MICROBIOLOGICAL AND CHEMICAL QUALITY OF FRESH AND FROZEN WHOLE TROUT AND TROUT FILLETS

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ABSTRACT

The rainbow trout (*Oncorhynchus mykiss*) is considered as an important fish in the freshwater aquaculture and play a significant role in the human diet. The final quality of fish depends on the chemical and microbiological quality of fish at the time of freezing as well as on other factors including storage temperature and processing. The purpose of the study was to determine the microbiological status of 30 samples cooled and frozen trouts collected from approved farm in the Turiec region, territory of middle Slovakia. Total viable counts (TVCs), psychrotrophic bacteria, *Pseudomonas* spp. and also total volatile base-nitrogen (TVB-N) and pH were measured in samples of fresh (1. and 7. day of storage at $0 - 2\ °C$) and frozen whole trout and trout fillets. Frozen samples were stored at -18 °C during 1, 3, and 6 months. Samples were collected from the skin, muscles (sterile) and muscles after filleting. The microbiological quality of samples varied between fresh and frozen (6th month of storage) regarding TVCs and also between samples taken from the skin and muscles after filleting compared to muscle samples collected sterile regarding all tested bacteria. A large number of bacteria (pathogens and spoilage bacteria) enter with the raw material and in particular the skin contamination had a negative impact on the increase of microbial load in fillets. All processing techniques and procedures including filleting therefore must be designed and aimed to minimise contamination and growth of microorganisms in fish. However, based on the results of TVB-N analysis, differences between fresh and frozen samples were found, but all the samples were suitable for human consumption.

Keywords: total viable counts; psychrotrophic bacteria; *Pseudomonas*; total volatile base-nitrogen, rainbow trout

INTRODUCTION

The subsurface flesh of live, healthy fish is considered sterile and should not present any bacteria or other microorganisms. On the contrary, as with other vertebrates, microorganisms colonise the skin, gills and the gastrointestinal tract of fish. The number and diversity of microbes associated with fish depend on the geographical location, the season and the method of harvest. In general, the natural fish microflora tends to reflect the microbial communities of the surrounding waters (**Fernandes, 2009**). The fish could be also contaminated after being caught or during transportation to retail markets. Fish quality is influenced by many factors as the source, cooling methods, processing, packaging, storage conditions. Especially, during the filleting of fish as practised in filleting plants or shops, it is impossible to avoid contamination of the initially virtually sterile fish flesh (**Shewan, 1961**). After contamination and replication of microorganisms, decay occurs and the consumption becomes dangerous (**Alparslan et al., 2014**).

The quality and freshness of fish are rapidly deteriorated through microbial and biochemical mechanisms (**Al-Jasser and Al-Jasass, 2014**). The autochthonous bacterial flora of fish is dominated by Gram-negative genera including: *Acinetobacter*, *Flavobacterium*, *Moraxella*, *Shewanella* and *Pseudomonas*. Members of the families *Vibrionaceae* (*Vibrio* and *Photobacterium*) and the *Aeromonadaceae* (*Aeromonas* spp.) are also common

aquatic bacteria (**Huss, 1995**). Fish quality is mainly assessed through the total aerobic plate counts and counts of bacteria with public health relevance. A monitoring of these microorganisms has been suggested as a measure of fish quality. Psychrotrophs are these bacteria that grow well at or below 7 °C and have their optimum temperature for growth between $20 - 30$ °C. Some psychrotrophic pathogens can grow in the refrigerated food with little or no obvious change of sensory characteristics. *Pseudomonas* species are important spoilage microorganisms in many chilled food products especially fish in which they become the dominant microflora during chill storage (**Gram, 1993**). Thus, their presence in food creates a great risk as they lead to food poisoning and spoilage of food (**Jay, 2000**). *Pseudomonas* spp. mediated spoilage is characterised by unpleasant odours from the production of ketones, aldehydes, esters and sulphur-containing compounds such as methyl sulphide (**Vogel et al., 2005**).

Food spoilage can be considered as any change that renders the product unacceptable for human consumption (**Sivertsvik et al., 2002**). Spoilage of fish starts upon death due to autoxidation (oxidation of unsaturated lipids), reactions caused by activities of the fish's own enzymes, and metabolic activities of microorganisms present in the fish. Over time, loss of the fresh characteristics may be simply measured by comparative visual and smell analysis. Bacterial activity results in unpleasant odour due to

conversion of amino acids into biogenic amines, sulfides, organic acids etc. **(Velu et al., 2013)**. However, volatile bases are the best-characterised chemical indicators of fresh fish spoilage. Evaluation of Total volatile base-nitrogen (TVB-N), or a specific fraction of the volatile bases, for example the TMA fraction, using Conway diffusion chambers allows determination of changes of mg-N/100 g fish.

