Dietary nucleotides enhance growth performance, feed efficiency and intestinal functional topography in European Seabass (*Dicentrarchus labrax*)

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Bowyer, P.H., El-Haroun, E.R., Hassaan, M., Salim, H. and Davies, S.J. 2019. Dietary nucleotides enhance growth performance, feed efficiency and intestinal functional topography in European Seabass (*Dicentrarchus labrax*). *Aquaculture Research*.

2 May 2019

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2 topography in European Seabass (*Dicentrarchus labrax*)

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Abstract

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30   Nucleotides, nucleosides and nucleic acids (NU) have many critical functions in supporting

31 life and increasing evidence suggests that exogenous supply can benefit the health of mammals

32 and fish. For these reasons, a 6-week feeding trial was conducted on juvenile European seabass

33 (*Dicentrarchus labrax*) with diets containing 0%, 0.15% and 0.3% inclusion of a NU mixture

34 (Laltide®) derived from the yeast *Saccharomyces cerevisiae*. At the end of the study no significant

35 differences were found in fish performance, although a tendency towards better performance was

36 indicated in fish fed the Nu0.3 diet. In relation to histological assessment, a significantly greater

37 perimeter ratio; internal to outer (IP/OP) was observed in the posterior intestine of fish fed

38 supplemental NU. Microvilli heights in the posterior intestine were also shown to be significantly

39 promoted in fish fed NU diets ($P < 0.05$). Goblet cell abundance was shown to be unaffected by

40 the inclusion of NU in the diet ($P > 0.05$). Overall, this study indicates that orally administered

41 NU may be effective promoters of gut functional topography with marginal associated

42 improvements to fish performance. Nonetheless, longer exposure and/or commercial scale

43 application, and in diets that were challenging in use of high inclusion levels of plant by-products

44 would potentially amplify improvements in production characteristics, in turn benefiting fish

45 culturists.

46 KEYWORDS*:* Aquaculture, Intestine, Microvilli, Morphology, Nucleotides, Nucleosides

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1. INTRODUCTION

With an ever-increasing volume of aquaculture activities around the globe comes growing pressure to maximize the efficiency of finfish production whilst at the same time ensuring sustainability. Typically, being the main expenditure in intensive finfish aquaculture, feeds must be formulated to promote fish performance, maintain the health of fish and be economically viable; they must also increasingly conform to the emerging paradigm of extending beyond satisfying basic nutritional requirements (Meng, Ma, Ma, J., Han, D. Xu, Zhang & Mai, 2016; Li and Gatlin, 2004; Hassaan, Mahmoud, Jarmolowicz, El-Haroun, Mohammady & Davies, 2018). However, modern aquaculture continues to face an array of nutritional restrictions which threaten optimal culture. The ever-increasing inclusion of alternative feed ingredients, particularly plant proteins (PP), is associated with amino-acid and fatty-acid deficiencies as well as increases in the presence of anti-nutritional factors (ANF) in diets. PP-derived ANF have been extensively studied in commercial finfish and have often been shown to negatively interact with the digestion process, be it via decreasing nutrient availabilities or impingement upon gastrointestinal physiology and function. Of particular concern is gut enteritis, which results in structural damage to the intestinal absorptive area, caused by saponins in high PP inclusions which have now become common-place (Francis, Makkar, & Becker, 2001). As the site of nutrient assimilation, and to some extent defence against pathogens, damage to the gut can result in marked detriment to the health and production of livestock. Maintaining and promoting gut health is therefore of utmost importance. Several studies on fish confirmed the role of nucleotides on beneficial in growth performance (Ferreira, Pinho, Vieira, & Tavarela, 2010; Jarmołowicz, Rożyński, Kowalska, & Zakęś, 2018; Azeredo, Machado, Kreuz, Wuertz, Oliva-Teles, Enes, & Costas, 2018), physiological response and antioxidant capacity (Tahmasebi-Kohyani, Keyvanshokooh, Nematollahi, Mahmoudi & Pasha-

74 Zanoosi, 2011), and intestinal health (Cheng, Buentello & Gatlin, 2011).Yeast derived nucleotides

75 have long been acknowledged as valuable agricultural feed ingredients, applied in bovine, poultry

76 and porcine diets; however, their application in aquafeeds remains much less significant (Ferreira

77 et al., 2010; Jarmołowicz et al., 2018; Azeredo, Machado, Kreuz, Wuertz, Oliva-Teles, Enes,

78 Costas, 2018). Although the attention yeasts traditionally received was based upon their attractive

79 protein content, more recently their application as functional feed ingredients have been

80 investigated in endeavours to exceed basic nutritional requirements (Li & Gatlin, 2005). The

81 driving force behind this research appears to be in part due to the numerous nutritionally beneficial

82 compounds present within yeast cells such as mannan-oligosaccharides, β-glucans and nucleotides

83 (NUT) (Li & Gatlin, 2004; Berto, Pereira, Mouriño, Martins, & Fracalossi, 2016). Further

84 investigations into the potential benefits of these individual components are required, in order to

85 understand the nutritive qualities of yeast as an ingredient and to evaluate the feasibility of

86 supplementing yeast-derived products into finfish diets. Nucleotides are present in ingredients of

87 plant or animal origin as well as yeast cells as free nucleotides and nucleic acids (Fegan 2006).

88 Nucleotides participate in many biochemical processes that are indispensable to the support of life.

89 They are crucial in the storage, transfer and expression of genetic information, stand as activated

90 intermediates of energy transport in cells (e.g. ATP), are important components of certain

91 coenzymes (e.g. coenzyme A) and are also fundamental biological regulators (e.g. cyclic AMP)

92 (Cosgrove 1998; Sanchez-Pozo 1998). Because endogenous synthesis occurs, and the NUT

93 contents of dead cells can be recycled through salvage pathways, signs of deficiency are not

94 typically developed in higher vertebrates or fish thus NUT have traditionally been considered to

95 be non-essential nutrients (Grimble, 1996; Sanchez-Pozo, 1998; Li & Gatlin, 2006). However,

96 numerous research publications have successively suggested that dietary supplementation of NUT

97 may be of significant benefit to consumers under certain conditions, particularly stress (VanBuren

98 and Rudolph, 1997; Hess and Greenberg, 2012).

