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Diploma thesis

Bacterial contamination of fish products – microbiological and MALDI TOF approach

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1 THESES INTRODUCTION

This diploma thesis is focused on the microbiological contamination of raw fish products. The fresh fish is very sensitive to spoilage. That and the fact that the fish are one of the main sources of protein in food for humans are the reasons why the quality, proper handling and storing of the fish are crucial. It is estimated that one-fourth of the annual food supply and 30 % of all landed fish are lost due to microbial activity (Ghaly et al., 2010).

The human population worldwide reached almost 7.6 billion people in the year 2018 and to this number is yearly added about 8 million new lives (World Population Review, 2021).

With the growing population, there is a significantly rising demand for protein and consequently is growing the world's appetite for fish as fish area crucial source of nutrition and an important part of the diet for many people worldwide. In 2017 the fish provided about 3.3 billion people with almost 20 % of their animal protein intake per capita, which makes 17 % of total animal protein consumed globally (The fish site, 2020). In 2018 according to Fao (fao.org, 2021), the average amount of consumed fish per capita reached 18 kg.

It is also true that, according to the WHO, unsafe food will cause an average of 600 million cases of outbreaks from food borne illness, which is up to 420 cases that will cause the death of the consumer (WHO, 2021).

From what has been said above, it follows that quality and health safety for consumers is extremely important in the case of fish and fish products. After decease, autolytic, oxidative, and microbiological processes take place in fish meat. According to González et al. (1999) the metabolic activity of bacteria plays an important role in the spoilage process. Unfortunately, several stains of microorganism causing food spoilage are potentially dangerous and can be a threat to human health (Samples et al., 2014). In fish and fish products, the frequently reported pathogenic bacteria involved in food-borne infections and intoxication are *Listeria monocytogenes, Clostridium botulinum, Salmonella spp., Aeromonas spp., Plesiomonas shigelloides, Staphylococcus aureus, Shigella spp., Escherichia coli,* and *Yersinia enterocolitica*. Therefore, it is crucial to ensure sufficiently fast, accurate and simple methods for determining the quality and safety of raw and processed fish.

The growth medium recommended for enumerating microorganisms on food, including the fish is a plate count agar. According to the work of Broekaert et al. (2011), there are few microorganisms (even of genera potentially dangerous to human) unable to grow on that media. Namely are the genera *Photobacterium*, *Shewanella*, *Vibrio*/*Aliivibrio*, *Pseudoalteromonas*, *Psychrobacter* and *Pseudomonas.* However, they are abundant in fish tissue and grow very well on it. Other types of agar are also used, though, most of them are bovine based and therefore the composition of the nutrients in them may be different from what is required by microorganisms present on fish tissue.

Another aspect we considered was the commonly used incubation temperature. According to the Association for the Advancement of Medical Instrumentation recommendations, all cultures should be incubated at 37 ° C for 48 h (Arduino et al. 1991). However, since fish are poikilothermic animals, and in temperate conditions, the water practically never reaches such values (2007-2021 meteocentrum.cz), it can be assumed that even bacteria naturally occurring in fish are adapted to lower temperatures. And since the storage of fish and fish products also takes place at lower temperatures, the question is whether the cultivation of samples at 37 ° C is sufficiently indicative of the bacterial microflora present on fish, and of its evolution over time, at normal storage temperatures.

In this thesis, we tried to compare microorganism species grown on agar plates prepared from the broth of three different fish species, and in three different temperatures. For control plates, we used typical Plate count agar. The common carp (*Cyprinus carpio)* meat as a traditional and widely used fish in the Czech Republic was selected as a source of microorganisms for inoculation on agar.

The colonies of grown microorganisms were identified with the use of MALDI-TOF MS, the obtained data were analysed and compared against the control plate.

2 THEORETICAL INTRODUCTION

Nowadays it is necessary to transport food from one place of production to another where it is processed and even further, where it is sold and consumed. As the spoilage of fish meat is rather rapid after the fish is caught and slaughtered, the proper handling, pretreatment and preservation are necessary to increase the shelf life of the fish and protect the health of people. It is estimated that one-fourth of the annual food supply and 30 % of all landed fish are lost due to microbial activity (Ghaly et al., 2010).

Another important point of view is maintaining the attractivity of the product for the final customer. All the changes in fish meat can alter its texture, flavour, aroma, and nutritional value.

According to González et al. (2000) changes in the quality of freshwater raw fish stored in chilled and on-air, are results of enzymatic changes (autolysis), non-enzymatic reactions (rancidity), and metabolic activity of bacteria.

Vácha (2000) introduced one more postmortem process: increased mucus production. This process runs simultaneously with the early stages of autolysis. During the life of fish protective mucus is produced on the surface of fish skin. The production of mucus visibly intensifies directly after the death of the fish. The mucus is rich in nitrogen compounds and therefore is a perfect source of energy for microorganisms from the environment. It is important to ensure proper handling with slaughtered fish to protect the skin of fish from rupturing, breaking or other damage. Any damage to the skin can allow the microorganisms to enter the muscles, spread, and accelerate the spoilage. Production of the mucus stops with the onset of the rigor mortis.

Shawyer and Pizzaly (2003) introduced four stages of spoilage differenced by the sensory changes of fish meat. Progress of these phases and major changes of meat in time is shown in Figure1. The first two phases are mostly connected with autolytic changes and maturing of the fish meat. The fish is still rated as fresh. In the third phase, the quality of meat deteriorates gradually, with the beginning and spreading of microbial activity. In this phase, the fish meat is still acceptable, though not rated as fresh. The fourth phase is typical of voluminous microbial activity and the meat is inedible. Of course, the time of changes is not exact, it always depends on specific conditions. The main factors affecting the autolytic and microbial processes according to Fraser and Sumar (1998) are the degree of microbial growth and flora, the storage temperature and packaging.

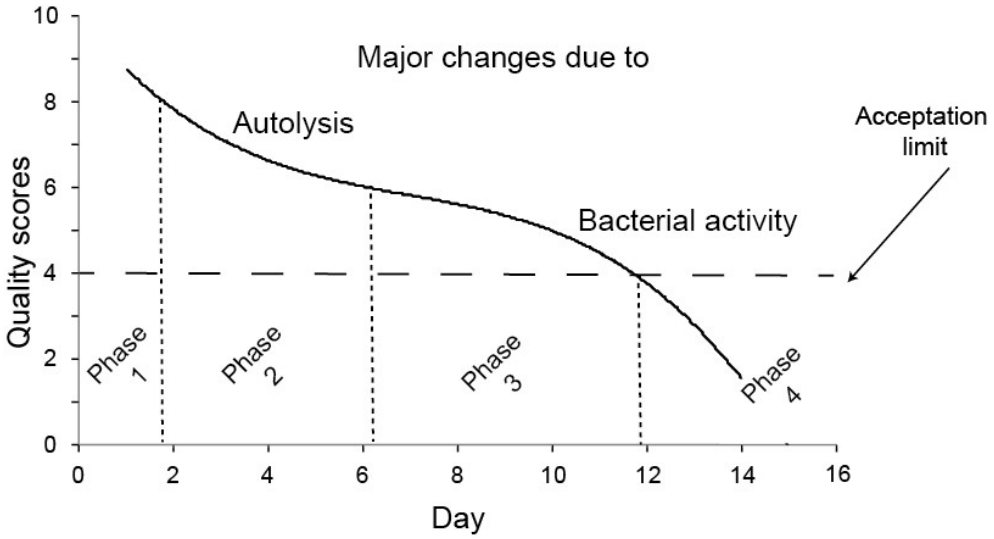

Figure 1. Progression of bacterial spoilage with four phases of spoilage according to Shawyer and Pizzaly (2003). Passed from Ghaly (2010), modified.

For purposes of this thesis, I decided to separate the postmortem changes of fish and fish meat by Ghaly (2010) into three major steps caused by different mechanisms: T

- Enzymatic autolysis
- Oxidation
- Microbial growth

2.1 ENZYMATIC AUTOLYSIS

The sources of energy for the muscle of living fish are glycogen (carbohydrate) and fat. These substances are oxidated, end eventually changed into carbon dioxide, water, and energy-rich substance – adenosine triphosphate (ATP). During that process is glycogen in glycogenolysis broken down to glucose which is the actual source of energy for the muscles. Fat is disintegrated into fatty acids, enters the Citric acid cycle, and is used for the production of ATP (Ophardt, 2003).

The amount of glycogen in fish muscle differs according to the species of fish, the size and age of fish, condition, slaughtering technic, and other factors. The level of glycogen in the muscle is at the lowest when the fish is hungry - for example from holding - or stressed (Vácha, 2000).

After the heart stops, the oxygen is no more pumped into the muscles and the only pathway of production of energy is glycolysis. Glycolysis is an extremely inefficient

process for ATP production. This process produces only two moles of ATP from one mole of glucose compared to 36 moles of ATP obtained from one mole of glucose, using all end products in the mitochondria of living breathing fish (Huss, 1995).

According to Watabe et al. (1991), glycolysis regenerates ATP and at the same time, lactate is produced as a final product. Theoretically, when restoring three moles of ATP, two moles of lactate are formed. Lactate production proceeds simultaneously and in proportion to the rigor mortis process.

2.1.1 Rigor mortis

Contractions and relaxations in animal muscle are controlled by calcium and enzyme ATP–ase – occurring in every muscle cell, while both actions use ATP as the source of energy. In a moment the animal is slaughtered the breathing stops providing the oxygen, but the muscles continue consuming ATP. When the intracellular amount of ATP decreases under the critical point, the fish enters the Rigor mortis (FAO, 1995).

Post-mortem stiffness or "rigor mortis" is defined by the decline of ATP to zero, zero per cent extensibility, an ultimate pH that is reached, and the production of lactic acid that has plateaued (Warner, 2016).

Rigor mortis has been thoroughly studied in slaughter animals including fish. According to Borgstrom (2012) fish generally stay in rigor mortis for a shorter period than mammals. The onset of rigor mortis and its duration depends on many factors: mostly condition of the fish, amount of stress shortly before and directly in the time of slaughter, the temperature at which slaughtered fish are stored, and others. For the fish stored on ice, rigor mortis starts 1 – 7 hours after death and last 30 – 120 hours.

Glycogen is a source of energy in living muscle and after the death of the fish experience together with phosphor creatine anaerobic glycolysis forming ATP, lactic acid or three hydrogen phosphorus acid (Hao et al., 2020). As a result of creating these acids, the pH of fish meat drops from the usual degree.

Borgstrom (2012) stated that the usual value of pH immediately slaughtered fish at approximately about 7. The value can slightly differ according to condition, species, and age of the fish, etc. During the rigor mortis pH gradually decline but even in the most acidic state reaches maximally pH 6.2 - 6.5. These values of pH are insufficient for the inhibition of microbial growth. That is one of the reasons why is fish meat more susceptible to microbial attack (Huss, 1988). Even though the amount of glycogen in

muscles and consequently the value of pH can vary depending on the species, age and physical condition of the fish, it can never reach the values of the muscles of mammals, which means as low as pH 5.1 or lower. Generally, the fish with white meat reach higher final pH, while the pH in fish with red meat reaches lower (Lougovois and Kyrana, 2005).

According to Mukundan et al. (1986), the pH of slaughtered fish can be lowered even more, due to the breakdown of lipids. The lipids are broken down by a set of lipases, including phospholipase. These enzymes naturally occur in almost all fish, especially in the fatty species, and the species with red meat. The primary products of lipolysis are glycerol and fatty acids. These acids even though weak, can significantly lower the pH of the tissue. The combination of lactic acid and fatty acids can bring the pH down to between 5 and 6.

The meat of fish does not stay in this stiff and acidic state for long. When the stiffening resolve, the values of pH rises back to the original 7. The increasing of pH in this stage is the result of denaturation of proteins, alternatively onset of spoilage or combination of both processes (Borgstrom, 2012).

Even according to Munro (2008), the re-increase in pH value is caused by early denaturation and decomposition of muscles. At the same time, however, he admits that the exact mechanism is still unknown.

2.1.2 Enzymatic changes of ATP

Autolytic changes in fish meat mainly affect the texture of the meat. At the initial stage of spoilage, the enzymatic processes produce no off-odour or off-flavour, nevertheless, they do negatively affect the time of shelf life of fish (Samples et al., 2014).

