TURUN AMMATTIKORKEAKOULU
TURKU UNIVERSITY OF APPLIED SCIENCES

Bachelor's Thesis

ISOLATION AND IDENTIFICATION OF RAINBOW TROUT SPOILING MICROBIOTA

Susanna Virta

Biotechnology and Food Technology

2009

TURKU ABSTRACT
UNIVERSITY OF APPLIED SCIENCES

Degree Program: Biotechnology and Food Technology	
Author: Susanna Virta	
Title: Isolation and identification of rainbow trout spoiling microbiota	
Specialization line: Food Technology	Instructors: Kai Rosenberg M. Sc., Jukka Kaitaranta PhD, Fandi Ibrahim PhD
Date: December 2009	Total number of pages: 54

Fish is highly perishable. Fish is packed in a vacuum or in modified atmosphere packages to increase preservability. Modified packaging combined with cold storage alters the microbiota of rainbow trout through inhibiting the growth of mesophilic and aerobic bacteria, while anaerobes and psychrophiles can still grow. Some pathogens can grow in vacuum packages due to the diminished competition between bacteria. The target of this thesis was to isolate spoilage bacteria from vacuum-packed rainbow trout and identify them using DNA sequencing. The study was part of a research project at the Functional Foods Forum, the University of Turku, aiming to extend the shelf-life of vacuum-packed rainbow trout using commercial lactic acid bacteria as a protective culture.

The purpose was to cultivate rainbow trout samples in selective media at different spoilage stages. The DNAs of the isolated colonies were to be amplified using polymerase chain reaction (PCR) and then identified by sequence analysis. Samples of rainbow trout were brought from the fish processing company, Jokisen Eväät Oy. The samples were stored in a refrigerator (4 °C) and cultivated in 11 different media at the best before date, and 3 days and 6 days after the best before date. At least 10 colonies from each selective media were isolated. PCR was used to amplify a piece of DNA from the isolated colonies. The amplification was verified using gel electrophoresis. The PCR products were then purified by means of a DNA isolation kit. The actual sequencing was carried out at the Turku Centre of Biotechnology.

Most of the sequences had a smaller than 95 % compatible in the bacterial sequence database. Nevertheless, *Brochothrix thermosphacta*, lactic acid bacteria and *Enterobacteriaceae* dominated the microbiota of spoiled vacuum-packed rainbow trout. Pathogens such as *Listeria monocytogenes* were not found in the rainbow trout samples. One *Bacillus* genus bacterium was sequenced. *Bacillus cereus* may cause food poisonings.

Keywords: rainbow trout, spoiling, bacteria

Deposit at: Turku University of Applied Sciences Library

TURUN AMMATTIKORKEAKOULU

TIIVISTELMÄ

Koulutusohjelma: Bio- ja elintarviketekniikka	
Tekijä: Susanna Virta	
Työn nimi: Pilaantuneen kirjolohen bakteerien eristys ja tunnistus	
Elintarviketekniikka	Ohjaajat: FK Kai Rosenberg, Fil. tri Jukka Kaitaranta, Fil. tri Fandi Ibrahim
Opinnäytetyön valmistumisajankohta: 17.12.2009	Sivumäärä: 54

Kalat ovat helposti pilaantuvia. Kaloja pakataan vakuumiin ja suojakaasupakkauksiin säilyvyyden lisäämiseksi. Suojakaasuun ja vakuumiin pakkaaminen sekä kylmäsäilytys estävät mesofiilisten ja aerobisten bakteerien kasvua, jotka tavallisesti vallitsevat kirjolohen elimistössä. Kuitenkin psykrofiiliset ja anaerobiset bakteerit voivat lisääntyä vakuumipakkauksissa. Vakuumipakkauksissa myös patogeeniset bakteerit voivat lisääntyä, koska bakteerien välistä kilpailua on vähemmän. Tämän opinnäytetyön tavoitteena oli selvittää vakuumipakatun pilaantuneen kirjolohen mikrobifloora. Työ on osa Turun yliopiston Funktionaalisten elintarvikkeiden kehittämiskeskuksen tutkimusta, jonka tarkoituksena on lisätä vakuumipakatun kirjolohen säilyvyyttä kaupallisilla maitohappobakteereilla.

Työn tarkoituksena oli eristää pilaantuneen kirjolohen sisältämät bakteerit ja tunnistaa ne sekvenoimalla. Työ suoritettiin Funktionaalisten elintarvikkeiden kehittämiskeskuksen tiloissa. Kirjolohet haettiin Jokisen Eväät Oy:stä ja niitä säilytettiin jääkaapissa (4 °C). Kirjolohinäytteet otettiin kaloista viimeisenä käyttöpäivänä sekä 3 ja 6 päivää viimeisen käyttöpäivän jälkeen. Kirjolohisuspensionäytteet levitettiin 11 erilaiseen kasvatusmaljaan pintalevitysmenetelmällä. Kasvaneita pesäkkeitä eristettiin 10 kappaletta kultakin maljalta. PCR suoritettiin eristettyihin pesäkkeisiin DNA:n lisäämiseksi. DNA-määrät mitattiin geelielektroforeesilla. Varsinainen sekvenointi puhdistetuista DNA-näytteistä suoritettiin Biotekniikan keskuksessa.

Suurin osa sekvensseistä täsmäsi vain alle 95 % bakteerien tietopankin sekvensseihin. Tuloksista voidaan kuitenkin päätellä, että *Brochothrix thermosphacta*, maitohappobakteerit ja *Enterobacteriaceae* dominoivat pilaantuneen kirjolohen bakteerikantaa. Yhtään *Listeria monocytogenes* -bakteeria ei näytteistä sekvenoitu. Yksi *Bacillus* -suvun bakteeri kuitenkin löydettiin. *Bacillus cereus* aiheuttaa ruokamyrkytyksiä.

Hakusanat: kirjolohi, pilaantuminen, bakteerit

Säilytyspaikka: Turun ammattikorkeakoulun kirjasto

CONTENTS

EMPIRICAL PART

1 INTRODUCTION

The consumption of fish in Finland in 2007 was 4.9 kg for domestic and 11.7 kg for imported fish per person as filleted weight. The total consumption of fish was 13 % higher than the previous year and it is increasing constantly. In Finland, the most consumed fish are salmon and rainbow trout. The consumption of rainbow trout was 1.1 kg for domestic and 0.8 kg per person for the imported rainbow trout in 2007. (1)

Fish is classified as perishable food. Fresh fish spoils easily due to microbial growth as well as enzymatic and chemical reactions. The rapid spoilage is challenging for fish processing. Fish must be iced immediately after catching and sold within the next few days. To increase the shelf-life of the product, fisheries pack the products in a vacuum or in protective gas or use other chemicals. Other methods of extending the preservability include salting. On the other hand, consumers are becoming more and more aware of health risks and prefer products without added salt or other food additives, so-called lightly preserved foods (2). It would be valuable to increase the shelf-life of fish or fish products by natural and biological means. Extending the shelf-life even by only one day will have a significant commercial impact. (3)

Spoiling could be delayed by beneficial lactic acid bacteria. Lactic acid bacteria produce bacteriocins and organic acids, mainly lactic acid that reduces the pH. These agents prevent and inhibit harmful spoilage bacteria. The preservability of lactic acid bacteria is based on their ability to grow in a vacuum and in protective gas packages, and at high salinity and low temperature. (3)

In order to examine the effects of probiotic bacteria in the shelf-life of fish products, the spoiling microbiota must be known. Therefore, this graduation thesis is focused on the spoilage bacteria of rainbow trout in addition to the basic principle of biopreservation. This study is part of a research project aiming to improve the preservation of fish products by biological methods. The study is carried out at the Functional Foods Forum, the University of Turku, Finland in collaboration with a fish processing company, Jokisen Eväät Oy.

2 SPOILAGE OF FISH

Fish spoilage is due to autolysis, oxidation, dehydration and bacterial activity. The high water content, neutral pH and the high total number of autolysis enzymes in fish tissue influence the susceptibility to spoilage. There is a rigor mortis phase before the actual spoilage. Rigor mortis is caused by the enzyme activity in fish. After dying, the fish blood circulation and oxygen supply to the cells stop. However, the enzymes of the flesh keep functioning by creating lactic acid from the flesh glycogen. After the glycogen is worn out, enzymes stop functioning and the fish becomes rigid and hard. (4)

2.1 The effects of spoilage on fish

At the time of rigor mortis, the fish is relatively fresh. Fresh fish has a mild smell, glossy eyes and red gills. The spoilage affects the appearance, smell, taste and texture of the fish. Spoiled fish is no longer rigid, but limp. The eyes of the fish are bleary, its gills have gone pale and the smell of the fish is unpleasant and rotten. Fish tends to become slimy and sticky on the surface. The intestines of the spoiled fish will burst even if the fish is preserved at 0 °C. The metabolites of the microbes diffuse into the fish and cause the spoiling effects. This can evoke acids, NH_3, harmful amines, H_2S or even botulin, depending on the microbiota. The effects of spoilage on the appearance and on the sensory evaluation of fish are shown in Table 1. (4, 5)

Table 1. The effects of spoilage on the appearance and sensory evaluation of fish (4).

Variable	**Fresh fish**	**Spoiled fish**
Smell	fresh and typical for the fish	first indefinable then unpleasant
Colour	bright	pale
Slime layer of skin	clear and translucent	cloddy and bleary
Muscles	firm, elastic and flexible	limb
Abdomen wall	normal	burst or discoloured by gall
Eyes	clear	turbid and sagged
Gills	pink	slimed, grey and pasted on
Anus	small and narrow	slack and widened

2.2 Enzymatic and chemical spoilage

The spoilage of raw fish is first caused by enzymatic autolysis followed by microbiological spoilage. In autolysis, the enzymes and ATP-related compounds disintegrate and tissues become soft. These degradation products operate as microbial nutrient: enzymes disperse into surrounding tissues, leading to intestinal or abdominal peritoneum puncture. Degradation reactions develop faulty odours and flavours. (4)

The fish fat is mostly composed of polyunsaturated fatty acids, especially fatty fish like salmonoids, which become oxidized and fester easily. Oxidation and hydrolysis develop faulty odours and flavours in the fish as well. Both enzymes and microbes in the fish intestines and tissues hydrolyze fatty acids. Stringing, fileing, light and heat accelerate the hydrolysis. On the other hand cooling, antioxidants, the removal of oxygen and freezing, except on fatty fish, decelerate the hydrolysis. (2, 4)

There are a lot of non-protein nitrogen compounds in fish. These include among others trimethylamineoxide (TMAO), amino acids and nucleotides. TMAO is an odourless nitrogen compound that many bacteria can convert it to trimethylamine (TMA). The latter is the usual compound in spoiled fish that causes bad smell. (2)

2.3 Microbiological spoilage

Many variables influence the rate of spoilage and microbial growth in fish. Fish flesh is naturally sterile. Microbes get into the flesh from the surrounding environment and the fish intestines after the fish have died. Therefore, it is very important how the fish is preserved until it is consumed. Microbes have various optimal conditions to extend their growth. The fish condition (fish diseases), species, season (time of spawn), nourishment, environment, the temperature, fishing manner (stress level) and handling all affect the rate of microbial growth. A small fish has a high pH after death. Thin skin and high fat content increase the rate of bacterial growth. In addition, fish fillets have inferior preservability to the whole fish, because the flesh is more exposed to bacteria. The microbes of fish are situated in the slime layer of skin, gills and intestines. Because the spoiling starts from the intestines, cleaning the fish is very critical to minimize contamination. That and draining of the blood also reduces the enzymes, bacteria and bacterial breeding grounds and therefore slows down the rate of spoiling. Microbial spoilage causes slime, faulty odours, flavours and colours and eventually the fish becomes inedible. (2, 6, 7)

The biota of fresh fish is reflective of the waters they grow in. Fresh and warm-water fishes have different microbiota from salt water or cold-water fishes. Warm-water fish tend to have more of a mesophilic and gram-positive biota and cold-water fish have mostly gram-negative bacteria in their biota. Rainbow trout is expected to have more gram-negative and psychrophile or psychrotroph bacteria because of its cold-water surroundings. Commonly appearing bacteria genera are *Pseudomonas*, *Flavobacterium*, *Moraxella*, *Acinetobacter*, *Shewanella*, *Photobacterium*, *Vibrionaceae*, and *Aeroemonadaceae*. Gram-positive organisms such as *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus*, and *Corynebacterium* can also be found in rainbow trout. Bacteria need nourishment, warmth, moisture, the suitable acidity and oxygen to grow. Because of these terms, food products are naturally good breeding grounds for bacteria. On the other hand, the competition between bacteria limits their growth in foods. When all water and nutrients have run out, they can no longer multiply. Some of the

conditions can be influenced. Still the existence of bacteria cannot be entirely removed, but only reduced by controlling the conditions of handling and preservation (4, 6, 7)

2.3.1 Temperature

The temperature is the most significant way to control the growth of bacteria because it requires minimal modifications of the product. Bacteria can be divided into four groups based on their optimal growth temperature. These groups are listed in Table 2. (4)

Table 2. Grouping of bacteria according to optimal growth temperature (4).

Groups of bacteria	**Temperature (°C)**
Psychrophiles	10 – 15
Psychrotrophs	20 – 30
Mesophiles	30 – 40
Thermophiles	55 – 65

In the case of rainbow trout, the most commonly found bacterial groups are psychrophiles and psychrotrophs. The minimum temperature for psychrophiles is between -5 and +5 °C and maximum between 15 and 20 °C. The minimum and maximum temperatures for psychrotrophs are between -5 °C and 35 °C. Therefore, fish should be stored at temperatures close to 0 °C, below the optimum growth temperatures of most bacterial strains, in order to increase the fish preservability. (8). Fish can also have other bacteria in their biota than psychrophiles and psychrotrophs; therefore, icing or preserving at refrigerator temperature is the best way to prevent all bacterial growth in a fish product. Correctly preserved at 0 °C, fish lasts 2-3 times better than fish preserved at +5 °C. At high temperatures (15 – 20 °C), fish is preserved only for a day. Rainbow trout spoils in two days at the temperature of 10 – 15 °C (Fig. 1.). (4)

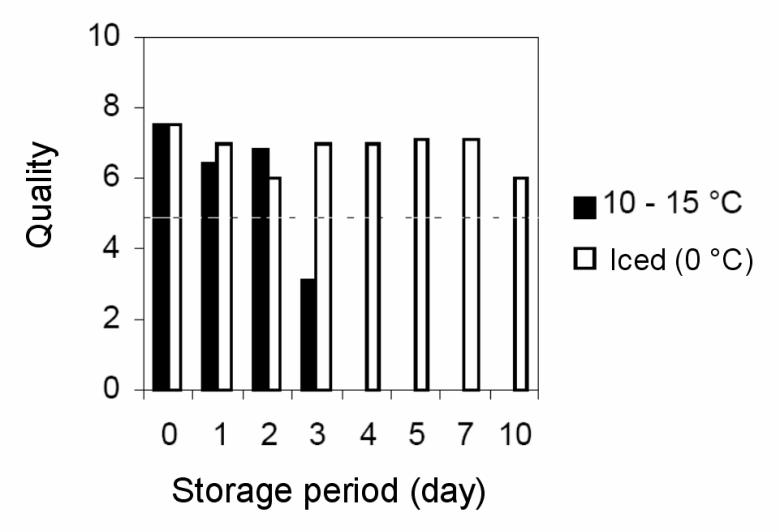

Figure 1. The preservability of rainbow trout at 10 – 15 °C and iced (Quality 0 – 10, when ≤ 5 discarded, 5 – 7 satisfactory, 7 – 9 good and ≥ 9 excellent), (4).

2.3.2 Water activity

Water is a necessity for bacterial growth and proliferation. Water should be in available form for bacteria to use it. One of the crucial characteristics that describe the microbial spoilage of food is the free and unbound total number of water in food, also known as water activity (a_w). Water activity is defined by the ratio of the water vapour pressure of food substrate to the vapour pressure of pure water at the same temperature. The low water activity of a food product causes higher osmotic pressure around the growing bacteria. Some bacteria react to this by altering their intracellular water activity. This way, the bacterial cell can protect itself from the osmotic pressure. When the pressure rises too much, the cell dies. Water activity is described with numerical values from zero to one. Number one means that all water in the food is freely in use for microbes. Most bacteria have the approximate water activity minimum of 0.91, but there are some exceptions, which can grow in lower moistures. For example, *Staphylococcus aureus* can live at water activity 0.83. Other minimum values for certain bacteria are shown in Table 3 (7).

