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Raw frozen Antarctic krill (*Euphausia superba*) as an alternative feed source for cuttlefish *Sepiella japonica* in artificial breeding systems

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Abstract

The study aims to evaluate the feasibility of completely replacing raw frozen shrimp *Palaemon gravieri* diets with raw frozen Antarctic krill (*Euphausia superba*) in the diets of cuttlefish *Sepiella japonica*. To address the knowledge gap, we conducted a 60-day feeding trial. At the end of the experiment (day 60), the cuttlefish *Sepiella japonica* eating *Palaemon gravieri* (SJP) grew significantly faster than those eating *Euphausia superba* (SJE), with the specific growth rate $(SGR)_{SJP}$ (7.92%) > $(SGR)_{SJE}$ (7.09%). Approximately 33.3% and 20.0% mortality was observed in the SJE and SJP, during the course of the experiment respectively. Some important fatty acids (i.e. n-3 and n-6 PUFAs) were elevated in SJE with respect to SJP. Replacement of Antarctic krill increased the diversity of the gut microbiome composition in the SJE group. Fluoride accumulated in the ink sac and cuttlebone of cuttlefish in SJE. Overall, these findings imply that PUFA-rich Antarctic krill could replace *P. gravieri* shrimp for feeding cuttlefish *S. japonica*.

KEYWORDS

Antarctic krill, feed replacement, fluoride, gut microbiome, *Sepiella japonica*, shrimp

1 | INTRODUCTION

Since the 1950s, the cuttlefish (*Sepiella japonica*) has become an important fish species in China. The average annual catch of Zhejiang Province (the main producing area of this species) was 22,543 tons, and the highest catch was 62,225 tons since the 1950s (Dong, 1991). Due to overfishing, *S. japonica* has been at the margin of extinction in the East China Sea since 2005. Thanks to advances in breeding techniques, *S. japonica* can be artificially reared in aquaculture systems (Reid, Jereb, & Roper, 2005; Zheng et al., 2010) and the optimal growth period is from February to August. In some artificial farming, *Palaemon gravieri* is generally used as feed for cuttlefish. According to the laws and regulations of fisheries in Zhejiang province (China), the summer fishing moratorium covers May to September. During this period, it is hard to obtain sufficient *P. gravieri* for cuttlefish breeding. Not only is the price of *P. gravieri* high, but the prices of alternative feeds also increase commensurately. As a result, there is a problem in keeping up with the recent surge in demand for factory farmed *S. japonica*.

Antarctic krill (*Euphausia superba*) are the main prey for numerous predators in the Southern Ocean ecosystem (Olsen et al., 2006). They have been caught since the 1970s, and catches have stood at over 200,000 tonnes since 2009 (Nicol & Foster &Kawaguchi, 2012). The utilization of krill as an aquaculture feed has been investigated for several species, such as the Atlantic salmon (*Salmo salar*) (Hatlen et al., 2017; Olsen et al., 2006), rainbow trout (*Oncorhynchus mykiss*) (Yoshitomi & Aoki &Oshima, 2007; Yoshitomi, Aoki, Oshima, & Hata, 2006), mink (*Neovison vison*) (Krogdahl et al., 2015), Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Moren et al., 2007). Xu et al. (2017) held the view that Antarctic krill meal improved the reproductive performance of tongue sole (*Cynoglossus semilaevis*). However, the fluoride of Antarctic krill largely derived from the exoskeleton is one of its potential anti-nutritional factors (Soevik & Braekkan, 1979; Yoshitomi et al., 2007). Although dietary fluoride of Antarctic krill influences the growth performance, decreasing the fluoride content has been successfully applied in the use of krill to replace fish meal for rainbow trout (Yoshitomi et al., 2007; Yoshitomi & Nagano, 2012). In long-term experiments where salmon have fed on Antarctic krill for up to 3 years (Grave, 1981), fluoride in the muscle of the salmon marginally increases, and nonetheless does not exceed that observed in wild fish species from the North Sea (up to 670 mg/kg of dry matter). According to the European Union standard, the maximum recommended level of fluoride in the feed is 150 mg/kg dry feed (Directive, 1999). However, little is known about the krill replacement for use in cuttlefish diets.

With the advancement in next-generation sequencing (NGS), 16S rDNA gene-based bacterial microbiota profiles can be used to describe microbiota diversity (Escalas et al., 2017; Sun et al., 2014). Microbial community dynamics impact on the growth performance and immunity of aquatic organisms, for example in fish (Bruijn, Liu, Wiegertjes, & Raaijmakers, 2018) and mussels (Vezzulli et al., 2017). Sequencing results of different complete diet treatments fed to salmon illustrated that members of the genera *Aliivibrio*, *Vibrio* and *Photobacterium* became predominant (Zarkasi et al., 2017). In this study, we performed a factorial experiment to evaluate the effect of the complete replacement of *E. superba* on cuttlefish *S. japonica*, compared to the control fed *P. gravieri*. Raw frozen *P. gravieri* and *E. superba* were used for a 60-day feeding trial (including maturation and spawning). Response variables included growth performance, nutritional level, gut microbial community (GMC), toxicity and oxidative stress markers, and fluoride content. The findings will contribute to the development of alternative strategies for the feeding of krill in cuttlefish aquaculture.

2 | MATERIALS AND METHODS

2.1 | Experimental design

In August 2017, a total of 300 healthy cuttlefish larvae (average body weight/BW: 5.57 ± 0.40 g, average mantle length/ML: 3.27 ± 0.11 cm) were collected from the same population and immediately transferred to the breeding laboratory in the Marine Fisheries Research Institute of Zhejiang, China. They were randomly divided into two groups (each group consisted of 150 individuals). One group (SJE) was fed with the frozen Antarctic krill, *E. superba* (average body length/BL: 6.51 ± 0.62 cm, average body weight/BW: 1.77 ± 0.11 g), and the other group (SJP) was fed with frozen shrimp *P. gravieri* (average BL: 6.83 ± 0.74 cm, average BW: 1.93 ± 0.12 g). Each treatment had three replicates, and each replicate had 50 individuals. The animals were independently cultivated in aquaponic polycarbonate tanks (Φ = 1.3 m, depth = 0.7 m) and maintained indoors with aerated seawater (25°C, salinity: 28 ppt) during the course of the experiment (60 days). Each tank was strongly aerated by an air stone and two airlifts. The experimental diets were hand-fed three times a day. Based on our previous evidence (Ping, Yu, Zhang, & Shi, 2017), a feeding volume of 5% of BW per day per tank was adopted. To avoid frequent stress, we adjusted the feeding rate based on each weight measurement. Allowing for mortality, three individuals were randomly collected from each test tank, and a total of nine individuals or tissues were sampled to evaluate the performance metrics. All animal welfare and experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2016) and were approved by the Animal Care and Use Committee of the Marine Fisheries Research Institute of Zhejiang.