ATP is processed by series of dephosphorylation and deamination and finally broken down to hypoxanthine and ribose. The process of degradation shows Figure 2.

According to Huss (1988), this breaking down of ATP comes through in every kind of fish, what differs is the speed of the process. The majority of ATP deteriorated to some of the metabolites in approximately 24 hours, regardless of species. Usually, in most fish, the catabolism of ATP results in a short period of accumulation of inositol monophosphate (IMP). This substance contributes to the desirably pleasant and fresh flavour of fish muscle (Ocaño-Higuera, 2009).

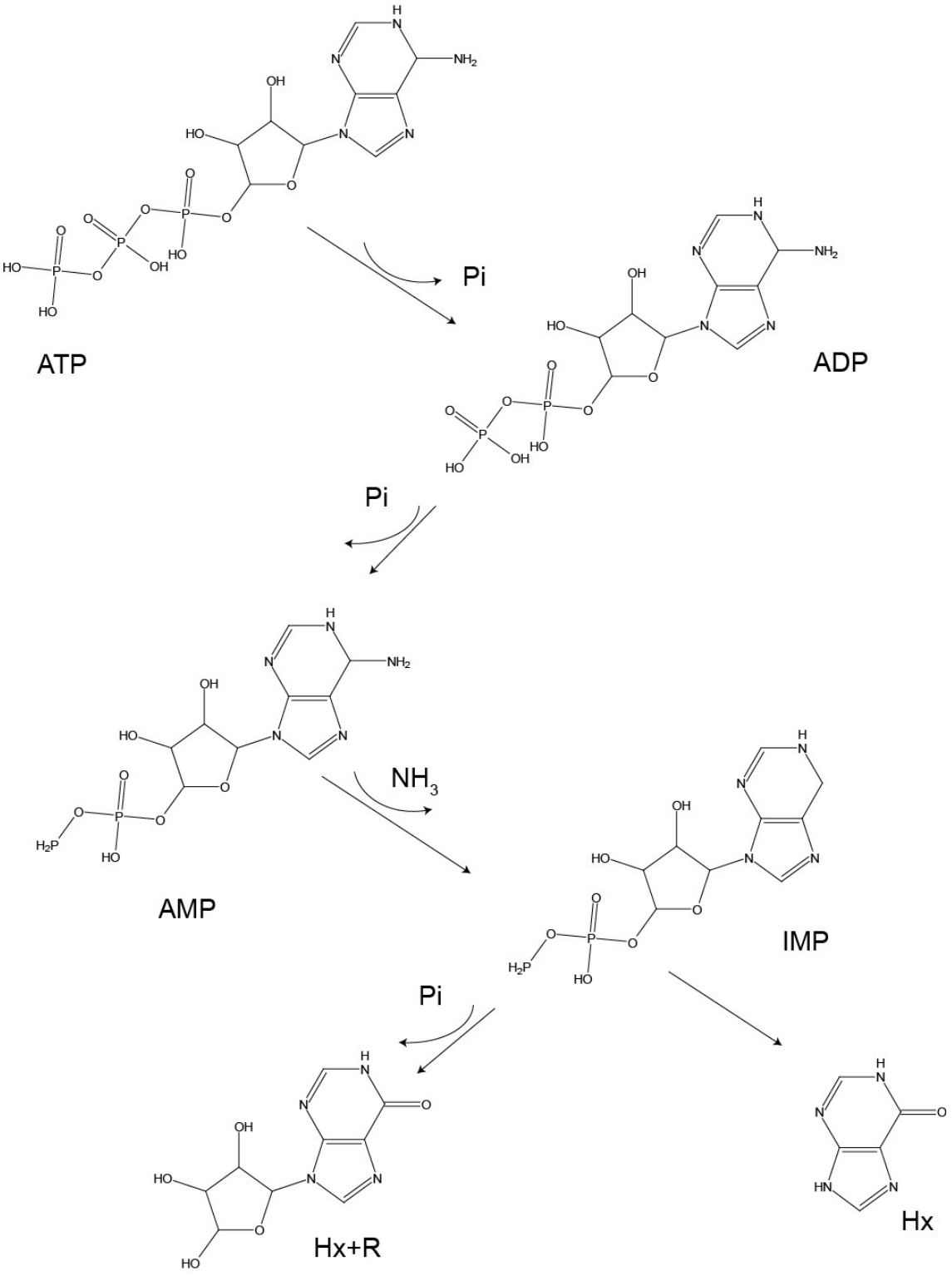

Figure 2. Autolytic degradation of ATP (Huss, 1988). ATP – adenosine triphosphate, ADP – adenosine diphosphate, AMP – adenosine monophosphate, IMP – inositol monophosphate, Hx + R – hypoxanthine + ribose, Hx - hypoxanthine

The continuing enzymatic processes gradually decrease the amount of IMP and increase the amount of neutral-tasting hypoxanthine-ribose (Hx+R) or even bitter tasting hypoxanthine (Hx). The amount of some of these components in meat issued in tests of freshness (Lakshmanan, 2000).

One of the most often mentioned methods of testing freshness is the so-called K-value. Figure 3 shows the formula for the calculation of the K-value. This method has been developed and is widely used in Japan. The K-value expresses the relationship between the amount of Hx+R and Hx and the total amount of ATP – related compounds (Huss, 1988).

$$K\% = \frac{(Hx+R)+Hx}{ATP+ADP+AMP+IMP+(Hx+R)+Hx} x100$$

Figure 3. The formula for calculation of K-value. Passed from Samples et al. 2014.

Because the amount of ATP and ADP rapidly decrease and mostly disappear in 24 hours, and even the amount of AMP drops to unmeasurable values, is sometimes instead of K-value used K_1-value. It simplifies the calculation, as is shown in Figure 4, and makes the value clearer, considering developments of amounts of all ATP-related metabolites. From the formula is obvious that the K_1-value increases with the storage time (Samples et al., 2014).

$$K_1\% = \frac{(Hx+R)+Hx}{IMP+(Hx+R)+Hx} x100$$

Figure 4. The formula for calculation of K_1-value. Passed from Samples et al. 2014.

There are many methods for the quantitative determination of amounts of ATP and its metabolites. Unfortunately, most of them are time-consuming or the results are limited to the amount of one compound. However, there is one method, which produces a rapid quantitative evaluation of ATP and its breakdown products, for the calculation of K-value or K_1-value: the high-performance liquid chromatography - HPLC, with a commercially available column and UV detection (Agric, 1985; Veciana-Nogues et al., 1997).

The value of K immediately after capture is usually below 10%, the K-value of

20% is considered an optional freshness point, and a K-value of 60% is the latest point of rejection. When the K-value is above 60% the fish is spoiled and poses a health risk to the potential consumer (Cheng et al., 2015).

2.1.3 The enzymatic changes of proteins

Several proteolytic enzymes are present in the muscle of fish, especially in small circular organelles called lysosomes. These lysosomes are very acidic, rich in enzymes, and in a living organism, they are important for the degradation of dead cells, viruses, microorganisms and for the production of energy (Gahl et al., 2001).

According to Mukundan et al. (1986) are the lysosomes in fish chiefly rich in cathepsins and proteases. The amount of cathepsins in the fish body is not distributed evenly, but the overall amount is at least 10 times higher than in mammals.

In the lysosomes of fish tissue is usually about 13 different types of cathepsins. Cathepsins in tissue break down proteins into peptides, oligopeptides, and smaller polypeptide protein fragments. The post-mortem activity of cathepsins results in the weakening and degradation of several structural muscle proteins, and that activity leads to tissue softening (Ahmed et al., 2015).

Between pH 5 and 6, which can in fish tissue be reached during rigor mortis, are dissolved the membranes of lysosomes and cathepsins and proteases are released to cells. Since the fish are poikilothermal animals, their proteases and cathepsins are active even at a significantly lower temperature than the mammalian enzymes. As a result, given the optimal conditions of pH and temperature, the cathepsins and proteases can digest all peptides in fish muscle in less than 24 hours (Mukundan et al., 1986, Karmas, 1978).

Other enzymes also play their role in post-mortem changes in fish tissue. Among them are important calpains, responsible for digesting of part of myofibril, or collagenases causing deterioration of the fine collagenous fibrils. The main result of these processes is softening of the fish tissue (Huss, 1995).

There are the major autolytic changes and the enzymes causing them shown in Table 1. There is also shown one more relatively important autolytic process in this table, a process that can cause significant economic losses for fishing companies - the belly bursting.

Table 1. The main autolytic changes in chilled fish, and enzymes causing them (Huss, 1995), adapted.

Enzyme(s)	Substrate	Changes Encountered
glycolytic enzymes	glycogen	production of lactic acid, pH of tissue drops, loss of water-holding ability in muscle high-temperature rigor may result in gaping
autolytic enzymes, involved in nucleotide breakdown	ATP ADP AMP IMP	loss of fresh fish flavour, gradual production of bitterness with Hx (later stages)
cathepsins	proteins, peptides	softening of tissue making processing difficult or impossible
chymotrypsin, trypsin, carboxy-peptidases	proteins, peptides	autolysis of visceral cavity in pelagic (belly- bursting)
calpain	myofibrillar proteins	softening, melt-induced softening in crustaceans
collagenases	connective tissue	gaping of fillets softening

The belly bursting is a problem mostly for fish in the period of high feeding. The digestive tract of these fish is full of digestive enzymes, which can easily dissolve tissues and cause spoilage soon after catch. In the digestive tract are also high numbers of bacteria, these bacteria proliferate in dissolved intestines and cause the production of gases – mostly CO_2 and H_2. The gasses concentrate and increase the inside pressure on the fish belly and in the final consequence can cause belly bursting (Lakshmanan, 2000). This problem increases with long-term chilled storing, or with freezing and thawing of a whole, not gutted fish (Ghaly et al., 2010).

2.2 OXIDATION

Autooxidation unfavourably correlates with tissue colour, flavour, texture and in the case of unsaturated FA and cholesterol can even result in the production of potentially toxic compounds (Gray, 1996, Addis and Park, 1989).

2.2.1. *Oxidation of lipids*

Oxidative spoilage is mostly connected with lipids and therefore is the risk of developing this spoilage process highest at the fat or oily fish like mackerel and herring (Ghaly et al., 2010). The quantity of fat is also connected with the colour of fish meat. Red fish meat has a higher volume of lipids and hem substances than white fish meat.

From the stated above is clear that red meat is more prone to oxidation, and consequently spoilage, than white meat. Sometimes in the fish processing plants is the red meat cut out to prolongate the shelf-life of the rest of white meat (Samples et al., 2014).

According to Samples (2013), the oxidation of lipids is always a present hazard in meat and fish and their products. The lipids of fish are especially rich in unsaturated FA, with a high portion of polyunsaturated fatty acid (PUFA), and in consequence receptive to oxidation. Oxidation results in the production of off-flavours, rancid taste, and even the development of many substances which can endanger human health.

The oxidation of unsaturated fatty acid (UFA) proceeds in a three-stage free radical mechanism: Initiation, propagation, termination, as is it showed in Figure 5. The process involves the reaction of oxygen with the double bonds in UFA. Oxygen must be activated for this reaction, primary oxygen activators in animal tissues are transition metals (Ghaly, 2010). These transition metals are mostly iron and copper (Hultin, 1994).

$$RH + X\bullet \rightarrow R\bullet + XH \quad (a)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \quad (b)$$
$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$

$$\left.\begin{array}{l} R\bullet + ROO\bullet \rightarrow \\ 2\ ROO\bullet \rightarrow \\ 2R\bullet \rightarrow \end{array}\right\} \text{Nonradical products} \quad (c)$$

Figure 5. Three stages of lipid oxidation: Initiation (a), propagation (b), termination (c). RH = Lipid, X• = free radical. Passed from Gardner (1989).

In animal tissue, glycerol lipids occur mostly in two forms as polar lipids – including first of all phospholipids and as neutral lipids – triacylglycerols (TAGs), a small number of diacylglycerols and monoacylglycerols.

