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Chapter 9

Assessment of Proximate and Bioactive Lipid Composition of Black Sea Mussels (*M. galloprovincialis*) from Bulgaria

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.71909

Abstract

Farmed marine mussels from genera *Mytilus* are important for the human diet by providing high levels of proteins, omega-3 polyunsaturated fatty acids (PUFAs), fat soluble vitamins and carbohydrates. Recently, black mussels are commercially important species from the Bulgarian Black Sea. The aim of this study was to assess the seasonal changes in proximate composition and to focus on the lipid bioactive components such as fatty acids, cholesterol, fat-soluble vitamins (A, E and D_3), and carotenoids (astaxanthin, beta-carotene) in farmed mussels (*M. galloprovincialis*) from the northern part of the Bulgarian Black Sea coast. All analyzed samples presented high protein and low lipid content. The fatty acids (FA) profile was characterized by the highest amount of PUFA, as 22:6 omega-3 (n-3) dominated, regardless of the seasons. In all seasons, the content of n-3 was significantly higher than the omega-6 PUFA. The amounts of cholesterol were in the range 62.3 (summer) to 78 (autumn) mg 100^{-1} g ww. The highest amounts of vitamin D_3 (3.1 μg 100^{-1} g ww), vitamin E (2525 μg 100^{-1} g ww), astaxanthin (0.470 mg 100^{-1} g ww), and beta-carotene (0.445 mg 100^{-1} g ww) were found in the summer season. The studied mussel aquaculture from Bulgaria presented a high beneficial potential in all seasons in terms of human health protection.

Keywords: *M. galloprovincialis*, astaxanthin, cholesterol, fat soluble vitamins, seasonal changes, omega-3PUFA

1. Introduction

The phylum Mollusca represents one of the most diverse groups of marine animals. The Bivalves comprise some of the best-known invertebrates including the mussel species, represented in all

marine environments. Nowadays, mussels are harvested commercially and are of considerable significance for aquaculture worldwide. Farmed marine mussels from the Mytilidae family, especially genera *Mytilus*, are important for the human diet in the provision of high levels of proteins, omega-3 polyunsaturated fatty acids (PUFAs), fat-soluble vitamins, and carbohydrates. In recent years, the functional properties of mussel lipids have been investigated and few dietary supplements, based on lipid extracts of mussels, have been presented at the market [1, 2]. Due to these facts, the importance of marine mussels as a source for bioactive substances with anti-inflammatory, antimicrobial, and lowering cholesterol level agents, is increasing rapidly. In addition, mussels have recently become one of the most commercially important species from the Bulgarian Black Sea [3]. The assessment of the proximate composition and the lipid qualities may facilitate consumer acceptance and predict the market feasibility of aquaculture mussels in our region. However, the information about the nutritional qualities of mussels from the Bulgarian Black Sea waters, based on their chemical composition, fat-soluble pigments, cholesterol and PUFA contents is very limited. In this article, we studied the seasonal changes of mussel primary metabolites as proteins, lipids, and carbohydrates with a focus on lipid bioactive components such as fatty acids, cholesterol, fat-soluble vitamins (A, E and D_3) and pigments (astaxanthin, beta-carotene) in farmed mussels (*Mytilus galloprovincialis*) from the Northern part of the Bulgarian Black Sea coast.

2. Materials and methods

2.1. Sample collection

All mollusk samples were purchased from two mussel farms in spring (March 2015), summer (July 2015), and autumn (October 2015). The mussel farms are located in one of the most ecologically non-polluted areas along the Northern part of the Bulgarian Black Sea coast (Kavarna). The samples were immediately frozen at −20°C and stored in a fridge. The biometric characteristics as mean weight (g) and mean length (cm) were determined (**Table 1**).

Average 40 specimens of mussels (from each season and each farm) were used for a proximate, fatty acid and fat-soluble vitamins, cholesterol and pigments analysis. All shucked mussels were cut into small pieces and homogenized at 800 rpm for 5 min, using a Moulinex blender.

	Spring (n = 45)	Summer (n = 43)	Autumn (n = 46)
Mean weight	11.0 ± 0.5	12.0 ± 0.5	13.0 ± 0.5
Mean length	4.5 ± 0.3	5.5 ± 0.5	6.0 ± 0.5
Habitat		Demersal	
Food habits		Herbivorous	

n, number of specimens; SD, standard deviation.

Table 1. Biometric characteristics of mussel samples (mean ± SD).

2.2. Standards and reagents

Fatty Acid Methyl Esters (FAME) Mix standard (SUPELCO FAME, Mix C4-C24), and nonadecanoic acid and methyl ester nonadecanoic acid standards were purchased from Sigma–Aldrich™. Pure solid substances of all-trans-retinol, cholecalciferol, alpha-tocopherol, astaxanthin, beta-carotene, and total-cholesterol are HPLC-grade reagents, purchased from Sigma-Aldrich™. All used chemicals were of analytical, HPLC, and GC grade (Scharlau, Scharlau Sourcing Group, Spain).

2.3. Proximate composition analysis

The homogenized mussel tissues (2.000 ± 0.005 g) were dried at 105 ± 2°C in an air oven for 16–18 hours to a constant weight [4]. The moisture was calculated as weight loss. The crude protein content was determined by the Kjeldahl method [5]. The total lipids (TLs) were estimated according to Bligh and Dyer procedure [6] and the results were presented as g per 100 g wet weight (g 100 g^{-1} ww). The carbohydrates were determined according to [7]. The method was based on the treatment of the mussels' tissue with a methanolic KOH solution, followed by acid hydrolysis of starch to glucose. The glucose quantity was determined through the oxidation with a bivalent copper from a copper reagent and was then converted into starch. The energy values were calculated by multiplying fat, protein, and carbohydrate with appropriate coefficients (4.0 kcal/g for proteins and carbohydrates and 9.0 kcal/g for lipids) [8].

