Effects of LAB fermentation on the quality of grass carp Fillet

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Abstract: Over a 7 days’ fermentation process, LAB (lactic acid bacteria) fermentation improved sensory characteristics such as hardness, springiness, adhesiveness, chewiness and resilience. The effects of the two LAB strains combination showed better than that of either single strain because of their synergy in growth and acid production. The results demonstrated that LAB cultures could be developed as fermentation starter and bio-preservation to improve the quality of grass carp in storage, and combination of synergetic LAB strains more promising.

1. Introduction

The spoilage in fish process is complicated in which chemical and microbiological mechanisms occupy a large part. Biogenic amines (BAs) are present in living organisms at low levels for some essential biological functions. The key BA histamine is regarded as an index to identify the freshness of fish and fish products, while the reduction of BAs tyramine, putrescine in several sausages were reported recently. Total volatile basic nitrogen (TVB-N) is the most useful and important index for determining the spoilage extent of fish products, indicating that the fish product is fresh when it is in the range of 15-25 mg/100g. Accordingly, BAs play an important role in the formation of TVB-N.

Fish protein decomposition also makes fish body loose and sensory characteristics worse. The growth of contaminant microorganisms is the substantial cause of fish product spoilage.

In China, the total fish amount of freshwater is more than that of ocean. The storage of freshwater fish is mainly based on low temperature and freezing preservation which is of high cost. Grass carp is a most popular farmed herbivorous freshwater fish in a wide range of aquatic environments. Developing its preservation technology with good cost performance is an urgent need for Chinese freshwater fishery.

Fermentation could suppress the growth of spoilage microbe, substantially decrease pH quickly and inhibit TVB-N production. Lactic acid bacteria (LABs), which is generally accepted as safety, produce metabolites such as lactic acid, hydrogen peroxide, diacetyl, acetoin and other organic acids, acting as bio-preservatives by inhibiting spoilage microorganisms and modifying the intrinsic properties of food. Considerable research has focused on improving fish quality and extending shelf life by LAB fermentation. Yin et al (2005) used Lactobacillus plantarum BCRC10069, Lactococcus lactis subsp. Lactis BCRC12315, Lactobacillus helveticus BCRC14092 as culture to inhibit TVB-N production and pathogenic microorganism growth on mackerel mince. Liu et al (2010) used LAB for...
bighead carp surimi to suppress the growth of spoilage bacteria by decreasing pH rapidly, thus inhibiting TVB-N formation and giving fish products (e.g. minced fillet and fish sauce) special aroma. LABs, some yeasts and the group of Gram-positive catalasepositive cocci (including micrococci and staphylococci) could contribute to sausage ripening or improvement of flavor of fish muscle extracts (Hugas & Monfort, 1997; Qiu et al., 2001).

LABs differ in growth and metabolism characteristics, thus having different effects on the accumulation of BAs and TVB-N. The combination of LABs may have synergistic effect that stimulates their own growth and acid production. Streptococcus thermophilus and Lactobacillus bulgaricus grow in association in yogurt, the latter decomposing the milk proteins to provide the free amino acids and peptides for the former growth[11][11]. Diverse co-culture systems were gotten by cultivating two LAB strains in a bio-reactor, and having different growth rate and acidification characteristics depending on various initial concentration ratios between the two strains [14]. Appropriate LABs could be applied in fish preservation. The main purpose of this research was to study the effects of the selected LAB fermentation on grass carp fillets, so as to develop LAB starter culture for high-quality fermentation and bio-preservation of grass carp.

2. Materials and methods

2.1. Preparation of starter cultures

Lactobacillus plantarum ZJU10012 and Lactobacillus acidophilus ZJU10023 were stored in our laboratory. When used as starter culture, the LABs were separately subcultured twice in MRS medium at 37 ℃ for 48 h, making the viable cell count of each strain higher than 1×10⁸ CFU mL⁻¹.

2.2. Preparation of samples

The fish were purchased from Wenzhou Village in Hangzhou. The grass carp was washed in running tap water and then gutted, eviscerated and deboned, after which the scale, pin bones, debris and tissues were removed. Then fish were cut into fillets with 15-20 g.

Before of the experiments, four bottles with 100 mL brine of 5 g salt (w/v) and 5 g sucrose (w/v) each were made by strict sterilization in 121 ℃ for 20 min. The activated strains were centrifuged at 3000 × g for 20 min. The lower level precipitation was taken and washed with sterilization water, flushed 2-3 times and diluted into 1×10⁸ cfu/mL.