Phospholipids are essential constituents of membranes of cells and organelles ensuring membrane fluidity. TAGs serve as an energy source. Phospholipids are more unsaturated due to their functionality and therefore prone to oxidation. In addition to

this, free fatty acids can occur in tissue due to enzymatic deterioration of acylglycerols and phospholipids. The oxidative reactivity is in general TAGs > phospholipids > FFAs (Samples, 2013).

According to Gray et al. (1996) is the influence of lipid oxidation on the loss of quality of meat and meat products well proved. Lipid oxidation also correlates with the pigmentation of the tissue. In the red meat fish like for example Atlantic salmon (*Salmo salar*) the autooxidation cause discolouring. On the other hand, in the white meat fish accumulation of secondary metabolites and coaction of Maillard reaction can cause browning of the meat (Samples et al., 2014).

Maillard reaction or non-enzymatic browning are interchangeable terms used to indicate a cascade of reactions involving on one side amino acids, peptides and proteins and the other side, reducing sugars. This reaction affects to great extend the sensory and nutrition characteristic of meat (Arnoldi, 2004).

2.2.2. ***Oxidation of proteins***

In addition to the oxidation of lipids in the fish tissue also proceed oxidation of proteins. The main problem resulting from these processes is a change in the structure of the fish meat. These changes influence the physical qualities of the tissue, alter sensory features and texture of the meat (Samples, 2014).

Protein oxidation is induced by direct reaction with reactive oxygen species (ROS), or by indirect reaction with by-products of oxidative stress. ROS can cause oxidation and consequently changes in the whole protein structure – side chains amino acids as well as the backbone of the protein. The results of the activity of ROS are protein fragmentation and the creation of cross-chain protein linkage.

Oxidative stress is on the other hand caused by an imbalance between production and accumulation of ROS in cells and the ability of a biological system to produce antioxygenic substances (Zhang et al., 2013). Oxidative stress influences living organism and does not affect the meat.

The rate of oxidative degradation of amino acids is affected by the position of amino acids within the protein. Some amino acids are more exposed to ROS and hence faster affected by protein oxidation. Amino acids with highly perceptive sulphur centres - methionine and cysteine are within the first oxidated even in mild oxidative

conditions. In presence of transition metals are promptly oxidated also residues of tryptophan (Estévez, 2011).

Oxidation of proteins has a negative effect on the nutritional quality of the meat and therefore their selling potential. Foremost the water-holding ability, decrease in solubility and emulsification of protein and increase oxidation of lipids. Protein oxidation can even affect the digestibility of certain proteins and thereby influence the nutritious value of the meat (Samples et al., 2014).

2.3 MICROBIAL GROWTH

All raw foods are populated by a wide variety of microorganisms. The multiplication and activity of microorganisms cause spoilage of many foods and are also the most common cause of food poisoning and foodborne illness. Bacterial and microorganism growth is the major threat for easily perishable foods (including fish), and therefore a major concern of food producers (Singh and Anderson, 2004). It is estimated that 25 % of all harvested food is lost due to the action of microorganisms (Gram and Dalgaard, 2002).

Fish muscles have ideal conditions for the rapid development of bacteria and therefore rapid spoilage. Samples et al. (2014) gives three main reasons why is it so:

- Fish are poikilotherm organisms and therefore thanks to a wide temperature range (0-40 °C), they allow the survival of a large number of bacteria.
- The pH of fish muscle is relatively high (mostly > 6), only in the rigor mortis stage it drops slightly and for a short time. This decrease is not sufficient to suppress bacterial growth.
- Fish muscle contains a relatively high amount of non-protein nitrogen. This form of nitrogen is readily soluble in water and is an ideal breeding ground for bacterial development

According to Vácha and Buchtová (2005), the development of bacterial microflora is also helped by higher water content and at the same time a lower content of connective and adipose tissue in the muscle of fish, compared to other farm animals.

2.3.1. Microorganisms of alive or freshly caught fish

There are always high numbers of microorganism on the living and freshly caught fish. The numbers vary enormously rather according to the living conditions than the species of the fish (Huss, 1995). The total amount of the colony-forming units (CFU) on the surface (skin) of the fish is in the range of 10^2-$10^7 \times cm^{-2}$ (Huss, 1995; Liston, 1980). Other rich sources of microorganism in the fish body are gills and intestines. Schewan (1962) stated that gills and intestines both contain 10^3-10^9 $CFU \times g^{-1}$.

The wide range of microbial colonization is due to the different living conditions of fish. The numbers of $CFU \times cm^{-2}$ are lowest, on the fish skin living in clean, cold waters. In contrast, the microbial population of fish living in warm, polluted water is at the upper end of the range (Huss, 1988).

The bacterial microflora of fish is divided into two basic groups: autochthonous bacteria and allochthonous bacteria. Autochthonous bacteria are microorganisms inherent in fish, commonly found on fish. Conversely, allochthonous bacteria are non-native to fish and fish or fish products must be contaminated by them from the external environment (Samples et al., 2014).

As is written above, the total amount of microorganisms depends on the environmental conditions, but not only the total amount, the species spectrum differs as well. In the cold, clean waters the dominating microorganisms are psychotropic gram-negative species, contained in genera *Acinetobacter, Flavobacterium, Moraxella, Shewanella, Pseudomonas, Vibrionaceae* and *Aeromonadaceae*. From the gram-positive species are aquatic microorganisms mostly engaged in genera: *Bacillus, Micrococcus, Clostridium, Lactobacillus* (Fraser and Sumar, 1998, Huss, 1995). In fresh-water fish from polluted waters can be found members of *Enterobacteriaceae*, who are otherwise common in terrestrial mammals (Lougovois and Kyrana, 2005). The main genera of microorganisms in living or freshly slaughtered fish are shown in Table 2, the main allochthonous bacteria with the primary source of contamination, are in Table 3.

As for tropical fish, it was long time believed that the composition of the microorganisms differs from the composition of species occurring in temperate waters. It was supposed that the dominant genera of tropical waters are *Bacillus* and *Micrococcus.* However, it was declared by Huss (1995), that works of numerous researches (Acuff et al.,1984; Gram et al., 1990; Lima dos Santos 1978; Surendran et

al., 1989) proved that the composition of species of microorganism in tropical and temperate waters are similar, just with a higher load of gram-positive and enteric bacteria in the warmer waters (Fraser and Sumar, 1998).

There is one more factor affecting the composition of microbial flora in the aquatic environment, and it is the salinity of the waters. While bacteria *Aeromonas spp.* is typical for freshwaters, the genera of *Vibrio* and *Photobacterium* need sodium for their growth and reproduction, therefore they are typical for the marine environment. A little confusing situation is around *Shewanella putrefaciens.* Even though it is characterised as sodium requiring, it can be isolated from freshwaters as well (Huss, 1995).

Table 2. The main genera of microorganisms found on living or freshly caught fish. Passed from Huss (1995), adapted.

Gram-negative	**Gram-positive**	**Comments**
Pseudomonas	*Bacillus*	
Moraxella	*Clostridium*	
Acinetobacter	*Micrococus*	
Shewanella putrefaciens	*Lactobacillus*	
Flavobacterium	*Coryneforms*	
Cytophaga		
Vibrio *Photobactrium* *Aeromonas*		*Vibrio* and *Photobacterium* are typical of marine waters; *Aeromonas*is typical of freshwater
Escherichia colli		Can occur in polluted waters, in tropical waters can survive for long periods
Salmonella		

Table 3. The main allochthonous bacteria and their primary source. Passed from Samples et al., (2014), adapted.

Microorganism	**Primary source**
Listeria monocytogenes	Soil, birds, sewage sludge, estuaries, mud
Staphylococcus aureus	ubiquitous, humane origin
Salmonella spp.	the digestive tract of terrestrial vertebrates
Shigella spp.	Humane origin
Escherichia coli	faecal contamination
Yersinia enterocolitica	ubiquitous

As mentioned above, the species composition, as well as the number of microorganisms present on fish, depends on the environment in which the fish live (Gram and Huss, 2000). Besides, not all microorganisms present on the fish's body are involved in the spoilage process. According to Fraser and Sumar (1998), and Huss (1995), some microorganisms present on the body of fish, even in large numbers, do not participate in the process of spoilage at all. This phenomenon is shown in Figure 6. Therefore, we distinguish two different terms: spoilage flora and spoilage bacteria.

Spoilage flora is microorganisms present on the fish when it spoils, whereas spoilage bacteria are microorganisms directly causing the spoilage.

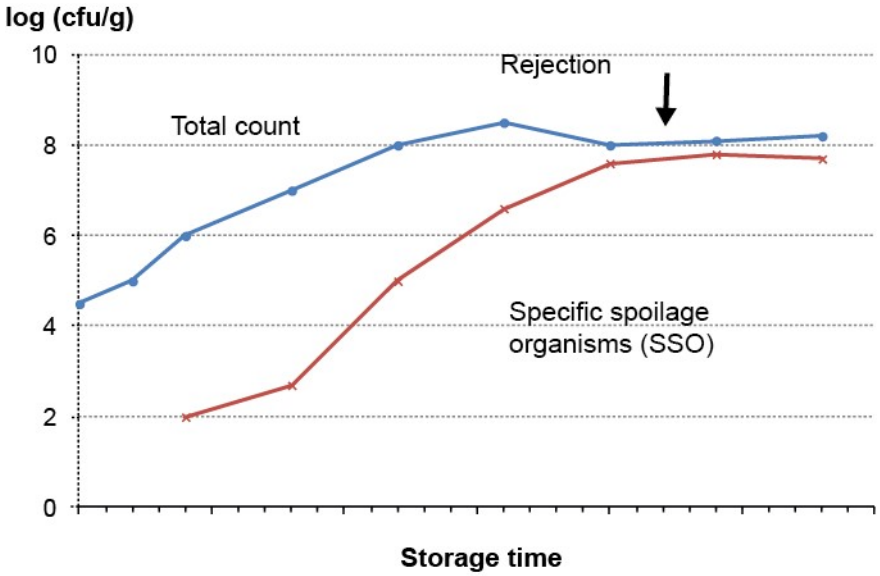

Figure 6. Development of the total count of bacteria and volume of specific spoilage organisms during the storage. Passed from Huss, 1995.

2.3.2 ***Spoilage microorganism***

Spoilage of the fish and food, in general, is always a consequence of microbial growth. All the raw and slightly processed food is initially contaminated with a wide spectrum of microorganism. But only a selection of these microorganisms can grow to high numbers. “Spoilage association” is a term specifically fabricated for these intensely growing microorganisms. The specific mechanism why some bacteria predominate over another, often closely related bacteria is not fully understood yet (Gram and Huss, 1996).

The muscle of live and freshly caught fish is considered sterile, thanks to a functioning immune system. However, after the death of the fish, the immune system collapses and nothing prevents the bacteria from developing (Huss, 1995). Microorganisms then enter the body of the fish through the skin and the gills, and the body cavity is contaminated with microorganisms of the digestive tract. The most common strains of microorganisms in the fish intestine tract are gram-negative *Vibrio, Achromabacter, Pseudomonas, Xanthomonas,* and gram-positive *Clostridium spp*., in marine fish. In the fresh-water fish is the situation similar, only *Vibrio* is replaced with *Aeromonas spp*. which is dominant in the intestines of these fish (Lougovois and

Kyrana, 2005). Enzymatic autolysis products such as soluble proteins, low molecular weight components, etc. provide bacteria with an excellent feeding ground (Fraser and Sumar, 1998). According to Lougovois and Kyran (2005), the mucus of fish is thanks to the presence of free amino acids, mucopolysaccharides, trimethylamine oxide (TMAO), etc., ideal for the development of bacteria and therefore intensive microbial activity occurs on the surface of fish.

The intensity of microorganisms growth in the body of a slaughtered fish has four phases:

- Lag phase - in this phase there is no increase in the number of microorganisms
- Exponential phase - bacteria multiply exponentially
- Stationary phase - there is no further increase in the number of microorganisms, but there is no decrease.
- The nutrients needed for the growth of a given microorganism have been depleted, and thus the number of bacteria decreases.

The duration of individual phases depends on many factors – storage temperature, the species of fish, age, type of bacteria etc. (Sternisa et al., 2018).

