Molecular characterization of *Flavobacterium* spp. isolated from Rainbow trout (*Oncorhynchus mykiss*) farmed in Southern Africa and development of a PCR-based tool for differentiating between isolates

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Declaration:

1. I hereby present this thesis in full fulfilment of the requirements for the degree of Master of Science in the Faculty of Science (Molecular and Cell Biology Department) at the University of Cape Town.
2. I declare that the work presented in this dissertation is my own, except for the work of others which is properly referenced.
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DEDICATION AND ACKNOWLEDGEMENTS.

This work is dedicated to my late father Mathume Jack Komane (Mokone wa nakana tša pudi), my mother Chuba Anna Ntabo, my late grandmother Ragosebo Komane, my late sister Mologadi Grace Komane. And last but not least I dedicate this work to my son Mathume Thatego Komane.

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Contents

Molecular characterization of *Flavobacterium* spp. isolated from Rainbow trout (*Oncorhynchus mykiss*) farmed in Southern Africa and development of a PCR-based tool for differentiating between isolates

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Abstract

Bacterial fish diseases caused by yellow-pigmented, filamentous bacteria of the genus *Flavobacterium* are among those that lead to significant losses in the international aquaculture industry. An increasing number of *Flavobacterium* spp. have been isolated in association with diseased fish within the aquaculture industry. In salmonids, well known flavobacterial diseases include bacterial cold-water disease (*F. psychrophilum*), rainbow trout fry syndrome (*F. psychrophilum*), bacterial gill disease (*F. branchiophilum*, *F. aquatile*), and columnaris disease (*F. columnare*). Conventional diagnosis of *Flavobacterium* spp. is based on physico-chemical tests, but these tests are time-consuming and labor-intensive, and they are unable to distinguish between closely related species due to morphological similarities. Furthermore, little information exists on the diversity of fish associated *Flavobacterium* spp. from southern Africa. Recently, numerous, yellow-pigmented, filamentous bacteria were isolated from diseased rainbow trout farmed in South Africa and neighboring Lesotho. The aim of the present study was to elucidate the genotypic and phylogenetic diversity of the *Flavobacterium* spp. associated with these fish and to develop a new molecular system for rapid and accurate identification and differentiation of the isolates. In order to do this, the genotypic and phylogenetic diversity of ninety bacterial isolates, mostly yellow-pigmented, obtained from the gills, skin ulcers, liver, and kidneys of diseased *Oncorhynchus mykiss* was assessed following PCR and sequencing of the 16S rRNA gene. A BLAST search of the GenBank database revealed that 47 of the 16S rRNA gene sequences showed high similarity to several *Flavobacterium* spp.; 6 showed high similarity to

Chryseobacterium spp., and 19 non-*Flavobacterium* isolates were identified, which included, amongst others, *Hafnia* spp. and *Aeromonas* spp. Fifteen isolates were excluded from further analysis due to poor DNA sequence data, whilst four isolates showed high similarity to uncultured bacteria and they were also excluded from further analysis. Isolate OM-46 was excluded from the phylogenetic analysis of *Flavobacterium* spp. since only the forward 16S rRNA sequence was available. Phylogenetic analysis based on the maximum likelihood method confirmed the allocation of 46 isolates as *Flavobacterium* spp., and 6 isolates as *Chryseobacterium* spp. Differential identification of the *Flavobacterium* spp. was achieved following PCR amplification of a hypervariable region of the 16S rRNA gene followed by high-resolution melt (HRM) analysis. Nine *Flavobacterium* isolates, presumed to be different species based on the phylogenetic analysis were identified and successfully differentiated using high resolution melt analysis. The present study has identified new *Flavobacterium* spp. (OM-13, OM-39, OM-51, OM-82, and OM-84) from rainbow trout farmed in southern Africa and developed a tool that may be useful for the management of flavobacterial diseases worldwide.

1.Introduction

Global aquaculture has experienced significant growth in recent years due to an increase in food fish demand and decreasing wild fish stocks (Nadarajah & Flaaten, 2017; Subasinghe & Bondad-Reantaso, 2001). Since 1970 the aquaculture sector has experienced an average growth rate of 8.9% yearly, whilst both capture fisheries and land meat production recorded 1.2% and 2.8% average growth respectively within the same period (Subasinghe & Bondad-Reantaso, 2001). In South Africa, the aquaculture industry has also experienced a rapid increase in growth, with the freshwater aquaculture industry experiencing 85% growth in production between 2006 and 2015 (DAFF, 2017). However, the rapid growth of aquaculture has been greatly hampered by diseases (Rodger, 2016). Annual economic losses caused by diseases in the aquaculture industry are estimated to be billions of US dollars worldwide (Subasinghe & Bondad-Reantaso, 2001).

Bacteria, fungi, viruses, and parasites are serious pathogens that hinder production in both freshwater and marine aquaculture (Pridgeon, 2012). However, infectious bacterial diseases are the most prevalent challenge hampering production in fish aquaculture (Dhar *et al*., 2014). Bacteria belonging to the genera *Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Pseudomonas*, *Vibrio*, *Lactococcus*, and *Streptococcus* are amongst those reported to be responsible for significant disease outbreaks in aquaculture (Altinok *et al*., 2007; Karsidani *et al*., 2010; Kerie *et al*., 2019; Loch & Faisal, 2015; Nielsen *et al*., 2001; Pasnik *et al*., 2009; Zorrilla *et al*., 2003). The genus *Flavobacterium* contains some of the most important pathogens of cultured fish such as *F. psychrophilum*, *F. columnare,* and *F. branchiophilum* (Bernardet & Nakagawa, 2006; Mcbride, 2014).

Chryseobacterium, a genus closely related to *Flavobacterium*, contains bacteria such as *C. indologenes, C. scopthalmum,* and other related *Chryseobacterium* spp. that have also been described as emerging pathogens of fish (Kämpfer *et al*., 2019; Loch & Faisal, 2018; Loch & Faisal, 2015; Zamora *et al*., 2012a).

1.1. Flavobacterial diseases affecting rainbow trout

Various members of the family *Flavobacteriaceae* (Phylum Bacteroidetes) are responsible for causing flavobacterial diseases of fish (Loch & Faisal, 2015). Historically *F. psychrophilum*, *F. columnare*, and *F. branchiophilum* are the most widely recognized causative agents of flavobacterial diseases (Loch & Faisal, 2015). However, there have also been reports of other *Flavobacterium* spp. associated with diseased fish, including *F. johnsoniae*-like isolates (Flemming *et al*., 2007), *F. succinicans* (Anderson & Ordal, 1961), and *F. hydatis* (Strohl & Tait, 1978).

Amongst the well-established flavobacterial pathogens, *F. psychrophilum* is responsible for bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS), *F. branchiophilum* causes bacterial gill disease (BGD), and *F. columnare* causes columnaris disease (Faisal *et al*., 2017; Starliper, 2011, 2012). The severity and outcome of flavobacterial diseases is dependent on various interactions between the host, environment, and pathogen factors. Factors such as overcrowding, water temperatures, size and susceptibility of the host, and virulence of the eliciting strain have been shown to affect the severity of flavobacterial diseases (Decostere *et al*., 2001; Good *et al*., 2008; Marancik *et al*., 2014; Pacha & Ordal, 1967; Wagner & Oplinger, 2014).

1.1.1. Bacterial Cold-Water Disease and Rainbow Trout Fry Syndrome

Bacterial cold-water disease affects a wide range of cold, freshwater fish species. The disease mostly occurs when water temperatures are below 16°C and is most acute when temperatures are below 10°C (Starliper, 2011). BCWD affects all ages of wild and farmed fish populations, however, trout and salmon fry weighing 0.5 to 5.0g appear to be more susceptible to BCWD (Starliper, 2011). Several environmental factors such as salinity, water temperature, poor water quality, and overcrowding have been demonstrated to enhance the establishment and severity of BCWD (Fredriksen *et al*.,2016; Nematollahi et al., 2003; Starliper, 2011). *F. psychrophilum*, the causative agent of BCWD, is currently regarded as the main disease-causing bacteria negatively affecting the production of salmonids globally (Duchaud *et al*., 2018).

F. psychrophilum has a wide host range and it has been isolated from both wild and cultured non-salmonid fish species including diseased eel (*Anguilla anguilla*) and Cyprinids (Lehmann *et al*., 1991), Pale chub (*Zacco platypus*) (Iida & Mizokami, 1996), Sea lamprey (*Petromyzon marinus* L.) (Elsayed *et al*., 2006), Ayu (*Plecoglossus altivelis*) (Izumi *et al*., 2003), Farmed pech (*Perca fluviatilis* L.) (Lonnstrçm *et al*., 2008), and others (Cipriano & Holt, 2005; Starliper, 2011). *F. psychrophilum* grows optimally at temperatures ranging from 15-18°C, but it can also grow at temperatures as low as 4°C (Bernardet & Bowman, 2006).

Coho salmon (*Onchorynchus kisutch*), Rainbow trout (*Onchorynchus mykiss*), Atlantic salmon (*Salmo salar*), and Ayu (*Plecoglossus altivelis*) seem to be more susceptible to infections caused by *F. psychrophilum* than other fish species (Dhar *et al*., 2014). The bacterium has been recovered from external sites (skin ulcers, and skin lesions), internal organs (spleen, liver, kidney, and the brain), and from milt, mucus, and ovarian fluid of infected fish (Ekman *et al*., 1999; Madsen & Dalsgaard, 2000; Van Vliet, 2016). The clinical signs of BCWD and the rate of mortality are dependent on the size and age of the affected fish (Nematollahi *et al*., 2003).

In rainbow trout fry weighing 2.0 g and less, epizootics of BCWD or RTFS (as it is named in fry) begin within the 6^{th} or 7^{th} week after the fry start feeding, resulting in about 60-90% mortality if left untreated. Affected fry swim at the surface of the water and show lethargy, inappetence, melanosis, exophthalmia, and abdominal distention due to ascites (Loch & Faisal, 2015). In rainbow trout fingerlings, signs of RTFS are frequently comparable with those of BCWD (Loch & Faisal, 2015), where the erosion of tissues, particularly involving the caudal peduncle or caudal fin (Fig 1.1a), and skin lesions (Fig 1.1b) are classic characteristics of BCWD (Barnes, 2011). A common sign of BCWD is the growth of an opaque deposit around the margin of the caudal fin, accompanied by progressive necrosis (Barnes, 2011).

1.1.2. Columnaris Disease

Flavobacterium columnare is the organism responsible for columnaris disease, and this bacterium is regarded as one of the most problematic and harmful disease-causing organisms within the freshwater finfish aquaculture industry (Bandilla *et al*., 2006; Suomalainen *et al*., 2005). The disease is named columnaris due to the column-like bacterial cells that are commonly seen in wet mounts or culture of tissue from infected fish (Bader & Starliper, 2002; Declercq *et al*., 2013). Columnaris is more prominent when fish are stressed by environmental factors, such as high rearing densities and water temperatures above 20°C (Suomalainen *et al*., 2005).

Clinical manifestations of the disease are amongst others mostly dependent on the virulence of the infecting strain, host factors such as age, and environmental factors such as water temperature. Strains with low virulence tend to induce infections that progress slowly at temperatures above 21°C, resulting in massive damage to the fish before death, whereas highly virulent strains cause little to no tissue damage before rapid death of the fish (Declercq *et al*., 2013). In young fish columnaris disease develops acutely severely damaging the gills of the infected fish, whilst in adult fish the disease might be acute, subacute, or chronic with yellowish arears slowly appearing on the gills, which eventually leads to gill necrosis and complete gill destruction (Bernardet & Bowman, 2006; Pacha & Ordal, 1967).

Various host stressors and predisposing factors including skin abrasions (Bader *et al*., 2003, 2006), feed deprivation (Shoemaker *et al*., 2003), static challenge water (Thomas-Jinu & Goodwin, 2004a), high stocking densities (Iguchi *et al*., 2003), and temperature stress (Suomalainen *et al*., 2005; Thomas-Jinu & Goodwin, 2004b) have been shown under experimental conditions to increase the susceptibility of fish to columnaris disease (Mohammed & Arias, 2014). Cells of *F. columnare* can be recovered externally from gills and lesions, and internal organs especially the kidneys of infected fish (Bader & Starliper, 2002). Lesions on the fins of infected fish usually become necrotic, whilst those on the skin develop a yellowish, mucoid material. Lesions also develop on the gills of infected fish causing subacute disease and mortality (Arias *et al*., 2004).

The gill lesions often start as epithelial proliferation and mucous cell hyperplasia, which blocks the spaces between adjacent lamellae. As the disease progress, congestion of the gill lamellae, inflammation, and oedema results in epithelial lifting, leading to lamellar fusion causing extensive hemorrhaging and circulatory failure, as well as necrosis (Loch & Faisal, 2015). The skin lesions differ according to fish species. In scaleless fish, such as catfish, the infection begins as small circular lesions with a blue-grey necrotic centre and a margin of red inflamed skin, caused by the infiltration of red cells. In scaled fish, lesions start at the edge of the fins and spread towards the body giving an appearance of a saddle, hence the name saddleback disease. The scales loosen and slough off as the integument disintegrates (Buller, 2014).

1.1.3. Bacterial Gill Disease

Flavobacterium branchiophilum is the main organism responsible for causing bacterial gill disease (BGD), even though other organisms such as *F. hydatis*, and *F. succinicans* have been recovered from the gills of fish displaying clinical signs of BGD (Good *et al*., 2015; Strohl & Tait, 1978). *F. branchiophilum* is considered not to cause systemic infections in fish since it is rarely isolated from the internal organs of fish (Sundell *et al*., 2014). BGD is marked by an accumulation of bacteria on the gills causing irritation of the gills, and gill clubbing impairing the respiratory function of the infected fish (Touchon *et a*l., 2011; Wakabayashi *et al*., 2009).

BGD outbreaks commonly occur at the beginning of summer with increasing water temperatures and when most hatcheries have a high number of fingerlings (Starliper, 2012). Environmental factors such as overcrowding, high levels of ammonia, and low oxygen levels are predisposing factors that enhance the colonization of the bacterium to the gills (Bullock, 1972). Fish affected by BGD become lethargic, swim near the surface, and display signs of inappetence (Meyer *et al*., 1983). Gross clinical signs include red swollen gills, dark body coloration, and congested eyes (Starliper, 2012; Swain *et al*., 2007). Bacterial gill disease affects numerous freshwater fish worldwide including rainbow or steelhead trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), black-spotted trout (*Oncorhynchus clarki lewisi*), Atlantic salmon (*Salmo salar*), chinook salmon (*O*. *tshawytscha*), yamame or masou salmon (*O*. *masou*), sockeye salmon (*O*. *nerka*), sheatfish or wels catfish (*Silurus glanis*) and goldfish (*Carassius auratus*) from countries such as Canada, Japan, Hungary, Korea, and the USA (Buller, 2014; Starliper, 2012).

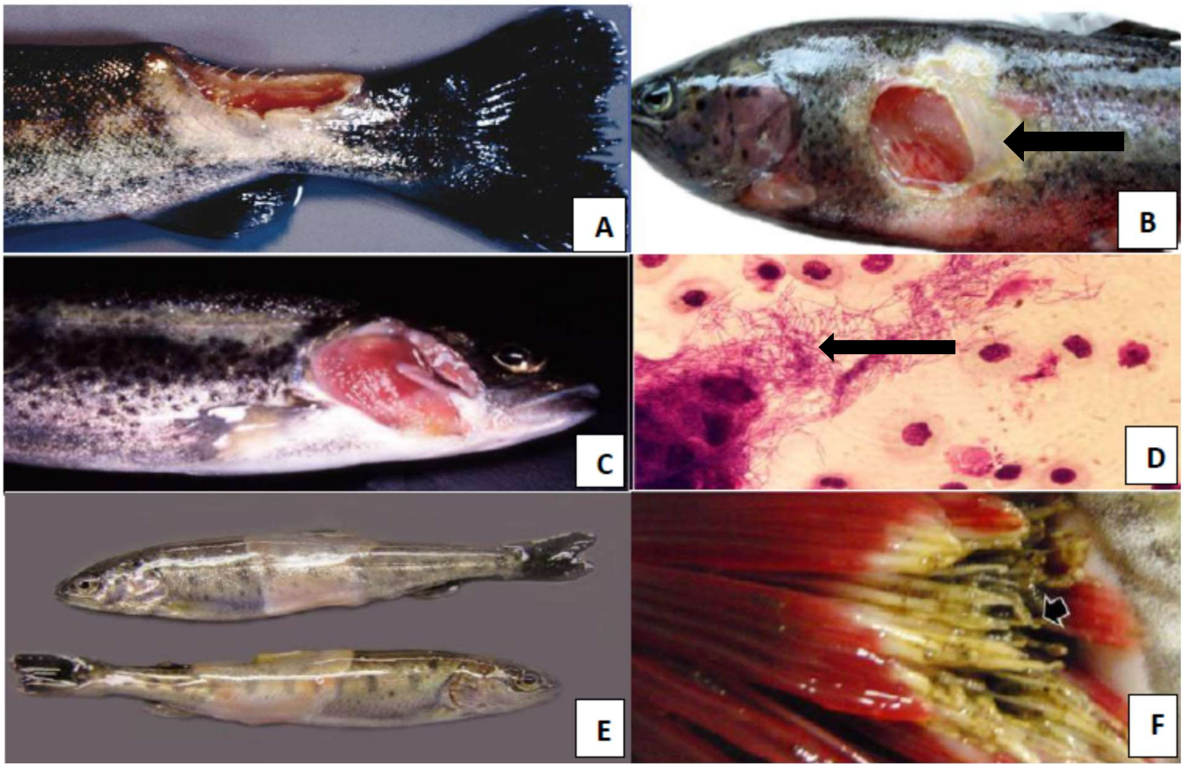

Figure 1.1 Common pathology displayed by rainbow trout infected by *Flavobacterium* species. **(a)** Erosion of skin and muscle tissues of the caudal peduncle caused by *F. psychrophilum* infection, leading to a deep ulcerative lesion that exposes the spinal cord. **(b)** Yellowish discoloration (Arrow) caused by grossly appreciable mats of the yellow-pigmented bacterium (*F. psychrophilum*) around a deep skin ulceration. **(c)** Yellow discoloration at the ventral portions of the gill arches, gill pallor, and gill mottling in a rainbow trout fingerling infected with *F. branchiophilum*. **(d)** Thin, long rods of (Arrow) *F. branchiophilum* on rainbow trout fingerling gills. **(e)** Saddle-back lesions on rainbow trout caused by *F. columnare*. **(f)** Acute necrosis of the gill filaments of a Chinook salmon due to infection with *F. columnare* (Note yellowish discoloration, arrow). *Adapted from;*(Loch & Faisal, 2015; Rodgers, 1997).

1.2. History and Taxonomy of *Flavobacteriaceae*

The family *Flavobacteriaceae* (Phylum Bacteroidetes) was proposed for the first time in 1985, and it was later reported in *Bergey's Manual of Systematic Bacteriology* in 1989 (Bernardet & Nakagawa, 2006). The family was later validated in 1992, and in 1996

Bernardet and colleagues published an emended description of the family and the type genus (*Flavobacterium*) (Bernardet *et al*., 1996; Bernardet & Bowman, 2006). *Flavobacteriaceae* as described by Bernardet *et al*. (1996) included the genera *Chryseobacterium*, *Bergeyella*, *Weeksella*, *Riemerella, Myroides,* and *Tenacibaculum* (Bernardet *et al.,* 1996*)*. In 2002, Bernardet and colleagues revised the description of the family *Flavobacteriaceae* and included "new" genera, which were previously excluded in the family, namely: *Cellulophaga*, *Coenonia*, *Psychroserpens,* and *Gelidibacter*, as well as *Polaribacter*, *Psychroflexus*, *Salegentibacte*r, and *Zobellia* (Bernardet *et al*., 2002).

The family *Flavobacteriaceae* belongs to the phylum Bacteroidetes, and it is the largest family within Bacteroidetes. Members of the family *Flavobacteriaceae* are Gram-negative, do not form endospores, and are rod-shaped chemoorganotrophs, with a predominant respiratory metabolism. Few members of *Flavobacteriaceae* are capnophilic and facultative anaerobes that prefer high levels of carbon dioxide and low levels of oxygen, most members are however aerobes (Mcbride, 2014). Members of *Flavobacteriaceae* vary in cell shapes, some cells are filamentous and flexible while others are spherical or coccoid (Bernardet & Nakagawa, 2006).

Most members of the family have a shining yellow to light yellow colony pigmentation that is caused by the presence of flexirubin and carotenoid pigments (Bernardet & Nakagawa, 2006). However, the colonies of members of certain genera, such as *Bergeyella*, *Coenonia*, *Ornithobacterium,* and *Weeksella, are* non-pigmented (Bernardet & Nakagawa, 2006). *Flavobacteriaceae* are widely distributed and besides diseased fish, they have also been recovered from dairy products and food processing plants (de Beer *et al*., 2005; Tsoeu *et al*., 2016), human cerebrospinal fluid (Holmes *et al*., 1986), marine agar from Antarctica (Bowman, 2000), and other sources such as hospital equipment, diseased frogs, diseased birds, and diseased plants (Bernardet & Nakagawa, 2006).

1.3. The genus *Flavobacterium*

1.3.1 History of the genus *Flavobacterium*

The genus *Flavobacterium* was established in 1923 to classify yellow-pigmented rods that were non-spore-forming, Gram-negative, with weak carbohydrate metabolism. The genus was poorly defined, which lead to many poorly defined bacterial species being included in the genus *Flavobacterium* (Bernardet *et al*., 1996). The genus was later revised in 1979 to include only aerobic, clinical, environmental, and food bacteria that were Gram-negative, chemoorganotrophic, non-motile, oxidase-, and catalase-positive, and produced yellow to orange colonies (Bernardet & Bowman, 2006).

Flavobacterium breve was proposed as the new type species of the genus to substitute *F. aquatile*; however, this proposition was rejected and *F. aquatile* remained the type species of the genus (Bernardet *et al*., 1996). Several bacteria that were originally allocated to the genus *Flavobacterium* were redefined and moved to other genera, which included amongst others *Cytophaga*, *Empedobacter*, and *Weeksella*. Another genus, *Chryseobacterium* was later proposed to include some of the bacteria that had been included in the genus *Flavobacterium* (Bernardet *et al*., 2006).

1.3.2. Characteristics of *Flavobacterium* species

Cells of *Flavobacterium* spp. are usually single, moderately curved, rod-shaped with spherical or slightly thin ends. Cells of some *Flavobacterium* spp. do form chains for example, cells of *F. denitrificans* have been reported to form chains of about 3-4 cells. Cell diameter is usually within the range of 0.3 to 0.5 µm, with cell length of 2 to 5 µm. However, cells of some *Flavobacterium* spp. such as *F. plurextorum* have been shown to be 0.7 µm wide in contrast to the 0.3 to 0.5 µm width range. Some species have rod-shaped cells measuring approximately 1µm in length, whilst other species have flexible, long cylindrical single cells that are 10 to 40 µm long (Bernardet & Bowman, 2015; Zamora *et al*., 2013).

