Antibacterial activity in fish mucus from farmed fish

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Master of Science in International Fisheries Management

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Abstract

Mucus samples were collected from five different farmed Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) in order to study the content of antibacterial activity in the mucus and to compare the activity of mucus samples of the two different fish species. The mucus samples were diluted in buffer, desalted and adjusted to different protein concentrations in the mucus samples, A search for antibacterial activity in the mucus was performed for different protein concentrations, against four different bacterial strains *Escherichia coli*, *Listionella anguillarum*, *Corynebacterrium glutamicum* and *Staphylococcus aureus.*Protein concentrations in cod mucus samples were found higher than the salmon mucus samples when tested by different protein assays, Bicinchonic assay (BCA) and absorbance based method with measurement in spectrophotometer at A280 nm. Antibacterial activity was detected in mucus samples of both fish species, against the four bacterial strains. The bacteria *C. glutamicum was* the most sensitive, while *S. aureus* was the least sensitive against the different mucus samples. Furthermore, mucus samples of cod contained more antibacterial active substances than the mucus samples of the salmon. The results of this study indicate that that the mucus of salmon and cod contain one or several components with antibacterial activity. These bioactive substances may play an important role in the ability of mucus to defend salmon and cod against pathogenic bacteria or microorganisms. However, more investigation is necessary to confirm the antibacterial activity in cod and salmon mucus and to purify and characterize the active components.

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Acronyms

ATCC	American Type Culture Collection
BCA	Bicinchonic acid
BSA	Bovine Serum Albumin
MHB	Mueller Hinton Broth
ISA	Infectious Salmon Anaemia
NGO	Non- Government Organizations
OD	Optical Density
EUS	Epizootic Ulcerative Syndrome
WSSV	White Spot Syndrome Virus
SEMBV	Systemic Epidermal and Mesodermal Baculo Virus
NaAc	Sodium Acetate
RPM	Rotation Per Minute
MW	Molecular Weight
UV	Ultra Violet

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1. Introduction

Aquaculture is the cultivation of aquatic animals, such as fish or shellfish, or of plants, such as seaweed, in a controlled and sometimes enclosed body of water. During the past three decades, aquaculture has developed to become the fastest growing food producing sector in many parts of the world and has become the characteristic feature of today's fishery industry. In aquaculture, according to the intensive system of production, to reduce the cost, fish are kept in high densities and the possibility for exposure to pathogens, which can be bacteria, parasites, or virus, throughout production cycle is becoming high (Laidler et al. 1999). Under such conditions, the problems of the infectious fish diseases become serious and have considerable effects on aquaculture. In fact, bacterial and viral diseases of farmed fish have lead to high moralities and reduced economical income for the fish farming industry (Munro 1993, Pilcher and Fryer 1980). Furthermore, (Zhang and Austin 2000) stated that fish diseases, particularly those caused by bacterial pathogens are the most important causes of losses among fish farm stocks. Many pathogens have caused severe, even catastrophic, losses in aquaculture industry. For example, the economical loss due to IPNV (Infectious Pancreatic Necrosis Virus) is large in the salmon farming industry, and outbreaks may occur both in Atlantic salmon juveniles in fresh-water and in post-smolts period (www.aquanet.ca/sommer.htm). Infectious salmon anaemia (ISA) virus nearly put the Norwegian salmon aquaculture in a real crisis in recent years. Also, bacterial infectious diseases, like vibriosis (*Listionella anguillarum* earlier named *Vibrio anguillarum*) and furunculosis (*Aeromonas salmonicida*), have caused serious problems for the salmon industry in Scandinavia (Egidius 1987).

Today, total 140 million tons of aquatic products consumed world wide, of which more than 28% is farmed. An enormous amount of aquatic products over 37 million tons in 2000 alone, and the percentage is growing annually. The dramatic expansion of fish farming has been matched by an intense period of research into the biology of farmed species. The range of species cultured is large and growing. Though aquaculture is very

profitable, it is also serious economic threat when diseases outbreaks. Avoiding disease epidemics in aquaculture is very important to get economic benefit and it is only possible when fish species are reared in a good environmental condition and given priority in fish welfare.

1.1. Fish Welfare

The welfare of an animal is its state as regard its attempts to cope with its environment. This means that welfare is a characteristic of an individual animal which can vary from good to very poor and which can be assessed scientifically (Broom 1998). Fish welfare or animal right law is related to farmed fish. It means that fish have right to live a life as good as possible and express its natural behavior as much as possible and free from negative experiences. Fish are in intimate contact with their environment through the large surface of their gills, skin and of necessity they defecated into the medium in which they live, so water quality (in terms of dissolved oxygen, CO_2, ammonia and pH) and the presence of contaminants (organic and inorganic pollutants) are probably the most critical aspects of the environment for fish welfare and also the best defined (Mellor and Stafford 2001). Optimal conditions vary markedly between species; for example, catfish do poorly in clear water, whereas salmon do poorly in cloudy water and cyprinid fish are very tolerant of low dissolved oxygen levels where as salmonid fish are not (Kramer 1987). The flow characteristics of the fish's natural habitat are also of importance. A degree of environmental complexity may be important, depending on the species concerned. Conditions that produce unacceptable levels of anxiety, fear, distress, boredom, sickness, pain, thirst, hunger and so on should be minimized in fish as in other vertebrates.

1.2. Biology of Atlantic salmon *Salmo salar* (*S.salar*)

The Atlantic salmon is an anadromous fish spends its early life in freshwater and then migrates to sea on growing, only to return to reproduce (Fig.1). In the wild, the young fish may spend several years in freshwater until they reach an appropriate size. In mid

April to early May, young Atlantic salmon, called smolts, undergo physiological changes that adapt them to living in sea water. They migrate seawards. Some of the salmon migrate to feeding areas probably not beyond the continental shelf and some may actually remain in coastal waters within the influences of the rivers where they were born, but the majority of the salmon migrate well beyond the continental shelf and spend much of their sea life in the rich feeding grounds before returning to freshwater to reproduce or spawn. Survival rates for fish in the wild are low, with values of less than 1 percent common but sufficient to sustain the population if not over harvested. A few salmon will stay for anything up to 5 years at sea and can reach an incredible 13-18 kg or even more.

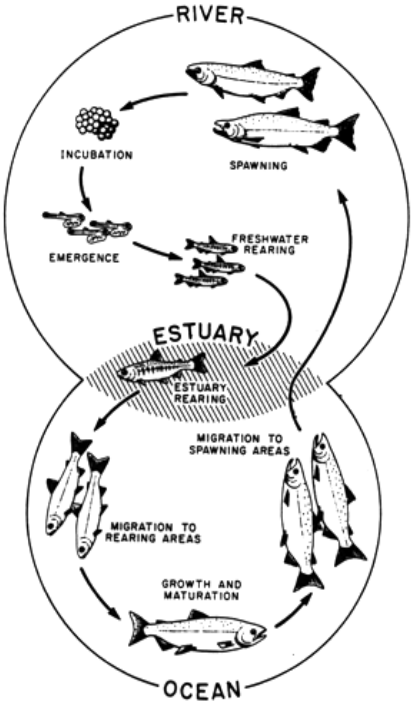

Fig.1. Showing life cycle of *Salmo salar* (*S.salar*) in wild environment.
Source-http://www.fish.washington.edu/hatchery/salmon.html.

1.2.1. Life cycle of cultured salmon

The life cycle of the cultured salmon reflects the natural cycle. Production begins with the removal of eggs from mature females, a process called stripping. Eggs are then

fertilized with the sperm from male salmon, taken in a similar fashion. The eggs are incubated under controlled conditions in a freshwater hatchery until they hatch. The young salmon, called fry, are nurtured throughout the year until they are physiologically capable of responding to the increasing light conditions of springtime, that is, they smolt. Prior to transport to sea cages, the smolts are vaccinated against common salmon diseases. In cages they are reared in good environmental condition with feeding until they grow market size.

Norway produces almost 50% of total world production of farmed salmon. In 1971, production was approximately 600 tons. In the year 2003 it was half a million tons (http://www.intrafish.com/laws-and regulations/report_bc/v4c_ii.htm). In recent years many pathogens have caused severe losses in salmon aquaculture industry and Atlantic salmon are susceptible to numerous bacterial, viral, and fungal diseases (Roberts 1993).

1.3. Biology of Atlantic cod (*Gadus morhua)*

The Atlantic cod (*G. morhua)* is a cold water species that is distributed on the continental shelves and in the coastal waters of the northern North Atlantic (Ruzzante et al. 1996). Adult cod prefer water temperatures from 2 to 8°C. Young cod have special enzymes that act as anti-freeze, which enables them to survive in sub-zero environments.

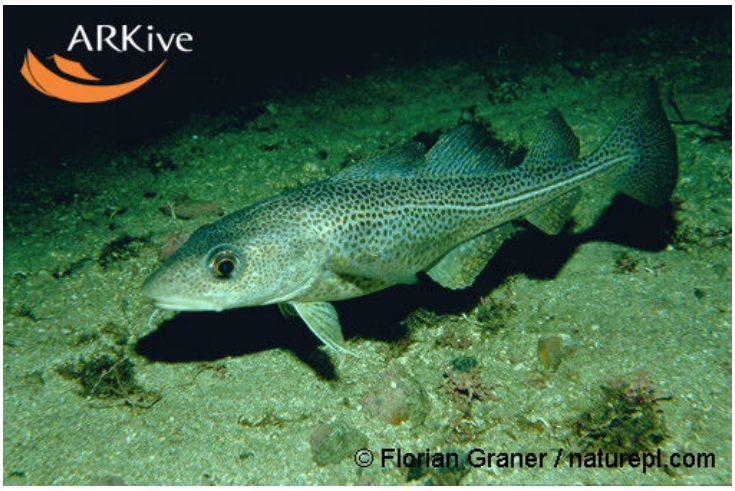

Fig. 2. The Atlantic cod *(G. morhua).* Source: www.arkive.org.

Cod spawn on the offshore banks in late winter and spring. Gradually, the eggs float to the surface layer. When they hatch, the tiny larvae live for a week or two on the contents of their yolk sacs. When this is used up, they feed on plankton for several months, and then start settling to the bottom. All this time they've been drifting wherever the shifting currents take them. When they finally settle, they may find themselves in a place where life is good.