In fish caught from cold waters, kept on ice, some bacteria practically skip the lag phase and immediately enter the exponential growth phase. This is mainly because these bacteria are adapted to the cold environment, therefore lowering the temperature does not prevent their development. After storing on ice for 2-3 weeks the number of bacteria presented reaches 10^8-10^9 CFU$\times g^{-1}$ on cm^{-2} (Huss, 1995). That is the time when spoilage becomes apparent. In comparison, the microorganisms of fish tissue stored at ambient temperatures reach the level of 10^7-10^8 CFU$\times g^{-1}$ in approximately 24 hours (Huss, 1995).

The psychotropic, cold-tolerant bacteria are mostly gram-negative strains. For most fish, the longer they are stored on ice, the more dominant are *Pseudomonas* and *Shewanella* strains. The strains of *Psychrobacter* (*Moraxella*), *Acinetobacter* and *Flavobacterium* are often also present although in much smaller numbers (Lougovois and Kyrana, 2005). The spoilage microorganisms are often only 1 % of initial microflora but grow significantly fast from the beginning and in time of spoilage may form 30 % of the bacterial load (Fraser and Sumar, 1998).

The situation is different for microorganisms of fish from warm waters. Because most of these microorganisms are thermophilic, and therefore need higher temperatures

for optimal growth and multiplication, the lag phase can last 1 - 2 weeks when stored on ice. After this time is elapsed the number of microorganisms begin to grow exponentially. At the time of spoilage, the microorganisms load of warm-water fish is essentially the same as that of cold-water fish (Huss 1995).

The activity of individual bacteria in slaughtered fish depends not only on the storage temperature but also on the type of bacteria. Each bacterial strain has a different level of activity and rate of reproduction. The activity of the most common spoilage bacteria in fish was summarized by Hui (1992), as is shown in Table 4.

According to Lougovois and Kyran (2005), most microbial activity takes place on the surface of the fish and deeper layers are penetrated primarily by microbial enzymes and other products of microbial activity. Since most of the activity takes place on the surface, it is important to prevent skin damage (for example, by inappropriate hunting techniques) and thus the disruption of the natural barrier, in order to prevent microorganisms from penetrating deeper into the meat.

The penetration of microbial enzymes from the surface into the deeper layers of the meat, as well as the penetration of nutrients from the meat to the surface, is more intense on fish with thin, fine skin, and therefore spoilage is progressing faster on these fish (Huss, 1995).

In the aerobic environment, the main bacteria causing spoilage in fish stored on ice are *Shewanella putrefaciens* and *Pseudomonas spp*., in ambient temperatures is it *Vibrionaceae* and (especially in polluted waters) *Enterobacteriaceae.* When stored in an anaerobic environment, the growth of *Pseudomonas spp.* is suppressed, but *Shewanella putrefacins* and *Photobacterium phosphoreum* can continue to prosper and cause spoilage (Lougovois and Kyrana, 2005).

Table 4. The activity of the main microorganism involved in Fish spoilage. Source Hui (1992), adapted.

Spoilage activity	**Microorganisms**
High	*Pseudomonas putrifaciens, Pseudomonas fluorescens* and other *Pseudomonas, Shewanella putrefaciens*
Moderate	*Moraxella, Acinetobacter* and *Alcaligenes*
Low, active only in specific conditions	*Aerobacter, Lactobacillus, Flavobacterium, Micrococcus, Bacillus* and *Staphylococcus*

2.3.1 Chemical changes caused by spoilage bacteria

As is written above, spoilage bacteria like *Pseudomonas putrifaciens* and *Pseudomonas fluorescens* multiplies rapidly at the beginning of spoilage and start to produce huge amounts of proteolytic and hydrolytic enzymes. The formed enzymes begin to break down proteins into peptides and amino acids, and these are further broken down into simpler substances. Lipids are degraded to fatty acids, glycerol, and other components. (Fraser and Sumar, 1998).

Although fish tissue contains few carbohydrates, it is rich in low molecular weight, non-protein nitrates (NPN), which are a source of energy and nitrogen for bacteria. These NPNs include in particular: free amino acids, creatine, nucleotides, TMAO and urea (Leroi and Joffraud, 2011).

These chemical compounds are transformed by the action of bacteria in decaying fish into new substances: trimethylamine, volatile sulphur compounds, aldehydes, ketones, esters, low molecular weight compounds (Huss, 1995), amines, biogenic amines (putrescin, cadaverine, histamine), etc. Many of these compounds produce extremely unpleasant off-odours and off-flavours, causing the fish to become unacceptable (Lougovois and Kyrana, 2005).

The composition of these unpleasant odours is not always the same but differs according to the type and origin of the fish. The resulting sensory perception is also different. While marine fish from temperate waters are characterized by the odour of fishy, rotten and hydrogen sulphide, the odour of fresh-water and tropical fish is characterised mostly as fruity and sulfhydryl (Gram and Huss, 1996; Lima dos Santos, 1978; Gram et al., 1989). This difference is due to the difference in body composition of these fish and the presence of different strains of spoilage bacteria, and thus to the formation of different volatile products. The basic spoilage bacteria, together with the volatile substances which arise from their activities, are clearly shown in table 5, as was summarized by Church (1998). In table 6 on the other hand are shown metabolic products of bacteria together with the substrate from which they are typically formed.

Table 5. typical bacterial spoilage compounds. Passed from Church (1998).

Specific spoilage bacteria	Spoilage compounds
Shewanella putrifaciens	TMA, H_2S, CH_3SH, $(CH_3)_2S$, HX
Photobacterium phosphoreum	TMA, HX
Pseudomonas spp.	Ketones, aldehydes, esters, non - H_2S sulphides
Vibrionacaea	TMA, H_2S
Aerobic spoilers	NH_3, acetic, butyric and propionic acid

TMA: trimethylamine; H_2S: hydrogen sulphide; CH_3SH: methylmercarptan; $(CH_3)_2S$: dimethylsulphide; HX: hypoxanthine, NH_3: ammonia

Volatile sulphur-containing components such as hydrogen sulphide (H_2S), methyl mercaptan $(CH_3)SH$ and dimethyl sulphide $(CH_3)_2S$ are formed by the conversion of the amino acids cysteine and methionine. Hydrogen sulphide is produced from cysteine by *Shewanella putrifaciens* and possibly *Vibrionaceae*. *Shewanella putrifaciens* is also behind the production of $(CH_3)SH$ and $(CH_3)_2S$ by the processing of methionine. All these sulphur-containing substances are extremely strongly off-smelling even in small concentrations and therefore are strongly connected to the quality of the fish (Huss, 1995).

TMAO has an important osmoregulatory function in the body of fish - mostly marine. This compound is present in the body of fish in a concentration of 1 – 2 %, and it is pH neutral and non-toxic in live fish (Fraser and Sumar, 1998). After the death of the fish, in the environment with deficiency of oxygen, this substance is used by some gram-negative bacteria such as *Shewanella putrifaciens*as an electron acceptor, when the bacteria continue aerobic metabolism (Huss, 1995). During this process, TMAO is reduced to TMA. Meanwhile, is TMAO also dissociated by the endogenous enzymes to form dimethylamine (DMA), and formaldehyde (FA). While TMAO is completely odourless, the volatile amines formed in process of its reduction, contribute to the odour of stale fish and have a significant effect on quality (Fraser and Sumar, 1998).

The reduction of TMAO to TMA is often accompanied by the formation of hypoxanthine (Hx). Hx, as mentioned above, is formed by autolysis of ATP, but can also be caused by the action of bacteria. Bacterial Hx production from Hx+R (inosine), or IMP (inosine monophosphate), may even be higher than autolytic. The bacterial strains responsible for the formation of Hx are mainly: *Shewanella, Pseudomonas* and *Photobacterium* (Gram and Huss, 1996).

Simultaneously with the reduction of TMAO, the degradation of amino acids to NH_3 also takes place in an anaerobic environment (Huss, 1995). Ammonia is produced by bacterial degradation of urea and other non-protein nitrogenous substances (Fraser and Sumar, 1998). Especially with a longer storage period, there can be a notable increase in the concentration of NH_3. At the same time, the lower fatty acids such as acetic, butyric, and propionic acid are accumulated in fish tissue (Huss, 1995).

Samples et al.(2014) also mentions the content and formation of biogenic amines in fish meat. It is mainly the formation of histamine by bacterial or enzymatic decarboxylation of histidine. The increased content of histamine can be dangerous for humans and causes the so-called scombroid fish poisoning. According to Fraser and Sumar (1998) is a histamine product of purely bacterial activity. The bacteria responsible for the decarboxylation of histidine are primarily *Vibrio spp., Proteus morganii,* and *Klebsiela pneumoniae.* Because these bacteria are only able to grow at temperatures from 8-15 ° C, the formation of histamine is associated with poor storage conditions. Histamine content is also one of the basic indicators of fish freshness. Other low molecular weight amines such as putrescine, cadaverine, spermidine, spermine, and tyramine are formed in a similar manner from ornithine, lysine, arginine and tyrosine, however, these substances do not cause any problems in a healthy person (Samples et al., 2014).

Table 6. The off-odour compound by bacteria activity during the ice storage of fish. Passed from Huss (1995).

Substrate	**Compound produced by bacterial activity**
TMAO	TMA
Cysteine	H_2S
Methionine	CH_3SH, $(CH_3)_2S$
Carbohydrates and lactate	Acetate, CO_2, H_2O
Amino acids (glycine, serine, leucine)	Esters, ketones, aldehydes
HxR, IMP	Hx
Amino acids, urea	NH_3

TMAO: trimethylamine oxide; H_2S: tydrogensulphide; CH_3SH: methylmercarptan; $(CH_3)_2S$: dimethylsulphide; HX:hypoxanthine, NH_3:ammonia

2.4 MALDI – TOF MS

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) over the last decade has become a versatile and powerful tool for the analysis of biomolecules. In principle, when analysing a sample using MALDI-TOF MS, it is necessary to apply the sample to a detection plate and add a matrix. These are mostly crystals forming small organic compounds. The matrix forms crystals, into which the analysed sample is also incorporated. After irradiation with a laser beam, the structure of these crystals disintegrates, and a cloud of particles is released. The particles gain velocity depending on their mass (m) and charge size (z). The energy of the particles is thus directly proportional to the magnitude of the charge and indirectly proportional to the mass. The detector captures ions and then calculates $m \times z^{-1}$ according to the time of flight (TOF) (Matoušková, 2017). According to the resulting spectral fingerprint, it is possible to identify the analysed sample, without the need for further analyses (Jurinke et al., 2004). The output of TOF measurements is the so-called peptide/protein mass fingerprints (PMF) (Singhal, 2015).

MALDI - TOF approach for identification of microorganism species is based on the comparison of a single colony microbial spectral PMF to the library spectra. According to Singhal et al. (2015), PMFs are mainly created from ribosomal proteins, which make up 60-70% of the dry weight of the microbial cell. This procedure, where whole cells were used for analysis, has already been described by Holland et al. (1996). Lay (2001) claims that although in a complex whole-cell environment it is possible to isolate only a certain fraction of proteins with this approach, the PMF thus obtained is sufficient to identify the microorganism. The same results were reported also by Claydon et al. (1996). Sample preparation is crucial for successful measurements on MALDI-TOF MS. Since the samples are properly prepared the MALDI Biotyper software (Bruker) is employed to obtain a sufficient peak mass list and PMFs from each sample. The data are clustered to reduce its amount. It is performed using a software tool integrated into the MALDI Biotyper. This software enables principal component analysis (PCA), which can indicate the homogeneity or heterogeneity of a given data set. PCA clustering, by the virtue of the reduction and visualization of acquired data, can often show those relationships and clusters that would not be obvious.

There are several ways to identify microorganisms using MALDI-TOF MS. However, the currently widely used method is comparing the results with the

microbiological library, mainly because it is sufficiently reliable, easy to perform, and fast (Sandrin et al., 2012).

The principle of determining the microorganism is very simple. It is a comparison of the PMF of an unknown organism with the PMFs in the database. Using algorithms, MALDI-TOF MS compares the similarity of the sample with the library data and expresses the probability of correct identification. In the case of a low probability of accurate identification of the microorganism, it can also offer alternative species to which the measured values could correspond (Jang and Kim, 2018). The resulting score indicates the degree of accuracy of the identification. The manufacturer states that the accurate identification to the species level has a score ≥ 2.0, intermediate score ≥ 1.7 and < 2 means accurate identification to the genus level, score <1.7 is unreliable identification (Bizziny et al., 2010).