The activity of bacterial enzymes could be minimised by observing hygiene rules, proper processing, conservation and low temperatures. Cooling and freezing are widely used methods for preservation of fish. However, cooling could not prevent spoilage but shelf-life could be prolonged **(Shamsuzzaman et al., 2011)**. Maintenance of product temperature at -18ºC will not permit bacterial growth or enzymic spoilage. The conditions and freezing process most favourable for good-quality products will, however, also be those that have the least effect on the viability of microorganisms.

The purpose of this survey was to evaluate the microbiological status of fresh and frozen rainbow trout and to assess the degree of bacterial colonisation, amount of TVB-N and pH of fresh and consequently frozen whole fish and fillets.

MATERIAL AND METHODOLOGY

Sample collection

The study was conducted with 30 samples cooled and frozen fish products. Fresh fish were collected from approved farm Rybarstvo Požehy Ltd. located in the Turiec region, territory of middle Slovakia. After catching the fish were slaughtered, vacuum packed and cooled and then transported to the laboratory for analysis. The fresh samples have been stored at 0 – 2 °C (12 pcs) and 18 fish have been frozen immediately after receiving in the laboratory and stored 1, 3 and 6 month at -18 °C. The samples were taken from the fresh fish on 1. (fresh 1) and 7.day (fresh 7) of storage for microbiological examination from the skin (first step), muscle sterile (second step) and from the muscle after filleting (third step). The same procedure was repeated also for frozen fish on 1. (frozen 1), 3. (frozen 3), and 6. (frozen 6) month of storage. At the same time samples were taken for estimation of pH and determination TVB-N. All the analysis were performed on 6 samples in each group.

Sample analysis

In the beginning, swabs were taken from the skin and then 10 grams of muscle tissue were collected aseptically and placed into a Stomacher bag. Ninety ml sample diluent were added, and samples were homogenised at 256 rpm for 1 min. Tenfold dilutions were performed in tubes with 9 ml sample diluent. The total viable counts (TVCs) was determined using the pour plate method according to **ISO 4833 (2003)** and plates were incubated at 30 °C for 48 – 72 h. Horizontal method was used for enumeration of psychrotrophic bacteria **(ISO 6730, 2005)**, and colonies were counted in a solid medium after incubation at 6.5 °C for 10 days. **ISO 13720 (2010)** specifies a method for the enumeration of presumptive Pseudomonas spp. present in meat and meat products, including poultry. The results are presented as log cfu/g.

The pH of the meat was measured by a digital pH meter with glass electrode (AMA digit AD 140, Germany). TVB-N values were determined using micro diffusion Conway method and expressed as mg TVB-N/100 g rainbow trout flesh **(Conway and Byrne, 1933)**.

Statistical analysis

Group means and standard deviations were calculated using column statistics, followed by one-way ANOVA analysis of variance, Tukey's multiple comparison test **(GraphPad Prism 5, 2007)**; and treatments were considered significantly different at $p < 0.05$.

RESULTS

Total viable counts (TVCs) in fresh and frozen fish taken from skin, muscle and fillet are presented in Table 1. The highest microbial loads were established in frozen samples after six month of storage. Contamination of fish samples analyse during first three months of frozen storage was comparable to bacterial load in fresh samples. Slight increase in TVCs was found in fresh samples after 7 days of storage under chilling conditions. The amount of TVCs in samples collected sterile from muscles have been significantly lower ($p < 0.05$) compared to bacteria number on the skin and in the muscle samples collected after filleting.

Most contaminated with psychrotrophic bacteria were samples taken from skin of fresh trouts within 7 days of storage under chilling conditions and high load of bacteria

Table 1 Total viable counts (log CFU.g^{-1}) in fresh and frozen fish and fillets.

Sample	Fresh 1	Fresh 7	Frozen 1	Frozen 3	Frozen 6
Skin	4.75 ±0.15	5.4 ±1.0	4.75 ±0.25	4.8 ±0.2	7.05 ±0.45
Muscle	2.7 ±0.2	2.75 ±0.25	2.1 ±0.4	2.2 ±0.2	5.05 ±0.36
Fillet	4.55 ±0.35	4.85 ±0.45	3.7 ±0.3	4.1 ±0.3	6.05 ±0.55

Table 2 Psychrotrophic bacteria (log CFU.g^{-1}) in fresh and frozen fish and fillets.

