IDENTIFICATION AND CHARACTERIZATION OF FLAVOBACTERIACEAE FROM FARMED NILE TILAPIA (*Oreochromis niloticus*) AND AFRICAN CATFISH (*Clarius gariepinus*) IN UGANDA

AMONO RACHEAL

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS IN HEALTH OF AQUATIC ANIMAL RESOURCES OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

2017

ABSTRACT

Bacteria under family *Flavobacteriaceae* (in this study were also referred to as Flavobacteria) are important pathogens of fish, people, many other animals and plants. In this study, Flavobacteria from Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarius gariepinus*) were identified and characterized from the selected farms in Uganda. Gill and skin swabs were obtained from a total of 119 fish from 19 farms and were dissected aseptically to sample internal organs. The samples were inoculated onto Sheih media and incubated at 25°C for 48 hours. The suspected isolates were identified by colon characteristics, conventional biochemical tests and API 20 NE kits. The isolates were grouped into eight based on colon characteristic similarity. One isolate was selected per group for 16S rRNA gene sequencing and identified using the EZbiocloud.net ID software. Phylogenetic analysis of selected isolates was performed using the 16S rRNA gene sequences in BioEdit and MEGA 7.0.2 software. Basing on extrapolation of sequence analysis of the selected isolates, out of the 86 isolates, *Myroides marinus* was the most predominant species taking up 4 of the 8 groups (60 isolates) in 13 farms. The rest of the groups comprised of; *Acinetobacter pitti*, one group (6 isolates) in 6 farms, *Chryseobacterium gambrini* 2 groups (3 isolates) in 3 farms and one isolate was unidentified, in 3 farms. However, a total of 16 isolates did not grow on sub culturing. Phylogenetic analysis indicated that *M. marinus* isolates grouped with other *M. marinus* isolates from gene bank with significant intra-species diversity which was also observed with *C. gambrini* isolates. All the sampled farms had at least one isolate of a Flavobacterium from Tilapia and/or Catfish. Pathogenicity studies should be conducted on the isolates to establish their importance as fish pathogens and transmission dynamics so that an appropriate control measure can be recommended.

DECLARATION

I, RACHEAL AMONO, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for a higher degree award in any other institution.

_____ _____

Racheal Amono Date

(MSc. Candidate)

This work has been done under the supervision of;

_____ _____

Prof. Christopher J. Kasanga Date

(College of Veterinary Medicine and Biomedical Sciences

Sokoine University of Agriculture)

_____ _____

Prof. Denis K. Byarugaba. Date

(College of Veterinary Medicine, Animal Resource

and Biosecurity, Makerere University)

COPYRIGHT

ACKNOWLEDGEMENT

I am so grateful to my supervisors Prof Denis K. Byarugaba and Prof. Christopher J. Kasanga for their support throughout this research. I also take the opportunity to appreciate Prof. Robinson H. Mdegela and Prof. S. Mutoloki for the help during the planning and writing of the research findings. Am grateful to TRAHESA NORHED project for the scholarship, and the TRAHESA PhD. students for their assistance whenever approached, to mention, Dr. S. Wamala, Mr. E. Mwega and Dr. K. Mugimba. I am grateful to the help given by the technical staff of Makerere University for some guidance during the bacteriology laboratory work and molecular analysis. These include Mr. L. N. Musisi, Dr. D. Kahwa, Mr. D. Kamya, Mr. A. Kalyebi, Mr. J. Kasirye, Mr. J. Mayega and Mr. S. Ochwo. I acknowledge the assistance of Dr. J. Walakira of Kajansi NAFIRRI for the help and support during sample collection.

DEDICATION

I dedicate this work to my personal Lord and Savior, Jesus Christ that has given me reason to live on earth.

TABLE OF CONTENTS

ABSTRACT ..... **ii**

DECLARATION ..... **iii**

COPYRIGHT ..... **iv**

ACKNOWLEDGEMENT ..... **v**

DEDICATION ..... **vi**

TABLE OF CONTENTS ..... **vii**

LIST OF TABLES ..... **xi**

LIST OF FIGURES ..... **xii**

LIST OF ABBREVIATIONS AND ACRONYMS ..... **xiii**

CHAPTER ONE **1**

1.0 INTRODUCTION **1**

1.1 Background 1

1.2 Problem statement and study justification 3

1.3 Objectives 4

1.3.1 General objective 4

1.3.2 Specific objectives 4

1.4 Research questions 4

CHAPTER TWO **5**

2.0 LITERATURE REVIEW **5**

2.1 Aquaculture in Uganda 5

2.2 History of Flavobacterial diseases in fish 6

2.3 Classification of bacteria in Family *Flavobacteriaceae* 8

2.3.1 The genus *Flavobacterium* 9

2.3.2 The genus *Chryseobacterium* 9

2.3.3 The genus *Myroides* 9

2.3.4 The genus *Elizabethkingia* 10

2.4 Flavobacteriacea fish pathogens and diseases 10

2.4.1 The genus *Flavobacterium* 11

2.4.2 The genus *Chryseobacterium* 12

2.4.3 The genus *Myroides* 13

2.4.4 The genus *Elizabethkingia* 13

2.5 Hosts of Flavobacteria 14

2.6 Culture and characteristics of *Flavobacteriaceae* organisms 14

2.6.1 Genus *Flavobacterium* 15

2.6.2 Genus *Chryseobacterium* 16

2.6.3 Genus *Elizabethkingia* 16

2.7 Diagnosis of bacteria under family *Flavobacteriaceae* 16

2.7.1 The Genus *Chryseobacterium* 17

2.7.2 The genus *Flavobacterium* 17

2.7.3 The genus *Myroides* 18

2.8 Molecular characterization of bacteria based on 16S rRNA gene 18

2.9 Challenges when working with family *Flavobacteriaceae* 19

CHAPTER THREE 21

3.0 MATERIALS AND METHODS 21

3.1 The study area 21

3.2 Study design 23

3.3 Sampling 23

3.4 Isolation of bacteria under family *Flavobacteriaceae* 23

3.5 Morphological identification of Flavobacteria colonies 24

3.6 Identification of Flavobacteria by Biochemical tests 24

3.7 Molecular identification of Flavobacteria 24

3.7.1 DNA extraction for Flavobacteria sequencing 25

3.7.2 PCR process for the extracted DNA 25

3.7.3 Electrophoresis 25

3.7.4 Purification of the PCR products and sequencing 26

3.8 Data analysis 26

CHAPTER FOUR 27

4.0 RESULTS 27

4.1 Biodata for the sampled farms 27

4.2 Symptoms encountered in the fish samples 28

4.3 Culture and isolation of Flavobacteria 29

4.4 Colony characteristics 29

4.5 Biochemical test results 31

4.5.1 Biochemical test results for the sequenced isolates 31

4.5.2 General biochemical test results for the groups 32

4.5.3 API test results 33

4.5.4 Comparison of conventional and API 20NE biochemical test results 34

4.6 Electrophoresis results 36

4.7 Identification and occurrence of the study isolates 37

4.7.1 Identification of isolates using Ezbiocloud.net 37

4.7.2 Identification of the 86 isolates 39

4.7.3 Occurrence of Flavobacteria on the farms 40

4.8 Phylogenetic analysis 41

CHAPTER FIVE 43

5.0 DISCUSSION 43

CHAPTER SIX 51

6.0 CONCLUSION AND RECOMMENDATIONS 51

6.1 Conclusion 51

6.2 Recommendations 51

REFERENCES 53

APPENDICES 71

LIST OF TABLES

Table 1: Biodata of the selected farms 27

Table 2: Biochemical test results of the sequenced isolates..... 31

Table 3: General biochemical test results of the groups 32

Table 4: API 20NE results 33

Table 5: Comparison of API 20NE and conventional tube test results for selected isolates..... 35

Table 6: Identification of isolates and their occurrence in fish 38

LIST OF FIGURES

Figure 1: Map of Uganda and study area 22

Figure 2: Lesions encountered on catfish 28

Figure 3: Colony characteristics of the study isolates 30

Figure 4: Electrophoresis results for the 16S rRNA gene 36

Figure 5: Identification based on the extrapolation of results of sequenced isolates 39

Figure 6: Occurrence of isolates on the selected farms 40

Figure 7: Phylogenetic relatedness of the isolates based on the 16S rRNA gene 41

LIST OF ABBREVIATIONS AND ACRONYMS

%	Percent
µg	Micrograms
µm	Micro meter
16S	Sixteen sub-unit
BGD	Bacterial Gill Disease
BLASTN	Basic Local Alignment Search Tool Nucleotide
bp	Base pair
CDL	Central Diagnostic Laboratory
Cg	*Clarius gariepinus*
COVAB	College of Veterinary Medicine Animal Resource and Biosecurity
DFR	Department of Fisheries Resources
et al.,	And others
e.g	For example
FAO	Food and Agriculture Organization
FAT	Fluorescent Antibody Test
Kg	Kilograms
Km^2	Square kilometer
L	Littre
MAAIF	Ministry of Agriculture Animal Industry and Fisheries
ml	Milliliter
MLSA	Multilocus sequence analysis

MLST	Multilocus sequence typing
NAFIRI	National Fisheries Research Institute
NCBI	National Center for Biotechnology and Information
NDP	National Development Program
NMBU	Norwegian University of Life Sciences
O.n	*Oreochromis niloticus*
^{o}C	Degrees Celsius
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
rDNA	Ribosomal Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal ribonucleic acid
Spp	Species
TSI	Triple Sugar Iron
UBOS	Uganda Bureau of Statistics
USDA	United States Department of Agriculture

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Agriculture is the backbone of Uganda`s economy with aquaculture as one of the major enterprises highly growing, yet still with enormous potential for production (NDP11 2015/2016-2019/20). The Department of Fisheries Resources (DFR) 2012 annual report estimated over 25,000 earthly ponds producing over 100,000 tons annually and over 50 hatcheries in Uganda. However, increase in aquaculture is accompanied with an increased risk of diseases. In a research for common fish diseases and parasites affecting wild and farmed tilapia and catfish in central and western Uganda, over 70% of fish farms sampled had a high incidence of four bacterial pathogens including *Pseudomonus sp*., *Aeromonas sp*., *Vibrio sp*. and *F. columnare* of family *Flavobacteriaceae* (Walakira *et al*., 2014). Many Flavobacteria are opportunistic pathogens to fish with a worldwide geographical distribution yet limited studies have been conducted in Africa (Abowei and Briyai, 2011; Ekpo *et al*., 2013; Loch and Fasial, 2015).

There are multiple bacterial species within family *Flavobacteriaceae* that have been incriminated in the devastating losses in the wild and farmed fish stocks around the world. These cause Flavobacterial diseases which among others include columnaris disease, cold water disease, bacterial gill disease and many other emerging Flavobacterial diseases (Farmer, 2004; Bernardet *et al*., 2006; Aly, 2013; Loch, 2014; Loch and Fasial, 2015). Flavobacterial disease outbreaks are notoriously challenging

to prevent and control even though a lot of research has been carried out for nearly 100 years. They are known for their great economic and ecological effects (Wagner *et al*., 2002; Welker *et al*., 2005). Fish that recover from some Flavobacterial diseases remain carriers and shed the bacteria into the environment which makes them more dangerous in aquaculture (Welker *et al*., 2005). Loch and Faisal (2015) stated that there are recent reports of previously uncharacterized Flavobacteria to systemic infections and mortality events in fish stocks. These were reports from Asia, Europe, Africa, North and South America which poses a great concern since it contributes to the difficulties in the diagnosis and chemotherapeutic treatment of Flavobacterial fish diseases (Lorenzen *et al*., 1997; Flemming *et al*., 2007; Kämpfer *et al*., 2011; Kämpfer *et al*., 2012; Aly, 2013; Loch and Fasial, 2015).

Phylogenetic analysis of Flavobacterial fish pathogens is critical for the appropriate control of infections caused especially given the fact that Uganda is having a high growth rate in aquaculture (MAAIF, 2004). Information about the occurrence of Flavobacterial diseases in Uganda is not well documented but there are several undocumented cases (unpublished, NAFIRI, Kajansi). The fact that Uganda`s aquaculture is growing at a fast rate predisposes to increased incidences of columnaris disease and other Flavobacterial diseases due to stress conditions that usually accompany the intensification of aquaculture (Pridgeon and Klesius, 2012). The occurrence of diseases caused by Flavobacterial pathogens in countries with high aquaculture production like America, Europe and Asia (Shotts and Starliper, 1999; Farmer, 2004; Zamora *et al*., 2012; Loch and Fasial, 2014), could be one of the indications that Uganda will at one time face the same problem. It is therefore

important to proactively study the occurrence of species prevalent in the country and with further studies on their pathogenicity. It may be possible to develop and implement appropriate control measures such as vaccination using tailored vaccines.

1.2 Problem statement and study justification

Intensification of aquaculture has been accompanied by frequent disease out breaks with bacterial pathogens as one of the major problems contributing to high fish mortalities and low production. Walakira *et al.* (2014) found out that in Uganda, over 70% of the fish farms sampled had a high incidence of *Pseudomonus sp*., *Aeromonas sp*., *Vibrio sp*. and *F. columnare. Flavobacterium columnare* is just one of the many bacteria in family *Flavobacteriaceae* that has caused mortalities in a big number of fish species in farms and hatcheries worldwide (Shotts and Starliper, 1999; Farmer, 2004). In the United States, *F. columnare* is the second most important fish pathogen after *Edwardsiella ictalluri*, to cause mortalities in channel catfish (*Ictalurus punctatus*) leading to annual losses estimated at 30 million dollars (Hawk *et al*., 1992; Wagner *et al.,* 2002; Shoemaker *et al*., 2011).

The high rate of growth of intensive aquaculture in Uganda with the usual water quality problems highly predispose fish to Flavobacteriosis. Flavobacteria pathogens have been highly studied in Europe, America, and Asia but poorly studied in Africa. In Africa, Flavobacteriosis has been reported in Nigeria, Egypt, South Africa and Kenya (Aly, 2013; Ekpo *et al*., 2013; Flemming *et al*., 2007). There is limited information about occurrence and characterization of Flavobacteria in Africa. Information from this study will aid in; forming a basis for further research in the

Flavobacteriacea strains, understanding the differences in Flavobacteriosis occurrence, forming strategies to be used for future management of Flavobacteriosis in aquaculture by the government and providing a basis for research for therapeutics and vaccination control strategies for the diseases.

1.3 Objectives

1.3.1 General objective

To identify and characterize *Flavobacteriaeae* associated with *Clarius gariepinus* and *Oreochromis niloticus* in selected farms in Uganda

1.3.2 Specific objectives

I. To determine the occurrence of *Flavobacteriaceae* in *Oreochromis niloticus* and *Clarius gariepinus* in the selected farms in Uganda
II. To determine the molecular characteristics of *Flavobacteriacea* isolates from *Oreochromis niloticus* and *Clarius gariepinus* in the selected farms in Uganda, using the 16S rRNA gene.

1.4 Research questions

I. What is the occurrence of *Flavobacteriaceae* in *Oreochromis niloticus* and *Clarius gariepinus* in the selected farms in Uganda?
II. What genetic relationship exists between the species of *Flavobacteriacea* present in the selected farms in Uganda?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aquaculture in Uganda

Uganda is a well-watered country with 15.1% (36,527,4 km^2) of its area dedicated to swamp or open water (UBOS, 2014). It has been estimated that Uganda's growth in aquaculture at over 200% as the private sector (MAAIF, 2004). The top major fish species reared in Ugandan aquaculture are Nile tilapia (*Oreochromis niloticus*) and North African catfish (*Clarias gariepinus*) (FAO, 2005). There is a high production from cage fish farming of mainly Tilapia, which is expected to be rolled out to all water bodies including ponds in Uganda. Production ranges between 1,500 kg per hectare per year for subsistence farmers to 15,000 kg per hectare per year for emerging commercial fish farmers (Kifuko, 2015). With improved market prices for fish, government intervention for increased production and stagnating supply from capture fisheries, aquaculture has begun to attract entrepreneurial farmers seeking to exploit the business opportunity provided by the prevailing demand for fish (FAO, 2012).

There are over 50 hatchery operators in the country with good hatchery establishments and capacity to produce quality fish seeds for supply and distribution (DFR, 2012). The recent growth of regional fish demand left 5 out of 20 fish processing factories out of business and 15 operating at less than 40% of their installed licensed processing capacities. This is because of the demand for fish getting higher than production and creating shortage of fish as raw materials to the fish processing factories (FAO, 2012). This recent expansion in fish demand and aquaculture has also resulted in the transformation of around 30% of the smallholder

subsistence ponds into profitable small-scale production units. This increase in aquaculture production predisposes to disease outbreaks by opportunistic pathogens like Flavobacteria.