2.2 | Growth performance

Based on the method of anaesthesia of Collins and Nyholm (2010), cuttlefish were randomly captured from each test tank and soaked into the anaesthesia solution (2% solution of ethanol) for 10 min. For each sampling day (0, 10, 20, 30, 40, 50 and 60 days after feeding), we randomly collected three individuals from each tank to evaluate growth performance. The BW was measured using an electronic balance and the ML was measured by a calliper in the laboratory of the Marine Fisheries Research Institute of Zhejiang. After measuring, all the cuttlefish were transferred into fresh seawater for recovery. The specific growth rate (SGR) was calculated by the formula (1) at the last sampling day. Moreover, we dissected each cuttlefish to measure liver weight (LW) and muscle weight (MW) at the 1st, 3rd and 5th sampling session. Gonadal weight (GW) was only sampled after feeding at 40 and 60 days of trial. Condition factor (CF), hepatosomatic index (HI), meat content ratio (MCR) and gonadosomatic index (GI) were evaluated by the following formulas:

$$SGR = \frac{\ln BW_f - \ln BW_i}{days} \times 100\%, \quad (1)$$

where $\ln BW_f$ = the natural logarithm of the final body weight; $\ln BW_i$ = the natural logarithm of the initial body weight.

$$CF = \frac{BW}{ML^3} \times 100\%, \quad (2)$$

$$HI = \frac{LW}{BW} \times 100\%, \quad (3)$$

$$MCR = \frac{MW}{BW} \times 100\%, \quad (4)$$

$$GI = \frac{GW}{BW} \times 100\%. \quad (5)$$

2.3 | Evaluation of nutritional levels

To evaluate the nutrient content of the shrimp (*E. superba* and *P. gravier*) and cuttlefish (SJE and SJP), the moisture content, crude protein, crude fat, ash content, amino acid composition and fatty acid composition were measured after 60 days of feeding. The moisture content, referenced to the Chinese national standard determination GB/T 5009.3-2003 (NHFPC/PRC, 2003a), was calculated by weight loss after drying until constant weight. Crude protein, referenced to GB/T 5009.5-2003 (NHFPC/PRC, 2003b), was measured using the Kjeldahl Nitrogen method (nitrogen coefficient = 6.25). Crude fat, referenced to GB/T 5009.6-2003 (NHFPC/PRC, 2003c), was determined gravimetrically after extraction with diethyl ether. The ash content, referenced to GB/T 5009.4-2003 (NHFPC/PRC, 2003d), was determined after ignition at 550°C.

Amino acid composition in the muscles was referenced to GB/T 5009.124-2003 (NHFPC/PRC, 2003e) and determined using acid hydrolysis and ninhydrin reaction methods using an amino acid analyser (Sykam S433D) at Nanjing Jiancheng Bioengineering Institute. Amino acid score (AAS) and chemical score (CS) were calculated referenced to the FAO/WHO model and Egg standard. The equations were as follows:

$$AAS = W_{aa}(S.\,japonica)/W_{AA}(FAO/WHO) \times 100\%, \quad (6)$$

$$CS = W_{aa}(S.\,japonica)/W_{AA}(Egg) \times 100\%. \quad (7)$$

The fatty acid composition was referenced to GB/T5009.168-2016 (NHFPC/PRC, 2016) and quantitatively measured by gas chromatography with mass spectrometric detection (Agilent 5975C-7890A, USA) at Nanjing Jiancheng Bioengineering Institute.

2.4 | Gut slice observation and gut microbiota diversity

To explore whether Antarctic krill causes an alteration in gut morphology and adherent microbiome assemblages, we performed a rectum slice and 16S rRNA amplicon sequencing. Only animals fed for 60 days were considered. Intestine tissues (mainly small intestine and rectum) of cuttlefish were removed and fixed within 24 hr in Bouin's fluid according to the method of Owen (1970). Samples were collected after 4 hr of feeding. Following paraffin embedding and dehydration, the samples were embedded in ester wax and 5–8 μm sections were routinely stained with haematoxylin and eosin (HE) and examined under a Leica microscope. Considering the small rectum size of the cuttlefish and the minimum sample amount required for DNA extraction, we randomly selected two individuals per tank, separated their gastrointestinal tracts, gently squeezed the digesta out and rinsed the entire intestine twice with PBS. Two homogenized samples were then presented as a single experimental unit. Each treatment had three experimental units. All digesta and filtrate samples were collected and subsequently stored at −80°C.

2.5 | DNA extraction, amplification and sequencing

The DNA of gut microbiota was extracted from the same amount (250 mg) of each sample according to the manufacturer's alternative protocol for the PowerSoil® DNA Isolation Kit. The primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were designed based on the V3-V4 region of the 16S rRNA gene (Xu, Tan, Wang, & Gai, 2016). The PCR was conducted in 20 μl reaction mixtures, including 4 μl 5 × FastPfu Buffer, 2 μl dNTPs, 0.8 μl 338F, 0.8 μl 806R, 10 ng template DNA and 0.4 μl TransStart Fastpfu DNA Polymerase (TransGen Biotech), and the final volume was made up with double distilled H_2O. The PCR amplification was performed on an ABI GeneAmp® 9700, with the following amplification conditions: 2 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, with a final extension of 5 min at 72°C. Triplicate reaction mixtures were pooled per sample, and the PCR products were gel-purified using the AxyPrep DNA Gel Extraction Kit (Axygen) and quantified using the QuantiFluor-ST Fluorescence quantitative system (Promega). The mixtures were then pyrosequenced using a Roche 454 Genome Sequencer FLX Titanium platform (Majorbio Bio-Pharm Technology Co. Ltd.).

2.6 | Pyrosequencing data analysis

The raw sequencing reads were uploaded and analysed in Majorbio I-Sanger Cloud Platform of Shanghai MEIJI Biotechnology. After subsampling, the cleaned reads were clustered into operational taxonomic units (OTUs) using a 97% identity threshold by Usearch (Edgar, 2010). Taxonomy was assigned according to the SILVA 132 database. To compare alpha-diversity between different treatments, a series of subsets of each library were chosen to calculate their respective community richness and community diversity. The taxonomic distributions of SJE and SJP at the phylum level were analysed based on the method of Ondov, Bergman, and Philiippy (2011). Heatmap analysis and a microbial community pie-plot at multi-levels were conducted using R-g plots for Linux with the clustering of the different samples. The analysis was carried out without data transformation and focused on the scaling of original data.

2.7 | Toxicity and oxidative stress markers determination

Three cuttlefish from each tank were anesthetized, and blood was withdrawn from the back venous vessel using a 1 ml syringe with a 4.5-gauge needle, according to the method of Yin et al. (2013). The blood was immediately placed in a sterile 1.5 ml tube on ice and centrifuged at 1,800 *g* for 10 min at 4°C. The liver was directly removed and stored in the 4°C freezer. Five toxicity and oxidative stress markers including superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), alanine transaminase (ALT) and aspartate aminotransferase (AST) were determined from the blood and liver of all the samples after 60 days of feeding. Laboratory methods were based on the manufacturer's protocol for respective assay kits and were performed at the Wuhan Servicebio Technology Company Limited laboratory.

