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Regio-specific lipid fingerprinting of edible sea cucumbers using LC/MS

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Abstract

Sea cucumbers are a rich source of bioactive compounds and are gaining popularity as nutrient-rich seafood. They are consumed as a whole organism in Pacific regions. However, limited data are available on the comparison of their lipid composition and nutritional value. In this study, untargeted liquid chromatography/mass spectrometry was applied to comprehensively profile lipids in the skin, meat, and intestinal contents of three color-distinct edible sea cucumbers. Multivariate principal component analysis revealed that the lipid composition of the intestinal contents of red, black, and blue sea cucumbers differs from that of skin, and meats. Polyunsaturated fatty acids (PUFAs) are abundant in the intestinal contents, followed by meats of sea cucumber. Lipid nutritional quality assessments based on fatty acid composition revealed a high P:S ratio, low index of atherogenicity, and high health promotion indices for the intestinal contents of red sea cucumber, suggesting its potential health benefits. In addition, hierarchical cluster analysis revealed that the intestinal contents of sea cucumbers were relatively high in PUFA-enriched phospholipids and lysophospholipids. Ceramides are abundant in black skin, blue meat, and red intestinal samples. Overall, this study provides the first insights into a comprehensive regio-specific profile of the lipid content of sea cucumbers and their potential use as a source of lipid nutrients in food and nutraceuticals.

Keywords: Lipids, liquid chromatography, mass spectrometry, nutritional indices, polyunsaturated fatty acids, intestinal contents, cluster correlations

1. Introduction

Marine invertebrates, such as sea cucumbers, are a valuable source of bioactive lipids. Their lipid composition is influenced by various factors such as species (Ridzwan et al., 2014), season (Feng et al., 2021), geographic conditions (Zhao et al., 2020), and diet (Gianasi et al., 2017; Yu et al., 2016). These creatures can be found in deep-sea and benthic regions around the world. Sea cucumbers contain bioactive compounds such as triterpene glycosides, polysaccharides, phenols, and lipids (Xu et al., 2018), which makes them highly valuable. Consequently, it has been widely used as a traditional tonic in Asian countries (Hossain et al., 2020). Various compounds isolated from sea cucumber have been found to exhibit anticancer (Janakiram et al., 2015; Ru et al., 2022; Sajwani, 2019), antiangiogenic (Tian et al., 2005; Wargasetia & Widodo, 2017), anticoagulant (He et al., 2020), anti-inflammatory (Ding et al., 2018; Olivera-Castillo et al., 2018), antihypertensive (Sadegh Vishkaei et al., 2016), antithrombotic (S. Chen et al., 2012), antioxidant (Hossain et al., 2022), antitumor (Tian et al., 2005; J.-J. Zhang & Zhu, 2017), and wound healing properties (Zheng et al., 2022).

The health benefits of consuming sea cucumber depend on the several factors including quality of their lipids. Intake of saturated fatty acids (SFA) has been linked to increased serum total cholesterol and low-density lipoprotein cholesterol (Ruiz-Núñez et al., 2014), making it a risk factor for cardiovascular coronary heart disease (Givens, 2009). Conversely, consuming ω-3 polyunsaturated fatty acids (PUFA) has been shown to reduce cardiovascular mortality (Khan et al., 2021) and the risk of developing cognitive impairment (Wood et al., 2022). In contrast, excessive intake of ω-3 fatty acids from marine sources has been linked to a higher risk of developing atrial fibrillation (Gencer et al., 2021). It is important to replace SFA with PUFA (Schwingshackl et al., 2021) and

to balance their daily intake (Kang et al., 2005). Sea cucumbers may be a good source of both SFA and ω-3 PUFAs after fish. Analysis of fatty acid composition in the body wall of sea cucumber (*Stichopus japonicus*) revealed that their relative abundance varies according to season (For example in spring: SFA < PUFA < MUFA and autumn: PUFA < SFA < MUFA) (Feng et al., 2021) with MUFAs and PUFAs being account for 36.9-47.3% and 27.5-36.7% of FAs respectively. The ω3-fatty acids such as EPA (19.2%) and DHA (19.2%) were the dominant species in *S. japonicus*. However, in another study using sea cucumbers species from Malaysia showed that abundance of ω6-fatty acid, arachidonic acid (42.41%) in *Stichopus horrens* but DHA and EPA were 0.17% and 0.32% respectively (Ridzwan et al., 2014). Further, cultured sea cucumber (*Apostichopus japonicus*) showed a high abundance of PUFA (35.26-46.47%), with EPA being predominant species (10.04-16.04%) and ω6/ω3 ratios were also found to be in a range of 0.52-1.88, which is good for health (Li et al., 2021). This has increased the interest and demand for sea cucumbers as they can be used in the development of nutritional supplements and functional food ingredients. However, it should be noted that sea cucumber *Apostichopus japonicus* is an endangered species, according to the IUCN Red List of Threatened Species in 2010 (Mercier & Hamel, 2013).

Two species of sea cucumbers *Apostichopus armatus* (blue and black) and *Apostichopus japonicus* (red) were selected for this study as they are widely consumed in Japan and have the limited information about their comprehensive lipid profiles. Previous studies have focused on analyzing the lipid composition and nutritional significance of *A. japonicus* species, but the studies focusing on *A. armatus* species is very limited. Moreover, these studies are limited to the analysis of specific lipid molecular species, such as fatty acids and phospholipids (Liu et al., 2021; Ramalho et al., 2020; Wang et al.,

2020; Xing et al., 2021) and nutritional significance knowledge such as ω6/ω3 ratios (0.52-1.88), atherogenic (0.20-0.36), thrombogenic indices (0.21-0.29) in *A. japonicus* species (Li et al., 2021). Recently, non-targeted mass spectrometry approaches have been developed to provide lipid profiles of sea cucumber and seafood (Cui et al., 2022; Hu et al., 2023). However, research on species- and region-specific lipid profiles and nutritional assessments of sea cucumbers has been limited. In this study, we used high-performance liquid chromatography with linear-ion trap-orbitrap mass spectrometry (HPLC/LTQ-Orbitrap-MS) to provide a comprehensive lipid profile of sea cucumbers collected from different regions in Japan. We analyzed the correlations between the lipids in the skin, meat, and intestinal contents of three types of sea cucumbers (black, red, and blue) and assessed their nutritional values to discuss their potential health benefits.

2. Materials and Methods

2.1 Materials

LC/MS grade solvents such as chloroform, isopropanol, and methanol were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Additionally, 1M ammonium acetate, which is an additive to the LC/MS eluent, was purchased from Sigma-Aldrich (St. Louis, USA). Zirconium oxide ceramic bulk beads with a diameter of 1.4 mm (catalog number 15-340-159, Fisherbrand) were purchased from Thermo Fisher Scientific (Tokyo, Japan). These beads were used for the homogenization of sea cucumber samples. EquiSPLASH LIPIDOMIX and the oleic acid-d_9 quantitative standard for mass spectrometry were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). The internal standard mixture was prepared using methanol by mixing EquiSPLASH Lipidomix of 1 µg/mL of each of the following: phosphatidylcholine (PC) (15:0-18:1(d7)), phosphatidylethanolamine (PE) (15:0-18:1(d7)), phosphatidylglycerol (PG) (15:0-

18:1(d7)), phosphatidylserine (PS) (15:0-18:1(d7)), phosphatidylinositol (PI) (15:0-18:1(d7)), lysophosphatidylethanolamine (LPE) (18:1(d7)), lysophosphatidylcholine (LPC) (18:1(d7)), sphingomyelin (SM) (d18:1/18:0(d9)), ceramide (Cer) (d18:1/15:0 (d7)) and 10 μg/mL of oleic acid (d9).