There are about one hundred and fifty different kinds of cod fish. The Atlantic cod is the largest. It may reach a length of 180 cm (6 ft.) and a weight of over 40 kg. The cod is normally a deep water fish but comes into shallower waters to spawn. This usually happens between January and March. Some of the important spawning grounds for cod are found around the Lofoten Islands off the coast of Norway, along the coasts of Greenland and Iceland. The water temperature plays an important part in the reproductive process. The cod have their own favorite temperature range for spawning. If this changes, they may not spawn at all. Also, the eggs and young fish are very sensitive to heat and cold, and temperature changes can kill them.

Atlantic cod (*G. morhua*) is an economically and historically important species in the northern Atlantic and in the Norwegian fishery. During the last couple of years, there has been a trend of introducing new non-salmonid fish species for farming purposes of intensive culture in several countries, for example in Iceland, Canada, Norway and Scotland (Tilseth 1990). The Norwegian aquaculture industry, which is based on salmonids, will consist of other species than salmon and trout. In the future, Atlantic cod (*Gadus morhua*), halibut *(Hippoglossus hippoglossus*), spotted wolfish (*Anarhichas minor* O.). In recent years, cod producers have made progress in this field and in 2002 more than three million cod have been produced in Norway (www.intrafish.com/intrafish-analysis). Furthermore, the interest in the intensive culture of Atlantic cod has increased due to a reduced supply from the wild fishery, which leads to a high demand for this species on the market. However, cultured cod has showed cannibalism in the early stages of culture and susceptibility to infectious diseases (Espelid

et al. 1991), (Wiklund and Dalsgaard 1998). Therefore, in the context of an expanding industry, many issues are raised in cod health and disease control measures to enhance protection of cod against the infectious diseases.

1.4. Aquaculture in Bangladesh

A total of 230 rivers with their tributaries and branches criss-cross the country, with extensive floodplains along their banks; inland waters occupy about 36% land area of Bangladesh. In addition, there is a 710 km long coastal belt, 12 nautical miles of marine territorial waters extending from the coast and 200 nautical miles Exclusive Economic Zone, covering much of the Bay of Bengal (www.cia.gov/cia/publications/factbook).

Based on habitat, there are two types of aquaculture in Bangladesh, freshwater and coastal aquaculture; there is no marine aquaculture. Freshwater aquaculture is comprised mainly of pond aquaculture, especially poly culture of carps of native and exotic origin. The major indigenous cultured species are Catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*) and kalbaush (*L. calbus*). The exotic species in aquaculture are silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), Pangas (*Pangasius sutchi*). Thai silver barb (*Barbodes gonionotus*), tilapia (*Oreochromis sp.*) and hybrid magur native (*clarias batrachus* x African catfish *clarias garipinus*). Aquaculture of *Hilsa ilisha* (National fish) is not started yet. A number of technologies have been developed by universities and non-government organizations (NGOs).

In earlier days, people were depended mainly on natural waters for fish. But because of declining fish catch in nature as a result of increased fishing efforts related to a growing population and environmental degradation, people began culturing fish in closed waters. Therefore, pond fish production per hectare increased considerably from 800kg in 1985 to 2,550 kg in 2000. This is the result of intensification in culture systems by applying fertilizer and supplemental feed and better management practices (Hussain et al. 2002) .

Coastal aquaculture is comprised primarily of shrimp farming. With the lucrative international shrimp market, there has been a rapid expansion of shrimp culture in the coastal areas of both the southeast and southwest regions. The area of land for shrimp farming increased from 50,000 hectare to 141,00 hectare in the last two decades.

Despite the vast potential of aquaculture in Bangladesh, there are some environmental, biological and social problems. Exotic fish species have been introduced without any comprehensive studies of their biology, ecology and compatibility with native species. As elsewhere in the region, Epizootic Ulcerative Syndrome (EUS) is a common problem for both farmed and wild fish species. Occurrence white spot syndrome virus (WSSV) (reported as systemic epidermal and mesodermal baculovirus -SEMBV) alone caused a 44.4% production loss in 1996 to the shrimp industry (http://www.fao.org/documents).

1.4.1. Hilsha fishery in Bangladesh

Hilsha is the national fish of Bangladesh. It is found only in wild environment and the fish is anadromous, with a life cycle that follows the general pattern of breeding upstream in fresh water and the larvae hatching from the free-floating eggs. The immature young stages grow in river channels and then descend to the sea for a period of feeding and growth before returning to the rivers as mature breeding adults to complete the cycle. The hilsa is a highly fecund fish. A large-sized female may produce up to 2 million eggs. Although hilsas spawn more or less throughout the year, they have a minor spawning season during February- March and a major season in September- October. Immature hilsa fish (6 - 10 cm), known as jatka, are extensively caught during their seaward migration in some of the major rivers of the country.

Hilsa is primarily a plankton feeder and its food includes blue-green algae, diatoms, desmids, copepods, cladocera, rotifers, etc. The feeding habit may vary according to the season and age of the fish. Environmental degradation and unknown diseases is responsible for its vast unavailability in Bangladesh.

Fig.3. Hilsha fish (*Tenualosa ilisha*).
Source- www.banglapedia.search.com.bd.

Hilsa (*ilish)* any of the *Tenualosa* of the family Clupeidae, order Clupeiformes. Locally known, as Ilish. Body length may reach up to 60 cm, but commonly found specimens measure 35 to 40 cm. A large-sized hilsa weighs about 2.5 kg. Females grow faster, and are usually larger than males. The hilsa is known to be a fast swimmer, and attains maturity in one to two years.

1.5. Immunology of fish

Fish are a diverse group of animals, highly specialized for their aquatic existence and comprising almost half the number of vertebrate species in existence today. Fish are in intimate contact with their environment, which can contain very high concentrations of bacteria and viruses. Many of these are saprophytic, some are pathogenic and both are capable of digesting and degrading the fish's tissues. However, under normal conditions the fish maintains a healthy state by defending itself against these potential invaders by a complex system of innate defense mechanisms. The immune system is composed of numerous organs and cells that act together in a dynamic network in the defense against infection, disease and foreign substances. The vertebrate immune system is composed of two types of immunity, innate and adaptive. Adaptive immunity emerged early in vertebrate evolution, at some time during the division of the jawless lamprey and the cartilaginous fishes (Sima and Vetvicka 1993). Innate immunity developed before the divergence of vertebrates and invertebrates, and most multicellular organisms (e.g. invertebrates) depend on it completely (Kimbrell and Beutler 2001). In all vertebrates, initial penetration by microorganisms into the body is firstly encountered by innate

defense mechanisms, making innate immunity a pivotal barricade against early infection. An adaptive immune response is called for when the innate host defenses are defeated or by passed and when the elimination of a new infection is unsuccessful.

Many of the innate immune mechanisms of higher vertebrates have been identified in fish. Immune responses in ectothermic vertebrates display many analogies to those of higher vertebrates (Ellsaesser et al. 1985).

1.6. Mucus as an immunological factor in fish

The mechanical barrier of the skin impedes entry of the majority of microorganisms into the body (Bressler and Bressler 1989). Mucus membranes lining the alimentary, respiratory, and urogenital tracts are equipped with a layer of mucus which functions to entrap foreign microorganisms out of the body. In addition to these physical and anatomical barriers, non-pathogenic organisms, referred to as the natural flora of a host, often compete with potentially pathogenic microorganisms for attachment sites and nutrients on the surfaces of epithelial cells of mucosal surfaces (van der Waaij 1984). Innate mechanisms are both constitutive and responsive (i.e.-existing or inducible) and provide protection by preventing the attachment, invasion or multiplication of microbes on or in the tissues. The mucus layer is suggested to be multifunctional by displaying traits and actions important in e.g.osmoregulation, reduction of friction and diseases resistance (Shephard 1994). The major components of the mucus layer are produced by goblet cells and these cells start to differentiate in the basal part of the epidermis, and then grow in size and move towards the surface where they release their content (Pickering 1977). The mucus is a dynamic coat, which passively flows over and covers the fish (Powell et al. 1992), Mucus slime is the material that makes fish slippery. Its ‘slipperiness’ is the result of its high water content and the presence of high-molecular weight, gel-forming, macromolecules. In most vertebrates, including fish, the predominant gel-forming macromolecules are glycoproteins (Fletcher et al. 1976). Other known ingredients include: lysozyme the components of the innate immune system (Bullock and Roberts 1980), immunoglobulins (Fletcher et al. 1976), complement

(Harrell et al. 1976), carbonic anhydrase (Wright et al. 1989), lectins (Shiomi et al. 1990), crinotoxins (Cameron and Endean 1973), calmodulin (Flik et al. 1984), C-reactive protein (Ingram and Alexander 1981), and proteolytic enzymes (Hjelmeland et al. 1983).

The skin of fish is a dynamic tissue whose cellular make up is known to be influenced by factors such as season, stress, diseases, development stage and environmental conditions (Blackstock and Pickering 1982). A great deal of research supports the notion that layers of mucus accumulate on the skin and gills of fish that are stressed by disease, adverse environmental conditions and handling (Handy and Eddy 1991). The skin surfaces support considerable concentration of gradients, particularly for sodium and chloride in fresh water or seawater. Fish biologists suspected that mucus might be involved in ion regulation. The abundance of goblet cells on fish surface may also be correlated with environmental salinity (Laurent 1984) and this has contributed to the view that mucus somehow supports ion regulation by fish. In general the abundance of goblet cells on gills and non-gill surface decreases as salinity increases (Burden 1956, Ahuja 1970, Laurent 1984).

Antibacterial activity in mucus has been demonstrated in several fish species (Austin and Mcintosh 1988) yet this activity seems to vary from fish species to fish species and can be specific towards certain bacteria (Noya et al. 1995).The objective of the study is –

- To compare the protein concentration in fish mucus from farmed fish.
- To get theoretical and practical knowledge of testing antibacterial activity in mucus from farmed fish (*S. salar* and *G. morhua).*
- To study antibacterial activities in mucus of two farmed fish species, cod and salmon, against some bacterial strains.
- To test the influence of the different samples (at different concentration of protein dilution) on the bacterial growth for different bacteria.

2. MATERIALS AND METHODS

2.1. Experimental animals and sample collection

Mucus samples of the five Atlantic salmon (*Salmo salar*) were collected from Kårvika Aquaculture Research Station and five Atlantic cod (*Gadus morhua*) samples for mucus were collected from Havbruks stasjonen, Tromsø, Norway. The fish samples were collected from salt water rearing condition. The sampled fish were killed with a sharp blow to the head. The fish were placed with the ventral side of the body facing downward in the "surgery-bed" and cutaneous mucus from the dorsal side of the fish was collected by a cell-scraper and transferred to a tube with 200 µl 50 mM NaAc, pH 6.0 (See appendix-8.2.2). Samples were kept on ice during transportation to the lab and kept frozen to avoid bacterial growth and degradation at –80^{o}C until used. Samples in the collection tubes were weighed prior to and after collection of mucus by weight machine (METTLER AJ 100, Switzerland).