2.2 History of Flavobacterial diseases in fish

In 1917-1919, Davis at the U.S. Fisheries biological station in Fairport, Iowa, observed multiple fish mortality events that he associated with an unidentified bacterium (Davis, 1926). In 1926 and 1927, Davis reported multiple disease outbreaks in fingerling brook trout and steelhead (*Oncorhynchus mykiss*) reared in Vermont and New York, which he attributed to an unknown bacterium that was associated with damage to the gills (Davis, 1927). He noted slow chronic mortalities that increased with time when temperatures began to rise. Davis noted that the bacteria formed "luxuriant growth over the surface of the gills" that coincided with increased mucus production, clubbing of the gill lamellae, and proliferation of gill epithelium causing fusion of the secondary lamellae. Otherwise, fish appeared normal until death (Davis, 1926; Davis, 1927).

Bullock (1972) in his research observed similar disease outbreak and isolated yellow pigmented bacteria from affected fish but was unable to reproduce the disease experimentally. Wakabayashi (1980) however successfully recovered a yellow pigmented bacterium from hatchery-reared salmonids from Japan and Oregon that was distinct from those used in the studies of Bullock (1972) and successfully reproduced the disease. This bacterium was classified as *Flavobacterium branchiophila* (Wakabayashi *et al.*, 1989), which became *F. branchiophilum* (Bernardet *et al.,* 1996). It is now widely believed that *F. branchiophilum* is a causative agent of bacterial gill disease (BGD) (Bullock, 1990). However,

environmental parameters and other bacteria are also believed to play a role in some outbreaks of BGD.

A third unidentified yellow-pigmented bacterium was associated with serious disease in rainbow trout (*O. mykiss*) fingerlings reared at the national fish hatchery in Lee town, West Virginia (Davis, 1946). Although Davis was unable to isolate this bacterium, he observed huge numbers of non-motile bacterial rods in scrapings taken from deep ulcerations present on the caudal peduncle of affected fish. These bacteria did not form the characteristic "columns" associated with *F. columnare* (Davis, 1946). Soon thereafter, Borg (1948) reported a similar pathological condition among diseased hatchery-reared juvenile Coho salmon (*O. kisutch*) from Washington. In this case, a bacterium was successfully isolated from the kidneys and external lesions of systemically infected fish. Borg reproduced the disease in experimentally challenged fish, with signs that included ulcerations at the caudal peduncle that went so deep so as to almost detach the tail from the body (Bog, 1960). While this bacterium was initially placed in the order *Myxobacterales* and named *Cytophaga psychrophila*, it was reclassified as *Flexibacter psychrophilus* (Bernardet *et al.*, 1986) and later as *Flavobacterium psychrophilum* (Bernardet *et al.* 1996). As its name implies, *F. psychrophilum* grows best at low temperatures (around 15 °C) and frequently causes disease when water temperatures are below 10 °C. In North America, the term low temperature disease and bacterial cold-water disease are used to describe outbreaks associated with this bacterium (Holt, 1987) cited by Loch and Fasial, (2015), whereas outbreaks in Europe are commonly referred to as rainbow trout fry syndrome (Lorenzen *et al.*, 1997; Lorenzen *et al*., 1991).

2.3 Classification of bacteria in Family *Flavobacteriaceae*

Taxonomy and speciation of this family has undergone many revisions. (Ordal and Rucker, 1944; Bernardet *et al.,* 1986; Bernardet *et al.,* 1996) cited by Loch and Fasial, (2015).

Family*:* *Flavobacteriaceae*

Phylum: *Bacteroidetes*

Class*:* *Flavobacteriia*

Order: *Flavobacteriales*

The above classification was first suggested by Jooste (1985) and was verified in 1992 by Reichenbach, its formal description was published by Bernardet and other researchers (Bernardet *et al.,* 1996) cited by Loch and Fasial (2014). Genera within family *Flavobacteriaceae* include; *Flavobacterium, Chryseobacterium, Bergeyella, Empedobacter, Capnocytophaga, Elizabethkingia, Tenacibaculum, Ornithobacterium, Weeksella, Riemerella, Myroides* and *Tenacibaculum* (Vandamme *et al.,* 1994; Bernardet *et al.,* 1996; Vancanneyt *et al.,* 1996), cited by Loch, 2014. Bernardet *et al.* (2006) published minimal standards for describing new taxa in *Flavobacteriaceae* family and more genera were included in the family that is; *Coenonia, Psychroserpens Gelidibacter, Polaribacter, Psychroflexus, Salegentibacter, Cellulophaga,* and *Zobellia* (Gosink *et al.,* 1998; Johansen *et al.,* 1999; Barbeyron *et al*., 2001). The number of genera currently are more than 100 (Bernardet and Bowman, 2006; Kim *et al.,* 2012; Loch and Fasial, 2015). The genera within the family encompass pathogens of fish, amphibians, reptiles, birds, and mammals, including humans (Bernardet *et al*., 2006).

2.3.1 The genus *Flavobacterium*

This genus has species that include; *F. johnsoniae, F. flevense, F. branchiophilum, F. columnare, F. aquatile, F. pectinovorum, F. hydatis, F. succinicans, F. psychrophilum,* and *F. saccharophilum* (Frankland and Frankland, 1889; Bernardet *et al.,* 1996). Of these, *F. aquatile* is the original species in the genus and in 2002 when minimal standards for describing novel *Flavobacteria* were set, many more species (over 100) have been added (Bernardet *et al*., 2002; Bernardet *et al*., 2006).

2.3.2 The genus *Chryseobacterium*

The genus *Chryseobacterium* was created by Vandamme and other scientists (Vandamme *et al*., 1994) for six bacterial taxa which by then, were classified as members of the genus *Flavobacterium.* These included *F. meningosepticum, F. indoltheticum, F. balustinum, F. indologenes, F. scophthalmum* and *F. gleum.* With improved microbiological diagnostic techniques and molecular diagnostics, the genus now contains more than 80 described and proposed species (Kim *et al.,* 2012).

2.3.3 The genus *Myroides*

The reclassification of *Flavobacterium odoratum* led to the creation of genus *Myroides* (Vancanneyt *et al*., 1996) cited by Bernardet *et al*. (1996). *Flavobacterium odoratum* was excluded from genus Flavobacterium because of some characteristics like halotolerance, good growth at 37, lack of gliding motility and several differences in fatty acid profile (Bernardet *et al*., 1996). Species in genus *Myroides* have been found both in many terrestrial and aquatic environments. There are two medically relevant species that is, *Myroides odoratimimus* and *Myroides odoratus*, although they have been found to be causative agents in various infections. *Myroides*

odoratimimus is an important opportunistic human pathogen with ability to develop resistance to antibiotics. *Myroides* species have been reported in wet environments, sea water and insect guts (Starliper, 2011; Shotts and Starliper, 1999; Wakabayashi *et al.,* 1989). *Myroides Odoratus* and *M. Odoratimimus* have been isolated from Tilapia and grey mullet (Anderson and Ordal, 1961).

2.3.4 The genus *Elizabethkingia*

Many *Elizabethkingia spp*. have been isolated from diverse ecological niches, including eutrophic lakes, soil, freshwater sources and spent nuclear fuel pools. Only two species have been identified to date i.e., *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* (Bernardet *et al*., 2006). In the aquatic environment, two challenges may be posed by *E. meningoseptica*, i.e., ability of these multidrug-resistant species to resist drug action following antimicrobial treatment and persistence in tanks due to biofilm community formation, leading to disease and associated economic losses and their potential role as opportunistic human pathogens. The ability of these organisms to act as potential zoonotic pathogens, via transmission from fish and fish farm environments to immuno-compromised workers and consumers should not be underestimated (Jacobs and Chenia, 2011).

2.4 *Flavobacteriacea* fish pathogens and diseases

Davis in 1922 was the first to report Flavobacterial diseases and ever since then, these diseases have been documented as serious threat to both wild and farmed fish (Bernardet *et al*., 2006). Flavobacteriosis presents with many lesions which present relatively similar among the different species and these include; Branchititis that presents with epithelial hyperplasia of the secondary lamellae and secondary lamellar

fusion, monocytic infiltrate and mucus cell hyperplasia; monocytic myositis, hemorrhage within the muscle, liver, adipose tissue, and ovaries, spongiosis of white matter of the brain, multifocal edema within the granular cell layer of the cerebellar cortex, and renal tubular degeneration and necrosis; skin erosions; hemorrhages on fins; necrosis of gills; erosion of skin, yellowing of the skin (Loch and Fasial, 2015). Subacute and chronic Flavobacteriosis brings about lingering mortalities that may cause continuous economic losses (USDA, 2003). However, acute Flavobacteriosis can cause cumulative mortalities up to 70% and above among the affected fish stock. The fish that survive may suffer with spinal abnormalities and poor growth (Austin and Austin, 2007).

Originally, three bacteria within the family *Flavobacteriaceae* were known for Flavobacteriosis namely, *Flavobacterium columnare* (causative agent of columnaris disease), *Flavobacterium branchiophilum* (cause of bacterial gill disease) and *Flavobacterium psychrophilum* (the etiology for bacterial cold-water disease) (Wakabayashi *et al*., 1989; Shotts and Starliper, 1999; Nematollahi *et al*, 2003; Bernardet *et al*., 2006; Starliper, 2011). With time, many more species under the genera *Chryseobacterium, Myroides* and *Flavobacterium* have been documented as fish pathogens or potential fish pathogens (Chinnarajan *et al.,* 2015; Loch and Fasial, 2015; Austin and Austin, 2007). Examples of fish pathogens or potential fish pathogens under the different genera are listed below under different genera, among which some have just been newly discovered.

2.4.1 The genus *Flavobacterium*

This is the genus that contains the main three Flavobacteria causing fish mortalities (*F. columnare, F. branchiophilum* and *F. psychrophillum*). It however now contains

many more species among which include; *F. succinicans, F. johnsoniae, F. hydatis* (formerly *Cytophaga aquatilis), F. oncorhynchi, F. araucananum, F. collinsii, F. branchiarum, F. branchiicola* as well as other uncharacterized yellow-pigmented bacteria (Anderson and Ordal, 1961; Christensen, 1977; Strohl and Tait, 1978; Bernardet *et al.* 1996; Austin and Austin, 2007; Zamora *et al.,* 2013).

There are other Flavobacteria-like organism under genus *Flavobacterium* associated with different lesions and these include among others; *F. johnsoniae*-like bacterium associated with external lesions on the gills, jaws, skin, and fins in multiple farmed salmonids in Finland, farmed longfin eels (*Anguilla mossambica*), rainbow trout (*Oncorhynchus mykiss*) and koi (*C. carpio*) in South Africa, in cultured Russian sturgeon (*Acipenser gueldenstaedtii*) in Turkey, in farmed rainbow trout in Korea (Rintamäki-Kinnunen *et al*., 1997; Flemming *et al.,* 2007; Karatas *et al*., 2010 ; Suebsing *et al*., 2012).

2.4.2 The genus *Chryseobacterium*

There are numerous emerging *Chryseobacterium* species being discovered to be associated with diseased fish and a few studies have also been conducted to understand their pathology (Loch and Fasial, 2015). Some of the *Chryseobacterium* species documented as pathogens include; *C. viscerum, C. piscicola, C. chaponense, C. viscerum, C. balustinum, C. scophthalmum, C. oncorhynchi, C. aahli and C. indologenes* (Mudarris *et al*., 1994; Vandamme *et al.,* 1994) cited by Loch (2015). Chryseobacteria isolated from fish and are known to cause human infections include; *C. hominis, C. shigense, C. indologenes* in farmed yellow perch (*P. flavescens*) in the United States was confirmed to be pathogenic to perch via experimental challenge (Pridgeon *et al*., 2012).

2.4.3 The genus *Myroides*

Myroides spp. are gram negative aerobes and have been reported as opportunistic pathogens in humans, they however, had not been known to be fish pathogens until in 2015 (Chinnarajan *et al.,* 2015). *Myroides odoratimimus* was isolated from the gut of *Mugil cephalus* and showed potential infectivity to the experimental grey mullet. *Myroides odoratimimus* acted as an ultimate pathogen with significant symptoms in healthy juveniles of *Mugil cephalus*. Inoculum isolated from the infected fishes were cultured and selected colonies were reinjected into healthy juveniles of *Mugil cephalus*. Characterizations of the re-isolated bacteria were the same as those of the isolated *M. odoratimimus* from naturally infected mullet. Re-isolated *M. odoratimimus* came from the liver, muscle and gut of moribund fish. The *M. odoratimimus* infection and pathogenicity was confirmed with that of the innate immunity indicator tests such as respiratory burst activity and Super oxide dismutase activity effects with that of the challenged fish (Chinnarajan *et al.,* 2015).

2.4.4 The genus *Elizabethkingia*

Elizabethkingia meningoseptica has been isolated from various eukaryotes among which include dogs, amoebae, turtles, birds, frogs, cats, and fish (Bernardet *et al*., 2006). The first *E. meningoseptica* infection in fish was identified in farmed koi carp with hemorrhagic septicemia and skin lesions. Fish-associated members of the genus *Elizabethkingia* may represent as normal bacterial flora that colonize the mucus or belong to the surface of the skin and gills and intestine of healthy fish or as opportunistic pathogens or spoilage organisms (Bernardet *et al*., 2006).

2.5 Hosts of Flavobacteria

Organisms under family *Flavobacteriaceae* live in extremely diverse habitats which range from marine to fresh aquatic environments, foods, soils, processing plants, veterinary and human hospitals (Bernardet *et al*., 2006). Many organisms under family *Flavobacteriaceae* are pathogenic to many organisms including fish, plants, invertebrates, amphibians, reptiles, birds and mammals including humans (Segers *et al*., 1993; Haburjak and Schubert, 1997; Bernardet and Nakagawa, 2006; Xie *et al.*, 2009; Hernandez-Divers *et al*., 2009; Li *et al.*, 2010; Bernadet *et al*., 2011).

2.6 Culture and characteristics of *Flavobacteriaceae* organisms

According to Bernadet (1986), the characteristics of this family include; Gram negative short to long filamentous rods, non-spore forming, generally non-flagellated, motile via gliding or non-motile. Colonies strongly adhere to the surface of agar (for *F. columnare*), colony shape range from round and convex to flat and rhizoid. Most colonies contain a non-diffusible yellowish to orange pigment due to the presence of carotenoid and/or flexirubin although some do not pigment. The pigment presence can be detected using KOH whereby the colonies turn to pink or brown. They grow under aerobic conditions although some genera grow under micro-aerobic or anaerobic conditions (Bernadet *et al.,* 1996). Nitrate reduction may occur but typically, nitrates are not reduced. They are generally oxidase and catalase positive with some exceptions (Gosink *et al*., 1998). Most genera contain species that degrade proteins (e.g gelatin and casein), carbohydrates (e.g, esculin, starch, carboxymethylcellulose chitin pectin) and lipids like tween (Gosink *et al*., 1998). Species under this family may be mesophilic, halophilic, halotolerant or

psychrophilic. Fatty acids that are branched monounsaturated, branched saturated and branched hydroxy C_{15} to C_{17} are often present in large amounts (Gosink *et al*., 1998).

2.6.1 Genus *Flavobacterium*

Members under this genus *Flavobacterium* are Gram negative rods that range from1.0 to 40.0 μm in length and 0.3 to 0.5 μm in diameter. All species are non-motile or display gliding motility. Optimal growth occurs between 20 to 30°C although growth can also occur below 20°C. Psychrophilic species actually grow better in the range of 15-20°C. Colonies are pale to bright yellow pigmented and may contain non-fluorescent, non-diffusible, carotenoid and/or flexirubin pigments. *F. psychrophilum, F. columnare, F. branchiophilum* don`t grow on trypticase soy agars (TSA) but majority of *Flavobacteria* species grow on TSA and nutrient agar (Frankland and Frankland, 1889). *Flavobacterium spp*. vary in the ease with which they are cultured on microbiological media. Some freshwater fish-pathogenic species are fastidious and require special culture media like Sheih's medium, cytophaga agar, Hsu-Shotts medium, tryptone yeast extract salts medium (Anacker and Ordal, 1955; Bullock *et al*., 1986; Holt, 1987; Starliper. *et al*, 2007) cited by Loch and Fasial (2015). Some of these media and their derivatives are made more selective by incorporation of antibiotics like, tobramycin, polymyxin-B, neomycin, and/or vancomycin. The antibiotics avoid overgrowth by less fastidious bacteria that may also be present in an inoculum, especially from external lesions of fish. Most fish pathogenic Flavobacteria grow optimally between 15 to 25 °C (Bernardet *et al*., 2006).

