Norwegian University
of Life Sciences

**Master's Thesis 2019 60 ECTS**

Faculty of Chemistry, Biotechnology and Food Science (KBM)

# *Characterization of Lactococcus garvieae from rainbow trout (Oncorhynchus mykiss) in Spain and Turkey.*

Anja Nygaard
MSc Biotechnology, Microbiology

## Acknowledgement

The work presented in this thesis was carried out as part of the two-year master's program in microbiology at the Faculty of Chemistry, Biotechnology and Food science of the Norwegian University of life Sciences, supervised by Professor Leiv Sigve Håvarstein. The work was carried out at PHARMAQ AS under supervision of Dr. Are Klevan.

I want to thank everyone who helped me in my work with this thesis. First and foremost, I would like to thank my supervisor Dr. Are Klevan for all your availability and guidance in this process, and providing good insight into microbiology and phylogeny. I am so grateful for all your time and that you kept my spirit up through all the experiment work. I would also like to thank Professor Leiv Sigve Håvarstein for all the helpful remarks and advices. Furthermore, thank you to Dr. Børge Nilsen-Fredriksen for your valuable advices during the writing process. And thank you to Edel Anne Norderhus, Director Product Development, R&D, who gave me the opportunity to take this thesis at PHARMAQ AS, as part of my job.

Last but not least, I would like to thank my family for being so supportive and patient. Their encouragement has been invaluable. Thank you, I am beyond grateful.

Ås, May 2019
Anja Nygaard

## Abstract

*Lactococcus garvieae* is an emerging pathogen that causes mortalities in various farmed fish species, resulting in significant economic losses in aquaculture worldwide. The Gram-positive bacteria is pathogenic to both freshwater- and marine fish and occurs usually when the water temperature increases to about 16 ºC. In the Mediterranean *L. garvieae* affects the fresh water species rainbow trout (*Oncorhynchus mykiss)* and causes hyperacute haemorrhagic septicemia.

Even though *L. garvieae* is considered an emerging pathogen, there are few studies performed to characterize this pathogen with respect to pathogenicity and virulence factors. Most studies on the virulence factors have been performed in *L. garvieae* strains of the marine species yellowtail (*Seriola* sp.) in Japan were the pathogen have been reported to exist since mid-1950s. The first outbreak in rainbow trout in the Mediterranean was reported in 1988. Based on current literature, the virulence factors in strains from these two fish species does not seem to be the same, and rainbow trout seem to be more sensitive for the pathogen with acute disease and raised mortality. Different typing techniques has been performed on *L. garvieae* from different hosts, but few studies have compared isolates from fish species only.

The aim of the current study was to obtain increased knowledge of the virulence factors and the phylogenetic relationship in *L. garvieae,* and to compare isolates from different geographical regions and fish species. Furthermore, there was also of interest to find a rapid and reliable identification method. Comparison of different phenotypic and genotypic methods, showed that sequencing of the *gyrB* was considered reliable. However, a simple PCR method published in 1998 seem to be the fastest method.

To the best of our knowledge this is the first multilocus sequence analysis study (MLSA) of *L. garvieae* based on strains isolated only from fish. It revealed two main groupings of the strains that strengthen the proposed theory of how the pathogen was introduced to Europe from Japan. It could also explain why the vaccines used in aquaculture in the Mediterranean does not fully prevent outbreaks of the disease.

In this study the major virulence factor, the capsule gene cluster, present in *L. garvieae* strains from yellowtail, was not found in strains from rainbow trout. However, few other virulence factors were detected in this work, and further investigation regarding virulence factors need to be done.

## Sammendrag

*Lactococcus garvieae* er et patogen som forårsaker økende dødelighet i ulike fiskearter i oppdrettsnæringen og gir betydelige økonomiske tap i akvakultur over hele verden. Den Gram-positive bakterien er patogen for både ferskvanns- og saltvannsfisk, og forekommer vanligvis når vanntemperaturen øker til ca. 16 ºC. I Middelhavet gir *L. garvieae* sykdom i ferskvannsarten regnbueørret (*Oncorhynchus mykiss*) og forårsaker hyperakutt septikemi.

Til tross for at sykdom forårsaket av *L. garvieae* er et økende problem i akvakulturen, er det gjort få studier på karakterisering når det gjelder sykdomsbildet og virulensfaktorer. De fleste studier på virulensfaktorer er blitt utført på *L. garvieae*-stammer fra den marine arten yellowtail (*Seriola* spp.) i Japan, hvor patogenet har eksistert siden midten av 1950-tallet. Det første utbruddet i Middelhavet på regnbueørret ble rapportert i 1988. Basert på nåværende litteratur, så har man ikke funnet de samme virulensfaktorene i stammer fra disse to fiskeartene, og regnbueørret ser ut til å være mer følsom for patogenet med akutt sykdom og økt dødelighet.
Ulike metoder på karakterisering har blitt utført på *L. garvieae* fra forskjellige verter, men få studier har sammenlignet isolater fra kun fiskearter.

Målet med dette studien var å få bedre kunnskap om virulensfaktorer og fylogenetisk slektskap i *L. garvieae,* og sammenligne stammer fra forskjellige geografiske områder og fiskearter. Videre var det også av interesse å finne en rask og pålitelig identifikasjonsmetode. Sammenligning av forskjellige fenotypiske og genotypiske metoder viste at sekvensering av *gyrB* var en pålitelig metode. Men den raskeste metoden var en enkel PCR-metode publisert i 1998.
Så vidt vi vet, er dette den første MLSA (multilocus sequence analysis)-analysen av *L. garvieae* med stammer som kun er isolert fra fisk. Det avslørte to hovedgrupper av stammene som styrker den foreslåtte teorien om hvordan patogenet ble introdusert fra Asia til Europa. Grupperingene kan også forklare hvorfor vaksinene som brukes i akvakultur i Middelhavet, ikke fullt ut forhindrer utbrudd av sykdommen.
I dette studiet ble genene for kapselen, som er ansett som den avgjørende virulensfaktoren i *L. garvieae*-stammer fra yellowtail, ikke funnet i stammer fra regnbueørret. Imidlertid ble det oppdaget få andre virulensfaktorer i dette arbeidet, og ytterligere undersøkelser vedrørende virulensfaktorer må gjøres.

## Abbreviations

| | |
|---|---|
| BLAST | Basic Local Alignment Search Tool |
| BT | Bootstrap |
| bp | Base pair |
| CPSs | Capsular polysaccharides |
| dATP | Deoxyadenosine 5’-triphosphate |
| dCTP | Deoxycytidine 5’-triphosphate |
| ddNTP | di-deoxynucleotide triphosphate |
| dGTP | Deoxyguanine 5’-triphosphate |
| dsDNA | Double stranded DNA |
| dTTP | Deoxythymidine 5’-triphosphate |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphates |
| EPS | Exopolysaccharides |
| EtBr | Ethidium bromide |
| FAO | Food and Agriculture Organization of the United Nations |
| IS | Insertion sequence |
| kb | Kilo bases |
| LAB | Lactic acid bacteria |
| MEGA | Molecular Evolutionary Genetics Analysis |
| MLSA | Multilocus sequence analysis |
| MLST | Multilocus sequence typing |
| NCBI | National Centre for Biotechnology Information |
| PFGE | Pulsed field gel electrophoresis |
| RAPD | Random amplified polymorphic DNA |
| RFLP | Restriction fragment length polymorphism |
| ssDNA | Single stranded DNA |

# Table of contents

# 1. Introduction

The work in this thesis was performed on *Lactococcus garvieae* isolated from rainbow trout (*Oncorhynchus mykiss*) with clinical signs of infection, from fisheries in Spain and Turkey. This introduction will give a brief review of the genus *Lactococcus* in general, with emphasis on *L. garvieae*.

## 1.1 The Genus Lactococcus

The genus *Lactococcus* is in the phylum *Firmicutes*, the class *Bacilli*, the order *Lactobacillales* and the family *Streptococcaceae*. Bacteria in this genus are described as Gram-positive spherical or ovoid coccus that usually occur in pairs and short chains (Figure 1-1). They are non-motile, facultative anaerobic, non-spore forming bacteria that does not produce catalase or oxidase. They usually grow at 10 ºC, but not at 45 ºC (Teuber, 2009). Cultivation is usually done in 4 % (w/v) sodium chloride, except for *Lactococcus lactis* subsp. *cremoris* which tolerates only 2 % (w/v) sodium chloride (Teuber, 2009). They are chemo-organotroph and have a fermentative metabolism. *Lactococcus* species do not have a full functional respiratory system and obtain energy from substrate-level phosphorylation. They are homofermentative, which means they produce lactic acid as the major or only product during glucose fermentation. Due to oxygen metabolizing enzymes like superoxide dismutase or NADH oxidases, and specific enzyme activity from the Krebs cycle, *Lactococcus* can grow in the presence of oxygen even though they do not have a full Krebs cycle (citric acid cycle) and respiratory system (Duwat et al., 2001; Teuber, 1995).

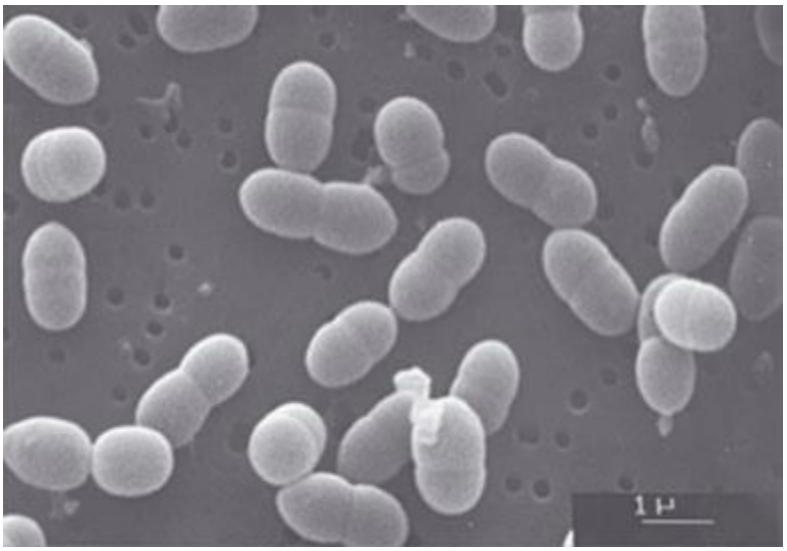



*Figure 1-1. Electron microscopy of Lactococcus lactis subsp. lactis. Figure is derived from (Neve et al., 1987).*

### *1.1.1 Classification and phylogeny*

*Lactococcus* can be isolated from spontaneously fermented milk. Some members of the genus are of high importance to the dairy industry as they are part of commercial starter cultures used for fermented milks, sour cream, fermented butter, unripen cheeses (cottage cheese and cream cheeses) and matured cheeses (Caballero et al., 2003). In 1857, Louis Pasteur was the first to claim that lactic fermentation of raw milk was caused by a microbe (Teuber & Geis, 2006). In his attempt to prove Pasteur's theory, Joseph Lister (1873) was the first to obtain and scientifically describe a pure bacterial culture. He named the bacterial culture *Bacterium lactis*, now known *Lactococcus lactis*. Similarity among strains discovered in fermented dairy products, made Löhnis (1909) rename this to *Streptococcus lactis*. Orla-Jensen (1919) used a set of criteria (cellular morphology, mode of glucose fermentation, sugar utilization patterns and temperature ranges of growth) that divided lactic acid bacteria (LAB) into four genera; *Lactobacillus, Pediococcus, Leuconostoc* and *Streptococcus.* His taxonomy divided the mesophilic lactic streptococci to *Streptococcus lactis* and *Streptococcus cremoris*. Mesophilic bacteria grow best at moderate temperatures, typically 20-45 ºC.

A classification based on a system developed by Rebeca Lancefield (1933) divided catalase-negative and coagulase-negative bacteria into groups based on the carbohydrate composition of bacterial antigen in the cell wall. This put the lactic streptococci in the group N, clearly separated them from enterococci (group D) and the pathogenic streptococci (groups A, B, C). A reclassification of the genus in 1985 based on nucleic acid hybridization and comparative immunological studies divided the genus into three genera: *Streptococcus, Enterococcus* and *Lactococcus* (Schleifer et al., 1985). DNA-DNA hybridization is considered the gold standard

to distinguish between bacterial species and is a method were you incubate a mixture of DNA from the species you want to compare (Sibley & Ahlquist, 1981). When the mixture is heated, the DNA-strands are dissociated, and after a cooling step, new hybrid double-stranded DNAs are formed. To measure the degree of similarity between the species, the DNA is bound to a column and heated in small steps. High degree of similarity needs more energy to separate the strains (Sibley & Ahlquist, 1981). Based on DNA-DNA hybridization in 1987, *Lactococcus lactis* and related organisms formed a single DNA homology group as seen in Figure 1-2, (Schleifer & Kilpper-Bälz, 1987). Furthermore, due to phenotypic differences the group was subdivided into three subspecies: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, Lactococcus *lactis* subsp. *hordniae*. Subspecies are too distantly related to be regarded as the same species, but too closely related to be regarded as a different species. Table 1-1 summarizes biochemical and chemical characteristics in the genus Lactocuccus found by Schleifer et al. (1985).

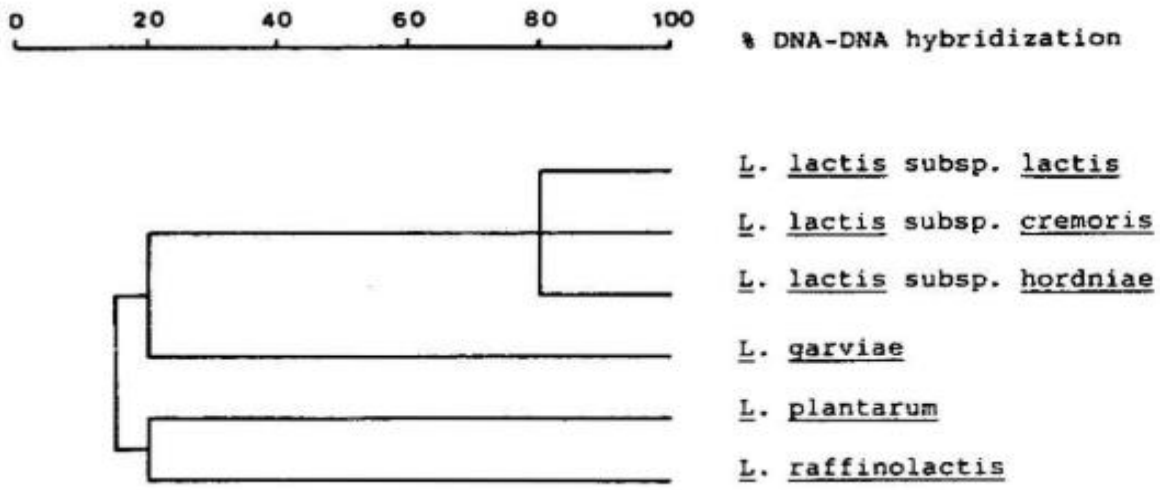



*Figure 1-2. Dendrogram from 1987 of lactococci based on DNA-DNA hybridization studies under optimal conditions. Picture from (Schleifer & Kilpper-Bälz, 1987).*

Table 1-1. Biochemical and chemical characteristics in the genus Lactocuccus found by Schleifer et al. (1985) dividing the genus into species and subspecies.

| | | | Acid production from | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species/subspecies | Murein type | Hydrolysis of Arginine | Galac-tose | Lactose | Maltose | Meli-biose | Meli-zitose | Raffin-ose | Ribose |
| *L. lactis* subsp. *lactis* | Lys-D-Asp | + | + | + | + | - | - | - | + |
| *L. lactis* subsp. *cremoris* | Lys-D-Asp | - | + | + | - | - | - | - | - |
| *L. lactis* subsp. *hordniae* | Lys-D-Asp | + | - | - | - | - | - | - | - |
| *L. garvieae* | Lys-Ala-Gly-Ala | + | + | + | v | v | - | - | + |
| *L. plantarum* | Lys-Ser-Ala | - | - | - | + | - | + | - | - |
| *L. raffinolactis* | Lys-Thr-Ala | v | + | + | + | + | v | + | v |

v: variable reaction, +: positive reaction, -: negative reaction

Recent comparative genomic analysis of the Lactococcus genus has shown that the genus is relatively small and diverse (Yu et al., 2017). Figure 1-3 shows the phylogenetic relationship among species and subspecies within the *Lactococcus* genus based on concatenated amino acid sequences of 643 core genes and 16S rRNA sequences (Yu et al., 2017). Both the concatenated tree and the 16s rRNA tree are divided into two branches with *L. garvieae* in the same branch as *L. lactis.*

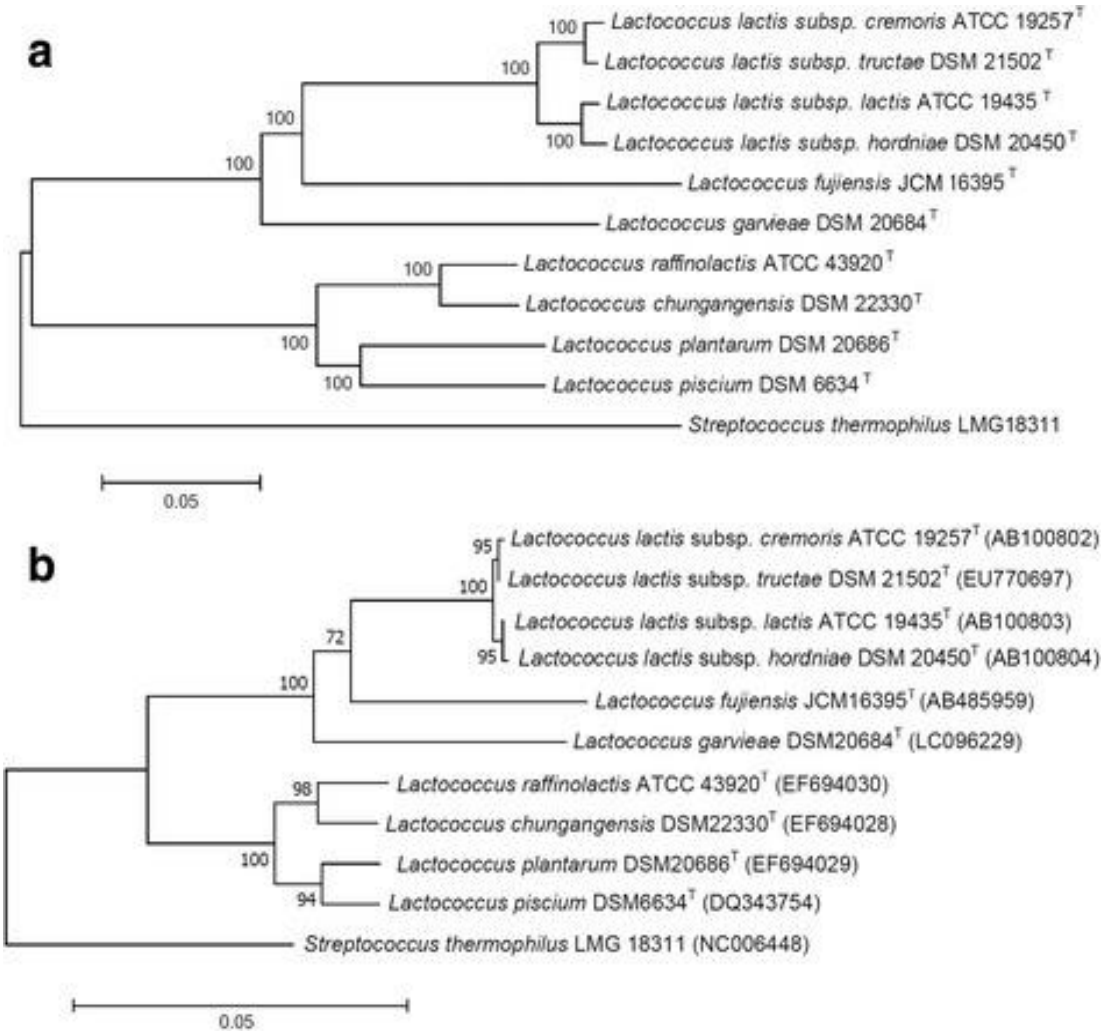



*Figure 1-3. Phylogenetic tree of the relationship among species and subspecies in the genus Lactococcus based on* ***a)*** *concatenated amino acid sequences of 643 core genes and* ***b)*** *16S rRNA sequences. The trees are constructed by maximum-likelihood and neighbor-joining methods, respectively. Streptococcus thermophilus served as outgroup. The scale bar indicates 0,05 changes in substitutions per site. Figure derived from (Yu et al., 2017)*

Currently (May 2019) there are 14 species and 6 subspecies in the genus *Lactococcus* according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN), as summarized in Table 1-2. (http://www.bacterio.net/lactococcus.html)

Table 1-2. Recognized species and subspecies in the genus *Lactococcus* as of May 2019 according to LPSN.

| Species | Subspecies | Source | References |
|---|---|---|---|
| *L. lactis* | *lactis* | Fermented milk | (Lister, 1873; Schleifer et al., 1985) |
| | *cremoris* | Fermented milk | (Orla-Jensen, 1919; Schleifer et al., 1985) |
| | *hordniae* | Leaf hopper | (Latorre-Guzman et al., 1977; Schleifer et al., 1985) |
| | *tructae* | Intestinal mucus of brown trout and rainbow trout | (Pérez et al., 2011) |
| *L. garvieae* | | Bovine mastitis | (Collins et al., 1983; Garvie et al., 1981; Schleifer et al., 1985) |
| | *bovis* | Indian bison, Wild gaur | (Varsha & Nampoothiri, 2016) |
| | *garvieae* | | |
| *L. plantarum* | | Pea plant | (Collins et al., 1983; Meucci et al., 2015; Schleifer et al., 1985) |
| *L. raffinolactis* | | | (Orla-Jensen & Hansen, 1932; Schleifer et al., 1985) |
| *L. chungangensis* | | Sludge foam | (Cho et al., 2008) |
| *L. formosensis* | | Fermented broccoli stems | (Chen et al., 2014) |
| *L. fujiensis* | | Vegetable matter | (Cai et al., 2011) |
| *L. hircilactis* | | Goat milk | (Meucci et al., 2015) |
| *L. laudensis* | | Goat milk | (Meucci et al., 2015) |
| *L. nasutitermitis* | | Termite gut | (Yang et al., 2016) |
| *L. petauri* | | Abscess of a sugar glider | (Goodman et al., 2017) |
| *L. piscium* | | Salmonid fish | (Williams et al., 1990) |
| *L. reticulitermitis* | | Termite gut | (Yuki et al., 2018) |
| *L. taiwanensis* | | Cummingcordia | (Chen et al., 2013) |

### *1.1.2 Clinical significance*

Members of the genus *Lactococcu*s have mainly been isolated from food-related sources and are **g**enerally **r**egarded **a**s **s**afe (GRAS). However, there are some species with clinical significance. *L. lactis* have been isolated from human urinary tract, wound infections and patients with endocarditis (Aguirre & Collins, 1993; Mannion & Rothburn, 1990; Zechini et al., 2006). *L. lactis* subsp. *hordniae* is commonly associated with infection in leafhopper (Latorre-Guzman et al., 1977). *L. piscium* has been isolated in spoiled vacuum-packed chilled meat (Sakala et al., 2002) and in one reported incident the strain has been isolated in diseased rainbow trout (Williams et al., 1990). *L. garvieae* is considered an emerging pathogen in veterinary and human medicine (Facklam & Elliott, 1995; Teixeira et al., 1996; Vela et al., 2000). The disease in fish is called Lactococcosis and has spread in several countries and is the cause of significant economic losses in aquaculture worldwide (Vendrell et al., 2006). Even

though there has been findings of *L. garvieae* in dairy (Alegría et al., 2009; Fortina et al., 2003; Foschino et al., 2006; Yu et al., 2015), this species is considered mainly a fish pathogen as well as the causative agent for mastitis in cows and buffalos (Teixeira et al., 1996; Teuber, 2009).

## 1.2 Lactococcus garvieae

*Lactococcus garvieae,* initially named *Streptococcus garvieae (Collins et al., 1983),* was first isolated as a causative agent of bovine mastitis (Garvie et al., 1981) and has been identified as reference strain ATCC 43921. As mentioned before, there was a reclassification in 1985 of the *Streptococcus* genus based on nucleic acid hybridization and comparative immunological studies, which transferred *L. garvieae* to the *Lactococcus* genus along with other lactic streptococci like *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris (Schleifer et al., 1985).*

Since the first report in 1981 (Garvie et al.), *L. garvieae* has been isolated from pigs with pneumonia and cows and water buffaloes with subclinical mastitis (Collins et al., 1983; Teixeira et al., 1996; Tejedor et al., 2011). In humans, *L. garvieae* causes a variety of infections and because of the increasing number of infections it is now considered an opportunistic pathogen (Chan et al., 2011; Russo et al., 2012; Suh et al., 2016). However isolation of *L. garvieae* in faeces from healthy humans raises the question that not all humans are susceptible to *L. garvieae* infections or that not all strains are pathogenic to humans (Kubota et al., 2010).

The first infection by a Gram-positive coccus in rainbow trout (*Oncorhynchus mykiss)* was reported in Japan in 1958 (Hoshina, 1958) and the causative agent was identified as a *Streptococcus*. It was suggested in 1991, for the purpose of bringing together several Gram-positive isolates from streptococcosis outbreaks in Japanese yellowtail (*Seriola* sp.), as a new species *Enterococcus seriolicida* (Kusuda et al., 1991) and ATCC 49156 was suggested as type strain. In 1988 the first outbreak of lactococcosis was reported from Spanish farms, and in the following years the agent was described as *Enterococcus seriolicida* (Vendrell et al., 2006). *E. seriolicida* was later reclassified as a junior synonym of *L. garvieae* (Eldar et al., 1996).

### *1.2.1 Lactococcosis in aquaculture*

According to the Food and Agriculture Organization of the United Nations (FAO) there is increasing demand for seafood for human consumption worldwide. The fact that this demand cannot be satisfied solely by wild fisheries, has made an increasing expansion in fish farming the past century (FAO, 2018b). Since 1980, capture fisheries have been relative static while aquaculture has had a substantial growth in supply of fish for human consumption (Figure 1-4). The world aquaculture production was 80 million tons in 2016, which is 47% of the total global fish production (FAO, 2018a).

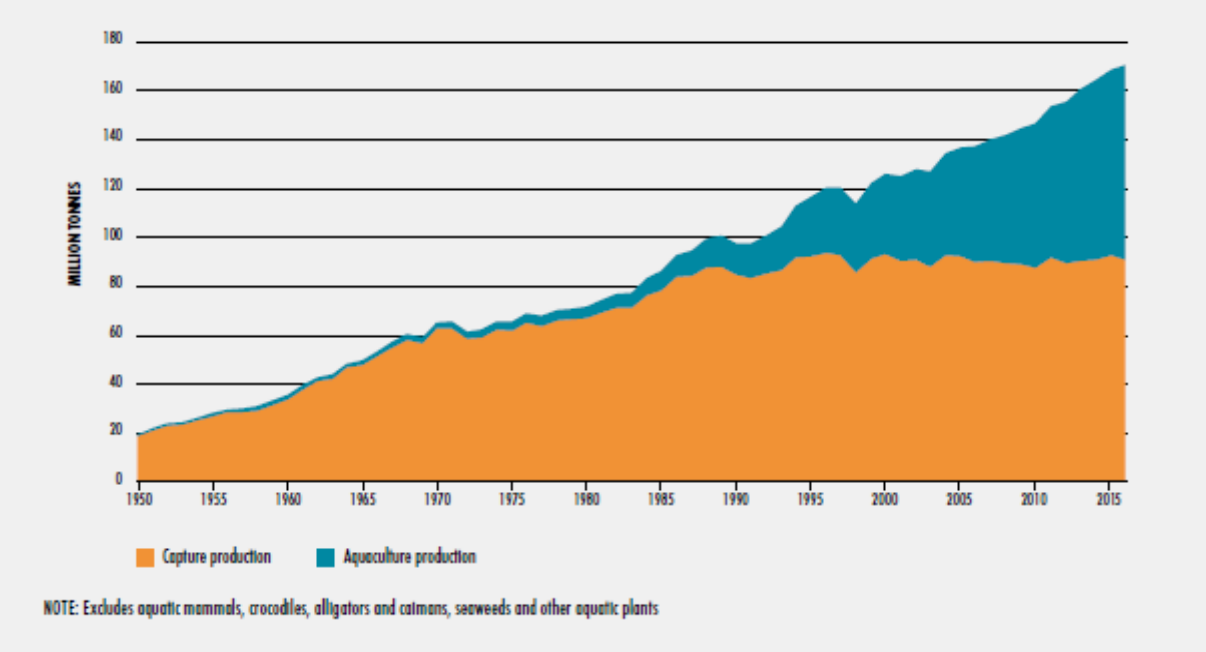



*Figure 1-4. World capture fisheries and aquaculture production from 1950 to 2016. Figure derived from (FAO, 2018b)*

In the growing aquaculture industry, losses due to infectious diseases by viruses, bacteria, protozoa and trematodes is increasing (Austin & Austin, 2012). In marine fish, pathogenic bacteria have been described in the majority of existing taxonomic groups, but only a small number of these pathogens cause important economic losses in cultured fish worldwide. According to Toranzo et al. (2005) the most important bacterial diseases in economically important fish in aquaculture are diseases caused by Gram-negative bacteria like *Vibrio* sp. (vibriosis), *Aeromonas salmonicida* (furunculosis), *Moritella viscosa* (winter ulcer), *Photobacterium damselae* subsp. *piscicida* (photobacteriosis), *Pasteurella skyensis* (pasteurellosis), *Piscirickettsia salmonis* (piscirickettsiosis) and *Tenacibaculum maritimum* (flexibacteriosis).

The most important Gram-positive bacteria are *Mycobacterium marinum* (mycobacteriosis), *Renibacterium salmoninarum* (BKD), some *Streptococcus* sp. (streptococcosis) and *Lactococcus garvieae* (lactococcosis) (Toranzo et al., 2005).

The latter disease, lactococcosis, usually occurs when the water temperature rises to about 16 ºC. According to epidemiological data, carrier fish remains asymptomatic when the water temperature is less than 13 ºC (Algöet et al., 2009; Vendrell et al., 2006). Figure 1-5 show distribution of *L. garvieae* and pathogenic *Streptococcus* species over the continents (Mishra et al., 2018). *L. garvieae* has been isolated from both marine species and fresh water species (Table 1-3) and the disease is considered an emerging disease in aquaculture and is also a potential zoonotic pathogen (Vendrell et al., 2006). It is reported that the most sensitive fish species which suffers acute disease with raised mortality, is rainbow trout (Ghittino & Muzquiz, 1998; Vendrell et al., 2006).

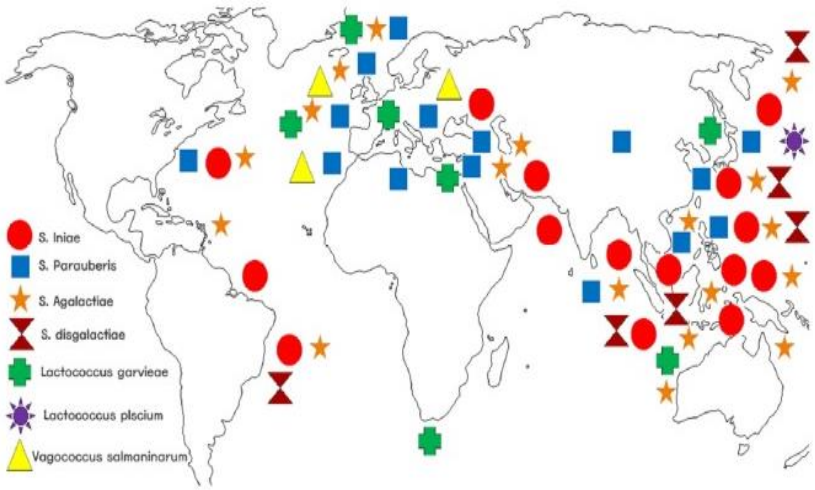



*Figure 1-5. Distribution of major Streptococcal pathogens. L. garvieae can be seen as green plus sign. Other pathogens are Streptococcus iniae (red circle), S. parauberis (blue square), S. dysgalactiae (brown inverted box), S. agalactiae (orange star), Vagococcus salmoninarum (yellow triangle) and Lactococcus piscium (violet sun). The picture is derived from (Mishra et al., 2018).*

Table 1-3. List of different aquatic host of *L. garvieae* found in literature

| **Host** | **Country** | **Reference** |
|---|---|---|
| Rainbow trout, *Oncorhynchus mykiss* (Walbaum) | South Africa | (Boomker et al., 1979; Meyburgh et al., 2018) |
| | Australia | (Bragg & Broere, 1986) |
| | UK | (Bark & McGregor, 2001; Carson et al., 1993) |
| | Taiwan | (Chang et al., 2002) |
| | France | (Eyngor et al., 2004) |
| | Bulgaria, Israel, Portugal | (Pereira et al., 2004) |
| | Greece | (Savvidis et al., 2007) |
| | Iran | (Milad et al., 2014; Sharifiyazdi et al., 2010) |
| | Spain | (Aguado-Urda et al., 2011; Domenech et al., 1993; Palacios et al., 1993) |
| | Italy | (Reimundo et al., 2011) |
| | Turkey | (Didinen et al., 2014) |
| | India | (Shahi et al., 2018) |
| Japanese eel, *Anguilla japonica* (Temminck & Schlegel) | Japan | (Kusuda et al., 1991) |
| Red sea wrasse, *Coris aygula* (Lacépède) | Israel | (Colorni et al., 2003) |
| Brazil Nile tilapia, *Oreochromis niloticus L.* | Brazil | (Evans et al., 2009) |
| Pintado, *Pseudoplathystoma corruscans* (Spix & Agassiz) | Brazil | (Evans et al., 2009) |
| Olive flounder, *Paralichthys olivaceous* (Temminck & Schlegel) | Japan | (Kawanishi et al., 2006) |
| Yellowtail amberjack, *Seriola dumerili* (Risso) | Japan | (Kawanishi et al., 2006) |
| Kingfish, *Seriola quinqueradiata* (Temminck & Schlegel) | Japan | (Kawanishi et al., 2006) |
| Grey mullet, *Mugil cephalus L.* | Taiwan | (Chen et al., 2002) |
| Catfish *Silurus glanis L.* | Italy | (Ravelo et al., 2003) |
| Freshwater prawn *Macrobrachium rosenbergii* (De Man) | Taiwan | (Chen et al., 2001) |
| Bottlenose dolphin *Tursiops truncatus* (Montagu) | Kuwait | (Evans et al., 2006) |
| Common octopus, *Octopus vulgaris* (Cuvier) | Italy | (Fichi et al., 2015) |

### *1.2.2 Clinical signs and symptoms*

Lactococcosis in rainbow trout is reported as a sudden onset hyperacute systemic disease (Austin & Austin, 2012). Early symptoms are erratic “head up” -swimming, anorexia, lethargy and melanosis. External signs are characterized by uni- or bilateral exophthalmia, and dark pigmentation, swollen abdomen, anal prolapses and hemorrhaging of the eye (Bragg & Broere, 1986; Eldar & Ghittino, 1999). Figure 1-6 shows hemorrhaging of the eye in rainbow trout. Internally there is severe hemorrhaging and congestion of the blood vessels (Eldar & Ghittino, 1999).

Clinical signs of *L. garvieae* in yellowtail (*Seriola* sp.) are accumulation of ascetic fluid in peritoneal cavity, damage to the liver, kidney, spleen and intestine (Austin & Austin, 2012; Kusuda et al., 1991).

*Figure 1-6. Hemorrhaging in the eye of a rainbow trout caused by L. garvieae. Picture from Austin & Austin (2012)*

## 1.3 Characterization of *L. garvieae*

### *1.3.1 Morphology, phenotypic and biochemical characteristics.*

*L. garvieae* is described as Gram-positive ovoid coccus that usually occur in pairs and short chains. They are non-motile, facultative anaerobic, non-spore forming bacteria that produces α-hemolysis on blood agar (Gibello et al., 2016), but there has also been reports on β-hemolysis (Domenech et al., 1993). *L. garvieae* does not produce catalase or oxidase. Growth temperatures ranges from 4 ºC to 45 ºC with optimal growth temperature at 37 ºC (Vendrell et al., 2006), however some has weak and slow growth at 45 ºC (Domenech et al., 1993; Kusuda et al., 1991). It can grow in 0-6.5 % (w/v) sodium chloride at pH 4.5-9.6 and the mol % G + C of the DNA is 44 (Austin & Austin, 2012; Boomker et al., 1979; Eldar et al., 1996; Kusuda et al., 1991). Phenotypic characteristics are listed in Table 1-4.

Table 1-4. Phenotypic, physiological and biochemical characteristic of *L. garvieae* (Vendrell et al., 2006)

| **Characteristic** | **Reaction** | **Characteristic** | **Reaction** |
|---|---|---|---|
| Cell morphology | Ovoid cocci | **Production of:** | |
| Gram stain | + | Arginine | + |
| Motility | - | Ornithine | - |
| Lancefield group | N | Lysine | - |
| **Growth:** | | **Acid from:** | |
| 4 ºC | + | Glycerol | - |
| 20 ºC | + | Raffinose | - |
| 37 ºC | + | Arabinose | - |
| 45 ºC | + | Sorbitol | + |
| pH 9.6 | + | Mannitol | + |
| 6.5 % NaCl | + | Cellobiose | + |
| Hemolysis | α | Galactose | + |
| Catalase | - | D-Glucose | + |
| Oxidase | - | Maltose | + |
| TSI | A/A- | Trehalose | + |
| Oxidative/fermentative | F | D-Mannose | + |
| Nitrate reduction | - | Inositol | - |
| Citrate | - | Lactose | (+) |
| Urea | - | Ribose | V |
| Indol production | - | Sucrose | V |
| Esculin | + | Adonitol | - |
| VP | + | Glycogen | - |
| $H_2S$-production | - | Melibiose | - |
| Arginine dihydrolase | + | Melezitose | - |
| Pyrrolidonyl arylamidase | + | Starch | - |
| Alkaline phosphotase | - | Tagatose | V |
| β-Glucorinase | V | L-Rhamnose | - |
| Leucine arylamidase | + | D-Xylose | - |
| Sodium Hippurate hydrolisis | - | Salicin | + |

V: variable reaction. +: positive reaction, -: negative reaction
(): weak or slow reaction. A/A-: acidification of medium and $H_2S$ not produced.

*L. garvieae* is phenotypically very similar to *L. lactis* and they can barely be differentiated by biochemical and antigenic characteristic. It is possible to differentiate them a with specific Polymerase Chain Reaction (PCR) (Elliott & Facklam, 1996; Zlotkin et al., 1998) and *L. garvieae* is reported to be resistant to clindamycin, whereas *L. lactis* is not (Elliott & Facklam, 1996) .

*L. garvieae* strains have been divided into biotypes based on fermentation of sugars and presence of enzymes (Eldar et al., 1999; Vela et al., 2000; Vela et al., 1999). Eldar et al. (1999) established three biotypes for *L. garvieae* based on fermentation of tagatose, ribose and sucrose (saccharose) and Vela et al. (2000) established thirteen biotypes based on fermentation of sugars (saccharose, tagatose, mannitol and cyclodextrin) and presence of the enzymes pyroglutamic acid arylamidase and N-acetyl-β-glucosaminidase.

### *1.3.2 Genotypic characterization*

Phenotypically and genetically *L. garvieae* is described as heterogenous (Eldar et al., 1999; Reguera-Brito et al., 2016; Vela et al., 2000). Identification of clonal genetic groups in trout has been found in genotypic studies using pulsed field gel electrophoreses (PFGE) (Kawanishi et al., 2006; Tsai et al., 2012; Vela et al., 2000). But among isolates from other hosts, environment or food, PFGE and multilocus sequence typing (MLST) has revealed major heterogeneity (Reguera-Brito et al., 2016; Tejedor et al., 2011). Various typing techniques used for molecular characterization for *L. garvieae* are listed in Table 1-5.

Table 1-5. Various typing techniques used for molecular characterization of *L. garvieae*

| **Techniques** | **Country** | **Source** | **Reference** |
|---|---|---|---|
| RFLP ribotyping | Japan, Italy, Spain, United Kingdom | Yellowtail, trout, cattle | (Eldar et al., 1999) |
| | Israel, Spain, France, Italy, Greece, Bulgaria | Rainbow trout | (Eyngor et al., 2004) |
| RAPD analysis | Spain, Italy, Portugal, England, Turkey, France, Japan, United Kingdom | Rainbow trout, catfish, yellowtail, cow | (Ravelo et al., 2003) |
| PFGE | Spain, Portugal, France, Italy, Brazil, USA, UK, Japan | Trout, yellowtail, water, buffalo, human, cow | (Vela et al., 2000) |
| | Japan, Spain, Italy, Belgium, United Kingdom | Yellowtail, amberjack, kingfish, trout, cow, pig, cat, dog, horse | (Kawanishi et al., 2006) |
| | Spain | Trout, cow, pig | (Tejedor et al., 2011) |
| | Taiwan | Freshwater prawn, tilapia, bullfrog, Japanese eel, yellowtail, grey/basket and borneo mullet, yellowfin seabream, grouper, cattle, human blood, squid, | (Tsai et al., 2012) |
| MLST/MLSA | United Kingdom, Italy, Spain, Japan | Rainbow trout, trout, yellowtail, Bovine mastitis, meat products, poultry, turkey, beef, salad, cow milk, cow cheese, celery, broccoli, wheat flour, human blood, mallard duck intestines | (Ferrario et al., 2013) |
| MLSA | Italy, Spain, France, USA, United Kingdom, | Rainbow trout, yellowtail, trout, poultry meat, turkey meat, human feces, dairy product, beef meat, cow milk, cow cheese, human urine, human blood, human native mitral valve, human ascetic fluid, human gall bladder, bovine mastitis, salad, celery, broccoli, wheat flour | (Reguera-Brito et al., 2016) |

## 1.4 Virulence factors in L. garvieae

Bacteria has a wide repertoire of virulence factors to help them to attach to a host, entry into and exit out of cells, immunoevasion (evasion of the immune response of the host), immunosuppression (inhibition of the immune response of the host) and obtain nutrition from the host. Infections caused by *L. garvieae* are increasing in both veterinary and human medicine. Despite this, there is a general lack a of knowledge and limited studies on the pathogenic mechanisms (Meyburgh et al., 2017; Ture & Altinok, 2016). Studies on hemolytic activity and the proteins that were involved, was done in the early 80's, and showed that exotoxins played a role in the pathogenesis of infections in yellowtail (Kimura & Kusuda, 1982). Investigations on the relevance of siderophores in iron uptake has shown that siderophore may play a role in the virulence of *L. garvieae* (Schmidtke & Carson, 2003). Kawanishi et al. (2007) investigated the relevance of a capsule, and for a long time the presence of a capsule was considered the major virulence factor in *L. garvieae*. However, the capsule that was identified in *L. garvieae* from yellowtail in Japan, has not been detected in *L. garvieae* in rainbow trout from Turkey, Spain, Italy, France and USA, nor in all *L. garvieae* that caused disease in yellowtail in Japan (Nelson et al., 2016; Nishiki et al., 2016; Oinaka et al., 2015; Ture et al., 2015; Ture & Altinok, 2016) suggesting that other potential virulence factors may play a role in *L. garvieae* infections.

### *1.4.1 Capsule formation*

Some bacteria produce exopolysaccharides (EPSs) in the form of mucus which is released into the medium and some bacteria produces polysaccharides that forms a capsule. A capsule is a gelatinous outer layer secreted by the bacteria that surrounds and adheres to the cell wall and cannot be readily washed off (Wen & Zhang, 2015). Capsules prevents phagocytosis and therefore plays a significant role during infection and invasion in bacterial pathogens. *Streptococcus pneumonia* is a well-known pathogen were the capsule plays an important role in the pathogenicity (Mäkelä & Butler, 2008). The capsule in most bacteria are composed of polysaccharides, but some are composed of polypeptides and in most cases the capsular polysaccharides (CPSs) are negatively charged (acidic) (Wen & Zhang, 2015). The production of EPS in lactococci are common and an important trait for production of sour milk and cheese (von Wright & Tynkkynen, 1987).

Early work on *L. garvieae* from yellowtail in Japan, divided it into two antigenic types, KG+ and KG–, by biochemical tests (Kitao, 1982). Antibody to KG– agglutinated to both KG– and KG+ isolates, but antibody to KG+ agglutinated only to KG+ isolates.

Kitao (1982) suggested that an envelope-like substance surrounding the KG– isolates inhibits the agglutination and makes them more pathogenic in yellowtail than the KG+ isolate. Electron microscopy by Yoshida (1997) verified the presence of a capsule in the KG– isolates and reports verifies that a capsule increases the resistance to opsonophagocytosis by yellowtail macrophages (Ooyama et al., 2002; Yoshida et al., 1996).

A capsule gene cluster consisting of 16.5 kb, has been located in *L. garvieae* isolated from yellowtail. It is thought to be located on a genomic island due to the fact that there are insertion sequences, IS982 elements, flanked on both end of the fifteen genes in the gene cluster (Morita et al., 2011). Eight of the fifteen genes (*epsRXABCD* and *cpsLW*) in the capsule gene cluster were conserved in the EPS biosynthesis gene cluster of three *L. lactis* strains isolated from human faeces. *L. lactis* K147 had seven of the fifteen genes, only missing epsR (Figure 1-7).

