Freshwater-adapted sea bass *Dicentrarchus labrax* feeding frequency impact in a lettuce *Lactuca sativa* aquaponics system

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Abstract

The aim of this study is to investigate the effect of 3 daily fish feeding frequencies, 2, 4 and 8 times per day (FF2, FF4, and FF8, respectively) on growth performance of sea bass (*Dicentrarchus labrax*) and lettuce plants (*Lactuca sativa*) reared in aquaponics. 171 juvenile sea bass with an average body weight of 6.80 ± 0.095 g were used, together with 24 lettuce plants with an average initial height of 11.78 ± 0.074 cm over a 45-day trial period. FF2 fish group showed a significantly lower final weight, weight gain and specific growth rate than the FF4 and FF8 groups. Voluntary feed intake was similar for all the three feeding frequencies treatmens ($p>0.05$). No plant mortality was observed during the 45-day study period. All three aquaponic systems resulted in a similar leaf fresh weight and fresh and dry aerial biomass. The results of the present study showed that the FF4 or FF8 feeding frequency contributes to the more efficient utilization of nutrients for better growth of sea bass adapted to fresh water while successfully supporting plant growth to a marketable biomass.

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Keywords: aquaponics; feeding frequency; juvenile sea bass; lettuce; water reuse;

1. Introduction

Global population growth, climate change, soil degradation, water pollution and food security management are the main problems related to food production for human consumption that the world is facing. Aquaponic culture is an innovative and sustainable method for both fish and plant production and is environmentally friendly in relation to aquaculture fish and soil monocultures [1]. The flexibility of an aquaponic system allows it to grow a large variety of vegetables, herbs, ornamental and aquatic plants to cater to a broad spectrum of consumers. Aquaponic products are organic and pesticide free,

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with a small environmental footprint [2]. Aquaponic growth contributes to water resource management, biodiversity conservation and energy savings [3]. In aquaponics, soil is not needed, and only a small amount of water is required as the systems do not typically discharge or exchange water under normal operation but instead recirculate and reuse water very effectively. Thus, aquaponic systems can be set up in areas that have traditionally poor soil quality or contaminated water [2].

Freshwater fish, especially tilapia species, carp, perch and catfish, are the main cultured species [4, 5, 6], along with some crustacean species such as *Cherax quadricarinatus* [7] and *Procambarus* spp. [8]. Recent research by Knaus & Palm [9] suggests multispecies cultivation is more efficient in an aquaponic system.

The basic principle of aquaponics is the biochemical oxidation of ammonia to nitrite and nitrate through the autotrophic bacteria *Nitrosomonas* sp. and *Nitrobacter* sp., respectively. Ammonia released by fish through their metabolism is oxidized by nitration into nitrate ions [10]. Nitrate is not toxic for fish and is useful for plants. Finally, the water is transferred back to the fish tanks and is nitrate-free [11]. Fish, plants and bacteria must coexist in balance in an aquaponic system. Therefore, the system type, the size of the filter, fish species, fish biomass and plant species and biomass should be carefully chosen. Proper combination of fish and plants leads to successful production without downgrading water quality [11]. The total biomass of fish should be calculated in comparison with plant biomass and the oxidizing capacity of the filter [12]. If fish and plant biomasses are in appropriate proportions, the fish-produced daily ammonia is sufficient to meet 80% of the daily plant nutrition needs [13]. Lower plant biomass will lead to nutrient accumulation in the systems, as a higher plant biomass will lead to slower plant growth [14].

Fish feed supplies most of the essential nutrients required for optimal plant growth with the exception of Ca, K and Fe, which are usually inadequate and must be supplemented in aquaponic systems [15]. Nitrogen and phosphorus in an aquaponic system are derived from fish food. Therefore, the rate of ammonia production depends on food quantity, its protein composition and the feeding frequency [16]. Approximately 5% of feed is not consumed by the farmed fish, and the remaining 95% is ingested and digested. From the total amounts of nitrogen and phosphorus contained in the consumed food, only 30-40% is used by fish for their metabolism and growth [17]. The remaining 60-70% is released in the form of faeces, urine and ammonia [17]. The protein content of the fish diet differs between fish species and ages. A high protein content leads to better diet convertibility and improves fish growth [18]. Approximately 1 kg of fish feed containing 30% crude protein releases approximately 27.6 g of N, and 1 kg of fish biomass releases 90.4 g of N and 10.5 g of P [17]. Many carnivorous fish species are less able to utilise dietary carbohydrates and cellulose contained in plant cells [19]. A plant-based protein fish diet can lead to higher plant biomass compared to an animal-based protein fish diet, but the growth rate of the carnivorous fish will be lower [20].

Fish show daily patterns of deamination of proteins and nitrogenous wastes related to their nutritional status and feeding rhythms. The feeding frequency and the feeding time affect ammonia production and the catabolism of proteins [21]. According to Gelineau et

al. [22], ammonia production and protein catabolism are lower in fish fed at dawn than in those fed at midnight. Generally, growth and feed conversion increase with feeding frequency. The optimal feeding frequency is very important to ensure optimum fish growth, survival, improved immunity and stress resistance [23]. Feed loss and faecal waste is the largest contributor to solid waste in fish culture. The amount and relative composition of faecal material will be determined by the indigestible nutrients of the diet. An increased feeding frequency can lead to increased fish growth rates and increased amounts of excretion but lower food digestibility and water quality degradation [24, 25]. Plants also show daily rhythms in nitrogen uptake. According to Steingrover et al. [26], the nitrate concentration in the leaves increases during the night, as the uptake rate of nitrate by the roots increases at that time. Therefore, an increased feeding frequency contributes to more efficient plant nutrition [27].