2.2 Sea cucumber samples

The classification of red, blue, and black sea cucumbers distributed in Japan is controversial, but in recent years, the idea that black and blue are the same species (*Apostichopus armatus*) and red is a separate species (*Apostichopus japonicus*) has gained traction (Kanno et al., 2006; Woo et al., 2017; Yamana, 2020). This study also follows the above classification. Red sea cucumbers (*Apostichopus japonicus*) from Awaji, Hyogo, Japan (34°20'31"N 134°53'43"E), and black and blue sea cucumbers (*Apostichopus armatus*) from Hamasaka, Hyogo, Japan (35°37'49"N 134°26'35"E), were purchased from Yamayone Fresh Fish Co., Ltd and Hanamitsu Co., Ltd via the Internet (https://www.rakuten.co.jp/). Samples were transported to the laboratory as live organisms, and their skin, meat, and intestinal contents were collected within 10 min, and stored at -80°C until sample pretreatment. Each variety of sea cucumber was subjected to five consecutive analyses from the same animal.

2.3 Lipid extraction

First, each part of the sea cucumber (skin, meat, and intestinal content) was weighed (200 mg) and placed into a 2 mL Eppendorf tube. The samples were then homogenized using 1 mL of methanol for two sets of 30 s each, using a homogenizer (Bead Mill 4, Fisherbrand, Tokyo, Japan). Total lipids were extracted using a slightly modified Folch method (Folch et al., 1957; Gowda et al., 2022). To begin, 100 μL of homogenate (20

mg) was transferred to 1.5 mL Eppendorf tubes (n=5). Next, 100 µL of an internal standard mixture was added and vortexed at 3500 rpm for 30 s. Following this, 400 µL of chloroform and 100 µL of Mili-Q were added and vortexed for 5 min. After centrifugation of the lipid extracts at 3500 rpm for 5 min, the chloroform layer was transferred to a new Eppendorf tube. The remaining aqueous layer was re-extracted by adding 400 µL of chloroform. The combined chloroform layers containing lipids were then evaporated in a centrifugal evaporator at 4°C under vacuum. After complete evaporation, 100 µL of methanol was added, vortexed at 3500 rpm for 1 min, centrifuged at 20,630×*g* for 10 min at 4°C, and transferred to an LC/MS vial.

2.4 LC/MS analysis

Lipids were separated using an HPLC system (Shimadzu, Kyoto, Japan) equipped with an Atlantis T3 C18 column (2.1 × 150 mm, 3 µm, Waters, Milford, USA). The column temperature was maintained at 40 °C, and the flow rate and injection volume were set at 200 µL/min and 10 µL, respectively. To separate the lipids, three mobile phases were used: (A) Milli-Q (10 mM ammonium acetate), (B) isopropanol, and (C) methanol. Gradient elution was applied in either positive or negative mode, as follows; Positive mode: 0-1 min, 30 % B, and 35 % C; 1-9 min, 82.5 % B, and 15 % C; 9-15 min, 95 % B, and 5 % C; 15-25 min, 95 % B, and 5 % C; 25-26 min, 30 % B, and 35 % C; 26-30 min, 30 % B, and 35 % C. Negative mode: 0-1 min, 30 % B, and 35 % C; 1-9 min, 75 % B, and 15 % C; 9-21 min, 82.5 % B, and 15 % C; 21-25 min, 95 % B, and 5 % C; 25-26 min, 30 % B, and 35 % C; 26-30 min 30 % B, and 35 % C. The different gradients for positive and negative ionization mode were selected based on the ionization sensitivity and elution profiles of lipid species. Finally, mass spectrometry was performed using an LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific Inc., San Jose, CA, USA) in both

positive and negative ionization modes. The electron spray ionization (ESI) conditions were as follows; capillary temperature of 330 °C, nitrogen sheath gas flow rate of 50 units, and auxiliary gas flow rate of 20 and 30 units for positive and negative modes, respectively. In the positive ionization mode, the source and capillary voltages were set to 4 kV and 25 V, respectively, while in the negative ionization mode, 3 kV and 10 V, respectively. To obtain high-resolution mass spectra, the scan range of MS^1 was set at *m/z* 160 to 1900 in negative mode and *m/z* 100 to 1750 in positive mode in Fourier transform mode with a resolution of 60,000 and a collision energy of 40 V was set to acquire MS/MS spectra.

2.5 Data processing for identification of lipids and nutritional indices

Raw data alignment, peak extraction, identification, and peak area integration were performed using MS-DIAL Ver 4.6. The parameters were set as previously reported (Gowda et al., 2021). The molecular species of lipid and fatty acyl composition of phospholipids, sphingolipids were identified by confirming the MS and MS/MS spectra. For relative quantitation, similar lipids subclasses or internal standards of typical classes were used for the analytes. The concentrations of the analytes were calculated by considering the peak area ratio of analyte to the internal standard and multiplying it by the amount of internal standard added. The data normalization was performed by the weight of the sample taken for the analysis. The free fatty acid composition data were used to evaluate the quality of lipid nutrition. Nutritional indexes were calculated using the following equations (J. Chen & Liu, 2020) ;

(1) PUFA/SFA ratio (P:S)

$$P:S = \Sigma PUFA / \Sigma SFA.$$

(2) Index of Atherogenicity(IA)

IA = [C12:0 + (4 × C14:0) + C16:0] / ΣUFA.

(3) Hypocholesterolemic/Hypercholesterolemic ratio (HH)

HH = (cis−C18:1 + ΣPUFA) / (C12:0 + C14:0 + C16:0).

(4) Health Promotion Index (HPI)

HPI = ΣUFA / [C12:0 + (4× C14:0) + C16:0].

2.6 Statistical analysis

Principal component analysis (PCA) and cluster correlation analysis were performed using MetaboAnalyst version 5.0. Visualization of the data was performed with Graph Pad Prism 8 by representing means and standard errors (n=5). To identify statistically significant differences between the groups, one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used. Statistical significance was denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant.

3 Results and Discussion

3.1 Lipid annotation and principal component analysis

An untargeted lipidomics using HPLC/LTQ-Orbitrap-MS was applied for the identification and characterization of lipids in sea cucumber. A total of 133 lipid molecular species were detected in both positive and negative ion modes depending on their ionization ability. A detailed list of lipid metabolites and their concentrations are provided in supporting information **Table S1**. Principal component analysis (PCA) revealed the differences in the lipid composition of the meat, intestinal content, and skin samples, as depicted in **Figure 1A.** The score plot in **Figure 1A** showed that the meat and skin had almost similar lipid compositions, whereas the intestinal contents had different

lipid compositions. The intestinal contents of black and blue sea cucumbers were nearly identical, whereas the intestinal contents of red sea cucumbers were different. The loading plot in **Figure 1B** shows that certain lipid molecular species such as FA 18:0, FA 20:4, FA 20:5, phospholipids, and lysophospholipids containing unsaturated fatty acids had large positive scores for PC1 and contributed to group separation. Overall, PC1 and PC2 accounted for 91.6% of the total model variance, with PC1 explaining 83.4% of the variance. Previous studies have compared the lipid profiles of various edible sea cucumbers. However, the analysis is limited to specific lipids such as fatty acids and phospholipids (Liu et al., 2021; Ramalho et al., 2020; Wang et al., 2020; Xing et al., 2021). In addition, few studies have focused on body part-specific lipid composition in sea cucumbers (S. Zhang et al., 2023). This is the first study that to reveal the regio-specific distribution of lipids in three varieties of sea cucumber.

3.2 Distribution of free fatty acids in sea cucumbers

There are three types of free fatty acids, which are classified based on the basis of the number of double bonds in their structure. These are saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). **Figure 2A** shows the percentage distribution of the total concentrations of SFA, MUFA, and PUFA in different body parts of each sea cucumber species. The distribution of SFA was almost the same in the meat and skin, ranging from 25.73 to 32.36 %, but slightly higher in the intestinal contents, ranging from 40.08 to 42.71 %. In all sea cucumbers, MUFA and PUFA were highly concentrated in the intestinal contents, with MUFA ranging from 78.12 to 82.80 % and PUFA ranging from 66.07 to 77.70 %.