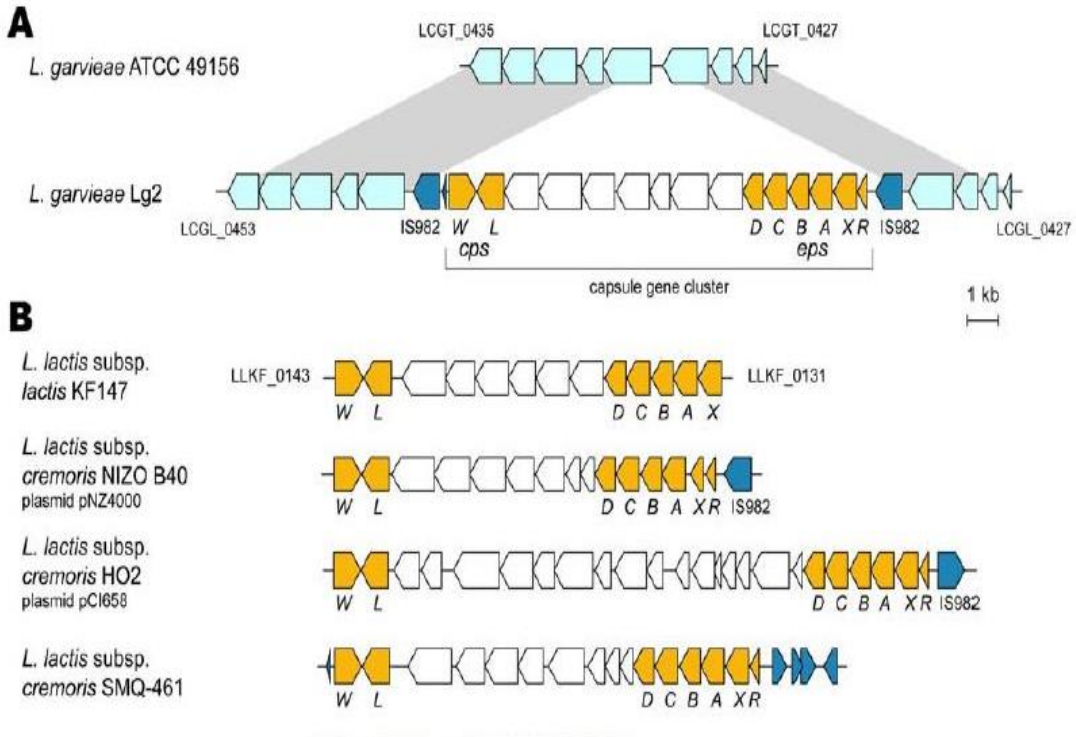



*Figure 1-7. Arrangement of the genes in the gene cluster in L. garvieae isolate Lg2 in comparison to the corresponding region in L. garvieae ATCC 49156 and L. lactis strains. Orientations are shown by arrows and genes colored orange are conserved genes in the gene cluster, blue color are transposase genes and white color are other genes in the capsule gene cluster. Grey bars are orthologous regions.* ***A:*** *Arrangement of the genes in the gene cluster in L. garvieae isolate Lg2 in comparison to the corresponding region in L. garvieae ATCC 49156.* ***B****: The capsule gene cluster in L. lactis strains. Figure from (Morita et al., 2011)*

Morita (2011) also reported that subculturing *L. garvieae* Lg2 resulted in the less virulent Lg2-S with a single deletion in the *cpsL* (conserved hypothetical protein) and *epsD* (glycosyltransferase) indicating the importance of these to genes in the capsular gene cluster. The Lg2-S was KG+ and was non-capsulated, whereas Lg2 was KG– and capsulated.

The polysaccharide capsule has been widely regarded as the major virulence factor in *L. garvieae*, but recent reports shows that non-capsulated strains Lgper and ATCC 49156 are pathogenic to rainbow trout with 89% and 98% mortality, respectively (Ture et al., 2014). Furthermore, there have been no detections of a capsule gene cluster in several pathogenic strains of *L. garvieae* from both rainbow trout from Spain, Turkey, Italy, France, USA, India, South- Africa and from yellowtail in Japan (Meyburgh et al., 2018; Nelson et al., 2016; Nishiki et al., 2016; Oinaka et al., 2015; Shahi et al., 2018; Ture et al., 2014; Ture et al., 2015; Ture & Altinok, 2016). Hence the presence of a capsule in *L. garvieae* cannot be the only virulence factor directly linked to pathogenicity.

### *1.4.2 Other virulence factors*

A presence of a hemolytic toxin in the culture supernatant was detected in an early study of a non-Lancefield *Streptococcus* sp. from yellowtail (most likely *L. garvieae*) (Kusuda & Hamaguchi, 1988; Meyburgh et al., 2017) .

*L. garvieae* isolated in dairy are found to be non-pathogenic to fish and humans and differs from fish-isolates in their ability to assimilate lactose. *L. garvieae* from fish are unable to assimilate lactose, but in spite of that they are able to grow in milk (Fortina et al., 2009). The genes for utilization of lactose was initially thought to be specific to dairy isolates, but they were also found in strains from other sources like cows, humans, birds and pigs (Aguado-Urda et al., 2010).

Several genes encoding putative virulence factors were identified in *L. garvieae* Lg2 from yellowtail (Morita et al., 2011) and Miyauchi et al. (2012) proposed a list of possible virulence genes responsible for *L. garvieae* virulence in addition to the capsule gene cluster (Table 1-6).

Table 1-6. Putative virulence genes in *L. garvieae* Lg2 identified by Miyauchi et al. (2012) and studied by Ture & Altinok (2016)

| Gene | Role in virulence | Reference |
|---|---|---|
| *Hemolysin* 1 (*hly*1) | Destroys the cell membrane and causes lysis of red blood cells | (Kawanishi et al., 2007) |
| *Hemolysin* 2 (*hly*2) | | |
| *Hemolysin* 3 (*hly*3) | | |
| *NADH oxidase* | Enzymes that serves for survival of pathogen in aerobic environments. | (Mitchell, 2003) |
| *Superoxide dismutase* (*sod*) | | |
| *Phosphoglucomutase* (*pgm*) | Enzyme interconverts glucose-6-phosphate and glucose-1-phosphate and play a role in polysaccharide capsule production. | (Hardy et al., 2000) |
| *Adhesin* PsA (*adh*PsA) | Divalent metal-ion-binding lipoprotein in the ABC transport system. | (Kadioglu et al., 2008; Mitchell, 2003) |
| *Adhesin* Pav (*adh*Pav) | Sequence similarity to pneumococcal surface adhesins. Adhesins facilitates adherence to other cells or surfaces | |
| *Enolase* (*eno*) | | |
| *LPxTG*-containing surface protein (*LPxTG*-1) | Protein for covalent anchoring to the peptidoglycan matrix. | (Marraffini et al., 2006) |
| *LPxTG*-containing surface protein (*LPxTG*-2) | | |
| *LPxTG*-containing surface protein (*LPxTG*-3) | | |
| *LPxTG*-containing surface protein (*LPxTG*-4) | | |
| *Adhesin* (*adh*) | Adhesins facilitates adherence to other cells or surfaces | (Kadioglu et al., 2008; Mitchell, 2003) |
| *Adhesin* cluster | | |

## 1.5 Aim of the study

Based on current literature, species of *L. garvieae* are considered to be phenotypically and genetically heterogenous bacteria, consisting of clonal groups. To date, few studies have been performed to characterize *L. garvieae* from diseased fish with respect to pathogenicity and virulence factors. The virulence factor that has been most studied is the capsule gene cluster discovered in *L. garvieae* from yellowtail in Japan, but a capsule does not seem to be present in virulent *L. garvieae* from rainbow trout.

Based on this, the study presented herein have two main objectives:

1. Identify *L. garvieae* strains isolated from clinical outbreaks in farmed rainbow trout in Spain and Turkey using various phenotypic end genotypic methods.
2. Screen the strains for virulence factors, study their phylogenetic relationship and compare features of the new isolates to strains isolated from other geographical regions and fish species.

The different methods used will be further described under chapter 3.

# 2. Materials

## 2.1 Bacterial strains

Table 2-1. List of bacterial strains and their origin used in this work.

| Species | Strain name | Country | Source | Reference | Accession no. |
|---|---|---|---|---|---|
| *L.garvieae* | AL 20 869 | Turkey | Rainbow trout | PHQ[1] | - |
| *L.garvieae* | AL 20 870 | Turkey | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 20 906 | Spain | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 20 909 | Spain | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 20 910 | Spain | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 20 911 | Spain | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 21 045 | Turkey | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 21 046 | Turkey | Rainbow trout | PHQ | - |
| *L.garvieae* | AL 21 225 | Spain | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 226 | Spain | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 227 | Spain | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 228 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 229 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 230 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 231 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 232 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 233 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 234 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 235 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 236 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 237 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 238 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 239 | Turkey | Rainbow trout | This work | - |
| *L.garvieae* | AL 21 240 | Turkey | Rainbow trout | This work | - |
| *S. agalactiae* serotype III | AL 12110 | Malaysia | Tilapia | PHQ | - |
| *S. agalactiae* serotype Ib | AL 12111 | Costa Rica | Tilapia | PHQ | - |
| *S. iniae* | AL 12112 | Vietnam | Barramundi | PHQ | - |
| *S. agalactiae* serotype Ia | AL 12113 | Malaysia | Tilapia | PHQ | - |
| *L.garvieae* | ATCC 49156 | Japan | Yellowtail | (Morita et al., 2011) | KE136467 |
| *L.garvieae* | Lg2 | Japan | Yellowtail | (Morita et al., 2011) | NC_017490 |
| *L.garvieae* | 8831 | Spain | Rainbow trout | (Aguado-Urda et al., 2011) | NZ_AFCD00000000 |
| *L.garvieae* | UNIUD074 | Italy | Rainbow trout | (Reimundo et al., 2011) | NZ_AFHA00000000 |
| *L.garvieae* | PAQ102015-99 | USA | Rainbow trout | (Nelson et al., 2016) | NZ_LXWL00000000 |
| *L.garvieae* | LG9 | Italy | Rainbow trout | (Ricci et al., 2012) | NZ_AGQY00000000 |
| *L.garvieae* | 122061 | Japan | Yellowtail | (Nishiki et al., 2016) | NZ_AP017373 |
| *L. lactis* subsp. *lactis* | KF147 | New Zealand | Mung bean sprout | (Siezen et al., 2010) | NC_013656.1 |
| *L. lactis* subsp. *cremoris* | A76 | France | Cheese | (Bolotin et al., 2012) | CP003132 |

[1]PHQ: Provided by PHARMAQ AS.

Light blue rows are strains where only the gene sequences were used and not live material or DNA. The dark blue row; no living material from Lg2, but DNA was kindly provided Dr. Mustafa Ture, Central Fisheries and Research Institute, Turkey.

## 2.2 Primers

Table 2-2. Primers used in this work, their sequence and product size, description and reference. Working concentration for the primers were 25 µM and all were produced by Thermo Fisher.

| Primer | Sequence (5'-3') | Product size (bp) | Description and use | Reference |
|---|---|---|---|---|
| pLG-1 | CATAACAATGAGAATCGC | 1100 | ID-PCR, target gene 16S rRNA | (Zlotkin et al., 1998) |
| pLG-2 | GCACCCTCGCGGGTTG | | | |
| 27F | AGAGTTTGATCMTGGCTCAG | 1500 | 16S rRNA sequencing | (Lane, 1991) |
| 1492R | ACCTTGTTACGACTT | | | |
| gyrB-F | CATGCTGGTGGTAAATTTGG | 1464 | Detection galactose permease for identification and MLSA | (Fortina et al., 2003) |
| gyrB-R | GTCATCCATTTCTCCTAAACC | | | |
| als-F | ATTCGGCTCAGACTTAGTTG | 1076 | Detection of α-acetolactate synthase for MLSA | (Menendez et al., 2007) |
| als-R | TTCAGCTGCTTCAACATCAA | | | |
| gapC-F | AAGTTGGTATTAACGGTTTCG | 974 | Detection of glyceraldehyde-3-phosphatedehydrogenase for MLSA | (Ferrario et al., 2012) |
| gapC-R | AAGTGTACGAACGAGGTTAG | | | |
| rpoC-F | TTGGTCCACAAAAGGACTGG | 1377 | Detection of RNA polymerase β'-subunit for MLSA | (Ferrario et al., 2013) |
| rpoC-R | TCACGTCCTTTTGCTTCCAT | | | |
| galP-F | TGGGGAAAATTTAAACCTTGG | 1070 | Detection of galactose permease for MLSA | (Fortina et al., 2009) |
| galP-R | ATCATCAGAACGGCTGGAAG | | | |
| Cap2012-F | TGCTGTCATCATATTGTGTCCA | 16500 / 750 | Detection of capsule gene cluster | (Miyauchi et al., 2012) |
| Cap2012-R | GGCTATGGCATTAGTCAGGAAG | | | |
| 1020-F | ACCTTCACTTGCATTCATAGGGT | 304 | Multiplex PCR for detection of capsule | (Ture & Altinok, 2016) |
| 1323-R | TTGTCCCAGAGGGTTCTCCT | | | |
| 851-F | TAGGAGGTGTTCCTGGGAGG | 549 | | |
| 1399-R | TGTCCCACTCCTACTGTCGT | | | |
| 6329-F | AAAAACGGAGGGCAACAAGC | 785 | | |
| 7175-R | CACTTGTACAGGCCACTGGT | | | |
| 5358-F | TGGAGGGTATTGCCTACCGA | 650 | | |
| 6007-R | CCACAGCAGCTTCTTCACCT | | | |
| epsC-F | ACCGGGGATAGATGATGGGG | 217 | Detection of capsule gene cluster | This work |
| epsC-R | TCTCACCTCTTGTCCGGGTA | | | |
| epsR-F | ACCACCGGCTAAAGGAACTA | 206 | Detection of capsule gene cluster | This work |
| epsR-R | TGCAGAACTGTCATTAGGCTCA | | | |
| cpsW-F | ACCAGTGGCCTGTACAAGTG | 382 | Detection of capsule gene cluster | This work |
| cpsW-R | TGCTTCTATAGCCTACGCTGC | | | |
| AF-F | CAGCCAGCACCAGGTTATGA | 358 | Virulence factor Adhesin | (Ture & Altinok, 2016) |
| AF-R | CTCCTGCGTTGACATGGACT | | | |
| LP1-F | GTGAACGTGGAGCTTCCAGA | 878 | Virulence factor LPxTG-1 | (Ture & Altinok, 2016) |
| LP1-F | CCACTCACATGGGGGAGTTC | | | |
| LP4-F | GGGAGCACCGGATTCACTTT | 928 | Virulence factor LPxTG-1 | (Ture & Altinok, 2016) |
| LP4-R | ACAAAGCCGCAGACCTTACA | | | |

## 2.3 DNA-ladders

Table 2-3. DNA-ladders used in this work.

| Name | Concentration (µg/ml) | Producer | Product no. | Lot no. |
|---|---|---|---|---|
| 1 kb ladder[1] | 500 | New England BioLabs | N3232S | 1401701 |
| PCR marker[1] | 300 | New England BioLabs | N3234L | 0081401 |
| E-Gel 1 kb Plus ladder | 25 | Invitrogen | 10488090 | 00662854 |

1.Ladders from New England BioLabs are diluted 1 µl ladder, 1 µl loading buffer, 4 µl sterile MQ water.

## 2.4 Kits

Table 2-4. Kits used in this work and description of usage.

| Name | Description and use | Producer | Product no. | Lot no. |
|---|---|---|---|---|
| API Rapid ID 32 STREP | Identification of streptococci and related organism | Biomerieux | 32600 | 06576_E |
| VP A + VP B | Reagents needed for Rapid 32 STREP | Biomerieux | 70572 | 1006566720 |
| FB | Reagent needed for Rapid 32 STREP | Biomerieux | 70562 | 1006615550 |
| NIN | Reagent needed for Rapid 32 STREP | Biomerieux | 70491 | 1006324260 |
| QIAquick Gel Extraction | Gel extraction and cleanup | Qiagen | 28704 | 148024525 |

## 2.5 Computer programs

Table 2-5. Computer programs used in this work and description of usage

| Name | Description and use | Available from |
|---|---|---|
| BLAST | Identification and searching of sequences | https://blast.ncbi.nlm.nih.gov/Blast.cgi |
| MEGA 7.0.26 | Alignment of sequences | https://www.megasoftware.net/ |
| CLC Main workbench 8.0 | Modeltest and phylogenetic trees | https://www.qiagenbioinformatics.com/products/clc-main-workbench/ |
| Primer design tool | Design of primers | https://www.ncbi.nlm.nih.gov/tools/primer-blast/ |
| api*web*™ identification software | Identification of Rapid ID 32 STREP results | https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin |

## 2.6 Media, solutions, chemicals and consumables.

Table 2-6. List over media, solutions, chemicals and consumables used in this work with a short description of their use.

| Name | Description and use | Producer | Product no. | Lot no. |
|---|---|---|---|---|
| Blood agar 2% NaCl | Growth of bacteria and check for hemolysis | Media production Norwegian Veterinary institute | 06.002.00 | 180528-01 |
| Colombia agar with 5 % sheep blood | Bacteria culturing prior to API Rapid ID 32 Strep | Bio-Rad | 63784 | 64219254 |
| Crystal violet solution | Negative staining | Sigma-Aldrich | 109218 | HX85246118 |
| Dulbecco's Phosphate Buffered Saline | DNA isolation | Sigma-Aldrich | D8537 | RNBF5231 |
| E-Gel Sybr safe 1.2% | Gel electrophoresis | Invitrogen | G521801 | S281118 |
| ExoSAP-IT | PCR product clean up reagent | Affymetrix | 78201 | 4346501 |
| Gel Loading dye | Gel electrophoresis | New England BioLabs (NEB) | B7021S | 0011208 |
| Glycerol 85 % | Glycerol stocks of bacteria | Sanivo Pharma AS | 3057 | 17J054/3 |
| Hydrogen peroxide ($H_2O_2$) 35 % | Test for the enzyme catalase | Merck | 108600 | K46680900 |
| Illustra PuReTaq Ready-To-Go PCR Beads | PCR | GE Healthcare | 9799143/ 16890225 | 27955702 27955701 |
| InstaGene Matrix | DNA isolation | Bio-Rad | 7326030 | L007422B |
| Isopropanol | Gel extraction and cleanup | SIGMA | 59304 | BCBV7093 |
| Potassium hydroxide (KOH) | Distinguish between Gram-positive and Gram-negative | Merck | 105032 | B0475332112 |
| Nigrosine | Negative staining | Sigma-Aldrich | 1159240025 | FN1384524811 |
| Nuclease free water | PCR | Ambion | AM9530G | 1611318 |
| Oxidase | Diagnostic test for the detection of the cytochrome oxidase activity of microorganisms | Merck | 40560 | HC606296 |
| SeaPlaque™ Agarose | Gel electrophoresis | Lonza / Ficher Scientific | BMA50100 | 0000748142 |
| SYBR™ Safe DNA gel stain | Gel electrophoresis | Invitrogen | S33102 | 1988948 |
| 10 X TAE buffer | Gel electrophoresis | Invitrogen | 15558-042 | 2038353 |
| Tryptic Soy broth (TSB) | Growth of bacteria | Media production Norwegian Veterinary institute | 02.094.00 | 180608-01 |

## 2.7 Laboratory equipment

Table 2-7. List over equipment and machines used in this work. Common laboratory equipment is not listed.

| Equipment | Model | Producer |
|---|---|---|
| Autoclave | Matachana S1000 | Thune Products AS |
| Biofreezer (< -70 °C) | FORMA 8600 | Thermo Scientific |
| Heating block I | QBT2 | Grant |
| Heating block II | Dri-Block DB-2D | Techne |
| Electrophoresis power supply | PAC 300 | BioRad |
| Electrophoresis tank | Wide Mini-Sub Cell GT | BioRad |
| E-Gel™ Power Snap Electrophoresis Device | G8100 | Invitrogen |
| Gel documentation | GelDoc-1000 | BioRad |
| Incubator (22 °C) | Minitron | Infors |
| Microscope I | DMLS | Leica |
| Microscope II | DMRB | Leica |
| Microwave oven | M430 JET | Whirlpool |
| Spectrophotometer | PICOPET01 | Picodrop |
| Pipettes | Research Plus (0,5-10 µl, 10-100 µl, 100-1000 µl) | Eppendorf |
| Tabletop centrifuge I | MICRO STAR 17 | VWR |
| Tabletop centrifuge III | 5424 R | Eppendorf |
| Thermal cycler | S1000 | BioRad |
| Vortexer | LAB DANCER S40 | VWR |
| Weight | AG204 | Mettler Toledo |

## 2.8 Solutions and buffers made in the laboratory

3 % KOH

3 g ml KOH

Adjust to 100 ml with sterile MilliQ water

3 % $H_2O_2$

1 ml 35 % $H_2O_2$

Adjust to 11.7 ml with sterile MilliQ water

1 X TAE

100 ml 10 X TAE buffer

900 ml sterile MilliQ water

1 % Agarose gel

Dissolve 0.5 g agarose in 50 ml 1 x TAE buffer in the micro wave. Cool down before adding one drop of SYBR™ Safe DNA gel stain. Cast the gel.

DNA ladders from New England Biolabs (NEB)

10 µl ladder

10 µl loading buffer

40 µl Sterile MilliQ water.

# 3. Methods

## 3.1 Origin of samples

The strains isolated in this work was sampled from one Spanish and four Turkish rainbow trout farms that experienced disease. The fish were farmed in fresh water, the size ranged from 15 – 500 g and the water temperature ranged from 13-18 ºC. Records of clinical signs were limited to include exophthalmos (bulging of the eye anteriorly out of the orbit), which is a general clinical sign found during infection with a range of fish pathogens. Samples from the kidney, liver and spleen were streaked out on blood agar containing 2 % NaCl and shipped to PHARMAQ AS, Oslo, Norway. This was done by the personnel at each fish farm. The following sections will describe the methods for phenotypic (chapter 3.2) and genotypic (chapter 3.3) characterization. In addition, eight isolates provided by PHARMAQ AS were also identified in this work.

## 3.2 Phenotypic and biochemical identification

Upon arrival, the plates were examined macroscopically for *L. garvieae*-like colonies. The blood plates had multiple colonies that had grown into each other. Colonies that were small, white and non-motile coccus in duplets and chains were streaked out on new plates to obtain pure cultures. Alpha hemolytic cultures were tested for catalase, oxidase and finally API Rapid ID 32 STREP test. Positive *L. garvieae* isolates, according to these tests, were inoculated in TSB in sterile tubes and placed in incubator at 22 ± 2 °C with 100 rpm shaking for approx. 24 hours. Glycerol stocks were made by adding sterilized glycerol to a final concentration of 15 %, distributed 1 ml in ampoules and placed in a biofreezer at < -70 °C. Figure 3-1 shows a flow chart of the methods used for phenotypic identification in this thesis. Chapter 3.2.1 to 3.2.5 describes the methods in more details.

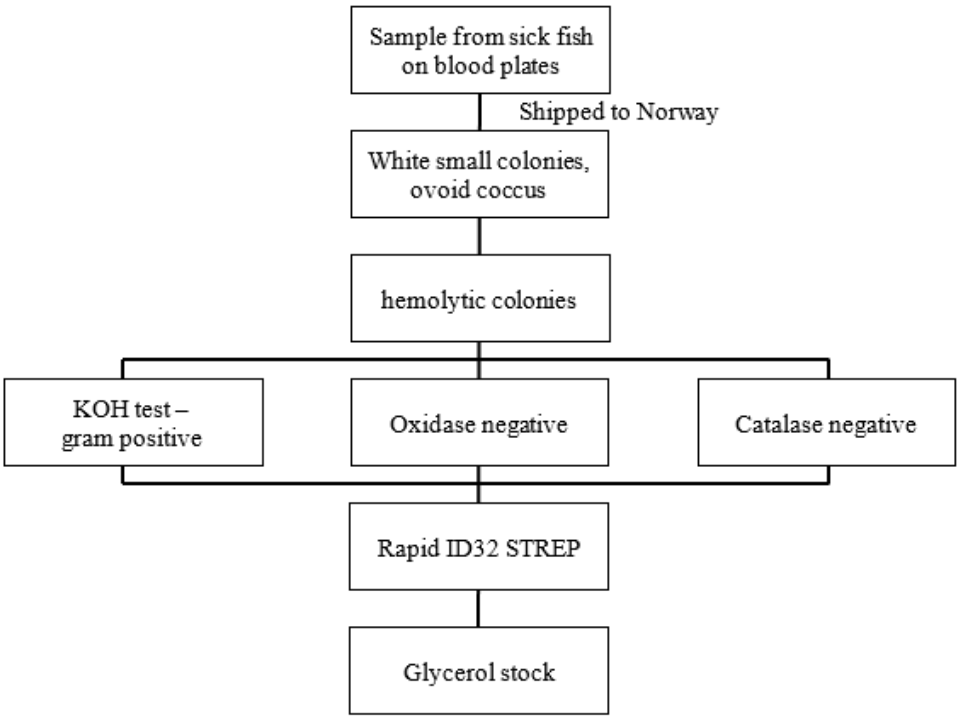



*Figure 3-1. Flow chart for biochemical characterization of L. garvieae used in this work.*

### *3.2.1 Hemolysis*

Hemolysins are lipids or proteins produced by pathogens that destroys the cell membrane of red blood cells and thereby causes lysis (Wilson et al., 2002) Hemolysis can be observed either as green color on blood plates (α-hemolysis), completely lysis of the blood cells that causes a halo on blood plates (β-hemolysis) or non-hemolysis (γ-hemolysis).

*L. garvieae* colonies appears as small, light/white circular and smooth colonies on blood plates which are α-hemolytic. Figure 3-2 shows an example of morphology and α-hemolysis on blood plate of ATCC 49156. All the isolates were streaked out on blood plates to check for hemolysis.

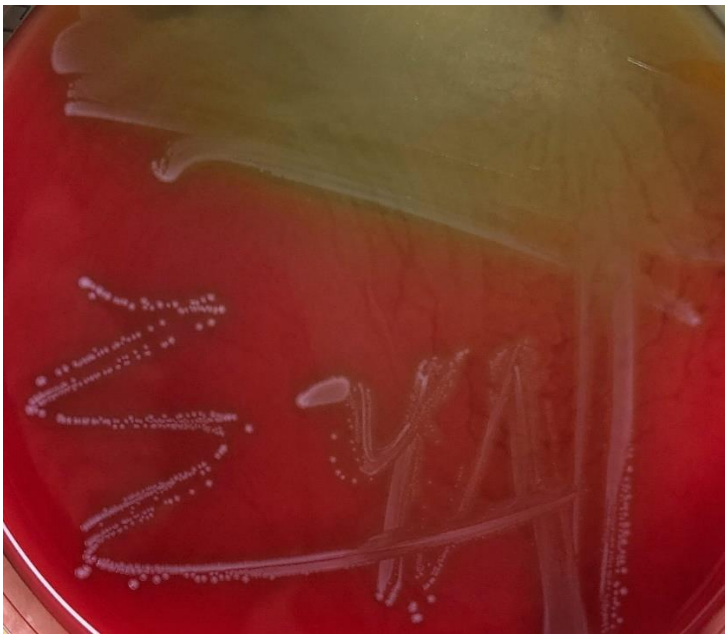


*Figure 3-2. α-hemolysis of L. garvieae ATCC 49156 on blood plate.*

### *3.2.2 KOH-test*

Gram staining is a procedure to distinguish between Gram-positive and Gram-negative bacteria by coloring these cells red or violet. A quicker method to distinguish between G+ and G- is the KOH-test (Buck, 1982) although it has some disadvantages. It does not say anything about cell morphology, the culture sample needed is bigger than for Gram staining and it does not detect Gram-variable bacteria, only Gram-positive and Gram-negative. KOH dissolves the peptidoglycan layer of the cell wall in gram-negative bacteria which causes lysis and releases the cell contents including the DNA, whereas Gram-positive bacteria is unaffected.

One drop of aqueous 3 % KOH was placed on a slide. A visible amount of bacterial growth from an agar plate was transferred to the drop with a sterile loop, and mixed thoroughly. If the isolate was Gram-negative, the suspension became markedly viscid, due to the released DNA, within 5 to 60 s by raising the loop about 1 cm from the slide. If there was no gelling observed, the isolate was Gram-positive. *L. garvieae* is Gram-positive and should be unaffected of KOH.

### *3.2.3 Oxidase reaction*

To determine if the isolates utilizes oxygen in the electron transport chain, the isolates were tested for the presence or absence of cytochrome oxidase. Cytochrome oxidase oxidizes cytochrome C in the electron transport chain. At the same time $O_2$ is reduced to $H_2O$.

A visible amount of bacterial growth from an agar plate was transferred and smeared well into the test strip which is saturated with N,N-dimethyl-p-phenylenediamine and alpha-naphthol. If

the enzyme cytochrome oxidase is present (Gram-negative bacteria) the N,N-dimethyl-1,4-phenylene diamine and alpha-naphthol react to indophenol blue. Hence production of a blue color indicated presence of the enzyme and no color indicated that the bacteria had no cytochrome oxidase. *L. garvieae* is oxidase negative and therefor produces no color on the strip.

### *3.2.4 Catalase test*

The enzyme catalase is present in all aerobic organisms and protects the cells from oxidative damage. Catalase neutralizes the bactericidal effect of $H_2O_2$: $2\ H_2O_2 \rightarrow 2H_2O + O_2$.
To determine if the isolates has the enzyme, one drop of 3 % $H_2O_2$ was placed on a glass slide. A small amount of bacterial colonies were transferred to the drop with limited mixing. Production of bubbles in 5-10 seconds was regarded a positive test result. No appearance of bubbles, or appearance after 30 seconds (due to degradation of $H_2O_2$ by other enzymes) were considered negative. *L. garvieae* should be catalase negative.

### *3.2.5 API Rapid ID 32 STREP*

Further biochemical characterization was done with Rapid ID 32 STREP (Biomerieux) which is a standardized system for identification of most streptococci and related genera in 4 hours. In addition to the strain isolated in this work, 8 isolates provided from PHARMAQ AS and ATCC 49156 as control, were also tested on API Rapid ID 32 STREP. This system consists of 32 dehydrated substrates and checks the presence of specific enzymes or if the bacterium consumes different sugars. After inoculation with the bacterial culture, a change in color indicates positive reaction, Figure 3-3. The strains were streaked out on Colombia agar containing 5 % sheep blood and incubated at 23 ± 2 °C for about 24 hours. With a sterile inoculation loop, the growth from the agar was harvested into 2 ml API® Suspension Medium. The turbidity was adjusted to 4 McFarland standard and 55 µl was dispensed into each cupule. The lid was placed on the strip and incubated at 22 ± 2 °C for 4 hours. After incubation 1 drop of VP A and VP B was put into the VP test (test 0.0), 1 drop of FB reagent into tests APPA to GTA (tests 0.1-0.5) and 1 drop of NIN reagent was put in the HIP test (test 0.6). After 5-10 minutes the test was registered by visual inspection. Number of positive and negative tests were noted and identification was obtained using api*web*™ identification software.


*Figure 3-3. Example of Rapid ID 32 Strep strip showing all reactions positive. Picture from Biomeriex (https://biomerieuxdirect.com/industry/Bacteriology/ID-AST-Auto/ID-AST-Semi-automated-%3A-ATB-system/ATB-Galleries-ID/ATB-Identification-Streptococci/API%26reg-RAPID-ID-32-STREP-%2825-STRIPS%29/p/32600) . Information retrieved 28.04.2019.*

## 3.3 Genotypic identification

Different techniques were employed for genotypic identification and characterization of the strains. Figure 3-4 shows a flow chart of the methods used in this thesis. Chapter 3.3.1-3.3.9 describes molecular methods for identification of *L. garvieae*. Chapter 3.4 describes other molecular techniques for further characterization.

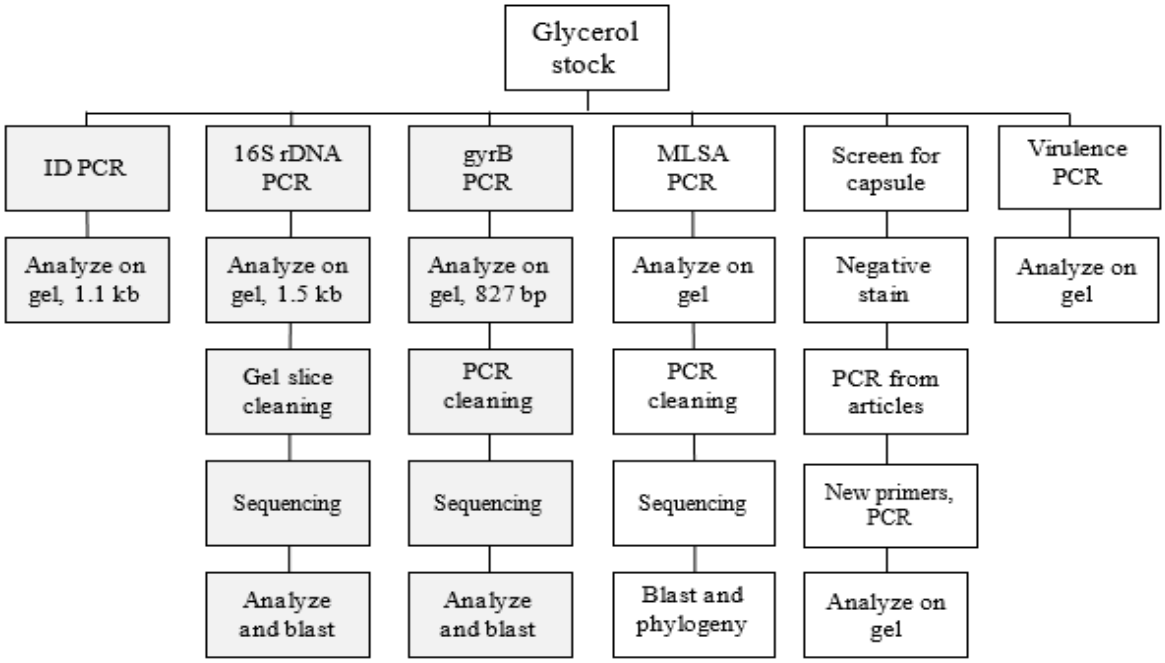



*Figure 3-4. Flow chart for various genotypic techniques used for identification and characterization of L. garvieae used in this thesis. Grey boxes are methods for identification. White boxes are methods used for further characterization of the strains,*

### *3.3.1 Isolation of genomic DNA*

Genomic DNA was isolated using InstaGene™ Matrix (Bio-Rad, 2018).
The InstaGene™ Matrix is composed of 6 % Chelex resin and a magnetic stir bar. Boiling in the presence of the matrix results in cell lysis. The Chelex resin binds to PCR inhibitors, rather than DNA. This prevent DNA loss due to irreversible DNA binding. Centrifugation pellets the Chelex resin with the PCR inhibitors whereas the supernatant contains the purified DNA.

The strains were streaked out on blood agar with 2 % NaCl and incubated at 22 ± 2 °C for about 24 hours or more until good growth was achieved. Two-three single colonies were dissolved in 500 µl PBS and centrifuged at 13 000 rpm for 1 minute. The supernatant was discarded and the pellet dissolved in 100 µl InstaGene™ Matrix before incubation at 56 °C in 15 ± 1 minutes. The sample was vortexed before incubating at 100 °C for 8 ± 1 minutes. The sample was vortexed a second time before centrifugation at 13000xg for 1 minute. 70 µl of the supernatant was transferred to a new clean Eppendorf tube and stored at -20 °C.

### *3.3.2 Polymerase Chain Reaction*

Polymerase Chain Reaction (PCR) is a widely used method for *in vitro* amplification of DNA sequences. Since the method was developed by Kary Banks Mullis in 1983 (Mullis et al., 1986), it has become one of the most widely used methods within molecular biology (Bartlett & Stirling, 2003). For a PCR reaction one need a mixture which consist of a primer pair, DNA template, deoxyribonucleotide triphosphates (dNTPs) and a heat resistance DNA polymerase in a buffered environment with divalent cations ($Mg^{2+}$). PCR involves a series of heating and cooling steps in a machine called thermal cycler. One cycle consists of three steps. The first step is the denaturation step where the mixture is heated to 94-95 °C. The high temperature causes the hydrogen bonds between the bases in the double stranded DNA (dsDNA) to break and separate. The two single stranded DNA (ssDNA) serve as templates for production of new strands of DNA. The next step is the annealing step where the mixture is cooled down to 50-65 °C. This allows for the primers to attach by hydrogen bonds to complementary sites on the DNA template. Primers are short single strands of DNA, typically 15-30 bases, designed to bind to either side of the section of DNA you want to copy, and serves as starting point for DNA synthesis. The specific temperature in the annealing step depends on the melting temperature of the primers.

The third step in the PCR cycle is the elongation step. The mixture is typically heated to 72 °C and this enables the polymerase to synthesize new complementary strand of DNA by using the dNTPs (dATP, dTTP, dGTP, dCTP) in the PCR mixture. The time spent on this step depends on the length of the fragment to be amplified and the polymerase.
The result after these three steps is two dsDNA originating from one. The new synthetized strands will serve as new templates in the next cycle. These three steps are repeated typically 20-40 times which can yield millions of identical copies of the DNA sequence of interest.

In this work, PuReTaq Ready-To-Go PCR Beads from GE Healthcare were used (GE-Healthcare, 2018) . The benefit with PuReTaq Ready-To-Go PCR Beads is saving time, there are less risk of pipetting errors and contamination and it can be stored at room temperature.
The beads are pre-dispensed in PCR tubes and contains necessary reagents for 25 µl reactions except for template, primers and water which need to be added.

*3.3.2.1 Protocol for PCR*

All the PCR-reaction in this work followed the same protocol except for the PCR-programs. Each PuReTaq bead contains stabilizers, BSA, dATP, dCTP, dGTP, dTTP, PuReTaq DNA polymerase and reaction buffer. When a bead is reconstituted to a 25 µl final volume, the concentration of each dNTP is 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, and 1.5 mM $MgCl_2$ . Template-specific primers, DNA and water was added to a final volume of 25 µl as described below.

1. A PCR reaction mix was made in pre-dispensed PCR tubes with beads and kept on ice.

   22 µl Rnase free water
   0.5 µl forward primer (25 µM)
   0.5 µl revers primer (25 µM)
   2 µl template (~ 25 µM)
   25 µl total in each tube

2. PCR Programs

   PCR reactions were amplified in a Thermal cycler with programs from articles or programs customized in this work, Table 3-1 and Table 3-2.

   For PCR programs for detection of capsule with primers made in this work (chapter 3.4.2.2) and for detection of virulence genes (chapter 3.4.3), it was necessary to do a

gradient PCR. This is a method to find the optimal annealing temperature for the primers. The Thermal cycler was programed to have different annealing temperatures in different rows. This made it possible to have one program running with annealing temperatures in a range of up to eight temperatures.

Table 3-1. Different PCR programs used for identification.

| | **ID PCR (Zlotkin et al., 1998) Chapter 3.3.4** | | | **16S program This work Chapter 3.3.5** | | | ***gyrB* (Ture & Altinok, 2016) Chapter 3.3.6** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Initial denaturation** | 94 °C | 3 min. | | 94 °C | 10 min. | | 95 °C | 5 min. | |
| **Denaturation** | 94 °C | 60 s | x35 | 94 °C | 30 s | x35 | 94 °C | 45 s | x30 |
| **Annealing** | 55 °C | 60 s | | 50 °C | 40 s | | 58 °C | 45 s | |
| **Elongation** | 72 °C | 90 s | | 72 °C | 90 s | | 72 °C | 60 s | |
| **Final extension** | 72 °C | 10 min | | 72 °C | 10 min | | 72 °C | 7 min | |
| **Temporary storage** | 4 °C | ∞ | | 4 °C | ∞ | | 4 °C | ∞ | |

s: seconds. min: minutes

Table 3-2. Different PCR programs used for further characterization

| | **MLSA Scheme (Ture & Altinok, 2016) Chapter 3.4.1** | | | **Identification of capsule, Long range PCR (Miyauchi et al., 2012) Chapter 3.4.2.2** | | | **Identification of capsule, Multiplex PCR (Ture & Altinok, 2016) Chapter 3.4.2.2** | | | **Identification of capsule, Primer design This work Chapter 3.4.2.2** | | | **Identification of virulence genes (Ture & Altinok, 2016) Chapter 3.4.3** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Initial denaturation** | 95 °C | 5 min. | | 95 °C | 3 min. | | 95 °C | 5 min. | | 95 °C | 5 min | | 94 °C | 3 min. | |
| **Denaturation** | 94 °C | 45 s | x30 | 95 °C | 30 s | x30 | 94 °C | 30 s | x30 | 94 °C | 30 s | x30 | 94 °C | 30 s | x35 |
| **Annealing** | 56/ 58 °C | 45 s | | 62 °C | 60 s | | 56 °C | 30 s | | 67/64 /62°C | 30 s | | 68/59/ 54 °C | 30 s | |
| **Elongation** | 72 °C | 60 s | | 68 °C | 5 min | | 72 °C | 60 s | | 72 °C | 60 s | | 72 °C | 60 s | |
| **Final extension** | 72 °C | 7 min | | 72 °C | 10 min | | 72 °C | 10 min | | 72 °C | 10 min | | 72 °C | 10 min | |
| **Temporary storage** | 4 °C | ∞ | | 4 °C | ∞ | | 4 °C | ∞ | | 4 °C | ∞ | | 4 °C | ∞ | |

s: seconds. min: minutes

### *3.3.3 Agarose Gel Electrophoresis*

Agarose gel electrophorese is a method for separating molecules based on their size and charges and can be used for separation and purification of DNA fragments, for instance PCR-products (Sambrook & Russell, 2006). Loading buffer is added to the DNA samples to be analyzed and loaded to the top of the gel. Loading buffer have higher density and helps the sample to settle

in the well. It also contains dye so it is possible to track the movement during the run time. By adding an electric currant, the DNA, which is negatively charged, will migrate through the agarose gel towards the positive electrode. Small DNA fragments will migrate faster than bigger fragments. How fast the molecules will migrate through the gel depends on the size and conformation, the concentration of agarose, type of buffer used and the amount of voltage applied (Sambrook & Russell, 2006).
To be able to analyze the DNA fragments, SYBR™ Safe DNA gel stain is added to the gel. The stain binds to nucleic acids and is fluorescent under UV-light or blue-light. This makes it possible to visualize the migration of the DNA through the gel.
To determine the size of the DNA fragment to be analyzed, a ladder with known DNA-fragments is separated along with the samples.

In this thesis E-gel™ agarose gel, 1.2 % from Invitrogen was used when analyzing PCR-products (Invitrogen, 2018). Except when analyzing 16S rDNA PCR-products, a 1 %-agarose gel was made.

#### *3.3.3.1 Protocol for agarose gel electrophoresis*

1. E-gel™ agarose gel, 1.2 %

   The E-gel™ agarose gel was removed from the package, the comb was removed from the cassette and the gel was inserted into the E-gel power base. Samples (5µl of PCR product plus and 15 µl sterile water ) were loaded to each well in the gel. Molecular DNA weight marker (5 µl ladder and 15 µl sterile water) was loaded in the first and/or last well. Empty wells was loaded with 20 µl sterile water. The gels were analyzed in the E-gel Power base at time predefined for 1.2% agarose gels set by the manufactures. If needed, the gels were run for longer time. Visualization of the gel was done under UV-light in Gel Doc-1000 (Biorad)

2. 1 % -gel for analyzing 16S rDNA-PCR products

- 50 ml 1x TAE buffer was added to 0.5 g agarose and heated in a microwave oven until completely dissolved
- After cooling down to approximately 60 °C, 5 µl SYBR™ Safe DNA gel stain was added and mixed well without making air bubbles in the solution.

- The solution was transferred carefully to a gel casting tray with a sample comb to solidify.
- When completely solidified, the gel was transferred to a gel chamber, the comb was removed and 1xTAE buffer was added to the chamber until it completely covered the gel.
- PCR-products were added 5 µl loading buffer before loaded in the wells. 10 µl of DNA ladder (appendix A) was loaded into the first well.
- Electrophoresis was run at 90 V and after sufficient migration the gel was visually inspected and photographed in UV-light in Gel Doc-1000 (Biorad)
- Band at approximately 1500 bp compared to the ladder was cut out of the gel under UV-light with a scalpel for further purifying, ref chapter 3.3.5.

### *3.3.4 Identification of L. garvieae by PCR*

There are different molecular techniques based on PCR that can be used for identification of fish pathogens. Zlotkin et al. (1998) developed a PCR assay for the identification of *L. garvieae* targeting the 16S rRNA gene. The assay gave a PCR-product of 1100 base pair (bp) for *L. garvieae* but was negative for the phenotypically similar *L. lactis* and other fish pathogen.
All isolates in this study were analyzed by the PCR assay described by Zlotkin (1998). One *Streptococcus iniae* and three serotypes of *Streptococcus agalactiae* were included as negative controls. *L. garvieae* ATCC 49156 was included as a positive control. The PCR reaction mix was made as described in chapter 3.3.2.1 with primers pLG-1 and pLG-2, and amplified in a Thermal cycler at the following program as described by Zlotkin et al. (1998):

| | | |
|---|---|---|
| 94 °C | 3 min | |
| 94 °C | 1 min | x35 |
| 55 °C | 1 min | |
| 72 °C | 1,5 min | |
| 72 °C | 10 min | |
| 4 °C | ∞ | |

The PCR products were analyzed on E-gel™ agarose gel, 1.2 %, to verify a positive result at 1100 bp for *L. garvieae*.

### *3.3.5 16S rDNA PCR and sequence preparation*

The 16S ribosomal RNA (16S rRNA) is part of the 30S subunit and is present in all bacteria (Woese, 1987). The gene encoding 16S rRNA (16S rDNA) consist of highly conserved and

variable regions. The conserved regions allow primers to be designed to target all bacteria. When primers bind to a conserved region, it can amplify the 16S rRNA gene through a variable region, which makes it possible to determine various species down to genus level. Due to the slow rate of evolution in the conserved regions 16S rDNA sequencing is used in identification and phylogenetic studies. Before sequencing, a PCR must be performed to amplify the 16S rRNA gene.

