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The biogenic amines and bacterial changes of farmed rainbow trout (*Oncorhynchus mykiss*) stored in ice

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Abstract

The biogenic amine content and related bacterial changes (*Pseudomonas* spp., psychrotrophic and mesophilic counts) in whole farmed rainbow trout (*Oncorhynchus mykiss*) were monitored during ice storage for 18 days (at 0, 3, 6, 9, 12, 15 and 18 days). Levels of putrescine, cadaverine and histamine, and bacterial loads, increased ($P < 0.05$) during storage, but tyramine was not detected in any of the tested samples. Concentration of putrescine ranged from 0.4 initially to 8.97 μg/g, and psychrotrophic microorganisms were dominant. The best linear regressions (correlations) were for putrescine and *Pseudomonas* spp. and psychrotrophs ($r = 0.98$), and for cadaverine with *Pseudomonas* spp. ($r = 0.82$). Putrescine content was a good quality marker. Histamine was detected only at later stages of storage and was therefore less suitable than the other biogenic amines as freshness indicator.

Keywords: Rainbow trout; Biogenic amines; Bacterial counts; Freshness indicator

1. Introduction

Fish is an extremely perishable food commodity. Deterioration of fish, either marine or freshwater, occurs mainly as a result of enzymatic and microbial activities, which lead to loss of quality and spoilage (Arashisar, Hisar, Kaya, & Yanik, 2004; Liston, 1980). Analysis of spoilage metabolites may be significantly faster than microbial methods as a quality index and give more information about eating quality and freshness of fish (Gram & Dalgaard, 2002; Huss, 1995).

Biogenic amines are nonvolatile compounds formed at low levels in fish as a result of microbial decarboxylation of amino acids, and their presence is related to spoilage (Huss, 1995; Mietz & Karmas, 1977; Rodriguez, Besteiro, & Pascual, 1999). The importance of biogenic amines is related to their toxicological effects and their advantages for the assessment of the quality of fish and fish products (Lehane, 2000; Ozogul, Taylor, Quantick, & Ozogul, 2002). Mietz and Karmas (1977) proposed a biogenic amine index (BAI) to evaluate the quality of rockfish, salmon, lobster and shrimp. Fran and Sims (1986) proposed that putrescine, cadaverine and histamine have good potential as chemical indices of degree of decomposition in tuna. Jorgensen, Dalgaard, and Huss (2000) found good correlation of BAI with sensory analysis in cold-smoked salmon (*Salmo salar*), but they stated that the biogenic amines are not necessarily the causal agents of spoilage off-flavors. The main decarboxylase-active bacteria can be mesophilic or psychrophilic (Taylor & Summer, 1986). Krizek, Vacha,

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Vorlova, Lukasova, and Cupakova (2004) found the best correlation between putrescine and sensory levels and total mesophilic count in carp (*Cyprinus carpio*) flesh. Putrescine and spermidine were proposed as freshness indicators in rainbow trout (Chytiri, Paleologos, Savvaidis, & Kontominas, 2004b) and Mediterranean sea bass, *Dicentrarchus labrax* (Paleologos, Savvaidis, & Kontominas, 2004). Many studies on fish biogenic amines have been reported in marine species (Ozogul, Ozogul, & Gokbulut, 2006; Ruiz-Capillas & Moral, 2001). There have been a few studies on biogenic amines changes in rainbow trout during various storage conditions (Arashisar et al., 2004; Chytiri et al., 2004b; Ozogul & Ozogul, 2004). The results were variable, since moment of capture, stomach contents at death and microbial flora on the live fish which may vary seasonally can influence decomposition (Rodriguez et al., 1999). Trout are mainly stored in ice. Given their commercial importance, this study was undertaken in order to further evaluate the use of biogenic amines for quality assessment. The objectives of the study were the determination of changes in levels of biogenic amines in ice-stored rainbow trout and the correlation of such changes with changes in bacterial counts under the same conditions.

2. Materials and methods

2.1. Sample preparation and storage conditions

Freshwater rainbow trout, *Oncorhynchus mykiss* (average weight and length, 345 ± 50 g and 250 ± 17 mm, respectively) were obtained from an aquaculture farm located at Brudjerd, in western Iran. The fish were fed fish meal-based diet purchased from Chineh Company (Tahran, Iran). The formulation and composition of the diet are given in Table 1. Fish samples were harvested in February 2005 and slaughtered by immersion in ice-cold water (hypothermia). They were then packed in an insulated styrofoam box containing ice, and delivered to the laboratory 6-h post-capture. Three fish were sampled immediately (day zero), and the rest were covered with ice and stored. The ice/fish (3:1, w/w) ratio was maintained constant throughout the experiment. The box was provided with an outlet for water drainage. After 0, 3, 6, 9, 12, 15 and 18 days, three randomly chosen fish were removed from the ice and analyzed in triplicate.