Sample	Fresh 1	Fresh 7	Frozen 1	Frozen 3	Frozen 6
Skin	4.8 ±0.1	5.2 ±0.8	4.8 ±0.1	4.5 ±0.6	4.9 ±0.5
Muscle	2.75 ±0.05	2.75 ±0.25	2.05 ±0.05	1.9 ±1.14	1.75 ±0.45
Fillet	4.55 ±0.45	4.95 ±0.55	3.8 ±0.2	4.1 ±0.4	3.9 ±0.1

Table 3 *Pseudomonas* spp. (log CFU.g^{-1}) in fresh and frozen fish and fillets.

Sample	Fresh 1	Fresh 7	Frozen 1	Frozen 3	Frozen 6
Skin	1.5 ±1.06	1.63 ±0.96	2.63 ±0.08	2.65 ±0.26	3.33 ±0.37
Muscle	1.13 ±1.41	1.18 ±0.7	0	0	0
Fillet	1.28 ±1.68	1.83 ±1.45	2.53 ±0.13	2.73 ±0.38	2.85 ±0.57

Table 4 presents the results for pH and TVB-N (mg.100g^{-1}) in fresh and frozen fish.

Sample	Fresh 1	Fresh 7	Frozen 1	Frozen 3	Frozen 6
pH	6.51 ±0.03	6.64 ±0.05	6.79 ±0.01	6.78 ±0.05	6.75 ±0.02
TVB-N	12.75 ±0.52	13.96 ±0.34	17.03 ±0.1	17.03 ±0.21	18.74 ±1.71

was also found in the fillets examined at the same time. The amount of psychrophilic bacteria in samples collected sterile from muscles have been significantly lower ($p < 0.05$) compared to bacteria number on the skin and in the muscle samples collected after filleting. Freezing did not cause the increase in the number of bacteria in all samples (Table 2).

Non-significant increase in microbial counts was observed in samples of fresh trout after seven days of storage (0 – 2 °C). Frozen fish samples taken from the skin and muscles after filleting exhibited higher number of *Pseudomonas* spp. compared to cooled fish samples. The amount of *Pseudomonas* spp. in samples collected sterile from muscles have been significantly lower ($p < 0.05$), in frozen samples has been absented, compared to bacteria number on the skin and in the muscle samples collected after filleting (Table 3).

Results of pH value and TVB-N values in samples of rainbow trout are shown in Table 4. There was no significant difference ($p > 0.05$) in pH values, however slightly higher pH values were found in frozen samples. Significantly higher TVB-N concentrations ($p < 0.05$) were recorded in frozen fish compared to fresh ones, but all the values were below the 30 mg.100g^{-1} considered to be value when spoilage become obvious.

DISCUSSION

Quality of meat products is affected by the quality of raw meat, storage temperature and handling conditions. Current challenges and concerns related to consumption of meat products may be divided into those associated with microbial pathogens and into other meat safety issues. Major challenges related to microbial pathogens include foodborne illness outbreaks, associated product recalls, regulatory compliance, and issues related to microbiological control (**Kunová et al., 2014**). The complex concept of fish quality consists of safety, nutritional value, availability, integrity, freshness, eating quality, product size and type (**Abbas et al., 2008**). Cooling and freezing are the usual methods for conservation of fish. Freezing of fish has the advantage of providing the consumer with unprocessed fish that retain to a much greater extent the flavour, odour, appearance and texture of the freshly caught fish (**Jeon et al., 2002**).

The most widely accepted microbiological criteria for chilled and frozen raw fish are those set for aerobic plate counts (APC) at 25 °C proposed by the International Commission on Microbiological Specifications for Foods (**ICMSF, 1986**). An increase of APC to levels in excess of 10^6 cfu/g is usually indicative of inadequate refrigeration, long storage under refrigeration or one of the former prior to freezing. According to **Broekaert et al., (2011)** loads of $10^7 – 10^8$ CFU.g^{-1} make spoilage organoleptically detectable.