Fig.4. Collection of mucus sample from salmon by cell-scraper.

2.2. Preparation of mucus from fish

Prior to preparation and analysis, mucus samples were thawed and kept on ice. By Pipetting up and down with a 200 µl pipette and vortex dispersed the mucus samples. Samples were centrifuged (SRL, A 14, 20125 Jouan, Italy) at 4^0 C for 15 minutes at 14,000 rpm and the supernatant were transferred to new tubes. The pellet was resuspended again with 300 µl 50 mM NaAc buffer, pH 6.0 and centrifuged again at 14,000 rpm for 15 minutes. The supernatant were transferred to the same tube with the first supernatant and the prepared samples were stored at -80^0C until analyzed.

2.2.1. Sample desalting by Spin column

Pierce Protein Desalting Columns are designed to desalt or exchange buffer of protein samples with volumes from 30 to 120 µl. Protein Desalting spin columns have over stepping desalting characteristics with > 95% retention of salts and small molecules while providing good recovery of protein greater than 7,000 MW (Molecular Weight). Columns were inverted to suspend slurry and placed column in 1.5-2.0 ml micro centrifuge collection tube. To remove excess liquid from the column centrifuge at 4700 rpm for 1 minute. Placed column in a fresh collection tube, remove cap and apply 30-120 µl of mucus sample to the center of the compacted resin bed. Centrifuge again at 4700 rpm for 2 minutes. Desalted samples were collected in collection tubes and stored at -80^0 C until use.

2.3. Measurement of protein concentration in fish mucus

Components in mucus especially proteins are tested by different methods.

2.3.1. Protein measurement by BCA kit:

The Pierce BCA (Bicinchonic acid), (Pierce, Rockford, IL, U.S.A.) protein assay is a detergent-compatible formulation based on bicinchonic acid for the colorimetric detection and quantitation of total protein. Dilutions (1:2) of the sample were made by adding 55 µl of sample and 55 µl of dH_2O in a vial. Prepared 1:2 dilution samples (50 µl) were put 96-well microtitre plates (Sarsted, Inc.Newton, NC 28658, U.S.A) in the planned wells. Adding 25 µl of that samples and 25 µl of distilled H_2O made 1:4 dilutions. Working reagent (200 µl) was added in each well. Standards solutions (Bovine Serum Albumin Standards 25-2000 µg/ml) were made as per procedure beside the unknown wells. Microtitre plates were shaked for 30 seconds and put at 37^oC for 30 minutes. Assign the micro plate essay in Soft Max program (2002) for the measurement of protein concentration in absorbance at 560 nm after cooling the plate at room temperature.

2.3.2. Protein measurement by two different Nano Drop methods

Proteins, unlike nucleic acid, can exhibit considerable diversity. The A280 method is applicable to purified proteins exhibiting absorbance at 280 nm in a Nano Drop instrument (ND-100 Spectrophotometer). It doesn't require generation of a standard curve and is ready for quantitation of protein samples at startup. This module displays the UV spectrum, measures the protein's absorbance at 280 nm (A280) and calculates the concentration (mg/ml). The Nano Drop ND-1000 Spectrophotometer will accurately measure protein samples up to 100 mg/ml (BSA) without dilution. To do this, the instrument automatically detects the high concentration and utilizes the 0.2 mm path length to calculate the absorbance. The measurements were done according to the manufactures manual (V3.1.0 User's manual).

Samples (A280 BSA and A280 Lysozyme) were taken in a marked tube. Nano Drop pedestal was cleaned before measure the concentration by using distilled water and ethanol. Desired manual was set up in the computer programme. Distilled water (2 µl)

was used as blank. Samples (2 µl) were measured in the same way. Program showed the concentration of the sample automatically. That means the software calculates the concentration as if the sample were pure BSA or lysozyme, instead of all the different proteins and other molecules of the mucus.

2.4. Bacteria and growth of bacteria

Listionella anguillarum (La). AL 104- the bacterium *L. anguillarum* is a polarly flagellated, Gram-negative, curved rod. The temperature and quality of the water, the virulence of the *L. anguillarum* strain and stress on the fish are important elements influencing the onset of disease outbreaks. *Escerichia coli (Ec).* ATCC 25922- a species of Gram-negative aerobic bacteria that is widely used in microbiological and genetics research. *Cornebacterium glutamicum (Cg).* CCUG 27702-is a Gram-positive, non-pathogenic and fast growing soil bacterium with special biotechnological importance. *Staphylococcus aureus (Sa).* ATCC 9144- Staphylococci are Gram-positive spherical bacteria. *S. aureus* should always be considered a potential pathogen. They were grown at room temperature in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, USA).
One bacterial colony from the MHB plate was transferred to 5 ml MH (Mueller Hinton) solution and shaked over night in a shaker machine at room temperature. The next morning 20 µl of that solution was transferred again into 5 ml MH and shaked for 2 hours. Optical density (Absorbance) was measured after transferring 20 µl of that solution to 10 ml MH solutions by taking 5-20 µl of that sample in a Spectrophotometer. These suspensions were used in the antibacterial testing.

2.5. Antibacterial activity testing

The antibacterial test was performed in 100- well microtitre plates (Lab systems, HC2 Patpend, Finland). Fifty micro liters of distilled water was transferred to each well except the positive control wells and the first row of the plate. Cecropin (anti microbial peptides) was used in the positive control wells. Hundred micro liters of specific samples were transferred in the first row wells of the micro plate. All samples were diluted in water to a

protein concentration of 250 µg/ml and serial two fold dilutions were made till 8th row of the plate. Fifty micro liter of 10 ml MH solution containing bacterial sample were transferred to each well in the micro plate. For control group 50 µl of specific bacterial sample and 50 µl of distilled water was added. Bacterial growth was assayed by Easy Bioscreen Experiment Programme (48 hours for Cod mucus and 72 hours for Atlantic salmon mucus samples). Antibacterial activity was determined when the optical density of the growth control reached an absorbance equal to 420-580 nm. The materials and methods were shown as a flow chart in Fig.5.

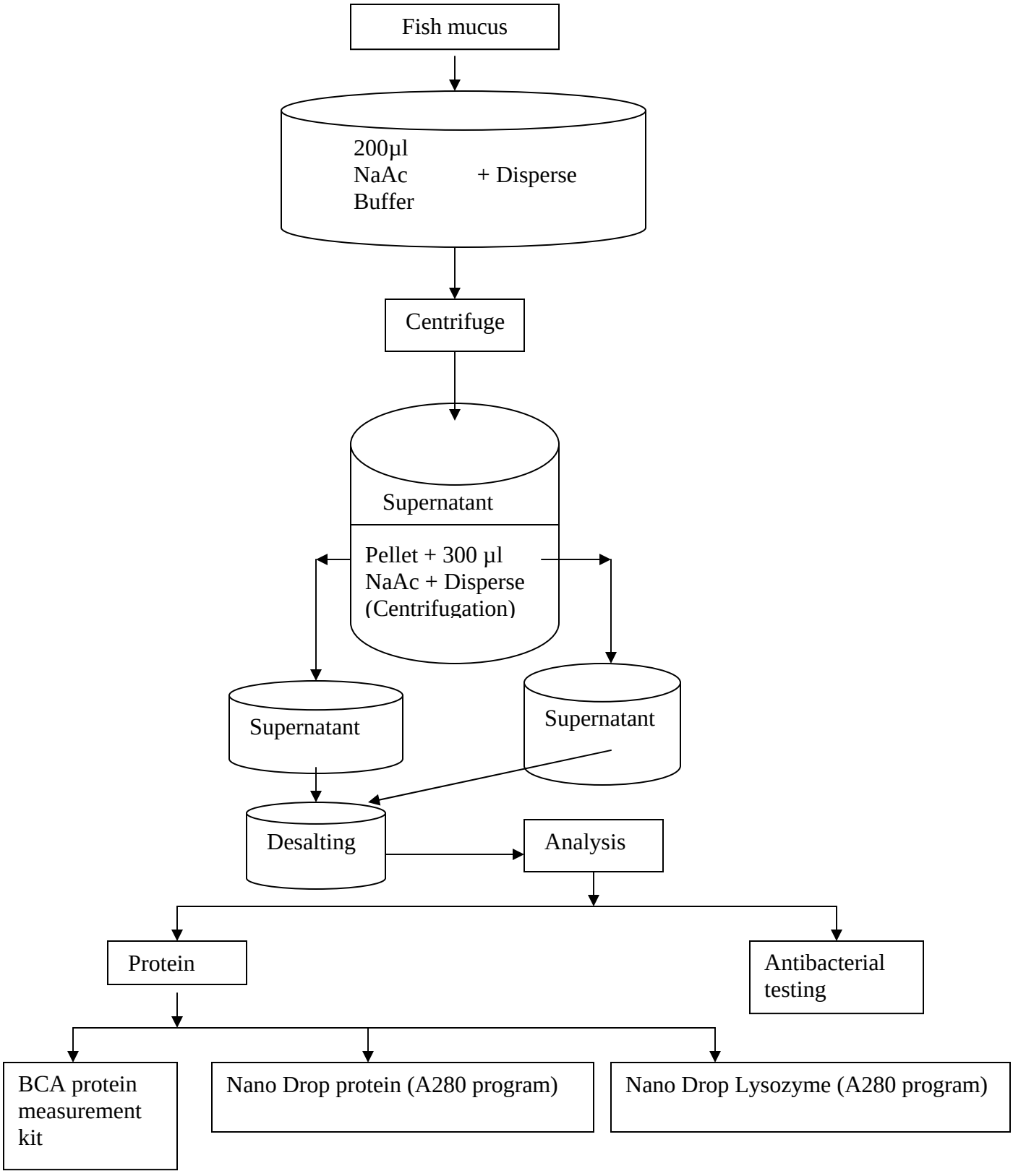

Fig.5. Flow chart of the mucus sample preparation and analysis.

