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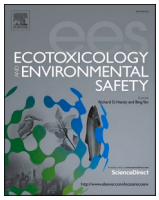

Dietary soybean oil aggravates the adverse effects of low salinity on intestinal health in juvenile mud crab *Scylla paramamosain*

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ABSTRACT

Salinity is one of the important factors affecting the physiological state of crustaceans in marine environments. Lipid plays major roles in energy supply and is main sources of essential fatty acids for membrane integrity, which is critical in adaptations to changes in salinity. Here we evaluated the effects of salinity (medium, 23 ppt and low, 4 ppt) and dietary lipid source (fish oil, FO and soybean oil, SO) on intestinal health of the marine crustacean mud crab *Scylla paramamosain*. The results indicated that low salinity and dietary SO (LSO group) significantly affected intestinal histomorphology, with a significant decrease of intestinal fold height and width as well as down-regulation of intestinal mRNA levels of tight junction genes compared to crab reared at medium salinity and fed FO diets (MFO group). Crabs reared at low salinity and fed SO showed an increased inflammatory response in intestine, which stimulated a physiological detoxification response together with apoptosis compared to crab in the MFO group. Low salinity and SO diets also could be responsible for multiply the pathogenic bacteria of Photobacterium and inhibit the beneficial bacteria of Firmicutes and Rhodobacteraceae in intestine, and act on a crucial impact on the development of intestinal microbial barrier disorders. The results of microbial function predictive analysis also support these inferences. The findings of the present study demonstrated that soybean oil as the main dietary lipid source could exacerbate the adverse effects of low salinity on intestinal health of mud crab, and provided evidence suggesting that dietary lipid source and fatty acid composition may play vital roles in intestinal health and the process of adaptation to environmental salinity in marine crustaceans.

1. Introduction

Salinity is a crucial element in marine environment and influences physiological and metabolic condition of marine organisms in various ways, including growth (Xiong et al., 2020), development (Fridman et al., 2012), reproduction (Yen and Bart, 2008), osmoregulation (Abou Anni et al., 2016) and energy metabolism (Zhou et al., 2020). However, seawater salinity can decrease frequently due to tidal fluctuations, typhoons or heavy rains, especially in estuaries areas (Ma et al., 2019). Meanwhile, aquaculture production of euryhaline crustaceans has recently expanded from the coastal environment to inland regions with a supply of underground low saline water (water salinity around 1–6 ppt) in order to make use of the cleaner water resource and economic progress in these areas (Li et al., 2017). In response to the low salinity environment, marine crustaceans must develop the particular adaptations and biological properties (Péqueux, 1995). Many studies in osmoregulation have shown that the response of marine crustaceans includes changes in ion channel enzyme, bioamine and free amino acid to adjust the balance of osmotic pressure, with gill being the main functional tissue (McNamara and Faria, 2012). But in the aspect of intestinal barriers and health effected by salinity, the related research is less on euryhaline crustaceans (Liu et al., 2020; Zhang et al., 2016).

Dietary lipids able to positively impact on the adaptation of crustaceans in low salinity water (Chen et al., 2015). Firstly, lipids provide energy to hold the bodily humor stabilization as well as the osmotic balance (Tseng and Hwang, 2008). Furthermore, omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) can increase the unsaturation of cell membranes required to guarantee the regular operation of

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membrane proteins and ion transport (Monroig and Kabeya, 2018). Fish oil (FO), with a high content of n-3 LC-PUFA, has traditionally been the foremost lipid source utilized in aquatic feed due to its high-efficiency energy conversion and LC-PUFA (Betancor et al., 2015). However, terrestrial vegetable oils, such as soybean oil (SO) have been increasingly used in commercial aquafeed as a consequence of the limited availability of FO to supply the increasing demand for this product (Montero et al., 2015). Inevitably, using vegetable oils as the dietary lipid source may raise nutritional issues, due to its lack of LC-PUFA and imbalances proportion of n-3/n-6 fatty acids (FAs) (Turchini et al., 2009). Therefore, there is a pressing need to understand how diverse dietary lipid sources influence low salinity adaptation in aquatic organisms. Some studies in Pacific white shrimp *Litopenaeus vannamei* cultured under low salinity water demonstrated that using soybean oil as the main dietary lipid source led to retarded growth performance and poor survival rate compared to shrimp fed a blend of oils as the main lipid source (Chen et al., 2015). The same authors also showed that increased dietary n-3 PUFA contents would enhance the osmoregulation ability of Pacific white shrimp cultured under low salinity (Chen et al., 2019). Consistently, dietary supplementation with n-3 PUFA also increased the survival of mud crab maintained in a low salinity environment (Dan and Hamasaki, 2010).

Intestinal health is important for optimal growth, related to intestinal barrier functions in aquatic organisms (Ding et al., 2021; Song et al., 2017). The intestinal barrier composed of multilayer of defensive system comprising a physical barrier, epithelial immune system and endogenous microbiota (Jutfelt et al., 2007). The physical barrier main constitute by enterocyte, tight junctions and peritrophic membrane (Suo et al., 2017), while intestinal immune barrier can activating intestinal inflammation and inflammatory-induced apoptosis to break the invasion of pathogens (Chen and Tang, 2013). The intestinal microbiome also plays a pivotal role in regulating host immune homeostasis and metabolic functions (Qiao et al., 2019), and the three intestinal barriers also interact with each other to maintain intestinal operation and homeostasis (Limbu et al., 2019). In order to adapt to low salinity environment, intestinal fluid transport increases by increased epithelial paracellular permeability and enhanced the activities of NaK-ATPase (Sundell et al., 2003). Augmented permeability of intestine maybe promote the transfer of possibly harmful substances, such as disease germ and virus (Jutfelt et al., 2007). Meanwhile, intestine is the first tissue exposed to the diet and, therefore, dietary FAs profile probably liable to change the composition of enterocyte membranes (Jutfelt et al., 2007). In addition, vegetable oils have different FAs compositions compared with fish oils, which will cause a series of metabolic alterations including changed membrane lipid fluidity, suppressed epithelial enzyme activities and impaired intestinal barrier (Barton et al., 1992; Chen et al., 2020; Gao et al., 2020). Overall, both low salinity and vegetable oils have the potential risk of damaging the intestinal barrier. Nevertheless, the potential influence of salinity and dietary lipid source on the intestinal health of crustaceans is largely unknown.

Mud crab, *Scylla paramamosain*, is a euryhaline species with good ability to adapt to different salinity, and is widely distributed in estuarine, shallow sea, inner bay and mangrove systems (Li et al., 2018) that frequently encounter decreased water salinity. Mud crab is also one of the new and developing species in aquaculture suitable for the exploration of inland low-salinity water culture. Thus, this species could provide an excellent model to study the physiological and biochemical changes in response to various osmotic stresses and, in addition, the study of adaptation to low salinity in mud crab could have great practical significance. Hence, the aims and objectives of the this study were to expand our understanding of the effects of altered salinity on intestinal health of marine crab and also explore lipid nutrition strategies to improve the adaption to low environmental salinity. To achieve this, mud crabs were fed for 6 weeks with two diets containing FO or SO as added lipid at two water salinities, medium salinity 23 ppt and low salinity 4 ppt. The salinity values were chosen according to the optimal growth salinity range for mud crab (medium salinity) and the prescribed minimum salinity to guarantee survival in mud crab (FAO, 2011). Analyses of intestinal structure, inflammatory reactions, apoptosis and microbiota composition were conducted in order to assess impacts on the intestinal barrier condition of mud crab.

2. Material and methods

2.1. Compliance with ethic guidelines

The terms in animal welfare and settlement executed in this study were agreed by Animal Research and Ethics Committees of Ningbo University.

2.2. Experimental diets

Two isonitrogenous and isolipidic experimental diets (48% protein, 10% lipid) containing either FO or SO (non-genetically modified product) as main lipid sources were formulated to meet the nutrient requirements of mud crab juveniles (NRC, 2011) (Table S1). FAs profile of experimental lipid sources or diets are shown in Tables S2 and S3, respectively. Peruvian fishmeal, soybean meal, krill meal and soybean protein concentrate were used as protein sources, whereas wheat flour was used as the main carbohydrate source. The feeds were prepared refer to the process depicted in detail previously (Luo et al., 2020b), stored in vacuum-packed bags and −20 °C in order to keep high-quality until used in feeding trial.

