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1 Impact of Nucleotide enriched diets on the production of gilthead seabream, *Sparus aurata*
2 fingerlings by modulation of liver mitochondrial enzyme activitity, antioxidant status, immune
3 gene expression, and gut microbial ecology

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30 **Abstract**

31 The objective of this study was to evaluate the effect of fish meal replacement with a
32 mixture of plant, and animal protein supplemented with a commercial nucleotide
33 NucleoforceFish™, on growth, feed utilization, whole-body composition, biometric indices; liver
34 mitochondrial enzyme activities, selected gene expression and influence on the gut microbiome of
35 gilthead seabream, *Sparus aurata.* Six hundred and thirty gilthead seabream with an average initial
36 body weight of 0.358 ± 0.002 g/fish, were used. Six experimental diets were formulated to be
37 isonitrogenous, 45% crude protein (CP) and isocaloric 20 MJ/kg gross energy (GE). The control
38 diet (D1) contained 25% of fish meal (FM_{25}) and had no NucleoforceFish™ added ($FM_{25}/_{0}$), diets
39 2-3 contained FM at a level of 25% and supplemented with 250 ($FM_{25/250}$) and 500 ($FM_{25/500}$)
40 mg/kg NucleoforceFish™, respectively. Diets 4-6 were a non-fishmeal formulation and had no
41 NucleoforceFish™, (diet 4, $FM_{0/0}$), 0%FM with 250mg/kg NucleoforceFish™ (diet 5, $FM_{0/250}$),
42 and 0%FM with 500mg/kg NucleoforceFish™ (diet 6, $FM_{0/500}$). The results revealed that dietary
43 nucleotides supplemented at 250mg/kg or 500mg/kg enhanced final body weight, weight gain, and
44 specific growth rate of seabream either with dietary level of 25% FM diet (FM_{25}) or non-fishmeal
45 diet (FM_0). All mitochondrial liver complex enzyme activities were recorded as having a
46 significant positive response to the nucleotide dietary supplement. Gilthead seabream fingerlings
47 fed $FM_{0/250}$ diet for 150 days had significantly elevated liver mitochondrial enzyme (Complex III,
48 Complex IV, Complex I and II) activities. In addition, gilthead seabream fed $FM_{0/500}$ had
49 significantly increased the gene expression of TCR-B and IL1-*B*, while fish fed FM25/250
50 increased significantly the gene expression of IGF1, while fish fed FM25/500 increased
51 significantly the gene expression of Hepcidin (Hep) and the iron homeostasis hormone. There was
52 a marked influence on specific bacteria comprising the gut microbiome namely *Photobacterium*;

damselae; *Vibrio anguillarum*; *Lactobacillus plantarum*; *Lactobacillus Enterococcus faecium subtilis*. It can be concluded that $FM_{25/250}$ and $FM_{25/500}$ diets are expressing the best result, which explains that FM must be in the feed of gilthead seabream, and addition of NucleoforceFish™ at 250 mg/kg feed may further increase the performance.

Keywords: Fish meal, Nucleotide Supplementation, Mitochondrial & metabolic enzymes, financial analysis.

1. Introduction

The production of marine fish has increased to meet the growing demand of global seafood (FAO, 2018). Marine fish species need high levels of protein in their diets which is usually provided by fish meal obtained from wild fisheries or by-products of the animal processing industries. Fish meal is an optimal protein source for aquafeeds because of its nutritional value in terms of essential amino acids, and high palatability to fish. Elimination or reduction of the contribution of fish meal in marine fish diets was the gateway for nutritionists to provide economic and environmental benefits by reducing the cost of feed for fish farmers and reducing the pressure on species harvested for fish meal production and also serve as important resources in the marine food web (Tacon et al., 2011, Kok et al., 2020). Currently, a variety of vegetable and animal protein sources are used for substitution in aquafeeds with different inclusion rates (Rossi et al., 2021; Deng et al., 2011; Santigosa et al., 2011; Wang et al., 2012; Estruch et al., 2018; Hassaan et al., 2021; Hosseini et al., 2020; Goda et al 2020a,b; Davies et al., 2020; Hassaan et al., 2020; Goda et al ., 2019) The common obstacle that nutritionists challenged regarding using alternative ingredients was changes in the immune status of marine fish by the introduction of a host of anti-nutritional factors especially in many plant by-products (Sitjà-Bobadilla et al., 2005). Not only the immune status can be altered by the fish meal replacement but also the intestinal absorption can be affected

76 by these replacements as discussed by Santigosa et al. (2011). Consequently, aid solutions were
77 recommended to solve this situation by using several feed additives among which are nucleotides
78 (Ringø et al., 2012; Reda et al., 2018; Cardinaletti et al., 2019; Ashouri et al., 2020; Hossein et al.,
79 2016; Hassaan et al., 2018).

80 Various researchers have studied nucleotides (NT) as functional nutrients for many species,
81 especially mono-gastric animals such as those for aquaculture and poultry (Jung and Batal, 2012;
82 Hossain et al., 2019). As such, nucleotides have been viewed as semi-essential nutrients; under
83 specific conditions of rapid growth, stress and disease where *de novo* synthesis is not sufficient to
84 meet physiological and metabolic demand. In this respect, Ki and Sung (2019) investigated the
85 attributes of supplemental dietary nucleotides on intestinal health and growth performance of
86 newly weaned pigs to examine primarily the effects on gut health with positive findings. Recently,
87 Mohamed et al. (2020) investigated the impact of exogenous dietary nucleotides in ameliorating
88 *Clostridium perfringens* infection by controlled challenge and elevating the intestinal barrier gene
89 expression in broiler chicken with good results for a commercial nucleotide source (Nucleoforce
90 poultry^{TM}). These workers also observed significant improvement in microscopic lesion scores,
91 intestinal histomorphology, intestinal barriers (occludin and mucin mRNA expression) and growth
92 parameters in broilers fed nucleotide supplementation. With respect to fish, Ferreira et al. (2020)
93 found a positive influence of genes expression related to lipid metabolism and oxidative stress
94 modulating of rainbow trout (*O.Mykiss*) fed diets contained *Saccharina latissima*. Furtermore,
95 Safari et al. (2015) found that crayfish fed diets contained dietary nucleotide enhanced the growth
96 performance, digestibility and immune responses. Also, Ashouri et al. (2020) found that Asian
97 seabass fed diets supplemented with dietary sodium alginate and *Pediococcus acidilactici*

98 improved liver antioxidant enzymes, intestinal lysozyme gene expression, histomorphology,
99 microbiota, and digestive enzyme activity.

100 Animals can obtain their required amount of nucleotides exogenously or endogenously (Reda
101 et al., 2018). Because of the active synthesis of NT mainly in the liver, most animals appear to be
102 almost independent of exogenous NT (Jyonouchi, 1994). For aquatic animals, a positive
103 correlation between the addition of nucleotides and growth performance was also witnessed in
104 many aquatic species (Oliva-Teles et al., 2006; Xu et al., 2015; Jin et al., 2018; El-Nokrashy et al.,
105 2020). Most recently, Bowyer et al. (2019) reported the benefits of supplementation of a
106 commercial nucleotide product on the intestinal morphology of sea bass, *Dicentrachus labrax*. The
107 information on the effect of nucleotides on feed efficiency, metabolism, and mitochondrial enzyme
108 complexes for sea bream is scarce. Dietary nucleotides positively affect fat metabolism, immunity,
109 tissue development, and repair. The immune system and intestinal tract are unable to meet the
110 needs of cellular nucleotides exclusively by synthesizing *de novo* and prefer to use 'the rescue'
111 pathway to restore nucleotides and the nucleotide base of blood and system (Gil, 2020). The
112 molecular mechanisms through which dietary nucleotides modify the immune system are
113 practically unknown. The small intestine plays a key role in the regulatory effects of nucleotides
114 on the immune response. Dietary nucleotides have been shown to promote gene expression in the
115 small intestine and may affect the biosynthesis of protein as well as the interaction of external
116 nucleosides, and their receptors may also contribute to the modification of the expression of
117 several genes. Additionally, mitochondria play a central role in energy metabolism and are the
118 principal organelles of intracellular reactive oxygen species (ROS) generation. As such, they
119 contain a complement of factors that can also promote cell death, thus displaying a shared platform
120 for metabolism and apoptosis (Vakifahmetoglu-Norberg et al., 2017).

121 To maintain optimal function of mitochondria, an adequate supply of many micronutrients
122 should be sustained (Wai and Langer, 2016). The complex relationship of the gastrointestinal tract
123 and gut mucosal interface in terms of optimized nutrition and immune-competence in fish is of the
124 vital importance. Burrells et al. (2001a) reported that dietary supplementation with nucleotides
125 induced an increased resistance against pathogens in rainbow trout. These workers specifically
126 showed that a combined inclusion level of 0.03%, mitigated challenge infections with bacterial,
127 viral and rickettsial diseases as well as ectoparasitic infestation. Cheng at al. (2011) demonstrated
128 the effectiveness of the influence of dietary nucleotides on immune responses and intestinal
129 morphology of red drum, *Sciaenops ocellatus* showing higher enterocyte height and microvilli
130 height. For salmonids, Hunt et al. (2016) investigated the effects of dietary nucleotide yeast
131 supplemenation on immune responses and related antioxidant enzyme activities of rainbow trout
132 juveniles (*Oncorhynchus mykiss*) with promising findings. Ringø et al. (2012) presented a
133 comprehensive review on the utilization of immunostimulants and nucleotides in aquaculture
134 covering many scenarios.