The relative amounts of SFA, MUFA, and PUFA in different types of sea cucumbers

238 and their body parts are presented in **Figure 2B**. The black sea cucumber intestinal
239 contents had the highest levels of SFAs and MUFAs (99.63 ± 1.40 mg/100 g of intestinal
240 content, 25.98 ± 0.12 mg/100 g), followed by the blue sea cucumber intestinal contents
241 (91.42 ± 0.90 mg/100 g, 21.05 ± 0.34 mg/100 g). On the other hand, the skin of blue sea
242 cucumbers had the lowest amount of SFAs (56.94 ± 0.52 mg/100 g of skin) and MUFAs
243 (1.39 ± 0.02 mg/100 g). The intestinal contents of red sea cucumber had the highest
244 amount of PUFAs (44.51 ± 0.68 mg/100 g), whereas the meat of black sea cucumber had
245 the lowest amount (1.80 ± 0.03 mg/100 g of meat). The intestinal content of sea cucumber
246 is rich in PUFAs, making it a valuable source of nutrition.
247 The concentrations of arachidonic acid (ARA, 20:4, ω-6) and eicosapentaenoic acid
248 (EPA, 20:5, ω-3), the major PUFAs in each body part detected is shown in **Figure 2C**. In
249 the meat, the highest level of ARA was found in blue sea cucumber (7.46 ± 0.07 mg/100
250 g), followed by red sea cucumber (5.77 ± 0.06 mg/100 g). In contrast, EPA was almost
251 equally abundant in blue and red sea cucumbers (2.91 ± 0.03 mg/100 g, 2.89 ± 0.04
252 mg/100 g) and least abundant in black sea cucumbers (0.41 ± 0.01 mg/100 g). Regarding
253 intestinal contents, the highest level of ARA was found in red sea cucumber (17.24 ± 0.39
254 mg/ 100g), whereas EPA was almost equally abundant in black and red sea cucumbers
255 (19.18 ± 1.70 mg/100 g, 21.16 ± 0.28 mg/100 g) and lowest in blue sea cucumber (12.91
256 ± 0.16 mg/100 g). In the skin, ARA was equally abundant in black and red sea cucumbers
257 (4.96 ± 0.03 mg/100 g, 4.76 ± 0.06 mg/100 g) whereas blue sea cucumbers had the least
258 amount (1.19 ± 0.01 mg/100 g). On the other hand, EPA was highest in red sea cucumbers
259 (3.25 ± 0.06 mg/100 g) while blue sea cucumbers had the lowest amount (0.51 ± 0.01
260 mg/100 g). Consuming high levels of ω-6 PUFAs and low levels of ω-3 PUFAs can lead
261 to inflammation and numerous diseases, including cardiovascular disease, atherosclerosis,

Alzheimer's disease, and obesity (Patterson et al., 2012). A lower ratio of ω-6/ω-3 fatty acids can help reduce the risk of chronic diseases (Simopoulos, 2002). The intestinal contents of sea cucumber have a higher concentration of EPA than ARA, indicating that it is a promising food that does not require concern over excessive consumption of ω-6 PUFAs. Sea cucumber consume mainly phytoplanktonic cells and occasionally small crustaceans and a variety of eggs and larvae (Hamel & Mercier, 1998). There are reports suggest that the availability of bioactive compounds in sea cucumbers may be associated with the existence of algal materials in the diet (Hossain et al., 2023) and experiments were conducted whether substrate colour could influence the body wall of sea cucumber (Sun et al., 2020). Studies on the effects of different feed ingredients on the lipid profile of sea cucumbers have shown that the levels of ω3 and ω6 fatty acids in sea cucumber tissue vary depending on the type and combination of feeds (Wen et al., 2016). These findings may suggest that the body colour of the sea cucumber and its lipid composition truly depends on the nature of diet.

3.3 Assessment of the lipid nutritional quality in sea cucumbers

The effects of lipids on cardiovascular health can be analyzed using several nutritional indices such as the P:S ratio, IA, HH, and HPI (Refer to Section 2.5). **Figure 3** shows the results of the four nutritional indices, calculated from the lipid profiles obtained in this study. Among these indices, the PUFA/SFA (P:S) ratio is the most commonly used to evaluate the effects of dietary lipids on cardiovascular health. A P:S ratio in the range of 1-1.5 is ideal for reducing the risk of cardiovascular disease (Kang et al., 2005). This index was developed on the basis the hypothesis that dietary PUFAs can lower serum cholesterol levels, whereas SFAs contribute to elevated serum cholesterol levels (J. Chen

& Liu, 2020). In this study, the highest P:S ratio was found in the intestinal contents of red sea cucumber (0.52 ± 0.01), followed by the intestinal contents of black sea cucumber (0.35 ± 0.02), and lowest in the meat of black sea cucumber (0.03 ± 0.00). Therefore, the consumption of red sea cucumber intestinal contents could lower the risk of cardiovascular diseases. However, the P:S ratio of sunflower oil (4.75-4.94), fish (0.50-1.79) was reported to have higher values compared to sea cucumber, but dairy products (0.02-0.175) have relatively lower than the sea cucumber (J. Chen & Liu, 2020). The index of atherogenicity (IA) indicates the potential for free fatty acids to increase the incidence of atheroma (Ulbricht & Southgate, 1991). The highest IA value was observed for the meat of black sea cucumber (5.73 ± 0.13), followed by the skin of blue sea cucumber (4.75 ± 0.08). The intestinal contents had much lower IA values and are comparable with sunflower oil (0.09-0.11) and fish (0.21-1.41). However, dairy products (1.42-5.13) have relatively similar to that of skin and meat of the sea cucumber (J. Chen & Liu, 2020).

The skin and meat are the major edible parts of the sea cucumber, but their consumption was suggested to increase the risk of atheroma formation. The hypo/hypercholesterolemic (HH) ratio (hypocholesterolemic fatty acids (cis-18:1 and PUFA) and hypercholesterolemic fatty acids (C 12:0, C 14:0, C 16:0)), which indicates the effect of free fatty acid composition on cholesterol (J. Chen & Liu, 2020), was highest in the intestinal contents of red sea cucumber (2.14 ± 0.02) and lowest in the meat of black sea cucumber (0.13 ± 0.00). In other hand, camelina oil (11.2-15.0) and fish (0.87-4.83) reported to have higher HH ratio, but dairy products (0.32-1.29) have lower values compared to intestinal contents of red sea cucumber (J. Chen & Liu, 2020). The Health Promotion Index (HPI) was developed to assess the nutritional value of dietary lipids (S.

310 Chen et al., 2004). HPI values were highest for the intestinal contents of red sea cucumber
311 (2.79 ± 0.03), followed by black (2.29 ± 0.09) and blue sea cucumber (1.96 ± 0.03),
312 indicating that these parts are healthier than other body parts of the sea cucumber.
313 Moreover, the HPI values for intestinal contents of sea cucumber is relatively higher
314 compared to the dairy products (0.16-0.68) (J. Chen & Liu, 2020).
315
316 **3.4 Analysis of phospholipid composition in sea cucumbers**
317 Phospholipids (PLs) are essential components of cell membranes in various organisms
318 and can influence signal transduction and enzymatic processes (Lordan et al., 2017). The
319 consumption of dietary PLs has been shown to lower the risk of coronary heart disease,
320 cancer, metabolic syndrome, and renal impairment (Küllenberg et al., 2012). The relative
321 concentration of each PL, which was identified in this study, was visualized by heat maps,
322 as shown in **Figure 4A**. Dark red indicates a high concentration of PLs, whereas dark
323 blue indicates a low concentration. Red areas were found in the intestinal contents of all
324 sea cucumbers, suggesting that these parts have relatively high concentrations of PLs.
325 The intestinal content of red sea cucumber showed a different gradient pattern compared
326 with those of blue and black sea cucumbers. This difference was due to
327 phosphatidylinositol (PI(18:0/20:4, PI(20:1/20:4), PI(19:0/20:4), PI(20:0/20:4)) and
328 phosphatidylethanolamine (PE(O-18:1/20:1), PE(O-18:1/21:1), PE(20:0/20:3), PE(O-
329 18:1/22:1)). **Figure 4B** shows the composition of PLs, including MUFA and PUFA, in
330 different parts of the sea cucumber. The blue sea cucumber had a higher amount of PLs,
331 including MUFA (5.44 ± 1.34 mg/100 g of meat) and PUFA (11.09 ± 0.44 mg/ 100 g), in
332 its meat portion. The intestinal contents of PL containing unsaturated fatty acids did not
333 differ significantly for either MUFA (41.25 ~ 47.72 mg/ 100 g) or PUFA (52.36 ~ 57.76

mg/ 100 g). In the skin, black sea cucumber had the highest content of both MUFA (3.74 ± 0.83 mg/100 g) and PUFA (14.70 ± 0.24 mg/ 100 g).

