The determination of the leaching ratios of microdiets containing algae used as direct and indirect in aquaculture

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ABSTRACT
The purpose of the study was to determine the biochemical compositions and the leaching ratios according to different times (1 minute, 3 minutes, 5 minutes and 15 minutes) of microdiets containing Spirulina sp., Chlorella sp. and Sargassum sp. in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm). The lowest and highest ash, lipid and protein values of algae tested were 3.29 ± 0.031% (Chlorella sp.)–27.94 ± 0.023% (Sargassum sp.), 0.91 ± 0.024% (Sargassum sp.)–8.91 ± 0.04% (Spirulina sp.) and 20.69 ± 0.07% (Sargassum sp.)–56.98 ± 0.216% (Chlorella sp.), respectively. The 2532 Da ≥ leaching ratio of Chlorella sp. was lower than those of Sargassum sp. and Spirulina sp. (p < .05). The highest and lowest values for microdiets containing Spirulina sp. and Sargassum sp. were 2532 Da ≥ and 2532–13,000 Da, respectively. However, the highest and lowest values for microdiets containing Chlorella sp. were 13,700–67,000 Da and 67,000 Da ≤, respectively. In conclusion, the alginate method showed good performance because the biochemical compositions of microdiets produced reflected the ash, lipid and protein levels of algae tested. Results cautioned that the use of Spirulina sp. and Sargassum sp. in microdiets may yield the high leaching ratios containing 2532 Da ≥ molecular weight. However, the use of Chlorella sp. results in the high leaching ratios containing 13,700–67,000 Da molecular weight.

1. Introduction
Aquaculture sector is highly dependent on fish meal and fish oil supply from wild fisheries. Because fish meal is regarded as the best dietary protein source due to the excellent balance of essential amino acids and essential fatty acids (Tacon 1993). For sustainable aquaculture as mentioned by Higgs et al. (1995) replacing fish meal with more sustainable ingredients of either animal or vegetable sources is necessary. According to Lovell (1998), feed ingredients containing 20% or more crude proteins are considered protein sources. The sustainability of the aquaculture sector may be threatened by its present over-dependence on fish meal and fish oil (FAO 2002). Naylor et al. (2009) indicated that the aquafeed cost represents the variable cost of the highest fish production. The reducing of feed cost can be achieved by the use of cheap and sustainable feed ingredients. Therefore, trials have been done to evaluate cheap and sustainable plant-based protein sources. However, the main obstacles to the use of high amounts of plant protein sources in fish diets are low protein quality due to the amino acid imbalances, inadequate nutritional profile to meet fish requirements and the presence of antinutritional factors reducing the activity of fish digestive enzymes (Alexis 1997; Tacon 1997; Francis et al. 2001; Krogdahl et al. 2003). In addition, Drew et al. (2007) stated that it should be paid attention to digestibility and fish development in the use of plant resources. However, algae with high protein content and high production rate have become the centre of attraction due to the possibility of these aquatic plants as an alternative protein source for aquaculture sector. The average protein level in macroalgae is around 8–15% dry matter, while the average lipid content is only 1–3%. Nakagawa and Montgomery (2007) revealed that protein and lipid contents of microalgae were 30–50% and 40%, respectively. The addition of small amounts of algae meal to aquafeeds resulted in considerable effects on growth, feed utilization, lipid metabolism, body composition, stress responses, liver function, disease resistance, and carcass quality (Nakagawa et al. 1984; Nakagawa et al. 1986; Koh-Ichi Satoh et al. 1987; Nakagawa et al. 1997; Hashim and Maat-Saat 1992; Nakagawa et al. 1997).

Chlorella sp. is a single-celled alga that grows in fresh water. It contains 60% protein, essential amino acids, and various vitamins and minerals (Bengwayan et al. 2010). Tartiel (2005) found that crude protein, fat and ash contents of Chlorella spp. were 46.7%, 14.8%, and 17.5%, respectively. Nakagawa and Montgomery (2007) reported Spirulina sp. is one of the most frequently used microalgae in aquatic feeds due to its high contents of protein, vitamins, essential amino acids, minerals, essential fatty acids and antioxidant pigments such as carotenoids. Spirulina contains high protein contents (up to 70%) and lipids (7–16%) (Vonshak 1997). Spirulina replaced up to 40% of fish meal protein in tilapia (Oreochromis mossambicus) diet (Olvera-Novoa et al. 1998) and even higher replacement of...
Spirulina was possible in common carp (Cyprinus carpio) (Nan-deesha et al. 1998). Several studies have been conducted using dried Spirulina as a feed supplement (Jaime-Cevallos et al. 2005; Hanel et al. 2007; Dernekbasi et al. 2010; Ghaeni et al. 2011).