2.3. Feeding trial conditions

Feeding trial was carried out in the experimental feed Center of Ningbo University Meishan Campus (Ningbo, China) and juvenile mud crabs were procured from a local farm. Before the feeding trial, crabs were cultured under 50 L recirculation aquaculture system (RAS) aquaria (48.3 × 28.4 × 38 cm) for two weeks to acclimate to the laboratorial environment. Water was continuously purified by a series of filtration treatments including mechanical and bio-filter systems followed by ultraviolet (UV) treatment with controlled water temperature. Crabs were exposed to an artificial light regime of approximately 12 light and 12 dark (8:00 a.m. to 8:00 p.m.). The low salinities water were obtained by diluting natural seawater with freshwater. Crabs in the low salinity groups were acclimated from 23 ppt to 4 ppt salinity by decreasing 2 ppt/day over approximately 10 days before the feeding trial. During this period, crabs fed with the same diet (45% protein, 10% lipid, with blended vegetable and fish oil as the lipid source). Sum of 180 crabs with healthy and actively eating were chosen as experimental crabs (sex ratio was 1:1; initial weight 22.08 ± 0.82 g; 90 crabs adapted to medium salinity and 90 crabs adapted to low salinity) were randomly distributed into 180 aquaria with one crab in each aquarium to prevent crab aggression. Each experimental treatment (MFO, moderate salinity, fed FO diet; MSO, moderate salinity, fed SO diet; LFO, low salinity, fed FO diet; LSO, low salinity, fed SO diet) was randomly allocated for three replicates (n = 45 crabs per treatment). During the 6-week experimental period, crabs were hand-fed once daily at 6:00 p.m. with a daily ration of 3–6% of crab weight, with feeding ration adjusted daily according to actual consumption and residual feed in order to maintain a level of apparent satiation. Faeces and uneaten feed were removed by siphoning daily, 40% of the aquarium water was renewed every two days to keep the water quality. Parameters involved in water quality were measured weekly, the mean values as follows (mean ± SEM): salinity (low salinity group 4.00 ± 0.20 ppt and medium salinity group 23.00 ± 0.50 ppt), temperature 24.5 ± 1.10 ℃, dissolved oxygen 7.50 ± 0.88 mg/L, ammonia nitrogen 0.02 ± 0.00 mg/L, and pH 7.35 ± 0.21.

2.4. Sample collection

After feeding experiment, all crabs fasted 24 h and anaesthetized with MS-222 (20 mg/L). The same segment of middle intestine (0.5–1.0 cm) was dissected from two crabs per replicate and the intestinal contents removed before being placed in 2 ml microfuge tubes containing 4% paraformaldehyde solution for histological examination (1 crab per replicate, n = 3). Middle intestine was rapidly collected from another four crabs per replicate and stored at −80 °C before enzymatic activity and qPCR assay (pools of 2 crabs per sample, n = 6). The whole intestines of a further four crabs per replicate were aseptically dissected and stored at −80 °C before intestinal microbial assay (pools of 4 crabs per replicate, n = 3). Hemolymph from the same four crabs were sampled and incubated for 24 h at 4 °C prior to centrifugation (1467×*g*, 10 min), and stored at − 80 °C before analysis of enzymatic activity (pools of 2 crabs per sample, n = 6).

2.5. Intestinal morphology analysis

Analysis of intestinal morphology was conducted refer to methods showed by Xiong et al. (2018). Briefly, following intestine tissue fixation in 4% paraformaldehyde, embedded in paraffin, sliced of 4 μm were stained with H&E, then tested the intestinal fold height, fold width and muscularis thickness using a light microscope (Olympus, DP72) and ImageJ software. For each tissue sample, 10 measurements were taken (n = 30).

2.6. Enzymatic activity analysis

Intestine samples were weighed and homogenized in 9 vol of ice physiological saline, followed centrifuged as above. The activities of alkaline phosphatase (AKP) and acid phosphatase (ACP) in hemolymph supernatant and intestinal homogeneous supernatant were assayed using related assay kits (Jiancheng Bio. Inst.) and Multiskan spectrum (Thermo) refer to the corresponding protocol.

2.7. Gene expression analysis

RNA extraction, reverse transcription and real-time quantitative PCR were conducted according to methods described in detail previously (Luo et al., 2020b). Briefly, RNA was extracted and reverse transcribed by TRIzol Reagent (Vazyme, China) and HiScript® RT SuperMix Reagent kit (Vazyme, China), respectively. Specific primers are shown in Table S4. Reaction components and cycle conditions of PCR amplification described on previous study (Luo et al., 2020b). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with the β-actin used as the housekeeping gene and the MFO group used as the control/reference group.

2.8. Intestinal microbial analysis

DNA extraction and quality verification, PCR amplification, high-throughput sequencing and bioinformatic analysis were performed refer to the previous study (Sun et al., 2018). Briefly, total bacterial community DNA of crab intestine was isolated with a TIANamp Micro DNA Purification Kit (Tiangen, China). Quality and quantity of DNA was verified by NanoDrop 2000 and 1% agarose gel electrophoresis. Bacterial DNA was used as the template for 16S rRNA gene amplification with universal primers 343F (5′-TACGGRAGGCAGCAG-3′) and 798R (5′-AGGGTATCTAATCCT-3′). All PCR reactions and thermal cycling conditions followed those described in detail previously (Sun et al., 2018). Purified PCR products were subjected to high-throughput sequencing on an Illumina HiSeq 2500 platform (USA).

Processing of Illumina sequencing data was conducted according to methods described in detail previously (Sun et al., 2018; Yuan et al., 2019a). Briefly, the raw pair-end readings obtained were subjected to quality control procedures using Quantitative Insights into Microbial Ecology software (QIIME, version 1.17) (Caporaso et al., 2010). The qualified reads were clustered to generate operational taxonomic units (OTUs) at the 97% similarity level using UPARSE (version 7.1) (Edgar, 2013), and chimeric sequences identified and removed using UCHIME (Edgar, 2016). Taxonomic richness and diversity estimators were determined using the Mothur software. Ace (http://www.mothur.org/wiki/Ace/) and Chao1 (http://www.mothur.org/wiki/Chao/) were used to reflect community richness. Diversity was assessed using Shannon (http://www.mothur.org/wiki/Shannon/) and Simpson (http://www.mothur.org/wiki/Simpson/). All these indices were estimated based on operational taxonomic unit (OTU) abundance matrices. The mean of the estimated richness was used for comparisons among samples.

Microbial function was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, http://galaxy.morganlangille.com/) (Langille et al., 2013) and the detail referred to the methods of Zhao et al. (2015). The OTUs were mapped to database at 97% similarity by QIIME's command. OTU abundance was normalized automatically using 16S rRNA gene copy numbers from known bacterial genomes in Integrated Microbial Genomes (IMG). The predicted genes and their functions were aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The predicted genes of average expression level of TOP 500 were selected for volcano plot analysis, with fold-change >1.5 and P value <0.05 selected as the filtering criteria for significant differential genes. Two-side Welch's *t*-test was used for two-group analysis of the TOP 15 functional pathways of microbes.

2.9. Statistical analysis

For principal component analysis (PCA) and heat map visualization analysis, all data were homogenized using the online program APT-BioCloud (http://cloud.aptbiotech.com). Differences among the means were tested by one-way ANOVA followed by Tukey's multiple-range test. Data throughout the text are expressed as means ± SEM as indicated, and asterisks indicating differences between treatment groups. In addition, the differences among water salinity (4 and 23 ppt) and diet (FO and SO) were analyzed by two-way ANOVA followed by Tukey's multiple comparison test (Results as shown in Table S5). The 200 OTUs with the highest abundance levels in crab intestine were selected for Pearson correlation analysis with intestinal structure, inflammation and apoptosis parameters, with the 30 OTUs with the highest r values further analyzed.