135 Gut integrity and improved function is also a well recognized attribute of dietary nucleotide
136 addition to the diets of fish. Burrells (2001b) showed that fish fed nucleotides displayed a healthier
137 intestinal system indicated by 18-21% increase of the lateral branching and height of the intestinal
138 mucosal folds and also positive effects on vaccination, sea water transfer, growth rates and
139 physiology of Atlantic salmon (*Salmo salar L.*). It has been reported that improved morphology of
140 the intestine is a contributing factor to the improved growth of fish due to more efficient nutrient
141 digestion and absorption. Xu et al. (2015) in their studies to elucidate the effects of dietary yeast
142 nucleotides on growth, nonspecific immunity, intestine growth and intestinal microbiota of

143 juvenile hybrid tilapia *Oreochromis niloticus x Oreochromis aureus* reported several production
144 related attributes.

145 The role of nucleotides in influencing the key mechanisms of cellular communication has not
146 been delineated and requires attention. For these reasons, a group of genes of interest was targeted
147 for their expression in response to nucleotide supplementation in the diet for seabream. TCR, IL1-
148 *B*, and IGF1 and immunoglobulin M IgM, and Interleukin1 beta T-Cell receptor proteins, are
149 known to be important. Additionally, Hepcidin, an important peptide with antimicrobial properties
150 and iron regulation capacity, was also selected. With the advent of new molecular sequencing
151 techniques, it is easier to understand how the immune system of fish, may be orchestrated by the
152 complex microbial communities that live in symbiosis with their hosts (Daniela Gomez et al.,
153 2013). An even more paucity exists for the effect of dietary nucleotides on the gut microbial
154 community, so the following important selection of gut-associated bacteria that may support
155 immune function, i.e., *Photobacterium damselae*, *Photobacterium damse*, *Vibrio anguillarum*,
156 *Lactobacillus plantarum*, *Lactobacillus subtilis*, *Lactobacillus subtilis,* and *Enterococcus faecium*
157 were determined (Li et al., 2015). The mechanism involved in the growth-promotion effect of
158 nucleotides in fish is still unclear. Given the importance of Gilthead seabream to the aquaculture
159 industry of the Mediterranian and Adriatic regions we need more information with regard to this
160 species.

161 The objective of this study was to evaluate the effect of the reduction and elimination of
162 dietary fishmeal using alternate ingredients and supplementation of a commercial nucleotide
163 source (NucleoforceFish™) on growth, feed utilization, biometric indices; liver mitochondrial
164 enzyme activities and selected gene expression levels relating to specific immune function and
165 specific genes associated to the gut microbial profile. The synergistic interplay of these factors

166 warranted investigation. Additionally, an economic analysis of gilthead seabream, *Sparus aurata*
167 fingerling production, was also determined with dietary nucleotide incorporation to assess cost
168 benefits.

169 **2. Materials and Methods**

170 **2.1. Fish and experimental facilities**

171 Five hundred and forty seabream fingerlings with an average initial body weight of 0.358 ±
172 0.002 g/fish, were obtained from El-Shref Hatchery, Alexandria, Egypt. Prior to the start of the
173 experiment, fish were acclimated to the experimental conditions for two weeks. Fish fed
174 commercial seabream diet (44% crude protein and 18% fat) at a level of 10 % of body weight. The
175 daily ration was divided into two equal amounts and offered two times a day. The fingerlings were
176 stocked into six cement ponds (each with 18 m^3) at the National Institute of Oceanography and
177 Fisheries (NIOF). Each cement pond was installed with three equal net-enclosures (each of 0.5
178 m^3), and each net-enclosure was stocked with 30 fish. Three replicate net enclosures were
179 randomly assigned to each treatment. Ponds were supplied with saline water (32ppt) from
180 underground saltwater well. Water quality parameters measured and maintained within the
181 optimum range for seabream. The turnover rate of water was 20%/pond/day and fish were held
182 under natural light (12:12 h light: dark schedule). The experiment was carried
183 out in 2017 and continued for 150 days.

184 **2.2. Experimental diets and feeding regime**

185 The ingredients used in this experiment were purchased from a commercial feed
186 manufacturer except Poultry By-product Meal (PBM) that was obtained from a rendering plant-
187 based in Egypt. NucleoforceFish™ was obtained from Bioiberica® Spain, and is a balanced
188 concentrate of free nucleotides (>24%) and active precursors obtained from dried yeasts

189 (*Saccharomyces cerevisiae*). It has the following nutrient specification: Protein 20.34%; Protein
190 Nitrogen 3.25% Non-protein Nitrogen (mainly from nucleotides) 2.09%; crude ash 3.38% and
191 crude fiber 0.10%.

192 Six experimental diets were formulated to be isonitrogenous, 45% crude protein (CP) and
193 isocaloric 20 MJ/kg gross energy (GE). The control diet (D1) contained 25% of fish meal (FM_{25})
194 and had no NucleoforceFish™ added ($FM_{25/0}$), diets 2-3 contained FM at a level of 25% and
195 supplemented with 250 ($FM_{25/250}$) and 500 ($FM_{25/500}$) mg/kg NucleoforceFish™, respectively.
196 Diets 4-6 were a non-fishmeal composition and had no NucleoforceFish™, (diet 4, $FM_{0/0}$), 0%FM
197 with 250mg/kg NucleoforceFish™ (diet 5, $FM_{0/250}$), and 0%FM with 500mg/kg
198 NucleoforceFish™ (diet 6, $FM_{0/500}$). The diets were processed by blending the dry ingredients into
199 a homogenous mixture for 10 minutes. Oil source and hot water were added, and then diets were
200 made in an electric kitchen meat grinder without using additional heat (cold-pressed). All diets
201 were air-dried for 4 hrs after that oven-dried at 45°C for 12 hrs. Dried diets were sieved using
202 different feed sievers to provide the right pellet size for fish.The chemical composition of the
203 experimental diets is presented in Table (1). The fish were fed twice a day, 6 days a week at rates
204 starting from 10% of their body weight at the beginning of the experiment, and finished with 5% at
205 the end.

206 **2.3. Sample collection and analytical procedures**

207 **2.3.1. Chemical analysis and Growth performance**

208 The chemical compositions of the experimental diets were measured according to the
209 procedures of AOAC (2000). Dry matter was determined after drying the samples in an oven (105
210 °C) for 24 h. Ash by incineration at 550°C for 12 h. Crude protein was determined by micro-
211 Kjeldhal method, N% × 6.25 (using Kjeltech autoanalyzer, Model 1030, Tecator, Höganäs,

212 Sweden) and crude fat by soxhlet extraction with diethyl ether (40 – 60 °C). Fish were weighed
213 every month, and the feeding rates were re-adjusted depending on the fish weight and the fish's
214 health status. Growth performance was measured, according to Lu et al. (2016). Final Body
215 Weight (FBW), Weight Gain (WG), Specific Growth Rate (SGR):, Feed Conversion Ratio (FCR),
216 Protein Productive Value (PPV) and Energy Utilization, (EU, %), Economical Conversion Rate
217 (ECR) were calculated using the following equations:

218 FBW= Final fish biomass (g)/fish number; WG = Final Body Weight (g) - Initial Body Weight
219 (g); Specific Growth Rate (%/day): $SGR=100 \times (\ln Wt - \ln Wo)/ n$, Where: Wo: Initial Mean
220 Weight of fish (grams); Wt: Final Mean Weight of fish (grams); n: Experimental period (days); ln:
221 natural logarithm; FCR = Feed Intake (g)/Weight Gain (g); Protein Productive Value (PPV,
222 %)=$100 \times (P_t - P_0$/protein intake (g)); Where: P_0: Protein content in fish carcass at the start, P_t:
223 Protein content in fish carcass at the end; Energy Utilization, EU (%): EU=100 × (Energy Gain
224 (Kcal)/ Energy Intake (Kcal)), ECR = Cost of diet ($/kg) × FCR according to the following
225 equation described by Bonaldo et al. (2010).

226 **2.3.2 Isolation of liver mitochondria**

227 The liver mitochondria isolation was performed in a medium A containing 10 mM
228 KH_2PO_4, 250 mM sucrose, and 5 mM ethylenediaminetetraacetate (EDTA) adjusted to pH 7.4.
229 Two fish from each net-enclosure were randomly chosen, anesthetized with 50 ppm Tricaine
230 Methanesulfonate (MS-222), and sacrificed with a sharp blow to the head. Liver samples were
231 dissected immediately and placed in ice-cold extraction medium A. Approximately 3 g of liver
232 tissue was homogenized in 10 volumes of medium A with a handheld Teflon/glass homogenizer
233 (Potter-Elvejhem). The homogenate was centrifuged at 600×g for 10 min at 4 °C. The superficial
234 lipid layer was removed, and the remaining supernatant was centrifuged at 9000×g for 10 min. The

235 pellet obtained from the second spin was washed three times in medium A and re-suspended in a
236 small volume of medium A plus 1 mg mL^{-1} Bovine Serum Albumin (BSA) according to Suarez
237 and Hochachka (1981). Isolation and preparation of mitochondrial fractions from muscle and
238 intestine were performed in medium B containing 120 mM KCl, 20 mM HEPES (4-(2-
239 Hydroxyethyl) piperazine-1-ethanesulfonic acid), 2 mM $MgCl_2$, 1 mM ethylene glycol tetra-acetic
240 acid (EGTA) and 5 mg mL_{-1} BSA adjusted to pH 7.4. Muscle and intestine samples (3–5 g) were
241 dissected, chopped finely with a pair of scissors, and homogenized separately using a Potter-
242 Elvejhem grinder in 10 volumes of medium B. The homogenate was centrifuged at 600×g for 10
243 min, and the supernatant was filtered through four layers of cheesecloth to remove fat and fibrous
244 tissue. The supernatant was centrifuged at 17000×g for 10 min, and the pellet containing the
245 mitochondria was re-suspended in 10 volumes of medium B and centrifuged at 7000×g for 10 min.
246 The pellets were re-suspended in 10 volumes of medium C (300 mM sucrose, 2 mM HEPES, 0.1
247 mM EDTA adjusted to pH 7.4) and centrifuged at 3500×g for 10 min. The mitochondrial fraction
248 was finally suspended in a small volume of medium C (Birch-Machin and Turnbull, 2001; Kirby
249 et al., 2007).