2.6.2 Genus *Chryseobacterium*

Chryseobacterium spp. are gram negative straight rods with a width of about 0.5 μm and length that ranges between 1 and 3 μm. They are non-motile, non-flagellated and no gliding motility. They are generally catalase and oxidase positive. Colonies are flexirubin pigmented appearing pale to a bright golden yellow in color. *Chryseobacterium spp*. grow well at 4–42 °C on commercial media like, blood, nutrient, TSA, brain heart infusion, marine and Mueller Hinton agars (Bernardet and Grimont, 1989: Bernardet *et al.,* 2006), at salinities of up to 5%, depending upon the species. Most are resistant to numerous antibiotics and are strongly proteolytic (Holmes *et al*., 1923). *Chryseobacterium spp*. are resistant to a wide-ranging spectrum of antibiotics, including polymyxins, aminoglycosides tetracyclines, erythromycin, chloramphenicol, linezolid, and many β-lactams. They are also known to be intermediately sensitive to vancomycin and clindamycin and vary in their sensitivity to trimethoprim-sulfamethoxazole (Fraser and Jorgensen, 1997; Kirby *et al.,* 2004; Bernardet *et al*., 2006; Chou *et al.,* 2011).

2.6.3 Genus *Elizabethkingia*

This genus contains two species namely; *E. miricola and E. meningoseptica.* The characteristics of the members include; Gram-negative, aerobic, non-motile rods forming colonies that may display a light-yellow pigmentation or may be non-pigmented (Bernadet *et al*., 2006).

2.7 Diagnosis of bacteria under family *Flavobacteriaceae*

Davis, (1926) noted that affected fish displayed “dirty-white or yellowish areas” on the body, whereby lesions developed and caused death within 24–72 hrs. Fins (especially the caudal fin) were eroded and in more severe cases, only “mere stubs”

remained. There was also necrosis of the gills visible as white patches that spread rapidly, causing death. The author also observed mortalities in wild fishes of the Mississippi River associated with this bacterium (Davis, 1926).

2.7.1 The Genus *Chryseobacterium*

Presumptive identification of a *Chryseobacterium spp.* is often based upon phenotypic characters (e.g. Gram negative, non-motile rods that produce bright yellow colonies due to the presence of flexirubin-type pigments; possess oxidase and catalase activities; produce a *Chryseobacterium spp*. profile on commercial galleries (Bernardet *et al.*, 2005), after which a definitive identification is based upon polyphasic characterization, including biochemical, morphological, and physiological characterization, fatty acid profiling, and sequence/phylogenetic analyses (Bernardet *et al*., 2002). Additional techniques that have most recently been utilized to identify *Chryseobacterium spp*. include matrix assisted laser desorption ionization-time of flight mass spectrometry and PCR amplification of the 16S rRNA gene using universal bacteria primers 27f (5'-*AGAGTTTGATCCTGGCTCAG*-3 and 1492R (5'-*GGTTACCTTGTTACGACTT*-3') and internal transcribed spacer (ITS) rDNA and subsequent sequence analysis (Daroy *et al.*, 2011; Fernández-*Olmos et al*., 2012).

2.7.2 The genus *Flavobacterium*

In addition to bacterial culture and subsequent identification via phenotypic tests, many other means for detection and identification were developed. Whole-cell agglutination, fluorescent antibody tests, enzyme-linked immunosorbent assays, *in situ* hybridization, loop-mediated isothermal amplification, polymerase chain reaction (PCR), immunomagnetic separation in conjunction with flow cytometry, quantitative

PCR, and DNA array-based multiplex assay are used to detect and identify *F. psychrophilum, F. columnare,* and/or *F. branchiophilum* (Daroy *et al.,* 2011). It is noteworthy, however, that few diagnostic reagents specific for the lesser-known fish associated Flavobacteria exist, which makes their identification more difficult and laborious (Bernadet *et al*., 2002; Loch and Fasial, 2015).

2.7.3 The genus *Myroides*

Culture and biochemical characteristics of bacteria under genus *Myroides* may be used in the diagnosis. These are yellow pigmented colonies, gram negative rods that are oxidase and catalase positive, produce flexirubin pigments. They are non-fermentative bacilli and negative for indole production (Vancanneyt *et al*., 1996). PCR could also be used in the diagnosis of bacteria under genus *Myroides* (Daroy *et al.,* 2011).

2.8 Molecular characterization of bacteria based on 16S rRNA gene

The study of bacterial taxonomy and phylogeny using 16 S rRNA gene sequencing is the most common housekeeping genetic marker (Avendaño-Herrera *et al.,* 2006). This is because: (i) the function of the 16S rRNA gene has not changed over time. This indicates that random sequence changes are a more accurate measure of evolution (time). (ii) its occurrence in nearly all bacteria, often existing as operons or a multigene family. (iii) the 16S rRNA gene is large enough with 1,500 bp, which is good for informatics purposes (Avendaño-Herrera *et al.,* 2006). Use of the 16S rRNA is not perfect and thus mistakes may be found in gene sequencing leading to misidentifications of bacteria (Starliper, 2011). Misidentifications are due to the 16S rRNA gene sequencing having low phylogenetic power at species level and

unsatisfactory power for some genera of different bacteria species (USDA, 2003). Therefore, DNA relatedness and sometimes biochemical tests are required for absolute resolution to these classification problems (Starliper, 2011). Therefore, proper identification of microbial organisms using the 16S rRNA needs the use of harmonious set of guidelines. The recommendations made by Drancourt in 2000 have been recommended for including full 16S rRNA gene sequences whenever possible (Loch and Fasial, 2015).

2.9 Challenges when working with family *Flavobacteriaceae*

Many of the Flavobacteria pathogenic to fish are fastidious and grow only on poor nutrient media (Bernardet *et al*., 2006). Less fastidious bacteria have to be hindered from growing by adding antibiotics. Care has to be given to the osmotic conditions, ratio and brand of ingredients incorporated into these media, otherwise this can affect the ability to cultivate some Flavobacteria (Lorenzen, 1993; Cipriano, 1994; Michel, 1999) cited by Loch and Fasial (2015). Another critical factor is the incubation conditions like temperature and oxygen availability which otherwise could impede culture of the organisms. Some species have a slow generation time. Members of family *Flavobacteriaceae* are being discovered and described at a rapid pace which necessitates a wide and a constant search for literature to include new species and be up to date in the changes in nomenclature (Kämpfer *et al.*, 2009).

Flavobacteria are ubiquitous in aquatic habitats and on/in the skin, gills, mucus, and intestines of fish (Bernardet *et al*., 2006), whereby some isolates from healthy fish are also implicated as facultative fish pathogens. Therefore, the roles of many

Flavobacteria in fish health is not well understood (Anderson and Ordal, 1961; Mudaris and Austin, 1989). It may not be clear whether external Flavobacteria are transient inhabitants of fish or whether they are normal constituents of their bacterial flora. Whereas some new species are truly fish pathogens, others have been proposed to play a mutualistic role with their host like some *Chryseobacterium spp*. strains (Kämpfer *et al.,* 2009; Lauer *et al.,* 2007). There is difficulty with experimental challenge models to study pathogenicity of fish-pathogenic Flavobacteria (Liu *et al.,* 2001). Another challenge is the lack of specific diagnostic reagents needed to identify many fish associated Flavobacteria outside of those commonly associated with fish disease (Bernadet *et al*., 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The study area

The study was conducted on selected farms in the districts of Wakiso, Kampala, Lira, Arua, Nebbi and Kole (Kole is a new district that has just been formed from Lira district). These are in the Northern, west Nile and central part of Uganda. The government in partnership with Food and Agriculture Organization (FAO) launched a project to promote fish farming in West Nile and northern and central regions of Uganda. The government of Uganda is running a project to support fish farmers through fish farming in these districts through NAFIRI (National Fisheries Research Institute) at Kajansi hatcheries. Therefore, aquaculture is more in these areas, thus the selection of these districts as the study area for this research.

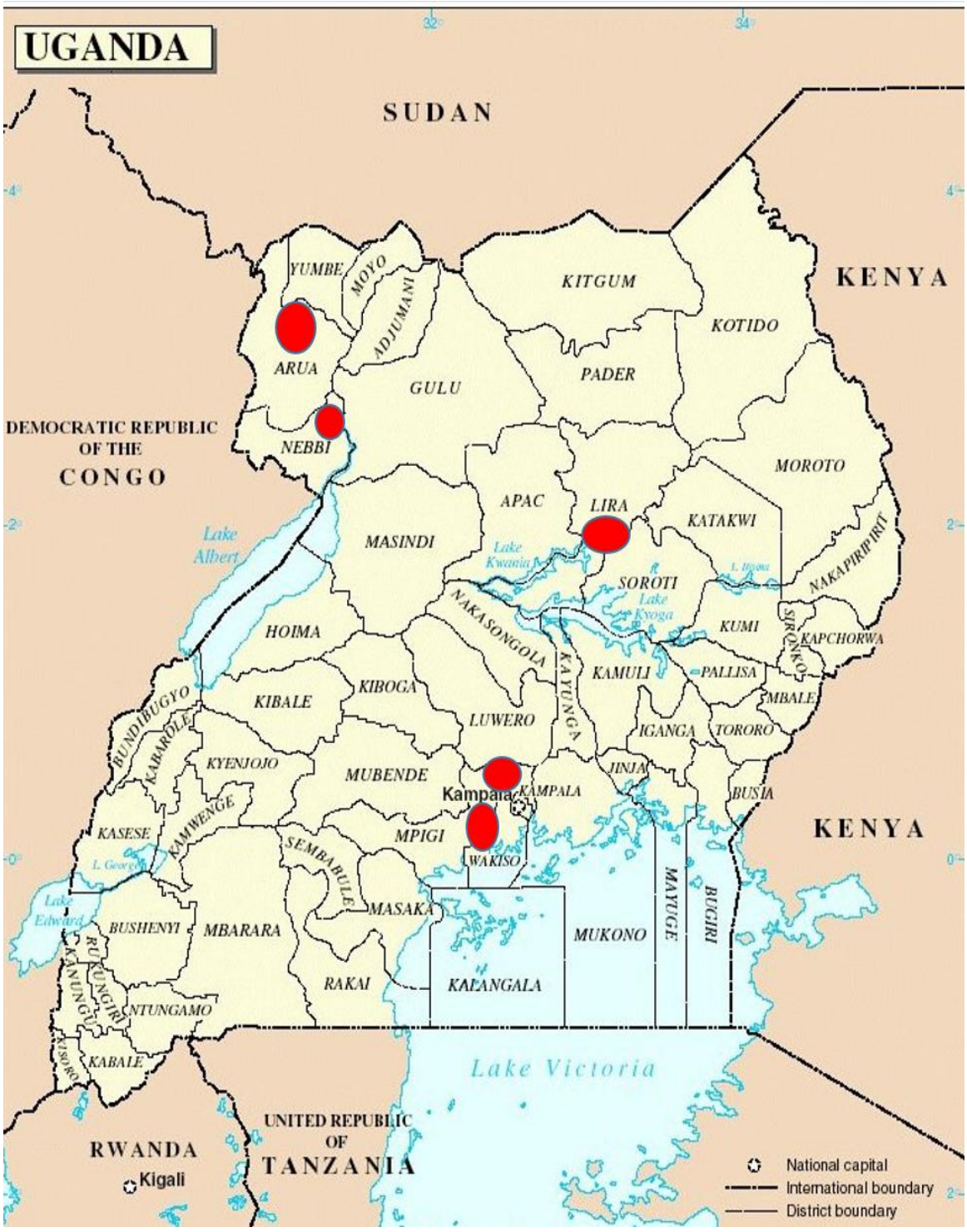


Figure 1: Map of Uganda and study area

3.2 Study design

This was a cross sectional study to isolate and identify *Flavobacteriaceae* isolates from African catfish and Nile tilapia in selected farms in Uganda. Bacteria were isolated from fish collected between October 2016 and March 2017. These were identified as Flavobacteria basing on growth colony characteristics (color, elevation, margin texture, colony consistency), biochemical tests and sequencing of the 16S rRNA gene.

3.3 Sampling

Convenience and purposive sampling techniques were used in this study. Purposive sampling was done based on disease history, presence of disease, availability of farms and accessibility to the farms. Disease presence was indicated by abnormal fish mortalities and fish lesions like ulcerations, hemorrhages, swollen belly, ophthalmia. Five fish per pond per fish species were targeted for those with both Tilapia and catfish. However, in some cases the number of sampled fish was less than 5 for catfish due to challenges in capturing them. A total of 119 fish were collected from 19 farms. Fish with abnormal lesions were given priority although asymptomatic fish were also sampled in case of no sick fish or insufficient number of symptomatic fish. Live fish in water troughs were transported to the College of Veterinary Medicine Animal Resources and Biosecurity (COVAB) Central Diagnostic Laboratory (CDL).

3.4 Isolation of bacteria under family *Flavobacteriaceae*

Samples of internal organs were taken aseptically including kidneys, liver, spleen and these were homogenized by cutting into smaller pieces using sterile surgical blade and then inoculated into sheih broth. Swabs were also obtained from skin, lesions and

gills using a sterile swab stick and inoculated on Shieh`s agar. The samples were incubated at 25 for 48 hours. Liver, kidney and spleen were pooled into Shieh broth for 24 hours before culturing on Shieh agar supplemented with tobramycin at a concentration of 0.001g/L.

3.5 Morphological identification of Flavobacteria colonies

The phenotypic characterization of the isolates was based on colony morphology and consistency, Gram staining, and standard biochemical tests. All yellow colonies were considered. Shieh agar and Shieh broth were made as in the table in the appendix 1. Cellular morphology was determined by Gram staining and viewed under a microscope whereby gram-negative rods were considered (magnification, x 100).

3.6 Identification of Flavobacteria by biochemical tests

Colonies were grown in peptone water for 48 hours and motility was determined under light microscope (magnification, x 100). Other biochemical tests included; presence of flexirubin type pigments using 1% KOH, cytochrome oxidase, catalase, TSI (Triple Sugar Iron Agar) tests (Sebastião1 *et al*., 2010). API 20NE test kits from Biomerieurix were also used both at Makerere University and Norwegian University of Life Sciences (NMBU) as screening tests to further identify some isolates before sequencing.

3.7 Molecular identification of Flavobacteria

The isolates were preserved on Sheih agar slants and transported at room temperature to the microbiology laboratory at the Norwegian University of Life Sciences. The bacteria were sub-cultured on agar (BHI agar media was used from DIFCO Laboratories, and Merck KGaA Germany and the suspected *Flavobacteriaceae*

colonies were divided into eight groups basing on colony morphology similarity (basing on colony color, size, elevation, margin) and one colony per group was selected for sequencing.

3.7.1 DNA extraction for Flavobacteria sequencing

Genomic DNA was extracted from the 8 selected isolates at the Gen-lab NMBU where further molecular analysis was performed. Genomic DNA isolation was done using QIAamp DNA mini kit (Qiagen). The manufacturer`s protocol was followed as stated in the appendix 2 and all spin steps used a bench top Minispin centrifuge.

3.7.2 PCR process for the extracted DNA

The 16S rRNA genes were amplified by PCR using universal bacteria primers 27f (5'-*AGAGTTTGATCCTGGCTCAG*-3 and 1492R (5'-*GGTTACCTTGTTACGACTT*-3').

Each PCR reaction was performed in a final volume of 25µL containing: 2.5µL of 10X reaction buffer (50MM, 75MM Tris-HCL pH 9.0), 2MM $MgCl_2$, 20MM $(NH_4)_2SO_4$), 0.5 µL. 10MM deoxyribonucleotide mix, 0.2 µL of DNA template, and 16.8 µL of sterile ultrapure water. PCR reactions were performed by icycler (from BIO-Rad) under the following conditions: Initial denaturation at 94 °C for 3 mins, followed by 30 cycles of amplification as follows; denaturation at 94°C for 30s, annealing at 56°C for 30s and extension at 72°C for 2mins, followed by a final extension step at 72°C for 5 minutes and left to stand at 4°C until analysis.