Most authors report the accuracy of bacterial identification up to the species level of about 90 %, for all of them. The experiment performed by Bizziny et al. (2010) has to be mentioned. They performed measurements using MALDI-TOF on 1,371 known samples (bacteria and yeast) and the accuracy of determination up to the level of the species reached 93.2 %, 5.3 % of the samples were correctly determined at the genus level, and 4.9 % of samples were misidentified.

As mentioned above, sample preparation is essential for accurate and repeatable measurements with MALDI-TOF MS. Any modification or shortening of the procedure can affect the measurement output (Pavlovic et al., 2013).

3 THE GOAL OF THE THESIS

Fish is considered a very valuable source of quality proteins, healthy fats, and other important nutrients. However, fish muscle is also an easily perishable material, often even when properly stored. Both autolytic and bacterial changes in fish tissues can cause loss of nutritional value, endanger consumer health, or even inedibility and thus significant economic losses. Therefore, it is important to strive to improve control techniques for both the freshness, quality, and health safety of fish and fish products.

Most methods for controlling bacterial strains in meat are currently developed for mammals and therefore mainly industrially produced bovine based agars are used, and the cultivation of samples takes place at 37 ° C. These conditions may not be optimal for all bacterial strains occurring in fish, and therefore it can be assumed that not all of the microorganisms contained in the cultured sample sufficiently grow during cultivation.

The diploma thesis aims to find out how are affected microbial communities gained from the carp fillets at different storage period and the shelves temperature. Furthermore, agars originated from different fish species will be evaluated for potential microbial preferences.

4 STUDY WORKFLOW

The work on this diploma thesis was divided into 4 basic phases

Phase I.– Preparatory

Phase II.– Inoculation of Petri dishes and bacterial growth

Phase III. – MALDI-TOF

Phase IV.– Chemical analytical approaches – determination of ATP and lactate

The flow diagram of stages II. and III. is shown in Figure 7.

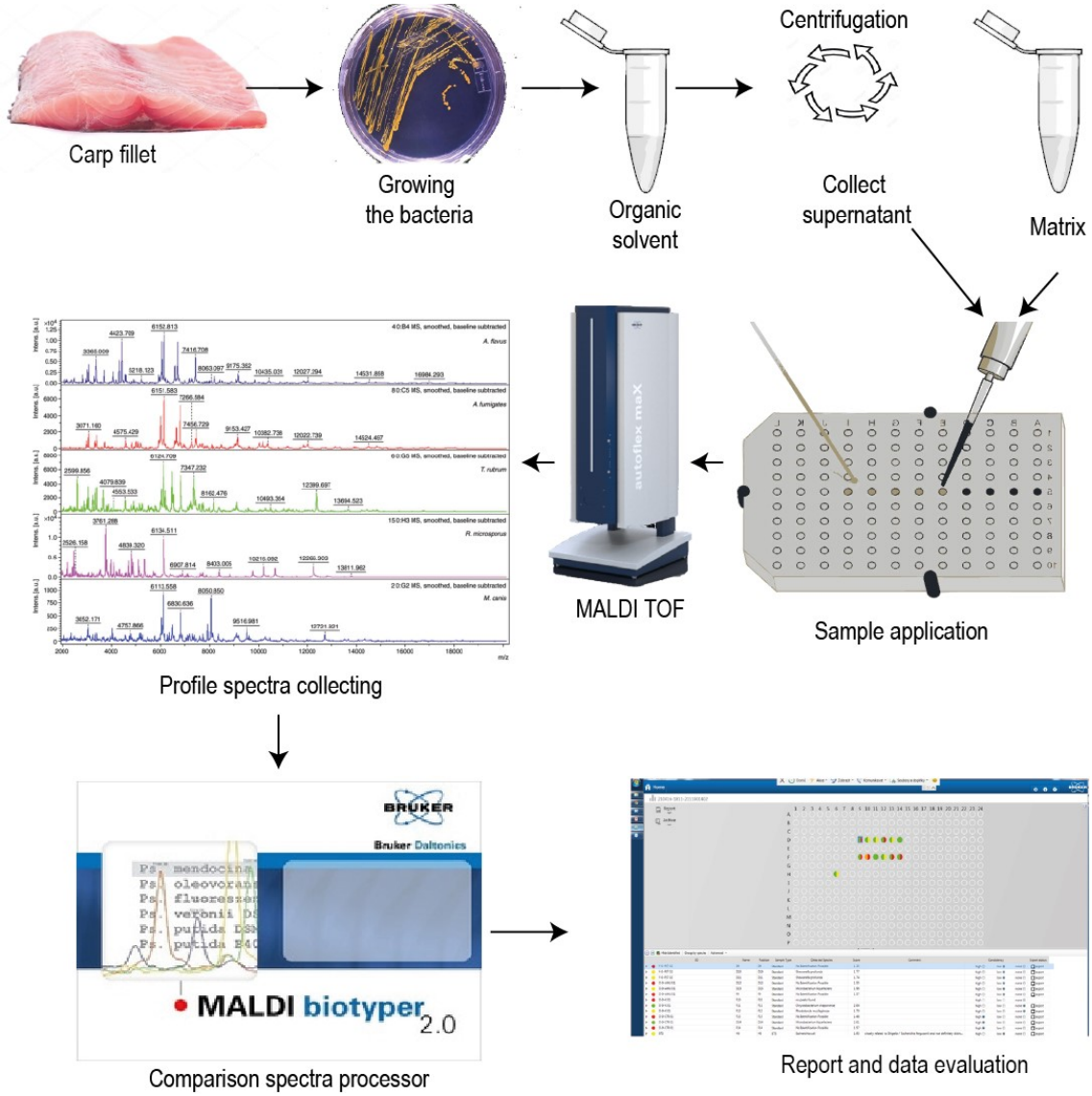

Figure 7. Detailed workflow diagram of the second and third working phase

4.1 PHASE I - PREPARATORY

4.1.1 Agar preparation

First, it was necessary to prepare broth from selected species of fish. After careful consideration, taking into account the availability of fresh freshwater fish in the conditions of the Czech Republic, the following fish species were selected:

- common carp *(Cyprinus carpio)* as a representative of freshwater omnivore
- white grass carp *(Cternopharyngodon idella)* as a representative of freshwater herbivore
- rainbow trout *(Oncorhynchus mykiss)* as a representative of freshwater carnivore

The meat and bones of these fish were cooked at 90 ° C for 3 hours. The obtained pure, filtered broth was used to replace the distilled water in and Bovine – based agar was used 1.5 g × l^{-1}.The samples of particular broths were then analyzed for amino acid and fatty acid profiles. Bovine-based plate count agar (Sigma-Aldrich) prepared with distilled water (1.5 g × l^{-1}) was considered as a control. The agar was then autoclaved and poured into marked Petri dishes under sterile conditions in a flow box. After the agar had cooled and solidified, the plates were ready for use.

4.1.2 Sample preparation

After the inoculation medium was prepared, it was necessary to obtain samples of fish for inoculation. Common carp *(C. carpio)* was used as a model fish. This species was chosen mainly because it is the best-selling Czech freshwater fish, and at the same time this species is the main fish bred in aquaculture in the Czech Republic (Samples et al., 2014).

Market carps weighing 2.5 - 3 kg were percussive stunned, exsanguinated by cutting the gill arches and, after evisceration and washing, cut into fillets circa 600 g. The left fillets were used for the microbial contamination during the storage experiment, the right fillets were then used for the determination of ATP and lactate. The first samples were collected immediately after filleting by cutting circa 100g of the fillet. The left fillet sample was used as a source of microbial cultures and the right filet sample was stored at - 80 ° C for further analytical analysis. The same sampling method was used after 72 and 120 hours of the experiment from fillets kept on ice (0 ° C), fridge

(4 ° C), and thermal chamber set to 8 ° C to simulate marked shelf. Each group have 10 repetitions. Since the work in a sterile environment, handle microorganism and preparation samples for the MALDI TOF is very time-consuming it was unable to process more frequent sampling. Totally were collected 90 samples for microbial investigation (360 Petri dishes) and 90 samples for further chemical analysis.

4.2 PHASE II – INOCULATION OF PETRIS DISHES AND BACTERIAL GROWTH

Immediately after processing at time 0, the samples were quickly tempered at desired temperatures and the first series of inoculations was performed. Three different storage temperatures were chosen to test the bacterial contamination during storage: 0 ° C for storage on ice, 4 ° C for storage in the fridge and 8 °C for storage on the market shelf. The method of inoculation is outlined in figure 8. The remaining fillets after sampling were then placed in incubators to maintain the correct temperature. The same procedure was used for inoculation of additional plates at 72 and 120 hours after slaughter.

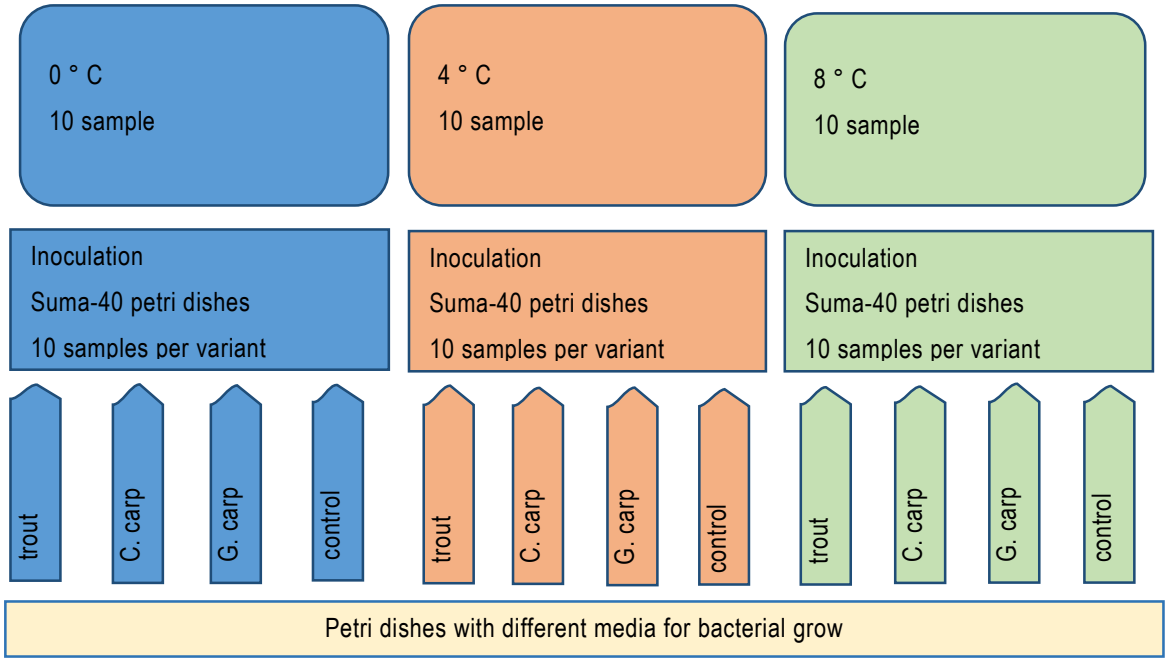

Figure 8. Schema of planned inoculations. Done in times 0, 72, 120 hours after slaughter.

When inoculating agar, it is very important to follow the exact technological procedure to avoid undesired contamination and at the same time to be able to separate

individual colonies after the growth of bacteria. To prevent contamination by microorganisms from the environment and possibly from the hands of the vaccinator, the whole process takes place in a laboratory fume hood, using disposable rubber gloves.

We used sterile cotton swabs to inoculate the agar plates. We first wiped them thoroughly on a sample of meat (muscle and skin) and then transferred them to one spot on the prepared Petri dish. Then the cotton swab was thrown away, and we continued to work with the laboratory loop.

The laboratory loop must be sterilized by tanning over a torch before each use. With the loop thus prepared, we then spread the material applied with a cotton swab in a zigzag pattern gradually over the entire Petri dish. The inoculation procedure is shown in Figure 9. The purpose of this procedure is to stretch the applied material as much as possible to gradually dilute it and thus separate the individual colonies.