3. Results

3.1. Intestinal structure

Two-way ANOVA showed that both salinity and diet significantly affected intestinal structure ($P < 0.05$) (Table S5). Specifically, the histological sections of the intestine (Fig. 1) showed crabs in LSO group had a significant lower height of intestinal folds compared to crabs in MFO group ($P < 0.05$) (Fig. 1e and Table S6). The width of the intestinal folds also decreased significantly in both experimental groups maintained at low salinity ($P < 0.01$) (LFO and LSO; Fig. 1f and Table S6). However, no significant differences in the thickness of the intestinal muscularis were observed across the four experimental groups ($P > 0.05$) (Fig. 1g).

In addition, diet and, especially salinity significantly affected the mRNA expression of intestinal structure protein ($P < 0.05$) (Table S5). Accordingly, low salinity groups (LFO and LSO) had the significant lower mRNA levels of *CLAUDIN* and *Peritrophin-44-like* (*PT-44*) in low salinity groups (LFO and LSO) compared to the MFO group ($P < 0.05$) (Fig. 1h). In addition, significantly lower gene expression of *ZO-1* was observed in LSO-fed crabs when compared to crabs fed MFO ($P < 0.01$)

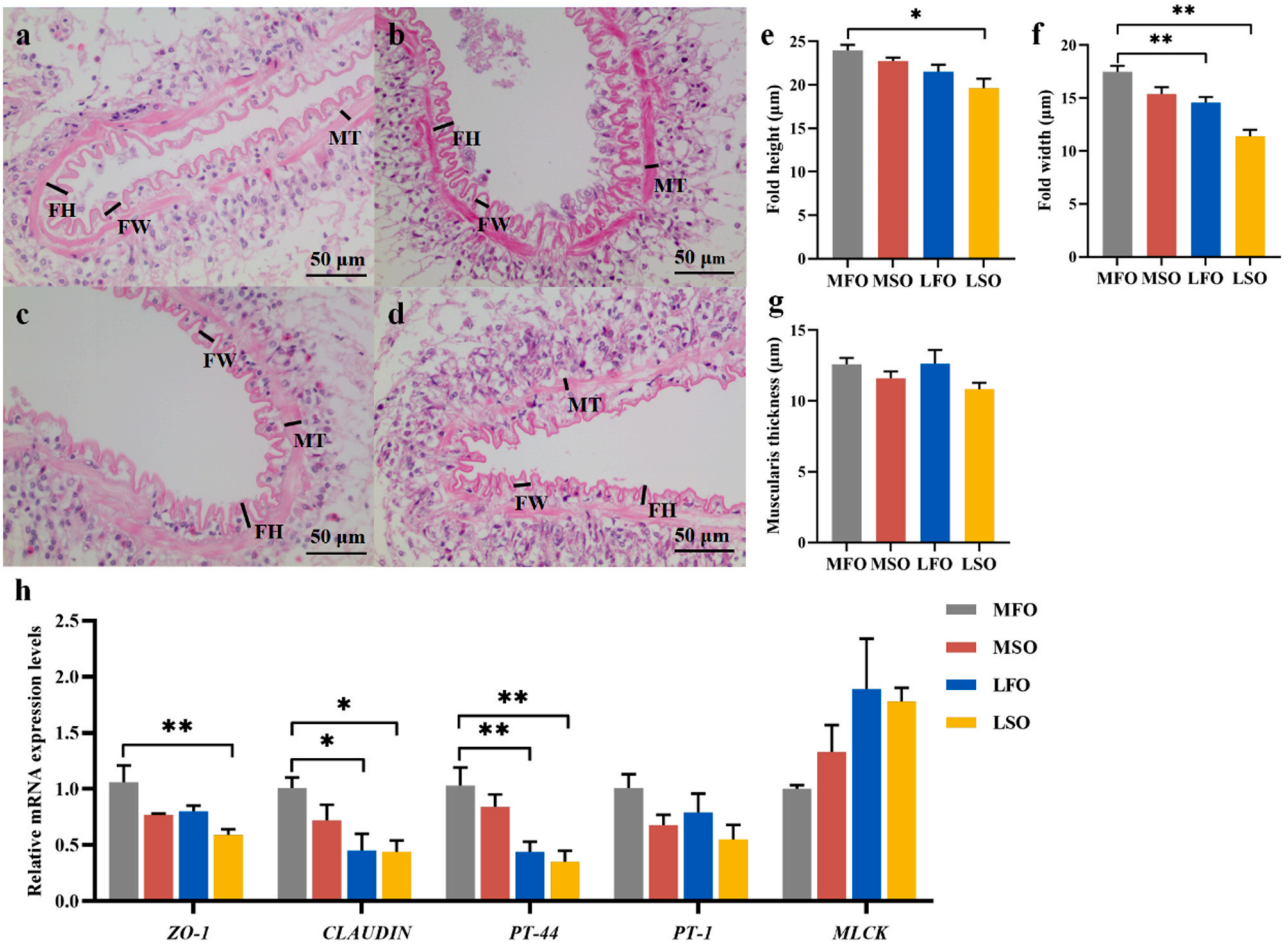

Fig. 1. The intestinal histological structure and mRNA expression involved into physical barrier in mud crab. Histological sections of intestine are shown in MFO (a), MSO (b), LFO (c) and LSO (d) groups under 200× magnification, respectively (n = 3). The marked black lines indicate fold height (FH) with data provided in panel (e), fold width (FW) with data provided in panel (f), and thickness of intestinal muscularis (MT) with data provided in panel (g) (n = 30). (h) relative mRNA expression of *ZO-1*, *CLAUDIN*, *PT-44*, *PT-1* and *MLCK* (n = 6). Asterisks (* and **) express remarkable ($p < 0.05$) and greatly remarkable ($p < 0.01$) differences among medium salinity with fish oil diet (MFO) and medium salinity with soybean oil diet (MSO), low salinity with fish oil diet (LFO) and low salinity with soybean oil diet (LSO).

(Fig. 1h). However, no remarkable differences were found in the mRNA levels of both *Peritrophin-1-like* (*PT-1*) and *myosin light chain kinase* (*MLCK*) ($P > 0.05$) (Fig. 1h).

3.2. Intestinal inflammation related enzymatic activity and gene expression

The enzymatic acticity and mRNA levels of several genes involved in intestinal inflammation were significantly affected by salinity and, to a lesser extent, diet ($P < 0.05$) (Table S5). In detail, activities of AKP in hemolymph were dramatically increased in LSO group than other group ($P < 0.05$). Low salinity dramatically decreased the activities of AKP in intestine than medium salinity ($P < 0.05$). And the activities of ACP in intestine were dramatically decreased in LSO group than MFO group ($P < 0.05$). Moreover, crabs fed LSO had significantly lower expression levels of anti-inflammatory cytokines (*TGF* and *ALF3*) than crabs fed MFO, particularly in the case of *ALF3*, where highly significant differences were observed ($P < 0.05$). In addition, LSO-fed crabs displayed generally higher mRNA of pro-inflammatory cytokines genes (*LITAF*, *RAB6A*, *RELISH*) than carbs fed MFO, albeit it was only significant in the case of *RELISH* ($P < 0.05$) (Fig. 2).

3.3. Intestinal apoptosis related gene expression

Salinity significantly regulated in mRNA levels related to CASPASE apoptotic signal pathway whereas diet had much less impact ($P < 0.05$) (Table S5). The specific data showed that low salinity and dietary SO (LSO group) led to significantly increased intestinal genes expression of *CASPASE7* and *CASPASE8* than medium salinity group ($P < 0.05$), regardless of the dietary treatment (MFO and MSO groups) (Fig. 3a). In addition, expression level of *CASPASE3* was dramatically up-regulated in LSO group than MFO group ($P < 0.05$) (Fig. 3a). Additionally, low salinity significantly increased mRNA levels of anti-apoptotic protein (*BCL2*), and decreased mRNA levels of apoptosis regulator (BCL2 associated x protein, *BAX*) compared to crabs reared at medium salinity ($P < 0.05$) (Fig. 3b). There was a trend towards increased expression of *P38 MAPK* in intestine in the LSO group, although not statistically significant ($P > 0.05$) (Fig. 3b).

