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Novel perspective on spoilage metabolism of refrigerated large yellow croaker (*larimichthys crocea*) under air-packaging and modified atmosphere-packaging: Insights into adenosine triphosphate degradation

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ABSTRACT: This study evaluated the spoilage potential of dominant bacteria (*Shewanella putrefaciens* and *Pseudomonas fluorescens)* on the effect of the deterioration of adenosine triphosphate-related compounds in refrigerated large yellow croaker fillets under air-packaging and modified atmosphere packaging (MAP). In this work, the total viable count (TVC), adenosine triphosphate-related compounds, and related enzyme activities were characterized in refrigerated large yellow croaker fillets inoculated spoilage bacteria. Macrogenomic analysis was employed to explore the contribution of packaging methods to metabolic pathways and related enzymes. The result demonstrated that the TVC of *Shewanella putrefaciens* reached 7.85 log CFU/g on day 21, exhibiting stronger potential for growth in MAP. The results showed that *Shewanella putrefaciens* had a higher potential to degrade adenosine triphosphate-related compounds and produce related enzyme activities under MAP conditions than *Pseudomonas fluorescens*. The initial value of HxR during storage in the AIRP group was 0.76 μmol/g, which reached a peak on day 3 (1.71 μmol/g), and the level of HxR decreased to 0.38 μmol/g on day 12. The MAP group showed a similar trend to the AIRP group, but the peak appeared later than the AIRP group. The ability to degrade hypoxanthine riboside (HxR) in *Shewanella putrefaciens* exhibited a significant difference in air packaging. In addition, macrogenomic analysis indicated that the degradation of IMP was primarily performed by 5'-nucleotidase (5'-NT). This study enhances our comprehension of the role of microorganisms in fish spoilage and provides valuable insights for the development of novel preservation methods.

Keywords: Large yellow croaker; Modified atmosphere packaging; Adenosine triphosphate; ATP-degrading related enzymes; Macrogenomic

1. Introduction

The large yellow croaker is not only delicious and nutritious but also highly favored by people. However, being rich in protein, carbohydrates, and other nutrients, a fresh large yellow croaker serves as an ideal medium for the growth of microorganisms[1]. These microorganisms that are present under specific storage conditions and are involved in the spoilage process of fish and produce spoilage products are known as

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specific spoilage organisms (SSO)[2]. Studies have shown that the loss of freshness in fish is associated with the growth and reproduction of SSO[3]. These microorganisms can use flavor substances to obtain nutrients and grow, including inosine monophosphate (IMP) and flavor amino acids[4]. Different storage methods such as modified atmosphere packaging (MAP), temperature/pressure treatments, essential oils, and natural polymers are effective in inhibiting the growth of microorganisms, thus extending the shelf life of fish and maintaining its fresh taste[5]. However, the impact of different storage methods on the loss of fish freshness due to microorganisms is not well understood. Therefore, it is crucial to explore the specific role microorganisms play in the deterioration of fish freshness.

Adenosine triphosphate (ATP) can be degraded to adenosine diphosphate (ADP), adenosine monophosphate (AMP), IMP, hypoxanthine riboside (HxR), hypoxanthine (Hx), xanthine (Xa) and uric acid (Ua). Studies have shown that changes in the levels of ATP-related compounds have a significant impact on the flavor of fish, such that AMP can suppress bitterness, while acting as a flavor enhancer to produce pleasant sweet, and savory flavors[6]. IMP serves as an important flavor substance in aquatic products and helps to maintain the flavor of aquatic products. However, the accumulation of HxR and Hx led to the loss of freshness and deterioration of flavor in fish[7]. In addition, previous studies have confirmed strong correlations between ATP-related compounds and traditional freshness indicators (sensory score, total volatile basic nitrogen, total viable count, etc.)[8]. For instance, IMP content exhibited a strong correlation with sensory evaluation, reflecting the freshness of grass carp in super-ice storage[9]. Meanwhile, some studies pointed out that Hx content also has a strong association with freshness indexes, revealing positive potency in the sensory evaluation of fish[10]. Li et al. (2020) explored the relationship between K value and freshness indexes. However, although the relationship between ATP-related compounds and traditional freshness metrics has been confirmed by many studies, ATP degradation-related studies are still relatively scarce. The degradation of ATP involves a range of enzymes, including those from the fish's endogenous enzymes and those from microbial secretory enzymes. However, the activity of enzymes produced by SSO in different environments remains unknown. Therefore, it is essential to investigate the contribution of SSO to the degradation of ATP-related compounds in various environments, particularly under high CO_2 concentrations.

Modified atmosphere packaging has been widely used in food preservation technology. Research has shown that changing the ratio of the various atmospheric gases can reduce the rate of microbial reproduction and decelerate the rate of oxidation and enzymatic reactions[11]. The antibacterial capability of CO_2 allows MAP to inhibit the growth of a large number of microorganisms, while low O_2 simultaneously inhibits the growth of aerobic microorganisms[12]. Some studies have shown that *Pseudomonas* spp. can be effectively inhibited by MAP, while *Shewanella* spp. can grow slowly[13]. However, the enzymatic activity contributed by high concentration CO_2 MAP on SSO during fish storage needs to continue to be explored in depth.

This study aims to further investigate the microbial activity and the impact of different packaging methods on the degradation of ATP-related compounds in large yellow croaker fillets. Microbial growth, ATP-related compounds, and enzyme activities were examined in fillets under various packaging methods,

with inoculation of specific spoilage bacteria. To achieve this, metagenomic analysis was used to identify the role of relevant enzymes in the degradation of ATP-related compounds. This research provides new insights for controlling the quality and flavor of refrigerated large yellow croaker fillets.

2. Material and Methods

2.1 ATP-related Compounds on Air-packaging and Modified Atmosphere Packaging

2.1.1 Fish Fillets Preparation

Large yellow croakers (*Larimichthys crocea*; weight of (0.65 ± 0.23) kg; length of (35.5 ± 3.7) cm, n=30) were purchased from Luchao Gang seafood markets (Shanghai, China), and then transported alive to the laboratory in oxygenated clean water. Stunned large yellow croaker by striking them on the head with a wooden club, as directed by the World Organization for Animal Health (2012) (The fish were kept in an oxygenated environment for 24 hours before being stunned). Subsequently, as instructed, large yellow croaker was gutted and washed repeatedly with clean water. Immediately slice the large yellow croaker into fillets of similar size (60 ± 8) g [14]. Wiped the large yellow croaker fillets with sterile paper towels and packed them individually into high-density polyethylene (HDPE) bags. The AIRP group of 20 fish fillets was packed with HDPE bags in air and the MAP group of 28 fillets was packed evacuated and inflated the HDPE bags with an atmosphere gas ratio of CO_2/N_2: 80/20, the gas-product ratio in the packaging was 1:30 through the gas conditioning machine. The packing bags have an O_2 permeability of 19.95 $cm^3/m^2/day$ and a CO_2 permeability of 164.87 $cm^3/m^2/day$[15]. All samples were stored at (4 ± 0.5) ℃ in the refrigerator, the experimental samples were collected on days 0, 3, 6, 9, 12, 15, 18, and 21.