2.4. Fatty acid analysis

Fatty acid composition of total lipids at edible mussel tissue was determined by GC of the corresponding methyl esters. The residual lipid fraction was methylated by base-catalyzed transmethylation, using 2 M methanolic KOH and n-hexane according to [9]. To determine the analytical recoveries, the samples of homogenized tissue were spiked with a methanolic solution containing C19:0 (1 mg/ml). Gas chromatography analysis was performed by a FOCUS GC, autosampler A 3000, Polaris Q MS detector (Thermo Scientific, USA). The capillary column was a TR-5 MS (Thermo Scientific, USA), 30 m, 0.25 mm i.d. Helium was used as a carrier gas at flow rate 1 ml/min. Chromatographic separation of fatty acids methyl esters was performed under the following temperature regime: 40°C initial temperature for 4 min, followed by 10°C increase per minute until 235°C were reaches, temperature increase up to 280°C with a stay at this level for 5 min. The sample volume was 1 μl. The injector was a split/splitless injector operated in the split mode (1:10). Peak identification was measured by: retention time (RT) based on fatty acid methyl esters (FAME) mix standard (SUPELCO F.A.M.E. Mix C4-C24), and mass spectra (ratio *m*/*z*) compared to the internal Data Base (Thermo Sciences Mass Library; Thermo Corporation, Waltham, USA). FAMEs were quantified by the method of external standard. The FA content was expressed as a percentage of total FAs content [10].

2.5. Extraction of fat soluble vitamins, cholesterol, pigments, and HPLC analysis

The edible tissue of the mussels from the three different farms was used to evaluate its astaxanthin, beta-carotene, and cholesterol content. The extraction and quantity analysis

was performed by the method of Dobreva et al. [11]. An aliquot of the homogenized sample (1.000 ± 0.005 g) was weighed into a glass tube with a screw cap, 1% of methanolic L-ascorbic acid and 0.3 M methanolic KOH were added. Six parallel samples of edible tissue from each mussel farm were prepared and subjected to saponification at 50°C for 30 min. The fat-soluble components of interest were extracted with two portions of n-hexane: dichloromethane = 2:1 solution. The combined extracts were evaporated under a nitrogen flow and the dry residue was dissolved in methanol: dichloromethane and injected (20 μl) into the HPLC/UV/FL system. All fat-soluble compounds were analyzed simultaneously by an HPLC system, equipped with an RP analytical column (Synergi Hydro-RP (80 Å, 250 × 4, 6 mm, 4 μm)). Astaxanthin, beta-carotene, and cholesterol were identified by UV detection. The mobile phase composition was ACN:MeOH:iPrOH = 75:20:5 v/v, with the flow rate being 1 mL/min. The qualitative analysis was performed by comparing the retention times of pure substances: at λ_{max} = 208 nm for cholesterol, at λ_{max} = 450 nm for beta-carotene, and λ_{max} = 474 nm for astaxanthin. The quantitation was performed by external calibration, comparing the chromatographic peak areas of the corresponding standards (Astaxanthin, Supelco; Cholecalciferol, Supelco, and Beta-carotene, Supelco). The results were expressed as μg per 100 g wet weight (μg·100 g^{-1}ww).

2.6. Nutritional quality indices

Nutritional qualities were estimated by several indices and ratios of fatty acid composition: the indices of atherogenicity (AI), thrombogenicity (TI), cholesterolemic index (h/H), n-6/n-3 and PUFA/SFA ratios, according to Simopoulos [12]. Ulbricht and Southgate [13] suggest two indices, AI and TI, which might better describe the atherogenic and thrombogenic potential of different unsaturated FA. AI indicates the relationship between the sum of the main saturates and that of the main unsaturated, the former being considered pro-atherogenic (favoring the adhesion of lipids to cells of the immunological and circulatory systems), and the latter being anti-atherogenic (inhibiting the aggregation of plaque and diminishing the levels of esterified FA, cholesterol and phospholipids, thus preventing the appearance of micro- and macrocoronary diseases). TI shows the tendency to form clots in the blood vessels and is defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic FAs (MUFA, n-6 PUFA, and n-3PUFA). The cholesterolemic index (h/H) presents the functional effects of different PUFAs of the cholesterol metabolism (hypo- and hyper-cholesterolemic effect) and is calculated in accordance with the method, described elsewhere [14]. In addition, the hyperlipidemic and atherogenic potential of mussel lipids, related to cholesterol, SFA, and unsaturated FA content, were determined. To assess the dietary effect of the mussel lipid consumption on serum cholesterol levels, two indices were calculated: cholesterol/SFA index (CSI) and cholesterol index (CI) [15, 16].

2.7. Statistical analysis

All analytical estimations were performed in triplicate. The results were expressed as a mean and standard deviation (mean ± SD). The obtained data were analyzed using Graph Pad Prism 5.0 software. An unpaired t-test statistical analysis was performed to estimate the differences between the analyzed samples. Thus, the comparison was made for proximate compositions,

individual FA, FA groups, fat-soluble vitamins, cholesterol and carotenoids, and nutritional quality indices. The differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Proximate composition

The proximate composition of edible mussel tissue varied with the season (**Table 2**). The assessment of the nutritional quality based on the macronutrients content in black mussel was conducted in accordance with Commission Regulation (EC) No. 116/2010 [17]. As water is the main component of mussel tissue, the levels of moisture are ranged between 73.35 and 77.15%, being higher in the summer samples in comparison to other seasons.