99 In aquaculture, NUT were initially recognized as feeding stimulants (Hughes, 1991; Kasumyan

100 and Doving, 2003). Their potential as functional nutritional supplements in fish diets has also since

101 been explored. Supplementation of NUT has been demonstrated to have immunomodulatory

102 effects in numerous species (Low, Wadsworth, Burrells & Secombes, 2003; Lin, Wang, & Shiau,

103 2009; Cheng et al., 2011; Tahmasebi-Kohyani et al., 2011; Welker, Lim, Yildirim-Aksoy, &

104 Klesius, 2011; Kenari, Mahmoudi, Soltani, & Abediankenari, 2013; Peng, Xu, Ai, Mai, Liufu, &

105 Zhang, 2013), including reports of improved resistance against bacterial, rickettsia, viral and

106 ectoparasitic pathogens (Burrells, Williams, & Forno, 2001a; Li, Lewis, & Gatlin, 2004).

107 Furthermore, modulation of stress responses has also been reported (Kenari et al., 2013; Palermo

108 Cardinaletti, Cocci, Tibaldi, Polzonetti-Magni, & Mosconi, 2013). Effects of NUT

109 supplementation on fish performance have appeared somewhat more variable, although growth

110 promotion has been observed in salmonids (Burrells, Williams, Southgate & Wadsworth, 2001b;

111 Tahmasebi-Kohyani et al., 2011). To date, gut morphological analysis of finfish exposed to

112 supplementary NUT has only been conducted in Atlantic salmon (*Salmo salar*) (Burrells et al.,

113 2001b), red drum (*Sciaenops ocellatus*) (Cheng et al., 2011) and turbot (*Scophthalmus maximus*)

114 (Peng et al., 2013) but benefits to functional gut structure following NUT dietary supplementation

115 have been reported throughout. However, no studies to date have reliably assessed gut structure at

116 the ultrastructural level of microvilli. This is despite their delicate nature and arguably their

117 heightened susceptibility of being impaired compared to gut macrostructure, such as intestinal

118 folds. The potential of supplementary NUT as functional feed additives for improving gut structure

119 warrants further research considering the attractive but sparse research findings in aquaculture to

120   date. The present study was conducted to identify any effects of orally administered graded levels

121   of a NUT-based product (Laltide®) derived from the yeast *Saccharomyces cerevisiae* on the

122   performance and intestinal morphology of juvenile European seabass being a very valuable fish

123   farmed in the Mediterranean.

124   2. MATERIALS AND METHODS

125   2.1. Diet formulation and proximate analysis

126   A basal diet (Control) was formulated to satisfy all known nutrient requirements of European

127   seabass (Lim 2003). Two experimental diets were subsequently formulated with the inclusion of

128   the NU ingredient, Laltide®, obtained from Lallemand Animal Nutrition UK; Spring Lane North.

129   Malvern Link. Worcestershire. WR14 1B. Laltide® was added to the basal mix at 0.15% (Nu0.15)

130   and 0.3% (Nu0.3) inclusion. This product contains bioavailable nucleotides (including 5'-

131   nucleotides), as well as nucleosides, nucleic acids and nucleotide containing adducts.

132   Proximate composition of experimental diets was determined using standard AOAC (2000)

133   procedures: dry matter (105 °C to constant weight), ash (incinerated at 550°C to constant weight),

134   crude protein (Nx6.25) by the Kjeldahl method after an acid digestion (Gerhardt Kjeldatherm KB8

135   S and Gerhardt Vapodest 50) and crude lipid extracted with hot petroleum-ether (Gerhardt

136   Soxtherm). All analyses were performed in triplicate, bar ash content which was run in duplicate.

137   2.2. Experimental system and animals

138   Juvenile European seabass (approximately 50 g) were obtained from Selonda UK ltd and held for

139   a 6-week conditioning period in a salt-water recirculating system at the aquaculture nutrition

140   research facility. During conditioning, fish were fed EWOS Sigma 50 at 1-2% body weight per

141   day as a maintenance diet. After conditioning, the stock fish were graded by size. Selected fish

142   averaging 62.19 g ± 0.42 in weight were stocked into 9 tanks (triplicate groups per treatment) in

143   groups of 20 individuals, having an average total biomass of 1245g ± 8.37 per tank (15.37 kg/m³

144   ± 0.1). Adequate water quality was ensured throughout the trial with biological and mechanical

145   filtration, alongside UV sterilization and protein skimming. Water used in the system was natural

146   seawater with additional $NaHCO_3$ as a buffer. During the trial, pH was 6.16. Air was supplied

147   using a low-pressure side channel blower (Rietschle, UK ltd) via air stones and maintained

148   dissolved oxygen (DO) levels at 7.38 mg/l. Water temperature was controlled by an inline heater

149   (Elecro Titanium) at 21.5 °C ±1.0 throughout the trial. Tanks were illuminated with a photoperiod

150   of 12 h light: 12 h dark with timer-controlled overhead fluorescent light array. Fish were weighed

151   collectively in bulk from each as tank at weeks 0, 1, 3, 5, and 6 of the feeding trial. Feeding was

152   performed twice a day at 2% bodyweight for the first 12 days followed by 2.6% for the rest of the

153   trial duration, based upon acceptance of the feed.

154   2.3. Sampling

155   Sampling for tissue was performed 6 weeks after the start of the trial, including a 24h starvation

156   period. Fish were randomly selected and anesthetized by immersion in buffered tricaine methane

157   sulphonate (MS-222, 200 mg/l); they were subsequently euthanized by a sharp blow to the head

158   and pithing of the brain. Four fish per tank were sampled for carcass composition and K-factor

159   calculations, a further two fish per tank were sampled for light and electron microscopy.

