Spoilage of tilapia by Pseudomonas putida with different adhesion abilities

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A B S T R A C T

Four Pseudomonas putida strains isolated from spoiled tilapia were divided into three adhesion abilities—high, medium, and low—by an in vitro mucus model. Four strains had no significant difference in spoilage ability to the inoculated fish fillets. However, according to the in vivo experiment, the spoilage caused by the four P.putida was positively correlated with their adhesion abilities. High adhesion strains not only caused more TVB-N in chilled fish, but also activated the spoilage activity of intestinal flora. The diversity of intestinal flora and the changes in volatile components in fish were detected by high-throughput sequencing and SPME-GC/MS. The strains with high adhesion abilities significantly changed the intestinal flora, which led to a significant increase in low-grade aldehydes, indole, and esters in flesh of fish, as well as the production of a fishy and pungent odor. The intestinal adhesion ability of spoilage bacteria was considered the key factor in spoilage of chilled fish.

1. Introduction

In the production, distribution and storage of meat products, the loss caused by microbial accounts for about 20% of the total loss of food. (FAO, 2011; Höll et al., 2016). Fish products are extremely perishable and sensitive to microbial growth, while some microflora are suitable for survival and take part in the spoilage process, producing spoilage odors and off-flavor metabolites (Gram and Dalgaard, 2002).

Pseudomonas spp. are Gram-negative aerobic bacteria and very common in fresh foods, the genus was subdivided into five rRNA similarity groups based on DNA-DNA hybridization studies. The species that are closely related to the spoilage of meat products are in group I, include Pseudomonas fragi, Pseudomonas fluorescens, and Pseudomonas lundensis and others (Ercolini et al., 2010; Mellor et al., 2011; Liu et al., 2015). P.putida is considered the main component of these spoilage microbial with an incidence between 56.7% and 79.0% on spoilt meat (Tryfinopoulou et al., 2002; De Jonghe et al., 2011; Doulgeraki and Nychas, 2013; Mohareb et al., 2015). Markers of microbial spoilage include total number of bacteria, chemical indicators including total volatile basic nitrogen (TVB-N), trimethylamine (TMA), and volatile organic content (VOC)and enzyme-producing capacity (Jaffr et al., 2011; Ge et al., 2017; Parlapani et al., 2017; Silbane et al., 2018; Papadopoulou et al., 2020; Huang et al., 2021). Recent studies discussed the molecular mechanism of spoilage-related enzymes and quorum sensing (Zhu et al., 2016; Wang et al., 2021).

Our previous studies found that the adhesion and spoilage ability of Shewanella spp. in mariculture large yellow croaker in vivo and in vitro were significantly higher than those of Pseudomonas spp. (Zhang et al., 2019). Therefore, we speculated that adhesion ability plays an important role in fish spoilage. Some Pseudomonas spp. were isolated from spoiled tilapia. By comparing the adhesion ability and spoilage ability in vitro, four Pseudomonas putida strains were selected for in vivo test. Combined with the intestinal flora diversity and volatile compounds of fish, the role of adhesion of spoilage bacteria in the spoilage of chilled tilapia was discussed. The results of this study may provide a new strategy for further revealing the spoilage process and the mechanism of spoilage bacteria. The results may also provide a theoretical reference for the development of preservatives.

2. Materials and methods

2.1. Materials and strains

Pseudomonas spp. were isolated from spoiled tilapia, through 16 S rRNA construct a phylogenetic tree and physiological and biochemical identification, they were identified as P.putida and registered in GenBank, as shown in Table A. Four strains of P.putida were LP-3, LP-4, LS-6 and PF01. The fresh tilapias were donated by Fuzhou Aquaculture Institute of Food Science and Technology, College of Biological Science and Technology, Fuzhou University, Fuzhou, 350108, China.