2.2. Chemical analysis

The dorsal half of each fish (without skin or bones) was taken. Then the samples of three fish homogenized in a Waring laboratory blender for 60 s. Then three portions of 50 g of homogenized fish flesh were analyzed for biogenic amines (putrescine, cadaverine, histamine and tyramine) by HPLC. The process involved 5% TCA (trichloroacetic acid) extraction (Mietz & Karmas, 1978) followed by derivatization with benzoyl chloride (Redmond & Tseng, 1979).

Table 1
Formulation and composition of the commercial diet

Ingredients	(%)
Fish meal	48.04
Meat meal	3.74
Soybean meal	20
Wheat flour	20
Wheat bran	0.59
Fish oil	0.5
Soybean oil	0.5
Choline chloride	0.25
Vitamin premix[a]	1
Mineral premix[b]	1
Antioxidant	0.02
Anti phages	0.25
Dicalcium phosphate	1.5
Vitamin C	0.1
Filler	2.5
Proximate analysis (%)	
Protein	39.46
Lipid	13.89
NFE[c]	21.43
Ash	11.4
Fiber	4.47

[a] Vitamin premix provided per kg of diet: vitamin A (retinyl acetate), 2500 IU; vitamin D_3, 2000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K (menadione sodium bisulphate), 1 mg; ascorbic acid (ascorbyl monophosphate), 50 mg; vitamin B_{12}, 0.02 mg; D-biotin, 0.14 mg; choline chloride, 1000 mg; folic acid, 1 mg; niacin, 10 mg; vitamin B_5 (calcium pantothenate) , 20 mg; pyridoxine, 5 mg; riboflavin, 6 mg; thiamin, 1 mg.

[b] Mineral premix provided per kg of diet: sodium (sodium chloride), 1200 mg/kg; iron (ferrous sulphate), 13 mg/kg; manganese (manganese sulphate), 32 mg/kg; zinc (zinc sulphate), 60 mg/kg; copper (copper sulphate), 7 mg/kg; iodine (potassium iodide), 8 mg/kg.

[c] Nitrogen free extraction.

The mobile phase was an isocratic mixture of methanol:water (62:38 by volume) and the flow rate was 1.1 mL/min at room temperature.

2.2.1. Reagents

The standard biogenic amines include putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tyramine hydrochloride were obtained from Fluka Biochemica (Buchs, Switzerland). Methanol, chloroform, butanole, diethyl ether, and *n*-heptane were LC grade (E. Merck); benzoyl chloride (*for synthesis*), HCl, NaCl, and NaOH were extra pure and all of them have been purchased from E. Merck (Darmstadt, Germany). Double distillated and deionized water was used for dilution and chromatographic separation.

2.2.2. Apparatus

High-performance liquid chromatography (HPLC) used Waters 1525 (Waters Company, Milford, MA, USA) apparatus equipped with a UV-detector Waters 2487 set at 254 nm. The column was reversed phase, C_{18} Waters Spherisorb ODS-2 (250×4.60 mm; particle diameter, 5 μm) which was supplied Waters Spherisorb pre-column cartridge (10 mm, length) packed with the same material,

both of them were obtained from Waters (Milford, MA, USA). A 20 μL loop was used.

2.3. Microbiological analyses

A sample was taken from the flesh of the anterior-dorsal region of each fish. Then 5 g of fish flesh were transferred aseptically to 45 mL sterile physiological saline (0.85% NaCl) and homogenized. Serial dilutions of each homogenate were carried out with the same diluent (1:10, by vol.). For microbial enumeration, 0.1-mL samples of serial dilutions were spread on the surface of dry media. Psychrotrophic bacteria were counted using king agar medium, after incubation for 48 h at 21 °C (ISIRI No. 2629, 1991). *Pseudomonas* spp. were enumerated on cetrimide agar, after incubation for 48 h at 37 °C (ISIRI No. 4791, 2000). For the mesophilic viable count, a 1.0-mL sample was inoculated into 10 mL of molten (45 °C) nutrient agar. After setting, a 10-mL overlay of molten medium was added, and then incubated for 48 h at 37 °C (ISIRI No. 356, 1997). Microbiological data were transformed into a logarithm of the number of colony forming units per gram (log CFU/g).

2.4. Statistical analyses

Data were subjected to one-way analysis of variance (ANOVA). The least significant difference (LSD) was used for differences between means. Linear regression was calculated to determine correlation of biogenic amines with bacterial counts. Significance of differences was defined at $P < 0.05$. All statistical tests were done using the statistical software for Windows SPSS, version 12.0. The experiments were performed in triplicate.