The effect of chilling (0 – 2 ºC) on the quality deterioration of whole ungutted aquacultured rainbow trout was studied by integrated evaluations of microbiological, biochemical, and sensory attributes. The counts of aerobic mesophilic, psychrotrophic bacteria and *Pseudomonas* increased exponentially. Aerobic mesophilic and psychrotrophic bacteria grew exponentially from an initial load of 3 – 5 log$_{10}$ CFU.g^{-1} reaching 7.6 log$_{10}$ CFU.g^{-1} on day 15 (**Ninan et al., 2011**). The initial values are comparable with our results in samples taken from skin and fillets. However on day 7 increase of bacteria count has not been significant and fish were evaluated as a fit for human consumption. It is widely accepted that the initial microbial load of fresh water varies depending on water conditions and temperature. In our study, mesophilic and psychrotrophic counts in fresh fish tissues were close to or lower than 5.7 log CFU.g^{-1} recommended by **ICMSF (1986)** for whole fresh water fish. In their experiments **Diler et al. (2000)** demonstrated that mesophilic bacterial counts on trout skin varied between 2 and 7 log CFU.g^{-1}. Our studies showed average total viable microbial counts was 4.75 – 5.4 log CFU.g^{-1} on the skin and in the fillet and only in frozen trout after six months of storage was higher (7.05 log cfu/g). Lower results have been achieved by **Gonzales et al. (1999)** established TVCs of 2.90 log CFU.g^{-1} in rainbow trout. However most of the authors reported the similar TVCs in rainbow trout to be increased from a baseline of 4.0 log CFU.g^{-1} up to 7.04 log CFU.g^{-1} during storage (**Rezaei and Hosseini, 2008**). According to **Özogul et al. (2013)** a TVC load of 3.59 log CFU.g^{-1} is a parameter for high quality of trouts.

The high psychrotrophic count of frozen fish may be attributed to the contamination of raw materials which come in contact with fish unsatisfactory sanitation during handling, processing and distribution as well as inadequate

chilling and/or freezing which increase the existing microorganisms. *Pseudomonas* species are widely distributed in nature, unsanitized equipment's, pouted water and fishermen hands especially during harvesting, transportation and storage are considered as the source of fish contamination. The contamination of *Pseudomonas* organisms may be attributed to the heavily contamination boats and boxes which transfer the organisms to fish during cleaning. Accordingly, the consumption of such frozen fish contaminated with different members of psychrotrophic bacteria particularly and *Pseudomonas* species may constitute, at times public health hazards (**Hassan et al., 2014**). *Pseudomonas* spp. may only represent the minority of the total microflora at the beginning, but become the dominant at the end of the shelf life. The quantity of present SSOs can be used to predict the residual shelf life of products causing identification of this organism as a main concern. The shelf life of any food product can be detected from beginning of storage period and ending when the SSOs reach the least spoilage level. *Pseudomonas* spp. decrease the shelf life of food products and so their quality by producing lipolytic and proteolytic enzymes which is the main cause of food spoilage during the storage (**Franzetti and Scarpellini, 2007**). **Khidhir et al. (2014)** found that *Pseudomonas* spp. was 3.77 log $CFU.g^{-1}$ for silver carp sold in Sulaimania markets, Iraq. Higher count was recorded by **Begum et al. (2010)** who collected fish samples from the super shop in Bangladesh, and reported that the highest count of *Pseudomonas* spp. was found in Tilapia (5.94 log $CFU.g^{-1}$). The counts of psychrotrophic bacteria and *Pseudomonas* were even lower in frozen samples compared to fresh samples. The similar results were found in our previous study, when thawing and repeated freezing in double frozen fish samples led to an increase in TVCs, but the counts of psychrotrophic bacteria in frozen was lower over the period of storage compared to fresh fish (**Popelka et al., 2014**).

The amount of TVCs, psychrotrophic bacteria and *Pseudomonas* in samples collected sterile from muscles have been significantly lower ($p <0.05$) compared to bacteria number on the skin and in the muscle samples collected after filleting. Fish product safety is influenced by many factors as origin of the fish, product characteristics, processing mode and cooking. During cold storage of fish in a chill room or in melting ice these bacteria will grow, and together with those acquired by contact with contaminated surfaces on board ships or ashore they form the natural spoilage flora in fish. During filleting more spoilage bacteria will contaminate the fish flesh from debris and slime on filleting boards and knives and from the filleters' hands (**van den Broek et al., 1984**). To attain the best possible bacteriological condition of fish fillets it is essential to start with freshly caught fish, consistently cooled in ice of good bacteriological quality (**Shewan, 1961**). Before filleting, the fish must be washed thoroughly in clean drinking water to keep contamination of the fish flesh during filleting as low as possible. Filleting itself must be done on cutting boards made of synthetic material under running water. The cutting boards and other utensils must be cleaned and disinfected daily and the fillets must be cooled immediately after cutting. These measures in combination represent good

manufacturing practice (GMP). The numbers of TVCs did not exceed 10^6 $CFU.g^{-1}$ (**van den Broek et al., 1984**).