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2665-9271/© 2022 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
2.2. Bacterial adhesion ability in vitro

Mucus was prepared using the method published by Chen et al. (2008), with slight modifications. The intestinal mucus of tilapia was harvested by scraping off the inner surface of the intestines with a spatula to remove the mucous gel layer covering the intestine. Finally, the mucus was homogenized in sterile 0.01 mol L⁻¹ PBS and centrifuged twice at 10,000 g for 30 min at 4 °C to remove particulate matter. The supernatant was filtered through 0.22-μm filters. The mucus samples were adjusted to 0.5 mg protein mL⁻¹ with sterile 0.01 mol L⁻¹ PBS and stored at −20 °C until use. The protein concentration was determined by the Lowry method (Hartree, 1972).

FITC staining was performed according to a previously published method (Vinderola et al., 2004). Fresh cultures of each strain were washed thoroughly with sterile water, placed in a sterile Petri dish, and irradiated with ultraviolet light for 20 min. Sterile fish fillets were washed thoroughly with sterile water, placed in a sterile 50-mL centrifuge tube, and immediately stored at 4 °C for 3 min. VOCs were extracted with an aging SPME fiber for 30 min. The adhered bacteria were released and lysed with 150 μL of 1% SDS for 3 min, VOCs were extracted with an aging SPME fiber for 30 min. The non-adhered bacteria were flushed twice with sterile physiological saline. The adhered bacteria were released and lysed with 150 μL of 1% SDS solution for 2 min. Then, genomic DNA was extracted and total number of bacteria were used as a blank control.

2.3. Spoilage potential evaluation

The fresh tilapia (approx. 500 g) were transported to the laboratory on ice from Fuzhou Aquaculture Institute, China, within 6 h of fishing. They were subsequently scaled; their gills and guts were removed. The fish were cleaned and filleted (2.0 ± 0.1 g each after cleaned). Then, the fillets were washed thoroughly with sterile water, placed in a sterile Petri dish, and irradiated with ultraviolet light for 20 min. Sterile fish fillets were soaked in a bacterial suspension of P. putida (approx. 10⁶ CFU mL⁻¹) for 20 s, drained, and placed in a 50-mL sterile centrifugal tube. The tube was stored at 4 °C. Sterile fish fillets without inoculated bacteria were used as a blank control.

A fish sample (2 g) was homogenized with 18 mL of sterile saline solution for 2 min. Then, genomic DNA was extracted and total number of bacteria was determined by qPCR. The fish fillets were homogenized. TVB-N was determined following the method described by Malle and Poumeyrol (1989).

The SPME method published by Mičko-Krajnik et al. (2016) was used for reference, with slight modifications. Samples (2.0 g) were transferred to a 15-mL glass vial, followed by the addition of 5.00-mL NaCl and 2 μg/mL three methyl pyridine (TMP). After equilibration at 60 °C for 3 min, VOCs were extracted with an aging SPME fiber for 30 min. Finally, the fiber was inserted into the GC injection port immediately and analyzed at 250 °C for 3 min. The absolute concentration was obtained by calculating the ratio of the measured volatile to the peak area of TMP, assuming that the absolute correction factor of each volatile was 1.0 (Equation (2)).

\[
\text{Volatile component concentration (μg/g) = } \left( \frac{A_{i} \times 0.4}{A} \right) / 2
\]

where \(A_{i}\) is the peak area of each volatile component; \(A\) is the peak area of the volatile component TMP; 0.4 is the amount of TMP added; and 2 is the mass of the sample (g).

2.4. Feeding experiment

Two hundred tilapia, with an average body weight of 20 g, were randomly divided into a control group or a treatment group supplemented with P.putida (either the LP-3 group, LP-4 group, LS-6 group, or PF01 group). There were three replicates per group, and 20 tilapia per replicate. Feed was supplied twice a day (09:30 and 17:30 h). During the experiment, 0.01% bacterial suspension with water volume fraction was added daily, the final bacterial content was approximately 1.0 × 10⁶ CFU/mL. The cultivation water was fresh water, with a temperature range of 22 °C–24 °C. Approximately 30% of cultivation water was changed daily, and unconsumed feed and fish feces were purged. After one week of adaptation with basal feed, the feeding experiment began and lasted one week.