250 The mitochondrial suspensions were divided into aliquots, immediately and frozen in
251 liquid nitrogen and stored at −80 °C for the spectrophotometric measurement of the activities of
252 individual complexes I–IV.

253 **2.3.3 Measurements of liver mitochondrial enzyme activities**

254 The activities of respiratory chain enzymes were determined at 28°C using a SmartSpec
255 spectrophotometer after the mitochondrial membrane was ruptured by two freezes (in liquid
256 nitrogen) and melting (in ice-cold water) cycles. All duplicate analyses in a Spectrosil Quartz
257 Cuvette were executed to a final volume of 1 ml for each reading.

258 All enzyme activities were expressed as a function of mg mitochondrial protein for an ml
259 unit with one unit of enzyme activity corresponding to the appearance of 1 μmol of the product or
260 consumption of 1 μmol of the substrate per minute. Complex I (NADH: Ubiquinone
261 Oxidoreductase, EC 1.6.5.3) activity was analyzed following the decrease in absorbance due to
262 oxidation of NADH at 340 nm with 425 nm as the reference wavelength (extinction coefficient for
263 NADH=6.81mM^{-1} cm^{-1}, to account for the contribution of ubiquinone1 to the absorbance at 340
264 nm) according to Birch-Machin and Turnbull (2001) and Kirby et al. (2007). Briefly, NADH
265 (0.13mM), ubiquinone1 (65 μM), and antimycin A (2 μgm L^{-1}) were added to the assay medium
266 containing 25mM potassium phosphate buffer, 5 mM $MgCl_2$, pH 7.2, 2 mM KCN, and 2.5 mg
267 mL^{-1} BSA and the absorbance change recorded for 2 min. Mitochondria (20– 50 μg protein) were
268 added, and the NADH: ubiquinone oxidoreductase activity was measured for 4 min before the
269 addition of rotenone (2 μgmL^{-1}), after which the activity was measured for an additional 3 min.
270 Complex I activity was the rotenone-sensitive NADH: ubiquinone oxidoreductase activity.
271 Complex II (Succinate: ubiquinone1 oxidoreductase, EC 1.3.5.1) activity was measured by
272 following the decrease in absorbance due to oxidation of 2,6-dichlorophenolindophenol (DCPIP)
273 at 600 nm according to the procedure of Birch-Machin and Turnbull (2001) and Kirby et al.
274 (2007).

275 Briefly, mitochondria (10–50 μg protein) were pre-incubated in assay medium containing
276 25 mM potassium phosphate buffer, 5 mM $MgCl_2$, pH 7.2, plus 20 mM sodium succinate, at 28 °C
277 for 10 min. Antimycin A (2 μg mL^{-1}), rotenone (2 μg mL^{-1}), KCN (2 mM), and DCPIP (50 μM)
278 were added, and a baseline rate was recorded for 3 min. The reaction rate was started with 65 μM
279 ubiquinone1, and the enzyme-catalyzed reduction of DCPIP was measured for 4 min. The specific

280 activity of this complex was determined using the DCPIP extinction coefficient of ε=19.1 mM^{-1}
281 cm^{-1}.

282 Mitochondrial complex III (ubiquinol-cytochrome c reductase, EC 1.10.2.2) activity was
283 measured by monitoring cytochrome c reduction using decylubiquinol at 550 nm with 580 nm as
284 the reference wavelength (Jeejeebhoy, 2002). Briefly, mitochondria were pre-incubated at 28 °C in
285 an assay medium containing potassium phosphate (50 mM, pH 8), 0.1 mM EDTA, 2 g L^{-1} defatted
286 BSA, 3 mM sodium azide and 60 μM ferricytochrome c for 3 min. The reaction was initiated by
287 adding 0.1 mM L^{-1} decylubiquinol, and the enzyme activity was measured for 4 min. The non-
288 enzymatic reduction of cytochrome c was measured after the addition of 10 mg L^{-1} of antimycin
289 A, and an extinction coefficient factor of ε=19 mM^{-1} cm^{-1} was used to calculate absolute changes.

290 Complex IV (cytochrome c oxidase, EC 1.9.3.1) activity was measured by following the
291 oxidation of reduced cytochrome c as a decrease in absorbance at 550 nm with 580 nm as the
292 reference wavelength (Birch-Machin and Turnbull, 2001). Cytochrome c subunit II (15 μM) and
293 dodecyl maltoside (0.45 mM) were added to the assay medium containing potassium phosphate
294 (20 mM, pH 7), and the non-enzymatic rate was recorded. Mitochondria (5–15 μg of protein) were
295 added, and the complex IV activity was measured as the apparent first-order rate constant after
296 fully oxidizing cytochrome c subunit II by the addition of few grains of potassium
297 hexacyanoferrate. The specific activity was calculated using an extinction coefficient factor of ε=19.1
298 mM^{-1} cm^{-1}.

299 **2.3.4 Measurements of antioxidant parameters**

300 Malondialdehyde (MDA) content was examined as an indicator of lipid peroxidation,
301 which is based on the 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA) reactivity,
302 with the results expressed as nmol/g tissue (Jain et al., 1989). The glutathione (GSH) content was

303 determined spectrophotometrically by monitoring the chromophoric product resulting from the
304 reaction of the 5,50-dithiobis-(2- nitrobenzoic acid) with GSH in the presence of NADPH and
305 glutathione reductase at 412 nm (Wu et al. 2011). The GSH content was expressed as nmol/g
306 tissue. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decrease in H_2O_2
307 concentration at 240nm, according to Aebi (1984). The reaction mixture contained 50mM-
308 potassium phosphate buffer (pH 7·0) and 10mM-H_2O_2 freshly added.

309 Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the ferricytochromic
310 method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction was
311 monitored at 550nm, according to McCord and Fridovich (1969). The reaction mixture consisted
312 of 50mM-potassium phosphate buffer (pH 7.8), 0·1mM-EDTA, 0·1mM-xanthine, 0·012mM-
313 cytochrome, and 0·025 IU/ml xanthine oxidase. Activity is reported in units of SOD/mg of
314 protein. Here, one unit of activity is defined as the amount of enzyme necessary to produce a 50%
315 inhibition of the ferricytochrome c reduction rate.

316 **2.3.5 Gut microbiota qRT-PCR**

317 At all sampling points, the entire intestine was excised and immediately placed in
318 RNAlater^{TM} (Sigma-Aldrich) at 4 ºC for 24 hours before they were frozen at -20 ºC. The DNA
319 was extracted from the collected intestines for use as a PCR template. The intestines of each fish
320 were shaken with 300 µL of lysis buffer provided in the pathogen DNA Extraction Kit (Intron
321 Biotech, South Korea). The DNA was extracted according to the manufacturer's instructions. The
322 concentration of the DNA was then quantitated on a NanoDrop ND-1000 Spectrophotometer
323 (Thermo Fischer, USA).

324 The amount of specific bacterial DNA (coding for 16S rRNA) (*Photobacterium damselae,*
325 *Vibrio anguillarum, Lactobacillus plantarum, Lactobacillus subtilis and Enterococcus faecium)* in

326 the intestinal samples was measured by qPCR for each fish with amplification of the 16S rRNA
327 gene hypervariable region. Isolated DNA samples were diluted to 80 ng/µl for normalization and
328 used as a template in the qPCR reaction. The PCR reactions were setup in 25 µl reaction volumes
329 containing 1 µl of the forward and reverse primers as displayed in Table 2 (10 µM), 0.5 µl of
330 $MgCl_2$ (25 mM), 12.5 µl of 2X RealMode SYBR® Green Mix (Intron Biotech, South Korea), 80
331 ng of DNA template and 9 µl of nuclease-free water. The PCR reactions were carried out in a
332 CFX96 real-time PCR detection system (Bio-Rad, USA). Reaction times and cycling conditions
333 were 94°C for 2 min, 40 cycles of 94°C for 30 s and 60°C for 50 s. A melting curve analysis
334 further terminated the run.

335 **2.3.6. Gene expression analysis**

336 Liver samples were obtained, stored at −80 °C until analysis. For the mRNA extraction, the
337 RNeasy Mini Kit (Qiagen) was used. Liver samples of 15mg inserted in a microcentrifuge tube,
338 homogenized and centrifuged at 5000 ×g for 15s. The supernatant was collected and mixed with
339 70% ethanol. After the extraction of RNA, cDNA was synthesized using the HiSenScript RH
340 cDNA Synthesis Kit (Intron Biotech, South Korea) following the manufacturer's protocol. Real-
341 time PCR was done using SYBR select Master Mix kit (Thermo Fisher Scientific, Lithuania) using
342 the following primers (TCR, IL1-*B*, Hepcidin, and IGF1) as shown in Table 2. *β*-Actin (ACTB)
343 was used as housekeeping gene for mRNA gene expression normalisation (Table 2). Amplification
344 was performed using the CFX96 Real-Time PCR System (Biorad, USA) with the protocol as
345 follows: Initial 3 min denaturation at 94 °C, 40 cycles of 94 °C for 20 s, and 60 °C for 30 s. each
346 assay was done in triplicate. SYBR Green was used as a fluorogenic dye that exhibits little
347 fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-

348 stranded DNA. Spectrophotometric measurements at 260 nm was used to assess the concentration
349 of the House Keeping Gene and target amplicon DNA for mRNA expression.