3.7.3 Electrophoresis

The PCR products were then run on 1% ultra-pure agarose (Invitrogen, Thermo Fisher Scientific) using Power Pac 300 (BioRad) at 100Volts for 60 minutes with Gene Ruler^{TM} 1 kb Ladder. The gels prestained with syberSafe (source) were

visualized using Safe Imager™ (Invitrogen) and bands of interest excised with a scalpel blade. Gel pictures were captured using ChemiDoc™ XRS Molecular imager (Bio Rad).

3.7.4 Purification of the PCR products and sequencing

The PCR products were purified using QIAquick Gel extraction kit (Quiagen) following manufacturer's instructions as stated in appendix 2. The Purified PCR were quantified, and quality checked using Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific inc.) and sent for sequencing by sanger sequencing technology and technique at GATC Biotech, Germany using the same primers as those used for PCR above.

3.8 Data analysis

Data was summarized and stored in Microsoft excel version 10. BLAST searches were done online to get similar sequences from the gene banks using NCBI website. The obtained sequences from isolates in this study were edited using bioedit and aligned with those retrieved from gene banks using *Claustal W* algorithm in MEGA version 7.0 software. The alignments were used to construct phylogenetic tree using Neighbor Joining method using Kimura-2-parameter model. Identification of the sequences was also done using EZBiocloud.net ID software online.

CHAPTER FOUR

4.0 RESULTS

4.1 Biodata for the sampled farms

Biodata sheet in appendix 4 was designed to collect information from the sampled fish farms. Table 1 below summarizes the 19 farms according to the status of the farmers (small scale or large scale), which was based on number of culture units (number of ponds/cages/tanks). It includes sources of water used by the 19 farms, the number / percentage of farms that had experienced a disease outbreak, species of fish cultured on the 19 farms and type of culture system(s) used on the 19 farms.

Table 1: Biodata of the selected farms

Status of farmer	**No. of units**	**Species of fish**	**Sources of water**	**History of disease**	**culture systems**
-16 small scale farms	-2 - 5 units for small scale	-koi carp -Silver carp	-lake -river -underground -streams	-5 farms (26.31%) with disease outbreak /history.	-13 farms with only earthen ponds
-3 large scale farms	-over 20 units for large scale	-African Catfish -Tilapia			-3 farms with only cages -2 farms with tanks and ponds -1 farm with tanks only

4.2 Symptoms encountered in the fish samples

Both symptomatic and asymptomatic fish were sampled and some of the lesions encountered in the symptomatic fish included: hemorrhages on skin, fins, barbells, yellow skin, skin erosions, swollen belly, eroded tail fin, pale liver. Figure 2 below shows some of the lesions.

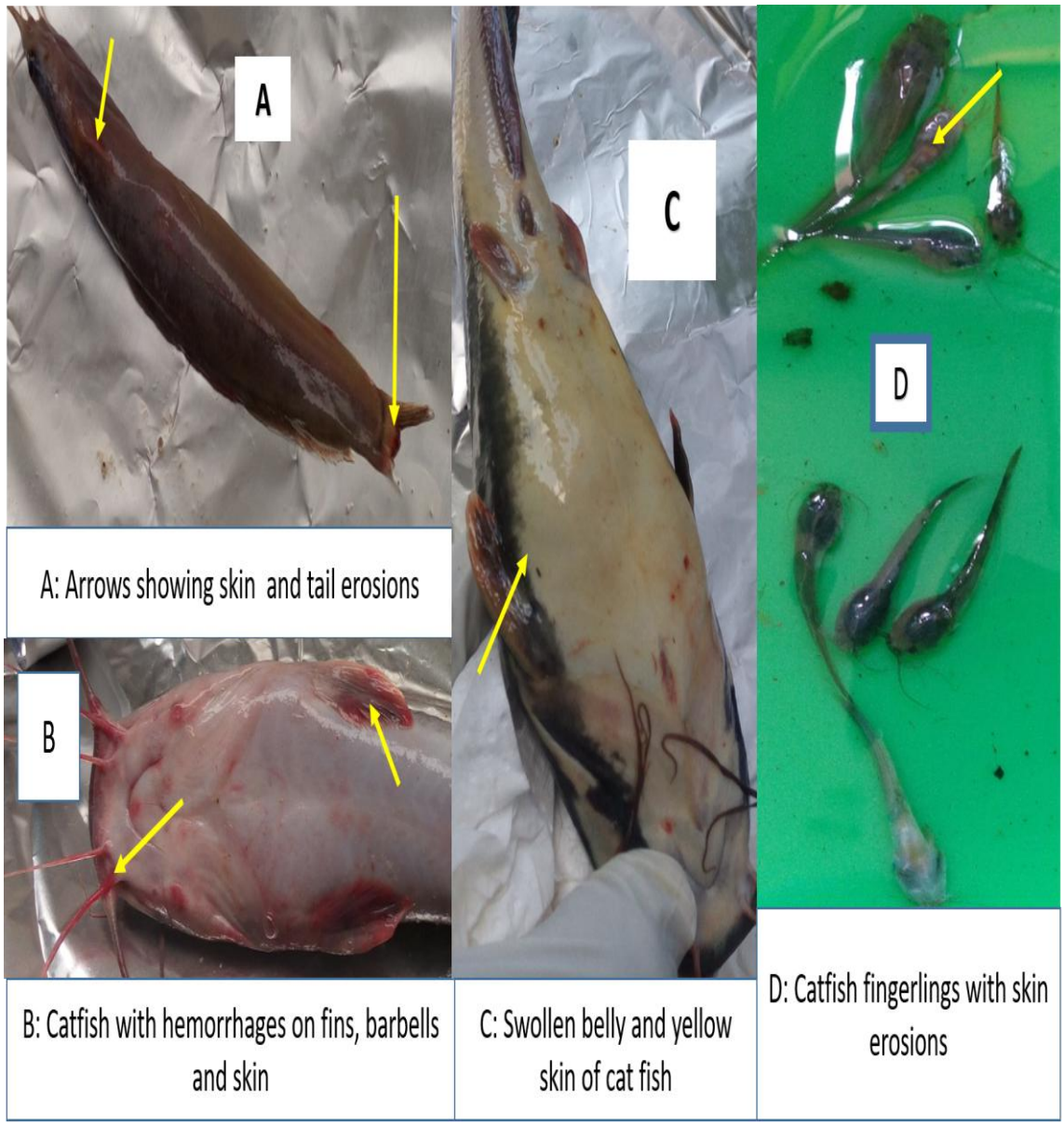


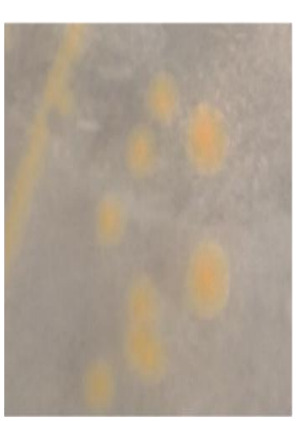
Figure 2: Lesions encountered on catfish

4.3 Culture and isolation of Flavobacteria

Culturing the pooled organs in Sheih broth followed by streaking the broth on Sheih agar always gave fewer types of colonies (sometimes only one) per sample compared to direct streaking of the gill and skin swabs on agar. A total of 86 isolates were got from the 119-fish sampled, with colonies ranging from pale yellow, bright yellow, deep yellow to orange and had a fruity odor and of varying sizes ranging from small to large. The 86 isolates were grouped into 8 groups based on colony growth characteristic similarities (color, elevation, margin texture, size of colonies) and one representative isolate from each group was considered for sequencing.

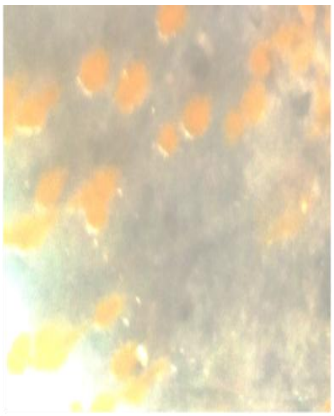
4.4 Colony characteristics

A total of 86 isolates were got with colonies ranging from pale yellow, bright yellow, deep yellow to orange and had a fruity odor and of varying sizes ranging from small to large. These were grouped into 8 and one colony per group selected. Figure 3 below shows some of the colonies selected for sequencing but missing the colony for isolate 4.



Isolate 1:Soft, sticky, bright yellow, flat, large size, smooth

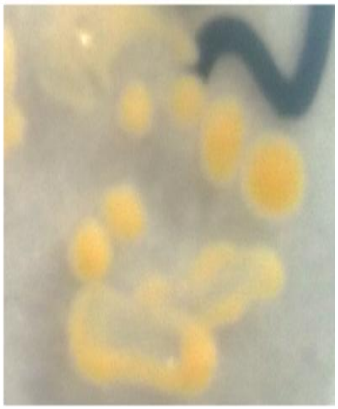
Isolate 2: soft, large, yellow, flat, gelatinous



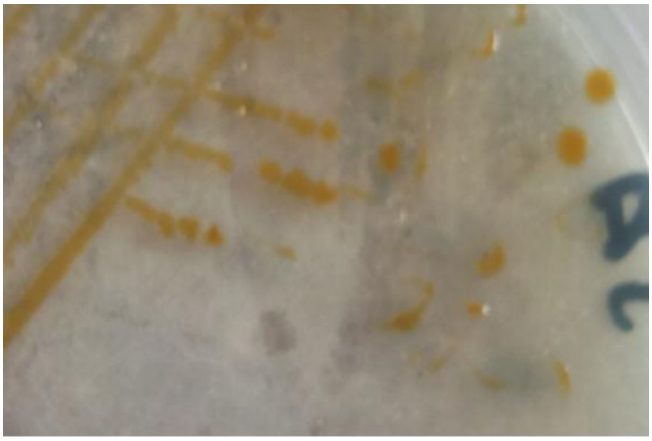
Isolate 3: Yellow medium size, flat, glistening, flat

Isolate 5: Pale yellow, round, flat, medium size, shinny

Isolate 6:Soft, sticky, yellow, flat, medium size, irregular



Isolate 7: Large, yellow, round, smooth, flat, soft



Isolate 8: Small, orange round, raised

Figure 3: Colony characteristics of the study isolates

4.5 Biochemical test results

4.5.1 Biochemical test results for the sequenced isolates

The biochemical tests results for gram reaction, catalase activity, oxidase activity, presence of flexirubin pigment, Congo red absorption, production of hydrogen sulphide (H_2S), gelatinase activity, indole production, motility, glucose and sucrose fermentation and gas production from glucose are summarized in table 2 below. Some colonies produced H_2S but after storage and sub-culturing and their TSI test did not give off H_2S.

Table 2: Biochemical test results of the sequenced isolates

Isolate	Catalase	Oxidase	Flexirubin Pigment	Congo red absorption	H_2S	Urease	Gelatinase	Indole productio	Motility	Glucose fermentati	Gas off glucose	Sucrose fermentati
Isolate 4	(+)	(+)	(+)	(+)	_	_	(+)	_	_	_	_	_
Isolate 1	(+)	(+)	(+)	(+)	_	(+)	_	(+)	_	_	_	(+)
Isolate 5	(+)	(+)	(+)	(+)	_	_	_	_	_	_	_	_
Isolate 6	(+)	(+)	(+)	(+)	_	_	(+)	_	(+)	_	_	_
Isolate 3	(+)	(+)	(+)	(+)	_	(+)	(+)	_	_	_	_	_
Isolate 7	-	(+)	(+)	(+)	_	(+)	_	_	_	_	_	_
Isolate 8	(+)	(+)	(+)	(+)	_	_	(+)	_	_	(+)	(+)	_
Isolate 2	(+)	(+)	(+)	(+)	_	(+)	(+)	_	_	_	_	_

4.5.2 General biochemical test results for the groups

Table 3 below summarizes the biochemical test results of the isolates in the groups from which the sequenced isolates were obtained. Some groups had only one isolate (i.e groups 6 and 5) while one group had two isolates (group 8). The group from which isolate 8 was got had two isolates but biochemical tests results of the other isolates are missing. Isolate 8 thus has a star in the table below to indicate missing results.

Table 3: General biochemical test results of the groups

Representative Sequenced isolate	**1**	**2**	**3**	**4**	**5**	**6**	**8***
No. of isolates in the group	14	3	30	13	1	1	2
Flexirubin	92.9 (+)	100 (+)	93.3 (+)	76.2 (+)	(+)	(+)	(+)
Catalase	100 (+)	100 (+)	96.7 (+)	100 (+)	(+)	(+)	(+)
Oxidase	85.7 (+)	66.7 (-)	86.7 (+)	70.0 (+)	(+)	(+)	(+)
Congo red	100 (+)	100 (+)	93.3 (+)	70.0 (+)	(+)	(+)	(+)
Urease	100 (+)	100 (+)	60.0 (+)	76.9 (+)	(-)	(-)	(-)
TSI	92.9 (-)	100 (-)	83.3 (-)	84.6 (-)	(-)	(-)	(+)
H2S	100 (-)	100 (-)	96.7 (+)	100 (-)	(-)	(-)	(-)
Gliding motility	92.9 (-)	66.7 (-)	93.3 (-)	84.6(-)	(+)	(-)	(-)
Indole production	71.4 (-)	100 (-)	70.0 (-)	53.8 (-)	(-)	(-)	(-)
Gelatin hydrolysis	50.0(+)	100 (+)	73.3 (+)	92.3 (+)	(+)	(+)	(+)
Glucose fermentation	92.9 (-)	100 (-)	96.7 (-)	92.3 (-)	(-)	(-)	(+)
Gas from glucose fermentation	100 (-)	100 (-)	100 (-)	92.3 (-)	(-)	(-)	(+)
Sucrose fermentation	92.9 (-)	100 (-)	93.3 (-)	92.3 (-)	(-)	(-)	(-)

4.5.3 API test results

The API test results shown in the table below were for some selected isolates most of which were not sequenced directly or did not regrow on sub culturing thus. Those that were not sequenced are named by letters and a few of which were included in the groupings from which a representative isolate was chosen for sequencing. Some isolates tested using the API 20NE kits gave codes which had unacceptable profiles and therefore were not identified as shown in table 4 below

Isolates A, B, C, D, E and F did not grow on sub-culturing while isolates G, H and I were not sequenced but were included in the groupings.

Table 4: API 20NE results

ISOLA-TE	GROUP	IDENTIFICATION	PERCENTAGE IDENTIFICATION
A	NR	Unacceptable profile	N/A
B	NR	Unacceptable profile	N/A
C	NR	Unacceptable profile	N/A
D	NR	*C. indolgenes*	90.6
E	NR	*Acinetobacter sp.*	60
F	NR	*C. indolgenes*	99.9
G	1	*Myroides sp.*	64
H	1	*Weeksiela sp.*	37
1	1	*Myroides sp.*	64
I	3	*C. indolgenes*	49

KEY: NR- Not represented in the groupings since did not grow on sub-culturing

N/A- Not applicable

The API test results for isolates 1 and G at 64 gave a correct genus identification even though the percentage identity was still considered low while for isolates H and I whose percentage identification were below average, and the identification was not correct.

4.5.4 Comparison of conventional and API 20NE biochemical test results

The biochemical tests compared between the conventional laboratory method and the API 20NE kits were glucose fermentation, presences of urease activity (URE), gelatin hydrolysis (GEL) by gelatinase, oxidase activity (OX) and indole production (TRP). There were minimal differences in the test results observed between the two methods (not more than two tests out of the five tests per isolate) as observed in the table 5 below.

Table 5: Comparison of API 20NE and conventional tube test results for selected isolates

ISOLATE	Test method	GLU	URE	GEL	OX	TRP
A	API	(-)	(+)	(+)	(+)	(+)
	Conventional	(-)	(-)	(+)	(+)	(+)
B	API	(+)	(-)	(+)	(+)	(+)
	Conventional	(-)	(+)	(+)	(+)	(+)
3	API	(-)	(+)	(+)	(+)	(-)
	Conventional	missing	(+)	(+)	(+)	(-)
C	API	(-)	(-)	(+)	(+)	(+)
	Conventional	(-)	(-)	(-)	(+)	(+)
D	API	(-)	(-)	(+)	(-)	(+)
	Conventional	(-)	(-)	(+)	(+)	(+)
E	API	(-)	(+)	(+)	(-)	(-)
	Conventional	(-)	(+)	Missing	(+)	(+)
F	API	(-)	(+)	(+)	(-)	(+)
	Conventional	(-)	(+)	(+)	(+)	(+)
1	API	(-)	(+)	(+)	(-)	(-)
	Conventional	(-)	(+)	(+)	(+)	(+)
G	API	(-)	(+)	(+)	(-)	(-)
	Conventional	(-)	(+)	(+)	(+)	(-)
H	API	(-)	(-)	(+)	(-)	(-)
	Conventional	(-)	(+)	(+)	(+)	(-)
I	API	(-)	(+)	(+)	(-)	(-)
	Conventional	(-)	(+)	(+)	(+)	(-)

4.6 Electrophoresis results

The figure 4 below shows the electrophoresis results with the bands of sizes of approximately 1500bp (indicated by an arrow) obtained using universal bacterial primers 27F and 1492R.