Sea bass (*Dicentrarchus labrax*) has not been used frequently in aquaponics. It is a euryhaline species, ideal for aquaponics with low salinity water in combination with edible or aromatic plants. Several studies have shown that sea bass can survive and grow in brackish water [28] and successfully be adapted to freshwater [29]. Increased mortality in freshwater adaptation can sometimes be detected [30-32]. The habitat of fish plays an important role in their welfare and growth. The adaptation of a euryhaline fish from sea water to fresh water can affect its food digestibility [33]. The frequency of feeding can affect fish's nitrogen and energy utilization. In sea bass, a feeding frequency of 1–3 meals per day promotes better growth performance and food consumption rates (FCR) [34], but this can vary with the time of year, fish size, fish feed and the production system.

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The aim of this study was to evaluate the effect of 3 daily feeding frequencies (2, 4 and 8 meals) on water quality and growth performance and histology of sea bass (*Dicentrarchus labrax*) adapted to freshwater in an aquaponic system. In addition, it examines which is the most efficient feeding frequency for sea bass in an aquaponic system that ensures the combined maximum growth performance of sea bass and lettuce plants (*Lactuca sativa*). No extra Ca, K or Fe was added to the aquaponic system.

2. Material and Methods

2.1. Aquaponic system and experimental set-up

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Three autonomous aquaponic systems with a total volume of 500 L per system were constructed. Each system consisted of 3 glass fish tanks (50 cm x 50 cm x 50 cm) with a 100 L water volume each and a 26 L hydroponic cultivation tank (112 cm x73 cm x 20 cm) paved with clay pebble (8–16 mm) substrate. Each aquaponic system was supported by a biological sump filter (100 cm x 50 cm x 48 cm) with a total volume of 184 L, which had a significant contribution in the nitrification process by increasing filter

efficiency. The salinity of each aquaponic system was gradually decreased during a 60-day period by reducing 4 units of salinity once a week until it was stabilized from 35 ppt to <1 ppt.

The sump filter was divided into three sections, with most of the filter covered with suitable media providing a specific surface area (SSA) for nitrifying bacteria to colonize. The mechanical filter covered an area of 1250 cm^2 and consisted of three layers of fibreglass material, creating in this way a 30 cm thick layer to retain the solid residues from the fish tanks (uneaten food and faeces). The biological filter covered an area of 2150 cm^2 and was fixed by a mixed media of 20 L of porous cylindrical substrate K1 (10 mm diameter each), a 10 L ceramic ring (15 mm diameter each) and 10 L bioballs (30 mm diameter each). A pump (Aqua Medic OR 2500 L/h, 38 W, 2.6 m h_{max}) was placed in the last part of the filter to supply the aquaponic system with water through the filter (Q=6.27 L/min). Clay pebble substrate of the hydroponic tank also provided sufficient biofiltration, increasing the efficiency of the system. In each system, a high-pressure sodium 400 W lamp (Sylvania) was placed at a distance of 65 cm from the surface of the grow beds to ensure the appropriate exposure of plants to light. A 10 h light, 14 h dark photoperiod (winder photoperiod) was set up. An air-lift pump was used to recycle the water through a filter bed during the experiments (adjusted flow 1.53 L/min), thus creating a filtration speed (V) of 1.79 cm/min. The oxygen levels fluctuated between 75% and 80% saturation. The system was arranged in such a way that water flowed via gravity from the hydroponic tank to the fish tank and then into the sump filter. The setup period of the systems lasted 2 months to develop the biological filter. According to Hirayama [35], 40-60 days are necessary for the establishment of bacteria and the satisfactory oxidation of ammonia to nitrate ions.

At the beginning of the experiment, an initial period of 24 h was used to permit any trace of chlorine to escape. Ten grains of a previously conditioned freshwater aquarium's filter bed were introduced to each aquaponic system, serving as inocula for nitrification bacteria. A total of 0.2 g of NH_4Cl as an ammonia source was added and dissolved in each system. Water temperature (ºC) and pH of fish tanks were recorded daily, while oxygen concentration (mg/L), electrical conductivity (mS/cm) and salinity were recorded every three days. Temperature, pH and the oxygen concentration were measured with multimeter sensors (Hach, HQ40d); electrical conductivity was measured using a multimeter (Crison, CM35); and salinity was measured using an optical refractometer (ATC).