Due to time savings and sequencing problems (chapter 4.4), only six of the 24 species in this study were identified by sequencing their 16S rDNA. PCR reaction mix was made as described in 3.3.2.1 with primers 27F and 1492R, and amplified in a Thermal cycler at the following program:

| | | |
|---|---|---|
| 94 °C | 10 min | |
| 94 °C | 30 sec | x35 |
| 55 °C | 40 sec | |
| 72 °C | 1 min | |
| 72 °C | 10 min | |
| 4 °C | ∞ | |

The PCR-products were analyzed on 1% agarose gel and PCR-products at approximately 1500 bp was cut out from the gel with a scalpel put in a tube. DNA was extracted from the agarose using Qiaquick Gel extraction kit from Qiagen. The gel slice was weighed and 3x gel volume of QG buffer was added to the tube and incubated at 50 ºC for 10 minutes. After vortexing, 1x gel volume of isopropanol was added before the volume was transferred to spin column. After centrifugation at 1600g for 1 minute in room temperature, the filtrate was discarded and 500 µl QG buffer was added to the column. After centrifugation at 1600g for 1 minute in room temperature, the filtrate was discarded. The column was added 2x375 µl PE buffer with centrifugation between each time. The column was moved to a new tube and added 50 µl EB buffer. After a final centrifugation step the filtrate was stored at 4-8 ºC before sent to Sanger sequencing at LGC Genomics GmbH, Germany.

### *3.3.6 gyrB PCR and sequence preparation*

The marker gene 16S rRNA is highly conserved and is universal in all bacteria, but the lack of variability in the 16S rRNA gene does not allow for identification of closely related species or subspecies. To identify bacteria to more than genus level, sequencing of a suitable housekeeping gene could be a better approach. Housekeeping genes are genes that have higher genetic variation than 16S rRNA gene. They are needed for basic cellular function and they

typically occur in a single copy. Some housekeeping genes are transcribed relatively constant. In this work the housekeeping gene *gyrB* was used. The *gyrB* gene encodes the subunit B protein of DNA gyrase, a type II DNA topoisomerase. This gene is universally distributed among bacterial species and plays an essential role in DNA replication (Mun Huang, 1996; Watt & Hickson, 1994).

Prior to sequencing, a PCR must be performed to amplify the relevant housekeeping gene using specific PCR primers. Primers and PCR programs are from Ferrario et al. (2013).

All 24 isolates were identified by sequencing *gyrB*, as well as ATCC 49156 as a positive control. PCR reaction mix was made as described in 3.3.2.1 with primers from Table 2-2, and amplified in a Thermal cycler at the following program (Ferrario et al., 2013):

| | | |
|---|---|---|
| 95 °C | 5 min | |
| 94 °C | 45 sec | x30 |
| 58 °C | 45 sec | |
| 72 °C | 60 sec | |
| 72 °C | 7 min | |
| 4 °C | ∞ | |

5 µl of the PCR-products were analyzed on E-gel™ agarose gel, 1.2 %, to verify the presence of a positive PCR-product at 1464 bp. The rest of the PCR- reaction was purified (chapter 3.3.7) before submitted for Sanger sequencing at LGC Genomics GmbH, Germany.

### *3.3.7 Purification of PCR products*

PCR products analyzed on E-gel, were purified using ExoSAP-IT™ *Express* PCR Product Cleanup Reagent (Affymetrix, 2018), before Sanger sequencing.

Exonuclease I combined with Shrimp Alkaline Phosphatase (SAP) in a buffer removes excess primers and dNTPs from the post PCR product. 2 µl ExoSAP-IT™ reagent was added to 5 µl PCR product and incubated at 37°C for 15 minutes, followed by inactivation at 80°C for 15 minutes. The treated PCR products was stored at 2-8°C. This purification method was used on PCR products amplifying the *gyrB* gene for identification (chapter 3.3.6) and purifying other housekeeping genes used in Multiple Locus Sequence Analysis (MLSA) in chapter 3.4.1 before Sanger sequencing.

### *3.3.8 Sanger sequencing*

DNA sequencing is a method to determine the order of nucleotides (adenine, guanine, cytosine and thymine) in DNA. Chain termination, or Sanger method, was developed in 1977 by Fredrik Sanger and colleagues and has been the most widely used method since (Sanger et al., 1977).

The method requires a template, DNA primer, DNA polymerase, deoxynucleosidetriphosphates (dNTP) and modified di-deoxynucleotidetriphosphate (ddNTPs). ddNTPs differs from dNTP in the sense that they lack the 3´OH-group that forms phosphodiester bonds with the next nucleotide, thereby terminating the DNA strand elongation.

All the ingredients are mixed into one tube, but the four ddNTPs are added in a smaller amount than the ordinary nucleotides. The mixture is heated to denature the template DNA and separate the strands. Next the mixture is cooled so the primer can bind to the single stranded DNA. The temperature is raised again so the DNA polymerase can synthesize new DNA. DNA polymerase continues to add nucleotides until a dideoxy nucleotide is added and the reaction is terminated. This process is repeated in multiple cycles. Since ddNTPs are randomly incorporated into a sequence, the result will be DNA fragments of different length (Sambrook & Russell, 2006). When first developed by Sanger et al. (1977), the DNA fragments were analyzed visually by polyacrylamide gel electrophoreses. Now the ddNTPs are fluorescently labelled and can be detected in an automated sequencing machine.

The fragments are run through a long, thin tube with gel matrix (capillary gel electrophoresis). Short fragments move faster than long fragments. At the end of the tube the fragments are illuminated by a laser and the attached dye is detected. The colors of dyes are registered and the result is a series of peaks in fluorescence in a chromatogram. The DNA sequence is read from the sequence in the chromatogram.

In this work the sequencing was done by LGC Genomics GmbH in Germany. Purified PCR product was sent to LGC Genomics along with primers for the amplified gene. The resulting chromatogram was downloaded for further analyses described in chapter 3.3.9 and 3.4.1.1.

### *3.3.9 Analyzing 16S rDNA and gyrB sequences for identification*

The sequences from 16S rDNA and *gyrB* were analyzed by downloading the ABI-files from LGC Genomics into CLC workbench version 8.0 (Qiagen, 2011). The chromatograms from each strain from the forward and reverse sequences were assembled into a contig and visually checked for poor sequence regions. Poor sequence regions at the ends were removed. The trimmed contigs were exported as FASTA-files and used as input in BLAST (Basic Local Alignment Search Tool) and the program nucleotide BLAST at NCBI (National Centre for Biotechnology Information). For the *gyrB*-alignments, megaBLAST (optimized for highly similar sequences) and the database nucleotide collection (nr/nt) were used. For 16S rRNA alignments, megaBLAST and the database 16S ribosomal RNA sequences were used. The best

hits in megaBLAST search were used. Species description, accession number, maximum score, total score, query cover and E-value was noted.

## 3.4 Further characterization of strains

### *3.4.1 Multi Locus Sequence Analysis*

Phylogenetics is the study of evolutionary history and relationship between organisms which is shown as a phylogenetic tree or diagram. A gene, usually 16S rRNA or a housekeeping gene, can be used for constructing a phylogenetic tree. But it has become more common to analyze multiple protein-encoding housekeeping genes for taxonomic relationship (Adékambi & Drancourt, 2004; Christensen & Olsen, 1998; Holmes et al., 2004; Naser et al., 2005; Thompson et al., 2005; Wertz et al., 2003).

Multi locus sequence analysis (MLSA) is a technique first introduced by Gevers et al. (2005). In MLSA multiple sequences of housekeeping genes, usually 5-7, are joined together end-on-end which then creates a single concatenated sequence. This concatenated sequence is used for phylogenetic relationships. MLSA, when constructing a concatenated tree, provides an objective method to cluster strains within a genus and reduces the chance of coincidence that is present when using a single gene approach (Gevers et al., 2005).

For the MLSA scheme, five housekeeping genes from 16 strains were sequenced. Housekeeping genes was: *als, gapC, gyrB* (chapter 3.3.6 ), *rpoC and galP* (Ferrario et al., 2013).

PCR reaction mix was made as described in 3.3.2.1 with primers from Table 2-2, and amplified in a Thermal cycler at the following program (Ferrario et al., 2013):

| | | |
|---|---|---|
| 95 °C | 5 min | |
| 94 °C | 45 sec | ⎫ |
| 56/58 °C | 45 sec | ⎬ x30 |
| 72 °C | 60 sec | ⎭ |
| 72 °C | 7 min | |
| 4 °C | ∞ | |

Annealing temperature was 56 °C for *gapC* and 58 °C for *als, galP and rpoC*.

The PCR-products were analyzed on E-gel™ agarose gel, 1.2 %, to verify a PCR-products at 1076 bp (*als*), 974 (*gapC*), 1377 (*rpoC*) and 1070 (*galP*). The rest of the PCR- reaction was purified as described in chapter 3.3.7 before submitted for Sanger sequencing at LGC Genomics GmbH, Germany.

#### *3.4.1.1 Analyzing sequences for MLSA*

The sequences were assembled, quality checked and trimmed in CLC workbench version 8.0, before exporting as FASTA files and used in BLAST search as described for *gyrB* in chapter 3.3.9 (megaBLAST and the database nucleotide collection (nr/nt)).

The contigs were imported and aligned using ClustalW (Thompson et al., 1994), edited in MEGA 7.0.26 (Molecular Evolutionary Genetics Analysis) (Kumar et al., 1994) and adjusted to proper reading frame. The alignments were employed as input in modeltest (Posada & Crandall, 1998) in CLC workbench, a test for selecting the best fit model of nucleotide substation. Phylogenetic trees were constructed by Maximum-Likelihood (Pfanzagl, 2011) in CLC workbench based on the result of the modeltest.

#### *3.4.1.2 Searching for sequences for MLSA*

As input in the phylogenetic trees, sequences for the genes *als*, *gapC, gyrB, rpoC* and *galP* for *L. garvieae* strains 8831, UNIUD074, PAQ102015-99, LG9, ATCC 49156, Lg2, and 122061, in addition to *L. lactis* subsp. *lactis* KF147 and *L. lactis* subsp. *cremoris* A76 (Table 2-1), were found using nucleotide BLAST search at NCBI.

FASTA-file for the gene *als* from AL 20 869, or any of the 16 strains sequenced above, was uploaded in nucleotide BLAST and used as input in megaBLAST against the database "whole-genome shotgun", limited to organism *Lactococcus garvieae* ATCC 49156.

The result was checked for best hit, query coverage and percent identity and saved as a FASTA-file for gene *als* in ATCC 49156. To double check if the sequence found for *als* in ATCC 49156 was right, the FASTA-file was used as input at nucleotide BLAST using megaBLAST and the database nucleotide collection (nr/nt). This procedure was repeated for all the genes in the six strains.

### *3.4.2 Presence of exopolysaccharides (EPS)*

Phenotypic and genotypic approaches used to investigate if the isolates had a capsule or not were as follows:

#### *3.4.2.1 Phenotypic detection of capsule*

Capsules stain poorly with reagent used in simple staining so it is difficult to see the

capsule in a regular light microscope. Negative staining is a two-step staining method where the background is stained with india ink, nigrosine or congo red, but the cells and capsule remains unstained. By counterstaining with dyes like crystal violet or methylene blue, the bacterial cell wall get stained and again, the capsule remains unstained. In a light microscope the capsule appears colorless with stained cell against dark background (Duguid, 1951).

A small drop of Nigrosine was placed on a slide. A loopful of bacterial culture was transferred to the drop and mixed well into the dye. Another slide was used to drag the mixture into a thin film along the first slide. The slide was left to air dry for 5-7 minutes. The smear was covered with crystal violet for 1 minute. This stained the cells but not the capsules. The crystal violet was drained by tilting the slide at a 45-degree angle and left standing until the stains were drained and air dried. The smear was examined microscopically (100x) for the presence of encapsulated cells as indicated by clear zones surrounding the cells.

#### *3.4.2.2 Genotypic detection of capsule*

As described earlier, Morita et al. (2011) found that presence of a capsule was a major virulence factor in yellowtail in Japan, and they identified a 16.5 kb capsule gene cluster composed of 15 genes.

PCR-methods have been developed to identify the presence of this gene cluster (Miyauchi et al., 2012; Ture & Altinok, 2016):

- Miyauchi et al. (2012) developed a long-range PCR with primers that binds outside the gene cluster, Figure 3-5. If the isolate is positive for a capsular gene cluster the PCR product will give a band of ~16.5 kb on agarose gel. A band of ~750 bp, indicates absence of the capsule gene cluster.

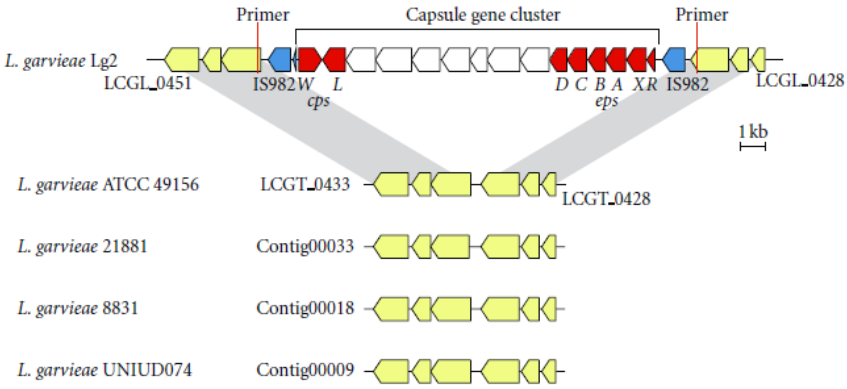



*Figure 3-5. Genomic location of the gene cluster of L. garvieae Lg2 in comparison to the corresponding locations in other L. garvieae strains. Arrows indicate the genes and their orientations; blue are transposase genes, red are genes conserved in the capsule gene cluster of various L. lactis strains and white are other genes in the capsule gene cluster. Gray bars are orthologous regions. Primer binding site are indicated by red lines. Picture derived from (Miyauchi et al., 2012)*

All 24 isolates were analyzed with these primers for presence of capsule gene cluster. *L. garvieae* Lg2 was included as a positive control and ATCC 49156 and $H_2O$ as negative controls.

The PCR reaction mix was made as described in chapter 3.3.2.1 with primers cap2012-F and cap2012-R, and amplified in a Thermal cycler at the following program (Miyauchi et al., 2012):

| | | |
|---|---|---|
| 95 °C | 3 min | |
| 95 °C | 30 sec | x30 |
| 62 °C | 60 sec | |
| 68 °C | 5 min | |
| 72 °C | 10 min | |
| 4 °C | ∞ | |

- Ture et al. (2016) developed a multiplex PCR for detection of the capsular gene cluster. Presence of the capsule gene cluster will, after analyzing the PCR product on agarose gel, give four bands of 785 bp, 650 bp, 549 bp and 304 bp. No band indicates that the strain is lacking capsular gene cluster.

  All the 24 isolates were analyzed with this multiplex PCR. *L. garvieae* Lg2 was included as a positive control and ATCC 49156 and $H_2O$ as negative controls.

  The PCR reaction mix was made as described in chapter 3.3.2.1 with primers from Table 2-2, except for the amount of water which was reduced from 22 µl to 19 µl in each tube to compensate for four primer pairs (4 µl). The PCR reaction was amplified in a Thermal cycler at the following program (Ture & Altinok, 2016):

| | | |
|---|---|---|
| 95 °C | 5 min | |
| 95 °C | 30 sec | ⎫ |
| 56 °C | 30 sec | ⎬ x30 |
| 72 °C | 60 sec | ⎭ |
| 72 °C | 10 min | |
| 4 °C | ∞ | |

As described earlier, the capsule gene cluster characterized in *L. garvieae* Lg2 was thought to be located on a genomic island because there are insertion sequences (ISs) flanked on both end of the 15 genes in the gene cluster (Morita et al., 2011). Theoretically this means that the capsule gene cluster could be inserted in different genomic loci than Lg2. If that was the case, the primers from Miyauchi et al.(2012) would not amplify the capsule gene cluster since the primers are designed to bind outside the ISs (Figure 3-5). To check if this was the case, primers were designed for *cpsW, epsR* and *epsW*, Figure 3-5, using National Center for Biotechnology Information, NCBI primer-BLAST. PCR product size was set to minimum 70 bp and maximum 400 bp. Anything else was default settings in the primer-Blast program. The top results from the primer design were used for PCR.

The PCR reaction mix was made as described in chapter 3.3.2.1 with primers from Table 2-2. Gradient PCR was performed with temperatures from 50-68 ºC to obtain the right annealing temperature.

Program *epsC* /*epsR* /*cpsW*:

| | | |
|---|---|---|
| 95 °C | 5 min | |
| 94 °C | 30 sec | ⎫ |
| 67/64/62 °C | 30 sec | ⎬ x30 |
| 72°C | 60 sec | ⎭ |
| 72 °C | 10 min | |
| 4 °C | ∞ | |

Visualized on E-gel™ agarose gel, 1.2 %, positive *cpsW* gene gave band at 382 bp, positive *epsR* gave a band at 206 bp and positive *epsC* gave a band at 217 bp.

### *3.4.3 Detection of other putative virulence factor*

Several genes encoding putative virulence factors were identified in *L. garvieae* Lg2 from yellowtail listed in Table 1-6 (Morita et al., 2011).

Ture et al. (2016) developed primers for these virulence factors and three of them were tested in this thesis, *LPxTG*-1, *LPxTG*-4 and *adhesin*. *LPxTG*-1 and *LPxTG*-4 are proteins for covalent anchoring to the peptidoglycan matrix (Marraffini et al., 2006) and adhesin facilitates adherence to other cells or surfaces (Kadioglu et al., 2008; Mitchell, 2003).

The PCR reaction mix was made as described in chapter 3.3.2.1 with primers from Table 2-2. Gradient PCR was performed with temperatures from 48-68 ºC to obtain the right annealing temperature.

The PCR reaction was amplified in a Thermal cycler at the following program (Ture & Altinok, 2016):

Program *LPxTG*-1/ *LPxTG*-4/*adhesin*:

| | | |
|---|---|---|
| 94 °C | 3 min | |
| 94 °C | 30 sec | ⎫ |
| 68/59/54 °C | 30 sec | ⎬ x30 |
| 72 °C | 60 sec | ⎭ |
| 72 °C | 10 min | |
| 4 °C | ∞ | |

Visualized on E-gel™ agarose gel, 1.2 %, positive *LPxTG*-1 gave band at 878 bp, positive *LPxTG*-4 gene gave a band at 928 bp and positive *adhesin* gave a band at 358 bp.

# 4. Results

## 4.1 Incoming plates and isolation of pure cultures

A total of 25 blood plates from various locations in Turkey (25 plates) and Spain (3 plates) were sent to PHARMAQ AS, Oslo, Norway. Each plate was divided into three equal sectors and consisted of colonies from kidney, liver and spleen from one fish. The plates from Turkey generally had multiple bacterial growth with different morphology (Figure 4-1), while the three plates that were received from Spain, had pure cultures. White small colonies were checked microscopically and ovoid cocci were streaked onto new plates to obtain pure cultures. Sixteen pure cultures were given a unique strain number, AL 21 225-AL 20 240, as listed in Table 4-1 before subjected to further characterization. In addition, glycerol cultures of strain AL 20 869, AL 20 870, AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045 and AL 20 046 in Table 4-1 provided from PHARMAQ AS, were also characterized biochemically and genotypically.

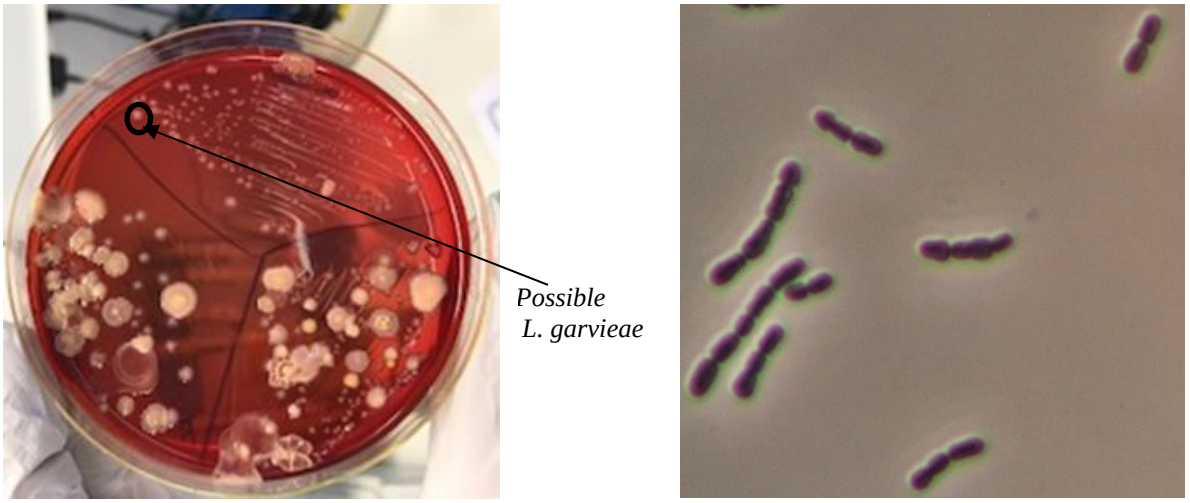



*Figure 4-1. Left; Example of one of the blood agar plates with multiple bacterial colonies from kidney, liver and spleen from sick fish. Arrow shows small white colony that was possible L. garvieae and was picked and streaked out on a new agar plate to obtain pure culture. Right picture; The colony from a blood plate visualized using 100x magnification.*

Table 4-1. Strains isolated in this work and strains provided by PHARMAQ AS, their origin and their given strain number.

| Country | Farm[1] | Fish species | Organ | Strain no. | Reference |
|---|---|---|---|---|---|
| Turkey | T2 | Rainbow trout | Liver | AL 20 869 | PHQ[2] |
| Turkey | T2 | Rainbow trout | Spleen | AL 20 870 | PHQ |
| Spain | S2 | Rainbow trout | Kidney | AL 20 906 | PHQ |
| Spain | S2 | Rainbow trout | Kidney | AL 20 909 | PHQ |
| Spain | S2 | Rainbow trout | Kidney | AL 20 910 | PHQ |
| Spain | S2 | Rainbow trout | Kidney | AL 20 911 | PHQ |
| Turkey | S2 | Rainbow trout | Kidney | AL 21 045 | PHQ |
| Turkey | S2 | Rainbow trout | Liver | AL 21 046 | PHQ |
| Spain | S1 | Rainbow trout | Kidney | AL 21 225 | This work |
| Spain | S1 | Rainbow trout | Kidney | AL 21 226 | This work |
| Spain | S1 | Rainbow trout | Kidney | AL 21 227 | This work |
| Turkey | T1 | Rainbow trout | Kidney | AL 21 228 | This work |
| Turkey | T2 | Rainbow trout | Kidney | AL 21 229 | This work |
| Turkey | T2 | Rainbow trout | Liver | AL 21 230 | This work |
| Turkey | T2 | Rainbow trout | Liver | AL 21 231 | This work |
| Turkey | T2 | Rainbow trout | Liver | AL 21 232 | This work |
| Turkey | T3 | Rainbow trout | Liver | AL 21 233 | This work |
| Turkey | T3 | Rainbow trout | Liver | AL 21 234 | This work |
| Turkey | T3 | Rainbow trout | Spleen | AL 21 235 | This work |
| Turkey | T3 | Rainbow trout | Spleen | AL 21 236 | This work |
| Turkey | T3 | Rainbow trout | Spleen | AL 21 237 | This work |
| Turkey | T3 | Rainbow trout | Kidney | AL 21 238 | This work |
| Turkey | T3 | Rainbow trout | Kidney | AL 21 239 | This work |
| Turkey | T3 | Rainbow trout | Kidney | AL 21 240 | This work |

1. Farm T1 indicates Turkey 1, T2 is Turkey 2 and so on. Farm S1 is Spain one.
2. PHQ: Provided by PHARMAQ AS

## 4.2 Phenotypic and biochemical characterization

The sixteen strains isolated in this work (Table 4-1) were subjected to biochemical testing. All the pure cultures were α-hemolytic, gram-positive, oxidase negative and catalase negative. The 16 strains in addition to 8 strains provided from PHARMAQ (Table 4-1) and ATCC 49156 as a control, were tested on Rapid ID 32 STREP. All strains were identified as *L. garvieae.* All of the isolates were positive for ADH (arginine dihydrolase), βGLU (β-glucosidase), MAN (mannitol), TRE (trehalose), VP (Voges Proskauser, acetoin production), APPA (alanyl-phenylalany-proline-arylamidase), PyrA (pyroglutamic acid arylamidase), MAL (maltose), TAG (tagatose). AL 20 869 was the only one negative for SAC (saccharose). AL 20 910 and AL 21 045 were, in addition to the above-mentioned sugars, also positive for Hip (hydrolysis of hippurate). The same was AL 20 870, AL 20 906, AL 20 909 and AL 20 911 in addition to being positive for MBDG (methyl-βD glucopyranoside).

AL 21 046, AL 21 225, AL 21 226, AL 21 230-AL 21 234, AL 21 236- AL 21 240 were positive for MBDG, but negative for HIP. The control ATCC 49156 was positive for ADH, βGLU, TRE, VP, APPA, PyrA. Results are listed in Table 4-2 and examples of test-strips can be observed in Figure 4-2 . Examples of results using api*web*™ identification software can be seen in Figure 4-3. In api*web*™ identification software, the results are denoted as “excellent”-

, “very good”-, “good”-, “acceptable”-, “doubtful”- and “unacceptable profile”. The frequency of occurrence is measured by % ID and a T-index. The % ID is percentage of reactions observed for one taxon relative to reactions observed in all taxon. T- index is reactions observed divided with most typical profile. “Tests against” is a test that has an opposite result of the typical profile. For more info: https://apiweb.biomerieux.com/jsp/help_ident/index.jsp. Of the 25 isolates tested, 13 had excellent identification as *L. garvieae* with 99.9 % ID and no tests against. 5 isolates had excellent identification *L. garvieae* with 99.9 % ID and MβG 85% as tests against. 4 isolates had doubtful identification *L. garvieae* with 99.7 % ID and HIP 0% as tests against and 2 isolates had doubtful identification *L. garvieae* with 99.7 % ID with MβG 85% and HIP 0% as tests against. ATCC 49156 was identified as excellent identification of *L. garvieae* with 99.9 % ID and MAN 75%, MAL 75%, MβG 85% as tests against (Table 4-2).

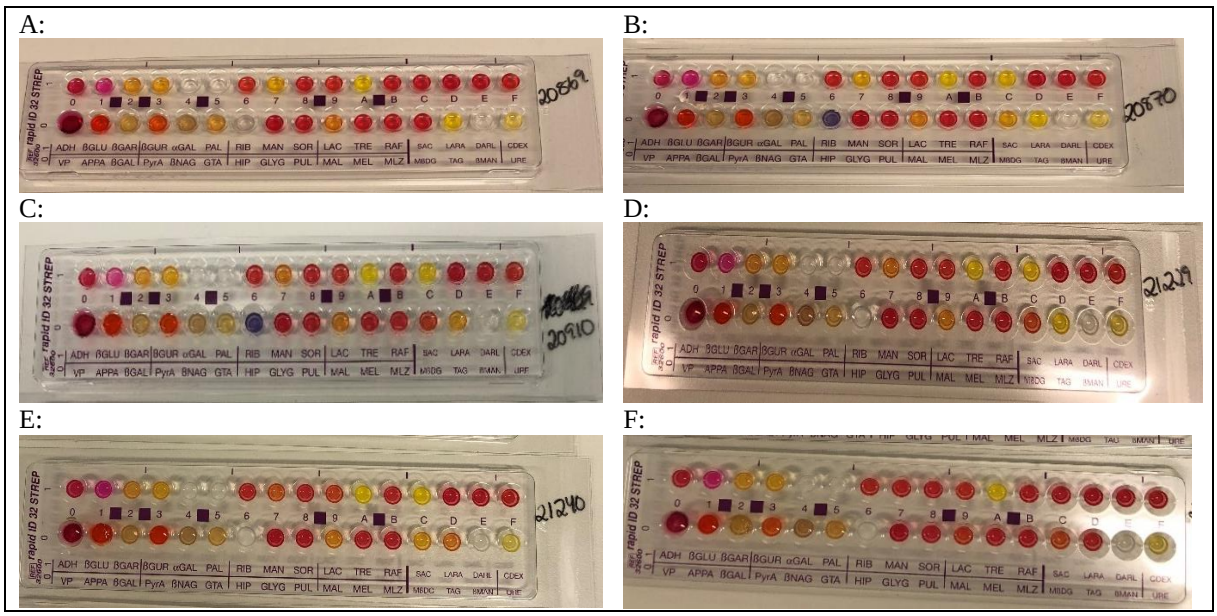
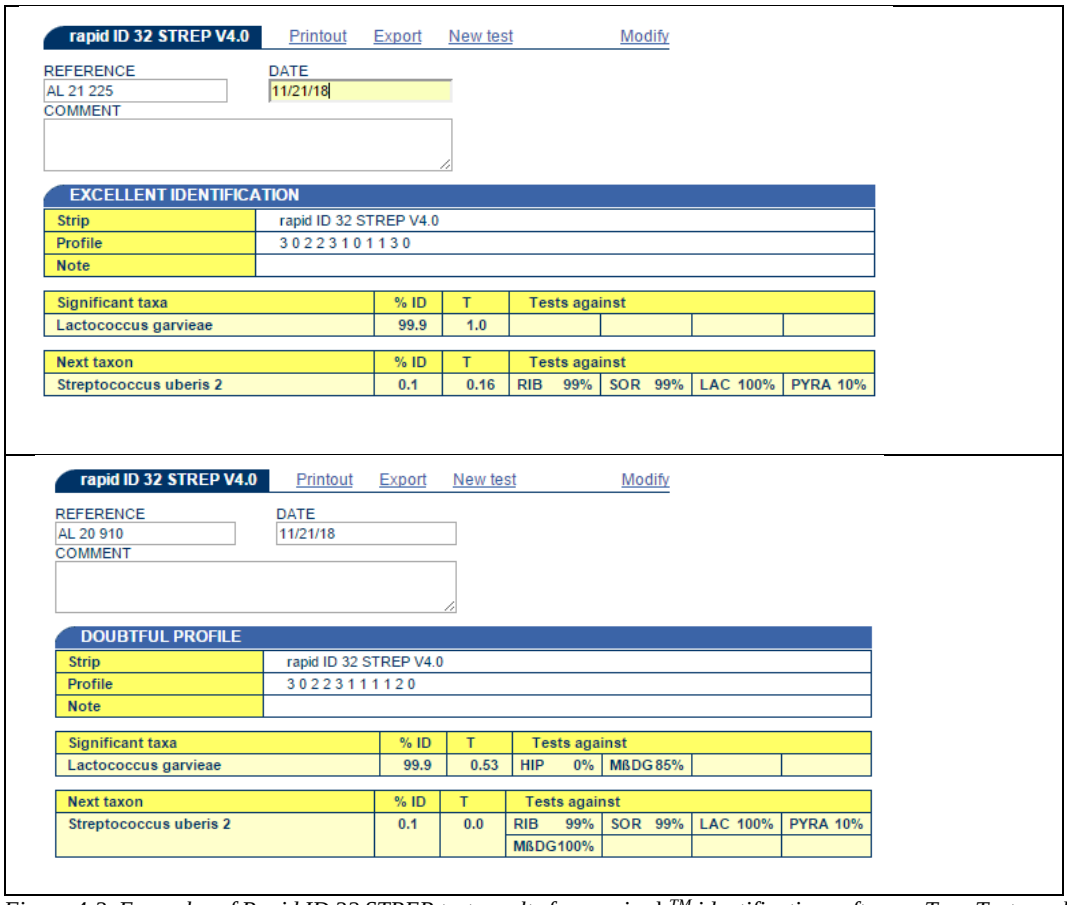



*Figure 4-2. Examples of Rapid ID 32 STREP test results.* ***A:*** *AL20 869 was positive for ADH (arginine dihydrolase), βGLU (β-glucosidase), MAN (mannitol), TRE (trehalose), VP (Voges Proskauser, acetoin production), APPA (alanyl-phenylalanyl-proline-arylamidase), PyrA (pyroglutamic acid arylamidase), MAL (maltose), TAG (tagatose).* ***B:*** *AL20 870 was positive for ADH, βGLU, MAN, TRE, SAC (saccharose), VP, APPA, PyrA, HIP (hydrolyses of Hippurate), MAL, MBDG (methyl-βD glucopyranoside), TAG. The same result was noted for AL20 906, AL20 909 and AL 20 911.* ***C****: AL20 910 was positive for ADH, βGLU, MAN, TRE, SAC, VP, APPA, PyrA, HIP, MAL, TAG. The same result was noted for AL21 045.* ***D****: AL21 229 was positive for ADH, βGLU, MAN, TRE, SAC, VP, APPA, PyrA, MAL, TAG. The same result was noted for AL21 227, AL21 228 and AL21 235.* ***E:*** *AL21 240 was positive for ADH, βGLU, MAN, TRE, SAC, VP, APPA, PyrA, MAL, MBDG, TAG. The same result was noted for AL21 046, AL21 225, AL21 226, AL21 230-AL21 234, AL21 236- AL21 239.* ***F:*** *ATCC 49156 was positive for ADH, βGLU, TRE, VP, APPA, PyrA.*



rapid ID 32 STREP V4.0 Printout Export New test Modify

REFERENCE: AL 21 225 DATE: 11/21/18

COMMENT:

**EXCELLENT IDENTIFICATION**

| | |
|---|---|
| Strip | rapid ID 32 STREP V4.0 |
| Profile | 3 0 2 2 3 1 0 1 1 3 0 |
| Note | |

| Significant taxa | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactococcus garvieae | 99.9 | 1.0 | | | | |

| Next taxon | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Streptococcus uberis 2 | 0.1 | 0.16 | RIB 99% | SOR 99% | LAC 100% | PYRA 10% |

rapid ID 32 STREP V4.0 Printout Export New test Modify

REFERENCE: AL 20 910 DATE: 11/21/18

COMMENT:

**DOUBTFUL PROFILE**

| | |
|---|---|
| Strip | rapid ID 32 STREP V4.0 |
| Profile | 3 0 2 2 3 1 1 1 1 2 0 |
| Note | |

| Significant taxa | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactococcus garvieae | 99.9 | 0.53 | HIP 0% | MßDG 85% | | |

| Next taxon | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Streptococcus uberis 2 | 0.1 | 0.0 | RIB 99% | SOR 99% | LAC 100% | PYRA 10% |
| | | | MßDG 100% | | | |

*Figure 4-3 .Examples of Rapid ID 32 STREP test results from apiweb™ identification software. Top:* ***Test result for AL 21 225 with excellent identification as L. garvieae with 99.9 % ID and no tests against. Bottom****: Test result for AL 20 910 with doubtful identification as L. garvieae with 99.7 % ID and HIP 0% as tests against. The frequency of occurrence is measured by % ID and a T-index. The % ID is percentage of reactions observed for one taxon in relative to reactions observed in all taxon. T-index is reactions observed divided with most typical profile. Tests against is test that has an opposite result of the typical profile.*

Table 4-2. Rapid ID 32 STREP test results from apiweb™ identification software.

| AL no | Profile | Identification | Significant taxa | % ID[1] | T[2] | Test against[3] |
|---|---|---|---|---|---|---|
| 20 869 | 30 223 101 020 | Excellent | *Lactococcus garvieae* | 99.9 | 0.91 | MβG 85% |
| 20 870 | 30 223 111 130 | Doubtful | *Lactococcus garvieae* | 99.7 | 0.63 | HIP 0% |
| 20 906 | 30 223 111 130 | Doubtful | *Lactococcus garvieae* | 99.7 | 0.63 | HIP 0% |
| 20 909 | 30 223 111 130 | Doubtful | *Lactococcus garvieae* | 99.7 | 0.63 | HIP 0% |
| 20 910 | 30 223 111 120 | Doubtful | *Lactococcus garvieae* | 99.9 | 0.53 | HIP 0%, MβG 85% |
| 20 911 | 30 223 111 130 | Doubtful | *Lactococcus garvieae* | 99.7 | 0.63 | HIP 0% |
| 21 045 | 30 223 111 120 | Doubtful | *Lactococcus garvieae* | 99.9 | 0.53 | HIP 0%, MβG 85% |
| 21 046 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 225 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 226 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 227 | 30 223 101 120 | Excellent | *Lactococcus garvieae* | 99.9 | 0.91 | MβG 85% |
| 21 228 | 30 223 101 120 | Excellent | *Lactococcus garvieae* | 99.9 | 0.91 | MβG 85% |
| 21 229 | 30 223 101 120 | Excellent | *Lactococcus garvieae* | 99.9 | 0.91 | MβG 85% |
| 21 230 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 231 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 232 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 233 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 234 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 235 | 30 223 101 120 | Excellent | *Lactococcus garvieae* | 99.9 | 0.91 | MβG 85% |
| 21 236 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 237 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 238 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 239 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| 21 240 | 30 223 101 130 | Excellent | *Lactococcus garvieae* | 99.9 | 1.0 | |
| ATCC 49156 | 30 023 100 000 | Excellent | *Lactococcus garvieae* | 99.9 | 0.79 | MAN 75%, MAL 75%, MβG 85% |

[1] % ID is percentage of reactions observed for one taxon in relative to reactions observed in all taxon.

[2]T-index is reactions observed divided with most typical profile.

[3]Tests against is test that has an opposite result of the typical profile.

Colors indicate same results.

## 4.3 Characterization by ID-PCR

All isolates gave the expected 1100 bp amplicon that is specific to *L. garvieae* when using the PCR assay from Zlotkin et al. (1998) and thereby confirmed the identify for all the isolates as *L. garvieae*. Analysis for *Streptococcus iniae* and three serotypes (Ia, Ib and III) of *Streptococcus agalactiae* were negative (Figure 4-4).

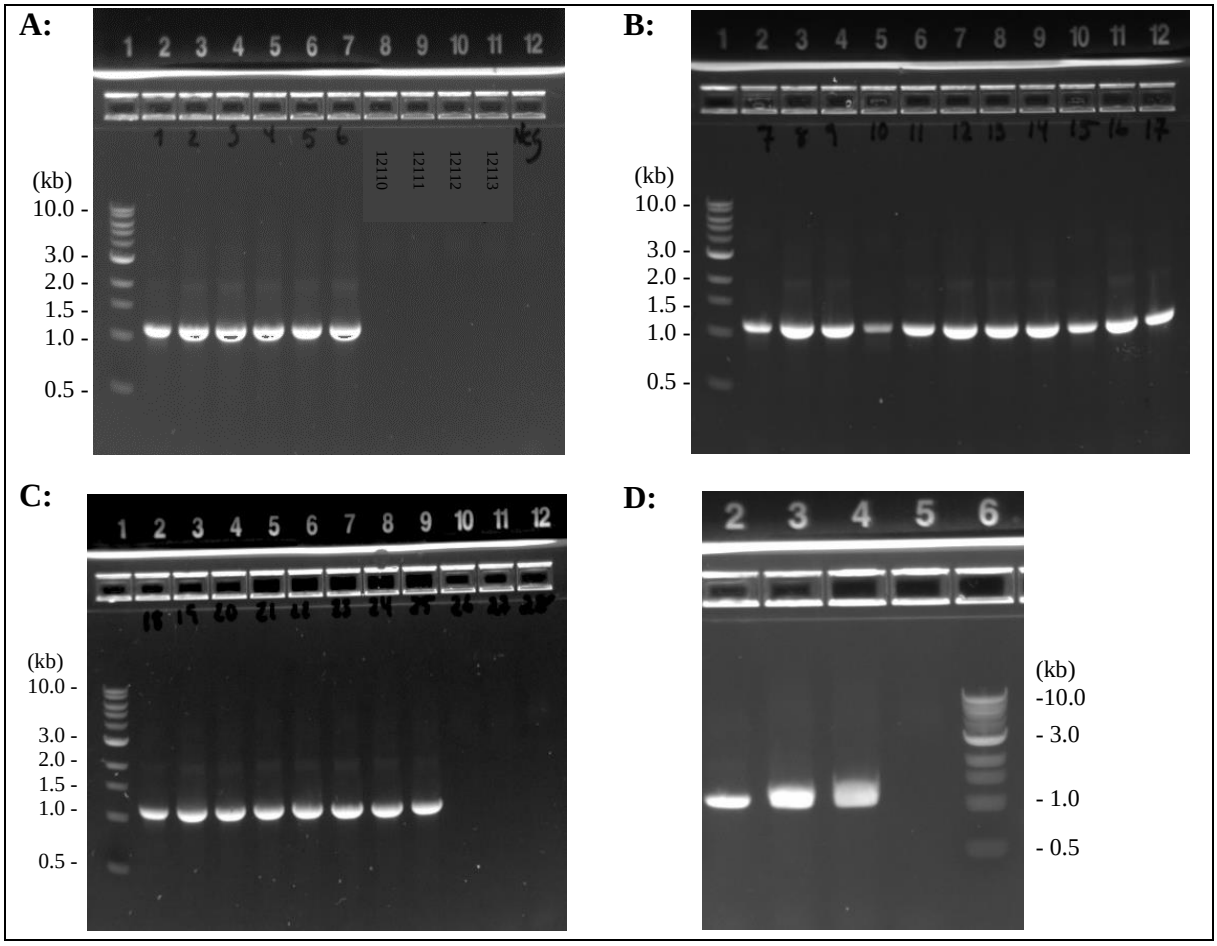



*Figure 4-4. Identification of L. garvieae by PCR. PCR products, 1100 bp, visualized under UV-light on 1.2 % agarose gel A-D.* ***Gel A:*** *Lane 1; 1 kb NEB ladder. Lane 2-7; AL 20 869-AL 20 911. Lane 8; AL 12110 S. agalactiae serotype III. Lane 9; AL 12111 S. agalactiae serotype Ib. Lane 10; AL 12112 S. iniae. Lane 11; AL 12113 S. agalactiae serotype Ia. Lane 12; Negative control.* ***Gel B****: Lane 1; 1 kb NEB ladder. Lane 2-11; AL 21 045-AL 21 233.* ***Gel C:*** *1 kb NEB ladder. Lane 2-8; AL 21 234-AL 21 240. Lane 9; ATCC 49156. Lane 10; Negative control. Lane 11-12: empty.* ***Gel D****: Lane 2; ATCC 49156. Lane 3; Lg2 2µl DNA. Lane4; Lg2 1µl DNA. Lane 5; negative control. Lane 6; 1 kb NEB ladder.*

## 4.4 Identification of isolates by 16S rDNA PCR and sequencing

Six isolates were identified by sequencing the 16S rRNA gene. AL 21 225 and AL 21 226 was isolated from one location in Spain and AL 21 228, AL 21 229, AL 21 230, AL 21 234 was from three locations in Turkey (Table 4-1). Bands at 1500 bp (Figure 4-5) was excised from the gel and purified before sequencing by LGC Genomics GmbH.

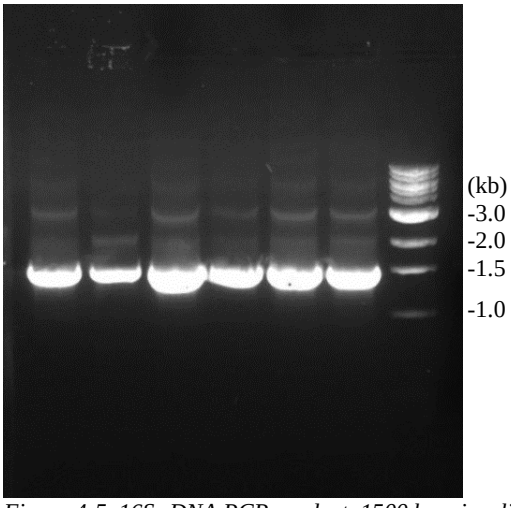



*Figure 4-5. 16S rDNA PCR product, 1500 bp, visualized under UV-light on 1 % agarose gel. Lane 1-6; AL 21 225, AL 21 226, AL 21 228, AL 21 229, AL 21 230, AL 21 234. Lane 7; 1 kb NEB ladder. Lane 8; Negative control. Band of 1500 bp was cut out and purified.*

Unfortunately, after sequencing, some of the sequences (forward and/or reverse abi-files) were not possible to assemble into one contig. Some of the isolates were sequenced twice, without any improvement of the result. This resulted in different length of the contigs. Unfortunately, it was not possible to perform an assembly using a forward and a reverse sequence read for all contigs, and instead two reverse sequences was assembled into one contig. This was the case for AL 21 226 and AL 21 230 and resulted in shorter contigs for these two strains. The contigs (sequences in appendix B) were used in megaBLAST search in NCBI against the 16S ribosomal RNA sequence database. All the isolates were identified as *L. garvieae* with identity > 99.3 % and the same best hit (Table 4-3). Percent coverage was 95 percent for AL 21 229, 99 percent for AL 21 225, AL 21 226, AL 21 228, AL 21 230, for 100 percent for AL 21 234. Percent coverage is a number for how much the query sequence cover all the target sequence. E- value describes how many times one would expect a match by chance. The E-value was zero for all the strains.

Due to time savings and sequencing problems no other strains were identified by sequencing their 16S rDNA.