After 24 h of fasting at the end of the experiment, all fish were caught and immediately stored at 4 °C. At storage for 1 d and 3 d, the fish were washed with sterile water to reduce contamination by commensal bacteria. Digestive tracts were aseptically removed and homogenized in a sterile homogenization bag. The bags were stored at −20 °C for DNA extraction. The fish were scaled; their gills and guts were removed. The fish were cleaned, filleted (2.0 ± 0.1 g each after cleaned), and stored at −20 °C for the TVB-N test.

2.5. DNA extraction, qPCR reaction, and high-throughput sequencing analysis

Total DNA was extracted from the intestines using a fecal genomic DNA extraction kit and bacterial genomic DNA extraction kit (Tiangen, China). The qPCR reaction consisted of 2.0 μL of 10-fold diluted DNA, 10 μL of SYBR Green, and 0.8 μL of each primer (10 μM) in a total volume of 20 μL. The PCR program comprised 45 cycles at 95 °C (35 s), 60 °C (30 s), and 72 °C (30 s), followed by melt curve generation. Melt curves were analyzed to check the specificity of amplification. Gene-specific primers were designed as:

- Pseudomonas, Pse-F, CTGCATCATGCGCGGTGACACATTT, Pse-R, GTGGCATGCGTGGCGGCTCCGATGATC, were used to qPCR for Pseudomonas.
- Bacterial primers, 27-F, AGAGTTTGATCCTGGCTCAG, 1492-R, GGTACCTTGTTACGACTT, were used to qPCR for Pseudomonas.
- Bacterial primers 341-F (50-CCCTACGGGAGGCAGCAG-30) and 806-R (50-GGATCTTGAAGTTTGATCCTGGT) were used to amplify the V3–V4 region of bacterial 16S rRNA genes. The sequencing library of bacterial 16 S rRNA genes was generated for high-throughput sequencing, employing the TruSequ DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA). Next, the library was sequenced on an Illumina HiSeq2500 platform by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

2.6. Statistical analysis

The results were expressed as mean ± standard deviation, and the probability value of \(P < 0.05\) was considered significant. Significance analysis for all graphs was obtained by comparative analysis within and between groups. An LSD test with Statistical Package for Social Sciences
24.0 (SPSS for Windows, SPSS Inc., Chicago, IL, USA) was used for significance analysis. GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used to make the graphs. Bidirectional orthogonal partial least squares (O2PLS) modeling was used to elucidate the relationships between functional microbiota and flavor formation; PLS-DA was performed using SIMCA-14.1 software (UMETRICS, Malmo, Sweden). STAMP (Statistical Analysis of Metagenomic Profiles) was used to perform difference analysis.

3. Results and discussion

3.1. In vitro adhesion and spoilage by Pseudomonas putida

3.1.1. In vitro mucus adhesion ability of P. putida

As shown in Fig. 1, the adhesion abilities of the four P. putida strains in fish intestinal mucous were divided into high adhesion strain LP-3, medium adhesion strains LP-4 and LS-6, and low adhesion strain PF01 by an in vitro mucus model.

In the process of bacterial adhesion to the host, it first contacts the mucus layer and then adheres to intestinal epithelial cells (Tassell and Miller, 2011). Therefore, in vitro mucus model is an effective model to evaluate bacterial adhesion. In vitro mucus model was used to measure the adhesion of candidate probiotic lactic acid bacteria (LAB) to carp intestinal mucus and it was found that there was a strong correlation between mucus adhesion in vitro and colonization ability in vivo (Sugimura et al., 2011). We found the difference of adhesion ability of four strains of P. putida in vitro and speculated it would lead to differences in fish spoilage.

3.1.2. Analysis of spoilage ability in vitro

The four strains of P. putida were inoculated in fresh, sterile tilapia fillets, and the changes in the total number of bacteria and TVB-N during cold storage were measured. The growth of the four strains was similar, except that PF01 grew slowly in a later stage (3 d) (Fig. 2A). P. putida LS-6 produced the highest TVB-N, followed by LP-3 and LP-4, and then finally PF01 (Fig. 2B). However, there was no significant difference in spoilage ability (p > 0.05).