350 The relative gene expressions relative to β-Actin (ACTB) were calculated by the delta delta
351 ct values. The expression stability was analyzed using geNorm and NormFinder software.

352 **2.4 Statistical analysis**

353 All data were analyzed using the SAS software, version 6.03 (Statistical Analysis System,
354 1996). Two-way ANOVA was used for analyzing the individual effects of fishmeal and nucleotide
355 inclusion levels and the interaction between them. Duncan's multiple range test was used to
356 compare differences between treatment means when significant F values were observed (Duncan,
357 1955). All percentage data were arc-sin transformed prior to analysis (Zar 1984). However, data
358 are presented untransformed to facilitate comparisons.

359 **3. Results**

360 **3.1. Growth performance and feed efficiency**

361 The growth performance of seabream is presented in (Table 3). The data obtained showed
362 that the growth performance was ($P \leq 0.05$) affected either by the addition of nucleotides or the
363 dietary fishmeal replacements and /or the interaction between two variables. The highest growth
364 performance was recorded for fish fed $FM_{25/250}$ and $FM_{25/500}$ diets, while the lowest growth values
365 were recorded for FM_0 and $FM_{0/250}$ groups. Dietary nucleotides supplemented at 250mg/kg or
366 500mg/kg enhanced the final body weight of seabream either FM_{25} or FM_0 compared to non-FM-
367 N group ($FM_{0/0}$). The same trend was observed for WG and SGR. In terms of feed efficiency,
368 Table 3 illustrates a significant difference in feed utilization indices between treatments ($P \leq 0.05$)
369 with positive effects when dietary nucleotides was added. Fingerling seabream fed nucleotides-
370 enriched diets recorded significantly ($P \leq 0.05$) better FCR values compared to non-nucleotides

371 enriched diets, either FM_0 or FM_{25}. These results revealed that supplementation of the FM_{25} diet
372 with 500 mg/kg NucleoforceFish™ ($FM_{25/500}$) obtained the best significant ($P \leq 0.05$) FCR
373 values. An improvement in dietary protein and energy utilization efficiency in terms of PPV and
374 EU was observed. PPV increased by 32.9, and 17.8% for fish fed $FM_{25/500}$, and $FM_{25/250}$ diets,
375 respectively, compared to ($FM_{25/0}$), while it was 18.6 and 13.7% for $FM_{0/500}$, and $FM_{0/250}$,
376 respectively, compared with non-FM-N diet ($FM_{0/0}$). Energy utilization was improved
377 significantly ($P \leq 0.05$) by 44.7, and 28.8% for fish fed the $FM_{25/500}$, and $FM_{25/250}$ diets,
378 respectively, compared with the non-nucleotides enriched 25% FM diet ($FM_{25/0}$), while 21.0 and
379 14.3% values were recorded for fish fed the $FM_{0/500}$, and $FM_{0/250}$ diets, respectively, compared
380 with FM-N free diet ($FM_{0/0}$).

381 **3.2. Liver enzyme activity**

382 Table 4 shows that complex I liver enzyme activities gave a significant response ($P \leq 0.05$)
383 to the dietary nucleotides enriched diets either FM-free or 25% FM contents and the interaction
384 between them ($P \leq 0.05$). Complex I values ranged from 13.6 to 8.9 mL units/mg mitochondrial
385 protein. The highest significant complex I activity values were recorded in $FM_{25/500}$ and $FM_{25/0}$,
386 respectively. Complex II and III activity ranged from 62.25 to 42.5 and 212.64 to 151.48 mL
387 units/mg mitochondrial protein, respectively. Fish fed the diets $FM_{25/250}$, and $FM_{25/500}$ recorded the
388 highest significant values for either complex II or III activity compared to other treatments.
389 Complex IV ranged from 304.7 to 250.92 mL units/mg mitochondrial protein. Complex IV
390 showed higher significant values for the $FM_{25/0}$ diet group.

391 **3.3 Gene expression**

392 The gene expression results of IGF1, TCR-B, IL1-*B*, and Hepcidin of fish fed diets
393 containing different levels of nucleotides and fish meal presented in Table 5. The results showed

394 that the highest gene expression (*P*≤0.05) of IGF1 and Hepcidin was recorded for fish fed $FM_{25/250}$,

395 while the highest gene expression (*P*≤0.05) of TCR-B and IL1-*B* were recorded for fish fed $FM_{0/0}$

396 and $FM_{0/500}$, respectively.

397 **3.4. Gut micobiome**

398 Table 6 shows the gut microbiome abundance of seabream fed different experimental diets.

399 The obtained data showed that the selected gut microbial communities were significantly affected

400 either with NucleoforceFish™ enriched or FM-free tested diets. The highest abundance of

401 *Photobacterium damselae* was observed in FM_0 diets supplemented with 250 and 500 mg

402 NucleoforceFish™. Furthermore, the lowest abundance was associated with the absence of

403 nucleotides $FM_{25/0}$ diet. Furthermore, *Vibrio anguillarum* was also dominating in fish fed with

404 FM_0 supplemented with 500mg NucleoforceFish™; however, the lowest richness was linked to

405 the $FM_{0/0}$ diet. On the other hand, the highest abundance of the *Lactobacillus plantarum* and

406 *Lactobacillus subtilis* was revealed in fish fed on $FM_{25/0}$, but the lowest abundance was associated

407 with $FM_{0/0}$. Finally, the highest richness *Enterococcus faecium* was recognized in fish fed on

408 $FM_{0/500}$; nevertheless, the lowest was connected to the $FM_{25/250}$ diet.

409 **3.5. Activity of oxidative enzymes**

410 Table 7 shows the activities of superoxide dismutase (SOD), catalase (CAT) (U/mg

411 protein), glutathione (GSH) and malondialdehyde (MDA) (nmol/g tissue). Superoxide dismutase

412 and CAT results significantly improved in both $FM_{25/500}$ and $FM_{25/250}$ with 554.5, 363.7 U/mg

413 protein, and 268.1, 218.4 U/mg protein, respectively. Meanwhile, they were decreased in a

414 significant way in $FM_{0/0}$ diets with 138.5 and 75.5 U/mg protein. Reduced GSH and showed a

415 significant decrease in both $FM_{0/0}$ and $FM_{0/250}$ diets with 1491.7 and 2179.3 nmol/g tissues,

416 associated with a significant increase in MDA values recorded in the same two diets with 19.88
417 and 17.80, correspondingly.

418 3.6. Economic Evaluation

419 Table 8 presents the data on the economic conversion rate (ECR). The cost of one kg of
420 $FM_{25/0}$ diet increased by 3.32, 6.64, 13.86, 17.18 and 20.50% in the $FM_{25/500}$, $FM_{25/250}$, $FM_{0/0}$,
421 $FM_{0/250}$, and $FM_{0/500}$ diets, respectively. The lowest values of ECR were recorded for $FM_{0/500}$, and
422 $FM_{25/500}$ diets, respectively, compared with either $FM_{0/0}$ or $FM_{25/0}$ diets, which recorded the highest
423 ECR values.

424 4. Discussion

425 Nucleotides play a crucial role in cellular metabolism, including the storage, transfer, and
426 expression of genetic information, standing as activated intermediates of energy transport in cells
427 (e.g., ATP, NAD+), (Krüger and Mariët, 2018). Nucleotides are synthesized *de novo* in most fish
428 tissues, but with low quantity under certain conditions (Do Huu, 2016). Therefore, they are
429 considered as being mostly non-essential nutrients but sometimes becoming semi-essential (Li and
430 Gatlin, 2006).

431 Inclusions of nucleotides have a significant benefit to the body under certain conditions,
432 particularly under physiological stress (Hess and Greenberg, 2012). Fish meal has a considerable
433 content of nucleotides, but unfortunately, fish meal is becoming a limited, expensive natural
434 resource. Most aquafeed formulas without fish meal contain relatively low amounts of nucleotides
435 (Do Huu et al., 2012). To combat this issue, considerable research is now being performed toward
436 the replacement of fish meal with other alternative products that contain high levels of nucleotides.