It has been reported that dietary PL can transport fatty acids (FAs) to the plasma membrane, which can alter its composition (Küllenberg et al., 2012). The composition of long-chain unsaturated fatty acids (UFAs) in PL is important for its health function. This includes the composition of ARA (proinflammatory), EPA, and docosahexaenoic acid (DHA) (anti-inflammatory), as shown in **Figure 4C**. PL, which includes ARA, was found to be the most abundant in all parts of the sea cucumber. In the meat, blue sea cucumber had the highest ARA content (8.08 ± 0.29 mg/100 g), whereas in the skin, black sea cucumber had the highest ARA content (10.47 ± 0.22 mg/100 g). There was no significant difference in ARA content among the three types of sea cucumber with regard to the intestinal contents. DHA was also detected, although its content was relatively low.

Marine phytoplankton are a good source of both ω-3 and ω-6 fatty acids (Taipale et al., 2020). Sea cucumber consume mainly phytoplanktonic cells and occasionally small crustaceans and a variety of eggs and larvae (Hamel & Mercier, 1998). Due to the phytoplankton in their diet, we could detect ω-3 and ω-6 fatty acids in different body parts of sea cucumber. The intestinal contents of the Chinese sea cucumber (*Apostichopus japonicus*) were found to contain higher concentrations of DHA in PE, PC, and PS than other tissues (Ermolenko et al., 2022). The study also revealed that in sea cucumbers with a deficiency in the ω-3 diet, their PL molecular species profile is altered, and ω-3 PUFAs are significantly reduced. Therefore, the sea cucumbers analyzed in this study may have been exposed to an ω3-deficient diet. Further research is necessary to comprehensively analyze the nutritional status and lipid composition of other edible sea cucumbers.

Hierarchical clustering heat maps were used to visualize the concentrations of

lysophospholipids (LysoPL) in sea cucumbers. Visualization of LysoPL, such as lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), lysophosphatidylserine (LPS), and lysophosphatidylinositol (LPI), is represented in a heat map and shown in **Figure 4D**. The concentrations of LysoPL were found to be relatively high in the intestinal contents of sea cucumbers compared with other body parts. LPE and ether-linked LPE were relatively high in the intestinal contents of red sea cucumber and blue sea cucumber meats. In a recent study, ether-type LPE and ether-type LPC were detected in the body wall of the sea cucumber (*Apostichopus japonicus*) (Hu et al., 2023). On the other hand, only ether-type LPE could be detected in this study. Blue and black sea cucumbers exhibited similar LysoPL profiles. The similarity of the profiles may be attributed to the fact that these two species of sea cucumbers are considered to be the same species.

3.5 Hierarchical cluster analysis of ceramides and hexosylceramides in sea cucumbers

The sphingolipids such as ceramide (Cer) and hexosylceramide (HexCer) detected in sea cucumber were visualized using heat map, and the results are shown in **Figure 5**. The results shows that phytoceramides (ex: Cer (t18:0/24:0(2OH)) were found to be abundant in the intestinal contents of red sea cucumber compared to other regions. Black sea cucumber skin and blue sea cucumber meat were relatively rich in the same odd-chain ceramide molecular species (ex: Cer (d17:1/24:0(2OH)). It is known that dietary ceramides and glucosylceramides can prevent and alleviate insulin resistance (Yang et al., 2021) and phytoceramides are widely used in cosmetic products. The abundance of ceramides in sea cucumber reveals that they are potential sources of several unique

ceramides including unnatural ones such C17 carbon chain. This study is the first to compare lipid profiles in different body parts of three varieties of sea cucumbers collected across different regions of Japan. However, our study has the following limitations, the analysis results are semi-quantitative not absolute levels, and the factors that could influence the lipid composition, such as season and temperature are not considered in this investigation. Although we performed the positive ionization mode analysis, triacylglycerols were not detected in the sea cucumber samples. Our study is limited to three varieties from two different species of sea cumber, further studies involving other species are essential to draw better conclusions about lipid abundances.

4. Conclusion

The lipid profiles of the skin, meat, and intestinal contents of three varieties of sea cucumbers were analyzed using HPLC/LTQ-Orbitrap MS. The red sea cucumber has the highest health promotion indexes and HH ratio, suggesting that it may be the most beneficial to human health. The distribution of lipid profiles among different sea cucumbers and their body parts was analyzed using multivariate analysis. The results showed that intestinal contents of the sea cucumber are rich in functional lipids such as polyunsaturated fatty acids and phospholipids compared to other regions. These lipids may contribute to improved human health when they are consumed through diet. This study employed untargeted LC/MS technique to comprehensively analyze regio-specific lipid profiles in sea cucumbers. In the future we aim to apply this technology to other foods to better understand their lipidome.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability Statement

The data will be available from the corresponding author upon a reasonable request.

Credit authorship contribution statement:

Y.M: Formal analysis, Visualization, Methodology, writing-original draft. S.G.B.G: Conceptualisation, Supervision, resources, Writing-original draft. D.G.: Data curation, Visualization, resources, Writing- review & editing. H.C.: Supervision, Writing- review & editing. S-P.H.: Supervision, Resources, Writing- review & editing.

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Figures

Fig. 1. Principal component analysis (PCA) of untargeted lipidomics data of sea cucumbers **A.** PCA score plot and **B.** PCA loadings plot showing the lipid molecular species responsible for group separation.

Fig. 2. Analysis of free fatty acids in sea cucumbers **A**. Percentage distribution of free fatty acids **B**. Free fatty acids composition based on unsaturation. ($p < 0.05$ is considered statistically significant. The significant groups are as follows; SFA: a1-[b1, e1, h1] i.e. group a1 is significantly different with the groups shown in square bracket, b1-[c1, d1, e1, f1, g1, h1, i1], c1-[e1, f1], d1-[e1, f1, h1], e1-[f1, g1, i1], f1-[g1, h1, i1], g1-[h1], h1-[i1]. MUFA: a2- [b2, c2, d2, e2, g2, h2, i2], b2-[c2, d2, e2, f2, g2, h2, i2], c2-[d2, e2, f2, g2, h2, i2], d2-[e2, f2, g2, h2, i2], e2-[f2, g2, h2, I2], f2-[g2, h2, i2], g2-[h2], h2-[i2]. PUFA: a3- [b3, c3, d3, e3, f3, g3, h3, i3], b3-[c3, d3, f3, g3, i3], c3-[d3, e3, f3, g3, h3, i3], d3-[e3, f3, g3, h3, i3], e3-[f3, g3, h3, i3], f3-[g3, h3, i], g3-[h3, i3], h3-[i3]). **C.** ARA and EPA composition in skin, meat, and intestine of three varieties of sea cucumbers. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test, * $p < 0.05$, ** $p < 0.01$*** $p < 0.001$, **** $p < 0.0001$.

Fig. 3. Assessment of the nutritional indices of sea cucumbers. **A.** PUFA/SFA (P:S) ratio, **B.** Index of Atherogenicity (IA), **C.** Hypo/hypercholesterolemic ratio (HH), **D.** Health promotion index (HPI).

Fig. 4. A. Heatmap of phospholipids, **B.** Composition of monounsaturated and polyunsaturated fatty acids in phospholipids, **C.** Composition of representative polyunsaturated fatty acid molecular species in phospholipids. **D.** Heatmap of lysophospholipids (clustering method: ward, distance measure: Euclidean). One-way analysis of variance (ANOVA) and Tukey's multiple comparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, *n.s.* not significant.

Fig. 5. Hierarchical cluster correlation analysis of ceramides (Cer) and hexosylceramides (HexCer) (clustering method: ward, distance measure: Euclidean).