2.1.2 ATP-Related Compounds

In order to obtain accurate changes in the ATP-related compounds of large yellow croaker fillets during storage, we examined the content of ATP-related compounds by HPLC. Followed the method of Li et al (2019) with minor modifications, 5.0 g of sample was accurately weighed, homogenized by adding 10 mL of 10% perchloric acid (P112072, Aladdin, China), centrifuged at 7200 r/min for 10 min at 4℃ and the supernatant was removed. Repeated the above procedure for the precipitate and combine the supernatants. Added 15 mL of ultrapure water to the supernatant and adjusted the pH of the supernatant to 6.5 with 10 mol/L and 1 mol/L of KOH solution and 5% and 10% perchloric acid solutions, left for 30 min, then removed the supernatant from a 50 mL volumetric flask, and fixed the volume to 50 mL with ultrapure water. The filtrate was stored in a liquid-phase injection vial at -80 ℃ in a freezer for measurement.

The sample was measured by high-performance liquid chromatography (Waters E2695, Co., Milford, CT, USA) on a VP-CDSC18 (46 mm×150 mm) column with 0.05 mol/L phosphate buffer at pH 6.0. The sample volume was 10 μL, the flow rate was 1 mL/min, the column temperature was 30 ℃, and the detection wavelength was 254 nm, ATP, ADP, AMP, IMP, HxR and Hx were calculated concerning the standard samples.

2.1.3 Macrogenomic Analysis

Total microbial genomic DNA was extracted from large yellow croaker fillets samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcr, GA, U.S.). After measuring the quality and concentration of DNA, primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the hypervariable region V3-V4 of the bacterial 16S rRNA gene on an ABI GeneAmp® 9700 PCR thermal cycler. PCR amplification cycling conditions were as follows: initial denaturation at 95 ℃ for 3 min, followed by denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, 30 cycles of extension at 72 ℃ for 45 s, single extension at 72 ℃, and 10 ℃ until discontinued. Quantitation was performed using a Quantus™ Fluorometer (Promega, USA), and paired-end sequencing was performed on the Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocol of Majorbio Bio-Pharm Technology Co. (Shanghai, China)[16-17].

2.1.4 Strain Preparation

After 12 days of AIRP storage, 5 g of large yellow croaker fillet was mixed with 180 mL of sterile saline and tapped to homogenize for 2 min. The resulting homogenate was serially diluted to 2.0-3.0 CFU/mL with sterile saline and spread onto plate counting agar. The plates with 40-100 colonies were selected with isolation and purification and a total of 60 colonies were obtained. The SSOs were stored in a -80℃ freezer. The SSOs were activated with tryptic soy broth (TSB). Then 0.2 mL of the bacterial solution was inoculated into tubes containing 10 ml of TSB and incubated in a constant temperature shaker for 9 h (30 ℃, 200 r/min). After which 2 mL TSB culture was centrifuged (10,000 g for 4 min) to collect bacteria cells. Collected bacteria cells were subjected to DNA extraction using Bacterial Genomic DNA Extraction Kit (Beijing Solarbio Science & Technology Co., Ltd. Beijing, China). The primers used to amplify the 16S rRNA gene fragment (approximately 1400 bp) were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and primer 1495R (5′-CTACGGCTACCTTGTTACGA-3′). The gene sequencing was performed by Sangon Biotech Co., Ltd. (Shanghai, China)[18]. The gene sequences were aligned with the EZtaxon database (https://www.ezbiocloud.net/)[18]. The 60 species of spoilage organisms were identified and analyzed to finally obtain two SSOs, including *Pseudomonas fluorescens* (35.2% of the total) and *Shewanella putrefaciens* (13.2% of the total)[19]. They were stored in a -80℃ refrigerator. The two SSOs were activated with tryptic soy broth (TSB). Then 0.2 mL of the bacterial solution was inoculated into tubes containing 10 ml of TSB and incubated in a constant temperature shaker for 9 h (30 ℃, 200 r/min). The bacterial concentration after incubation reached approximately 8.0-9.0 log CFU/mL and the bacterial solution was kept as a reserve.

2.2 Inoculation of Sterile Fish Fillets with SSO

2.2.1 Preparation of Sterile Fish Fillets

Large yellow croaker was sliced into similar-sized fillets ((60 ± 9) g, n=50) immediately. The fish fillets were soaked in 5% (w/v) formalin solution for 40 s and then sterilized by rinsing three times with sterile water. The sterile fish fillets was then stored at 4 ℃ until bacterial inoculation [18].

2.2.2 Sterile Fish Fillets Inoculation

There were four sets of fillets treated differently for each packing method (air packaging and modified atmosphere packaging). ① The control group (referred to as groups A and M) involved treating the fish fillets in the same manner as described in section 2.1.1. However, a slight modification was made: the fish fillets were immersed in a sterile 0.9% NaCl solution for 10 min before packaging[20]. ② For the sterile group (AN group/MN group), the fish fillets were sterile fillets and untreated. ③ The fish fillets in groups AP and MP were inoculated with a diluted solution of *Pseudomonas fluorescens* (at a concentration of 6.0 log CFU/mL). They were then washed repeatedly with sterile water for 10 min to achieve an inoculum level of 3-5 log CFU/g. ④ Similarly, the fish fillets in groups AS and MS were inoculated with a diluted solution of *Shewanella putrefaciens* (at a concentration of 6.0 log CFU/mL). These fillets were also washed repeatedly with sterile water for 10 min to attain an inoculum level of 3-5 log CFU/g. The air packaging group (consisting of groups A, AN, AP, and AS) was packed using HDPE bags in ambient air, while the MAP group (comprising groups M, MN, MP, and MS) had their HDPE bags evacuated and filled with a gas mixture of CO_2 and N_2 in a ratio of 80/20. Subsequently, all samples were evacuated, and the packed fillets were stored in a refrigerator at a temperature of (4 ± 1) ℃. For analysis, three fillets were randomly selected from each group and tested on days 0, 3, 6, 9, and 12.

2.2.3 Total Viable Count (TVC)

The plate count method was used to determine the microbial load of large yellow croaker fish during refrigeration. A 5 g fish sample was weighed, followed by the addition of 45 mL of sterile saline. The mixture was homogenized for 5 minutes, and then 1 mL of the homogenate was serially diluted 10-fold with sterile saline. Two suitable dilutions of sample homogenate were selected and 100 μL of homogenate was drawn onto a Plate Count Agar plate and spread evenly[21]. The plates were incubated in an incubator at 30 ℃ for 48 hours and then counted. Microbial counts are expressed as log CFU/mL with a minimum limit of 2 log CFU/mL.

