Suppressive Effects of Green Tea Polyphenols on Microbial Growth and Volatile Basic Nitrogen Content in Round Form Yellowtail (*Seriola quinqueradiata*) Meat during Ice Storage

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Suppressive effects of green tea polyphenols (GTP), which contain (+)-catechin, (-)-epicatechin, (-)-gallocatechin, (-)-epigallocatechin, (-)-gallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin gallate, on microbial growth and volatile basic nitrogen (VBN) content in the cultured fish meat were investigated. The moist pellets supplemented with different concentrations of GTP (0, 0.02 and 0.2% (w/w)) were fed to young yellowtails (*Seriola quinqueradiata*) of 0-year fish. These fish were killed on the 4th week after feeding. The numbers of microbial and psychrophilic bacteria in their meat were counted during ice storage of the round fish. The contents of VBN, which are known as their bacteria metabolites, content in the meat, were also determined. The increase of microbial counts and psychrophilic bacteria counts in their meat were suppressed by GTP feeding. The increase in VBN of their meat were also suppressed. The feeding of 0.2% GTP were more effective than that of 0.02% GTP. Thus, it was suggested that GTP were effective to control microbial growth and to maintain freshness of the meat during delivery of cultured fish.

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Recently, it has been well investigated that green tea polyphenols (GTP), which are polyphenolic compounds in green tea (*Camellia sinensis* L.), have valuable chemical and biological properties¹⁾. There are especially numerous studies on antimicrobial effects of GTP.

We reported that GTP have antibacterial activities against various pathogenic bacteria in livestock industries²⁾. SAKANAKA *et al.*^{3), 4)} reported that GTP

have antibacterial activities against cariogenic and periodontal bacteria. They also described that GTP exhibit antimicrobial effects against thermophilic spore-forming bacteria⁵⁾. Furthermore, it was indicated that the inhibition on the extracellular release of Vero toxin from enterohemorrhagic *Escherichia coli* O157 : H7 also occurs⁶⁾. Thus, GTP have been used as useful and effective additives in various foods and animal feed.

With the progress of aquacultural technology, the price of expensive fish has sufficiently been reduced so that they have become a part of our regular diet. Accordingly, there is currently great interest in the production of higher quality cultured fish since consumers always prefer natural, fresh and tasty fish⁷⁾. For this reason, progress in the technology of maintaining freshness during delivery from the fish farm to the consumer has also recently gained much attention⁸⁾. It is likely that GTP are suitable feed material for cultured fish. However, little is known about the biological effects of GTP on cultured fish.

The present study aims at investigating the suppressive effects on microbial growth during ice storage in the meat of round form cultured yellowtails. The suppressive effect of GTP on the increasing in content of volatile basic nitrogen (VBN) in fish meat, which are bacterial metabolites, was also examined at the same time.

Materials and Methods

1. Fish

Yellowtails (*Seriola quinqueradiata*) used in this experiment were juvenile fish (0-year fish) within one year after hatching, and were approximately each weighed 12 grams. They were obtained from a marifarm of Kowaura in Mie Prefecture on June 1998.

They were cultured in net cages (5 m × 5 m × 5 m), which were located in the cove of Owase Bay at the Ozone Fish Culture Ground of Owase Branch, Fisheries Research Institute of Mie (Owase, Mie, Japan), and had been fed on commercial aquacultural extruded pellets (Hamachi Special EP, Marubeni Feed Co., Ltd., Chiyoda, Tokyo, Japan) until the starting our the experiment. The values of chemical analysis of aquacultural feed used was 48.1% crude protein, 10.2% crude fat, 2.9% crude fiber, 14.8% crude ash, 2.5% calcium, 1.5% phosphorus. The specimens of yellowtail used in this experiment were cultivated such that the young fish possessed an average fork length of 32.0cm and average body weight of 596 g at the start of the experiment.

2. Test material

The GTP (a mixture of 6 kinds of polyphenolic

Table 1 Specification of Green Tea Polyphenols (Teapecus®B)

Item	Specifications
Color	Yellowish brown or brown
Appearance	Powder
Moisture	Less than 6.0%
Ash	Less than 16.0%
Polyphenols ¹	25.0~26.0%
Free Reducing Sugar	13.0~14.0%

1 : Including (–) – epigallocatechin gallate, (–) – epigallocatechin, (–) – epicatechin gallate, (–) – epicatechin, (+) – galocatechin and (+) – catechin.

compounds in green tea) tested in this study were commercially available as Teapecus® B (Taiyo Kagaku Co. Ltd., Yokkaichi, Mie, Japan). The specifications of GTP used are shown in Table 1. Teapecus® B containing 25.5% polyphenolic compounds was prepared as the feed mix. Chemical structures of polyphenolic compounds containing in Teapecus® B are shown in Fig.1.