160   2.4. Fish performance and condition

161   Growth performance, feed efficiency and fish condition calculations were performed as follows:

162   Weight gain = (final weight (g) − initial weight (g)) / 100; Specific growth rate (SGR) =

163   $\left(\frac{LnW_1 - LnW_2}{T}\right)$ x 100; where LnW_1 and LnW_2 are the initial and final natural logarithmic weights,

164   respectively, and T is the number of days in the feeding period; Feed conversion ratio (FCR) = feed

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

166   K-factor (K-F) = 100 (weight (g))/(total fish length (cm)^3).

167   2.5. Carcass composition

168   Carcass chemical analysis of fish was conducted in identical manner to proximate analysis of feed

169   according to standard AOAC (2000) procedures.

170   2.6. Intestinal morphology assays

171   2.6.1. Light Microscopy

172   The gastrointestinal tracts of fish were removed from the body cavity and a section of the posterior

173   intestine was removed, these were fixed in 10% formalin and kept at 4°C for 48 hours then

174   transferred to 70% ethanol at 4°C for storage. After storage, samples were further dehydrated in

175   incremental ethanol concentrations and embedded in paraffin wax according to standard

176   histological techniques. Samples were sectioned at 5μm thickness (Leica RM2235 microtome),

177   dried in an oven overnight and subsequently auto- stained with haematoxylin and eosin (HE) or

178   periodic acid Schiff (PAS) (Leica Autostainer XL). Slides were mounted with cover slips using

179   DPX and left to dry. Micrographs of HE and PAS stained samples were then captured at 1, 4 and

180   10X magnifications (Leica DMIRB microscope and Olympus E410 digital SLR camera).

181   2.6.2. Transmission electron microscopy (TEM)

182   Small sections of the distal end of the posterior intestine were excised and placed in gluteraldehyde

183   and stored at 4°C. Samples were immersed twice in 0.12 sodium cacodylate buffer (pH 7.2) at

184   15minute intervals. The samples were then placed in osmium tetroxide (OsO$_4$) for 2 hours and

185   subsequently immersed in fresh OsO$_4$ twice at 15minute intervals. Dehydration of samples was

186   then conducted by immersion in 30, 50, 70, 90 and 100% ethanol at 15minute intervals. Samples

187   were then placed in incremental absolute ethanol: Agar low viscosity resin mixes (70:30, 50:50,

188　30:70, 0:100) at 24-hour intervals. Samples were subsequently embedded in BEEM® capsules

189　with fresh resin. Sectioning was performed using a Reichert-Jung Ultracut E ultratome and

190　Microstar diamond knife. Sections were stained with saturated uranyl acetate solution and lead

191　citrate. Micrographs of brush borders at 10,000X magnification were captured using a JEOL 1200

192　ex2 transmission electron microscope with a built in Soft Imaging System Megaview 3 camera.

193　2.6.3.　Microscopic analysis

194　Image J 1.43 was used for all image analysis in this study. For HE-stained samples, intestinal fold

195　length (FL) was measured from the base to the extremity of folds using an intersecting line.

196　Perimeter ratio (PR) was also calculated from HE-stained samples as follows: PR = IP / OP; where

197　OP is the outer mucosal perimeter of the gut and IP is the inner absorptive surface perimeter.

198　Goblet cell abundances in PAS-stained samples were calculated by measuring the perimeter of

199　folds and subsequently counting the number of goblet cells present within this distance. The

200　abundance measurement calculated and used was number of goblet cells per mm of fold perimeter.

201　From TEM micrographs, the lengths of 40 visibly complete microvilli were measured per fish,

202　from different locations. All analyses were performed blind.

203　2.7. Statistical analysis

204　One-way Analysis of variance (ANOVA) and *post hoc* Fisher's LSD was used for the analysis of

205　normally distributed data. Kruskal-Wallis was used for the analysis of non-normally distributed

206　data in association with Mann-Whitney U-tests and Bonferroni correction. Statistical analyses

207　were performed using IBM SPSS Statistics 20 (Chicago, IL, USA).

208　3.　RESULTS

209　3.1. Fish performance and condition

210 No statistically significant differences in mean weight gain (WG; P = 0.126), specific growth rate

211 (SGR; P = 0.267), feed conversion ratio (FCR; = 0.42), protein efficiency ratio (PER; P =0.232)

212 or condition factor (K-F; P = 0.662) were found among fish fed the experimental diets (Table 2).

213 There was a distinct trend however for seabass fed the Nu1.5 treatment to perform better than the

214 control and Nu3.0 for all parameters measured.

215 3.2. Carcass Proximate Analysis

216 No significant difference in mean moisture (P = 0.737), crud protein (CP; P =0.498), crude lipid

217 (CL; P = 0.927) or ash content (P = 0.377) was found among fish fed the experimental diets (Table

218 3).

219 3.3. Intestinal morphology assays

220 3.3.1. Perimeter ratio

221 Although no significant difference in Perimeter ratio between Nu0.15 and Nu0.3 (PR; P = 0.08)

222 was observed (Table 4), Significant differences were observed to lie between control diet fed and

223 Nu0.15 (P = 0.02) and control diet and Nu0.3 (P = 0.04).

224 3.3.2. Fold length (FL)

225 No significant difference in FL was observed between the three treatments (*P* = 0.08). Figure 2

226 displays the Juvenile European seabass posterior intestinal folds. (A, B) fed Control (C) fed Nu0.15

227 (D) fed Nu0.3. H&E stained. FL = fold length, FB = fold branching. Scale bar: 100μm.

228 Fold length (μm) was determined as 337.6 ± 80.0 (control group) 479.2 ± 163.5 (Nu1.5) and 431.0

229 ± 27.6 for seabass receiving Nu0.3

230 3.3.3. Goblet cell abundance

231 No statistically significant difference in mean goblet cell abundance ($P = 0.10$) was indicated

232 among treatments (Table 4). Figure 3 displays the Juvenile European seabass posterior intestinal

233 folds displaying goblet cells (10X magnification). (A, B) fed control (C) fed Nu0.15 (D) fed Nu0.3.

234 PAS stained. GC = goblet cells. Scale bar: 100μm. Cells ranged in number from 45 to 54 per

235 mucosal fold.