Four experimental groups were set. 2-3 pieces of grass carp fillets were placed into each one of the four bottles above. The first bottle (100 mL) was inoculated with 3 mL (v/v) Lactobacillus acidophilus and 3 mL (v/v) lactobacillus plantarum together. The second and third (both were 100ml) were inoculated with 3 mL Lactobacillus acidophilus and 3 mL lactobacillus plantarum, respectively. The fourth was inoculated nothing as control group.

The samples were then fermented at 32 ℃, relative humidity 80% for 144 h, and taken every 24 h for analysis.

2.3. Microorganism analysis

Total viable counts (TVC) were determined according to the method of Song et al. [26]. LAB counts (LABC) were determined in Man Rogosa Sharpe (MRS) media. Enterobacteriaceae counts (EC) were determined in Violet red bile glucose agar (VRBG) media in 37 ℃ for 24 h. Results were expressed as log colony forming units per gram sample (log CFU g⁻¹ sample).

2.4. Determination of pH

Fish flesh (10 g) was dispersed in 100 mL of distilled water and stirred for 30 min, then the mixture was filtered. The pH of the filtrate was measured using a pH metre [27].
2.5. **Determination of BAs**

The extraction of BAs in fish fillets was according to Hu et al (2007) with appropriate modification\(^9\)\(^{12}\). BAs were extracted from grass carp with 5% (w/v) trichloroacetic acid twice. Pre-column amine derivatisation was done with dansyl-chloride (De Mey et al., 2012). 10 μL volume was injected into HPLC (LC-2010A SHIMADZU) each time for analysis.

The HPLC column was phenomenex C\(_18\) (250×4.6 mm, Synergi 4u Hydro-RP 80A). The HPLC operating condition was: column temperature was 30 ℃, flow rate was 1 mL/min, sample volume injected was 10 μL, 254 nm UV detector.

Standard BAs, namely, histamine, putrescine, tyramidine, spermidine, spermine were purchased from Sigma-Aldrich.

2.6. **Determination of TVB-N**

TVB-N was measured using microdiffusion methodology. TVB-N value was determined according to the consumption of hydrochloric acid. It referred to Conway micro-diffusion technique (Cobb & Thompson, 1973).

2.7. **Texture profile analysis (TPA)**

TPA measurements were carried out with TA-XT2i Stable Micro Systems Texture analyzer. Fermented grass carp fillet was cut into 2 cm thick to detect its hardness, springiness, adhesiveness, cohesiveness, chewiness and resilience with probe P50. TPA measurement conditions were: measurement speed is 2 mm/s, test rate is 10 mm/s, after-test rate is 10 mm/s, strain 40%, time interval between first and second stroke 5 s.

2.8. **Data analysis**

All statistical analyses were performed using SPSS statistic program (version 16.0 for windows, SPSS 2010). Comparison of values was carried out using analysis of variance (ANONA).

3. **Results and discussion**

3.1. **PH**

In the first 24 h fermentation period, pH of the sample fermented by the mixture starter culture decreased from 5.56 to 3.38. In the cases of fish fillets fermented with lactobacillus plantarum ZJU10012 and Lactobacillus acidophilus ZJU10023, pH values declined from 5.23 to 3.56 and 5.37 to 3.68, respectively. The control sample showed slight change in pH whose 24 h’ value was 4.29. It was in agreement with the previous studies that LAB can ferment fish and produce lactic acid and acetic acid etc, thus decreasing pH\(^2\)\(^{28}\).

According to Fig.1, both lactobacillus plantarum ZJU10012 and Lactobacillus acidophilus ZJU10023 can make pH decline independently, while the mixture of the two can make pH dropping more quickly. Relevant reports have showed similar synergism between different strains, such as Streptococcus thermophilus and Lactobacillus bulgaricus\(^11\), Lactobacillus helveticus and Lactobacillus delbrueckii\(^14\).
3.2. Microorganism analysis

The changes in the microbial flora are shown in Table 1. The TVC of the control sample reaches to 11.44±0.16 log cfu/g after 144 h, which was of significant difference with that of the samples fermented by LABs (p<0.05). The TVC of the sample fermented by the mixture starter was less than that of the samples fermented by single strain either Lactobacillus plantarum ZJU10012 or Lactobacillus acidophilus ZJU10023 (p<0.05).

LABC got rapid increase during fermentation, especially in the samples fermented by LABs, and highest in the sample fermented by the mixture starter (p<0.05). In the 0-48 h, The LABC of the sample fermented by the mixture starter reached to 9.72±0.16 log cfu/g, which was one order of magnitude more than that of the samples fermented by single strain either Lactobacillus plantarum ZJU10012 or Lactobacillus acidophilus ZJU10023 (p<0.05), while control sample was just in the level of 6.92±0.12 log cfu/g. It implied that LABs inoculated in the fish fillets could adapt to the real conditions and became the dominant microorganism during the fermentation process[17].