Table 3. Approximate minimum a_w values for some specific organisms (7).

Organism	**a_w**
Pseudomonas spp.	0.97
Acinetobacter spp.	0.96
Bacillus subtilis	0.95
Vibrio parahaemolyticus	0.94
Clostridium botulimun	0.94
Most Gram-negatives	0.97
Most Gram-positives	0.90

Most fresh foods have a water activity between 0.8 and 0.99. Fresh fish has water activity close to the upper end, therefore it is very perishable. The methods of influencing the amount of free water comprise drying, freezing, and the addition of salt or sugar. Salted fish was a water activity value of 0.87. Adding salt and lowering the a_w effectively prevents some bacteria from growing. There is a linear relationship between water activity and sodium chloride concentration. The relations between the total number of salt and the value of water activity are shown in Table 4. (7, 9)

Table 4. Relationship between water activity and concentration of salt (9).

a_w	NaCl %
0.995	0,9
0.99	1,7
0.98	3,5
0.96	7
0.94	10
0.92	13
0.90	16
0.88	19
0.86	22
0.84	25

2.3.3 pH

Most microorganisms grow the best at pH values between 6.6 and 7.5, whereas only a few grow at a pH below four. Rainbow trout and other fish have a pH value range between 6.2 and 6.6 and this makes it a good medium for bacterial growth. Halibut is an exception because it has a pH value of 5.6, and therefore it has better preservability than most other fish. Live fish have a pH value around neutral, but rigor mortis causes it to drop to a minimum of 6.2. Since fish have lesser glycogen storages to create lactic acid compared with meat, the pH does not drop enough to inhibit the growth of some bacteria. pH values for the growth of some foodborne pathogens are shown in Table 5. (7)

Table 5. pH growth ranges of common food-poisoning bacteria in fish (7).

Bacterium	**pH range**
Listeria monocytogenes	4.2 – 9.7
Yersinia enterocolitica	4.2 – 9.0
Clostridium botulinum	4.5 – 8.4
Bacillus cereus	5.0 – 9.5
Shigella spp.	5.0 – 9.3
Vibrio parahaemolyticus	5.0 – 11.0
Vibrio cholerae	5.0 – 9.6

Unfavourable acidity restrains the functions of enzymes and affects the transportation of nutrients into cells. Therefore, bacteria attempt to affect the acidity of their surroundings. Some of the off-odours are a consequence of bacterial actions to neutralize the pH. (8)

2.3.4 Oxidation-reduction potential

When a substance loses electrons, it becomes oxidized, and when it receives electrons, it respectively reduces. This migration of electrons causes electric potential, which can be measured. The reaction is also known as oxidation-reduction potential or redox (Eh) potential. In foods, the Eh potential is expressed in millivolts. If the food is oxidized, the value is positive, and if it is reduced, the value is negative. Aerobic bacteria grow at positive Eh, anaerobic at negative Eh, and facultative anaerobes or aerotolerants can grow in both. *Clostridium* is a genus of anaerobic bacteria; *Bacillus* is a genus of aerobic bacteria. Lactobacilli are an example of microaerobic bacteria that can grow in slightly reduced conditions. Bacteria affect the Eh of their environments during growth just as they do with pH. Aerobics can lower the Eh while anaerobes cannot. Some microorganisms can produce H_2S as their metabolic by-product, which has the capacity of lowering Eh. Because H_2S reacts readily with O_2, it will accumulate only in anaerobic conditions. (9, 10)

Microbial growth in food reduces its Eh, but food products also have SH groups, ascorbic acid, sugars, and other antioxidants that have a reducing effect. TMAO on the other hand is known to cause a high redox potential in the fish flesh. Chopping, grinding or mincing on the other hand will increase the access of air to the fish, increasing its oxidation-reduction potential. The Eh of fish before rigor mortis is on the positive side around 200 mV. Anaerobic bacteria do not multiply until the rigor mortis phase has stopped. This is because of the high Eh. After rigor mortis, the Eh of fish tends to reduce. In addition, the method of preservation has an effect on the Eh. Fish is commonly preserved in a vacuum or gas where the oxygen is limited and Eh can be down to –200 mV. This way the growth of aerobic spoilage bacteria is inhibited. However, anaerobic bacteria can grow in a vacuum, and therefore the risk of, for example *Clostridium botulinum*, is higher. Obligate or strict aerobes are those that are respiratory bacteria and generate most of their energy from oxygen. These dominate the surface of food where air is readily available. Examples include *Pseudomonas fluorescens*, which grows at Eh between +100 - +500 mV, and other oxidative gram-negative rods which produce slime and off-odours on fish and meat surfaces. (8, 10)

2.3.5 Nutrient content

The growth of microbes demands water, a source of energy, a source of nitrogen, corresponding growth factors and minerals. All of these are found in foods. The approximate chemical composition of rainbow trout is 63.4 % of water, 17.4 % of protein, 16.5 % of fat and 1 % of ash. There are no carbohydrates in fish. Microorganisms may also require B vitamins in low quantities. The sources of energy are usually sugars, alcohols and amino acids, but microbes rarely use fats. Amino acids are also a source of nitrogen. Not all microbes can degrade proteins to amino acids and peptides, so they have to use free amino acids. Gram-positive bacteria are an example because they do not synthesize amino acids themselves. In general, gram-positive bacteria are the least synthesizing. Gram-negative bacteria and molds are able to synthesize most or all of their requirements. Microbes can be divided on the grounds of nutrient use as well. Heterotrophs need organic carbon molecules for division and organotrophs use carbon dioxide as their main carbon source. Most of the bacteria in

food products use easily soluble compounds like carbohydrates and sugars as a nutrient and for growing. Microbes use and dissociate proteins when the growth accelerates and carbohydrates run out. The bacteria in fish, and especially in rainbow trout, are mostly gram-negative bacteria as mentioned previously. Therefore, rainbow trout spoils easily because gram-negatives can use and degrade the proteins in fish. The fragmentation of proteins cause off-odour compounds and the microbes that dissociate proteins are called spoilage bacteria. (8, 9, 11, 12)

3 PRESERVATION OF FISH

Because fish tend to spoil easily and do not preserve as raw, there are ways to increase the shelf-life. The best way to prevent spoilage is preserving fish at low temperature at all times. Fish must be put to ice immediately after catching and quickly transported to processing. Keeping the fish in higher temperature than refrigerator temperature rapidly spoils the fish as shown before. There are other methods of increasing the preservability in addition to cold storage.

3.1 Conventional preservation methods

The oldest way of preserving fish is by salting. Salting affects the fish water activity, which is one of the main reasons bacteria can grow and spoil the fish. Most gram-positive bacteria have the water activity minimum of 0.90 and gram-negative have the A_w minimum of 0.97. Using salting, the water activity can drop from 0.99 to 0.87, which effectively suppresses the growth of bacteria. In addition to the lowered water activity, some bacteria do not tolerate salt itself. Vacuum and protective gas packages are most commonly used in fresh fish products. In these products, the spoiling organisms are different from fresh products that are not packaged in this way. Packaging fish products in to a vacuum package increases the shelf-life when combined with cold storage. Raw fish fillet preserves unspoiled in a refrigerator for three to four

days assuming that it has been handled correctly. The same fish packed in a vacuum stays fresh for 6 days. Preservation methods and their effects that are used in food industry are shown in Table 6. (8, 12, 13)

Table 6. Some preservation procedures and their mechanism of effect. †

Procedure	**Influence to microbes**
Cooling	Low temperature to retard growth
Freezing	Low temperature and reduction of a_w to prevent growth
Drying and curing	Reduction of water activity to delay or prevent growth
Vacuum and oxygen-free MAP*	Low oxygen tension to inhibit aerobes and delay growth of facultative anaerobes
CO_2-enriched packaging	Specific inhibition of some bacteria by carbon dioxide
Addition of acids	Reduction of pH value and possible inhibition by the specific acid
Lactic fermentation	Reduction of pH value by microbial actions and possible inhibition by the lactic and acetic acids formed
Addition of preservatives	Inhibition of specific group of micro-organisms
Pasteurization	Delivery of heat to inactivate target micro-organisms
Application of high hydrostatic pressure	Pressure-inactivation of vegetative bacteria, yeasts and moulds

† Adapted from Adams & Moss, 2000 (12)

** MAP = 'modified-atmosphere packaging'*

Other procedures are used in food technology, like emulsification that limits nutrients within the aqueous droplets in foods, but are not suitable for fish products. Some of the procedures shown above are not suitable for raw fish products for obvious reasons. For example, pasteurization is meant for cooked fish therefore it is not suitable for raw products. In addition, irradiation, that could inactivate target microorganisms with delivered ionizing radiation, is not used because of food law restrictions. Many of fish

preservation procedures are combined with each other. Usually, MAP is used with cold-storage.

Salting causes curing and when mixed with spices and sugar, the fish becomes rawpickled in a couple of days. Rawpickled or 'gravad' rainbow trout is softened and is not so susceptible to spoil by bacteria than raw rainbow trout. Smoke-curing also influences the shelf-life of fish, mostly due to the increased salt content. Cold-smoking is prepared at approximately 30 – 32 °C for 2 – 3 days and warm-smoking at 105 – 120 °C for 2 - 3 hours. Regardless, the most significant method of improving preservation is packaging fresh raw fish to vacuum or gas (CO_2) compined with cold-storage. The displacement of oxygen or deoxygenation inhibits aerobic bacteria from growing. Gram-negative bacteria are sensitive towards carbon dioxide, but gram-positive bacteria can be resistant and able to grow in those conditions. (10)

3.2 Biopreservation

The conventional fish preservation procedures are not always sufficient. The carbon dioxide in protective gas packed products prevents the growth of *Pseudomonas* and *Shewanella*. Still on the other hand some pathogens in fish that could spoil the fish already in low concentrations and can cause serious food poisonings, grow better in a vacuum and protective gas packages when the growth of rival bacteria has been inhibited. *Listeria monocytogenes*, *Clostridium botulinum* and *Staphylococcus aureus* are examples of these. *C. botulinum* is commonly found in fresh water fish like whitefish. *Listeria* can be found in salt water fish like salmonoids (fresh and rawpickled) and *S. aureus* contamination is linked to smoked fish. (14)

Biopreservatives are not synthetic and therefore more accepted by public. These natural preservatives can be for example oregano oil, which have been found to increase the shelf-life of swordfish by 5 days (13). Other biopreservatives could be beneficial microbes that prevent spoilage bacteria from growing. Some lactic acid bacteria have been found to have inhibitory effects on spoilage bacteria. (15)

Lactic acid bacteria (LAB) have major potential for use in biopreservation because they are safe to consume and because they naturally dominate the microflora of most foods during storage. Non-spoiling LAB inhibits the growth of other bacteria due to the formation of lactic acid and bacteriocins or the competition for nutrients. This may contribute to their selection during the spoilage of lightly preserved seafood products. Lactic acid bacteria are gram-positive, nonsporing, nonrespiring cocci or rods that produce lactic acid. The genera of LAB are shown in Table 7. (15)

Table 7. The genera of lactic acid bacteria (15).

Lactic acid bacteria genera			
Aerococcus	*Enterococcus*	*Lactosphaera*	*Streptococcus*
Alloiococcus	*Globicatella*	*Leuconostoc*	*Tetragenococcus*
Carnobacteria	*Lactobacillus*	*Oenococcus*	*Vagococcus*
Dolosigranulum	*Lactococcus*	*Pediococcus*	*Weissella*

The inhibition effects of lactic acid bacteria are highly studied, because different LAB can have different effects on spoilage bacteria. The antagonistic effect of nisin on *L. monocytogenes* has been demonstrated as well as pediocins from *Pediococcus pentosaceus* and *P. acidilactidi* (16). Bacteriocins from *Lactobacillus bavaricus* transiently affect *L. monocytogenes* especially at low temperatures. Similar effect was also observed with bacteriocin from *Carnobacterium piscicola* (16). Similar studies have been made concerning the pathogen inhibition ability of probiotic bacteria in a vacuum and modified atmosphere packed salmonoids. Inoculation with *Lactobacillus sakei* showed the best preservative effect among inoculated treatments in vacuum-packed filleted rainbow trout (17). *Enterococcus faecium* was the best biopreservative candidate from five different LAB for controlling and inhibiting *Listeria innocua* growth in vacuum-packed cold-smoked salmon (18). The association *Lactobacillus casei-Lactobacillus plantarum* was the most effective in cold-smoked salmon as *Listeria innocua* counts decreased of 3.2 log cfu/g compared with the control (19). Adding *Carnobacterium divergens* is a promising way to inhibit the growth of

pathogenic bacteria such as *Listeria monocytogenes* with low effect on the quality of the salmon product (20). The presence of bacteriocin production in *Carnobacterium piscicola* enhances its inhibition of *Listeria monocytogenes* in salmon (21). Most effective bacteria seem to be *Lactobacillus sakei*, *Enterococcus faecium*, *Lactobacillus plantarum*, *Carnobacterium divergens* and *Carnobacterium piscicola*. There can be significant value for increasing the shelf-life of rainbow trout products by using pathogen inhibitory LAB moreover vacuum or carbon dioxide package. Besides biopreservation do not require large investments for technical equipment. A solution, which contains the LAB, can be sprayed on the surface of the fish or the fish can be dipped to the solution before packaging into vacuum or gas packages. If the shelf-life of the product is increased even for one day, there might be commercial benefits to the fish producer. Extension of shelf-life has an ecological value also, because it will reduce losses due to spoilage. (15, 22)

4 RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Rainbow trout is by far the most farmed fish in Finland. It is originated from the north side of the Pacific and the rivers and lakes that run there from North America. The living territory of rainbow trout reached from the west side of the Rocky Mountains of Alaska to Northern Mexico. Rainbow trout spread all around Europe in the late 19th century and nowadays it can be found almost all around the world. Fertilized fish eggs were restocked to natural waters also in Finland, but it was unsuccessful. Despite many attempts, rainbow trout did not become wild and naturally prolific. The reason might be that rainbow trout have to compete for living space with many other fish species. Another possible reason could be the acidity of water at the time of spawn. (23)

Rainbow trout was named after the recognisable broad pink and violet line on the side of the fish (Fig. 2.). Rainbow trout is very similar to salmon and trout. It belongs to the *Salmonidae* family. It has the same kind of spots and sturdy figure as other salmonoids.

Rainbow trout can be distinguished from other salmonoids by the colour, the smaller head and the small cut in the tail fin. (23)

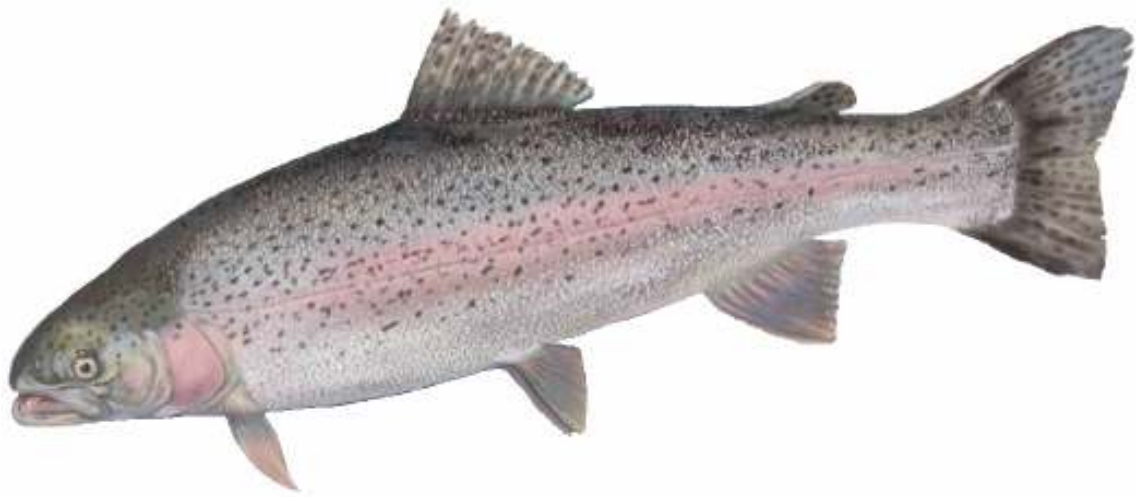

Figure 2. Rainbow trout has a very recognisable pink line and dots on the side (23).