TVB-N, TMA (Trimethylamine) and K value are often used to indicate the spoilage ability (Park et al., 2021; Peleg, 2016). Research showed that there was a correlation between adhesion and biofilm formation (Garrett et al., 2008; Muhammad et al., 2020). For example, flagella can promote adhesion, colonization, and biofilm formation, resulting in a large number of bacteria (Conrad, 2012; Bruzaua et al., 2015). On the contrary, the adhesion ability of PF01 was weak, which made it difficult to form biofilm on fish fillets, so the total number of bacteria was less. However, it could be seen that PF01 showed individual strong spoilage ability.

3.2. In vivo adhesion and spoilage of Pseudomonas putida

3.2.1. P. putida colonization in fish intestine and muscle

After feeding tilapia with four P. putida strains for one week, the number of Pseudomonas and total bacteria in intestine and flesh of tilapia were determined by qPCR at 0 d and 8 d of storage to characterize the adhesion of Pseudomonas in vivo. Compared with the control, four P. putida strains adhered to the fish intestines, and the adhesion of PF01 in the intestine was significantly lower than that of other strains (as shown in Fig. 3A, at 0 d). The results showed that P. putida could adhere to fish intestines, and PF01 had the lowest adhesion in vivo and in vitro. After eight days of cold storage, the number of Pseudomonas in intestine was significantly higher than that in the control group, and there was no significant difference except that PF01 group was slightly lower. It showed that Pseudomonas has been fully bred during storage. There was no difference in the number of Pseudomonas in the flesh at 0 day, but the count of Pseudomonas in the flesh of PF01 was lower than other treatment groups at 8 d. It was consistent with adhesion ability in vitro mucus model. Because the adhesion ability is related to the bacterial motility, the diffusion ability of bacteria with low adhesion is weak (Nejidat et al., 2008; Navarrete et al., 2019; Suchanek et al., 2020). Therefore, Pseudomonas was also the lowest in the flesh of PF01. There was no significant difference in the total number of bacteria among the groups, which indicated that the addition of P. putida did not affect the total number of bacteria.

3.2.2. Quantitative analysis of TVB-N production and spoilage ability of P. putida in fish

The tilapia inoculated with P. putida were stored at 4 °C. TVB-N in the flesh was measured at 0 d and 8 d. As shown in Fig. 4, TVB-N in the treatment group was significantly higher than that in the control group at 0 d (P < 0.05). There was no significant difference in TVB-N content between LP-3, LP-4 and LS-6. The concentration in all samples was approximately 24.59 mg/100 g, and the content of TVB-N produced in PF01 group was lower than the other three groups. TVB-N in each group exceeded 30 mg/100 g at 8 d, and TVB-N concentration in LP-3 and LP-4 was the highest, followed by LS-6, and then PF01. Thus, TVB-N content is positively correlated with adhesion ability.

The spoilage ability of spoilage bacteria is related to the activity of protease and growth ability (Bekker et al., 2015; Shao et al., 2021). Our study found that strains with similar spoilage ability on fish fillets have different spoilage potential in vivo due to their adhesion and colonization ability.