The cells of *Flavobacterium* spp. are non-flagellated, gliding motility varies amongst cells of *Flavobacterium* spp. for example cells of *F. aquatile*, *F. columnare*, and *F. flevense*, displays gliding motility on Anacker and Ordal's agar. Whilst some *Flavobacterium* spp. such as *F. branchiophilum*, *F. granuli* and *F. limicola* displayed no gliding motility on the same agar (Bernardet & Bowman, 2006). No poly-β-hydroxybutyrate granules are present within cells. Colony morphology varies with the media used for culture; on solid nutrient-rich media some colonies are rounded, shiny with whole or rough edges; On nutrient poor solid media, nearly all species produce cells that are even, broad, sometimes sticky with irregular, rhizoid, or thin edges (Bernardet & Bowman, 2006).

The cells produce carotenoid and or flexirubin pigments which are responsible for the pale to bright yellow colony pigmentation. The majority of *Flavobacterium* spp. have a strictly aerobic, and respiratory metabolism that uses oxygen as the final electron acceptor (Bernardet & Bowman, 2015). The production of acid from carbohydrates varies amongst *Flavobacterium* spp. Some species including *F. aquatile*, *F. branchiophilum*, and *F. johnsoniae* produce acid from carbohydrates (Bernardet & Bowman, 2015). Whilst other *Flavobacterium* spp. including *F. psychrophilum*, *F. columnare*, and *F. limicola* are unable to produce acid from carbohydrates (Bernardet & Bowman, 2015). The degradation of other polymers such as gelatin, casein, starch, chitin, and carboxymethyl cellulose is also varied amongst *Flavobacterium* spp. (Bernardet & Bowman, 2006, 2015).

1.3.3. Habitat and Ecology

Flavobacterium spp. are found naturally in a broad range of ecosystems ranging from aquatic ecosystems such as freshwater lakes and rivers to seawater, polar lakes, and muddy soil (Bernardet & Bowman, 2006). Species such as *F. johnsoniae, F. flevense, F. saccharophilum*, and *F. limicola* are known to inhabit cool freshwater bodies such as rivers, lakes, and groundwater. While *F. psychrolimnae*, *F. micromati*, *F. hibernum*, and *F. xanthum* are commonly found in microbial mats and lakes in the Antarctic (Bernardet & Bowman, 2006). Some *Flavobacterium* spp. such as *F. granuli,* can grow at temperatures of about 37°C, others such as *F. indicum* can grow at higher temperatures of 40 to 45°C, even though members of this genus are commonly considered psychrophilic or psychrotolerant (Loch & Faisal, 2015). Optimum growth occurs at 20 to 30°C for most species, however, the psychrophilic members, such as *F. psychrophilum*, grow better at 15 to 20°C (Loch & Faisal, 2014).

Most *Flavobacterium* spp. are part of the chemoheterotrophic bacteria that mineralize organic compounds and polysaccharides in aquatic environments (Bernardet & Bowman, 2006). The three fish pathogens *F. psychrophilum*, *F. branchiophilum*, and *F. columnare* are commonly isolated from external sites, such as skin lesions, and internal organs of diseased fish, but are rarely recovered from water even though *F. psychrophilum* has been demonstrated to survive in water (Bernardet & Bowman, 2006, 2015; Madetoja *et al*., 2003).

1.4. Pathogenicity of *Flavobacterium* spp. in fish

1.4.1. Flavobacterium psychrophilum

F. psychrophilum was isolated for the first time from diseased salmonids in the United States of America (USA) in 1948, and the geographical distribution of the pathogen appeared to be limited to the USA and Canada (Bernardet & Bowman, 2006; Jarau *et al*., 2018; Starliper, 2011). Currently *F. psychrophilum* is thought to have a global distribution and it has been recovered from several countries including Chile, Japan, Turkey, United Kingdom, Italy, and Denmark (Bernardet & Bowman, 2006; Duchaud *et al*., 2018; Ngo *et al*., 2018; Saticioglu *et al*., 2019). While it is not clear how the pathogen spread worldwide, and its geographical origin is unknown, it has been suggested that *F. psychrophilum* might have spread globally as a result of the international trade of live fish, and fish eggs and gametes (Knupp *et al*., 2019; Nilsen *et al*., 2014). Salmonid fish that survived an infection caused by *F. psychrophilum*, and other fish species such as eels (*Anguilla anguilla*) that suffer moderate infections, are considered to be responsible for introducing the bacterium in rivers and subsequently to susceptible salmonids (Izumi *et al*., 2003; Madetoja *et a*l., 2000; Madsen & Dalsgaard, 2000). Dead, infected fish have also been shown to release high numbers of *F. psychrophilum* into the water which may infect other fish through disrupted skin tissue (Madetoja *et al*., 2000).

Several virulence factors, including adhesins, exotoxins, proteases, and endotoxins, contribute to the pathogenicity of *F. psychrophilum*. Adhesins facilitate the colonization and invasion of the host tissue by initiating or enhancing the attachment of the bacterium to host cells and tissue (Cipriano & Holt, 2005). *F. psychrophilum* cells have been shown to be enveloped by a thin loose, polysaccharide layer that has been suggested to facilitate motility and adhesion to host cells (Cipriano & Holt, 2005; Nematollahi *et al*., 2003). The polysaccharide layer is suggested to be involved in gliding by acting as an adhesin that allows bacterial cells to easily glide on the host tissue while keeping the cells attached to the surface. This behavior allows the bacterium to colonize specific tissue sites, and release toxins and protease enzymes that aid in causing disease and degrade host tissue (Dalsgaard, 1993).

F. psychrophilum has been shown to degrade chondroitin sulfate and hyaluronic acid, which forms part of the connective tissue of fish. The enzymes responsible for this degradation have been suggested to play a role in the severe skin and muscle necrosis that is common in infected fish (Bernardet & Bowman, 2006). The bacterium produces other extracellular enzymes that enhance virulence by degrading elastin, collagen, gelatin, fibronectin, and other important components of muscle and connective tissue (Bernardet & Bowman, 2006; Cipriano & Holt, 2005).

1.4.2. Flavobacterium columnare

Flavobacterium columnare has long been known as an important pathogen of different cultured, wild, and ornamental fish globally. The bacterium was originally isolated from diseased sockeye salmon (*Oncorhynchus nerka*) during an outbreak of columnaris disease in 1944 (Bernardet & Bowman, 2006; Declercq *et al*., 2013). This pathogen is broadly distributed worldwide and infects different fish species, including channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), perch (*Perca fluviatilis*), brook trout (*Salvelinus fontinalis*), Pacific salmon (*Oncorhynchus kisutch*), and others (Avendaño-Herrera *et al*., 2011; Declercq *et al*., 2013; Morley & Lewis, 2010; Řehulka & Minařík, 2007; Wagner *et al*., 2002). Genetic diversity has been demonstrated among *F. columnare* strains and they are commonly allocated into different genetic groups (LaFrentz *et al*., 2019; Triyanto & Wakabayashi, 1999). Isolates belonging to genetic group I have been associated with outbreaks in salmonid aquaculture, whilst different genetic groups are responsible for epidemics in warm water fish species, including catfish (*Clarius gariepinus*) (Li *et al*., 2017).

Flavobacterium columnare strains have been shown to produce extracellular protease with casein and gelatin degrading abilities, and these extracellular proteases have been suggested to work in concert with the chondroitin degrading enzyme (Chondroitin sulfate lyase) to produce the severe lesions caused by columnaris disease (Bernardet & Bowman, 2006; Newton *et al*., 1997; Suomalainen *et a*l., 2006). Adhesion of *F. columnare* cells to fish tissue has been demonstrated to be linked to the disease-causing ability of the bacterium. For example, Decostere *et al*. (1999) found that strains with high virulence attached easily and strongly to gill arches in contrast to those with low virulence. In another study, Bader *et al*. (2005) observed a decrease in virulence when fish were experimentally challenged with an *F. columnare* mutant that was adhesion defective. The attachment of *F. columnare* cells to tissue is facilitated and enhanced by a lectin-like part of the capsule, and cells with high virulence have a thick lectin-like layer, while those that are of low virulence have a thin layer of this component (Decostere *et al*., 1999).

1.4.3 *Flavobacterium branchiophilum*

Flavobacterium branchiophilum was first recognized as a pathogen of salmonids that suffered from gill disease in Japan (Bernardet & Bowman, 2006). This bacterium infects a variety of fish including rainbow trout, cyprinids, sheatfish, and silver carp. It has also been recovered from diseased fish in several countries, including Korea, Hungary, and Canada (Bernardet & Nakagawa, 2006; Ko & Heo, 1997; Ostland *et a*l., 1995). Virulent and non-virulent *F. branchiophilum* strains have been shown to readily attach themselves to the gills. However, only the virulent strains proceed to colonize the gills and cause disease (Ostland *et al*., 1995). *F. branchiophilum* produces extracellular products that include a protease, a phosphatase, and a phosphoamidase, and these products were able to produce gill lesions in an experimental infection involving young rainbow trout that resembled the lesions seen in *F. branchiophilum* natural infections (Ototake & Wakabayashi, 1985).

Analysis of the complete genome of *F. branchiophilum* strain FL-15 revealed various proteins that are suggested to play a key role in the virulence of the bacterium by enhancing adhesion, colonization, and disrupting osmoregulation by the gills (Touchon *et* *a*l., 2011). Fimbriae or pili on the cell surface of *F. branchiophilum* have also been suggested to enhance virulence by attaching the bacterium to gill epithelium followed by secretion of hemagglutinating extracellular enzymes that contribute to the fusion of the gill lamella leading to impairment of vital processes such as uptake of oxygen, and urea excretion (Sundell *et al*., 2014; Touchon *et al*., 2011).

1.5. The genus *Chryseobacterium*

1.5.1. History of the genus *Chryseobacterium*

The genus *Chryseobacterium* belongs to the family *Flavobacteriaceae*, phylum Bacteroidetes (Bernardet *et al*., 2015)*. Chryseobacterium* was proposed by Vandamme *et al*. (1994) to accommodate bacteria that were removed from the genus *Flavobacterium* (Bernardet & Nakagawa, 2006; Vandamme *et a*l., 1994). The newly proposed genus was made up of *C. balastinum, C. indologenes, C. scopthalmum, C. gleum, C. indoltheticum,* and *C. meningosepticum* all of which were previously members of *Flavobacterium* (Loch & Faisal, 2015; Vandamme *et al*., 1994)*.* The two earliest described members of the genus, *C. balustinum,* and *C. indoltheticum,* were not chosen as type species since their description was inadequate and they were both represented by a single strain. *C. gleum* was subsequently designated to be the type species of the genus since it was adequately characterized (Vandamme *et al.,* 1994*)*.

Phylogenetic analysis based on the 16S rRNA gene revealed that *C. meningosepticum* was distantly related to the other *Chryseobacterium* spp. and as a result, it was also not considered as a type species of the genus (Kämpfer *et al*., 2019; Vandamme *et al*., 1994). Based on phylogenetic analysis, both *C. meningosepticum* and *C. miricola* (Li *et al*., 2003) were assigned to a new genus *Elizabethkingia*, under the names *E. meningosepticum* and *E. miricola* (Charimba *et al*., 2012; Kim *et al*., 2005). Since its inception, the genus *Chryseobacterium* has been continuously growing, some of the new species that have been added since the description of the genus include *C. shigense* (Shimomura *et al*., 2005), *C. vrystaatense* (de Beer *et al*., 2005), *C. piscium* (de Beer *et al*., 2006), *C. oncorhynchi* (Zamora *et al*., 2012a), *C. ureilyticum* (Herzog *et al*., 2008), *C. viscerum* (Zamora *et al*., 2012b), and *C. pennipullorum* (Oosthuizen *et al*., 2019).

1.5.2. Characteristics of the genus *Chryseobacterium*

Chryseobacterium spp. stain Gram-negative, they display no motility, and the cells appear as single, straight rods with disc-shaped edges. The cells measure approximately 1 – 3 µm in length and 0.5 µm in width. However, the cells do vary in shape and size displaying pleomorphism (Bernardet *et al*., 2015). *Chryseobacterium* spp. colonies appear shiny on solid media, due to a characteristic yellow to orange pigmentation which is caused by flexirubin pigments (Bernardet *et al*., 2015). Several unpigmented and pale-yellow *Chryseobacterium* spp. have been described; *C. anthropi*, (Bernardet *et al*., 2015) *C. hominis* (Vaneechoutte *et al*., 2007), and *C. pallidum* (Herzog *et al.,* 2008). Colonies are disc-shaped, bulging, semi-transparent, and glossy with whole margins (Kämpfer *et al*., 2019). The presence of flagella and movement by gliding has not been reported in any of the *Chryseobacterium* spp. (Bernardet *et al*., 2015). *Chryseobacterium* spp. are chemoorganotrophic organisms, strictly aerobic, with a respiratory type of metabolism using oxygen as the terminal electron acceptor (Bernardet *et al*., 2015).

Most environmental isolates of *Chryseobacterium* spp. grow well at 5 °C, whilst other isolates grow well at 15 – 30 °C, and few others grow at higher temperatures of up to 37 °C, human clinical isolates do not grow at 5 °C but 15 – 37 °C (Bernardet *et al*., 2015). Most *Chryseobacterium* spp. do not grow at 42 °C, however, some *C. gleum* strains have been reported to grow at 42 °C (Bernardet & Nakagawa, 2006). *Chryseobacterium* spp. are easy to grow on routine growth media such as blood agar, tryptic soy agar (TSA), and different nutrient agars (NA), and most species grow on media with 2 – 5% sodium chloride content, such as Difco marine 2216 agar (Bernardet *et al*., 2006; Kämpfer *et al*., 2019). *Chryseobacterium* spp. test positive for catalase and oxidase, can oxidize carbohydrates, and hydrolyze esculin, but not agar. Production of proteolytic enzymes and high antibiotic resistance has been reported amongst *Chryseobacterium* spp. (Kämpfer *et a*l., 2019).

1.5.3. Habitat and Ecology

Chryseobacterium spp. inhabit various natural environments including rivers and freshwater lakes, soil from temperate, tropical, and polar regions, glaciers, and drinking water (Kämpfer *et al*., 2019). *Chryseobacterium* spp. were found to be amongst the bacteria recovered from groundwater in Germany (Ultee *et al*., 2004), whereas another study in South Africa identified *Chryseobacterium* spp. from treated and untreated drinking water (Bernardet *et al*., 2006; Pavlov *et al*., 2004). Other members of the genus have been isolated from food such as unpasteurized cow's milk (Hantsis-Zacharov *et al*., 2008), butter (Tsoeu *et al*., 2016), raw chicken (De Beer *et al*., 2005), and from a lactic acid beverage (Shimomura *et al*., 2005).

Other *Chryseobacterium* spp. have been recovered from industrial environments. For example, *C. defluvii* was recovered from wastewater of a sequence batch reactor (Kämpfer *et al*., 2003), whilst isolates of *C. soli* were amongst the bacterial community that formed biofilms in a paper mill machine in northern Germany (Zumsteg *et al*., 2017). *Chryseobacterium* spp. have also been isolated from apparently healthy freshwater and marine fish. For example, *C. piscum* was originally isolated from freshly caught marine fish in South Africa (de Beer et al., 2006; Kämpfer *et al*., 2019). Some *Chryseobacterium* spp. have been recovered in association with diseased fish and have been suggested as emerging pathogens of fish (Bernardet *et al*., 2005; Abraham *et al*., 2017; Loch & Faisal, 2015).

1.5.4 *Chryseobacterium* spp. as pathogens

Chryseobacterium spp. have been recovered from numerous diseased organisms that include soft ticks (Burešová *et al*., 2006), turtles (Hernandez-Divers *et al.*, 2009), bullfrogs (Mauel *et al*., 2002), and fish (Shahi *et al*., 2018). *Chryseobacterium indologenes* has been associated with human clinical cases including immunocompromised patients with pneumonia and bacteraemia (Shahul *et al*., 2014; Tsouvalas *et al*., 2020). An increasing number of *Chryseobacterium* spp. have been recovered in association with diseased fish, and some are pathogenic to fish, whilst others are regarded as emerging pathogens of both marine and freshwater fish (Abraham *et al*., 2017; Loch & Faisal, 2015; Zamora *et al*., 2012c).

Some of the *Chryseobacterium* spp. isolated from diseased fish include *C. indologenes*, isolated from diseased cultured yellow perch (*Perca flavescens*) displaying gross skin lesions below the dorsal fin and caudal peduncle (Pridgeon *et al*., 2013); *C. aahli*, isolated from lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*) (Loch & Faisal, 2014a); and *C. chaponense* from Atlantic salmon (*Salomo salar*) displaying external lesions (Ka *et al*., 2011). The majority of these *Chryseobacterium* spp. were able to produce disease related symptoms when used in experimental trials on apparently healthy fish. For example, when apparently healthy yellow perch were challenged with *C. indologenes* via intraperitoneal injection, the fish displayed inappetence and loss of balance as well as swelling above the anal fin; symptoms that were similar to those of fish naturally infected with *C. indologenes.*

Abraham *et al*. (2017) challenged fry of Pacu (*Piaractus brachypomus*) with a *Chryseobacterium* sp. PLI_2 isolate by abrasion-bath treatment and the fry displayed gross clinical signs of disease, such as lethargy, sluggish behaviour, erratic swimming, hanging, excessive mucus secretion, lesions on the caudal peduncle, opercular haemorrhages, scale loss, skin ulceration, skin discoloration, skin peeling, and pale and inflamed kidneys. Shahi *et al*. (2018) found that *C*. *scophthalmum* isolates from golden mahseer (*Tor putitora*) produced several extracellular enzymes such as hemolysin, DNase, gelatinase, and protease which are important for pathogenesis. It is possible that *Chryseobacterium* spp. could enhance their invasiveness in a host by using these extracellular proteins, which is a common trait amongst Gram-negative bacteria (Dumpala *et al*., 2010).

1.6. Diagnosis of flavobacterial diseases

Accurate and rapid diagnosis of disease-causing agents plays an important part in the management of diseases in aquaculture, as rapid diagnosis allows for appropriate action to be taken when the disease-causing pathogen is first detected (Adams & Thompson, 2011). Various diagnostic methods are available for the detection, isolation, and identification of pathogens in aquaculture. These methods include traditional methods such as culture, immunodiagnostic techniques such as the fluorescent antibody technique (FAT), and molecular methods based on PCR (Polymerase chain reaction) (Adams & Thompson, 2011).

1.6.1. Conventional methods

Conventional methods that rely on bacteriology, virology, parasitology, and mycology are extensively used by attending veterinarians for the detection and identification of pathogens (Adams & Thompson, 2008). Culture and histopathology are the most used techniques of conventional diagnostic methods. Culture methods rely on the inoculation of specimen samples on different media and incubation for a specific period and under specific conditions awaiting the growth of the pathogen. Differential and selective media are commonly used to enhance identification and presumptive diagnosis of the pathogen, and biochemical tests are often performed to further confirm and identify pathogens to a species level (Laupland & Valiquette, 2013).

Traditional methods such as culture are useful in that the causative organism is often isolated and its identity can be confirmed (Adams & Thompson, 2008). Culture techniques also allow for other important discriminatory analyses, such as antimicrobial susceptibility testing, to be performed. However, these methods can lack the specificity required to discriminate between certain species within a genus and may always not allow for the detection of intra-species or inter-species phenotype diversity. Moreover, these methods can be time consuming, especially for the detection of pathogens that are hard to culture and extremely slow-growing, such as *F. branchiophilum,* which requires up to 12 days for growth on culture media (Adams, 2004; Bernardet & Bowman, 2006).

Flavobacterium species are fastidious, slow-growing, and their growth is easily suppressed by other bacteria. As a result, *Flavobacterium* spp. are usually overgrown by other fast-growing bacteria, which may lead to these bacteria being overlooked during sample processing using culture based methods, resulting in incorrect diagnoses (Strepparava *et al*., 2012). While simple to perform, conventional culture techniques are usually slow, and labor-intensive, and they don't always lead to a conclusive identification of the pathogen (Adams, 2009).

In contrast to culture, histopathology is not focused on the isolation of the causative agent, but rather the microscopic examination of diseased animal tissue to look for pathological changes associated with a disease or a particular invading pathogen (Saukko & Pollak, 2013). The procedure normally involves fixing of collected tissue using a fixative, then embedding the fixed tissue in paraffin wax after which they are thinly sliced, stained with a dye then mounted on a glass slide to be observed under a microscope (Paul, 2017). Histopathology is advantageous in diagnosing diseases, especially in cases where the causative agents of the disease have not been identified or in cases where specific laboratory methods to detect the pathogen are yet to be developed (Adams & Thompson, 2008).

Histopathology also enables observation of the morphological changes of the infected organs that are caused by the invading pathogen. Unlike culture, histopathology can detect hard to culture and/ or slow-growing microorganisms (Gupta *et al*., 2009). It is often difficult to detect low levels of infections using histopathology, and in this regard extensions of histopathology, such as immunohistochemistry, are often employed (Gupta *et al*., 2009). Histopathology however lacks the sensitivity and specificity that is offered by other diagnostic techniques, such as the PCR based techniques (Kent *et al*., 2013).

1.6.2. Immunodiagnostics

Immunodiagnostic techniques such as the fluorescent antibody test (FAT), indirect fluorescent test (IFAT), immunohistochemistry (IHC), and enzyme linked immunosorbent assay (ELISA) are frequently used for diagnostic purposes in aquaculture. In contrast to traditional culture methods, immunological methods are rapid and can be performed without first isolating the pathogen (Adams & Thompson, 2008). Many immunodiagnostic tests uses polyclonal antibodies (PAbs) because they are less expensive, more stable under different reaction conditions (e.g. Salt concentration), and can be produced rapidly in comparison to monoclonal antibodies (MAbs) (Lipman *et al*., 2005). The heterogenous nature of PAbs enables them to recognize several antigenic epitopes as such they are less likely to be affected by small changes in the structure of an epitope resulting in an increased affinity for the antigen. However, this heterogeneity of PAbs often lead to cross reactivity with antigens of related pathogens (Adams, 2004; Lipman *et al*., 2005). For example, Faruk *et al*. (2002) developed a polyclonal rabbit anti-*F. psychrophilum* serum for the virulent strain *F. psychrophilum* B97026P1. Although the serum was able to detect different *F. psychrophilum* strains in an ELISA assay, it cross-reacted with *F. branchiophilum* and other fish pathogenic bacteria, such as *Aeromonas salmonicida*.

Both FAT and IFAT utilize fluorescent-labeled antibodies that are directed against specific target antigens of the pathogen (Odell & Cook, 2013). Fluorescent antibody technique uses antibodies that are labeled with fluorescence to bind directly to the target antigen, while IFAT uses an unlabeled primary antibody to bind to the antigen of interest, thereafter another labeled antibody directed against the primary antibody is used to detect the first antibody (Odell & Cook, 2013). Both techniques are commonly used for the detection of fish pathogens. For example, Lorenzen and Karas (1992) developed an IFAT based diagnostic tool that was used to screen fish tissues using serial dilutions of a rabbit serum raised against *F. psychrophilum.* Although the assay was able to detect *F. psychrophilum,* it was not highly specific as the rabbit serum used showed some cross-reactivity with *F. columnare* (Lorenzen & Karas, 1992).