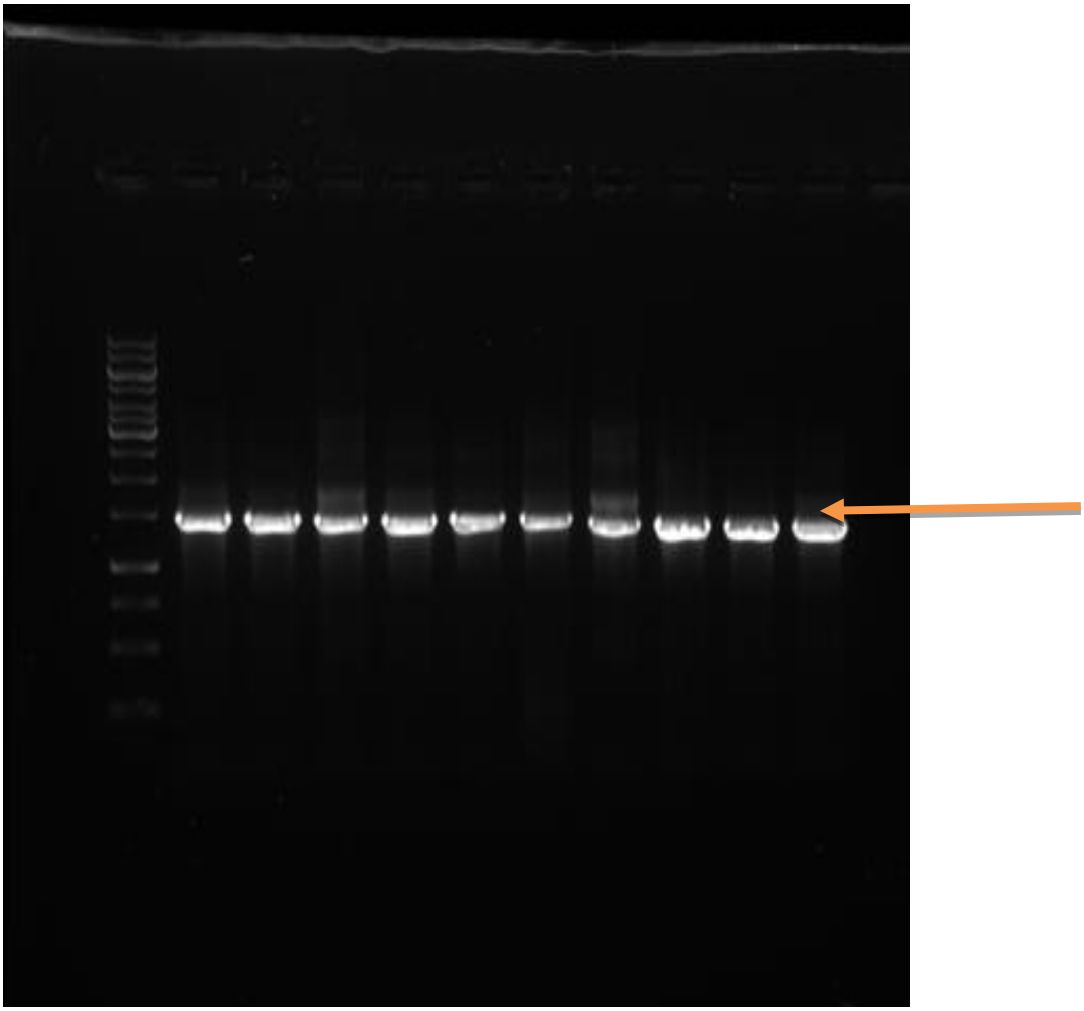


Figure 4: Electrophoresis results for the 16S rRNA gene

4.7 Identification and occurrence of the study isolates

4.7.1 Identification of isolates using Ezbiocloud.net

The commonest species that was isolated was *M. marinus* and the closest strain to the isolates was *M. marinus* JS 08 (GQ857652) at a percentage similarity of 99.0 to 99.79% (for the different group isolates) using Ezbioclod.net. These were isolated on 15 farms out of the 19 sampled farms. The least common species isolated were those closely similar to *M. odoramitimus*, with closet strain as *M. odoratimimus* CCUG 39352 at percentage similarity of 86.7% and *C. gambrini* with closest strain as *C. gambrini* DSM 18014 at a percentage similarity of 98.37 to 97.82% (for the different selected isolates) using Ezbiocloud.net.

Table 6 below shows the identification of the isolates, the health status, species of fish (*Oreochromis niloticus* (O.n) or *Clariaus gariepinus* (C.g) and site of fish from which they were isolated, culture system and water source of the farms from which the isolates were obtained.

Table 6: Identification of isolates and their occurrence in fish

Isolate	Status of fish	Percentage similarity and Closest strain using EZBiocloud.net	Species of fish	Site on sampled fish	Culture System	Water source
5	Symptomatic fish	*Chryseobacterium gambrini* DSM 18014 (98.37%)	O.n	Pooled organs, skin, gills	Pond	stream
1	Symptomatic and symptomatic	*Myroides marinus* JS 08 (99.49%)	Cg	Pooled liver, spleen, gills	Tank, pond	Rain, tap water
3	Assympt-omatic	*Myroides marinus* JS 08 (99.0%)	Cg, O.n	Pooled kidney, liver, Spleen, Skin Gills	Pond	Stream
6	Assympt-omatic	*Myroides odoratimimus* CCUG 39352 (86.7%)	O.n	Pooled organs, liver, spleen Kidney	Pond, Tank	Lake
8	Symptomatic and symptomatic	*Chryseobacterium gambrini* DSM 18014 (98.19%)	Cg, O.n	Pooled organs, liver, spleen Kidney	Tank	Tap water, rain water,
2	Assympt-omatic	*Myroides marinus* JS 08 (99.79%)	Cg, O.n	Gills and skin	Pond	Stream
7	Symptom-atic fish	*Acinetobacter pittii* CIP 70.29 (99.36%)	O.n	Gills, skin	Pond, cage	Lake
4	Assympt-omatic fish	*Myroides marinus* JS 08 (99.79%)	Cg	Pooled organs, liver, spleen, kidney	Pond, tank	Lake

4.7.2 Identification of the 86 isolates

Figure 5 below shows the composition of the isolates based on extrapolation of the results of the sequenced isolates.

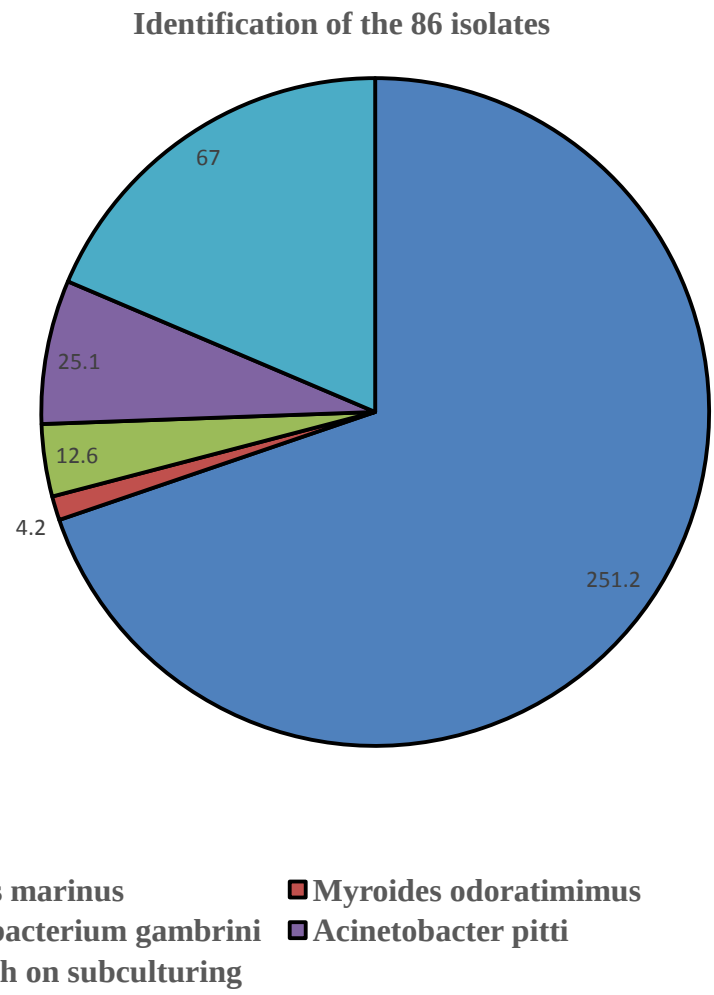


Figure 5: Identification based on the extrapolation of results of sequenced isolates

4.7.3 Occurrence of Flavobacteria on the farms

Out of the 19 sampled farms, *Myroides marinus* was the commonest while the unidentified isolate was the least common. The isolates were distributed on the farms as summarized in figure 6 below.

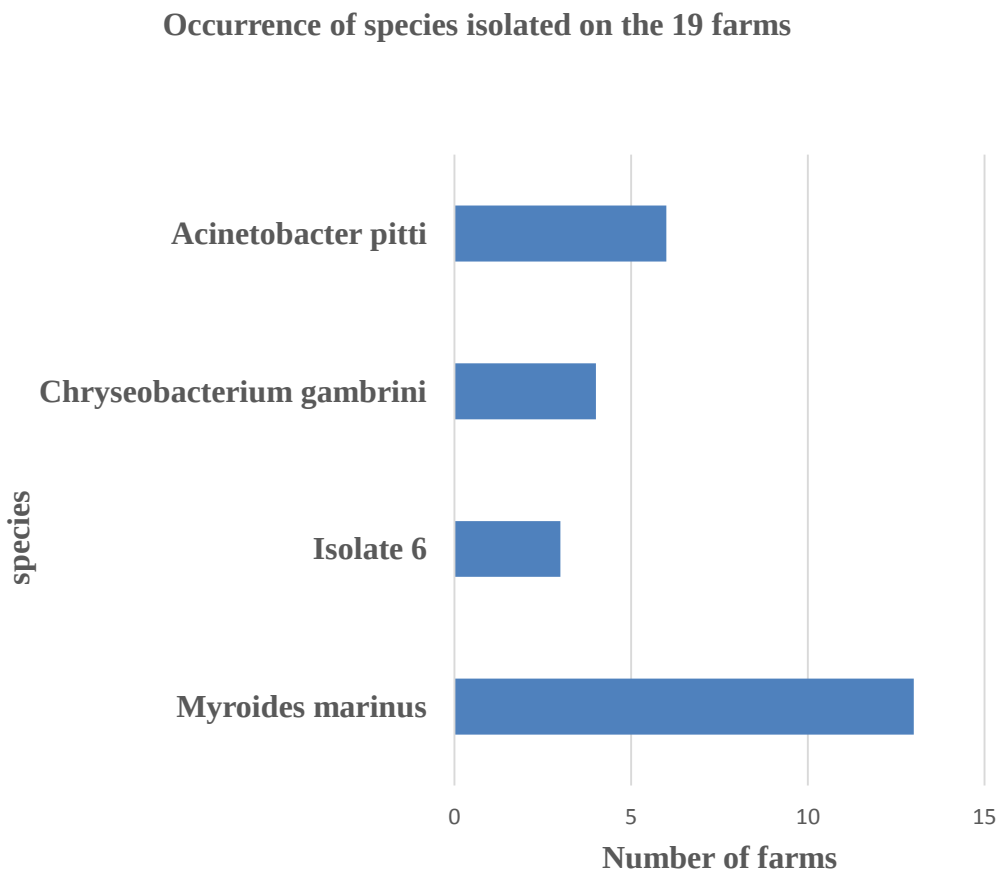


Figure 6: Occurrence of isolates on the selected farms

4.8 Phylogenetic analysis

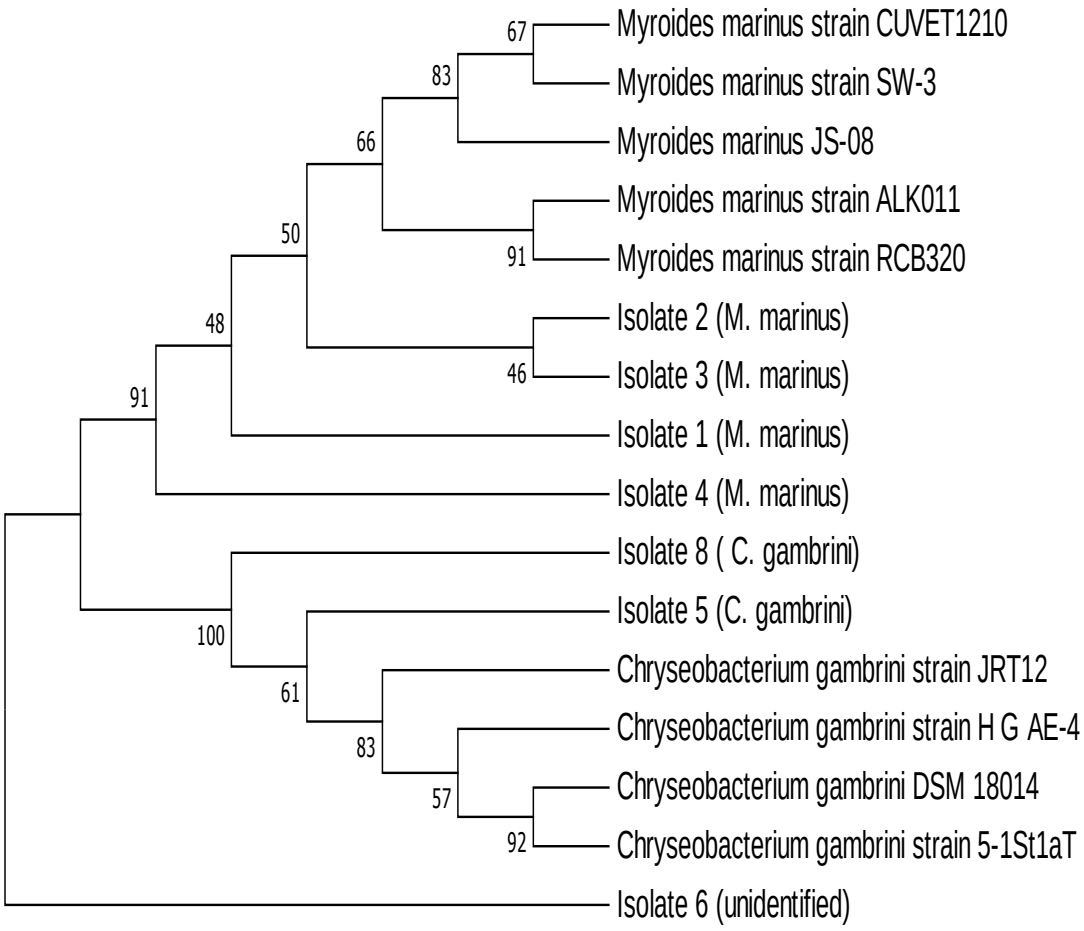


Figure 7: Phylogenetic relatedness of the isolates based on the 16S rRNA gene

Key: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.51734957 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura,1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 989 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, 2016).

The isolates 1,2,3 and 4 were grouped with the other *M. marinus* isolates obtained from the gene bank. Isolate 2 and 3 were more closely related to each other and to the reference strain *M. marinus JS 08* compared to isolate 1 and 4. Isolate 4 was furthest from the reference strain of all the *M. marinus* isolates. Therefore, there is diversity in the phylogenetic relatedness between the isolates 1,2,3 and 4. Isolate 6 did not cluster with any of the other isolates. Isolates 8 and 5 were grouped with the other *C. ganbrini* isolates obtained from the gene bank. Isolate 5 was more closely related to the reference strain compared to isolate 8.