2.2. Experimental design, fish rearing and plant growth conditions

Juvenile seabass individuals of 1-2 g were transported from a local fish breeding station (SELONTA SA) located at Tapies-Pelasgia at the University of Thessaly, in special transport bags with oxygenation. Upon the arrival of the juvenile fish at the laboratory, they were placed for 3 hours in aquariums filled with water of the same salinity (25‰) as the transport water. Thereafter, salinity was gradually reduced by removing seawater and simultaneously adding fresh water to the desired salinity of 20 ‰. Every 7 days, the salinity was gradually reduced by 4 ppt. During adaptation, fish were fed to satiation twice a day. The temperature, pH and electrical conductivity (EC) were recorded daily. Total ammonia (mg/L), nitrites (mg/L) and nitrates (mg/L) in the water column were measured weekly (Api, Test Kit). Adaptation of fish to salinity <1 ppt

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lasted 60 days. Upon successful adaptation of the fish to fresh water, 19 fish were placed in each fish tank of the aquaponic systems and left for 15 days before the beginning of the experiment to permit their acclimation. At the end of acclimation, their weight and total length were measured. Fish were placed in the aquaponic fish tanks in such a way that there were no statistically significant differences in initial weights and lengths among aquaponic fish tanks.

At the end of the acclimation a total of 171 individuals juvenile sea bass, *Dicentrarchus labrax*, with an average body weight of 6.80 ± 0.095 g and an average body length of 8.62 ± 0.048 cm, were placed in the aquaponic fish tanks (19 individual/tank). All experimental procedures were conducted according to the guidelines of the EU Directive 2010/63/EU regarding the protection of animals used for scientific purposes and were applied by FELASA accredited scientists (functions A–D). The experimental protocol was approved by the Ethic Committee and conducted at the registered experimental facility (EL-43BIO/exp-01) of the Laboratory of Aquaculture, Department of Ichthyology and Aquatic Environment, University of Thessaly (n. 18402/05-09-2019).

Fish were fed daily at 5% of their body weight with a commercial floating pellet diet (55% protein and 15% crude fat). Meals were distributed throughout the day (24 hours) at three different feeding frequencies (FF) of 2, 4 and 8 meals/day over 45 days. Each aquaponic system was represented by all three feeding frequencies (fig. 1). Feeding was performed in a semi-automatic way. Feeding until 18:00 h (1 meal for FF2, 2 meals for FF4 and 4 meals for FF8) was performed by hand, and the other meals were performed with automatic feeders. The feeding rate was adjusted to fish weight every 15 days. Fish tanks were cleaned every day by siphoning, and uneaten food was removed. Daily food consumption per fish tank is calculated by the difference between the amount fed and the amount of uneaten feed collected (corrected for leaching losses). At the end of the experiment, fish were anaesthetized with Tricaine methansulfonate (MS 222), and their final fish body weights and lengths were measured.

Lettuce plants (*L. sativa* var. Musena) were grown in an unheated greenhouse until the 6-true-leaf stage. Five days prior to their transfer to the aquaponic system, Fe (Fe-DTPA), Ca (foliar application) and K (KOH) fertilization was performed. A total of 24 lettuce seedlings were chosen, showing no statistically significant differences in their morphometric characteristics (height, number of leaves). Eight lettuce plants were evenly placed in each hydroponic bed, 20 cm apart. Plant positions were carefully selected to ensure the homogeneity of the light environment; thus, each plant was exposed to 400-500 μmol m^{-2} sec^{-1} of photosynthetically active radiation (PAR). The artificial light was supplied by a 400 W HPS lamp placed 65 cm above each growing area and accompanied by a timer for accurate control of the photoperiod (10 h light: 14 h dark). Plant height as well as the number of leaves were monitored every 15 days.

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2.3. Water quality indicators

Ammonium (NH_4^+), ammonia (NH_3), nitrate and phosphate ions were monitored once a week before the daily first fish feeding. Water samples were taken at the water inlet point (GBin) and at the exit point (GBout) of the hydroponic cultivation tank. All

measurements were performed using a Hach DR3900 model photometer with special pre-weighted reagents.

To control the efficiency and the function of the filter bed, the hydraulic loading ratio (HLR), the recycled ratio (r), the hydraulic retention time of the water in the filter bed (HRT), the specific surface area of the filter (SSA), the volume of filter media (V_{media}) were calculated according to the equations described by Endut et al. [12] and Huguenin & Colt [36].

HLR (m/day) = flow rate (Q) /total surface area of the trough

HRT (min)= (surface area water x depth x porosity of gravel trough/flow rate)

SSA (m^2/m^3) = Surface area of filter media/volume of the filter media

Vmedia (m^3) = surface area of the filter media/SSA

r=volume of recycled water/volume of the system

Production rate of ammonia nitrogen (PTAN) was calculated according to the below equation described by Dediu et al. [37].

PTAN (mg/g fish/h) = $(C_e-C_i) * Q/W$,

where: C_e, C_i inlet and outlet ammonia concentration (mg/L), W: mean fish body weight in the tank (g), Q: flow rate(L/h).

2.4. Fish and plants growth performance indicators

At the end of the 45-days, fish growth performance was calculated as below,

- SGR (%/day) = $[(\ln W_{fin} - \ln W_i)/\Delta t]x100$
- WG (gr) = $W_{fin} - W_i$
- Voluntary Feed Intake (VFI, % W day^{-1}) = 100xfood consumed (g)/$[(W_{in}+W_{fin})/2x\Delta t]$
- Feed Conversion Ratio (FCR) = Feed consumed/WG
  where W_{in} and W_{fin} are the initial and final weight of the fish respectively, and Δt is the duration of the experiment in days.