Seaweeds, classified as green, red and brown based on their pigmentation. Kumar et al. (2008) indicated that seaweeds are excellent dietary sources of vitamins, proteins, carbohydrates, minerals and other bioactive compounds. Several studies on the nutritional compositions of seaweeds have been conducted (Wassef et al. 2001; Ma et al. 2005; Casas-Valdez et al. 2006; Valente et al. 2006; Ortiz et al. 2006; Marsham et al. 2007; Matan-jun et al. 2009). Investigations carried out on seaweeds have shown promising results for the use of seaweeds as partial replacement of fishmeal or protein hydrolysate in aquafeeds. Seaweeds are rich in proteins, vitamins, carbohydrates, fibre, lipids and minerals. When fresh, they are 75–85% water and 15–25% organic components and minerals. Dry matter is 65–85% organic substances and 30–35% ash (FAO 2005). Sargassum is a good source of minerals, vitamins, carbohydrates and some amino acids. Yangthong et al. (2014) revealed that microdiets produced in different sizes with the alginate method had high leaching ratios, especially 2532 Da. Diken (2017) revealed the molecular weight profiles of algae such as Spirulina, Sargassum and Chlorella used in the current study. However, a study on the leaching ratios of microdiets containing feed ingredients such as Spirulina, Sargassum and Chlorella is not available.

The purpose of this study was to determine the biochemical compositions and the leaching ratios according to four different times (1 minute, 3 minutes, 5 minutes and 15 minutes) of microdiets containing Spirulina, Chlorella and Sargassum in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm).

2. Materials and methods

2.1. Feed ingredients and microdiet production

The individual behaviours of Spirulina. (Fuzhou Wonderfun Biological Technology Co. Ltd.–China). Chlorella. (Akuamaks, Turkey) and Sargassum sp. (Fuzhou Wonderfun Biological Technology Co. Ltd.–China) used as direct or indirect in aquaculture sector were tested in the formulations of microdiets produced with alginate methods in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm). Microdiets were produced according to Yufera et al. (2005) (Table 1).

2.2. Proximate compositions

Spirulina sp. and Chlorella sp. were tested in the current study as alternative feed ingredients and microdiets produced. Proximate compositions, such as ash and protein of feed ingredients and microdiets produced in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm), were determined according to the AOAC (2000) procedures. Also, lipid analyses were performed according to the chloroform–methanol extraction method described by Bligh and Dyer (1959).

2.3. Leaching ratios of feed ingredients and microdiets

The leaching ratios of feed ingredients and microdiets produced in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) were performed according to Boza et al. (1994). Firstly, samples were stirred in the phosphate buffer (pH = 8.10 mg/ml), centrifuged and the supernatant was filtered with 0.22 μm syringe filter and analysed HPLC-Gel Filtration Chromatography for determination of the leaching ratios. Samples were injected in a chromatograph with a TSK-Gel G2000 SWXL column. The eluent was 0.1 mol/sodium sulphate in the 0.1 mol/l phosphate buffer at a flow rate of 1 ml/min, and column effluent was monitored for UV light absorption at 230 nm. Based on the retention time of molecular weight standards, four fractions were defined: 67,000 Da < 67,000 – 13,700 Da, 13,700–2532 Da and 2532 Da. The molecular
weight standards (from SIGMA) were bovine albumin (67,000 Da), ribonuclease A (13,700 Da), insulin chain A (2532 Da), val-alal-a-la-phe (407 Da), tyr-tyr-tyr (508 Da), tryptophan (204 Da), tyrosine (181 Da) and p-aminobenzoic acid (137 Da).

2.4. Statistical methods

In the present study, biochemical compositions and the leaching ratios in four different times (1 minute, 3 minutes, 5 minutes and 15 minutes) of feed ingredients and microdiets (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) produced with the alginate method in the laboratory scale were given as mean ± standard error (SE). The measurements were carried out in triplicates. The experimental data were subjected to one-way (ANOVA) and mean ± standard error (SE) differences by using the SPSS 15.0 statistical package (SPSS 1993).