The graphical views showing comparison of the isolates to their reference strains are shown in appendix 4 below. The isolates 1,2,3 and 4 differed from the reference strain *M. marinus JS 08* GQ857652 at regions between 221 and 223, 591, but most especially between 1097 and 1302. Isolate 4 had the greatest differences of the four isolates. The isolates 5 and 8 differed from the reference strain *C. ganbrini* JGI1096583 in the regions between 270 and 277, 978 and 996. Isolate 8 had more nucleotide differences to the reference strain compared to isolate 5.

CHAPTER FIVE

5.0 DISCUSSION

Flavobacteria are some of the major fish pathogens of importance in aquaculture worldwide (Loch and Fasial, 2015; Bernardet *et al*., 2006; Starliper, 2011; Shotts and Starliper, 1999; Nematollahi *et al.,* 2003; Wakabayashi *et al*., 1989). Previous studies in Uganda by Walakira *et al.* (2014) indicated that *F. columnare* had a high prevalence in the selected farms in central and western Uganda. This study determined the occurrence of Flavobacteria in fish farms and their molecular characterization as a way to better understand the Flavobacterial diseases.

In this study, all the selected farms had at least one bacterium from the family *Flavobacteriacea* isolated and some had more than one colony type of the isolates. Some of these Flavobacteria like the *Myroides species,* have potential to cause disease in laboratory experiments but have not yet been reported to cause disease in the natural (Chinnarajan *et al.,* 2015). Sixteen isolates did not grow on sub culturing and thus were not represented in the sequencing of the selected isolates in this study. The exact reason why they would not regrow was not known but there is a probability transportation conditions could have caused their death. Most isolates were found in both tilapia and catfish especially from gills and skin swabs. This is not surprising given the fact that Flavobacteria are ubiquitous, common in the environment and thus could have been due to contamination from the environment. Flavobacteria can be found in diverse habitats, including; freshwater streams, lakes, marine environments and sediments and are so numerous, still emerging under different genera every other year (Loch and Fasial, 2015; Qu *et al.* 2009; Lee *et al*., 2010; Yoon *et al.* 2011).

Many genera have emerging pathogens that include *Chryseobacterium, Tenacibacterium, Ornithobacterium, Elizabethkingia* and these include pathogens of reptiles, humans, birds, mammals and those of fish health importance (Loch and Fasial, 2015).

Seven out of the eight selected representative isolates in this study were closely related to family *Flavobacteriaceae*, grouped under the genera *Myroides* and *Chryseobacterium* as shown by the phylogenetic tree in figure 7. These are some of the genera with the commonest species that have been reported to be associated with sick fish and even causing disease in fish (Loch and Fasial, 2015). Blast results of the sequences of the isolates got in this study were closely similar to many other Flavobacteria isolates from soil, water, sewage, earthworm, plants, humans and aquatic animals including fish. Flavobacterial fish pathogens are known to be opportunistic and thus take advantage of stress in fish otherwise are not troublesome in their environments and hosts.

Sixteen out of the 19 farms in this study were small scale farms some of which were getting water source from the wild. Previous studies of problems facing small scale farmers in Asia, Particularly Thailand ranked disease second to lack of funds (Chinabut *et al*, 2002). The lack of funds is somehow associated with creating stressful conditions especially due to the associated poor water quality issues like limited oxygen, high nitrogen wastes and increased water turbidity. Lack of funds could lead to poor water quality issues through limiting availability of good water quality management equipment and services. Poor water quality brings about stress and this makes opportunistic pathogens like in family *Flavobacteriacea* to take advantage and cause disease.

The isolates in this study were grouped into 8 based on colony morphology similarity and only one per group was sequenced. This was due to limited resources, but it would have been better if each isolate had been sequenced and identified individually because there is a possibility that different species or strains were grouped together. Some isolates identified as same species were morphologically different (figure 3) and had some differences in their biochemical reactions for the tests that were carried out (table 2), for example, isolates 1, 3, 2 and 4 that still turned out to group with the reference strain *Myroides marinus* JS 08 (bootstrap values above 60%) and were identified as *Myroides marinus* (table 6).

The colony morphological and biochemical differences could be due to differences in the strains which was not well studied here. The fact that some of the isolates had phylogenetic relationship and yet were found in different farms in different parts of the country, could be an indication of similar source. Most of the sampled farms had previously received fingerlings from Kajansi through a government project to support fish farmers in Uganda, thus could be a common source. Isolate 6 was not closely related to any of the other isolates in this study, not even to *M. odoratimimus* which was the closest possible species. Although the closest strain was *M. odoratimimus,* the percentage similarity of 86.7% is low and thus the isolate is a bacterium probably not under family *Flavobacteriaceae.*

Isolate 7 although with colony and biochemical characteristics similar to Flavobacteria, was identified as *Acinetobacter pitti* using EZtaxon ID software. The biochemical tests of many colonies in this study tentatively suggested *F. columnare* but were ruled out by the API kits and 16S rRNA gene sequencing. There were differences in the biochemical characteristics of isolates between and within the

groups formed as shown in tables 3 and 4 above. This could be because of differences in species or strains among the isolates in each group. The colony characteristics (color, size, elevation colony margins) similarity used to group the isolates is not sufficient to differentiate the bacteria species or strains of Flavobacteria. For example, isolates 1, 2 and 3 were all identified as *M. marinus* but have different colony growth characteristics as shown in figure 3. Graphical views in the appendix 3 revealed differences in their nucleotides between the isolates 1, 2, 3 and 4 and thus could be due to differences in the strains.

Similarly, isolates 5 and 8 where both identified as *C. gambrini* but had differences in biochemical test results for example isolate 8 fermented glucose, produced acid on TSI and did not have gliding motility while isolate 5 did not ferment glucose, no acid production in TSI and had gliding motility.

API 20NE kits when used in this study could rule out *F. columnare* even though morphological and biochemical tests suggested otherwise. The comparison of identification by API kits and 16s RNA gene sequencing was not well studied here, although both API kits and 16S RNA gene sequencing did not identify any of the major Flavobacteria. The API test results for isolates G and I at 64% identity gave a correct genus identification even though the percentage identity was still considered low while for isolates H and I whose percentage identification were below average; the identification was not correct compared to sequence identification. The API results in this study had generally low percentage identities and were not reliable. Adley and Saieb (2005) compared biomeriueux API 20NE and Remel RapiD NF Plus in the identification systems of type strains of *Ralstonia picketti*. Only 29 out of 48 isolates were identified and the API 20NE was considered inconsistent. However, use

of API kits (API NE and API ZYM) in a study by Farmer proved to be useful in the identification of *F. columnare* (Farmer, 2004). API NE kits when used in this study could rule out *F. columnare* even though colony morphology on sheih agar and biochemical tests suggested otherwise. The comparison of identification by API kits and 16s RNA gene sequencing was not well studied here, although both API kits and 16S RNA gene sequencing did not identify any of the major Flavobacteria. There were minimal differences in the five test results observed between the two methods (not more than two tests out of the five tests per isolate) as observed in the table 5. However, the number of samples tested, and the number of the biochemical tests compared were both too small to be reliable for a conclusion.

The findings in this study have differed from those of the previous studies done in Uganda which have indicated a high incidence of *F. columnare* (Walakira *et al*., 2014). In this study, there is however a high occurrence of bacteria under family *Flavobacteriaceae* with the exception of *F. columnare*. There is a possibility that the presumed *F. columnare* in Walakira *et al.* (2014) study could have been different species under the different genera of family *Flavobacteriaceae*. The physiological, morphological and biochemical analysis of the suspected *F. columnare* colonies in that study probably led to a misdiagnosis. The diagnosis of lesser – known Flavobacteria in fish is difficult and laborious (considering *F. columnare, F. branchiophilum* and *F. psychrophillum* as the major Flavobacteria (Loch and Fasial, 2015). This is because there are few diagnostic reagents specific for the lesser-known fish associated Flavobacteria organisms. Diagnosis is further made more difficult by the fact that Flavobacteria are being discovered at a high rate and their classifications keep on changing (Bernardet *et al.*, 1996; Qu *et al.*, 2009; Lee *et al*., 2010, Yoon *et*

al., 2011; Loch and Fasial, 2015). Varga *et al.* (2016) similarly conducted a survey for incidence of *F. columnare* in wild and cultured freshwater fish species in Hungary. Twenty-five isolates from wild and cultured freshwater fishes were identified as *F. columnare* by specific PCR. However, both the fragment lengths and the results of PCR-RFLP genotyping with BsuRI (HaeIII) and RsaI restriction enzymes were not convincing enough regarding *F. columnare* classification. Sequencing of the 16S ribosomal RNA gene revealed that 23 isolates belonged to the species *F. johnsoniae* and two represented *Chryseobacterium spp*. thus showing that misidentification of Flavobacteria is easily possible (Varga *et al.,* 2016).

Similarly, some Flavobacteria isolates recovered by Loch in his study, did not have descriptions of characterized Flavobacteria and yet they were recovered from fishes with symptoms of Flavobacteriosis (Loch, 2014). Therefore, the fact that different Flavobacteria including the uncharacterized Flavobacterial species cause similar lesions in fish and other aquatic animals makes diagnosis of these species more complex. It is possible that morphological, biochemical and physiological tests are not sufficient to confirm the pathogen *F. columnare*.

The commonest of the Flavobacteria isolated in the selected farms in this study was *M. marinus* as indicated in the table 3 and Figures 2 and 3 above. The isolates were got from both symptomatic and asymptomatic fish for example isolates (table 6 above). This coincides with the research by Davis who isolated Flavobacteria from fish that did not show any gross pathological lesions (asymptomatic), but only had microscopic gill lesions (Davis, 1927; Davis, 1926). Clinical signs in the symptomatic fish included skin erosions, hemorrhages, yellowing of the skin, swollen

belly and fin erosions as shown in figure 2. Some of the isolates from symptomatic fish with skin erosions for example isolates 1 and 8 were recovered from catfish fingerlings (*Clariaus gariepinus*) that were reported to be experiencing abnormal mortalities for a week. The isolate 8 was identified as *C. gambrini.* Loch in his study stated that *Flavobacterium sp.* and *Chryseobacterium spp* were a major cause of fry and fingerling mortalities in Michigan State (Loch, 2014). For this case however, it requires further experimental studies to tell if the isolates were the causative agents for the skin erosions and death of the catfish fingerlings since there is a possibility of mixed infection.

A previous study by Loch has shown different Flavobacteria species being isolated from both symptomatic (with hemorrhages, skin and fin erosions, gill necrosis) and asymptomatic fish, some of which were just emerging fish pathogens (Loch, 2014). Other than the three-main fish disease causing Flavobacteria, other emerging Flavobacteria have also been found to cause hemorrhages, erosions on the skin and fins (Loch and Fasial, 2015). The Original Flavobacteria known to be causing fish health issues were the *F. columnare, F. branchiophilum, F. psychrophilum* but there are many other *Flavobacteriacea* causing disease in fish. The newly identified Flavobacteria vary in the degree of virulence for example, Flavobacteria isolates were proposed as a novel species, whereby *C. aahli sp*. nov., was found to be mildly pathogenic to fish under laboratory conditions while *F. spartani sp*. nov., was rather more pathogenic (Loch, 2014). Thus, it is important to study pathogenicity of emerging Flavobacteria.

Some farmers reported poor growth of fish. This could be due to many other factors that could include but not limited to poor management, genetic factors, reproduction in Tilapia and diseases. However, Flavobacteriosis is one of the diseases that could lead to poor growth of fish that survive the infection. Acute Flavobacteriosis was reported to contribute to poor growth in fish that survive which sometimes present with spinal abnormalities (Austin and Austin, 2007).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

I. All the sampled farms had at least one isolate of Flavobacterium from Tilapia and/ or Catfish. *Myroides marinus* was common in the selected farms in this study isolated on 13 farms which is 68.4% of the 19 farms. However, *C. gambrini* (on 4 farms) and the unidentified isolate 6 (on 3 farms) were not very common in the selected farms.

II. None of the major Flavobacteria (*F. columnare, F. branchiophilum* and *F. psychrophillum*) was identified in this study.

III. The routinely used biochemical and morphological growth characteristics were not sufficient to identify Flavobacteria.

IV. Phylogenetic analysis indicated that *M. marinus* isolates grouped with other *M. marinus* isolates from gene bank although intra-species diversity was observed, a similar situation observed with *C. gambrini* isolates.

6.2 Recommendations

i) Sequencing and characterization of individual isolates should be done for better identification.

ii) Wider research should be done to establish the prevalence of Flavobacteria and Flavobacteriosis in Uganda.

iii) There is need to conduct Pathogenicity and virulence studies of the isolates in this study.

iv) There is need to study the relationship between the water quality and other stress factors on fish farms and occurrence of Flavobacteria.

v) There is a need to study the strains of these isolates.

vi) Most of the closely related isolates were obtained from the gills and skin which could be due to contamination from the environment, therefore there is a need to study environmental factors in relation to the different *Flavobacteriacea* isolated in this study.

REFERENCES

Abowei, J. F. N. And Briyai, O. F. (2011). A Review of Some Bacteria Diseases in Africa Culture Fisheries. *Asian Journal of Medical Sciences* 3(5): 206-21.

Adley, C. C. and Saieb, F. M. (2005). Comparison of biomeriueux API 20NE and Remel RapiD NF Plus, identification systems of type strains of *Ralstonia pickettii*. *Letters in Applied Microbiology* 41(2): 136-140.

Aly, S. M. (2013). A Review of Fish Diseases in the Egyptian Aquaculture Sector; Working Report. *Canal University, Ismailia*, Egypt 65:42.

Anacker, R. L. and Ordal, E. J. (1955). Study of a bacteriophage infecting the *myxobacterium Chondrococcus columnaris*. *Journal of Bacteriology* 70(6):738.

Anderson, R. L. and Ordal, E. J. (1961). *Cytophaga succinicans* sp. n., a facultatively anaerobic, aquatic myxobacterium. *Journal of Bacteriology* 81(1):130.

Austin, B. and Austin, D. A. (2007). Bacterial fish pathogens: diseases of farmed and wild fish, 4th ed. Praxis Publishing Ltd.; United Kingdom. pp. 81-183.

Avendaño-Herrera, R., Toranzo, A. E. and Magariños, B. (2006). Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Diseases of Aquatic Organisms* 71(3):255–266.

Barbeyron, T., L'Haridon, S., Corre, E., Kloareg, B. and Potin, P. (2001). *Zobellia galactanovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and classification of *Cytophaga uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 51(3):985–97.

Bernardet, J.-F., Hugo, C. J. and Bruun, B. (2011). Bergey's manual of systematic bacteriology; Genus VII. *Chryseobacterium*. New York. 4:180–196.

Bernardet, J-F. and Nakagawa, Y. (2006). The prokaryotes: An introduction to the family *Flavobacteriaceae*. Springer-Verlag., New York 7:455–480.

Bernardet, J-F. and Bowman, J. P. (2006). The prokaryotes: a handbook on the biology of bacteria (The genus Flavobacterium). Springer-Verlag., New York, 7:481–531.

Bernardet, J-F. and Grimont, P. A. (1989). Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. Rev. *International Journal of Systematic Bacteriol*ogy 39(3):346–354.

Bernardet, J-F., Hugo, C. and Bruun, B. (2006). The genera *Chryseobacterium* and *Elizabethkingia* the prokaryotes: a handbook on the biology of bacteria, 3rd edition. Springer., 638–76.

Bernardet, J-F., Nakagawa, Y. and Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *International Jounal of Systematic and Evolutionary Microbiology* 52(3): 1049–1070.

Bernardet, J-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. and Vandamme, P. (1996). Cutting a Gordian knot: Amended classification and description of the genus *Flavobacterium*, amended description of the family *Flavobacteriaceae* and proposal of *Flavobacterium hydatis* nom. nov. *International Journal of Systematic Bacteriol*ogy 46(1):128–148.

Bernardet, J-F., Vancanneyt, M., Matte-Tailliez, O., Grisez, L., Tailliez, P. and Bizet, C. (2005). Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals. *Systematic Applied Microbiology*, 28(7):640–660.

Borg, A. F., (1960). Studies on myxobacteria associated with diseases in salmonid fishes. *Wildlife Diseases* 8: l–85.

Borg, A.F., (1948). Studies on Myxobacteria associated with diseases in salmonid fishes, University of Washington.

Bullock, G. (1990). Bacterial gill disease of freshwater fishes, vol. 19. Washington (DC): United States Fish and Wildlife Service Fish Disease Leaflet.