In a separate study, Vatsos *et al*. (2002) developed an IFAT assay that was able to detect *F. psychrophilum* preserved in stream water for a month, demonstrating that the technique can be used to detect viable but non-culturable bacteria. Although both IFAT and FAT are rapid, and simple to perform, a fluorescent or confocal microscope is required to analyze the results and staff need to be trained to differentiate specific and non-specific staining to correctly interpret results (Adams & Thompson, 2011). Another problem associated with FAT for detecting *F. psychrophilum* is the low antibody reactivity displayed by some *F. psychrophilum* strains, and high cross-reactivity with other *Flavobacterium* species and bacteria belonging to other genera (Vatsos *et al*., 2002).

Immunohistochemistry, an extension of histology, is an easy diagnostic method that enables the pathology associated with a particular infection to be visualized using a light microscope (Kabiraj *et al*., 2015). Whilst the technique is useful for visualizing the localization of the pathogen in animal tissues, cross-reactivity of the anti-sera used in the technique is often a problem. For example, Evensen and Lorenzen (1996) developed an immunohistochemistry assay to detect *F. psychrophilum* in naturally infected and experimentally infected rainbow trout and although the assay was successful in detecting *F. psychrophilum* cells in both naturally and experimentally infected fish, the antisera used in the experiments cross-reacted with *Aeromonas salmonicida* (Evensen & Lorenzen, 1996). FAT is considered to be more sensitive than IHC, and to increase the sensitivity of the technique biotin-streptavidin is commonly used to amplify the signal of the target antigen (Adams & Thompson, 2011).

The enzyme-linked immunosorbent assay is regarded as the benchmark of immunodiagnostic techniques. The assay is versatile in that it can be used to detect and quantify either the antigen or antibody of interest (Alhajj & Farhana, 2021). During direct ELISA the antigen(s) of interest is fixed to a solid medium and linked with a primary antibody; the primary antibody is then linked with a reporter enzyme, which is used to measure the activity of the target antigen after incubation with a substrate (Walker, 1987). Sandwich ELISA is slightly different to direct ELISA in that the protein of interest is sandwiched between a capture antibody and the primary antibody (Walker & Crowther, 2009). The assay has been used in the detection of fish pathogens such as *F. psychrophilum*.

For example, Mata & Santos (2001) developed a sandwich ELISA assay that used whole-cell antigens and unabsorbed rabbit antisera to detect *F. psychrophilum* in fish tissue. The latter method was able to differentiate between *F. psychrophilum* serotypes without the need for antisera absorption and had a detection limit of about 1 x 10^4 cells.ml^{-1}. Lindstrom *et al*. (2009) also developed a quantitative ELISA that was able to detect *F. psychrophilum* in spiked and naturally infected kidney tissue samples of coho salmon (*Oncorhynchus kisutch*). This quantitative ELISA was, however, unable detect *F. psychrophilum* in the ovarian fluid of coho salmon (Lindstrom *et al*., 2009). ELISA has also been used successfully in monitoring bacterial kidney disease caused by *Renibacterium salmoninarum* in fish such as chinook salmon (*Oncorhynchus tshawytscha*) (O'Connor & Hoffnagle, 2007). Although immunodiagnostic methods are useful for the detection of pathogens in pure culture and infected fish tissues, their sensitivity threshold limits their use in environmental samples and in tissue samples where the pathogen levels are extremely low (Adams, 2004).

1.6.3 PCR-based diagnostic methods

The polymerase chain reaction (PCR) is widely used as a molecular diagnostic tool in the diagnoses of various diseases by researchers and clinical practitioners (Carriço *et al*., 2013). Variations of the original PCR technique have made it possible to directly detect pathogens in diverse environments such as water, soil, tissue, and food samples (Adams & Thompson, 2006). The use of PCR and/or its derivatives for the diagnoses of bacterial fish pathogens is on the rise in aquaculture, and several PCR based techniques have been developed for this purpose (Adams, 2009). PCR diagnostic methods have a better sensitivity threshold in comparison to other diagnostic methods, and PCR based methods are not affected by a change in protein expression, since they target nucleic acids rather than proteins as is the case in immunodiagnostic methods (Adams & Thompson, 2008; Morris *et al*., 2002).

Improvements on the standard PCR technique have made it possible to differentiate dead and live pathogens during diagnosis and to identify pathogens to species level (Adams & Thompson, 2008). For example, Wu *et al*. (2015) were able to differentiate between dead, viable non-culturable, and actively growing cells of *Vibrio cholerae* using propidium monoazide combined with quantitative PCR. This modified PCR technique relies on the treatment of the bacterial cells before DNA extraction/PCR with the intercalating dye propidium monoazide. The dye penetrates compromised membranes of dead bacterial cells and inhibits DNA amplification but has little effect on the DNA of membrane-intact live bacterial cells (Zhang *et al*., 2015).

Real-time PCR or quantitative PCR (qPCR) is widely used in the diagnosis of finfish diseases. This method is advantageous compared to standard PCR because there is no need for post PCR sample processing, eliminating the risk of post PCR sample contamination and false positives. Two of the most common qPCR methods use a TaqMan probe and SYBR green dye to detect and quantitate target DNA in real-time, both of which rely on the detection of fluorescence emitted during the PCR process (Adams & Thompson, 2012). Orieux *et al*. (2011) developed a qPCR assay using SYBR green I to detect *F. psychrophilum* from organ tissue of diseased rainbow trout, and the assay was able to detect about two bacterial cells per reaction using *F. psychrophilum*-specific primers that targeted a variable region within the 16S rRNA gene of the bacterium.

Toyama *et al*., (1994) developed a nested PCR technique that targeted the 16S rRNA gene of *F. psychrophilum*, and Izumi & Wakabayashi (1997) subsequently used the method to screen and detect *F. psychrophilum* from juvenile ayu and Coho salmon eggs (Manji, 2008). Recently, Lewin *et al*., (2020) developed a multiplex digital droplet PCR (ddPCR) that was used successfully to detect *F. psychrophilum* and *Yersinia ruckeri* (Causative agent of red mouth enteric disease in salmon) in water samples collected from salmon smolts aquaculture facilities in Norway. This assay was sensitive enough to detect between 1 – 240 gene copies. mL^{-1} sample water for *F. psychrophilum* and 1-40 gene copies. mL^{-1} sample for *Y. ruckeri* (Lewin *et al*., 2020). In another study, Misaka and colleagues developed a quantitative reverse transcriptase PCR (qRT-PCR) which was able to detect live *F. psychrophilum* cells by targeting the *gyr*B mRNA of the pathogen in kidney tissue samples of chum salmon (*Oncorhynchus keta*) fry during a BCWD outbreak in Japan (Misaka *et al*., 2020).

Other PCR-based techniques that have been used successfully to detect *Flavobacterium* spp. and other fish pathogens include a TaqMan based PCR assay for detection of *F. psychrophilum* (Del Cerro *et al*., 2002), a terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene (used to detect *F .psychrophilum*, *Aeromonas salmonicida* and *Renibacterium salmoninarum*) (Nilsson & Strom, 2002), and a multiplex PCR technique combined with DNA array hybridization used for the simultaneous detection of *F. branchiophilum*, *F. columnare* and *F. psychrophilum* (Lievens *et al*., 2011).

SYBR green qPCR is the most cost-effective and easy to use method in comparison to TaqMan qPCR because there is no need to design a specific probe as is the case with TaqMan qPCR. However, TaqMan is more specific since the probe will only bind to the target of interest during PCR, reducing the occurrence of false positives which may occur in SYBR green qPCR since the dye is not sequence-specific but rather binds to any double stranded DNA (Tajadini *et al*., 2014). The 16S rRNA gene is often targeted for the identification and discrimination of bacterial pathogens using PCR based techniques since 16S rRNA genes contain species-specific regions amongst bacterial species and due to its universal distribution among bacteria (Drancourt *et al*., 2000). The gene is also in high abundance in all bacterial species, which facilitates detection via 16S rRNA targeting PCR techniques.

Another PCR based technique that has been widely used is high resolution melt analysis (HRMA), a post PCR technique that is used to identify sequence variations within a DNA fragment of interest (Foroni *et al*., 2017). The melt analysis occurs after PCR amplification of the fragment of interest whereby the amplicon is denatured through small temperature increments (0.1 - 0.5 °C) and the fluorescence of the melting amplicon is closely monitored until complete melting of the amplicon is reached, at which point the fluorescence sharply drops (Foroni *et al*., 2017). The collected melt curve data of the various amplicons is then processed using a specific melt curve analysis software to generate unique melt curves that are based on the characteristic melt profile of each amplicon (Farrar & Wittwer, 2017; Wittwer *et al*., 2003).

HRMA exploits saturating fluorescent dyes that do not inhibit amplification and the differences in melt temperature amongst the amplicons caused by nucleotide differences between amplicons of the different microorganisms (Borer *et al*., 1974; Farrar & Wittwer, 2017). After normalization of fluorescence, a fluorescence difference plot is generated by plotting the normalized fluorescence of each amplicon against temperature. One melt curve is commonly chosen as a reference and the fluorescence difference between the other melt curves and the reference is plotted against temperature to generate a difference curve, which allows for differentiation of the amplicons (Farrar & Wittwer, 2017; Farrar *et al*., 2009).

To date, HRMA has not been used for the identification and differentiation of *Flavobacterium* spp., but this method has the potential to differentiate fish associated *Flavobacterium* spp. HRMA has been successfully used in the identification and differentiation of bacterial pathogens and other microorganisms. For example, Pugliese *et al*. (2017) used HRMA to differentiate between three distinct *Pseudo-nitzschia* spp. recovered in the Mediterranean Sea. In another study HRMA was used to identify bacterial strains of *Lactobacillus casei*, the technique enabled the researchers to further discriminate between the closely related *Lactobacillus* spp.; *L. casei*, *L. paracasei*, and *L. rhamnosus* (Iacumin *et al*., 2015). Robinson *et al*. (2006) used HRMA to identify amoebae belonging to the genus *Naegleria* and the technique was able to differentiate *N. fowleri, N. lovaniensis, N. italica, N. australiensis, N. gruberi, N. byersi, N. carteri*, and *Willaertia magna*.

Other studies have used HRMA to identify and differentiate *Cydia pomonella* granulovirus genotypes (Hinsberger *et al*., 2019), discriminate 10 ungulate species (Sika deer, Sambar deer, Red deer, Fallow deer, Rusa deer, Feral sheep, Alpine chamois, Himalayan tahr, Feral goat, and White-tailed deer) in New Zealand (Ramón-Laca *et al*., 2014), and to identify and discriminate 100 clinically important bacteria, including *Staphylococcus aureus*, *Listeria monocytogens*, and *Streptococcus agalactiae* (Yang *et al*., 2009). These studies and others (Cheng *et al*., 2006; Gago *et al*., 2011; Janavicius *et al*., 2010) demonstrate that high resolution melt analysis is a cost-effective, rapid, and sensitive technique that could be used to identify and discriminate various organisms, including bacteria.

High resolution melt analysis allows sensitive detection and identification of specific pathogens through analysis of their respective amplicons in a closed tube setting which minimizes sample contamination and experimental errors that are often introduced during post-processing steps (Winder *et al*., 2011). This in turn shortens the turn-around time of tests results, an important factor when dealing with an infectious disease outbreak such as BCWD. In contrast to 16S rRNA phylogeny, HRMA is not dependent on 16S rDNA sequencing of PCR amplicons, which is often time consuming, labor intensive, costly, and unsuitable for high-throughput systems. Sequencing is, however, sensitive, and accurate (Winder *et al*., 2011).

Because HMRA can detect even a single base pair difference within a sequence it offers an improved resolution, which is critical in differentiating closely related microorganism such as the *Flavobacterium* spp. (Reed *et al*., 2007). In contrast 16S rRNA gene phylogenetic analysis is often limited in providing resolution of closely related organisms (Peeters & Willems, 2011). The reliability of HRMA on melt profiles of DNA fragments however can also serve as a limitation since the melt profile of a fragment is a function of its GC content, length, and sequence. For example, it may be difficult for HRMA to detect a base change(s) that does not significantly affect the GC content of the fragment and using larger fragment sizes (>200bp) may interfere with the ability of the technique to detect variances (Tong & Giffard, 2012).

1.7. Management of bacterial diseases in aquaculture

1.7.1. Antibiotics

Antimicrobial treatment continues to remain one of the primary means of prevention and treatment of bacterial infections and for minimizing major economic losses caused by mass mortalities of aquaculture species. Antimicrobial agents are used intensively and often indiscriminately in aquaculture; in the United States of America alone about 68 kg of antimicrobial agents are used for every 420 kg of harvested salmon (FAO, 2005). The continued use of antibiotics in the treatment of infectious bacterial diseases in aquaculture is one of the key factors that has led to the emergence of bacterial fish pathogens with increased resistance to antimicrobial agents (FAO, 2005; Sekkin & Kum, 2011).

Increased resistance to commonly used antimicrobial agents, such as Oxolinic acid, by fish pathogens such as *A. salmonicida*, *E. tarda*, and *P. damselae* has been reported (Miranda *et al*., 2013). *F. psychrophilum* has been demonstrated to display patterns of increasing resistance to some of the commonly used antimicrobial agents that have been used for its treatment (Bruun *et al*., 2000). For example, in the study conducted by Bruun *et al*. (2000), 97% of the Danish *F. psychrophilum* isolates tested showed increased resistance to Oxolinic acid (OXO).

Another study by Saticioglu *et al*. (2019), which assessed the antimicrobial sensitivity of twenty-five Turkish *F. psychrophilum* isolates, reported that 88% of the isolates displayed lowered sensitivity to Oxolinic acid and Enrofloxacin (ENR); whilst 80% displayed reduced susceptibility to Oxytetracycline (OXY). Several antibiotics are approved for use in the treatment of trout and other salmonid bacterial infections in South African aquaculture, including Florfenicol, Sulfadimethoxine, Ormetoprim, Oxytetracycline-dihydrate, and Oxytetracycline-hydrochloride (Kutu *et al*., 2017).

1.8. Objectives of the study

In South Africa and Lesotho, numerous, yellow-pigmented bacteria have been isolated from rainbow trout displaying disease symptoms associated with infection by pathogenic *Flavobacterium* spp. Several *Flavobacterium* spp. are well-known pathogens of salmonids and are responsible for economically important diseases, such as bacterial cold-water disease (BCWD), rainbow trout fry syndrome (RTFS), and bacterial gill disease (BGD). These diseases have been associated with mass mortalities on salmonid farms globally and have caused major economic losses. The majority of *Flavobacterium* spp. are morphologically similar and hard to differentiate on routinely used isolation media (Chen *et al*., 2017; Loch & Faisal, 2014). Different strains can also vary in their pathogenicity, hence rapid and specific methods are required to discriminate against different species of fish associated flavobacteria and to inform effective treatment strategies.

Several other bacterial pathogens are also routinely isolated from disease trout in South Africa and Lesotho including, amongst others, *Streptococcus, Lactococcus, Aeromonas,* and *Vibrio* species (Kutu, 2017). Hence the first objective of this study was to (1) identify and characterize the bacteria that have been isolated from diseased rainbow trout in South Africa and neighboring Lesotho using genotypic and phylogenetic analysis. The preliminary identifications based on culture-based methods and some sequencing data suggest that many of the bacteria isolated from diseased fish in South Africa and Lesotho were *Flavobacterium* spp. If the DNA sequencing and phylogenetic analysis from objective one supported these preliminary findings, the second objective of the study was then to (2) develop a PCR-based method that will allow for rapid and accurate discrimination between the *Flavobacterium* spp. isolated from diseased trout.

2. Materials and Methods

2.1. Bacterial Isolation and phenotypic characterization

Ninety, mostly yellow-pigmented bacteria were isolated from diseased rainbow trout (*Oncorhynchus mykiss*) displaying gill necrosis, skin ulcers, and systemic disease, from ten freshwater fish farms in the Western cape, South Africa (n=7) and Lesotho (n=3) in 2018 (during February, March, April, July, and August). The bacteria were isolated from the head kidney, skin ulcers, and other lesions, gills, and eyes of diseased rainbow trout. The bacteria were isolated by the Wemmershoek Diagnostic Laboratory in Paarl, South Africa. Pure cultures of each bacterium were submitted to the molecular laboratory at the DEFF Marine Research Aquarium (MRA), where they were immediately sub-cultured onto *Flavobacterium psychrophilum* agar (FLPA) plates (Tryptone, 4.0 $g.l^{-1}$; yeast extract, 0.4 $g.l^{-1}$; $CaCl_2.2H_2O$, 0.2 $g.l^{-1}$; $MgSO_4.7H_2O$, 0.5 $g.l^{-1}$; Bacteriological Agar 10.0 $g.l^{-1}$) supplemented with 0.5 $g.l^{-1}$ D(+)-glucose (Cepeda *et al*., 2004). Bacterial cultures were incubated at 4 – 15 °C for 5 – 7 days. For long-term storage, stock cultures were stored in 20% (v/v) glycerol-supplemented tryptone soy agar (TSB) at -80 °C.

2.2. Genomic DNA extraction

Genomic DNA was extracted from each of the ninety isolates using a modified heat-lysis method (Dashti *et al*., 2009). Cells picked from a single colony of each bacterium were suspended in a mixture of 300 µl of Tris-EDTA buffer (TE, pH 7.6) in a 1.5 mL microcentrifuge tube containing 0.04 g Chelex® 100 sodium beads (Sigma Aldrich, CAS no: 11139-85-8) and lysed at 95 °C for 15 min (Chelex®-100 is comprised of negatively charged microscopic beads that chelate metal ions, which are required as catalysts or cofactors in enzymatic reactions). Following lysis, the cell suspension was cooled rapidly on ice for 5 min and centrifuged at 10,000 × *g* for 30 seconds. An aliquot of the supernatant (~100 µl), containing the bacterial genomic DNA, was transferred to a new sterile 1.5 ml microcentrifuge tube. The quality and concentration of DNA in each sample was evaluated using the Lasec® Jenway Genova Nano spectrophotometer (Genova Nano, Jenway., Bibby Scientific Ltd UK). All tubes containing isolated DNA were stored at -20 °C until needed for polymerase chain reaction (PCR) amplification.

2.3. DNA sequencing and molecular phylogeny

2.3.1. DNA sequencing

To amplify the small subunit (SSU) rRNA gene of each of the isolates the universal primers 16S F-fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S R-Rp2 (5'-ACGGCTACCTTGTTACGACTT-3') were used as forward and reverse primers, respectively (Weisburg *et al.*, 1991). PCR reaction mixtures of 25 µl each were prepared using 2 µl genomic DNA (~40 ng); 12.5 µl of 2 × Ampliqon master mix (Cat no: A140303), containing 0.2 U.µl^{-1} DNA Polymerase, 1.5 mM $MgCl_2$, and 0.4 mM of each dNTP; 200 nM of each primer; and 8.5 µl of PCR grade water. The Labnet Multigene™ thermal cycler (Labnet International Inc.) was used for PCR amplification and consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of DNA denaturation at 95 °C for 45 sec, annealing at 57 °C for 30 sec and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 10 min. A non-template control (PCR-grade H_2O instead of genomic DNA) was used as a negative control to ensure that there was no amplification except for the bacterial DNA.

The amplified PCR products and the negative control were electrophoresed (80 V for 30 min) on 0.8% (w/v) Tris-Acetate-EDTA (TAE) agarose gels containing ethidium bromide and the PCR products (~1.5 kb fragment expected) were visualized using UV illumination. A 1-kb ladder (Roche Applied Science, Indianapolis, IN, USA) was used as a molecular weight marker on each gel to verify reaction specificity and fragment size. The resulting PCR products were purified using a PCR purification kit (Roche) and the purified products were submitted to the Stellenbosch University Central DNA sequencing unit (Stellenbosch, Western Cape, South Africa) for DNA sequencing. Sequencing was conducted using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI3730xl Genetic Analyzer (Applied Biosystems) according to the sequencer manufacturer's instructions. Both the forward and reverse primers, 16S F-fD1 (1.1 µM) and 16S R-Rp2 (1.1 µM), were used for cycle sequencing.

The software CLC Main Workbench version 6.8.4 (Qiagen Bioinformatics) was used to edit and assemble the DNA sequences obtained for each isolate. Consensus sequences of approximately 1500 base pairs (bp) in length were obtained and saved in FASTA format. Percent similarity searches were carried out using the BLASTN algorithm (Altschul *et al.*, 1990) provided by the Internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/). To compare the isolates to published type sequence material in the GenBank database, the database search was limited to compare isolates with sequences from type material only and to exclude uncultured sample sequences.

2.3.2. Molecular phylogenetic analysis of *Flavobacterium* species

The 16S rRNA gene sequences of 45 study isolates that shared high sequence similarity with *Flavobacterium* spp. type strains were aligned using CLC Genomic Workbench version 6.8.4 (www.qiagenbioinformatics.com) with type strain sequences from fifty-eight (58) known *Flavobacterium* taxa (Table 2.1) downloaded from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Three outgroup species, *Weeksella virosa* (NR_074495.1), *Capnocytophaga ochracea* DSM 7271 (NR_027581.1), and *Croceibacter atlanticus* HTCC 2559 (NR_029064.1) were also downloaded from the GenBank database and used to anchor the phylogenetic tree. Following alignment, sequence overhangs were removed, and the alignment was exported into Phylip format for construction of maximum-likelihood (ML) trees using Molecular Evolutionary Genetics Analysis (MEGA; Version X) (Kumar *et al*., 2018).

The best possible model for ML phylogenetic analysis was assessed using the Smart Model selection (SMS v1.8.4) in PhyML (Lefort *et a*l., 2017). Briefly, the sequence alignment in Phylip format was uploaded on the PhyML online execution platform (http://www.atgc-montpellier.fr/phyml/). The following changes were made to the default settings: 1) DNA was selected as the data type; 2) to select substitution model the automatic model selection by SMS was chosen; 3) Akaike Information Criterion (AIC) was chosen as the selection criterion; 4) GTR (General time-reversible) was selected as the best optimal substitution model including invariable sites and discrete gamma distribution of 4 categories (GTR + G + I); 5) the proportion of invariable sites was estimated (0.480) and on the remaining sites a gamma distribution with a shape of 0.617 with four categories applied; 6) NNI (Nearest neighbour interchange) was selected as the type of tree improvement; 7) the initial tree was BioNJ (Neighbour-joining) (Gascuel, 1997); 8) aLRT SH-like (approximate likelihood ratio test and Shimodaira–Hasegawa) was selected for branch support (Anisimova *et al*., 2011). The reliability of the inferred phylogenetic trees was assessed using the bootstrap test (Felsenstein, 1985) with 1000 bootstrap re-samplings. Tree files were viewed and edited using Mega X (Kumar *et al*., 2018).