236 3.3.4. Microvilli height

237 Statistical analysis of microvilli heights indicated a highly significant difference among the

238 experimental treatments ($P < 0.05$). Pairwise comparisons revealed significant differences in

239 median microvilli height between Nu0.15 ($P < 0.02$), Nu0.15 and Nu0.3 ($P < 0.02$) and Nu0.3 (P

240 < 0.02) (Table 4). Figure 1 shows the posterior intestinal Microvilli of juvenile European seabass

241 (A, B) fed Control (C) fed Nu0.15 (D) fed Nu0.3. MV = microvilli. Scale bar: 5μm. Microvilli

242 height (μm) ranged from 1.71 (control) to 2.04 (Nu0.15) to 1.77 (Nu0.3)

243 4. DISCUSSION

244 Since interest in the exogenous supply of nucleotides, nucleosides and nucleic acids (NU) for

245 medical applications and livestock nutrition began, improved growth performance has been

246 reported by several authors. The present results showed that European seabass fed diet

247 supplemented with 0.15% (Nu0.15) and 0.3% (Nu0.3) trended towards improved growth

248 performance, feed utilization (WG, SGR, FCR, PER) and condition factor (Table 2). The current

249 results were marginally consistent with previous findings obtained by (Burrells, Williams,

250 Southgate & Wadsworth, 2001b) who found that inclusion of 0.03% nucleotide (NT) diet fed to

251 Atlantic salmon (*Salmo salar*) significantly improved growth after just 3 weeks. Similarly,

252 incorporating 0.25% Ascogen® a NT containing dietary supplement, into the diets of rainbow trout

253 (*Oncorhynchus mykiss*) was reported to significantly improve growth performance (Adamek,

254   1994). Growth performance enhanced with 0.25% NT in Caspian brown trout (*Salmo trutta*

255   *caspius*) (Kenari et al., 2013). Furthermore, in grouper (*Epinephelus malabaricus*), 0.15% dietary

256   NT also indicated significant improvements to weight gain (Lin et al., 2009). Recently, growth

257   performance of pikeperch (*Sander lucioperca*) were significantly improved with yeast extract

258   supplemented (Jarmołowicz et al., 2018). In addition, Performance of juvenile red drum

259   (*Sciaenops ocellatus*) also did not significantly improve with diets containing Ascogen® (Li, Burr,

260   Goff, Whiteman, Davis, Vega, Neill & Gatlin, 2005; Li, Gatlin & Neill, 2007a). Later studies in

261   red drum revealed that orally administering 0.5% and 1% Ascogen® had a tendency to improve

262   survival and weight gain of fish but again no significant differences were apparent (Cheng et al.,

263   2011). Despite non-significant findings, this study would seem to suggest a tendency for improved

264   fish performance with dietary inclusion of NU. Interestingly, Cheng et al. (2011) suggested that

265   dietary inclusion of NT may be more beneficial on WG of the fish at 0.5% than 1%. Increasing the

266   inclusion of Ascogen® in the diets of rainbow trout and goldfish (*Carassius auratus*) from 0.25%

267   to 0.5% displayed a similar yet more extreme scenario with a reversal from enhancement to

268   depression of growth (Hamackova, Kouril, Adamek, Vachta & Stibranyiova, 1992). The findings

269   of this investigation may also appear to show that in terms of maximising performance, the

270   optimum inclusion of the supplement is not at its highest tested level. Despite a lack of statistical

271   significance, this indication arises from Nu0.15 fed fish consistently displaying the best

272   performance characteristics; followed by Nu0.3 fed fish, whilst control fish displayed the least

273   desirable production characteristics. Overall, the fish performance results of this study are largely

274   in correspondence with the body of research in this field as improved performance of fish in NU

275   nutrition trials remains rather marginal when considering adult and juvenile fish (Li & Gatlin,

276   2004; 2006).

277   No statistically significant differences in moisture, CP, CL or ash of fish carcasses were

278   identified between treatments after the feeding trial, corresponding to Li et al. (2004) whom

279   similarly observed no significant changes to whole body composition of hybrid striped bass fed

280   supplementary NT. The present results were consistent with those reported by JarmoŁowicz,

281   Zakęś, Siwicki, Kowalska, Hopko, GŁĄbski, & Partyka (2012) who found that Juvenile pikeperch

282   Sander lucioperca (L.) fed diets supplemented with brewer's yeast has no significant changes on

283   body chemical composition. Also, no significant differences were found in chemical composition

284   in Nile tilapia (Lunger, Craig, & McLean, 2006; Hassaan et al., 2018) fed died supplemented with

285   nucleotides.

286   Significantly greater perimeter ratio and Microvilli heights ($P \leq 0.05$) were observed in the

287   posterior intestine of seabass fed supplemental NU, while Goblet cell abundance was shown to be

288   unaffected by the inclusion of NU in the diet (Figure 1-4). The present results are consistent with

289   Adjei Morioka, Ameho, Yamauchi, Kulkarni, Al-Mansouri, & Yamamoto, (1996) who found that

290   a nucleotide-nucleoside mixture supplemented into the diets of mice could increase villus height

291   and decrease gut damage. Enhancement of villus height has similarly been described in rats fed

292   0.8% nucleosides, with increases reported as greatest in the posterior section of the gut (Uauy,

293   Stringel, Thomas & Quan, 1990). Furthermore, research has shown the growth of human and rat

294   intestinal cell lines to benefit greatly from supplementary exogenous provision of DNA-Na+

295   derived from fish soft roe, RNA derived from yeast and deoxy-mononucleotides from yeast (Holen

296   & Jonsson, 2004). In fish, increases in intestinal fold length in response to dietary NT were first

297   suggested by Burrells et al. (2001a) as the explanation behind enhanced growth of Atlantic salmon.

298   Interestingly, despite continuing work into fish NU nutrition, morphological analyses of the

299   gastrointestinal tract have remained sparse. Nonetheless, evidence of fold length enhancement in

300 the posterior intestine was shown in red drum fed a NT containing product (Cheng et al., 2011).