Plant growth performance was calculated:

- Stem height (cm)
- Number of leaves
- Leaf fresh weight (gr) = Total fresh weight of leaves/ Number of leaves
- Total fresh aerial biomass (gr) = Total fresh weight of leaves + Stem fresh weight
- Total dry aerial biomass (gr) = Total dry weight of leaves + Stem dry weight
- Total produced biomass (kg/m^2) = Total fresh weight of aerial part/cultivated area
- Root dry biomass (gr)

265 2.5. Fish histology and gut microbiota structure

266 Euthanasia of animals followed the EU Directive 2010/63/EU and FELASA
267 guidelines and performed through an overdose of Tricaine methansulfonate (MS 222,
268 300+ mg/L). At the end of the experiment, five fish per tank were removed for
269 histopathological examination. Fish were placed immediately on ice after euthanization.
270 Samples of liver, midgut, kidney and gill were dissected from each fish. Tissue samples
271 were fixed in Davidson' fixative for 24 h at 4ºC followed by dehydration in graded series
272 of ethanol, immersion in xylol and embedding in paraffin wax. Thin sections of 4-7 μm
273 were mounted, deparaffinized, rehydrated, stained with Hematoxylin-Eosin, mounted
274 with Cristal/Mount and examined for alterations with a microscope (Axiostar plus Carl
275 Zeiss Light Microscopy, Carl Zeiss Ltd, Gottingen, Germany) under a total magnification
276 of 100X and 400X. A semi - quantitative grading system was used in order to quantify
277 the histopathological alterations of the examined tissues [38]. Severity grading used the
278 following system: Grade 0 (not remarkable), Grade 1 (minimal), Grade 2 (mild), Grade 3
279 (moderate), Grade 4 (severe).

280 Fish midgut microbiota were withdrawn at days 0, 15 and 45 from each feeding
281 frequency treatment in triplicate. Midgut samples were removed after dissection and
282 DNA was extracted with DNA Mini kit (QIAGEN, Germany). Bacterial diversity was
283 assessed by targeting the V3-V4 region of the 16S rRNA gene on the Illumina MiSeq
284 2x300 bp platform and bioinformatics analysis was performed as described in Panteli et
285 al. [39].

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286

287 2.6. Statistical Analysis

288 Values are presented as means ± standard error of the mean (S.E.M.). Data were
289 tested for normality and homogeneity with Kolmogorov–Smirnov and Levene' s tests,
290 respectively. To determine any significant differences between different feeding
291 frequencies treatments, one-way ANOVA was used, followed by Tukey's post-hoc test.
292 Independent t-tests were considered statistically significant at $p < 0.05$. Statistical
293 analyses were carried out using the software package IBM SPSS Statistics V22.

294

295 **2. Results**

296 3.1. Abiotic Factors

297 Temperature was kept constant at 20 ºC for each aquarium. The mean pH value
298 was 6.75±0.073, 6.76±0.073 and 6.77±0.703 for FF2, FF4 and FF8 respectively, while
299 the dissolved oxygen levels were 8.59 ± 0.052 mg / L, 8.50 ± 0.059 mg / L and 8.52 ±
300 0.062 mg / L (Table1). In all aquaponic systems the electrical conductivity was 1.28 ±
301 0.006mS / cm while the average salinity was 0.64 ± 0.003ppt (Table 1). There were no
302 significant differences ($p > 0.05$) in the means of NH_4^+, NH_3, nitrate ions, and pH
303 concerning the water quality in all of the 3 systems (Table 2).

304 The NH_4^+, NH_3, NO_3^- and PO_4^{3-} fluctuation at the water inlet point (GBin) and at
305 the exit point (GBout) of the hydroponic cultivation tank is shown in Figure 2

306 respectively for all of the three systems. The pH fluctuation for all of the three feeding
307 frequencies (FF2, FF4, FF8) was similar (fig. 3).

308 Hydraulic loading rate (HLR), the recirculation rate (r), the retention time of the water
309 into the filter bed (HRT), the flow rate (Q), ammonia production rate (P_{TAN}) the specific
310 surface area of the filter bed (SSA), the volume of filter media (V_{media}) and the filter
311 volume (V) summarized in Table 3 were not statistically different ($p>0.05$).

312

313 3.2. Fish growth performance, histology and midgut microbiota

314 The fish growth performance is illustrated in Table 4. At the start of the study,
315 there were no significant differences in the means of sea bass initial body weight (gr)
316 and length (cm), (t-test, $p > 0.05$) for all the feeding frequencies groups (Table 4). At the
317 end of the 45-days study period FF2 group showed significant lower final weight, weight
318 gain, specific growth rate and final length than FF4 and FF8 groups, ($p<0.05$), (Table 4).
319 Voluntary feed intake and FCR was similar for all the three feeding frequencies
320 ($p>0.05$), (Table 4). Survival rate for FF2, FF4 and FF8 was 77.2±25.96%, 96.5±1.75%,
321 and 96.5±1.75% respectively.