2.2.4 ATP-related Compounds

The treatment is the same as in 2.1.3

2.2.5 Determination of AMP Deaminase (AMPD), Acid Phosphatase (ACP), Alkaline Phosphatase (AKP), 5'-Nucleotidase (5'-NT), and Xanthine Oxidase (XOD) Activity

One gram of fish samples were accurately weighed, added to 9 mL of 0.9% saline and homogenized for 30 s using a homogenizer to make a 10% tissue homogenate. A portion of the homogenate was taken to measure protein concentration by bicuculline, and the remaining homogenate was centrifuged at 3000 r/min for 10 min. The supernatant was collected and used to determine enzyme activities. The activities of ACP, AKP, and XOD were determined using the ACP, AKP, and XOD assay kits (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China) in accordance with the method described by Li et al. (2022). 5′-NT activity was determined using the 5′-NT assay kit (Beijing Solabao Technology Co., Ltd., Beijing, China) and AMPD activity was determined using the AMPD assay kit (Shanghai KEAIB Biotechnology Co., Ltd.,

Shanghai, China). Enzyme activity was determined in conformity with the assay kit method and expressed in units per liter (U/gprot).

2.3 Statistical Analysis

Four fillets were chosen randomly from each group and analyzed for macrogenomic analysis, Three fillets were chosen randomly from each group and analyzed for ATP-related compounds and related enzymes. The experimental data were analyzed for significance using IBM SPSS Statistics 25 software (SPSS Inc., Chicago, IL, USA), and the experimental values were expressed as mean ± standard deviation (mean ± SD) and plotted using Origin 8.5 software (Origin Lab Co., Northampton, MA, USA). All data were subjected to one-way ANOVA followed by the Duncan method with a significance level of 5%.

3. Results and Discussion

3.1 Macrogenomic Analysis

3.1.1 ATP-Related Compounds on Different Packaging

In our previous studies, the changes in fish fillets were measured by gas packing with different CO_2 ratios (CO_2/N_2: 60/40, 70/30 and 80/20)[17]. It was found that the longest shelf life and the slowest quality deterioration of fish fillets were observed at gas packing gas ratio of 80/20. Therefore, this study compares the microbiological changes in the experimental groups with AIRP and MAP ratios of 80/20[19]. The experiments showed that MAP treatment effectively inhibited microbial growth. The AIRP and MAP groups exhibited unacceptable at day 12 and 21, respectively. Previous study pointed out that the SSOs in the AIRP group were *Pseudomonas* spp. and *Shewanella* spp., while the MAP group was predominantly dominated by *Shewanella* spp.[12, 15, 19].

The results (Fig.1) showed that the content of ATP, ADP and AMP decreased rapidly in the initial three days. ATP is initially at a maximum of 2.46 μmol/g and it degraded substantially to about 0.5 μmol/g in the initial three days. The initial content of ADP and AMP was low, which might be due to the fast converting of ADP and AMP to IMP during the degradation process. Meanwhile, there was no significant difference in the ATP, ADP and AMP contents between the AIRP group and MAP group. It could be caused by the rapid conversion of ATP and the little role of specific spoilage microorganisms during the conversion of ATP to IMP[22].

IMP is an important flavor substance in fish. Based on Figure 1D, the degradation of IMP to Hx was slower than the degradation of ATP to IMP, and both groups showed similar levels of IMP in the early stages. In the later stages of storage, IMP decomposition occurred more rapidly in the AIRP group and more slowly in the MAP group, likely due to microbial activity. During the early stages of storage, IMP in the fish fillets was degraded by enzymes responsible for its breakdown, and there was no significant difference in IMP content between the AIRP and MAP groups at this time. However, in the later storage period, IMP degradation accelerated, with the AIRP group showing a faster rate of degradation than the MAP group. This could be

attributed to the faster proliferation of microorganisms at this stage, with specific spoilage bacteria promoting the degradation of IMP.

The degradation of IMP continued in the middle and late stages of fish storage to produce HxR and Hx, which are generally considered to be spoilage substances and their degradation products (Hx, Xa, Ua) [23]. Their presence severely reduced the quality and flavour of fish products, with accumulated Hx contributing to the bitter taste of meat. According to Figure 1E, the initial value of HxR in the AIRP group was 0.76 μmol/g during storage, peaks on day 3 (1.71 μmol/g) and the HxR content decreased to 0.38 μmol/g on day 12. The MAP group exhibited a similar trend to the AIRP group, but the peak occurred later, possibly because the AIRP group had a higher microbial load during the pre-storage period compared to the MAP group. These results suggest that the degradation of HxR is linked to specific spoilage bacteria. In the later stages of storage, the gradual decline in IMP content and further degradation of HxR caused its accumulation rate to slow, leading to a decrease in its overall content. Based on Figure 1F, Hx showed an increasing trend throughout the storage period, with rapid accumulation in the AIRP group and slow accumulation in the MAP group. The accumulation of Hx was mainly associated with microorganisms, which is similar to the study of Liu et al (2018).

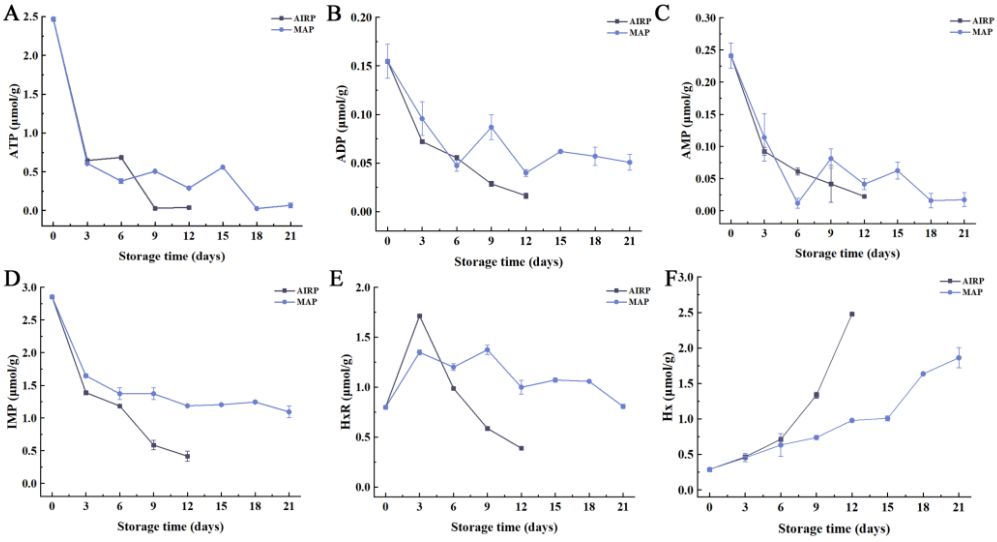


Figure 1. Changes in ATP-related compounds of large yellow croaker fillets during storage at 4°C in different packaging methods. (A) Adenosine triphosphate (ATP); (B) adenosine diphosphate (ADP); (C) adenosine monophosphate (AMP); (D) inosine monophosphate (IMP); (E) hypoxanthine ribonucleoside (HxR); (F) hypoxanthine (Hx). (AIRP: Air-packed group; MAP: Modified atmosphere packaging.)