301 However, Cheng et al. (2011) did not detect any significant differences in fold height at distal and

302 mid intestinal locations. This study's analysis did not show any statistically significant difference

303 between posterior intestinal fold lengths of fish fed the experimental diets, but numerical

304 observation would appear that display elongation of folds could be present in NU fed fish. It is of

305 note that there was a rather large variability between individuals, particularly in the Nu0.15

306 treatment, which could mask effects. Perimeter ratios were significantly increased in both Nu0.15

307 and Nu0.3 compared to the control group. This suggests that the functional absorptive inner

308 epithelial layer (perimeter) of the intestine was enlarged following NU supplementation. To the

309 author's knowledge, this is the first instance that this analysis has been performed in animals and

310 in particular fish fed an exogenous supply of NU. The results of this can nonetheless be treated as

311 comparable to previous discussions of fold length promotion.

312   Even though NU is increasingly being seen as promising supplementary nutrients for increasing

313 absorption area in the gut by promoting macrostructure (fold/villus length), their potential to

314 enhance absorption area at an ultrastructural level, by promoting microvilli length, seems to have

315 been largely neglected. In red drum (*Sciaenops ocellatus*), microvilli height was reported as

316 significantly increased in the distal, mid and proximal intestine by exogenous supplementation of

317 NT (Cheng et al., 2011). It was also reported that supplemental NT increased microvilli height in

318 Turbot (Peng et al., 2013). Although nucleotides do appear effective promoters of absorptive

319 surface area in the gut, the use of light microscopy in the study of gut micromorphology must be

320 treated with great care due to its low degree of accuracy. The findings of this study give a clear

321 and accurate indication that dietary inclusion of NU can have prominent positive effects on

322 microvilli height, via the use of electron microscopy. It is noteworthy that the results of this

323 investigation indicated that higher inclusion levels of dietary NU may begin to have slightly more

324 negligible effects on microvilli height. Height and assumed proliferation were greater in Nu0.15

325 than Nu0.3 fish, although Nu0.3 remained more beneficial to microvilli structure than no

326 exogenous supply of NU. Interestingly, this is in contrast with findings by Cheng et al. (2011) who

327 identified microvilli height to be greater in fish fed a 1% inclusion of NT as appose to 0.5%.

328 However, non-linear responses in growth, haematological parameters and stress responses have

329 been reported in Caspian brown trout (*Salmo trutta caspius*) (Kenari et al., 2013).

330 Goblet cell abundance was not significantly different between treatments, but again numerical

331 trends may be apparent with reduced average abundances of cells in NU-exposed individuals.

332 Overall there is a distinct lack of comparable research into how dietary provision of NU may affect

333 goblet cell abundance and continuing future research into goblet cell responses would be a great

334 contribution to our knowledge of both mammalian and piscine NU nutrition.

335 Dietary NUT is partly absorbed in the gut as NS through a variety of mechanisms; they are then

336 incorporated into body tissues (Hess & Greenberg, 2012), but it has been suggested as particularly

337 important in development and proliferation of tissues with rapid cell turnover, such as the liver

338 and gut, (Norton, Leite, Vieira, Bambirra, Moura, Penna & Penna, 2001; Hess & Greenberg, 2012).

339 This explains why inclusion of the commercial supplement resulted in observed improvements to

340 functional perimeter and microvilli height. Improvements in intestinal morphology are commonly

341 associated with improvements to animal performance as increases in structural complexity create

342 a greater surface area for digestion and absorption of nutrients (Burrells et al., 2000a). Despite the

343 non-significant findings, the fish performance trends in this study appear to be supported closely

344 by the intestinal functional topography findings of this study.

345 Conclusion

346　It is now widely accepted that exogenous supply of nucleotides alongside nucleosides and nucleic

347　acids can be of great benefit to the health of the consumer. These results indicate that dietary

348　provision of combined nucleotides, nucleosides and nucleic acids derived from yeast can positively

349　influence the functional gut topography of European seabass, at both a macro and ultrastructural

350　level. Gut morphology was seen to improve with nucleotide supplementation, increasing

351　absorption surface area for digestion and assimilation of nutrients; tendencies of improved fish

352　performance seemed to reflect this promotion. The results also appeared to show that dosage is an

353　important consideration with lower inclusions outperforming higher ones in certain aspects. This

354　will have important consequences in the use of high inclusion rates of plant proteins in carnivorous

355　fish species such as seabass where the challenges of modern feed formulations may lead to gut

356　enteritis and related lesions. The supplementation of nucleotides could mitigate these effects.

357　The potential of nucleotides in improving gut structural integrity and functional topography in

358　aquaculture remains relatively unexplored and should be investigated further with appropriate

359　modern technologies.

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1   **TABLE 1** Compositions of experimental diets with graded levels of NU inclusion.

Ingredient (% inclusion)	Experimental diets		
	Control	Nu0.15	Nu0.3
Fish meal [a]	62.93	62.93	62.93
Corn Starch [b]	17.82	17.82	17.82
Maize gluten [c]	10.00	10.00	10.00
Fish oil [d]	7.25	7.25	7.25
Vitamin/Mineral Premix [e]	2.60	2.60	2.60
NU [f]	-	0.15	0.30
Analysed composition (% of feed)			
Crude Protein (CP) N*6.25	49.26 ± 1.09	49.84 ± 0.39	49.73 ± 0.54
Crude lipid (CL)	13.33 ± 0.95	12.94 ± 0.34	12.47 ± 0.09
Ash	9.45 ± 0.03	9.61 ± 0.08	9.55 ± 0.16
Moisture	4.80 ± 0.05	4.83 ± 0.05	4.79 ± 0.04

2   Control: No inclusion of NU, Nu 0.15: 1.5g kg^{-1} inclusion of NU, Nu0.3: 3g kg^{-1} inclusion of NU
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4   [a] Herring meal LT94, CC Moore, UK
5   [b] Unmodified starch from maize, Sigma Aldrich, UK
6   [c] Glutalys®, Roquette Frères, Fr
7   [d] Biomar, Dk
8   [e] PNP Fish 2%, Premier Nutrition, UK
9   [f] Laltide®, Lallemand UK (Biotal), UK
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TABLE 2 Fish performance and condition factor of European seabass fed incremental levels of
Laltide® over 6 weeks (n=3).