322 Liver histopathology of all the feeding frequency groups revealed mild (grade 2)
323 accumulation of lipid droplets in liver cells with some of the nuclei of the liver cells to be
324 pushed by the lipid droplets to the edge of the cells (Table 5, fig. 4). Midgut and kidney
325 microscopic examination showed no histopathological alterations (grade 0) in any of the
326 feeding frequency groups (Table 4, fig. 4-5). Minimal (grade 1) gill histopathological
327 alterations were detected in FF2 and FF4 groups (Table 5), while mild (grade 2)
328 histopathological alterations were detected in FF8 group (Table 5). Epithelium
329 detachment at the secondary lamellae and hyperplasia of primary lamellae were
330 detected in some cases of all the 3 groups (fig. 5).

331 A total of 2506 bacterial operational taxonomic units (OTUs) were found in all
332 samplings. The lowest number of OTUs occurred on day 0 (106±36.0). The average
333 number of OTUs on days 15 and 45 ranged between 232±166.7 and 467±129.0.
334 Permutanional Analysis of Variance (PERMANOVA) of the OTUs relative abundance
335 indicated that there were no statistically significant differences between sampling points
336 and feeding frequency (Table 6).

337

338 3.3. Plant growth performance

339

340 The plant growth characteristics are presented in Table 7. At the end of the 45-
341 days study period, plants in all systems exhibited similar leaf fresh weight, total fresh
342 weight of leaves, total fresh and dry aerial biomass (Table 7). Nevertheless, plants in
343 system 2 showed inferior root growth and significantly lower number of leaves
344 compared to system 1 and 3 (Table 7). Additionally, plants in system 3 significantly
345 outweigh all the others in stem length.

346

347

4. Discussion

4.1. Abiotic Factors

In the present study an experimental aquaponic system for Mediterranean fish (sea bass) and a vegetable (lettuce) was studied for a duration of 45 days. To the authors' knowledge, this is one of a few studies using sea bass in aquaponic systems [40-42] and the first one to use three different feeding frequencies per day in the same aquaponic system. A successful aquaponic system provides important benefits, such as water quality control, high fish and plant growth performances, plant and fish disease management, and eliminating environmental impacts [38]. Such systems require less than 5% of freshwater to be renewed due to evaporation or losses from daily functioning [43, 44]. Plant growth and production are indirectly related to feeding strategies, fish metabolic condition and microbial activity. Feeding rate and frequency affects nutrient availability in solution inside the system. Increased feeding frequency for fish contributes to more efficient plant nutrition [27, 45] as amounts of nitrate are available to the water for a longer period during the day.

Since the late 1980s, sea bass has become increasingly important in Europe, particularly for the Mediterranean region, with a steady increase in demand [46]. The present study showed that the adaptation of sea bass in a fresh water aquaponic system together with cultivation of leafy vegetable lettuce is possible. Sea bass is an euryhaline specie. Direct transfer from sea to freshwater shows increased mortality [31, 47]. However, fish gradually adapted over a period of one month [48] do not show any mortality. The mean value of salinity during the experiment was estimated at 0.64 ± 0.003 ppt in all treatments.

In an aquaponic system, the water temperature setting is dependent on the fish and plant species. For sea water-cultured sea bass, temperatures 19-22 °C show the maximum food utilization and growth rate [49, 50]. According to Barnabé [51] and Lanari et al. [52], higher weight gain can be achieved for sea bass at temperatures between 22 -28 °C. In the present study, water temperature was kept constant at 20 ºC, meeting the requirements of both sea bass and lettuce plants. The management of pH is also necessary in aquaponic systems. Plants, fish and bacteria require different pH ranges. Plants require a pH value between 5.5 and 6.5 to enhance the uptake of nutrients, and the optimal pH range for bacteria is 7.0-8.0, while the recommended pH for aquaculture is 6.5–8.5 [11]. So, an optimal pH range for an aquaponic system appears to be 6.5-7.0. Values higher than 7.0 cause reduced micronutrient and phosphorus solubility, and plant uptake of certain nutrients is restricted in the aquaponic environment [53]. In our study, pH showed a downward trend (fig. 3) for all the three feeding frequencies with mean values of 6.75-6.77. This downward trend is not unexpected, as the accumulation of nitrates (effective oxidation of ammonia) tends to make the aquatic environment more acidic. The mean value of pH is lower than 7.0 and within the tolerance levels for aquaponics. It is obvious that both pH and temperature are important parameters for the optimization of aquaponic production both for fish welfare/health issues and for plant needs.

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Dissolved oxygen (DO) is the primary water quality consideration for aquaponic systems as in other aquaculture units. Oxygen levels of 7-8 mg/L ensure adequate ventilation for sea bass respiration [25, 34], while oxygen levels > 5 mg/L strengthen the plant's root system, nutrient uptake as well as the nitrification process [54, 55]. In general, the recommended limit for DO levels in fish culture is 6 ppm for cold water fish and 4 ppm for warm water fish [56]. In the present study, oxygen levels were 8.59±0.052, 8.50±0.059 and 8.52±0.062 for FF2, FF4 and FF8, respectively.