2.8 | Fluoride content

Fluoride, referenced to GB/T 5009.18-2003 (NHFPC/PRC, 2003f), was measured spectrophotometrically as H_2SiF_6 after staining with alizarin complex and lanthanum (III) nitrate at 580 nm at the laboratory of Greentown Testing Co. Ltd. These samples included included feed (*E. superba* and *P. gravieri*), integral cuttlefish (SJE and SJP) and some tissues (i.e. muscle, liver, ink sac and cuttlebone of cuttlefish) after 60 days of feeding.

2.9 | Statistical analyses

To evaluate the differences in growth of cuttlefish fed shrimp or krill, a two-factor repeated measure ANOVA was performed using time as repeated measure and dietary treatment means for each tank. The mean over all time points was used to compare treatments using Student's *t* test. All analyses were performed in R (version 3.3.3., 2019).

3 | RESULTS

3.1 | Growth performance

To characterize the variation in growth on SJE and SJP, the relevant indexes (SGR, CF, HI, MCR and GI) were calculated. Mortality was 33.3 ± 8.3% versus 20.0 ± 4.0% in the SJE and SJP groups, respectively, during the 60 day feeding trial. Body weight and mantle length of the cuttlefish differed significantly from the mean

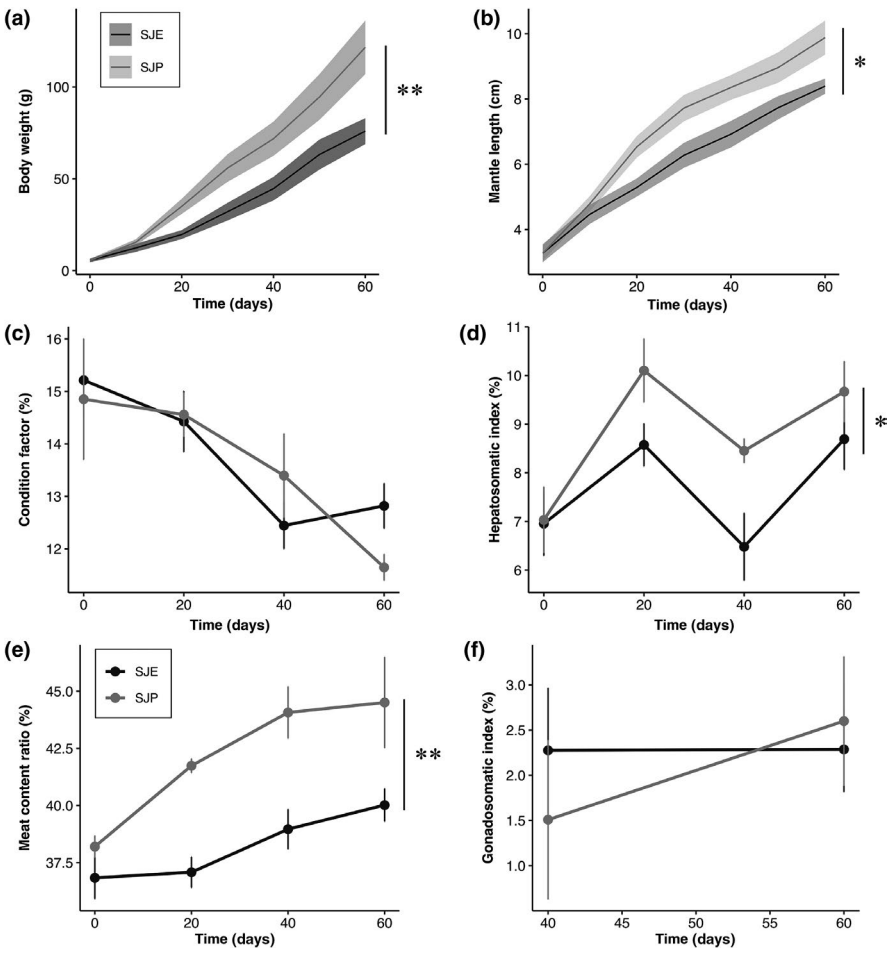

FIGURE 1 The growth performance in SJE-/SJP-treated group. (a,b) Body weight and mantle length of cuttlefish during 60 days after feeding. The line is the mean value, and the shade is confidence interval (CI). *$p < .05$; **$p < .01$. (c–f) The percentage of condition factor, hepatosomatic index, meat content ratio and gonadosomatic index during 60 days after feeding. SJE: *Sepiella japonica* was fed by *Euphausia superba*; SJP: *S. japonica* was fed by *Palaemon gravieri*. Each tank, three individuals were randomly collected; totally, nine individuals were sampled to evaluate growth performance. The data are represented Mean ± *SE*. *$p < .05$; **$p < .01$

TABLE 1 Comparison nutrients composition in all animals (n = 9)

Item	ω (Moisture content, %)	ω (Ash content, %)	ω (Crude protein, %)	ω (Crude fat, %)
ES	51.33 ± 0.21**	5.56 ± 0.26	39.29 ± 0.65**	3.82 ± 0.25
PG	70.64 ± 0.16	6.44 ± 0.26	18.87 ± 0.21	4.05 ± 0.28
SJE	75.11 ± 0.25	4.91 ± 0.21	18.15 ± 0.32	1.82 ± 0.11
SJP	73.49 ± 0.30	4.87 ± 0.08	19.07 ± 0.30	2.56 ± 0.15*

Note: SJE: *Sepiella japonica* was fed by *E. superba*, SJP: *Sepiella japonica* was fed by *P. gravieri*.

Abbreviations: ES, *Euphausia superba*; PG, *Palaemon gravieri*.

*p < .05,

**p < .01.

TABLE 2 Amino acids analysis of muscle of experimental animals on dry matter basis (mg/g)

	ES		PG		SJE		SJP	
Amino acid	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*
Ala	32.42	0.27	32.33	0.26	35.16	0.5	38.3	0.27
Arg	37.42	0.17	43.95	0.35	72.12	0.75	52.04	0.32
Asp	56.87	0.51	56.14	0.4	68.68	0.58	70.73	0.36
Cys	5.6	0.13	4.12	0.16	6.4	0.03	6.37	0.14
Glu	81.32	0.27	86.86	0.29	113.54	1.17	116.52	0.25
Gly	34.86	0.4	34.44	0.24	31.81	0.16	33.15	0.46
His	25.25	0.19	40.46	0.09	15.9	0.53	18.74	0.11
Ile	25.19	0.59	21.07	0.55	26.36	0.22	25.62	0.06
Leu	40.81	0.44	37.2	0.06	52.1	0.27	52.64	1.27
Lys	43.55	0.28	39.4	0.13	59.69	0.44	58.39	0.02
Met	12.98	0.43	12.52	0.35	16.11	0.14	16.7	0.08
Phe	15.98	0.5	21.08	0.28	16.3	0.18	25.16	0.21
Pro	21.72	0.64	16.61	0.27	25.45	0.41	36.71	0.45
Ser	22.01	0.35	22.79	0.44	30.59	0.13	31.78	0.51
Thr	22.67	0.2	20.51	0.46	28.59	0.22	29.86	0.4
Tyr	16.95	0.45	17.8	0.53	16.31	0.29	20.05	0.36
Val	26.31	0.23	22.55	0.29	23.32	0.15	23.35	0.01
EAA	187.49		174.33		222.47		231.72	
FAA	205.47		209.77		249.19		258.7	
TAA	521.91		529.83		638.43		656.11	
EAA/TAA	0.359		0.329		0.348		0.353	
EAA/NEAA	0.561		0.49		0.535		0.546	
FAA/TAA	0.394		0.396		0.39		0.394	

Note: Tryptophan was not measured. SJE: *Sepiella japonica* was fed by *E. superba*; SJP: *Sepiella japonica* was fed by *P. gravieri*.