Starter dominated the fermentation process gives lower EC. The data shows that at the finish point, the EC of the samples fermented by LAB got to 2.8-3.1 log cfu/g, especially the mixture starter sample only 2.8±0.24 log cfu/g (p<0.05), while in the control sample it has increased to 5.14±0.17 log cfu/g (p<0.05). LABs have relatively wide inhibition spectra, competing with the surrounding spoilage microbe such as enterobacteria and pseudomonas and overwhelming them by the actions of bacteriocins and the rapid drop of pH[16][22].

The results of TVC, LABC and EC indicated that LABs could inhibit the growth of spoilage microbe especially the amino-positive microorganism enterobacteria, may due to the actions of lactic acid and bacteriocins produced by LAB. For the control of spoilage microbe, mixture starter culture fermentation showed better effect than either single strain culture fermentation, which is reasonably deduced because of the synergistic effect of the two strains. Acid production was more rapid when mixed cultures of the two strains were inoculated than when single strain was used independently, which could be due to some compounds causing symbiotic growth of the LAB strains[24][6][11]. The crucial point of LAB fermentation used for bio-preservation is whether LABs can occupy the dominant position by competing with contaminant microorganisms in the initial stage.

3.3. BAs

BAs occur in a wide range of foods especially meat products. The compounds are of interest for two reasons: firstly as quality indicators and secondly high levels of BA can present a toxic risk to certain consumers[29].

Chromatogram of sample 1 (fermented with the mixed starter culture) are shown in Fig.2. BAs contents of 5 samples are shown in Table 2. BAs were very low in fresh fish fillets or raw material, as reported by Pons-Sanchez-Cascado et al. (2005)[21]. During 72 h’ fermentation, a distinctly
sharp rise occurred in sample control, the content of each BA was far more than the 3 samples fermented by LABs. BAs were accumulated in spontaneously fermented sausage [8]. Many enterobacteriaceae and pseudomonas possess histamine, lysine and ornithine decarboxylase activities, which can produce considerable levels of histamine, cadaverine and putrescine [8][13]. Histamine, as the most important BA, was still in security target in the sample fermented by the mixture starter, which was below 50 mg/kg [23], and in the samples fermented by single strain exceeded safety index slightly. Putrescine and tyramine showed similar situation to histamine in the samples. Low concentrations of the three BAs in the samples fermented by LAB (p<0.05) could be due to the inhibition of enterobacteriaceae and pseudomonas which is in agreement with the microorganism analysis results above. The high contents of histamine, putrescine and tyramine in the control might be produced from the decarboxylation of endogenous decarboxylase enzymes naturally occurring in fish or high levels of spoilage bacteria like enterobacteriaceae [12].

The levels of spermidine and spermine varied slightly during the fermentation, ranging between 1.01 mg/kg to 2.13 mg/kg, which could be due to that the two BAs are not synthesized by microbial decarboxylation of amino acids[8][12]. Even so, samples with LAB inoculation showed better than the control sample, especially the sample fermented by the mixture starter (p<0.05), implying that LAB could inhibit the accumulation of spermidine and spermine, and synergism of LABs played an important role.

Table 1: Changes of microbe in grass carp fillets fermented with/without inoculated LAB

|                | 0h   | 24h  | 48h  | 72h  | 96h  | 120h | 144h |
|----------------|------|------|------|------|------|------|------|
| **Total bacteria (log cfu/g)** |      |      |      |      |      |      |      |
| 1              | 6.06±0.18 | 6.30±0.25 | 6.36±0.17 | 7.40±0.18 | 7.62±0.19 | 8.34±0.10 | 8.52±0.03 |
| 2              | 6.42±0.22 | 6.98±0.14 | 7.54±0.12 | 8.87±0.09 | 8.84±0.16 | 9.35±0.19 | 9.58±0.16 |
| 3              | 6.47±0.28 | 7.05±0.22 | 7.74±0.25 | 8.59±0.23 | 9.21±0.24 | 9.87±0.27 | 10.59±0.22 |
| 4              | 6.27±0.21 | 7.26±0.19 | 8.27±0.06 | 9.12±0.20 | 9.98±0.15 | 10.78±0.16 | 11.44±0.16 |