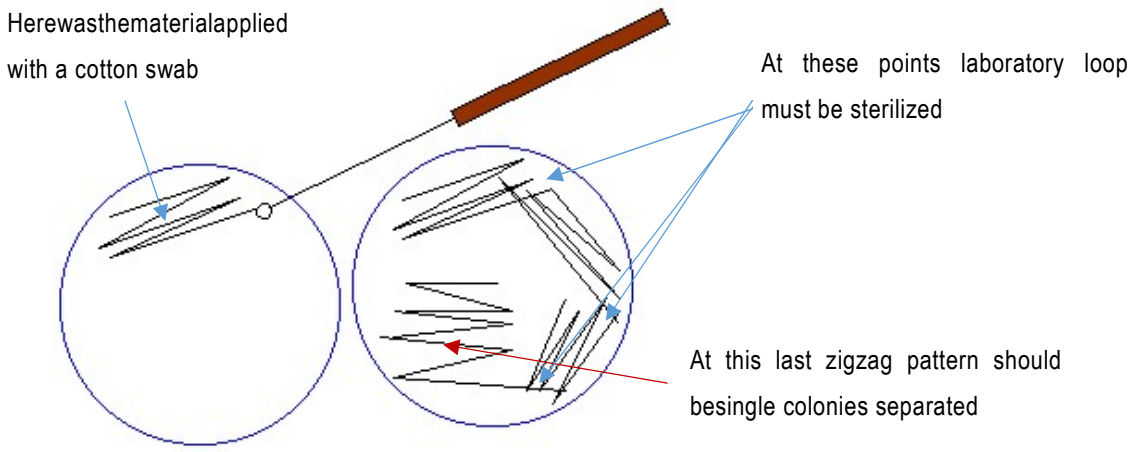

Figure 9. The application of analysed material on Petri dish. The laboratory loop must be sterilised repeatedly during this process. Passed from: Weber scientific manual adapted.

4.3 PHASE III –MALDI – TOF MS

The appearance of the first colonies was dependent on the incubation temperature. The first single colonies appeared on plates incubated at 8 ° C. Over time, they began to appear on other inoculated plates, the last on plates incubated at 0 ° C. After the discovery of the colonies, it was necessary to start the analysis by MALDI-TOF MS.

In our work, the aim was to take a sample of one bacterial colony with a sterile tip, place it in a vial with an organic solvent (Burker standard solvent 50:2.5:47.5 ACN:

trifluoroacetic acid: water v:v:v), pipetting down and up 10 times to mix the solvent with sample, centrifuge and use the supernatant for sampling on a metal analytical plate - MSP 96 target polished steel (Bruker Daltonics)

Along with the supernatant samples, it was also necessary to apply a standard (bacteria with a well-known PMF) to calibrate the instrument. After all, samples had dried at room temperature, each (including the standard) was covered with a layer of the matrix solution. For the matrix, we used a solution of crystallin cinnamic acid and HCCA (α-Cyano-4-hydroxycinnamic acid). The samples prepared in this way had to dry thoroughly on air again at room temperature.

When everything was sufficiently dry, all that remained was to insert the analytical plate into the Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser and operating in positive ion mode operate under MALDI Biotyper® Compass version 4.1.100 (Bruker Daltonics, Bremen, Germany) automatic control. 500 laser shots (40-60 % laser intensity) were used for sample spectra accumulation. Bacterial Test Standard (Bruker Daltonics, Bremen, Germany) was used for external mass calibration. The obtained results were further processed also by the MALDI Biotyper software, i.e. the so-called clustering was performed.

4.4 PHASE IV - CHEMICALANALITICALAPPROACHES – DETERMINATION OF ATP AND LACTATE

4.4.1 ATP analysis

Determination of ATP and its derivates was performed according to Vecian-Nogues et al. (1996). Extraction from the 0.1 g of meat was achieved by Ultratorrax T18 basic (Ika, Staufen im Breisgau, Germany) homogenizator in 5ml ice-cold 0.6 M perchloric acid (Penta). The homogenate was centrifuged in Hettich Micro 200 R centrifuge at 4°C and set to 3000 g per 10 minutes. Collected supernatant approx 3 ml was neutralized by adding 0.4 ml 4 M KOH to pH 6-7. Then the neutralized supernatant was last in the fridge for 30 minutes. The homogenates were filtrated using Whatman filtration paper no.1 and filtrates were stored at – 80 ° C before analysis.

HPLC system consisted of the autosampler, thermostatic chamber, UV and DAD detector all Ultimate 3000, Thermo Scientific Dionex (Germering Germany). The separation of analytes was performed on chromatographic column C18 RP Hypercarb 100x 3mm, 3µm (Thermo Science) by injecting 7µl of stored extract. 0.08 M KH_2PO_4 and 0.12 M K_2HOP_4 in water was used as the first mobile phase (A), acetonitrile (HPLC grade) was the second one (B). The flow was 0.5 ml×min^{-1} and the gradient chromatography was used as follows: 0-2 min. 10 0% A, 9-13 min 75 % A and 15 % B, 13-18 min 50 % A and 50 % B. After that 5 minutes of equilibration was done by 100 % A. The temperature of the column was maintained at 35 ° C and the UV detector was set to 260 nm. Standards of ATP, ADP, AMP, IMP, inosine, adenosine, and hypoxanthine were purchased from Sigma Aldrich. The data was processed in Dionex Chromeleon 7.1.2.1713 (Thermo fisher scientific).

4.4.2 Lactate analysis

Determination of lactate was performed according to Au et al. (1989). Extraction from the 1 g of meat was achieved by Ultratorrax T18 basic (Ika, Staufen im Breisgau, Germany) homogenizator in 10ml of acetonitrile (HPLC grade). The homogenates were filtrated using Whatman filtration paper no.1 and evaporated by a stream of nitrogen. The extract was then dissolved in 3 ml of 0.03 M H_3P0_4 and stored in -80 ° C before HPLC analysis.

HPLC system consisted of the autosampler, thermostatic chamber, UV and DAD detector all Ultimate 3000, Thermo Scientific Dionex (Germering Germany). The separation of analytes was performed on chromatographic column C18 RP lichrocart 250x4 mm, 5 µm (Thermo Science) by injecting 7 µl of stored extract. 0.03 M phosphate buffer, acetonitrile and water in ration 80:8:12 (v:v:v) was used as a mobile phase with isocratic flow 0.5 ml×min^{-1}. The temperature of the column was maintained at 45 ° C and UV detector was set to 210 nm. Lactate standard was obtained from Sigma Aldrich. The data was processed in Dionex Chromeleon 7.1.2.1713 (Thermo fisher scientific).

5 RESULTS

5.1 PHASE I

The first stage was the preparation of agar, all work associated with this stage took place on the premises of the Faculty of Fisheries and Water Conservation in České Budějovice. Firstly, at the fish processing plant and in the nutrition laboratory.

Market carp for samples were purchased from the company Lesy a rybníky města České Budějovice s.r.o. Processing to the fillet stage took place at the fish processing plant.

5.2 PHASE II

Inoculation took place over 6 consecutive days, according to the established schedule. After each inoculation, the Petri dishes were placed upside down in incubators at 0 ° C, 4 ° C and 8 ° C. Due to low incubation temperature the incubation lasted for several days. The first colonies available for the MALDI TOF experiment were occurred after 7 days at 8 ° C, 9 days at 4 ° C and 12 days at 0 ° C.

5.3 PHASE III

The appearance of the first colonies was dependent on the incubation temperature. The first single colonies appeared on plates incubated at 8 ° C (9days). Over time, they began to appear on other inoculated plates, the last on plates incubated at 0 ° C (12 days). However, what differed was not only the time that the first colonies began to appear but also the species composition of these colonies, as is described lower. After the discovery of the colonies, it was necessary to start the actual analysis by MALDI-TOF MS.

First, it was necessary to prepare the samples for measurement on MALDI-TOF MS and proceed to the measuring. At this stage of the work, the extreme importance of careful preparation of samples for measurement has shown. Even though the beginnings were very demanding, in the end, we managed to successfully master the process and began to obtain the first measured data.

At this point, it is necessary to mention another important result - at an incubation temperature of 0 ° C, about a third of the plates were without any microbial growth even

after a prolonged incubation period (30 days). This fact testifies not only to the quality of the tested product but also to the quality of our work with the material. Furthermore, we also observed some contamination caused by the handling of Petri dishes and agar, but these contaminations occurred very rarely.

The summary results acquired by measurement on MALDI-TOF MS are shown in attachments. In general, the diversity of identified microbial genera and species was significantly higher in samples inoculated at 0 and 72 hours after slaughtering of fish than in samples inoculated at 120 hours after.

It is clear from the tables that the conclusively dominant genus of bacteria, regardless of the time of inoculation or incubation temperature, is the genus *Pseudomonas*. In the group inoculated 120 hours after slaughter, we were no longer able to identify other species of bacteria than *Pseudomonas spp*., with only one exception - in the case of rainbow trout agar, when incubated at 0 ° C, it was possible to identify the species *Shewanella profunda*.

While *Pseudomonas spp*. occurred abundantly regardless of inoculation time and incubation temperature, some species occurred only on a small number of samples and only under specific inoculation and incubation conditions.

For example, gram-negative *Chryseobacterium chaponense* was isolated on carp and trout agar inoculated at 0 and 72 hours. This bacterium was first isolated from Atlantic salmon *(Salmo salar)* reared on a lake farm in Chile (Kämpfer, 2011). In our conditions, however, it grew surprisingly better on carp agar than on agar made of trout, which is more closely related to salmon, and the method of breeding and nutrition are more similar. When inoculated after 72 hours, this bacterium has grown only on trout agar incubated at 0 ° C. However, it appeared on carp agar at all monitored temperatures.

Another interesting bacterial species that appeared only on trout agar and most on control agar, and only when inoculated at time 0, is *Janthinobacterium lividum*. According to Tabor-Godwin et al. (2009), is this dark violet (almost black) bacterium common in soil and water bodies and on very rare occasions can cause septicemia in humans.

Interestingly, we managed to grow *Sphingomonas aurantiaca* twice on control agar. It was on agars inoculated at times 0 and 72, incubated at 0 ° C. This dust-occurring bacterium was first isolated by Busse et al. (2003) in Finland, in a cow stable, after spreading bales of straw and hay. Because this bacterium is found in dust, its

presence indicates airborne contamination of Petri dishes. However, the occurrence of this type of bacterium was limited to only two dishes and therefore cannot be considered statistically significant.

In our samples, we also identified one representative of the yeast - the species *Rhodotorula mucilaginosa*. It is a single-celled yeast that typically forms orange to red colonies. It is commonly found in soil, water, milk and even air. *Rhodotorula spp*. has a strong affinity for plastic and can therefore attack various medical equipment (eg catheters), toothbrushes, bath curtains, etc. In the event of an attack on medical equipment, there is also a danger for patients (Wirth and Goldani, 2012).

If we look at the tables in general, it can be seen that the most abundant species identified after inoculation at time 0 were *Janthinobacterium lividum* and *Pseudomonas fluorescens*. At time 72, the most frequently identified microorganisms were *Pseudomonas tolaasi, Pseudomonas antarctica, Pseudomonas brenneri, Pseudomonas grimontii* and *Pseudomonas veronii* (all these species of *Pseudomonas* belong in *P. fluorescens* group). The samples inoculated at time 120 had the highest incidence of *Pseudomonas veronii, Pseudomonas tolaasi* and *Pseudomonas fragi* (has been placed in the *P. chlororaphis* group). However, in none of the groups were the differences in the incidence of identified microorganisms significantly different.

If we look at the results in terms of the abundance of identified species, then most species in all groups were identified on control agar (with minor exceptions), followed by carp agar. The frequency of species on trout agar and grass carp agar is similar, and it cannot be said that more or fewer bacterial species have been identified on any of these media.

5.4 **PHASE IV.**

5.4.1 ATP analysis

The results of the analysis of the development of the content of ATP and its metabolites in meat over time are shown in the graphs in Figure 10. The figure shows three different graphs dedicated to observed temperature. It is clear from the graphs that the rate of ATP metabolism is significantly affected by temperature.