Abbreviations: EAA, essential amino acids except for Tryptophan; ES, *Euphausia superba*; FAA, flavor amino acids; NEAA, unessential amino acids; PG, *Palaemon gravieri*; TAA, total amino acids.

value between the two groups (p < .01, Figure 1a,b). The SGR_{SJP} (7.92%) was greater than the SGR_{SJE} (7.09%) after 60 days of feeding. The moisture content of *E. superba* (51.33 ± 0.21%) was significantly different to *P. gravieri* (70.64 ± 0.16%) (p < .05, Table 1), and the crude protein content of *E. superba* (39.29 ± 0.65%) was more than twofold that of *P. gravieri* (18.87 ± 0.21%). The CF_{SJE} decreased from an initial 15.21% to 12.82% at 60 days while the CF_{SJP} decreased from 14.85% to 11.65% (Figure 1c). The HI in both groups fluctuated but it was significantly higher in the SJP than the SJE group (p < .05, Figure 1d). The MCR_{SJE} increased from 36.84% to 40.02%, and the MCR_{SJP} increased from 38.20% to 44.51% (p < .01, Figure 1e). The GI was measured from 40 days after feeding. The GI_{SJE} remained at 2.28%, while that of GI_{SJP} rose from 1.51% to 2.60% (Figure 1f).

3.2 | Effects of the diets on amino acid and fatty acid levels of cuttlefish

The amino acid (AA) content of muscle was similar under the two feeding regimes (Table 2). SJE and SJP groups were both rich in glutamic acid, (113.54 and 116.52 mg/g respectively). The EAA/TAA (essential amino acids/total amino acids) and FAA/TAA (flavour amino acids/total amino acids) ratios in SJE and SJP were 34.8% versus 35.3% and 39.0% versus 39.4% respectively. Both the SJE and SJP groups were rich in lysine with the content (373.04 and 364.96 mg/g N respectively), exceeding the FAO/WHO standard (340 mg/g N, Table 3).

Table 4 shows the fatty acid composition of *the shrimp* (*E. superba and P. gravier*) *and cuttlefish* (*SJE and SJP*). *E. superba* was richer in n-3 polyunsaturated fatty acids (n-3 PUFA) (47.97% of total fatty acids), compared to *P. gravieri* (35.13% of total fatty acids).As a result, the content of n-3 PUFA in SJE (58.58% of total fatty acids) was higher than in SJP (39.48% of total fatty acids, $p = .347$). Levels of eicosapentaenoic acid (EPA; C20:5; n-3) and docosahexaenoic acid (DHA; C22:6; n-3) were higher in *E. superba* than in *P. gravieri*, which may lead to the significantly up-regulated EPA and DHA (over 50% of total fatty acids) in SJE compared with SJP ($p < .01$). Interestingly, a relatively small amount of the n-6 PUFA gamma-linolenic acid (GLA; C18:3; n-6) was found in *E. superba* (1.73% of total fatty acids), contributing 0.50% to n-6 PUFA in the SJE. Heneicosylic acid (C21:0) was observed only in SJE (0.18% of total fatty acids).

3.3 | Effects of the diets on the gut microbiome community

Figure 2 shows the slices of proximal intestine (small intestine) and distal intestine (rectum) of cuttlefish. The intestine tissue was thin, and the bowel wall was composed of a mucous, submucosa and muscle layer. Gut fold-forming cristae, especially two protruding rod-shaped long crests, crossed the lumen. More goblet cells were distributed in the midgut of SJP, and more goblet cells were distributed in hindgut of SJE.

To assess the variation in digestion and absorption of SJE and SJP, we examined the diversity in gut microbiota based on 16S rRNA sequences. A total of 125,348 and 123,777 reads were identified. The dominant length distributions were approximately 436 and 430 bps. We then evaluated a series of diversity metrics (Table 5) based on the rarefied OTU clustering (97% identity). There were a total of 82 OTUs in the SJE and 59 OTUs in the SJP, where 47 OTUs were shared between the two feed treatments. The alpha-diversity in microbiota assemblage of SJE and SJP is shown in Figure 3. Predominant species belonged to the class Alphaproteobacteria (SJP 76% vs. SJE 51%). The second largest group consisted of Mycoplasma, at 21% (SJP) and 45% (SJE). Acidobacteria, mainly of the genus *Rhodococcus*, were tenfold higher in the SJE (1%) when compared to the SJP (0.1%). The most abundant species in the SJP group were *Mycoplasma* (9.52%), *Leisingera* (4.76%), *Nautella* (4.76%), *Kordiimonas* (3.57%), *Tenacibaculum* (3.57%), *Acinetobacter* (2.38%) and *Alphaproteobacteria* (2.38%), while SJE was dominated by *Mycoplasma* (6.61%), *Bacteroidales_S24-7_group_norank* (4.96%), *Burkholderia–Paraburkholderia* (4.96%), *Leisingera* (3.31%), *Nautella* (3.31%), *Tenacibaculum* (3.31%) and *Blautia* (2.48%). The genera *Bacteroides* (2.38%), *Flavobacteriaceae*_unclassified (2.38%), *Muricauda* (2.38%), *Rhodospirillaceae*_uncultured (2.38%), *Flaviramulus* (1.19%), *Gsoil-1167_norank* (1.19%), *Ruminococcaceae_UCG-002* (1.19%) and *Sva0725_norank* (1.19%) were always found in the SJP. Four genera consisting of [*Eubacterium*]*_rectale_group* (1.65%), *Cyanobacteria_norank* (1.65%), *Enterobacter* (1.65%) and *S085_norank* (1.65%) were only found in the SJE.

3.4 | Effects of the diets on the level of toxicity and oxidative stress markers

The blood and liver of cuttlefish (SJE and SJP) were sampled to measure the activity of ALT, AST, GSH, MDA and SOD

TABLE 3 Evaluation of amino acids in SJE and SJP (mg/g N, wet basis, $n = 9$)

			SJE			SJP		
	FAO/WHO model	Egg standard	Mean	AAS	CS	Mean	AAS	CS
Ile	250	331	164.76	0.66	0.5	160.12	0.64	0.48
Leu	440	534	325.62	0.74	0.61	328.99	0.75	0.62
Lys	340	441	373.04	1.1	0.85	364.96	1.07	0.83
Met + Cys	220	386	140.69	0.64	0.36	144.22	0.66	0.37
Phe + Tyr	380	565	203.81	0.54	0.36	282.55	0.74	0.5
Thr	250	292	178.71	0.71	0.61	186.63	0.75	0.64
Val[a]	310	410	145.77	0.47	0.36	145.96	0.47	0.36

Note: Tryptophan was not measured. SJE: *Sepiella japonica* was fed by *Euphausia superba*; SJP: *Sepiella japonica* was fed by *Palaemon gravieri*; AAS: amino acid score referenced to FAO/WHO model; CS: chemical score referenced to Egg standard. Nitrogen coefficient = 6.25.