Fish Performance & Condition	Experimental diets		
	Control	Nu0.15	Nu0.3
Weight gain (WG)	28.57 ± 0.26	32.67 ± 0.97	29.77 ± 1.96
Specific Growth rate (SGR)	0.91 ± 0.05	0.97 ± 0.06	0.92 ± 0.01
Feed conversion ratio (FCR)	2.19 ± 0.15	2.04 ± 0.17	2.15 ± 0.07
Protein efficiency ratio (PER)	1.02 ± 0.02	1.11 ± 0.06	1.04 ± 0.09
K-factor (K-F)	1.13 ± 0.01	1.15 ± 0.10	1.17 ± 0.07

29 **TABLE 3** Proximate chemical composition (mean % of total on wet matter basis) of whole carcass
30 of European seabass fed incremental levels of Laltide® for 6 weeks.
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Carcass	Experimental diets		
	Control	Nu0.15	Nu0.3
Moisture	64.52 ± 1.25	64.78 ± 1.50	64.67 ± 1.48
Crude protein (CP)	17.28 ± 0.36	17.38 ± 0.22	17.52 ± 0.29
Crude lipid (CL)	13.09 ± 0.49	13.02 ± 0.64	12.96 ± 0.68
Ash	4.02 ± 0.16	4.13 ± 0.16	4.18 ± 0.17

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44   **Table 4.** Morphological characteristics of posterior intestine of European seabass fed incremental

45   levels of Laltide® for 6 weeks.

Intestinal parameter	Experimental diet		
	Control	Nu0.15	Nu0.3
Fold length (µm)	337.6 ± 80.0	479.2 ± 163.5	431.0 ± 27.6
Perimeter ratio	3.03 ± 0.19 [b]	4.17 ± 0.13 [a]	4.00 ± 0.14 [a]
Goblet cell abundance[1]	54.0 ± 12.0	44.0 ± 6.02	45.0 ± 5.47
Microvilli height (µm)	1.71 ± 0.32 [a]	2.04 ± 0.34 [c]	1.77 ± 0.31 [b]

46   [a,b,c] Means possessing different superscripts in the same row are significantly different at $P < 0.05$.

47   [d,e,f] Medians possessing the same superscript in the same row are not significantly different at P

48   <0.017

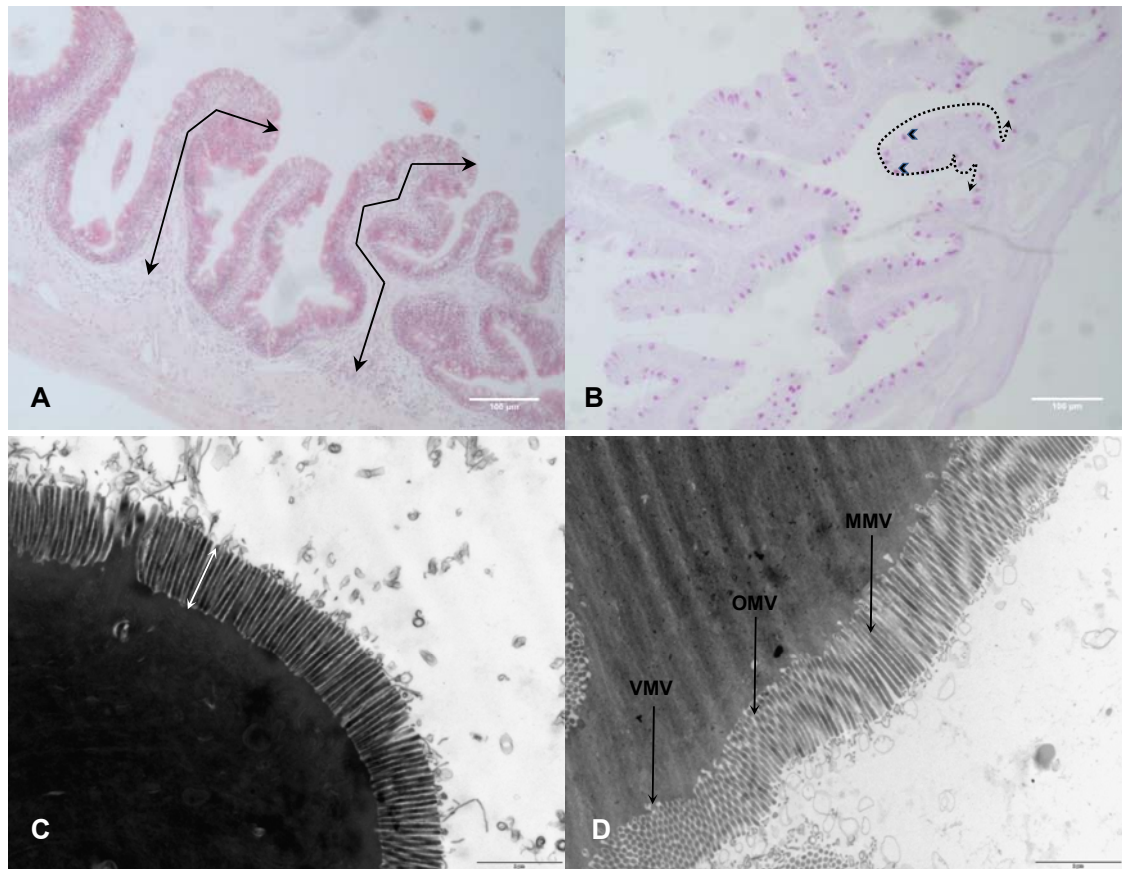49   [1] values expressed as no. cells per mm of fold border.