Table 4-3. Results from BLAST of contigs from 16S rDNA contigs against 16S ribosomal RNA sequence database in NCBI.

| Isolate AL no. (query) | Contig length (bp) | Result BLAST (subject) | Max score[1] | Total score[2] | Query cover[3] (%) | E-value[4] | Ident[5] (%) | Accession |
|---|---|---|---|---|---|---|---|---|
| 21 225 | 1462 | Lactococcus garvieae strain JCM 10343 16S ribosomal RNA, partial sequence | 2630 | 2630 | 99 | 0.0 | 99.52 | NR_113268.1 |
| 21 226 | 375 | Lactococcus garvieae subsp. bovis strain BSN307 16S ribosomal RNA, partial sequence | 688 | 688 | 99 | 0.0 | 100 | NR_152050.1 |
| 21 228 | 1452 | Lactococcus garvieae strain JCM 10343 16S ribosomal RNA, partial sequence | 2619 | 2619 | 99 | 0.0 | 99.65 | NR_113268.1 |
| 21 229 | 932 | Lactococcus garvieae strain JCM 10343 16S ribosomal RNA, partial sequence | 1605 | 1605 | 95 | 0.0 | 99.32 | NR_113268.1 |
| 21 230 | 463 | Lactococcus garvieae subsp. bovis strain BSN307 16S ribosomal RNA, partial sequence | 850 | 850 | 99 | 0.0 | 100 | NR_152050.1 |
| 21 234 | 929 | Lactococcus formosensis strain 516 16S ribosomal RNA, partial sequence | 632 | 632 | 100 | 0.0 | 99.43 | NR_114366.1 |

[1] Max score is score of single best aligned sequence
[2] Total score is sum of scores of all aligned sequences.
[3] Query cover describes how long the query sequence and the target sequences are, relative to each other. If the target sequence covers the whole query sequence, then the query cover is 100%.
[4]E value (expected value) describes how many times on would expect a match by chance in a database of that size. The lower the E value is, the more significant the match.
[5]Percent identity (Ident %) describes how similar the query sequence is to the target sequence. The higher the percent identity is, the more similar.

## 4.5 Identification of isolates by *gyrB* PCR and sequencing

All the 24 isolates and ATCC 49156 as a positive control, were identified by sequencing gyrB. After PCR, a band at approx. 1.5 kb was observed (Figure 4-6) as expected when using the PCR assay from Ferrario et al. (2013). Primers for gyrB should give a band of 1464 bp. PCR products were purified and sent to LGC Genomics GmbH for sequencing.

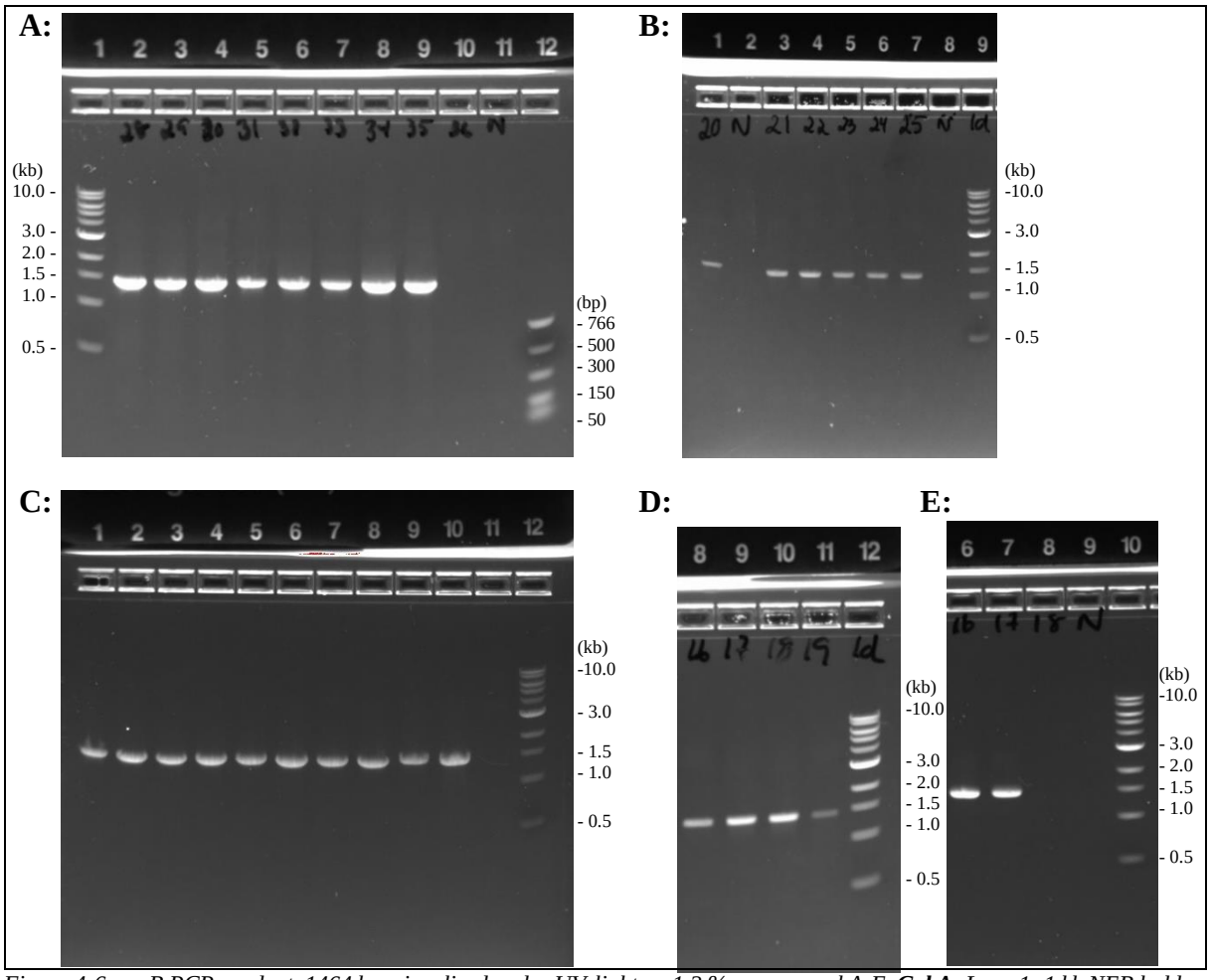



*Figure 4-6. gyrB PCR product, 1464 bp, visualized under UV-light on 1.2 % agarose gel A-E.* ***Gel A:*** *Lane 1; 1 kb NEB ladder. Lane 2-7; AL 21 225-AL 21 230. Lane 8-9; AL 21 233-AL 21 234. Lane 10; empty. Lane 11; Negative control. Lane 12; PCR marker NEB.* ***Gel B****: Lane 1; AL 21 045. Lane 2; Negative control. Lane 3-8; PCR products with rpoC primers from chapter 4.6. Lane 9; 1 kb NEB ladder.* ***Gel C:*** *Lane 1; AL 21 046. Lane 2-3; AL 21.231-A L21 232. Lane 4-9; AL 21 235-AL 21 240. Lane 10; ATCC 49156. Lane 11; Negative control. Lane 12: 1 kb NEB ladder.* ***Gel D****: Lane 8-11; AL 20 906-AL 20 911. Lane 12; 1 kb NEB ladder.* ***Gel E****: Lane 6-7; AL 20 869-AL 20 870. Lane 8; empty. Lane 9; Negative control. Lane 10; 1 kb NEB ladder.*

The forward and reverse sequences for each isolate were assembled into one contig (see sequences in appendix C and G). The contigs were exported as FASTA files and used as input in megaBLAST search in NCBI against the nucleotide collection database.

All isolates were identified as *L. garvieae* with identity > 98.7 % and the same best hit; complete genome for strain JJJN1, Lg2 and ATCC 49156 (Table 4-4). Percent coverage was 100 percent for all the strains, and the E-value was zero for all the strains. One of the results from megaBlast was *L. garvieae* gyrase subunit B (*gyrB*), with accession no GU324261.1.. There was little information about this sequence, so it was considered not reliable.

Table 4-4. Results from BLAST of *gyrB* contigs against nucleotide collection database in NCBI.

| Isolate AL no. (query) | Contig length (bp) | Best hit (subject) | Max score[1] | Total score[2] | Query cover[3] (%) | E-value[4] | Ident (%)[5] | Accession |
|---|---|---|---|---|---|---|---|---|
| 20 869 | 789 | Lactococcus garvieae strain JJJN1 chromosome, complete genome | 1402 | 1402 | 100 | 0.0 | 98.73 | CP026502.1 |
| 20 870 | 849 | | 1048 | 1048 | 100 | 0.0 | 100 | |
| 20 906 | 827 | | 1048 | 1048 | 100 | 0.0 | 100 | |
| 20 909 | 831 | | 1530 | 1530 | 100 | 0.0 | 99.88 | |
| 20 910 | 761 | | 1400 | 1400 | 100 | 0.0 | 99.87 | |
| 20 911 | 764 | | 1406 | 1406 | 100 | 0.0 | 99.87 | |
| 21 045 | 868 | | 1048 | 1048 | 100 | 0.0 | 100 | |
| 21 046 | 868 | Lactococcus garvieae Lg2 DNA, complete genome | 1048 | 1048 | 100 | 0.0 | 100 | AP009333.1 |
| 21 225 | 654 | | 1203 | 1203 | 100 | 0.0 | 99.85 | |
| 21 226 | 812 | | 1495 | 1495 | 100 | 0.0 | 99.88 | |
| 21 227 | 813 | | 1496 | 1496 | 100 | 0.0 | 99.88 | |
| 21 228 | 720 | | 1330 | 1330 | 100 | 0.0 | 100 | |
| 21 229 | 819 | | 1507 | 1507 | 100 | 0.0 | 99.88 | |
| 21 230 | 918 | | 1690 | 1690 | 100 | 0.0 | 99.89 | |
| 21 231 | 799 | | 1471 | 1471 | 100 | 0.0 | 99.87 | |
| 21 232 | 746 | Lactococcus garvieae ATCC 49156 DNA, complete genome | 1048 | 1048 | 100 | 0.0 | 100 | AP009332.1 |
| 21 233 | 699 | | 1291 | 1291 | 100 | 0.0 | 100 | |
| 21 234 | 716 | | 1323 | 1323 | 100 | 0.0 | 100 | |
| 21 235 | 899 | | 1655 | 1655 | 100 | 0.0 | 99.89 | |
| 21 236 | 1012 | | 1864 | 1864 | 100 | 0.0 | 99.90 | |
| 21 237 | 821 | | 1864 | 1864 | 100 | 0.0 | 99.90 | |
| 21 238 | 731 | | 1048 | 1048 | 100 | 0.0 | 100 | |
| 21 239 | 798 | | 1048 | 1048 | 100 | 0.0 | 100 | |
| 21 240 | 798 | | 1469 | 1469 | 100 | 0.0 | 99.87 | |
| ATCC 49156 | 970 | | 1543 | 1543 | 100 | 0.0 | 100 | |

[1] Max score is score of single best aligned sequence
[2] Total score is sum of scores of all aligned sequences.
[3] Query cover describes how long the query sequence and the target sequences are, relative to each other. If the target sequence covers the whole query sequence, then the query cover is 100%.
[4]E value (expected value) describes how many times on would expect a match by chance in a database of that size. The lower the E value is, the more significant the match.
[5]Percent identity (Ident %) describes how similar the query sequence is to the target sequence. The higher the percent identity is, the more similar.

## 4.6 MLSA

Sixteen of the isolates from this study and nine isolates collected from NCBI Genbank were included in a MLSA scheme from Ferrario et al. (2013). After PCR and gel electrophoresis, single band of expected size was observed for every primer pair. Figure 4-7 shows examples of agarose gels for *als, gapC, rpoC and galP* for eight isolates. See appendix D, Figure D-1, for the remaining data. Agarose gels for *gyrB*, are shown in Figure 4-6, chapter 4.5.

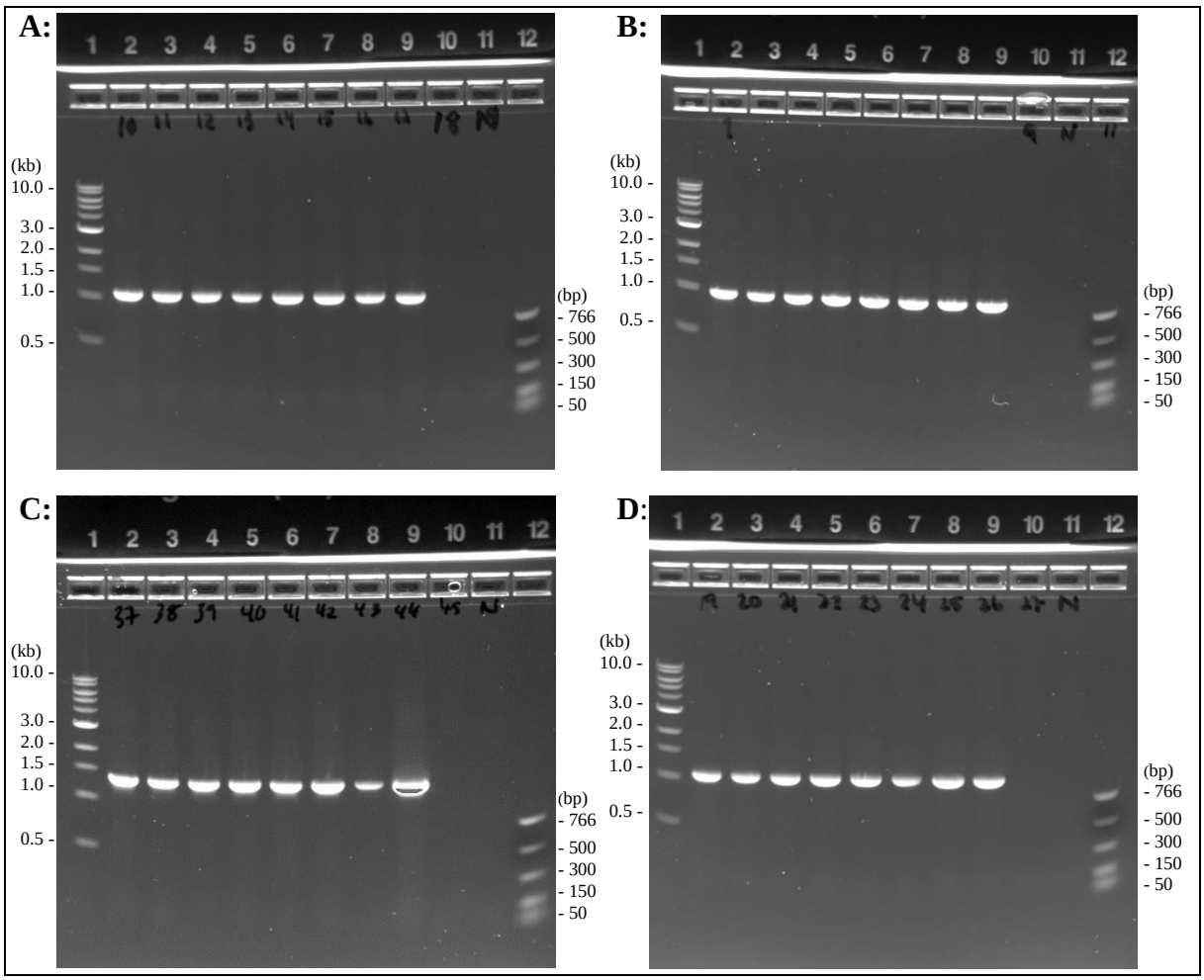



*Figure 4-7. PCR product of MLSA genes visualized on agarose-gel A-D for eight of the sixteen isolates.* ***Gel A****: gene als, ~1kb.* ***Gel B****: gapC, ~1 kb.* ***Gel C****: rpoC, ~1.5 kb.* ***Gel D****: galP, ~1 kb. All the gels had strains in the same order: Lane 1; 1 kb NEB ladder. Lane 2-7; AL 21 225-AL21 230. Lane 8-9; AL 21 233-AL21 234. Lane 10; empty. Lane 11; Negative control. Lane 12; PCR marker NEB.*

PCR products were purified and submitted to sequencing to LGC Genomics GmbH. The forward and reverse sequences for each isolate were assembled into one contig. See contig size after assembly and trimming in CLC in Table 4-5. The contigs were exported as FASTA files and used in megaBLAST search in NCBI against the nucleotide collection database to verify the identity and gene. All isolates were identified as *L. garvieae* with identities ranging from

> 94 % for *galP* to > 99 % for *gapC* (Table 4-6). Percent coverage was 99-100 for all the strains, E-value was zero and max score and total score was the same (not shown in the table).

One of the results from megaBLAST for the gene *gapC* was; *Lactococcus garvieae* glyceraldehyde-3-phosphate dehydrogenase gene, complete cds, with accession no FJ524849.1. There was little information about this sequence, so it was considered not reliable.

Table 4-5. Size of the contigs after assembly and trimming in MEGA.

| Strain, AL no. | Contig size (bp) | | | | |
|---|---|---|---|---|---|
| | ***als*** | ***gapC*** | ***gyrB*** | ***rpoC*** | ***galP*** |
| 20 869 | 970 | 865 | 789 | 1063 | 962 |
| 20 870 | 972 | 858 | 849 | 957 | 958 |
| 20 906 | 991 | 857 | 827 | 1063 | 977 |
| 20 909 | 540 | 807 | 831 | 1021 | 977 |
| 20 910 | 991 | 857 | 761 | 766 | 977 |
| 20 911 | 926 | 857 | 764 | 1077 | 977 |
| 21 045 | 925 | 808 | 868 | 705 | 977 |
| 21 046 | 973 | 866 | 868 | 810 | 958 |
| 21 225 | 974 | 856 | 654 | 807 | 977 |
| 21 226 | 933 | 806 | 812 | 978 | 977 |
| 21 227 | 540 | 872 | 813 | 996 | 977 |
| 21 228 | 991 | 872 | 720 | 850 | 891 |
| 21 229 | 991 | 824 | 819 | 983 | 912 |
| 21 230 | 980 | 873 | 918 | 832 | 977 |
| 21 233 | 933 | 859 | 699 | 819 | 977 |
| 21 234 | 945 | 815 | 716 | 963 | 750 |

The contigs for each gene were aligned using ClustalW, edited in MEGA and adjusted to proper reading frame. Table 4-7 shows the resulting alignment length for each gene, and the alignments are shown in appendix E, Figure E-1 to E-5. The alignments were employed as an input in modeltest in CLC workbench and the best substitution model for the gene alignments are listed in Table 4-7. The substitution models were used to construct phylogenetic trees by Maximum-Likelihood in CLC workbench as seen in Figure 4-8, Figure 4-9 and Figure 4-10.

Table 4-6. Summary of results from BLAST of *als*, *gapC*, *rpoC* and g*alP* contigs against nucleotide collection database in NCBI.

| Gene (query) | Result BLAST, Best hit, (subject) | Query cover[1] [%] | Ident[2] [%] | Accession |
|---|---|---|---|---|
| *als* | Lactococcus garvieae clone IV alpha-acetolactate synthase (als) gene, partial cds | 100 | > 97 | EF450031.1 |
| | Lactococcus garvieae DNA, complete genome, strain: 122061 | | | |
| | Lactococcus garvieae strain JJJN1 chromosome, complete genome | | | AP017373.1 |
| | Lactococcus garvieae Lg2 DNA, complete genome | 100 | > 96 | CP026502.1 |
| | Lactococcus garvieae ATCC 49156 DNA, complete genome | | | AP009333.1 |
| | | | | AP009332.1 |
| *gapC* | Lactococcus garvieae strain JJJN1 chromosome, complete genome | 100 | > 99 | CP026502.1 |
| | Lactococcus garvieae Lg2 DNA, complete genome | | | AP009333.1 |
| | Lactococcus garvieae ATCC 49156 DNA, complete genome | | | AP009332.1 |
| *rpoC* | Lactococcus garvieae strain JJJN1 chromosome, complete genome | 98-100 | > 96 | CP026502.1 |
| | Lactococcus garvieae Lg2 DNA, complete genome | | | AP009333.1 |
| | Lactococcus garvieae ATCC 49156 DNA, complete genome | | | AP009332.1 |
| *galP* | Lactococcus garvieae strain JJJN1 chromosome, complete genome | 99-100 | > 94 | CP026502.1 |
| | Lactococcus garvieae Lg2 DNA, complete genome | | | AP009333.1 |
| | Lactococcus garvieae ATCC 49156 DNA, complete genome | | | AP009332.1 |
| | Lactococcus garvieae galactose operon, complete sequence | | | EU153555.1 |

[1] Query cover describes how long the query sequence and the target sequences are, relative to each other. If the target sequence covers the whole query sequence, then the query cover is 100%.
[2] Percent identity (Ident %) describes how similar the query sequence is to the target sequence. The higher the percent identity is, the more similar.
E-values was zero for every sequence (not shown in table).

Table 4-7. Predicted best fit nucleotide substitution model by modeltest in CLC workbench for each of the five gene-alignment used in this study and the concatenation of the five genes used in MLSA.

| Gene | Alignment length (bp) | Substitution model[1] |
|---|---|---|
| *Als* | 540 | GTR + G + T |
| *gapC* | 763 | GTR + T |
| *gyrB* | 567 | GTR + G + T |
| *rpoC* | 705 | GTR + G + T |
| *galP* | 750 | HKY + G + T |
| Concatenated | 3325 | GTR + G + T |

[1] GTR, General Time Reversible, a substitution model that has six substitution types, one parameter for each pair of nucleotides (six in total). HKY, Hasegawa-Kishino-Yano, a two-parameter substitution model allowing for transition/transversion. +G, denotes the rate variation (4 categories). +T, denotes topology variation.

The five phylogenetic trees showed that most of the isolates from this work were grouped together and thus assumed to be identical. Only AL 20 869 was different. All trees displayed a clear distance from *L. lactis* subs. *lactis* and *L. lactis* subs. c*remoris,* that served as outgroups, compared to the other strains in the trees. *L. garvieae* strains from yellowtail were separated in two groups where ATCC 49156 and Lg2 were always clustered together. Strains from rainbow trout were clustered in one major group with some internal differences. Strain Lg9 and UNIUD074 were clustered with either rainbow trout or yellowtail depending on what gene that was being analyzed. AL 20 869 is in every tree either on a branch alone or together with yellowtail, making this strains different from the other isolates in this work.

The alignments were shorter in length (Table 4-7) for each gene than Ferrario et al.(2013) got in their MLSA scheme (803 to 830 bp). New alignment was made for als without the two strains that had the shortest contigs, AL 20 909 and AL 21 227 (Table 4-5). Alignment was made using ClustalW, edited in MEGA and adjusted to proper reading frame, see appendix F, Figure F-1. The new alignment was 860 bp as opposed to 540 for the first *als* alignment. The same substitution model was used to construct a new phylogenetic tree by Maximum-Likelihood in CLC workbench, appendix F, Figure F-2.
The two trees for *als* are in general very similar, but a slightly different grouping was seen for AL 20 869 and Lg2, however the bootstrap values in the new tree is lower for this particular grouping. New alignment were also made for *gyrB* without the two shortest contigs, but the new alignment was just 618 bp. Since the new alignment was not much longer than the first one, no new tree was constructed.

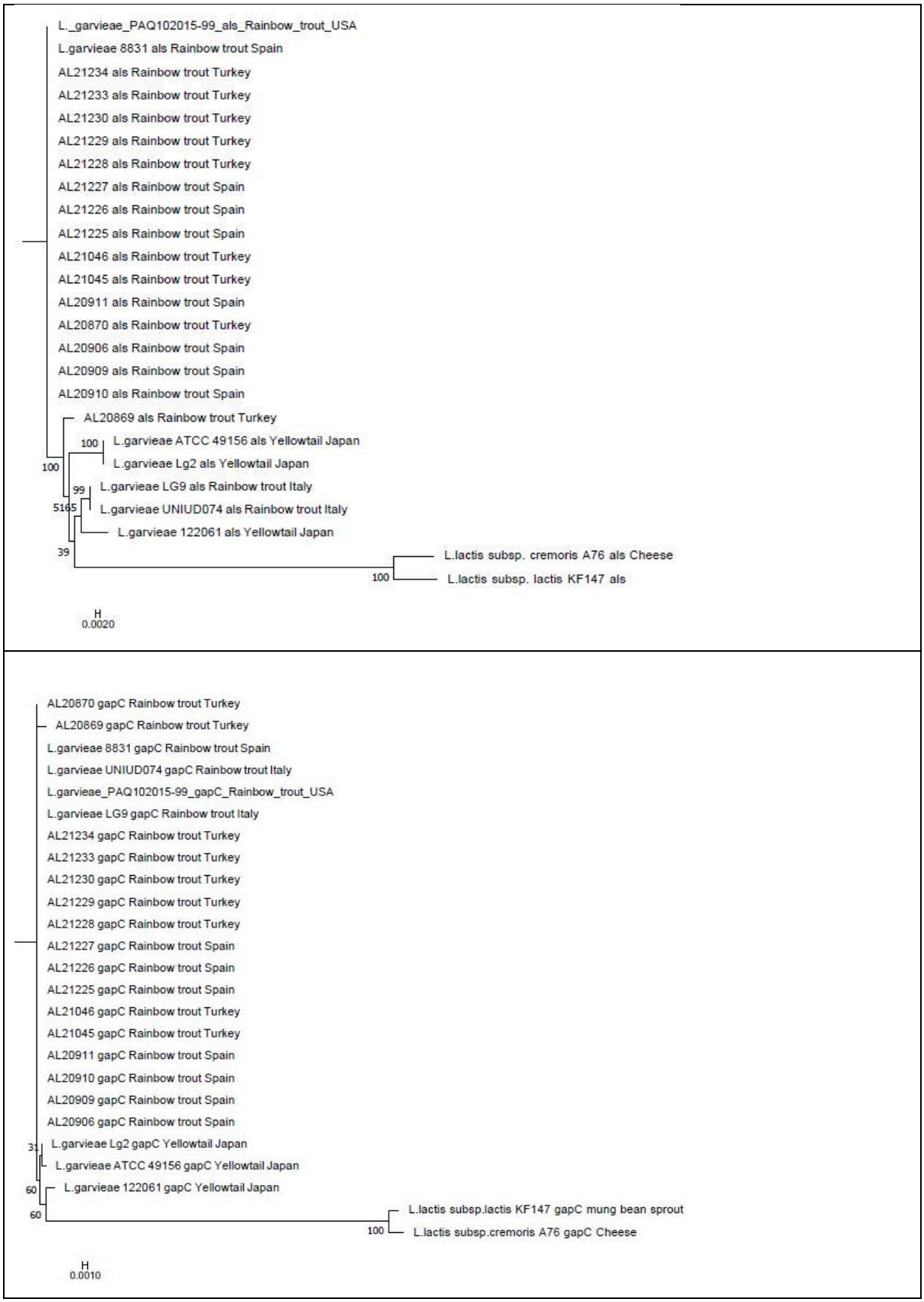



*Figure 4-8. Phylogenetic relationship between 25 strains based on individual analyses of als 540bp (top picture) and gapC 763 bp (lower picture). The trees were constructed by maximum-likelihood method. Lactococcus lactis subsp. lactis and Lactococcus lactis subsp. cremoris served as outgroups. The scale bar indicates nucleotide sequence variation of 0.2 % for als and 0.1 % for gapC.*

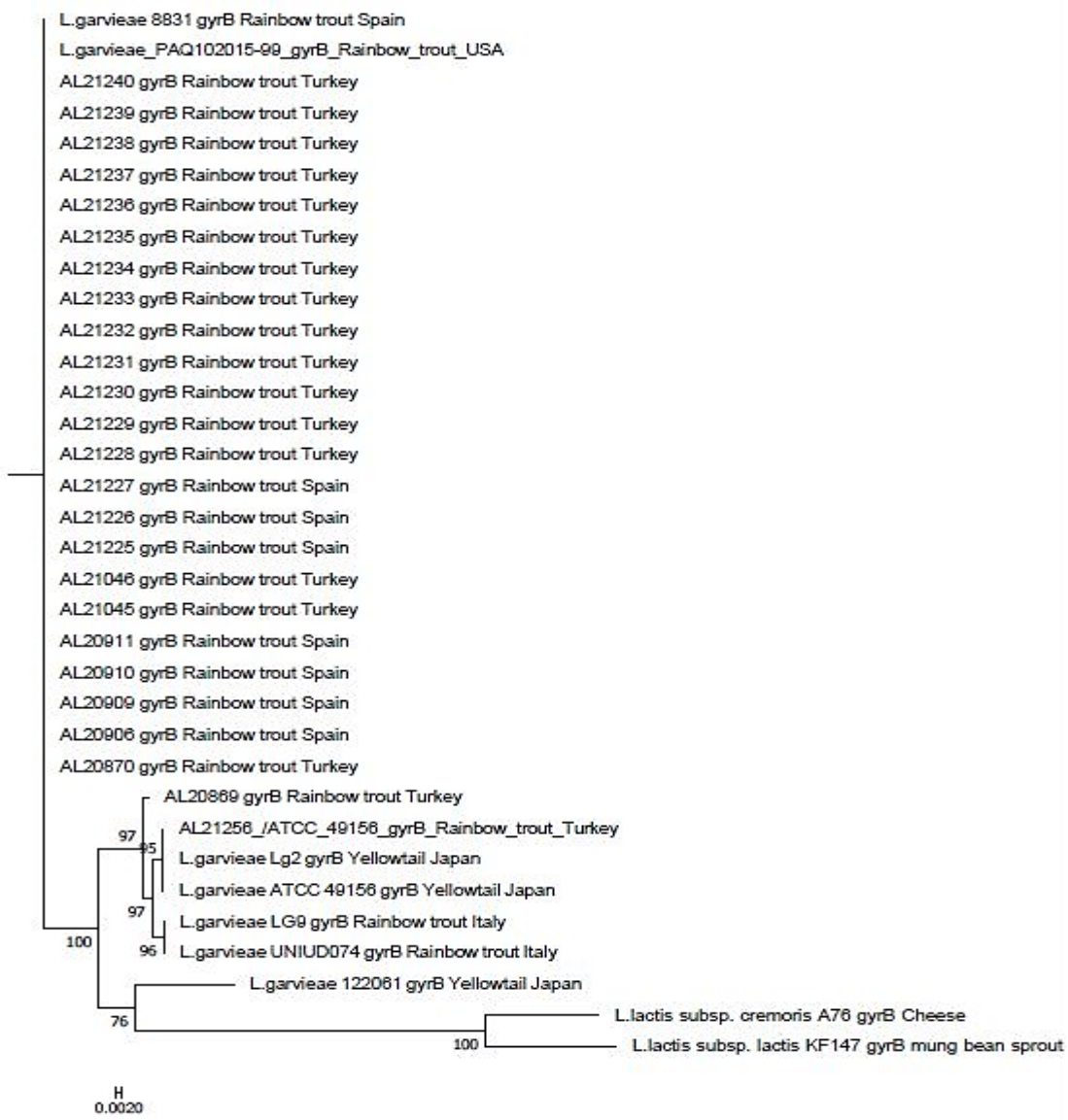



*Figure 4-9. Phylogenetic relationship between 34 strains based on analyses of gyrB, 567bp. The tree was constructed by maximum-likelihood method. Lactococcus lactis subsp. lactis and Lactococcus lactis subsp. cremoris served as outgroups. The scale bare indicates nucleotide sequence variation of 0.2 %.*

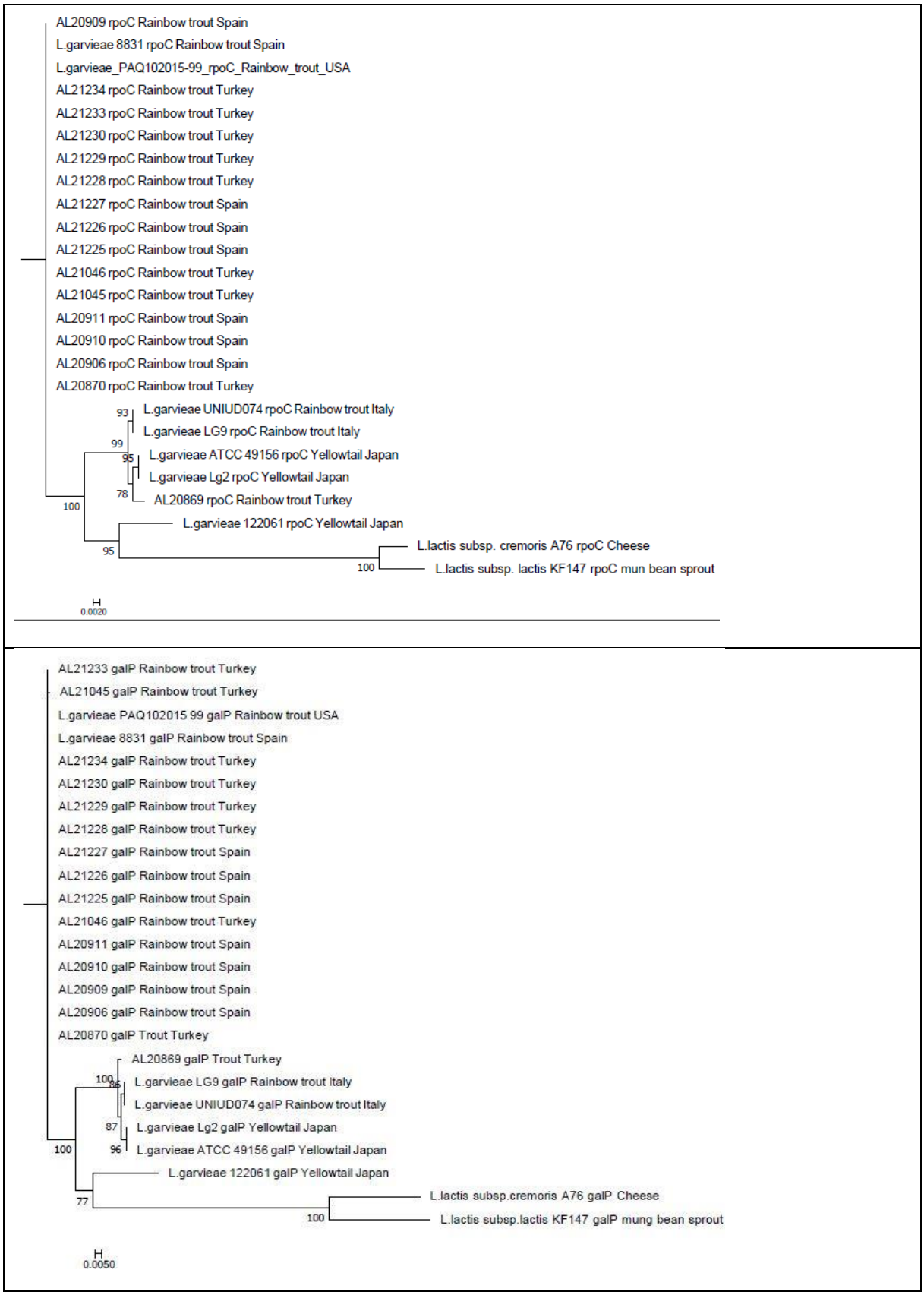



*Figure 4-10. Phylogenetic relationship between 25 strains based on individual analyses of rpoC 705bp (top picture) and galP 750bp (lower picture). The tree was constructed by maximum-likelihood method. Lactococcus lactis subsp. lactis and Lactococcus lactis subsp. cremoris served as outgroups. The scale bar indicates nucleotide sequence variation of 0.2 % for rpoC and 0.5 % for galP.*

For the MLSA scheme the contigs from each strain were joined together end-on-end in the order; *als, gapC, gyrB, rpoC* and *galP,* which then created a single concatenated sequence that was 3325 bp in length. The concatenated sequences were aligned using ClustalW, edited in MEGA and adjusted to proper reading frame (Table 4-7). The concatenated sequences of five genes are shown in appendix G. The alignment was employed as an input in modeltest in CLC workbench and the best substitution model for the concatenated alignment is shown in Table 4-7. The substitution model was used to construct a phylogenetic tree by Maximum-Likelihood in CLC workbench as seen in Figure 4-11.

The same clustering that could be seen in the single gene phylogenetic trees, could also be seen in the phylogenetic tree from the 3325 bp concatenated sequence of the five loci, Figure 4-11. Almost all the isolates from Turkey and Spain identified in this work were clustered in one group along with strain 8831 from rainbow trout in Spain and PAQ102025 from USA. The second cluster is divided into two branches; AL20 869, LG9 and UNIUD074, Lg2 and ATCC 49156 on one branch and 122061, *L. lactis* subsp. *lactis* and *L. lactis* subsp. c*remoris* in the second branch.

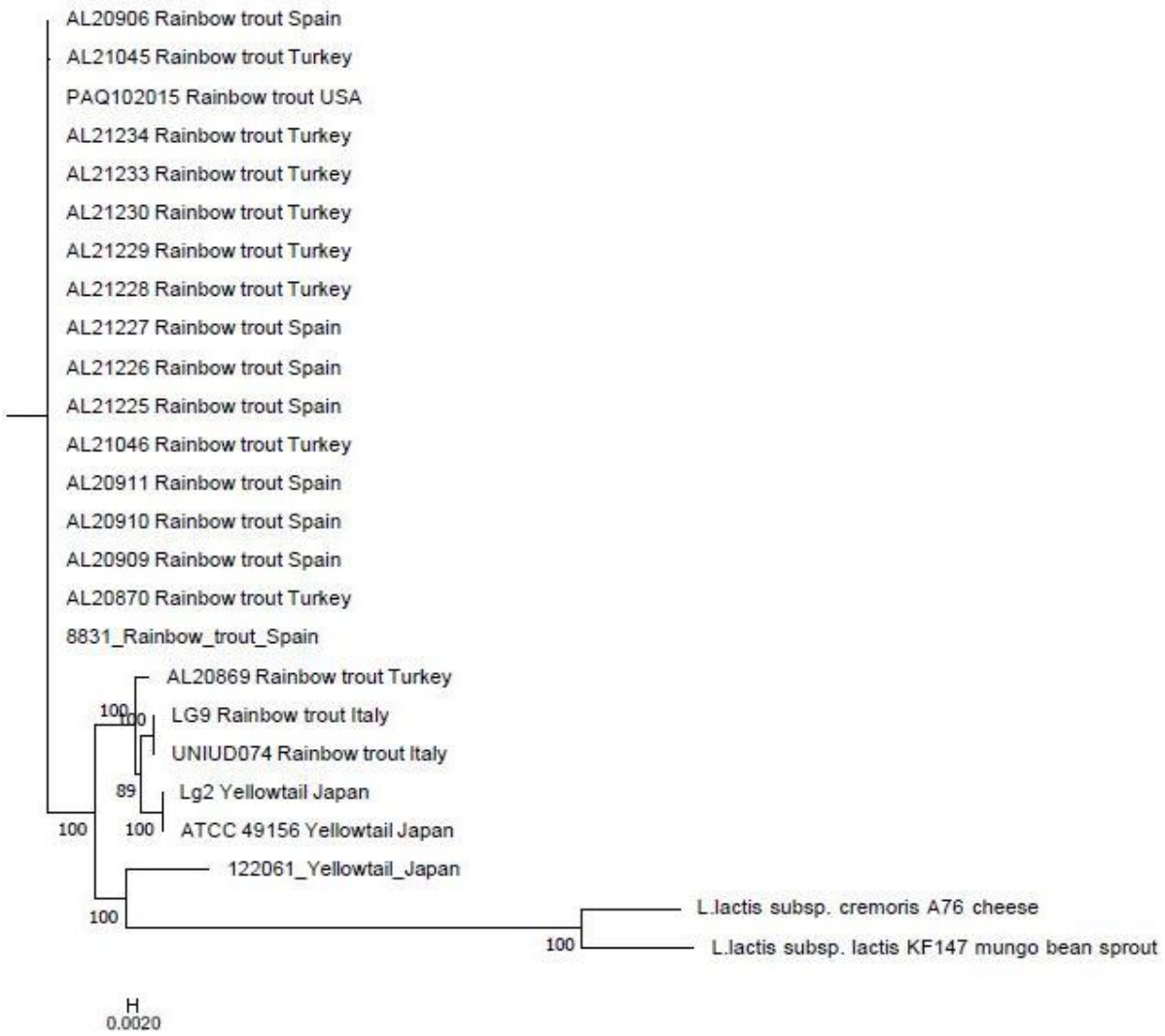



*Figure 4-11. Phylogenetic tree between 25 L. garvieae strains constructed from the 3325 bp concatenated DNA sequences of the five loci (als, gapC, gyrB, rpoC and galP). The tree was constructed by maximum-likelihood method. Lactococcus lactis subsp. lactis and Lactococcus lactis subsp. cremoris served as outgroups. The scale bar indicates nucleotide sequence variation of 0.2 %.*

## 4.7 Characterization of exopolysaccharides (EPS)

### *4.7.1 Phenotypic detection of capsule*

Negative staining with nigrosine was performed on 25 isolates, including ATCC 49156 as a negative control. None of the isolates exhibited a halo surrounding the bacteria cells, hence none of the isolates were capsulated. Figure 4-12 shows an example of negative stain of AL 20 870,

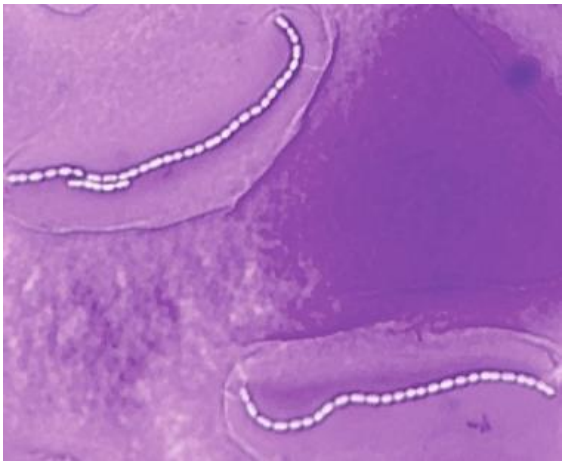


*Figure 4-12. Negative staining of AL 20 870 using nigrosine, visualized under 100x magnification showing no capsule. All the isolates exhibit the same form as this isolate.*

### *4.7.2 Genotypic detection of capsule*

Three different approaches were performed to investigate if the isolates in this work had a capsule gene cluster as described in Lg2 by Miyauchi et al. (2012) ;

1) PCR MLSA from Miyauchi et al. (2012),

2) Multiplex PCR from Ture & Altinok (2016)

3) Primer designed in this work to detect internal genes of the capsule gene cluster.

1) Figure 4-13 shows PCR products visualized on agarose-gels were every isolate had a PCR amplicon at approx. 750 bp indicating no capsular gene cluster. The positive control for gene cluster, Lg2, also had a band at approx. 750 bp which should have been a band at 16.5 kb.

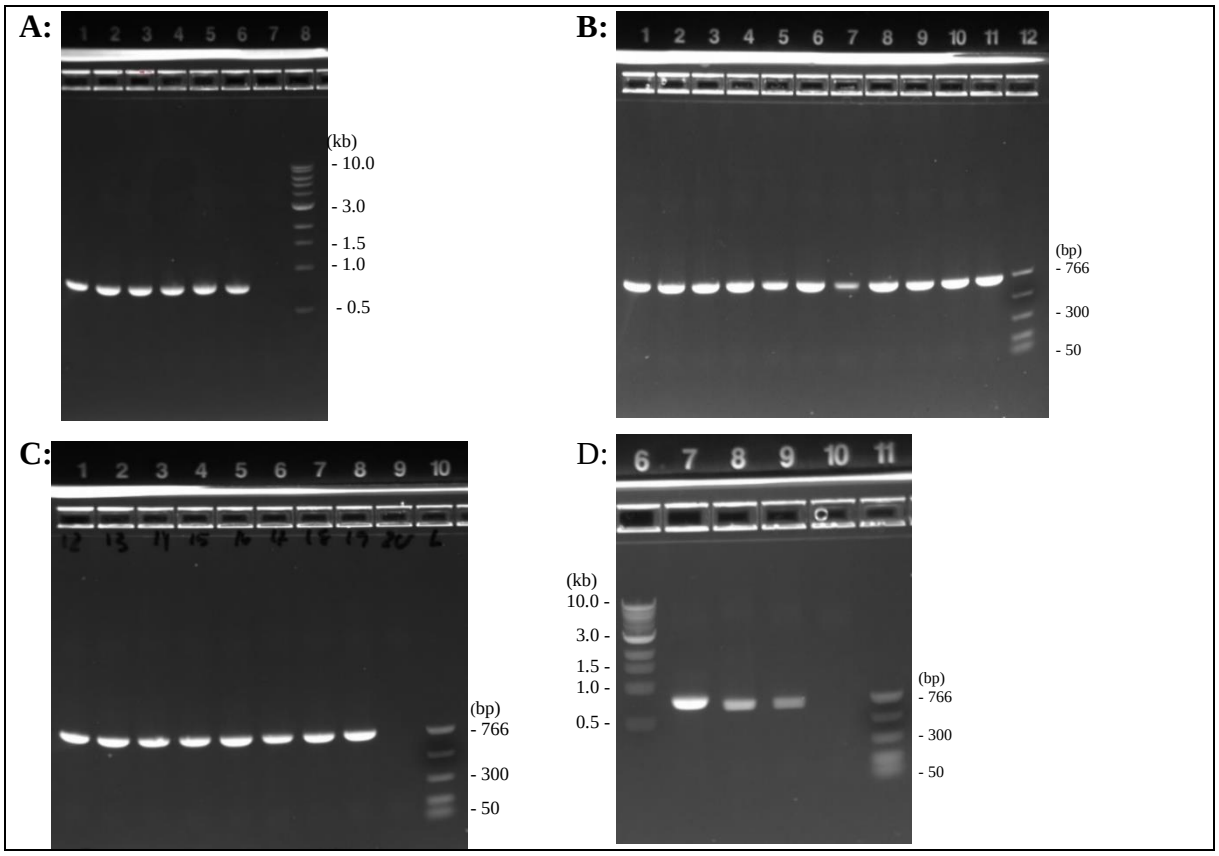



*Figure 4-13. Identification of capsule gene cluster according to Miyauchi et al.(2012). PCR-products visualized on agarose-gel A-D. All the amplicons were 750 bp* ***Gel A:*** *Lane 1-6; AL 20 869, AL 20 906, AL 21 225, AL 21 228, AL 21 234, ATCC 49156. Lane 7; negative control. Lane 8; 1 kb NEB ladder.* ***Gel B:*** *Lane 1-11; AL20 870, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046, AL21 226, AL21 227, AL21 229-AL 21 231. Lane 12; PCR marker NEB.* ***Gel C****: Lane 1-11; AL 21 232, AL 21 233, AL21 235-AL21 240. Lane 9 negative control. Lane 12; PCR marker NEB.* ***Gel D:*** *Lane 6; 1 kb NEB ladder. Lane 7; ATCC49156. Lane 8; Lg2 2µl DNA Lane 9; Lg2 1µl DNA. Lane 10; negative control. Lane 11; PCR marker NEB.*

2) Figure 4-14 shows PCR products from the multiplex PCR assay visualized on agarose-gels. Only the positive control Lg2, had positive bands and gave four bands of 785 bp, 650 bp, 549 bp and 304 bp. All the other isolates had no PCR product, indicating no capsular gene cluster.