4.1 Cultivation of rainbow trout in Finland

The cultivation of rainbow trout for food purposes started properly in the 1950. Rainbow trout is quite resilient when it comes to the surroundings. It can grow in warmer temperature and lesser oxygen content than domestic salmon or trout. However when the conditions are not optimal for rainbow trout, it eats less and therefore does not grow well. The water temperature should be 0-21 °C, the optimal being 16-18 °C. The oxygen content of the water should be 5-12 mg/l and pH 6.2-8.5. Usually rainbow trout is farmed under these conditions in lakes, rivers or other water areas, but the temperature of the water is colder than the optimal. (24, 25)

Rainbow trout spawns in the spring, after the water temperature has risen to 10 °C. The female lays 800 -1000 fish eggs of 4 mm in diameter in a pit. The young hatch out a month after the spawning. They eat animal plankton, shellfish and insects. Rainbow trout uses other fish as its main nutriment after reaching 35 – 40 cm in length. The maximum length for rainbow trout is about 60 cm and it can live up to eight years. In fish farms, a rainbow trout at the age of 5 – 6 years is a rare individual. (23)

Farming was moved from inland waters to coastal waters in 1980. In 2005, 11.612 million kg of rainbow trout was cultivated in coastal waters and 2.081 million kg in inland waters. The total number of domestic cultivated rainbow trout is decreasing while the imported total number, from Norway and Sweden, is increasing. A peak for domestic production was reached in 1990 when nearly 19 million kg of rainbow trout was produced. (6, 26)

Finns eat approximately 3 kg of rainbow trout per person per year and the total number is increasing every year. In total Finnish people eat up to 17 kg of fish per person per year, which is the same as Norwegians. Finnish rainbow trout is the most eaten fish species in Finland, but the total number of Norwegian salmon is slowly reaching it. Most Finns buy the fish or fillets fresh and prepare it at home. Rainbow trout is sold fresh, but it is available in frozen, canned and fresh processed forms as well. (1, 6)

5 BACTERIA OF RAINBOW TROUT

The Nordic rainbow trout lives in cold-water conditions, its pH is neutral, and the water activity value of 0.99, in which case the common bacteria of rainbow trout are gram-negative, facultative anaerobic and psychrophile or psychrotroph bacteria. The most often found bacteria in fresh and spoiled fish are *Aeromonas, Pseudomonas, Shewanella* and *Vibrio*. The bacteria that are known to occur in fish are shown in Table 8. (2, 27)

Table 8. The genera of bacteria in fresh and spoiled fish (27).

Bacteria	**Gram**
Aeromonas	-
Alcaligenes	-
Bacillus	+
Corynebacterium	+
Enterobacter	-
Enterococcus	+
Escherichia	-
Flavobacterium	-
Lactobacillus	+
Listeria	+
Microbacterium	+
Moraxella	-
Photobacterium	-
Pseudomonas	-
Psychrobacter	-
Shewanella	-
Vibrio	-
Weissella	+

The common bacteria genera in the microbiota of rainbow trout are *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Pseudomonas* and *Carnobacterium* (28). However, the microbiota varies in fish according to its environment. *Aeromonas* and *Enterobacteriaceae* dominated the bacterial population structure and other general bacteria in the Baltic Sea rainbow trout were *Acinetobacter*, *Pseudomonas*, *Shewanella*, *Plesiomonas* and *Proteus* (28). Motile *Aeromonads* and *Carnobacterium* were the dominant psychrotrophs in addition to *Plesiomonas shigelloides*, *Bacillus* and coryneforms that were found in the Mediterranean rainbow trout (29). Lactic acid bacteria that appear in rainbow trout are *Lactobacillus sakei*, *L. curvatus*, *Carnobacterium piscicola* and *C. divergens* (30).

5.1 Food poisoning bacteria

There are some bacteria in rainbow trout that can cause foodborne illness. Some of these cause mild diarrhoea even in large concentrations. There can also be pathogens, which can cause serious food poisonings due to diminished competition. In general, seafoods have a few notable microbes or toxins that cause food poisonings: histamine in tuna, parasites and listeria in raw fish, viruses, vibrios and clam toxins in oysters and clams and botulin and listeria in a vacuum-packed fish. *Clostridium botulinum* is very common bacteria in the environment, but the *C. botulinum* food poisonings connected with rainbow trout are rare. (31)

5.1.1 *Aeromonas*

Aeromonas genus bacteria are common bacteria in the environment and appear especially in natural waters. *Aeromonas* are also found in pipeline waters, which is how it can get to a wide range of foods. *Aeromonas* are a transient component in the microbiota of humans and animals and they are very common in fish, because the bacteria grow in fish natural environment. *Aeromonas* genus is nowadays divided into 14 different species. *Aeromonas* are *A. hydrophila*, *A. sorbia*, *A. caviae*, *A. salmonicida*, *A. veronii*, *A. jandaei*, *A. schubertii*, *A. trota* and *A.eucrenophila*, from which some are divided into two or three strains. (32, 33, 34)

Aeromonas are gram-negative, facultative anaerobic, catalase and oxidase positive rods. Most *Aeromonas* ferment glucose and move with single polar flagellum. *Aeromonas* do not tolerate high salinity or acidity. The salt concentration should be less than 5 % and pH approximately over 6.0 for the bacteria to grow. *Aeromonas* are significant bacteria in food preservation, because most strains can grow in chilled conditions as low as minus 0.1 °C, but some strains are mesophilic. The optimal growth temperature is 28 °C, but the temperature growth ranges between species from <5 ° to 45 °C. The growths of Aeromonas in modified atmosphere packages depend also on the nature and number of competing microbiota. *Aeromonas* are easily killed with heat or irradiation.

In a study, *Aeromonas* were killed in 2 min at 55 °C compared with 15 min for *S. aureus* and *Salmonella*. Some species of *Aeromonas* produce toxins such as aerolysin, cytolysin, Asao toxin, β-hemolysin, which can be heat stable. (32, 33, 34)

Aeromonas can cause intestinal infections to immunocomprimised. Only two of 50 volunteers got diarrhoea from $5 \cdot 10^5$ high dose. The research results regarding *Aeromonas* foodborne illness show that log 10^6 to >log 10^7/g dose can cause diarrhoea after 24 h, but the results are not consistent which species are the cause. *A. veronii* (strain HG8), *A. hydrophila* (HG1 and HG3) and *A. caviae* (HG4) have been associated with gastroenteritis. Gastroenteritis occurs commonly on children under five year old, but it is usually mild with watery diarrhoea. *A. hydrophila* is very usual bacterium in healthy fish, but it is a pathogen for some fish species and can cause hemorrhagic septicemia on them. (32, 33, 34)

5.1.2 *Bacillus*

Bacillus genus bacteria are gram-positive, aerobic, spore-forming rods. It is very common in soil and in water. *B. cereus*, *B. licheniformis*, *B. subtilis* and *B. pumilus* can cause food poisonings, but *Bacillus cereus* is the most common to cause foodborne illness. *B. cereus* generates spores that are very durable for extreme conditions and most of the food poisonings are due to faults in heating, cooling or preservation. Foodstuff should be preserved in chilled conditions and heated properly, when prepared, to destroy the *B. cereus* spores. (33)
B. cereus is facultative anaerobic and has large vegetative cells, which can be over 0.9 µm in width. Growth temperature ranges from eight to 55 °C with optimal around 28-35 °C. Nowadays psychrotrophs strains have been found, that can grow in 4-6 °C. Growth limiting pH ranges 4.5 – 9.3 and the minimum water activity is 0.95. (35, 36)

B. cereus causes two different food poisonings; enterotoxins cause diarrhoea and kereulide-toxin causes vomiting. Enterotoxins such as hemolysine BL, nonhemolysis enterotoxin and cytotoxin K causes intestinal infection with abdominal pain and profuse watery diarrhoea as symptoms. About 10^5 – 10^7 cfu/g doses can cause the diarrhoea

within 8-16 hours that lasts for 12-24 hours. The heat labile enterotoxins consist in the small intestine and affect the symptoms. The typical foods for high *B. cereus* growth that causes intestinal infections are meat, fish, vegetables, milk products and other perishables. The emetic syndrome is caused typically by the kereulide, which is formed in the food by the *Bacillus* bacteria. The intoxication causes nausea and vomiting within one to five hours from consuming, lasting between 6 to 24 hours. The total number of bacteria for producing enough kereulide to cause food poisoning is 10^5-10^8 cfu/g. Once the kereulide have been formed in the food, it does not perish even if the food is heated. Foods that have usually caused emitic food poisoning are cooked rice, pasta and noodle foods. (32, 33, 35, 36)

5.1.3 *Listeria*

The *Listeria* genus consists of six bacteria species: *L. gray*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri*. *Listeria monocytogenes* is a known animal and human pathogen. *Listeria* has been isolated from fresh and salt water, soil, sewage sludge, decaying vegetation and silage. *Listeria* appears also in healthy animals and humans, 1-10 % of population emit it to faeces. Due to its frequency *Listeria* come across in meat and fish as well. (32, 35)

L. monocytogenes is a gram-positive facultatively anaerobic, catalase-positive, oxidase-negative, non-sporeforming rod with 0.4-0.5 μm in diameter and 0.5-2.0 μm in length. *Listeria* possesses peritrichous flagella and has very characteristic tumbling motility. *L. monocytogenes* can grow in both aerobic and anaerobic conditions and it survives easily in a vacuum and protective gas packages. *L. monocytogenes* can be divided into 13 different serotypes, but only three of the serotypes (1/2a, 1/2b and 4b) cause 90 % of the foodborne illnesses. *Listeria* are salt tolerant, they are able to grow in 10 % sodium chloride. *L. monocytogenes* can grow in the water activity of 0.92, but it survives and preserves even in lower water activity. The minimum pH for *Listeria* to grow is 4.4 and maximum pH is 9.6. In addition, the temperature area of growth is wide, *Listeria* can grow in temperatures ranging from -0.4 to 45 °C. Foodstuffs that increase microbe proliferation are especially soft cheeses and vacuum packed cold cuts meat and fish

products. *Listeria* have been found in 10-20 % of raw pickled and other fish products in Finland (37). More than 100 cfu/g have been found in a vacuum packed fish products. (16, 32, 35)

L. monocytogenes bacterium is very invasive and can proliferate in host cells. The bacterium penetrates to the epithelial cell and is carried along to the local lymph gland. The liver is the main target of the infection. In healthy body, the T-cell response destroys *L. monocytogenes* in the next few days from the infection. If not, the infection can spread through blood to the central nervous system and in the placenta with pregnant women. The disease-causing mechanism of *L. monocytogenes* is very complex. This foodborne illness called listeriosis is very severe to young children, elderly, immunocomprimised and pregnant, because it has a mortality rate of 20-25 %. The symptoms are usually feverish general infection, vomiting, nausea, stomach cramps, diarrhoea, a severe headache, constipation, persistent fever, stiff neck, the loss of balance and convulsions. Listeriosis can also lead to meningitis. Listeriosis during pregnancy causes miscarriage or neonatal death. (16, 32, 35, 37)

Listeria can also cause a non-invasive form of the disease that has feverish gastroenteritis as a symptom and is not as severe as the invasive form. Below 100 bacteria concentration per gram in food is not considered meaningful to food poisoning risk, but the infective dose for the risk groups is yet unknown. The listeriosis incubation period varies from 2 to 70 days. *L. monocytogenes* is a common ground bacterium, so the connection between the infection and the food that causes the infection is hard to define. *L. monocytogenes* ability to cause the listeriosis is quite weak; therefore a large total number of the bacterium does not harm a healthy adult. In Finland, there is 0-tolerance for *L. monocytogenes* in milk products. There have been two epidemic incidents concerning *L. monocytogenes* transmitted food poisoning; other from butter and another from vacuum-packed cold-smoked rainbow trout. (16, 32, 33, 35)

5.1.4 *Vibrio*

Vibrio bacteria are gram-negative, facultative anaerobic, oxidase-positive rods, which do not form spores. *Vibrio* move with a polar flagella and they are generally found in the coasts of seas. At least 10 pathogenic species are known which eight of them spreads through food (Table 9.). (33)

Table 9. Vibrio species associated foodborne diseases (33).

Species	**Disease**
V. cholerae, O1 and O139	Cholera
V. cholerae, non-O1 and non-O139	Diarrhoea, gastroenteritis
V. mimicus	Diarrhoea, gastroenteritis
V. hollisae	Diarrhoea
V. fluvialis	Diarrhoea
V. furnissii	Diarrhoea
V. vulnificus	Diarrhoea, septicaemia
V. parahaemolyticus	Gastroenteritis, otitis media

Some *Vibrio* are halophilic and almost every one endures high salinity. The sodium chloride concentration should range between 1-6 % for *Vibrio* to grow. On the other hand *Vibrio* are sensitive to heating and acidity. Heating for a few minutes in 60 °C is enough to destroy them. The optimal growth temperature is 37 °C and ranges between 5 – 43 °C. Some strains can grow in refrigerator temperature. The vehicle foods among all Vibrio species are generally seafoods, because *Vibrio* need salt to grow. (33, 35)

The *V. cholerae* strains O1 and O139 cause cholera, which is an intestinal infection. The symptom is violent watery diarrhoea that can lead to dehydration by fluid loss in a matter of hours. Cholera always needs to be treated and it can be vaccinated against by Dukoral®. Cholera is caused by cholera toxin (CT-toxin), which alters the mucous membrane of bowel to be permeable for water. Cholera does not appear in the countries

of good hygiene and for example, the cases that have occurred in Finland are all connected with travelling in cholera-areas. The infective dose of *V. cholerae* is between $10^4 – 10^{11}$ bacteria. Its incubation time is from 6 hours to 5 days and the symptoms lasts for two or three days after treatment has been started. (32, 38)

The *V. cholerae* strains O2 – O138 or non-O1 and non-O139 strains does not cause cholera. These strains and *V. minicus*, *V. holisae*, *V. fluvialis* and *V. furnissii* species cause vibriosis that is mild diarrhoea. The incubation time ranges from 6 hours to 4 days for the conventional diarrhoea caused by vibriosis. The symptoms vary from mild to intence diarrhoea and sometimes gastroenteritis. The infective dose is $10^6 – 10^9$ bacteria and the symptoms last usually for one day. (33, 38)

V. parahaemolyticus causes gastroenteritis, but sometimes it can lead to a chronic inflammation of the ear. The disease is caused by THD toxin, which is formed in the intestines. Symptoms are diarrhoea, stomach pain, nausea and headache and can last for 1 – 8 days. The infectional dose is $10^5 – 10^7$ bacteria and incubation time is from 2 hours to two days, but commonly it is 12 hours. The bacterium is destroyed by heating. It is common in Japan, where a lot of raw fish foods are consumed. (32, 35)

V. vulnificus bacterium penetrates to blood circulation and causes septicaemia. The bacterium produces endotoxin in the blood, which rapidly decreases the blood pressure. The bacterium needs high iron concentration to proliferate in the blood. The risk groups for *V. vulnificus* causing food poisoning are people who have a increased iron concentration level and over 50-year-old men. 80 % of the food poisonings have been among men and it has been noted that estrogen protects from *V. vulnificus* endotoxin. The incubation time varies from 7 hours to several days. Symptoms are fever, chills, nausea and hypotension, but sometimes abdominal pain, vomiting and diarrhoea. The disease is highly dangerous, approximately 60 % of the patiens die in a few days if the antibiotic treatment is not started. Necrosis is also related to the disease and limbs are often amputated. *V. vulnificus* foodborne cases are always connected to salt water oysters and other seafood that are eaten raw, but *V. vulnificus* is not found in cold water areas. (35, 38)

5.2 Spoiling bacteria

Different bacteria cause spoilage changes in different products. The bacterium that causes the most of the changes in a certain product is called the specific spoiling organism (SSO). The seafood SSOs produce ammonia, biogenic amines, organic acids and sulfur-compounds from aminoacids, hypoxantine from ATP degradation products, and acetate from lactate. TMA is produced by some bacteria capable of using TMAO in anaerobic respiration. The typical spoilage compounds that are produced by bacteria are shown in Table 10. (39, 40)

Table 10. Typical spoilage compounds, substrates and producing bacteria in fish (40).