Fig. 1

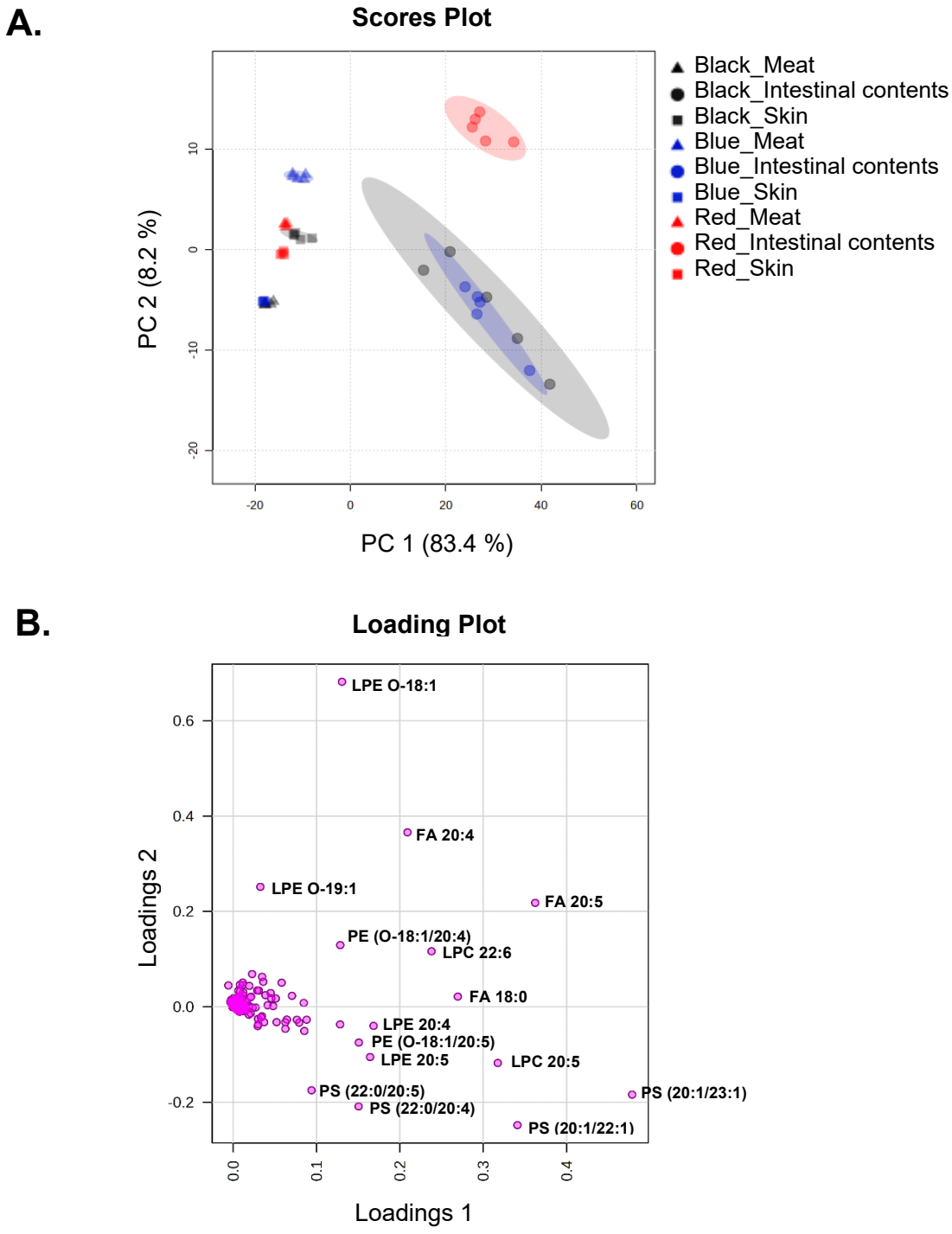

A.
Scores Plot
Black_Meat
Black_Intestinal contents
Black_Skin
Blue_Meat
Blue_Intestinal contents
Blue_Skin
Red_Meat
Red_Intestinal contents
Red_Skin
PC 2 (8.2 %)
PC 1 (83.4 %)
B.
Loading Plot
LPE O-18:1
FA 20:4
LPE O-19:1
FA 20:5
PE (O-18:1/20:4)
LPC 22:6
FA 18:0
LPE 20:4
PE (O-18:1/20:5)
LPE 20:5
LPC 20:5
PS (22:0/20:5)
PS (20:1/23:1)
PS (22:0/20:4)
PS (20:1/22:1)
Loadings 2
Loadings 1

Fig. 2

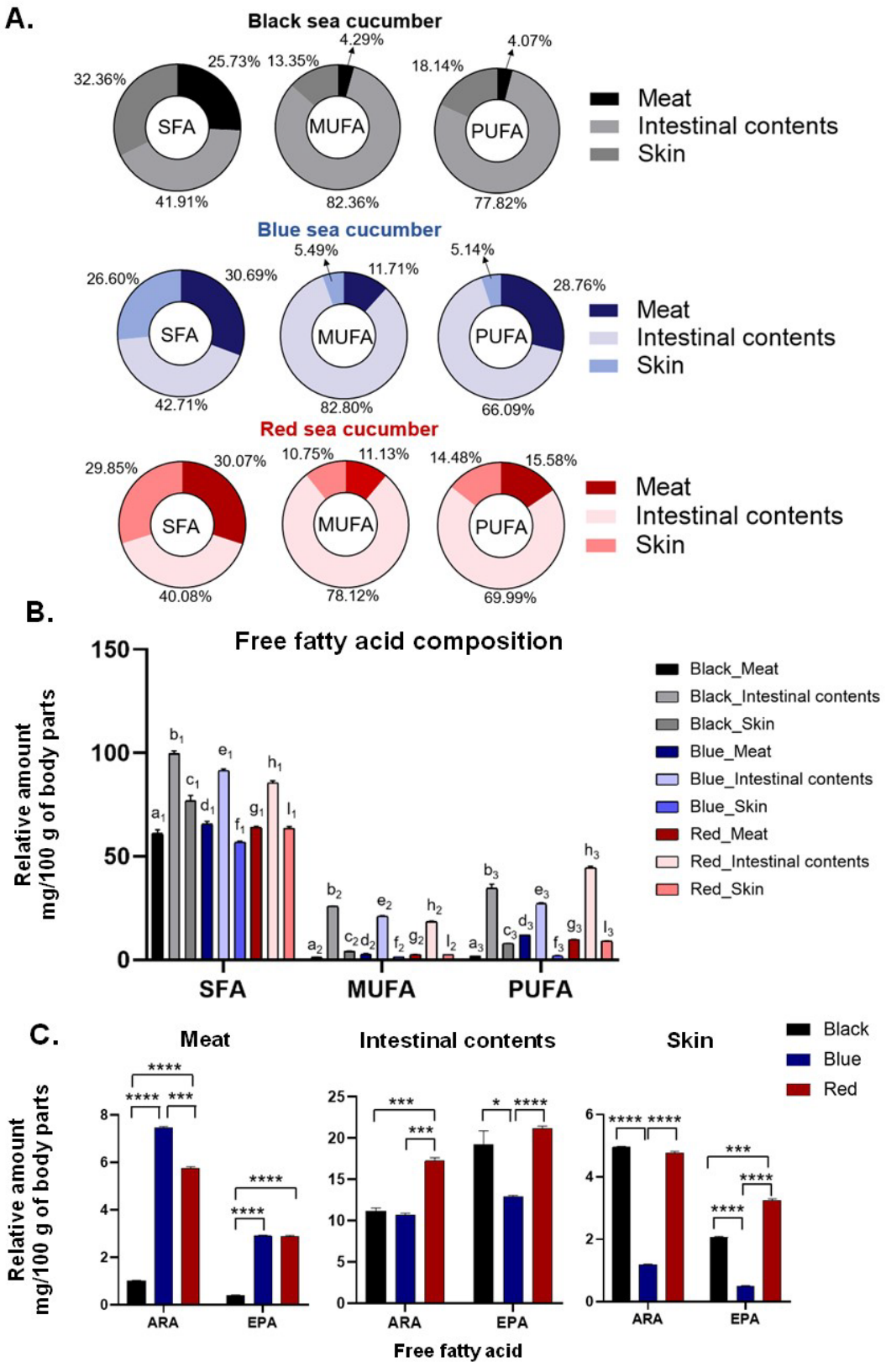

A.
Black sea cucumber
32.36%
25.73%
41.91%
SFA
13.35%
4.29%
82.36%
MUFA
18.14%
4.07%
77.82%
PUFA
Meat
Intestinal contents
Skin
Blue sea cucumber
26.60%
30.69%
42.71%
SFA
5.49%
11.71%
82.80%
MUFA
5.14%
28.76%
66.09%
PUFA
Meat
Intestinal contents
Skin
Red sea cucumber
29.85%
30.07%
40.08%
SFA
10.75%
11.13%
78.12%
MUFA
14.48%
15.58%
69.99%
PUFA
Meat
Intestinal contents
Skin
B.
Free fatty acid composition
Relative amount
mg/100 g of body parts
150
100
50
0
SFA
MUFA
PUFA
Black_Meat
Black_Intestinal contents
Black_Skin
Blue_Meat
Blue_Intestinal contents
Blue_Skin
Red_Meat
Red_Intestinal contents
Red_Skin
C.
Meat
Intestinal contents
Skin
Black
Blue
Red
Relative amount
mg/100 g of body parts
ARA
EPA
Free fatty acid