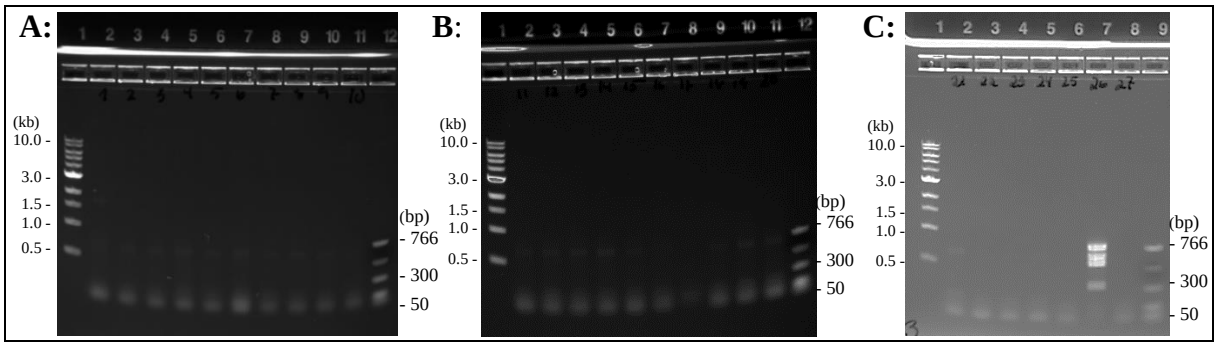



*Figure 4-14. Multiplex PCR for detection of capsule gene cluster according to Ture & Altinok (2016). PCR-products visualized on agarose-gel A-C. The weak bands at 50 bp are primer dimers.* ***Gel A:*** *Lane 1; 1 kb NEB ladder. Lane 2-11; AL20 869, AL20 906, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046, AL 21 225, AL21 226. Lane 12; PCR marker NEB.* ***Gel B****: Lane 1; 1 kb NEB ladder. Lane 2-11; AL21 227-AL21 236. Lane 12; PCR marker NEB.* ***Gel C****: Lane 1; 1 kb NEB ladder. Lane 2-8; AL 21 237-AL21 240. Lane 9; PCR marker NEB.*

3) Three primer pairs were designed to check for the presence of *epsC*, *epsR* and *cpsW*. A gradient PCR was necessary for *epsC* and *cpsW* to achieve the right annealing temperature. Temperatures tested in the gradient PCR ranged from 50 to 68 °C for *epsC*, and 50 to 64 °C cpsW. Resulting in an annealing temperature at 67 °C for *epsC* and 62 °C for *cpsW*. Annealing temperature for *epsR* was 64°C and did not need a gradient PCR. The Lg2 band was weak since DNA was diluted 50 %. A small amount of DNA from Lg2 was kindly provided Dr. Mustafa Ture, Central Fisheries and Research Institute, Turkey, that were used for many PCR reactions in this work and there was only a small amount of DNA left for this PCR test.

Figure 4-15 shows PCR products for the gene *epsR* visualized on agarose-gels. Only the positive control Lg2, had positive band at predicted size at 206 bp. All the other isolates had no PCR product indicating no *epsR* gene in these isolates.

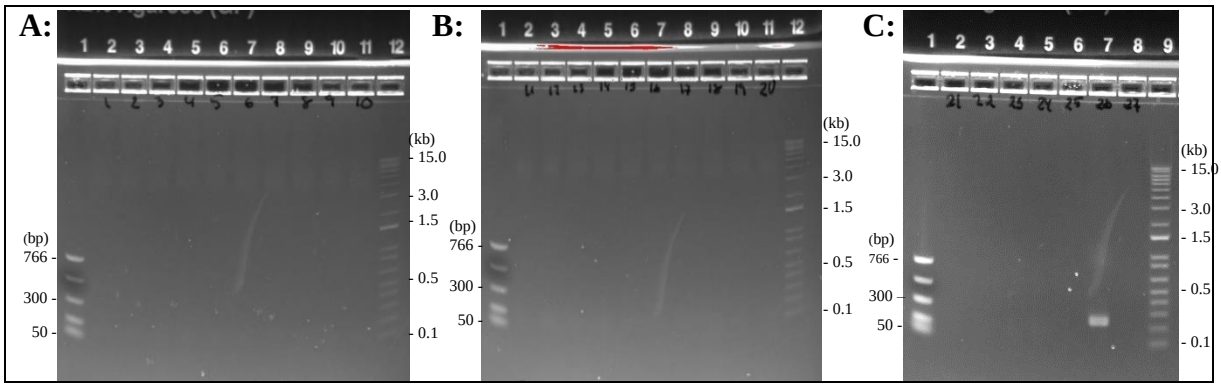



*Figure 4-15. Detection of gene epsR inside the capsule gene cluster. Only the positive control Lg2, had positive band at predicted size at 206 bp. PCR-products visualized on agarose-gel A-C.* ***Gel A:*** *Lane 1; PCR marker. Lane 2-11; AL20 869, AL20 906, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046, AL 21 225, AL21 226. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel B****: Lane 1; PCR marker NEB. Lane 2-11; AL21 227-AL21 236. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel C****: Lane 1; PCR marker NEB. Lane 2-5; AL 21 237-AL21 240. Lane 6; ATCC 49156. Lane 7; Lg2. Lane 8; Negative control. Lane 9; 1 kb Plus ladder Invitrogen.*

Figure 4-16 shows PCR products for the gene *epsC* visualized on agarose-gels. Only the positive control Lg2, had positive band at predicted size at 217 bp. All the other isolates had no PCR product indicating no *epsC* gene in these isolates.

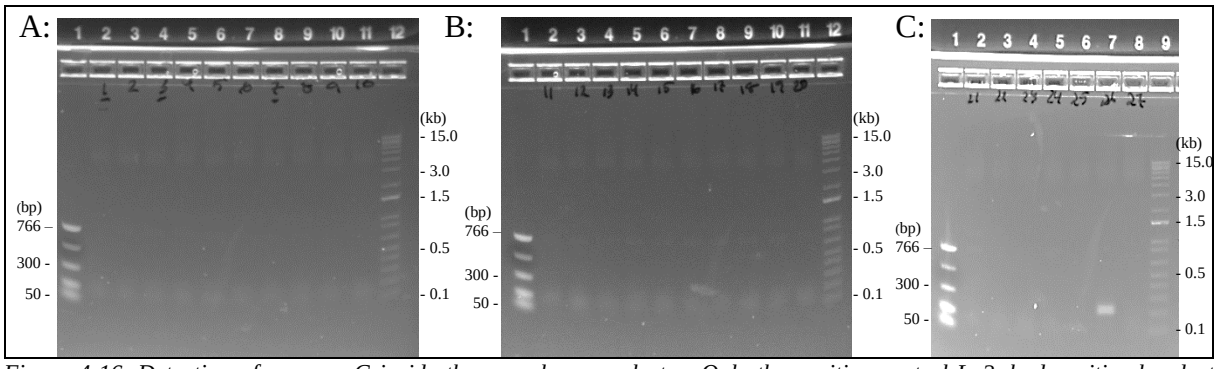



*Figure 4-16. Detection of gene epsC inside the capsule gene cluster. Only the positive control Lg2, had positive band at predicted size at 217 bp. PCR-products visualized on agarose-gel A-C.* ***Gel A:*** *Lane 1; PCR marker. Lane 2-11; AL20 869, AL20 906, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046, AL 21 225, AL21 226. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel B****: Lane 1; PCR marker NEB. Lane 2-11; AL21 227-AL21 236. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel C****: Lane 1; PCR marker NEB. Lane 2-5; AL 21 237-AL21 240. Lane 6; ATCC 49156. Lane 7; Lg2. Lane 8; Negative control. Lane 9; 1 kb Plus ladder Invitrogen.*

Figure 4-17 shows PCR products for the gene *cpsW* visualized on agarose-gels. Only the positive control Lg2, had positive band at predicted size at 382 bp. All the other isolates had no PCR product indicating no *cpsW* gene in these isolates.

Only Lg2 among all the strains had positive PCR products for *epsR, epsC* and *cpsW* which are genes inside the capsular gene cluster (Morita et al., 2011) indicating that only Lg2 have a capsular gene cluster.

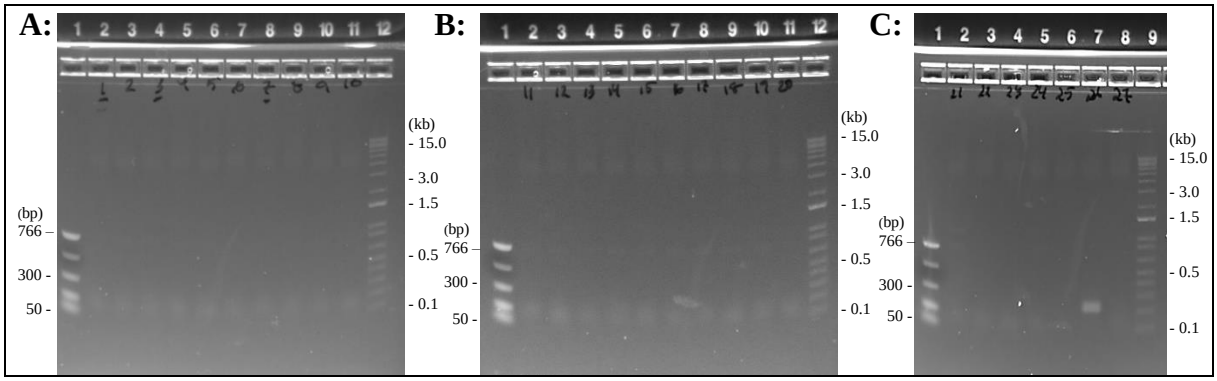



*Figure 4-17. Detection of gene cpsW inside the capsule gene cluster. Only the positive control Lg2, had positive band at predicted size at 382 bp. PCR-products visualized on agarose-gel A-C.* ***Gel A:*** *Lane 1; PCR marker. Lane 2-11; AL20 869, AL20 906, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046, AL 21 225, AL21 226. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel B****: Lane 1; PCR marker NEB. Lane 2-11; AL21 227-AL21 236. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel C****: Lane 1; PCR marker NEB. Lane 2-5; AL 21 237-AL21 240. Lane 6; ATCC 49156. Lane 7; Lg2. Lane 8; Negative control. Lane 9; 1 kb Plus ladder Invitrogen.*

## 4.8 Putative virulence genes

The 24 isolates were tested if they had the virulence genes *LPxTG*-1, *LPxTG*-4 and *adhesin.* It was necessary to do gradient PCR for *LPxTG*-1 and *LPxTG*-4 to achieve the right annealing temperature. Temperatures tested in gradient PCR ranged from 50 to 65 °C for *LPxTG*-1, and 50 to 68 °C *LPxTG*-4. Resulting in annealing temperature at 68 °C for *LPxTG*-1 and 59 °C for *LPxTG*-4. The PCR reaction of *adhesin* had an annealing temperature at 54 °C as described by Ture & Altinok (2016). Figure 4-18, Figure 4-19 and Figure 4-20 shows results from PCR products for the genes visualized on agarose-gels. Isolate Lg2 and ATCC 49156 were positive for all three genes. Isolate AL20 869 was positive for LPxTG-1 and *adhesin*. No other isolates were positive for the genes tested.

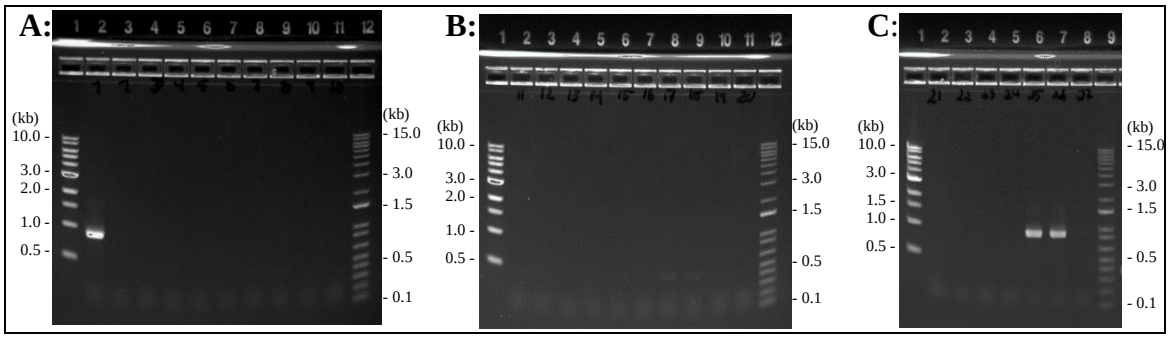



*Figure 4-18. Identification of possible virulence gene LPxTG-1. PCR product of 878 bp indicate presence of virulence gene LPxTG-1. Positive isolates AL 20 869, ATCC 49156 and Lg2 had a band just below 1 kb. PCR-products visualized on agarose-gel A-C.* ***Gel A:*** *Lane 1; PCR marker. Lane 2-11; AL 20 869, AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045, AL 21 046, AL 21 225, AL 21 226. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel B****: Lane 1; PCR marker NEB. Lane 2-11; AL 21 227-AL 21 236. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel C****: Lane 1; PCR marker NEB. Lane 2-5; AL 21 237-AL 21 240. Lane 6; ATCC 49156. Lane 7; Lg2. Lane 8; Negative control. Lane 9; 1 kb Plus ladder Invitrogen.*

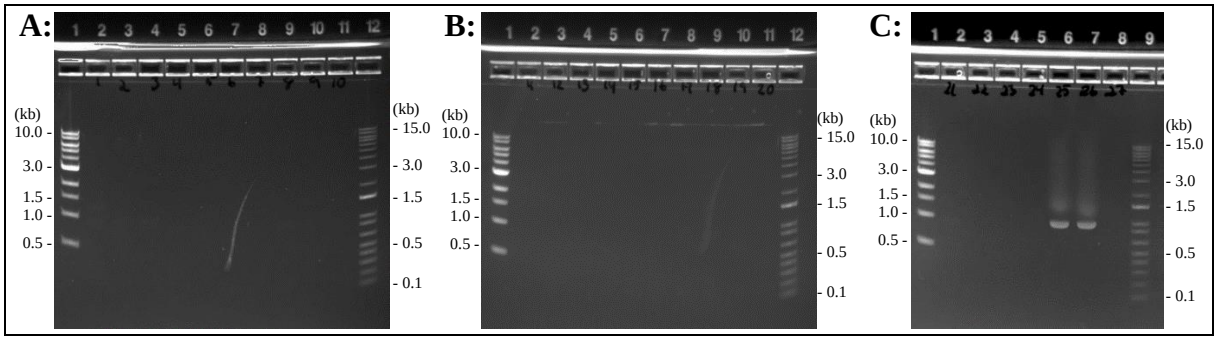



*Figure 4-19. Identification of possible virulence gene LPxTG-4. PCR product of 928 bp indicate presence of virulence gene LPxTG-4. Positive isolates ATCC 49156 and Lg2 had a band just below 1 kb. PCR-products visualized on agarose-gel A-C.* ***Gel A:*** *Lane 1; PCR marker. Lane 2-11; AL20 869, AL20 906, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046, AL 21 225, AL21 226. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel B****: Lane 1; PCR marker NEB. Lane 2-11; AL21 227-AL21 236. Lane 12; 1 kb Plus ladder Invitrogen.* ***Gel C****: Lane 1; PCR marker NEB. Lane 2-5; AL 21 237-AL21 240. Lane 6; ATCC 49156. Lane 7; Lg2. Lane 8; Negative control. Lane 9; 1 kb Plus ladder Invitrogen.*

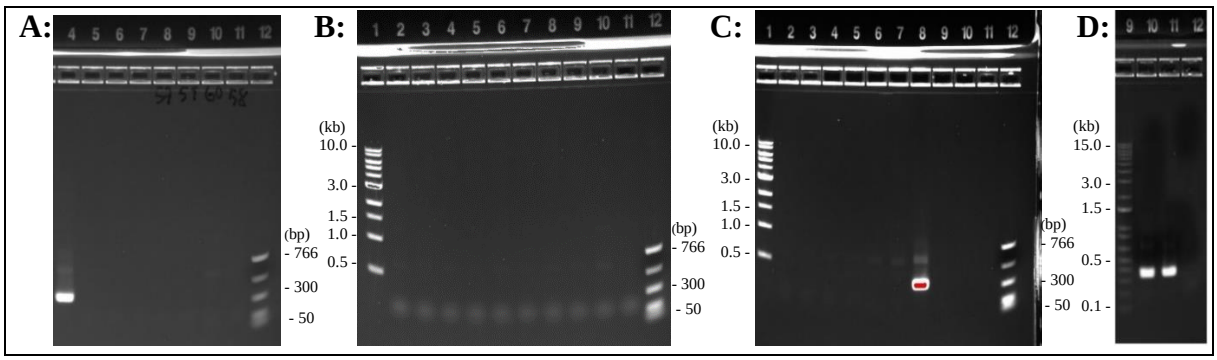



*Figure 4-20. Identification of possible virulence gene adhesin. PCR product of 358 bp indicate presence of virulence gene adhesin. Positive isolates AL 20 869, ATCC 49156 and Lg2 had a band at approx. 350 bp. PCR-products visualized on agarose-gel A-D.* ***Gel A:*** *Lane 4-11; AL20 869, AL20 906, AL20 909, AL20 910, AL20 911, AL21 045, AL21 046. Lane 12; PCR marker.* ***Gel B****: Lane 1; 1 kb NEB ladder. Lane 2-11; AL 21 225-AL 21 234. Lane 12; PCR marker.* ***Gel C****: Lane 1; 1 kb NEB ladder. Lane 2-7; AL 21 235-AL 21 240. Lane 8; ATCC 49156. Lane 9; negative control, lane 10-11; empty. Lane 12; PCR marker.* ***Gel D****: Lane 9; 1 kb Plus ladder Invitrogen. Lane 10; ATCC 49156. Lane 11; Lg2. Lane 12; negative control.*

# 5. Discussion

From clinical outbreaks in farmed rainbow trout in one location in Spain and three in Turkey, 16 isolates, along with 8 strains provided by PHARMAQ AS, were biochemically and genotypically identified as *L. garvieae.* The 24 strains were screened for virulence factors and they were all negative for the virulence gene *LPxTG*-4. Only one strain, AL 20 869, was positive for the virulence genes *adhesin* and *LPxTG*-1. None of the strains had a capsule or a capsular gene cluster. Their phylogenetic relationship revealed that all except from one isolate, AL 20 869, were clustered in one group. The isolate from Turkey that was different, clustered with isolates from rainbow trout from Italy and yellowtail from Japan

## 5.1 Phenotypic and biochemical identification

The classical biochemical methods oxidase, hemolysis, catalase and miniaturized system like Rapid ID 32 STREP identified all 24 isolates to be *L. garvieae*. However, six of the isolates had a "doubtful profile" according to Rapid ID 32 STREP and api*web*™ identification software. Methyl-βD-glucopyranoside (MβG 85%) and sodium hippurate (HIP 0%) was the two tests that was identified as "tests against" normal results for *L. garvieae* according to api*web*™ identification software. Isolates that only had MβG 85% as "test against" had an "excellent profile", indicating that HIP was a reaction of significant value when it came to identification with this system. This is also indicated by the percent value behind HIP stating that 0 % of *L. garvieae*-strains should be positive. In the acidification reaction MβG, 85% of all *L. garvieae* should be positive for this reaction. Positive hydrolyzation of hippurate was also reported in rainbow trout from Spain and catfish from Italy even though this is an uncommon characteristic (Ravelo et al., 2001). The same findings have been reported in strains from prawn in Taiwan (Cheng & Chen, 1998). *L. garvieae* was first isolated as a causative agent of bovine mastitis (Garvie et al., 1981) and this raises the question that a negative reaction for hippurate was set as an input in api*web*™ identification software on the basis of result from this strain and others isolated from bovine and similar animals. Now, when more and more *L. garvieae* strains from aquaculture have been isolated and analyzed to be positive for hydrolyzation of hippurate, maybe the reaction in api*web*™ identification software should be considered altered.

Sucrose was positive for all the strains except for AL 20 869 and ATCC 49156. Apart from these three reactions, every other test gave the same result, indicating a high-level homogeneity among the strains. The work presented herein corroborate the results by Ravelo et al. (2001)

that found high biochemical homogeneity from strains from different origin, but more strains should be collected from the Mediterranean area to validate the results further.

Eldar et al. (1999) established three biotypes for *L. garvieae* from acidification of tagatose, ribose and sucrose (saccharose) with biochemical (fermentation of carbohydrates) and enzymatic tests. In the current work, none the strains produced acid from ribose. According to Eldar et al. (1999) all *L. garvieae* biotypes produces acid from ribose, making it impossible to divide *L. garvieae* strains found in this work, into any biotype with this system. All of the strains tested by Eldar et al. (1999) were positive for hydrolyzation of hippurate as, mentioned above, was an essential reaction in API rapid ID STREP. This biotyping system was created in 1999, and since then several *L. garvieae* strains have been isolated which were both negative for ribose (Vela et al., 2000) and positive for hydrolyzation of hippurate (Cheng & Chen, 1998; Pereira et al., 2004; Ravelo et al., 2001). However, Vela et al. (2000) found that all the strains in their study were negative for acidification of ribose with the API Rapid ID 32 Strep, whereas the same strains were positive with API 50 CH. Strains in this work have not been tested for ribose with other systems than Rapid ID 32 Strep, and it should be taken into account that there is a possibility that they may be positive for ribose if the strains was tested with API 50 CH or other tests.

Vela et al. (2000) established thirteen biotypes based on acidification of sugars (saccharose, tagatose, mannitol and cyclodextrin) and presence of the enzymes pyroglutamic acid arylamidase and N-acetyl-β-glucosaminidase. In this work all the strains were positive for mannitol, tagatose and pyroglutamic acid arylamidase. All strains, except for AL 20 869, were positive for sucrose. According to these authors and this biotyping system, AL 20 869 is biotype 3 and all the others are biotype 1. However in this work the control, ATCC 49156, was not positive for cyclodextrin as is should have been according to Vela et al. (2000) making this strain different from any of the 13 biotypes.

Rapid ID 32 Strep consists of 32 phenotypic reactions that check the presence of specific enzymes or whether the bacterium consumes different sugars. If the dilution of the culture is inaccurate or incorrect, this may bias the results. Too few cells in the culture could result in the bacteria using too long time to consume the sugar so that there is no pH change that causes color change at the time of reading. Too few cells can also cause a delay in enzymatic reaction, so that the color reaction is incomplete when the results are read. Another source of error is that reading the color code is subjective. The use of miniaturized systems has previously been

reported to give variable results. Ravelo et al. (2001) demonstrated that the culture medium and the right initial inoculum is important to achieve right identification with Rapid ID 32 Strep. They concluded that in order to avoid misidentification as *L. lactis* subsp. *lactis* and *Streptococcus uberis*, repetitive and accurate identification were only achieved using adjusted inoculum from blood agar. Vela et al. (2000) discovered variability in the results of acidification of ribose between Rapid ID 32 Strep and API 50 CH.

The overall results of the biochemical characteristic of the strains in this study showed homogeneity among the strains in spite if these potential sources of error. However it is necessary to take precautions to differentiate biotypes based on acidification of sugars with miniaturized systems like Rapid ID 32 Strep and conventional tests must be used in addition to get an accurate identification (Ravelo et al., 2001). Biochemical identification is cheap, it does not require expertise and it is relative fast. However, as described above, biochemical identification could produce variable results, making it less accurate and less discriminating than genotypic identification. This variability should be taken into account, and the use of biochemical testing should be considered as an additional test and not the primary test for defining identity and relationship.

## 5.2 Genotypic identification

Use of 16S rRNA gene sequencing is widely used for both phylogeny and taxonomy because it its present in all bacteria, its function over time has not changed and the gene is large enough (1500 bp) for statistically relevant information with approximately 50 functional domains (Patel, 2001). In this study, six strains were 16S rRNA gene sequenced. These six strains were selected from different locations from Spain and Turkey. AL 21 225 and AL 21 226 were isolated from one location in Spain and AL 21 228, AL 21 229, AL 21 230, AL 21 234 were from three different locations in Turkey (Table 4-1). The initial plan was to sequence all the 24 isolates by 16S rRNA gene sequencing, but due to time savings and sequencing problems no other strains were identified by sequencing their 16S rDNA.

The PCR gave the expected amplicon of 1500 bp on 1 % agarose gel, however there were issues related to sequencing the PCR-product. For AL 21 226 and AL 21 230 the chromatogram for the forward sequences had poor start followed by weak sequence (low peaks) and some peaks were overlapping. Se example for chromatogram for AL 21 230 forward sequence in

Figure 5-1. A second sequencing did not improve the results, consequently two reverse sequences were assembled to one contig for these two isolates. The result was contigs that was shorter than it would have been if with the forward and reverse assembling was possible.

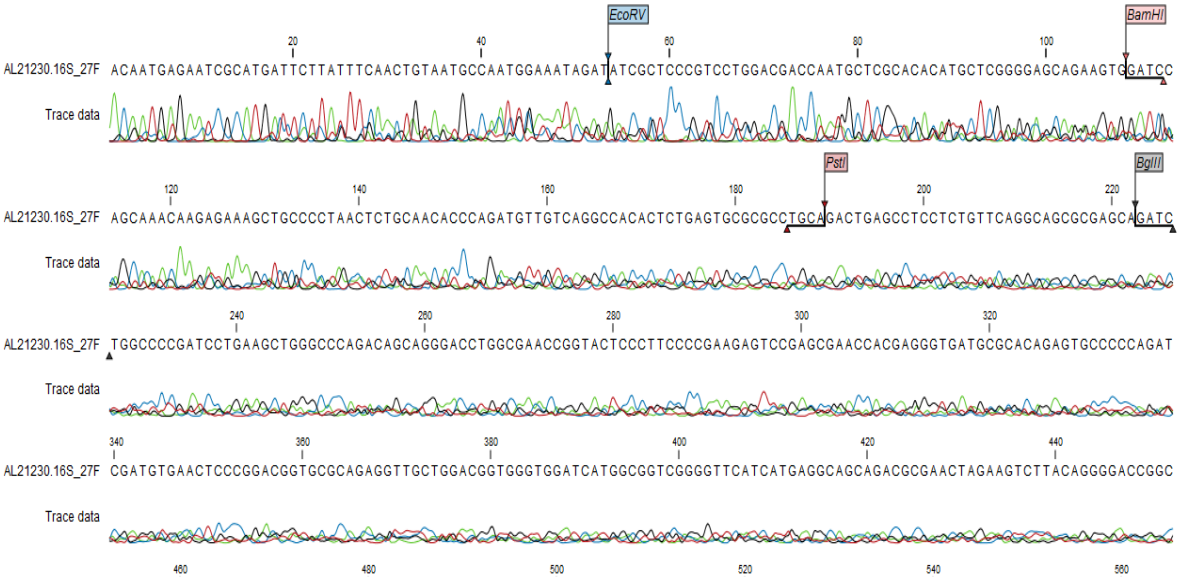



*Figure 5-1. Part of chromatogram from forward sequence of AL 21 230 in CLC workbench. The chromatogram had a poor start followed by weak sequence (low peaks) and some peaks were overlapping.*

There are several possible explanations to these issues. One potential cause is the primers. The primer could bind to itself or other primers could be present. If the PCR clean up did not remove all the primers before sequencing, the reverse primer could dominate the reaction. Another cause could be that the forward primer mismatches primer site. A new forward primer could solve the problem.

Secondly there could be template issues. A secondary template could be present. In this work the PCR products were analyzed on 1 % agarose gel and single bands at 1500 bp were observed. However, if there was a secondary template contaminant present that generates a PCR product close in size to 1500 bp, it would not be detected on a 1 % agarose gel. However, analyzing the PCR products on an agarose gel which have a better resolution (higher percent agarose), could reveal if this was the case. It is also important to have sufficient amounts of DNA when sequencing large fragments like 1500 bp, and insufficient amounts of DNA template could be a problem. Insufficient amount of DNA results in low peaks throughout the chromatogram. The chromatograms of the forward sequences for AL 21 226 and AL 21 230, both started with higher peaks followed by weak peaks, thus the amount of DNA probably wasn't the issue here. Another source of error could be the step were the PCR amplicon of 1500 bp was cut out of the gel and purified. It is important not to cut away any DNA to avoid loss of DNA during the cleaning step. DNA-Extraction from the gel slice involve multiple centrifugation a washing

steps which also could have an effect of the DNA. It is also important to be quick when excising the DNA out of the gel as UV light degrades DNA.
Thirdly, the issue with sequencing could also be related to the high G-C content making the gene folded and difficult to sequence. Chang et al. (2002) sequenced a smaller part (529 bp) of the 16S rRNA gene in *L. garviae* from Taiwan and got 100 % sequence similarity to the reference strain in GeneBank. For identification purposes, Patel stated (2001) that the region in the 16S rRNA gene that was most heterogenic was the first 500 bases of the 5' end and therefore sequencing this is sufficient for identification purposes.

Sequencing the 16S rRNA gene is a well-known method used in identification and phylogenetic studies. However, even if the sequencing is successful the nucleotide databases could be a potential source of error. There have been reports of misidentifications when sequencing the 16S rRNA gene (Boudewijns et al., 2006). One issue is the deposited sequences in the public databases, such as GenBank. Anyone can deposit and name sequence data, which may lead to sequence errors, incomplete sequences and insufficient strain characterization (Drancourt et al., 2000; Patel, 2001). Another problem is that there are discussions on universally criteria for the level of sequence homology required between an unknown isolate and sequences in the databases to conclude on genus and species of bacterial isolates for 16s rRNA gene sequence (Clarridge, 2004). However, it is most common to operate with 97 % sequence identity for 16s rRNA gene to conclude that it is the same genus (Stackebrandt & Goebel, 1994).
The gene for 16S rRNA can have multiple copies within one isolate that can differ from each other. For example *Aeromonas veronii* have up to six copies of this gene that differs up to 1.5 % (Janda & Abbott, 2007). Heterogenicity among 16S rRNA genes along with the database issues highlighted above, show that this technology has its limitations with respect to identifying unknown isolates down to species level, especially for some genera.
Drancourt et al. (2000) formed several identification criteria and recommendations for 16S rRNA gene sequencing. These authors emphasized the importance of including full 16S rRNA gene sequences into databases whenever possible, and they defined identification to the species level as a 16S rDNA sequence identity of $\geq$ 99 % and to the genus level as a 16S rDNA sequence identity of $\geq$ 97 % to the prototype strain in GenBank.

Sequencing of another housekeeping gene than 16S rRNA could be a better approach for species identification. Less-conserved genes that only exist in one copy offers a higher discriminatory power than 16S rRNA gene (Adékambi & Drancourt, 2004; Mollet et al., 1997;

Wang et al., 2007). However, there is no universal primers for housekeeping genes that targets all bacteria. Therefore, sequencing of the 16S rRNA gene or some other identification approach must be employed first, before determining what kind of primers to use. In this work *gyrB* sequencing was done on all the isolates, including ATCC 49156. The *gyrB* gene encodes the subunit B protein of DNA gyrase, a type II DNA topoisomerase. It is universally distributed among bacterial species and plays an essential role in DNA replication (Mun Huang, 1996; Watt & Hickson, 1994). Sequencing the *gyrB* gene has been used in several phylogenetic studies, indicating that this gene is a suitable marker for phylogenetic as well as taxonomic studies, for closely related species (Fukushima et al., 2002; Kasai et al., 2000; La Duc et al., 2004; Niemann et al., 2000; Yamamoto & Harayama, 1996; Yamamoto & Harayama, 1998; Yamamoto et al., 1999; Yanez et al., 2003).

The sequencing of *gyrB* did not involve the additional step of cutting the PCR product out of the gel and the subsequent cleaning step, thereby removing this source of error. All the contigs used as input in megaBLAST against the nucleotide collection database were *L. garvieae* with the same best hit and identity > 98.7 % (Table 4-4). One of the results from megaBlast was *L. garvieae* gyrase subunit B (*gyrB*), with accession no GU324261.1. As mentioned above, anyone can deposit and name sequence data into the database, and it is important to check if the result from BLAST is reliable. There was no information of the strain from which *L. garvieae* gyrase subunit B (*gyrB*) was sequenced from and there was no published data on the work, hence this result was considered not reliable.

Identification by the PCR-method developed by Zlotkin et al. (1998) is a method that has been widely used and claims to be specific for *L. garvieae*. In this study it was positive for all strains including ATCC 49156, and gave the expected 1100 bp amplicon that is specific to *L. garvieae*. It was negative for *S. iniae* and three serotypes of *S. agalactiae* which are close related to *L. garvieae*. Figure 5-2 shows the relationship between *L. garvieae,* various *Streptococcus* sp. and other related species in a phylogenetic tree based on 16S rRNA genes (Abu-Elala et al., 2019).

Under development of this method the PCR-assay was tested on deceased fish from three continents (Asia, Australia and Europe) and was negative for *L. lactis*, *Streptococcus iniae*, *Streptococcus difficile*, *Lactococcus piscium, Vagococcus salmoninarum* and *Aeromonas salmonicida* (Zlotkin et al., 1998). Primers for this PCR-method is targeting the 16S rRNA gene, so sequencing of this PCR amplicon to check for specificity against *L. garvieae* should be considered to verify if it is truly specific against *L. garvieae*.

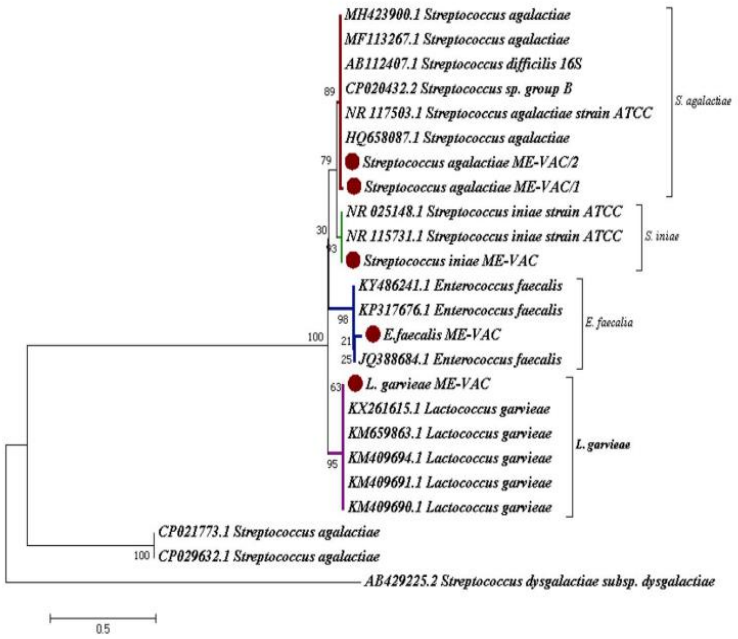



*Figure 5-2. Neighbor-joining phylogenetic tree of 16S rRNA genes from L. garvieae, Streptococcus species and other related species, pathogenic to Nile tilapia in Egypt. Picture derived from (Abu-Elala et al., 2019)*

## 5.3 Phylogeny and MLSA

Phylogenetics is the study of evolutionary history and relationship between organisms. In bacteria, this involves studies of DNA and different genes in relation to each other. The result is a phylogenetic tree that is a diagram of the evolutionary history. A phylogenetic tree can be unrooted or rooted, were the latter show a common ancestor in the tree which is placed as a point for comparison. The 16S rRNA gene is the universal marker gene for basic evolutionary analysis. However, as mentioned earlier, the 16S rRNA gene does not always allow species discrimination and thereby is not optimal for analyzing taxonomic and phylogenetic relationship. To study the phylogenetic relationship based on a protein-coding gene (housekeeping gene) would have a better resolution power. However, when studying a single protein coding gene, the phylogenetic result would reflect the evolution in only this particular gene which does not necessary reflect the true picture. There may be random recombination in the gene to be analyzed and this will give a wrong phylogenetic relationship.

It has become more common to analyze multiple protein-encoding housekeeping genes for taxonomic relationship (Adékambi & Drancourt, 2004; Christensen & Olsen, 1998; Holmes et al., 2004; Naser et al., 2005; Thompson et al., 2005; Wertz et al., 2003). Multi locus sequence analysis (MLSA) is a term first introduced by Gevers et al. (2005). Sequencing the 16S rRNA gene first, is necessary to have an identification down to the genus level. Then MLSA can be

used for further discrimination. MLSA is a method based on multi locus sequence typing (MLST) which is a typing method were typically 5-7 housekeeping genes are sequenced and the different allele profiles found is defined as different sequence types (Maiden et al., 1998). Sequencing of a minimum of five housekeeping genes, universally distributed, present as single copies and located at distinct chromosomal loci is regarded by the *ad hoc* committee for re-evaluation of the species definition, as a method of great promise for prokaryotic systematics (Stackebrandt et al., 2002). In MLSA, the sequenced housekeeping genes are joined together end-on-end which then creates a single concatenated sequence. This concatenated sequence is used for phylogenetic relationships. Construction of a phylogenetic tree based on one particular gene can give a completely different grouping than if another gene is analyzed. This is because of the rate of evolutionary changes in two genes can be different. MLSA, when constructing a concatenated tree, provides an objective method to cluster strains within a genus and reduces the chance of coincidence that is present when using a single gene approach (Gevers et al., 2005). In MLST the use of seven loci has become the norm (Maiden et al., 1998), but in MLSA 4-5 genes can be used although it is important to bear in mind that the more genes the less problematic the bias (De Vos, 2011).

In this work the five housekeeping genes *als, gapC, gyrB, rpoC* and *galP* were used in a MLSA-scheme (Ferrario et al., 2013). These authors had seven unlinked genes in their scheme that were all polymorphic and equally distributed in the genome sequences (minimum distance 18kb). The loci had different evolution rates indicated by the number of alleles varying between eight in *gapC* (the most conserved locus) and fourteen in *rpoC* (Table 5-1).
In this work, *atpA* and *tuf* were not analyzed because there were problems amplifying the gene with the primers suggested by Ferrario et al. (2013). Since number of genes recommended for MLSA is lower than for MLST (De Vos, 2011) it was decided that no further work should be done in order to optimize the PCR for these two genes. Number of alleles and polymorphic sites for *atpA* and *tuf* are covered by the five genes in the MLSA scheme in this study shown in Table 5-1.

Table 5-1. Five housekeeping gene, with observed polymorphism used in phylogenetic analyses from Ferrario et al. (2013)

| Locus | Size (bp) | No alleles | No. Polymorphic sites |
|---|---|---|---|
| *als* | 811 | 11 | 110 |
| *atpA* | 803 | 9 | 92 |
| *tuf* | 809 | 12 | 37 |
| *gapC* | 821 | 8 | 23 |
| *gyrB* | 827 | 11 | 164 |
| *rpoC* | 830 | 14 | 79 |
| *galP* | 812 | 13 | 186 |

The single phylogenetic trees of each gene (Figure 4-8, Figure 4-9 and Figure 4-10) were almost similar. All the isolates found in this study, except for AL 20 869, are clustered in one group. This indicate high similarity amongst these strains. AL 20 869 is in every tree either on a branch alone or together with yellowtail, making this strain different from the other isolates in this work. *L. garvieae* strains from yellowtail were separated in two groups where ATCC 49156 and Lg2 were always clustered together. All the strains from rainbow trout in this work, except for the mentioned AL 20 869, were clustered in one major group together with strain 8831 (Spain) and PAQ102015-99 (USA). Strain Lg9 and UNIUD074 from rainbow trout in Italy, were clustered with all these strains from rainbow trout in the *gapC* tree (Figure 4-8), but in all the other phylogenetic trees they were closer related to strains from yellowtail.

The alignments were shorter in length (Table 4-7) for each gene than Ferrario et al.(2013) got in their MLSA scheme (Table 5-1). The alignment for *gapC, rpoC* and *galP* was only slightly shorter (763, 705, 750 bp), but for *als* and *gyrB* the difference was greater.
For future work new sequencing should be done to get longer alignments in the same size range as Ferrario et al.(2013). To have an indication that the length was not causing big differences in the groupings of the tree, new alignment was made for *als* (appendix F, Figure F-1) without the two strains that had the shortest contigs, AL 20 909 and AL 21 227 (Table 4-5). The new alignment was 860 bp as opposed to 540 for the first *als* alignment.
The two trees for *als* (Figure 4-8 and appendix F, Figure F-2) are in general very similar, but a slightly different grouping was seen for AL 20 869 and Lg2, however the bootstrap (BT) values in the new tree is lower for this particular grouping. The bootstrapping values in the trees indicates how many times out of 100 the same branch is observed when repeating the

phylogenetic reconstruction on a re-sampled set of the data. The higher the BT value, the more robust it is.
New alignment was also made for *gyrB* without the two shortest contigs, but the new alignment was just 618 bp as opposed to 567 bp. Since the new alignment was not much longer than the first one, no new tree was constructed. As mentioned above, for future work sequencing the strains that had short contigs after assembling and trimming (Table 4-5) should be done to verify the phylogenetic trees.

The same clustering into groups as the *als, gyrB, rpoC and galP* trees, could be seen in the phylogenetic tree from the 3325 bp concatenated sequence of the five loci (Figure 4-11). Concatenated trees increases the number of phylogenetically informative characters and thereby resolve any previously unresolved branches (Gevers et al., 2005).
In the concatenated tree in this study the clusters were supported by higher bootstrap values than in the single-gene tree and were therefore more robust.

ATCC 49156 is not pathogenic to yellowtail, but it is a pathogen in rainbow trout (Ture et al., 2014). The virulent strain Lg2, found in yellowtail, has a capsule and a capsule gene cluster, whereas ATCC 49156 and the virulent strain 122061, from yellowtail, does not have a capsule (Nishiki et al., 2016). Since the virulent and capsulated Lg2 always are clustered with the avirulent, non-capsulated ATCC 49156, the phylogenetic trees do not seem to reflect their pathogenicity to yellowtail.
Strain Lg9 and UNIUD074 are both from rainbow trout in Italy and they are in all the trees, except for *gapC* tree, closer related to isolates from yellowtail, than isolates from the Mediterranean. Ferrario et al. (2013) had the same clustering in their MLSA scheme where Japanese and Italian isolates were closer related than Italian and Spanish isolates, even though they analyzed seven genes and in this work we analyzed five genes. Eldar et al. (1999) showed that the Italian and Japanese isolates had the same phenotypic traits (biotype 1) and that they were different from the Spanish isolates (biotype 3). RFLP ribotyping also revealed the same *Eco*RI ribotype for all Italian and Japanese strains and they raised the possibility that the disease has spread from Japan to Italy through the import of livestock or fish food (Eldar et al., 1999).

It is interesting that one isolate, AL 20 869, differs from all the other ones sampled in this work in the phylogenetic trees. This same strain was also different in the biochemical testing. The

two strains, AL 20 869 and AL 20 870, were sampled at the same time, from the same farm in Turkey, however the latter are identical with all the other strains isolated in this work. This indicates that the two phylogenetic groups can be present at the same time in a disease outbreak and raises the question if they really are different when it comes to virulence and pathogenicity in fish.

In this work the *L. garvieae* strains were isolated from three fish farms in Turkey and two in Spain, which is not enough data to give a clear picture of the reality. In the future, with more *L. garvieae* strains being whole genome sequenced and deposited into databases and by collecting and analyzing more strains from outbreaks in aquaculture from different geographical areas, the phylogenetic relationship could change. It is necessary to characterize a large number of isolates to produce a robust tree to cluster the strains right within a genus (Gevers et al., 2005).