Abbreviations: DAA, delicious amino acids; EAA, essential amino acids except for Tryptophan; NEAA, unessential amino acids; TAA, total amino acids.

[a]The first limiting amino acid.

TABLE 4 Composition of fatty acids in PG, ES, SJE and SJP (%, *n* = 9)

	ES		PG		SJE		SJP	
Fatty acid	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*
C12:0	0.33	0.02	0.29	0.12	—	—	0.12	0.05
C13:0	0.12	0.03	0.16	0.01	5.35	0.08	—	—
C14:0	16.8	1.5	5.32	0.51	0.17	0.05	3.56	0.17
C14:1	0.33	0.05	—	—	—	—	—	—
C15:0	1.13	0.4	1.48	0.08	29.45	1.51	1.42	0.26
C16:0	9.08	0.59	26.75	0.35	0.97	0.12	29.22	1.41
C16:1	1.86	0.42	—	—	—	—	—	—
C17.632	3.2	0.23	7.23	0.56	1	0.06	11.89	0.24
C17:0	0.53	0.13	3.17	0.05	1.23	0.62	3.17	0.29
C17:1	—	—	1.55	0.49	—	—	0.25	0.02
C18:0	4	0.27	8.23	0.02	0.19	0.03	6.79	1.1
C18:1	9.66	0.02	10.56	0.35	2.09	0.3	2.06	0.72
C18:3n-3	5.24	0.21	12.67	0.84	—	—	0.28	0.11
C18:3n-6	1.73	0.35	—	—	0.5	0.1	—	—
C20:0	0.33	0.02	0.13	0.01	0.29	0.15	2.04	0.38
C20:1	2.93	0.09	—	—	—	—	—	—
C20:3n-3	—	—	—	—	3.79	0.22	—	—
C20:5n-3 (EPA)	16.33	0.35	13.12	1.43	27.26	0.32	20.61	1.08
C21:0	—	—	—	—	0.18	0.01	—	—
C22:6n-3 (DHA)	26.4	1.53	9.34	0.91	27.53	0.11	18.58	0.34

Note: SJE: *Sepiella japonica* was fed by *E. superba*; SJP: *Sepiella japonica* was fed by *Palaemon gravieri*; —: undetected.

Abbreviations: ES, *Euphausia superba*; PG, *Palaemon gravieri*.

(Figure 4). GSH and SOD in liver were significantly elevated (39.49 ± 5.16 μmol/g protein and 71.45 ± 4.28 U/mg protein respectively) compared to SJP (6.15 ± 0.61 μmol/g protein and 38.78 ± 1.56 U/mg protein) ($p < .05$). The activity of ALT in the blood of SJE was significantly reduced to 65.92 ± 14.71 U/L compared to SJP (107.34 ± 19.75 U/L ($p < .05$).

3.5 | Fluoride content

To explain the effect of the fluoride on growth performance, the fluoride content of *P. gravieri* and *E. superba* individuals, as well as the integral cuttlefish (SJE and SJP) were measured after 60 days of feeding (Figure 5a). With respect to *P. gravieri* (60 mg/kg), the fluoride content of *E. superba* was extremely high (264 mg/kg; $p < .01$). The fluoride concentration in the SJE group (11.67 mg/kg) was threefold higher than the SJP group (4 mg/kg; $p < .01$). Fluoride was measured in the muscle, liver, ink sac and cuttlebone of cuttlefish after 60 days of feeding (Figure 5b). The fluoride was concentrated mainly in the ink sac and cuttlebone of cuttlefish (SJE; both more than 140 mg/kg), with cuttlefish (SJE) significantly more fluoride than SJP ($p < .01$).

4 | DISCUSSION

Chinese cuttlefish (*S. japonica*) are intensively produced in some southeast Chinese coastal provinces (e.g. Zhejiang, Fujian, Guangdong). Given that production will intensify even further to meet the increased demand for cuttlefish, it is necessary to investigate a partial or total replacement of the shrimp, which are becoming concomitantly more expensive to use as a feed ingredient Feeding frozen shrimp is common in the artificial breeding of cuttlefish, but is associated with a seasonality and an expense. Cuttlefish seem to be able to adjust digestive enzyme activity according to their diet (Perrin, Bihan, & Koueta, 2004). Hence, to address the industrial and commercial demands of the cuttlefish (*S. japonica*) aquaculture system, this study investigated the replacement of raw frozen *P. gravieri* with raw frozen Antarctic krill *E. superba*. In this report, the growth performance of cuttlefish (SJE) was inferior to SJP, which suggests that complete replacement of the raw *P. gravieri* in the diet reduced growth efficiency of the cuttlefish. In agreement with our results, complete substitution of fish meal (FM) with Antarctic krill meal in Atlantic salmon (*Salmo salar* L.) diets led to a decrease in the SGR during days 110–126 after feeding (Rungruangsak-Torrissen, 2007). Additionally, weight gain, feed intake, specific growth rate and feed efficiency in Yellowtail (*Seriola quinqueradiata*) fed 100%

Antarctic krill meal were shown to be markedly lower than with partial replacement (0.0% and 15.4%) (Yoshitomi & Nagano, 2012). Previous studies have also concluded that total replacement with krill generally led to stagnant growth (Koops, 1979; Rungruangsak-Torrissen, 2007). However, partial replacement is an alternative strategy, producing either better performance (Olsen et al., 2006) or no effect at all (Yoshitomi et al., 2006). This is the first study to evaluate the effect of the use of Antarctic krill for the feeding of cuttlefish (*S. japonica*). Subsequent research will focus on graded supplementation with the krill in cuttlefish feeding.

In the present study, even though abundant glutamic acid was detected in SJE and SJP, there was no significant difference in essential amino acid profiles of the two groups. Other studies have also considered the replacement of fish meal with krill, for example in rainbow trout (Yoshitomi et al., 2006). The raw *E. superba* and cuttlefish (SJE) are a good source of fatty acids. EPA and DHA (over 50% total fatty acids) are markedly elevated in SJE. Antarctic krill and krill oil, in particular, is known to be rich in n-3 PUFAs (mainly DHA and EPA) and n-6 PUFAs (Castrogómez, Holgado, Rodríguezalcalá, Montero, & Fontecha, 2015; Gigliotti, Davenport,