Fig. 3

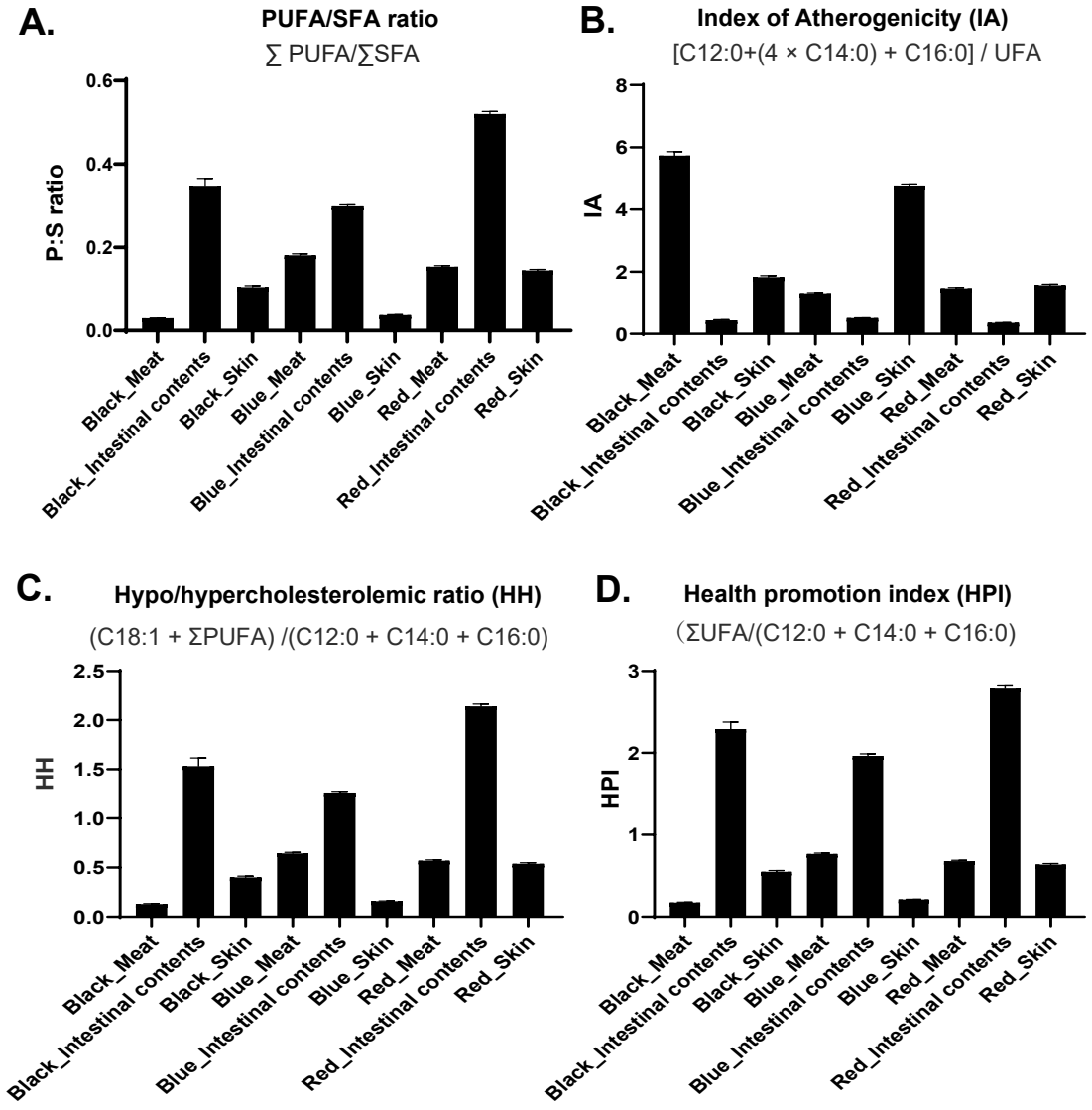
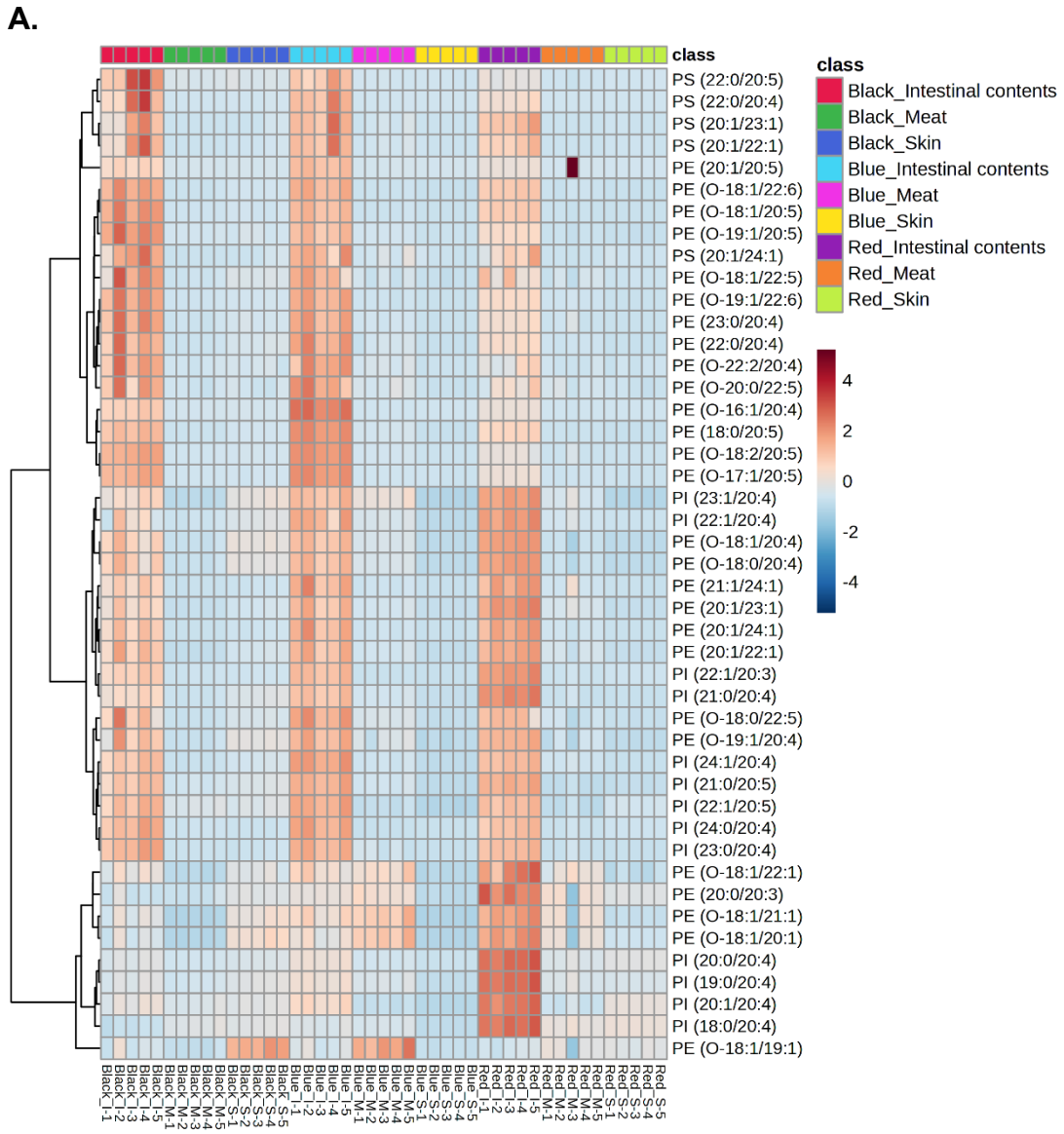
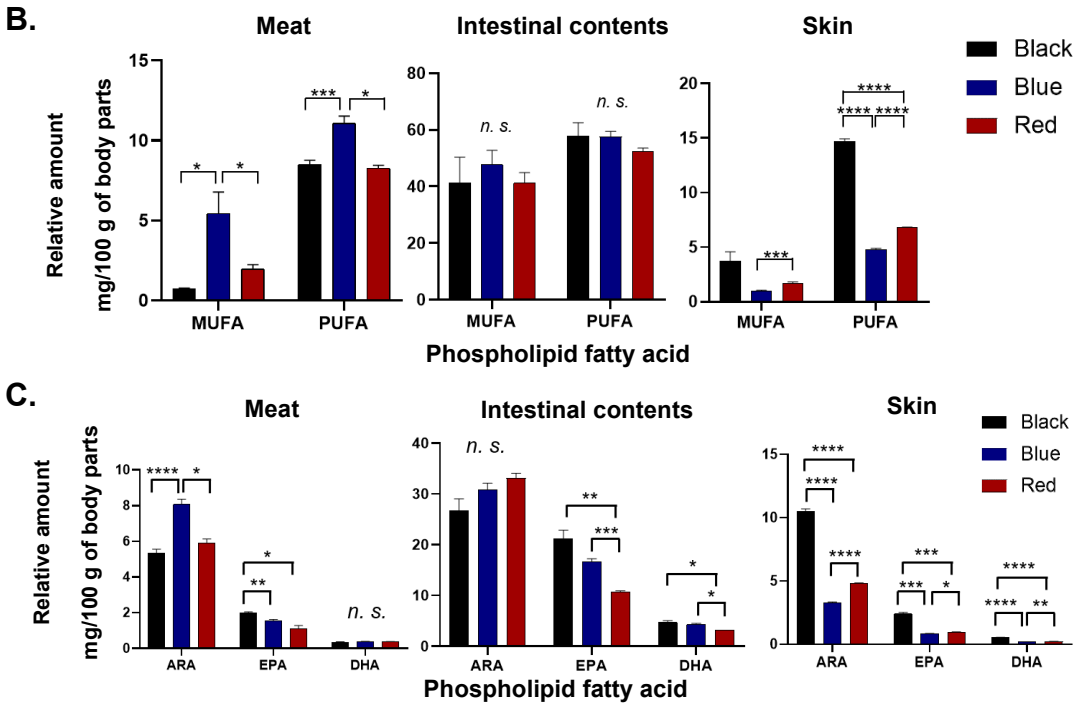