3.4. Intestinal microbial communities

Proteobacteria, Tenericutes, Fusobacteria and Firmicutes were dominant in intestine of *S. paramamosain*. The proportion of Proteobacteria, ranged from 39.4% to 56.7%, followed by Tenericutes ranging from 4.3% to 33.0% (Fig. 4a). Crabs fed with SO diets significantly increased the abundance of Fusobacteria than crabs fed with FO diets

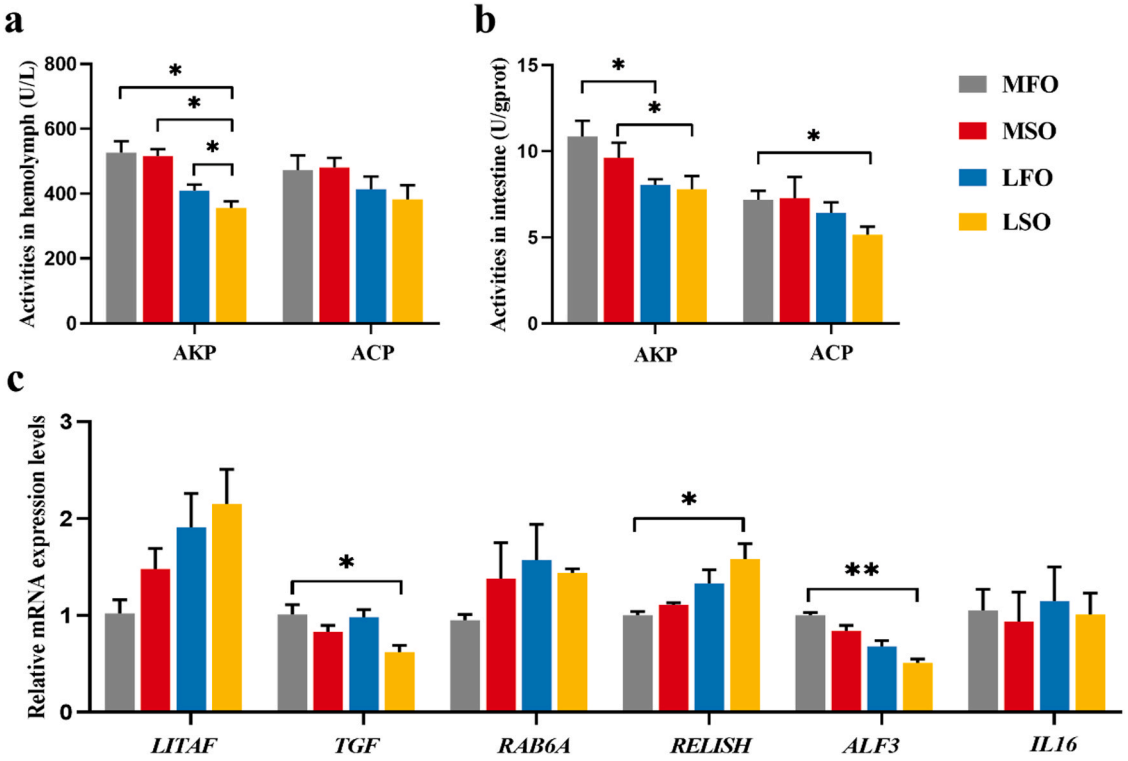

Fig. 2. The enzymes activities and genes expression levels related to inflammation in mud crab (n = 6). The activities of AKP and ACP in hemolymph and intestine, respectively (a and b). mRNA expression of inflammation related genes including *LITAF*, *TGF*, *RAB6A*, *RELISH*, *ALF3* and *IL16* in intestine (c). Asterisks (* and **) express remarkable ($p < 0.05$) and greatly remarkable ($p < 0.01$) differences among medium salinity with fish oil diet (MFO) and medium salinity with soybean oil diet (MSO), low salinity with fish oil diet (LFO) and low salinity with soybean oil diet (LSO).

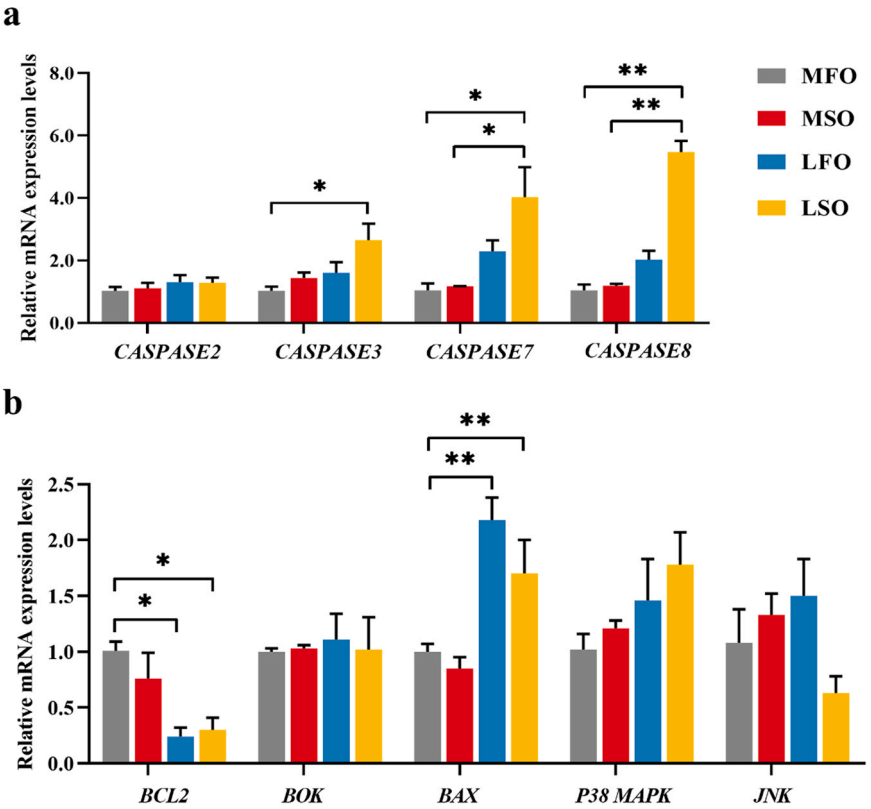

Fig. 3. The expression levels of apoptosis related genes in mud crab intestine (n = 6). (a) *CASPASE 2, 3, 7 and 8.* (b) *BCL2, BOK, BAX, P38 MAPK and JNK.* Asterisks (* and **) express remarkable ($p < 0.05$) and greatly remarkable ($p < 0.01$) differences among medium salinity with fish oil diet (MFO) and medium salinity with soybean oil diet (MSO), low salinity with fish oil diet (LFO) and low salinity with soybean oil diet (LSO).