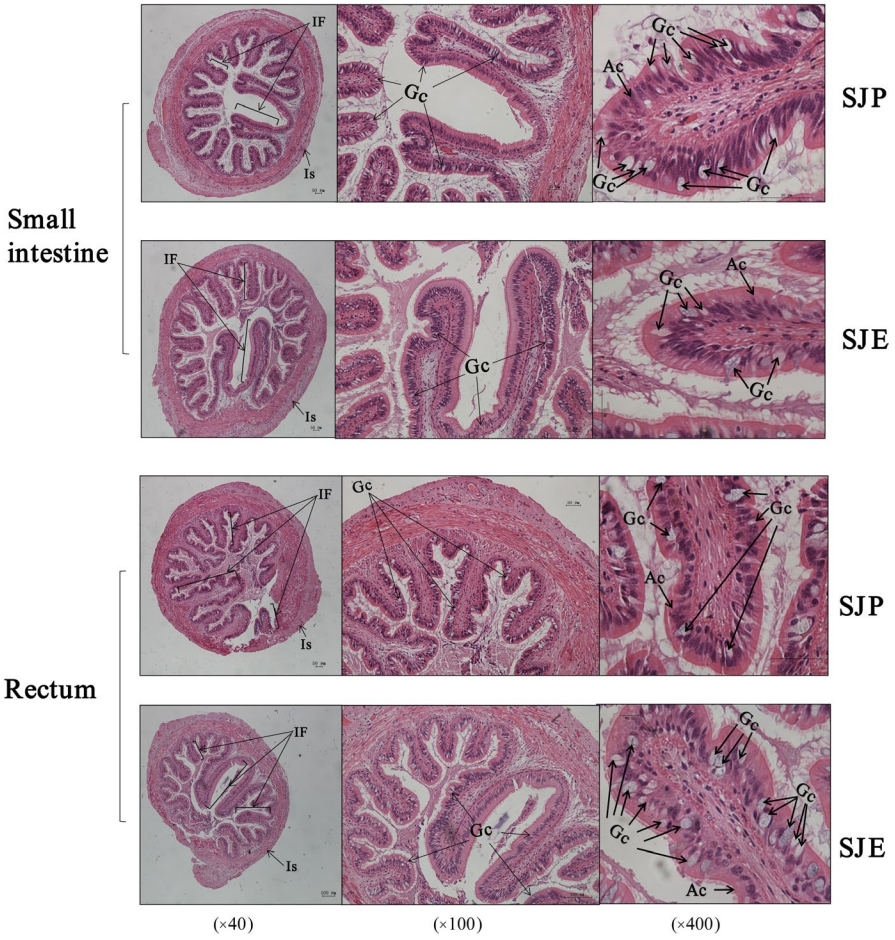

FIGURE 2 Cross-section of the midgut and hindgut of SJE and SJP at 60 days after feeding. SJE: *Sepiella japonica* was fed by *Euphausia superba*; SJP: *Sepiella japonica* was fed by *Palaemon gravieri*. The samples were embedded in ester wax, and sections 5–8 Mtm thick were routinely stained with HE stain. The cross-sections were examined in an Leica microscope. Ac, absorption cell; Gc, goblet cells; IF, intestinal folds; Is, intestinal serosa

TABLE 5 The diversity index after subsampling in SJE and SJP based on OTUs clustering at the 0.97 level of homology

ID	Subid	Reads	OTUs	Coverage	Ace	Chao	Shannon	Simpson
SJE	LX1	58,471	45	0.999779	60 (50, 85)	52 (47, 69)	0.7 (0.7, 0.71)	0.631 (0.627, 0.635)
	LX2	58,471	76	0.999969	77 (76, 83)	77 (76, 86)	1.49 (1.48, 1.5)	0.356 (0.353, 0.360)
SJP	ZC1	58,471	34	0.999807	68 (50, 107)	43 (36, 71)	1.01 (1, 1.02)	0.522 (0.518, 0.526)
	ZC2	58,471	50	0.99988	55 (51, 68)	53 (51, 66)	0.76 (0.75, 0.77)	0.677 (0.672, 0.682)

Note: SJE: *Sepiella japonica* was fed by *Euphausia superba*; SJP: *S. japonica* was fed by *Palaemon gravieri*. We randomly selected two individuals in each tank as a whole sample, separated their guts, gently squeezed digesta as well rinsed the entire intestine with PBS twice. Each treatment has two replicates (total individuals $n = 12$).

Beamer, Tou, & Jaczynski, 2011; Yoshitomi et al., 2006)). Raw *E. superba* provides a good source of dietary lipid and facilitates the increase of PUFAs in the cuttlefish. The evidence presented thus far supports the idea that the higher the substitution ratio with *E. superba*, the higher the PUFA content (Yoshitomi et al., 2007). Consequently, the ingestion of cuttlefish in the human has several benefits. These PUFAs regulate various biological processes and support various processes, like non-specific immune status, improving physiological functions in the brain (Sugiura et al., 2009), treating or alleviating inflammation (Belluzzi, 2002; Oliver, Mcgillicuddy, Phillips, Toomey, & Roche, 2010) and ameliorating or reducing oxidative stress (Rukkumani, Aruna, Varma, Rajasekaran, & Menon, 2004).

To assess whether a high lipid feed source could modify the non-specific immune status when fed to cuttlefish, toxicity and oxidative stress markers (ALT, AST, GSH, MDA and SOD) were measured. Compared to the levels of markers in SJP, a low level of ALT in the blood and a high level of GSH and SOD were found in the liver of cuttlefish (SJE). The marker activities may be associated with high lipid feed source. For example, dietary PUFAs may down-regulate the level of plasma ALT (Sekiya et al., 2003). Li et al. (2018) found that Antarctic krill oil (AKO) could reduce β-amyloid (Aβ) accumulation in the hippocampus by decreasing the MDA content and increasing the activity of SOD and glutathione peroxidase (GSH-Px) in SAMP8 mice. MDA in the SJE was graphically lower than in SJP and GSH was high both in the liver and serum of SJE cuttlefish. In the literature, n-3 PUFAs regulate the expression of PON1 and the activity of HCTLase in the liver and serum and restore PON1 and GSH (especially liver GSH) activity in parallel (Varatharajalu, Garige, Leckey, Gong, & Lakshman, 2010). Zhan, Zhou, and Shan (2009) indicated that PUFAs display synergistic anti-oxidation activity after synthesis of PUFA-modified SOD. Thus, PUFAs may be invoked in the activities of these toxicity and oxidative stress markers.