**LAB (log cfu/g)**

|                | 0h   | 24h  | 48h  | 72h  | 96h  | 120h | 144h |
|----------------|------|------|------|------|------|------|------|
| 1              | 6.70±0.15 | 8.23±0.21 | 9.72±0.16 | 10.65±0.09 | 9.50±0.17 | 8.29±0.14 | 7.51±0.13 |
| 2              | 6.57±0.27 | 7.48±0.17 | 8.65±0.21 | 8.72±0.16 | 8.67±0.21 | 7.77±0.20 | 6.47±0.21 |
| 3              | 6.35±0.19 | 7.39±0.20 | 8.44±0.31 | 8.76±0.20 | 8.63±0.16 | 7.72±0.14 | 6.63±0.20 |
| 4              | 5.74±0.16 | 6.33±0.21 | 6.92±0.12 | 7.42±0.21 | 7.51±0.22 | 6.66±0.28 | 5.72±0.36 |

**Enterobacteria (log cfu/g)**

|                | 0h   | 24h  | 48h  | 72h  | 96h  | 120h | 144h |
|----------------|------|------|------|------|------|------|------|
| 1              | 0.56±0.14 | 1.37±0.09 | 2.24±0.14 | 2.38±0.16 | 3.17±0.35 | 3.01±0.13 | 2.80±0.24 |
| 2              | 0.75±0.17 | 1.49±0.22 | 2.50±0.23 | 2.67±0.19 | 3.45±0.07 | 3.33±0.10 | 3.02±0.21 |
| 3              | 0.65±0.18 | 1.58±0.14 | 2.68±0.19 | 2.61±0.13 | 3.41±0.07 | 3.39±0.21 | 3.08±0.14 |
| 4              | 1.16±0.15 | 2.51±0.18 | 3.26±0.26 | 3.91±0.13 | 4.67±0.15 | 4.91±0.15 | 5.14±0.17 |

1: sample fermented with the two LABs mixture;
2: sample fermented with *Lactobacillus acidophilus* ZJU10023;
3: *Lactobacillus plantarum* ZJU10012;
4: sample control;

Data in the table are mean±SD from triplicates. Values with different superscript letters in the same column are significantly different at 0.05 levels.
Fig.2 Chromatogram of the sample fermented by the mixed starter culture
1 putrescine; 2 histamine; 3 internal standard; 4 tyramine; 5 spermidine; 6 spermine

Table 2 Changes of BAs in grass carp fillets inoculated with/without inoculated LABs

| Time (h) | samples               | Putrescine (mg/kg) | histamine (mg/kg) | tyramine (mg/kg) | spermidine (mg/kg) | spermine (mg/kg) |
|---------|-----------------------|-------------------|------------------|-----------------|-------------------|-----------------|
| 0       | Fresh grass carp      | 13.57±0.02        | 32.18±0.01       | 37.05±0.27      | 1.01±0.12         | 1.57±0.09       |
| 72      | 1                     | 41.21±0.23        | 45.17±0.11       | 37.54±0.17      | 1.07±0.21         | 1.92±0.10       |
|         | 2                     | 47.38±0.26        | 51.20±0.09       | 43.76±0.21      | 1.21±0.15         | 2.03±0.21       |
|         | 3                     | 55.91±0.14        | 60.10±0.15       | 50.83±0.13      | 1.34±0.10         | 1.96±0.13       |
|         | 4                     | 405.14±0.37       | 713.59±0.19      | 321.46±0.11     | 1.86±0.09         | 2.13±0.16       |

1: sample fermented with the two LABs mixture; 2: sample fermented with *Lactobacillus acidophilus* ZJU10023; 3: *Lactobacillus plantarum* ZJU10012; 4: sample control;
Data in the table are mean±SD from triplicates. Values with different superscript letters in the same column are significantly different at 0.05 levels.

3.4. TVB-N

TVB-N is the most useful and important index for identifying the spoilage in fresh and fermented fish products, indicating that the fish is fresh when it is in the range of 15-25 mg/100g. Fig.3 showed that TVB-N in the control sample increased rapidly after 72 h, although 5% salt could have some effects for inhibiting its accumulation.[10]. In contrast, slight increase has been observed in the samples with LAB fermentation, both single LAB or mixed LAB and the latter better. After 7 days’ storage, the TVB-N of the sample fermented by the mixture starter increased to 18.291 mg/100g, which is less than that of the samples fermented by single strain either *Lactobacillus plantarum* ZJU10012 or *Lactobacillus acidophilus* ZJU10023 in each period (p<0.05). Spoilage organisms, especially enterobacteriaceae and pseudomonas belonging to amino-positive microorganism, use free amino acids and nitrogenous compounds to produce TVB-N[15]. Low pH values suppress the growth of amine-positive microbe, especially enterbacteriaceae, and consequently reduce BA formation, thus TVB-N formation inhibited. The results showed that the better growth of LAB leads to the lower value of TVB-N, coinciding with pH situation and microorganism analysis present above.
Fig. 3 Changes of TVB-N in grass carp fillets inoculated with/without inoculated LABs