Bullock, G., (1972). Studies on selected Myxobacteria pathogenic for fishes and on bacterial gill disease in hatchery-reared salmonids. *Technical Paper of US Bureau of Sport Fisheries and Wildlife* 60: 3–30.

Bullock, G., Hsu, T-C. and Shotts, J. E. (1986). Columnaris disease of fishes. Service United States Fish and Wildlife service.

Chinabut, S., Somsiri, T., and Danayadol, Y. (2002). Impacts of disease in small-scale aquaculture in Thailand: case studies, Primary Aquatic Animal Health Care in Rural, Small-scale, Aquaculture Development. FAO Fish. Tech. Pap. No. 406.

Chinnarajan, R., Govindaswamy, R. V., Rajasabapathy, R., Logeshwaran, V. and Sreepada, R. A. (2015). Infection and pathogenecity of *Myroides odoratimimus* (NIOCR-12) isolated from the gut of grey mullet (*Mugil cephalus* Linnaeus, (1758)). *Journal of Microbiology* 88: 22-28.

Chou, D-W., Wu, S-L., Lee, C-T., Tai, F-T. and Yu, W-L. (2011). Clinical characteristics, antimicrobial susceptibilities, and outcomes of patients with *Chryseobacterium indologenes* bacteremia in an intensive care unit. *Japanese Journal of Infectious Di*seases 64(6):520–524.

Christensen, P. J. (1977). The history, biology, and taxonomy of the *Cytophaga* group. *Journal of Microbiology* 23(12):1599–1653.

Cipriano, R. C. and Teska, J. (1994). Effects of medium composition on the growth of two fish pathogens, *Cytophaga columnaris* and *Cytophaga psychrophila*. *Biomedical Letters* 49(193):7–12.

Daroy, M., Lopez, J., Torres, B., Loy, M., Tuaño, P. and Matias R. (2011). Identification of unknown ocular pathogens in clinically suspected eye infections using ribosomal RNA gene sequence analysis. *Clinical Microbiology and Infections* 17(5):776–779.

Davis, H. (1926). A new gill disease of trout. *Transactions of the American Fisheries Society* 56(1):156–160.

Davis, H. S. (1927). Further observations on the gill disease of trout. *Transactions of the American Fisheries Society* 57(1):210–216.

Davis, H. S. (1922). Government Printing Office; A new bacterial disease of fresh-water fishes.

Davis, H. S. (1946). Care and diseases of trout United States fish and wildlife service research report 12: *US Government Printing Office*, Washington, DC.

Department of Fisheries Resources annual report 210/2011, Ministry of Agriculture and Animal Industry and Fisheries. (2012). Government printer, Uganda [http://www.agriculture.go.ug/userfiles/DFR%20ANNUAL%20REPORT%202012.pdf] visited 13/8/2016.

Ekpo, I. A., Agbor, R. B., Osuagwu, A. N., Ekanem, A. P., Okpako, E. C. and Ekanem B.E, (2013). Relationship Between Bacteria Associated with Fish Pond Sediment and water. *The Fish Journal of current research in Science*, 1:50-54.

Food and Agriculture Organisation (FAO). (2005).National Aquaculture Sector Overview. Uganda. National Aquaculture Sector Overview Fact Sheets. *FAO Fisheries and Aquaculture Department* [online]. Rome. Updated 19 July 2005. [http://www.fao.org/fishery/countrysector/naso_uganda/en site visited 10/11/2017] visited on 13/8/2016.

Food and Agriculture Organisation (FAO). (2012). REPORT/RAPPORT: SF-FAO/2012/06: Capacity building workshop on conducting aquculture as a business, Uganda. [http://www.fao.org/3/a-az012e.pdf] visited on 6/9/2016.

Farmer, B., (2004). Improved methods for the isolation and characterization of *Flavobacterium columnare,* MSc Dissertation. Louisiana State University, Agricultural and Mechanical College, Baton Rouge, LA, USA.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fernández-Olmos, A., García-Castillo, M., Morosini, M-I., Lamas, A., Máiz, L. and Cantón, R. (2012). MALDI-TOF MS improves routine identification of non-fermenting Gram-negative isolates from cystic fibrosis patients. *Journal of Cystic Fib*rosis 11(1):59–62.

Flemming, L., Rawlings, D. and Chenia, H. (2007). Phenotypic and molecular characterisation of fish-borne *Flavobacterium Johnsoniae* -like isolates from aquaculture systems in South Africa. *Research in Microbiology* 158(1):18–30.

Frankland, G. C. and Frankland, P. F. (1889). Ueber einige typische Mikroorganismen im Wasser und im Boden (Typical microorganisms in water and soil). *Medical Microbiology and Immunology* 6(1):373–400.

Fraser, S. L. and Jorgensen, J. H. (1997). Reappraisal of the antimicrobial susceptibilities of *Chryseobacterium* and *Flavobacterium* species and methods for reliable susceptibility testing. *Antimicrobial Agents and Chemotherapeutics* 41(12):2738–2741.

Gosink, J. J., Woese, C. R. and Staley, J. T. (1998). *Polaribacter gen*. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group and reclassification of *'Flectobacillus glomeratus'* as *Polaribacter glomeratus* comb. nov. *International Journal Systematic Bacteriology*. 48(1):223–235.

Haburjak, J. and Schubert, T., (1997). *Flavobacterium breve* meningitis in a dog. *Journal of American Animal Hospital Association* 33(6):509–512.

Hawke, J. P. and Thune, R. L. (1992). Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. *Journal of Aquatic Animal Health* 4(2):109–113.

Hernandez-Divers, S. J., Hensel, P., Gladden, J., Hernandez-Divers, S. M., Buhlmann, K. A. and Hagen, C. (2009). Investigation of shell disease in map turtles (*Graptemys spp*.). *Journal of Wild Life Diseases* 45(3):637–652.

Holmes, B., Owen, R. and McMeekin, T. (1984). Genus *Flavobacterium*, Bergey's manual of systematic bacteriology 1:353–361.

Holt, R. A., (1987). *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish (PhD Thesis). Oregon State University, Corvallis.

Jacobs, A. and Chenia, H. Y. (2011). Biofilm formation and adherence characteristics of an *Elizabethkingia meningoseptica* isolate from *Oreochromis mossambicus*. *Annals of Clinical Microbiology and Antimicrobials* 10:16.

Johansen, J., Nielsen, P. and Sjoholm, C. (1999). Description of *Cellulophaga baltica* gen nov, sp nov and *Cellulophaga fucicola* gen nov, sp nov and reclassification of *Cytophaga lytica* to *Cellulophaga lytica* gen nov. International *Journal of Systematic Bacteriology* 49(3):1231–40.

Jooste, P. J. (1985). The taxonomy and significance of *Flavobacterium-Cytophaga* strains from dairy sources. *Bloemfontein, South Africa:* University of the Orange Free State.

Kämpfer, P., Fallschissel, K. and Avendan-Herrera, R. (2011). *Chryseobacterium chaponensesp*. nov., isolated from farmedAtlantic salmon (*Salmo salar*). *International Journal of Systematic and Evolutionary Microbiology* 61(3):497–501.

Kämpfer, P., Lodders, N., Martin, K. and Avendan-Herrera, R. (2012). *Flavobacterium chilensesp*. nov. and *Flavobacterium araucananum sp*. nov., isolated from farmed salmonid fish. *International Journal of Systematic and Evolutionary Microbiology* 62(6):1402–8.

Kämpfer, P., Lodders, N., Vaneechoutte, M. and Wauters, G. (2009). Transfer of *Sejongia antarctica*, *Sejongia jeonii* and *Sejongia marina* to the genus *Chryseobacterium* as *Chryseobacterium antarcticum* comb. nov., *Chryseobacterium jeonii* comb. nov. and *Chryseobacterium marinum* comb. nov. *International Journal of Systematic Evolutionary Microbiolology* 59(9):2238–2240.

Kämpfer. P., Vaneechoutte, M., Lodders, N., De-Baere, T., Avesani, V. and Janssens, M. (2009). Description of *Chryseobacterium anthropi* sp. nov. to accommodate clinical isolates biochemically similar to *Kaistella koreensis* and *Chryseobacterium haifense*, proposal to reclassify *Kaistella koreensis* as *Chryseobacterium koreense* comb. nov. and emended description of the genus *Chryseobacterium*. *International Journal of Systematic Evolutionary Microbiolology* 59(10):2421–2428.

Karatas, S., Ercan, D., Steinum, T. M., Turgay, E., Memis, D. and Candan, A. (2010). First isolation of a *Flavobacterium johnsoniae* like bacteria from cultured Russian sturgeon in Turkey. *Journal of Animal Veterinary Advances* 9(14):1943–1946.

Kifuko, R. (2015). The State of Cage Fish Farming in Uganda: Actors Enabling Environment Challenges and Way forward. *International Journal of Education and Research.* 3(3).

Kim, O-S., Cho, Y-J., Lee, K., Yoon, S-H., Kim, M. and Na, H. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *International Journal of Systematic and Evolutionary Microbiology* 62(3):716–21.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111-120.

Kirby, J. T., Sader, H. S., Walsh, T. R. and Jones, R. N. (2004). Antimicrobial susceptibility and epidemiology of a worldwide collection of *Chryseobacterium spp*.: report from the SENTRY antimicrobial surveillance program (1997–2001). *Journal of Clinical Microbiol*ogy 42(1):445–448.

Kumar, S., Stecher G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33:1870-1874.

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A. and Harris, R. N. (2007). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *Multidisciplinary Journal of Microbial Ecology* 2(2):145–157.

Lee, S. H., Kim, J. M., Lee, J. R., Park, W. and Jean, C. O, (2010). *Flavobacterium fluviin sp*. nov., isolated from stream sediment. *International Journal of Systematic Evolutionary Microbiolology* 60 (2): 353-357.

Li, H., Qiao, G., Gu, J-Q., Zhou, W., Li, Q. and Woo, S-H. (2010). Phenotypic and genetic characterization of bacteria isolated from diseased cultured sea cucumber *Apostichopus japonicus* in northeastern China. *Diseases of Aquatic Organisms* 91(3):223–235.

Lin, Y-T., Jeng, Y-Y., Lin, M-L., Yu, K-W., Wang, F-D. and Liu, C-Y., (2010). Clinical and microbiological characteristics of *Chryseobacterium indologenes* bacteremia. *Journal of Microbiology, Immunology and Infections* 43(6):498–505.

Liu, H., Izumi, S. and Wakabayashi, H. (2001). Detection of *Flavobacterium psychrophilum* in various organs of *ayu Plecoglossus altivelis* by in situ hybridization. *Fish Pathology* 36: 7-11.

Loch, P. T. and Faisal, M. (2015). Emerging Flavobacterial infections in fish: A review. *Journal of advanced research* 6(3): 283–300.

Loch, P.T. (2014). Identification of novel Flavobacteria from Michigan and assessment of their impacts on fish health. Michigan State University, United States of America pp. 112-241.

Loch, T. and Faisal M. (2014). *Chryseobacterium aahli* sp. nov., isolated from lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*), and emended descriptions of *Chryseobacterium ginsenosidimutans* and *Chryseobacterium gregarium*. *International Journal of Systematic Evolutionary Microbiolology* 64(5):1573–1579.

Lorenzen, E. (1993). The importance of the brand of the beef extract in relation to the growth of *Flexibacter psychrophilus* in Anacker and Ordals medium. *European Association of Fish Pathology* 13:64–5.

Lorenzen, E., Dalsgaard, I. and Bernardet, J-F., (1997). Characterization of isolates of *Flavobacterium psychrophilum* associated with cold-water disease or rainbow trout fry syndrome I: phenotypic and genomic studies. *Diseases of Aquatic Organisms* 31(3):197–208.

Lorenzen, E., Dalsgaard, I., From, J., Hansen, E., Horlyck, V. and Korsholm, H., (1991). Preliminary investigations of fry mortality syndrome in rainbow trout, *European Association of Fish Pathol*ogy. vol. 11.

Marak, S., Sarchianaki, E. and Barbagadakis, S. (2012). *Myroides odoratimimus* soft tissue infection in an immunocompetent child following a pig bite: case report and literature review. *The Brazilian Journal of Infectious Diseases* 16 (4): 390–392.

Michel, C., Antonio, D. and Hedrick, R. P. (1999). Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. *Research in Microbiology* 150(5):351–8.

Ministry of Agriculture Animal Industry and Fisheries (MAAIF). (2004). The National Fisheries Policy. 1st edition. Department of Fishery Resources. Kampala, Uganda.

Mudarris, M. and Austin, B. (1989). Systemic disease in turbot *Scophthalmus maximus* caused by a previously unrecognized *Cytophaga*-like bacterium. *Diseases of Aquatic Organisms* 6(3):161–166.

Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B. and Bernardet, J. (1994). *Flavobacterium scophthalmum sp*. nov., a pathogen of turbot (*Scophthalmus maximus*). International *Journal of Systematic Bacteriology* 44(3):447–53.

Nematollahi, A., Decostere, A., Pasmans, F. and Haesebrouck, F. (2003). *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases* 26(10):563–74.

Ordal, E. J. and Rucker, R. R. Pathogenic myxobacteria. Proc Soc Exp Biol Med 1944:15–8.

Pacha, R. and Porter, S. (1968). Characteristics of Myxobacteria isolated from the surface of freshwater fish. *Jounal of Applied Microbiology*. 16(12):1901–1906.

Pridgeon, J. W., Klesius, P. H. and Garcia, J. C. (2012). Identification and virulence of *Chryseobacterium indologenes* isolated from diseased yellow perch (*Perca flavescens*), *Jounal of Applied Microbiology* 7; 48.

Qu, J. H., Yuan, H. L., Li, H. F. and Deng, C. P. (2009). *Flavobacterium Cauense sp*. nov., isolated from sediment of eutrophic lake. *International Journal of Systematic Evolutionary Microbiolology* 59 (11):2666-2669.

Rintamäki-Kinnunen, P., Bernardet, J.-F. and Bloigu, A. (1997). Yellow pigmented filamentous bacteria connected with farmed salmonid fish mortality. *Aquaculture* 149(1):1–14.

Sebastião, F. A., Pilarski, F. and Lemos, M. V. F. (2010). Isolation and molecular characterization of *Flavobacterium columnare* strains from fish in Brazil. *Journal of Bacteriology Research* 2(3): 22-29.

Second National Development Plan II 2015/16 - 2019/20. (2015). [http://library.health.go.ug/publications/leadership-and-governance/second-national-development-plan-ii-201516-201920] site visited on 27/ 07/ 2016.

Segers, P., Mannheim, W., Vancanneyt, M., De-Brandt, K., Hinz, K.-H. and Kersters K. (1993). *Riemerella anatipestifer* gen. nov., comb. nov., the causative agent of septicemia *anserum exsudativa* and its phylogenetic affiliation within the *Flavobacterium-Cytophaga* rRNA homology group. *International Journal of Systematic Bacteriology* 43(4):768–776.

Shoemaker, C. A., Klesius, P. H., Drennan, J. D. and Evans, J. (2011). Efficacy of a modified live *Flavobacterium columnare* vaccine in fish. *Fish and Shellfish Immununology* 30:304–308.

Shotts, E. and Starliper, C. (1999). Flavobacterial diseases: columnaris disease, cold-water disease and bacterial gill disease (Fish diseases and disorders: viral, bacterial and fungal infections). CABI Publishing; New York (NY), 3:559–576.

Starliper, C. E. (2011). Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *Journal of Advanced Research* 2(2):97–108.

Starliper, C. E., Marcquenski, S. and Noyes, A. (2007). Development of an improved medium for primary isolation of *Flavobacterium psychrophilum*, cause of bacterial coldwater disease. Great Lakes Fishery Commission Research Report.

Strohl, W. R. and Tait, L. R. (1978). *Cytophaga aquatilis sp*. nov., a facultative anaerobe isolated from the gills of freshwater fish. *International Journal of Systematic Bacteriology* 28(2):293–303.

Suebsing, R. K. and Kim, J. H., (2012). Isolation and Characterization of *Flavobacterium johnsoniae* from Farmed Rainbow Trout *Oncorhynchus mykiss*. *Canadian Journal of Fisheries and Aquatic Sciences* 15(1):83–89.

Uganda Bureau of Statistics (UBOS). (2014). [www.ubos.org/onlinefiles/uploads/ubos/.../Statistical%20Abstract%202004.pdf] site visited on 10/11/2017.

United States Department of Agriculture. (2003). Reference of 2002 U.S. Catfish health and production practices. Fort Collins, Colorado: Center for Epidemiology and Animal Health.