The higher concentrations of NH_3, NH_4^+ and NO_3^- at the inlet point (GBin) than at the exit point (GBout) of the hydroponic cultivation tank indicate that the lettuce plants absorbed nutrients through the water. According to Von Wiren et al. [57], the nitrogen form that plants absorb depends on the temperature. Low temperatures generally increased the reliance of plants on ammonium as a mineral nitrogen source. Buzdy et al. [3] reported higher ammonia (NH_4^+ and NH_3) removal than nitrate by lettuce in an aquaponic system. Xu et al. [58] determined that ammonium was the preferred nitrogen source when nitrogen concentrations were low, while nitrate was preferred when concentrations were high. In the present study, all forms of nitrogen (NH_3, NH_4^+ and NO_3^-) at the exit point of the hydroponic cultivation tank exhibited lower concentrations after the 29th day (fig. 2), indicating better absorbance from the lettuce plants after this day. In general, there were no major differences between the inlet point and exit point (fig. 2). This is in accordance with the fact that lettuce has low ability to remove inorganic nitrogen [3]. Phosphate absorbance increased after the 32nd day (fig. 2). According to Buzby et al. [3], phosphate removal rates are increased when the plants are young and decrease over time. The type of substrate (clay pebbles) may affect the measurement of the nutrient concentrations at the exit point of the hydroponic cultivation tank. According to Meinken [59], nutrients can be absorbed (through diffusion) by clay pebbles and can be released back into the water circulation. This can be clearly seen from the 21st – 28th days, when both nitrate and phosphate concentrations were higher at the exit point than the inlet point (fig. 2). In the 0-14 day time period, the phosphate concentration was also higher at the exit point than the inlet point (fig. 2). The gradual rise of nitrate levels proved the efficiency of the filter in oxidizing the produced ammonia. In the present study, the daily supply of 20-25 gr of fish food efficiently provides the necessary nutrients for plants. During the experiment, the water supply (Q) was adjusted to 6.27 L/min and the filtering speed (V) to 1.79 cm/min, ensuring the successful nitration and maximum efficiency of the filter [60].

The mean hydraulic loading rate (HLR) and hydraulic retention time (HRT) for all aquaponic systems ranged from 0.95 to 0.96 m/d and 7.46 to 7.49 min, respectively, indicating the efficiency of the filter performance and the nutrient removal efficiency. The HRT has an impact on the ammonia removal efficiency, alkalinity production, sulphate production and C/N ratio in the denitrification process [61, 62]. HLR has an impact on fish and plant production and nutrient removal [42]. Chen et al. [63] suggested that the best HLR for a freshwater aquaponic system is 1.28 m/day. Endut et al. [12] reported that better growth performance of fish in a freshwater aquaponic system was observed at a higher HLR (2.56 m/d) than the HLR used in the present study. Nevertheless, Vlahos et al. [38] reported that better growth performance of gilthead seabream and rock samphire was observed at an HLR of 1.84 m/d, which was higher than the HLR of

the present study. The hydraulic loading rate (HLR) affects the production process of plants and fish and the daily nutrient removal efficiency and influences the contact time of the nutrients and microbial communities in the plants that grow in the bed [12, 38]. High values of HLR affect nutrient cycling in the hydroponic tank and reduce the nutrient contact time with bacteria in contrast with a lower HLR [64].

According to El-Sayed and Kawanna [65], photoperiod is a factor that has a direct effect on the selected crop and does not exert a significant effect on fish growth. Liang & Chien [27] came to a different conclusion. An increased photoperiod (24 hours of lighting) leads to increased fish growth compared to 12 hours of lighting [27]. A high light intensity and long photoperiod can favour both plant and fish growth and can improve water quality [66]. In the present study, the photoperiod was adjusted to 10 hours of light and 14 hours of darkness, simulating the winter season and enhancing lettuce growth [67].

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4.2. Fish growth performance, histology and gut microbiota

During the 45-day trial period, the fish food was distributed throughout the day (24 hours). Sea bass is an easily adjustable species in different feeding habitats [68]. According to Sanchez-Vazquez et al. [69], sea bass show seasonal preference in feeding hours, preferring the morning during spring and summer and the evening during winter. Azzaydi et al. [70] showed higher SGR and lower FCR in night feeding during the winter months (0.26 ± 0.01%/day and 2.65 ± 0.08, respectively) in an RAS system compared to morning feeding (0.19 ± 0.01%/day and 3.73 ± 0.17, respectively).

The feeding frequency did not affect fish survival, with 77.2±25.96%, 96.5±1.75%, and 96.5±1.75% survival being observed under the FF2, FF4 and FF8 treatments, respectively. On the day 16th an unexplained fish mortality was observed (10 fish) for the FF2 group. This was probably due to anaesthesia fish handling. Consequently, it had no relation with the feeding procedures. In sea bass, a feeding frequency of 1–3 meals per day can deliver good growth and FCR performance [71]. For juvenile's sea bass (5.2-6.8 g) a feeding frequency of two times per day seems to be the minimum with good growth results and was followed to previous studies [72-74]. Nevertheless, in this study feeding frequency of 2,4 and 8 meals per day was tested in order to examine how it affects the daily nitrate fluctuation for better plant nutrition in an aquaponic system. In a study by Biswas et al. [25], Asian sea bass fed 1, 2, 3 and 4 times a day in brackish water (3.2-4.1‰) showed the highest survival at 3 times (75.89 ± 4.17%) compared to other treatments. Similar results were reported for the fish species of *Epinephelus tauvina*, *Aristichthys nobilis* and *Sparus aurata* [75-77]. Vlahos et al. [38], working with *Sparus aurata* in an RAS aquaponic system under the salinities of 8‰ and 20‰ and a feeding frequency of 3 times per day, reported survival rates of 99% and 97%, respectively. In the present study, the survival rate of FF2, FF4 and FF8 feeding frequency treatment were higher than those for Asian sea bass and *Aristichthys nobilis* [25, 76]. The survival rates under FF4 and FF8 were similar to those reported for *Epinephelus tauvina* and *Sparus aurata* [38, 75, 77].