There was no significant difference in the pH values, however slightly higher pH values were found in frozen samples (6.75 – 6.79) compared to fresh fish on day 7 (6.64). Significantly higher TVB-N concentrations were recorded in frozen fish compared to fresh ones, but all the values were below the 30 $mg.100g^{-1}$. In the study performed by **Ninan et al. (2011)** the pH values increased from an initial value of 6.74 to 7.13 on day 15 of chilled storage. The pH values indicating bacterial growth and production of volatile basic compounds such as ammonia by fish spoilage bacteria. Increase in pH due to accumulation of alkaline compounds through autolytic activities and microbial metabolism has been reported. Many microbes including *Pseudomonas* produce ammonia during amino acid metabolism. TVB-N values exceeded 27.87 $mg.100g^{-1}$ on day 14 when the psychrotrophic counts exceeded 10^7 $CFU.g^{-1}$. *Pseudomonas* also displayed the typical growth pattern of psychrotrophic bacteria without a lag phase increasing from initial counts of 3.0 to 5.02 log $CFU.g^{-1}$ on day 15. Based on the TVB-N and microbiological limits, the shelf life of trout at 0 – 2 ˚C was 9 – 12 days. In this study, the values exceeded the limit of acceptability of 25 $mg.100g^{-1}$ proposed by **Stansby and Olcott (1963)**. However, higher concentration of 30–35 mg TVB-N.$100g^{-1}$ flesh is considered the limit of acceptability for ice-stored cold water fish by **Connell (1995)**. Critical limits of 25, 30 and 35 $mg.100g^{-1}$ of TVB-N were established for different groups of fishes (**Commission Regulation 2074/2005**), but no limit for acceptability has been established for rainbow trout. However, comparing results for different fish species, does not show correlation between muscle pH and the amount of volatile bases contained within the fish at rejection. Neither does the change in pH during storage correlate well with the production of TVB-N (**Fernandes, 2009**). During spoilage, the majority of volatile bases are produced from the soluble non-protein nitrogen of the fish (free amino acids and other low-molecular-weight nitrogenous compounds), as significant proteolysis is observed only during the latest stages of spoilage and after rejection. For some fish species, a correlation can be made between the spoilage of the fresh fish and the production of TVB-N.

Post mortem spoilage of food products can be caused by chemical, enzymatic or microbial activities and is accompanied by the formation of compounds responsible for changes in odour, flavour and texture of fish meat. One of the chemical markers of spoilage in fish is the total volatile basic nitrogen, including ammonia, trimethylamine (TMA) and dimethylamine (DMA), the concentrations of which increase with spoilage by either bacterial or enzymatic degradation. The total volatile basic nitrogen is produced during degradation of proteins and non-protein nitrogenous compounds, mainly as a result of microbial activity (**Özogul and Özogul 2000**). **Commission Regulation (EC) 2074/2005** set the limits for TVB-N in sea fish, however, no limits are available for fresh-water fish. Since TVB-N is produced mainly during bacterial decomposition of fish meat, the higher content of TVC of samples throughout the period of frozen storage could account for higher TVB-N values of rainbow trout.

CONCLUSION

Taking into consideration the obtained results and the recommendations of the International Commission on Microbiological Specifications for Foods, it could be concluded that all the samples were fit for human consumption in respect to method and time of storage and processing. The highest microbial contamination was observed in samples taken from the skin of cooled and frozen trout, followed by fillets, and lowest microbial load was in samples of muscles collected sterile. The higher number of contaminated processed fish products, both cooled and frozen, and the *Pseudomas* spp. counts could pose a risk for human health after consumption of undercooked fish.

Contaminated fish could be dangerous and the efficient bacteriological control of hygiene is important to ensure acceptable levels of contamination and prevention of food intoxications. In the fish processing chain managing risks should be based on scientific knowledge of the microbiological hazards and the understanding of the primary production, processing and manufacturing technologies and handling during fish storage and transport, retail and catering.

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