2.3.3. Phylogenetic analysis of *Chryseobacterium* species

Six *Chryseobacterium* 16S rDNA sequences determined in this study were aligned using CLC Genomic Workbench version 6.8.4 (www.qiagenbioinformatics.com) with seven known *Chryseobacterium* taxa (Table 2.1) downloaded from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Two outgroup species, *Weeksella virosa* (NR_074495.1*)*, and *Capnocytophaga ochracea* DSM 7271 (NR_027581.1) were also downloaded from the GenBank database and used to anchor the phylogenetic tree. Following multiple sequence alignment (MSA) the sequence overhangs were removed and the alignment was exported into Phylip format for construction of maximum-likelihood (ML) trees using the same conditions described above (2.3.2).

Table 2.1 *Flavobacterium and Chryseobacterium taxa used for the 16S rDNA phylogenetic analysis. The type strain sequences were downloaded from the GenBank database.*

***Flavobacterium* spp.**	**GenBank Accession number**
F. branchiarum strain 57B-2-09	NR 145954.1
F. branchiarum strain CECT 7908	MT760281.1
F. luteum strain P3160	MH100900.1
F. chryseum strain CCM 8826	MT760255.1
F. cupreum	MH100899.2
F. collinsii strain 983-08	NR 145952.1
F. aquidurense strain WB 1.1-56	NR 042470.1
F. aquidurense strain DSM 18293	NR 118475.1
F. saccharophilum strain NCIMB 2072	NR 115479.1
F. saccharophilum strain DSM 1811	AM230491.2
F. saccharophilum strain NBRC 15944	NR 112839.1
F. piscis strain 412R-09	NR 133746.1
F. hydatis strain NBRC 14958	NR 113710.1
F. hydatis strain DSM 2063	NR 114993.1
F. olei strain R-10-9	NR 157625.1
F. reichenbachii strain WB 3.2-61	NR 114983.1
F. pectinovorum strain DSM 6368	NR 114994.1
F. tructae strain 47B-3-09	HE774301.1
F. tructae strain 435-08	NR 133749.1
F. spartansii strain T16	NR 133748.1
F. hercynium strain WB 4.2-33	NR 042520.1

Table 2.1 Continued

F. chungangense strain CJ7	NR 044581.1
F. plurextorum strain 1126-1H-08	NR 133747.1
F. plurextorum strain JM59	MN758819.1
F. oncorhynchi strain 646B-08	FR870076.1
F. oncorhynchi strain 631-08	NR 117031.1
F. johnsoniae strain:IFO 15970	AB078043.1
F. johnsoniae strain UW101	NR 042496.1
F. glaciei strain 0499	NR 043891.1
F. granuli strain NBRC 102009	NR 114020.1
F. granuli strain Kw05	NR 041052.1
F. succinicans strain 8	NR 042498.1
F. tiangeerense strain 0563	NR 116159.1
F. branchiophilum strain BGD -7721	NR 104713.1
F. branchiophilum strain: QI14	AB871648.1
F. swingsii strain WB2.3-68	NR 115083.1
F. psychrophilum strain NBRC 100250	NR 113883.1
F. psychrophilum strain IFO 15942	NR 040914.1
F. psychrophilum strain NCIMB 1947	NR 117852.1
F. psychrophilum strain AQ Fpsy 001 2012 A4215	KC404865.1
F. pectinovorum strain NBRC 15945	NR 104717.1
F. branchiicola strain 59B-3-09	NR 145953.1
F. circumlabens	MH100898.2
F. chilense strain LM-09-Fp	NR 108512.1

Table 2.1 continued

F. psychrophilum strain FPCH6	MN653382.1
F. psychrophilum strain CH22	MT249244.1
F. psychrophilum strain CH14	MT249236.1
F. psychrophilum strain CH31	MT249253.1
F. psychrophilum strain CH18	MT249240.1
F. psychrophilum strain FPCH8	MN653384.1
F. psychrophilum strain FPCH6	MN653382.1
F. tegetincola strain A103	NR 044804.1
F. collinsii strain IMCC34522	MG456789.1
F. aquatile strain DSM 1132	NR 042495.1
F. columnare strain: ATCC 49513 (= JIP 49/87)	AB023660.1
***Chryseobacterium* spp.**	**GenBank Accession number**
C. ureilyticum VersM10.91	LN812942.1
C. indologenes LMG 8337	NR_042507.1
C. ureilyticum G186	JX100826.1
C. vrystaatense R-23566	NR_042370.1
C. balustinum LMG NBRC15053	NR_113721.1
C. tenax EP105	NR_041912.1
C. indoltheticum LMG 4025	NR_042926.1

2.4. High resolution melt analysis (HRMA)

2.4.1. *Flavobacterium* genus-specific primer design

The 16S rRNA gene region of the genus *Flavobacterium* was selected to design genus-specific real-time quantitative PCR primers. The almost complete 16S rRNA gene sequence of ten *Flavobacterium* species representing the *Flavobacterium* spp. most commonly isolated from diseased rainbow trout (Table 2.2) were downloaded from the GenBank database to find conserved regions for designing genus-specific primers. The primers would target conserved regions of the genus that flank a hypervariable region within the 16S rRNA gene that could be used as a target to differentiate between *Flavobacterium* spp. following real-time quantitative PCR, using high-resolution melt analysis (HRMA). The CLC Genomic Workbench version 6.8.4 software package was used to align the 16S rRNA gene sequences of the ten *Flavobacterium* spp. using the multiple sequence alignment option with the following settings: 1) gap open cost was set at 10.0; 2) gap extension cost at 1.0; 3) end gap cost set at free, and 4) the alignment stringency was set at very strong (slow). To visualize the base pair differences amongst the sequences identical residues were shown as dots. The hypervariable regions, with conserved flanking regions, were identified by eye.

The primers for the conserved flanking regions were then designed using the GenBank NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) Primer-Blast™ design tool (Ye *et al*., 2012) and standard criteria were utilized for the primer design (Bustin & Huggett, 2017; Lorenz, 2012). The 16S gene sequence of *F. psychrophilum* IFO 15942 (NR_040914.1) was used as the input sequence during primer design. The following changes were made to the default settings on Primer-Blast™: 1) forward primer range was set from position 92 to 115 of the input sequence; 2) reverse primer range was set from position 214 to 235; 3) PCR product size was set to a minimum of 70 bp and maximum of 300 bp; 4) the primer melting temperature (Tm) range was set between 55 °C and 65 °C, with an optimum Tm of 60 °C and a maximum Tm difference of 3 °C; and 5) the specificity check was enabled to search for primer pairs specific to the intended PCR template, and the specificity stringency was left at default settings allowing for two total mismatches and to ignore targets that have six or more mismatches.

Candidate primers were blasted against the GenBank database, using the Basic Local Alignment Search Tool (BLAST) analysis tool (National Center for Biotechnology Information) to ensure specificity of the primers to the genus *Flavobacterium* and to ensure that there is no cross-reactivity with any other bacterial species. The designed primers were synthesized by Integrated DNA Technologies (IDT) (www.idtdna.com).

Table 2.2 The GenBank accession numbers of *Flavobacterium* spp. commonly isolated from diseased rainbow trout, used for designing genus-specific primers to be used in the high resolution melt analysis (HRMA) following real-time quantitative PCR amplification.

***Flavobacterium* species**	**Accession number**
F. psychrophilum IFO 15942	NR_040914.1
F. branchiophilum BGD-7721	NR_104713.1
F. columnare ATCC 49512	DQ005508.1
F. aquatile DSM 1132	NR_042495.1
F. succinicans NBRC 14905	NR_113693.1

F. collinsii 983-08	NR_145952.1
F. tructae 435-08	NR_133749.1
F. hydatis DSM 2063	NR_114993.1
*F. johnsoniae UW*101	NR_042496.1
F. saccharophilum DSM 1811	AM230491.2
F. chilense LM-09-Fp	NR_108512.1

2.4.2. Optimization of real-time PCR cycling conditions

PCR cycling conditions for the newly designed SSU gene primers were optimized to identify optimal PCR cycling conditions for detection specificity. Genomic DNA of *F. psychrophilum* was used for the optimization step and was diluted using TE buffer to a concentration of ~30 ng. μL^{-1} for all subsequent optimization assays. DNA concentration was determined using the Lasec® Jenway Genova Nano spectrophotometer Genova NANO, Jenway, Bibby Scientific Ltd., UK). The PCR reactions were carried out in a 25 µl reaction volume containing ~30 ng (1 µl) of *F. psychrophilum* FPCH6 genomic DNA, 12.5 µl of 2× Bio-Rad Precision melt supermix (Cat no: 172-5110; containing dNTPs, iTaq™ DNA polymerase, $MgCl_2$, and EvaGreen dye), 200 nM of each primer, and 10.5 µl PCR grade water.

Amplification was monitored using a Bio-Rad CFX96™ real-time PCR detection system on a C1000™ thermal cycler and consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing for 45 s, elongation at 72 °C for 45 s, 70 cycles of 60 °C for 10 s with an increment of 0.5 °C $cycle^{-1}$. Data collection and real-time analysis occurred at the annealing step of each cycle, whereas melt curve data collection and analysis occurred at each increment in the latter 70 cycles. To determine the conditions giving the highest specificity the annealing temperatures (55 to 65 °C) and the PCR cycle numbers were varied.

2.4.3. Specificity of the SSU gene primers

To confirm the specificity of the designed 16S rRNA gene primers for the genus *Flavobacterium* and to test for cross-reactivity with other bacterial species, real-time quantitative PCR amplification was performed using genomic DNA isolated from pure cultures of eleven isolates recovered in this study that were closely related to 11 different *Flavobacterium* spp. (*F. psychrophilum* NBRC 100250*, F. olei* R-10-9*, F.chungangense* CJ27*, F.saccharophilum* DSM1811*, F. tructae* 47B-3-09*, F.cupreum, F.plurextorum* JM59*, F. collinsii* 983-08*, F.hercynium* WB.4.2-33*, F.circumlabens, and F.plurextorum* 112-1H-08), an isolate closely related to *Chryseobacterium indoltheticum* and other non-*Flavobacterium* bacteria (*Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas aeruginosa,* and *Vibrio anguillarum*).

The identity of *A. hydrophila*, *E. coli*, *P. aeruginosa*, and *V. anguillarum* were determined via 16S rRNA sequencing by colleagues at the molecular laboratory at the DEFF Marine Research Aquarium (MRA). A non-template control (PCR-grade H_2O instead of genomic DNA) was analyzed as a negative control in each reaction to ensure that there was no amplification except for the bacterial DNA. The specificity assay was performed using the optimized real-time PCR conditions described above.

2.4.4. Optimization of template DNA concentration for high resolution melt curve analysis

The genus specific primers designed in this study flank a highly variable region of the 16S rRNA gene of the genus *Flavobacterium*, producing a 146 bp (base pairs) amplicon that could be used to generate the discriminating melting curves following high-resolution melt-curve analysis (HRMA). Since the concentration of template DNA utilized during HRMA is critical in acquiring reproducible melt curves (Fortini *et al*., 2007), we prepared 10-fold serial dilutions of the genomic DNA (from working stocks of 30 ng.µl^{-1} of each test strain) obtained from the 11 isolates recovered in this study that were closely related to *Flavobacterium* spp. (*F. psychrophilum* NBRC 100250, *F. olei* R-10-9, *F.chungangense* CJ27, *F.saccharophilum* DSM1811, *F. tructae* 47B-3-09, *F.cupreum*, *F.plurextorum* JM59, *F. collinsii* 983-08, *F.hercynium* WB.4.2-33, *F.circumlabens,* and *F.plurextorum* 112-1H-08 identifications based on phylogenetic results of this study); it was prepared by adding 10 µl of genomic DNA to 90 µl of PCR-grade H_2O. Four dilutions (10^0 – 10^{-4}; representing a DNA concentration of 30 ng – 0.03 ng. µl^{-1}) were prepared for each genomic DNA sample.

The final real-time quantitative PCR assay was performed using the optimized cycling conditions described above (2.4.2) with the following adjustments: 3 µl of the template the diluted DNA was used for each reaction and melt curve data were collected at 75 to 85 °C with 0.2 °C increments. The raw melting curve data obtained from the Bio-Rad CFX96™ were processed by the Bio-Rad (www.bio-rad.com/hrm) Precision Melt Analysis ™ software (Catalog # 184-5020). The resulting melting curves were normalized to relative values of 100% (pre-melting phase) or 0% (post-melting phase) to eliminate differences in fluorescence intensity and background fluorescence between wells. Reactions were performed in quadruplicate for all isolates. A non-template control (PCR-grade H_2O instead of genomic DNA) was analyzed as a negative control in each reaction.

To visualize the curves the analysis option of the software were set as follows: 1) Auto detect melt region was turned off; 2) Pre-melt range was set at 75.0 to 75.2 °C; 3) Post-melt range was set at 85.0 to 85.2 °C; 4) Zoomed to melt region was turned on; 5) Temperature-shifted view turned off; 6) Temperature shift bar height was set at 0.20 °C; 7) Melt curve shape sensitivity for cluster detection was set at 50; and finally, 8) The Tm difference threshold for cluster detection was set at 0.15 °C.

3. Results

3.1. Bacterial isolation and characterization

Of the ninety bacterial isolates investigated in this study, forty-seven (47) were isolated from diseased rainbow trout on three farms in Lesotho (25 bacterial isolates from Farm A, 20 from farm B, and 2 from Farm C), whereas the remaining bacteria (n=43) were isolated from disease trout on seven farms in the Western Cape province of South Africa; Farm D (n=1 bacterial isolates), E (n=2), F (n=1), G (n=10), H (n=12), I (n=11), and J (n=6) (Table 3.1 below). The bacteria were isolated from a variety of infected tissues/organs, including the head kidney (n=43), gills (n=3), skin ulcers (n=10), skin lesions (n=18), liver (n=10), and the eyes (n=6) (Table 3.1). Although isolates were collected during summer (Feb), autumn (March, April, and May), and winter (July, and August) of 2018, most of the isolates were recovered from diseased fish during the winter period (~63%) and in autumn (~30%) when the water temperatures were lower, whereas the lowest number of bacteria were isolated during summer (~4%) (Fig. 3.1). A total of fifty isolates exhibited yellow colony pigmentation (Fig. 3.2), thirty-seven were orange, two were light orange, and one colony had a bright yellow pigmentation.

All ninety isolates were incubated at 15 °C on FLP media, only eighty-three of the bacterial isolates grew on FLP media at 15 °C (Table 3.1). Seven of the isolates did not grow at 15 °C on FLP and were incubated at 4 °C on FLP, only three of the seven isolates grew at 4 °C. The remaining four isolates that did not grow on FLP at both 4 °C and 15 °C were able to grow on TSA at 24 °C. Table 3.1 below provides detailed information on the isolation date, location, and growth media utilized for each isolate.

Table 3.1 Details of the ninety bacterial isolates obtained from diseased rainbow trout examined from farms in South Africa and Lesotho in this study, including the isolation date, the farm where the bacteria were isolated and its location, growth media used for the isolation, and incubation temperature for growth/isolation and colony pigmentation. The identification made by the veterinary diagnostic laboratory is also provided, together with the size of the edited 16S rRNA gene sequence obtained for each isolate and the top three type strain sequence similarities as obtained from the BLAST search of the GENBANK database. The percentage sequence similarity is provided in brackets.

Isolation date	**Farm and location**	**Isolates**	**Media and temperature**	**Organ**	**Colony pigment**	**Presumptive identification***	**16S rDNA size (bp)**	**Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)**
February 2018	Farm B, Lesotho	OM-8	FLP (4 °C)	Head kidney	Yellow	*Flavobacterium psychrophilum*	885	*Flavobacterium tructae* 435-08 (99.99%). *Flavobacterium* sp. P5626 (99.44%). *F. luteum* P3160 (99.32%).
		OM-12	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	868	*Flavobacterium* sp. strain AD-85 (99.66%). Uncultured bacterium clone 86_84 (99.40%). *Flavobacterium* sp. HL-20 (99.60%).
		OM-13	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	1381	*F. chryseum* (99.41%). *F. cupreum* (98.84%). *F. saccharophilum* NBRC 15944 (98.62%).
		OM-26	FLP (15 °C)	Head Kidney	Orange	*Flavobacterium* spp.	1394	*Chryseobacterium psychrotolerans* TSBY 57 (98.91%). *C. tenax* DSM 16811(98.85%). *C. ginsengiterrae* DCY 78 (98.21%).
March 2018	Farm I, WC, SA	OM-3	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1377	*F. psychrophilum* NBRC 100250 (99.56%). *F. psychrophilum* NCIMB 1947(99.56%). *F. psychrophilum* IFO 15942 (99.42%).
		OM-16	FLP (15 °C)	Head kidney	Light orange	*Flavobacterium* spp.	1381	*F. tructae* 435-08(99.71%). *F. spartansii* T16 (99.56%). *F. aquidurense* WB 1.1-56 (98.55%).

bp = Base pairs; FLP = *F. psychrophilum* media, WC = Western Cape, SA = South Africa, SSU= Small subunit, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
	Farm H, WC, SA	OM-39	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1384	*Flavobacterium luteum* P3160 (99.33%). *F. cupreum* (98.77%). *F. saccharophilum* NBRC 15944 (98.56%).
March 2018	Farm B, Lesotho	OM-15	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	ND	ND
		OM-9	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	ND	ND
		OM-17	FLP (15 °C)	Head kidney	Light orange	*Flavobacterium* spp.	1378	*F. tructae* 435-08 (99.78%). *F. spartansii* T16 (99.49%). *F. aquidurense* WB 1.1-56 (98.62%)
		OM-18	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	ND	ND
		OM-19	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	573	*Brevundimonas* sp. 2F40 (100%). uncultured bacterium (100%). uncultured bacterium (100%).
		OM-20	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	(500)^{F}	*B. staleyi* M0438 (100%). *Brevundimonas* sp. C3502 (100%). *Brevundimonas* sp. Tibet-IK53 (100%).
		OM-21	FLP (4 °C)	Head kidney	Orange	*F.psychrophilum*	546	*Mycoplana* sp. CB2015-107-DE_0018 (100%). *B. staleyi* HMF4667 (100%). *Brevundimonas* sp. strain R-10-15 (100%).
		OM-22	FLP (4 °C)	Head kidney	Orange	*F.psychrophilum*	550^{R}	*Brevundimonas sp.* R-10-10 (100%). Uncultured *Brevundimonas* sp. clone 306 (100%). Uncultured bacterium cloneXZ7_P8 (100%).

ND= BLAST search not done due to poor DNA sequence data, FLP= *F. psychrophilum* media, ^{R} = Only reverse sequence available (Poor DNA sequence data), ^{F} = only forward sequence available due to Poor DNA sequence data), bp = base pairs, SSU= Small subunit, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
March 2018	Farm B, Lesotho	OM-1	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1400	*Massilia aurea strain* EWF2 (99.54%). *M. aurea* strain b3 (99.54%). Unidentified microorganism clone xplvp464r2a2027 (99.54%).
April 2018		OM-6	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	880	*M. aurea* b3 (99.80%). *Massilia* sp. B2 (99.60%). *M. aurea* SJL26 (99.60%).
		OM-14	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	1320	*Brevundimonas* sp. R-10-10 (100%). *Brevundimonas* sp. clone *306 (100%).* Uncultured bacterium cloneXZ7_P8 (100%).
		OM-27	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	889	*Massilia* sp. B2 (99.85%). *M. aurea* SJL26 (99.85%). Uncultured bacterium clone BJ201305-115 (99.85%).
		0M-28	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	874	*Flavobacterium psychrophilum* NBRC 100250 (100%). *F. psychrophilum* NCIMB 1947(100%). *F. psychrophilum* IFO 15942 (99.89%).
		OM-29	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1400	*Massilia* sp. 9B11b1 (100%). Uncultured bacterium clone B5-59 (100%). Beta *proteobacterium* Wuba26 (100%).
		OM-30	FLP (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	1384	*F. psychrophilum* NBRC 100250 (100%). *F. psychrophilum* NCIMB 1947(100%). *F. psychrophilum* IFO 15942 (99.89%).