437 The results observed in the current investigation showed that nucleotide-enriched diets
438 with 250mg/kg or 500mg/kg enhanced growth performance and feed utilization and metabolic

439 indices of sea bream fed both fish meal and non-fish meal control diets respectively. These results
440 are consistent with Hossain et al (2016) who demostrated positive effects of nucleotides on growth,
441 blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream
442 (*Pagrus major*). Furthermore, Hassaan et al (2018) found that Nile tilapia fed diets contained four
443 levels of yeast extract rich in nucleotides and β- glucan increased the weight gain linearly while
444 the feed conversion ratio declined linearly with increasing levels of yeast extract. These latter
445 authors also stated that in addition, fish fed diets supplemented with enriched nucleotide product
446 improved Hepatic function and promote liver and gut restoration. In addition,Tahmasebi-Kohyani
447 et al. (2012) reported inclusion of 0.1% or 0.2% nucleotide to the diet promoted improved weight
448 gain and feed efficiency in fingerling rainbow trout. Xu et al. (2015) found that fingerling hybrid
449 tilapia fed a 0.60% nucleotide supplemented diet exhibited the highest growth and feed utilization
450 indices compared to a nucleotide free control diet. Also, Yin et al. (2015) concluded that
451 *Ancherythroculter nigrocauda* (a Chinese cyprinid species) fed experimental diets containing yeast
452 nucleotide with a concentration of 0.45% showed the highest values of final weight, SGR, PER
453 and the best value of FCR compared with other diets. Moreover, Hossain et al. (2019) reported the
454 advantage of inclusion nucleotide on growth performance and immune system in different fish
455 species. Following the same trend, Selim et al. (2020) found that the highest growth performance
456 and feed utilization efficiency were recorded in fish fed 2.5 g NT/kg diet. However, the present
457 results for seabream are inconsistent with Ridwanudin et al (2019) who found that rainbow trout
458 fed diets containing low-fish meal content supplemented with nucleotide clearly showed that there
459 was no positive effect of dietary nucleotides on growth of rainbow trout. Furthermore, nucleotide
460 supplementation in juvenile turbot, *Scophthalmus maximus* failed to affect the growth either in a

461 positive or negative pattern, this finding could be due to species effect or the insufficient dose of
462 nucleotide employed (Fuchs et al., 2015).

463 The present results could be attributed to a different scenario as follows: i) the inclusion of
464 Nucleoforce Fish™ that has good palatability, flavor, and aromatic characteristics, which may
465 cause better attractability, feed intake and development of the intestine of the nucleotide-
466 supplemented juvenile seabream. As a consequence, better growth response induction and feed
467 utilization of the tested diets is obtained, ii) Inclusion of nucleotide alters intestinal bacteria that
468 enhances nutrient assimilation and consequently improves growth performance and feed utilization
469 (Bower et al., 2019; Gupta et al 2019 and Hosseinfer et al., 2020)., iii) Nucleotide fortification
470 improves the haemato-biochemical parameters as serum alanine amino-transferase (ALT),
471 aspartate aminotransferase (AST), Alkaline Phosphatase (ALP); v) stimulatory effects of digestive
472 enzymes as lipase, amylase, and growth hormone which direcly has positive effects on growth
473 performance and feed efficiency (Ridwanudin et al 2019).

474 The present study revealed a positive effect of nucleotides on liver mitochondrial enzyme
475 complex activities, which are consequently associated with the positive effect on feed utilization
476 parameters FCR ($n=3$, $r^2=0.99$), PPV ($n=3$, $r^2=0.93$) and energy utilization ($n=3$, $r^2=0.94$). Fish fed
477 the diets $FM_{25/500}$ was superior in the expression of Complex I by 13.62% and associated with the
478 best values of FCR (1.95) which supports the hypothesis that enhancement of mitochondrial
479 function is correlated with enhancing growth and feed utilization, The activity levels of Complex
480 II enzymes for fish fed the diets of $FM_{25/250}$ were highly significantly different compared with
481 other diets (Table 4). Similarly, the best FCR ratios, PPV and EU values, were recorded with
482 either fish fed the diets $FM_{25/250}$ or $FM_{25/500}$, which both were superior to either FM_{25} or FM_0 diets
483 supporting the current study findings as they are linked to their best expression to Complex II.

484 The present results are in parallel with (Bottje and Carstens, 2009; Eya et al., 2013). Moreover,
485 Eya et al. (2013) indicated that correlating enhancement of performance and feed efficiency with
486 mitochondrial enzyme complexes in many animals is vital to decrease the cost expenditure for
487 energy production at the celluar level.

488 In terms of Hepatic gene expression, expression of IGF1 in diet $FM_{25/250}$ was statistically
489 elevated than in the livers of seabream from the other treatments. This finding was in accordance
490 with the FBW with the same diet as it showed an enhanced growth pattern. The present results are
491 consistent with Hossain et al (2016) who found positive effects of nucleotide on growth and
492 growth factor gene expression of juvenile red sea bream. Furthermore, Larva Lanes et al. (2012)
493 found an enhancement of gene expression of IGF1 of rainbow trout fed diets enriched with
494 nucleotides, which will positively affect the growth rate. On the contrary, Do Huu, (2016) found
495 that excessive use of nucleotide (10%) negatively affects the growth of rainbow trout under their
496 experimental conditions.

497 T cell receptors B (TCR-B) protein in nature complexes are found in the plasma
498 membranes of immune cells to recognize antigens and are responsible for interaction with major
499 histocompatibility complex (MHC) considered as a very important indicator for cell-mediated
500 immunity (Estevez et al., 2018). Nucleotide supplementation elevates stress tolerance in
501 aquaculture as it enhances and modulates cell-mediated immunity as well as innate immunity,
502 consequently increases the fish capability to face any infection (Ringø et al., 2012). Our findings
503 were in accordance with the previously mentioned hypothesis in which $FM_{25/250}$ and $FM_{25/500}$ diets
504 showed the least up-regulation of TCR-B with statistical difference other than the rest of the diets
505 following then $FM_{0/250}$ and $FM_{0/500}$. Furthermore, Torrecillas et al. (2015) and Zhou et al. (2018)
506 suggested that the usage of soybean meal-based diets showed down-regulation of TCR-B; this was

507 not in the same line with our findings where FM_0 diet was statistically the highest up-regulation of
508 TCR-B in particular with $FM_{0/500}$ treatment with the apparently strongest nucleotide modulating
509 influence. This could be due to differences in diet formulations between the two studies as ours
510 was supplemented with poultry by-product meal while the other study was totally formulated with
511 soybean meal and other plant protein sources. As a result of activation of pathogen associated
512 molecular patterns (PAMPs) via host pattern recognition receptors (PRRs) Interleukin 1-beta (IL-
513 1β) is produced, (Zou and Secombes, 2016).

514 According to Angosto et al. (2012) the mechanism of IL-1β is different in teleost fish from
515 mammals, yet it still plays a vital role in regulating the inflammatory progression, furthermore it
516 has a role in fish muscle metabolism and growth, as it controls muscle mass and insulin growth
517 factor binding protein (IGFBP), (Pooley et al., 2013; Heidari et al., 2015). In the present study, IL-
518 1β was significantly up-regulated in $FM_{0/500,}$ $FM_{0/250}$ and $FM_{25/250}$ seabream liver compared to the
519 remaining dietary treatments fed to seabream. These findings are in accordance with Reda et al.,
520 2018 with the same product tested in tilapia. In contrast, Reyes-Becerril et al. (2008) reported IL-
521 1β expression in liver was down-regulated in sea bream using live yeast, *Debaryomyces hansenii*.

522 The up-regulation of IL-1β via nucleotide supplementation needs further clarification
523 either it is up-regulated due to increase in the inflammatory action or it is due to it is positive effect
524 on IGF expression. Interestingly, in our study the significant up-regulation of IL-1β expression in
525 $FM_{0/500}$ could be due to the compensatory effect of enriched diets with NucleoforceFish™ in the
526 absence of dietary fish meal. In this connection, Sitjà-Bobadilla et al., (2005) reported that
527 increasing the inclusion of dietary plant protein sources in FM-free diets may negative affects the
528 immune system of fish.

529 Hepcidin (Hep) an antimicrobial peptide, is one of the main mediators in the innate
530 immune system expressed in liver (Cuesta et al., 2008). Gene expression of Hep is up-regulated as
531 a result of lipopolysaccharide (LPS) presence, iron overload and bacteria (Yang et al., 2007).
532 Pereiro et al. (2012) reported that Hep is up-regulated due to bacterial infection not because of iron
533 surplus.

534 Although Hep showed antimicrobial activity against most bacteria, according to Cuesta et
535 al. (2008) the viability of *Photobactrium damselae* and Vibrio *anguillarum* was not affected yet
536 growth inhibition of nearly all strains of pathogenic bacteria was achieved in tha latter study.

537 Our findings showed significant differences in Hep relative gene expression values
538 between all experimental treatments, where $FM_{25/500}$ showed the highest relative up-regulation and
539 $FM_{25/0}$ showed the lowest degree of up-regulation.

540 The present results are consistent with Guo et al. (2019) who found zebra fish, *Danio rerio*
541 fed diets supplemented with nucleotides modulate the regulation of Hep which may consequently
542 enhance the immune system. On the contrary, Reyes-Becerril et al. (2008) found a down-
543 regulation of the Hep gene in liver when the diet was supplied by live yeast, *Debaryomyces*
544 *hansenii* to gilthead seabream. The effect of absence of dietary fish meal on Hep was reported by
545 Wang et al. (2020) for juvenile hybrid grouper (*Epinephelus fuscoguttatus*♀× *Epinephelus*
546 *lanceolatus*♂). Their results exhibited down-regulation of Hep gradually with the decrease of fish
547 meal inclusion where the diet with 34% fish meal showed the lowest significant decline in Hep.
548 Their results were in partial accordance with our outcomes as diet $FM_{25/0}$ was observed to present
549 the most relative down-regulation of Hep. The significant up-regulation for Hep for fish receiving
550 the 500mg/Kg supplementation of NucleoforceFish™ in the presence and absence of fishmeal
551 was notable and a trend for significant up-regulation with increased nucleotide addition was

552 detected. This finding suggests that nucleotides could support the absence of FM by substitution
553 with terrestial animal and plant based ingredients but only to a certain limit.