A.
PUFA/SFA ratio
∑ PUFA/∑SFA
P:S ratio
0.6
0.4
0.2
0.0
Black_Meat
Black_Intestinal contents
Black_Skin
Blue_Meat
Blue_Intestinal contents
Blue_Skin
Red_Meat
Red_Intestinal contents
Red_Skin
B.
Index of Atherogenicity (IA)
[C12:0+(4 × C14:0) + C16:0] / UFA
IA
8
6
4
2
0
C.
Hypo/hypercholesterolemic ratio (HH)
(C18:1 + ΣPUFA) /(C12:0 + C14:0 + C16:0)
HH
2.5
2.0
1.5
1.0
0.5
0.0
D.
Health promotion index (HPI)
（ΣUFA/(C12:0 + C14:0 + C16:0)
HPI
3
2
1
0

Fig. 4


A.
class
PS (22:0/20:5)
PS (22:0/20:4)
PS (20:1/23:1)
PS (20:1/22:1)
PE (20:1/20:5)
PE (O-18:1/22:6)
PE (O-18:1/20:5)
PE (O-19:1/20:5)
PS (20:1/24:1)
PE (O-18:1/22:5)
PE (O-19:1/22:6)
PE (23:0/20:4)
PE (22:0/20:4)
PE (O-22:2/20:4)
PE (O-20:0/22:5)
PE (O-16:1/20:4)
PE (18:0/20:5)
PE (O-18:2/20:5)
PE (O-17:1/20:5)
PI (23:1/20:4)
PI (22:1/20:4)
PE (O-18:1/20:4)
PE (O-18:0/20:4)
PE (21:1/24:1)
PE (20:1/23:1)
PE (20:1/24:1)
PE (20:1/22:1)
PI (22:1/20:3)
PI (21:0/20:4)
PE (O-18:0/22:5)
PE (O-19:1/20:4)
PI (24:1/20:4)
PI (21:0/20:5)
PI (22:1/20:5)
PI (24:0/20:4)
PI (23:0/20:4)
PE (O-18:1/22:1)
PE (20:0/20:3)
PE (O-18:1/21:1)
PE (O-18:1/20:1)
PI (20:0/20:4)
PI (19:0/20:4)
PI (20:1/20:4)
PI (18:0/20:4)
PE (O-18:1/19:1)
Black_I-1
Black_I-2
Black_I-3
Black_I-4
Black_I-5
Black_M-1
Black_M-2
Black_M-3
Black_M-4
Black_M-5
Black_S-1
Black_S-2
Black_S-3
Black_S-4
Black_S-5
Blue_I-1
Blue_I-2
Blue_I-3
Blue_I-4
Blue_I-5
Blue_M-1
Blue_M-2
Blue_M-3
Blue_M-4
Blue_M-5
Blue_S-1
Blue_S-2
Blue_S-3
Blue_S-4
Blue_S-5
Red_I-1
Red_I-2
Red_I-3
Red_I-4
Red_I-5
Red_M-1
Red_M-2
Red_M-3
Red_M-4
Red_M-5
Red_S-1
Red_S-2
Red_S-3
Red_S-4
Red_S-5
class
Black_Intestinal contents
Black_Meat
Black_Skin
Blue_Intestinal contents
Blue_Meat
Blue_Skin
Red_Intestinal contents
Red_Meat
Red_Skin
4
2
0
-2
-4


B.
Meat
Intestinal contents
Skin
Black
Blue
Red
Relative amount
mg/100 g of body parts
MUFA
PUFA
n. s.
Phospholipid fatty acid
C.
Meat
Intestinal contents
Skin
Black
Blue
Red
Relative amount
mg/100 g of body parts
ARA
EPA
DHA
n. s.
Phospholipid fatty acid