3.1.2 Macrogenomic Analysis

By using macrogenomic analysis, we mapped the main pathways of ATP metabolism (Figure 2). By comparing the expression activity of ATP-degrading related enzymes in two different packaging methods, the effect of different packaging methods on ATP metabolism can be inferred. According to the macrogenomics results, the highest expression during ATP metabolism was 5'-nucleotidase (EC: 3.1.3.5). It was shown that 5′-NT, ACP (EC: 3.1.3.2) and AKP (EC: 3.1.3.1) were considered to be the main enzymes involved in IMP degradation and they are all phosphate hydrolases [6]. It indicates that phosphate hydrolase is active during storage for the AIRP groups，and MAP groups, the degradation of IMP is the main degradation process.

There is no significant difference in the expression of 5′-NT between the two packaging methods ($P \leq 0.05$). As shown by the graph of the test for differences between metabolic pathway groups, indicates that even in the MAP group, there are microorganisms capable of producing 5′-NT in large quantities, and this spoilage microorganism is presumed to be of the genus *Shewanella* based on biodiversity analysis ($P \leq 0.05$). In contrast, AMPD (EC: 3.5.4.6), PNP (2.4.2.1) and XOD (EC: 1.17.3.2) showed variability ($P \leq 0.05$). This suggests that the aeration packaging affected the ATP metabolism of fish during storage, and that this effect may have been achieved by affecting the species and number of bacteria.

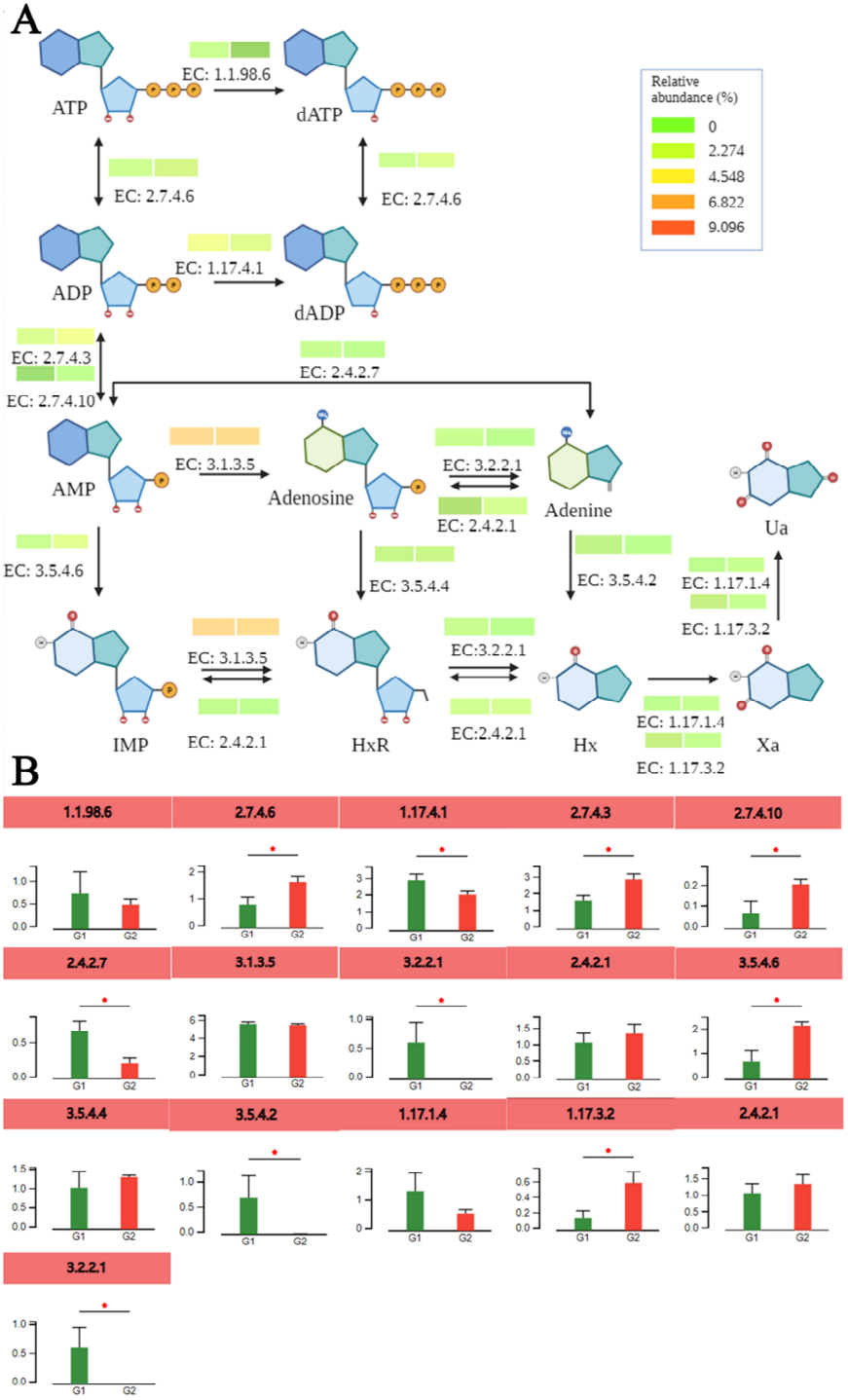


Figure 2. Differences in ATP metabolic pathways between groups in different packaging methods of refrigeration large yellow croaker fish. A: Annotation of macrogenomic results with the KEGG database revealed the ATP degradation pathway of SSO. B: The difference between groups of metabolic pathways in the KEGG pathway. (AIRP: Air-packed group (G1) vs MAP: Modified atmosphere packaged group (G2)) .

3.2 The Degradation of ATP-Related Compounds of Fish by Specific Spoilage Bacteria

3.2.1 TVC

Two specific spoilage bacteria (*Pseudomonas fluorescens* and *Shewanella putrefaciens*) isolated from large yellow croaker fillets were inoculated to investigate the changes in ATP-related compounds and ATP-degrading related enzymes under different packaging methods. As shown in Figure 3, the initial TVC of inoculated groups were higher than 3.0 log CFU/g indicating successful inoculation. The sterilized groups AN and AM had a small number of bacteria present, but remained below 2.0 log CFU/g (lowest limit of detection) during whole storage, indicating that excluded the microbial background in this work[18]. Based on Figure 3A, microorganisms in the modified atmosphere packaging rose rapidly in all groups except the not inoculated group, exceeding acceptable limit (7.0 log CFU/g) on day 12. In contrast, microbial growth in the M group was slow, similar to the previous section. *Shewanella putrefaciens* continued to grow and multiply normally during storage in MAP group, while *Pseudomonas fluorescens* was inhibited. The aerobic activity of *Pseudomonas fluorescens* may prevent it from surviving in a hypoxic environment, whereas *Shewanella putrefaciens* is partially anaerobic and can grow and multiply in an anaerobic environment. This is similar to the results of the biodiversity analysis, where the spoilage microorganisms in the AIRP group were *Pseudomonas* and *Shewanella*, while the spoilage microorganisms in the MAP group were *Shewanella putrefaciens*[24].