## 5.4 Exopolysaccharides and virulence genes

As previously mentioned there is a general lack of knowledge and limited studies on the pathogenic mechanisms for *L. garvieae* (Meyburgh et al., 2017; Ture & Altinok, 2016).
The presence of a capsule was considered the major virulence factor in *L. garvieae* (Kitao, 1982; Morita et al., 2011), but there was no capsule detected in this study. The phenotypic method for detecting a capsule, negative staining with nigrosine, is not optimal. It can be very difficult to visualize the capsule in the microscope and wrong conclusions are easily drawn. Therefore, genotypic approaches were done in addition to negative staining, to check if any of the isolates had a capsule.
The PCR-method from Miyauchi et al. (2012) is designed to amplify the gene cluster and the primers flank both ends of the gene cluster including the insertion sequences (Figure 3-5). All the isolates had a 750 bp PCR amplicon, including the positive control Lg2. The control should have had a PCR product of 16.5 kb, which is the size of the gene cluster. To amplify such a large sequence, it is important to have the right Taq polymerase that is optimized for Long Range PCR and can amplify large sequences. In this study it was only used PuReTaq Ready-To-Go PCR Beads which has Taq polymerase already incorporated in the beads. This Taq polymerase is not designed to amplify such large PCR products. DNA from LG2 was kindly provided by Dr. Mustafa Ture, but we don't know how the DNA was extracted. If the DNA was extracted from a single or multiple colonies or culture. If it was from multiple colonies, some of the colonies could have lost their ability to produce a gene capsule under culturing. It

has been reported that subculturing of *L. garvieae* Lg2 resulted in the less virulent Lg2-S with a single deletion in the *cpsL* (conserved hypothetical protein) and *epsD* (glycosyltransferase) and thereby could not produce a capsule any more. However, if a deletion had occurred in the gene cluster, the method from Miyauchi et al. (2012) would still be able to amplify the capsule gene cluster. That is if it's not completely lost. Since the gene cluster is flanked by insertions sequences, it is a possibility that the gene cluster could be inserted another place or even lost. In this case a PCR amplicon of 750 bp could appear. In the article from Ture & Altinok (2016) it is possible to see a weak band at 750 bp for Lg2 that might support this. However, the most likely reason why the 16.5 bp PCR product did not amplify in this work, is the Taq polymerase and the fact that it cannot amplify such large PCR products.

Ture & Altinok (2016) designed a multiplex PCR targeting the gene cluster, to avoid this Long Range PCR, and thereby making a more robust method. With this method all the isolates were negative except for the control Lg2.
Because the capsule gene cluster are flanked by insertion sequences (Morita et al., 2011), theoretically this means that the capsule gene cluster could be inserted in different genomic loci. If that was the case, the primers from Miyauchi et al.(2012) would not amplify the capsule gene cluster since the primers flank both the gene cluster including the ISs. But it will be positive for the multiplex PCR by Ture et al. (2016). To double check the multiplex result, primers were designed for *cpsW*, *epsR* and *epsW*, which are genes within the gene cluster. No bands were detected with either of the three primer-pairs, thereby verifying that the isolates had no capsules.

All the strains that were isolated in this study was from diseased fish with symptoms of lactococcosis suggesting that all the strains were virulent. However, none were positive for capsule and capsule gene cluster. There has been reports that ATCC 49156 which is avirulent to yellowtail (Kawanishi et al., 2006), could be highly virulent for rainbow trout (Ture et al., 2014). This indicates that the presence of a capsule in *L. garvieae* cannot be the only virulence factor directly linked to pathogenicity. Since all the strains were lacking a capsule and a capsule gene cluster, other virulence genes were investigated to see if there were similarities between the isolates. Several genes encoding putative virulence factors, an addition to the capsule gene cluster, were identified in *L garvieae* Lg2 from yellowtail (Miyauchi et al., 2012). Ture et al. (2016) investigated the putative virulence genes and found that all of the 34 isolates in their study were positive for *hemolysins* 1, 2, and 3, NADH oxidase, phosphoglucomutase, *adhesin*

*Pav, LPxTG*-containing surface proteins 2 and 3, superoxide dismutase, *adhesin PsaA*, *enolase*, and *adhesin* clusters 1 and 2. Only *Adhesin, LPxTG*-1 and *LPxTG*-4 varied between the isolates. Therefor these three genes were targeted in this study to find putative virulence genes. Isolate Lg2 and ATCC 49156 were positive for all three virulence genes. Isolate AL20 869 was positive for *LPxTG*-1 and *adhesin*. No other isolates were positive for *adhesin, LPxTG*-1 and *LPxTG*-4.

For a strain to have a pathogenic status it is necessary to have the right virulence genes in combination to cause disease in a specific host species (Gilmore & Ferretti, 2003). All the isolates were hemolytic on blood plates indicating that they were positive for hemolysin. Other than that, it was only AL20 869 that was positive for the virulence genes *LPxTG*-1 and *adhesin*, and none were positive for capsule gene cluster. As mentioned, all of the strains isolated in this work were from rainbow trout with signs of lactococcosis. Thereby indicating that all the strains would have some virulence genes. Perhaps the selected three virulence genes in this work were absent in most of the strains, but they could still have one or more of the other virulence genes suggested by Miyauchi et al (2012) that were not checked. The specificity of primers could be checked more as well as the PCR-program.
Further testing must be done to have a better understanding of the virulence in these isolates and maybe find some similarities.

## 5.5 Future vaccine possibilities

Diseases in fish can be controlled by chemical antibacterial treatment (Lewin, 1992). However, the use can cause antibiotic resistance so vaccination is considered the best option for controlling diseases in farmed fish. Fish farmers that experience lactococcosis in rainbow trout in the Mediterranean uses autogenous formalin-inactivated vaccines (obtain form a specific area and only allowed to use in specified areas) or a commercial formalin-inactivated vaccine. There have been reports on protection of 80 – 90 % with intraperitoneal injection (Bercovier et al., 1997) and with persistent up to 5 months with adjuvant vaccines (Vendrell et al., 2007). However, both the autogenous and the commercial vaccines were used in the fish farms from where the *L. garvieae* strains in this work were isolated from. This raises the question if there is a need for a new vaccine.
The phylogenetic study in this work revealed clustering of two groups of *L. garvieae*. One group consists of isolates only from rainbow trout and another group with isolates from both rainbow trout and yellowtail. AL 20 869, is the only one of the sampled strains in this work

that was in the group with strains from yellowtail. It was also the only one that was positive for the virulence genes tested. It could be that the vaccines that are available today for fish farmers to use, consist of *L. garvieae* from one of the groups and there is no cross-protection. For a future vaccine against this pathogen it would be of interest to see if isolates from rainbow trout in both groups gave the same result in a virulence study in fish and to see if it was necessary with a vaccine with two strains or if there are cross reaction between the groups. However more isolates need to be collected from different geographical areas and phylogenetically analyzed to see if the groupings in this work is the actual true picture of *L. garvieae* strains in aquaculture. The strains in this work were isolated from two countries and four fish farms, and many of the strains were from the same farm. This could bias the result, so more strains from different geographical areas and should be analyzed. It would also be of interest to investigate more on the virulence factors in *L. garvieae*, as the results in this work did not reveal so much. More of the putative virulence genes found by Miyauchi et al. (2012) should be investigated. Knowledge of the virulence factors in *L. garvieae* may be of interest both on a general basis, but also for a possible other direction with respect to vaccine. Instead of a vaccine with attenuated or inactivated microbes, DNA vaccines is upcoming as a new promising technique. DNA vaccines contain DNA that encodes specific antigens that stimulates the immune response in the host (Liu, 2003). In order for this to work, it is necessary to have knowledge of which proteins (antigens) cause disease or create a reaction in the immune system. Hence more investigation of the virulence genes in *L. garvieae* is needed.

# 6. Conclusion and future perspective

*L. garvieae* is an emerging pathogen in both fresh water and marine fish, and causes significant loss in aquaculture worldwide. In spite of this, there are few studies that have been performed to characterize *L. garvieae* from diseased fish with respect to pathogenicity and virulence factors. There are vaccines available for rainbow trout in the Mediterranean, though it does not seem to give full protection.

In this work *L. garvieae* strains were sampled from one Spanish and four Turkish rainbow trout farms that experienced disease. The biochemical characteristic showed homogeneity among the strains and all were identified as *L. garvieae.* However it is necessary to take precautions before dividing the strains into biotypes based solely on biochemical reactions, especially with miniaturized identification systems. In spite of this, biochemical methods is a fast method for identifying *L. garvieae* and can be a used as a test in addition to genotypic identification.

Sequencing the 16S rRNA gene is widely used for both phylogeny and taxonomy. In this work, there were sequencing problems that may be related to the primers. Further work must be done to optimize the primers and the method so this method can be used for unknown species in the future. However it was possible to get a positive identification in spite of these issues. Sequencing the housekeeping gene *gyrB*, proved to be a promising gene for identification instead of 16S rDNA in *L. garvieae*, and is recommended for future identification. In this work there were results from the database at NCBI, that were not reliable. Anyone can deposit and name sequence data into the database, so it must be taken into consideration when analyzing sequences.

Phylogenetic relationship revealed similarity among the strains that were isolated. All except for one isolate were identical and in the same phylogenetic group. The strain that was different from the others, AL 20 869, was isolated from the same farm and at the same time as AL 20 870 which was clustered with all the other strains in this work. The single phylogenetic trees of each gene were almost similar. However, phylogenetic relationship based on one single protein coding gene, would reflect the evolution in only this particular gene which does not necessary reflect the true picture. Hence, analysis of multiple housekeeping genes (MLSA) is recommended to get a more reliable phylogenetic relationship. The phylogenetic study in this work showed that strains from rainbow trout in Italy were more closely related to strains from yellowtail in Japan than they were to strains from other countries in the Mediterranean. These findings supports the view of a possible route of the pathogen from Asia to Europe.

The *L. garvieae* strains that were isolated in this work were all from fish farms were vaccines has been used against *L. garvieae*. The phylogenetic results in this work showed a possible clustering of two groups, and it raises the question if there is a need for a multiple vaccine containing strains from both groups. More isolates from different locations and other countries have to be phylogenetic analyzed to be sure of the groupings in the phylogenetic tree found in this work.

For a long time, the presence of a capsule was considered the major virulence factor in *L. garvieae,* but there was no capsule detected in this study either by phenotypic or genotypic methods. This supports findings from others, on strains from rainbow trout. There was only one isolate positive for other putative virulence genes tested and it was the same isolate that was different in the phylogenetic studies. The fact that all of the strains were isolated from diseased fish with lactococcosis, suggests that there are virulence genes present in the strains. Further testing on other genes must be done to have a better knowledge of the pathogenicity and virulence factors.

In the recent years there has been increasing published research on this pathogen. The fact that *L. garvieae* is an emerging pathogen in aquaculture will hopefully result in even more research studies and reports in the future. Increasing insight of this pathogen is necessary for the aquaculture industry as it causes major economic losses in cultured rainbow trout in the Mediterranean as well as other fish species worldwide.

# 7. Bibliography

Abu-Elala, N., Samir, A., Wasfy, M. & Elsayed, M. (2019). Efficacy of injectable and immersion vaccines against streptococcal infections in broodstock and offspring of Nile tilapia (Oreochromis niloticus). *Fish & shellfish immunology*.

Adékambi, T. & Drancourt, M. (2004). Dissection of phylogenetic relationships among 19 rapidly growing Mycobacterium species by 16S rRNA, hsp65, sodA, recA and rpoB gene sequencing. *International journal of systematic and evolutionary microbiology*, 54 (6): 2095-2105.

Affymetrix. (2018). *ExoSAP-IT™ Express PCR Product Cleanup Reagent*. https://www.thermofisher.com/order/catalog/product/75001.200.UL: Thermo-Fisher Scientific.

Aguado-Urda, M., Lopez-Campos, G. H., Fernandez-Garayzabal, J. F., Martin-Sanchez, F., Gibello, A., Dominguez, L. & Blanco, M. M. (2010). Analysis of the genome content of Lactococcus garvieae by genomic interspecies microarray hybridization. *BMC Microbiol*, 10: 79. doi: 10.1186/1471-2180-10-79.

Aguado-Urda, M., Lopez-Campos, G. H., Gibello, A., Cutuli, M. T., Lopez-Alonso, V., Fernandez-Garayzabal, J. F. & Blanco, M. M. (2011). Genome sequence of Lactococcus garvieae 8831, isolated from rainbow trout lactococcosis outbreaks in Spain. *J Bacteriol*, 193 (16): 4263-4. doi: 10.1128/JB.05326-11.

Aguirre, M. & Collins, M. (1993). Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology*, 75 (2): 95-107.

Alegría, Á., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S. & Mayo, B. (2009). Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk. *International journal of food microbiology*, 136 (1): 44-51.

Algöet, M., Bayley, A., Roberts, E., Feist, S., Wheeler, R. & Verner‐Jeffreys, D. (2009). Susceptibility of selected freshwater fish species to a UK Lactococcus garvieae isolate. *Journal of fish diseases*, 32 (10): 825-834.

Austin, B. & Austin, D. A. (2012). *Bacterial fish pathogens*: Springer.

Bark, S. & McGregor, D. (2001). The first occurrance of Lactococcosis in farmed trout in England. *Trout News*: 9-10.

Bartlett, J. M. S. & Stirling, D. (2003). A Short History of the Polymerase Chain Reaction. In Bartlett, J. M. S. & Stirling, D. (eds) *PCR Protocols*, pp. 3-6. Totowa, NJ: Humana Press.

Bercovier, H., Ghittino, C. & Eldar, A. (1997). Immunization with bacterial antigens: infections with streptococci and related organisms. *Developments in biological standardization*, 90: 153-160.

Bio-Rad. (2018). *Nucleic acid purifiifation*. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT544.pdf: Bio-Rad Laboratories, Inc.

Bolotin, A., Quinquis, B., Ehrlich, S. D. & Sorokin, A. (2012). *Complete genome sequence of Lactococcus lactis subsp. cremoris A76*: Am Soc Microbiol.

Boomker, J. D. F., Imes, G., Cameron, C. M., Naude, T. & Schoonbee, H. (1979). Trout mortalities as a result of Streptococcus infection.

Boudewijns, M., Bakkers, J. M., Sturm, P. D. & Melchers, W. J. (2006). 16S rRNA gene sequencing and the routine clinical microbiology laboratory: a perfect marriage? *Journal of clinical microbiology*, 44 (9): 3469-3470.

Bragg, R. & Broere, J. (1986). Streptococcosis in rainbow trout in South Africa. *Bulletin of the European Association of Fish Pathologists (Denmark)*.

Buck, J. D. (1982). Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Applied and Environmental Microbiology*, 44 (4): 992-993.

Caballero, B., Trugo, L. & Finglas, P. (2003). *Encyclopedia of food sciences and nutrition: Volumes 1-10*: Elsevier Science BV.

Cai, Y., Yang, J., Pang, H. & Kitahara, M. (2011). Lactococcus fujiensis sp. nov., a lactic acid bacterium isolated from vegetable matter. *International journal of systematic and evolutionary microbiology*, 61 (7): 1590-1594.

Carson, J., Gudkovs, N. & Austin, B. (1993). Characteristics of an Enterococcus‐like bacterium from Australia and South Africa, pathogenic for rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Fish Diseases*, 16 (4): 381-388.

Chan, J., Woo, P., Teng, J., Lau, S., Leung, S., Tam, F. & Yuen, K.-Y. (2011). Primary infective spondylodiscitis caused by Lactococcus garvieae and a review of human L. garvieae infections. *Infection*, 39 (3): 259-264.

Chang, P., Lin, C. & Lee, Y. (2002). Lactococcus garvieae infection of cultured rainbow trout, Oncorhynchus mykiss in Taiwan and associated biophysical characteristics and histopathology. *Bull. Eur. Assoc. Fish Pathol*, 22: 319-327.

Chen, S.-C., Lin, Y.-D., Liaw, L.-L. & Wang, P.-C. (2001). Lactococcus garvieae infection in the giant freshwater prawn Macrobranchium rosenbergii confirmed by polymerase chain reaction and 16S rDNA sequencing. *Diseases of Aquatic Organisms*, 45 (1): 45-52.

Chen, S. C., Liaw, L. L., Su, H. Y., Ko, S. C., Wu, C. Y., Chaung, H. C., Tsai, Y. H., Yang, K. L., Chen, Y. C. & Chen, T. H. (2002). Lactococcus garvieae, a cause of disease in grey mullet, Mugil cephalus L., in Taiwan. *Journal of fish diseases*, 25 (12): 727-732.

Chen, Y.-s., Chang, C.-h., Pan, S.-f., Wang, L.-t., Chang, Y.-c., Wu, H.-c. & Yanagida, F. (2013). Lactococcus taiwanensis sp. nov., a lactic acid bacterium isolated from fresh cummingcordia. *International journal of systematic and evolutionary microbiology*, 63 (7): 2405-2409.

Chen, Y.-s., Otoguro, M., Lin, Y.-h., Pan, S.-f., Ji, S.-h., Yu, C.-r., Liou, M.-s., Chang, Y.-c., Wu, H.-c. & Yanagida, F. (2014). Lactococcus formosensis sp. nov., a lactic acid bacterium isolated from yan-tsai-shin (fermented broccoli stems). *International journal of systematic and evolutionary microbiology*, 64 (1): 146-151.

Cheng, W. & Chen, J.-C. (1998). Isolation and characterization of an Enterococcus-like bacterium causing muscle necrosis and mortality in Macrobrachium rosenbergii in Taiwan. *Diseases of Aquatic Organisms*, 34 (2): 93-101.

Cho, S.-L., Nam, S.-W., Yoon, J.-H., Lee, J.-S., Sukhoom, A. & Kim, W. (2008). Lactococcus chungangensis sp. nov., a lactic acid bacterium isolated from activated sludge foam. *International journal of systematic and evolutionary microbiology*, 58 (8): 1844-1849.

Christensen, H. & Olsen, J. E. (1998). Phylogenetic relationships of Salmonella based on DNA sequence comparison of atpD encoding the β subunit of ATP synthase. *FEMS microbiology letters*, 161 (1): 89-96.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*, 17 (4): 840-862.

Collins, M., Farrow, J., Phillips, B. & Kandler, O. (1983). Streptococcus garvieae sp. nov. and Streptococcus plantarum sp. nov. *Microbiology*, 129 (11): 3427-3431.

Colorni, A., Ravelo, C., Romalde, J., Toranzo, A. & Diamant, A. (2003). Lactococcus garvieae in wild Red Sea wrasse Coris aygula (Labridae). *Diseases of aquatic organisms*, 56 (3): 275-278.

De Vos, P. (2011). Multilocus sequence determination and analysis. In vol. 38 *Methods in Microbiology*, pp. 385-407: Elsevier.

Didinen, B., Yardimci, B., Onuk, E., Metin, S. & Yildirim, P. (2014). Naturally Lactococcus garvieae infection in rainbow trout (Oncorhyncus mykiss Walbaum, 1792): new histopathological observations, phenotypic and molecular identification. *Revue de Medecine Veterinaire*, 165 (1-2): 12-19.

Domenech, A., Prieta, J., Fernandez-Garayzabal, J., Collins, M. D., Jones, D. & Dominguez, L. (1993). Phenotypic and phylogenetic evidence for a close relationship between Lactococcus garviae and Enterococcus seriolicida. *MICROBIOLOGIA-MADRID-*, 9: 63-63.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.-P. & Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of clinical microbiology*, 38 (10): 3623-3630.

Duguid, J. (1951). The demonstration of bacterial capsules and slime. *The Journal of pathology and bacteriology*, 63 (4): 673-685.

Duwat, P., Sourice, S., Cesselin, B., Lamberet, G., Vido, K., Gaudu, P., Le Loir, Y., Violet, F., Loubière, P. & Gruss, A. (2001). Respiration capacity of the fermenting

Bacteriumlactococcus lactis and its positive effects on growth and survival. *Journal of bacteriology,* 183 (15): 4509-4516.

Eldar, A., Ghittino, C., Asanta, L., Bozzetta, E., Goria, M., Prearo, M. & Bercovier, H. (1996). Enterococcus seriolicida is a junior synonym of Lactococcus garvieae, a causative agent of septicemia and meningoencephalitis in fish. *Current Microbiology,* 32 (2): 85-88.

Eldar, A., Goria, M., Ghittino, C., Zlotkin, A. & Bercovier, H. (1999). Biodiversity of Lactococcus garvieaeStrains Isolated from Fish in Europe, Asia, and Australia. *Applied and Environmental Microbiology,* 65 (3): 1005-1008.

Eldar, A. a. & Ghittino, C. (1999). Lactococcus garvieae and Streptococcus iniae infections in rainbow trout Oncorhynchus mykiss: similar, but different diseases. *Diseases of Aquatic Organisms,* 36 (3): 227-231.

Elliott, J. & Facklam, R. (1996). Antimicrobial susceptibilities of Lactococcus lactis and Lactococcus garvieae and a proposed method to discriminate between them. *Journal of clinical microbiology,* 34 (5): 1296-1298.

Evans, J., Klesius, P. & Shoemaker, C. (2009). First isolation and characterization of Lactococcus garvieae from Brazilian Nile tilapia, Oreochromis niloticus (L.), and pintado, Pseudoplathystoma corruscans (Spix & Agassiz). *Journal of fish diseases,* 32 (11): 943-951.

Evans, J. J., Pasnik, D. J., Klesius, P. H. & Al-Ablani, S. (2006). First report of Streptococcus agalactiae and Lactococcus garvieae from a wild bottlenose dolphin (Tursiops truncatus). *Journal of wildlife diseases,* 42 (3): 561-569.

Eyngor, M., Zlotkin, A., Ghittino, C., Prearo, M., Douet, D. G., Chilmonczyk, S. & Eldar, A. (2004). Clonality and diversity of the fish pathogen Lactococcus garvieae in Mediterranean countries. *Appl Environ Microbiol,* 70 (9): 5132-7. doi: 10.1128/AEM.70.9.5132-5137.2004.

Facklam, R. & Elliott, J. (1995). Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clinical microbiology reviews,* 8 (4): 479-495.

FAO. (2018a). *FAO Yearbook. Fishery and Aquaculture Statistics 2016.*

FAO. (2018b). *The State of World Fisheries and Aquaculture, 2018-Meeting the sustainable development goals.* Rome: Agriculture Organization of the United Nations. Fisheries Department.

Ferrario, C., Ricci, G., Borgo, F., Rollando, A. & Fortina, M. G. (2012). Genetic investigation within Lactococcus garvieae revealed two genomic lineages. *FEMS Microbiol Lett,* 332 (2): 153-61. doi: 10.1111/j.1574-6968.2012.02591.x.

Ferrario, C., Ricci, G., Milani, C., Lugli, G. A., Ventura, M., Eraclio, G., Borgo, F. & Fortina, M. G. (2013). Lactococcus garvieae: where is it from? A first approach to explore the evolutionary history of this emerging pathogen. *PLoS One,* 8 (12): e84796. doi: 10.1371/journal.pone.0084796.

Fichi, G., Cardeti, G., Perrucci, S., Vanni, A., Cersini, A., Lenzi, C., De Wolf, T., Fronte, B., Guarducci, M. & Susini, F. (2015). Skin lesion-associated pathogens from Octopus vulgaris: first detection of Photobacterium swingsii, Lactococcus garvieae and betanodavirus. *Diseases of aquatic organisms*, 115 (2): 147-156.

Fortina, M., Ricci, G., Acquati, A., Zeppa, G., Gandini, A. & Manachini, P. (2003). Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the Toma piemontese. *Food Microbiology*, 20 (4): 397-404.

Fortina, M. G., Ricci, G. & Borgo, F. (2009). A study of lactose metabolism in Lactococcus garvieae reveals a genetic marker for distinguishing between dairy and fish biotypes. *Journal of food protection*, 72 (6): 1248-1254.

Foschino, R., Picozzi, C., Borghi, M., Cerliani, M. & Cresci, E. (2006). INVESTIGATION ON THE MICROFLORA OF CAPRINO LOMBARDO CHEESE FROM RAW GOAT MILK. *Italian journal of food science*, 18 (1).

Fukushima, M., Kakinuma, K. & Kawaguchi, R. (2002). Phylogenetic analysis of Salmonella, Shigella, and Escherichia coli strains on the basis of the gyrB gene sequence. *Journal of clinical microbiology*, 40 (8): 2779-2785.

Garvie, E. I., Farrow, J. A. & Phillips, B. A. (1981). A taxonomic study of some strains of streptococci which grow at 10 C but not at 45 C including Streptococcus lactis and Streptococcus cremoris. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene: I. Abt. Originale C: Allgemeine, angewandte und ökologische Mikrobiologie*, 2 (2): 151-165.

GE-Healthcare. (2018). *Nucleic Acid Sample Preparation for Downstream Analyses. Handbook.*

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., de Peer, Y. V., Vandamme, P., Thompson, F. L., et al. (2005). Re-evaluating prokaryotic species. *Nature Reviews Microbiology*, 3: 733. doi: 10.1038/nrmicro1236.

Ghittino, C. & Muzquiz, J. (1998). La estreptococosis de la trucha arco iris en Espan¬ a. Resumen de lo expuesto en la reunion de piscicultores de Zaragoza del 26 de enero de 1998. *Revista AquaTIC*, 2.

Gibello, A., Galan-Sanchez, F., Blanco, M. M., Rodriguez-Iglesias, M., Dominguez, L. & Fernandez-Garayzabal, J. F. (2016). The zoonotic potential of Lactococcus garvieae: An overview on microbiology, epidemiology, virulence factors and relationship with its presence in foods. *Res Vet Sci*, 109: 59-70. doi: 10.1016/j.rvsc.2016.09.010.

Gilmore, M. S. & Ferretti, J. J. (2003). The thin line between gut commensal and pathogen. *Science*, 299 (5615): 1999-2002.

Goodman, L. B., Lawton, M. R., Franklin-Guild, R. J., Anderson, R. R., Schaan, L., Thachil, A. J., Wiedmann, M., Miller, C. B., Alcaine, S. D. & Kovac, J. (2017). Lactococcus petauri sp. nov., isolated from an abscess of a sugar glider. *International journal of systematic and evolutionary microbiology*, 67 (11): 4397.

Hardy, G. G., Caimano, M. J. & Yother, J. (2000). Capsule Biosynthesis and Basic Metabolism inStreptococcus pneumoniae Are Linked through the Cellular Phosphoglucomutase. *Journal of Bacteriology*, 182 (7): 1854-1863.

Holmes, D. E., Nevin, K. P. & Lovley, D. R. (2004). Comparison of 16S rRNA, nifD, recA, gyrB, rpoB and fusA genes within the family Geobacteraceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology*, 54 (5): 1591-1599.

Hoshina, T. (1958). A Streptococcus pathogenic to fish. *J. Tokyo Univ. Fish.*, 44: 57-68.

Invitrogen. (2018). *E-Gel™ Agarose Gels*.

Janda, J. M. & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology*, 45 (9): 2761-2764.

Kadioglu, A., Weiser, J. N., Paton, J. C. & Andrew, P. W. (2008). The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nature Reviews Microbiology*, 6 (4): 288.

Kasai, H., Tamura, T. & Harayama, S. (2000). Intrageneric relationships among Micromonospora species deduced from gyrB-based phylogeny and DNA relatedness. *International journal of systematic and evolutionary microbiology*, 50 (1): 127-134.

Kawanishi, M., Yoshida, T., Yagashiro, S., Kijima, M., Yagyu, K., Nakai, T., Murakami, M., Morita, H. & Suzuki, S. (2006). Differences between Lactococcus garvieae isolated from the genus Seriola in Japan and those isolated from other animals (trout, terrestrial animals from Europe) with regard to pathogenicity, phage susceptibility and genetic characterization. *J Appl Microbiol*, 101 (2): 496-504. doi: 10.1111/j.1365-2672.2006.02951.x.

Kawanishi, M., Yoshida, T., Kijima, M., Yagyu, K., Nakai, T., Okada, S., Endo, A., Murakami, M., Suzuki, S. & Morita, H. (2007). Characterization of Lactococcus garvieae isolated from radish and broccoli sprouts that exhibited a KG+ phenotype, lack of virulence and absence of a capsule. *Letters in applied microbiology*, 44 (5): 481-487.

Kimura, H. & Kusuda, R. (1982). Studies on the pathogenesis of streptococcal infection in cultured yellowtails, Seriola spp.: effect of crude exotoxin fractions from cell‐free culture on experimental streptococcal infection. *Journal of Fish Diseases*, 5 (6): 471-478.

Kitao, T. (1982). The methods for detection of Streptococcus sp., causative bacteria of streptococcal disease of cultured yellowtail (Seriola quinqueradiata). *Fish Pathology*, 17 (1): 17-26.

Kubota, H., Tsuji, H., Matsuda, K., Kurakawa, T., Asahara, T. & Nomoto, K. (2010). Detection of human intestinal catalase-negative, Gram-positive cocci by rRNA-targeted reverse transcription-PCR. *Appl. Environ. Microbiol.*, 76 (16): 5440-5451.

Kumar, S., Tamura, K. & Nei, M. (1994). MEGA: molecular evolutionary genetics analysis software for microcomputers. *Bioinformatics*, 10 (2): 189-191.

Kusuda, R. & Hamaguchi, M. (1988). Extracellular and intracellular toxins of Streptococcus sp. isolated from yellowtail. *Bull Eur Assoc Fish Pathol*, 8: 9-10.

Kusuda, R., Kawai, K., Salati, F., Banner, C. & Fryer, J. (1991). Enterococcus seriolicida sp. nov., a fish pathogen. *International Journal of Systematic and Evolutionary Microbiology*, 41 (3): 406-409.

La Duc, M. T., Satomi, M., Agata, N. & Venkateswaran, K. (2004). gyrB as a phylogenetic discriminator for members of the Bacillus anthracis–cereus–thuringiensis group. *Journal of microbiological methods*, 56 (3): 383-394.

Lancefield, R. C. (1933). A serological differentiation of human and other groups of hemolytic streptococci. *Journal of experimental medicine*, 57 (4): 571-595.

Lane, D. (1991). *16S/23S rRNA sequencing*. Nucleic acid techniques in bacterial systematics.

Latorre-Guzman, B., Kado, C. & Kunkee, R. (1977). Lactobacillus hordniae, a new species from the leafhopper (Hordnia circellata). *International Journal of Systematic and Evolutionary Microbiology*, 27 (4): 362-370.

Lewin, C. (1992). Mechanisms of resistance development in aquatic microorganisms. *Chemotherapy in Aquaculture: from Theory to Reality. Office International des Epizooties, Paris, France*: 288-301.

Lister, J. (1873). Memoirs: A Further Contribution to the Natural History of Bacteria and the Germ Theory of the Fermentative Changes. *Journal of Cell Science*, 2 (52): 380-408.

Liu, M. (2003). DNA vaccines: a review. *Journal of internal medicine*, 253 (4): 402-410.

Löhnis, F. (1909). Die Benennung der Milchsaürebakterien. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg*, 22: 553-555.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K. & Caugant, D. A. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences*, 95 (6): 3140-3145.

Mannion, P. & Rothburn, M. (1990). Diagnosis of bacterial endocarditis caused by Streptococcus lactis and assisted by immunoblotting of serum antibodies. *Journal of Infection*, 21 (3): 317-318.

Marraffini, L. A., DeDent, A. C. & Schneewind, O. (2006). Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol. Mol. Biol. Rev.*, 70 (1): 192-221.

Menendez, A., Fernandez, L., Reimundo, P. & Guijarro, J. A. (2007). Genes required for Lactococcus garvieae survival in a fish host. *Microbiology*, 153 (Pt 10): 3286-94. doi: 10.1099/mic.0.2007/007609-0.

Meucci, A., Zago, M., Rossetti, L., Fornasari, M. E., Bonvini, B., Tidona, F., Povolo, M., Contarini, G., Carminati, D. & Giraffa, G. (2015). Lactococcushircilactis sp. nov. and

Lactococcuslaudensis sp. nov., isolated from milk. *International journal of systematic and evolutionary microbiology*, 65 (7): 2091-2096.

Meyburgh, C. M., Bragg, R. R. & Boucher, C. E. (2017). Lactococcus garvieae: an emerging bacterial pathogen of fish. *Dis Aquat Organ*, 123 (1): 67-79. doi: 10.3354/dao03083.

Meyburgh, C. M., Bragg, R. R. & Boucher, C. E. (2018). Detection of virulence factors of South African Lactococcus garvieae isolated from rainbow trout, Oncorhynchus mykiss (Walbaum). *Onderstepoort Journal of Veterinary Research*, 85 (1): 1-9.

Milad, A., DEHKORDI, A. E., YAGHOUBZADEH, Z., SADRABAD, E. K. & BABAALIAN, A. (2014). Isolation and Characterization of Lactococcus garvieae from Diseased Rainbow Trout (Oncorhynchus mykiss, Walbaum) Cultured in Northern Iran Based on the Nucleotide Sequences of the 16s rRNA Gene. *Walailak Journal of Science and Technology (WJST)*, 12 (6): 533-538.

Mishra, A., Nam, G.-H., Gim, J.-A., Lee, H.-E., Jo, A. & Kim, H.-S. (2018). Current Challenges of Streptococcus Infection and Effective Molecular, Cellular, and Environmental Control Methods in Aquaculture. *Molecules and cells*, 41 (6): 495.

Mitchell, T. J. (2003). The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nature Reviews Microbiology*, 1 (3): 219.

Miyauchi, E., Toh, H., Nakano, A., Tanabe, S. & Morita, H. (2012). Comparative Genomic Analysis of Lactococcus garvieae Strains Isolated from Different Sources Reveals Candidate Virulence Genes. *Int J Microbiol*, 2012: 728276. doi: 10.1155/2012/728276.

Mollet, C., Drancourt, M. & Raoult, D. (1997). rpoB sequence analysis as a novel basis for bacterial identification. *Molecular microbiology*, 26 (5): 1005-1011.

Morita, H., Toh, H., Oshima, K., Yoshizaki, M., Kawanishi, M., Nakaya, K., Suzuki, T., Miyauchi, E., Ishii, Y., Tanabe, S., et al. (2011). Complete genome sequence and comparative analysis of the fish pathogen Lactococcus garvieae. *PLoS One*, 6 (8): e23184. doi: 10.1371/journal.pone.0023184.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986). *Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction*. Cold Spring Harbor symposia on quantitative biology: Cold Spring Harbor Laboratory Press.

Mun Huang, W. (1996). Bacterial diversity based on type II DNA topoisomerase genes. *Annual review of genetics*, 30 (1): 79-107.

Mäkelä, P. H. & Butler, J. C. (2008). History of pneumococcal immunization. In *Pneumococcal vaccines*, pp. 19-29: American Society of Microbiology.

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. & Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of Enterococcus species based on rpoA and pheS genes. *Microbiology*, 151 (7): 2141-2150. doi: doi:10.1099/mic.0.27840-0.

Nelson, M. C., Varney, J. S., Welch, T. J. & Graf, J. (2016). Draft genome sequence of Lactococcus garvieae strain PAQ102015-99, an outbreak strain isolated from a

commercial trout farm in the Northwestern United States. *Genome announcements*, 4 (4): e00781-16.

Neve, H., Geis, A. & Teuber, M. (1987). Conjugation, a common plasmid transfer mechanism in lactic acid streptococci of dairy starter cultures. *Systematic and applied microbiology*, 9 (1-2): 151-157.

Niemann, S., Harmsen, D., Rüsch-Gerdes, S. & Richter, E. (2000). Differentiation of clinical Mycobacterium tuberculosis complex isolates by gyrB DNA sequence polymorphism analysis. *Journal of clinical microbiology*, 38 (9): 3231-3234.

Nishiki, I., Oinaka, D., Iwasaki, Y., Yasuike, M., Nakamura, Y., Yoshida, T., Fujiwara, A., Nagai, S., Katoh, M. & Kobayashi, T. (2016). Complete genome sequence of nonagglutinating Lactococcus garvieae strain 122061 isolated from yellowtail in Japan. *Genome announcements*, 4 (4): e00592-16.

Oinaka, D., Yoshimura, N., Fukuda, Y., Yamashita, A., Urasaki, S., Wada, Y. & Yoshida, T. (2015). Isolation of Lactococcus garvieae showing no agglutination with anti-KG-phenotype rabbit serum. *魚病研究*, 50 (2): 37-43.

Ooyama, T., Hirokawa, Y., Minami, T., Yasuda, H., Nakai, T., Endo, M., Ruangpan, L. & Yoshida, T. (2002). Cell-surface properties of Lactococcus garvieae strains and their immunogenicity in the yellowtail Seriola quinqueradiata. *Diseases of aquatic organisms*, 51 (3): 169-177.

Orla-Jensen, A. D. & Hansen, P. A. (1932). The Bacteriological Flora of Spontaneously Soured Milk and of Commercial Starters for Butter Making. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene*, 86: 6-29.

Orla-Jensen, S. (1919). *The lactic Acid Bacteria*. Copenhagen, Denmark: Host & Son.

Palacios, M., Zamora, M., Velazquez, J., Zamora, E. & Duran, A. (1993). Streptococcosis in rainbow trout(Oncorhynchus mykiss) in Spain. *Bollettino Societa Italiana di Patologia Ittica*, 5 (13): 11-16.

Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis*, 6 (4): 313-321.

Pereira, F., Ravelo, C., Toranzo, A. & Romalde, J. (2004). Lactococcus garvieae, an emerging pathogen for the Portuguese trout culture. *Bulletin of the European Association of Fish Pathologists*, 24 (6): 274-279.

Pérez, T., Balcázar, J. L., Peix, A., Valverde, A., Velázquez, E., de Blas, I. & Ruiz-Zarzuela, I. (2011). Lactococcus lactis subsp. tructae subsp. nov. isolated from the intestinal mucus of brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss). *International journal of systematic and evolutionary microbiology*, 61 (8): 1894-1898.

Pfanzagl, J. (2011). *Parametric statistical theory*: Walter de Gruyter.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics (Oxford, England)*, 14 (9): 817-818.

Qiagen. (2011). *CLC Genomics Workbench*: Workbench.

Ravelo, C., Magariños, B., Romalde, J. L. & Toranzo, A. E. (2001). Conventional versus miniaturized systems for the phenotypic characterization of Lactococcus garvieae strains. *Bulletin-European Association of Fish Pathologists*, 21 (4): 136-144.

Ravelo, C., Magarinos, B., López-Romalde, S., Toranzo, A. E. & Romalde, J. L. (2003). Molecular fingerprinting of fish-pathogenic Lactococcus garvieae strains by random amplified polymorphic DNA analysis. *Journal of clinical microbiology*, 41 (2): 751-756.

Reguera-Brito, M., Galán-Sánchez, F., Blanco, M. M., Rodríguez-Iglesias, M., Domínguez, L., Fernández-Garayzábal, J. F. & Gibello, A. (2016). Genetic analysis of human clinical isolates of Lactococcus garvieae: relatedness with isolates from foods. *Infection, Genetics and Evolution*, 37: 185-191.

Reimundo, P., Pignatelli, M., Alcaraz, L. D., D'Auria, G., Moya, A. & Guijarro, J. A. (2011). Genome sequence of Lactococcus garvieae UNIUD074, isolated in Italy from a lactococcosis outbreak. *J Bacteriol*, 193 (14): 3684-5. doi: 10.1128/JB.05210-11.

Ricci, G., Ferrario, C., Borgo, F., Rollando, A. & Fortina, M. G. (2012). Genome sequences of Lactococcus garvieae TB25, isolated from Italian cheese, and Lactococcus garvieae LG9, isolated from Italian rainbow trout. *Journal of bacteriology*, 194 (5): 1249-1250.

Russo, G., Iannetta, M., D'Abramo, A., Mascellino, M. T., Pantosti, A., Erario, L., Tebano, G., Oliva, A., D'Agostino, C. & Trinchieri, V. (2012). Lactococcus garvieae endocarditis in a patient with colonic diverticulosis: first case report in Italy and review of the literature. *Microbiologica-Quarterly Journal of Microbiological Sciences*, 35 (4): 495.

Sakala, R. M., Hayashidani, H., Kato, Y., Hirata, T., Makino, Y., Fukushima, A., Yamada, T., Kaneuchi, C. & Ogawa, M. (2002). Change in the composition of the microflora on vacuum-packaged beef during chiller storage. *International journal of food microbiology*, 74 (1-2): 87-99.

Sambrook, J. & Russell, D. W. (2006). *The condensed protocols from molecular cloning: a laboratory manual*.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences*, 74 (12): 5463-5467.

Savvidis, G., AnatohotiS, C., Kanaki, Z. & Vafeas, G. (2007). Epizootic outbreaks of Lactococcosis disease in rainbow trout, Oncorhynchus mykiss (Walbaum), culture in Greece. *Bulletin of the European Association of Fish Pathologists*, 27 (6): 223-228.

Schleifer, K., Kraus, J., Dvorak, C., Kilpper-Bälz, R., Collins, M. & Fischer, W. (1985). Transfer of Streptococcus lactis and related streptococci to the genus Lactococcus gen. nov. *Systematic and Applied Microbiology*, 6 (2): 183-195.

Schleifer, K. & Kilpper-Bälz, R. (1987). Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. *Systematic and Applied Microbiology*, 10 (1): 1-19.

Schmidtke, L. & Carson, J. (2003). Antigen recognition by rainbow trout (Oncorhynchus mykiss) of whole cell proteins expressed by Lactococcus garvieae when obtained directly from fish and under iron limited culture conditions. *Veterinary microbiology*, 93 (1): 63-71.

Shahi, N., Mallik, S. K., Sahoo, M., Chandra, S. & Singh, A. K. (2018). First report on characterization and pathogenicity study of emerging Lactococcus garvieae infection in farmed rainbow trout, Oncorhynchus mykiss (Walbaum), from India. *Transbound Emerg Dis*, 65 (4): 1039-1048. doi: 10.1111/tbed.12843.

Sharifiyazdi, H., Akhlaghi, M., Tabatabaei, M. & Mostafavi Zadeh, S. (2010). Isolation and characterization ofLactococcus garvieae from diseased rainbow trout (Oncorhynchus mykiss, Walbaum) cultured in Iran. *Iranian Journal of Veterinary Research*, 11 (4): 342-350.

Sibley, C. G. & Ahlquist, J. E. (1981). The relationships of the Accentors (Prunella) as indicated by DNA-DNA hybridization. *Journal für Ornithologie*, 122 (4): 369-378.

Siezen, R. J., Bayjanov, J., Renckens, B., Wels, M., van Hijum, S. A., Molenaar, D. & van Hylckama Vlieg, J. E. (2010). Complete genome sequence of Lactococcus lactis subsp. lactis KF147, a plant-associated lactic acid bacterium. *Journal of bacteriology*, 192 (10): 2649-2650.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International journal of systematic and evolutionary microbiology*, 44 (4): 846-849.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C., Nesme, X., Rosselló-Mora, R., Swings, J. & Trüper, H. G. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International journal of systematic and evolutionary microbiology*, 52 (3): 1043-1047.

Suh, Y., Kim, M. J., Jung, J. S., Chong, Y. P., Kim, C. H., Kang, Y., Sohn, J. W. & Yoon, Y. K. (2016). Afebrile multi-valve infective endocarditis caused by Lactococcus garvieae: A case report and literature review. *Internal Medicine*, 55 (8): 1011-1015.

Teixeira, L. M., Merquior, V. L. C., Vianni, M. d. C. E., Carvalho, M. d. G. S., Fracalanzza, S. E., Steigerwalt, A. G., Brenner, D. J. & Facklam, R. R. (1996). Phenotypic and genotypic characterization of atypical Lactococcus garvieae strains isolated from water buffalos with subclinical mastitis and confirmation of L. garvieae as a senior subjective synonym of Enterococcus seriolicida. *International Journal of Systematic and Evolutionary Microbiology*, 46 (3): 664-668.

Tejedor, J., Vela, A., Gibello, A., Casamayor, A., Domínguez, L. & Fernández‐Garayzábal, J. (2011). A genetic comparison of pig, cow and trout isolates of Lactococcus garvieae by PFGE analysis. *Letters in applied microbiology*, 53 (6): 614-619.

Teuber, M. (1995). The genus Lactococcus. In Wood, B. J. B. & Holzapfel, W. H. (eds) *The Genera of Lactic Acid Bacteria*, pp. 173-234. Boston, MA: Springer US.

Teuber, M. & Geis, A. (2006). The Genus Lactococcus. In Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (eds) *The Prokaryotes: Volume 4: Bacteria: Firmicutes, Cyanobacteria*, pp. 205-228. New York, NY: Springer US.

Teuber, M. (2009). Bergey's Manual of Systematic Bacteriology. In *Bergey's Manual of Systematic Bacteriology*, pp. 711-722.

Thompson, F., Gevers, D., Thompson, C., Dawyndt, P., Naser, S., Hoste, B., Munn, C. & Swings, J. (2005). Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl. Environ. Microbiol.*, 71 (9): 5107-5115.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research*, 22 (22): 4673-4680.

Toranzo, A. E., Magarinos, B. & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, 246 (1-4): 37-61.

Tsai, M. A., Wang, P. C., Liaw, L. L., Yoshida, T. & Chen, S. C. (2012). Comparison of genetic characteristics and pathogenicity of Lactococcus garvieae isolated from aquatic animals in Taiwan. *Dis Aquat Organ*, 102 (1): 43-51. doi: 10.3354/dao02516.

Ture, M., Haliloğlu, H. İ., Altuntaş, C., Boran, H. & Kutlu, İ. (2014). Comparison of Experimental Susceptibility of Rainbow Trout (Oncorhynchus mykiss), Turbot (Psetta maxima), Black Sea Trout (Salmo trutta labrax) and Sea Bass (Dicentrarchus labrax) to Lactococcus garvieae. *Turkish Journal of Fisheries and Aquatic Sciences*, 14 (2): 507-513.

Ture, M., Altinok, I. & Capkin, E. (2015). Comparison of pulsed ‐ field gel electrophoresis and enterobacterial repetitive intergenic consensus PCR and biochemical tests to characterize L actococcus garvieae. *Journal of fish diseases*, 38 (1): 37-47.

Ture, M. & Altinok, I. (2016). Detection of putative virulence genes of Lactococcus garvieae. *Dis Aquat Organ*, 119 (1): 59-66. doi: 10.3354/dao02981.

Varsha, K. K. & Nampoothiri, K. M. (2016). Lactococcus garvieae subsp. bovis subsp. nov., lactic acid bacteria isolated from wild gaur (Bos gaurus) dung, and description of Lactococcus garvieae subsp. garvieae subsp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 66 (10): 3805-3809. doi: doi:10.1099/ijsem.0.001268.