Compound	**Substrate**	**Producing bacterium**
TMA	TMAO	*S. putrefaciens*, *P. phosphoreum*, *Vibrionaceae*, *Enterobacteriaceae*
H_2S	Cysteine	*S. putrefaciens*, *Vibrionaceae*
CH_3SH, $(CH_3)_2S$	Methionine	*S. putrefaciens*, *Pseudomonas* sp.
Ketones, esters, aldehydes, NH_3	Other amino acids	*Pseudomonas* sp., *Enterobacteriaceae*, lactic acid bacteria
Hypoxanthine	IMP, inosine	*S. putrefaciens*, *Pseudomonas* sp., *P. phosphoreum*, *Enterobacteriaceae*
Acids	Carbohydrates, lactate	*S. putrefaciens*, *Enterobacteriaceae*, lactic acid bacteria

All of the compounds above cause faulty odours and flavours in the fish. The SSO of fresh and aerobic preserved fish are *Pseudomonas* spp. and *S. putrefaciens*. The same fish packed in a vacuum have LAB, *S. putrefaciens*, *Brothothrix thermosphacta* and *P. phosphoreum* as the spoilage bacteria. Fish products that are packed in protective gas can have LAB, *B. thermosphacta* and *P. phosphoreum* as their spoilage bacteria. Rawpickled fish have LAB, H_2S producing bacteria such as *Enterobacter* and *Aeromonas* spp. and *S. putrefaciens* as spoilage bacteria. It is common that the spoilage

bacteria in processed fish products are LAB, which uses the amino acid and proteins in the fish and can grow in anaerobic conditions. (2, 39, 40)

6 CULTIVATION AND ISOLATION

Cultivation techniques are widely used to examine microorganisms in foods. The most common cultivation method by far is a standard plate count (SPC) for viable cells or colony-forming units (cfu). It is possible to determine approximate numbers or types of microorganisms with SPC. To perform a SPC method, the food sample is homogenized. The solid food sample and diluent are put in a special plastic bag. The Stomacher device homogenizes by pounding the specimen bag with two paddles at desired speed. Pounding affects by releasing the microorganisms into the diluent. Homogenizing is used rather than blending, because it is less lethal to the bacteria. The homogenized sample is then serially diluted and plated onto a suitable medium solidified with an agar. The agars major component is agarose, which has the ability to form gel in low concentrations (1.0 – 2 %). The agars with selective media are autoclaved depending on the medium, and poured molten to a Petri dish. The function of the medium is to provide adequate nutrition for the growth of the bacteria that are enumerated. They can also include inhibitory agents to be more selective and because of that, some media or their compounds cannot be autoclaved. Agar solidifies below 40 °C temperature making it possible to mix it with the samples. Sterile moulted agar dishes are suitable for samples to be plated onto and incubated at appropriate temperature and time. After incubation, visible colonies are counted and the total number of bacteria in the sample can be evaluated. (41, 42)

In order to identify the spoilage microbiota of rainbow trout, fish samples were cultivated in selective media with standard plate count. The samples were vacuum-packed rainbow trout fillet in three different spoilage stages. The sample packages were stored in a refrigerator until analysis. The packages came from the same batch and they were packed in the morning in the fish processing company Jokisen Eväät Oy. The

samples were cultivated in eleven different culture media and in standard plate medium in four ways. The selective media determined the counts of *Aeromonas hydrophila*, *Bacillus cereus*, *Brochothrix thermosphacta*, *Enterobacteriaceae*, *Enterococcus*, H_2S producers, *Pseudomonas*, *Lactobacillus*, *Weissella*, *Listeria*, and *Vibrio*. All of the media were self-prepared from purchased powders or kits and casted to Petri dishes. The commercial media are designed so the agar would support the growth of the bacterium that is being determined by inhibiting the growth of other bacteria. However separate other bacteria species or genera might grow on it.

According to the manufacturer labelling, the vacuum-packed raw rainbow trout is consumable for 6 days after packaging. The cultivations were performed at the best before date (BBD) ($< 10^7$ cfu/g), at 3 days (+3 d) and 6 days (+6 d) after the best before date. At best before date, the sample rainbow trout fillet was fresh smelling and looked somewhat as edible as the packaging day fish fillets evaluated with a sensory impression. Three days after the best before date, the spoilage smell was still quite mild, but the color of the flesh was slightly paler. After six days the smell and appearance was obviously spoiled and slime had formed on the surface of the flesh. The smell resembled rotten eggs and ammonia. The samples were prepared for bacterial cultivation by conventional microbial methods and based on the NMKL 96/2003 method of bacteriological examination from fresh and frozen seafood. Approximately 50 grams of sample fish in 100 ml of peptone water (Difco, 218071) were homogenized at 230 rpm for 5 minutes. Homogenized fish suspension was serially diluted from 10^{-1} to 10^{-8} as shown in the Fig. 3. The rest of the homogenized fish suspensions were stored in 40 % glycerol in -86 °C for future later analysis.

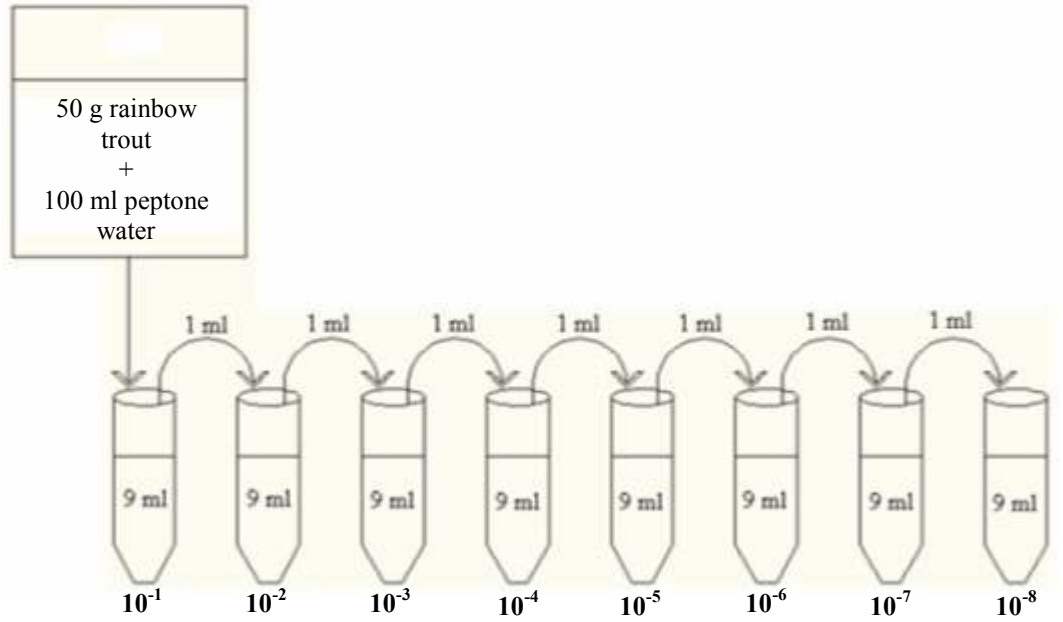

Figure 3. Serial dilution chart of homogenized fish sample.

6.1 Total count of bacteria

The total bacterial count was determined by cultivating samples on plate count agar (Difco, 247940). The total count gives the total number of microorganisms, comprising bacteria, yeasts and moulds, in a sample. The total bacterial count should be below 10^7 cfu/g in edible fish. 10^6 cfu/g is the limit for good quality and 10^7 cfu/g is the limit for tolerable quality (41). Food and fish that have exceeds this limit are considered to be spoiled and can cause food poisoning. The cultivations were made with the SPC method in two ways: Spread plate and pour plate.

6.1.1 Spread plate

The spread plate is the usual way of cultivating microbes on a culture dish. The sample (0.1 ml) was pipetted over the solidified agar and spread with a rod. The spread plates were incubated at three different conditions:

1. Aerobic at + 22 °C (room temperature) for 2-5 days
2. Aerobic at + 4 °C for approximately 10 days
3. Anaerobic at + 22 °C for 2-5 days

Most of the bacteria were observed to grow on aerobic and in room temperature incubated dishes. In refrigerator temperature, incubation determines the count of psychrotrophs. Anaerobic incubation was done to determine the count of anaerobic bacteria because the specimen rainbow trout was packed in a vacuum. The total counts (1.) were determined also from fresh samples at the packing packing (-6 d) and three days before the best before date (-3 d) to assure the microbial quality. The concentrations of total bacteria under three above-mentioned different incubation conditions are shown in Table 11.

Table 11. Total bacterial count (cfu/g) with spread plate methods.

Incubation method	**-6 d**	**-3 d**	**BBD**	**+3 d**	**+6 d**
1.	$1.5 \cdot 10^3$	$2.1 \cdot 10^3$	$4.1 \cdot 10^6$	$1.5 \cdot 10^7$	$3.3 \cdot 10^7$
2.	-	-	$3.2 \cdot 10^6$	$2.0 \cdot 10^7$	$3.1 \cdot 10^7$
3.	-	-	$5.0 \cdot 10^6$	$2.0 \cdot 10^7$	$2.0 \cdot 10^7$

(-6 d = six days before BBD, -3 d = three days before BBD, BBD = best before date, +3 d = three days after BBD, +6 d = six days after BBD)

The total counts show that at best before date sample the number of bacteria is close to the limit of edible fish. At packaging day and three days before BBD the total counts of bacteria was very low and evidently much lower than the limit of bad quality. The total counts of bacteria determined at aerobic (room temperature) for 2-5 days are illustrated in Fig. 4.

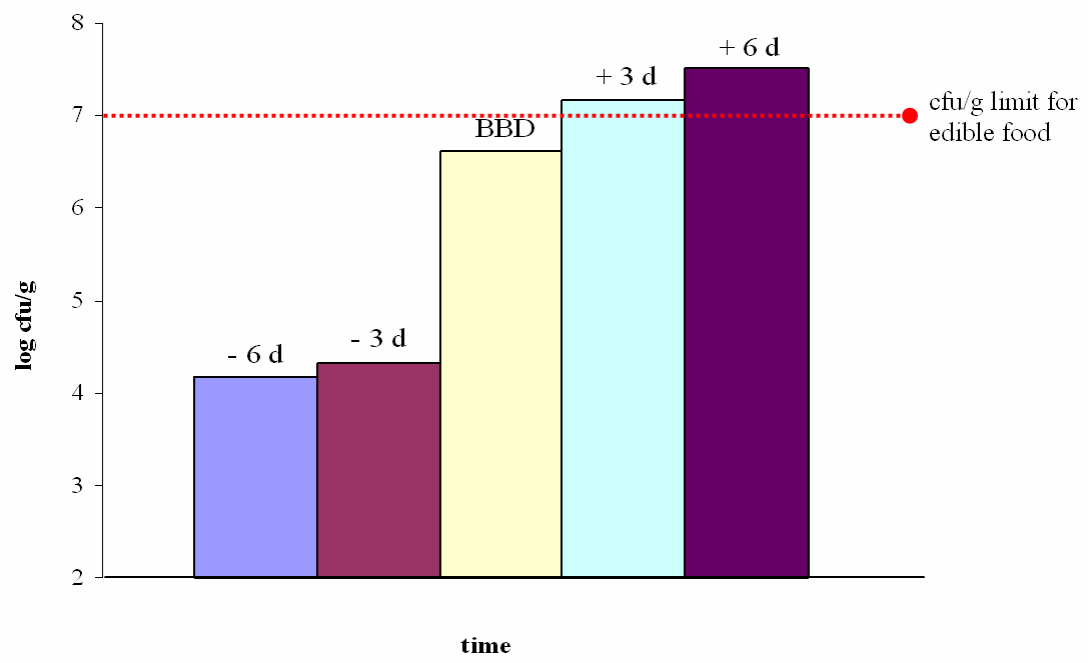

Figure 4. Total bacterial counts between packaging day and six days after BBD.

The total number of bacteria in anaerobically incubated (3) at the BBD sampling point is higher than the total counts incubated aerobically. The sample fish was packed in a vacuum, which is optimal for the growth of anaerobic bacteria. The growth of anaerobic bacteria did not increase six days after best before date. Perhaps some other bacteria or bacterial metabolites produced were inhibiting the growth of anaerobic bacteria, including LAB, to grow. The aerobic bacteria incubated in refrigerator temperature seem to increase exponentially although its growth was decelerating at six days after best before date sample (+6 d). The bacterial growths of these incubation ways are represented in Fig. 5.

6.1.2 Pour plate method

The pour plate method was done to determine the bacteria that can grow aerobically and anaerobically. In the pour plate method, 1 ml of the homogenized fish suspension was pipetted to the Petri dish and the molten standard agar is poured over it. In this method, the colonies of bacteria can grow in the agar instead of on the surface of the agar. The thermal shock of molten agar might harm the psychrotrophs that are typical in fish. This

method was used only in total bacterial count determination, because the colonies cannot be isolated by this method.

The pour plate dishes were incubated aerobically at room temperature for 2 – 5 days. The total number of bacteria determined with the pour plate method was $7.7 \cdot 10^6$ cfu/g for best before date sample, $2.8 \cdot 10^7$ cfu/g for + 3 days sample and $3.4 \cdot 10^7$ cfu/g for + 6 days sample. The counts in the pour plate method were higher than in any of the spread plated method. The total bacterial counts, both spread plates and pour plate, are shown in Fig. 5.

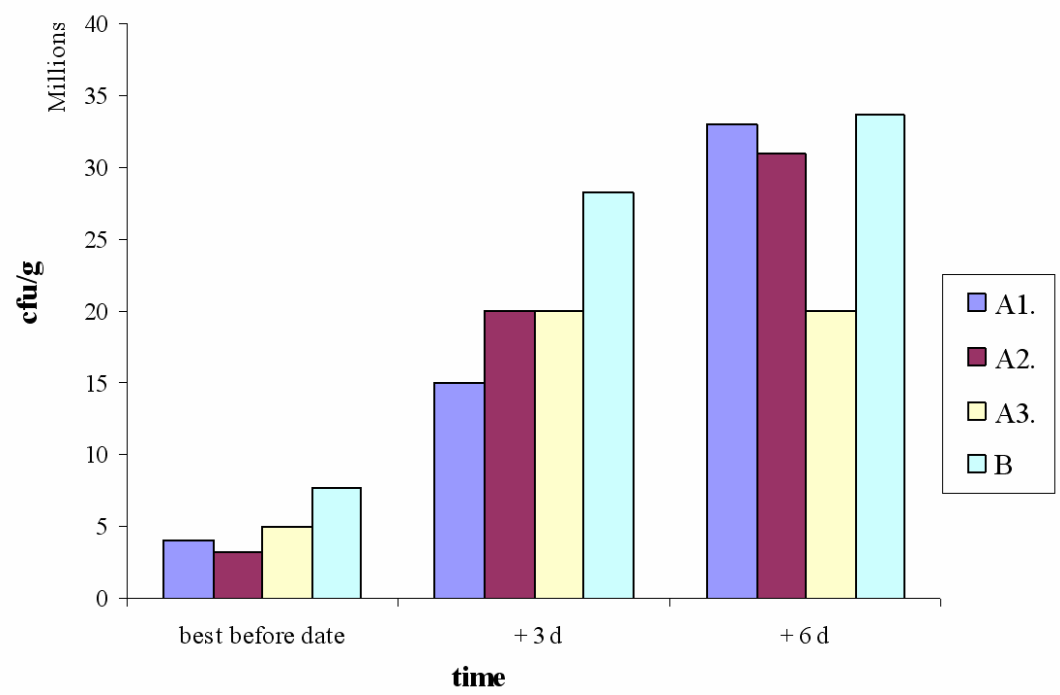

Figure 5. The counts of total bacteria (A = spread plate method, B = pour plate method, 1. = incubation aerobically at room temperature, 2. = incubation aerobically at refrigerator temperature, 3. = incubation anaerobically at room temperature).

The pour plate method determines the number of bacteria that can grow in facultative aerobic or anaerobic conditions. Most of the bacteria occurring in rainbow trout are facultative anaerobes that can grow in fish flesh near the surface. The pour plate method was carried out only in one of the cultivations, because the colonies needed to be isolated from the plates.

6.2 Selective media cultivations

The selective media were chosen based on the literature for the most commonly found bacteria on marine fish. All of the media were prepared according to the instructions of the manufacturers. The selective media and their incubation conditions are listed in Appendix 1.