Seafood products are considered "low fat" when containing below 3 g of lipids per 100 g wet weight (ww). In the present study, the range of the total lipids (TLs) content is between 1.40 and 2.89 g 100^{-1} g ww. The highest TL was found in spring mussels, whereas summer specimens presented twice lower values ($P < 0.001$). TLs that amount below 3 g 100^{-1} g ww were found in all analyzed seasons; therefore, Black Sea mussels can be classified as "low fat" food.

The lack of considerable variation in the protein content during the seasons is well illustrated in the results (see **Table 2**). According to [8], the seafood protein content below 15% is considered low. In this study, a significant decrease of the protein content was found in the autumn period as compared to the spring sample ($P < 0.001$). However, protein levels were significantly above 15% in all samples, and the analyzed mussels can be classified as protein-rich regardless of the season.

The observed seasonal pattern in the carbohydrate amounts showed the highest levels in the autumn season and the lowest in the summer period. The accumulated carbohydrates could be utilized under unfavorable conditions and the observed variation in the mussel tissue indicates that the level of mobilized carbohydrate reserves may vary widely and rapidly in response to fluctuation in environmental conditions [18, 19]. It was observed that in warmer seasons, the carbohydrate contents were higher than TL contents in the mussel

	Lipid	Protein	Carbohydrate	Moisture	Energy value
Spring	2.89 ± 0.10	19.92 ± 0.80	2.25 ± 0.08	73.35 ± 1.55	115.00 ± 5.50
Summer	1.40 ± 0.08[a]	18.30 ± 0.50	2.00 ± 0.06	77.15 ± 1.60[a]	94.50 ± 4.50[a]
Autumn	2.51 ± 0.12[c]	17.40 ± 0.55[b]	2.73 ± 0.10[b,c]	76.20 ± 1.40[b]	103.20 ± 5.20[b]

[a]$P < 0.001$ (spring vs. summer).
[b]$P < 0.001$ (spring vs. autumn).
[c]$P < 0.001$ (summer vs. autumn).

Table 2. Proximate composition in molluscs tissue, in g 100 g^{-1} ww and kCal 100^{-1} g ww (mean ± SD).

tissue. This may be explained by the higher mussels' metabolic activities during the summer-autumn period. Some studies suppose that the carbohydrate reserve may not be fully depleted during the mussel growth and remains relatively high throughout the spawning season [20, 21].

Further, on this study, it was demonstrated that seasonal changes of chemical composition largely depend on the mussel reproductive cycle. The moisture content is often used as an indication of the spawning time, hence the highest water content (July) correlated with the spawning period for the Black Sea mussels. The observed seasonal variation especially in TL and carbohydrates may be explained by the biochemical balance in relation to the mussels' reproductive activity. Mussels usually accumulate lipid reserves prior to gametogenesis. During the period of gametogenic development in spring (March and April) and autumn (September and October), the mussels showed two peaks of spawning after an important gonad ripeness. In this study, the accumulation of lipids and carbohydrates followed the main reproductive cycle in March and April (highest TL levels). The intensive spawning period between May and August was well correlated by the lowest TL and carbohydrates content found in the summer mussel samples. In addition, some authors [22] assume that the protein maximum and minimum levels also correspond to the mussel development phases (spawning and resting cycles). Similar seasonal variation in TL and protein and carbohydrate amounts of farmed and wild populations of *M. galloprovincialis* from Sinop (South Black Sea) and Romanian coast (North-west Black Sea) are reported by [21, 23]. The protein content in Bulgarian mussels was significantly higher than the values presented for mussel populations from the other parts of Black Sea (7–12%). For black mussel species from different regions such as the Adriatic Sea and Mar Grande of Taranto, different TL contents are reported in 2008 and 2010 [24, 25]. These patterns of temporal variability of TL in bivalve mollusks in previous studies result from several environmental factors acting simultaneously such as temperature, food availability, plankton composition, and physiological factors. Additionally, the differences mentioned above may be attributed to the longer reproductive periods in a warmer climate.

3.2. Fatty acid composition

The changes of fatty acid (FA) profile, main FA groups, n-3 and n-6 PUFA, EPA and DHA (as a percentage of the total FA and g 100 g^{-1} ww, mean ± standard deviation), FA ratios, and indices for the study period are shown in **Table 3**. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFAs) underwent statistically significant changes during the observed period ($P < 0.05$). SFA ranged from 25.60% (July) to 31% (October), MUFA showed minor changes and ranged between 16.0% (October) and 17.95% (July), while PUFA was the dominant group and ranged from 53.0% (October) to 56.5% (July). The bivalves are considered herbivores with phytoplankton as the main component of their diet and FA profile, respectively. However, several studies show that bivalves can use other food sources such as detritus, bacteria, micro zooplankton and meso-zooplankton [18, 20]. Orban et al. [20] and Zlatanos [26] presented a relative pattern PUFA > SFA > MUFA in the black mussel from the Adriatic coast and the local Mediterranean mussel farms. Our results