Vancanneyt, M., Segers, P., Torck, U., Hoste, B., Bernardet, J-F. and Vandamme, P. (1996). Reclassification of *Flavobacterium odoratum* (Stutzer 1929) strains to a New Genus, *Myroides*, as *Myroides odoratus* comb. nov. and *Myroides odoratimimus sp*. nov. *International journal of Systemic Bacteriology* 46(4):926–32.

Vandamme, P., Bernardet, J-F., Segers, P., Kersters, K. and Holmes, B. (1994). New perspectives in the classification of the Flavobacteria: description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* nom. rev. *International Journal of Systematic Bacteriology* 44(4):827–831.

Vaneechoutte, M., Kämpfer, P., De-Baere, T., Avesani, V., Janssens, M. and Wauters, G., (2007). *Chryseobacterium hominis* sp. nov., to accommodate clinical isolates biochemically similar to CDC groups II-h and II-c. *International Journal of Systematic Evolutionary Microbiolology* 57(11):2623–2628.

Varga, Z., Sellyei, B., Paulus, P., Papp, M., Molnár, K. and Székely, C. (2016). isolation and characterisation of Flavobacteria from wild and cultured freshwater fish species in Hungary. *Acta Veterinaria Hungarica* 64 (1): 13–25.

Wagner, B. A., Wise, D. J., Khoo, L. H. and Terhune, J. S. (2002). The epidemiology of bacterial diseases in food-size channel catfish. *Journal of Aquatic Animal Health* 14:263–272.

Wakabayashi, H. (1980). Bacterial gill disease of salmonid fish, *Fish Pathol*ogy, 14(4):185–189.

Wakabayashi, H. and Huh, G., (1989). Kimura N. *Flavobacterium branchiophila* sp. nov., a causative agent of bacterial gill disease of freshwater fishes. *International Journal of Systemic Bacteriol*ogy 39(3):213–216.

Walakira, J., Akoll P., Engole M., Sserwadda, M., Nkambo, M., Namulawa, V., Kityo, G., Musimbi, F., Abaho, I., Kasigwa, H., Mbabazi, D., Kahwa, D., Naigaga, I., Birungi, D., Rutaisire, J. and Majalija, S., (2014). Common fish diseases and parasites affecting wild and farmed Tilapia and catfish in Central and Western Uganda. *Uganda Journal of Agricultural Sciences* 15(2): 113 – 125.

Welker, L. T., Shoemaker, A. C., Arias, R. C. and Klesius, H. P. (2005). Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*. *Diseases of Aquatic Organisms*, 63 :129–138.

Xie, Z-Y., Zhou, Y-C., Wang, S-F., Mei, B., Xu, X-D. and Wen, W-Y. (2009). First isolation and identification of *Elizabethkingia meningoseptica* from cultured tiger frog, *Rana tigerina rugulosa*. *Veterinary Microbiology* 138(1):140–144.

Yoon, J. H, Park, S., Kang, S. J., Myung, S. C. and Kim, W., (2011). *Flavobacterium ponti sp.* nov., isolated from seawater. *International Journal of Systematic Evolutionary Microbiolology* 61 (1): 81-85.

Zamora, L., Fernández-Garayzábal, J., Palacios, M., Sánchez-Porro, C., Svensson-Stadler, L. and Domínguez, L. (2012). *Chryseobacterium oncorhynchi sp*. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Systemic Applied Microbiology* 35(1):24–29.

Zamora, L., Vela, A. I., Palacios, M. A., Sánchez-Porro, C., Svensson-Stadler, L. A. and Domínguez, L. (2012). *Chryseobacterium viscerum* sp. nov., isolated from diseased fish. *International Journal of Systematic Evolutionary Microbiolology* 62(12):2934–2940.

Zamora, L., Vela, A., Sánchez-Porro, C., Palacios, M., Domínguez, L. and Moore, E. (2013). Characterization of Flavobacteria possibly associated with fish and fish farm environment: description of three novel *Flavobacterium* species: *Flavobacterium collinsii* sp. nov., *Flavobacterium branchiarum* sp. nov., and *Flavobacterium branchiicola* sp. nov. *Aquaculture* 416:346–353.

APPENDICES

Appendix 1: Composition of Sheih agar and Sheih broth

Components	**Quantity in 1000ml distilled water (Sheih agar)**	**Quantity in 1000ml distilled water (Sheih broth)**
$FeSO_4.7H_20$	1ml	1ml
$CaCl_2.2H_2O$	1ml	1ml
KH_2PO_4	10ml	10ml
MgSO4	10ml	10ml
Peptone water	5g	5g
Yeast extract	2g	2g
European bacteriological agar	15g	-
Tobramycin	0.001g	0.001
Distilled water	1000ml	1000ml

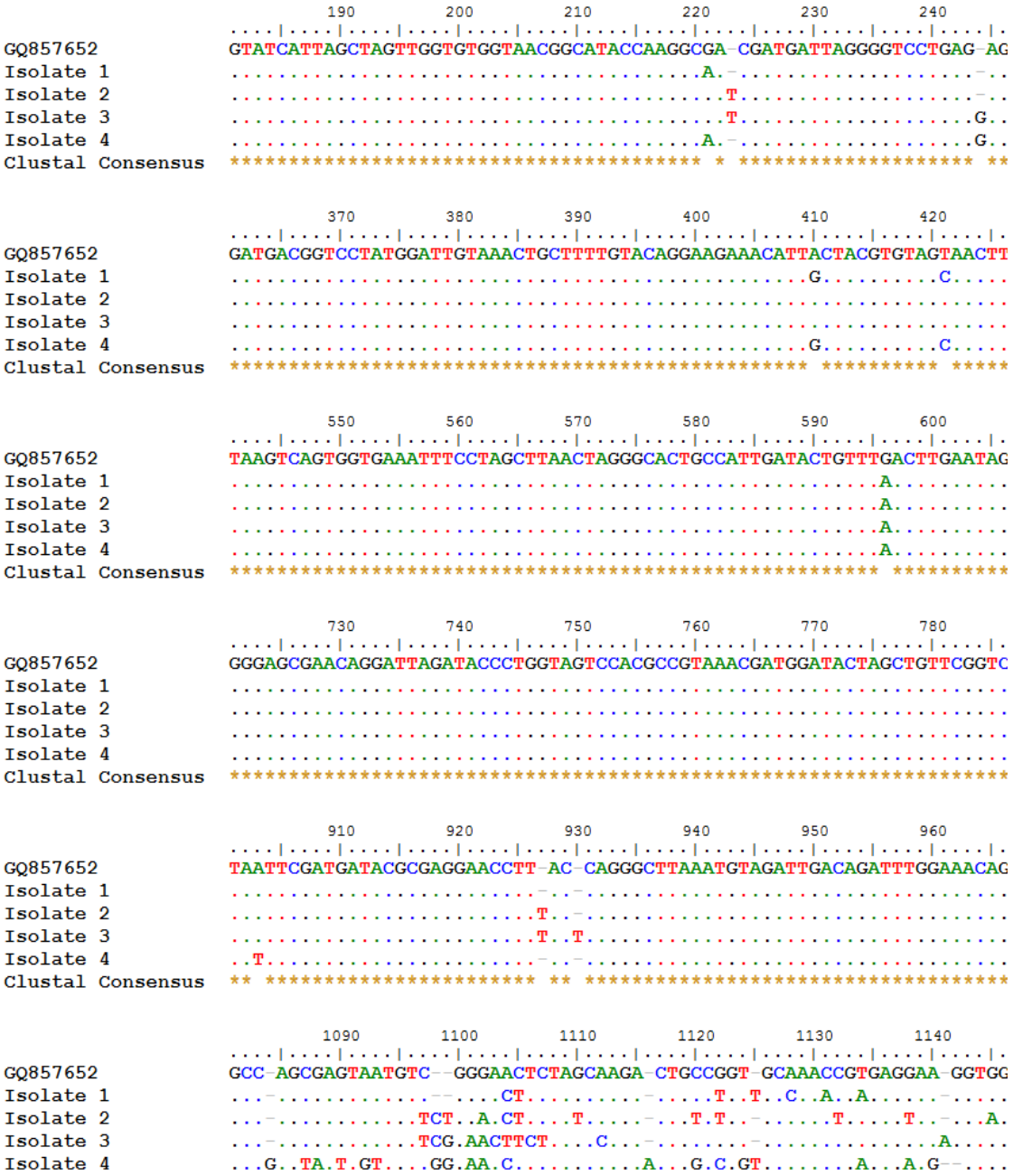
Appendix 2: Protocol for isolation of genomic DNA

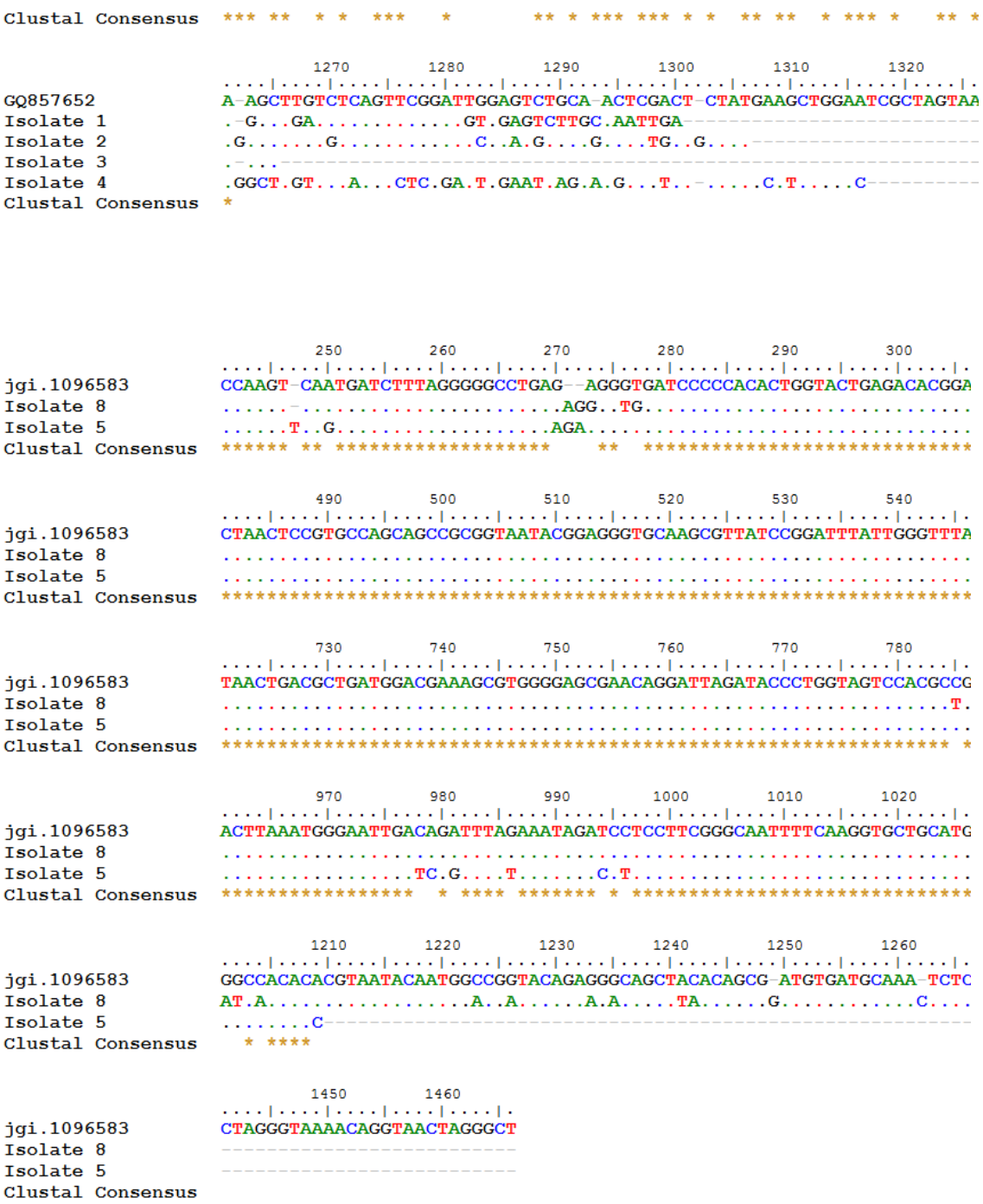
- ✓ Clean bench area. Set the water bath to 70 °C and thaw cell pellets on ice. (Do not use DYMO labels on tubes as they fade in the hot water bath)
- ✓ After thawing and before incubating the samples, add 1 µL of carrier nucleic acid (yeast RNA 10mg/ml, Thermo Fisher Cat. AM7118 preferred) for samples consisting of less than 200,000 cells. Carrier RNA is added as a co-precipitant to improve the amount of DNA recovered.
- ✓ Spin down each sample in a bench top centrifuge at the highest speed for 2 minutes, remove supernatant and resuspend the sample in PBS to attain a volume of 270 µl.
- ✓ Add 30 µl QIAGEN Protease to each sample. The final volume should be 300 ul. Vortex completely.
- ✓ If cells remain clumped, incubate at 70°C for 10 to 20 minutes, vortexing every few minutes, otherwise move on to step 6.
- ✓ Add 300 µl Buffer AL once cells have completely dissolved. Mix thoroughly by inverting the tube 10 times, followed by additional vigorous shaking for at least 1 minute.
- ✓ Incubate at 70 ^{O}C until no longer cloudy, about 10 to 30 min.
- ✓ Place a closed Buffer AE bottle on top of a heat block set at 50-60 for approximately 1-2 hours.
- ✓ Open tubes and add 300 µL ethanol (96-100%) to each sample and mix by inverting the tube 10 times, followed by additional vigorous shaking (10-20 seconds). Let the foam settle before opening the tube.

- ✓ Add the solution from step 9 onto a QIAamp Mini column. Close the cap and centrifuge at 3,750 rpm for 30 seconds.
- ✓ Discard the filtrate and add 750 µL Buffer AW1 to the column. Centrifuge at 3,750 rpm for 30 seconds.
- ✓ Repeat step 11 and dry the columns (removes all residual EtOH) on the bench top (about 30 minutes).
- ✓ If necessary, dry the outside of the column with Kim Wipe to remove residual EtOH. Place the column in a clean Eppendorf, and discard the tube containing the filtrate.
- ✓ Pipet 75 µl Buffer AE onto the membrane of the column and close the cap. Incubate at room temperature for 5 minutes, and centrifuge at 3,750 rpm for 2 minutes.
- ✓ Pipet an additional 75 µl Buffer AE onto the membrane of the column and close the cap. Incubate at room temperature for 5 minutes, and centrifuge at 3,750 rpm for 2 minutes, measure DNA concentration.

Appendix 3: Graphical view showing comparison of nucleotides between the identified sequences and the reference strains

Myroides marinus JS 08 represented by the accession number GQ 857652 was compared to the isolates 1, 2, 3 and 4 while *Chryseobacterium gambrini* DSM 18014 (accession number, jgi.1096583) was compared with isolates 8 and 5.




Clustal Consensus
1270 1280 1290 1300 1310 1320
GQ857652
Isolate 1
Isolate 2
Isolate 3
Isolate 4
Clustal Consensus
250 260 270 280 290 300
jgi.1096583
Isolate 8
Isolate 5
Clustal Consensus
490 500 510 520 530 540
jgi.1096583
Isolate 8
Isolate 5
Clustal Consensus
730 740 750 760 770 780
jgi.1096583
Isolate 8
Isolate 5
Clustal Consensus
970 980 990 1000 1010 1020
jgi.1096583
Isolate 8
Isolate 5
Clustal Consensus
1210 1220 1230 1240 1250 1260
jgi.1096583
Isolate 8
Isolate 5
Clustal Consensus
1450 1460
jgi.1096583
Isolate 8
Isolate 5
Clustal Consensus

Appendix 4: Biodata form

BIODATA FORM

Date…..……District…….

subcounty……. Parish……

Village…… Name of Farmer……...

Contact……Number of culture units……

Water source……..

Culture system(s) pond ☐ tank ☐ cage ☐

Purpose of farming Substance ☐ commercial ☐ both ☐

Species of fish …….

If both Tilapia and catfish Mixed ☐ separate ponds ☐ both ☐

History of disease outbreak Yes ☐ No ☐

Frequency of disease outbreak low ☐ high ☐ moderate ☐

Disease Common in which season Dry ☐ Rainy ☐

Other challenges experienced……..

…….