6.2.1 *Aeromonas hydrophila*

The total number of *Aeromonas hydrophila* was determined with selective medium (*Aeromonas* medium base, Oxoid CM0833) supplemented with ampicillin. The medium supports the growth of *Aeromonas* spp. and *Plesiomonas* spp. as well as *Enterobacteriaceae.* The addition of ampicillin at 5 mg/l is needed for differentiation to grow exclusively *Aeromonas*. (43)

The *Aeromonas* dishes were incubated aerobically at 37 °C for 24 hours. Dark green and unopaque colonies, which had a darker centre, were counted. They were 0.5 – 1.5 mm in diameter. *Plesiomonas* can also grow in the medium as smaller livid transparent colonies, but there were not any of those. *Aeromonas* were found $2.6 \cdot 10^4$ cfu/g from the best before date sample, $1.1 \cdot 10^5$ cfu/g from the + 3 days sample and $1.1 \cdot 10^4$ cfu/g from the +6 days sample. The colony counts from +6 d were smaller than the rests, which can be an indication of domination of some other bacteria diminishing the growth of *Aeromonas*.

6.2.2 *Bacillus cereus*

Bacillus cereus was determined from the samples with Chromogenic *Bacillus cereus* agar (Oxoid, 1036). The supplement in use was Polymyxin B (Oxoid, SR0099E). Polymixin B inhibits most gram-negative organisms and some gram-positive organisms including some non-cereus *Bacillus* species. *Bacillus thuringiensis* will also grow on this medium, because it is biochemically identical to *Bacillus cereus*. (44)

The culture dishes were incubated aerobically at + 37 °C for 24 hours. The colonies grow as blue and green colonies. Almost all of the colonies in the dishes were small dots on the surface of the agar and were not easy to isolate unlike usual colonies are. Although most were small dots in the agar, they were counted as colonies. Therefore the total number of *Bacillus cereus* were $4.8 \cdot 10^5$ cfu/g in best before date sample, $6.4 \cdot 10^5$ cfu/g in + 3 days sample and $4.6 \cdot 10^6$ cfu/g in + 6 days sample. Over 10^5 cfu/g could cause food poisoning and the best before date sample already exceeds the concentration limit. It is, nevertheless, improbable that the total number of *Bacillus cereus* exceeds 10^5 cfu/g in the BBD sample causing food poisoning (45).

6.2.3 *Brochothrix thermosphacta*

Brochothrix thermosphacta is a gram-positive, non-motile, facultatively anaerobic rod-shaped micro-organism which is not considered pathogenic. It is an economically important meat-spoilage organism that grows in a wide variety of food and produces off-odours metabolic end products. *Brochothrix thermosphacta* counts were determined with STAA Medium (Oxoid, CM0881). STAA Selective Supplement (Oxoid, SR0151) is added in the agar before casting. The selective supplement contains streptomycin sulphate, thallous acetate and actidione (cycloheximide). To increase the growth of *Brochothrix thermosphacta*, 12 ml per liter of glycerol were also added to the agar. Streptomycin sulphate inhibits some gram-positive organisms and most gram-negatives at higher concentrations while *Brochothrix thermosphacta* remains resistant. Thallous acetate inhibits most yeasts as well as many aerobic and facultatively anaerobic bacteria. The incorporation of cycloheximide inhibits yeasts and filamentous fungi. Still some *Pseudomonas* are able to grow on STAA media. (46)

STAA sample plates were incubated at 22 °C for 48 hours aerobically. The colonies in the agar grew as straw colored colonies, 0.5-1.0mm in diameter. The counts of *Brochothrix thermosphacta* were $7.4 \cdot 10^4$ cfu/g in BBD, $9.9 \cdot 10^4$ cfu/g in +3 d and $1.0 \cdot 10^5$ cfu/g in +6 d samples. The total numbers of *Brochothrix thermosphacta* agar colonies were much lower than for example *B. cereus* agar colonies. According to

literature *Brochothrix thermosphacta* is one of the usual SSO in vacuum-packed fish products, but the quantity might not be the highest of the spoilage microbiota.

6.2.4 *Enterobacteriaceae*

The total numbers of *Enterobacteriaceae* were determined with Violet red bile glucose agar (Oxoid, CM0485). The medium is recommended for the examination of *Enterobacteriaceae* in foodstuff. *Enterobacteriaceae,* which ferment glucose to produce acid and/or gas, grow best on VRBG agar. This group includes *salmonellae* and *shigellae*, which do not ferment lactose, and enterotoxigenic *Escherichia coli*. Also organisms, such as *Klebsiella* and *Citrobacter* can grow in the culture medium. The bile salts and crystal violet of the agar inhibits the growth of gram-positive bacteria. The medium is used for a reliable indication of process failure, under-processing or post-processing contamination in processed foods. The agar was not autoclaved and the most recent and fresh agar dishes were used for the determination. (47)

The sample dishes were incubated anaerobically at 35 °C for 24 hours. Round, purple-pink, 1-2 mm in diameter colonies were counted. The counts were $3.7 \cdot 10^4$ cfu/g in BBD, $1.3 \cdot 10^5$ cfu/g in +3 d and $1.1 \cdot 10^4$ cfu/g in +6 d samples. The low count in +6 d sample is probably due to other dominant bacteria in that stage of spoilage.

6.2.5 *Enterococcus*

Enterococcus are gram-positive, non-motile/motile, facultative anaerobic cocci. *Enterococcus* is a genus of lactic acid bacteria and two of the species are common organisms in the intestines of humans: *E. faecalis* and *E. faecium*. (46)

The medium for determining *Enterococcus* was Slanetz and Bartley agar (Oxoid, CM0377). Food samples can be examined for *Enterococcus* with Slanetz and Bartley agar, because it is very selective for enterococci. Petri dishes of Slanetz and Bartley agar with samples were incubated aerobically at 45 °C for 48 h. The colonies should have

been pink, reddish brown or dark red colonies to count. However, there were not any colonies to count in any of the dishes.

6.2.6 H_2S producers

The determinations of H_2S producer bacteria were conducted with Kligler Iron Agar (Difco, 211317). The H_2S producer bacteria can ferment dextrose and lactose releasing sulfides and causing the foul odors of rotten eggs. H_2S producing bacteria are among others *Salmonella*, *Enterobacter*, *Klebsiella*, *Morganella* and *Yersinia* species. Kligler iron agar contains double sugar (dextrose and lactose) agar, with ferric citrate as an indicator to detect hydrogen sulphide production. (48)

The H_2S producing bacteria formed yellow and red colonies to the agar after incubating anaerobically at 35 °C for 18 – 24 hours. The colonies were counted together. The counts were $5.0 \cdot 10^4$ cfu/g in BBD, $6.6 \cdot 10^4$ cfu/g in +3 d and $1.9 \cdot 10^6$ cfu/g in +6 d samples. The counts were six days after the best before date over 6 log higher than in + 3 day sample which indicate that the H_2S concentration has started to grow after the + 3 days from the best before date. In the + 6 day sample the rainbow trout sample already smelled unpleasant and the spoilage was obvious.

6.2.7 *Pseudomonas*

Pseudomonas aeruginosa and *Pseudomonas* spp. counts were determined with *Pseudomonas* agar (Oxoid, CM0559). Before autoclaving, 5 ml of glycerol was added to half liter of agar. After autoclaving the selective supplement (Oxoid, SR0103) was added in to the agar base. The selective supplement consisted of cetrimide and sodium nalidixate, that would inhibit the growth of *Klebsiella, Proteus* and *Providencia* spp. on the agar. Despite the selective supplement, *Aeromonas* species will also appear as pink/brown colonies, especially in samples of fish products. (49)

The dishes were incubated aerobically at 25 °C for 24 – 48 hours. The colonies were counted in UV-light because *Pseudomonas* form bluish-green or fluorescent colonies.

Light colored colonies were also formed on the agars, they were not counted as *Pseudomonas*. In best before date sample the total number of colonies were $8.4 \cdot 10^4$ cfu/g, in + 3 days sample $3.0 \cdot 10^5$ cfu/g and in + 6 days sample $2.8 \cdot 10^5$ cfu/g. In six days after the best before date the total number of colonies on the agar decreased from the + 3 days sample. *Pseudomonas* are known to cause off-odors and spoilage on fresh and aerobically preserved fish and they are common in all fish products as well. Probably in this case *Pseudomonas* were present, but other typically vacuum-packed fish spoilage bacteria became more dominant at the stage of spoilage causing the decrease of *Pseudomonas*.

6.2.8 *Lactobacillus*

Lactobacillus belongs to the lactic acid bacteria group. The total number of *Lactobacillus* was determined with MRS and APT agar. APT agar is also for determining the *Weissella* bacteria from samples. *Weissella* are also included in the lactic acid bacteria group.

6.2.8.1 *Lactobacillus* with MRS agar

The MRS media (Oxoid, CM0359) is designed for the examination of *Lactobacillus*. *Streptococcus, Pediococcus* and *Leuconostoc* species can also grow on the agar. They are gram-positive, catalase and oxidase negative and are fastidious in their nutritional requirements. Lactobacilli are microaerophilic and generally require layer plates for aerobic cultivation on solid media. The growth of leuconostocs and pediococci can be altered by pH adjustment. *Lactobacillus* are spoilage causing lactic acid bacteria, but there are non-spoilage species too. (50)

MRS agar plates were incubated anaerobically at 37 °C for 24 – 48 hours. The colonies were compact, small, opaque and white. The counts were $4.4 \cdot 10^4$ cfu/g in BBD, $1.1 \cdot 10^5$ cfu/g in +3 d and $1.9 \cdot 10^4$ cfu/g in +6 d sample. The total numbers of the colonies were relatively small comparing to other selective media counts. Depending

what lactic acid bacteria are found with identification, the *Lactobacillus* does not seem to be the most common bacteria in the spoiled samples of rainbow trout.

6.2.8.2 *Lactobacillus* and *Weissella* with APT agar

APT agar (Difco, 265430) was used for determining *Lactobacillus* and *Weissella*. *Lactobacillus* and *Weissella* are heterofermentative which require thiamine to be able to grow, but other bacteria that need thiamine can also grow on the agar. Thiamine is also known as B_1 vitamin. The total number of B_1 vitamin in rainbow trout is approximately 0.05 mg/ 100 g. (48)

APT agar dishes were incubated anaerobically at 37 °C for 24 – 48 hours. Light colored colonies formed on the agar. The colony counts in BBD were $1.4 \cdot 10^7$ cfu/g, $4.5 \cdot 10^7$ cfu/g in +3 d sample and $4.2 \cdot 10^6$ cfu/g in +6 d sample. The counts were one of the highest ones of the selective media counts. In six days after the best before date the total number of colonies decreased for no apparent reason other than the bacterial competition. Lactobacilli grown with APT agar might be the spoilage lactic acid bacteria that are known to be associated with spoiled vacuum-packed fish.

6.2.9 *Listeria*

Listeria counts were determined with Chromogenic *Listeria* Agar Base (Oxoid, CM1080). *Listeria monocytogenes* is the most common pathogenic *Listeria* species and has been shown to be pathogenic to both man and animals. *Listeria* possess chromogen enzyme that can be cleaved by ß-glucosidase. This is exploited in the agar, and other organisms that possess this enzyme, such as enterococci, are inhibited by the selective agents. Two different selective supplements (Oxoid, SR0227 & SR0228) were added to the agar after autoclaving. The other supplement contained lithium chloride, polymyxin B and nalidixic acid, inhibiting the growth of yeasts and moulds that may be present in the sample. *Listeria monocytogenes* were then further differentiated by their ability to produce the phospholipase enzymes with another supplement containing lecithin. (51)

The *Listeria* agar dishes were incubated aerobically at 37 °C for 24 hours (+/- 2 h). Blue colonies with light surrounds or a halo were supposed to count as *L. monocytogenes* and without the light surrounds as *L. inocua*. However, no colonies were found on the plates indicating that there were no *Listeria* bacteria in the samples of rainbow trout. *Listeria* is usually found in 10-20 % of the fish products in Finland (37).

6.2.10 *Vibrio*

The counts of *Vibrio* bacteria were made with Kligler Iron agar (Difco, 211317), supplemented with 1 % of NaCl. *Vibrio* bacteria are halophilic and need salinity in their breeding ground. The added salt combined with low temperature inhibits some of the H_2S producing bacteria that might occur on the agar. Still other such as *Photobacterium phosphoreum* and *P. fluorescens* can grow on the agar (48).

The agar dishes were incubated aerobically at 4 °C for 10 days. According to the instructions, the colonies of *Vibrio* were supposed to bioluminescence in the dark, but there were no luminous colonies detected. Growth of red colonies on the other hand was noticed and those colonies were counted. In best before date the counts were $2.3 \cdot 10^7$ cfu/g, in + 3 days $1.9 \cdot 10^7$ cfu/g and in + 6 days sample $1.3 \cdot 10^7$ cfu/g. The colony counts are decreasing along with the progress of the spoilage. Apparently, the presence of other bacteria inhibits the growth of *Vibrio*. Lactic acid bacteria are common in vacuum-packed fish products. *Vibrio* is sensitive towards acidity and that might be causing the decrease in colony count. However, it is not certain that the colonies were *Vibrio* since they were not bioluminescence.

6.3 Summary of the selective media bacterial counts

Among the selective media cultivations, APT and Vibrio had the highest colony count (over 10^6 cfu/g). At the sixth day after best before date, there were also more than 10^6 cfu/g of H_2S producer and *Bacillus* counts. All of the selective media that had visible

colonies are shown in Fig. 6. All counts of each sample and cultivations as well as incubation conditions are shown in Appendix 1.

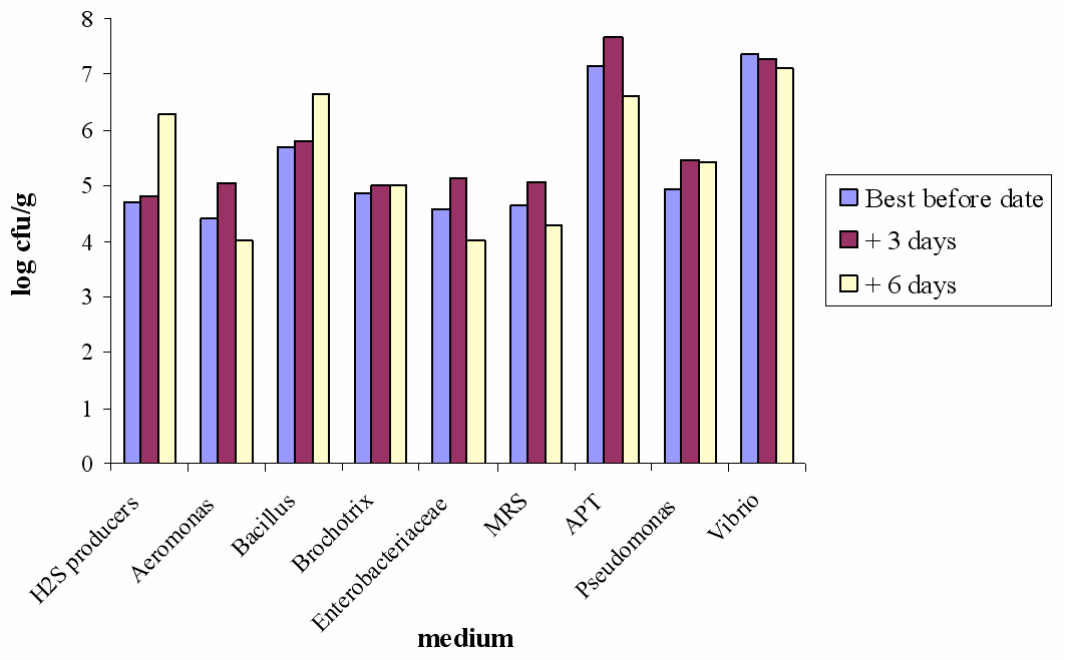

Figure 6. Bacterial growth in selective media.

6.4 Colony isolations

After the cultivations, 10-20 colonies were picked with an inoculation rod from the culture dishes of each selective media. The cultivations in the selective media were carried out with the spread plate method so that it would be possible to pick isolated pure colonies. Only colonies that were clearly round shaped were isolated. Colonies that were attached to each other or had rough borders were not picked to avoid contamination. From each selective media that had detectable growth colonies were isolated in different sizes and colors to have a large variation of bacteria to identify. The isolated colonies were cultivated in Cooked Meat Broth (Oxoid, CM0081) for two days in room temperature. After the broth cultivation, the bacterial cells were stored in 40 % glycerol in – 70 °C, for identification and future use in the field of research.

