains relatively low amounts of nucleotides, which in turn negatively affects fish growth. In this context, Jalili et al (2013) found that the complete replacement of FM with a mixture of plant proteins (wheat gluten, corn gluten, and soybean meal) reduced rainbow trout growth by 32.6% compared with 100% FM diet.

The present study revealed that increasing nucleotides level positively enhanced feed utilization induces (PER) compared with 0% nucleotide supplemented diets, especially at FM25 diets. This result is consistent with Tahmasebi-Kohyani et al (2012), who reported that 0.1% nucleotide added to rainbow trout diets improved feed efficiency. Lin et al (2009) said that growth performance and feed efficiency significantly improved in grouper *Epinephelus malabaricus* when fed nucleotide supplemented diet at 1.5 g kg^{-1} diet compared with the control diet.

Also, Yin et al (2015) stated that *Ancherythroculter nigrocauda* fed nucleotide enriched diets at 4.5 mg kg^{-1} feed showed the highest values of growth, PER, and the lowest value of feed conversion ratio (FCR) compared with nucleotide-free diet. Hossain et al (2016) concluded that the nucleotide-supplemented diet at 1.5 g kg^{-1} of juvenile red seabream, *Pagrus major* showed the best growth performance, feed utilization, and survival percent compared with the 0 g kg^{-1} feed using control diet contains 28% FM. Generally, as the tested product in the current study (Nucleoforce Fish™) has taste and aroma characteristics, this may have been caused by better attractability and development of the intestine of the nucleotide-supplemented larvae, and as a consequence, better utilization of the first exogenous feed. Increasing the values of HSI with increasing the dietary nucleotide levels in the present study indicates proper storage of macro and micronutrients and healthy condition of the liver as well as healthy clinical signs of fish. The state of the liver as measured with the HSI may serve as an initial practical bio-indicator to indicate the overall health status of fish and implicate different energy storage in fish (Garcia-Diaz et al 2006) which is a good indicator of recent feeding activity and the nutritional status of fish (Tyler & Dunn 1976; Hossain et al 2016). Condition factor (K) is one of the essential parameters that explain the physiological status of the fish (Salam & Davies 1992). The highest value recorded in the present study for K value was in favor of fish fed D6 (FM25/500) and FM25 diets compared with FM0 diets. Higher condition factors indicated good health with isometric growth, which is desirable for fish in fish farms (Ayode 2011). In the present study, higher survival rate for fish fed nucleotide supplemented diets attributed generally to the improved health condition, the enhanced resistance to parasitic, bacterial, and viral diseases (Ringø et al 2012; Kiron 2012; Herrera et al 2019), and might be attributed to high efficiency of fish to cope well with an acute stress challenge like handling as stated by Palermo et al (2013) in Senegalese sole *Solea senegalensis* fed nucleotides derived from yeast.

In the present study, the effects of dietary FM level irrespective nucleotides levels showed a significant improvement in feed utilization index (PER), survival, condition factor, and biometric indices in favor of FM25. This result agrees with Jalili et al (2013) whom found that the complete replacement of FM with a mixture of plant proteins reduced FCR by 27.1%, increased condition factor by 7.5%, reduced mortality by 10%, in rainbow trout feed diet contained 100% FM compared with FM free diet. Also, the previous authors found a significant correlation between FM contained diets, and both HSI and VSI. In agreement with our findings, the inclusion of plant protein in the rainbow trout diet resulted in significantly higher VSI (Palmegiano et al 2006) compared with FM inclusion diets.

Our results confirmed that the whole-body proximate composition was significantly influenced by dietary nucleotides supplementation and FM content. The increase in the level of nucleotides in seabream diets resulted in a significant increase in the fish body's protein content, especially in diets with FM0 content and a significant

increase in the fat content, especially in diets with FM25 content. Our results are in agreement with Selim et al (2019), who found that the nucleotide supplemented diets had significant effects on the whole-body chemical composition (protein and fat) of the Nile tilapia (*Oreochromis niloticus*). Also, our results concerning the content of protein are in agreement with Adamek et al (1996) for rainbow trout and Abtahi et al (2013) for *Huso huso.* Agreeing with our results, Carver (1994) stated that extracellular nucleotides and nucleosides might play an essential role in the synthesis of glycogen, protein, and lipid. Our results confirmed a negative correlation between the nucleotides level and ash content; also, a positive correlation with carcass energy in fish flesh was noted. This result is in agreement with Fuchs et al (2015). Fish fed FM0 diets in the current study showed higher content of lipids compared with FM25 fed groups. This result is congruent with Soltan et al (2008) in Nile tilapia and Havasi et al (2015) in *Silurus glanis.* They found a higher content of carcass lipid in the fish group fed a diet with zero percent FM (a mixture of animal and plant proteins) compared to FM diets. However, Liu (2016) found that FM and nucleotide supplementation did not significantly influence whole-body composition except for ash content of fish fed with 0% FM, which decreased significantly. However, significant interactions were detected between FM level and nucleotide levels affecting moisture, lipid, ash, and gross energy content that indicates the influence of nucleotide supplementation on whole body composition as a function of the FM level.