3. **Results**

3.1. Protein concentration in mucus samples

Mucus samples were collected from five Atlantic salmon and five Atlantic cod fish to measure the protein concentration of the fish mucus and to test the activity against bacteria The protein concentration of the mucus was measured by using BCA protein measurement kit and two different spectrophotometric measurement methods by a Nano Drop instrument.

3.1.1. Standard curves of protein concentration

The Standard curves of Ovalbumin and Bovine Serum Albumin (BSA) shown in (Fig. 6) is not optimal, because the values do not follow a straight line. The black line in the figure illustrates the standard curve of BSA that was used during this study.

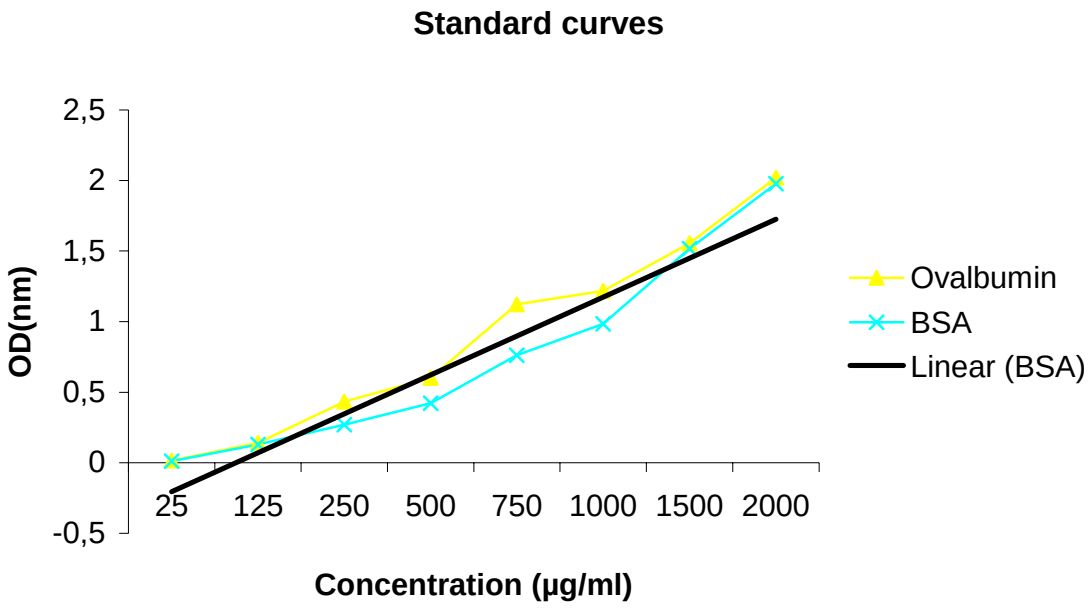

Fig.6. Standard curves for Ovalbumin and Bovine Serum Albumin (BSA).

3.1.2. Protein concentration in mucus samples of salmon

Mucus samples for salmon were weighed. Samples weight varies in different samples.

Table 1. Protein concentration in mucus samples from five different Atlantic salmons measured by BCA and Absorbance based method.

Mucus samples of the Atlantic salmon	Methods						
	BCA method *				Absorbance based method (Nano Drop)		
	Weight of mucus samples (g)	Protein conc. (mg/ml)	Mean protein conc. (mg/ml)	Std. Dev	A280 BSA[a] (mg/ml)	A280 BSA[b] (mg/ml)	A280 Lysozyme (mg/ml)
S-1	0.3	5.93 5.83	5.88	66.4	10.22	3.10	9.15
S-2	0.4	3.79 2.31 4.70 3.76	3.64	984.5	11.25	2.35	6.49
S-3	0.2	2.31 2.00 2.90 2.41	2.40	372.8	9.84	3.04	3.65
S-4	0.4	6.90 6.28	6.59	445.7	14.25	7.48	10.95
S-5	0.7	3.17 2.58 3.47 3.23	3.11	400.4	9.95	6.19	6.19
Avg.			4.32	453.9	11.10	4.43	7.28

* Bovine Serum Albumin (BSA) Standards 25-2000 (µg/ml).

BSA[a]- Original sample.

BSA[b]- Desalted sample.

Highest sample weight in salmon (0.7 g) found in S-5 and the lowest (0.2 g) in S-3 where as S-2, S-4 has the same weight (0.4 g).

Protein concentration in mucus samples from Atlantic salmon measured by different methods (Table 1). In salmon the highest protein concentration (6.59 mg/ml) by BCA method was measured in S-4 and the lowest (2.40 µg/ml) in S-3.Besides S-1, S-2, S-5 protein concentration were 5.88, 3.64 and 3.11 mg/ml respectively. On the other hand, Absorbance based method by Nano Drop highest protein concentration in salmon samples on BSA^a (14.25 mg/ml) and BSA^b (7.48 mg/ml) was measured in S-4, where as S-3 showed lowest value for BSA^b and BSA^a (3.04 and 9.84 mg/ml). In S-1, S-2 and S-5 protein concentration on BSA^a measured 10.22, 11.25 and 9.95 mg/ml respectively. However, it was found 3.10, 2.35 and 6.19 mg/ml in BSA^b A280 Lysozyme by absorbance method was highest (10.95 mg/ml) in S-4. It was mentionable that highest protein concentration was found in S-4 by both BCA and Absorbance based method.

The average mean protein concentration in salmon mucus by BCA* method was 4.32 mg/ml and the Std.deviation was 453.9. On the other hand by absorbance based method the average A280 BSA^a and A280 BSA^b was 11.10, 4.43 mg/ml, respectively. The average A280 lysozyme was found 7.28 mg/ml.

3.1.3. Protein concentration in mucus samples of cod

Mucus samples from cod were weighed before measured the protein concentration of the samples by different methods (Table 2). Weight of cod samples was found equal (0.4 g) in C-2, C-4 and (0.2 g) in C-3, C-5 where as in C-1 it was 0.3 g.

In cod samples mucus protein concentration was found higher in all samples by BCA and Absorbance based method. The highest protein concentration by BCA method was 15.84 mg/ml in sample C-2. On the other hand in C-1, C-4 and C-5 the protein concentrations were 13.49, 14.50 and 9.6 mg/ml, respectively. The lowest was measured 6.5 mg/ml in

C-3. However, protein concentration of the mucus samples in cod by Absorbance method showed little variance between BSA^a and BSA^b. C-2 was found highest protein concentration on BSA^a (38.28 mg/ml) and BSA^b (35.67 mg/ml).

Table 2. Protein concentration in mucus samples from five different Atlantic cods measured by BCA and Absorbance based method.

Mucus samples of the Atlantic Cod	Methods						
	BCA method*				Absorbance based method		
	Weight of the mucus samples (g)	Protein conc. (mg/ml)	Mean protein conc. (mg/ml)	Std.Dev	A280 BSA^a (mg/ml)	A280 BSA^b (mg/ml)	A280 Lysozyme (mg/ml)
C-1	0.3	13.54 13.43	13.49	77.9	27.12	25.02	9.04
C-2	0.4	16.64 15.03	15.84	1139.4	38.28	35.67	13.67
C-3	0.2	6.08 5.78 7.08 7.04	6.5	662.3	8.65	6.68	2.69
C-4	0.4	11.25 17.75	14.50	4594.9	24.24	25.03	9.18
C-5	0.2	7.75 11.95 9.09	9.6	2148	30.45	16.69	6.68
Avg.			11.99	1724.5	25.75	21.82	8.25

* Bovine Serum Albumin (BSA) Standards 25-2000 (µg/ml).

BSA^a- Original sample.

BSA^b- Desalted sample.

It is mentionable that C-3 showed the lowest protein concentration both for BSA^a and BSA^b. The protein concentrations were 8.65 mg/ml and 6.68 mg/ml, respectively. It was 27.12, 24.24 and 30.45 (mg/ml) in BSA^a for sample C-1, C-4 and C-5.Where as, it was 25.02, 25.03 and 16.69 (mg/ml) in BSA^b for the same sample. A280 Lysozyme by

absorbance based method was highest (13.67 mg/ml) in C-2 and the lowest was 2.69 mg/ml in C-3. For C-1, C-4 and C-5 it is 9.04, 9.18 and 6.68 mg/ml respectively.

The average mean protein concentration in cod mucus by BCA* method was 11.99 mg/ml and the Std.deviation was 1724.5. On the other hand by absorbance based method the average A280 BSA[a] and A280 BSA[b] was 25.75, 21.82 mg/ml, respectively. The average A280 lysozyme was found 8.25 mg/ml.

3.2. Antibacterial activity

Antibacterial activity (Inhibition of bacterial growth by chemical reaction in mucus) was tested in salmon and cod mucus samples by testing different bacterial strains, dilute samples to the same protein content and by monitoring growth of the bacteria.

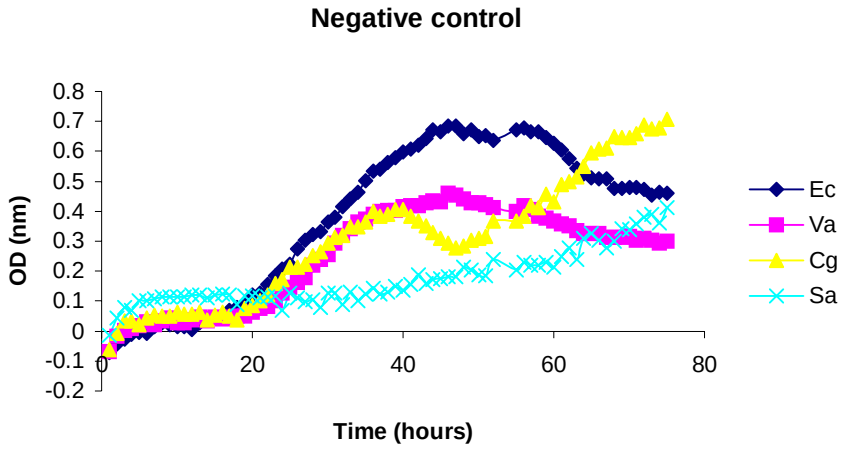

Fig.7. Bacterial growth along with water in salmon mucus plate samples.

Growth curves of different bacteria were measured against salmon and cod mucus samples in Easy Bioscreen Experiment Programme. Time period was 48 hours for Cod and 72 hours for Atlantic salmon fish mucus sample.