7 IDENTIFICATION

Conventionally bacteria are identified from food samples by a series of exclusionary analyses such as Gram staining, by examining cell morphology with a microscope or by determining catalase and oxidase activity etc. New molecular methods of identifying bacterium have been developed examples including sequencing. Sequencing can provide results of up to 100 % certainty and is suited for any pure cultured bacteria. (52)

7.1 Conventional methods

Bacterial identification is still an ongoing field and newer methods and techniques are being developed. Conventionally bacterial identification has been based on combined morphological, biochemical and physiological characteristics. These include: Gram stain (cell wall structure), growth temperature, ability to form heat stable spores, electron acceptors for respiration, photosynthetic ability, motility, cell shape, ability to use various carbon and nitrogen sources, and special nutritional requirements (e.g., vitamins). More recent methods include: fatty acid profile, protein profile, mole percent G+C in the genome, and molecular methods such as PCR, DNA hybridization, and 16S-rRNA gene sequence. (53)

7.2 Molecular methods

Different bacteria species have DNA sequences in their genotype that have remained unchangeable during evolution. By duplicating these (16S rRNA) sequences, the bacteria can be identified, that would otherwise be difficult, slow and sometimes impossible with classical bacteriological methods. DNA is composed of four nucleotides: adenine, cytosine, guanine and thymine. In sequencing, the order of the nucleotides is determined. Sequencing starts by denaturing the DNA sequence. The primer is allowed to anneal to the single-stranded DNA. DNA polymerase, primers and deoxynucleotides, the substrates, are needed for sequencing. The polymerase enzyme

starts to synthesize a new complementary DNA strand. The DNA polymerase adds deoxynucleotides (dATP, dCTP, dGTP and dTTP) after the primer in an order that is in accordance with the model sequence. For each nucleotide (A, C, G, T) there is a sequence reaction that also has one dideoxynucleotide (ddATP, ddCTP, ddGTP or ddTTP). The polymerase adds randomly either dNTP or ddNTP to the strand. If dideoxynucleotide is added, the reaction stops. Consequently, all possible sizes of new DNA strands are developed in four different reactions. The strands are separated by their size by capillary electrophoresis. The DNA strands are detected in the capillary electrophoresis when they pass a detector. Every dideoxynucleotide fluoresces a different color light enabled by the detector laser. The dideoxynucleotides then send fluorescent light at different wavelengths that can be measured with the detector. Each of differently sized strand-ending deoxynucleotides is found in the signal and the order of nucleotides in the sample sequence can be concluded. An example of sequencing data is illustrated in Fig. 7. (54, 55, 56)

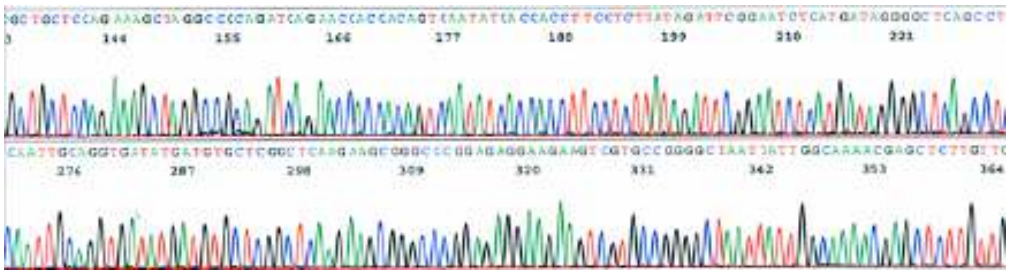

Figure 7. Part of fluorescent tag labelled DNA sequence (56).
Each nucleotide has it own colour signal; A=green, C=blue, G=black and T=red.

First the DNA of the sample bacteria, which have been isolated from a culture dish, needs to be duplicated by PCR (Polymerase Chain Reaction). The quantity of amplified DNA can be visualized as a band on an electrophoresis gel, the most common means of detecting PCR products. In sequencing, the purified DNA is labelled with fluorescent dyes. The order of the nucleotides can be determined, because each of them has a different fluorescence wavelength and emission can be measured by capillary

electrophoresis. The bacteria are identified by comparing the sequence order of the sample DNA nucleotides with the bacteria sequences data bank. (52, 54, 57)

7.2.1 PCR amplification

A DNA sequence, which serves as a model, heat stable DNA polymerase, primers, deoxynucleosides, a buffer, and divalent cations are needed to accomplish PCR. First the DNA sequence is heated to 95 °C for a few seconds so that the two strands of DNA are separated. After this DNA denaturation, the temperature is quickly lowered to 50 °C so that two primers (5' & 3') are annealed to each of the separated strands. Then the temperature is elevated to 72 °C, which activates the DNA polymerase enzyme. The enzyme synthesizes complementary DNA strands starting from the primers. Every single-strand reforms like the model double-stranded DNA sequence. The elongation takes less than a minute and the reaction can be started again from the denaturation. The reaction can be repeated as many as 40 times and every time the total number of DNA sequences doubles. After the last PCR cycle, the temperature is kept at 72 °C for 5-10 minutes to ensure that any remaining single-stranded DNA is fully extended. PCR products can be short-term stored at 4 °C (54, 55)

First, the sample bacteria were inoculated from the glycerol stocks with an inoculation rod to the selective media where they were cultivated. Ten isolates of six days after best before date colonies from each selective media that showed growths were inoculated. After the re-incubation, colonies were picked to a sterile eppendorf-tube with 50 µl of TE buffer (Tris-EDTA). The tubes were then heated in a microwave oven and the heating was continued in a heat block at 100 °C for 10 minutes to disperse the cells. The tubes were then centrifuged and supernatant, containing the DNA, was collected to use as a template in the PCR.

A Master Mix is needed to perform PCR and it includes TE buffer, 5' primer, 3' primer, $MgCl_2$ (divalent cation), dNTPs (deoxynucleotide triphosphates), DyNazyme II (DNA polymerase) and DNA-free water. The components came from a purchased PCR kit (DyNAzyme™ II PCR Master Mix, F-508S). It is critical to work accurately and

aseptically to avoid other foreign DNA. Surfaces were irradiated with ultraviolet light to reduce PCR contamination. Polymerase chain reaction was made from the samples, standard and negative control. 2 µl of templates and 50 µl of master mix were pipetted to wells in a special well plate intented for PCR. The well plate was then inserted into the PCR device to run the program. The program used is shown in Table 12.

*Table 12. PCR programme.**

Phase	**Time**	**Temperature (°C)**
Initial heating	2 min	95
DNA denaturation	15 s	95 (35 cycles)
Primer annealing	30 s	50
Elongation/replication	45 s	72
Final elongation	10 min	72
Short-term storage	∞	4

**The DNA denaturation, annealing and elongation phases were repeated 35 times.*

7.2.2 AGE and refining of the PCR products

Gel electrophoresis is a method of making sure that DNA was amplified by PCR and to determine the size of samples. The gel used for DNAs is agarose. Electrophoresis is based on electromotive force that can move the molecules through the gel. DNA chains have phosphate in them that is negatively charged. When the DNA sample is placed within an electric field, the phosphate is pulled towards the positive potential. Samples are pipetted into the gel and voltage is run through it. The samples migrate towards the positive side if they contain any DNA. If the molecular weight size of the DNA sample is high, it moves slower in the gel. To visualize the movement, fluorescent dye is added to the gel. For example, ethidium bromide is used to stain the DNA molecules. Molecular weight size markers are used for evaluating the sizes of the samples. The markers contain molecules of known sizes and by comparing the locations, the DNA

size of the samples can be determined. The molecules fluoresce because of the stain and they can be photographed under ultraviolet lighting. (55, 58)

After the PCR programme, agarose gel electrophoresis (AGE) was applied to verify the success of DNA duplication. A gel was prepared for AGE including agarose (1 %), ethidium bromide, and TE buffer. The gel was left to solidify in a special mould that forms wells in the gel. Samples were prepared mixing gel loading buffer and 8 µl of samples from the PCR. 8 µl of the sample mix was pipetted to the wells in the solidified gel holding the gel entirely under TE buffer. This work phase requires care not to break the gel. Only 10 samples could be measured at once, because of the size of the AGE device. The gel was put to the AGE device were 80 V tension is cut through the pipetted samples for 60 min. After that, the gel was put into an UV illuminator and the image was printed. If the PCR was successful, the duplicated sample would show as a light in the UV image. The stronger the band is, the brighter the light. All samples were approximately in the 500 bp point that was evaluated comparing them with the marker. If there was no band in the image, the PCR of that sample was not successful and PCR was done again to these kinds of samples. One of the UV images is shown if Fig. 8 as an example of the AGE image.

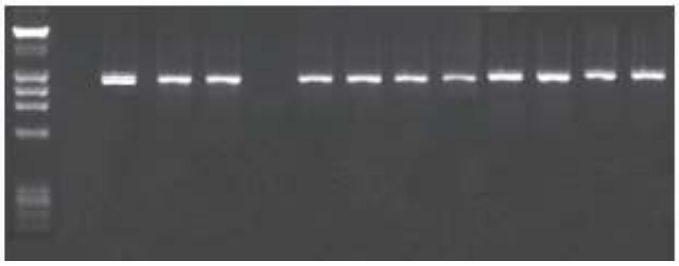

Figure 8. Ethidium-bromide-stained 1 % agarose gel displaying amplification products. From the left: marker, negative control, standard and ten samples.

After successful PCR was assured by AGE, the rest of the PCR products were refined with DNA purification kit (QIAquick PCR Purification Kit for the purification of PCR products, 28104). The kit contains QIAquick spin columns, buffer PB, buffer PE (concentrate), buffer EB, pH indicator I, collection tubes and loading dye. A

microcentrifuge was used for purifying the DNA from bacteria cell fragments. Working according to the instructions of the kit, it is possible to recover 90-95 % of the DNA and have up to 10 µg of DNA yield to each QIAquick column (59).

After the purification of the PCR products, the AGE was made yet again to evaluate the yield of DNA by a visual comparison of the AGE image given in the kit manual. The UV image of the kit manual is shown in Fig. 9. (59)

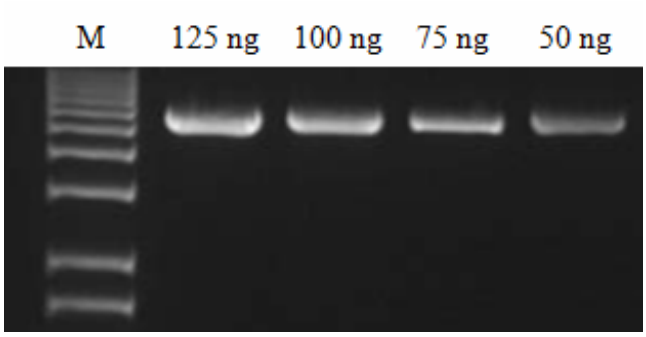

Figure 9. UV image of AGE of the PCR purification kit manual (57).

The DNA bands looked similar to the 50 ng band shown in the manual. Therefore, the DNA yields after the purification was approximately 50 ng/band. The concentrations were used to decide the total number that needs to be pipetted in the sequencing well plate for the sequencing.

7.3 DNA sequencing of rainbow trout isolated colonies

The DNA sequencing was made in the Turku Centre of Biotechnology, because they had the device needed for it. All the sample templates and primers (the same used in PCR) were prepared and pipetted according to the pipetting chart given by the Centre of Biotechnology to sequencing well-plate. The well plate was then brought to the Centre of Biotechnology. Personnel in the Centre of Biotechnology added necessary components and placed it in the sequencing instrument. The Centre of Biotechnology uses ABI PRISM 3130xl® Genetic Analyzer. It is a multi-color fluorescence-based

DNA analysis system that uses capillary electrophoresis to analyse the sample. The analyzer is fully automated from sample loading to data analysis, allowing DNA sequencing to be performed at medium-to-high throughput (60). The wavelength results were sent back by email in a few days. The results were compared with the bacteria sequence database for final identification.

First ten samples were analysed, but the results were quite poor. Most of the cases the signal was weak or there were more than one sequence. The Centre of Biotechnology had given a pipetting chart that advised the DNA concentration and the volume of the sample. In the first ten samples the templates were diluted to $^{1}/_{5}$ and the primers to $^{1}/_{2}$. When that did not give good enough results, the templates were diluted only to $^{1}/_{3}$. Again, the results varied from a weak signal to more than one sequence. In few cases, the poor data was caused by contamination (salts or other), but to use deionised formamide diluting the sample like advised in the trouble-shooting file, was not possible (45). The poor sequence data are illustrated in Fig. 10. (56)

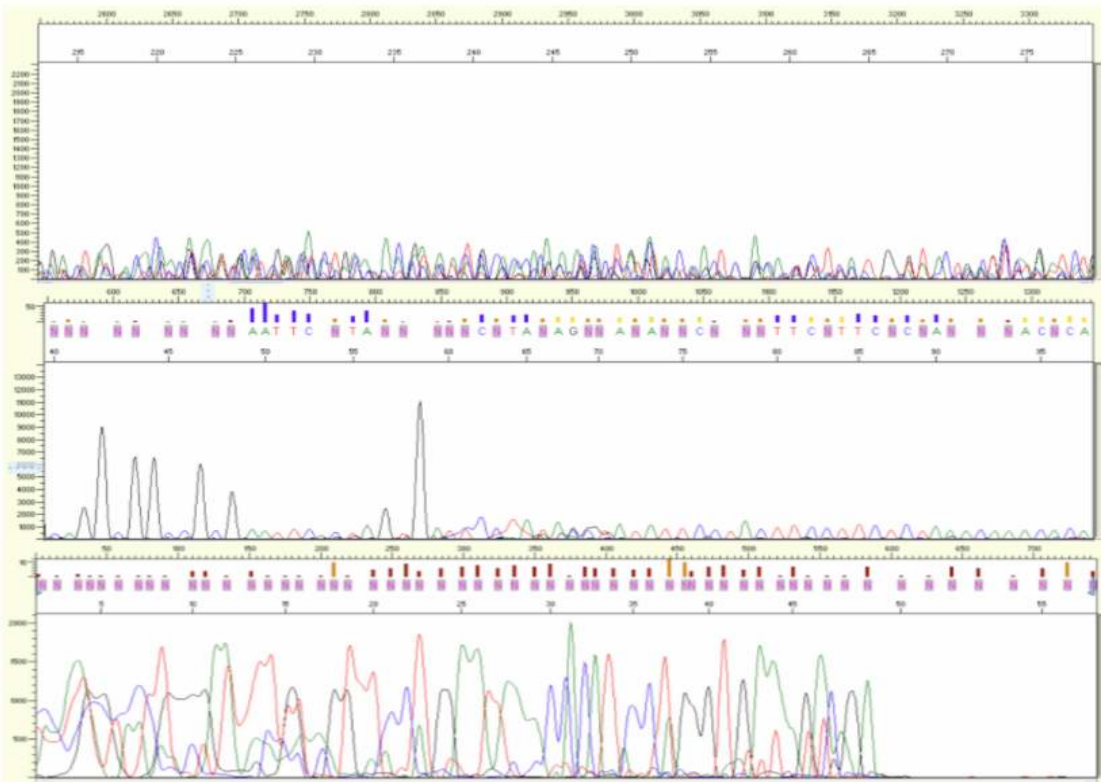

Figure 10. Poor sequence data from the first ten samples analysed.

Because the Centre of Biotechnology charges 10 € per sample for the sequencing service, trying every possible DNA template concentration was not possible. After the second batch of samples, that had $^1/_3$ diluted templates, were also not so successful, the DNA templates were decided not to be diluted at all. The previous sequence data results were evaluated according to trouble-shooting of sequencing and they seemed to fail because the template was insufficient. The trouble-shooting and recommended actions of sequencing are illustrated in Appendix 2. The third time the results were much better than before although there were some samples that went over the chart. The rest of the samples were analysed not diluting the templates and some that had given poor results before, were made again without the diluting. The pipetted volumes of water, primers and DNA templates are shown in Table 13.

Table 13. The concentrations of DNA templates used in sequencing.