Fig. 1 Chemical Structure of Green Tea Polyphenols

3. Experimental design

The cultured young yellowtail used in this study were 495 specimens in total. They were divided equally into three experimental groups, the control group (A), the 0.02% GTP (B) and the 0.2% GTP (C). Each group was separately cultured in net cages as the above mentioned in the cove of Owase Bay. The moist pellets possessing the compositions shown in Table 2 were fed to the three groups during the test period. The commercial formula feed (Hamachi Ace 50) were purchased from Sakamoto Feed Co., Ltd., Katori, Chiba, Japan. The chemical analysis values of the formula feed were 51.0% crude protein, 4.2% crude fat, 3.9% crude fiber, 14.8% crude ash, 1.3% calcium, 1.9% phosphorus. Fresh feed used was thawed spotlined sardine (*Sardinops melanostictus*) meat. A vitamin premix (Hamachi Aid W-N) for the yellowtail was purchased from Takeda Chemical Industries, Ltd., Chuo-Ku, Tokyo, Japan. The composition of the vitamin premix included 8.0×10^4 IU vitamin A, 1.6×10^4 IU vitamin D₃, 420mg d α -tocopherol acetate ester, 10mg vitamin K₃, 720mg vitamin B₁, 180mg vitamin B₂, 220mg vitamin B₆, 0.17mg vitamin B₁₂, 240 mg calcium D-pantothenate, 600mg nicotinamide, 18mg folic acid, 6.0 g choline chloride, 1.2mg D-biotin, 968 mg calcium ascorbate, and 600mg inositol per kg. Fish oil (Feed Oil MS) was purchased from Riken Vitamin Co., Ltd., Chiyoda-Ku, Tokyo, Japan. Fish oil was produced from the liver oil of alaska pollack (*Theragra chalcogramma*) and contained 0.02% butylhydroxytoluene. The feed was supplemented with a ratio of 0.02% GTP and 0.2% GTP to Groups B and C, respectively.

The moist pellets were prepared every two weeks and stored at -20°C until feeding. These pellets

were fed once every morning during the 4-week test period (from November 6 to December 3, 1998). The average water temperature at the depth of 2 m was nearly 20.6°C during the test period. The salinity and dissolved oxygen levels in the seawater ranged from 31.1 to 34.0 psu and 5.4 to 6.1mg/ℓ, respectively.

For the analysis of the fish meat, 50 fish of each group were dispatched instantly on the 4th week after feeding. These fish were put into expanded polystyrene insulated boxes with crushed ice and carried to our laboratory.

These round form fish were stored in crushed ice in a ratio of 1 : 1 (w/w) inside expanded polystyrene insulated boxes provided with holes at the bottom to drain out melting water. Crushed ice were added to these boxes appropriately until the day of analysis.

4. Preparation of fish meat for analysis

Five fish were sampled from each group and were filleted, skinned and boned, and the red meat was separated from the fillets on the day of each storage period. These fillets were cut into dorsal, ventral and abdominal-cavity portions. The dorsal meat was used in the analysis of this study. The ventral, abdominal-cavity and red meat was used in other studies. The dorsal meat was chopped into small pieces and minced with a Waring blender while cooling with ice water, and then the sample meat was thoroughly mixed to compensate for specimen differences. The different three portions of mixed meat was sampled three times, and each sample was measured three times, respectively. The average figure obtained from 9 measurements was used as representative data. Deviation among 9 measurements fell within 5 % in all experiments.

5. Estimation of freshness of the meat during their ice storage of round fishes

In this study, changes of total viable counts, psychrophilic bacteria counts and VBN contents in the meat during ice storage of round fish were investigated. Total viable bacteria and psychrophilic bacteria were counted on the day of dispatch, the 5th and 10th day after ice storage according to the method outlined by the Association of Official Analytical Chemists⁹⁾ and Pharmaceutical Society of

Table 2 Composition of Feed Components (%)

Group	Green Tea Polyphenols (GTP)	Fresh Feed ¹	Formula Feed	Vitamin Mix	Fish Oil
Group A	0	47.0	47.0	5.0	1.0
Group B	0.02	46.99	46.99	5.0	1.0
Group C	0.2	46.9	46.9	5.0	1.0

1 : Fresh feed was thawed spotlined sardine (*Sardinops melanostictus*) meat.

Japan¹⁰⁾. VBN contents were determined on the day of dispatch, the 1st, 3rd, 5th and 10th day after ice storage by the Yamagata method¹¹⁾. Measurements of microbial counts and determination of VBN contents for each sample were replicated three times.