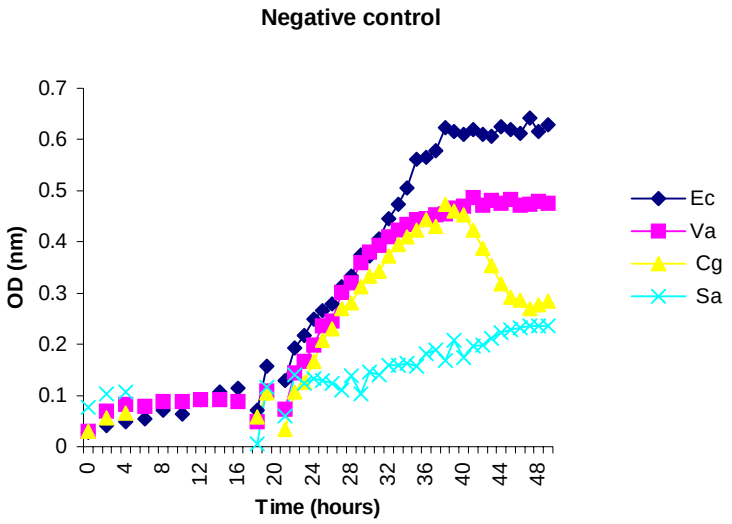

Fig.8. Bacterial growth along with water in cod mucus plate samples.

Control group (Bacterial growth along with water), and positive control (Bacterial growth along with cecropin) of the bacteria also assayed during the observation (Fig.7 and Fig.8). Negative control was showed as an average for specific bacteria.

3.2.1. Antibacterial activity in salmon and cod mucus samples

The salmon mucus samples showed antibacterial activity at a protein concentration 250 µg/ml except sample S- 4. Antibacterial activity against *L. anguillarum* showed at a protein concentration 250 µg/ml for all the mucus samples in Salmon mucus samples where as for S-1 and S-3 showed activity against *C.glutamicum* at protein concentration

62.5 µg/ml and 125 µg/ml. Against *S.aureus*, all the salmon samples showed activity at protein concentration 250 µg/ml where as S-3 showed activity at 125 µg/ml protein concentration (Table 3).

Table 3. Antibacterial activity in Atlantic salmon *(Salmo salar)* mucus sample tested against *Escherichia coli (Ec), Listionella anguillarum (La), Corynebacterrium glutamicum (Cg)* and *Staphylococcus aureus (Sa)*.

	Antibacterial activity			
Atlantic salmon mucus sample	*Ec*	*La*	*Cg*	*Sa*
S-1	+	+	+ + +	+
S- 2	+	+	+	+
S- 3	+	+	+ +	+ +
S- 4	-	+	+	+
S-5	+	+	+	+

Abbreviations:

- No antibacterial activity at a protein concentration ≥ to 250µg/ ml.
+ Antibacterial activity at a protein concentration of 250 µg/ ml.
+ + Antibacterial activity at a protein concentration of 125 µg/ ml.
+ + + Antibacterial activity at a protein concentration of 62.5 µg/ ml.
+ + + + Antibacterial activity at a protein concentration of 31.25 µg/ ml.

In cod mucus samples C-1 and C-2 there were no antibacterial activity against *E.coli* but other samples showed antibacterial activity at a protein concentration 250 µg/ml. Antibacterial activity against *L. anguillarum* showed at a protein concentration 250 µg/ml for all the samples in cod mucus samples except C-1 (≥250 µg/ml) and C-3 (62.5 µg/ml). C-3 showed antibacterial activity against *C.glutamicum* at 125 µg/ml and others showed at concentration 250 µg/ml. Only C-2 and C- 5 showed antibacterial activities at a protein

concentration 31.25 µg/ml against *S.aureus.*On the other hand C-1, C-4 showed at 250 µg/ml and C-3 showed activity at protein concentration 62.5 µg/ml, respectively (Table 4).

Table 4. Antibacterial activity in Atlantic cod (*Gadus morhua*) mucus sample tested against *Escherichia coli* (*Ec*), *Listionella anguillarum (La), Corynebacterrium glutamicum (Cg) and Staphylococcus aureus (Sa).* Abbreviations as in Table 3.

Atlantic cod mucus sample	*Ec*	*La*	*Cg*	*S.a*
C-1	-	-	+	+
C- 2	-	+	+	+ + + +
C-3	+	+ + +	+ +	+ + +
C- 4	+	+	+	+
C-5	+	+	+	+ + + +

The results show that antibacterial activity was found in both fish mucus sample (Table 3 and Table 4). However, when the antibacterial activities in two different fish mucus samples compared, wide differences were found. Four different strains of bacteria were tested. Among them *C.glutamicum* was the most sensitive, while *S.aureus* was the least sensitive. In cod mucus samples the highest activity was found mainly against *C.glutamicum*, where as in salmon mucus samples the highest activity was found *against L.anguillarum.*

3.3. Growth curves

Growth curves of four different bacteria (*E.coli*, *L.anguillarum*, *C.glutamicum and S.aureus*) against salmon and cod mucus samples were observed in different protein concentration (250, 125, 62.5 and 31.25 µg/ml) in relation to different dilutions. The results with the lowest protein concentration (highest dilution) was the most interesting

because then the sample contain little protein, but antibacterial components that still were active. Fig.9 - 12 showed the growth curves of the different bacteria against salmon and Fig.13 - 16 showed against cod mucus samples at a protein concentration 125 µg/ml.

3.3.1. Growth Curves of bacteria plus mucus samples of salmon adjusted to a certain protein concentration

Growth curves against salmon mucus were observed for 72 hours. In comparison to other salmon mucus samples and negative control, S-1 showed the inhibition against *E.coli*, *L.anguillarum* and *S.aureus* where as S-4 showed inhibition against *C. glutamicum.*

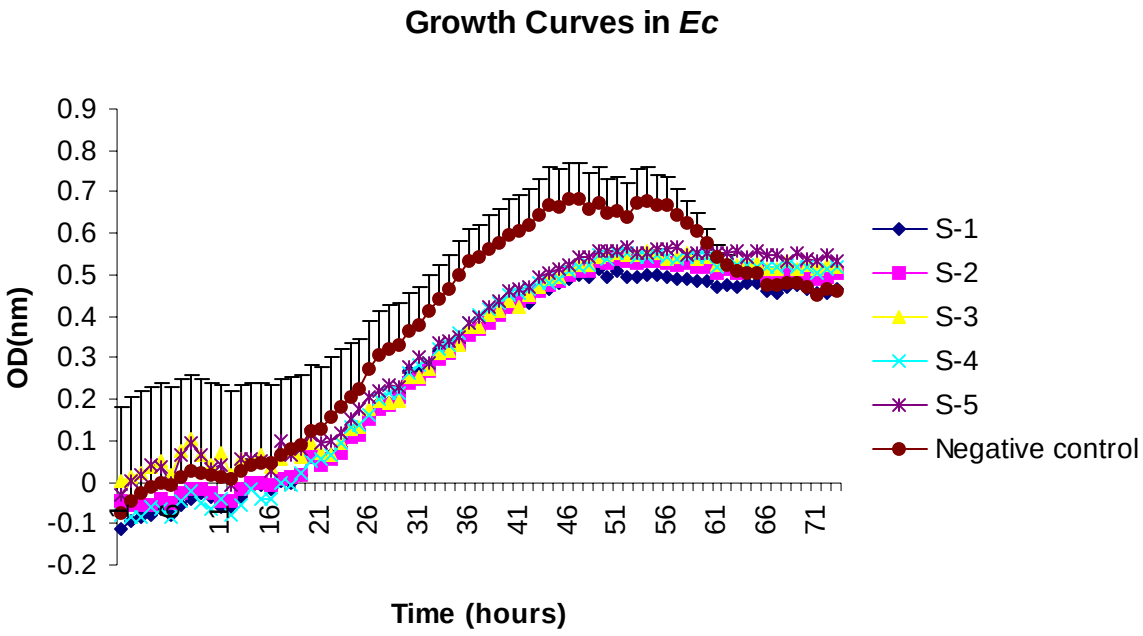

Fig.9. Antibacterial activity of mucus samples from five different salmon (S-1 to S-5) against *E.coli* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125 µg/ml.

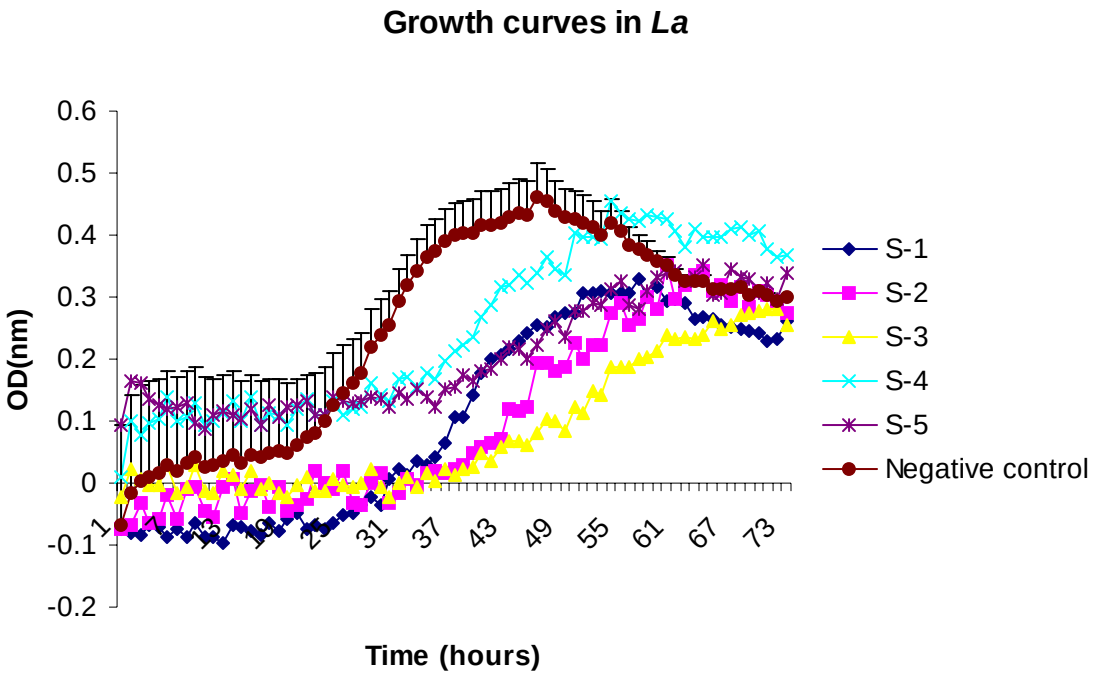

Fig.10. Antibacterial activity of mucus samples from five different salmon (S-1 to S-5) against *L.anguillarum* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125µg/ml.