Water µl	**Primers µl**	**Template µl**	**Concentration ng/µl**
4,4	0,6 + 0,6 (1/2 dilution)	1,0 (1/5 dilution)	10
4,4	0,6 + 0,6 (1/2 dilution)	1,0 (1/3 dilution)	16,7
0	0,6 + 0,6 (1/2 dilution)	5,4 (no dilution)	270

Some results had a convergent sequence in the database, but some had lower than 95 % match in the database. The best result of each sample was taken along and the ones that had lower than 95 % similarity in the data base are considered a bacterium in the same family as the identification result and the ones that had a result between 95 and 98 % are considered to belong in the same genus. Over 98 % of similarity in the database is a match of the bacterium species.

7.3.1 *A. hydrophila* agar isolates

Five out of the ten *Aeromonas* isolates identified with sequencing was *Serratia* genus bacteria. Although only one *Serratia* had over 95 % match. *Serratia* belongs to the

Enterobactericeae family. Other identified bacteria were *Hafnia* and *Yersinia* genera bacteria that also belong to the *Enterobacteriaceae* family. One isolate was identified as '*Marinobacter* sp./*Halomonas* sp.' (77.30 %) both found in seawater. None of the isolates were identified as the food poisoning causing *Aeromonas*. The medium supported the growth of *Aeromonas* as well as *Enterobacteriaceae*, but ampicillin was added for the differentiation of *Aeromonas*. The selective supplement must have been insufficient, because it did not prevent the growth of *Enterobacteriaceae*. (61, 62)

7.3.2 *B. cereus* agar isolates

Bacillus agar had the second most growth of the cultivated media in six days after the best before date sample. One isolate from *B. cereus* agar was identified as *Bacillus* species bacterium, but only 84.60 % similarity. It can be any bacteria from *Bacillaceae* family. All the other isolates were identified as *Carnobacteriaceae* that are a family of gram-positive lactic acid bacteria. Three of them were identified as *Carnobacterium divergens* and one as *Carnobacterium maltoromaticum*. (15, 61)

7.3.3 STAA agar isolates

STAA medium was used to determine the counts of *Brochothrix thermosphacta*. Three of the isolates could not be identified probably due to contamination. The rest were identified as *Brochothrix thermosphacta* or *Brochothrix* sp. bacteria similarity between 72.20 and 97.10 %. *Brochothrix thermosphacta* was assumed to be found in the sample, being the spesific spoilage organism of rainbow trout. (40)

7.3.4 VRBG agar isolates

Violet red bile glucose agar were used to determine the counts of *Enterobactericeae*. One of the isolates were identified as *Lactococcus lactis* (99.80 %). *Lactococcus lactis* is Gram-positive lactic acid bacteria. All else were identified as *Enterobacteriaceae*

family bacteria. Most were *Serratia* and *Hafnia* species, but the similarity percent only 74.10 – 93.10 %. (15)

7.3.5 Klinger iron agar isolates

Six of the isolates were identified as *Buttiauxella* species bacteria, but the similarities are low to confirm the species. *Buttiauxella* belongs to *Enterobacteriaceae* family and it is found in water areas. Other bacteria identified from the isolates also belong to *Enterobacteriaceaea* family. One isolate was identified as *Yersinia intermedia* with the match of 99.50 %. *Y. intermedia* belongs to *Enterobacteriaceae* family as well. Some *Enterobacteriaceae* family bacteria produce hydrogen sulphide gas. These bacteria are inter alia *Citrobacter freundii*, *Proteus vulgaris*, *Salmonella typhi* and some *Yersinia* species. (61, 62, 63)

7.3.6 *Pseudomonas* agar isolates

None of the *Pseudomonas* agar isolates were identified as *Pseudomonas*. All were *Enterobacteriaceaea* family bacteria including *Serratia*, *Rahnella*, *Hafnia* and *Yersinia* genera. *Yersinia kristensenii* (99.70 %) and *Yersinia aleksici* (100 %) were identified from the isolates. (62)

7.3.7 MRS agar isolates

MRS medium is designed for *Lactobacillus*. *Carnobacterium* (98.30 %), *Lactobacillus sakei* (98.90 % & 98.70 %) *Lactococcus lactis* (98.70 %) and *Lactobacillus curvatus* (99.10 %) species were identified from the isolates, which all are lactic acid bacteria. Other identified were *Streptococcaceae* (*Lactococcus* genus), *Lactobacillaceae* family. *Carnobacterium* genus bacteria were also identified from the isolates. (15)

7.3.8 APT agar isolates

APT agar was used to determine *Lactobacillus* and *Weissella* bacteria. The total number of APT colonies was the third highest in the six days after the best before date cultivation. *Carnobacterium maltaromaticum* (100 %) and *Hafnia alvei* (98.30 %) species were identified. Six of the isolates were identified as *Hafnia* genera or *Enterobactericeaea* family bacteria and two as *Carnobacteriaceae* family bacteria. *Lactobacillus* and *Weissella* were not identified from the isolates. (62)

7.3.9 *Vibrio* agar isolates

The growth of colonies in *Vibrio* agar was the highest in all of the selective media cultivations. Still any *Vibrio* bacteria were not identified from the isolates. Although *Serratia*, *Hafnia* and *Rahnella* species were again identified, the rests of the isolates identified belong to the *Enterobactericeaea* or *Carnobactericeae* family. (62)

7.4 Summary of identified bacteria

90 samples that were identified by sequencing, only 19 had more than 98 percentage probability of a match in the database. These bacteria are shown in Table 14.

Table 14. Identified bacteria with over 98 % of sequence similarity.

Nro	Selective media	Bacteria	Similarity
39	APT	*Hafnia alvei*	98.30 %
36	APT	*Carnobacterium maltaromaticum*	100.0 %
44	Bacillus cereus	*Carnobacterium divergens*	98.80 %
41	Bacillus cereus	*Carnobacterium divergens*	98.90 %
42	Bacillus cereus	*Carnobacterium divergens*	99.30 %
47	Bacillus cereus	*Carnobacterium maltaromaticum*	99.30 %
30	Kligler iron	*Yersinia intermedia*	99.50 %
1	MRS	*Carnobacterium sp.*	98.30 %
5	MRS	*Lactobacillus sakei*	98.70 %
9	MRS	*Lactococcus lactis subsp. lactis*	98.70 %
2	MRS	*Lactobacillus sakei*	98.90 %
8	MRS	*Lactobacillus curvatus*	99.10 %
12	Pseudomonas	*Rahnella sp.*	99.10 %
17	Pseudomonas	*Yersinia kristensenii*	99.70 %
20	Pseudomonas	*Yersinia aleksici*	100.0 %
78	Vibrio	*Serratia sp.*	98.80 %
73	Vibrio	*Rahnella sp.*	99.00 %
74	Vibrio	*Hafnia sp.*	99.10 %
89	VRBG	*Lactococcus lactis*	99.80 %

60 of the isolates had sequence similarity in the database lower than 95 % and only 8 had sequence similarity between 95 - 98 %. Major part of the identified sequences has the similarity below 95 percentages. It is not likely that the bacteria are not found in the database, but that there were problems in the sequencing. The problems may be because of various reasons that are listed in Appendix 2. Most likely the problems were because there was poor signal in some parts of the sequences. In order to have some kind of similarity, the poor signal was cut off from the sequence if they were situated either the end or the start part of the sequence. Still the percentage is not very high if the sequence that is being analyzed is very short. The shorter the DNA sequences the lower the percentage. Three of the sequences did not get results of any kind even after analyzed twice; all of the three were isolated from STAA agar. One of the Klinger iron agar

isolates was identified as “Uncultured bacterium” with 86.10 % match. The term uncultured bacterium comes from the database. The bacterium is therefore unknown.

The most common bacteria among the isolates that were identified were *Carnobacterium* spp. bacteria. The bacteria family *Carnobacteriaceae* was identified from *B. cereus*, MRS, APT, Klinger iron and *Vibrio* agar isolates. The growth of colonies was the highest ones in *Bacillus*, APT and *Vibrio* agar. In total 18 of the isolates belongs to the *Carnobacteriaceae* family, few were identified as some *Carnobacterium* species (*divergens*, *maltaromanticum*) or the genus *Carnobacterium*. *Carnobacterium* grow well at 0 °C and they are common in vacuum-packed fish products. *Carnobacterium* are gram-positive, rod-shaped lactic acid bacteria that have carnobacteriocin producing species. *Carnobacterium divergens* have been studied and it have been founded to inhibit the growth of *Listeria monocytogenes* in salmon products (20). The large total number of *Carnobacterium* spp. might explain why there was no growth on the *Listeria* agar and none was identified from the isolates. (15, 64)

Second commons were *Hafnia* and *Serratia* genera bacteria, which both had 14 isolates identified as those genera bacteria. *Hafnia* and *Serratia* bacteria both belong to *Enterobacteriaceaea* family. Both were identified from the isolates that came from the highest colony count media (APT, *Vibrio*). *Serratia* genus is non-faecal, aerobic, proteolytic and predominantly plant associated. Serratia cause spoilage mainly on refrigerated vegetables and meat products. *Serratia* have pathogenic species, but they do not cause foodborne illnesses. *Hafnia* are enteric bacteria that also cause spoilage on refrigerated vegetables and meat products. *Hafnia alvei* is commonly present in those products. (64)

Yersinia, *Brochothrix* and *Rahnella* genera were also identified from several isolates. *Yersinia* and *Rahnella* genera belong to *Enterobacteriaceaea* family, they are gram-negative, facultatively anaerobe rods. *Yersinia* genus bacteria have hydrogen sulfide producing species and one species (*Y. enterocolitica*) can cause food poisonings. *Y. enterocolitica* has been isolated from a wide range of animal intestines. The symptoms for yersiniosis are severe diarrhea, fever and gastroenteritis on children and

immunocomprimised. (65) *Brochothrix* were identified from the isolates of the STAA selective media intended for determining *Brochothrix thermosphacta* counts in a sample. *Brochothrix thermosphacta* is one of the SSO in vacuum-packed fish products. It is a gram-positive, non-sporeforming rod, which resemblances coryneform. *Brochothrix* is closely related to *Lactobacillus* and *Listeria*. *Brochothrix* utilizes glucose and glutamate and it can grow temperatures below 0 °C. (40, 64)

Other identified bacteria genera worth mentioning were *Buttiauxella* and *Lactobacillus* bacteria, which each was identified from six of the isolates. *Buttiauxella* also belong to the *Enterobacteriaceae* family like *Yersinia*, *Hafnia*, *Serratia* and *Rahnella*. Many of the isolates identified had lower results than 95 %. They can also be some other bacteria genera from *Enterobacteriaceae* family (e.g. *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella* and *Shigella*). *Enterobacteriaceae* are gram-negative, facultatively anaerobic and usually rod-shaped bacteria. They can produce spoilage compounds from TMAO, amino acids and lactate. *Lactobacillus* is a genus of gram-positive, facultative anaerobic or microaerophilic lactic acid bacteria. Some species of lactobacilli are homofermenters and those are used in yoghurt, cheese, wine etc. production. The heterofermentative *Lactobacillus* spp. and other lactic acid bacteria are common or sometimes dominant bacteria in vacuum-packed fish products. (29, 30, 39, 40, 66)

The counts for *Bacillus* in best before date sample exceeded the limit of food poisoning dose (10^5 - 10^8 cfu/g). Only one was identified as *Bacillus* genus bacterium out of ten *Bacillus* agar isolates that were sequenced. It is possible that the isolate identified was *B. cereus*. Still because only one isolate were identified as Bacillus, the total number of B. cereus does not presumably exceed the limit in best before date sample.

8 CONCLUSIONS

The total number of *Enterobacteriaceae* was the highest of the identified bacteria. *Enterobacteriaceae* can produce trimethylamine, ketones, esters, aldehydes, NH_3, hypoxanthine, and acids, which all cause spoilage effects. The identification of *Brochothrix* sp. was also significant finding although the counted colonies of STAA agar were not the highest ones of the cultivations. *Brochothrix thermosphacta* is known to be the specific spoilage organism in vacuum-packed fish. The lactic acid bacteria that were identified were *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, *Lactobacillus sakei*, *Lactobacillus curvatus*, and *Lactococcus lactis*. Lactic acid bacteria are also known to cause spoilage effects. Literature supports these identification results. As a conclusion *Enterobacteriaceae* and lactic acid bacteria dominated the microbiota of the sample rainbow trout.

The microbiota of rainbow trout varies between individuals and more so between the environments they live in. There were not any *Listeria monocytogenes* in the rainbow trout products that were sampled, but *Listeria* bacteria are still found at least in every tenth fish product.

It is still unknown which lactic acid bacteria cause spoilage and which inhibit the growth of pathogens. If a certain LAB is causing spoilage effects, it can be tested by measuring the total number of trimethylamine it produces in the fish. The next phase of the Functional Foods Forum research is to test which commercial lactic acid bacteria produce bacteriocins that can inhibit the growth of spoilage organisms and pathogens. The aim is also to test whether the added lactic acid bacterium will affect the microbiological and chemical quality of the fish. The profitable LAB will inhibit pathogens, but does not increase the total number of trimethylamine or change the sensory impressions in fresh rainbow trout. If that kind of lactic acid bacterium is found, it can extend the shelf-life of vacuum-packed rainbow trout products.

REFERENCES

1 Riistan- ja kalantutkimus (2008). Kalan kulutus [online, refered 26.12.2008]. Available at: http://www.rktl.fi/tilastot/talous_markkinatilastot/kalan_kulutus/.

2 Functional Foods Forum of Turku University (2008). Project planning: Improving the shelf-life of fish products by biological means.

3 Korkeala H. (2007). Elintarvikehygienia, p. 210-219. 1. edition. WSOY, Helsinki.

4 VTT Technical Research Centre of Finland (1997). Kalateollisuuden hygienia- ja pakkausopas [online, refered 12.12.2008]. Available at: http://www.vtt.fi/inf/pdf/tiedotteet/1997/T1847.pdf.

5 Mustaniemi, A. (1995). Kalan ja kalavalmisteiden valvontakampanja. Finnish Food Safety Authority, supervisory 4/1996. [online refered 12.12.2008] Available at: http://www.evira.fi/portal/fi/el__intauti-_ja_elintarviketutkimus/riskinarviointi/kaynnissa_olevat_projektit.

6 Laaksonen T. (2008). Elintarviketekniikka 3, lesson material. Turku University of Applied Sciences.

7 Jay J. M. (2000). Modern Food Microbiology, p. 101-110. 6. edition. Aspen Publishers Inc., Maryland.

8 Jay J. M. (2000). Modern Food Microbiology, p. 35-56. 6. edition. Aspen Publishers Inc., Maryland.

9 Korkeala H. (2007). Elintarvikehygienia, p. 17-22. 1. edition. WSOY, Helsinki.

10 Adams M. R. & Moss M. O. (1997). Food microbiology, p. 18-54. The Royal Society of Chemistry, Cambridge.

11 Niemi V. M. et al. (2004). Ruokaturvallisuuden käsikirja, p. 2-49. Art House Oy, Helsinki.

12 Adams M. R. & Moss M. O. (2000). Food Microbiology, p. 66. The Royal Society of Chemistry, Cambridge.

13 Niemi V. M. et al. (2004). Ruokaturvallisuuden käsikirja, p. 62-63. Art House Oy, Helsinki.

14 Finfood, MTK (2008). Tietoa kuluttajalle [online, refered 16.1.2009]. Available at: http://www.ruokatieto.fi/finfood/ff.nsf/0/a5d37582474a57d6c22570ba00261b6d/$FILE/TIKU.pdf.

15 Salminen S. & von Wright A. (1998). Lactic Acid Bacteria, p. 1-16. Marcel Dekker Inc., New York.

16 Doyle M. P. et al. (1997). Food Microbiology: Fundamentals and Frontiers, p. 335-352. American Society for Microbiology, Massachusetts.

17 Katikou P. et al. (2006). Relation of biogenic amines formation with microbiological and sensory attributes in Lactobacillus-inoculated vacuum-packed rainbow trout (*Oncorhynchus mykiss*) fillets. Journal of Agricultural and Food Chemistry, 54(12):4277-83.

18 Tomé E. et al. (2007). Growth control of Listeria innocua 2030c on vacuum-packaged cold-smoked salmon by lactic acid bacteria. International Journal of Food Microbiology, 121(3):285-94.