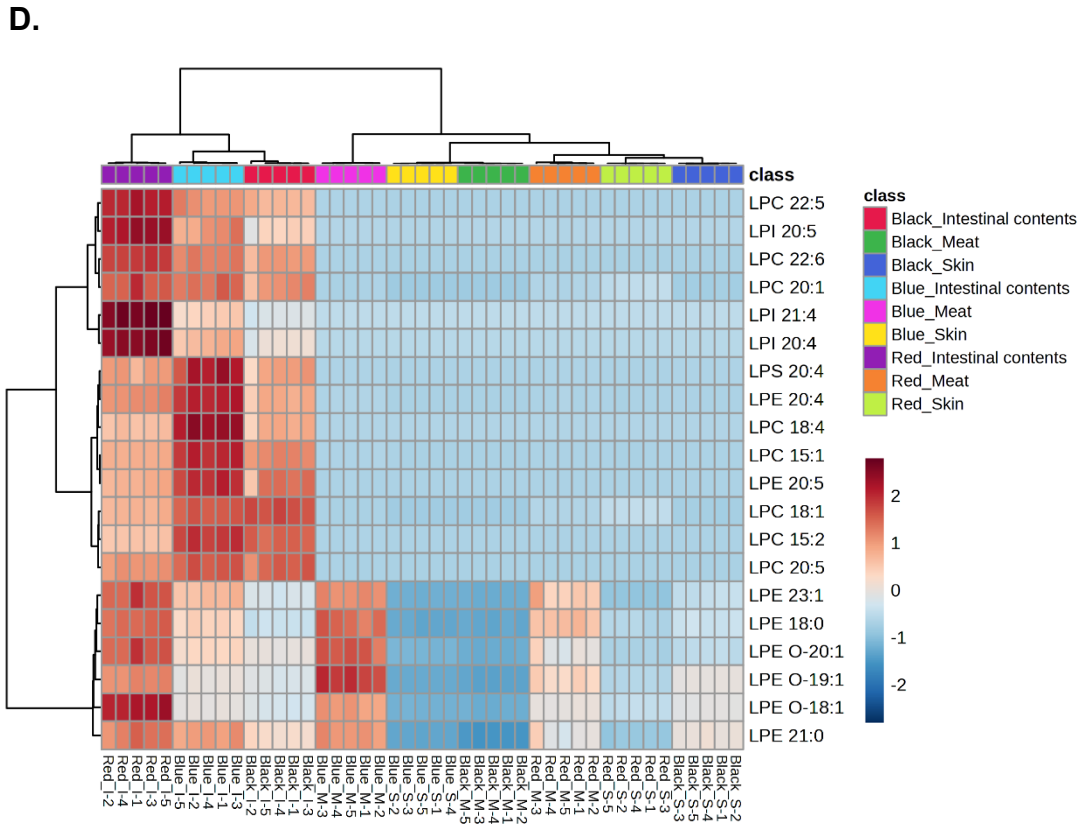
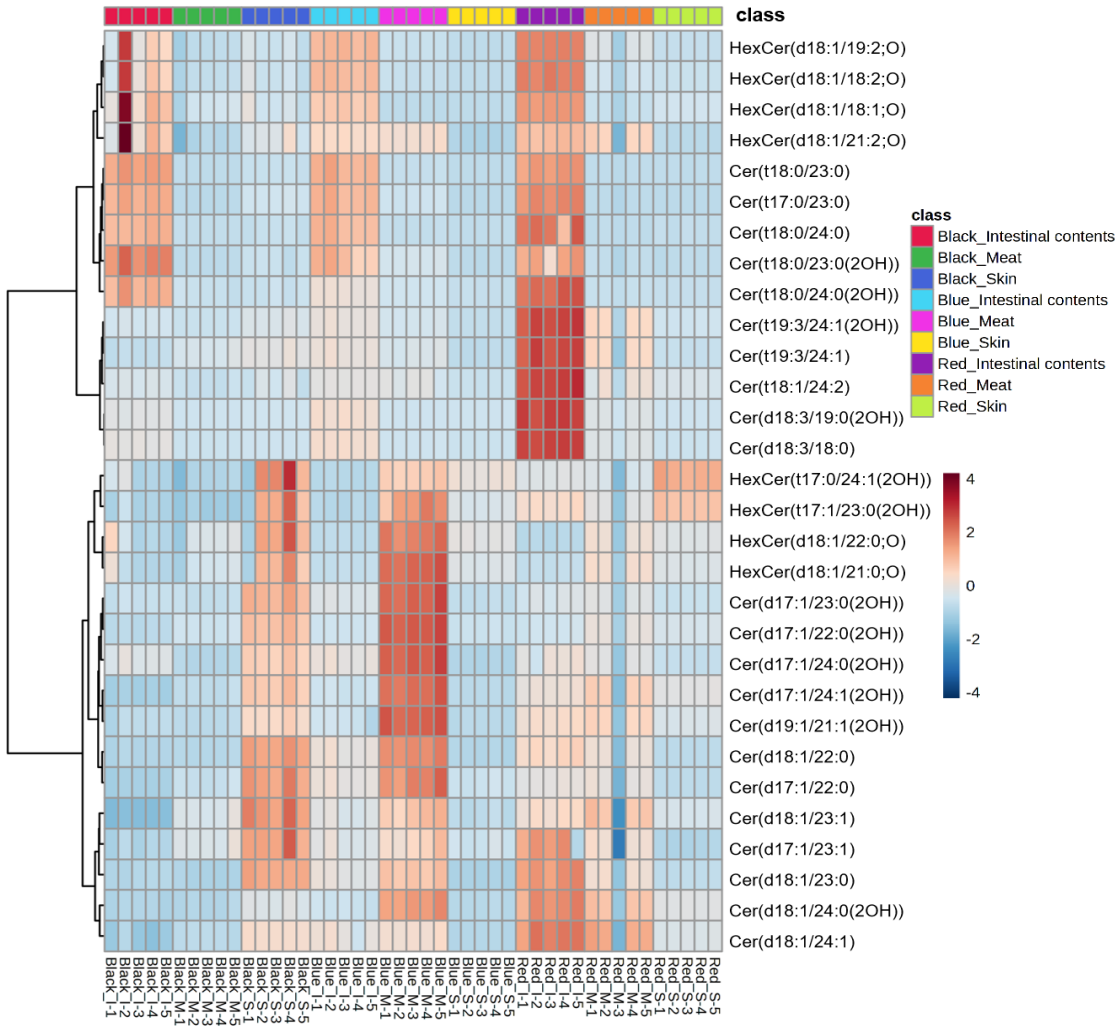

D.
class
LPC 22:5
LPI 20:5
LPC 22:6
LPC 20:1
LPI 21:4
LPI 20:4
LPS 20:4
LPE 20:4
LPC 18:4
LPC 15:1
LPE 20:5
LPC 18:1
LPC 15:2
LPC 20:5
LPE 23:1
LPE 18:0
LPE O-20:1
LPE O-19:1
LPE O-18:1
LPE 21:0
class
Black_Intestinal contents
Black_Meat
Black_Skin
Blue_Intestinal contents
Blue_Meat
Blue_Skin
Red_Intestinal contents
Red_Meat
Red_Skin

Fig. 5


class
HexCer(d18:1/19:2;O)
HexCer(d18:1/18:2;O)
HexCer(d18:1/18:1;O)
HexCer(d18:1/21:2;O)
Cer(t18:0/23:0)
Cer(t17:0/23:0)
Cer(t18:0/24:0)
Cer(t18:0/23:0(2OH))
Cer(t18:0/24:0(2OH))
Cer(t19:3/24:1(2OH))
Cer(t19:3/24:1)
Cer(t18:1/24:2)
Cer(d18:3/19:0(2OH))
Cer(d18:3/18:0)
HexCer(t17:0/24:1(2OH))
HexCer(t17:1/23:0(2OH))
HexCer(d18:1/22:0;O)
HexCer(d18:1/21:0;O)
Cer(d17:1/23:0(2OH))
Cer(d17:1/22:0(2OH))
Cer(d17:1/24:0(2OH))
Cer(d17:1/24:1(2OH))
Cer(d19:1/21:1(2OH))
Cer(d18:1/22:0)
Cer(d17:1/22:0)
Cer(d18:1/23:1)
Cer(d17:1/23:1)
Cer(d18:1/23:0)
Cer(d18:1/24:0(2OH))
Cer(d18:1/24:1)
class
Black_Intestinal contents
Black_Meat
Black_Skin
Blue_Intestinal contents
Blue_Meat
Blue_Skin
Red_Intestinal contents
Red_Meat
Red_Skin
4
2
0
-2
-4
Black_I-1
Black_I-2
Black_I-3
Black_I-4
Black_I-5
Black_M-1
Black_M-2
Black_M-3
Black_M-4
Black_M-5
Black_S-1
Black_S-2
Black_S-3
Black_S-4
Black_S-5
Blue_I-1
Blue_I-2
Blue_I-3
Blue_I-4
Blue_I-5
Blue_M-1
Blue_M-2
Blue_M-3
Blue_M-4
Blue_M-5
Blue_S-1
Blue_S-2
Blue_S-3
Blue_S-4
Blue_S-5
Red_I-1
Red_I-2
Red_I-3
Red_I-4
Red_I-5
Red_M-1
Red_M-2
Red_M-3
Red_M-4
Red_M-5
Red_S-1
Red_S-2
Red_S-3
Red_S-4
Red_S-5