554 Therefore, the interaction between the FM substitution and nucleotide supplementation
555 needs further research regarding the immune status of sea bream. Insulin-like growth factor 1
556 (IGF1) peptides released from the liver in nature regulates the growth of vertebrates via
557 stimulating cell division through the somatotropic axis (Tsai et al., 2014; Midhun et al., 2016).
558 Using nutrients that stimulate IGF1 is an old approach to enhance the growth in aquaculture as
559 well as deficiencies affecting the IGF1 expression decreases the performance and consequently
560 negatively affects the growth (Azizi et al., 2016).

561 To our knowledge, there is very scarce research regarding the effect of nucleotide
562 supplementation accompanied by FM limitation on the antioxidant response in sea bream diets.
563 The current trial evaluated the effect of nucleotide supplementation and FM replacement on SOD
564 and CAT as indicators for the increase of free radicals superoxide (O_2^-) and hydrogen peroxide
565 (H_2O_2) (Hassan, 1980). GSH, another non-enzymatic antioxidant marker indicator for antioxidant
566 stress and MDA, one of the metabolites of lipid peroxidation were measured to give a full picture
567 of oxidative stress (Requena et al., 1996). The present results showed a significant increase in
568 SOD and CAT in both FM_0, $FM_{25/250}$ and $FM_{25/500}$ in comparison to the other groups. This result is
569 in accordance with most of the literature where the increase in metabolic activity exerts an amount
570 of free radicals compensated by the production of SOD and CAT indicating that the supply of
571 nucleotides enhances the antioxidant activity (Zhao et al., 2017; Reda et al., 2018). On the
572 contrary, Wei et al. (2015) did not observe any differences in the expression of SOD and CAT as a
573 result of supplementation of dietary nucleotides in the diets of sea cucumber, *Apostickopis*
574 *japonicas*. Similarly, there was no significant difference in SOD and CAT activities reported when

575 a dietary commercial nucleotide yeast based protein (Nu-Pro®) (NP) additive was tested on
576 rainbow trout *(Oncorhynchus mykiss*) by Özlüer-Hunt et al., 2016 to evaluate 20 (NP 20), 40 (NP
577 40) and 60 % (NP 60) fish meal substitution with this nucleotide enriched product. Furthermore,
578 Zhou et al. (2011) stated that the replacement of FM by poultry by-product meal (PBM) in diets
579 for juvenile cobia, *Rachycentron canadum* does not affect the antioxidant defenses. In addition,
580 Hossain et al. (2016) reported that CAT levels were decreased by supplementation of nucleotides
581 in juvenile red sea bream, *Pagrus major*.

582 The highest MDA ($P \leq 0.05$) values observed in fish fed diets without nucleotide
583 supplemenattaion as $FM_{0/0}$ and $FM_{0/250}$ consistent with the increase in our results of lipid
584 peroxidation where there was no FM in the diets and the minimum amount of nucleotide addition.
585 The same results was observed by (Xu et al., 2015; Reda et al., 2018; Zhao et al., 2017), where
586 diets contained a high amount of nucleotides decreased the MDA level significantly in juvenile
587 hybrid tilapia, seabass, and yellow catfish, respectively.

588 The vertebrate intestinal environment is colonized by a complex microbiome community,
589 which plays a crucial role in host physiology and health (Pickard et al., 2017). The total or partial
590 replacement of fish meal in seabream diets is commonly accompanied by gut microbial
591 community changes (Estruch et al., 2015). However, the impact of nucleotide supplementation is
592 not fully elucidated. The Proteobacteria, Firmicutes and Actinobacteria dominate fish gut
593 microbial communities, therefore the present study focused on a group of these phyla, including
594 *Photobacterium damselae*, *Vibrio anguillarum*, *Lactobacillus plantarum*, *Lactobacillus subtilis*
595 and *Enterococcus faecium* (Egerton et al., 2018). The pathobiome is recently identified as a group
596 of host-associated organisms accompanied by a fundamental decrease of the animal health status,
597 owing to the interaction between these group members and hosts (Bass et al., 2019). Thus, the

598 present study presented two crucial species of seabream pathobiome; *Photobacterium damselae*
599 and *Vibrio anguillarum*. *Photobacterium damselae* (formerly *Vibrio damsela*) is one of the main
600 bacterial diseases affecting seabream, and it has been reported as an endemic infection in Egypt
601 (Essam et al., 2016). *Photobacterium damselae* was highly represented in FM_0 diets supplemented
602 with either 250 or 500 nucleotides than in comparison to other diets.

603 The total replacement of fish meal was reported to be linked to an increase in the
604 abundance of *Photobacterium* (Estruch et al., 2015). Differences in *Photobacterium* abundance
605 may be attributed to the differences in fibre between diets, and it might also suggest a change of
606 gut immune mechanisms of seabream (Estruch et al., 2015). Moreover, the nucleotide represents
607 an essential source of the building blocks of the bacterial nucleic acid (Reda et al., 2018). On the
608 contrary, a moderate abundance of *Photobacterium damselae* was observed in fish fed on $FM_{25/500}$
609 and $FM_{25/250}$ and the lowest abundance was associated with the absence of nucleotides. Besides,
610 *Vibrio anguillarum* was predominating in fish fed with FM_0 supplemented with 250 and 500
611 nucleotides. These results agreed with Estruch et al. (2015), who reported that the presence of
612 vibrio was only associated with the total replacement of fishmeal.

613 On the other hand, fish fed with $FM_{25/250}$ have shown a lower abundance of both species
614 suggesting that the supplementation of nucleotide is crucial for lowering some species of seabream
615 pathobiome. Lactic acid bacteria (LAB) play a pivotal role in finfish health and physiology, owing
616 to their role in gastrointestinal tract development and enhancement of digestive functions,
617 improving host immune response and disease resistance (Ringø et al., 2018). Furthermore, lactic
618 acid bacteria have been widely used as probiotics in aquaculture; thus, the current study targeted
619 three species of lactic acid bacteria. The highest abundance of the *Lactobacillus plantarum* and
620 *Lactobacillus subtilis* was detected in fish fed with $FM_{25/0}$. Although the replacement of fish meal

621 has been reported to negatively impact *Lactobacillus* abundance in seabream gut (Estruch et al.,
622 2015). A high abundance of *Lactobacillus* was revealed in fish fed on $FM_{0/250}$. The lowest
623 abundance of *Lactobacillus plantarum* and *Lactobacillus subtilis* was observed in fish fed on FM_0
624 and $FM_{25/500}$, respectively. On the other hand, the highest abundance of *Enterococcus faecium* was
625 identified in fish fed on FM0/500. However, previous studies suggested that *Enterococcus faecium*
626 was not detected in fish fed on FM substitutions (Torrecillas et al., 2017). Thus, this result reveals
627 the role of nucleotides in increasing the abundance of *Enterococcus faecium* within the seabream
628 gut microbiome. The moderate richness of *Enterococcus faecium* was detected in fish fed on
629 $FM_{25/500}$, but the lowest was connected to the $FM_{25/250}$ diet.

630 The increasing price of feed is considered one of the most important factors limiting
631 profitability in fish culture. The cost of all experimental supplemented nucleotide diets with and
632 without fish meal, remained low compared to the control treatment with no added nucleotide
633 ($FM_{0/0}$). This is due to the higher price and limited quantity of FM as a main ingredient in the
634 control diets. The elevated price of fish feed mainly responds to the cost of FM (Abdel Rahman, et
635 al., 2010), and therefore finding a relatively lower cost alternative ingredient has been an ongoing
636 research goal (Goda et al., 2020). Often in scientific research, technical results will appear to
637 favour a particular treatment, but what confirms and disproves the validity of technical results is
638 economical and financial analysis. Although the addition of NucleoforceFish™ has increased the
639 price of the diets compared to non-enriched N-diets, the value of ECR as a result of the inclusion
640 of dietary nucleotide has decreased, which means that the efficiency of the experimental enriched
641 diets with NucleoforceFish™ in terms of relative economic values.

In the present study, the data confirmed that the addition of NucleoforceFish™ to the feeds of gilthead seabream would have an apparent positive effect decreasing the ECR values, which are associated with increasing the economic return.

5. Conclusion

The data in this study indicated that the best overall dietary treatment performance was $FM_{25/500}$ that included fish meal at 25% with 500mg/kg of nucleotide addition. This was generally without adverse effects on fish quality parameters and the efficiency of growth and feed utilization. The supplementation of zero fish meal diets for seabream with NucleoforceFish™ as a commercial nucleotide supplement was also advantageous in compensation for a decrease in the abundance and richness of the gut microbiome without fish meal in formulated diets for Gilthead seabream. There was clear evidence of metabolic and health related benefits to the incorporation of dietary neucleotides for positive trends in metabolic enzymes and specific gene expression.

Use of nucleotides in aquaculture may support the economic performance. and aid the cost of production (investment volume). We must evaluate the cost benefits of such feed addtives in a wider context throughout the production cycle and for other marine species of commercial importance.

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Table 1. Formulation and proximate composition of the experimental diets for gilthead seabream (g/kg)

	Experimental Diets*					
	$FM_{0/0}$	$FM_{0/250}$	$FM_{0/500}$	$FM_{25/0}$	$FM_{25/250}$	$FM_{25/500}$
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 meal	134	134	134	100	100	100
Fish oil	118	118	118	121	121	121
Vitamins and mineral mixture [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
lipids, %	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
Price (US$/kg feed)	0.844	0.858	0.872	0.961	0.975	0.989

-$FM_{0/0}$ (0% fish meal; 0mg nucleotide), $FM_{0/250}$ (0% fish meal+250mg nucleotide), $FM_{0/500}$ (0% fish meal+500mg nucleotide), $FM_{25/0}$ (0mg nucleotide , 25% fish meal), $FM_{25/250}$ (25% fish meal+250mg nucleotide), $FM_{25/500}$ (25% fish meal+500mg nucleotide).