19 Vescovo M. et al. (2006). Inhibition of Listeria innocua growth by antimicrobial-producing lactic acid cultures in vacuum-packed cold-smoked salmon. Food Microbiology, 23(7):689-93.

20 Brillet A. et al. (2006). Effect of inoculation of Carnobacterium divergens V41, a bio-preservative strain against Listeria monocytogenes risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. International Journal of Food Microbiology, 104(3):309-24.

21 Nilsson L. (2004). The contribution of bacteriocin to inhibition of Listeria monocytogenes by Carnobacterium piscicola strains in cold-smoked salmon systems. Journal of Applied Microbiology, 96(1):133-43.

22 Giatrakou V. et al. (2008). Potential of oregano essential oil and MAP to extend the shelf-life of fresh swordfish: a comparative study with ice storage. Journal of Food Sciences, 73(4):M167-73.

23 Lehtonen H. (2003). Iso kalakirja, p. 82-85. WSOY, Porvoo.

24 Lehtonen H. (2003). Iso kalakirja, p. 12-16. WSOY, Porvoo.

25 Suomen kalankasvattaja liitto ry (2008). Kirjolohen kasvatus [online, refered 6.12.2008]. Available at: http://www.kalankasvattajaliitto.fi/page.php?page_id =172.

26 Seppälä J. et al. (2001). Kirjolohen tuotanto ja ympäristö, p. 529. The Environmental Administration of Finland, Helsinki.

27 Jay J. M. (2000). Modern Food Microbiology, p. 102-105. 6. edition. Aspen Publishers Inc., Maryland.

28 Huber I. et al. (2004). Pholygenetic analysis and in situ identification of the intestinal microbial community of rainbow trout. Journal of Applied Microbiology, 96(1):117-32.

29 González CJ. et al. (1999). Bacterial microflora of wild brown trout (*Salmo trutta*), wild pike (*Esox lucius*), and aquacultured rainbow trout (*Oncorhynchus mykiss*). Journal of Food Protection, 62(11):1270-7.

30 Lyhs U. et al. (2002). Identification of lactic acid bacteria from spoiled, vacuum-packaged 'gravad' rainbow trout using ribotyping. International Journal of Food Microbiology, 72(1-2):147-53.

31 Niemi V. M. et al. (2004). Ruokaturvallisuuden käsikirja, p. 59-63. Art House Oy, Helsinki.

32 Niemi V. M. et al. (2004). Ruokaturvallisuuden käsikirja, p. 187-219. Art House Oy, Helsinki.

33 Adams M. R. & Moss M. O. (1997). Food Microbiology, p. 157-219. The Royal Society of Chemistry, Cambridge.

34 Doyle M. P. et al. (1997). Food Microbiology: Fundamentals and Frontiers, p. 265-287. American Society for Microbiology, Massachusetts.

35 Korkeala H. (2007). Elintarvikehygienia, p. 35-106. 1. edition. WSOY, Helsinki.

36 Doyle M. P. et al. (1997). Food Microbiology: Fundamentals and Frontiers, p. 327-336. American Society for Microbiology, Massachusetts.

37 Merivirta L. & Kivisaari M. (2006). *Listeria monocytogenes* –bakteerin esiintyminen vähittäiskaupan vakuumipakatuissa kalatuotteissa. Finnish Journal of Veterinarian, 2006;112:6.

38 Doyle M. P. et al. (1997). Food Microbiology: Fundamentals and Frontiers, p. 228-264. American Society for Microbiology, Massachusetts.

39 Lone G. et al. (2002). Fish spoilage bacteria –problems and solutions. Environmental Biotechnology, 13:262-266.

40 Lone G. et al. (1996). Microbial spoilage of fish and fish products. International Journal of Food Microbiology, 33:121-137.

41 Nordic Committee on Food Analysis (2003) Bacteriological examination of fresh and frozen seafood NMKL 96/2003.

42 Jay J. M. (2000). Modern Food Microbiology, p. 179-182. 6. edition. Aspen Publishers Inc., Maryland.

43 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 16.1.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0833&org=2&c=UK&lang=EN

44 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 16.1.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM1036&org=9&c=UK&lang=EN

45 S. Vesterlund, Functional Foods Forum of Turku University (2008). Personal communication.

46 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 16.1.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=SR0162&org=14&c=UK&lang=EN

47 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 16.1.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0485&org=66&c=UK&lang=EN

48 Difco Microbiology (2005). Product catalogue. Massachusetts, USA.

49 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 28.2.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0559&org=152&c=UK&lang=EN

50 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 28.2.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0359&org=82&c=UK&lang=EN

51 Oxoid Ltd (2009). Oxoid – product detail. [online, refered 28.2.2009]. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM1084&org=92&c=UK&lang=EN

52 Doyle M. P. et al. (1997). Food Microbiology: Fundamentals and Frontiers, p. 695-703. American Society for Microbiology, Massachusetts.

53 F. Ibrahim, Functional Foods Forum of Turku University (2008). Personal communication.

54 Leisola M. et al. (2002). Bioprosessitekniikka, p. 64-70. WSOY, Helsinki.

55 Dieffenbach C. W. & Dveksler G. S. (1995). PCR primer: a laboratory manual, p. 509-525. CSHL Press, New York.

56 Sequence Data Evaluation (2009). [online, refered 28.2.2009] Available at: sequencingfacility.med.monash.edu.au/pdf/trouble_shoot.pdf

57 Weisburg W. G. et al. (1991). 16S Ribosomal DNA Amplification for Phylogenetic Study. Journal of Bacteriology, p. 697-703.

58 Pak Yang Chum and Chas Andre, Finnzymes Oy (2008).Application Note: High Temperature PCR with DyNAzyme™ II DNA Polymerase Provides Fast and Efficient Results.

59 Qiangen® (2008). QIAquick® Spin Handbook.

60 O. Rissanen, Turku Centre of Biotechnology (2009). Personal communication.

61 P. Singleton (1997). Bacteria in biology, biotechnology and medicine, p. 368-378. Wiley, Sussex.

62 J.P. Euzéby (2009). List of Prokaryotic names with Standing in Nomenclature. [online, referred 10.10.2009] Available at: http://www.bacterio.cict.fr/

63 Perry J. J. et al. (2002). Microbial Life, p. 749. SA inc., Massachusetts.

64 Jay J. M. (2000). Modern Food Microbiology, p. 19-24. 6. edition. Aspen Publishers Inc., Maryland.

65 Doyle M. P. et al. (1997). Food Microbiology: Fundamentals and Frontiers, p. 192-211. American Society for Microbiology, Massachusetts.

66 Jay J. M. (2000). Modern Food Microbiology, p. 114-118. 6. edition. Aspen Publishers Inc., Maryland.

Bacterial cultivations and counts

APPENDIX 1

Media	Bacteria	Incubation			Counts (cfu/g)		
		Oxygen level	Temperature	Time	BBD	+3	+6
Standard agar (method 1.)	Total bacteria	aerobic	22 °C	2-5 days	4,1E+06	1,5E+07	3,3E+07
Standard agar (method 2.)	Total psychrotrophs	aerobic	4 °C	~ 10 days	3,2E+06	2,0E+07	3,1E+07
Standard agar (method 3.)	Total anaerobes	anaerobic	22 °C	2-5 days	5,0E+06	2,0E+07	2,0E+07
Standard agar (method B)*	Total facultative aerobes/anaerobes	aerobic	22 °C	2-5 days	7,7E+06	2,8E+07	3,4E+07
Aeromonas agar	*Aeromonas hydrophila*	aerobic	30-35 °C	24 h	2,6E+04	1,1E+05	1,1E+04
Chromogenic Bacillus agar	*Bacillus cereus*	aerobic	37 °C	24 h	4,8E+05	6,4E+05	4,6E+06
STAA agar	*Brochotrix thermosphacta*	aerobic	22 °C	48 h	7,4E+04	9,9E+04	1,0E+05
VRBG agar	*Enterobacteriaceae*	anaerobic	37 °C	24 h	3,7E+04	1,3E+05	1,1E+04
Slanetz and Bartley agar	*Enterococcus*	aerobic	45 °C	48 h			
Kligler Iron agar	H2S producers	anaerobic	35 °C	18-24 h	5,0E+04	6,6E+04	1,9E+06
Pseudomonas agar	*Pseudomonas*	aerobic	25 °C	24-48 h	8,4E+04	3,0E+05	2,8E+05
MRS agar	*Lactobacillus*	anaerobic	37 °C	24-48 h	4,4E+04	1,1E+05	1,9E+04
APT agar	*Lactobacillus, Weissella*	anaerobic	37 °C	24-48 h	1,4E+07	4,5E+07	4,2E+06
Chromogenic Listeria agar	*Listeria*	aerobic	37 °C	24±2 h			
Kligler Iron agar + 1 % NaCl	*Vibrio*	aerobic	4 °C	~ 10 days	2,3E+07	1,9E+07	1,3E+07

* Pour plate method

Trouble-shooting of sequencing

Problem in the data	**Cause**	**Recommended actions**
No recognisable sequence, no defined peaks	• poor quality template or primer • failed sequencing reaction • failed primer annealing	• check template concentration • check primer design • check ethanol concentration
Poor data and weak signal, very low peak heights	• insufficient or poor quality template • poor primer design • poor clean-up	• check template concentration • check sequencing reagents and clean-up protocol • check primer design
Top-heavy sequence, very high peaks that fade off abruptly	• too much template used • too much primer used	• check template concentration • check primer concentration
Abrupt signal loss, very high peaks that stop abruptly	• secondary structure in the template • primer dimer contamination • high guanine-cytosine content	• use a primer that anneals at a different position • redesign primer to avoid primer dimer formation • check reaction variables
Multible sequences, lower peaks and more than one sequence	• multiple PCR products • multiple primers in reaction • primer-dimer contamination	• re-isolate the DNA from a pure colony and re-sequence • use a different primer • check PCR template on gel for single band

Trouble-shooting of sequencing

Problem in the data	**Cause**	**Recommended actions**
Repeat sequence, the gradual decrease of peak height	• repeated region	• sequence the complementary strand • use a primer that anneals at a different position
Slippage after homopolymer regions, overlapping sequence	• slippage in the sequence reaction	• sequence the complementary strand • use a primer that anneals at a different position • use an anchored primer
Delayed migration, peaks are not evenly spaced	• contaminating negative ions	• diluting the sample in deionised formamide and rerunning
Excess dye peaks, dye blobs	• incorrect estimation of template concentration • poor removal of unincorporated dye terminators	• check template concentration by agarose gel • use fresh ethanol and sodium acetate • do not leave reactions precipitating overnight
Pull-up peaks and very strong signal, very high peaks and signal	• incorrect estimation of template concentration	• diluting the sample in deionised formamide and rerunning • reduce the total number of template used

Identified bacteria

Selective media	Bacteria from database	Similarity	
Aeromonas	Serratia sp.	71.50 %	Family
Aeromonas	Serratia sp.	72.50 %	Family
Aeromonas	Rahnella sp.	74.60 %	Family
Aeromonas	Yersinia kristensenii	77.30 %	Family
Aeromonas	Marinobacter sp./ Halomonas sp.	77.30 %	Family
Aeromonas	Yersinia mollaretii	85.20 %	Family
Aeromonas	Serratia sp.	90.40 %	Family
Aeromonas	Serratia fonticola	92.70 %	Family
Aeromonas	Yersinia aleksici	94.90 %	Family
Aeromonas	Serratia sp.	96.20 %	Genus
APT	Hafnia sp.	81.20 %	Family
APT	Hafnia alvei	84.10 %	Family
APT	Hafnia alvei	85.80 %	Family
APT	Carnobacterium sp.	88.00 %	Family
APT	Carnobacterium sp.	88.40 %	Family
APT	Hafnia alvei	93.40 %	Family
APT	Hafnia alvei	93.70 %	Family
APT	Hafnia alvei	95.80 %	Genus
APT	Hafnia alvei	98.30 %	Species
APT	Carnobacterium maltaromaticum	100.0 %	Species
Bacillus cereus	Carnobacterium divergens	65.30 %	Family
Bacillus cereus	Carnobacterium divergens	77.30 %	Family
Bacillus cereus	Carnobacterium divergens	77.70 %	Family
Bacillus cereus	Carnobacterium divergens	83.90 %	Family
Bacillus cereus	Bacillus sp.	84.60 %	Family
Bacillus cereus	Carnobacterium divergens	87.70 %	Family
Bacillus cereus	Carnobacterium divergens	98.80 %	Species
Bacillus cereus	Carnobacterium divergens	98.90 %	Species
Bacillus cereus	Carnobacterium divergens	99.30 %	Species
Bacillus cereus	Carnobacterium maltaromaticum	99.30 %	Species
Kligler iron	Carnobacterium maltaromaticum	72.00 %	Family
Kligler iron	Buttiauxella agrestis	82.60 %	Family
Kligler iron	Buttiauxella sp.	83.00 %	Family
Kligler iron	Buttiauxella sp.	85.50 %	Family
Kligler iron	Buttiauxella sp.	86.00 %	Family
Kligler iron	Uncultured bacterium	86.10 %	Family
Kligler iron	Buttiauxella sp.	88.80 %	Family
Kligler iron	Buttiauxella sp.	88.80 %	Family
Kligler iron	Serratia sp.	89.30 %	Family
Kligler iron	Yersinia intermedia	99.50 %	Species
MRS	Lactobacillus curvatus/ Lactococcus lactis	90.30 %	Family
MRS	Lactobacillus curvatus/ Lactococcus lactis	91.00 %	Family
MRS	Lactococcus lactis subsp. lactis	92.20 %	Family
MRS	Lactobacillus sakei	95.40 %	Genus
MRS	Carnobacterium divergens	95.80 %	Genus

Identified bacteria

Selective media	Bacteria from database	Similarity	
MRS	Carnobacterium sp.	98.30 %	Species
MRS	Lactobacillus sakei	98.70 %	Species
MRS	Lactococcus lactis subsp. lactis	98.70 %	Species
MRS	Lactobacillus sakei	98.90 %	Species
MRS	Lactobacillus curvatus	99.10 %	Species
Pseudomonas	Yersinia bercovieri	86.20 %	Family
Pseudomonas	Rahnella aquatilis	87.00 %	Family
Pseudomonas	Rahnella sp.	87.50 %	Family
Pseudomonas	Hafnia sp.	90.00 %	Family
Pseudomonas	Serratia sp.	93.90 %	Family
Pseudomonas	Yersinia bercovieri	96.40 %	Genus
Pseudomonas	Hafnia sp.	97.10 %	Genus
Pseudomonas	Rahnella sp.	99.10 %	Species
Pseudomonas	Yersinia kristensenii	99.70 %	Species
Pseudomonas	Yersinia aleksici	100.0 %	Species
STAA	Brochothrix sp.	72.20 %	Family
STAA	Brochothrix termosphacta	75.50 %	Family
STAA	Brochothrix termosphacta	81.50 %	Family
STAA	Brochothrix termosphacta	87.30 %	Family
STAA	Brochothrix sp.	88.20 %	Family
STAA	Brochothrix termosphacta	96.60 %	Genus
STAA	Brochothrix termosphacta	97.10 %	Genus
STAA	non result		no result
STAA	non result		no result
STAA	non result		no result
Vibrio	Carnobacterium sp.	71.10 %	Family
Vibrio	Rahnella aquatilis	76.90 %	Family
Vibrio	Carnobacterium sp.	78.30 %	Family
Vibrio	Rahnella sp.	78.90 %	Family
Vibrio	Serratia sp.	86.60 %	Family
Vibrio	Serratia sp.	91.60 %	Family
Vibrio	Carnobacterium sp.	94.30 %	Family
Vibrio	Serratia sp.	98.80 %	Species
Vibrio	Rahnella sp.	99.00 %	Species
Vibrio	Hafnia sp.	99.10 %	Species
VRBG	Serratia sp.	74.10 %	Family
VRBG	Hafnia alvei	74.10 %	Family
VRBG	Enterobacteriaceaea	76.90 %	Family
VRBG	Serratia fonticola	83.10 %	Family
VRBG	Serratia sp.	84.40 %	Family
VRBG	Hafnia alvei	86.20 %	Family
VRBG	Hafnia sp.	87.60 %	Family
VRBG	Serratia sp.	87.90 %	Family
VRBG	Hafnia alvei	93.10 %	Family
VRBG	Lactococcus lactis	99.80 %	Species