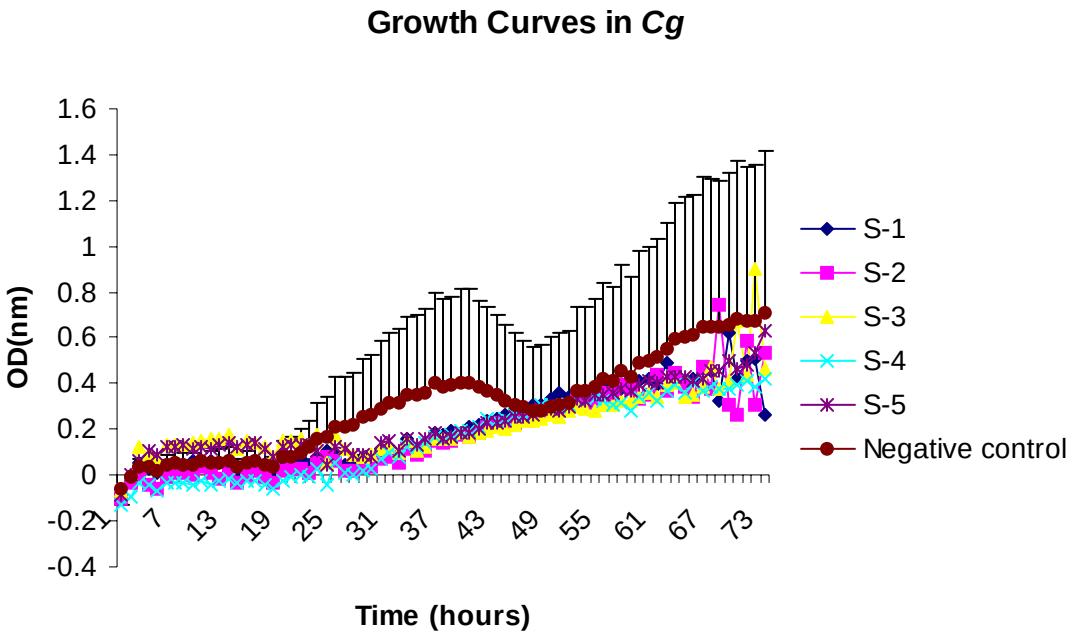

Fig.11. Antibacterial activity of mucus samples from five different salmon (S-1 to S-5) against *C.glutamicum* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125 µg/ml.

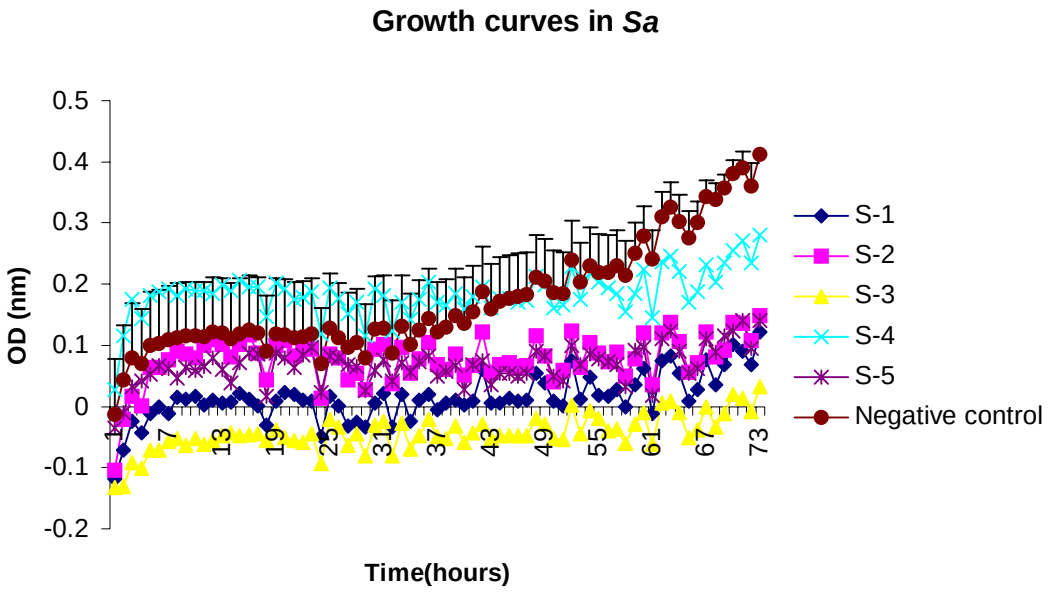

Fig.12. Antibacterial activity of mucus samples from five different salmon (S-1 to S-5) against *S. aureus* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125µg/ml.

3.3.2. Growth Curves of bacteria plus mucus samples of cod adjusted to a certain protein concentration

Growth curves of bacteria added mucus from different cod were observed for 48 hours. In comparison to other cod mucus samples and negative control, C-4 showed the inhibition against *E.coli* and *C. glutamicum*. On the other hand, C-3 and C-1 showed inhibition against *L. anguillarum* and *S.aureus*, respectively.

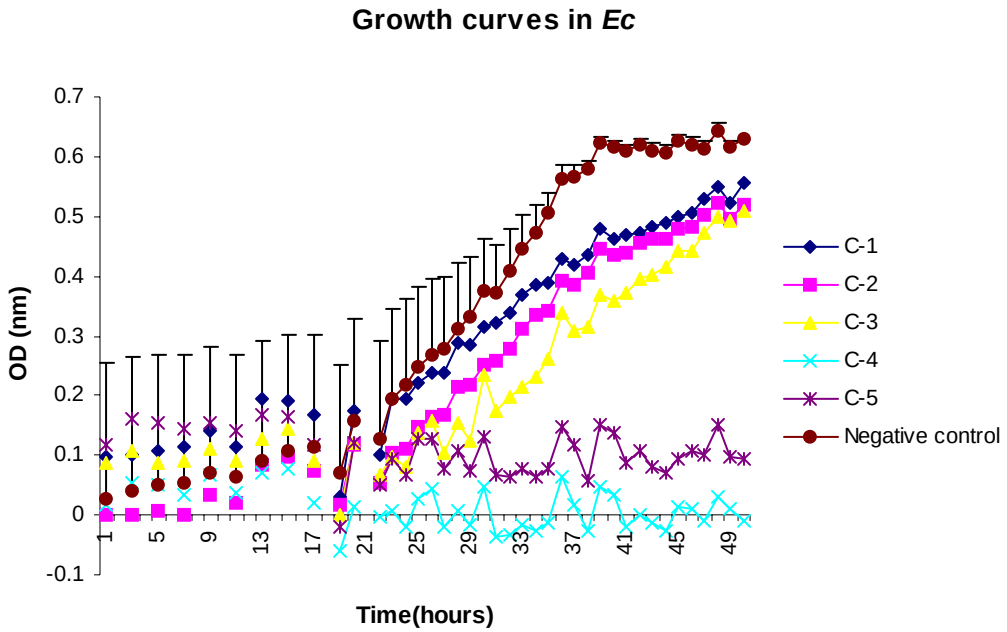

Fig.13. Antibacterial activity of mucus samples from five different cod (C-1 to C-5) against *E.coli* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125 µg/ml.

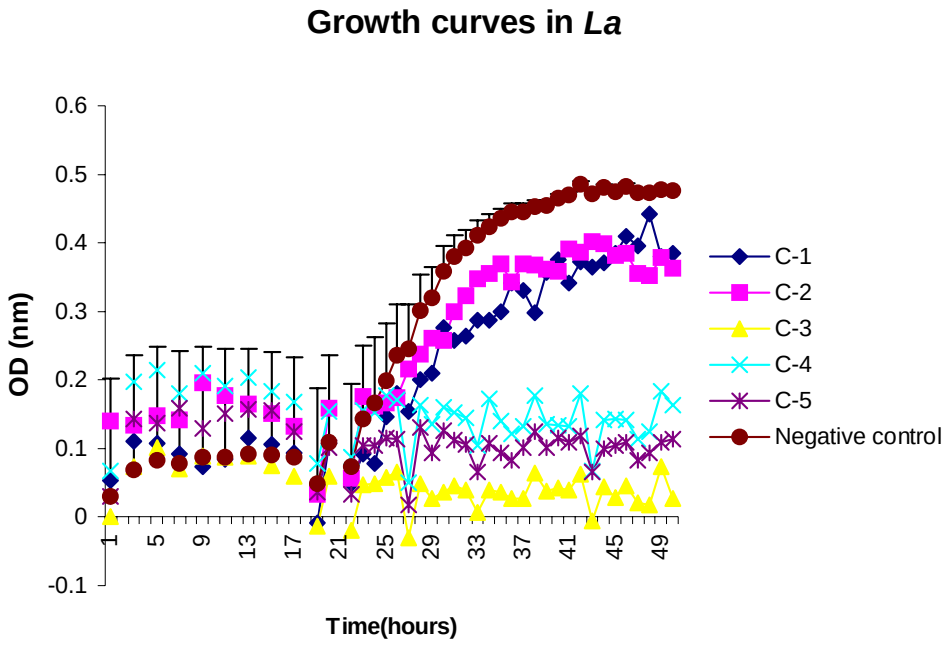

Fig.14. Antibacterial activity of mucus samples from five different cod (C-1 to C-5) against *L.anguillarum* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125 µg/ml.