Fatty acid	Spring	Summer	Autumn
C 8:0	0.26 ± 0.01	0.20 ± 0.01	0.11 ± 0.01
C 10:0	0.18 ± 0.01	0.35 ± 0.02	0.26 ± 0.01
C 12:0	0.36 ± 0.01	0.70 ± 0.02	0.88 ± 0.03[b]
C 13:0	Nd	Nd	Nd
C 14:0	2.93 ± 0.06	2.60 ± 0.08	2.40 ± 0.10[c]
C 16:0	19.69 ± 0.23	17.80 ± 0.35[a]	21.33 ± 0.85[b,c]
C 17:0	0.06 ± 0.01	Nd	1.06 ± 0.05
C 18:0	3.62 ± 0.08	3.24 ± 0.20	4.45 ± 0.15[b,c]
C 20:0	0.25 ± 0.01	0.23 ± 0.01	0.08 ± 0.01
C 21:0	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.01
C 22:0	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.01
C 24:0	0.56 ± 0.02	0.25 ± 0.02	0.24 ± 0.01
∑SFA	28.12 ± 0.55	25.60 ± 0.30	31.00 ± 0.85
C14:1n5	0.83 ± 0.03	0.62 ± 0.03	0.14 ± 0.01
C16:1n7	7.77 ± 0.15	8.20 ± 0.20[a]	6.00 ± 0.21[b,c]
C17:1n8	0.06 ± 0.01	Nd	0.18 ± 0.01[b]
C18:1n9	5.48 ± 0.10	7.10 ± 0.30[a]	5.22 ± 0.35[c]
C20:1n9	2.80 ± 0.06	1.60 ± 0.08[a]	4.00 ± 0.40[b,c]
C22:1 n9	0.10 ± 0.01	0.17 ± 0.01	0.12 ± 0.01
C24:1n9	0.39 ± 0.01	0.22 ± 0.01	0.34 ± 0.01
∑MUFA	17.43 ± 0.25	17.91 ± 0.18	16.00 ± 0.20
C18:3 n6	0.60 ± 0.02	0.80 ± 0.03	0.30 ± 0.01[c]
C18:2 n6	1.16 ± 0.07	2.00 ± 0.12[a]	1.05 ± 0.15[b,c]
C18:3 n3	1.05 ± 0.04	1.40 ± 0.09	0.65 ± 0.02[c]
C20:5 n3	6.90 ± 0.20	9.80 ± 0.24[a]	7.70 ± 0.85
C20:4 n6	10.55 ± 0.45	12.00 ± 0.30[a]	13.60 ± 0.69[c]
C 20:3 n6	1.15 ± 0.16	0.80 ± 0.03	2.00 ± 0.25
C 20:2 n6	0.41 ± 0.02	0.50 ± 0.02	0.60 ± 0.03
C 20:3 n3	1.72 ± 0.10	1.25 ± 0.11	0.90 ± 0.04
C 22:6 n3	30.39 ± 1.80	27.30 ± 0.54[a]	26.00 ± 0.88[b, c]
C 22:2 n6	0.53 ± 0.03	0.65 ± 0.01	0.20 ± 0.01

Fatty acid	Spring	Summer	Autumn
∑PUFA	54.45 ± 2.20	56.50 ± 2.10	53.00 ± 2.06
∑n 3	40.06 ± 1.70	39.75 ± 1.56	35.25 ± 1.30[b]
∑n 6	14.39 ± 0.85	16.75 ± 0.90	17.75 ± 1.05[b]
n6/n3	0.35 ± 0.02	0.42 ± 0.01	0.49 ± 0.02
PUFA/SFA	1.94 ± 0.12	2.20 ± 0.18	1.71 ± 0.10
DHA/EPA	4.40 ± 0.80	2.78 ± 0.25	3.38 ± 0.42
C16:1n7/C16:0	0.39 ± 0.02	0.46 ± 0.03	0.28 ± 0.02
CSI*	3.85 ± 0.50	4.04 ± 0.50	4.79 ± 0.38
CI*	3.73 ± 0.80	3.72 ± 0.43	4.06 ± 0.41
AI*	0.44 ± 0.01	0.39 ± 0.01	0.46 ± 0.01
TI*	0.19 ± 0.01	0.17 ± 0.01	0.22 ± 0.01
h/H*	2.45 ± 0.10	2.92 ± 0.15	2.28 ± 0.10
Fatty acid, g 100 g^{-1} ww			
∑SFA	0.731 ± 0.050	0.320 ± 0.030	0.722 ± 0.040
∑MUFA	0.453 ± 0.030	0.224 ± 0.025	0.373 ± 0.015
∑PUFA	1.416 ± 0.065	0.706 ± 0.040	1.235 ± 0.080
∑n3	1.042 ± 0.050	0.496 ± 0.020	0.821 ± 0.060
∑n6	0.374 ± 0.020	0.210 ± 0.015	0.414 ± 0.020
EPA	0.180 ± 0.012	0.123 ± 0.010[a]	0.179 ± 0.014
DHA	0.790 ± 0.045	0.341 ± 0.018[a]	0.606 ± 0.035
EPA + DHA	0.970 ± 0.048	0.464 ± 0.020	0.785 ± 0.050

[a]P < 0.001 (spring vs. summer).

[b]P < 0.001 (spring vs. autumn).

[c]P < 0.001 (summer vs. autumn).

*CSI = (1.01×SFA g 100^{-1} g ww) + (0.05×cholesterol mg 100^{-1} g ww); CI = 1.01(SFAg 100^{-1} g ww – 0.5PUFA g 100^{-1} g ww) + (0.06 cholesterol mg 100^{-1} g ww); AI = [(C12:0 + (4 × C14:0) + C16:0)]/(n6PUFA + n3PUFA+ MUFA); TI = (C14:0 + C16:0 + C18:0)/[(0.5MUFA) + (0.5n6PUFA) + (3n3PUFA) + (n3PUFA/n6PUFA)]; h/H = (C18:1n9 + C18:2n6 + C18:3n3+ C20:4n6 + C20:5n3 + C22:6n3)/(C14:0) + C16:0).