When stored at 0 ° C, the ATP content decreases very slowly and only at 120 hours after slaughter does it reach zero values. Along with the decrease in ATP, there is a

gradual increase in the Hx + R content, and about 50 hours after slaughter, both substances reach the same level. 60 hours after slaughter, the content of Hx + R exceeds the content of ATP, 70 - 80 hours after it reaches the peak and then its content begins to slowly decrease. Simultaneously with these processes, the Hx content begins to increase sharply and at the time of about 80 from slaughter, it reaches a peak concentration and even exceeds the original ATP content at time 0.

Graph B shows that when stored at 4 ° C, there is a much sharper decrease in ATP, the content of which reaches an unmeasurable value as early as about 70 hours after killing the fish. As in the first case, there is an increase in Hx + R and Hx as the ATP decreases. However, this increase is faster and both metabolites exceed the ATP content already 15 and 30 hours after killing, respectively. The growth rate is similar for both metabolites, and the concentration of Hx + R is higher until about 100 hours when its content is exceeded by the content of Hx. At 120 hours, the content of Hx + R gradually decreases, while Hx still increases sharply.

In the third group, stored at 8 ° C (graph C), the development is similar to the group stored at 4 ° C. It is only surprising that ATP reached zero or unmeasurable values about 20 hours later than in the previous group. However, the increase in Hx + R and Hx is as sharp as in 4 ° C, Hx exceeds the content of Hx + R about 90 hours after killing and continues to grow rapidly. At 120 hours, it reaches the highest values of all three monitored temperature groups.

From the above, it is clear that temperature has a significant effect on the post-mortem metabolism of ATP, and the most significant slowdown occurs when stored at 0 ° C, while the difference between storage at 4 ° C and 8 ° C is not so pronounced.

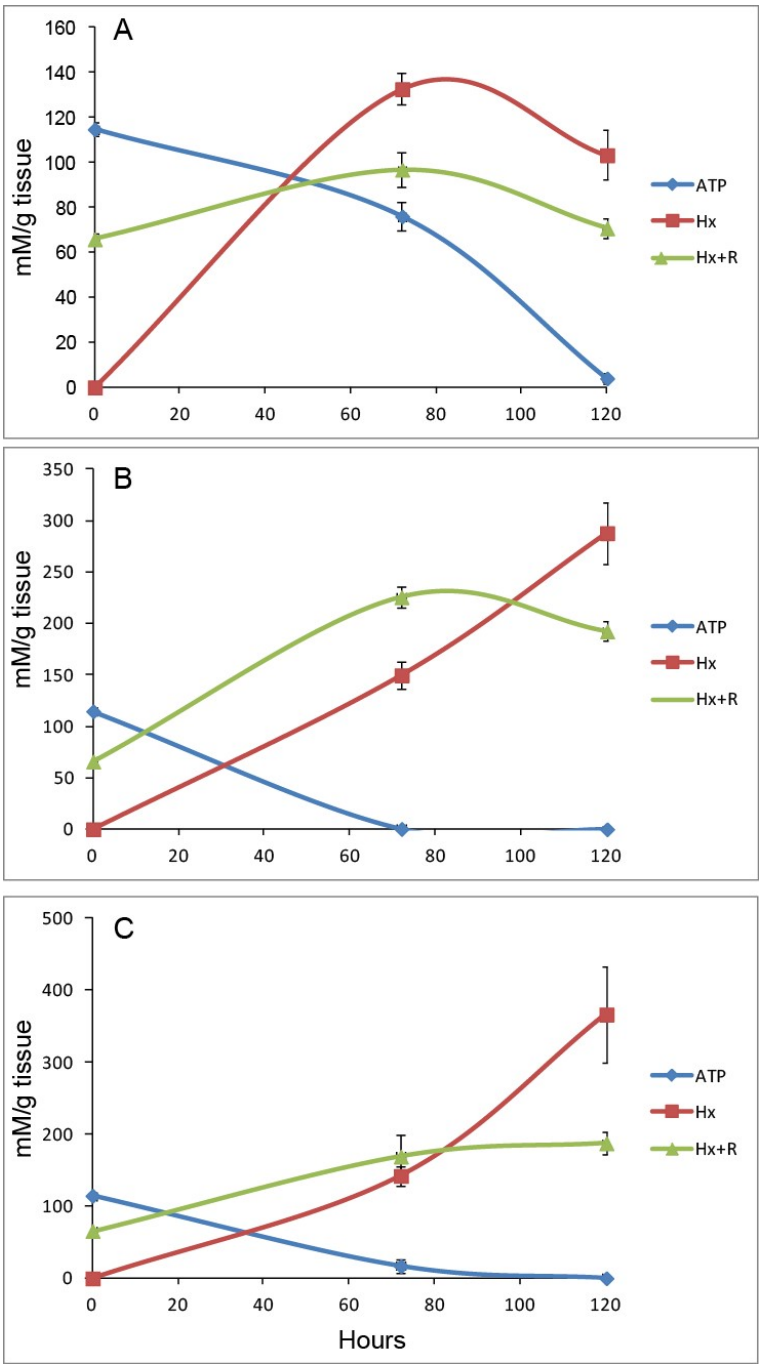

Figure 10: Graphs show changes in ATP, Hx, and Hx+R content in carp meat after 120 hours in three different temperatures A - at 0 ° C; B at 4 ° C and C at 8 ° C. (Hx – hypoxanthine, Hx+R hypoxanthine + ribose)

5.4.2 *Lactate analysis*

The development of lactate content is shown in Figure 11. The graph clearly shows that the slowest lactate is formed at 0 ° C and at 120 hours after slaughter it reaches only about half the lactate concentration than the other two groups studied. In the group stored at 4 and 8 ° C, the difference in growth is not significant, and within 120 hours of slaughter, both groups reach the same values.

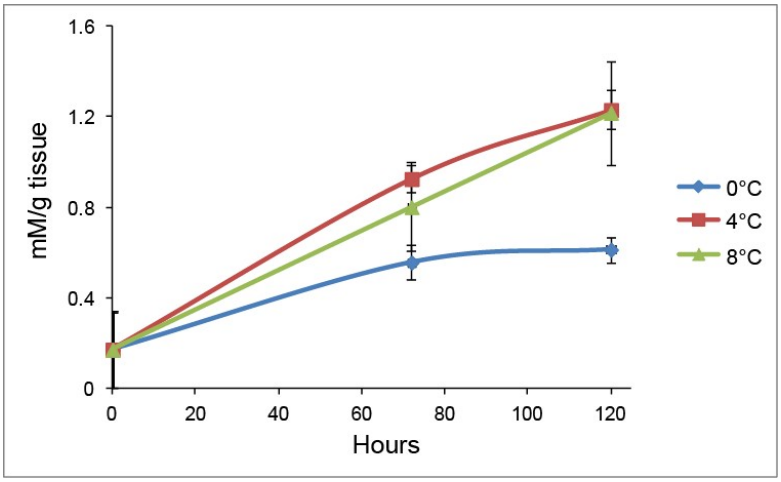

Figure 11. The graph shows the content of lactic acid in carp fillet during 120 storage period at different temperatures levels.

6 DISCUSSION

6.1 PHASE I – II

Most authors (Lay 2001, Sandrin et al., 2012, Singhal et al. 2015) cite working with MALDI-TOF MS as a very fast and simple method for identifying microorganisms. Although the resulting identification is really fast and the machine works automatically, sample preparation was positively time-consuming and MALDI calibration was initially an essential problem. However, after overcoming these initial problems, the experiment took place without significant problems and, as already written - the machine only needs to be programmed, calibrated and then it works on its own, completely unattended. Therefore, I agree with the opinion of the above-mentioned authors and I believe that if the analyses are performed by an experienced operator, this method can be really fast and effective.

6.2 PHASE III

As mentioned in the results, the most frequently identified microorganisms in all monitored groups were *Pseudomonas spp*, with the fact that at the last inoculation - 120 hours after the slaughter of the fish, only bacteria of the genus *Pseudomonas* grew. This is in line with the conclusions of Lougovois and Kyrana (2005), according to which the longer fish tissue is stored on ice, the more pronounced the representation of the genera *Pseudomonas* and *Shewanlla*. In our case, we identified the genus *Shewanella* only twice, namely the species *Shewanella profunda* on rainbow trout agar inoculated after 120 hours and incubated at 0 ° C. The question is whether the incidence of the genus *Shewanella* would increase if the experiment continued and further inoculation was performed 168 hours after slaughter or later.

According to the same authors, strains of *Psychrobacter* (Moraxella), *Acinetobacter* and F*lavobacterium* also occur in fish meat, but in significantly smaller amounts, which may be one of the reasons why we were not able to isolate and identify these species in our samples. According to Fraser and Sumar (1998), the bacteria *Pseudomonas putrifaciens* and *Pseudomonas fluorescens* begin to multiply very quickly after the fish is slaughtered. We were able to identify only *Pseudomonas fluorescens* as a specific species and the highest numbers on the Petri's dishes inoculated at 0 and 72 hours. It

also occurred in the group inoculated after 120 hours, but with a significantly lower incidence. On the other hand, we were able to identify a high number of bacteria belonging to *P. fluorescence* group.

No representative of allochthonous bacteria as described by Samples (2014) was found in the identified samples, which indicates our observance of proper hygienic procedures in the slaughtering and processing of fish. Huss (1995) lists two bacterial species: *Escherichia coli* and *Salmonella*, which can both occur in freshwater fish kept in mild to warm polluted waters. We did not find any trace of these bacteria either, so the breeding took place in fresh, clean water.

6.3 **PHASE IV**

6.3.1 ATP

The results of the ATP content were quite surprising to me. Huss (1988) and Samples et al. (2014) report that ATP stocks in fish muscle will drop to zero or unmeasurably low levels within 24 hours, even at low storage temperatures. However, this fact was not confirmed to us at all and in samples stored at 0 ° C the ATP values did not reach zero until after 120 hours, in samples stored at 4 and 8 ° C it happened earlier, but still, the time required for ATP disappearance from muscle 3 × (72 hours) or 4 × (96 hours) longer than stated by the authors.I cannot explain the reason for this significant difference.

Other analysed parameters undergo development exactly as described by Lakshmanan (2000). With a decrease in ATP, there is first an increase in Hx + R, and shortly afterwards, Hx, which is the largest with increasing storage time, begins to form.

6.3.2 Lactate

Watabeet al. (1991) in their research on sardines report that lactate levels increased continuously in correlation with an increase in rigor mortis. In this research, the muscle was divided into fast and slow. While there was an intense increase in lactate in fast muscle for the first 12 hours after the rigor mortis subsided, 24 hours after slaughter, the curve reversed and the lactate content in muscle began to decrease again.

In contrast, there was an increase in the lactate content in the slow muscle throughout the whole storage period. This is exactly what our results correspond to

when the amount of lactate does not grow so fast after a certain time, but it increases continuously throughout the storage period.

7 CONCLUSION

As mentioned in the introduction, fish is one of the essential sources of protein and many other healthy substances for human nutrition. At the same time, however, they are perishable foods and can therefore potentially become dangerous to human health. While shortly after slaughtering autolytic processes take place in fish tissue, at a later stage microbial communities develop, and their activity will degrade this valuable raw material.

It is clear from the above that strict adherence to technological and hygienic procedures and standards is essential in the management of fish as food. Because microbial contamination of food can be a threat to public safety, it has been necessary to develop methods to control food safety. These methods must be fast, sufficiently reliable, and easy to implement.

In my work, I tried to compare the growth of microorganisms on agars of different composition, at three different temperatures. The main goal of this work was to optimize the conditions for the growth of microorganisms occurring in fish and raw fish products.

The basic hypothesis was that microorganisms occurring in fish products could grow more and in greater species diversity on agars made directly from fish tissue and at lower temperatures. Lower temperatures correspond to the conditions of fish in the winter, when the experiment took place, and at the same time normal storage temperatures.

However, the hypothesis was not confirmed. It was not possible to prove a significant effect of the composition of the nutrient medium on the growth of bacteria. The main conditions affecting bacterial growth were the incubation temperature and the time from slaughtering of the fish to the sampling. Overall, the microorganisms were least successful when incubated at 0 ° C, with no statistically significant difference between samples stored at 4 and 8 ° C. Another important factor that affected the growth of bacteria was the time for which the samples were stored before the actual agar inoculation. Over time, the microbial community gradually changed so that, when

inoculated 120 hours after killing, the microbial community was formed almost exclusively of *Pseudomonas spp*.