Amino acid profile is more crucial than the whole crude protein levels in formulation of diets (Fegan et al 2006). Diet formulation with an optimal essential amino acid (EAA) accompanied by adequate protein content is a necessity to optimize the amino acid diet content for growth, thus reducing nitrogen excretion regardless the presence of fish meal in diet. The carnivorous fish, such as sea bream, use protein preferentially for fats or carbohydrates as an energy source (Peres & Oliva-Teles 1999; Estruch et al 2018). Retention of EAA is related with the fishmeal substitution as it was reported in previous studies that higher level of EAA retention is associated with diets with higher level of fishmeal replacement, our results being in accordance with these studies (Gómez-Requeni et al 2004; Sánchez-Lozano et al 2009; Martínez-Llorens et al 2012; Estruch et al 2018). The impact of using nucleotides supplementation significantly improved the essential amino acid body composition (Fegan et al 2006; Tie et al 2018). According to Fegan et al (2006), diets supplemented with NuPro® as an exogenous source of nucleotides at dose 250 mg kg^{-1} diet exhibited a substantial improvement in EAA body composition. Yet using excessive amount of nucleotides could have some negative impacts on essential amino acid body composition and consequently on growth (Do Huu 2016). In the current study FM0 diets improved significantly the content of AA compared with FM25. This might be attributed to the improved amino acid profile of the FM0 group diets as FM replaced with highly nutritive value diets contained a balanced content of amino acids (Table 1) which were reflected on the content of the AA in the fish body (Table 6). With increasing dietary nucleotide levels (irrespective of FM levels), the fish body exhibited higher significant ($P \leq 0.5$) content of protein, total AA, and amino acids compared with NT0 group.

However, the pattern of AA profile (g/100g DW protein) of juvenile gilthead seabream in the biochemical analyses of fish extraction (Figure 1) showed that FM0 diets (irrespective of nucleotide content) had higher retention than FM25 diets, furthermore diets with higher and zero nulceotide content (N500 and N0) were significantly lower in AA than diets with N250 supporting the hypothesis of Do Huu (2016). Therefore, the interrelation ship between fishmeal replacement and the dosage of nucleotide supplementation need further research. Thus, it is assumed that nucleotides are essential nutrients beneficial to fast-growing small fish.

Conclusions. The data in this study indicated that the best treatment was FM25/500. Taking into account that the FM25/500 diet reduced the usage of fishmeal in seabream feed by 25% and this eco-friendlier (more sustainable) without adverse effects on the efficiency of growth and feed utilization, and fish quality analysis. Also, the addition of nucleotide will reduce the cost of aquafeeds which is considered a determining factor for the economics of mariculture.

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Authors:
Asmaa M. El-Nokrashy, Department of Aquaculture, Faculty of Aquatic and Fisheries Science, Kafrelsheikh University, Kafr Elsheikh, Egypt, e-mail: drasmaa.elnokrashy2@gmail.com
Ramadan A. El-Banna, Department of Nutrition, Faculty of Veterinary Medicine, Cairo University, Egypt, e-mail: bannaram@yahoo.com
Bahgat M. Edris, Department of Nutrition, Faculty of Veterinary Medicine, Cairo University, Egypt, e-mail: Bmedrise2002@yahoo.com
Mohamed M. Abdel-Rahim, Aquaculture Division, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt, e-mail: mohamed_m_ar@yahoo.com
Miguel Jover Cerdá, Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology, Universitat Politècnica de València, València, Spain, e-mail: mjover@ dca.upv.es
Ana Tomás-Vidal, Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology, Universitat Politècnica de València, València, Spain, e-mail: atomasv@dca.upv.es
Radi A. Mohamed, Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt, e-mail: radiali_2007@yahoo.co.uk
Ashraf M. A. S. Goda, Aquaculture Division, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt, e-mail: goda_ashraf@yahoo.com

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