3. Results

Table 2 shows the proximate compositions of feed ingredients and microdiets. According to Table 2, the significant differences between ash, lipid and protein values of feed ingredients and microdiets produced in laboratory scale were observed ($p < .05$). The lowest and highest ash, lipid and protein values of feed ingredients were 3.29 ± 0.031% (Chlorella sp.)–27.94 ± 0.023% (Sargassum sp.), 0.91 ± 0.024% (Sargassum sp.)–8.04 ± 0.235% (Chlorella sp.), and 56.98 ± 0.216% (Chlorella sp.), respectively. Sargassum sp. and Chlorella sp. had the highest ash values. The lipid contents of microdiets containing Spirulina sp. and Chlorella sp. were higher than those of microdiet containing Sargassum sp. Protein contents of feed ingredients such as Spirulina sp., Chlorella sp. and Sargassum sp. used in the present study were 52.87 ± 0.193%, 56.98 ± 0.216% and 20.69 ± 0.07%, respectively.

Based on the retention time of molecular weight standards, four fractions for the leaching ratios were defined: 67,000 Da ≤, 67,000–13,700 Da, 13,700–2532 Da and 2532 Da ≥. The differences between the leaching ratios observed in four different times of microdiet groups containing Spirulina sp., Chlorella sp. and Sargassum sp. produced with the alginate method in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) were statistically significant ($p < .05$).

Figures 1–5 reveal the leaching ratios of Spirulina sp. and microdiets produced in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) containing Spirulina sp.,. The highest and lowest leaching ratios in four different times (1 minute, 3 minutes, 5 minutes and 15 minutes) of Spirulina sp. were observed in 2532 Da ≥ and 2532–13,000 Da, respectively. The 67,000 Da ≤ leaching ratios in different sizes of microdiets containing Spirulina sp. tended to increase. However, the 2532 Da ≥ leaching ratios in different sizes of microdiets containing Spirulina sp. were decreased.

Figures 6–10 show the leaching ratios of Sargassum sp. and microdiets produced in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) containing Sargassum sp. The highest and lowest leaching ratios in four different times (1 minute, 3 minutes, 5 minutes and 15 minutes) of Sargassum sp. were observed in 2532 Da ≥ and 2532–13,000 Da, respectively. The 67,000 Da ≤ and 13,700–67,000 Da leaching ratios in different sizes of microdiets containing Sargassum sp. tended to increase. However, the 2532 Da ≥ leaching ratios in different sizes of microdiets containing Sargassum sp. were decreased. According to the leaching ratios observed in four different times, the highest and lowest values for microdiets containing Spirulina sp. and Sargassum sp. produced in laboratory scale (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) were 2532 Da ≥ and 2532–13,000 Da, respectively. The 67,000 Da ≤ and 13,700–67,000 Da leaching ratios in different sizes of microdiets containing Sargassum sp. tended to increase. However, the 67,000 Da ≤ leaching ratios in different sizes of microdiets containing Spirulina sp. tended to increase.

Figures 11–15 give the leaching ratios of Chlorella sp. and microdiets produced in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) containing Chlorella sp.
300 μm, 300–500 μm and 500–800 μm) containing Chlorella sp. The highest and lowest leaching ratios in four different times (1 minute, 3 minutes, 5 minutes and 15 minutes) of Chlorella sp. were observed in 13,700–67,000 Da and 67,000 Da, respectively. According to the leaching ratios observed in four different times, the highest and lowest values for microdiets containing Chlorella sp. produced in laboratory scale (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) were

**Figure 1.** Leaching ratios in different times of Spirulina meal as feed ingredient (%).

**Figure 2.** Leaching ratios in different times of microdiet (100–200 μm) containing Spirulina meal as feed ingredient (%).

**Figure 3.** Leaching ratios in different times of microdiet (200–300 μm) containing Spirulina meal as feed ingredient (%).

**Figure 4.** Leaching ratios in different times of microdiet (300–500 μm) containing Spirulina meal as feed ingredient (%).
13,700–67,000 Da and 67,000 Da ≤, respectively. The 13,700–67,000 Da leaching ratios of microdiets containing *Chlorella* sp. tended to increase according to the leaching ratio of *Chlorella* sp. However, the 2532Da ≥ leaching ratios of microdiets containing *Chlorella* sp. tended to decrease.

### 4. Discussion

Until now, the knowledge about the leaching ratios of microdiets containing *Spirulina* sp., *Chlorella* sp. and *Sargassum* sp. is not available. In the present study, the leaching ratios of microdiets produced in different sizes with the alginate
method were revealed by HPLC-Gel Filtration Chromatography. Also, we determined the ash, lipid and protein contents of microdiets and feed ingredients. Results revealed that ash, lipid and protein levels of feed ingredients used in the ration reflected the biochemical compositions of microdiets produced with the alginate method in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm). Küşcu (2017) reported that the biochemical compositions of microdiets produced in the laboratory scale with the alginate method were similar to those of

Figure 9. Leaching ratios in different times of microdiet (300–500 μm) containing Sargassum meal as feed ingredient (%).