Z: Lactobacillus plantarum ZJU10012; S: Lactobacillus acidophilus ZJU10023

3.5. TPA

The control sample has presented bad appearance after 72 h’ storage that the acceptable degree is considerably low with heavy fishy smell and awkward gray color. While the samples inoculated with LAB, either mixture or single, showed good flavor and white color. As shown in Table 3, The TPA results indicated that the samples fermented with LAB were better than the control sample in hardness, springiness, adhesiveness, cohesiveness, chewiness and resilience, and the sample fermented by the mixture starter best. It was the more that the samples fermented with LAB showed better than raw material in some characteristics, the hardness value got sharply decrease and chewiness value got apparently increase.

| Table 3 Changes in TPA in grass carp fillets inoculated with/without mixed or single starter culture |
|---------------------------------------------------------------|---------------------------------------------------------------|
| Fresh grass carp (0 h) | 1 (72 h) | 2 (72 h) | 3 (72 h) | 4 (72 h) |
| Hardness (g) | 779±86.23 | 706.19±65.10 | 556.74±48.12 | 388.66±32.09 | 237.12±21.55 |
| Springiness | 0.84±0.01 | 0.54±0.02 | 0.68±0.026 | 0.70±0.013 | 0.78±0.011 |
| Adhesiveness (g.sec) | 0.823±0.12 | 0.632±0.023 | 0.65±0.021 | 0.645±0.011 | 0.736±0.010 |
| Cohesiveness | 1710±101.1 | 473.57±32.18 | 362.09±27.21 | 121.77±9.20 | 174.54±11.14 |
| Chewiness | 160±10.33 | 317.16±24.51 | 249.14±38.71 | 286.03±26.52 | 136.11±16.37 |
| Resilience | 0.817±0.01 | 0.251±0.01 | 0.26±0.02 | 0.276±0.019 | 0.314±0.013 |

1: sample fermented with the two LABs mixture;
2: sample fermented with Lactobacillus acidophilus ZJU10023;
3: Lactobacillus plantarum ZJU10012;
4: sample control;

Data in the table are mean±SD from triplicates. Values with different superscript letters in the same column are significantly different at 0.05 levels.

3.6 Conclusion

BAAs were main contributors of TVB-N. It was pointed out that the key BA histamine content should be in the range of 50-100 mg/kg in sausages processed according to “Good Manufacturing Practice” [19], and that TVB-N in the range of 15-25 mg/100g indicating that the fish is fresh.

In this study, the LABs could produce plenty of lactic acid, drastically reducing pH value and controlling spoilage microbes which lead protein transforming into BAAs and TPA change by
decomposition effect. The accumulation of BA histamine and TVB-N was apparently inhibited by the LAB fermentation, and the relation between the growth of LABs and the inhibition of fish spoilage was well in agreement with the results of each index. The control sample without inoculated with the LABs showed undesirable results that pH value failed to rapid decline, thus spoilage microbe growing fast, resulting in the index of BAs, TVB-N and TPA showing corrupted. In the samples fermented with the LABs, spoilage bacteria were obviously inhibited and TPA was in favorable level. It is of importance to notice that the sample fermented with the mixture of Lactobacillus plantarum ZJU10012 and Lactobacillus acidophilus ZJU10023 showed better results than that inoculated with either single strain independently, which may due to the combination of the two strains has significant synergistic effect to promote their growth and acid production. LAB combination with synergistic strains overweighted single LAB strain for fish fermentation and as bio-preservative.

In conclusion, the results showed that fermentation inoculated with the mixed starter cultures containing Lactobacillus plantarum ZJU10012 and Lactobacillus acidophilus ZJU10023 could decrease the pH quickly, thus suppressing the growth of contaminant microbe existing in the grass carp raw materials and inhibiting the accumulation of BAs and TVB-N. LABs, especially its combination with synergistic effect, could be used as fermentation starter and bio-preservative. Grass carp is one of the important freshwater fishery resource, the present research develops synergistic LAB as starter culture for its fermentation, and laying foundation for further study of applying LABs in freshwater fish bio-preservation and process technology.

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Conflict with interest
The authors declare no conflict of interest.

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