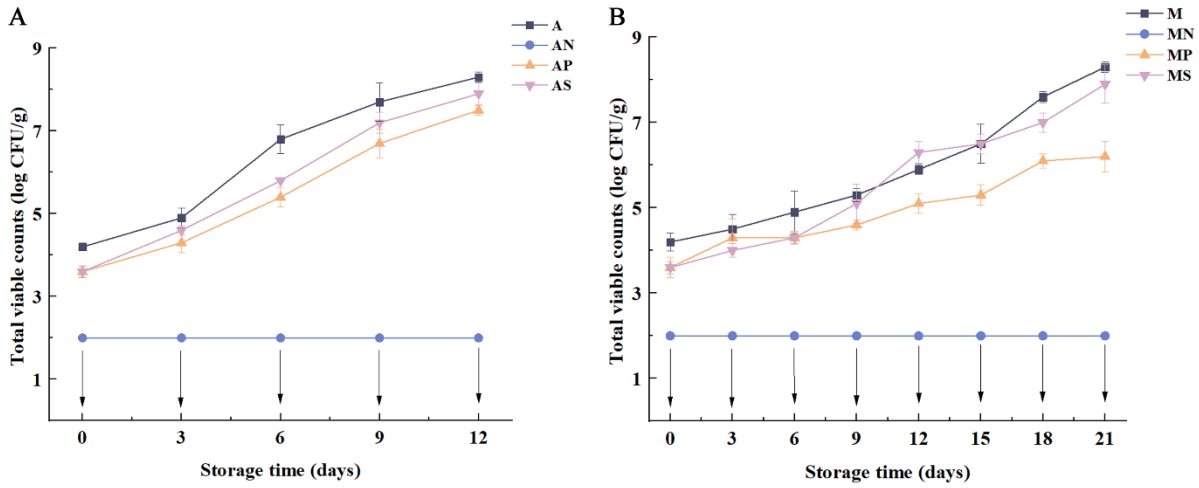


Figure 3. Changes in TVC of large yellow croaker fillets inoculated with different spoilage microorganisms during storage at 4 °C under different packaging methods. (A: Air-packed group with fish fillets without treatment; AN: Air-packed group with sterile fish fillets not inoculated SSO; AP: Air-packed group with sterile fish fillets inoculation with *Pseudomonas fluorescens*; AS: Air-packed group with sterile fish fillets inoculation with *Shewanella putrefaciens*; M: Modified atmosphere packaged group with fish fillets without treatment; MN: Modified atmosphere packaged group with sterile fish fillets not inoculated SSO; MP: Modified atmosphere packaged group with sterile fish fillets inoculation with *Pseudomonas fluorescens*; MS: Modified atmosphere packaged group with sterile fish fillets inoculation with *Shewanella putrefaciens*).

3.2.2 ATP Degradation in the Different Storage Methods

After the slaughter of fresh fish, and ATP loses its stable energy-supplying environment and is broken down by endogenous enzymes. During storage in fish, the degradation of ATP to AMP is short-lived and usually occurs within 1 day[24-25]. It has been shown that various enzymes are involved in the degradation of ATP to AMP, but these enzymes are endogenous to the fish and not related to microbial activity. As illustrated

in Figure 4, ATP, ADP, and AMP all declined rapidly within the first day of storage. During the initial storage period, all groups exhibited similar levels of ATP, ADP, and AMP. However, by day 1, the levels of ATP, ADP, and AMP decreased sharply across all groups, including the germ-free group, which followed a trend similar to the other groups. Notably, ADP and AMP levels were lower in the AIRP and MAP groups during the first three days, likely because these compounds are rapidly degraded as intermediates in the metabolic conversion of ATP to IMP.