3. Results

3.1. Biogenic amine analysis

The concentrations of biogenic amines in the muscles of rainbow trout stored in ice are given in Table 2. Of the four biogenic amines considered, only putrescine, cadaverine and histamine levels were determined. Tyramine was not detected in any sample. All of the determined amines increased significantly ($P < 0.05$) during storage. The initial concentration of putrescine was 0.4 μg/g. This increased slightly during the first 9 days, and then by a factor of 3 on day 12. The final maximum level of putrescine was 8.9 μg/g. Putrescine was the main amine formed, followed by cadaverine, which exhibited a similar increasing trend, with its concentration reaching 6.8 μg/g at day 18. However, the level of cadaverine did not increase rapidly like that of putrescine. Histamine was detected from the ninth day, and reached a level of only 1.6 μg/g at the end of the storage period ($P < 0.05$).

Table 2
The concentration of biogenic amines (μg/g muscle)[A] of rainbow trout (*O. mykiss*) stored on ice

Storage days	Putrescine	Cadaverine	Histamine	Tyramine
0	$0.42 \pm 0.17^{a,B}$	0.17 ± 0.16^{a}	ND	ND
3	1.00 ± 0.18^{ab}	0.39 ± 0.33^{ab}	ND	ND
6	1.47 ± 0.50^{b}	0.99 ± 0.40^{b}	ND	ND
9	2.23 ± 0.39^{c}	2.62 ± 0.61^{c}	0.38 ± 0.04^{a}	ND
12	6.62 ± 0.61^{d}	3.34 ± 0.77^{c}	0.36 ± 0.00^{a}	ND
15	7.05 ± 0.49^{e}	5.42 ± 0.41^{d}	1.23 ± 0.28^{b}	ND
18	8.97 ± 0.23^{f}	6.80 ± 0.21^{e}	1.61 ± 0.28^{c}	ND

[A] Data are expressed as mean ± standard deviation ($n = 3$). ND, Not detected.
[B] Different letters for mean values within a column denote significant differences ($P < 0.05$).

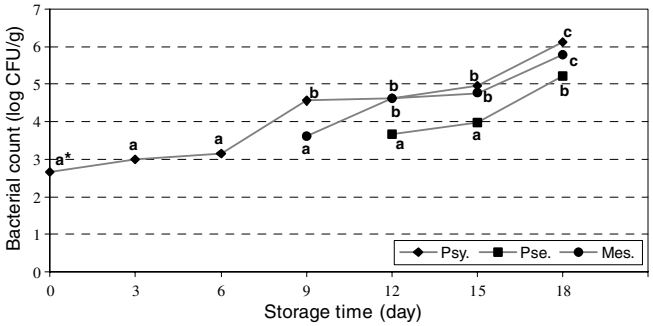

Fig. 1. Changes in bacterial counts in rainbow trout (*O. mykiss*) stored in ice. Abbreviations: Psy, psychrotrophs; Pse, *Pseudomonas* spp.; Mes, mesophilic viable count. * Different letters for each point denote significant differences ($P < 0.05$).

3.2. Microbiological analyses

Changes in bacterial counts of psychrotrophic bacteria, total mesophilic count and numbers of *Pseudomonas* spp. in whole rainbow trout during ice storage is shown in Fig. 1. All of these parameters increased significantly ($P < 0.05$) during storage. Psychrotrophs comprised the main bacterial load. From an initial count of 2.3 log CFU/g (day 0), with a stepwise trend, they finally reached 6.1 log CFU/g. Total mesophilic count attained a maximum level of 5.8 log CFU/g at the end of the storage period. *Pseudomonas* spp. count in trout samples reached 5.2 log CFU/g on day 18. In general, increasing trends of all bacterial counts were similar from day 12.

The best correlation (linear regression) for concentration of biogenic amines (μg/g) with bacterial counts (log CFU/g) was that for putrescine and *Pseudomonas* spp. and psychrotrophic counts ($r = 0.98$); and the next best was between cadaverine and *Pseudomonas* spp. counts ($r = 0.82$).

4. Discussion

Biogenic amines occur either as a physiological constituent of live fish or as a result of bacterial growth and spoilage (Paleologos et al., 2004). Formation of biogenic amines depends on aquaculture conditions, food (which affects

microbes), fish species, body composition, and storage and processing conditions (Krizek et al., 2004; Liston, 1980; Shahidi, 1994); and the presence of decarboxylase-active microorganisms and the availability of free amino acids (Bodmer, Imark, & Kneubuhl, 1999).