Results and Discussion

The changes of total viable counts of bacteria in fish meat during ice storage are shown in Fig. 2. The total viable counts were approximately same levels (0.17×10^3 CFU/g of meat in group A, 0.10×10^3 CFU/g in group B and 0.19×10^3 CFU/g in group C) among the three groups on the first day of dispatch. These counts in all groups increased with the passage of time during the storage period. In group A, the counts greatly increased to 0.47×10^3 CFU/g of meat and 1.62×10^3 CFU/g of meat, on the 5th and 10th day after their ice storage, respectively. In group B, the counts a little increased to 0.16×10^3 CFU/g of meat and 0.71×10^3 CFU/g of

meat, on the 5th and 10th day after ice storage, respectively. In group C, the counts also a little increase to 0.20×10^3 CFU/g of meat and 0.36×10^3 CFU/g of meat, on the 5th and 10th day after their ice storage, respectively. The total viable counts in group A had a higher tendency relatively than those in groups B and C on the 5th and 10th day after their ice storage. In group A, the total viable counts of bacteria showed a remarkable increase tendency on the 10th day after their ice storage, while only a slightly increased on the 5th day. In groups B and C, a little increase in them were observed during ice storage. In group C, stronger microbial growth inhibition than group B was observed.

Fig. 3 shows the changes of psychrophilic bacterial counts in the meat during their ice storage of round fishes. Psychrophilic bacterial counts at the day of dispatch were approximately same level in the three groups, (0.30×10^3 CFU/g of meat in group A, 0.21×10^3 CFU/g in group B and 0.43×10^3 CFU/g in group C). The counts among the three groups

Fig. 2 Changes of Total Viable Counts in Yellowtail Meat by the Feeding of Green Tea Polyphenols (GTP)

Fig. 3 Changes of Psychrophilic Bacteria Counts in Yellowtail Meat by the Feeding of Green Tea Polyphenols (GTP)

increased during the storage period. In group A, the counts drastically increased to 1.50×10^3 CFU/g of meat and 8.13×10^3 CFU/g of meat, on the 5th and 10th day after their ice storage, respectively. In group B, the counts slightly increased to 0.56×10^3 CFU/g of meat and 2.00×10^3 CFU/g of meat, on the 5th and 10th day after ice storage, respectively. In group C, the counts also a little increased to 0.49×10^3 CFU/g of meat and 1.5×10^3 CFU/g of meat, on the 5th and 10th day after their ice storage, respectively. The psychrophilic bacterial counts in group A showed an abrupt increase on the 5th and 10th day after ice storage. These results showed nearly the same tendency to that obtained in the total viable counts of bacteria. In group A, counts of the bacteria on the 10th day were observed much highly than those on the 5th day after ice storage. On the other hand, the increase of psychrophilic bacterial count in groups B and C during ice storage had not a tendency to be so pronounced. The growth inhibition of the psychrophilic bacteria tended to be more pronounced than that of the total viable bacteria.

One of the most serious problems in maintaining freshness of fish and shellfish is the deterioration of their quality by bacteria growth with the passage of storage time. As their spoilage is mainly caused by microbiological decomposition, an increase in bacteria growth is correlated to the degree of their spoilage. What is known of the relationship between the microbial counts and the freshness of fish, is as follows¹²⁾: fresh fish meat keep their total viable counts to less than 5×10^5 CFU/g of meat, the counts in the fish and shellfish meat stored chilled range from 10^5 to 10^6 CFU/g of meat, and spoiled meat show their bacteria counts to be more than 1.5×10^6 CFU/g of meat. Therefore, bacteria count tests are performed to estimate the incidence of microbiological contamination, the self life of foods or the risk of food poisoning. In particular, counts of psychrophilic bacteria in fish and shellfish are very important indexes because they easily increase even at low temperature where they are usually stored. For these reasons, the analysis of psychrophilic bacteria counts during storage of fish and shellfish

provides the most useful information. Thus, changes in total viable counts and psychrophilic bacteria counts were examined in this study.

Green tea polyphenols (GTP) used in this study are composed of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-galocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin gallate (Fig.1). Many studies have described that GTP, a mixture of the above chemicals, showed obvious antimicrobial effects against various kinds of bacteria, for example, were various domestic animal pathogens²⁾, cariogenic *Streptococcus mutans*³⁾, periodontal *Porphyromonas* spp.⁴⁾, thermophilic spore-forming bacteria⁵⁾ and intestinal *Clostridium* spp.^{13), 14)}.