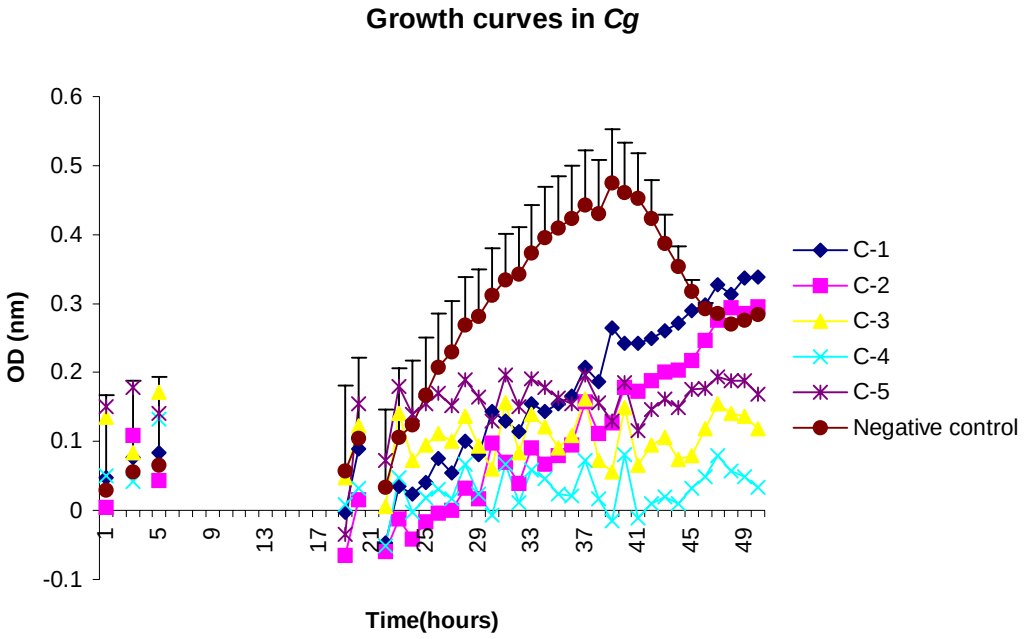

Fig.15. Antibacterial activity of mucus samples from five different salmon (S-1 to S-5) against *C.glutamicum* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125 µg/ml.

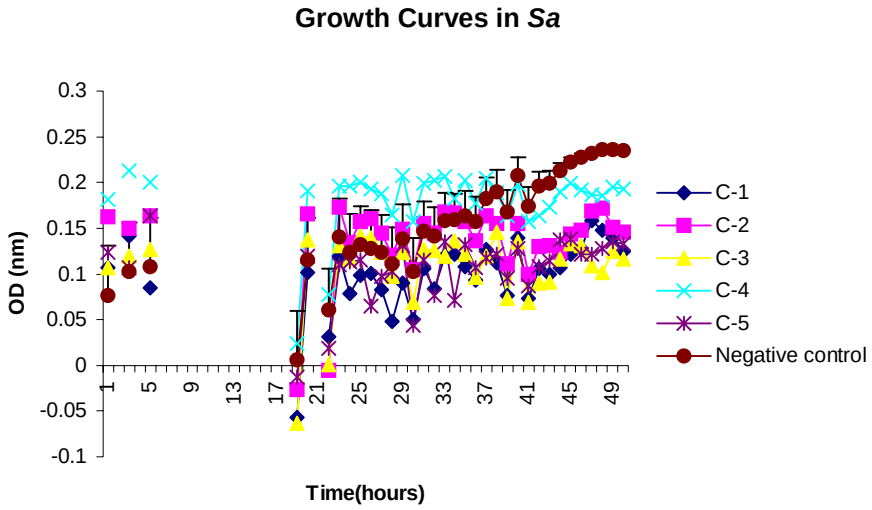

Fig.16. Antibacterial activity of mucus samples from five different salmon (S-1 to S-5) against *S.aureus* grown in MHB. The optical density at 420-580 nm was measured in a bacterial suspension of approximately 5 x 10^5 cells per well containing bacteria alone (negative control), or bacteria plus mucus of the different salmons adjusted to a protein concentration of 125 µg/ml.

4. Discussion

The biological interface between fish and their aqueous environment consists of a mucus layer composed of biochemical diverse secretions from epidermal and epithelial cells (Ellis 1999). This layer is thought to act as a lubricant (Rosen and Cornford 1971), to have a mechanical protective function, to be involved in osmoregulation and locomotion (Cameron and Endean 1973), to play a possible immunological role (Fletcher and Grant 1969) and to have some function in intra-species chemical communication (Saglio and Blanc 1989). Over the past years, it has also been shown that mucus plays a role in the prevention of colonization by parasites, bacteria and fungi and the antibacterial role of mucus has been known for many years (Austin and Mcintosh 1988). Fish mucus was found as a source of antimicrobial products (Hellio et al. 2002) .

Mucus protein concentration of salmon and cod mucus was conducted in this work**.** Protein concentrations are discussed in relation to the different protein measurements methods used. The results show that all the samples possess protein concentration. However, protein concentrations in cod mucus samples are higher than the salmon mucus samples. The optical density values obtained at 420-580 nm for the growth curves of the test bacteria added different samples and concentration of the salmon or cod is compared to the protein concentration. OD values are ignored when it crossed above 2 nm. Several dilutions are done to find the OD values in range especially in cod mucus samples as they might contain high protein. Average adjacent concentration use as a mean protein concentration and it helps to find out the Standard deviation of the concentration statistically.

Protein concentrations are higher in cod mucus samples in comparison with salmon mucus samples both in BCA method and Absorbance based method (Nano Drop). The highest protein concentration in cod mucus sample by BCA method is 15.84 mg/ml in C-2 where as it is 6.5 mg/ml in S-4. On the other hand it is also pointed out that cod sample showed highest protein concentration by Absorbance based method as well. It is 38.28 mg/ml (BSA^a) and 35.67 mg/ml (BSA^b) in sample C-3. The lowest protein concentration

by Absorbance based method is found in salmon sample and it is 9.84 mg/ml in BSA[a] (S-3) and 2.35 mg/ml in BSA[b] (S-2). From the work it was established that cod mucus samples contain more protein concentration than the salmon fish mucus sample.

The samples were also tested by Nano Drop to measure the protein concentration and it showed that protein concentration was always higher before desalting in both cod and salmon mucus samples. On the other hand, it was lower after desalting.

Mucus represents an important biological interface between Atlantic salmon and their aqueous environment (Fagan et al. 2003). The skin mucous layer and epidermis are important in fish defense because they are the first sites of interaction between the host and potential pathogens. Within these layers are many enzymes and antimicrobial proteins, which are thought to be involved in innate immunity of the fish (Dalmo et al. 1997). Differences in activities of antimicrobial enzymes, such as lysozyme and proteases, and how they relate to the structure and composition of mucus and epidermal layers, may also relate to the differences observed in disease resistance. Lysozyme, an antimicrobial, hydrophobic protein found in fish blood and tissues has been studied most extensively in association with disease resistance (Lie et al. 1989) and cortisol stress response (Fevolden and Roed 1993). Muona and Soivio (1992) identified seasonal decreases in plasma lysozyme activity in Atlantic salmon (*S. salar*) and sea trout (*S. trutta*) associated with temperature and the transformation (smoltification), which is the complex process of physiological, morphological, and behavioral change that enables anadromous juvenile salmonids to osmoregulate in sea water (Hoar 1976). Amore recent study reported for the first time mucus lysozyme activity in Pacific salmon during smoltification (Schrock et al. 2001). In this work, however, the lysozyme activity in the mucus samples did not measured.

The antibacterial activity in mucus samples of five salmon and five cod was tested in different protein concentrations, against four different bacterial strains (*E.coli*,*L.anguillarum*,*C.glutamicum* and *S.aureus*). Growth curves of the bacteria and water were considered as a negative controls, and were compared against the growth curves of bacterial cultures added in the different samples.

In salmon, the antibacterial activity in the mucus samples was evaluated at the time point of 48 hours of incubation and a 50% reduction of mean OD 420-580 nm values of the growth curves of bacteria and samples were regarded as antibacterial active when compared to negative controls. Activity at low protein concentrations (high dilution factors) means high antibacterial activity in a particular mucus sample. When the value was found less than the 50% of mean OD values than the activity was determined in certain protein concentration. For *E.coli* and water, the 50% of mean OD value was 0.26 and according to this, the antibacterial activity was detected in all samples at a protein concentration 250µg/ml except S-4. For *L.anguillarum* and *C.glutamicum* it was 0.19 nm and all the salmon samples showed activity at protein concentration 250 µg/ml. On the contrary for *C.glutamicum* S-1 and S-3 showed activity at 62.5 µg/ml and 125 µg/ml. *S.aureus*, the 50% of mean OD value was 0.10 nm and S-3 showed activity against bacteria at 125 µg/ml protein concentration ,others showed at 250 µg/ml.

Antibacterial activity against different bacterial strains in cod mucus samples was determined at a certain point time(35 hours) and 50% reduction of mean OD values of negative control. When the value was found less than the 50% of mean OD values then the activity was determined in certain protein concentration.For *E.coli* in cod mucus sample, the 50% of mean OD value was 0.23 nm.C-3,C-4 and C-5 inhibited the bacterial growth (*E.coli*) at protein concentration 250 µg/ml. It was mentionable that C-1 and C-2 showed no inhibition. The 50% of mean OD value for *L.anguillarum* was 0.22 and C-3 showed inhibition at 62.5 µg/ml and other showed inhibition at 250µg/ml except C-1(no inhibition).For *C.glutamicum* it was 0.21 and antibacterial activity was measured in all samples at 250 µg/ml except C-3 (125 µg/ml). However in *S.aureus* 50% reduction of mean OD values was 0.11 and antibacterial activity was determined in differenrt

concentration for all the samples. C-1 and C-4 showed inhibition at 250 µg/ml and C-2,C-5 showed at 31.25 µg/ml and it was remarkable that *S.aureus* was inhibited at protein concentration of 31.25 µg/ml.

From the results it is clear that antibacterial activity is detected in mucus samples of both fish species. However, when the antibacterial activities in the two different fish species were compared, wide differences were found. The effecct on four different bacterial strains were tested. Among them *C.glutamicum* was the most sensitive, while *S.aureus* was the least sensitive. Furthermore, in cod mucus samples, the highest activity was found against *C.glutamicum* where as in salmon mucus samples, the highest activity was found against *L. anguillarum.*It was also established that mucus samples of cod were more defensive to the bacterials strains rather than the mucus of the salmon samples.