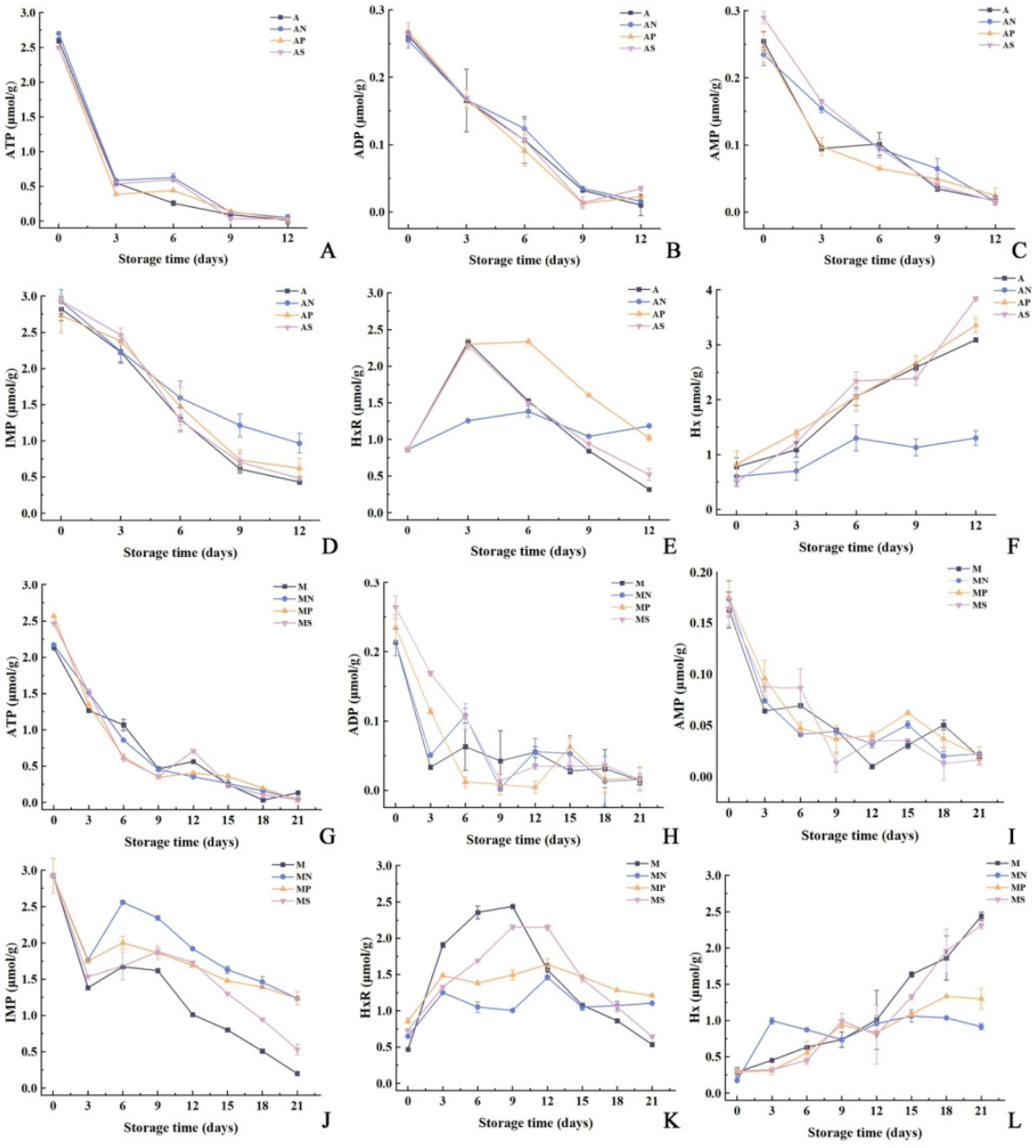


Figure 4. Changes in ATP-related compounds large yellow croaker fillets inoculated with different spoilage microorganisms during storage at 4 ℃ under different packaging methods. (A) Adenosine triphosphate (ATP); (B) adenosine diphosphate (ADP); (C) adenosine monophosphate (AMP); (D) inosine monophosphate (IMP); (E) hypoxanthine ribonucleoside (HxR); (F) hypoxanthine (Hx). ATP(G), ADP(H), AMP(I), IMP(J), HxR(K) and Hx(L) is Modified atmosphere packaged group (A: Air-packed group with fish fillets without treatment; AN: Air-packed group with sterile fish fillets not inoculated SSO; AP: Air-packed group with sterile fish fillets inoculation with *Pseudomonas fluorescens*; AS: Air-packed group with sterile fish fillets inoculation with *Shewanella putrefaciens*; M: Modified atmosphere packaged group with fish fillets without treatment; MN: Modified atmosphere packaged group with sterile fish fillets not inoculated SSO; MP: Modified atmosphere packaged group with sterile fish fillets inoculation with *Pseudomonas fluorescens*; MS: Modified atmosphere packaged group with sterile fish fillets inoculation with *Shewanella putrefaciens*).

IMP is a taste component and a flavor enhancer, and it is an important freshness substance for fish[26]. About Figure 4D, the most predominant nucleotide in fresh large yellow croaker is IMP, which is consistent with the study of rainbow trout[27]. IMP was at its highest level on day 0, probably due to the rapid degradation of ATP. According to Fig. 4D, in the first 6 days, IMP decreased rapidly in all samples of the AP group, but there was no significant difference. It indicated that at this time the degradation of IMP was mainly acted upon by endogenous enzymes which were not related to microorganisms at this time. On days 6-12, the degradation was faster in A and AS groups, coinciding with the time of microbial proliferation, indicating that microorganisms were involved in the degradation of IMP. According to Figure 4J, the rate of IMP degradation in M groups was similar in the A groups during the first 3 days of storage. After day 6, with the most pronounced decrease in the M and MS groups, which is the same as the data for the total number of colonies, with the M and MS groups having a high capacity for microbial growth and reproduction. On day 21 the M group only had 0.2 μmol/g, while the IMP level in the MN group remained at 1.41 μmol/g. In the MAP group, Pseudomonas fluorescens grew more slowly and contributed less to ATP degradation, and its IMP degradation trend was similar to that of the N group. This indicated that Pseudomonas fluorescens had a weaker ability to degrade ATP under unfavorable environmental conditions. In reference to Fig. 4, H. putrefaciens was more significant in promoting IMP degradation compared to Pseudomonas fluorescens, which is consistent with the findings of Li et al. (2022), who stated that the degradation of IMP to Hx was mainly dominated by bacterial activity. In addition, Jia et al. (2019) also found that the IMP content in samples inoculated with *Pseudomonas aeruginosa* and *Streptococcus putrefaciens* was significantly lower than that of germ-free samples, which further suggests that putrefaciens play a key role in the process of IMP degradation.

The IMP continued to be degraded in the middle and late stages of fish storage to HxR and Hx[11]. HxR and its degradation products (Hx, Xa, Ua) are generally considered to be spoilage substances and their presence severely reduces the quality and flavour of fish products[28]. According to Figure 4E, the initial content of HxR in group A was 0.86 μmol/g during storage. The HxR content first increased and then decreased, peaking on day 3 (2.28 μmol/g) and decreasing to 0.32 μmol/g on day 12. The HxR content of the AN group remained in a stable state during storage. AS group showed a similar trend to group A, but the decrease was faster in group A, probably because the AS group of large yellow croaker fillets at the same time had fewer colonies and lower enzyme-producing activity than group A. The AP group reached its peak HxR level between days 3 and 6 of storage, after which it began to decline. However, the rate of decline was slower compared to the A and AS groups. As shown in Figure 4K, the initial HxR content in group M was 0.5 μmol/g. HxR levels initially increased, peaked between days 3 and 9, and then began to decrease. In contrast, the HxR content in the MN and MP groups remained relatively stable throughout storage, likely due to the low number of spoilage microorganisms, which were unable to degrade HxR significantly. Group MS showed a similar trend to group A, but the interval of higher content in group MS was delayed by about 3 days compared to group M, probably because the MS group of large yellow croaker fillets in the same period had fewer colonies than group M, and the MAP had no effect on the spoilage of *Shewanella putrefaciens*. This was similar to the

results for group A. Based on previous studies and Figure 2, it is clear that the degradation of HxR is more directly related to the PNP enzyme, and these results suggest that the degradation of HxR is associated with specific spoilage bacteria, with spoilage *Shewanella putrefacien*s being more effective than *Pseudomonas fluorescens* in degrading HxR. In Jia et al. (2019) study, a trend of HxR rising first and then falling was also observed, which is related to microorganisms.