3.2.3. Intestinal flora diversity based on high-throughput sequencing results

The diversity of intestinal flora of tilapia stored for 0 and 8 d was determined by high-throughput sequencing. Principal component analysis (PCA) showed that, compared with the other three groups, the intestinal flora structure of PF01 group with weak adhesion was significantly different in the initial stage (0 d), while LP-3 with strong adhesion was significantly different from the other three groups in the spoilage stage (8 d). As shown in Fig. 5A, the medium and high adhesion groups (LP-3, LP-4, and LS-6) were grouped together, and Pseudomonas is the main bacterium in the initial storage stage indicating that P. putida exhibited high adhesion and had strong colonization ability in the fish intestinal tract. The intestinal flora structure of PF01 at 0 d was the most different from that of LP-3 at 8 d, as shown in Fig. 5B, the intestinal flora in PF01 at 0 d exhibited weak adhesion ability mainly consisted of Vibrionaceae, Rhizobiaceae, and Moraxellaceae except Fusobacteriaceae.
and the intestinal flora in LP-3 at 8 d exhibited high adhesion ability and mainly consisted of *Stretocococcaceae*, *Bacteroidaceae*, *Clostridiaceae*, and *Peptotrecoccaceae*. *Fusobacteriaceae* decreased during storage. *Aeromonas* increased during storage and had advantages in low adhesion PF01. *Clostridiaceae* and *Peptostreptococcaceae* increased significantly in high adhesion LP-3.

### 3.2.4. Volatile components in spoilage fish

Aquatic products are affected by the degradation of endogenous enzymes, lipid oxidation, microbial reproduction, and environmental factors, which reduce freshness and generate peculiar odors. The volatile components in tilapia were determined by SPME-GC-MS at 0 d and 8 d storage. Forty-eight volatile substances were detected, including alkanes, alcohols, aldehydes, ketones, esters, and aromatic compounds, as shown in Table B.

Content of volatile compounds was subjected to partial least squares discriminant analysis (PLS-DA) and calculated the value of variable importance in projection (VIP). 32 key volatile components (VIP > 1.0) that are associated with the process of spoilage were screened. PCA analysis of the volatile compounds is shown in Fig. 6A. Compared with the control, the addition of *P. putida* changed the flavor of fish at both the starting point and the end point of spoilage. At the beginning of storage (0 d), LP-3 was the farthest away from the control group, and LP-4, LS-6, and PF01 were close to the control group. After 8 d of storage, LP-3 and LP-4 were the farthest away from the control group, with similar odor characteristics, while LS-6 and PF01 were close to the control group. The results show that the higher the adhesion ability of *Pseudomonas putida*, the easier it was to change the flavor profile of fish (as shown by the arrow in Fig. 6A). The aldehydes in the *Pseudomonas* group increased significantly, include low-grade aldehydes, such as...
benzeneacetaldehyde and 3-methyl-butanaland hexanal. *Pseudomonas* spp. (mainly *Pseudomonas fragi* and *Pseudomonas fluorescens*) can produce many low-grade aldehydes (Bozarias and Parlapani, 2017). Studies have shown that low-grade aldehydes, such as hexanal, have unpleasant grassy and pungent odors, and are commonly found in freshwater fish (Hirano et al., 1992; Thiansilakul et al., 2010; Ma et al., 2020).

It was shown that butanal, indole and benzene were the differential volatile components of LP-3 on at 8 d (Fig. 6B). Indole was detected in the *Pseudomonas* treatment groups, especially in LP-3 group, but it was not detected in the control group. The content of indole was high in spoiled fish, and indole contributes to the pungent smell (Yang et al., 2011). Benzene, 1,3-bis(1,1-dimethylethyl)-, which is considered a marker of irradiated food, possibly because methyl radicals continue to bind to xylene radical during irradiation (Kim et al., 2005; Caja et al., 2008). It suggested that spoilage bacteria accelerated the oxidation of fish.

In general, the addition of high-adhesion *P.putida* accelerated the spoilage of fresh fish. The accumulation of low-grade aldehydes, indole, and esters resulted in the development of fishy and pungent odors, especially in the LP-3 and LP-4 groups.

The O2PLS method was used to investigate the correlation between microorganisms and volatile compounds of spoiled fish in different...

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**Fig. 4.** TVB-N value of tilapia inoculated with different *P.putida* and stored at 4 °C. Data was expressed as mean ± standard deviations (n = 3), and significance was measured by using the 2way-ANOVA. Different lowercase letters indicate significant differences at 0.05 level (p < 0.05).