FLP= *F. psychrophilum* media, bp= Base pairs, SSU= Small subunit, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
April 2018	Farm B, Lesotho	OM-31	FLPA (15 °C)	Head kidney	Orange	*Flavobacterium* spp.	887	*Flavobacterium psychrophilum* NBRC 100250 (99.89%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
	Farm J, WC, SA	OM-32	TSA (24 °C)	Head kidney	Yellow	*Flavobacterium* spp.	905	*Hafnia* sp. strain A1 (99.63%). *H. alvei* strain snnu 13 (99.63%). *H. alvei* strain 3.8.4E (99.63%).
		OM-33	TSA (24 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1411	*Aeromonas* sp. Y34A (100%). *Aeromonas* sp. Rem-amp251 (99.93%). Uncultured *Aeromonas* sp. Clone em.412 (99.93%).
		OM-34	TSA (24 °C)	Head kidney	Yellow	*Flavobacterium* spp.	988	*Hafnia* sp. 6B1 (99.36%). *H. alvei* 12D5 (99.36%). *Enterobacteriaceae* bacterium Pokym2-a (99.36%).
		OM-35	TSA (24 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1407	*Hafnia* sp. A1 (99.63%). *H. alvei* snnu 13 (99.63%). *H. alvei* 3.8.4E (99.63%).
		OM-36	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	889	*Enterobacteriaceae* bacterium Pokym2-a (99.36%). *H. alvei* M-T-MRS 56 (99.36%). *H. alvei* M-T-MRS 55 (99.36%).
		OM-37	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	980	*Enterobacteriaceae* bacterium Pokym2-a (99.36%). *H. alvei* M-T-MRS 56 (99.36%). *H. alvei* M-T-MRS 55 (99.36%).

bp= base pairs, WC= Western Cape, SA= South Africa, TSA= Tryptone soy agar, SSU= Small subunit, ND= Blast search not done due to poor DNA sequence data, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
April 2018	Farm A, Lesotho	OM-38	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	ND	ND
		OM-88	FLP (15 °C)	Head kidney	*Yellow*	*Flavobacterium* spp.	ND	ND
		OM-89	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	ND	ND
		OM-90	FLP# (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	ND	ND
May 2018	Farm I, WC, SA	OM-80	FLP (15 °C)	Skin lesion	Yellow	*Flavobacterium* spp.	ND	ND
July 2018	Farm H, WC, SA	OM-40	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1390	*Chryseobacterium vrystaatense* R-23566 (99.21%). *C.shigense* DSM 17126 (98.42%). *C. carnipullorum* 9_R23581(98.27%).
		OM-41	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	884	*Flavobacterium psychrophilum* NBRC 100250 (99.77%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
		OM-42	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1331	*C. indoltheticum* LMG 4025 (99.70%). *C. mulctrae* (99.02%). *C. cucumeris* GSE06 (98.86%).
		OM-43	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	792	*F. granuli* NBRC 102009 (98.40%). *F. granuli* strain Kw05 (98.18%). *F. branchiarum* strain 57B-2-09 (97.68%).
		OM-44	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	880	*F. branchiarum* 57B-2-09 (99.43%). *F. hydatis* NBRC 14958(99.21%). *F. hydatis* DSM 2063 (99.21%).

bp = base pairs, WC= Western Cape, SA = South Africa, ND = BLAST search not done due to poor DNA sequence data, FLP = *F. psychrophilum* media, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
July 2018	Farm H, WC, SA	OM-45	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	881	*Flavobacterium plurextorum* 1126-1H-08 (99.77%) *F. oncorhynchi* 631-08(99.55%) *Flavobacterium* sp. P5626(99.43%)
		OM-46	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	500[F]	*F. psychrophilum* AQ_Fpsy_001_2012_A4215 (99.80%). *F. psychrophilum* Fp-D (99.80%). *F. psychrophilum* Fp-C (99.80%).
		OM-47	FLP (15 °C)	Head kidney	Yellow	*Flavobacterium* spp.	1381	*F. plurextorum* 1126-1H-08 (99.86%) *F. oncorhynchi* 631-08(98.63%) *F. tructae* 435-08(98.43%)
August 2018	Farm E, WC, SA	OM-2	FLP (15°C)	Head kidney	yellow	*Flavobacterium* spp.	ND	ND
	Farm E, WC, SA	OM-4	FLP (15 °C)	Head kidney	Bright Yellow	*Flavobacterium* spp.	1380	*F. tructae* 435-08(99.78%) *F. spartansii* T16 (99.49%) *F. aquidurense* WB 1.1-56 (98.46%)
	Farm D, WC, SA	OM-78	FLP (15 °C)	Gills	Yellow	*Flavobacterium* spp.	889	*C.vrystaatense* R-23566 (99.89%) *C.bernardetii* G229 (98.65%) *C.oleae* CT348 (98.65%).
	Farm J, WC, SA	OM-5	FLP (15 °C)	Skin lesion	Yellow	*Flavobacterium* spp.	ND	ND
	Farm C, Lesotho	OM-79	FLP (15 °C)	Skin lesion	Yellow	*Flavobacterium* spp.	890	*F. psychrophilum* NCIMB 1947(99.89%) *F. psychrophilum* NBRC 100250 (99.77%) *F. psychrophilum* IFO 159429(99.77%)

bp= base pairs, WC = Wester Cape, SA = South Africa, FLP = *F.psychrophilum* media , ND= BLAST search not done due to poor sequence data, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
August 2018	Farm C, Lesotho	OM-76	FLP (15 °C)	Skin lesion	Yellow	*Flavobacterium* spp.	882	*Flavobacterium psychrophilum* NBRC 100250 (99.89%) *F. psychrophilum* NCIMB 1947(99.89%) *F. psychrophilum* IFO 15942 (99.89%)
	Farm H, WC, SA	OM-54	FLP (15 °C)	Liver	Yellow	*Flavobacterium* spp.	882	Uncultured bacterium clone SBfYyy35 (99.89%). Uncultured bacterium clone DUP6C10 (99.89%). Uncultured bacterium clone SINN459 (99.89%).
		OM-55	FLP (15 °C)	Liver	Yellow	*Flavobacterium* spp.	940	Uncultured bacterium clone TW5_79 (99.60%). Uncultured bacterium clone: RBC2-64 (99.60%). Uncultured bacterium clone: MK12 (99.60%).
		OM-77	FLP (15 °C)	Gills	Yellow	*Flavobacterium* spp.	867	*F. saccharophilum* DSM 1811 (100%) *F. saccharophilum* NBRC 15944 (100%) *F. collinsii* 983-08 (99.77%)
	Farm I, WC, SA	OM-56	FLP (15 °C)	Liver	Orange	*Flavobacterium* spp.	1379	*Flavobacterium* sp. P5626 (98.84%) *Flavobacterium* sp. P2683 (98.63%) *F. piscis* 412R-09 (98.55%)
		OM-57	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	1381	*Flavobacterium* sp. P5626 (98.84%) *Flavobacterium* sp. P2683 (98.63%) *F. piscis* 412R-09 (98.55%)
		OM-58	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	886	*Chryseobacterium lactis* KC1864 (99.32%) *C.jejuense* JS17-8(99.32%) *C.rhizosphaerae* RSB3-1(99.32%)
		OM-59	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	1384	*Flavobacterium* sp. P5626 (98.84%) *Flavobacterium* sp. P2683 (98.63%) *F. piscis* 412R-09 (98.55%)

bp= base pairs, WC = Western Cape, SA = South Africa, FLP = *F. psychrophilum* media, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
August 2018	Farm I, WC, SA	OM-60	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	879	*Flavobacterium collinsii* 983-08 (99.89%) *F. aquidurense* WB 1.1-56(99.89%) *F. saccharophilum* DSM 1811 (99.66%)
		OM-61	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	884	*F. collinsii* 983-08 (99.89%) *F. aquidurense* WB 1.1-56(99.89%) *F. saccharophilum* DSM 1811 (99.66%)
		OM-62	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	1398	*Flavobacterium* sp. P5626 (98.84%) *Flavobacterium* sp. P2683 (98.63%) *F. piscis* 412R-09 (98.55%)
		OM-63	FLPA (15 °C)	Liver	Orange	*Flavobacterium* spp.	885	*F. collinsii* 983-08 (99.89%) *F. aquidurense* WB 1.1-56(99.89%) *F. saccharophilum* DSM 1811 (99.66%)
	Farm G, WC, SA	OM-48	FLP (15 °C)	Skin Ulcers	yellow	*Flavobacterium* spp.	883	*F. psychrophilum* NBRC 100250 (99.77%) *F. psychrophilum* NCIMB 1947(99.77%) *F. psychrophilum* IFO 15942 (99.77%)
		OM-49	FLP (15 °C)	Skin Ulcers	yellow	*Flavobacterium* spp.	883	*F. psychrophilum* NBRC 100250 (99.77%) *F. psychrophilum* NCIMB 1947(99.77%) *F. psychrophilum* IFO 15942 (99.77%)
		OM-50	FLP (15 °C)	Skin Ulcers	yellow	*Flavobacterium* spp.	1380	*Flavobacterium* sp. P5626 (98.84%) *Flavobacterium* sp. P2683 (98.63%) *F. piscis* 412R-09 (98.55%)
		OM-51	FLP (15 °C)	Skin ulcers	yellow	*Flavobacterium* spp.	884	*Flavobacterium collinsii* 983-08 (99.77%) *Flavobacterium aquidurense* WB 1.1-56(99.77%) *Flavobacterium* sp. P2683(99.66%)
		OM-52	FLP (15 °C)	Skin ulcers	yellow	*Flavobacterium* spp.	882	*Aquitalea denitrificans* NY-09 (100%). *Aquitalea* sp. isolate: 17Y29 (100%). *A. magnusonii* PT109 (100%).

bp= base pairs, WC = Western Cape, SA = South Africa, FLP = *F.psychrophilum* media, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
August 2018	Farm G, WC, SA	OM-53	FLP (15 °C)	Skin ulcers	yellow	*Flavobacterium* spp.	886	*Flavobacterium psychrophilum* NBRC 100250 (99.77%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
		OM-67	FLP (15 °C)	Skin ulcers	Orange	*Flavobacterium* spp.	872	*F. psychrophilum* NBRC 100250 (100%). *F. psychrophilum* NCIMB 1947(99.89%). *F. psychrophilum* IFO 15942 (99.89%).
		OM-68	FLP (15 °C)	Skin ulcers	Orange	*Flavobacterium* spp.	887	*F. psychrophilum* NBRC 100250 (99.89%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
		OM-69	FLP (15 °C)	Skin ulcers	Orange	*Flavobacterium* spp.	886	*F. psychrophilum* NBRC 100250 (99.89%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
		OM-70	FLP (15 °C)	Skin ulcers	Orange	*Flavobacterium* spp.	880	*F. psychrophilum* NBRC 100250 (99.89%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
August 2018	Farm A, Lesotho	OM-64	FLPA (15 °C)	Lesion	Orange	*Flavobacterium* spp.	858	*F. olei* R-10-9 (99.30%). *F. saccharophilum* DSM 1811 (99.30%). *F. pectinovorum* NBRC 15945(99.30%).
		OM-65	FLP (15 °C)	Lesion	Orange	*Flavobacterium* spp.	879	*F. collinsii* 983-08 (100%). *F. aquidurense* WB 1.1-56(100%). *F. saccharophilum* DSM 1811 (99.77%).
		OM-66	FLP (15 °C)	Lesion	yellow	*Flavobacterium* spp.	ND	ND

bp= base pairs, WC= Western Cape, SA= South Africa, FLP= *F. psychrophilum* media, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
August 2018	Farm A, Lesotho	OM-23	FLP (15 °C)	Lesion	yellow	*Flavobacterium* spp.	1337	Uncultured bacterium clone EMIRGE_OTU_s1t2b_5552 (91.14%). Uncultured marine bacterium clone Station2_7_09 (89.42%). Uncultured bacterium clone MABRDTU36 (88.72%).
		OM-24	FLPA (15 °C)	Lesion	yellow	*Flavobacterium* spp.	868	*Flavobacterium psychrophilum* NBRC 100250 (99.77%). *F. psychrophilum* NCIMB 1947(99.77%). *F. psychrophilum* IFO 15942 (99.77%).
		OM-25	FLPA (15 °C)	Lesion	yellow	*Flavobacterium* spp.	899	Uncultured bacterium clone BJ201305-115 (99.89%); Uncultured bacterium clone BJ201305-50 (99.89%). Uncultured bacterium clone HL201309-96 (99.89%).
		OM-10	FLPA (15 °C)	Lesion	yellow	*Flavobacterium* spp.	1380	*Flavobacterium psychrophilum* NCIMB 1947 (99.49%). *F. psychrophilum* NBRC 100250 (99.35%). *F. psychrophilum* IFO 159429 (99.35%).
		OM-11	FLPA (15 °C)	Lesion	yellow	*Flavobacterium* spp.	1380	*Paracoccus* sp. P33 (100%). *P. marcusii* YT59 (100%). *P. marcusii* YT19 (100%).
		OM-7	FLP (15 °C)	Lesion	yellow	*Flavobacterium* spp.	ND	ND
		OM-71	FLP (15 °C)	Lesion	Orange	*Flavobacterium* spp.	ND	ND

bp= base pairs, FLP = *F. psychrophilum* media, ND= BLAST search not done due to poor sequence data, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
August 2018	Farm A, Lesotho	OM-72	FLP (15 °C)	Lesion	Orange	*Flavobacterium* spp.	886	*Massilia* sp. 185-1 (100%). *Massilia* sp. BAV3628 (100%). Uncultured bacterium clone BJ201305-85 (100%).
		OM-73	FLP (15 °C)	Lesion	Orange	*Flavobacterium* spp.	871	*Flavobacterium saccharophilum* DSM 1811 (99.31%) *F. pectinovorum* NBRC 15945 (99.31%) *F. saccharophilum* NBRC 15944 (99.31%)
		OM-74	FLP (15 °C)	Lesion	Orange	*Flavobacterium* spp.	883	*F. saccharophilum* DSM 1811 (99.66%) *F. saccharophilum* NBRC 15944 (99.66%) *F. hydatis* NBRC 14958 (99.55%)
		OM-75	FLP (15 °C)	Lesion	Orange	*Flavobacterium* spp.	890	*Chryseobacterium lactis* KC1864 (99.33%) *C.jejuense* JS17-8 (99.33%) *C.rhizosphaerae* (99.33%)
		OM-81	FLP (15°C)	Eyes	Orange	*Flavobacterium* spp.	1377	*F. psychrophilum* NCIMB 1947(99.49%) *F. psychrophilum* NBRC 100250 (99.42%) *F. psychrophilum* IFO 159429 (99.42%)
		OM-82	FLP (15°C)	Eyes	Orange	*Flavobacterium* spp.	1363	Uncultured *Flavobacterium* sp. clone V31 (99.93%). *Flavobacterium* sp. III-082-7 (99.85%). *F. saccharophilum* 2-2R (99.56%).
		OM-83	FLP (15°C)	Eyes	Orange	*Flavobacterium* spp.	1379	*F. branchiarum* CECT 7908 (98.96%) *F. branchiarum* 57B-2-09 (98.91%) *F. pectinovorum* DSM 6368 (98.19%)
		OM-84	FLP (15°C)	Eyes	Orange	*Flavobacterium* spp.	1380	*F. saccharophilum* NBRC 15944 (98.91%) *F. piscis* 412R-09 (98.77%) *F. chilense* LM-09-Fp (99.62%)

bp= base pairs, FLP = *F. psychrophilum* media, ND= BLAST search not done due to poor sequence data, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

Table 3.1 Continued

Isolation date	Farm and location	Isolates	Media and temperature	Organ	Colony pigment	Presumptive identification*	16S rDNA size (bp)	Top 3 type strain sequence similarities from BLAST search of the GenBank database (% identity shown in brackets)
		OM-85	FLP (15 °C)	Eyes	Orange	*Flavobacterium* spp.	1314	*Paracoccus* sp. strain P33 (100%). *P. marcusii* YT59 (100%). *P. marcusii* YT19 (100%).
August 2018	Farm A, Lesotho	OM-86	FLP (15 °C)	Gills	Orange	*Flavobacterium* spp.	1379	*Flavobacterium chungangense* CJ7 (98.55%). *F. hydatis* NBRC 14958 (98.42%). *F. hydatis* DSM 2063 (98.42%).
		OM-87	FLP (15 °C)	Eyes	Orange	*Flavobacterium* spp.	1379	*Flavobacterium* sp. CIP 105534 (98.53%). *F. tiangeerense* 0563 (98.34%). *Flavobacterium granuli* NBRC 102009 (97.97%).

bp= base pairs, FLP = *F. psychrophilum* media, *Identification based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media.

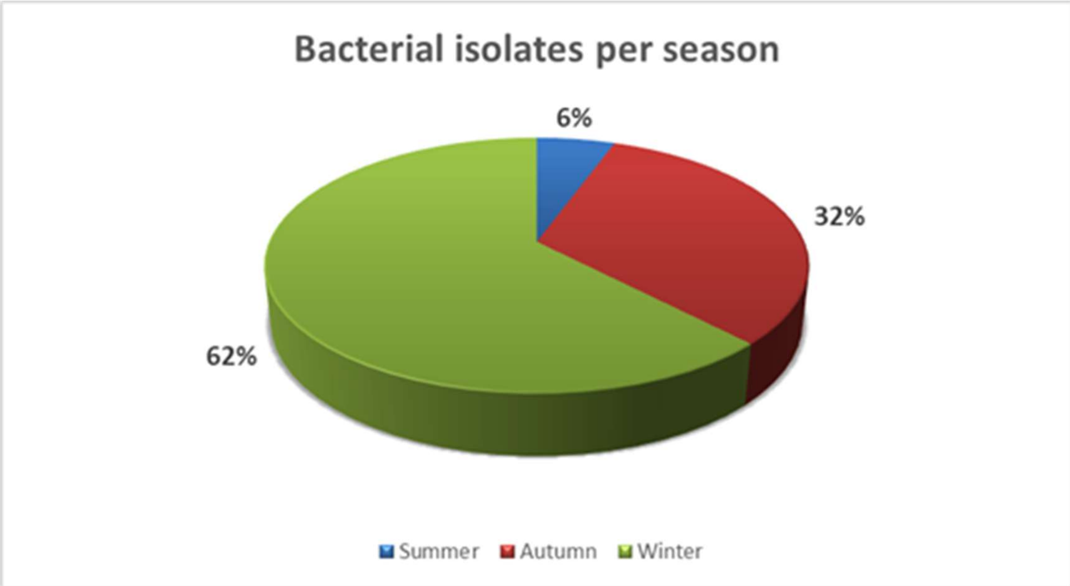

Figure 3.1 An overview of bacterial isolates (percentage) collected from South Africa and Lesotho during summer, autumn, and winter of the year 2018.

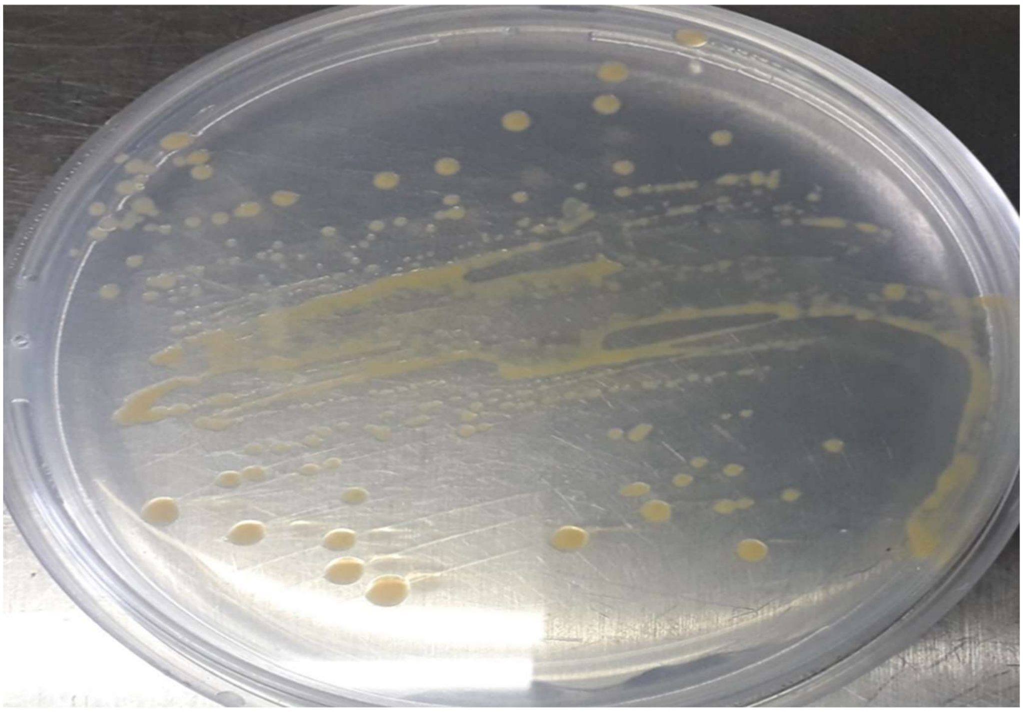

Figure 3.2 Characteristic yellow colonies formed by *Flavobacterium* spp. when grown on FLP media.

3.2. DNA sequencing and molecular phylogeny

To identify the bacteria cultured from infected trout tissues, the 16S rRNA gene of each isolate was subjected to PCR amplification and DNA sequencing. All DNA samples analyzed in this study produced a positive result in the PCR's, yielding bands of the expected size (~1.5 kb) for the 16S rRNA gene following gel electrophoresis (Fig. 3.3). No amplification occurred in the control samples (NTC that included PCR grade H_2O instead of genomic DNA), confirming the specificity of the PCR reactions. Nucleotide sequences of approximately 500 to 1400 bp in length were determined for the isolates. A BLAST search of the GenBank database confirmed some of the preliminary identifications made by the veterinary diagnostic laboratory (Table 3.1 above) since forty- seven out of the ninety bacteria analyzed in this study (~51%) were shown to share high 16S rRNA gene sequence similarity (≥99%) with bacteria from the genus *Flavobacterium* and six (~7%) of the sequences showed high sequence similarity with *Chryseobacterium* spp., which occur within the family *Flavobacteriaceae* and are also yellow pigmented.

However, nineteen of the sequences (~21%) showed sequence similarity with non-*Flavobacterium* isolates, which included, amongst others, *Hafnia* and *Aeromonas* species. Whilst 4 of the sequences (~4%) shared high sequence similarity with uncultured bacteria (Table 3.1). Unfortunately, poor quality DNA sequence data was acquired for fifteen (~17%) of the isolates (OM-2, OM-5, OM-7, OM-9, OM-12, OM-15, OM-18, OM-38, OM-46, OM-55, OM-71, OM-80, OM-88, OM-89, and OM-90) and they were subsequently excluded from further analysis.

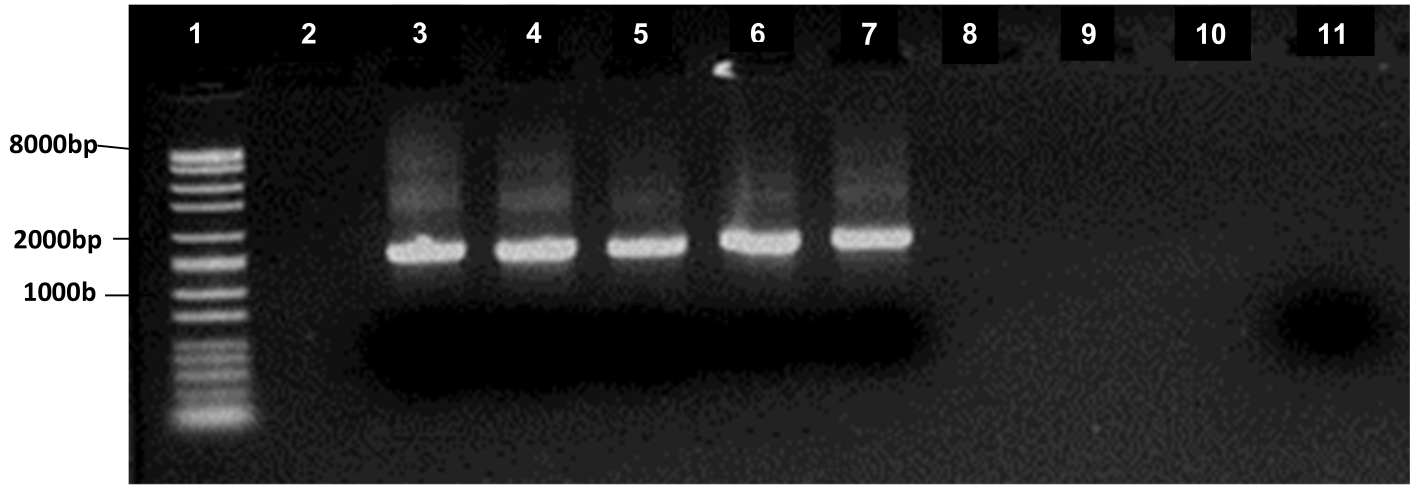

Figure 3.3 An example of the amplified SSU rRNA gene products obtained from some of the bacterial isolates examined in this study, separated by 0.8% agarose gel electrophoresis to verify reaction specificity and size of amplicons (expected band of ~ 1500bp). Lane 1: 1-kb molecular weight (MW) ladder (Roche Applied Science, Indianapolis, IN, USA), Lane 2: blank, Lane 3: OM-64, Lane 4: OM-10, Lane 5 OM-45, Lane 6: OM-47, Lane 7: OM-39, Lane 8-10: blank, Lane 11: Non-template control.