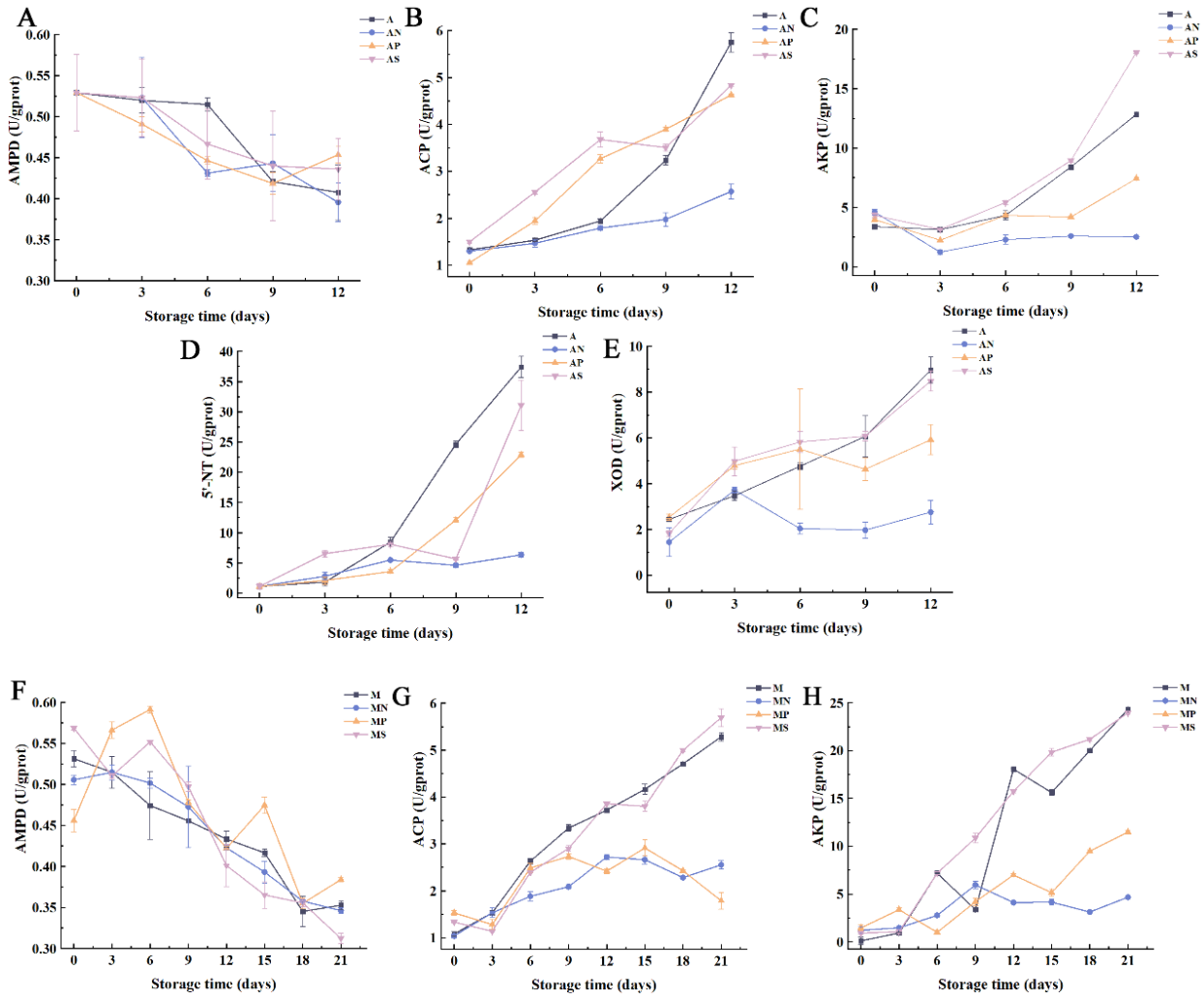
The Hx level in the AN group remained low, ranging from 0.5 to 1.2 μmol/g. In contrast, the A, AP, and AS groups showed a gradual increase, rising from approximately 0.5–1.0 μmol/g at the start to around 3.0–3.5 μmol/g by day 12. As shown in Figure 4, Hx levels in the MN group increased slowly at first before stabilizing. The final Hx accumulation in the MP group was lower than that in the MA and MS groups. Overall, Hx accumulation was lower in both the MN and MP groups, suggesting that the catabolism of HxR is slow at this stage, with no significant impact from endogenous enzymes. At this point, it can be assumed that the accumulation of Hx is mainly microbially related, a view supported by the *Pseudomonas fluorescens* and *Shewanella putrefaciens* groups. Li et al. (2023) research shows that with the degradation of HxR, the content of Hx increases, and the effective inhibition of the *Pseudomonas* can promote the accumulation of HxR faster than its degradation speed. Hx is also broken down into uric acid at a later stage of fish spoilage, and the extremely low levels of uric acid measured in this study suggest that the fish have not yet accumulated uric acid throughout the storage period, and perhaps after a longer period of sitting, the fish will produce uric acid.

3.2.3 Changes in ATP-Degrading Related Enzymes under Different Packaging Methods

Adenosine deaminase (AMPD) is an important catalytic enzyme for the accumulation of IMP, which mainly controls the formation of IMP during the degradation of ATP. Studies have shown that the main function of AMPD is to generate IMP and NH_3 by catalyzing the deamination of AMP[23]. As shown in Figure 5A, there was no significant difference in AMPD activity among the groups. AMPD activity peaked on day 3 and then gradually decreased to its lowest point by day 15. This pattern is consistent with the findings of Li et al. (2017), where AMPD activity increased during the pre-storage period, followed by a decline, with no significant differences in activity observed among the groups[24]. The results of the study were closely related to the rapid decrease of AMP in the pre-storage period of the fish, indicating that the degradation of AMP and the production of IMP are mainly the effect of the endogenous enzyme AMPD, and that specific spoilage bacteria have less influence on this biochemical process.

AKP, ACP and 5'-NT enzymes are the most important phosphate hydrolases involved in IMP degradation. It can catalyze the hydrolysis of IMP by hydrolyzing the phosphate bound to the 5'-position of pentose out to further degrade it to HxR[29-30]. As shown in Figure 5, during the first three days of storage, AKP and ACP activity were low. After 3 days of storage, there was a significant difference in AKP activity, with ACP activity in groups A, AP, and AS being significantly higher than that in group AN at the end of storage. This suggested that AKP was produced by microbial secretion in addition to the fish itself[31] and that *Shewanella putrefaciens* produced higher AKP activity than *Pseudomonas fluorescens* under air packing. As shown in Figure 5H, during storage, AKP activity was significantly higher in the M and MS groups than in the

MP and MN groups. However, the ACP activity of each group was lower compared to AKP. As shown in Figure 5G and H, AKP activity varied similarly to ACP, but AKP activity was up to about 24 U/gprot, much higher than ACP (up to 7 U/gprot). This indicates that ACP and AKP can be produced by microorganisms in addition to the fish itself during late storage, but ACP is produced in smaller amounts and AKP in larger amounts. This is due to differences in ACP and AKP contents in fish in the initial storage period and differences in SSO producing enzyme activities in the later storage period. It has been shown that the content of ACP and AKP varies in different fish and that the activity of AKP and ACP in late storage is closely related to the type of SSO[32-33]. Figure 2 depicted that the expression of AKP and ACP is lower, but AKP is higher than ACP, confirming this view. In the study of Li et al (2022), changes in AKP activity were closely correlated with changes in level of SSO, similar to the results of the present study. According to Li's report, IMP degradation in carp juice is related to AKP rather than 5'-NT, which is not consistent with this paper that 5'-NT is the main cause of IMP degradation. This might be because *Shewanella putrefaciens* had a higher level of AKP transcripts in the carp fish juice and a higher level of 5'-NT transcripts on the large yellow croaker fillets. It is noteworthy that based on the both experiments it is not difficult to find out that *Shewanella putrefaciens* has a higher level of 5'-NT gene. Meanwhile, the lower level of AKP expression in large yellow croaker than in carp may be due to the fact that the *Shewanella putrefaciens* used in the study of Li et al. (2022) was from freshwater fish, whereas the *Shewanella putrefaciens* used in this paper was from marine fish.