In this study, tyramine was not detected in any sample analyzed. Similar results were obtained by Dawood, Karkalas, Roy, and Williams (1988) in a study of chilled rainbow trout spoilage. In contrast, the other three biogenic amines increased during the storage time and putrescine was the main amine formed. Rodriguez et al. (1999) regarded a level of 5 μg/g putrescine as an early warning of autolytic degradation in ice-stored trout muscle. In our samples this concentration was obtained between days 9 and 12 of storage. Based on sensory and microbiological (limit of 7 log CFU/g) data, a putrescine value of 14 μg/g was proposed by Chytiri et al. (2004b) as an upper limit for spoilage initiation of whole ice-stored rainbow trout. In the present study, the level of putrescine did not reach this value. Dawood et al. (1988) also reported lower concentrations of putrascine in whole and eviscerated trout stored at 0 °C after pre-chilling for 6 h at 0, 10, 20 and 30 °C. Nevertheless, as putrescine showed a more-stepwise increase than the other amines, it may be useful as a freshness indicator in whole trout.

Cadaverine was present throughout the storage period, and reached the maximum concentration of 6.8 μg/g. Lower concentrations of cadaverine were reported by Dawood et al. (1988) in iced trout, and Paleologos et al. (2004) in Mediterranean sea bass (*D. labrax*). However, Krizek et al. (2004) found higher levels of cadaverine in carp flesh (≃92.5 μg/g), and Rodriguez et al. (1999) proposed cadaverine as indicator of alteration of muscle as a result of microbial activity.

A shelf life of whole rainbow trout stored in ice, as determined by sensorial and microbiological data, of 15–16 days has been proposed (Chytiri, Chouliara, Savvaidis, & Kontominas, 2004a). While total mesophilic count was lower than the limit of 7 log CFU/g (ISIRI No. 2394, 1993) in the present study, samples showed major signs of spoilage, such as putrefaction, odor and body burst at day 18. In this case, digestive enzyme interactions may have accelerated the muscle degradation, especially in latter stages of storage (Huss, 1995; Ozogul & Ozogul, 2000).

Histamine, a toxic amine and causative agent of histamine fish poisoning (Lehane, 2000), was detected from the ninth day, and had low concentration of 1.6 μg/g. This was consistent with the findings of Chytiri et al. (2004b). These authors suggested that low bacterial loads of Enterobacteriaceae (<6 log CFU/g), together with a low level of the histamine precursor histidinein live rainbow trout, would account for lower production of histamine in filleted trout samples. However, as free histidine increases in dead fish during storage, due to the action of endogenous and contaminating protease (Bodmer et al., 1999), this would account for the occurrence of small amounts of histamine in the latter days of storage. The concentration of histamine in the present study was lower by a factor of about 60 than the toxicological limit of 100 mg/100 g of fish proposed by Lehane and Olley (2000). It should be noted that histamine was not detected at all by some authors in studies of spoilage of trout in storage (Ozogul & Ozogul, 2004; Rodriguez et al., 1999). According to HACCP practice, histamine can be used as an indicator in the evaluation the quality of fresh fish (Hamada-Sato, Usui, Kobayashi, Imada, & Watanabe, 2005), but as previously stated by Chytiri et al. (2004b), as histamine is produced only in the latter stages of storage in trout, it is not suitable as a freshness indicator in this species.

In this study, the diamine putrescine had the best correlation with *Pseudomonas* spp. and psychrotrophic bacteria, and cadaverine with *Pseudomonas* spp. counts. In iced freshwater fish, *Pseudomonas* spp. are the specific spoilage organisms (Gram & Dalgaard, 2002) responsible for decarboxylation of lysine and ornithine, leading to formation of cadaverine and putrescine, respectively (Chytiri et al., 2004b). This is in agreement with our findings. During the experiment, this bacterial group remained small (5.2 log CFU/g) and this may be the reason for the relatively low formation of putrescine in the samples. Paleologos et al. (2004) discussed the role of proteolytic enzyme activity in formation of cadaverine in iced Mediterranean Sea bass. Such interactions may have influenced the formation of this diamine in the present study.

5. Conclusions

According to chemical and microbiological analyses, biogenic amine levels and bacterial loads increased during ice storage of rainbow trout. Rainbow trout cannot have important role in histamine poisoning, as levels of histamine were not high. It seems that monitoring putrescine levels, rather than those of histamine, may have a greater potential for evaluating freshness of rainbow trout. More research is required to elucidate relationships between biogenic amine changes and specific spoilage organisms, and the probable effects of external (e.g., aquaculture conditions and nutrition) and internal (e.g., autolytic interactions in formation of biogenic amines) factors.

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