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In an RAS and consequently in an aquaponic system, a properly selected diet must be managed in such a way as to meet the nutritional requirements of different fish and plant species. By selecting the appropriate food amount per day and appropriate feeding frequency, metabolic products (excretions) are reduced, fish growth is enhanced, and water quality ultimately improves [27]. The removal of fish metabolic products (nutrients) from the water is directly related to the quantity of the provided diet, the feeding frequency and the food quality. Nitrogen content in fish faeces ranges (10 to 40%), depending on the nitrogen content of the provided diet and the fish type [78]. In the present study, fish were fed daily at 5% of their body weight with a commercial floating pellet diet (55% protein and 15% crude fat). The observed WG at the end of the 45-day trial period was 10.66±0.401 g, 13.14±0.594 g and 13.85±0.498 g for FF2, FF4 and FF8, respectively, showing good growth for all of the feeding frequency groups. SGR was also high (2.11±0.047, 2.23±0.032 and 2.36±0.023 for FF2, FF4 and FF8, respectively). These results suggest that the provided food amount was appropriate, and they are in agreement with those of Eroldogan et al. [33], where sea bass with an initial weight 2.6±0.3 g cultured in seawater (40 ppm) and in fresh water (0.4 ppm) with six different feeding rates (2%, 2.5%, 3%, 3.5%, 4%, saturation) showed greater WG and SGR in fresh water and at a feeding rate of 3.5% until saturation. Türkmen et al. [79] also showed that sea bass fed at 5% of their body weight 4 and 8 times per day exhibited a higher SGR. In contrast, Waller et al. [40], working with sea bass fed daily to satiation, showed a lower SGR and FCR (1.5% and 0.93 respectively).

In aquaponic systems, increased feeding frequency seems to have positive effects on fish and plant growth. Liang and Chien [27], working in a tilapia-water spinach aquaponic system, reported that increasing feeding frequency increased both fish and plant production and lessened the accumulation of nitrogen and phosphorus nutrients in water. The same results were reported by Mohamed Abdelrahman [45] while studying the effect of different daily fish feeding frequencies (1, 2 and 3 times per day) in a tilapia and lettuce aquaponic system. In the present study, the higher WG, SGR and were achieved at FF4 and FF8 (no significant differences were detected between these two feeding frequencies). FCR and voluntary feed intake did not differ among the three feeding frequencies ($p>0.05$). Feeding four or eight times per day seems to have the best effects on fish growth. This result is in accordance with Biswas et al. [25], who showed that Asian sea bass (*Lates calcarifer*) cultured in brackish water had the best SGR when it was fed 3 or 4 times per day.

It is not clear if salinity is an important factor for the optimal growth of euryhaline species. Eroldogan and Kumlu [77] showed that sea bass juveniles cultured in fresh water, 10 and 20 ppt grew better than those at 30 or 40 ppt. In a second experiment of the same study [80], young sea bass grown in fresh water had higher WG than those grown in sea water, with a slightly higher FCR in sea water. Vlahos et al. [38] did not detect differences in the growth performance of seabreams in two different salinities (8 ppt and 20 ppt). Nozzi et al. [41] showed higher WG and SGR for sea bass in fresh water than in sea water. Even at extreme temperatures, sea bass seems to grow better in low salinity water. According to Islam et al [81], sea bass reared for 35 days followed by 10 days of extreme warm temperature (33 °C) showed higher weight gain and SGR at 12‰ and 6‰ salinity water than at 32‰. Weight gain and SGR were similar in 32‰

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and 2‰ salinity (8.45 g and 9.42 g weight gain, respectively, and 2.03 and 1.93 SGR, respectively). In our study, SGR was 2.11, 2.23 and 2.36, while weight gain was 10.66 g, 13.14 g, and 13.85 g under FF2, FF4, and FF8, respectively. These values are higher than those reported by Islam et al [81], probably because no temperature stress occurred. Yilmaz et al. [82], in a 60-day trial of the growth performance of sea bass in fresh water (0‰ salinity, 20ºC), reported a 1.1% SGR and 1.2 FCR, which SGR to be lower than the values in our study but FCR to be similar with our value in FF8 group.

In euryhaline fish, the kidney plays an important role in osmoregulation. Sea bass, trout, herring, and juvenile seabream can adapt to changes in salinity and are able to survive in both seawater and freshwater. According to Nebel et al. [29], sea bass juveniles that were successfully adapted to freshwater showed smaller collecting ducts than those cultivated in seawater. Vlahos et al. [38], when adapting seabream to lower salinity (8 ppt), did not detect histopathological alterations of the midgut, smaller collecting ducts, granulomas or dilation of Bowman space in the kidney, hyperplasia of primary/secondary lamellae or epithelial detachment of the secondary lamella in gills, while liver histopathology showed inflammation and steatosis. In the present study, midgut and kidney microscopic examination showed no histopathological alterations, while liver showed mild accumulation of lipid droplets, and the gills showed mild epithelial detachment at the secondary lamellae and mild hyperplasia of the primary lamellae. Similar results for gills were reported in previous studies [83, 84], thus indicating the high plasticity and gill remodelling of sea bass adapted from seawater to freshwater. Lipid accumulation in the liver seems to be more extensive in sea bass living in sea water than in sea bass acclimatized to fresh water [41].