Table 3. Comparison of FA profiles (FA, % of total FA), ratios, indices, EPA, and DHA contents of the edible tissue of black mussels (mean ± SD) during seasons.

are in agreement with the above mentioned authors. A deflection of this pattern (SFA > PUFA >MUFA) was presented by Badiu et al. [27] for wild mussel samples from the Baia Mamaia Zone-Park, Constanza (the Black Sea), and Cape Galata (the Bulgarian Black Sea) [28]. It is known that temperature and food availability are two of the most important factors regulating the growth of the marine bivalve mollusks.

The major SFA was palmitic acid (C16:0), followed by stearic acid (C18:0), which demonstrated highest levels in autumn (21.7 and 4.45%) and lowest in the summer season. Generally, three saturated FAs, which presented the next distribution C16:0 > C18:0 > C14 > 0 in all seasons, accounted for 90.0–93.0% of the total SFA. A possible explanation of the significant lowering of saturated FAs levels of energetic character in the summer season is that these acids were probably catabolized for the acquisition of the energy required for diverse metabolic functions as spawning, etc. In addition, some authors [29] pointed that elevated amounts of short-chain SFA such as C12:0, C14:0, and C16:0 SFAs displayed omnivorous feeding.

The main MUFAs were palmitoleic acid (C16:1n7), oleic acid (C18:1n9), and gondoic acid (C20:1n9). These FAs values are between 92% (spring) and 95% (autumn) of the total MUFAs and show C16:1n7 > C18:1n9 > C20:1n9 alignment in all seasons. According to Ref. [30], C16:1n7 and EPA (C20:5n3) are used as an indicator of diatom-based mussel diets. Another FA food behavior marker C16:1n7/C16:0 has been used to differentiate between diatoms versus phytoflagellate feeding. The phytoflagellate contained high levels of C16:0. Significant variation in C16:1n7/C16:0 ratio, from 0.28 (autumn) to 0.46 (summer), was observed and we could assume that in the summer season, diatoms prevail in mussels' food. Additionally, there was a significant increase of long-chain C20:1n9 ($P > 0.001$) in the autumn season. Some investigations reported that gondoic acid may be used as an indicative marker for zooplankton in the mussels' diet [31, 32]. Although mussels are herbivores species, several studies have demonstrated that species of micro- and mesozooplankton have been ingested by suspension form marine bivalves [25, 30]. A possible explanation of the raised levels of gondoic acid (two and a half times, $P < 0.001$) is that zooplankton may comprise the bigger part of the mussels' food in the autumn season. It is known that high levels of C 18:1 n9 are characteristic for deep-sea organisms as an adaptive response to high water pressure [32]. The lower amount of oleic acid in the Black Sea mussels is specific for specimens from shallow (especially warm) waters. The analyzed farmed mussels live at 12–18 m depths, which could explain the reported low levels of C18:1 n9 regardless of the season.

Among PUFA of black mussels, the docosahexaenoic acid (C22:6n3, DHA) was the predominant FA, followed by arachidonic acid (C20:4n6, AA) and eicosapentaenoic acid (C20:5n3, EPA) in all seasons. These FAs accounted for 87–89% of the total PUFA during the year. Significant seasonal variations were observed for DHA ($P < 0.001$) and EPA ($P < 0.001$) levels. Long-chain n3 PUFAs (LCPUFAs) showed lower levels in the autumn season, whereas AA presented highest values in the same season. These LCPUFAs are synthesized from the main mussels' food such as phytoplankton and microalgae in high quantities. Some authors [18] suppose that the water temperature may strongly affect the EPA and DHA levels. This statement has been confirmed by the fact that the Black Sea mussels lower their tissue DHA levels in warmer seasons (with 16% in autumn, $P < 0.001$) compared to the spring period. Due to the high biological activities of these n3 LCPUFAs, the DHA/EPA ratio characterized the deceleration of the mussel metabolism activities in the autumn period, which could be related to the higher water temperature and the reproductive cycle.

On the other hand, the DHA/EPA ratio is used to determine the degree of carnivory food, ingested by the mussels. As dinoflagellate contain greater amount of DHA and EPA is a specific marker for diatoms, the ratio could be used to assess the relative proportions of dinoflagellate and diatom contents in the mussels' food. Dinoflagellates prevail in the mussels' diet when this ratio is greater than 1 and diatoms are predominant when it is less than 1 [31–33]. In this study, we supposed that dinoflagellates are dominant in the analyzed spring-autumn period in the Black Sea mussels' food as DHA/EPA > 1. Other essential PUFAs as C18:3n3 and C18:2n6 were obtained in small amounts in the analyzed periods and ranged between 1.35 and 3.4%. The sum of both C18:3n3 + C18:2n6 was used as a terrestrial marker; therefore, the levels above 2.5% indicated a significant input of terrestrial material in the mussels' food [31]. Values above the cut-off levels (3.4%) were determined only for the summer period as for the other seasons, the sum of C18:3n3 + C18:2n6 was under 2.5% and it could be assumed that terrestrial matter was present in low levels in mussels' food from this Black Sea region.

The beneficial lipid quality of the mussel tissue was well displayed by the high levels of n3 PUFA. During the year, n3 FAs showed 35–40% of the total FAs, whereas n6 PUFA presented significantly lower values from 14 to 17.75%. The n6/n3 and PUFA/SFA ratios were used as indicators when comparing the relative nutritional values of sea food. The observed seasonal changes in these ratios are discussed in Section 3.4.

The fatty acid content in absolute amounts in g/100 g wet weight provides more useful and accurate information to assess the quality of mussels as food and to raise the consumers' interest. The European Food Safety Authority [34] recommends a daily intake of 0.500 g EPA + DHA n3 PUFA. Taking into account the above, the percentage values of this LCPUFA were recalculated to g/100 g of raw mussel tissue according to Ref. [35]. In the present study, the highest PUFA, n3 and EPA + DHA amounts were found in spring mussels compared to other seasons.