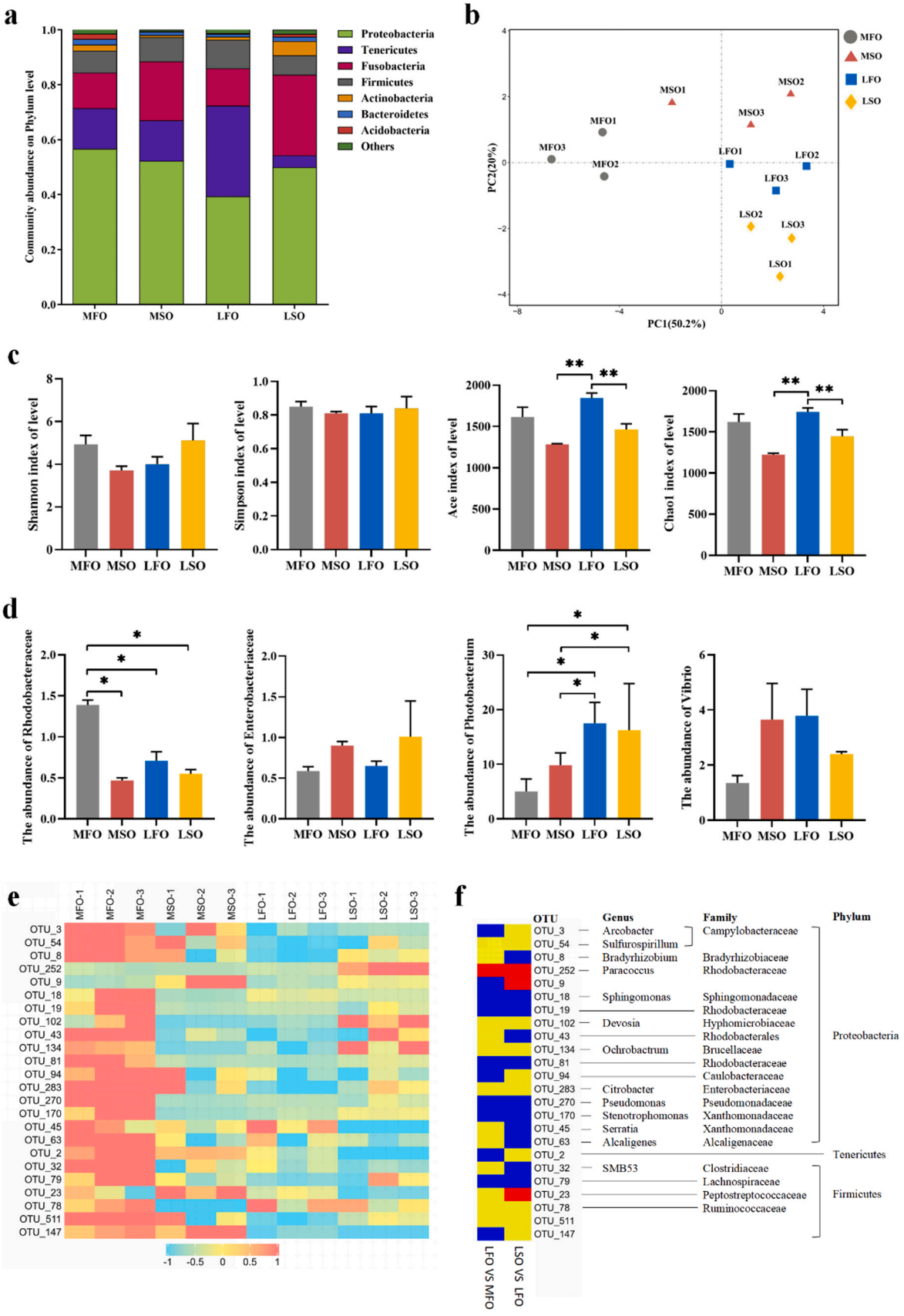

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Fig. 4. Comparisons of the intestinal microflora structure of mud crab (n = 3). (a) Microbial profile in different treatments at phylum level; (b) PCA analysis of all OTUs; (c) Community diversity and richness evaluated by Shannon, Simpson, Ace and Chao1 indices; (d) The abundance of Rhodobacteraceae, Enterobacteriaceae, Photobacterium and Vibrio; (e) The abundance of each OTU in the three replicates in each experimental group; (f) Comparison the abundance of OTUs of LFO to MFO, and LSO to LFO treatment groups, respectively. Red bars means the abundance of OTUs are significantly higher in LFO or LSO groups compared with control groups (MFO and LFO), and blue bar indicate the abundance of OTUs are significantly lower than control groups (Yellow, no significant difference). Asterisks (* and **) express remarkable ($p < 0.05$) and greatly remarkable ($p < 0.01$) differences among medium salinity with fish oil diet (MFO) and medium salinity with soybean oil diet (MSO), low salinity with fish oil diet (LFO) and low salinity with soybean oil diet (LSO) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

($P < 0.05$) (Fig. S1). With opposite trend were found in Tenericutes and Firmicutes, the significantly lowest abundance was found in LSO group ($P < 0.05$) (Fig. S1). The influence of salinity and dietary lipid sources on intestinal microbiota was examined by PCA, which showed OTUs were clustered of four groups according to salinity and dietary lipid source with PC1 and PC2 explaining 70.2% of the total variation (Fig. 4b). The estimators of microbiota community and diversity are shown a highly significantly decreased in Ace and Chao in MSO and LSO groups than LFO group ($P < 0.01$), but no statistical differences were Shannon and Simpson indices among the four groups ($P > 0.05$) (Fig. 4c). In addition, the relative abundances of Rhodobacteraceae and Photobacterium were influenced by salinity and dietary lipid sources ($P < 0.05$) (Table S5). Rhodobacteraceae was dominant in the MFO group and was significantly higher than other group ($P < 0.05$) (Fig. 4d). In contrast, the relative abundance of Photobacterium in low salinity group was higher than that in medium salinity group ($P < 0.05$) (Fig. 4d).

The twenty-four OTUs selected to particularly analyze according to: (1) there were significant differences among all the treatment; (2) OTUs abundance was excess for 0.02% (Fig. 4e). Among them, when crabs fed the same FO diets, eleven OTUs decreased significantly in crabs reared at low salinity compared to crabs reared at medium salinity ($P < 0.05$) (Fig. 4f), which located in Proteobacteria, Tenericutes and Firmicutes phyla. One OTU in Proteobacteria increased significantly in low salinity group than crabs cultured under medium salinity water ($P < 0.05$). Meanwhile, when crabs reared at low salinity water, crabs fed dietary SO showed significantly reduced abundance of eleven OTUs (distributed in the Proteobacteria, Tenericutes and Firmicutes), and significantly increased abundance of three OTUs (distributed in the Proteobacteria and Firmicutes) compared to crabs fed dietary FO ($P < 0.05$).

3.5. Intestinal microbial functional prediction

Further analysis was carried out by comparing the predicted microbial function in different treatment groups. In volcano plot analysis, 500 predictive genes with the highest average expression levels were identified as differentially expressed in pair-wise comparisons (LFO vs MFO, LSO vs LFO) (Fig. 5a and b). Among them 32 predictive genes were significantly increased and 138 were significantly decreased in LFO group than MFO group, while 20 predictive genes were significantly increased and 76 were significantly decreased in LSO group than LFO group ($P < 0.05$). Two-side Welch's *t*-test showed significant enrichment of genes involved in the metabolism of membrane transport, carbohydrate and amino acid metabolism in intestinal microflora of mud crab (Fig. 5c and d). Results also indicated that 6 functional pathways showed significant differences between the LFO and MFO groups ($P < 0.05$), including carbohydrate metabolism, cell motility, xenobiotics biodegradation, etc. (Fig. 5c). Differences between LSO and LFO groups were observed in 4 functional pathways, including membrane transport, energy metabolism, xenobiotics biodegradation and folding, sorting and degradation ($P < 0.05$) (Fig. 5d).

3.6. Pearson correlation analysis

Correlation between OTUs abundances in crab intestinal microflora and the significantly different parameters in intestinal structure,