AHN *et al.*^{13), 14)} reported that GTP inhibit the growth of intestinal *Clostridium* spp. *in vitro*. OKUBO *et al.*¹⁵⁾ demonstrated that GTP also decreased the number of human intestinal *Clostridium* spp. in human volunteer study, supporting the *in vitro* results reported by AHN *et al.*^{13), 14)}. We also described that intake of GTP prevented Holstein calves from digestive and respiratory organ diseases owing to improving intestinal microflora balance and inhibiting growth of pathogenic bacteria in the several field tests²⁾. Additionally, the growth inhibition of GTP against various fish pathogens, which were *Vibrio* spp., *Streptococcus* spp., *Pasteurella piscida*, *Edwardsiella tarda* and *Aeromonas* spp., has already been studied¹⁶⁾.

So far, various physiological effects besides antimicrobial properties of GTP have been found *in vivo* studies. It was reported that an oral intake of GTP suppressed the production of 8-hydroxydeoxyguanosine (8 OH-dG), which are produced by oxidative stress in colon¹⁷⁾. The production of 8-OH-dG is induced by giving 1, 2-dimethylhydrazine, resulting in colon cancer. Their study suggested that GTP could suppress physiological oxidation *in vivo*. YOKOZAWA *et al.*¹⁸⁾ reported that urinary methylguanidine, which is known as a uremic toxin and is produced by the oxidation of creatine, was decreased by oral administration of GTP to rats with induced renal failure. They also found that the level of serum methylguanidine in renal failure patients who have

been hemodialyzing decreased by the administration of GTP¹⁹⁾. In their studies, it was suggested that GTP was able to act as a radical scavenger to the hydroxyl radicals *in vivo*. It was also reported that the presence of GTP is determined in plasma and organs of both rats²⁰⁾ and human^{21)~23)} by oral administration of GTP. From these studies, it has been thought that administrated GTP might exist in organs. We have previously reported that the peroxide value (POV), thiobarbiturate value (TBA) and metmyoglobin formation ratio of meat during ice storage were suppressed in cultured yellowtail fed on GTP, and indicated that these effects were based on the possibility of suppressive effect of GTP on physiological oxidation in fish²⁴⁾.

In this study, changes of total viable and psychrophilic bacterial counts in fish meat during ice storage were suppressed by feeding of GTP. These results indicate that GTP might be incorporated into the fish body in the same manner as in the cases of rats and human. Furthermore, in this study, it was suggested that GTP could show antimicrobial effects in fish, while counts of both total viable and psychrophilic bacteria in all groups (groups A, B and C) were kept to less than 5×10^5 CFU/g of meat, indicating that the raw fish meat was edible even on the 10th day after ice storage. Based on these results above mentioned, it may be suggested that GTP improved intestinal microflora balance, or inhibited fish pathogens in fish. For these reasons, it was thought inhibiting effect of GTP might lead those results of microbial counts in two groups (groups B and C). Thus, GTP showed a tendency to stronger effect on the growth of total viable bacteria and psychrophilic bacteria in group C than that in group B. However, further work is needed to support these results obtained in this study. We are presently attempting to confirm our results.

The changes of volatile basic nitrogen (VBN) contents in the meat during ice storage of round fish are shown in Fig. 4. In all groups, the VBN was increased during ice storage, although the increasing rates showed a suppressive tendency in groups B and C compared with it of group A. In particular, on the 10th day after ice storage, VBN contents in

Fig. 4 Changes of Volatile Basic Nitrogen Contents in Yellowtail Meat by the Feeding of Green Tea Polyphenols (GTP)

groups B and C were observed obviously lower than that in group A (14.0mg/100 g of meat in group A, 12.3mg/100 g of meat in group B and 11.8mg/100 g of meat in group C, Fig. 4). The suppressing effect of GTP on the production of VBN was observed more strongly in group C than in group B.

In general, production of VBN is caused by the proliferation of various kinds of bacteria, and thus only a small volume of VBN is found at just after killing. VBN is comprised of ammonia, trimethylamine, dimethylamine, etc., and is produced by microbial enzymes which are generally known as reductase. The contents of VBN are used as an index for the freshness of fish meat. In this study, it was observed that the production of VBN in yellowtail meat during ice storage was suppressed by the feeding of GTP. The findings correspond to the results obtained in the bacteriological experiment (Fig. 1 and 2). It is suggested that the GTP fed to fish might express anti-bacterial effects, and subsequently the production of VBN was decreased.

Our experiment has suggested that the feeding of GTP to fish might be able to show a desirable influence on the freshness of fish meat during

storage. GTP may be available as an easy and preferable ingredient of feed to keep the freshness of cultured fish in the near future.

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緑茶ポリフェノール給与飼育した
ブリ氷蔵中における細菌増殖および
揮発性塩基態窒素量抑制効果

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