**Fig. 5.** (A) Principal component analysis of intestinal flora of tilapia inoculated with *P.putida* and stored at 4 °C for 0 d and 8 d. (B) Intestinal flora composition of tilapia inoculated with *P.putida* LP-3 and PF01 and stored at 4 °C for 0 d and 8 d.
Fig. 6. (A) Principal component analysis of VOCs (VIP value > 1) for tilapia inoculated with P. putida and stored at 4 °C for 0 d and 8 d. (B) Differences in volatile components in the flesh of tilapia from the LP-3 and control groups at 8 d. Data was expressed as mean proportion (n = 3), and significance was measured by using the Welch’s t-test (Two-sided).

Fig. 7. Network model of the correlation between microbial genera and volatile compounds. The grey line represents positive correlation, the red line represents negative correlation. The thicker the lines, the stronger the correlation. Correlation coefficients were obtained by Pearson’s correlation analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Pseudomonas treatment groups. A network model for the correlation between microbial genera and volatile compounds was drawn based on Cytoscape software. Twenty-four bacterial genera were highly correlated (|p| > 0.6) with volatile compounds (Fig. 7), and most of them had a positive correlation with volatile compounds, except Plesiomonas, Cetobacterium, Listonella and Bosea. This indicated that the change in microbial flora is accompanied by the formation of volatile compounds.

Combined with the results of high-throughput sequencing, Clostridiales and Bacteroidaceae were the dominant families in the LP-3 treatment group, while Clostridium was highly positively correlated with acid esters (pentanedioic acid, dimethyl ester, hexanedioic acid, dimethyl ester, dibutyl phthalate), which are usually the products of the esterification of carboxylic acids and alcohols generated by lipid oxidation. Many scholars believe that lipid oxidation is an important cause of fishy odors (Feng et al., 2012; Shahidi, 2016). Bacteroides has a highly positive correlation with 2,3-octanediol, a volatile substance related to lipid oxidation with an unpleasant grassy smell (Gomez et al., 2020). Study showed that Clostridiales and Bacteroidaceae promote β-oxidation processes, and β-oxidation leads to lipid oxidation (Coma et al., 2016). Therefore, we speculate that these bacteria promoted lipid oxidation and produced more ester acids with fishy aromas.

The above conclusions prove that P.putida with its high adhesion ability, not only has a strong spoilage factor, but can also lead to more serious spoilage by affecting the structure of the microflora.

4. Conclusion

Spoilage bacteria adhere and colonize on fish. The breed of spoilage bacteria led to fish spoilage. Therefore, this study aims to reveal the relationship between bacterial adhesion and spoilage. Four strains of P. putida isolated from tilapia showed no significant difference in their spoilage ability in fish fillet spoilage experiment. Through tilapia feeding experiment, four P.putida strains were added to the water and adhere to the fish, so as to compare their spoilage ability in vivo. The results confirmed a positive correlation between the intestinal adhesion ability of P.putida and the spoilage ability. Higher adhesion, more TVB-N. Moreover, the correlation between intestinal microflora and volatile components proves that inoculation with P.putida with a strong adhesion ability affects the intestinal microflora, especially Clostridiales and Peptostreptococcaceae, which cause the accumulation of low-grade aldehydes, indole, and esters resulted in the development of fishy and pungent odor. Therefore, it was shown in our work that the adhesion ability of P.putida was the premise of spoilage in fish, and the adhesion ability could be used as an important index of spoilage ability, especially for chilled whole fish.

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Ethical approval

All animal experiments comply with the ARRIVE guidelines and be carried out in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

CRediT authorship contribution statement

Wen Zhang: Conceptualization, Validation, Methodology, Writing -- original draft, Writing -- review & editing. Yunru Wei: Investigation, Formal analysis, Visualization, Writing -- original draft, Writing -- review & editing. Xinlin Jin: Validation, Investigation, Formal analysis, Data curation, Writing -- original draft. Xucong Ly: technical support, Visualization, Writing -- review & editing. Zhibin Liu: Methodology, technical support, Funding acquisition. Li Ni: Validation, Project administration, Supervision, Funding acquisition.

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. Supplementary data

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