[1]Vitamin and mineral mixture each 1-kg of 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] Nitrogen free extract (NFE) =100- (Protein%+lipids%+ Ash%+ Fiber %).

[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).

-DM= Dry matter.

Table 2. The primers using for determining TCR, IL-1β, Hepcidin and IGF1 are listed and were used relative to β-Actin (ACTB) and for bacteria .

Gene name	Gene abbreviation	Gene Bank number	Primer sequences (5' - 3')
Immunoglobulin M IgM	IgM	26691348	CAGCCTCGAGAAGTGGAAAC
		17547755	GAGGTTGACCAGGTTGGTGT
Hepcidin1	Hep	26691348	GCCATCGTGCTCACCTTTAT
		17547755	CCTGCTGCCATACCCCATCTT
Interleukin1 beta	IL-1β	28706519	GGGCTGAACAACAGCACTCTC
		26691348	TTAACACTCTCCACCCTCCA
T-Cell Receptor	Tcr	26691348	AAGTGCAATGCCAGCTTCTT
		28453393	TTGGCGGTCTGACTTCTT
Photobacterium damselae		28341681	CCTATGGGACATGAATGG
		27084008	GCTCTAGGCTAAATGAATC
Photobacterium damselae		27737014	ACATCATCCATTTGTTAC
Vibrio anguillarum		24649315	CCTTATCACTATCCAAATTG
Vibrio anguillarum		26840426	GAAAGAGCCCAAACCAAGTGATT
Lactobacillus plantarum		23386831	CTTCCCAGATAATTCAACTATCGCTTA
Lactobacillus plantarum		23386831	GGAATCTTCCACAATGGACG
Lactobacillus subtilis		30286089	CGCTTTACGCCCAATAAATCCGG
Lactobacillus subtilis		25973610	ATCAACCATGTTGATGTAGC
Enterococcus faecium		30286089	AAGGGATACCGGACAATTCA

Table 3. Growth performance and feed utilization efficiency of juvenile gilthead seabream, *sparus aurata* fed different experimental diets.

Items	FM_0			FM_{25}		
	NT_0	NT_{250}	NT_{500}	NT_0	NT_{250}	NT_{500}
IBW (g/fish)	0.35±0.01	0.36±0.01	0.36±0.01	0.35±0.01	0.36±0.01	0.36±0.01
FBW (g/fish)	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}
Weight gain (g/fish)	11.41 ± 0.23^{d}	12.02 ± 0.24^{cd}	12.78 ± 0.19^{bc}	12.25 ± 0.31^{c}	13.27 ± 0.18^{ab}	13.89 ± 0.21^{a}
SGR (%/fish/day)	1.947 ± 0.007^{d}	1.965 ± 0.005^{cd}	1.999 ± 0.013^{bc}	1.986 ± 0.014^{c}	2.022 ± 0.010^{ab}	2.043 ± 0.005^{a}
FCR	2.34 ± 0.03^{a}	2.17 ± 0.04^{b}	1.99 ± 0.02^{c}	2.23 ± 0.02^{b}	1.95 ± 0.05^{c}	1.81 ± 0.02^{d}
PPV (%)	16.16 ± 0.40^{c}	18.38 ± 0.56^{bc}	19.17 ± 1.34^{bc}	17.54 ± 0.26^{bc}	20.67 ± 1.46^{ab}	23.32 ± 1.64^{a}
EU (%)	13.02 ± 0.36^{bc}	14.88 ± 0.58^{bc}	15.75 ± 1.32^{ab}	12.42 ± 0.20^{c}	16.00 ± 1.10^{ab}	17.97 ± 1.26^{a}

	FM levels		NT Levels		
	0%	**25%**	**0mg**	**250mg**	**500mg**
IBW (g/fish)	0.35±0.1	0.36±0.1	0.35±0.1	0.36±0.1	0.36±0.1
FBW (g/fish)	12.43 ± 0.22^{b}	13.49 ± 0.25^{a}	12.18 ± 0.30^{c}	13.01 ± 0.22^{b}	13.70 ± 0.14^{a}
Weight gain (g/fish)	12.08 ± 0.21^{b}	13.13 ± 0.22^{a}	11.83 ± 0.29^{c}	12.65 ± 0.22^{ab}	13.34 ± 0.12^{a}
SGR (%/fish/day)	1.97 ± 0.01^{b}	2.02 ± 0.01^{a}	1.97 ± 0.01^{c}	2.00 ± 0.01^{ab}	2.02 ± 0.01^{a}
FCR	2.17 ± 0.03^{a}	2.00 ± 0.02^{b}	2.29 ± 0.02^{a}	2.06 ± 0.04^{b}	1.90 ± 0.02^{c}
PPV (%)	17.90 ± 0.95^{b}	20.51 ± 1.11^{a}	16.85 ± 0.38^{c}	19.53 ± 0.88^{b}	21.25 ± 1.42^{a}
EU (%)	14.55 ± 0.58^{b}	15.46 ± 0.95^{a}	12.72 ± 0.25^{c}	15.44 ± 0.85^{b}	16.86 ± 1.20^{a}

*Values are means ± SEM, n = 3.

**-$FM_{0/0}$ (0% fish meal; 0mg nucleotide), $FM_{0/250}$ (0% fish meal+250mg nucleotide), $FM_{0/500}$ (0% fish meal+500mg nucleotide), $FM_{25/0}$ (0mg nucleotide , 25% fish meal), $FM_{25/250}$ (25% fish meal+250mg nucleotide), $FM_{25/500}$ (25% fish meal+500mg nucleotide).

- Final body weight (FBW), Weight gain (WG), feed conversion ratio (FCR), protein productive value (PPV) and Energy utilization, (EU %).

-FM=fishmeal; NT = nucleotides.

Table 4. The specific activity (Activity Units/g Protein) of respiratory chain enzymes (Complex I, II, III, and IV) in liver mitochondria of juvenile gilthead seabream, *sparus aurata* fed different experimental diets.

Items	FM_0			FM_{25}		
	NT_0	NT_{250}	NT_{500}	NT_0	NT_{250}	NT_{500}
Complex I	8.98±0.18^{e}	9.59±0.32^{d}	10.32±0.14^{c}	12.40±0.11^{b}	19.16±0.16^{a}	13.62±0.16^{b}
Complex II	42.49±0.29^{e}	51.23±0.13^{c}	50.84±0.13^{c}	43.15±0.21^{d}	62.25±0.23^{a}	58.62±0.19^{b}
Complex III	151.48±1.71^{e}	165.76±0.95^{d}	178.95±1.84^{c}	152.95±0.42^{e}	212.64±1.45^{a}	198.35±0.47^{b}
Complex IV	250.92±1.57^{e}	261.31±1.27^{d}	268.43±1.09^{c}	268.50±1.25^{c}	296.31±1.55^{a}	290.44±0.50^{ab}
	FM levels			**NT Levels**		
	0%	**25%**		**0mg**	**250mg**	**500mg**
Complex I	9.63±0.21^{b}	11.73±0.13^{a}		10.69±0.14^{b}	9.38±0.24^{c}	11.97±0.13^{a}
Complex II	48.19±0.18^{b}	54.67±0.20^{a}		42.82±0.21^{c}	56.74±0.17^{a}	54.73±0.14^{b}
Complex III	165.40±1.31^{b}	187.98±0.64^{a}		152.22±1.11^{c}	189.20±1.01^{a}	188.65±1.23^{a}
Complex IV	260.22±1.29^{b}	285.08±1.01^{a}		259.71±1.27^{c}	275.81±1.43^{b}	282.44±0.87^{a}

*Values are means ± SEM, n = 3.

**Means in a row with different superscript letter are significantly different.

--$FM_{0/0}$ (0% fish meal; 0mg nucleotide), $FM_{0/250}$ (0% fish meal+250mg nucleotide), $FM_{0/500}$ (0% fish meal+500mg nucleotide), $FM_{25/0}$ (0mg nucleotide , 25% fish meal), $FM_{25/250}$ (25% fish meal+250mg nucleotide), $FM_{25/500}$ (25% fish meal+500mg nucleotide).

-FM=fishmeal; N = nucleotides.

-Complex I =NADH: Ubiquinone Oxidoreductase, EC 1.6.5.3, Complex II =Succinate: ubiquinone1 oxidoreductase, EC 1.3.5.1, Complex III = ubiquinol: ferricytochrome c reductase, EC 1.10.2.2, Complex IV= cytochrome c oxidase, EC 1.9.3.1.

Table 5. IGF1, TCR-B, IL-1β, and Hepcidin gene expressions in liver of juvenile gilthead seabream, *Sparus aurata* fed different experimental diets.