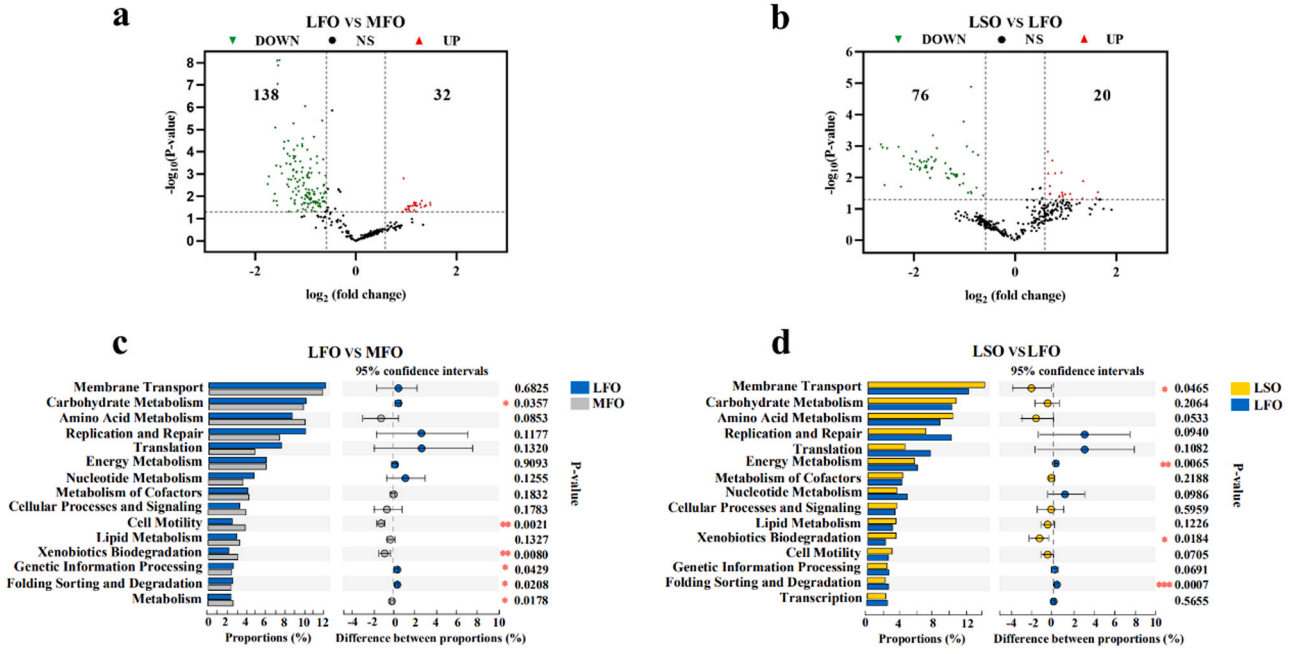

Fig. 5. Microbial functional categories associated with metabolism in intestine of mud crab (n = 3). (a and b) Differential expression analysis of the predicted functional genes of intestinal microorganisms when mud crab reared at LFO compared with MFO (a), and mud crab reared at LSO compared with LFO (b), respectively, by X-axis represents the fold change and Y axis represents the significance of predicted functional genes. (c and d) Comparison of functional pathways of microbes between LFO and MFO groups (c), and LSO and LFO groups (d), respectively, by two-side Welch's *t*-test.

inflammation and apoptosis is shown in a heat map and dendrogram (Fig. 6). The intestinal structure, inflammation and apoptosis parameters were themselves grouped into two main clusters: the parameters in cluster one were positively associated with (good) intestinal health, and included *TGF*, *CLAUDIN*, *PT-44*, Fold width, *ZO-1*, Fold height, *ALF3* and *BCL2*. On the contrary, the parameters in cluster two were negatively associated with (poor) intestinal health: *BAX*, *CASPASE7*, *CASPASE3*, *RELISH* and *CASPASE8*. The OTU groups could also be grouped into three subgroups: subgroup I included OTUs which showed negative correlations with these intestinal pro-health parameters and positive correlations with these intestinal anti-health parameters. In contrast, subgroup II showed an inverse correlation with subgroup I.

4. Discussion

The integrity of intestinal physical barriers are fundamental to intestinal health (Xiong et al., 2018). In current study, effects of salinity and dietary lipid source on intestinal structural integrity was evaluated by means of intestinal histology and expression of genes of intestinal structure - related proteins. The results of two-way ANOVA showed that crabs fed dietary SO and cultured in low salinity displayed decreased intestinal fold height and width, which would lead to reduced enterocyte absorptive area and, thus, which could subsequently result in impaired digestive ability (Luo et al., 2020a). The functionality of the intestinal physical barrier main depends intercellular junctions and intestinal structural proteins that, in turn, are dependent on tight junction and peritrophic proteins (Song et al., 2017; Terra, 2001). Shen et al. (2006) found that restrain MLCK would increase tight junction genes express. In this study, two-way ANOVA showed that low salinity significantly decreased the mRNA expression of *ZO-1*, *CLAUDIN*, *PT-44* and *MLCK,* suggesting that low salinity may partly rely on the down-regulation of the genes involved in intestinal structural proteins to impair intestinal physical barrier function in the intestines of crab. The negative effects of low salinity on tight junction genes mRNA levels might be partly explained by activated the inflammatory response. Evidence has shown that pro-inflammatory cytokines, such as TNF-α, down-regulated ZO-1 expression in Caco-2 cells (Jiang et al., 2015b). Meanwhile, the expression levels of *ZO-1*, *CLAUDIN* and *PT-44* were significantly lower in the LSO treatment group than MFO group in the present study. These results further confirmed that crab reared at low salinity water and fed dietary SO impaired the intestinal structural integrity of mud crab. We speculate the reasons for this result might be connected with role of n-3 LC-PUFA in establishment of the intestinal structure, as these essential FAs are key constituent in cytomembrane, affecting the function or activity of membrane-bound proteins participating in ion transport of intestinal cells (Fonseca-Madrigal et al., 2012). Furthermore, in low salinity conditions, activated ion transport relies even more on n-3 LC-PUFA to improve the intestinal membrane structure (Sundell et al., 2003). Soybean oil does not have n-3 LC-PUFA, therefore crabs fed dietary SO might not have their n-3 LC-PUFA requirements fully met.

The structural integrity of the intestine can also be damaged by inflammation (Han et al., 2020b; Qiao et al., 2019), hence, influencing of salinity and dietary lipid sources on intestinal inflammation were

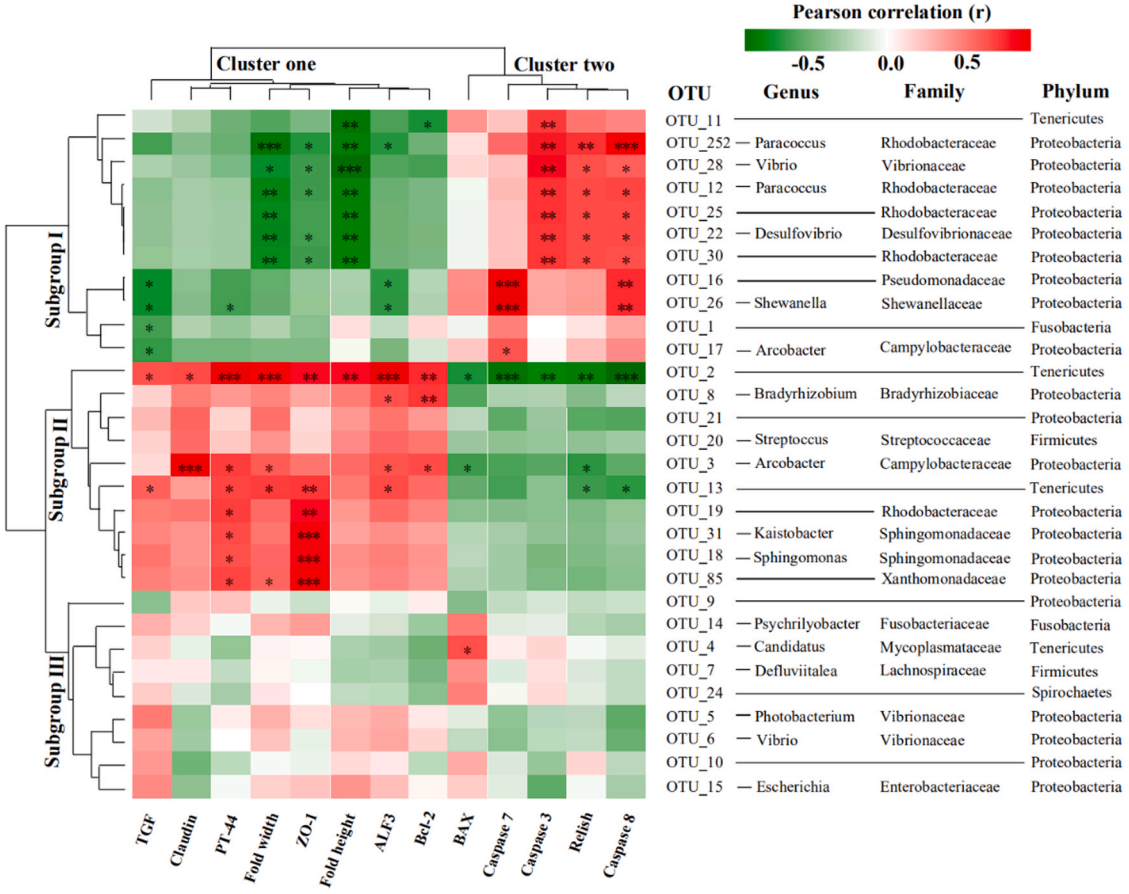

Fig. 6. Heat map characterize the Pearson correlation coefficients (r) between the abundances of the OTUs in crab intestine with intestinal structure, inflammation and apoptosis parameters. Red bars indicate that the abundance of OTUs show a positive correlation with intestinal structure, inflammation and apoptosis parameters, and blue bars indicate the abundance of OTUs show a negative correlation with intestinal structure, inflammation and apoptosis parameters. The asterisks (*, ** and ***) indicate significant ($0.5 < |r| < 0.6$), highly significant ($0.6 < |r| < 0.8$) and extremely significant ($|r| > 0.8$) correlations, respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