Figure 10. Leaching ratios in different times of microdiet (500–800 μm) containing Sargassum meal as feed ingredient (%).

Figure 11. Leaching ratios in different times of Chlorella meal as feed ingredient (%).

Figure 12. Leaching ratios in different times of microdiet (100–200 μm) containing Chlorella meal as feed ingredient (%).
commercial microdiets tested. When the biochemical compositions of feed ingredients such as *Spirulina* sp., *Sargassum* sp. and *Chlorella* sp. were compared with microdiets produced with the alginate method, the alginate method was more successful in terms of reflecting the biochemical compositions of the individual feed ingredients tested. In addition, the study data showed that *Chlorella* sp. and *Spirulina* sp. provided important contributions to the high protein and lipid levels to microdiets produced with the alginate method, while *Sargassum* sp. used in the study provided important contributions with high ash levels to microdiets.

Kuşcu (2017) revealed that the highest leaching ratios of microdiets produced with the alginate method were observed in 2532 Da≥. In the current study, the highest leaching ratios of microdiets containing *Spirulina* sp. and *Sargassum* sp. supported the results reported by Kuşcu (2017) but not the leaching ratios of microdiets containing *Chlorella* sp. Also, Kuşcu (2017) showed that the highest and lowest molecular weight distributions of microdiets produced with the alginate method in the study reflected the leaching ratios at different times. Molecular weight distributions belonging to the algae such as *Spirulina* sp., *Sargassum* sp. and *Chlorella* sp. reported by Diken (2017) were similar to the leaching ratios of feed ingredients such as *Spirulina* sp., *Sargassum* sp. and *Chlorella* sp. tested in the current study. According to the results obtained in the study, the highest leaching ratios of *Chlorella* sp. and microdiets containing *Chlorella* sp. were 13,700–67,000 Da. The 13,700–67,000 Da leaching ratio tended to increase in microdiets containing *Chlorella* sp. However, the 2532 Da≥ leaching ratios tended to decrease according to the leaching ratio of *Chlorella* sp. The results observed in algae tested and microdiets containing algae supported the relationships between the leaching ratios and molecular weight distributions of feed ingredients and microdiets.

Free amino acids (FAAs) seem to improve the performance of larvae when supplied at low levels in feeds, but not a
surplus of FAAs (Szlaminska et al. 1993, Carvalho et al. 1997, Cahu et al. 1999, Cahu and Zambonino Infante 1995, Lopez-Alvarado and Kanazawa 1995). The faster absorption of FAAs compared to protein-bound amino acids may lead to amino acid (AA) imbalances in the larval intestine and followed a decrease in protein utilization (Ronnestad et al. 2000). Zambonino Infante et al. (1997) showed that the partial substitution of whole protein by di- and tripeptides effectively improved larval development. Carvalho et al. (2003) indicated that protein macromolecules are digested into peptides and AAs in the intestine and then, di- and tripeptides are rapidly converted into AAs for absorption, a balance among the mentioned peptide groups seems to be important to optimize the protein utilization. Carvalho et al. (2003) suggested that the supplementation of diets with suitable levels of protein hydrolysates must be considered in order to achieve this purpose. In addition, it is suggested that artificial diets for fish larvae should have a molecular weight profile similar to that found in live food.

In conclusion, the alginate method had good performance because the biochemical compositions of microdiets produced in different sizes (100–200 μm, 200–300 μm, 300–500 μm and 500–800 μm) reflected the ash, lipid and protein levels of algae tested. The study data revealed that Chlorella sp. and Spirulina sp. provided important contributions with the high protein and lipid levels to microdiets produced with the alginate method, while Sargassum sp. used in the study provided important contributions with high ash levels to microdiets. Considering these data, it could be advised to use at moderate levels as ash, lipid and protein source instead of fish meal in aquafeeds. Results cautioned that the use of Spirulina sp. and Sargassum sp. in microdiet formulations may cause the high leaching ratios containing 2532 Da ≥ molecular weight. However, the use of Chlorella sp. in rations results in the high leaching ratios containing 13,700–67,000 Da molecular weight. When such data are available, the knowledge about leaching ratios of microdiets could be used to optimize feeding time in the larvae tank.

Disclosure statement
No potential conflict of interest was reported by the authors.

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