3.2.1. Phylogenetic analysis of *Flavobacterium* spp.

The 16S rDNA phylogenetic analysis confirmed some of the BLAST search results of the GenBank database for the forty-six bacterial isolates that shared high percentage similarity with members of the genus *Flavobacterium* (Fig 3.4a-c and Table 3.1). Seven clusters were apparent, with only some of the clusters, such as the *F. psychrophilum* cluster (Fig 3.4c), supported by high bootstrap values (>95%). The remaining clusters had bootstrap values that ranged between 50 and 82%. The first cluster was made up of seven isolates representing five different farms: Farm A (OM-83), Farm B (OM-8 and OM-17), Farm E (OM-4), Farm H (OM-43 and OM-44) and Farm I (OM-16) (Fig 3.4a). Isolates OM-4, OM-8, OM-16, and OM-17 formed a sub-cluster together with *F. tructae* strain 47B-3-09, supported by a bootstrap value of 81%. Sequence analysis revealed that isolates

OM-4, OM-8, OM-16, and OM-17 shared high 16S rRNA gene sequence similarity with *F. tructae* 435-08 (≥99.71%). Isolate OM-4 was isolated in the Western Cape province (South Africa) during winter (August), OM-8 was isolated in Lesotho during summer, whilst OM-16 and OM-17 were isolated during autumn (March) in the Western Cape (South Africa) and Lesotho, respectively.

Three isolates (OM-43, OM-44, and OM-83) within this cluster appeared to be related but distinct from *F. hercynium* strain WB 4.2-33, *F. branchiarum* strain 57B-02-09 and *F. branchiarum* strain CECT 7908, although their subcluster was not supported by a significant bootstrap value (<50%). Isolate OM-43 shared a high sequence similarity with *F. granuli* FI2G1(99.75%) and *F. granuli* NBRC 102009 (98.40%), whilst OM-44 shared high sequence similarity with *F. branchiarum* 57B-2-09 (≤99.43%), and OM-83 shared sequence similarity with *F. branchiarum* CECT 7908 (≤98.96%). Although OM-43 shared sequence similarity with both *F. granuli* FI2G1 and *F. granuli* NBRC 102009, it did not cluster with the two *F. granuli* type strains included in the phylogenetic analysis (*F. granuli* strain Kw05 and *F. granuli* strain NBRC 102009) (See Fig 3.4a and Table 3.1). Both isolate OM-43 and OM-44 were recovered on the same farm (Farm H) during winter (August) in South Africa, whilst OM-83 (Farm A) was recovered during winter (July) in Lesotho.

Three isolates (OM-13, OM-39, and OM-82) clustered with none of the type strains used in this study (Fig 3.4a and Table 2.1). Sequence analysis revealed that both OM-13 and OM-39 shared ≤99.41% sequence similarity with the type strains of *F. chryseum* and *F. luteum* respectively. Both isolates (OM-13 and OM-39) also shared a high sequence similarity of ≤99.85% with *Flavobacterium* sp. T101L.09.P.CHS.MI.H.Kidney.N, whilst

OM-82 shared 99.93% sequence similarity with an uncultured *Flavobacterium* sp. V31 clone. However, the clustering of these three isolates was not well supported (<50% bootstrap support). Isolate OM-39 was recovered in South Africa (Farm H, Western cape) in autumn (March), whilst both OM-13 (Farm B) and OM-82 (Farm A) were recovered in Lesotho during summer and winter, respectively.

The phylogenetic position of eleven isolates (OM-50, OM-51, OM-56, OM-57, OM-59, OM-60, OM-61, OM-62, OM-63, OM-65, and OM-88) was unclear and they shared a high 16S rRNA gene sequence similarity (≥99%) with *Flavobacterium* spp. including *F. collinsii* 983-08, *F. circumlabens*, and *F. saccharophilum* NBRC 15944 (Table 3.1 and Fig 3.4a). The majority of these isolates (n=9) were recovered from different farms in South Africa during winter (August), while the remaining 2 were recovered from Farm A in Lesotho during the same winter period. Isolates OM-51 (Farm G, South Africa) and OM-84 (Farm A, Lesotho) appeared to cluster together into a distinct subcluster with low bootstrap support (<50%), both isolates appeared to be related to *F. cupreum*; OM-51 shared ≤99.77% sequence similarity with the type strains; *F. collinsii* 983-0, *F. aquidurense* WB 1.1-56, and *F. cupreum*, whilst OM-84 shared ≤99.62% sequence similarity with the type strains; *F. saccharophilum* NBRC 15944, *F. piscis* 412R-09, and *F. chilense* LM-09-Fp (Table 3.1 and Fig 3.4a).

Isolate OM-77 clustered with the 3 *F. saccharophilum* type strains (DSM1811, NBRC15944, and NCIMB 2072) with strong bootstrap support (73%), and it shared 100% sequence similarity with *F. saccharophilum* NBRC 15944 and *F. saccharophilum* DSM 1811. Isolates OM-74 and OM-86 appeared to be related but distinct to *F. piscis* strain 412R-09, *F. hydatis* strain DSM 2063, and *F. hydatis* strain NBRC 14958, but was supported by a low bootstrap value (<50%). Both isolates shared sequence similarity with the type strains *F. saccharophilum* DSM1811 (99.66%) and *F. chungangense* (98.55%). Isolates OM-74 and OM-86 were both recovered from Farm A and Farm H respectively in August 2018.

Four isolates (OM-64, OM-73, OM-45, and OM-47) clustered (72% bootstrap support) with 15 *Flavobacterium* spp. type strains that included *F. olei* strain R-10-9, *F. branchiicola* strain 59B-3-09, *F. chungangense* strain CJ7, and *F. plurextorum* strain JM59 (Fig 3.4b). Isolates OM-64 and OM-73 formed a subcluster (98% bootstrap support) that appeared to be closely related to *F. olei* strain R-10-9, whilst OM-45 and OM-47 formed a subcluster (68% bootstrap support) that appeared to be closely related to *F. plurextorum* strain JM59 and *F. plurextorum* strain 1126-1H-08. Isolates OM-45 and OM-47 shared ≤99.86% sequence similarity with the type strain *F. plurextorum* strain 1126-1H-08, whilst both OM-64 and OM-73 shared ≤99.31% sequence similarity with the type strains *F. olei* strain R-10-9, and *F. saccharophilum* DSM1811 respectively. Isolate OM-87 clustered (96% bootstrap support) with 5 *Flavobacterium* spp. type strains that included *F. succinicans* strain 8, *F. glaciei* strain 0499, and *F. granuli* strain NBRC 102009. However, OM-87 appeared to be related to *F. succinicans* as it formed a low bootstrap (<50%) subcluster with both *F. succinicans* strain NBRC and *F. succinicans* strain 8.

Seventeen isolates clustered together with eleven strains of *F. psychrophilum* (see Fig 3.4c) supported by a high bootstrap value of 100%. Six different farms were represented by the isolates within this cluster, namely Farm A (OM-24, OM-81, OM-10), Farm B (OM-28, OM-30, and OM-31), Farm C (OM-79 and OM-76), Farm G (OM-48, OM-49, OM-53, OM-67, OM-68, OM-69, and OM-70), Farm H (OM-41), and Farm I (OM-3). Of the 17 isolates within the *F. psychrophilum* cluster 12 isolates (~ 70.6%) were isolated during the last month of winter (August 2018), whilst the remaining 5 isolates (~29.4%) were isolated during autumn (March to April). A BLAST search of the 16S rRNA gene of the 17 isolates revealed that all the isolates shared high sequence similarity (99 -100%) with *F. psychrophilum* type strains including *F. psychrophilum* strain NBRC 100250, *F. psychrophilum* strain IFO 15942, and *F. psychrophilum* strain NCIMB 1947(See Table 3.1 and Fig 3.4c).

3.2.2. Phylogenetic analysis of *Chryseobacterium* species

A maximum-likelihood (ML) analysis of the 16S rDNA confirmed the sequence comparison similarities for isolates OM-75, OM-58, OM-40, OM-78, OM-42, and OM-26 The phylogenetic analysis grouped the sequences into distinct clusters, all the clusters had high bootstrap support (≥56%) (Fig. 3.5). In the first cluster isolates OM-75 and OM-58 appeared to form a subcluster with the type strains, *C. ureilyticum* strain VersM10.91, and *C. rhizosphaerae* strain RSB3-1. Both OM-75 and OM-58 were recovered towards the end of the winter season (August 2018) from Farm A (Lesotho) and Farm I (Western Cape), respectively. The BLAST search of the GenBank database also revealed that these isolates had high sequence similarity (99.32%) with the type strains, *C. jejuense* strain JS17-8, *C. lactis* strain KC1864 and *C. rhizosphaerae* strain RSB3-1. Both isolates also shared 99% sequence similarity with the type sequence of *C. ureilyticum* strain versM10.91 (Table 3.1).

Two other isolates (OM-40 and OM-78) within the first cluster formed a subcluster with *C. vrystaatense* R-23566 and this subcluster exhibited a high bootstrap value with the ML analysis (bootstrap 99%), indicating the existence of a strong association between the two isolates and *C. vrystaatense*. Isolates OM-40 and OM-78 were recovered during the winter season of 2018 and exhibited a high sequence similarity with *C. vrystaatense* R-23566 (≥99.21%). Isolate OM-42 was recovered from Farm H during winter (July 2018) and showed a 99.70% sequence similarity with *C. indoltheticum* LMG 4025. The ML analysis confirmed the sequence similarity, with OM-42 forming a subcluster in the second cluster with *C. indoltheticum* strain LMG 4025 (bootstrap value of 99%).

Isolate OM-26 on the other hand was recovered from Farm B (Lesotho) during summer (February 2018) and showed a ≤98.95% sequence similarity with *C. psychrotolerans* strain TSBY 57,*C. tenax* strain DSM 16811, and *C. tenax* strain EP105. The latter finding was supported by the ML analysis, with isolate OM-26 clustering with the type strains of *C. psychrotolerans* strain TSBY 57,*C. tenax* strain DSM 16811, and *C. tenax* strain EP105 with the latter cluster exhibiting a high bootstrap value (88%).

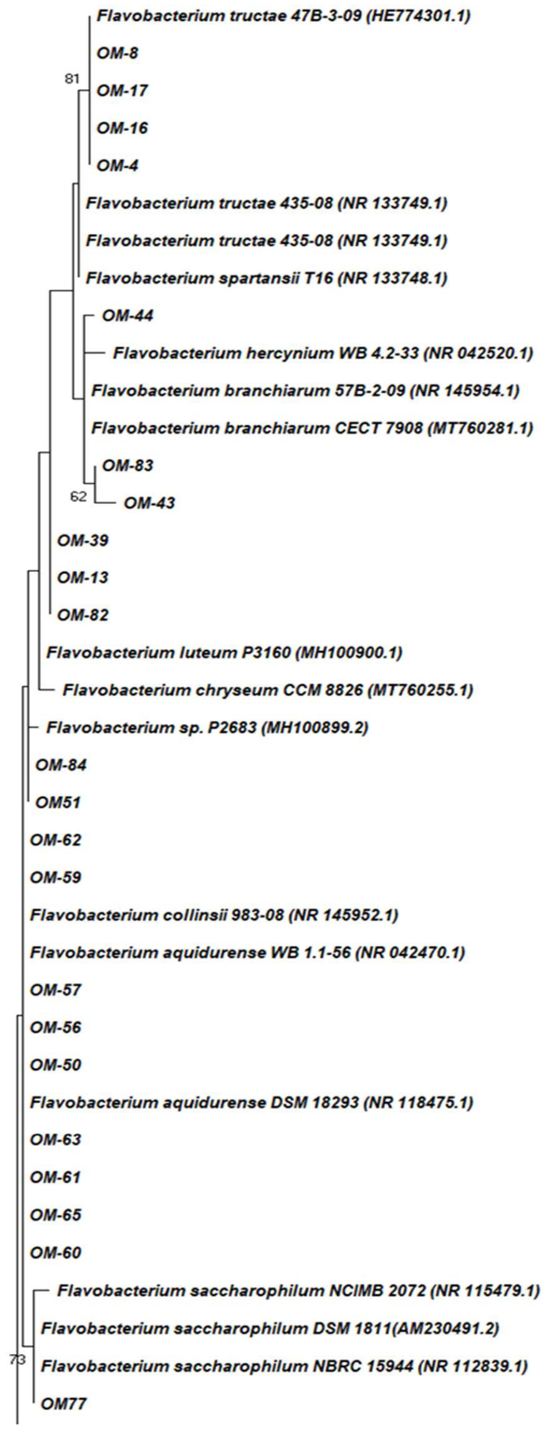

Figure 3.4a Subtree of the *Flavobacterium* phylogenetic tree depicting the phylogenetic relationship between 22 study isolates with *Flavobacterium* spp. type strain sequences. The numbers at branch nodes are bootstrap values shown as percentages of 1000 bootstrap replicates (only values 50% and above are shown). Scale bar represents 1% estimated substitutions.

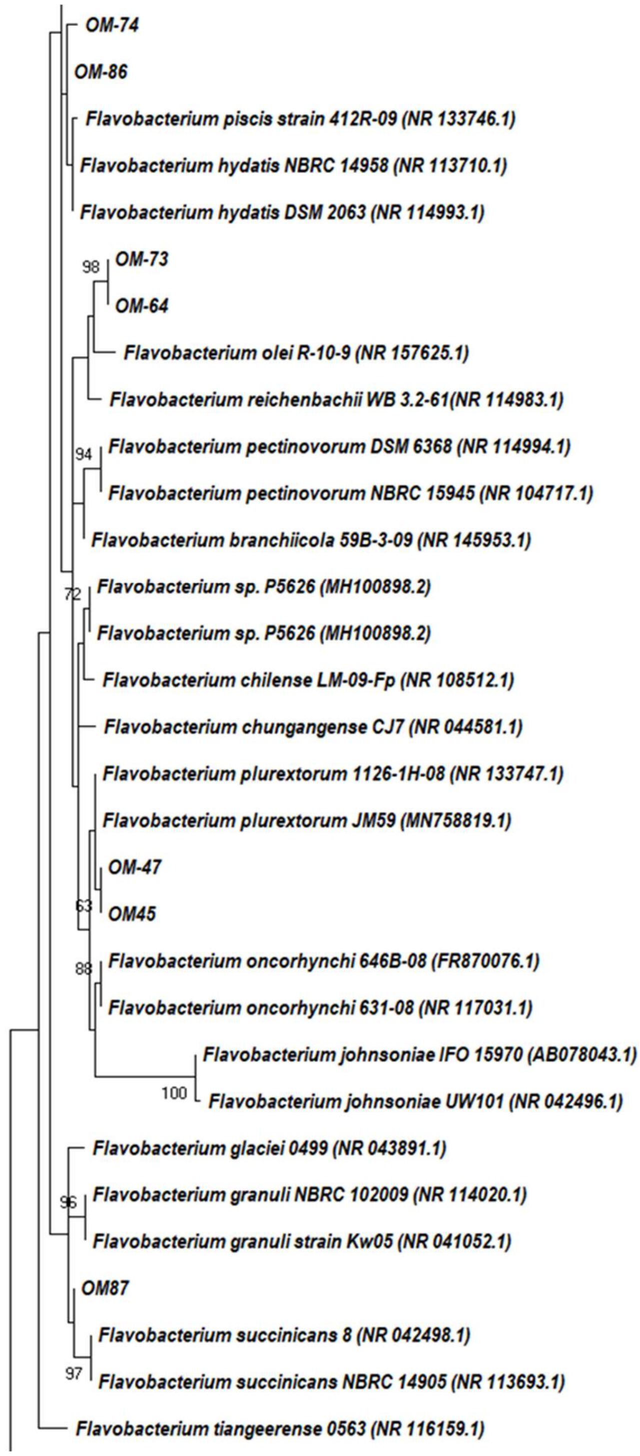

Figure 3.4b Subtree of the *Flavobacterium* phylogenetic tree based on maximum-likelihood analysis of the 16S rRNA gene sequence similarities depicting the phylogenetic relationship between 7 study isolates with *Flavobacterium* spp. type strain sequences. The numbers at branch nodes are bootstrap values shown as percentages of 1000 bootstrap replicates (only values 50% and above are shown). Scale bar represents 1% estimated substitutions.

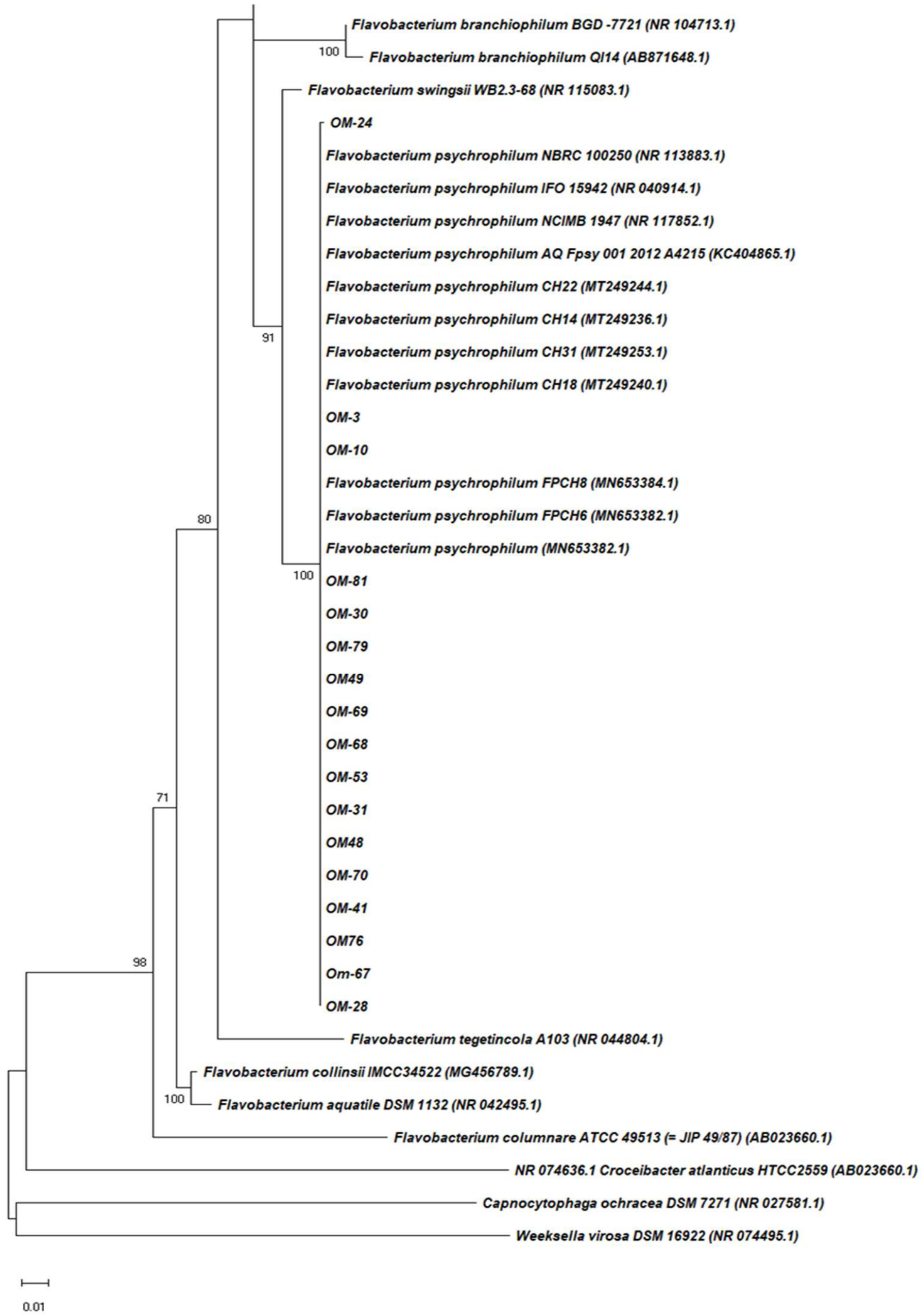

Figure 3.4c Subtree of the *Flavobacterium* phylogenetic tree based on maximum-likelihood analysis of the 16S rRNA gene sequence similarities depicting the phylogenetic relationship between 17 study isolates with *Flavobacterium* spp. type strain sequences. The numbers at branch nodes are bootstrap values shown as percentages of 1000 bootstrap replicates (only values 50% and above are shown). Scale bar represents 1% estimated substitutions. ***Weeksella virosa***, ***Capnocytophaga ochracea*** **DSM 7271** and ***Croceibacter atlanticus*** HTCC 2559 were used as outgroups to anchor the tree.

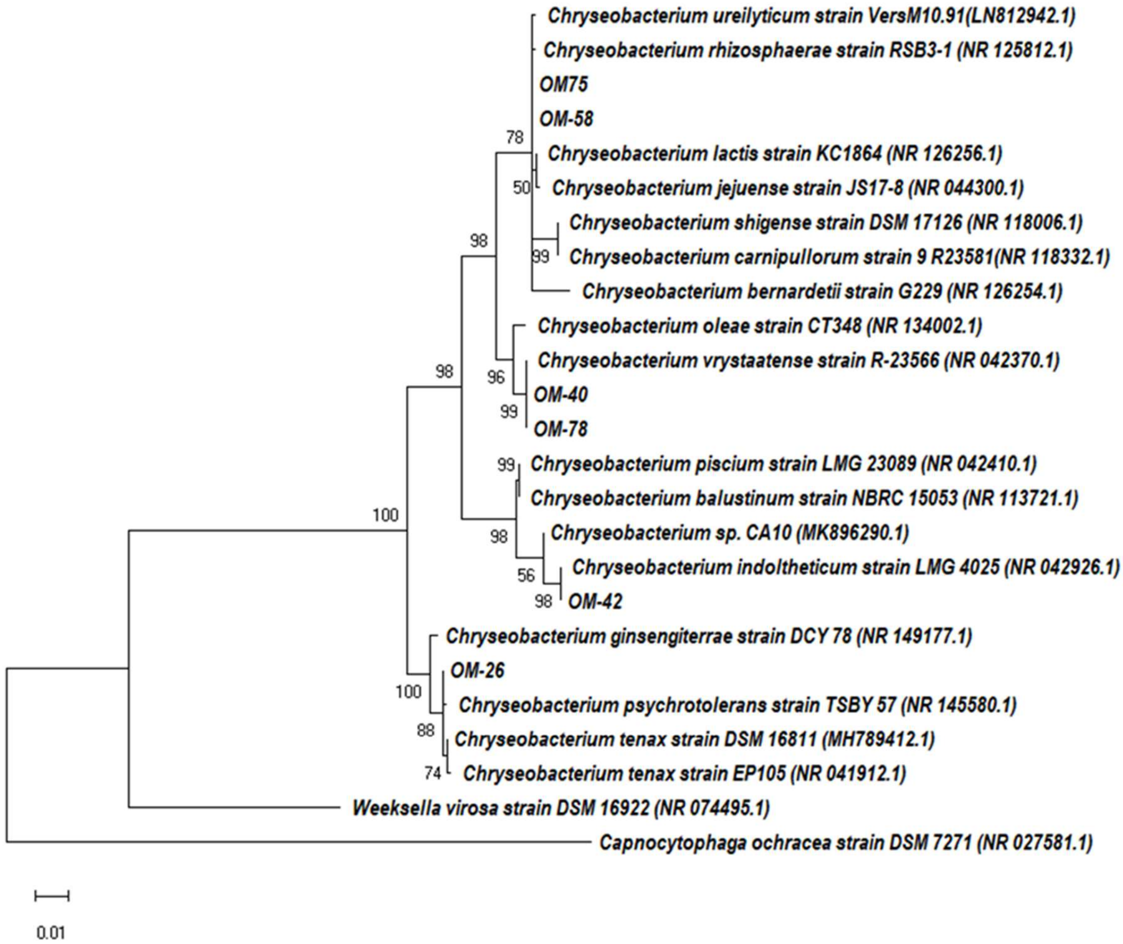

Figure 3.5 A maximum likelihood phylogenetic tree of isolates from diseased rainbow trout in South Africa and Lesotho and closely related *Chryseobacterium* spp. type strain sequences based on near complete 16S rRNA gene sequences ***Weeksella virosa*** *and* ***Capnocytophaga ochracea*** DSM 7271 were used as outgroups. The numbers at branch nodes are bootstrap values shown as percentages of 1000 bootstrap replicates (only > 50% are shown). Scale bar represents 1% estimated substitutions.