FIGURE 1 Transversal sections of juvenile European seabass posterior intestine depicting employed measurement techniques. (A) HE stained enteric section at 10X magnification. Arrows indicate example fold length measurements. (B) PAS stained enteric section at 10X magnification. Dashed arrowed line indicates example perimeter measurement and chevrons indicate the goblet cells. (C) Transmission electron micrograph of enteric section at 10,000X magnification. White arrow indicates example microvilli height measurement. (D) Transmission electron micrograph of enteric section at 10,000X magnification. VMV = vertically orientated microvilli (discounted), OMV = overlapping microvilli (discounted), MMV = visibly complete microvilli (measured)
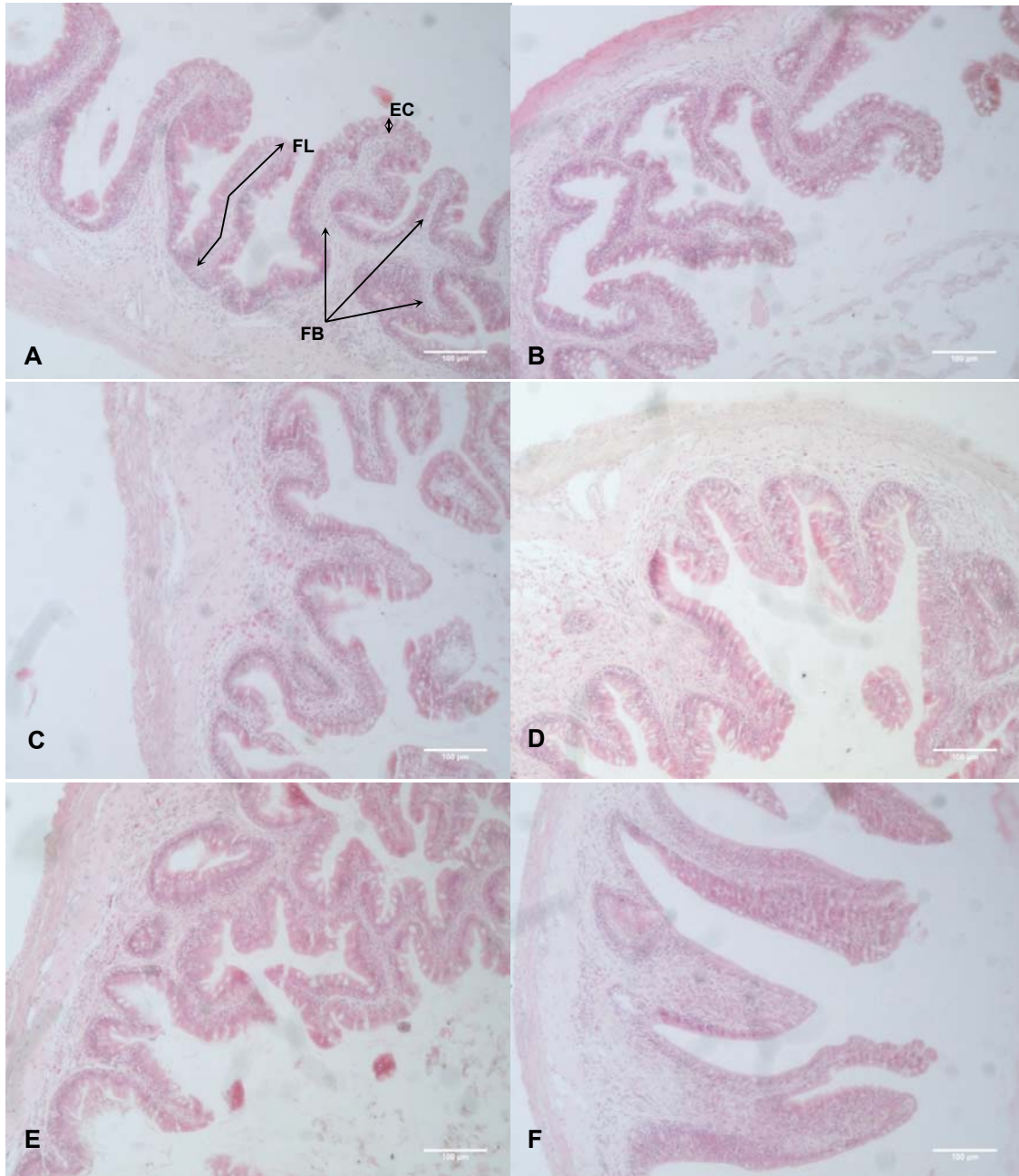
FIGURE 2 Juvenile European seabass posterior intestinal folds (10X magnification). (A, B) fed control diets, (C, D) fed Nu0.15 (E, F) fed Nu0.3 H&E stained. FL = fold length, FB = fold branching, EC = epithelial cells.
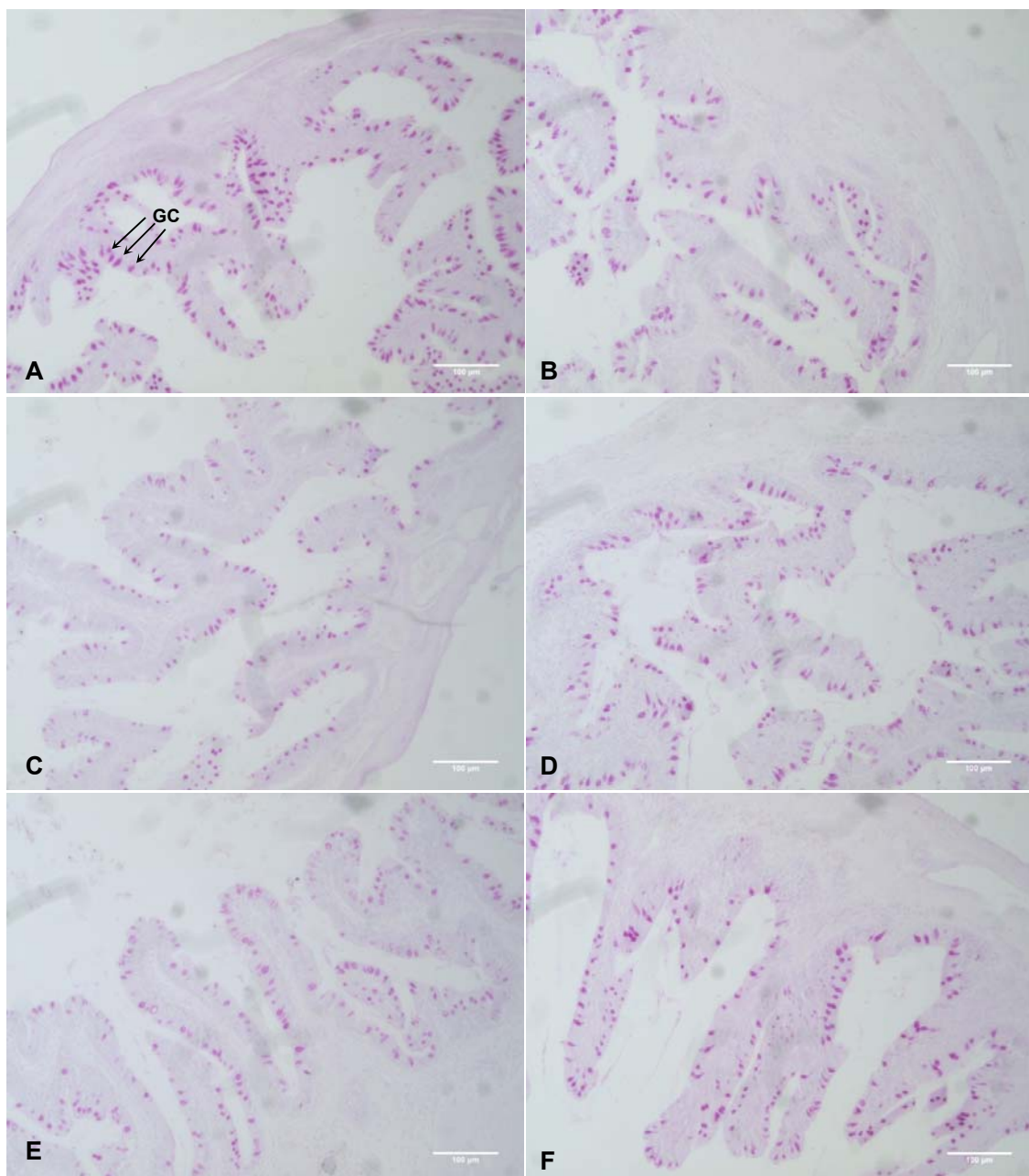
FIGURE 3 Juvenile European seabass posterior Nu0.15 (E, F) fed Nu0.3 PAS stained. GC = goblet cells.
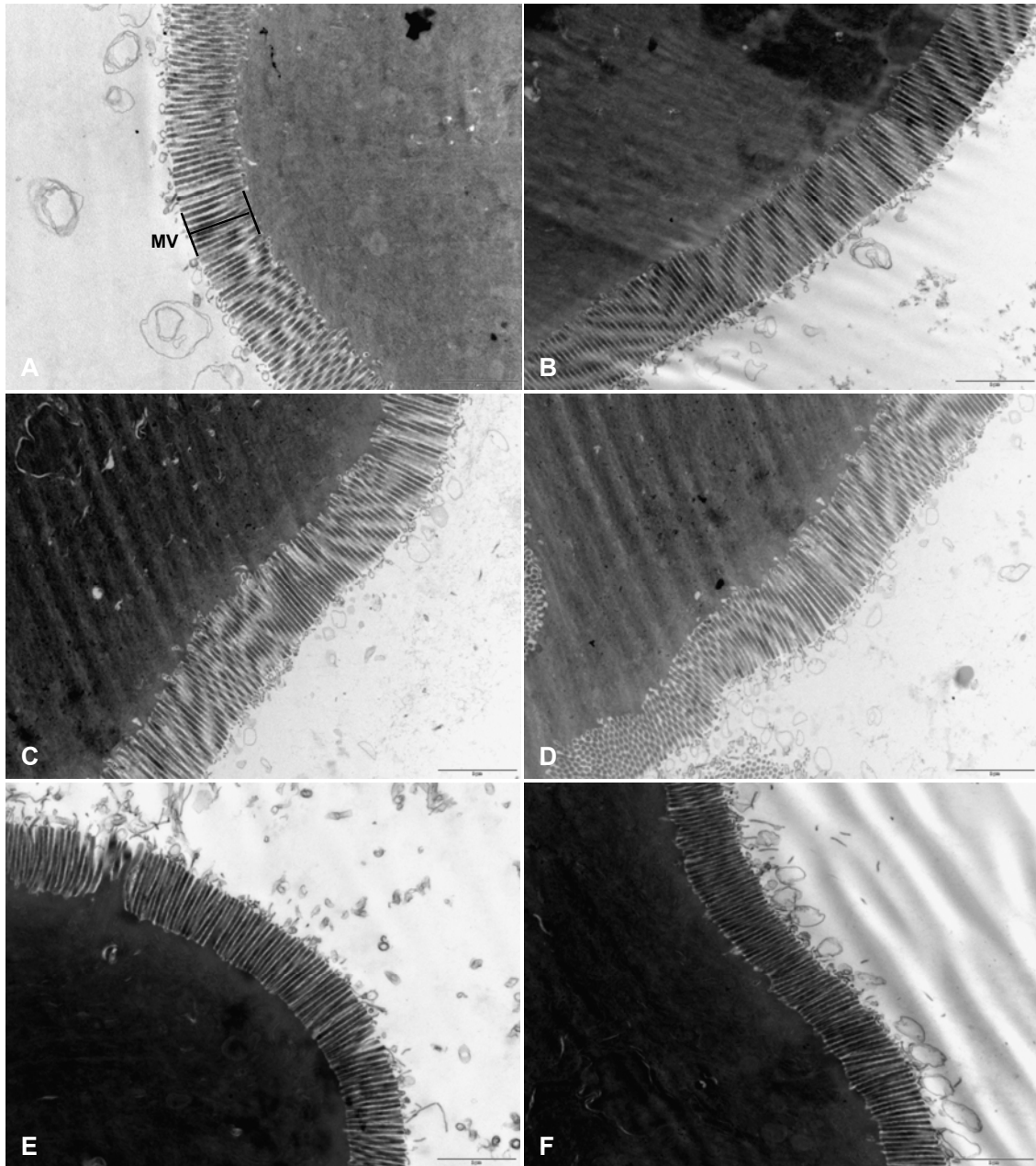
FIGURE 4 Posterior intestinal Microvilli (10000X magnification) of juvenile European seabass (A, B) fed C (C, D) fed Nu0.15 (E, F) fed Nu0.3 MV = microvilli. Scale bar = 5µm.