We further explored whether the variations in fatty acid profiles and non-specific immune performance are correlated with gastrointestinal tract structure as well as gut microbiome assemblages. There were no significant differences in the morphology of the midgut and hindgut slices between the SJE and SJP groups. The midgut and hindgut generally contain numerous goblet cells that are

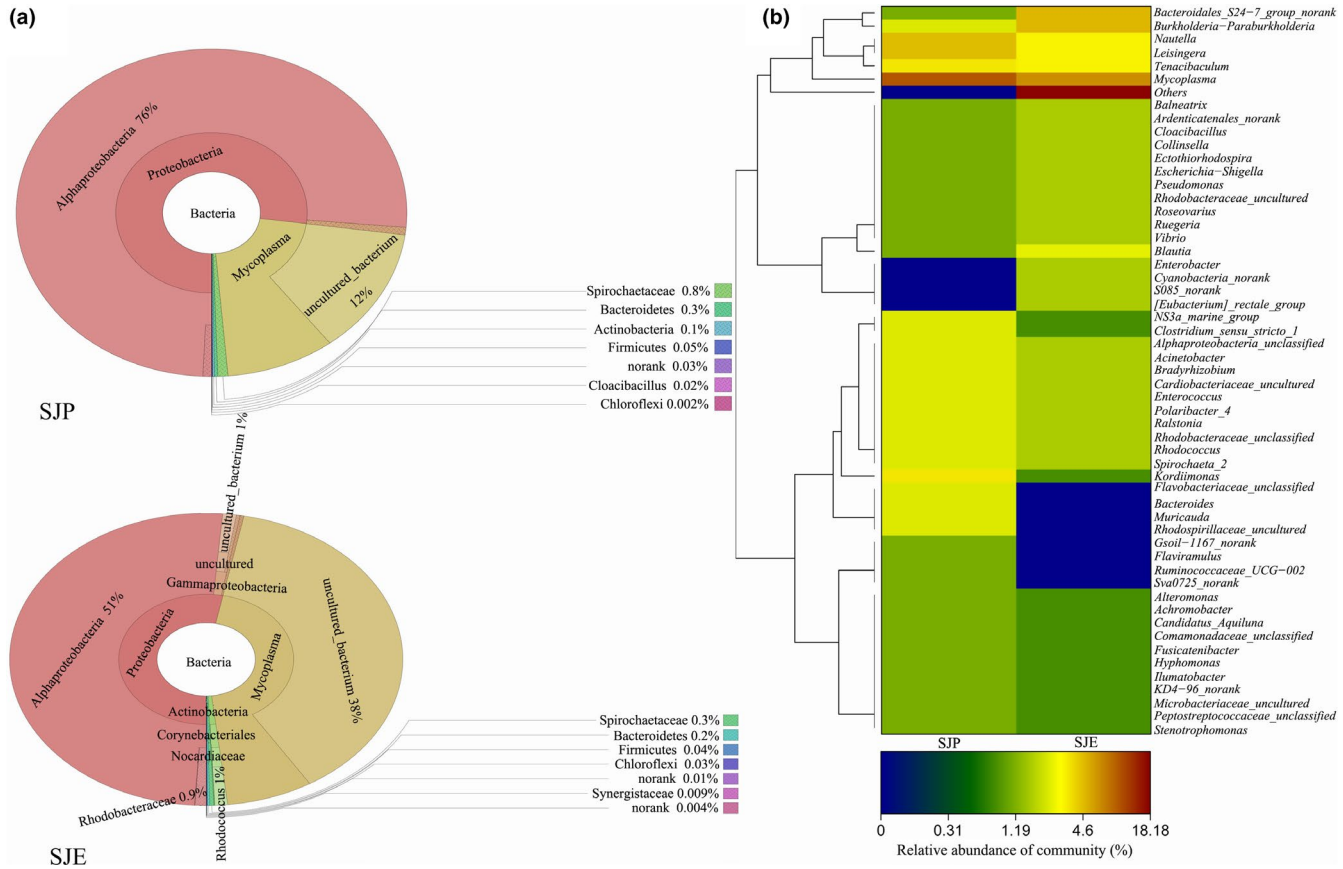

FIGURE 3 The alpha- and beta- diversity in SJE and SJP at 60 days after feeding. The mixtures were pyrosequenced using a Roche 454 Genome Sequencer FLX Titanium platform (Majorbio Bio-Pharm Technology Co. Ltd.). Subsampling of each treatment and control group was analysed. SJE: *Sepiella japonica* was fed by *Euphausia superba*; SJP: *Sepiella japonica* was fed by *Palaemon gravieri*. (a) The taxonomic distributions at phylum levels of SJE and SJP. (b) The hierarchical heatmap was built considering the abundant bacterial communities at the genus level. Considering the small size of the cuttlefish and the minimum sample amount for DNA extraction, we randomly selected two individuals in each tank as a whole sample, separated their guts, gently squeezed digesta as well rinsed the entire intestine with PBS twice. Each treatment has two replicates (total individuals n = 12)

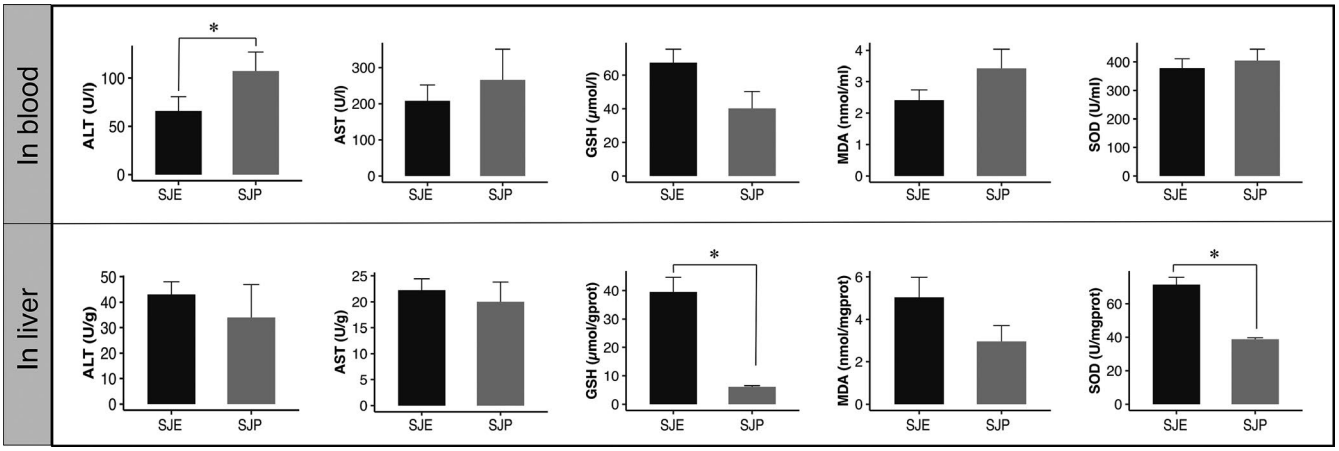

FIGURE 4 The level of 5 immunological enzymes in blood and liver of SJE and SJP at 60 days after. SJE: *Sepiella japonica* was fed by *Euphausia superba*; SJP: *Sepiella japonica* was fed by *Palaemon gravieri*. Superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), alanine transaminase (ALT) and aspartate aminotransferase (AST) were determined by according to the manufacturer alternative protocol of relative assay kits. The data are represented Mean ± *SE*. **p* < .05

thought to be involved in the active transport of potassium (Flower & Filshie, 1976), and production and maintenance of the protective mucus (Specian & Oliver, 1991). In this study, more goblet cells were found in the midgut of SJP and in the hindgut of SJE. We assume that the amount of goblet cells is related to the gut microbiota diversity. Sommer and Bäckhed (2013) indicated the gut microbiota affected and modulated goblet cells in humans, but the underlying mechanism is not known. Gut goblet cells are also associated with inflammatory responses.