3.3. qPCR primer specificity and high-resolution melt analysis

The forward primer Flavo_F (5'-ATCTACCTTTTACAGAGGGATAGC-3') and reverse primer Flavo_R (5'-GTAAGCCGTTACCTTACCAACT-3'), corresponding to conserved regions 92-115 and 224-235, respectively, of the reference sequence *Flavobacterium psychrophilum* strain IFO 15942 (NR 040914.1) 16S rRNA gene, flanked a hypervariable region of 107 bp (nucleotide position 116 – 223) of the reference sequence 16S rRNA gene. The 107 bp hypervariable region contained 4 – 17 base pair differences across the different *Flavobacterium* spp. used to design the primers (Table 2.2 and Fig. 3.6). To ensure primer specificity the annealing temperature of the primers was initially optimized via gradient PCR (50.0 ºC to 65.0 ºC) using two isolates, OM-24 and OM-42 that were closely related to *F. psychrophilum* and *Chryseobacterium indoltheticum* based on the phylogenetic analysis.

At annealing temperatures lower and greater than 62.5ºC, both DNA templates belonging to OM-24 and OM-42 were amplified. However, at an annealing temperature of 62.5ºC, only the DNA template belonging to OM-24 (related to *F. psychrophilum* NBRC 100250), was amplified; indicating that 62.5ºC is the optimal annealing temperature for specific amplification of DNA from *Flavobacterium* spp. Primer specificity was further confirmed following PCR amplification of DNA from isolates OM-24, OM-60, OM-73, (related to *F. psychrophilum* NBRC 100250, *F. collinsii* 983-08, and *F.* olei R-10-9 respectively) and OM-45, and OM-47 (both related to *F. plurextorum* JM59, and *F. plurextorum* 1126-1H-08) and agarose gel electrophoresis, which produced amplicons of the expected size of 146 bp (Fig. 3.7).

During real-time qPCR, primer pairs Flavo_F and Flavo-R, targeting the 16S rDNA of the genus *Flavobacterium* were found to be highly specific when tested against a panel of isolates phylogenetically related to *Flavobacterium* spp.: OM-24, *F. psychrophilum* NBRC 100250; OM-73, *F. olei* R-10-9; OM-86, *F. chungangense* CJ27; OM-77, *F. saccharophilum* DSM1811; OM-17, *F. tructae* 47B-3-09; OM-84, *F. cupreum (Flavobacterium sp.* P2683*)*; OM-47, *F. plurextorum* JM59; OM-60, *F. collinsii* 983-08; OM-45, *F. plurextorum* JM59; OM-57, *F. circumlabens (Flavobacterium sp.* P5626*)*; and OM-44, *F. hercynium* WB.4.2-33, successfully amplifying a single fragment and producing distinctive melt curves following HRMA (Fig. 3.8). There was no amplification for template DNA obtained from the non-*Flavobacterium* species, *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio anguillarum,* and *Chryseobacterium indoltheticum*, included in the assay; thereby confirming the specificity of the primers for bacteria within the genus *Flavobacterium*.

Varying DNA concentrations (40 ng – 0.03 ng) were tested to determine the optimal template DNA concentration per 25 µl PCR reaction volume for ensuring optimal discrimination of *Flavobacterium* spp. during HRMA. A DNA concentration of 3 ng per 25 µl PCR reaction volume provided the best discrimination between species and good reproducibility amongst PCR replicates (Fig. 3.9b). When a high template DNA concentration of 40 ng, as well as lower concentrations of 0.3 and 0.03 ng (data not shown), is included in a 25 µl reaction volume, isolates OM-17 (*F. tructae* 47B-3-09), OM-24 (similar to *F. psychrophilum* NBRC 100250), OM-45 (similar to *F. plurextorum* JM59), OM-47 (similar to *F. plurextorum* 1126-1H-08), OM-57 (similar to *F. circumlabens*), and OM-73 (*F. olei* R-10-9) clustered together and could not be differentiated (see red melt curves in Fig 3.9a), whilst replicates of OM-24 and OM-60 were allocated to different clusters by the software. Isolates OM-44 *(F. hercynium* WB.4.2-33), OM-77 *(F. saccharophilum* DSM1811), OM-84 (*F. cupreum)*, and OM-86 *(F. chungangense* CJ27) were allocated into different clusters and could be easily distinguished based on their melt curves (Fig 3.9a). Conversely, when a DNA concentration of 3 ng per 25 µl reaction volume was used, 9/11 (~82%) tested isolates were discriminated from one another and all replicate samples cluster with one another (Fig 3.9b).

Using the optimized real-time qPCR reaction (3ng template DNA) and cycling conditions, as well as the optimized HRMA conditions described above, eleven isolates that appeared to be closely related to different *Flavobacterium* spp. were chosen to confirm the ability of the real-time PCR and HRMA to differentiate between these species. Isolates OM-24 (*F. psychrophilum* NBRC 100250*)*, OM-44 (*F. hercynium* WB.4.2-33), OM-45 (similar to *F. plurextorum* JM59), OM-57 (*F. circumlabens*), OM-73 (*F. olei* R-10-9), OM-77 (*F. saccharophilum* DSM1811), OM-84 (*F. cupreum*), and OM-86 (*F. chungangense* CJ27) were all allocated to eight separate clusters represented by the different colours on the melt curve (Fig 3.9b and Table 3.2).

Isolates OM-17 and OM-45 could not be differentiated since they had the same Tm value (78.1 ºC); hence the software auto allocated them in the same cluster with average percent confidence of 99.0 and 98.9% respectively. The melt curve of OM-60 (Similar to *F. collinsii* 983-08) was used as the reference strain (red almost flat curve in fig 3.9b below). The melt curve of OM-45 (similar to *F. plurextorum* JM59) showed a similar melt profile with that of isolates OM-17 (*F.* tructae 47B-3-09) and OM-47 (similar *F. plurextorum* 1126-1H-08). Isolate OM-45 had a Tm of 78.3 ºC, whilst both OM-17 and OM-45 had a

Tm of 78.1 ºC which resulted in the three melt curves almost clustering together (fig 3.9b and Table 3.2). The isolates were auto-called into their respective clusters by the software with an average percentage confidence range of 99.3 – 99.7%.

The melt temperature (Tm) of the clusters ranged from 76.6 – 79.2 ºC with an average standard deviation range between 0 and 0.9. The melting temperature of all the samples showed a difference of 0.2 ºC in between replicates of the same isolate, except for replicates of OM-57, with one of the replicates having a melting temperature of 76.8ºC compared to 78.4 ºC for the other two replicates; hence the high Tm standard deviation of 0.9 compared to the low 0.1 standard deviation of the other melt curves (See table 3.2). The highest Tm difference between different isolates was 2.6 ºC between OM-84 (76.6 ºC) and OM-86 (79.2 ºC), whilst some of the isolates, such as OM-17(78.4 ºC) and OM-45 (78.3 ºC), had the lowest Tm difference (0.1 ºC) (Table 3.2). To visualize the melt curves, OM-60 was used as the reference strain, and as such 10 replicates of OM-60 were included in the melt curve study.

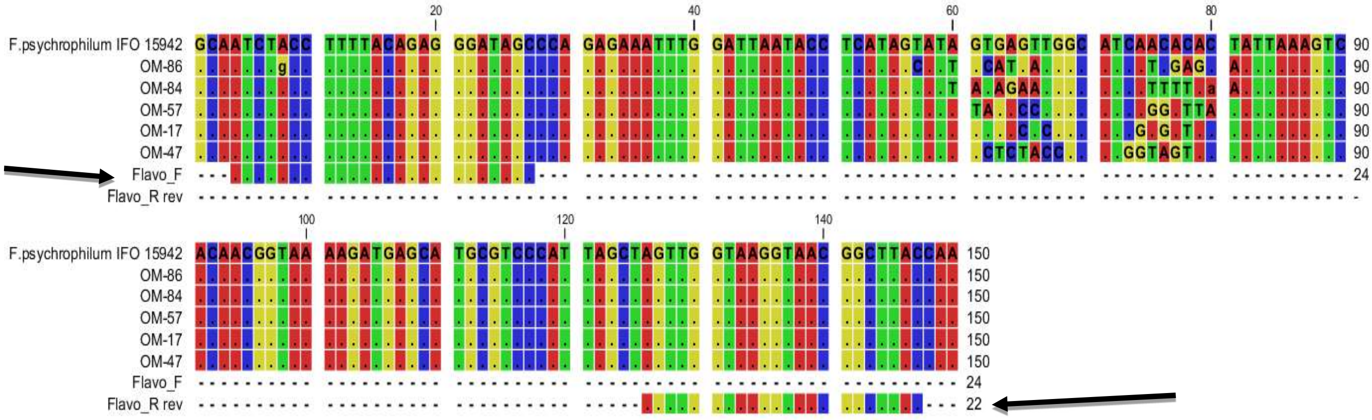

Figure 3.6 Alignment of 16S rRNA gene sequences of the reference strain *F. psychrophilum* IFO 15942 (NR_040914.1) and five of the eleven study isolates (OM-86, OM-84, OM-57, OM-17, and OM-47) showing sequence variances within the hypervariable region flanked by the primers Flavo_F and Flavo_R. The designed primers flanked a hypervariable region corresponding to nucleotide position 116 – 223 of the reference strain *F. psychrophilum* IFO 15942 (NR_040914.1). The dots represent identical base pairs, whilst the variant nucleotides are highlighted in different colors. The position of the primers is indicated by the arrows. This hypervariable region was used for differentiation of the isolates via high resolution melt analysis.

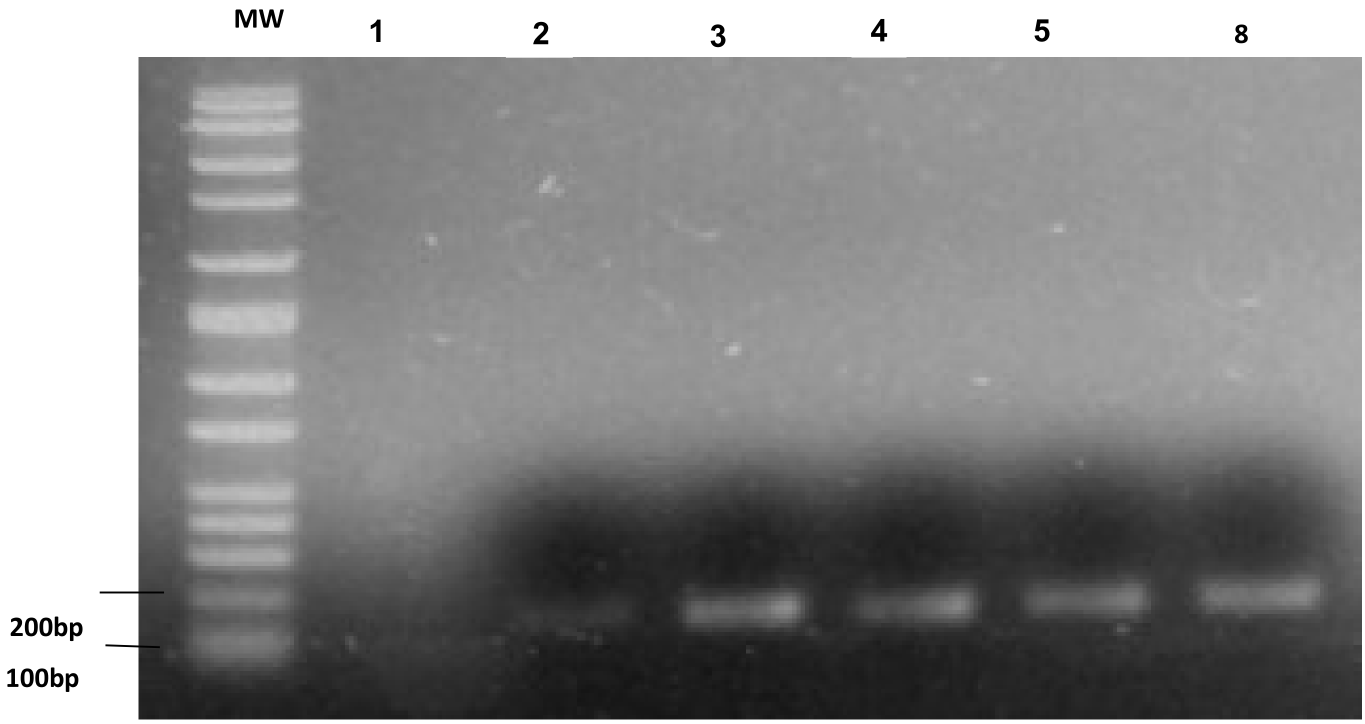

Figure 3.7 Gel electrophoresis confirming the sizes (~146 bp) of 5 selected *Flavobacterium* SSU rRNA gene amplicons produced using the designed primers Flavo_F and Flavo_R. MW = Molecular Weight ladder, Lane 1 = Blank, Lane 2 = *F. saccharophilum*, Lane 3 = *F. psychrophilum*, Lane 4= *F. collinsii*, Lane 5 = *F. plurextorum*, Lane 5 = *F. branchiarum*. Non-template control not visualized.

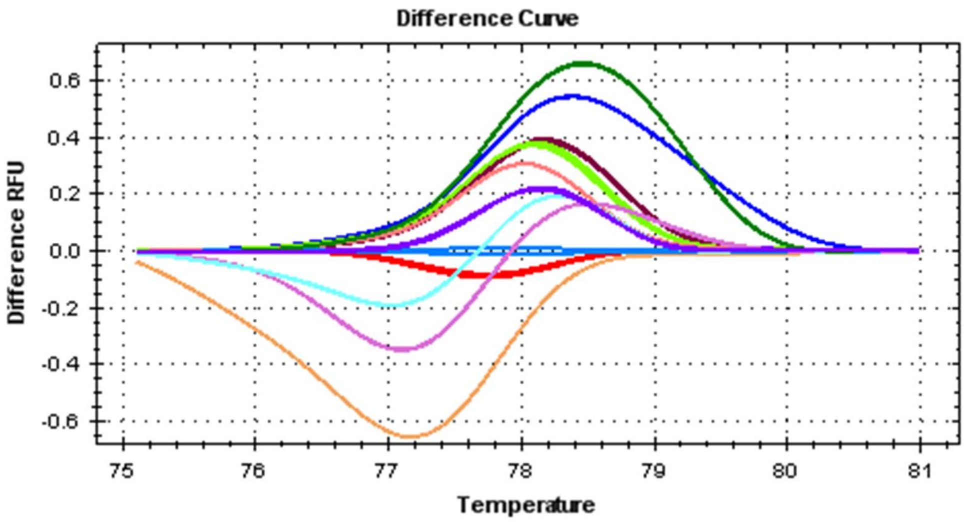

Figure 3.8 Melt curve results showing the specificity of the primer pair Flavo_F and Flavo_R to *Flavobacterium* spp., only DNA template samples (~3ng) of *Flavobacterium* spp. were successfully amplified. OM-17(*F. tructae* 47B-3-09)(■), OM-24 (*F. psychrophilum* NBRC 100250)(■), OM-44 (*F. hercynium* WB.4.2-33)(■), OM-45 (*F. plurextorum* JM59)(■), OM-47 (*F. plurextorum* 1126-1H-08)(■), OM-57 (*F. circumlabens*)(■), OM-60 (*F. collinsii* 983-08)(■), OM-73 (*F.* olei R-10-9)(■), OM-77 (*F. saccharophilum* DSM1811)(■), OM-84(*F. cupreum*)(■), and OM-86 (*F. chungangense* CJ27)(■). RFU= Relative fluorescence units.

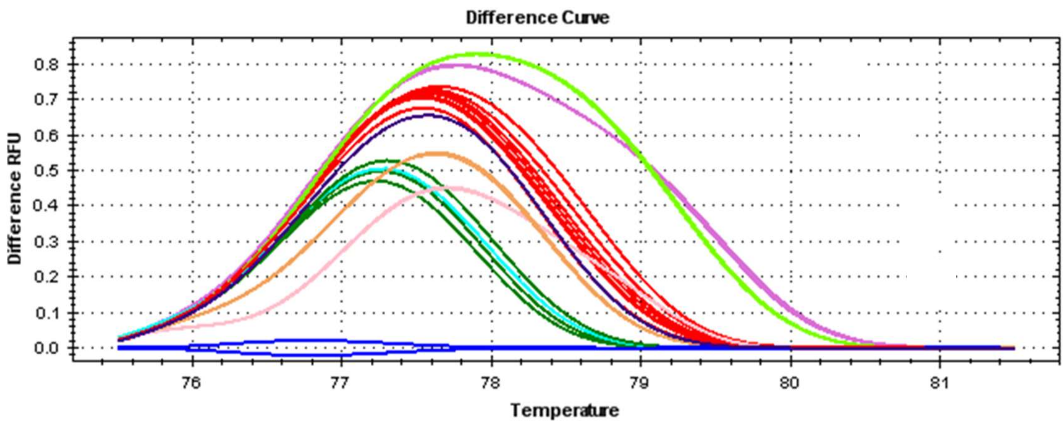

Figure 3.9a Results of a melt curve analysis performed using ~40 ng DNA concentration (compare with Fig 3.9b at DNA concentration ~3ng) of *Flavobacterium* spp. OM-24 (*F. psychrophilum* NBRC 100250) (■)(■), OM-77 (*F. saccharophilum* DSM1811) (■), OM-84(*F. cupreum*) (■), OM-60 (*F. collinsii* 983-08) (■)(■), OM-44 (*F. hercynium* WB.4.2-33) (■), OM-17(*F. tructae* 47B-3-09) (■), OM-45 (*F. plurextorum* JM59) (■), OM-47 (*F. plurextorum* 1126-1H-08) (■), OM-86 (*F. chungangense* CJ27) (■),OM-73 (*F. olei* R-10-9) (■), OM-57 (*F. circumlabens*) (■). OM-73(*F. olei* R-10-9) (■). RFU= Relative fluorescence units.

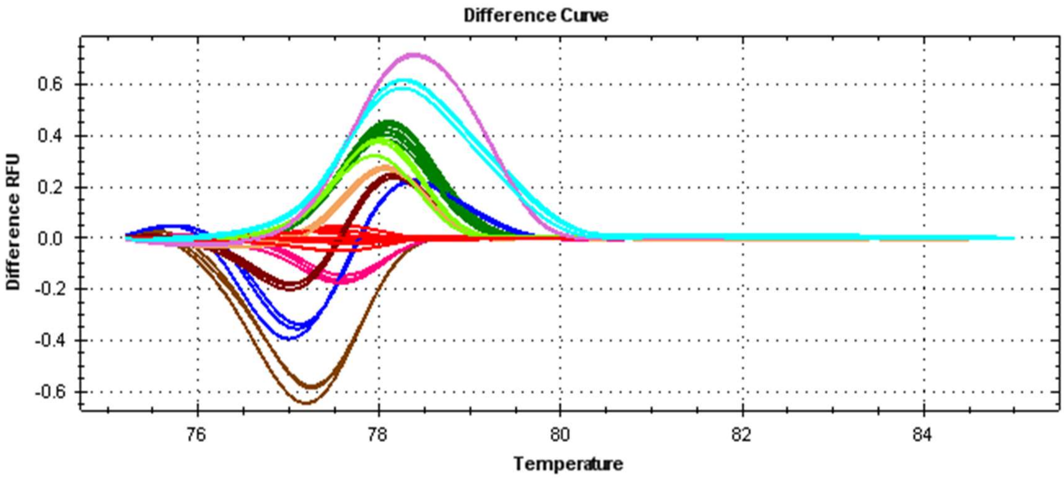

Figure 3.9b Melt curve analysis of 11 *Flavobacterium* spp. based on a 146 bp fragment of the 16S rRNA gene. To perform the analysis an optimal DNA concentration of 3 ng per 25 µl reaction volume was used for all samples, which provided improved discrimination of the isolates in contrast the analysis performed using ~40 ng DNA concentration (Fig 3.9a). OM-17 (*F. tructae* 47B-3-09)(■), OM-24(*F. psychrophilum* NBRC 100250) (■), OM-44 (*F. hercynium* WB.4.2-33*)* (■), OM-45 (*F. plurextorum* JM59) (■),OM-47 (*F. plurextorum* 1126-1H-08) (■), OM-57(*F. circumlabens*) (■), OM-60 (*F. collinsii* 983-08) (■), OM-73 (*F. olei* R-10-9) (■), OM-77(*F. saccharophilum* DSM1811) (■), OM-84 (*F. cupreum*) (■), and OM-86(*F. chungangense* CJ27) (■). RFU= Relative fluorescence units.

Table 3.2 *Melt* curve analysis data off the 11 *Flavobacterium* spp. used in the high- resolution melt curve analysis showing the isolates and *Flavobacterium* spp. that they shared high sequence similarity, the different clusters that they were allocated by the software, average melting temperature (Tm) amongst replicates of the same sample, average Tm standard deviation, and the average percent confidence of cluster allocation.