investigated in mud crab. The present study revealed that low salinity and SO diet reduced the activities of ACP and AKP in hemolymph and intestine of mud crab, which are key hydrolases impact on obliterated extracellular pathogen, and are considered crucial parts on innate immune and inflammation response in crustaceans (Zhou et al., 2018). In addition, a previous study related decreased of anti-inflammatory cytokines (*TGF* and *ALF3*) and increased of pro-inflammatory cytokines (*LITAF*, *RAB6A*, *RELISH* and *IL16*) with alteration of the immune barrier of crabs (Han et al., 2020a). In currently study, two-way ANOVA showed that the mRNA levels of *ALF3* were significantly affected by salinity and dietary lipid sources. As an antimicrobial peptide, *ALF3* strongly suppresses Gram-negative R-type bacteria, and can modulate inflammatory responses by down-regulating the release of pro-inflammatory cytokines (Han et al., 2020a; Zhang and Liu, 2011). As the lower expression of *ALF3* was also seen in LSO group, low salinity and SO diets may play an essential role to impair the intestinal integrity by decreasing the expression of antimicrobial peptide, *ALF3*. Additionally, SO diet and subjected to low salinity (LSO) displayed lower mRNA of anti-inflammatory cytokines (*TGF* and *ALF3*), and significantly higher expression levels of pro-inflammatory cytokine (*RELISH*), compared to animals kept at normal salinity and fed FO abundant in n-3 LC-PUFA (MFO), and the numerically highest mRNA levels of the pro-inflammatory cytokines (*LITAF* and *RAB6A)* observed in LSO group. Overall, the results indicated that crabs cultured at low salinity and fed dietary SO showed an activated inflammatory response in intestine, suggesting that both of low salinity and SO diet might induce inflammation. While this is the first study to evaluate influences of salinity and dietary lipid source on cytokine expression in crustaceans, the reasons why both factors could influence inflammation in mud crab intestine can be extrapolated from studies in other animals. Firstly, in Atlantic salmon, basal metabolism of the intestinal tract was increased in response to salinity change, and excessive metabolic load makes the intestine more vulnerable to pathogens and can increase the risk of inflammation (Jutfelt et al., 2007). Secondly, n-3 and n-6 PUFA act in contrary impact on inflammation, with n-3 PUFA having good anti--inflammatory impact, nevertheless n-6 PUFA are pro-inflammatory (Calder, 2006; Du et al., 2020). Hence, in order to regulate the synthesis of pro-inflammatory and anti-inflammatory mediators, which are crucial to keep the balance between n-6 and n-3 PUFA (Yang et al., 2016). The level of n-6 PUFA was high and n-3 PUFA low in SO, thus, SO diet compared to FO and the FO diet and, therefore, the proportion of n-6/n-3 PUFA was imbalanced and high, thus SO would likely promote the intestinal inflammatory response (Montero et al., 2015). Previous studies on *Solea senegalensis* and *Sparus aurata* have demonstrated that using SO as dietary lipid source up-regulated mRNA expression of intestinal pro-inflammatory cytokines (Montero et al., 2015, 2010). Therefore, it seems plausible that when crabs were reared under low salinity and fed the SO diet, significant impacts on intestinal inflammatory response were due to both factors having pro-inflammatory effects. While two-way ANOVA showed no interaction between low salinity and dietary SO in inflammatory response, this suggests that salinity and dietary lipid sources showed the independent and additive effects on activating the intestinal inflammatory process of crabs.

Previous studies have associated inflammatory reactions with the development of apoptosis (Haanen and Vermes, 1995). Apoptosis plays a crucial role in the control inflammation and eliminating neutrophils to prevent the spread of pathogenesis (Whyte et al., 1993). Apoptosis is characterized by DNA fragmentation, nuclear condensation and cell shrinkage among other cellular alterations (Jiang et al., 2015a). These characteristics are basically determined by the activity of CASPASES and other apoptosis-related proteins such as BCL2, BAX, BOK, P38 MAPK or JNK (Wang et al., 2020b). In this study, the levels *CASPASE3*, *CASPASE7* and *CASPASE8* mRNA were significantly increased in intestines of the LSO group. Particularly, two-way ANOVA showed that the mRNA levels of *CASPASE8* were high significantly affected by salinity and dietary lipid sources ($P < 0.001$). Which suggested that low salinity and dietary SO induced CASPASE expression that, in turn, promoted apoptosis in crab intestines. Other studies show that apoptosis can be initiated mainly by CASPASE pathway in mammalian regulated by up-regulating the transcription of pro-apoptotic genes (such as *BAX*) and anti-apoptotic genes (such as *BCL-2*) (Wang et al., 2020a). BCL-2 proteins are important gatekeepers of apoptosis, and BAX is considered to be a major activator of the apoptosis pathway and is a pro-apoptotic protein of BCL2 family (Gu et al., 2017). Interestingly, the genes expression of *BAX* was significantly increased, and those of *BCL2* were down-regulated in the intestine of crabs cultured at low salinity. Thus, the results indicated that low salinity potentially activated *BAX*, decreasing the expression of *BCL2*, and thus promoted the intestinal apoptotic process. These results may be partly explained by inflammation response noted above. Apoptotic cell death plays an important role in inflammatory processes and in the resolution of inflammatory reactions (Haanen and Vermes, 1995). And in Chinese mitten crab (*Eriocheir sinensis*), it also was reported that inflammation may promote the apoptotic process in hepatopancreas (Wang et al., 2020a). Similarly, our study showed that low salinity and SO diets could accelerate the intestinal inflammatory response by significantly down-regulated the mRNA levels of anti-inflammatory cytokine and up-regulated the mRNA levels of pro-inflammatory cytokine. Therefore, we presume that low salinity and SO diets get through activated the inflammatory response leading to the apoptotic process in intestine of crab. However, this hypothesis needs further investigation.

The function of the intestine could also be impaired by disturbance of the intestinal microbial complement (Qiao et al., 2019). Thus, impacts of salinity and dietary lipid sources on intestinal microbial communities were investigated in the mud crab. Microbiological barriers, as a crucial element on intestine healthy, which can block potentially hazardous substances into body, as well as play else critical role for host including digestive aids, energy production and immune response (Wang et al., 2018). Although aquatic animals are always in contact with the surrounding water, the composition of their gut microbiota has been reported to be rather different from the composition of microbial communities in the water they inhabit (Zhang et al., 2016). In this sense, the impact of salinity in constituting intestinal microbial composition has been extensively researched (Lozupone and Knight, 2007; Zhang et al., 2016). Several studies also found that dietary composition, such as lipid source, would affect intestinal microbiota (Zhang et al., 2014). In currently study, we systematically compared the shift in intestinal microflora of mud crab reared under different ambient salinity and fed different dietary lipid sources. A distinct change in the intestinal microflora structure was observed based on PCA analysis, indicating that salinity and dietary lipid source were crucial elements in shaping microbial community besides host phylogeny. Particularly, heat map analysis revealed that the abundance of 12 out of 24 OTUs in the intestine of mud crab were salinity dependent. For example, the lower abundance of Devosia, Pseudomonas and Pseudomonas were observed in low salinity group. And the abundance of 14 out of 24 OTUs in the intestine of mud crab were significantly influenced by dietary lipid sources, For example, the lower abundance of Bradyrhizobium, Sphingomonas and Pseudomonas were observed in SO diets group. These results were supported by the observations that those bacterial genera were associated with or prevalent in marine environments (Rungrassamee et al., 2014; Sun et al., 2018; Yuan et al., 2019a; Zhang et al., 2014, 2016).