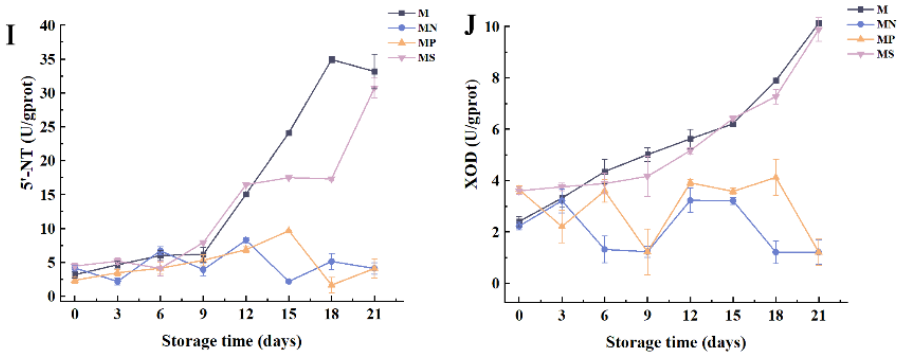


Figure 5. Changes in ATP-degrading related enzymes during storage of large yellow croaker fillets inoculated with different spoilage microorganisms at 4 °C under AIRP and MAP. Changes in AMPD (A), AKP (B), ACP (C), 5′-NT (D) and XOD (E) in AIRP and AMPD (F), AKP (G), ACP (H), 5′-NT (I) and XOD (J) in MAP(A: Air-packed group with fish fillets without treatment; AN: Air-packed group with sterile fish fillets not inoculated SSO; AP: Air-packed group with sterile fish fillets inoculation with *Pseudomonas fluorescens*; AS: Air-packed group with sterile fish fillets inoculation with *Shewanella putrefaciens*; M: Modified atmosphere packaged group with fish fillets without treatment; MN: Modified atmosphere packaged group with sterile fish fillets not inoculated SSO; MP: Modified atmosphere packaged group with sterile fish fillets inoculation with *Pseudomonas fluorescens*; MS: Modified atmosphere packaged group with sterile fish fillets inoculation with *Shewanella putrefaciens*).

As shown in Figure 5D, the initial value of 5'-NT activity in fresh large yellow croaker was 2.17 U/gprot, and there was no significant difference in the change of 5'-NT activity among the groups in the first 3 days of storage. 5'-NT activity was the highest in group A. Combined with the changing patterns of IMP content and ACP and AKP activities, the IMP content of group AN was almost unchanged in the late storage period, while the IMP content of the remaining groups decreased, indicating that IMP-degrading enzymes of spoilage bacteria origin were the main IMP-degrading enzymes. As shown in Figure 5I, group M 5'-NT was similar to A, but group M grew slowly, which was mainly related to the slower growth of spoilage microorganisms in group M, which could not multiply rapidly. group MN and MP 5'-NT remained at a lower level with lower activity, which was due to the inhibition of microorganisms. group MS 5'-NT was similar to group M, with a gradual increase in activity up to 34.9 U/gprot. According to the results of macrogenomics, 5'-NT is the main IMP degrading enzyme contributed by microorganisms. This is similar to the results of Li et al. (2020) that the metabolism of *P. versuta* and *S. putrefaciens* can produce nucleoside phosphorylases in bream meat in which 5'-NT plays an important role. Moreover, both AIRP and MAP groups had higher 5'-NT expression. The MAP group had higher levels than the AIRP group, indicating that *Shewanella putrefaciens*, was the main spoilage organism in the MAP group, was the main spoilage organism producing 5'-NT. Li et al. (2020) study showed that the growth of *Pseudomonas* and *Aeromonas* was inhibited, thus illuminating the low Hx content, suggesting that Pseudomonas and Aeromonas can produce IMP-degrading enzymes such as AKP, ACP, and 5'-NT.

XOD can catalyze the hydrolysis of Hx to produce uric acid. In this paper, the activity of XOD was measured to explore the changes of ATP-related compounds in large yellow croaker during the late storage period. According to Figure 4F and L, it can be seen that Hx tended to rise throughout the storage period with no downward trend, indicating that the amount of Hx degradation was low. However, as shown in Figure 5E and J, it is clear that XOD activity was also influenced by microbial activity. Before storage, there was no significant change in XOD activity in the A and M groups, as microorganisms had minimal impact on the fish

at that stage. However, as storage time progressed, the number of microorganisms in each group increased, and so did the XOD activity. The rise in XOD activity in the A and M groups was relatively modest throughout the storage period, likely due to the slow accumulation of Hx during the pre-storage phase. The activity of AN and MN was stable throughout the storage period. the slow increase of XOD in groups AP and MP indicated that the XOD production capacity of *Pseudomonas fluorescens* was weak even in the air environment. In contrast, the results of AS and MS indicated that XOD activity of *Shewanella putrefaciens* was dominant in the two different packaging groups. This is in agreement with the results of the macrogenome. In conclusion, microorganisms also contribute to XOD production, with spoiled *Shewanella putrefaciens* having a higher XOD production capacity than *Pseudomonas fluorescens* and being the main provider of enzyme activity. Li et al. (2022) found that *Aeromonas* spp. had a lower capacity to produce XOD than *Pseudomonas* spp. and *Shewanella* spp. In their report, Liu et al. (2018) showed that XOD activity could be detected in fish inoculated with SSO. Among them, *A. sobria* and *Ac. bohemicus* had lower XOD activity. Wen et al. (2023) demonstrated that Acinetobacter baumannii can produce xanthine oxidase while eliminating substrate inhibition, which can accelerate the degradation of purine content. The minimal amount of uric acid produced by the stored large yellow croaker in this study may be due to the low hydrolysis of Hx or the fact that Hx produces mainly Xa and does not yet produce significant amounts of Ua.

4. Conclusions

In this study, we investigated the function of spoilage bacteria in the biochemical degradation pathway of ATP-related compounds by inoculation to refrigerated large yellow croaker fillets under air packaging and MAP. MAP can effectively slow down the degradation of ATP-related compounds in fish fillets and maintain the freshness of fish. The degradation of ATP, ADP and AMP was independent of the factors in the packaging environment and spoilage microorganisms, subjecting to the AMPD ctivity. The degradation of ATP-related compounds and related enzyme activities is highly susceptible to the growth of spoilage bacteria. *Shewanella putrefaciens* exhibited strong potency in degradating ATP-related compounds, particularly in terms of IMP. This work provided valuable insights into the application of MAP and the importance of inhibiting *Shewanella putrefaciens* to preserve the flavor and quality of large yellow croaker fillets.

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Conflict of Interests

The authors declare they have no conflicts of interest.

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