The present results are in accordance with some previous investigations [23, 26, 28] of farmed Mediterranean and Black Sea mussels. Some authors [25] report significantly lower PUFA and n3 PUFA values for mussels from Mar Grande of Taranto (7.55–11.16%) in comparison to our findings. Orban et al. [20] and Badiu et al. [27] present higher EPA than DHA levels in wild black mussels from the Adriatic, the Tyrrhenian, and the Romanian Black Sea coasts. The observed discrepancy and variations of n3 LCPUFA contents could be related to the type of food available and ingested by the mollusks and the lipid metabolism of EPA to DHA.

3.3. Fat soluble vitamins, cholesterol, beta-carotene, and astaxanthin content

Fat soluble vitamins, cholesterol, and carotenes contents are expressed as an average and standard deviation (mean ± SD). The results are shown as microgram per 100 grams wet weight (μg 100^{-1} g ww) for fat soluble vitamins (A, D_3, and E) and as milligram per 100 grams wet weight (mg·100 g-1 ww) for cholesterol, astaxanthin, and beta-carotene (**Table 4**).

Fat soluble components	Spring	Summer	Autumn
Vit A (μg/100 g)	36.4 ± 4.0	50.2 ± 5.0[a]	47.0 ± 4.5
Vit D_3(μg/100 g)	2.7 ± 0.3	3.10 ± 0.5	2.5 ± 0.3
Vit E (μg/100 g)	2315.7 ± 50.0	2525.0 ± 55.0	1975.5 ± 45.0[b,c]
Cholesterol, mg	62.3 ± 0.50	68.0 ± 0.60[a]	75.00 ± 0.40[b]
beta-carotene, mg	0.409 ± 0.035	0.445 ± 0.030	0.228 ± 0.018[b,c]
Astaxanthin, mg	0.428 ± 0.040	0.470 ± 0.038	0.142 ± 0.025[b,c]
Percentage of the daily recommended intake of fat soluble vitamins			
Vit A	4.9%	6.7%	6.3%
Vit D_3	54%	62%	50%
Vit E	15.4%	16.8%	13.2%
Vit E/PUFA	1.63	3.60	1.60

[a]P < 0.001 (spring vs. summer).
[b]P < 0.001 (spring vs. autumn).
[c]P < 0.001 (summer vs. autumn).

Table 4. Seasonal variations in fat soluble vitamins, cholesterol, carotenoids, and RDI in edible mussel's tissue (mean ± SD).

In this study, significant seasonal changes in all fat soluble biologically active compounds were observed. The analyzed fat-soluble components with high antioxidant activity are vitamin E (alpha-tocopherol) and carotenoids (beta-carotene and astaxanthin). Vitamin E was found in highest levels in edible mussel tissue, followed by vitamin A and vitamin D_3, regardless of the season. Our previous investigation of wild and aquaculture *M. galloprovincialis* from the Bulgarian Black Sea coast shows a similar distribution of fat-soluble vitamins: vitamin E > vitamin A > vitamin D_3 and their contents are similar to the autumn mussel sample levels [36]. One possible reason for the observed high vitamin E content is that its amount reflects a higher degree of antioxidant protection, necessary for the n3PUFA-rich organisms [19]. Our study illustrated a strong correlation for the black mussel tissue: highest PUFA contents—highest vitamin E content (summer) and lowest PUFA—lowest amount of vitamin E (autumn). Discrepancies with our results are reported for fat soluble vitamin (A, D, E) contents in wild common clam *Donax cuneatus* from the Southeast coast of India [37]. Authors find that vitamin A (105.6 mg/g) and vitamin D (38.2 mg/g) dominate in clam tissue, whereas vitamin E content is drastically low (just 3.64 mg/g). The authors do not describe the sampling details, hence possible reasons for the observed differences could be sampling season, available food, environmental conditions, etc. Earlier investigations report significantly lower levels of vitamin E (790 μg/100 g) and similar values for vitamin A (38.7 μg/100 g) in aquaculture green-shell mussels from New Zealand [38]. Shulman and Soldatov [39] found the highest levels of alpha-tocopherol (vitamin E) and carotenoids for *M. galloprovincialis* from Northern Part of Black Sea (Sevastopol) in a warmer period, whereas in the cold months, their levels decrease. Our results showed a similar trend for all fat-soluble vitamins and carotenoids contents (except cholesterol) in mussel tissue.

The cholesterol content in the mussel edible tissue was found in significantly low levels regardless of the season (see **Table 4**). The cholesterol amounts increased slowly in the sequence: spring > summer > autumn. Earlier investigations report lower cholesterol levels for *M. galloprovincialis*—from 20 mg/100 g [40] from the Southern part of the Black Sea (Sinop) to 53 mg/100 g ww [28, 36] from the Bulgarian part of the Black Sea. Li et al. [41] reported similar seasonal changes of cholesterol content for cultured *Perna viridis* from Guangdong, China. The authors present a slow increase of cholesterol levels from 26% (spring) to 37% (autumn) of total sterols. A possible explanation is that the main diet of the mussels is plankton, which contains cholesterol precursors. The mussels can synthesize cholesterol from specific precursors, but cholesterol metabolism depends on different factors such as the reproductive cycle, sex, etc. [41]. According to the Bulgarian dietary standards [42], consumption of less than 300 mg per day of cholesterol may help maintain normal blood cholesterol levels and prevent cardiovascular disease. All analyzed mussel samples were characterized with low cholesterol content (RDA <300 mg/day, [42]) regardless of the season.