Vela, A. I., Vázquez, J., Gibello, A., Blanco, M. M., Moreno, M. A., Liébana, P., Albendea, C., Alcalá, B., Mendez, A., Domínguez, L., et al. (2000). Phenotypic and Genetic Characterization of *Lactococcus garvieae* Isolated in Spain from Lactococcosis Outbreaks and Comparison with Isolates of Other Countries and Sources. *Journal of Clinical Microbiology*, 38 (10): 3791-3795.

Vela, I., Liebana, P., Albendea, C., Gibello, A., Blanco, M., Moreno, M., Cutúli, M., García, J., Doméneche, A. & Domínguez, L. (1999). *Caracterización fenotípica y molecular de Lactococcus garvieae*. Proceedings of the XIII Congreso Nacional de Microbiologia de la Sociedad Española de Microbiologia. Granada, Spain.

Vendrell, D., Balcazar, J. L., Ruiz-Zarzuela, I., de Blas, I., Girones, O. & Muzquiz, J. L. (2006). Lactococcus garvieae in fish: a review. *Comp Immunol Microbiol Infect Dis*, 29 (4): 177-98. doi: 10.1016/j.cimid.2006.06.003.

Vendrell, D., Balcázar, J. L., Ruiz-Zarzuela, I., de Blas, I., Gironés, O. & Múzquiz, J. L. (2007). Safety and efficacy of an inactivated vaccine against Lactococcus garvieae in rainbow trout (Oncorhynchus mykiss). *Preventive veterinary medicine*, 80 (2-3): 222-229.

von Wright, A. & Tynkkynen, S. (1987). Construction of Streptococcus lactis subsp. lactis strains with a single plasmid associated with mucoid phenotype. *Applied and environmental microbiology*, 53 (6): 1385-1386.

Wang, L.-T., Lee, F.-L., Tai, C.-J. & Kasai, H. (2007). Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the Bacillus subtilis group. *International journal of systematic and evolutionary microbiology*, 57 (8): 1846-1850.

Watt, P. M. & Hickson, I. D. (1994). Structure and function of type II DNA topoisomerases. *Biochemical Journal*, 303 (Pt 3): 681.

Wen, Z. & Zhang, J.-R. (2015). Bacterial Capsules. In *Molecular Medical Microbiology (Second Edition)*, pp. 33-53: Elsevier.

Wertz, J., Goldstone, C., Gordon, D. & Riley, M. A. (2003). A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. *Journal of evolutionary biology*, 16 (6): 1236-1248.

Williams, A., Fryer, J. & Collins, M. (1990). Lactococcus piscium sp. nov. a new Lactococcus species from salmonid fish. *FEMS Microbiology Letters*, 68 (1-2): 109-113.

Wilson, J., Schurr, M., LeBlanc, C., Ramamurthy, R., Buchanan, K. & Nickerson, C. A. (2002). Mechanisms of bacterial pathogenicity. *Postgraduate medical journal*, 78 (918): 216-224.

Woese, C. R. (1987). Bacterial evolution. *Microbiological reviews*, 51 (2): 221.

Yamamoto, S. & Harayama, S. (1996). Phylogenetic analysis of Acinetobacter strains based on the nucleotide sequences of gyrB genes and on the amino acid sequences of their products. *International Journal of Systematic and Evolutionary Microbiology*, 46 (2): 506-511.

Yamamoto, S. & Harayama, S. (1998). Phylogenetic relationships of Pseudomonas putida strains deduced from the nucleotide sequences of gyrB, rpoD and 16S rRNA genes. *International Journal of Systematic and Evolutionary Microbiology*, 48 (3): 813-819.

Yamamoto, S., Bouvet, P. J. & Harayama, S. (1999). Phylogenetic structures of the genus Acinetobacter based on gyrB sequences: comparison with the grouping by DNA-DNA hybridization. *International Journal of Systematic and Evolutionary Microbiology*, 49 (1): 87-95.

Yanez, M., Catalán, V., Apraiz, D., Figueras, M. & Martinez-Murcia, A. (2003). Phylogenetic analysis of members of the genus Aeromonas based on gyrB gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, 53 (3): 875-883.

Yang, S. Y., Zheng, Y., Huang, Z., Wang, X. M. & Yang, H. (2016). Lactococcus nasutitermitis sp. nov. isolated from a termite gut. *International journal of systematic and evolutionary microbiology*, 66 (1): 518-522.

Yoshida, T., Eshima, T., Wada, Y., Yamada, Y., Kakizaki, E., Sakai, M., Kitao, T. & Inglis, V. (1996). Phenotypic variation associated with an anti-phagocytic factor in the bacterial fish pathogen Enterococcus seriolicida. *Diseases of Aquatic Organisms*, 25: 81-86. doi: 10.3354/dao025081.

Yoshida, T., Endo, M., Sakai, M. & Inglis, V. (1997). A cell capsule with possible involvement in resistance to opsonophagocytosis in Enterococcus seriolicida isolated from yellowtail Seriola quinqueradiata. *Diseases of aquatic organisms*, 29 (3): 233-235.

Yu, J., Wang, H., Zha, M., Qing, Y., Bai, N., Ren, Y., Xi, X., Liu, W., Menghe, B. & Zhang, H. (2015). Molecular identification and quantification of lactic acid bacteria in traditional fermented dairy foods of Russia. *Journal of dairy science*, 98 (8): 5143-5154.

Yu, J., Song, Y., Ren, Y., Qing, Y., Liu, W. & Sun, Z. (2017). Genome-level comparisons provide insight into the phylogeny and metabolic diversity of species within the genus Lactococcus. *BMC microbiology*, 17 (1): 213.

Yuki, M., Sakamoto, M., Nishimura, Y. & Ohkuma, M. (2018). Lactococcus reticulitermitis sp. nov., isolated from the gut of the subterranean termite Reticulitermes speratus. *International journal of systematic and evolutionary microbiology*, 68 (2): 596-601.

Zechini, B., Cipriani, P., Papadopoulou, S., Di Nucci, G., Petrucca, A. & Teggi, A. (2006). Endocarditis caused by Lactococcus lactis subsp. lactis in a patient with atrial myxoma: a case report. *Diagnostic microbiology and infectious disease*, 56 (3): 325-328.

Zlotkin, A., Eldar, A., Ghittino, C. & Bercovier, H. (1998). Identification of Lactococcus garvieae by PCR. *Journal of clinical microbiology*, 36 (4): 983-985.

# 8. Appendix

## Appendix Content

# Appendix A

## DNA-ladders

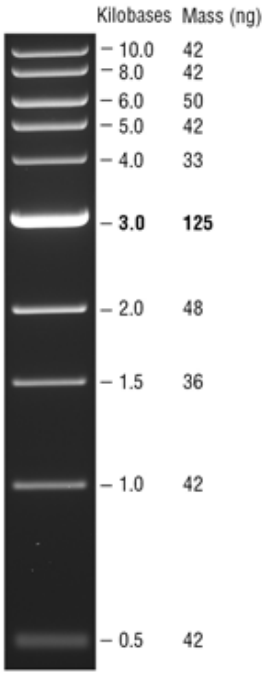



*Figure A.1. Image of 1 kb DNA ladder (NEB) visualized by ethidium bromide on 0.8 % TAE agarose gel. Mass values are for 0.5 µg/gel lane. This ladder is useful to estimate sizes of dsDNA-fragments ranging from 0.5 kb – 10 kb. Picture taken from* https://www.neb.com/products/n3232-1-kb-dna-ladder

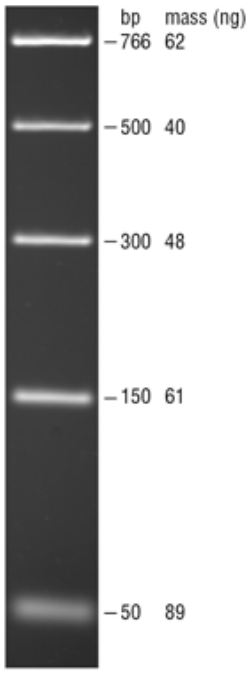



*Figure A.2. Image of PCR marker (NEB) visualized by ethidium bromide on 1.8 % TAE agarose gel. Mass values are for 0.3 µg/gel lane. This ladder is useful to estimate sizes of dsDNA-fragments ranging from 50 bp – 766 bp. Picture taken from* https://www.neb.com/products/n3234-pcr-marker

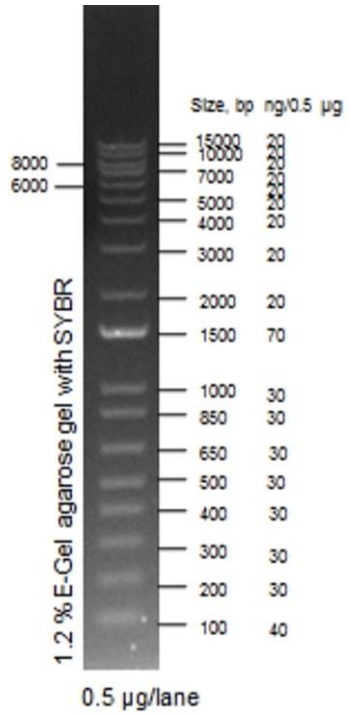



*Figure A.3. Image of E-Gel 1 kb Plus ladder (Invitrogen) visualized on 1.2 % E-Gel with SYBR. This ladder is useful to estimate sizes of dsDNA-fragments ranging from 100 bp – 15000 bp. Picture taken from* https://www.thermofisher.com/order/catalog/product/10488090

# Appendix B

Assembled and trimmed contigs from 16S rRNA gene sequencing, used as input in megaBLAST search in NCBI against 16S ribosomal RNA sequence database (chapter 4.4).

**>AL 21 225**
GGGAATGCGGCTGCCTATACATGCAAGTCGAGCGATGATTAAAGATAGCTTGCTATTTTTATGAAGAGCGGCGAACGGGTGA
GTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCA
TGATTCTTATTTGAAAGAAGCAATTGCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCA
AGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTC
TGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTACGTG
CCAGCAGGCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAAGTCT
GATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATG
TGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGCTGTAGGGAGCTATAAGT
TCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTT
AGAGATAAGGAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCC
AACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCG
CTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGA
GTACCCAAAGTAGGTTGCCTAACCGCAAGGAGGGCGCTCCTAAGGAAGCCGGCC

**>AL 21 226**
CTTAGGAGCGCCCTCCTTGCGGTTAGGCAACCTACTTTGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGGC
CCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCC
GAACTGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCC
CAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACT
TAATGATGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGAC

**>AL 21 228**
GGGAATGCGGCTGCCTATACATGCAAGTCGAGCGATGATTAAAGATAGCTTGCTATTTTTATGAAGAGCGGCGAACGGGTGA
GTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCA
TGATTCTTATTTGAAAGAAGCAATTGCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCA
AGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTC
TGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTACGTG
CCAGCAGGCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAAGTCT
GATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATG
TGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGCTGTAGGGAGCTATAAGT
TCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTT
AGAGATAAGGAGTTCCTTCGGGACACGGGATACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCC
ATCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCAT
TCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGAGTACCCAAAGTAGGTTGCCTAACCGCAAGGAGG
GCGCTCCTAAGGAAGCCGGCC

**>AL21229**
GGAGCGCCCTCCTTGCGGTTAGGCAACCTACTTTGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAAC
TGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGG
TCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACTTAAT
GATGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCAC
CACCTGTATCCCGTGTCCCGAAGGAACTCCTTATCTCTAAGGATAGCACGAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTT
GCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCA
GGCGGAGTGCTTAATGCGTTAGCTGCGCTACAGAGAACTTATAGCTCCCTACAGCTAGCACTCATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCTCCGGTG
TTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTCTCCCAGTTTCCAATGC
ACCCAATGGTTGAGCCACCGCCTTTTACATCAGACTTAAGAAACCTCCAGGCCTCGCGCTTACTCTTTGTAACTCCTTGAAAC
GCAAGCGCGTGCGCTAG

**>AL21 230**
CTTAGGAGCGCCCTCCTTGCGGTTAGGCAACCTACTTTGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGGC
CCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCC
GAACTGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCC
CAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACT

TAATGATGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCAT
GCACCACCTGTATCCCGTGTCCCGAAGGAACTCCTTATCTCTAAGGATA

**>AL21 234**
AGGAGCGCCCTCCTTGCGGTTAGGCAACCTACTTTGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCG
GGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAA
CTGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAG
GTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACTTAA
TGATGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCA
CCACCTGTATCCCGTGTCCCGAAGGAACTCCTTATCTCTAAGGATAGCACGAGTATGTCAAGACCTGGTAAGGTTCTTCGCGT
TGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCC
AGGCGGAGTGCTTAATGCGTTAGCTGCGCTACAGAGAACTTATAGCTCCCTACAGCTAGCACTCATCGTTTACGGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCTCCGGT
GTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTCTCCCAGTTTCCAATG
CACACAATGGTTGAGCCACTGCCTTTTACATCAGACTTAAGAAACTACCTGCGCTCGCTTTACGCCCAATAAATCGCGACAAC
GCTGGGGACGGACA

# Appendix C

Assembled and trimmed contigs from *gyrB* gene sequencing, used as input in megaBLAST search in NCBI against nucleotide collection (nr/nt) database (chapter 4.5). Following are the *gyrB* contigs for AL 21 231, AL 21 232, AL 21 235, AL 21 236, AL 21 237, AL 21 238, AL 21 239, AL 21 240. Contigs for the remaining sixteen strains are in appendix G, in the concatenated sequences for MLSA.

**>AL21231_gyrB_Rainbow trout_Turkey**
TCCACGTTCAAGATTTTACCACGGATGGGCAAAATAGCTTGGAATTCTCGATTACGGCCAGACTTAGCAGAACCACCAGCAG
AATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTTACCAGGCAAGTTAGAAATC
TCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGCAAGAATCCCTTTCTCAACA
ATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTATTGACGATACCTGTTACTTC
ACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGACAGCGGTCAAACCTTCACG
AACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGTTTATCGCACGCGTTAAAGC
TGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGATTGTTGAATGGTAAGTTCC
TGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCCTCTGTATAGATTGGCGTATCAAAAATAACTTCTTTGTT
TTCATTGATAAAGGAAACATAAGATTGAATCCCACCTTCATAGTGGAACATAGCATGATTGTCTTCTTGGCCTTCACGTTTATC
GGTAATAGAAATACGCAAACCACGGTTCAAGAAAGCCAACTCACGGA

**>AL21232_gyrB_Rainbow_trout_Turkey**
TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

**>AL21235_gyrB_Rainbow trout_Turkey**
ATCGACATCGGCATCGGTCATGATAACCAGTTTATGATACCGTGCTTTAGATAAATCATAGTCAGCACCAAAACCAGTTCCCA
TCGCTGTAAAGAGTGAACGGATTTCTTCATTTGCTAAAATCTTATCCATTGTCGCTTTTTCCACGTTCAAGATTTTACCACGGA
TGGGCAAAATAGCTTGGAATTCTCGATTACGGCCAGACTTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTC
TGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTTACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTA
CTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGCAAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCT
CAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTATTGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTT
GACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGACAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCT
TCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGTTTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTA
CCACCTTCATGCGTATTGATATTATTTGCAAATGACATGATTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACA
GTGATTCCATCTAACTCACCCTCTGTATAGATTGGCGTATCAAAAATAACTTCTTTGTTTTCATTGATAAAGGAAACATAAGAT
TGAATCCCACCTTCATAGTGGAACATAGCATGATTGTCTTCTTGGCCTTCACGTTTATCGGTAA

**>AL21236_gyrB_Rainbow trout_Turkey**
TCTTTTGTTTCCGAACCGACTTTGATTCCGTAAATTGGTGGTTGAGCAATATAAACATAACCGGCCTCAACTACAGGACGCAT
ATAGCGGTAAAAAAGAGTTAACAAGAGTGTACGAATGTGTGCCCCATCGACATCGGCATCGGTCATGATAACCAGTTTATGA
TACCGTGCTTTAGATAAATCATAGTCAGCACCAAAACCAGTTCCCATCGCTGTAAAGAGTGAACGGATTTCTTCATTTGCTAA
AATCTTATCCATTGTCGCTTTTTCCACGTTCAAGATTTTACCACGGATGGGCAAAATAGCTTGGAATTCTCGATTACGGCCAGA
CTTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTT
TACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTG
CAAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTA
TTGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATG
ACAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTG
TTTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATG
ATTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCCTCTGTATAGATTGGCGTA
TCAAAAATAACTTCTTTGTTTTCATTGATAAAGGAAACATAAGATTGAATCCCACCTTCATAGTGGAACATAGCATGATTGTC
TTCTTGGCCTT

**>AL21237_gyrB_Rainbow trout_Turkey**
TCGCTGTAAAGAGTGAACGGATTTCTTCATTTGCTAAAATCTTATCCATTGTCGCTTTTTCCACGTTCAAGATTTTACCACGGA
TGGGCAAAATAGCTTGGAATTCTCGATTACGGCCAGACTTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTC
TGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTTACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTA
CTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGCAAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCT
CAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTATTGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTT
GACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGACAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCT
TCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGTTTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTA
CCACCTTCATGCGTATTGATATTATTTGCAAATGACATGATTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACA
GTGATTCCATCTAACTCACCCTCTGTATAGATTGGCGTATCAAAAATAACTTCTTTGTTTTCATTGATAAAGGAAACATAAGAT
TGAATCCCACCTTCATAGTGGAACATAGCATGATTGTCTTCTTGGCCTTCACGTTTATCGGTAATAGAA

**>AL 21 238_gyrB_Rainbow_trout_Turkey**
TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

**>AL 21 239_gyrB_Rainbow_trout_Turkey**
TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

**>AL 21 240_gyrB_Rainbow trout_Turkey**
GTAAAGAGTGAACGGATTTCTTCATTTGCTAAAATCTTATCCATTGTCGCTTTTTCCACGTTCAAGATTTTACCACGGATGGGC
AAAATAGCTTGGAATTCTCGATTACGGCCAGACTTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTG
TTTAGGATCATTGGAAGAGCAGTCCGCAAGTTTACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCAC
GTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGCAAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCA
TAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTATTGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTT
CAAATTGTGGATTAGGGTGTTTAACAGAAATGACAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTA
TCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGTTTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCT
TCATGCGTATTGATATTATTTGCAAATGACATGATTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATT
CCATCTAACTCACCCTCTGTATAGATTGGCGTATCAAAAATAACTTCTTTGTTTTCATTGATAAAGGAAACATAAGATTGAATC
CCACCTTCATAGTGGAACATAGCATGATTGTCTTCTTGGCCTTCA

# Appendix D

Agarose gels of the remaining strains from MLSA PCR, chapter 4.6.

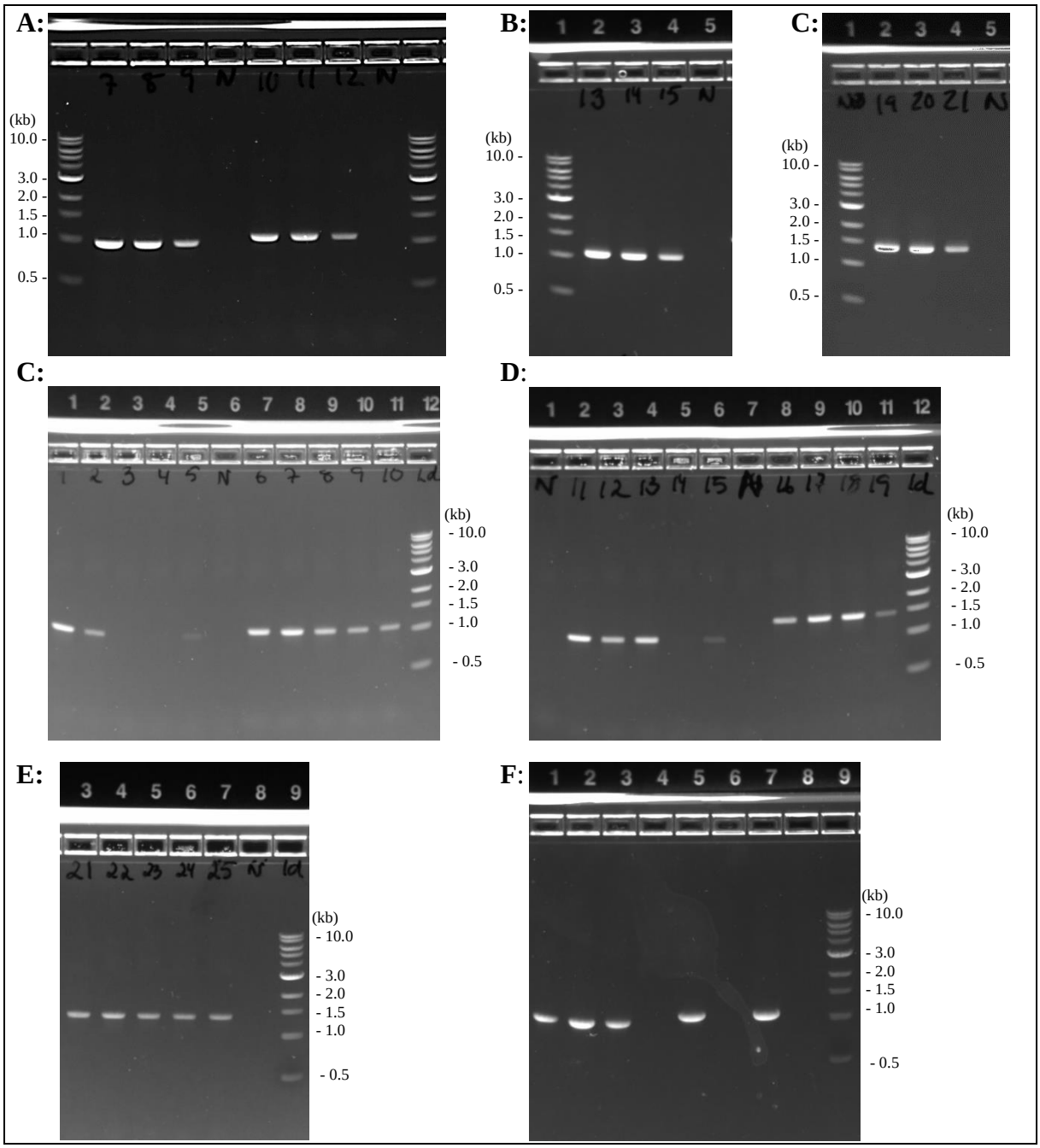



***Figure D-1:*** *PCR product of MLSA genes visualized on agarose-gel A-F for eight of the sixteen isolates.* ***Gel A****: Lane 1; 1 kb NEB ladder. Lane 2-4 gapC, ~1 kb; AL 20 869, AL 20 870, AL 21 046. Lane 5; Negative control. Lane 6-8 als ~1 kb; AL 20 869, AL 20 870, AL 21 046. Lane 9; Negative control. Lane 10; 1 kb NEB ladder.* ***Gel B****: Lane 1; 1 kb NEB ladder. Lane 2-4 galP ~1 kb; AL 20 869, AL 20 870, AL 21 046. Lane 5; Negative control.* ***Gel C****: Lane 1-5 gapC, ~1 kb; AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045. Lane 6; Negative control. Lane 7-11 als ~1 kb; AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045. Lane 12; 1 kb NEB ladder.* ***Gel D****: Lane 6; Negative control for als. Lane 2-6 galP, ~1 kb; AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045. Lane 7; Negative control. Lane 8-11 gyrB ~1.5 kb; AL 20 906, AL 20 909, AL 20 910, AL 20 911, Lane 12; 1 kb NEB ladder.* ***Gel E****: Lane 3-7 rpoC, ~1.5 kb; AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045. Lane 7; Negative control. Lane 8-11 gyrB ~1.5 kb; AL 20 906, AL 20 909, AL 20 910, AL 20 911, AL 21 045. Lane 8; Negative control. Lane 9; 1 kb NEB ladder.* ***Gel F****: Lane 1-3 gapC, ~1 kb; AL 20 910, AL 20 911, AL 21 226. Lane 4; Negative control. Lane 5 als ~1 kb; AL 21 046. Lane 6; Negative control. Lane 7 galP, ~1 kb; AL 20 911. Lane 8; Negative control. Lane 9; 1 kb NEB ladder.*

# Appendix E

Alignments of sequences used as input in modeltest in CLC workbench.

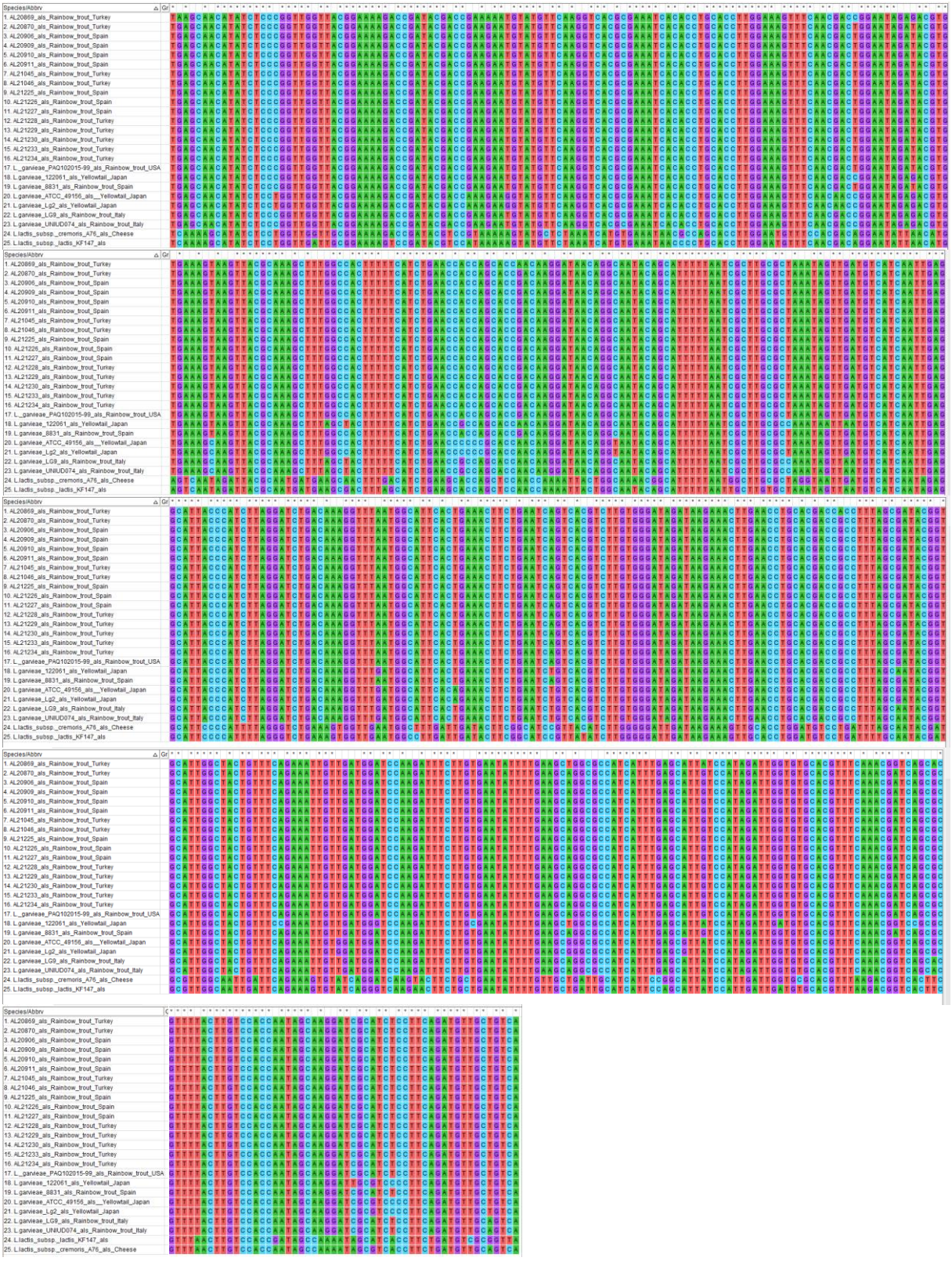


***Figure E-1.*** *Alignment of als-gene for 25 strains. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 540 bp*

***Figure E-2a.*** *Alignment of gapC-gene for 25 strains. More of the gene in figure E-2b next page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 763 bp.*

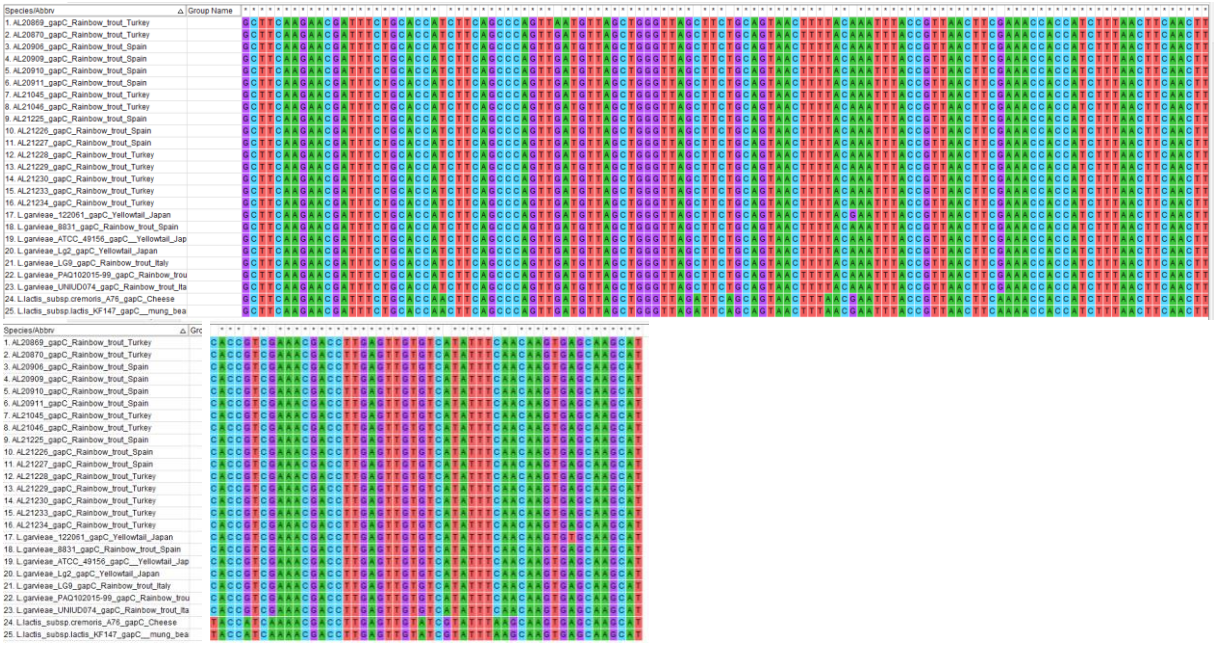


***Figure E-2b.*** *Alignment of gapC-gene for 25 strains, continuing from figure E-2a from previous page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 763 bp*

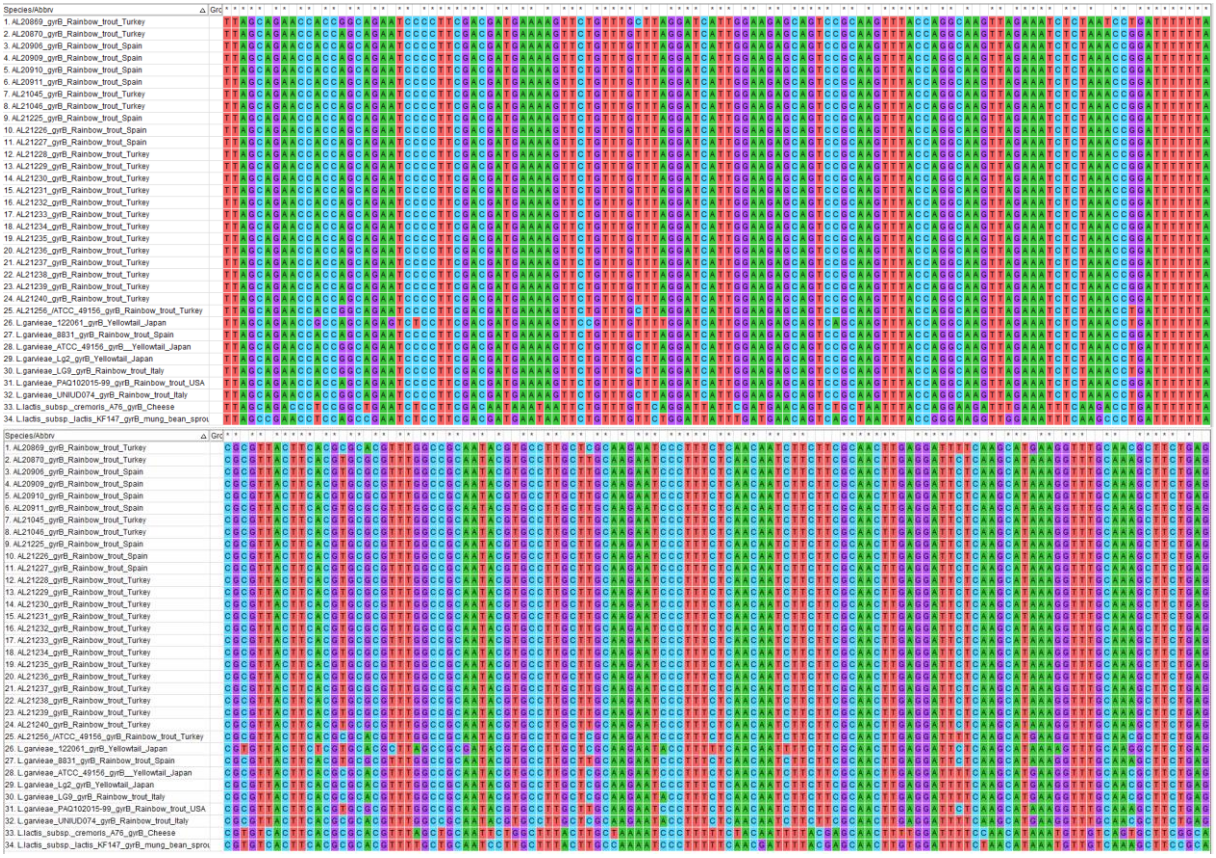


***Figure E-3a.*** *Alignment of gyrB-gene for 34 strains. More of the gene in figure E-3b next page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 5673 bp.*

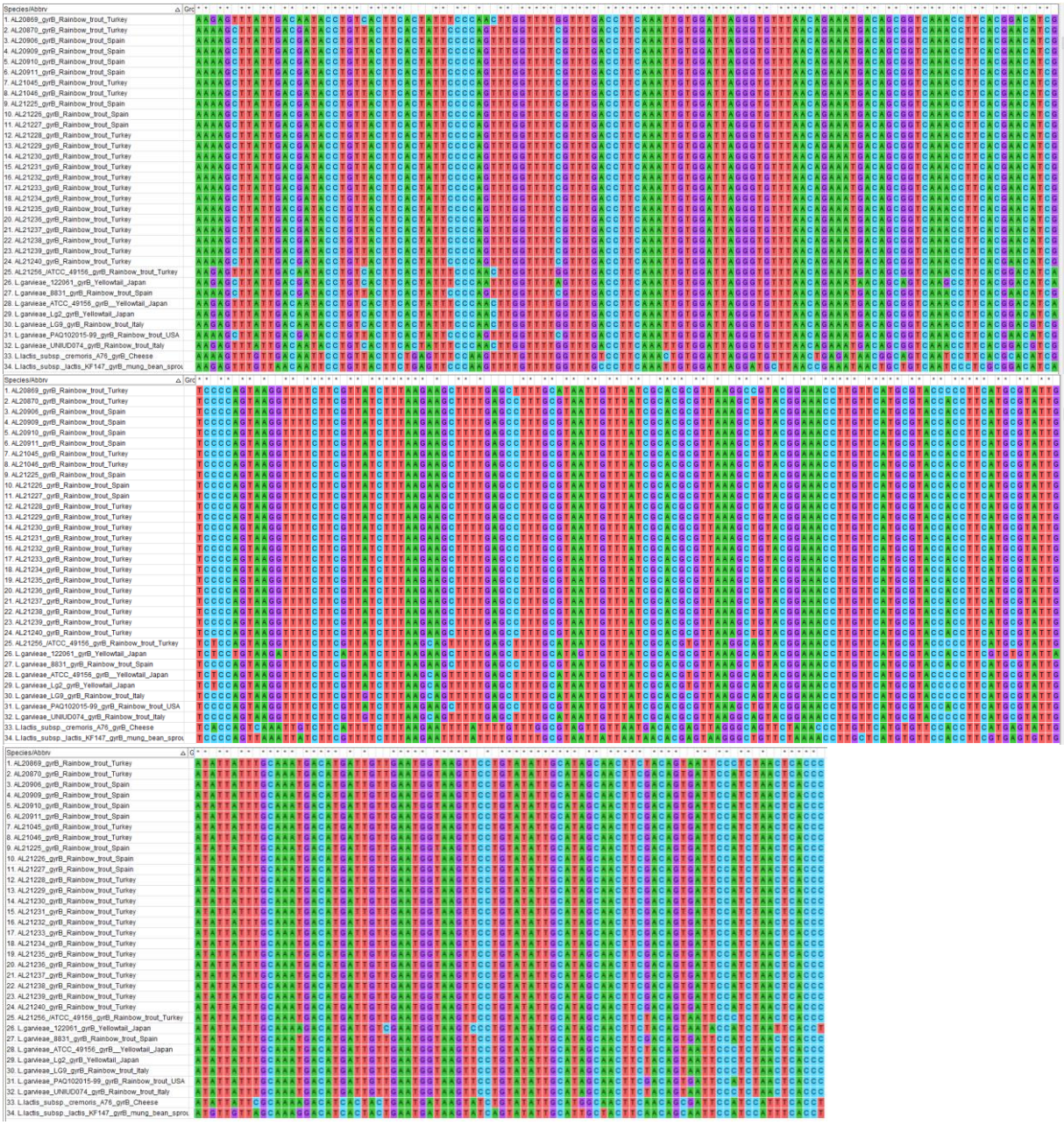


***Figure E-3b.*** *Alignment of gyrB-gene for 34 strains, continuing from figure E-3a from previous page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 567 bp*

***Figure E-4a.*** *Alignment of rpoC-gene for 25 strains. More of the gene in figure E-4b next page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 705 bp.*

***Figure E-4b.*** *Alignment of rpoC-gene for 25 strains, continuing from figure E-4a from previous page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 705 bp*

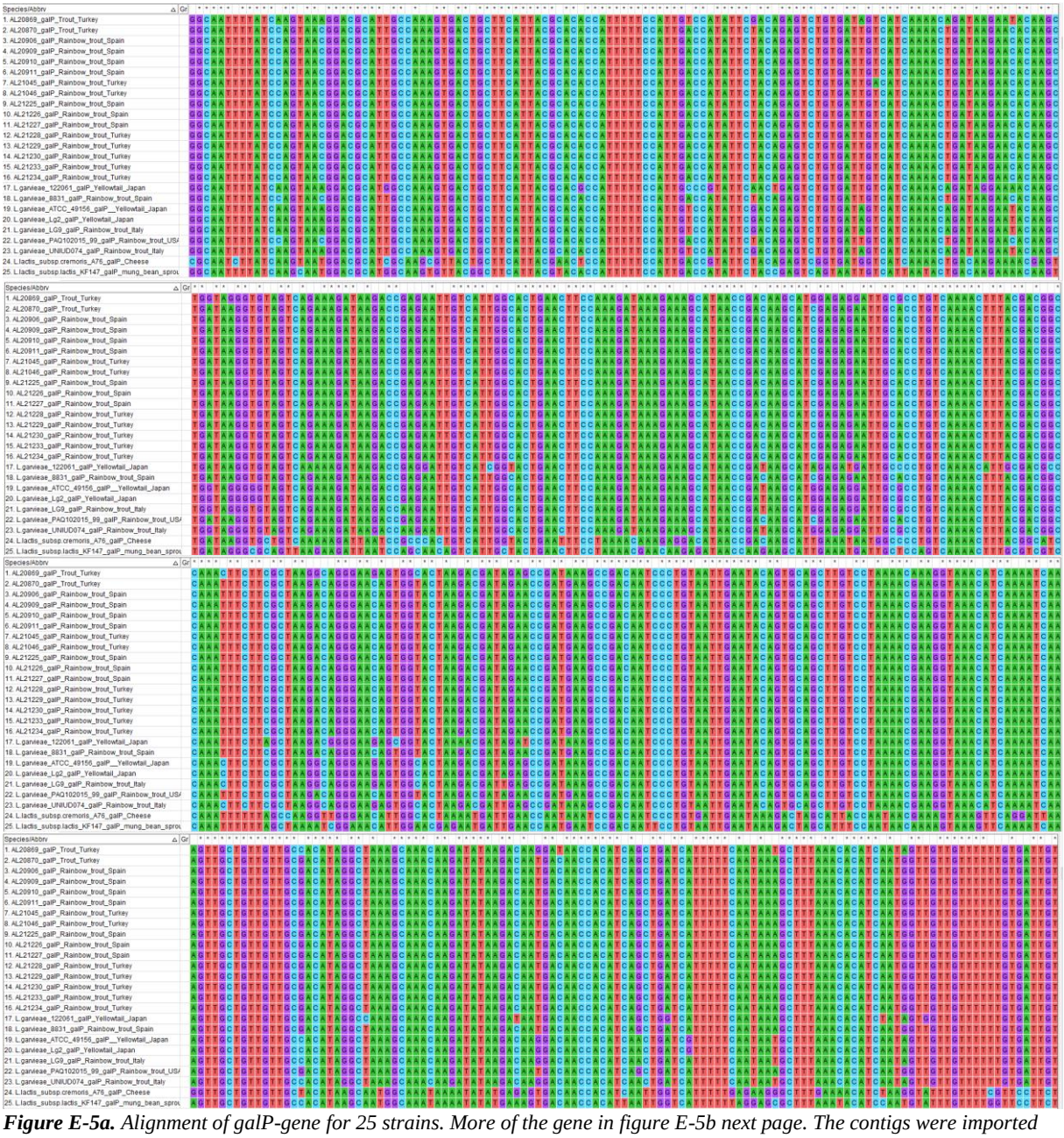


***Figure E-5a.*** *Alignment of galP-gene for 25 strains. More of the gene in figure E-5b next page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 750 bp.*

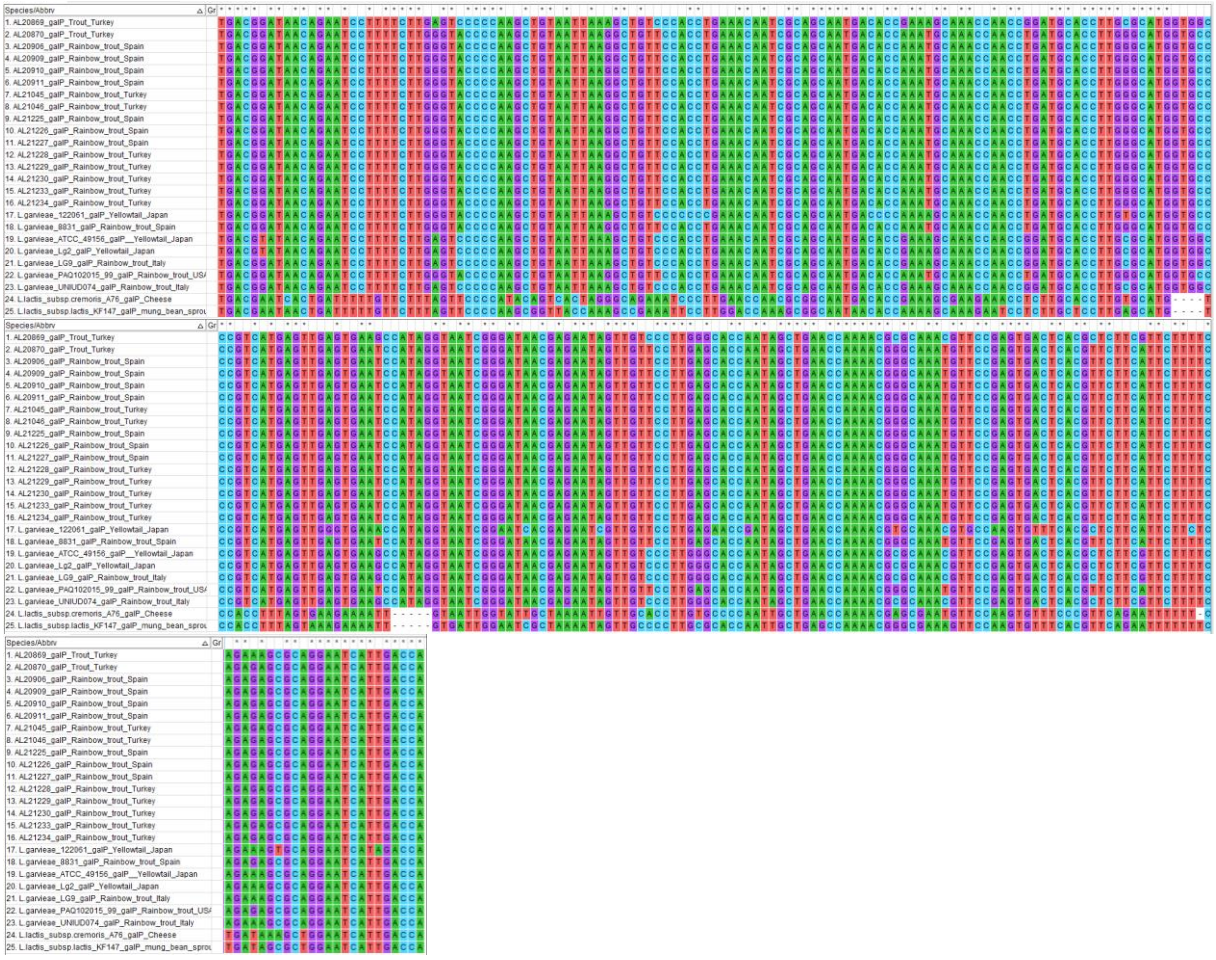


***Figure E-5b.*** *Alignment of galP-gene for 25 strains, continuing from figure E-5a from previous page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 750 bp*

# Appendix F

New alignment for als used as input in modeltest in CLC workbench, and phylogenetic tree.