Intestinal microbiota richness is an important indicator of host health (Qiao et al., 2019) and lower microbiota richness has been reproducibly observed in individuals with inflammatory intestinal disease (Ott et al., 2004). ACE and Chao1 are two crucial index in bacterial communities richness (Luo et al., 2020a), and the present study showed that crabs fed dietary SO showed significantly decreased microbiota species richness when compared to crabs fed dietary FO. Similar results have been previously described in Pacific white shrimp, which found that an increasing trends of Chao1 index were showed in shrimp fed with

FO diets compared to those shrimp fed the SO diets, but no significant difference were detected (Zhang et al., 2014). To some extent, the results suggested that using SO as the main dietary lipid source may cause intestinal inflammation by reducing the intestinal microbial richness of mud crab. On the other hand, intestinal inflammation may also be one of the reasons for the decreased richness of intestinal microbiome. Dietary lipid sources can significantly influence immunity and pathogenesis of diseases in aquatic animals, which may attribute to the HUFA supply in diet (Chen et al., 2016). In crustaceans, high levels of n-3 HUFA in diet can enhance the resistance to response the environmental stress (Rees et al., 1994), and a deficiency of n-3 HUFA has been proved to cause negative effect on immune defence (Chim et al., 2001). Close relationship between the intestinal bacteria and dietary FAs nutrition has been revealed, but the exact role of the intestinal bacteria in dietary FAs nutrition remains unknown. Thus, further work is needed to gather more information on how the intestinal bacterial structure and composition of aquatic animals is affected by various diets.

Illumina sequencing data suggested that the dominant phyla in mud crab were distributed in Proteobacteria, followed by Tenericutes, Fusobacteria and Firmicutes. Proteobacteria were the most prevalent members in each sample, which is consistent with previous studies in other aquatic animals (Sun et al., 2018; Wang et al., 2018; Yang et al., 2019; Yuan et al., 2019a; Zhou et al., 2018). We also found that the abundance of Firmicutes in the LFO group were significantly higher than the LSO group. Recent studies showed that Firmicutes plays a positive role in the homeostasis of intestinal mircoflora in rainbow trout *Oncorhynchus mykiss* (Ingerslev et al., 2014). Meanwhile, the high levels of Firmicutes in intestinal microflora were helped to disease resistance and immune response of Pacific white shrimp (He et al., 2017). To some extent, our results suggested that fish oils rise to a prebiotic effect on mud crab in low salinity environment by increasing their intestinal Firmicutes. Besides, Zhang et al. (2016) reported that the proportion of Bacteroidetes decreased in the intestines of Pacific white shrimp or Nile tilapia *Oreochromis niloticus* when the host facing salinity stress (hyposaline or hypersaline stress, respectively). However, this finding is not supported in mud crab, of this study, no significantly differences were found in the abundance of Bacteroidetes among different salinity treatments. Except for these common bacterial groups, salinity and dietary lipid sources affected some bacterial groups.

At the familiar level, Rhodobacteraceae belong to photosynthetic bacteria that contain abundant nutrients and functional factors (Zhou et al., 2007), are protective for enterocyte of crustaceans, suggesting that it could be potentially used as a probiotic (Zhang et al., 2014). In this study, significant higher of Rhodobacteraceae were found in the MFO group than other groups. Enterobacteriaceae have been found to be associated with a dysbiosis of the microbial community, characteristic of intestinal inflammatory disorders (Winter et al., 2013; Zhang et al., 2014). Crabs fed the FO diets had lower Enterobacteriaceae than crabs fed the SO diets, although, due to the limited samples and the intra-group variation, no significant differences were detected. The large number of Enterobacteriaceae may partly explain the intestinal inflammation in mud crab fed the SO diet. At the genera level, the relative abundance of Photobacterium in the low salinity group increased significantly compared to the medium salinity group. It is well known that many kinds of Photobacterium are pathogenic bacteria. Liu et al. (2016) investigated two pathogenic Photobacterium strains isolated from ridgetail white prawn *Exopalaemon carinicauda* and caused mortality of both ridgetail white prawn and Pacific white shrimp, suggesting that Photobacterium are a common pathogen of crustaceans. Above findings indicates that low salinity challenge has a direct relationship with the susceptibility of mud crab to Photobacterium infection.

Furthermore, the intestinal microbial functional prediction results showed that the majority of predicted genes were down-regulated but only relatively few predicted genes were up-regulated in LFO and LSO groups compared with MFO and LFO groups, respectively. More subdivide PICRUSt analysis showed that the pathway related to cell motility and xenobiotics biodegradation significantly decreased in low salinity group compared to medium salinity group, and the pathway related to energy metabolism and xenobiotics biodegradation significantly decreased in crabs fed SO diets compared to crabs fed FO diets. Cell motility is closely related to the characteristics of life process, and plays an important role in cell internal environment stability, injury repair and immunity (Li et al., 2005). Xenobiotics biodegradation to some extent determines the ability of stress resistance, detoxification and intracellular redox homeostasis of organisms (Zhang et al., 2020). Energy metabolism homeostasis is the basis of normal life activities of cells (Yuan et al., 2019b). The results of PICRUSt analysis suggested that low salinity impaired the normal functional processes of intestinal microbiota of mud crab, with these adverse effects worsening in crabs fed dietary SO. Some OTUs closely related to intestinal structure, inflammation and apoptosis parameters were screened out through Pearson correlation analysis in crab intestines in the present study. Results suggested that both low salinity and dietary soybean oil are likely to disrupt the intestinal physical and immune barriers by affecting the abundance of these OTUs. In future studies, these representative microorganisms species will be further studied.

5. Conclusion

The new findings from our study demonstrate that low salinity injures intestinal barriers of mud crab, and that using SO as single dietary lipid source exacerbated the adverse effects. Interestingly, the results of two-way ANOVA showed that there was no interaction between salinity and dietary lipid source on the vast majority of intestinal health parameters, which showed the independent and additive effects on intestinal health of mud crab. Significant alterations of intestinal histology and structural integrity biomarkers related to intestinal physical barrier were observed in crabs reared at low salinity and fed dietary SO. Moreover, alteration to intestinal immune barrier function caused by low salinity and dietary SO might be through activation of the intestinal inflammatory response and inflammation-induced apoptosis. Furthermore, low salinity and dietary SO could lead to dysregulation of the intestinal microbiota by increased the pathogenic bacteria of Photobacterium, and decreased the beneficial bacteria of Firmicutes and Rhodobacteraceae, which, in turn, can impact the functioning of intestinal inflammation of mud crab. This increases our understanding of mechanisms of environmental impacts on intestinal health of marine crustaceans, therein terrestrial vegetable oil and lower n-3 PUFA contents in diets may aggravate its adverse effects on intestinal health.

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CRediT authorship contribution statement

J.X.L., M.J. and Q.C.Z. conceived and designed the experiments. J.X.L., Y.Y.Z., J.J.L., Y.Y. and T.T.Z. performed the experiments. J.X.L. analyzed the data. J.X.L. and Y.Y.Z. contributed reagents/materials/analysis tools. J.X.L., T.T.Z., X.X.W., Y.Y., M.M.Z., X.Y.H and L.F.J. prepared the crab diets. J.X.L. conducted the crab feeding trial. J.X.L., T.T.Z., X.X.W., Y.Y. and Y.Y.Z. collected the samples. J.X.L., M.J., D.R.T., M.B.B. and Q.C.Z. wrote the paper. All authors contributed to and approved the manuscript.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112004.

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