Interest in the study of marine carotenoids increased after the discovery of the antitumor activity of beta-carotene and vitamin A [39]. Further on, astaxanthin has a beneficial effect on the human health due to its high antioxidant activity—10 times more than beta-carotene and 100 times more than alpha-tocopherol. Until recently, the effect of carotenoids on human health was focused mainly on beta-carotene. Information and clinical trials on the effect of the various carotenoids on the human endocrine and immune system, metabolism, etc., are scarce. De Carvalho and Caramujo [43] suppose that at present, natural beta-carotene accounts for 20% of the world demand, and a similar interest in natural astaxanthin is now emerging in the nutraceutical market. Thus, the information about the marine indigenous sources of these compounds is important for consumers. The main source of carotenoids in marine ecosystems is plankton microalgae. Pigments as beta-carotene and astaxanthin are synthesized in their cells and then distributed, and subjected to metabolic transformation in trophic chains. Most microalgae contain species-specific carotenoids. However, the composition of the main groups of these compounds remains unchanged within the microalgae classes. Shulman and Soldatov [39] report that the domination of diatoms in the composition of phytoplankton in the Black Sea ecosystem leads to an increase in pigments (such as beta-carotene, fucoxanthin, zeaxanthin, etc.), whereas astaxanthin is species-specific for dinoflagellates. The bivalves, which are filter-feeding mollusks, can accumulate carotenoids directly via dietary microalgae or after modification through metabolic reactions [43]. In this study, the astaxanthin content of the analyzed samples was lower in comparison to the beta-carotene amounts only in the autumn sample mussels, whereas in the spring and summer periods, it presented higher values (see **Table 4**). Therefore, we classified cultured Black Sea mussels as good sources of astaxanthin. In accordance with our previous conclusion based on DHA/EPA ratio, the observed higher astaxanthin levels in comparison with the beta-carotene content confirmed that dinoflagellates are dominant in the Black Sea mussels' food for the spring-summer period. No comparative study for seasonal changes of Black Sea mussel fat-soluble vitamins and carotenoids composition is available in the literature. Posleslova and Nehoroshev [44] reported the 0.5 mg·100 g^{-1} ww beta-carotene for wild Black mussels from the Northern part of the Black Sea (Sevastopol, The Ukraine). This result is consistent with our findings for the summer mussel samples (**Table 4**). Desnica et al. [45]

determined the average concentration of astaxanthin (1.5 mg·100 g^{-1} ww) in blue mussel *M. edulis* samples from Iceland. This result is higher than ours. The most likely reason for this difference is an algal availability in the region—a key factor for the astaxanthin and beta-carotene production in herbivorous mollusks [39, 45]. Earlier studies on edible mussel *M. edulis* found that beta-carotene can be converted to astaxanthin by oxidative (and reductive) metabolic processes. Other interesting properties of carotenoids are reported for bivalves from the littoral region of the Northern Black Sea coast. The authors present a correlation between carotenoid contents and pollution in the region. They suggest that mollusks with high carotenoid content in their tissue show higher resistance to environmental pollution and therefore, carotenoids may be an important part of oxygen metabolism in bivalves [46].

3.4. Assessment of the nutritional quality of the mussel lipids

The analyzed mussels have been assessed from a nutritional quality perspective. The evaluations of the lipid functional properties were based on fatty acids ratios, indices, contents of long-chain n3 PUFAs (EPA + DHA), recommended daily intake (RDI) for fat-soluble vitamins, cholesterol, and carotenoids contents. In addition, the seasonal changes of lipid quality indices, FA ratios, fat-soluble RDI, cholesterol, and carotenoid content were studied and discussed. There are two FA ratios—n6/n3 and PUFA/SFA, which are traditionally used in the estimation of the lipid quality. In this study, these ratios are significantly varied between seasons. An increase in the human dietary n6/n3 PUFA ratio is essential for preventing coronary heart disease by reducing plasma lipids and the risk of cancer [12]. In the present study, this ratio ranged from 0.35 to 0.49 in agreement with earlier published results for the Black Sea mussels [23.27, 36]. According to Ref. [12], the beneficial n6/n3 ratio for the human health is below 1 and the findings confirmed the high quality of mussel lipids. In addition, PUFA/SFA ratio described the FA balance in mussel lipids well. The Department of Health [47] recommends values of PUFA/SFA ratio which should be higher than 0.45. In this study, the PUFA/SFA ratio ranged from 1.7 to 2.2 (see **Table 3**) and we may conclude that all culture mussels have a well-balanced and beneficial FA profile regardless of the season.

As mentioned above, EFSA [34] recommends a daily intake of 0.500 g EPA + DHA n3 PUFA. A 100 g of mussel edible tissue contains from 0.464 to 0.970 g of EPA + DHA n3 PUFA and provides from 93% (summer) up to 194% (spring) of the recommended daily intake (**Table 3**). Thus, in accordance with [48], the analyzed farmed Black Sea mussels can be classified as high in omega-3 fatty acids regardless of the season. In our previous study [28], the reported results for EPA + DHA contents of Black Sea mussels are lower (0.45 g/100 g). Comparable information in literature for seasonal changes in long-chain n3 PUFAs contents in Black Sea mussel edible tissues was not found. The functional properties of mussel lipids were assessed by the following indices (**Table 3**): indices of atherogenicity (AI), thrombogenicity (TI), and cholesterolemic index (h/H). The mentioned indices were in the respective range: for AI—from 0.39 to 0.46; for TI—from 0.17 to 0.22; and for h/H—from 2.28 to 2.92. AI and TI seasonal variations showed opposite trends compared to h/H index. In the summer season, their values were the lowest, whereas h/H index presented the highest amounts in the same season. In contrast, a new investigation of wild black mussels, harvested in the Bulgarian part of Black Sea (Varna Bay), shows different levels for these indices [49]. The authors find

twice higher AI (up to 0.94) and higher TI (up to 0.37) values, but significantly lower h/H levels (up to 1.92) in comparison with our results. Moreover, the authors report different seasonal changes in FA distribution, especially between individual SFA and PUFA. The possible reasons for the observed discrepancies are different mussel populations (subtidal mussels) and locations, available food, sex, etc.