Isolate	***Flavobacterium* spp.**	**Cluster**	**Average Tm (°C)**	**Tm standard deviation**	**Average Percent confidence**
OM-60	*F. collinsii* 983-08		77.7	0.1	99.3
OM-77	*F. saccharophilum* DSM1811		77.5	0.1	95.4
OM-24	*F. psychrophilum* NBRC 100250		78.2	0	99.1
OM-57	*F. circumlabens*		77.9	0.9	99.0

Table 3.2 Continued

Isolate	***Flavobacterium* spp.**	Cluster	Average Tm (°C)	Tm standard deviation	Average Percent confidence
OM-45	*F. plurextorum* JM59		78.3	0.1	98.3
OM-17	*F. tructae* 47B-3-09		78.4	0	99.0
OM-86	*F. chungangense* CJ27		79.2	0	98.7
OM-73	*F. olei* R-10-9		79.0	0	98.9
OM-44	*F. hercynium* WB.4.2-33		76.7	0.1	96.0
OM-84	*F. cupreum*		76.6	0	99.7
OM-47	*F. plurextorum* 1126-1H-08		78.4	0	98.9

4. Discussion

4.1. Isolation and characterization of bacteria

All the 90 bacteria analyzed in this study were isolated from farmed rainbow trout that displayed clinical symptoms of fish disease that are typically associated with infections caused by *Flavobacterium* spp., such as erratic swimming, deep skin ulcers, lesions, lethargy, and darkened bodies. Based on the findings of the genotypic and phylogenetic analysis ~51% of the isolates appear to be closely related to several *Flavobacterium* spp. including *F. psychrophilum* FPCH6, *F. saccharophilum* strain NCIMB 2072, *F*. tructae 47B-3-09, and other undescribed *Flavobacterium* spp. (such as *Flavobacterium* sp. T417L.08.BNT.W. Gill.N). Whilst ~7% appeared to be closely related to *Chryseobacterium* spp. (*C. ureilyticum* strain verM10.91, *C. vrystaatense* strain R-23566, *C. indoltheticum* strain LMG 4025, and *C. tenax* strain EP105).

Most *Flavobacterium* spp., such as *F. psychrophilum*, are known to cause acute disease in fish at temperatures at or below 10°C (Starliper, 2011) and most *Flavobacterium* spp. have been shown to prefer cool to cold water temperatures (15 to 20 °C) (Bernardet & Nakagawa, 2006; Starliper, 2011). It was therefore not surprising to have recovered a large percentage of *Flavobacterium* spp. isolates (n=38; ~67%) and particularly *F. psychrophilum* strains during winter (13/18, ~72%) since the average water temperature on farms sampled in South Africa and Lesotho is 0 to 15 °C during winter, and 18 to 20 °C. Because of *Flavobacterium* spp. predilection to lower temperatures it will be important to increase screening for the pathogen during winter periods to ensure early detection and application of the necessary disease control measures such as quarantine. However, continuous screening throughout the year for *Flavobacterium* spp. and other pathogens is still important considering that pathogens such *F. columnare* are commonly cause disease during warmer periods even though it does cause disease at lower temperatures (12-14°C) (Starliper, 2011).

Colonies of *Flavobacterium* spp. have a distinctive yellow to bright orange pigmentation on solid media, due to the production of flexirubin and/or carotenoid type pigments (Bernardet & Bowman, 2015). This distinctive colony pigmentation, together with other morphological characteristics such as the production of flat, spreading colonies that sometimes adhere to the agar surface, is often used as a direct means of selecting suspected *Flavobacterium* spp. when grown on specific media (Bernardet & Bowman, 2006; Bernardet & Nakagawa, 2006; Loch & Faisal, 2015). Preliminary identification of many of the bacterial isolates received from Wemmershoek Diagnostic Laboratory in this study was primarily based on Gram-negative Bacilli producing distinctive yellow colonies on nutrient poor media, including Enriched Anacker and Ordal media (EAOA) (Anacker & Ordal, 1955) and solid FLP media, as well as growth at temperatures of 20 – 30 °C and 4 – 15 °C. The latter temperatures are known to be optimum for the growth of most *Flavobacterium* spp. (Bernardet & Bowman, 2006). Most of the isolates (92%) from this study grew at 15 °C on FLP media, except for three isolates that grew at 4 °C and four that grew at 20 °C, confirming findings from other studies on *Flavobacterium* spp. (Bernardet & Bowman, 2015; Flemming *et al*., 2007; Mcbride, 2014).

Chryseobacterium spp. are closely related to members of the genus *Flavobacterium* and belong to the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). Members of the genus *Chryseobacterium* also have a distinctive yellow to bright-orange colony pigmentation, similar to that of many *Flavobacterium* spp. colonies, making it difficult to identify either genera in mixed cultures based only on colony pigmentation (Bernardet & Bowman, 2015; Kämpfer *et al*., 2019). In our study, the genotypic and phylogenetic analysis of the 16S rRNA gene sequences of 6 isolates (OM-58, OM-78, OM-42, OM-26, OM-7, and OM-75) (Table 3.1) with yellow- to orange-pigmented colonies revealed that they were closely related to several *Chryseobacterium* spp. (99.28 – 99.89% sequence similarities).

Other phenotypic characterization methods, including biochemical characterization with the use of API ZYM profiles (Madetoja *et al*., 2001) and characterization of colony morphologies on specific growth media (Flemming *et al*., 2007), are commonly used to augment colony pigmentation in identifying *Flavobacterium* and *Chryseobacterium* spp. However, these methods are unable to identify newly isolated bacteria to the species level (Flemming *et al*., 2006). Aquaculture has been identified as a key role player in satisfying the local fish food demand in South Africa, and also to contribute to job creation and food security (DAFF, 2019). As the aquaculture industry continues to grow in South Africa the risk of bacterial diseases spreading between different geographical areas through activities such as movement of fish eggs, fingerlings, and broodstock between farms is a reality.

The results of this study clearly illustrate the limitations of traditional culture techniques in differentiating bacteria associated with diseased rainbow trout, particularly *Flavobacterium* spp. and *Chryseobacterium* spp. that may be responsible for causing diseases in South Africa and Lesotho. Further these results illustrate the need for increased screening of both symptomatic and asymptomatic fish, and sampling to detect *Flavobacterium* spp. and other fish pathogens that are known to devastate salmonid aquaculture industries worldwide. Development of molecular diagnostic tool(s) for detection and differentiation of bacterial pathogens such as *Flavobacterium* spp. will play a critical role in supporting the aquaculture industry in South Africa by providing a rapid, sensitive, and easy to use tool to detect and identify pathogenic organisms, especially *F. psychrophilum* which is one of the well-known pathogens devasting salmonid aquaculture globally, thus enabling development of effective fish health and welfare management practices that will contribute towards the growth and sustainability of the sector.

Several isolates appeared to be closely related to other non-*Flavobacterium* bacterial species (~21%) and some of the species that were identified have previously been implicated as either pathogens or opportunistic pathogens of fish (Nielsen *et al*., 2001; Orozova *et al*., 2014). Some of the isolated non-*Flavobacterium* spp. showed high sequence similarities (>99%) with members of the genera *Brevundimonas* (n=4), *Hafnia* (n=4), and *Aeromonas* (n=1). *Aeromonas* spp. are well-known pathogens of fish, causing diseases such as furunculosis (caused by *A. salmonicida*) and motile aeromonas septicemia (caused by *A. hydrophila*), whilst *Hafnia* spp., particularly *H. alvei*, has been associated with diseased fish suffering from epizootic haemorrhagic septicemia (Dalsgaard & Madsen, 2000; Gelev *et al*., 1990; Orozova *et al*., 2014; Padilla *et al*., 2015; Stratev & Odeyemi, 2017). Hence it is possible that some of the clinical symptoms displayed by the fish might also have been caused by these fish non-*Flavobacterium* isolates. Some of the diseases caused by these non-*Flavobacterium* bacteria such as *A. salmonicida* might be confused with flavobacterial diseases since most of the clinical symptoms displayed by fish are somewhat similar. For example, one of the symptoms caused by *A.salmonicida* in fish is focal skin lesions that often grow into deep skin ulcerations that exposes underlying muscles a symptom that is also observed in flavobacterial diseases (Buller, 2014; Loch & Faisal, 2015).

The results of our study also highlight that although *Flavobacterium* spp. are a cause for concern in freshwater fish aquaculture in South Africa there are other bacteria that might be pathogenic and or opportunistic pathogens of fish. Indeed, several other non-*Flavobacterium* bacteria such as *Lactococcus garviae* (Causative agent of Lactococcosis in trout) (Kutu *et al*., 2017), *Enterococcus faecium*, and *E. faecalis* (Causative agents of Enterococcosis) (Bekker *et al*., 2011) have been isolated from diseased rainbow trout in South Africa. Other non-*Flavobacterium* bacterial isolates from this study included *Brevundimonas* spp. (n=5), *Massillia aurea* (n=5) and *Paracoccus marcusii* (n=1). The isolation of the latter typically environmental bacteria from fish organs, such as the kidney, that are typically devoid of bacteria, warrants further investigation to understand the source and possible role that these bacteria may play in diseases of farmed rainbow trout (*Oncorhynchus mykiss*) in South Africa and Lesotho. To further characterize the isolates in this study, we pursued molecular/genotypic characterization by analyzing the 16S rRNA gene of the isolates as recommended for identification of suspected *Flavobacterium* spp. (Bernardet *et al*., 2002; Flemming *et al*., 2006).

4.2. DNA sequencing and Molecular phylogeny

The phylogenetic analysis and DNA sequencing data (Table 3.1 and Fig 3.5) in this study supports the allocation of the forty-six isolates (51%) as members of the genus *Flavobacterium*. Thirty-two of the isolates clustered with known *Flavobacterium* spp., including *F.* tructae 47B-3-09, *F. psychrophilum* strain NBRC 100250, and *F. branchiarum* strain 57B-2-09. Members of the genus *Flavobacterium* share a high degree of 16S rDNA sequence similarity, with up to 98.7% sequence similarity occurring between distinct *Flavobacterium* spp. (Bernardet & Bowman, 2006; Loch, 2012). The isolates in this study displayed a high degree of sequence similarity as expected for *Flavobacterium* spp., pairwise comparison of the forty-six isolates revealed that they shared as much as 97.16 -100% sequence similarity. The diversity of *Flavobacterium* spp. associated with diseased fish worldwide is well documented within fish health literature, for example Loch & Faisal (2015) and references therein provides a review of the different *Flavobacterium* spp. and *Chryseobacterium* spp. associated with diseased fish in different countries around the world.

However, information on the diversity of *Flavobacterium* spp. associated with diseased fish within the freshwater aquaculture industry in Southern Africa is limited. The results of this study illustrate the diversity of *Flavobacterium* spp. associated with diseased rainbow trout in South Africa and Lesotho. For example, 32 of the 46 isolates (~65.2%) in this study formed 7 distinct clusters with well-known *Flavobacterium* spp. upon phylogenetic analysis (Fig 3.4). The phylogenetic position of the 14 remaining isolates was unclear suggesting that these isolates could be novel *Flavobacterium* spp. However, further characterization is required to identify these potentially novel *Flavobacterium* spp.

associated with diseased fish and to understand their role in fish diseases. Other studies conducted elsewhere have implicated undescribed *Flavobacterium* and *Chryseobacterium* spp. as pathogens and or opportunistic pathogens of fish for example, Loch *et al.,* (2013) used undescribed *Flavobacterium* and *Chryseobacterium* spp. isolates to experimentally challenge rainbow trout and other fish species and the fish displayed disease symptoms such as necrotic ulceration, severe necrosis and hemmorhage of pectoral fin, and severe hemorrhage of the kidney (Loch *et al*., 2013).

In a previous study conducted by Flemming and colleagues several undescribed *Flavobacterium* spp. isolated from diseased fish in South Africa were able to produce clinical symptoms such as skin ulcers, caudal fin rot and gill necrosis, and mortality during experimental infection of Mozambique Tilapia (*Oreochromis mossambicus*) (Flemming *et al*., 2007). Whilst no experimental challenge of fish with any of our study isolates was performed, future studies elucidating the pathogenicity or lack thereof of these isolates are warranted. The information collected from this pathogenicity studies will be crucial in the implementation of disease control and fish health management strategies within the aquaculture industry in South Africa.

The results our study further suggests that the diversity of *Flavobacterium* spp. in South Africa is similar to that of *Flavobacterium* spp. Lesotho for example, in all the cluster that were formed upon phylogenetic analysis one or more isolates from farms located in South Africa and Lesotho are present . This similarity is not surprising as there is possibly movement of live fish or fish products such as eggs between the farms which could results in the transportation of bacterial pathogens from one geographical area to another as suggested by various other studies (Knupp *et al*., 2019; Nilsen *et al*., 2014). However, majority (9/17; ~53%) of the isolates in the *F. psychrophilum* cluster were recovered from farms in the Western Cape, South Africa. Suggesting that they might be a large number of *F. psychrophilum* isolates associated with diseased rainbow trout in South Africa compared to Lesotho however, large scale studies are needed to confirm this.

Of the 90 bacterial isolates investigated in this study, the BLAST search of the 16S rDNA sequences revealed that six of the isolates share high sequence similarity with several *Chryseobacterium* spp. The phylogenetic analysis confirmed that these isolates are closely related to *Chryseobacterium* spp., and five distinct clusters were evident following the ML analysis that were supported by strong bootstrap values (≥ 88%). *Chryseobacterium* spp. have been increasingly recovered from diseased fish, and some studies have demonstrated, via experimental infection, the ability of *Chryseobacterium* spp. to cause disease in fish (Abraham *et al*., 2017; Loch & Faisal, 2015; Pridgeon *et al*., 2013; Shahi *et al*., 2018). For example, *C. joostei* and *C. indoltheticum* were amongst the *Chryseobacterium* spp. that were isolated from diseased fish obtained from France, the USA, Belgium, and the United Kingdom (Bernardet *et al*., 2005).

The six *Chryseobacterium* isolates identified in this study were all recovered from diseased rainbow trout that presented with classical pathological symptoms of *Flavobacterium* disease, including skin lesions, skin ulcers, and darkened bodies. Isolates OM-26, OM-40, and OM-42 were all recovered from the head kidney, suggesting that they were the causal agent of disease in the infected fish. Conversely, isolate OM-58 was recovered from gill tissues and OM-75 from a skin lesion. No attempt was made in this study to determine the pathogenicity of the isolates; however, given the increased isolation of *Chryseobacterium* spp. in diseased fish, such studies are urgently needed.

The 16S rRNA gene has been extensively used as a molecular marker to study the taxonomy and phylogeny of bacteria because the gene is abundant in almost all bacteria, and it is highly conserved providing a better measure of evolutionary relationships amongst organisms (Janda & Abbott, 2007). Phylogenetic analysis of the 16S rRNA gene of *Flavobacterium* spp. was monumental in resolving some of the taxonomic confusion that existed within the genus *Flavobacterium* (Bernardet *et al*., 1996; Vandamme *et al*., 1994). Although the 16S rRNA gene is useful in the classification of bacteria several studies have indicated that it has low resolution or discriminatory power particularly for closely related bacteria with high sequence similarities (Gonçalves & Rosato, 2002; Mignard & Flandrois, 2006; Tokajian *et al*., 2016).

In this study, we were able to locate the phylogenetic position of 53 isolates from diseased rainbow trout using the 16S rRNA gene however, most of the groupings on the *Flavobacterium* spp. phylogenetic tree were not supported by significant bootstrap values (<50), and as such we were unable to provide clear identification of the isolates (Fig. 3.4). This finding was not surprising as other researchers have reported the same difficulty using the 16S rRNA gene, and they have used other markers to provide better discrimination. For example, Peeters & Williams (2011) used the gyrase B (*gyrB*) gene as a phylogenetic marker for *Flavobacterium* spp. and found out that this gene provides better discriminatory power in comparison to that provided by the 16S rRNA gene (Peeters & Willems, 2011).

In another study, Mun and colleagues used a multilocus sequencing analysis (MLSA) approach to differentiate five *Flavobacterium* spp. that could not be differentiated using the 16S rRNA gene. instead of using the 16S rRNA gene, they used six housekeeping genes: *atpA* (ATP synthase alpha subunit), *dnaK* (molecular chaperone protein), *glyA* (serine hydroxy-methyltransferase), *gyrB* (DNA gyrase, subunit B), *murG* (N-acetylglu-cosaminyl transferase), and tuf (elongation factor Tu)(Mun *et al*., 2013). Whilst the 16S rRNA gene was useful in providing a general understanding of the diversity of *Flavobacterium* spp. associated with diseased rainbow trout in South Africa and Lesotho it is not sensitive enough to differentiate between the closely related members of the genus *Flavobacterium* and as such more rapidly evolving markers such as the 16S-23S rRNA intergenic spacer region (ISR) or the use of multiple markers such as *gyrB*, *dnaK*, *tuf*, *murG*, *atpA*, and *glyA* is needed to provide better discriminatory power (Mun *et al*., 2013; Tokajian *et al*., 2016).

4.3. High resolution melt analysis

The 16S rRNA gene has been widely used to identify and discriminate between closely related microorganisms because it contains hypervariable and conserved regions that can be used to discriminate between microorganisms (Clarridge, 2004). Species-specific primers targeting unique regions of the 16S rRNA gene have been used to identify a variety of fish pathogenic bacteria, such as *F. columnare* (Darwish *et al*., 2004), *F. psychrophilum* (Toyama *et al*., 1994), and *A. hydrophila* (Nielsen *et a*l., 2001). In the present study, we designed *Flavobacterium*-specific primers to amplify a hypervariable region of the 16S rRNA gene to differentiate between the most common *Flavobacterium* spp. associated with diseased fish in aquaculture.

Since none of the non-*Flavobacterium* bacteria were amplified following real-time qPCR using the genus-specific primers (Flavo-F and Flavo-R) and all the *Flavobacterium* spp. that were included in this study produced a fragment of the expected size (146 bp), we can confirm that the primers are specific to the genus *Flavobacterium* at an annealing temperature of 62.5ºC. Further our primers were able to amplify DNA belonging to the closely related *Chryseobacterium* spp. at annealing temperatures below 62.5ºC, suggesting that our primers could be used to simultaneously detect fish associated *Chryseobacterium* spp. and *Flavobacterium* spp. providing an invaluable disease diagnostic tool considering that *Chryseobacterium* spp. have been implicated as potential or emerging pathogens of fish (Loch & Faisal, 2015, 2016; Zamora *et al*., 2012b).

In the present study, we found that 3 ng of DNA template was optimal for discrimination between species and yielded reproducible melt curves, which we could not achieve when using a higher template DNA concentration of 40 ng. This finding is in agreement with the study of Fortini *et al*., (2007) who showed that DNA quality and final concentration are important in yielding reproducible melt curves. We selected 11 isolates from our study that were closely related to *Flavobacterium* spp. including *F. saccharophilum* DSM1811*, F. psychrophilum* NBRC 100250*,* and *F. circumlabens* (Table 3.1 and Fig. 3.4a-c). We then used the 11 isolates to evaluate the ability of HRMA to discriminate the isolates based on the 146 bp fragment of the 16S rRNA gene and we were able to successfully discriminate 9/11 isolates (~82%) based on the distinctive melt curves that were produced following real-time PCR.

The ability of HRMA to differentiate closely related bacteria has been confirmed by other studies as well, such as the study of Jazi and colleagues who used HRMA to successfully discriminate between *Brucella abortus* and *B. melitensis* (Jazi *et al*., 2017). However, Cheng and colleagues were unable to discriminate *Streptococcus pyogenes* and *S. agalactiae* using the standard HRMA technique due to similar melt curves as a result of one base pair difference between these two closely related streptococci (Cheng *et al*., 2006). In our study, two isolates (OM-17 and OM-47) had the same Tm value (78.4 °C), and they could not be differentiated based on their melt curves, although they had 10 base pair differences within the 146 bp fragment. This was surprising since the base pair difference between the two isolates was presumed to be sufficient for discrimination via HRMA. Further sequence analysis of the 146 bp fragment of OM-17 and OM-47 revealed that they had the same guanine + cytosine (GC) content (40%), which could be part of the reason why they could not be separated; since GC content is one of the factors that determine the shape and position of a melt curve (Ririe *et al*., 1997).

Based on the phylogenetic analysis results (Fig. 3.4a and b), isolates OM-17 and OM-47 do not seem to be closely related, and as such we expected them to be easily differentiated by the HRM analysis. Except for isolate OM-17 and OM-47, the results of the HRM analysis appeared to support that most of these isolates are different as evidenced by the different melt curves (Fig. 3.9b). For example, isolate OM-45 and OM-47 clustered together on the phylogenetic tree with bootstrap support of 68% suggesting that they are closely related, and their melt curves were remarkably similar even though they were differentiated from each other (Fig 3.9b). However, it is important to state that the melt curve results cannot be used to infer any phylogenetic relationship between the isolates (Hjelmsø *et a*l., 2014). Whilst we do recommend HMRA as a rapid and sensitive diagnostic too, it is clear from the results of our study that further optimization is required to validate the HMRA technique developed in this study to enable the identification of isolates presumed to be *Flavobacterium* spp.

To achieve this *Flavobacterium* spp. type strains, obtained from a type collection such as ATCC (American type culture collection) (Berns *et al*., 1996), must be included in the preliminary optimization steps, which will enable us to identify the presumed *Flavobacterium* spp. by comparing the melt curves of the type strains with those of the unknown isolates. Iacumin *et al*. (2015) were able to identify 188/194 *L. casei* strains using HRMA by including generated melt curves of six known strains of the *L. casei* group. Thereafter, they compared the melt curves of the known strains with those of the unknown to identify them. In another study, Cheng *et al*. (2006) were able to identify 11/25 bacterial isolates by comparing the unknown isolates melt curves with the melt curve profiles of known bacteria.

Whilst HRMA appears to be a promising tool for discriminating *Flavobacterium* spp., most of the melt curves in our results have similar melt curve shapes that might lead to misidentification of unknown isolates. For example, isolates OM-17, OM-45, and OM-47 have similar melt curves that might be misidentified. To resolve this, perhaps other variations of HRMA or other techniques with better discriminatory power might be used. For example, Winchell *et al*. (2010) used a variation of the standard HRMA protocol by using 7 primer sets to amplify different markers of the genetically homogenous *Brucella* spp., and this technique was able to identify 152/153 unknown isolates.

The 16S rRNA gene is present in multiple copies in most bacterial species, and this could be a limiting factor of HRMA as a diagnostic tool when targeting the 16S RNA gene considering that studies have reported the existence nucleotide differences amongst copies of the 16S rRNA gene within some bacteria (Klappenbach *et al*., 2000; Rajendhran & Gunasekaran, 2011). For example, some isolates of *F.columnare* have been shown to contain up to 3 copies of the 16S rRNA gene with nucleotide variances amongst the multiple copies of the 16S rRNA gene (Lafrentz *et al*., 2014). This would serve as a limitation for the differentiation of Flavobacterium spp. using HRMA as isolates of the same Flavobacterium sp with sequence variations within the multiple copies of the 16S rRNA would possibly be assigned to different clusters based on their distinctive melt curves.

To overcome this limitation perhaps other variations of HRMA to target other genetic markers (e.g., gyrB gene) such as the one used by Winchell *et al*. (2010). Other typing techniques such as amplified fragment length polymorphism (AFLP) have also been used successfully to differentiate closely related bacteria. For example, Arias *et al*. (2004) were able to discriminate isolates of *F. columnare* using AFLP, which provided a higher resolution in comparison to restriction fragment length polymorphism (RFLP), and intergenic spacer region (ISR) sequencing. Overall based on the results of our study we do recommend HRMA as a powerful tool that can be used to identify and discriminate *Flavobacterium* spp. However, further optimization is needed to validate the HRMA tool presented in this study.

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