Items	FM_0			FM_{25}		
	NT_0	NT_{250}	NT_{500}	NT_0	NT_{250}	NT_{500}
IGF1	1.25 ± 0.21^{b}	0.93 ± 0.04^{c}	0.93 ± 0.04^{c}	0.84 ± 0.03^{c}	2.09 ± 0.04^{a}	1.10 ± 0.05^{bc}
TCR-B	1.28 ± 0.27^{c}	1.19 ± 0.01^{cd}	3.13 ± 0.12^{ab}	1.71 ± 0.02^{c}	0.91 ± 0.02^{d}	1.12 ± 0.33^{d}
IL-1β	0.89 ± 0.05^{d}	1.85 ± 0.08^{b}	2.38 ± 0.32^{a}	1.21 ± 0.02^{cd}	1.92 ± 0.07^{b}	1.59 ± 0.03^{bc}
Hepcidin	1.11 ± 0.02^{e}	1.41 ± 0.01^{d}	1.60 ± 0.01^{c}	0.51 ± 0.01^{f}	1.84 ± 0.06^{b}	2.08 ± 0.04^{a}
	FM levels			NT Levels		
	0%	25%		0mg	250mg	500mg
IGF1	1.04 ± 0.33^{b}	1.34 ± 0.56^{a}		1.04 ± 0.42^{b}	1.51 ± 0.61^{a}	1.02 ± 0.14^{b}
TCR-B	2.40 ± 0.43^{a}	1.72 ± 0.13^{b}		3.70 ± 0.75^{a}	1.05 ± 0.16^{c}	1.41 ± 0.62^{b}
IL-1β	1.70 ± 0.77^{a}	1.57 ± 0.32^{b}		1.05 ± 0.19^{c}	1.89 ± 0.18^{b}	1.98 ± 0.67^{a}
Hepcidin	1.37 ± 0.21^{b}	1.47 ± 0.72^{a}		0.81 ± 0.32^{c}	1.62 ± 0.24^{b}	$1.84\pm$o.26^{a}

*Values are means ± SEM, n = 3. Data is normalized to (1) for the control fishmeal nucleotide free diets

**Means in a row with different superscript letter are significantly different.

--$FM_{0/0}$ (0% fish meal; 0mg nucleotide), $FM_{0/250}$ (0% fish meal+250mg nucleotide), $FM_{0/500}$ (0% fish meal+500mg nucleotide), $FM_{25/0}$ (0mg nucleotide , 25% fish meal), $FM_{25/250}$ (25% fish meal+250mg nucleotide), $FM_{25/500}$ (25% fish meal+500mg nucleotide).

-FM=fish meal; N = nucleotides.

Table 6. Gut microbiota profile of juvenile gilthead seabream, *sparus aurata* fed different experimental diets.

Items	FM$_0$			FM$_{25}$		
	NT$_0$	**NT$_{250}$**	**NT$_{500}$**	**NT$_0$**	**NT$_{250}$**	**NT$_{500}$**
Photobacterium damselae	95.33±2.56^{a}	110.50±6.84^{a}	101.00±6.12^{a}	72.67±2.50^{b}	95.00±6.11^{a}	100.33±6.12^{a}
Vibrio anguillarum	55.50±1.98^{d}	162.67±1.21^{b}	213.17±1.10^{a}	120.83±6.17^{c}	111.50±6.85^{c}	155.83±5.40^{b}
Lactobacillus plantarum	77.50±3.49^{d}	132.00±7.59^{b}	115.50±8.63^{bc}	230.83±8.74^{a}	132.33±5.59^{b}	107.67±5.91^{c}
Lactobacillus subtilis	166.67±9.27^{b}	180.67±7.43^{b}	118.83±4.66^{c}	341.83±10.16^{a}	118.33±5.18^{c}	109.17±8.01^{c}
Enterococcus faecium	290.50±9.52^{c}	210.67±7.07^{e}	360.17±7.60^{a}	256.67±11.20^{d}	99.33±4.45^{f}	325.00±5.88^{b}

	FM levels		NT Levels		
	0%	**25%**	**0mg**	**250mg**	**500mg**
Photobacterium damselae	102.28±4.20^{a}	89.33±7.18^{b}	84.00±3.23^{c}	102.75±7.18^{a}	100.67±4.30^{b}
Vibrio anguillarum	143.78±5.58^{a}	129.39±7.84^{b}	88.17±3.03^{c}	137.08±9.76^{b}	184.50±4.27^{a}
Lactobacillus plantarum	108.33±3.08^{b}	156.94±2.24^{a}	154.17±9.26^{a}	132.17±5.82^{b}	111.58±6.11^{c}
Lactobacillus subtilis	155.39±8.39^{b}	189.78±7.00^{a}	254.25±8.57^{a}	149.50±5.57^{b}	114.00±7.75^{c}
Enterococcus faecium	287.11±7.60^{a}	227.00±3.89^{b}	273.58±5.76^{b}	155.00±3.57^{c}	342.58±4.36^{a}

*Values are means ± SEM, n = 3.

**Means in a row with different superscript letter are significantly different.

-FM$_{0/0}$ (0% fish meal; 0mg nucleotide), FM$_{0/250}$ (0% fish meal+250mg nucleotide), FM$_{0/500}$ (0% fish meal+500mg nucleotide), FM$_{25/0}$ (0mg nucleotide , 25% fish meal), FM$_{25/250}$ (25% fish meal+250mg nucleotide), FM$_{25/500}$ (25% fish meal+500mg nucleotide).

-FM=fishmeal; N = nucleotides.

Table 7. Specific activities of superoxide dismutase (SOD), catalase (CAT) (U/mg protein), reduced glutathione (GSH) and malondialdehyde (MDA) (nmol/g tissue) in the liver of gilthead seabream, *sparus aurata* fed different experimental diets.

Items	**FM₀**			**FM₂₅**		
	NT_0	**NT_{250}**	**NT_{500}**	**NT_0**	**NT_{250}**	**NT_{500}**
SOD (U/mg protein)	138.50±8.40[d]	154.60±18.60[d]	223.70±14.6[c]	220.30±12.00[c]	363.70±11.90[b]	554.50±15.10[a]
CAT (U/mg protein)	75.50±9.30[d]	99.40±11.60[cd]	128.40±8.50[c]	127.60±8.20[c]	218.40±9.60[b]	268.10±10.30[a]
GSH (nmol/g tissue)	1491.70±42.70[c]	2179.30±54.3[b]	2822.20±47.0[a]	2797.03±42.90[a]	2816.70±51.30[a]	2852.50±37.40[a]
MDA (nmol/g tissue)	19.88±0.30[a]	17.80±0.50[b]	9.88±0.30[d]	11.420±0.40[c]	10.88±0.50[cd]	9.97±0.40[d]

	FM levels		**NT Levels**		
	0%	**25%**	**0mg**	**250mg**	**500mg**
SOD (U/mg protein)	172.27±13.87[b]	379.50±13.00[a]	179.4±10.20[c]	259.15±15.25[b]	389.10±14.85[a]
CAT (U/mg protein)	101.10±9.80[b]	204.70±9.37[a]	101.55±8.75[c]	158.90±10.60[b]	198.25±9.40[a]
GSH (nmol/g tissue)	2164.40±48.00[b]	2822.17±43.87[a]	2144.50±42.80[c]	2498.00±52.80[b]	2837.35±42.20[a]
MDA (nmol/g tissue)	15.85±0.37[a]	10.76±0.43[b]	15.65±0.35[a]	14.34±0.5[b]	9.925±0.35[c]

*Values are means ± SEM, n = 3

**Means in a row with different superscript letter are significantly different.

-$FM_{0/0}$ (0% fish meal; 0mg nucleotide), $FM_{0/250}$ (0% fish meal+250mg nucleotide), $FM_{0/500}$ (0% fish meal+500mg nucleotide), $FM_{25/0}$ (0mg nucleotide , 25% fish meal), $FM_{25/250}$ (25% fish meal+250mg nucleotide), $FM_{25/500}$ (25% fish meal+500mg nucleotide).

-FM=fishmeal; N = nucleotides.

Table 8. Economic analysis of juvenile gilthead seabream, *Sparus aurata* production output cost/metrics fed different the experimental diets.

Items	FM_0			FM_{25}		
	NT_0	NT_{250}	NT_{500}	NT_0	NT_{250}	NT_{500}
Cost of Diet (kg/ $)*	0.844	0.858	0.872	0.961	0.975	0.989
Total consumed feeds(gm/fish)	26.68±0.89^{b}	26.06±0.56^{b}	25.39±0.56^{bc}	27.26±0.94^{a}	25.88±0.40^{bc}	25.13±0.39^{c}
ECR	1.98±0.26^{b}	1.86±0.23^{b}	1.74±0.19^{d}	2.14±0.36^{a}	1.90±0.16^{b}	1.79±0.24^{c}
	FM levels			**NT Levels**		
	0%	**25%**		**0mg**	**250mg**	**500mg**
Cost of Diet (kg/ $)*	0.86	0.98		0.90	0.92	0.93
Total consumed feeds (gm/fish	26.04±0.67	26.09±0.58		26.97±0.92^{a}	25.97±0.48^{b}	25.26±0.48^{c}
ECR	1.86±0.23 ^{b}	1.94±0.25 ^{a}		2.06±0.31 ^{a}	1.88±0.20 ^{b}	1.77±0.22 ^{c}

*Values are means ± SEM, n = 3.

**Means in a row with different superscript letter are significantly different.

--$FM_{0/0}$ (0% fish meal; 0mg nucleotide), $FM_{0/250}$ (0% fish meal+250mg nucleotide), $FM_{0/500}$ (0% fish meal+500mg nucleotide), $FM_{25/0}$ (0mg nucleotide , 25% fish meal), $FM_{25/250}$ (25% fish meal+250mg nucleotide), $FM_{25/500}$ (25% fish meal+500mg nucleotide).

-FM=fishmeal; NT = nucleotides.

One US$ = 18 Egyptian pound (L.E.).

FCR= Feed conversion ratio.

ECR=Economical conversion rate= Cost of diet ($/kg)* FCR.