Antibacterial activity in fish mucus and other marine organisms were detected by several authors. Antibacterial activity was conducted in the serum and mucus of rainbow trout (*Oncorhynchus mykiss*) and found a number of antibacterial factors increase in concentration following immunisation and that these probably played a role in protection against microbial disease (Rainger and Rowley 1993). (Ebran et al. 1999a), identified a strong antibacterial activity well correlated with pore-forming properties against several bacterial strains and these suggested that fish secrete antibacterial proteins able to permeabilize the membrane of the target cell and thus act as a defense barrier.(Haug et al. 2002) detected antibacterial activity in four marine crustacean decapods. Elutes from the solid phase extraction were tested for antibacterial activity against *E.coli, L.anguillarum, C.glutamicum* and *S.aureus* was detected in extracts from several tissues in all species tested, but mainly in the haemolymph and haemocyte extracts. *L.anguillarum* and *C.glutamicum* were found the most sensitive micro-organisms. However, (Hellio et al. 2002), studied antibacterial,antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus and found antifungal and antibacterial activities in the fish mucus. Two novel antibacterial muramidases were purified to homogeneity from skin of rainbow trout (*O.mykiss*) and these two muramidases probably contribute to epithelial defence of the fish against microbes, either alone or in synergism with antibacterial peptides. It was concluded that the mucus layer represents a hindrance with

L. anguillarum, while *A.salmonicida* seems to be able to penetrate through it (Svendsen and Bogwald 1997). Removal of the mucus layer on two occasions modifies the new mucus layer which simplifies the entrance of *L. anguillarum*. It was suggested that this was a result of a lowered concentration of compounds with antivibriotic activity. Wounds appear to be important invasion routes for *A.salmonicid.*An antimicrobial peptide was purified from skin secretions and epithelial cells of rainbow trout by cation exchange and reverses phase chromatography. This peptide may play a role in protection against intracellular or extracellular pathogens (Fernandes and Smith 2002). It was demonstrated that there were significant histological and biochemical differences between the skin and mucus of rainbow trout, coho and Atlantic salmon, which may change as a result of different environments. Variation in these innate immune factors is likely to have differing influences on each species response to disease processes (Fast et al. 2002). Hipposin is a new broad –spectrum histone –derived AMP (antimicrobial peptide) found in the skin mucus of Atlantic halibut and showed strong antimicrobial activity against several Gram-positive and Gram-negative bacteria (Birkemo et al. 2003). Mucus and blood sample from hatchery reared Atlantic salmon were studied over the peroid of smoltification. The protein profile result revealed that mucus profiles for individual fish with in sample groups were very similar and the changes in these mucus protein bands warrant further investigation to determine the changes coincide with the smoltification process (Fagan et al. 2003). Pleurocidin, a 25-residue a helical cationic peptide, isolated from skin mucous secretions of the winter flounder, displays a strong antimicrobial activity and appears to play a role in innate host defense. This peptide would be responsible for pore formation in the membrane of bacteria leading to lysis and therefore death (Saint et al. 2002). Sloughing of microbes in the mucus contains many antibacterial substances including antibacterial peptide, lysozyme, lectins and proteases (Barnes et al. 2003). The hydrophobic components of crude epidermal mucus of fresh water and sea water fish exhibit strong pore-forming properties, which were well correlated with antibacterial activity (Ebran et al. 1999b).

(Ebran et al. 2000) isolated glycosylated proteins from the hydrophobic supernatant of tench (*Tinca tinca*), eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*) mucus and suggested that fish secrete antibacterial glycoproteins able to kill bacteria by forming large pores in the target membrane.

The results were not tested by statistical programme except the standard deviation, as the number of sample and amount of mucus was less in quantity. The mucus samples were collected from 10 fish only (5 from Salmon and 5 from Cod). Furthermore, it was a pilot experiment and indication of results implies the repetition of the experiment. However, protein concentrations in salmon and cod mucus samples play an important role to inhibit the bacterial growth. Different authors analyzed salmon mucus against bacterial growth but there were no investigation on cod mucus against different bacterial strains. So it needs further investigation. It is also important to collect mucus from more fish species, fractionate mucus, purify and characterize the components in mucus with antibacterial activity.

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6. APPENDIX

6.2.1. Buffers and Solutions

1. Sodium acetate (NaAc) buffer (1M)

Materials

- Sodium Acetate powder (Sigma Chemical company).
- Distilled water.
- Spoon (Clean).
- Weight machine.
- Clean glass bottle 200 ml.
- Magnet.
- Magnet stirrer.

Methods

- Adding 13.6 g of NaAc and 80 ml of distilled water made NaAc buffer pH 6.0 was adjusted by pH meter (62 standard pH meter, Copenhagen, Denmark) and adding acid adjust the volume to 100 ml.
- Autoclave the buffer at 121^0C for 20 minutes. Store buffer at 4^0C until use.

2. Sodium acetate (NaAc) buffer (50 mM)

- The following formula is used to make 50mM of NaAc buffer-

  $C_W.V_W = C_S.V_S$

  C_W (Wanted concentration)=0.05

  C_S (Stock concentration)=1M

  V_W (Wanted volume) = 100 ml

  V_S (Volume of stock solution calculated)=? = 5 ml
- Add 5 ml NaAc buffer with 95 ml distilled water and the final solution is 50 mM NaAC. Store buffer at 4^0C until use.

6.2.2. Protocol for preparation of mucus from fish

Materials

- Pipette and tips.
- 50 mM Sodium acetate buffer P^H 6.0.
- Tubes.
- Eppendrof centrifuge (SRL, A 14, 20125 Jouan, Italy).

Methods

- Mucus sample should be on ice all the time to avoid bacterial growth and degradation of the sample.
- Take tubes according to samples and marked them.
- Disperse the mucus samples with the help of 200 µl pipette and vortex. Avoid spilling of the sample
- Centrifuge the samples for 15 minutes at 14,000 rpm. Keep samples parallel in the centrifugal machine. Before start the machine set up the desired requirements and time.
- Pipette out the all supernatant not touching the pellet/mucus of the samples and transfer them to new marked tube.
- Resuspend the pellet with 300 µl 50 mM Na Ac buffer pH 6.0 and disperse the pellet again.
- Centrifuge once more for 15 minutes at 14,000 rpm.
- Pipette out the supernatant to the same tube or new marked tubes. Prepared mucus samples may be stored at -80^0c until analysis.

6.2.3. Protocol for Prepare Bovine Serum Albumin (BSA) Standards

Materials

- Tubes
- Distilled water.
- 1 ml ampoule of BSA (2.0 mg/ml).
- Tissue paper.
- Still knife.

Methods

- Mark eight tubes as 2000,1500,1000,750,500,250,125 and 25 µg/ml.
- Keep distilled water in a tube for avoiding contamination.
- Take the ampoule from the kit box. Mark the ampoule bottle in its upper part with small still knife. Press the ampoule bottle softly with tissue paper. Pipette out 2000 µg/ml solution from the ampoule and transfer it to 2000 µg/ml marked tube.
- Find out the concentration and volume of water that should be added by using the equation ($M_1V_1 = M_2V_2$) and Volume of water added=(Sum of volume – add of concentration).

Calculation:

Wanted concentration (µg/ml)	The add of conc. (µl)	Volume of water added (µl)	Sum of the volume (minimum 200µl)
2000	200	0	200
1500	150	50	200
1000	100	100	200
750	75	125	200
500	50	150	200
250	25	175	200
125	12.5	187.5	200
25	2.5	197.5	200

* Copied from instructions BCA protein Assay Reagent Kit.

- Add water according to the calculation in each marked tubes.

- Add concentration according to the calculation in each marked tubes. Push the concentration gently to avoid air bubbles in the tubes. To avoid adding more concentration just add sample in one tubes and after transferring it to another row then start for second one.
- Keep the tubes in the freeze at 4^0C before use.

Protocol for the preparation of Working Reagent (WR)

- Prepare fresh WR by mixing 50 part of BCA (Bicinchoninic acid) Reagent A With 1 part of BCA Reagent B (50:1).
- Prepare sufficient volume of WR based on the number of samples to be assayed.
- The WR is stable for at least one day when stored in a closed container in room temperature.

Measurement of protein concentration in fish mucus

- After the preparation of the fish mucus sample it was kept in -80^0C for the measurement of different analysis (Protein concentration, antibacterial activity, lisozyme etc).
- Make 1:2 dilutions of the samples by adding 55 µl of sample and 55 µl of dH_2O in a vial for the accuracy of the experiment.
- First put the 1:2 dilution of prepared sample in the micro plate essay (50 µl) in each wall that planned before. It should be done in duplicate.
- Take 25 of that 1:2 dilution and put it in new wall. Put 25 µl of dH_2O in it .mix them gently and pipette out 25 of this solution. So it becomes 1:4 dilutions. Follow the above procedure for rest of the samples.
- Make working reagent according to the procedure for WR (Working Reagent). Plan before how much WR is needed. Add 200 µl of WR reagent in each planned wall.

- Make standard solutions as well beside the unknown cells as per procedure. Shake the micro plate essay for 30 seconds and put the plate at 37^{o}C for 30 minutes. Cool the plate at room temperature.
- Assign the micro plate essay in soft max program for the measurement of protein concentration in absorbance at 560 nm.

6.2.4. Antibacterial activity testing

Materials

- Micro plate.
- Spirit.
- Bacteria.
- Niddles.
- Mueller Hinton (MH) medium.
- Distilled water.
- Cecropin (CP).

Methods

- Switch on the chamber to pass air for filtration. Wash the table with ethanol for the removal of contamination.
- Collect the bacteria from deep freeze (-80^{0}C) and put it then into ice.
- Four agar plates with specific named bacteria should be ready by writing their names in the upper part of the plates.
- Switch on the burner and hit the niddles to collect bacteria samples from each marked vials. Scratch the niddle into the marked agar plate and cover the agar plate with laboratory film for safety.
- Niddle must be burned again after use, as there may be bacteria. Clean the working table again with 70% spirit. Switch off the burner and the chamber.
- Four agar plates covered with laboratory film kept in the table for two days to grow bacterial colonies. Use proper hand gloves to avoid contamination.
- Take one colony of selected specific bacteria by niddle and transfer it to 5 ml MH (Mueller Hinton) solution. Shake it over night in a shaker machine.

- Take 20 µl of that MH solution by pipette and transfer it to 5 ml MH solution (4 vials for 4 bacteria) and shake for 2 hours.
- Transfer 20 µl of that solution to 10 ml MH solution (4 vials for 4 bacteria). Observe the OD (Optical Density) value by taking 5-20 µl of that sample in a Specto photometric machine.

- Put 50 µl of distilled water in each well except the positive control wells and the first row of the plate. Put Cecropin in lieu of water in the positive control wells.

- Put 100 µl of specific sample in the first rows well of micro plate and then mix gently by pipette. Dilute the sample by transferring 50 µl of specific sample from 1 row to 8^{th} row and throw 50 µl of diluted sample from the 8^{th} row.

- Transfer 50 µl of 10 ml MH solution to each well in the micro plate.

- Mark the area for control group (negative control) and for this add 50 µl of specific bacterial sample with 50 µl of distilled water. For positive control add Cecropin and bacterial sample (50 µl) each. Bacterial growth was assayed by Easy Bioscreen Experiment Programme (3 days).