The hypercholesterolaemic-atherogenic potential of mussel lipids is related to their cholesterol content and FA profile. In the present chapter, cholesterol/SFA index (CSI) and cholesterol index (CI) were determined for the assessment of this potential. CSI was used to compare different types of food, whereas CI predicted the possible variation in an average individual serum cholesterol, which could be affected by individual portions of food. Seasonal variation was found for CSI (3.85–4.79) and CI (3.7–4.06). The calculated CSI and CI values are comparable with those calculated for red and pink shrimps from the Ionian Sea [50]. The low values of both indices found for the Black Sea mussel lipids indicated their high functional properties and protective role against the risk of cardiovascular disease [51].

The amount of fat-soluble vitamins provided by 100 g raw mussel tissue was calculated as a percentage of the average daily allowance (ADA) and was compared with the RDI, accepted in Bulgaria [42]. Bulgarian dietary standards for ADA are close to those accepted in the European Union [52] with the exception of the RDI for vitamin D3 (5 μg for adults in our country, while the recommendation of the European Union is 10 μg). According to the Dietary Standards in Bulgaria, the analyzed mussels could supply a low percentage of RDI of vitamin A (4.9–6.7%) and of vitamin E (13.2–15.4%). Substantial amounts of vitamin D3 were found in farmed mussels, where 100 g raw mussel tissue could provide between 50 and 62% of the average daily intake. Minor seasonal changes were found for the recommended daily intake values of vitamins A and E, whereas a more significant fluctuation was observed for vitamin D3. The highest RDI levels for all three fat-soluble vitamins were found in the summer season. An earlier investigation of farmed and wild mussels from Bulgaria [36] presents similar low amounts of aquaculture mussel vitamin A and E levels of RDI and lower values of vitamin D3 RDI in comparison with the present study.

The relationship between vitamin E and PUFA intake (for adults), presented as >0.5 for mg vitamin E/g total PUFA ratio, could also be used as a criterion for evaluation of the functional qualities of the mussel lipids (see **Table 4**). This ratio is based on the minimum requirement for vitamin E content, allowing for cellular synthesis and PUFA cellular membrane retention; and vitamin E amounts, required to protect and metabolize dietary PUFA [53, 54]. Based on the calculated ratio, which ranged between 1.6 (autumn) to 3.57 (summer), we can conclude that mussel lipids contained compounds with high biological activity and well-balanced Vit E/PUFA ratio.

4. Conclusion

The study investigated seasonal changes in the quantities of macro-components and fat-soluble biologically active compounds in the edible tissue of the aquaculture mussels from

http://dx.doi.org/10.5772/intechopen.71909

the Bulgarian Black Sea coast. Regardless of the observed variations in the chemical composition, the mussels are rich in proteins (average 18.5%) and contain low levels of total lipids and carbohydrates, and a low energy value (average 104 kcal/100 g). A well-expressed tendency of decreasing levels of total lipids and carbohydrates during the summer season (July) was established, which correlates well with the reproductive cycle of the Black Sea shellfish. Additionally, significant variations were found for bioactive lipid components such as fatty acids, fat-soluble vitamins, cholesterol, and carotenoids. High levels of n3 PUFAs (average 0.786 g/100 g) were found during the whole study period. Mussels are a rich source of EPA + DHA n3LCPUFAs (average 0.74 g/100 g), supplying 148% of RDI for these FAs. An interesting trend was determined for the summer season: the highest levels of all three fat-soluble vitamins and carotenoids were observed at the lowest total lipid values. Within the study period of catching and distribution of mussels to the Bulgarian markets, the levels of cholesterol that were subject to control were low (average 68.4 mg/100 g). The functional properties of the lipids were estimated by FA ratios, FA indices, and interactions between cholesterol and SFA (low CSI levels), and vitamin E and PUFA (high alpha-tocopherol/PUFA levels). The results demonstrated very good hypocholesterolemic (high h/H values), anti-atherogenic (low levels of AI), and anti-thrombogenic (low levels of TI) potential of the lipids. Valuable new information on changes in beta-carotene and astaxanthin contents in the intestinal tissue was provided. Beneficial carotenoid contents confirm the very good antioxidant potential of the mussel lipids. Although proximate and FA composition, fat-soluble vitamins, cholesterol, and carotenoids contents of mussel tissue are multifarious and strongly dependent on biotic and abiotic environmental factors, we can summarize that the present results illustrate well the high potential of mussels as healthy food. Moreover, mussel consumption could promote dietary recommendations for the consumption of low-fat and cholesterol, rich in n3 PUFA, vitamin D3, and astaxanthin foods. In addition to the study of the bioactive lipid composition of aquaculture black mussels, more detailed investigations devoted to the seasonal changes of different lipid classes as phospholipids, sterols, waxes, carotenoids, etc., are needed. The assessment of the proximate composition and the lipid quality of the black mussel edible tissue may promote their consumption. The findings concerning aquaculture mussels in Bulgaria may support consumers' dietary regimes and help them make healthy food choices.

Acknowledgements

This study was partially funded by National Science Fund (Grant No DM09/2 from 2016)

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