At the end of the research, tissue samples were tested for the development of ATP and its metabolites and lactate. The amount of these substances in the tissue gradually changes due to autolytic processes. In both cases, the effect of temperature on the course of these processes in the meat was demonstrated. As with the growth of microorganisms, these processes were significantly slowed down at a storage temperature of 0 ° C. At 4 and 8 ° C, the rate of autolysis underflow was also different, but these differences were less pronounced.

It follows from the above that in order to maintain the quality of fresh fish and fish products and to extend the shelf life, storage at the correct temperature is essential, in addition to following the principles of hygienic production practice. In our work, storage at 0 ° C has demonstrably slowed down the processes of autolysis and the development of microbial communities.

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9 ABBREVIATIONS

AA – amino acids

ADP – adenosine diphosphate

AMP – adenosine monophosphate

ATP – adenosine triphosphate

BA – biogenic acids

CFU – colony forming units

DMA – dimethylamine

FA – fatty acids

FA – formaldehyde

FFA – free fatty acids

HCCA – α-Cyano-4-hydroxycinnamic acid

HPLC – high-performance liquid chromatography

HPLC-UV – high-performance liquid chromatography-ultraviolet

Hx – hypoxanthine

HxR – inosine

IMP – inosine-monophosphate

MALDI-TOF MS – matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer

PCA – plate count agar

PCA – principal component analysis

PMF – peptide mass fingerprints

PUFA – polyunsaturated fatty acids

ROS – reacting oxygen species

UFA – unsaturated fatty acids

TAGS – triacylglycerols

TMA – trimethyl amine

TMAO – trimethyl amine oxide

10 LIST OF TABLES, FIGURES AND ATTACHMENTS

Figures:

Figure 1. progression of bacterial spoilage with four stages of spoilage according to Shawyer and Pizzaly (2003). Passed from Ghaly (2010), modified.

Figure 2. Autolytic degradation of ATP (Huss, 1988). ATP – adenosine triphosphate, ADP – adenosine diphosphate, AMP – adenosine monophosphate, IMP – inositol monophosphate, Hx + R – hypoxanthine + ribose, Hx - hypoxanthine

Figure 3. The formula for calculation of K-value. Passed from Samples et al. 2014.

Figure 4. The formula for calculation of K_1-value. Passed from Samples et al. 2014.

Figure 5. Three stages of lipid oxidation: Initiation (a), propagation (b), termination (c). RH = Lipid, X• = free radical. Passed from Gardner (1989).

Figure 6. Development of the total count of bacteria and volume of specific spoilage organisms during the storage. Passed from Huss, 1995.

Figure 7. Detailed workflow diagram of the second and third working phase

Figure 8. Schema of planned inoculations. Done in times 0, 72, 120 hours after slaughter.

Figure 9. The application of analysed material on Petri dish. The laboratory loop must be sterilised repeatedly during this process. Passed from: Weber scientific manual adapted.

Figure 10. Graphs show changes in ATP, Hx, and Hx+R content in carp meat after 120 hours in three different temperatures A - at 0 ° C; B at 4 ° C and C at 8 ° C. (Hx – hypoxanthine, Hx+R hypoxanthine + ribose)

Tables

Table 1. The main autolytic changes in chilled fish, and enzymes causing them (Huss, 1995), adapted.

Table 2. The main genera of microorganisms found on living or freshly caught fish. Passed from Huss (1995), adapted.

Table 3. The main allochthonous bacteria and their primary source. Passed from Samples et al., (2014), adapted.

Table 4. The activity of the main microorganism involved in Fish spoilage. Source Hui (1992), adapted.

Table 5. Typical bacterial spoilage compounds. Passed from Church (1998).

Table 6. The off-odour compound by bacteria activity during the ice storage of fish. Passed from Huss (1995).

Attachments

Attachment 1 – List of detected microbial species in Petri dishes. The samples were transferred to the Petri dishes immediately after slaughtering.

Attachment 2 - List of detected microbial species in Petri dishes. The samples were transferred to the Petri dishes after 72 hours of storage period.

Attachment 3 - List of detected microbial species in Petri dishes. The samples were transferred to the Petri dishes after 120 hours of storage period.

11 **ATTACHMENTS**

Attachment 1. Table of detected microbial species by MALDI TOF Biotyper – samples were collected immediately after fish slaughtering

Detected species	0°C				4°C				8°C			
	Carp	SilverCarp	Trouth	control	Carp	SilverCarp	Trouth	control	Carp	SilverCarp	Trouth	control
Chryseobacterium chaponense	*		**		*		*		**			
Janthinobacterium lividum				*							*	***
Microbacterium liquefaciens		*		*		*		*				
Microbacterium oxydans									*			*
Pseudomonas antartica					*			*		*		*
Pseudomonas brenneri		*								**		
Pseudomonas extremorientalis	*											
Pseudomonas fluorescens			*	*			*	*				*
Pseudomonas fragi									*			
Pseudomonas gessardii									*			
Pseudomonas grimontii		*		*						**		
Pseudomonas chlororaphis												
Pseudomonas koreensis				*								
Pseudomonas mandeli												
Pseudomonas marginalis												
Pseudomonas proteolytica				*								
Pseudomonas rhodesiae	*				*	*			*	*		
Pseudomonas taetroles												
Pseudomonas tolaasi		*					*				*	
Pseudomonas veronii					*							**
Rhodotorula mucilaginosa												
Schewanella profunda												
Sphingobacterium faecium												
Sphingomonas aurantiaca				*								

Attachment 2. Table of detected microbial species by MALDI TOF Biotyper – samples were collected 72 hours od storage period

Detected species	0°C				4°C				8°C			
	Carp	SilverCarp	Trouth	control	Carp	SilverCarp	Trouth	control	Carp	SilverCarp	Trouth	control
Chryseobacterium chaponense	**		**		*				*			
Janthinobacterium lividum												
Microbacterium liquefaciens		*		*		*		*				
Microbacterium oxydans												
Pseudomonas antartica					**					*		*
Pseudomonas brenneri		*				*	*	*				
Pseudomonas extremorientalis	*											
Pseudomonas fluorescens			*	*								*
Pseudomonas fragi									*			
Pseudomonas gessardii								*	*			
Pseudomonas grimontii				*				*		*	*	
Pseudomonas chlororaphis												
Pseudomonas koreensis				*								
Pseudomonas mandeli							**					
Pseudomonas marginalis												
Pseudomonas proteolytica				*								
Pseudomonas rhodesiae	*				*				*			
Pseudomonas taetroles												
Pseudomonas tolaasi		*				*		**			**	
Pseudomonas veronii					**							**
Rhodotorula mucilaginosa	*											
Schewanella profunda												
Sphingobacterium faecium								*				
Sphingomonas aurantiaca				*								

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Pseudomonas brenneri		*					*	*				
Pseudomonas extremorientalis	*											
Pseudomonas fluorescens				*								*
Pseudomonas fragi						*	*	*	*			
Pseudomonas gessardii												
Pseudomonas grimontii				*						*	*	
Pseudomonas chlororaphis			*									
Pseudomonas koreensis				*								
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Pseudomonas proteolytica				*								
Pseudomonas rhodesiae	*				*				*			
Pseudomonas taetroles								*				
Pseudomonas tolaasi		*				*					**	
Pseudomonas veronii			*		*				**			**
Rhodotorula mucilaginosa												
Schewanella profunda			**									
Sphingobacterium faecium												
Sphingomonas aurantiaca												

12 **ABSTRACT**

This diploma thesis was focused on microbial contamination of fish meat. Common carp (*Cyprinus carpio*) was chosen as a model fish. The work aimed to optimize the conditions for bacterial growth using four different growth media and three different temperatures. Incubation took place at 0, 72 and 120 hours after killing the fish and the samples were incubated at 0, 4 and 8 ° C. Agars selected from a broth of common carp (*Cyprinus carpio),* grass carp (*Ctenopharyngodon idella),* rainbow trout (*Oncorhynchus mykiss*) were used as growth media and a commonly used bovine-based agar was added as a control.

After incubation, the obtained bacterial colonies were analyzed by MALDI-TOF MS. At the same time, two chemical analyzes were performed on other meat samples stored under the same conditions. The first was to determine how the content of ATP and its metabolites (Hx + R and Hx) will change over time. The second analysis focused on the change in lactate content over time.

The work was divided into 4 phases. The most demanding phase of the whole project, both time and manual, was the preparation of samples for testing and MALDI-TOF MS.

The content of ATP and lactate in the meat was detected by high-performance liquid chromatography-ultraviolet (HPLC-UV).

The results of the work did not show the influence of the composition of the growth medium on the final microbial community. Temperature proved to be the most significant factor influencing the growth of microorganisms. Bacteria demonstrably grew more and faster at higher temperatures. The lowest and slowest growth was recorded for samples growing at 0 ° C.

The second important factor, mainly influencing the species composition of the bacterial community, was the time from the killing of the fish to the time of testing. While the community was grown on the media vaccinated at 0 and 72 hours after slaughter was relatively diverse, the media vaccinated at 120 hours after slaughter have already succeeded in isolating almost exclusively Pseudomonas *spp*.

Analyzes of the content of ATP and lactate have shown that at lower temperatures - respectively the temperature of 0 ° C there is not only a significant slowing down of bacterial growth but also a slowing down of the autolysis process. Differences in storage at 4 and 8 ° C were no longer significantly different.

Keywords: postmortem changes in meat, bacterial growth, MALDI-TOF MS, ATP, lactate, *Pseudomonas spp.*

13 **ABSTRAKT**

Tato diplomová práce byla zaměřena na mikrobiální kontaminaci rybího masa. Jako vzorová ryba byl vybrán kapr obecný (*Cyprinus carpio*). Cílem práce bylo optimalizovat podmínky pro růst bakterií za použití čtyř různých růstových médií a tří různých teplot. Inkubace proběhla v čase 0, 72 a 120 hodin od usmrcení ryby a vzorky byly inkubovány v 0, 4 a 8°C. Jako růstová média byly použity agary vyrobené z vývaru z kapra obecného (*Cyprinus carpio*), amura bílého (*Ctenopharyngodon idella*), pstruha duhového (*Oncorhynchus mykiss*) a jako kontrola byl přidám běžně používaný agar na bázi hovězího. Po inkubaci byly získané bakteriální kolonie analyzovány pomocí MALDI-TOF MS.

Zároveň byly na dalších vzorcích masa uchovávaných za stejných podmínek provedeny dvě chemické analýzy. Jejich cílem bylozjištění, jak se bude v čase měnit obsah ATP a jeho metabolitů (Hx+R a Hx), a laktátu.

Práce byla rozdělena do 4 fází. Nejnáročnější fází celého projektu, a to jak časově, tak manuálně, byla příprava vzorků pro testování na MALDI-TOF MS.

Obsah ATP a laktátu v mase byl detekován vysokoúčinnou kapalinovou chromatografií – ultrafialovým zářením (HPLC-UV).

Výsledky práce neprokázali vliv složení růstového média na konečné mikrobiální společenstvo. Jako nejvýraznější faktor ovlivňující růst mikroorganismů se ukázala být teplota. Bakterie prokazatelně více a rychleji rostly při vyšší teplotě. Nejnižší a nejpomalejší růst byl zaznamenán u vzorků rostoucích v nulové teplotě.

Druhým významným faktorem, ovlivňujícím především druhové složení bakteriálního společenstva, byl čas od usmrcení ryby do doby testování. Zatímco společenstvo vyrostlé na médiích očkovaných v čase 0 a 72 hodin od zabití bylo relativně pestré, na médiích očkovaných v čase 120 hodin od zabití už se podařilo izolovat téměř výlučně bakterie *Pseudomonas spp*.

Analýzy obsahu ATP a laktátu prokázali, že při nižších teplotách – respektive teplotě 0 °C dochází nejen k významnému zpomalení růstu bakterií, ale i ke zpomalení procesu autolýzy. Rozdíly při skladování ve 4 a 8 °C už nebyly signifikantně rozdílné.

Klíčová slova: posmrtné změny v mase, baktriální růst, MALDI-TOF MS, ATP, laktát, *Pseudomonas spp*.