One of the major limitations of aquaponic systems is related to the management of water quality to meet the requirements of the tank-reared fish, and cultivated crops are treated as the second step of the process [85]. According to Yavuzcan Yildiz et al. [11], a high level of suspended solids can affect the health status of fish, provoking damage to the gill structure, such as epithelial detachment, hyperplasia, lamella fusion and reduction of epithelial volume. Waste originating from the feed includes dissolved components (phosphorus and nitrogen-based nutrients) and suspended solids. Our results are similar to those reported by Yavuzcan Yildiz et al. [11]. Uneaten food and faeces were removed daily by siphoning, but a breakdown in small particles still occurred. These particles are potentially dangerous and very difficult to collect. According to Lekang [86], the small particles will normally dominate in re-use systems.

Feeding frequency had no statistically significant impact on the structure of the midgut microbiota, indicating a minimal observable impact on the sea bass gut bacterial community. In this study, we analysed the resident midgut microbiota, i.e., bacteria that replicate inside the host's gut tissue cells, and not the transient bacteria associated with the animal's digesta [87]. Moreover, it has been recently shown that even nutritionally similar diets in sympatrically reared fish species cannot override host genetics in shaping the resident gut microbiota [88]. Thus, the resident microbiota is expected to be less variable with pulses in the feed supply, as was the case in our study. However, small qualitative differences in the structure of gut bacterial communities have been reported for other fish after a short period after a single meal [89], and this remains to be investigated for freshwater-adapted sea bass.

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4.3. Plant growth performance

The successful cultivation of various plant species, including herbs, fruiting crops and leafy vegetables, in aquaponics has been well documented during the last decade of intensive relevant research. Lettuce is one of the most commonly studied species, mainly because it is a widely consumed vegetable worldwide with low to medium nutritional requirements, a short harvest period and its cultivation is convenient in terms of light and space [54]. Many studies have examined the performance of lettuce in aquaponic systems and furthermore have compared it with hydroponics and complemented aquaponics [90]. To the best of our knowledge, there are only two published experiments concerning the effect of the fish feeding frequency on lettuce growth. In an early study, Rakocy et al. [91] concluded that a higher fish feeding frequency had a positive effect on lettuce growth in aquaponics. In accordance with this finding, Mohamed Abdelrahman [45] reported that an increased feeding frequency contributes to a more efficient nutrient supply to lettuce. The outcome was a 13.7% increase in lettuce production (kg/m^2) when tilapias were fed thrice per day in comparison with once per day.

Despite the large number of published works involving lettuce in aquaponic systems, it is usually difficult to attempt comparisons of growth responses, mainly due to different experimental set-ups and physicochemical parameters, which greatly affect the results. Under this framework, the results of the present study concerning total fresh biomass production (3.22-3.97 kg/m^2) are intermediate between the low lettuce production of 47.9 g/m^2 reported by Castillo-Castellanos et al. [92] and the values of 4.97 kg/m^2 obtained in the study of Lennard and Leonard [67]. In the first case, the experiment included a tilapia-lettuce cultivation system (tilapia stocking density 7 kg/m^3, fed 4 times per day 3.5% of body weight), and in the second involved the cultivation of lettuce and Murray cod (feeding rate of 1-1.5% of body weight, 43% protein) for 21 days. The same growth period of 21 days in the work of Dediu et al. [37] yielded similar lettuce production as our system, though the latter lasted 45 days and was conducted with a 6 times-lower initial fish density. It is well established that different variants of the same plant species can react differently even though growth conditions are identical. Andriani et al. [6] (2017) co-cultivated lettuce and mixed fish species (catfish and Nile tilapia fed with 31-33% protein, 4% of body weight) for 49 days. They reported final fresh aerial biomass similar to the results of the present study, yet the different lettuce variety resulted in discrepancies in other growth characteristics due to the different plant architecture.

5. Conclusions

This study advances our understanding of aquaponic systems by establishing an effective productive system using fresh water and a high-demand fish (sea bass) and plant (lettuce). Aquaponic systems can have a positive effect on the increase of food production and food security. The results described in this study for a novel aquaponic system provide a comparison method and new research data that can be used as a reference for future research. Increasing the fish feeding frequency can increase fish

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growth and promote the growth of both aboveground parts (stem, leaves) and the root system. Additional research into aquaponic systems is needed to achieve high-quality food production with high commercial and nutritional value.

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Author contribution

P.B and E.M. designed the study. P.S., N.V., P.B. performed the experiments. K.A.K., E.N., E.M. performed the microbiome analysis and P.B., P.S. the histology analysis. P.S., P.B., E.L. N.K. performed the data analysis. P.B. N.K., E.M. drafted the manuscript. All authors checked and approved the final version of the manuscript.

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