The gastrointestinal tract, is known to harbour a diverse array of microbial species (Delport, Power, Harcourt, Webster, & Tetu, 2016). The microbiota are indispensable for host regulation of daily digestive functions (e.g. nutrient absorption and energy regulation) and maintenance of several metabolic processes such as innate immune defence, gut microbial metabolism and gut tissue maturation (Bevins & Martinporter &Ganz, 1999; Flint, Scott, Louis, & Duncan, 2012; Krajmalnik, Ilhan, Kang, & DiBaise, 2012; Nicholson et al., 2012; Tremaroli & Bäckhed, 2012). It is essential to better characterize the dominant commensal bacteria, community profiles and system characteristics that produce stable gut communities beneficial to health (Nicholson et al., 2012). In this study, most species belonged to the class Alphaproteobacteria in shrimp and krill, and the percentage of the total microbiome varied from 76% (SJP) to 51% (SJE). Based on the alpha-diversity metrics, the gut microbiome in the SJE group was more diverse than the SJP group. The most abundant species in the SJP were *Mycoplasma*, *Leisingera*, *Nautella*, *Kordiimonas* and *Tenacibaculum*, while the gut microbiota in the SJE group were dominated by *Mycoplasma*, *Bacteroidales_S24-7_group_norank*, *Burkholderia–Paraburkholderia*, *Leisingera*, *Nautella* and *Tenacibaculum*. Compared to SJP, the SJE exhibited an increased abundance of the genera *Bacteroidales_S24-7_group_norank* and *Burkholderia–Paraburkholderia*. The genus *Bacteroidales_S24-7_group_norank* belongs to the phylum Bacteroidetes. The S24-7 family is involved in host–microbe interactions known to impact on gut function and health (Ormerod et al., 2016), in addition to being actively involved in metabolizing chondroitin sulphate (Shang et al., 2016). In this study, more diversity of microbiota and higher n-3/n-6 PUFAs were identified in cuttlefish (SJE). It may be attributable to higher *Bacteroides* in the SJE (Andersen, Mølbak, Thymann, Michaelsen, & Lauritzen, 2011). The four genera [*Eubacterium*]*_rectale_group*, *Cyanobacteria_norank*, *Enterobacter* and *S085_norank* were found only in the SJE. *E. rectale* is a species commonly recovered from human faeces and produces mixtures of organic acids from carbohydrates as fermentation products (Oren, 2001). Hence, we can assume that the variation in gut microbiota is related to the absorption of polyunsaturated fatty acids. Our findings provide a basis for the future development of exogenous microbial additives for aquatic animals.

Fluoride in krill is inevitable and is derived mainly from the exoskeleton or carapace (Soevik &Braekkan, 1979; Wang, Xue, Wang, & Yang, 2011). Consequently, fluoride accumulates in the bone of animals that are fed krill. Several studies have reported that excess fluoride can affect the growth performance of animals (Nunes, Sá, & Sabry, 2011; Yoshitomi et al., 2007; Yoshitomi &Nagano, 2012). In this study, fluoride was concentrated mainly in the ink sac and cuttlebone of the cuttlefish. The cuttlebone is a rigid buoyancy tank that imposes a depth limit on *Sepia*, and whose morphology confers strength against implosion from hydrostatic pressure and, therefore, the greater the strength, the higher the cuttlebone density (Sherrard, 2000). When removing the krill exoskeleton, the fluoride content may be reduced (Jung et al., 2013; Yoshitomi & Nagano, 2012). Fluoride may affect the activity of antioxidant enzymes, including SOD and GSH. Dose- and time-dependent administration of fluoride decreased SOD and GSH and increased MDA in the carp juveniles (Chen et al., 2015). Gonadal retardation was observed in cuttlefish (SJE), and the GI_{SJE} was lower than GI_{SJP}. In a previous report, Wang et al. (2017) indicated that excessive fluoride intake can reduce oocyte developmental potential, indicating that removal of the exoskeleton

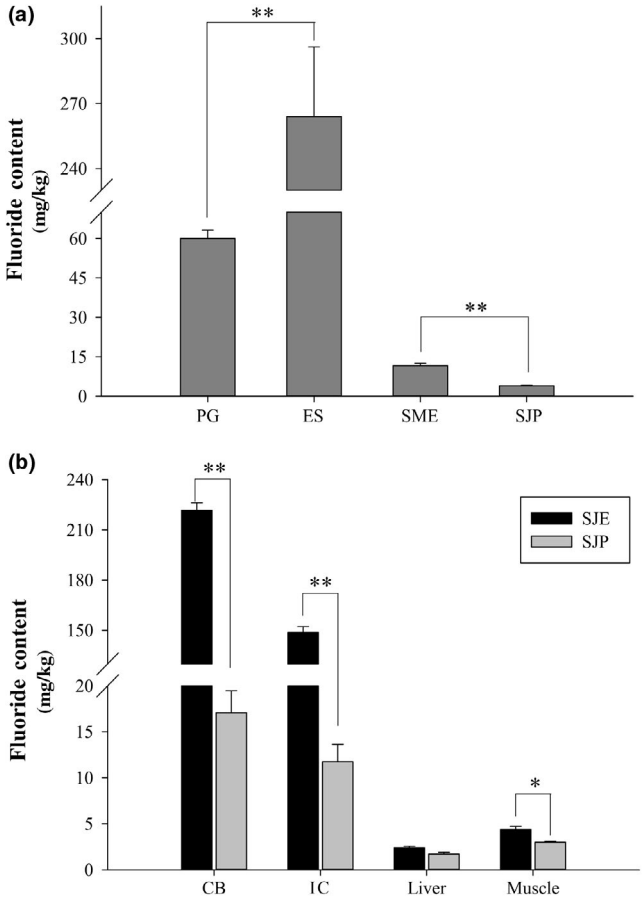

FIGURE 5 The fluoride content of *Euphausia superba*, *Palaemon gravieri*, SJE and SJP at 60 days after feeding. ES: *E. superba*; PG: *P. gravieri*; SJE: *Sepiella japonica* was fed by *E. superba*; SJP: *Sepiella japonica* was fed by *P. gravieri*. (a) The whole *P. gravieri* and *E. superba* and of the whole SJE and SJP at 60 days after feeding. (b) Some tissues and organic including muscle, liver, ink sac (IC) and cuttlebone (CB) of SJE and SJP at 60 days after feeding. Each tank, three individuals were randomly collected; totally, nine individuals were sampled to evaluate fluoride content. The data are represented Mean ± *SE*. $^{*}p < .05$; $^{**}p < .01$

or lowing the fluoride content would have to be considered if Atlantic krill were to be used as feed for cuttlefish in the future.

In conclusion, our findings suggest that the Antarctic krill *E. superba* can replace *P. gravieri* as feed for cuttlefish (*S. japonica*). *E. superba* enhanced PUFA (i.e. EPA and DHA) content and increased the gut microbial diversity of *S. japonica*. The variations in PUFAs may be associated with the alteration in gut microbiota assemblages. As follow-up studies, we will adopt partial replacement strategies of *E. superba* in the diet of cuttlefish to investigate growth, digestive ability, feed consumption, fluoride safety as well as the relationship between gut microbiota and PUFAs.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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