***Figure F-1a.*** *Alignment of als-gene for 23 strains (without AL 20 909 and AL 21 225), More of the gene in figure E-6b next page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 860 bp*

***Figure F-1b.*** *Alignment of als-gene for 23 strains, (without AL 20 909 and AL 21 225), continuing from previous page. The contigs were imported and aligned using ClustalW, edited in MEGA 7.0 and adjusted to proper reading frame. Alignment length is 860 bp*

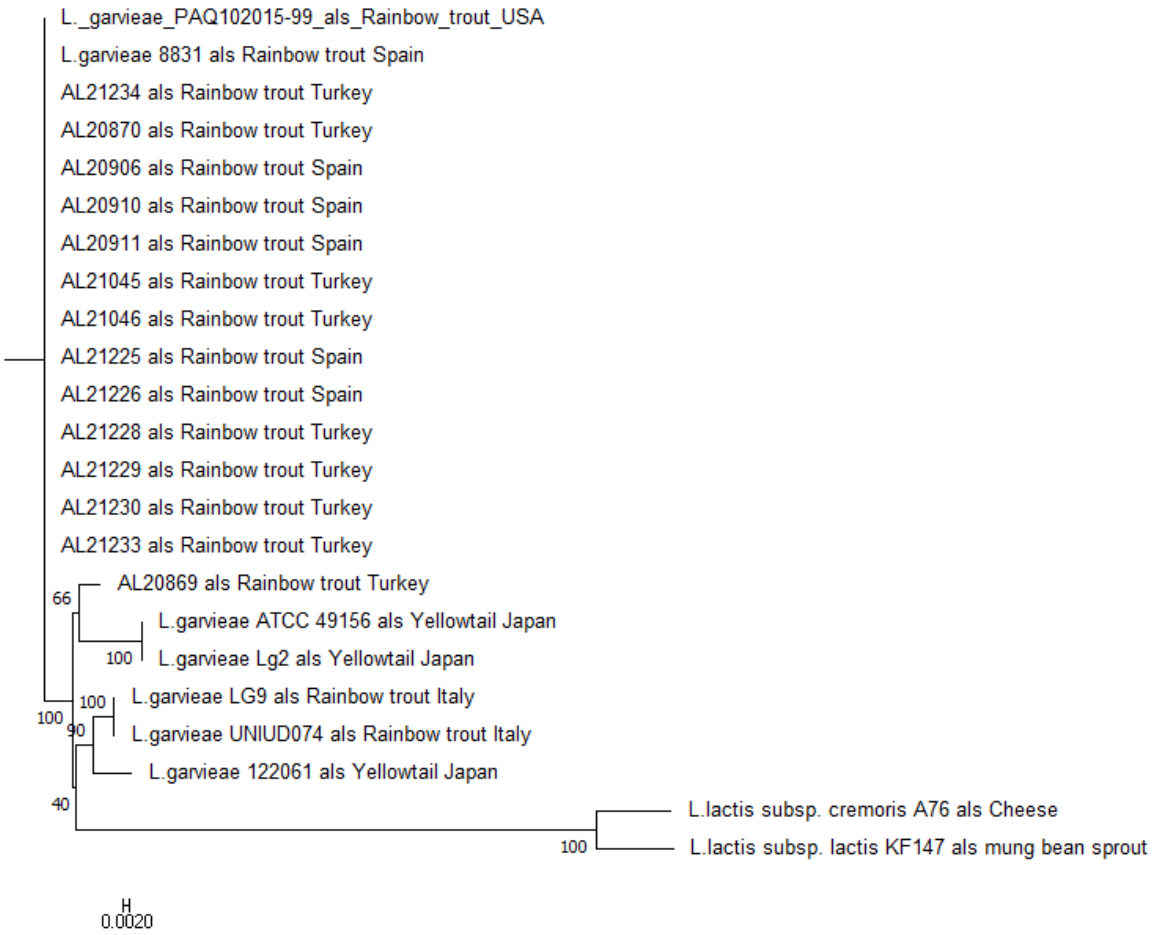



***Figure F-2***. *Phylogenetic relationship between 23 strains* (without AL 20 909 and AL 21 225 *based on individual analyses of als 860bp. The trees were constructed by maximum-likelihood method. Lactococcus lactis subsp. lactis and Lactococcus lactis subsp. cremoris served as outgroups. The scale bar indicates nucleotide sequence variation of 0.2 %.*

# Appendix G

Sequences for MLSA (chapter 4.6). Contigs were joined together end-on-end resulting in 3325 bp concatenated sequence of the five loci. The concatenated sequences were used for phylogenetic relationships. All the strains had the same order of genes; *als*, *gapC*, *gyrB*, *rpoC*, *galP* divided by gaps.

**>AL 20 869_Rainbow trout_Turkey**
TAAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAAAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACCGGAATAGAGACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCAACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCACCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCTGGCGCCATCATTTGAGCATTATCCATAGATTGGTGTGCACGTTTCAAACGGTCAGCACGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCGAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTAATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCGGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGCTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAATCCTGATTTTTTACGCGTTACTTCACGCGCACGTTTGGCCGCAATACGTGCCTTGCTCGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTTTCAAGCATGAAGGTTTGCAACGCTTCTGAGAAGAGTTTATT
GACAATACCTGTCACTTCACTATTTCCCAACTTGGTTTTGGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGAC
AGCGGTCAAACCTTCACGGACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCTTTTGCATAATTGTT
TATCGCACGCGTTAAGGCCGTACGGAAACCTTGTTCATGCGTACCCCCTTCATGCGTATTGATATTATTTGCAAATGACATGAT
TGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCTACAGTAATTCCCTCTAACTCACCC

TGGCGCTCTACTTTACGTTTAGCCGCACGGATGTTGGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATCTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACCGCGATAACGGAACGACCTGAG
TAGTCGACACGTTTACCCAACAAGTTTTGACGGAAACGTCCTTGTTTACCTTTCAGCATGTGGCTGAGAGATTTAAGCGGACG
ATTACCAGCACCTGTGATTGGACGACCACGACGACCATTGTCAATCAATGTATCCACAGCTTCTTGCAACATACGTTTTTCATT
TTGGACGATAATATTTGGGGCGTTAAGCTCCATCAAACGTTTCAAACGGTTGTTACGGTTGATTACACGACGATAAAGGTCAT
TCAAATCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAG
CACCATCCATGCCAATTTGTTACCAGATTTTTTGAAAGCTGTTAATACATCCAAACGACGAATGATTTTCACACGGCGTTGTCC
GCTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCTGC
ACCCATCTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGTAAAGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGTCCATATTCGACAG
AGTCTGTGATAGTCATCAAAACAGATAAGAATACAAGCTGGTAGGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGG
CACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATGGAGAGGATTGCGCCTGTCAAAACTTTACGACGGCCAAAC
TTCTTCGCTAAGGCAGGGAAGAGTGGCACTAAGACGATAGAGCCGATAAAGCCGACAATCCCTGTAATTGAATACAGTGCAG
CTTGTCCTAAAACAAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCCACATAGGCTAAAGCAAACAAGATATAAGACAA
GGATAACCACATCAGCTGATCATTTTTCAATAATGCTTTAAACACATCAATAGTTGTTGTTTTTTGTGATTGTTGACGGATAAC
AGAATCCTTTTCTTGAGTCCCCCAAGCTGTAATTAAAGCTGTCCCACCTGAAACAATCGCAGCAATGACACCGAAAGCAAACC
AACCGGATGCACCTTGCGCATGGTGGCCCGTCATGAGTTGAGTGAAGCCATAGGTAATCGGGATAACGAGAATAGTTGTCCC
TTGGGCACCAATAGCTGAACCAAAACGCGCAAACGTTCCGAGTGACTCACGCTCTTCGTTCTTTTCAGAAAGCGCAGGAATCA
TTGACCA

**>AL 20 870_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG

TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 20 906_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT

TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 20 909_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 20 910_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 20 911_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT

GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 045_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGACATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 046_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA

CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 225_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 226_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 227_Rainbow trout_Spain**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 228_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 229_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT

TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 230_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT

TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL 21 233_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>AL21234_Rainbow trout_Turkey**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>ATCC 49156_Yellowtail_Japan**
TGAGCAACATATCTCCTGGTTGGTTACGGAAAAGACCGATACGACCAAAGAAGGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACAACCGGAATAGAGACGTGTGAAAGCAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CCCCCGCACCAACAAGGATAACAGGTAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTGATGGCATTCACAGAAACTTCTGAATCTGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTGGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCGGGCGCCATCATTTGAGCGTTATCCATAGATTGGTGTGCACGTTTCAAACGGTCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCGTCCCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACAGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTATTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCGGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGCTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCTGATTTTTTACGCGTTACTTCACGCGCACGTTTGGCCGCAATACGTGCCTTGCTCGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTTTCAAGCATGAAGGTTTGCAACGCTTCTGAGAAGAGTTTATT
GACAATACCTGTCACTTCACTATTTCCCAACTTGGTTTTGGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGAC
AGCGGTCAAACCTTCACGGACATCATCTCCAGTAAGGTTTTCTTCGTTATCTTTAAGCAGTTTTTGAGCTTTTGCATAATTGTTT
ATCGCACGTGTTAAGGCAGTACGGAAACCTTGTTCATGCGTACCCCCTTCATGCGTATTGATATTATTTGCAAATGACATGATT
GTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCTACAGTAATTCCCTCTAACTCACCC

TGGCGCTCTACTTTACGTTTAGCCGCACGGATGTTGGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACCGCGATAACGGAACGACCTGAGT
AGTCGACACGTTTACCCAACAAGTTTTGACGGAAACGACCTTGTTTACCTTTCAGCATGTGGCTGAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCATTGTCAATCAATGTATCCACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTTGGGGCGTTAAGCTCCATCAAACGTTTCAAACGGTTGTTACGGTTGATTACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGT
ACCATCCATGCCAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACATCCAAACGACGAATGATTTTCACACGGCGTTGTCCG

CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCTGCA
CCCATCTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGTAAAGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGTCCATATTCGACAG
AGTCTGTGATAGTCATCAAAACAGATAAGAATACAAGCTGGTAGGGGGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGG
CACTGAACTTCCAAAGATAAAGAAAGCATAACCGATAAGCATGGAGAGGATTGCGCCTGTCAAAACTTTACGACGGCCAAAC
TTCTTCGCTAAGGCAGGGAAGAGTGGCACTAAGACGATAGAGCCGATAAAGCCGACAATCCCTGTAATTGAATACAGTGCAG
CTTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCCACATAGGCTAAAGCAAACAAGATATAAGACAA
GGACAACCACATCAACTGATCGTTTTTCAATAATGCTTTAAACACATCAATAGTTGTTGTTTTTTGTGATTGTTGACGTATAAC
AGAATCCTTTTCTTGAGTCCCCCAAGCTGTAATTAAAGCTGTCCCACCTGAAACAATCGCAGCAATGACACCGAAAGCAAACC
AACCGGATGCACCTTGCGCATGGTGGCCCGTCATGAGTTGAGTGAAGCCATAGGTAATCGGGATAACGAGAATAGTTGTCCC
TTGGGCACCAATAGCTGAACCAAAACGCGCAAACGTTCCGAGTGACTCACGCTCTTCGTTCTTTTCAGAAAGCGCAGGAATCA
TTGACCA

**>Lg2_Yellowtail_Japan**
TGAGCAACATATCTCCTGGTTGGTTACGGAAAAGACCGATACGACCAAAGAAGGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACAACCGGAATAGAGACGTGTGAAAGCAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CCCCCGCACCAACAAGGATAACAGGTAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTGATGGCATTCACAGAAACTTCTGAATCTGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTGGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCGGGCGCCATCATTTGAGCGTTATCCATAGATTGGTGTGCACGTTTCAAACGGTCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCGTCCCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACAGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCGGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGCTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCTGATTTTTTACGCGTTACTTCACGCGCACGTTTGGCCGCAATACGTGCCTTGCTCGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTTTCAAGCATGAAGGTTTGCAACGCTTCTGAGAAGAGTTTATT
GACAATACCTGTCACTTCACTATTTCCCAACTTGGTTTTGGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGAC
AGCGGTCAAACCTTCACGGACATCATCTCCAGTAAGGTTTTCTTCGTTATCTTTAAGCAGTTTTTGAGCTTTTGCATAATTGTTT
ATCGCACGTGTTAAGGCAGTACGGAAACCTTGTTCATGCGTACCCCCTTCATGCGTATTGATATTATTTGCAAATGACATGATT
GTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCTACAGTAATTCCCTCTAACTCACCC

TGGCGCTCTACTTTACGTTTAGCCGCACGGATGTTGGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACCGCGATAACGGAACGACCTGAGT
AGTCGACACGTTTACCCAACAAGTTTTGACGGAAACGACCTTGTTTACCTTTCAGCATGTGGCTGAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCATTGTCAATCAATGTATCCACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTTGGGGCGTTAAGCTCCATCAAACGTTTCAAACGGTTGTTACGGTTGATTACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGT
ACCATCCATGCCAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACATCCAAACGACGAATGATTTTCACACGGCGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCTGCA
CCCATCTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGTAAAGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGTCCATATTCGACAG
AGTCTGTGATAGTCATCAAAACAGATAAGAATACAAGCTGGTAGGGGGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGG
CACTGAACTTCCAAAGATAAAGAAAGCATAACCGATAAGCATGGAGAGGATTGCGCCTGTCAAAACTTTACGACGGCCAAAC
TTCTTCGCTAAGGCAGGGAAGAGTGGCACTAAGACGATAGAGCCGATAAAGCCGACAATCCCTGTAATTGAATACAGTGCAG
CTTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCCACATAGGCTAAAGCAAACAAGATATAAGACAA
GGACAACCACATCAACTGATCGTTTTTCAATAATGCTTTAAACACATCAATAGTTGTTGTTTTTTGTGATTGTTGACGTATAAC
AGAATCCTTTTCTTGAGTCCCCCAAGCTGTAATTAAAGCTGTCCCACCTGAAACAATCGCAGCAATGACACCGAAAGCAAACC
AACCGGATGCACCTTGCGCATGGTGGCCCGTCATGAGTTGAGTGAAGCCATAGGTAATCGGGATAACGAGAATAGTTGTCCC
TTGGGCACCAATAGCTGAACCAAAACGCGCAAACGTTCCGAGTGACTCACGCTCTTCGTTCTTTTCAGAAAGCGCAGGAATCA
TTGACCA

**>122061_Yellowtail_Japan**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACCGGAATAGAGACGTGTGAAAGTAAGTTACGCAAAGCTTTAGCTACTTTTTCATCTGAAC
CGCCAGCACCAACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCCAAATAATTAATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTGATGGCATTCACTGAAACTTCTGAATCTGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCAATACGGTAAGCATTGGCTACTGTTTCCGAAATTGTTGATGGGTCCAAGATTTCTTGCG
AATATTTTGAAGCTGGCGCCATCATTTGAGCATTATCCATAGATTGATGTGCACGTTTCAAACGGTCCGCGCGTTTTACTTGTC
CACCAATAGCAAGGATTGCGTCCCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCATGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC

CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCGCCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
GAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGTGCAAGCAT

TTAGCAGAACCGCCAGCAGAGTCTCCTTCGACGATGAAAAGTTCCGTTTGTTTTGGATCATTGGAAGAGCAGTCAGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCTGATTTTTTACGTGTTACTTCTCGTGCACGCTTAGCCGCGATACGTGCCTTGCTCGC
AAGAATACCTTTTTCAACAATTTTCTTCGCAACTTGAGGATTCTCAAGCATAAAAGTTTGCAAGGCTTCTGAGAAGAGCTTAT
TGACGATACCTGTCACTTCACTATTTCCCAATTTGGTTTTAGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATAA
CAGCAGTCAAGCCTTCACGGACATCATCTCCTGTAAGATTTTCTTCATTATCTTTAAGAAGCTTTTGAGCTTTTGCATAGTTGT
TTATCGCACGCGTTAAAGCAGTACGGAAACCTTGTTCATGCGTACCACCTTCGTGTGTATTAATATTATTTGCAAAAGACATG
ATTGTCGAATGGTAAGTCCCTGTATATTGCATAGCAACTTCTACAGTAATACCATCTAATTCACCT

TGGCGTTCAACTTTACGTTTAGCTGCACGAATGTTGGCAGCCATTTCACGTTTAACCAGTTCAGACATTACGAATGGTTTGAA
AAGTTCAATCGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCAGAGT
AGTCGACACGTTTACCCAACAAGTTTTGACGGAAACGTCCTTGTTTACCTTTCAGCATATGGCTGAGAGATTTAAGTGGACGG
TTACCGGCACCTGTGATTGGACGACCACGACGACCATTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGAACGATAATATTAGGGGCATTAAGCTCCATCAAACGTTTCAAACGATTGTTACGGTTGATAACACGACGGTAAAGATCATT
CAAATCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGGACATTGAGC
ACCATCCATGCTAATTTGTTACCAGATTTTTTGAAAGCTGTTAAAACATCCAAGCGACGGATGATTTTCACACGTCGTTGTCCA
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGAATAGCTTCTGCA
CCCATTTTTGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGTAAAGGACGCATGGCCAAAGTGACTGCTTCATTACGCACGCCATTTTTCCATTGCCCGTATTCAACTG
AGTCTGTGATTGTCATCAAAACAGATAGGAAAACAAGCTGATAAGGTGTAGTCAAAAAGATAAGACCGAGGATTGTCATCGG
TACTGAACTTCCAAAGATAAAGAAAGCATAACCGATAAGCATAGAGATGATTGCCCCTGTCAAAACATTGCGACGCCCAAAT
TTCTTAGCTAAGACGGGGAAGAGCGGTACTAAAACGATAGATCCGATAAAGCCGACAATCCCTGTAATTGAATACAGTGCAG
CTTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCCAAAGCAAACAAGATATAAGATAA
TGACAACCACATCAGCTGGTCATTTTTCAATAAAGCTTTAAACACATCTATAGTGGTTGTTTTTTGTGATTGTTGACGGATAAC
AGAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAAGCTGTCCCCCCCGAAACAATCGCAGCAATGACCCCAAAAGCAAACC
AACCTGATGCACCTTGTGCATGGTGCCCCGTCATGAGTTGGGTGAAACCATAGGTAATCGGAATCACGAGAATCGTTGTTCCT
TGAGAACCGATAGCTGAACCAAAACGTGCAAACGTGCCAAGTGTTTCACGCTCTTCATTCTTCTCAGAAAGTGCAGGAATCAT
AGACCA

**>LG9_Rainbow trout_Italy**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACCGGAATAGAGACGTGTGAAAGCAAGTTACGCAAAGCTTTAGCTACTTTTTCATCTGAAC
CGCCAGCACCAACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCCAAATAGTTAATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTGATGGCATTCACTGAAACTTCTGAATCTGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCAATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTATCCATAGATTGGTGTGCACGTTTCAAACGGTCAGCACGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCAGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCGGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGCTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCTGATTTTTTACGCGTTACTTCACGCGCACGTTTGGCCGCAATACGTGCCTTGCTCGC
AAGAATACCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTTTCAAGCATGAAGGTTTGCAACGCTTCTGAGAAGAGTTTATT
GACAATACCTGTCACTTCACTATTTCCCAACTTGGTTTTGGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGAC
AGCGGTCAAACCTTCACGGACGTCGTCCCCAGTAAGGTTTTCTTCGTTGTCTTTAAGCAGTTTTTGAGCTTTTGCATAATTGTT
TATCGCACGCGTTAAGGCAGTACGGAAACCTTGTTCATGCGTACCCCCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCTACAGTAATTCCCTCTAACTCACCC

TGGCGCTCTACTTTACGTTTAGCCGCACGGATGTTGGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACCGCGATAACGGAACGACCTGAGT
AGTCGACACGTTTACCCAACAAGTTTTGACGGAAACGTCCTTGTTTACCTTTCAGCATGTGGCTGAGAGATTTAAGTGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCATTGTCAATCAATGTATCCACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTTGGGGCGTTAAGCTCCATCAAACGTTTCAAACGGTTGTTACGGTTGATTACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCATGCCAATTTGTTACCAGATTTTTTGAAAGCTGTTAATACATCCAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCTGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGTAAAGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGTCCATATTCGACAG
AGTCTGTGATAGTCATCAAAACAGATAAGAATACAAGCTGGTAGGGTGTAGTCAGAAAGATAAGACCAAGAATTGTCATTGG

CACTGAACTTCCAAAGATAAAGAAAGCATAACCGATAAGCATGGAGAGGATTGCGCCTGTCAAAACTTTACGACGGCCAAAC
TTCTTCGCTAAGGCAGGGAAGAGTGGCACTAAGACGATTGAGCCGATAAAGCCGACAATCCCTGTAATTGAATACAGTGCAG
CTTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCCACATAGGCTAAAGCAAACAAGATATAAGACAA
GGACAACCACATCAACTGATCATTTTTCAATAATGCTTTAAACACATCAATAGTTGTTGTTTTTTGTGATTGTTGACGGATAAC
AGAATCCTTTTCTTGAGTCCCCCAAGCTGTAATTAAAGCTGTCCCACCTGAAACAATCGCAGCAATGACACCGAAAGCAAACC
AACCGGATGCACCTTGCGCATGGTGGCCCGTCATGAGTTGAGTGAAGCCATAGGTAATCGGGATAACGAGAATAGTTGTCCC
TTGGGCACCAATAGCTGAACCAAAACGCGCAAACGTTCCGAGTGACTCACGCTCTTCGTTCTTTTCAGAAAGCGCAGGAATCA
TTGACCA

**>PAQ102015_Rainbow trout_USA**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACTGGAATAGATACGTGTGAAAGTAAGTTACGCAAAGCTTTGGCCACTTTTTCATCTGAAC
CACCAGCACCGACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCTAAATAGTTGATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTAATGGCATTCACTGAAACTTCTGAATCAGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCGATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTGTCCATAGATTGGTGTGCACGTTTCAAACGATCAGCGCGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCTGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC
AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCAGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGTTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCGGATTTTTTACGCGTTACTTCACGTGCGCGTTTGGCCGCAATACGTGCCTTGCTTGC
AAGAATCCCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTCTCAAGCATAAAGGTTTGCAAAGCTTCTGAGAAAAGCTTAT
TGACGATACCTGTTACTTCACTATTCCCCAGTTTGGTTTTCGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGA
CAGCGGTCAAACCTTCACGAACATCGTCCCCAGTAAGGTTTTCTTCGTTATCTTTAAGAAGCTTTTGAGCCTTTGCGTAATTGT
TTATCGCACGCGTTAAAGCTGTACGGAAACCTTGTTCATGCGTACCACCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCGACAGTGATTCCATCTAACTCACCC

TGACGTTCAACTTTACGTTTAGCCGCACGGATGTTAGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACTGCAATAACTGAACGACCTGAGT
AGTCAACACGTTTACCCAACAAGTTTTGACGGAAACGCCCTTGTTTACCTTTCAGCATATGGCTAAGAGATTTAAGCGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCGTTGTCAATCAATGTATCAACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTAGGGGCGTTAAGCTCCATCAAACGTTTCAAACGATTATTACGGTTGATAACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCAAGCTAATTTGTTACCAGATTTTTTGAAGGCTGTTAATACGTCTAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCAGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCCAGTAACGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGACCATATTCTACAGA
GTCTGTGATTGTCATCAAAACTGATAAGAACACAAGCTGATAAGGTGTAGTCAGAAAGATAAGACCGAGAATTGTCATTGGC
ACTGAACTTCCAAAGATAAAGAAAGCATAACCGACAAGCATCGAGAGAATTGCACCTGTCAAAACTTTACGACGGCCAAATT
TCTTCGCTAAGACAGGGAACAGTGGTACTAAGACGATAGAACCGATGAAGCCGACAATCCCTGTAATTGAATACAGTGCAGC
TTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCGACATAGGCTAAAGCAAACAAGATATAAGACAAT
GACAACCACATCAGCTGATCATTTTTCAATAAAGCTTTAAACACATCAATGGTTGTTGTTTTTTGTGATTGTTGACGGATAACA
GAATCCTTTTCTTGGGTACCCCAAGCTGTAATTAAGGCTGTTCCACCTGAAACAATCGCAGCAATGACACCAAATGCAAACCA
ACCTGATGCACCTTGGGCATGGTGCCCCGTCATGAGTTGAGTGAATCCATAGGTAATCGGGATAACGAGAATAGTTGTTCCTT
GAGCACCAATAGCTGAACCAAAACGGGCAAATGTTCCGAGTGACTCACGTTCTTCATTCTTTTCAGAGAGCGCAGGAATCATT
GACCA

**>UNIUD074_Rainbow trout_Italy**
TGAGCAACATATCTCCCGGTTGGTTACGGAAAAGACCGATACGACCGAAGAATGTATGTTCAAGGTCACGCGAAATCACACC
TGCACCTTGGAAAGTTTCAACGACCGGAATAGAGACGTGTGAAAGCAAGTTACGCAAAGCTTTAGCTACTTTTTCATCTGAAC
CGCCAGCACCAACAAGGATAACAGGCAATACAGCATTTTTAATCGCTTGCGCCAAATAGTTAATGTCATCAATTGAGGCATTA
CCCATCTTAGGATCTGACAAAGGTTTGATGGCATTCACTGAAACTTCTGAATCTGTCACGTCTTGTGGGATAGATAAGAAACT
TGAACCTGCACGACCGCCTTTAGCAATACGGTAAGCATTGGCTACTGTTTCAGAAATTGTTGATGGATCCAAGATTTCTTGTG
AATATTTTGAAGCAGGCGCCATCATTTGAGCATTATCCATAGATTGGTGTGCACGTTTCAAACGGTCAGCACGTTTTACTTGTC
CACCAATAGCAAGGATCGCATCTCCTTCAGATGTTGCAGTCA

CGTCAAAGAGTGAAGAGTTAGAGATACCTACGATATCAGATGAAACGATTTCATCTTCGTTGTAACCGTAAGATTCGTTAGAT
GCTGCTTTCATAGCTGCATTTACTTCTTCAACAGTAACTTCTTTATCAAGAACTGATACAAGTTCTGTCAATGAACCAGTTGGA
ACTGGTACACGTTGTGCGTGACCTTGAAGTTTACCATTCAATTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTGA
GTTAGGTACGATGTTTTCAGCTGCAGCACGTGCACGACGGAAGTCACCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCAC
CAGTGTAACCGTGGATAGTTGTCATTGTACCAACTTTAAGACCGAAGTTTTTGTTCAAAGCATCAGCCATTGGTGCGAGACAG
TTAGTAGTACATGATGCACCAGAGATAACTGTTTCTTCACCAGTCAAGATTTCGTGGTTAGTGTTGAAAACGATAGTTTTAAC
ATCGTTTCCACCAGGTGCTGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCATCTTCAGCCCAGTTGATGTTAGCTGGGTTAGCTTCTGCAGTAACTTTTAC

AAATTTACCGTTAACTTCGAAACCACCATCTTTAACTTCAACTTCACCGTCGAAACGACCTTGAGTTGTGTCATATTTCAACAA
GTGAGCAAGCAT

TTAGCAGAACCACCGGCAGAATCCCCTTCGACGATGAAAAGTTCTGTTTGCTTAGGATCATTGGAAGAGCAGTCCGCAAGTTT
ACCAGGCAAGTTAGAAATCTCTAAACCTGATTTTTTACGCGTTACTTCACGCGCACGTTTGGCCGCAATACGTGCCTTGCTCGC
AAGAATACCTTTCTCAACAATCTTCTTCGCAACTTGAGGATTTTCAAGCATGAAGGTTTGCAACGCTTCTGAGAAGAGTTTATT
GACAATACCTGTCACTTCACTATTTCCCAACTTGGTTTTGGTTTGACCTTCAAATTGTGGATTAGGGTGTTTAACAGAAATGAC
AGCGGTCAAACCTTCACGGACGTCGTCCCCAGTAAGGTTTTCTTCGTTGTCTTTAAGCAGTTTTTGAGCTTTTGCATAATTGTT
TATCGCACGCGTTAAGGCAGTACGGAAACCTTGTTCATGCGTACCCCCTTCATGCGTATTGATATTATTTGCAAATGACATGA
TTGTTGAATGGTAAGTTCCTGTATATTGCATAGCAACTTCTACAGTAATTCCCTCTAACTCACCC

TGGCGCTCTACTTTACGTTTAGCCGCACGGATGTTGGCAGCCATTTCACGTTTAACCAATTCGGACATTACGAATGGTTTGAA
GAGTTCGATAGCCATTTCACGTGGGACACCACATTGGTACATTTTAAGTGTTGGACCAACCGCGATAACGGAACGACCTGAGT
AGTCGACACGTTTACCCAACAAGTTTTGACGGAAACGTCCTTGTTTACCTTTCAGCATGTGGCTGAGAGATTTAAGTGGACGA
TTACCGGCACCTGTGATTGGACGACCACGACGACCATTGTCAATCAATGTATCCACAGCTTCTTGCAACATACGTTTTTCATTT
TGGACGATAATATTTGGGGCGTTAAGCTCCATCAAACGTTTCAAACGGTTGTTACGGTTGATTACACGACGATAAAGGTCATT
CAAATCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGAAGATCTGGTGGAATAACTGGAAGCACATTAAGC
ACCATCCATGCCAATTTGTTACCAGATTTTTTGAAAGCTGTTAATACATCCAAACGACGAATGATTTTCACACGACGTTGTCCG
CTAACATTTTTCAATTCTTCTTTAAGTTCAGCAACTTCTGCATCAATATCAACATCGTTAAGCAAATCTTGGATAGCTTCTGCA
CCCATTTTCGCAACAAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGTAAAGGACGCATTGCCAAAGTGACTGCTTCATTACGCACACCATTTTTCCATTGTCCATATTCGACAG
AGTCTGTGATAGTCATCAAAACAGATAAGAATACAAGCTGGTAGGGTGTAGTCAGAAAGATAAGACCAAGAATTGTCATTGG
CACTGAACTTCCAAAGATAAAGAAAGCATAACCGATAAGCATGGAGAGGATTGCGCCTGTCAAAACTTTACGACGGCCAAAC
TTCTTCGCTAAGGCAGGGAAGAGTGGCACTAAGACGATTGAGCCGATAAAGCCGACAATCCCTGTAATTGAATACAGTGCAG
CTTGTCCTAAAACGAAGGTAAACATCAAAATCAAAGTTGCTGTTGTTGCCACATAGGCTAAAGCAAACAAGATATAAGACAA
GGACAACCACATCAACTGATCATTTTTCAATAATGCTTTAAACACATCAATAGTTGTTGTTTTTTGTGATTGTTGACGGATAAC
AGAATCCTTTTCTTGAGTCCCCCAAGCTGTAATTAAAGCTGTCCCACCTGAAACAATCGCAGCAATGACACCGAAAGCAAACC
AACCGGATGCACCTTGCGCATGGTGGCCCGTCATGAGTTGAGTGAAGCCATAGGTAATCGGGATAACGAGAATAGTTGTCCC
TTGGGCACCAATAGCTGAACCAAAACGCGCAAACGTTCCGAGTGACTCACGCTCTTCGTTCTTTTCAGAAAGCGCAGGAATCA
TTGACCA

**>L.lactis_subsp._cremoris_A76_cheese**
TCAAAAGCATATCTCCTGGTTGGTTGCGGAAAAGACCGATACGTCCGTAAAAAGTATGCTCTAAATCATGTGAAATAACGCC
AGCACCTTGGAATGTTTCCACGACAGGAATATTAACATGAGTCAATAGATTACGCAATGATGAAGCAACTTTGACATCTGAA
GCACCAGCTCCAACCAAAATTACTGGCAAAACGGCATTTTTAATGGCTTGCGCTAGGTAATTGATGTCATCAATAGAGGCATT
CCCCATTTTAGGGTCTGAAAGTGGTTGAATGGCTTTGATTGATACTTCGGCATCCGTTACATCTTGGGGGATTGATAAGAAAG
TTGCACCTGGATGTCCTGATTTAGCAATACGATAAGCGTTGGCAATTGATTCAGAAAGTGTATCAGGATCAAGTACTTCTGCT
GAATATTTTGTTGCTGATTGCATCATTCCGGCATTATCCATTGATTGGTGCGCACGTTTAAGACGGTCACTTCGTTTAACTTGT
CCACCAATAGCCAAAATAGCGTCACCTTCTGATGTTGCAGTCA

CATCAAAGAGTGAAGAGTTTGAGATACCAACGATATCAGATGAAACGATTTGGTCTTCGTTGTAACCAAATGATTCATTTGAA
GCAGCTTTCATAGCTGCGTTGATTTCGTCAACTGTAACTTCTTTATCAAGGATAGTAACAAGTTCAGTCAATGAACCAGTTGG
AACTGGTACACGTTGAGCATGTCCTTGAAGTTTACCTTGAAGTTCTGGCAACACAAGACCGATAGCTTTAGCAGCACCTGTTG
AGTTAGGTACGATGTTTTCAGCTGCAGCACGTGCGCGACGGAAATCTCCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCA
CCAGTGTAACCGTGAACTGTAGTCATTGTACCAACTTTGATACCGAATTGTTTGTTCAAAGTATCAGCCATTGGAGCAAGACA
GTTAGTTGTACATGAACCAGCTGAAATTACTGTTTCAGTTCCATCAAGTACTTCGTGGTTAGTGTTGAAAACGATTGTTTTAAC
ATCTGATCCACCAGGTGCAGTGATAACAACTTTCTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCAACTTCAGCCCAGTTGATGTTAGCTGGGTTAGATTCAGCAGTAACTTTAA
CGAATTTACCGTTAACTTCAAAACCACCATCTTTAACTTCAACTTTACCATCAAAACGACCTTGAGTTGTATCGTATTTAAGCA
AGTGAGCAAGCAT

TTAGCAGACCCTCCGGCTGAATCTCCTTCGACAATAAATAATTCTGTTTGTTCAGGATTATTCGATGAACAGTCTGCTAATTTA
CCAGGAAGATTTGAAATTTCAAGACCTGATTTTTTACGTGTCACTTCACGCGCACGTTTAGCTGCAATTCTGGCTTTACTTGCT
AAAATCCCTTTTTCTACAATTTTACGAGCAACTTTTGGATTTTCCAACATAAATGTTGTCAGTGCTTCGGCAAAAAGTTTGTTG
ACAATTCCTGTTACTTCTGAGTTTCCAAGTTTTGTTTTGGTTTGTCCTTCAAACTGTGGATTAGGGTGTTTAACTGAGATAACG
GCAGTCAATCCTTCACGCACATCGTCACCAGTCAAATTGTCTTCATTTTCTTTAAGAATTTTATTTTGTTTGGCGTAGTTGTTAA
TGACACGAGTTAGGGCAGTTCTAAACCCTTGTTCATGTGTTCCACCTTCATGAGTATTGATATTATTCGCAAAAGACATCACTA
CTGAATGATAAGTATCTGTATATTGCATGGCAACTTCAACAGCGATTCCATCCATTTCACCT

TGACGTTCAACTTTACGTTTAGCCGCACGAATATTAGCAGCCAATTCTTTTTTAACAAGTTGTGCCATTACAAATGGTTTGAAC
AACTCAATCGCCATTTCACGTGGAACACCACATTGGTACATTTTAAGTGTTGGACCGACAGCGATAACTGAACGTCCAGAATA
GTCAACACGTTTACCAAGCAAGTTTTGACGGAAACGTCCTTGTTTCCCTTTCAACATGTGTGAAAGAGATTTCAATGGACGGT
TACCAGCACCAGTGATTGGACGACCACGACGACCATTGTCAATCAAAGTATCAACAGCTTCTTGGAGCATCCGTTTTTCATTT
TGAACGATAATATTTGGAGCATTAAGTTCCATCAAACGTTTCAAACGGTTATTACGGTTGATAACACGACGATACAAGTCATT
CAAGTCAGATGTGGCAAAACGTCCACCATCTAATTGAACCATTGGACGCAAATCTGGTGGAATAACTGGTAATACGTTTAAG
ACCATCCAAGATAAAGCATTTCCTGATTTTCTGAAAGCTGACAAAACATCTAAACGACGAATAATTTTTACACGACGTTGTCC
AGTCACTGTTTTGAGTTCTTCTTTGAGTTCAGCAACTTCTTTGTCAATATCAACATCATTCAACAAATCTTGAATTGCTTCAGC
ACCCATTTTTGCAACGAATGAACCAAAACCATTTTTCAAA

CGCAATCTTATCAAGTAATGGACGCATCGCAAGCGTTACTGCTTCATTACGAACTCCATTTTTCCATTGACCGTATTCTACAGA
GTCGGTGATGGTCATCAAAACTGACAAGAAAACGAGTTGATAAGGTGCTGTCAAAAAGATTAATCCGCCCACTGTCATTGGT
ACTGAATTTCCTAAAACAAAGAGGACATAACCGACAAGCATTGAAATAATGGCCCCTGTCAAAACTTTACGGCATCCAAATT
TTTTAGCCAAGGTTGGGAACATTGGCACTAAAATGATTGAACCAATAAATCCGACAATCCCTGTGATTGAATAAAGACTAGC
ATTACCAATAACGAAAGTAAAGTTCAGGATTAAGGTTGCTGTTGTTGCTACATAAGCAATGGCAAATAAAATATATGAGAGT
GACAACCACATCAATTGGTCATTTTTGAGAAGGGCTTTGAAAACATCTAAGGTATTTGTTTTCGTTCCTTCTTGACGAATCACT

GATTTTTGTTCTTTAGTTCCCCATACAGTCACTAGGGCAGAAATCCCTTGAACCAACGCGGCAATGACACCGAAAGCGAAGAA
ACCTCTTGCACCTTGTGCATG----TCCACCTTTAGTGAAGAAAATT-----
GTAATTGGTATTGCTAAAATTGTTGCACCTTGTGCCCCAATTGCTGAACCAAAACGAGCGAATGTTCCAAGTGTTTCCCGTTC
AGAATTTTTT-CTGATAAAGCTGGAATCATTGACCA

**>L.lactis_subsp._lactis_KF147_mungo bean sprout**
TCAAAAGCATATCTCCTGGTTGATTGCGGAAAAGTCCGATACGTCCATAAAAAGTATGTTCTAAATCATGTGAAATAACCCCT
GCACCTTGGAATGTTTCAACGACAGGAATATTAACATGAGTCAATAGATTACGCAATGATGAAGCGACTTTAGCATCTGAAG
CACCAGCTCCAACCAAAATTACTGGCAATACAGCATTTTTAATTGCTTGTGCTAAATAGTTAATGTCATCAATAGAGGCATTC
CCCATTTTAGGGTCTGAAAGTGGTTGAATGGCCTTGATTGATACTTCGGCATCCGTTATATCTTGGGGGATTGATAAGAAAGT
TGCACCTGGATGTCCTGATTTTGCAATACGATAAGCGTTGGCAATTGATTCAGAAAGTGTATCAGGGTCAAGAACTTCTGCTG
AATATTTTGTTGCTGATTGCATCATTCCAGCATTATCCATTGATTGATGTGCACGTTTAAGACGGTCACTTCGTTTAACTTGTCC
ACCGATAGCCAAAATAGCATCACCTTCTGATGTCGCGGTTA

CATCAAAGAGTGAAGAGTTTGAGATACCAACGATATCAGATGAAACGATTTGGTCTTCGTTGTAACCAAATGATTCATTTGAA
GCAGCTTTCATAGCTGCGTTGATTTCGTCAACTGTAACTTCTTTGTTAAGGATAGTAACAAGTTCAGTCAATGAACCAGTTGG
AACTGGTACACGTTGAGCATGTCCTTGAAGTTTACCTTGAAGTTCTGGCAATACAAGACCGATAGCTTTAGCAGCACCTGTTG
AGTTAGGTACGATGTTTTCAGCTGCAGCACGTGCGCGACGGAAGTCTCCACCACGGTGTGGGCCATCAAGAGTCATTTGGTCA
CCAGTGTAACCGTGAACTGTAGTCATTGTACCAACTTTGATACCGAATTGTTTGTTCAAAGTATCAGCCATTGGAGCAAGACA
GTTAGTTGTACATGAACCAGCTGAAATTACTGTTTCAGTTCCATCAAGTACTTCGTGGTTAGTGTTGAAAACGATTGTTTTAAC
ATCAGATCCACCAGGTGCAGTGATAACAACTTTTTTAGCACCGTTAGCGTGCAAGTGTTGTTCAGCTTTTTCTTTAGTTGCGAA
GAAACCAGTTGCTTCAAGAACGATTTCTGCACCAACTTCAGCCCAGTTGATGTTAGCTGGGTTAGATTCAGCAGTAACTTTAA
CGAATTTACCGTTAACTTCAAAACCACCATCTTTAACTTCAACTTTACCATCAAAACGACCTTGAGTTGTATCGTATTTAAGCA
AGTGAGCAAGCAT

TTAGCCGAACCTCCAGCCGAATCTCCTTCGACGATGAATAATTCTGTTTGTTCTGGATTATTTGATGAACAGTCAGCTAATTTA
CCGGGAAGGTTGGAAATTTCAAGCCCTGATTTTTTACGTGTCACTTCACGCGCACGTTTTGCTGCAATCCTTGCTTTACTTGCC
AAAATCCCTTTTTCAACGATTTTACGAGCAACTTGTGGATTTTCTAACATAAATGTTGTCAAAGCTTCCGCAAAGAGTTTGTTA
ACAATTCCTGTTACTTCTGAGTTCCCAAGTTTTGTTTTGGTTTGCCCTTCAAATTGTGGATTAGGATGCTTAACCGAAATAACT
GCTGTCAATCCCTCGCGGACATCATCCCCAGTTAAATTATCTTCGTTTTCTTTAAGAATTTTATTTTGTTTTGCGTAATTATTAA
TAACACGAGTAAGGGCTGTTCTAAAACCTTGCTCATGTGTTCCACCTTCGTGAGTGTTGATGTTGTTAGCAAAGGACATCACT
ACTGAATGATAAGTATCAGTATATTGCATTGCTACTTCAACAGCAATTCCATCCATTTCACCT

TGACGTTCAACTTTACGTTTAGCCGCACGAATATTAGCAGCCAATTCTTTCTTAACAAGTTGAGCCATTACGAATGGTTTGAAC
AACTCAATCGCCATTTCACGCGGAACACCACATTGGTACATTTTGAGTGTTGGACCTACAGCGATAACTGAACGTCCAGAATA
GTCAACACGTTTACCAAGCAAGTTTTGACGGAAACGTCCTTGTTTCCCTTTCAACATGTGTGAAAGAGATTTCAATGGGCGGT
TACCAGCACCAGTGATTGGACGACCACGACGACCATTGTCAATCAAAGTATCAACAGCTTCTTGAAGCATCCGTTTTTCATTT
TGAACGATAATATTTGGAGCATTAAGTTCCATCAAACGTTTCAAACGGTTATTACGGTTAATAACACGACGGTATAAGTCATT
CAAGTCAGATGTGGCAAAACGTCCACCATCCAATTGAACCATTGGACGTAAATCTGGTGGAATAACTGGTAATACGTTTAAG
ACCATCCAAGATAAAGCATTTCCTGATTTTCTGAAAGCTGACAAAACGTCCAAACGACGAATAATTTTTACACGACGTTGTCC
AGTAACTGTTTTGAGCTCTTCTTTGAGTTCAGAAACTTCTTTATCAATATCAACATCGTTCAACAAATCTTGAATCGCTTCAGC
ACCCATTTTTGCAACGAATGAACCAAAACCATTTTTCAAA

GGCAATTTTATCAAGCAATGGACGCATGGCAAGTGTTACGGCTTCATTACGTACACCATTTTTCCATTGACCATATTCTACCGA
GTCAGTAATTGTCATTAATACTGACAAGAAAACAAGTTGATAGGGCGCAGTTAAGAAGATTAATCCAGCAACAGTCATTGCT
ACTGAACTTCCTAAAACGAACAAGAGATAACCAAGAAGCATTGAAATGATTGCTCCAGTCAAAACTTTGCGTCGTCCAAATT
TTTTAGCTAAAATCGGAAACATTGGAACGAGAATGATTGAACCAATGAATCCGACAATTCCTGTAATTGAATAAAGACTAGC
ATTTCCAATAACAAAAGTAAAGTTCAAAATCAAAGTTGCTGTTGTTGCCACATAAGCAATGGCAAATAAAATATATGAAAGT
GACAACCACATTAATTGGTCATTTTTTAGGAGCGCTTTAAATACATCCAATGTATTTGTTTTGGTTCCTTCTTGACGAATAACT
GATTTTTGTTCTTTAGTTCCCCAAGCGGTTACCAAAGCCGAAATTCCTTGGACCAAAGCGGCAATAACACCAAAAGCAAAGA
ATCCTCTTGCTCCTTGAGCATG----TCCACCTTTAGTAAAGAAAATT-----
GTGATTGGAATCGCTAAAATAGTTGCCCCTTGCGCACCAATTGCTGAGCCAAAACGGGCGAAAGTTCCAAGTGTTTCACGTTC
AGAATTTTTTTCTGATAGCGCTGGAATCATTGACCA


**Norges miljø- og biovitenskapelige universitet**
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway