OCCURENCES AND ANTIMICROBIAL SENSITIVITY OF BACTERIA IN RUFIJI TILAPIA AND ITS HYBRIDS WITH NILE TILAPIA FINGERLINGS AT DIFFERENT SALINITY LEVELS

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ABSTRACT

Diseases at hatchery stage are among challenges that hinder optimal mariculture development in Tanzania. Information on the occurrences of bacterial infections in mariculture hatchery in the country is limited. Thus, this study was conducted to determine the occurrence and antibacterial sensitivity of bacteria species in Rufiji tilapia and hybrids fingerlings of Nile tilapia female crossbreed with Rufiji tilapia male farmed at different salinity at Institute of Marine Science (IMS) - Mariculture Center in Pangani-Tanga. A total of 120 fingerlings (2.07-3.49g for Rufiji tilapia (RF), 6.61- 6.98g for hybrids (HB) and 250ml water samples were collected from three replicated tanks of about 30 m^2 set at water salinity level of 2, 15, 25 and 35. Bacterial loads were determined as CFU ml^{-1}. Isolated bacteria were identified to genus or species level. Molecular identification was done for *Staphylococcus* and *Bacillus spp*. Antibiotic sensitivity test was conducted using the following antibiotics; Tetracycline (TE30), Chloramphenicol (C30), Gentamycine (CN10), Amoxycilin (AMC30), Neomycine (N10), Ciproflaxcine (CIP5), Cefataxime(CTX30), Sulfamethoxazole (SMX50), Ampicillin (AMP10), Erythromycine (E15)and Penicillin G(P10). Bacterial loads (log10 CFUml-1) ranged from 6.34±0.12- 4.79±0.94 in HB; 5.99±0.70-4.44±0.45 in RF; 6.42±0.44-5.07±0.53 in Water with HB and 5.62±0.53-4.28±0.16 in Water with RF. There were no significant differences in bacterial loads between fish species at all levels of water salinity ($p>0.05$). A total of four genera were isolated namely; Staphylococcus, Escherichia, Micrococcus and Bacillus. Confirmation by PCR of 56 isolates showed; 69.4% positive for Staphylococcus spp, 5.6% *S. aureus,* 17 % *S. epidermidis*, 21.4% *Bacillus cereus* and 7.1% *B. subtilis.* Antimicrobial sensitivity test indicated that isolates were highly sensitive to Ciprofloxacin (100%) and Tetracycline (86.7%), and were

resistant to Sulfamethoxazole and Penicillin. Presence of bacteria species of which some are potentially pathogenic to fish may cause diseases and mortalities to fingerlings hindering mariculture development in the country. Biosecurity measures should be employed to reduce chances of bacterial contamination at hatchery.

DECLARATION

I **Jestina Obedi Ntaguda,** do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work, done within the period of registration and that it has neither been submitted nor is being concurrently submitted to any other institution.

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DEDICATION

I dedicate dissertation to God the almighty for his mercies, strength and grace to persevere to the end of the work. To my loving husband S. S. Bwasama, my twins (Aidan and Aiden) for their love, patients and support.

TABLES OF CONTENTS

ABSTRACT .... **ii**

DECLARATION .... **iv**

COPYRIGHT .... **v**

ACKNOWLEDGEMENTS .... **vi**

DEDICATION .... **vii**

TABLES OF CONTENTS .... **viii**

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

LIST OF FIGURES .... **xiii**

LIST OF APPENDICES .... **xv**

LIST OF ABBREVIATIONS .... **xvi**

CHAPTER ONE **1**

1.0 INTRODUCTION **1**

1.1 Background Information 1

1.2 Problem Statement and Study Justification 5

1.3 Objectives of the Study 6

1.3.1 Overall objective 6

1.3.2 Specific objectives 6

1.3.3 Research questions 7

CHAPTER TWO **8**

2.0 LITERATURE REVIEW **8**

2.1 Mariculture Sector in Tanzania 8

2.2 Bacteria Infections in Farmed Fish 9

2.2.1 Staphylococcus species 10

2.2.2 Bacillus species 11

2.2.3 Enterobacteriaceae species 12

2.2.4 Micrococcus species 13

2.3 Predisposing factors for occurrences of bacterial diseases in fish culture 14

2.4 Bacterial Load and Their Importance to Fish Health 14

2.5 Antibacterial Sensitivity Test 15

CHAPTER THREE 18

3.0 MATERIALS AND METHODS 18

3.1 Study Area 18

3.2 Study Design and Set Up 19

3.3 Study Setup and Sampling Strategy 19

3.3.1 Collection of fingerling samples 20

3.3.2 Water sample collection 20

3.3.3 Measurement of physicochemical water parameters 20

3.4 Bacteriological Analysis 20

3.4.1 Bacterial enumeration 20

3.4.1.1 Total viable bacterial count (TVC) from water sample 21

3.4.1.2 Total viable bacterial count (TVC) from fingerlings 21

3.5 Isolation and Identification of Bacteria from Water and Fish Samples 21

3.5.1 Biochemical tests 22

3.5.2 Molecular identification of selected isolates (*Staphylococcus spp and Bacillus spp*) 24

3.5.2.1 DNA Extraction 25

3.5.2.2 DNA amplification by PCR method 25

3.6 Antibacterial Sensitivity Test 28

3.7 Data Analysis 29

CHAPTER FOUR 30

4.0 RESULTS 30

4.1 Water Quality Parameters and Fish Biodata..30

4.2 Overall Bacteria Loads in Water and Fingerlings...30

4.3 Occurrences of Bacteria from Water and Fingerlings per Salinity.......................35

4.4 Bacteria Isolated from Water and Fingerlings per Salinity 36

4.5 Bacteria Isolated in Hybrids Fingerlings and Water Samples 37

4.6 Bacteria species isolated in Rufiji tilapia fingerlings and water per salinity.........38

4.7 Molecular Analysis 39

4.8 Antibacterial Sensitivity Test...43

CHAPTER FIVE 47

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.................47

5.1 Discussion...47

5.2 Conclusions...51

5.3 Recommendations 52

REFERENCES 54

APPENDICES..67

LIST OF TABLES

Table 1: Genus /species specific primer pairs used to identifystaphylococcus and bacillus species 27

Table 2: Antimicrobial agents used for sensitivity testing of the isolated bacteria 29

Table 3: The overall average of physical chemical water parameters measured at different salinity levels 30

Table 4: Bacterial load, means ± standard deviation (CFUml-1) in both water and Fingerlings samples per salinity levels 31

Table 5: Biochemical test 32

Table 6: Overall number of bacteria isolates per salinity in Fingerlings and Water sample (n=132).. 36

Table 7: Bacteria isolated in water and Fingerlings of Hybrids and Rufiji tilapia per genus showing high percentage in hybrids fingerlings (n=132) 37

Table 8: Overall bacteria isolated in Hybrids fingerlings per salinity (n)=58............. 37

Table 9: Percentages of bacteria isolated in water collected from Hybrids tanks per salinity (n) =15 38

Table 10: Bacteria isolated from Rufiji Tilapia fingerlings per salinity (n=51)............. 38

Table 11: Percentage of bacteria species isolated from water collected in Rufiji tilapia tanks (n)=8.. 39

Table 12: Results of the samples confirmed for *Staphylococcus spp* by PCR methods.. 40

Table 13: Results of the samples confirmed for *Bacillus spp* (using species specific primers) by PCR methods.. 41

Table 14: Results of antibiotic sensitivity tests showing fifteen bacteria species and the responses to antimicrobials.. 45

Table 15: Overall percentages of the bacterial isolates tested shown as sensitivity, intermediate and resistance per antibiotic agents used (n=15 isolates) 46

LIST OF FIGURES

Figure 1: Map showing Pangani River and the study area: Source from Google map. 19

Figure 2: Bacterial load (CFUml-1) in all samples collected indicating similar trend in bacterial for all groups tested. 32

Figure 3: Gram positive *Staphylococcus aureus* (A) and *Staphylococcus epidermids* (B) isolated from hybrids tilapia as seen under light microscope showing a cluster of cocci shape 33

Figure 4: *Micrococcus luteus* showing gram positive cocci (in tetra pairs) as seen under light microscope 33

Figure 5: Gram positive members of *Bacillus spp* (A) and gram negative *E.coli* (B) isolated from hybrids tilapia as seen under light microscope. 34

Figure 6: Test tube (A) coagulation due to enzymes (coagulase) produced by *S.aureus* in a rabbit plasma and test tube (B) showing the test tube containing *S.epidermids* a coagulase negative after four hours incubation at 28-37 ^{o}C 34

Figure 7: Bubbles produced by *Staphylococcus aureus* after adding 3% potassium hydroxide showing Positive catalase reaction (+ve) 35

Figure 8: A positive reaction of *Micrococcus sp* (A) to oxidase test showing deep blue color after rubbing to the Oxidase disc test and negative reaction of *Staphylococcus sp* (B) 35

Figure 9: Representative of 68% positive results for the first batch PCR amplification of staphylococcus spp at 370bp. 41

Figure 10: Represents positive results of PCR amplification of (A)*Staphylococcus epidermidis*, and (B) *S. aureus*. 42

Figure 11: Represents of positive results of PCR amplification of a positive products (A) *Bacillus cereus* at 71bp first batch and (B) *Bacillus subtilis*. 43

Figure 12: Antibacterial sensitivity test performed on *S. aureus* isolate (B), on Muller Hinton agar showing zones of inhibition after 24hrs incubation.. 44

LIST OF APPENDICES

Appendix 1: Experimental set up for Rufiji tilapia fingerlings at IMS –Mariculture Center –Pangani, Tanga..67

Appendix 2: Experimental set up for hybrids fingerlings at IMS –Mariculture Center –Pangani, Tanga 68

LIST OF ABBREVIATIONS

%	Percentage
<	Less than
>	Greater than
µl	Micro-litres
♀	Symbol for female fish
♂	Symbol for male fish
1F	First set of forward primer
2R	Second set of reverse primer
Bp	Base pair
CFU	Colony Forming Unit
CFU	Colony Forming Unit
DNA	Deoxyribose Nucleic Acid
e.g	For example
EDTA	Ethylenediaminetetra acetic acid
et al.,	and others
FAO	Food and Agriculture Organisation
Fig	Figure
G	Gram
HB	Hybrids fingerlings
HBW	Water sample collected from tanks containing Hybrids fingerlings
Hrs	Hours
MSA	Mannitol salt agar
^{o}C	Degree centigrade

PCR	Primarase Chain Reaction
pH	Hydrogen ion concentration
Ppt	Parts per thousands
RF	Rufiji Tilapia fingerlings
RFW	Water sample collected from tanks containing Rufiji tilapia fingerlings
S	Second (time)
Spp	Species
TRAHESA	Training and Research in Aquatic and Environimental health in Eastern and Southern Africa
TSB	Trypticase soy agar
TVC	Total viable bacterial count
USA	United States of America
ZOI	Zone of Inhibition

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Aquaculture is the fast growing industry purposely being practiced worldwide in order to meet increased demand of fish protein due to the growing world population. Traditionally fish stock from natural water bodies have been the main source of fish protein but population growth coupled with declining catches from natural sources like rivers, lakes and oceans have not been able to meet the demand. Fish farming has therefore been an alternative source. It is categorized into two types, which are mariculture and freshwater aquaculture (Olafsen, 2001; FAO, 2016; Panigrahi *et al.,* 2007). According to FAO (2016), all continents reported an increased share of aquaculture production trend in total fish production. Leading countries in producing fish from aquaculture systems include China, Philippines, India Vietnam, Bangladesh, Egypt, Norway, Japan, Indonesia, Thailand, Malaysia, Chile, Mexico, Ecuador, Brazil, and Colombia (FAO, 2016; Huicab-Pech *et al.,* 2017).

In Africa, the largest fish producer is Egypt because they started modern fish aquaculture as early as mid-1930s. In addition, the intensive and semi intensive farming practices adopted along the Nile delta reported to have been the major factor for their high success than other African countries (FAO, 2003). Most farming in Egypt occurs in fresh waters around the Nile delta though marine culture is practiced and the common farmed fish types are tilapia and mullet (Mmochi, 2015; FAO, 2016).

Global statistics indicates that Tanzania is among the sub Saharan countries that experienced good growth of aquaculture production in 2000s (FAO, 2016). Commonly

farmed fish in Tanzania are catfish and tilapia species. *Oreochromis niloticus, O. mossambicus, O. aureus, O. urolepis hornorum, O. urolepis urolepis, Tilapia rendalli* and *T. zilli* are among tilapia species commonly farmed in fresh water environment around in Tanzania (Lamtane *et al.,* 2008). In 2010s world mariculture production was estimated to be 53 million tonnes, of which seaweeds (wet weight) produced 44%, followed by mollusks 28%, finfish 23% and crustaceans 7% (Mmochi, 2015). The main reported fish producer was Asia that produced about 90%, and other African countries all combined contributed only 0.4% or 0.18 million tonnes (Mmochi, 2015). This low contribution might be associated with low investments in mariculture sector facing African country and lack of quality seeds for aquaculture development to compete to the world market (FAO, 2010). Marine fish culture is dominated by Atlantic salmon *(Salmo salar*) led by Norway, followed by Chile, United Kingdom, Canada and Ireland (Toranzo *et al.,* 2005). Other farmed fish species include gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax)* and turbot (*Scophthalmus maximus*) in countries such as Greece, Italy, France, Spain and Portugal, and yellowtail (*Seriola quinqueradiata*), ayu (*Plecoglossus altivelis),* flounder (*Paralichthys olivaceus*) and seabream (*Pagrus major*) in Japan (Toranzo *et al.,* 2005).

In 2012s, Tanzania both mainland and islands mariculture produced 89% of seaweeds and the remaining percent for finfish, shellfish products and mollusks (Mmochi, 2015). The total production of tilapia mariculture in Tanzania is not yet known (Mmochi, 2015). Several tilapia species including milkfish (*Chanos chanos)* started to be farmed in mariculture in Zanzibar and contributes to the economy of the country (Sullivan *et al.,* 2010). This species was reported to have better growth, survival, tolerance to a wide range of environmental parameters, and resistance to diseases, compared to other finfish mariculture species (Sullivan *et al.,* 2010; Mmochi, 2015). Other farmed species include

tilapia such as *O. urolepis urolepis* that has been reported to be tolerant to brackish and marine water under experimental conditions, with fastest growth at salinities between 20 and 25 ppt (Nehemia *et al.,* 2013). Rufiji Tilapia *(Oreochromis urolepis urolepis)* are reported to be second tilapia species among the cultured species after Nile tilapia in terms of growth performance that are of economic importance to aquaculture and fisheries sector (Lamtane *et al.,* 2008). It is also reported to be salinity tolerant species as compared to Nile tilapia *(O. niloticus)* and hence can grow well in fresh, brackish and marine waters (Mapenzi and Mmochi, 2016). Poor salinity tolerance of Nile tilapia *(O. niloticus)* limits its culture in brackish and marine water. The hybrids of *O. urolepis urolepis* ♂ and *O. niloticus* ♀ have been reported to be all males and with growth rates comparable to *O. niloticus* in fresh water, making the hybrid a proper candidate for farming in different water salinity levels (Mapenzi and Mmochi, 2016).

The demand of the hybrids is high because can grow fast and be farmed at a wide range of environmental parameters (Mmochi, 2016, personal observation). Despite of the diversity in salt tolerance and good growth performances, their production has been reported to be affected by diseases during early stage of their growth (fingerlings) among other challenges (Silva, 2010 and Nehemia *et al.,* 2013).

These limit production of the hybrid fingerlings to meet the required demand to expand aquaculture production and may pose risks of food borne diseases to consumers. Outbreak of bacterial diseases in fish farms has been reported to be the most significant limiting factors affecting fish culture worldwide (Noga, 2011; Austin and Austin, 2012; Wamala *et al.,* 2018). However, fry and fingerlings were reported to suffer more from marine bacterial infections such as T*enacibaculum maritimum* formally known as *Cytophaga marina Flexibacter maritimus and F.marinus* that led to marine

flexibacteriosis resulting to severe mortalities to younger fish in Gilthead seabream, turbot, sole, salmoids and red seabream reported in Taiwan, Scotland and Denmark (Toranzo *et al.,* 2005).

Furthermore, among of bacterial pathogens that are frequently reported to cause diseases and mortalities in fresh water tilapia fish includes *Aeromonas spp., Streptococcus spp., Edwardsiella spp., Flavobacterium spp., Vibrio spp., Francisella spp., Staphylococcus spp, Psedomonass spp* (Novotny *et al.,* 2004; Abowe *et al.,* 2011 and Huicab-Pech *et al.,* 2016). According to Toranzo *et al.* (2005) diseases that were classically considered as typical fresh water problem are currently reported as important problems also in mariculture. These may also be responsible for mortalities observed in hybrid fry and fingerlings in hatcheries in Zanzibar (Mmochi, A. J. personal communication, 2016). Findings elsewhere have reported bacterial infections in tilapia (*Oreochromis spp.*) hybrids. Toranzo *et al.* (2005) reported *S. iniae* infection in tilapia (*Oreochromis spp.*) hybrids in USA and Israel, and rainbow trout in Israel.

Understanding about antimicrobial sensitivity of bacteria is important for the proper management of the diseases they cause (Wamala *et al.,* 2018). The widespread and indiscriminate use of antibiotics has resulted in the development of antimicrobial resistance in pathogenic as well as commensal microorganisms (Biyela *et al.,* 2004). Worldwide, use of antimicrobial in aquaculture and the potential transmission of resistant bacteria between terrestrial and aquatic environments have been reported (Cabello, 2006). However, the occurrences of antimicrobial resistances are being reported in fish farms with no history of using antimicrobial (Hatha *et al*., 2005). The sources of these antimicrobial in the culture systems with no history of using antibiotics

are reported to be unclear (Rose *et al.,* 2009). Resistance genes may be horizontally or vertically transferred between bacterial communities in the environment (Sørum, 2006). However, several studies have focused on the transmission of antimicrobial resistance between humans and terrestrial food animals (Barton, 2000) and less attention to the aquatic ecosystem including fish. This has created inadequate information available about the antimicrobial susceptibility of the aquatic ecosystem that is also important aspect in the epidemiology of antimicrobial resistances (Biyela *et al.,* 2004; Wamala *et al.,* 2018). In Tanzania the available information suggests no use of antimicrobial in aquaculture, the use of animal wastes to fertilize ponds (Nonga and Katakweba, 2015) and the close interaction between humans, livestock and the aquatic ecosystem indicates a possible transmission of antimicrobial resistances to the aquatic environment. The widespread occurrence of naturally resistant bacteria in the aquatic environment and soil could also contribute to the passage of antibiotic resistance genes to fish bacteria (Cantas e*t al.,* 2013). The recipient bacterial communities may then act as a reservoir of these resistance genes.

However, no information is available on antimicrobial sensitivity for mariculture fish bacteria in Tanzania. Therefore, this study was conducted establish the occurrences and antimicrobial sensitivity of bacteria in Rufiji tilapia and its hybrids with Nile tilapia fingerlings farmed at different salinity levels and determine the antibiotic susceptibility of the isolated bacteria in at Institute of Marine sciences, Mariculture center in Pangani district, Tanga- Tanzania.

1.2 Problem Statement and Study Justification

The efforts to increase production of hybrid fingerlings are hindered by occurrences of diseases and mortalities at the hatchery stage (Nehemia *et al.,* 2013). However, the

demand of the hybrids of Rufiji tilapia male and Nile tilapia female to mariculture development is high because grows fast to the maximum growth of economic importance and can be farmed at a wide range of environmental parameters (both fresh, blackish and marine waters) (Mapenzi and Mmochi, 2016). The presence of bacterial infections in other fish species elsewhere at hatchery and adult stages has long been recognized (Austin and Austin, 2012), but less is known about the presence bacteria in Rufiji tilapia and their hybrids with Nile tilapia at the IMS-MC hatchery. Furthermore, information on the antimicrobial sensitivity for mariculture fish bacteria in Tanzania is not available (Nonga and Katakweba, 2015). No study has been conducted to assess the occurrence and antimicrobial sensitivity of bacteria in fingerlings of Rufiji tilapia and their hybrids with Nile tilapia in Tanzania. Therefore, the current study aimed to determine the occurrences of bacteria in *O. urolepis urolepis* (Rufiji tilapia) and its hybrids fingerlings crossbreed with *O. urolepis urolepis* ♂ (male) and *O. niloticus* ♀ (female) farmed at different water salinity levels. Findings would help to plan control measures for improving hybrid fingerlings production. It will also contribute in development of biosecurity principles.

1.3 Objectives of the Study

1.3.1 Overall objective

The overall objective of this study was to establish the occurrence and antimicrobial sensitivity of bacterial infections in *Oreochromis urolepis urolepis* (Rufiji tilapia) and hybrids of *O. urolepis urolepis* ♂ and *O. niloticus* ♀ fingerlings at different water salinity levels at IMS- Mariculture Center Pangani, Tanga.

1.3.2 Specific objectives

i. To determine bacterial loads in water, Rufiji tilapia and its hybrid fingerlings at different water salinity levels.

ii. To identify bacteria isolated in water, Rufiji tilapia and its hybrid fingerlings at different water salinity levels.

iii. To determine antimicrobial sensitivity of isolated bacteria from Rufiji tilapia and its hybrid fingerlings at different water salinity levels.

1.3.3 Research questions

i. Is there any significant difference between bacterial loads in water, Rufiji tilapia and its hybrid fingerings at different salinity levels?

ii. Is there any significant difference between bacteria species in water, Rufiji tilapia and its hybrid fingerings at different salinity levels?

iii. Are the pathogenic bacteria isolated sensitive to antimicrobials?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mariculture Sector in Tanzania

Mariculture involves farming of aquatic animals and plants in marine environments. In Tanzania mariculture production is dominated by the seaweed sector since the early 1990s (Mmochi, 2015). Prawn mariculture along the coast of Indian Ocean in Tanzania is also done at industrial scale (Troell *et al.,* 2011). Studies on finfish mariculture in Tanzania started in 2000s at Makoba Bay, Zanzibar (Msuya and Mmochi, 2007). Several finfish species observed to have tolerance to brackish and marine environments such as Rabbit fish (*Siganus sutor*) and Milkfish (*Chanos chanos*) were experimental upon (Nehemia *et al.,* 2013). According to Mmochi (2015) tilapia mariculture in Tanzania started between 2011-2014 as an experiment with tilapia acclimatization for marine tilapia farming. The increasing scarcity of freshwater, especially in the arid regions and the decline of capture fishery in water bodies such as ocean, lakes and rivers makes mariculture to become important. The decline is due to over exploitation, illegal fishing and pollution that have increased demands to develop aquaculture in brackish and seawater (Troell *et al.,* 2011; Mmochi, 2015).

Mariculture in Tanzania is at early developmental phase, with the exception of seaweed culture (especially in Zanzibar), that has grown substantially over the past decades (Troell *et al.,* 2011). Being at early development phase reported to be the most challenging activity among others in the country due to failure of extension systems to disseminate technical competence required for farming and marketing (Mmochi, 2015). Lack of good quality seeds for mariculture and genetic diversity among fish species are also reported to be a challenge to mariculture production in the country (Troell *et al.,*

2011; Mmochi 2015). The transportation of finfish for mariculture from one area to another is another challenges as they can contribute to the spread of diseases, facilitate biological invasions, or reduce genetic variability (Nehemia *et al.,* 2013; Mmochi, 2015).

Furthermore bacterial infections that were initially considered as typical of fresh water aquaculture, such as (Aeromonasis), bacterial kidney disease and some types of streptococcosis, vibriosis Pseudomonadiasis, Mycobacteriosis (fish tuberculosis) are reported to be the problems in some tilapia marine culture (Toranzo *et al,*. 2005). Weak business skills among farmers and lack of market access are reported to be main economic constraints on mariculture development (Mmochi, 2015). Therefore there is the need to diversify mariculture practices by introducing new candidate species and adaptation of culture methods to suit existing species (Msuya and Mmochi, 2007).

2.2 Bacteria Infections in Farmed Fish

Bacteria are among the unicellular organisms that have ability to cause diseases to living organisms. They are reported to be a major cause of infectious diseases in both wild and farmed fish. Pathogenic bacteria are categorized into two groups (Karimi, 2015). The first category being those bacteria that occur naturally in the environment and are capable of causing infections in healthy fish under stress such as *Aeromonas hydrophila, Vibrio parahaemolyticus, Vibrio anguilarum* and *Listeria monocytogenes (*Al-Harbi and Uddin, 2003). The other group is non-indigenous bacteria capable of causing infection in the existence of a primary pathogen or presence of predisposing factors such as stress. These include members of family Enterobacteriaceae (Huicab-Pech *et al.,* 2016). Pathogenic bacteria are also categorized into Gram negative like members of genus Aeromonas, Vibrio, Pseudomonas, Yersinia, Pasteurella, Edwardsiella

and Cytophaga and Gram positive such as, Staphylococcus, Streptococcus, Mycobacterium, Bacillus and Clostridium spp (Noga, 2011; Austin and Austin, 2012).

Most bacterial diseases are caused by Gram negative rods bacteria as compared to Gram positive bacteria (Brown, 1993). Transmission within the culture systems is through contacts with infected fish or vertically through eggs (Austin and Austin, 2012; Novotny, 2004). These pathogenic bacteria species have been isolated worldwide mostly in tilapia (*Oreochromis species*) farmed in Asia, China, Japan, Norway, Turkey and Malaysia (Austin and Austin, 2012).

Common reported bacteria causing infections and mortalities in tilapia includes*Flavobacterium columnare, Francisella spp*., *Aeromonas veronii* (Dong *et al.,* 2015), *Edwardsiella tarda*, *Aeromonas hydrophila*, *Streptococcus agalactiae*, *S. dysgalactiae, Streptococcus iniae*, and some of *Vibrio spp*. (Huicab-Pech *et al.,* 2016; Ismail *et al.,* 2016). *Staphylococcus spp* are reported to cause infections in red tilapia in Turkey (Canak and Timur, 2017). Infected fish present non-specific clinical signs such as septicaemia, haemorrhagic septicaemia, fins rot, ulcerations, exophthalmia, gill disease and skin lesions (Austin and Austin, 2012). Therefore to confirm the diagnosis requires laboratory examination. Some bacteria are implicated as normal flora as they do not cause any harm to fish but may pose health risks to the consumer if consumed undercooked or as raw fish.

2.2.1 Staphylococcus species

Staphylococcus species are Gram positive coccid-shape bacteria, non-spore, and non-motile, facultative anaerobic bacterium occurring in all environments. They are fish pathogens that can be found in the fish intestine and faeces but can also be isolated from

kidney, liver and spleen (Caretto *et al.,* 2005). Staphylococci bacteria cause important diseases that result to high mortalities in farmed fishes (Mousavi *et al.,* 2010). *Staphylococcus epidermidis, S. warneri, S. capitis* subspecie*s capitis and S. aureus* have been reported in some marine and freshwater fish in Japan, Taiwan, Turkey and Greece (Canak and Timur, 2017; Austin and Austin, 2012). In England, European sea bass (*Dicentrarchus labrax)* has been found to be infected by *S. xylosus, S. chromogens,* and *S. warneri,* marked by a dark body, ulceration, and necrosis either in fins or skin. Staphylococcus species are reported to be dominant bacteria in tilapia fish in Kenyir Lake, Malaysia (Marcel *et al.,* 2013).

Under experimental environment when rainbow trout, Black Sea trout and European sea bream were challenged with staphylococcus species, mortalities were first observed after the 4th day of injection and continued until the 20th day. However, the infected fish presented no clinical signs at initial stage of the infection (Canak and Timur, 2017). At later stage signs of infected fish were observed and included darkening of the skin, fin base haemorrhages, skin ulcers, anaemia in the liver and accumulation of a bloody fluid in the peritoneal cavity, ulceration on the tail and systemic disease characterized by septicaemia (Mousavi *et al.,* 2010).

2.2.2 Bacillus species

Bacillus spp. is Gram-positive, spore-forming, fermentative, and aerobic rod shaped bacteria (Ashe *et al.,* 2014). Bacillus species are reported to cause infections in various fish species worldwide. The first outbreak of the disease was reported at mortalities of about 10-15% of the infected fish in Nigeria (Austin and Austin, 2016). Diseased fish were characterized by weakness, lethargy, emaciation and generalized necrotizing dermatitis, with death occurring in a few days following infection. Other clinical signs

included blood-tinged fluid in the peritoneal cavity, petechial and focal necrosis in the liver and kidney, enlarged spleen being soft and friable and hyperaemic stomach (Oladosu *et al.,* 1994). Ferguson *et al*. (2001) demonstrated bacillary necrosis in farmed catfish (*Pangasius hypophthalmus*) from the Mekong Delta, Vietnam. Mortalities among infected fish were observed. Post-mortem lesions included presence of 1-3 mm diameter white necrotic and granulomatous areas in the kidney, liver, spleen and viscera. According to Austin and Austin (2012), Bacillus species are reported to cause septicaemia and bacillary necrosis in various freshwater fish species including cat fish (*Pangasius hypophthalmus*) in Nigeria and Vietnam.

Bacillus cereus was also demonstrated to cause Branchio-necrosis in common carp (*Cyprinus sp*.) and striped bass (*Moronesaxatilis*) in USA (Goodwin *et al.,* 1994). *Bacillus mycoides* caused an epizootic disease that occurred in channel catfish in Alabama during 1992. Infected fish were darker in color, inappetent, displaying pale areas or ulcers on the dorsal surface, focal necrosis of the epaxial muscle and opaque muscle (Orozova *et al*., 2017). Furthermore *Bacillus mycoides* caused ulceration in channel cat fish (*Ictalurus punctatus*) in Poland and USA (Goodwin *et al.,* 1994). *Bacillus subtilis* has been associated with branchio necrosis in common carp in Bulgaria (Ferguson *et al.,* 2001 and Austin and Austin, 2012).

2.2.3 Enterobacteriaceae species

Enterobacteriaceae is a large heterogeneous group of Gram negative, non-sporing rods often motile bacteria, whose natural habitat is the intestinal tract of human and animals (Oliveira, 2017). They contain a large number of genera that are biochemically and genetically related to one another. The family includes Escherichia, Shigella, Salmonella, Enterobacter, Klebsiella, Proteus and Citrobacter spp. (Elsherief *et al.,* 2014). According

to Oliveira (2017), members of Enterobacteriaceae are the major cause of infection in humans and are commonly found in fish as a normal flora. However, some members are reported to be opportunistic enteric bacterial pathogens such as Aeromonas, Escherichia, Klebsiella, Hafnia, Serratia, Plesiomonas, Shigella, Salmonella, Morganella and Yersinia (Surendraraj *et al.*, 2009). Enterobacteriaceae sp are reported to cause diseases in salmonids, sun fish (*Mola mola*), carp (*Cyprinus carpio*) in Europe, India, USA, Redpest, edwardsiellosis, emphysematous putrefactive disease, fin and tail disease, enteric septicaemia of catfish, haemorrhagic septicaemia in fresh water and marine waters fish species, enteric red mouth in salmonids in China, Indonesia, Japan, USA, Vietnam, Australia and Israel (Austin and Austin, 2012).

2.2.4 Micrococcus species

Micrococcus species are Gram positive coccid-shape bacteria, non-spore, and non-motile, facultative anaerobic bacterium. It grows well in environments with little water or high salt concentrations. They include members from family Micrococcaceae (Caretto *et al.,* 2005). Their characteristics are similar to staphylococcus species except that they show positive oxidase test.

Micrococcus species are mostly found in fish as a normal flora while some are pathogenic such as *Micrococcus luteus* reported to cause mortalities in rainbow trout during summer and spring (Mousavi *et al.,* 2010). The general clinical signs of the disease includes darkening of the skin, fin base haemorrhages, skin ulcers, anaemia in the liver and bloody fluid in the peritoneal cavity, ulceration on the tail, lesions on the skin and caudal fin and internal organs such as muscle, liver and spleen (Seyit *et al.,* 2005; Mousavi *et al.,* 2010; Canak and Timur, 2017). Micrococcus's infections were reported in red tilapia reared in cage-cultured system in Kenyir Lake, Terengganu and

Semantan River, Pahang, east of Malaysia Peninsular. The infected fish were reported to have no clinical sign but however, there were observed gross lesions, swollen or congested kidney and pale liver (Gisain *et al*., 2010; Marcel *et al*., 2013).

2.3 Predisposing factors for occurrences of bacterial diseases in fish culture

Bacteria diseases are widespread and can be of particular importance in fish culture systems. Karimi (2015) pointed out that, bacteria are everywhere, widely distributed in the environments. Bacteria exist as micro flora in water until certain environmental conditions such as poor water quality occur, which could impose a stress on fish, thereby making them vulnerable to infection, most especially by pathogenic bacteria. The occurrences and diversity of bacteria in a cultured fish depends on the habitat (Sichewo *et al.,* 2014) and are favoured by environmental conditions, fish health and virulence of the pathogen (Huicab-Pech *et al.,* 2016). Some bacteria such as *vibrio spp, Pasteurella spp, Streptococcus spp* are common in both salt water and freshwater (Austin and Austin, 2012; Noga, 2011), while *Aeromonas spp, flavobacterium and Pseudomonas spp* are only common in fresh water and while other like *Acinetobacter* are only in marine water (Silva, 2010). Furthermore, bacteria causing diseases are mostly associated with poor management of physicochemical parameters (Faruk, 2004) and failure of implementation of biosecurity practices (Brown, 1993). Bhatnagar and Devi (2013), pointed out that, any changes in environmental conditions surrounding the culture systems have influence on water quality and may cause stress to fish, triggering bacterial infection and disease outbreaks. Silva (2010), reported that, bacterial communities may also be attributed with process of feeding and drinking behavior of fish.

2.4 Bacterial Load and Their Importance to Fish Health

Bacteria load is the number and type of microorganisms contaminating an object or organism. Bacteria load in culture systems indicate their pathogenesis to fish (Ajayi and

Okoh, 2014; Hardi *et al.,* 2018). According to Hardi *et al.* (2011), bacteria with an LD_{50} below 10^{5} CFU mL-1 has been categorized as being pathogenic in which a density of about 10^{4} CFU mL-1 in a cultured tilapia experienced mortalities of up to 48% at day 7. According to experimental findings reported by Hardi *et al.* (2018), the number of bacteria causing mortality in fish is approximated to 10^4-10^8 CFU mL-1.

It was also explained that highest mortality was found in tilapia injected with Enterobacter sp., Listeria sp. and Streptococcus sp. at a density of 10^3 CFU mL-1 resulted in 40% mortality in tilapia *(O. niloticus).* Furthermore, Staphylococcus sp. reported to cause mortality of up to 80% at a density of 10^9 CFU mL-1, with an average LD_{50} at a density of 10^4 CFU mL^{-1} of bacteria. According to Hardi *et al.,* (2018) the number of bacteria causing mortality in fish was approximated to 10^4 -10^8 CFU mL^{-1}.

2.5 Antibacterial Sensitivity Test

Antibiotics are chemicals or form of chemotherapy that play an essential role in treatment of infections or diseases (Hall and Mah, 2017). They are categorized into natural or synthetic, where natural antibiotics are produced by living organisms such as bacteria and fungi and synthetic antibiotics produced by industrial chemical synthesis (Huicab-Pech *et al.,* 2016). Their actions depend on a type of bacteria, or on whether are Gram-positive or Gram-negative. Antibiotics exhibits two mechanism of action that have irreversible effects which are bacteriostatic and bactericidal (Burridge *et al.,* 2010). Mechanisms of action of antibiotics focus on key components such as inhibition of cell walls synthesis by glycopeptides and β- lactanms, alteration of cell membranes, inhibition of proteins synthesis by targeting the 30S or 50S subunits of bacterial ribosome, and inhibition of nucleic acids synthesis by inhibiting enzymes used in DNA replication (Burridge *et al.,* 2010). The effectiveness of antibiotics to bacteria depends

on the type of the pathogens whereby some bacterial pathogens produce proteineous toxins which allow them not to be affected by antibacterial agents (Hall and Mah, 2017).

However, those processes and components can be manipulated by bacterial pathogens to directly affect the functions of the cell components causing antibiotic resistance (Huicab-Pech *et al.,* 2016). Antibiotic resistance is the natural or acquired ability of a pathogen to survive in the presence of an antibiotic or chemical where at first it was susceptible to (Huicab-Pech *et al.,* 2016). This occurs by natural variations or acquired changes that occur in the target sites of antimicrobials that prevent the binding of drugs to the binding sites hence resistances. Those changes that occurs in the bindings sites are due to either spontaneous mutation of the bacterial gene on chromosomes or horizontal gene transfer in the environment, via natural transformation, transduction, or conjugation and enables resistant genes to move quickly throughout different bacterial populations and communities (Watts *et al.,* 2017).

The source and factors causing bacterial resistance gene in the culture system are commonly reported to be due to antibiotic usage (Halden, 2015). Multiple antibiotic resistance and sensitivity test reaction to one antibiotic agent are usually associated with antimicrobial use. It is demonstrated that the more often the antibiotics are being used in a culture system the more likely antibacterial resistance to occur (Tendencia and de la Peña, 2001). However, the occurrences of antibiotics residues are being measured and reported in fish farms with no history of using antibiotic (Hatha *et al*., 2005). The sources of these antibiotics in the culture systems with no history of using antibiotics are reported to be unclear (Rose *et al.,* 2009). Low but significant levels of tetracycline, macrolide (virginiamycin) and sulfonamide antibiotics have been detected in farmed trout (*Oncorhynchus* spp.), tilapia (*Oreochromis* spp.) and salmon with no history of

using antibiotics from 11 countries including the US, China, Mexico, Thailand, Scotland, and Canada (Done and Halden, 2015). These are reported to be due to terrestrial bacteria entering with antibiotic-resistant plasmids that might results for the prevalence of resistance genes in the culture systems (Subramanian *et al.,* 2008).

There are variations in resistance to different antibacterial agent within the same group of fish that may be due to the areas and the sources of fry stocked as well as the fish immunity (Muniruzzaman and Chowdhury, 2004). The causes of the variation in resistances to the same group of bacteria to same antibacterial agent are unclear (Rose *et al*., 2009). Furthermore, the consequences of antibacterial resistant in fish farm results in significant impacts to the immunity of fish as well as indigenous bacterial hence drug-resistant bacteria and reduced efficacy of antibiotic to treatment of those bacterial fish diseases (Liasi *et al*., 2009). Antibacterial resistance that occurs in fish culture might also have an impact to human health (Rhodes *et al*., 2000a). According to Sørum (2006) the exchange between antibiotic-resistance bacteria and transferable resistance genes in fish culture and disease-causing bacteria in human may occur resulting into antibiotic-resistant infections and hence reduced efficacy of antibiotic drugs.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out at the Institute of Marine Sciences (IMS) Mariculture Centre (MC) located at Bweni Village, Pangani District in Tanga Region (05° 26´ 0″ South, 38° 58´ 0″ East), on the north east coast of Tanzania. The site is drained by Pangani River whose basin is bordered by the Kilimanjaro and Meru Mountains and Pare and Usambara Mountain Ranges. It is well endowed with several species of terrestrial and marine ecosystems including mangroves. The Pangani River has a diversity of tilapia species including *O. panganikorogwe, O. panganipangani, O. Variabilis, T. rendalli* and *T. zillii.* Pangani is characterized by annual rainfall of above 1000 mm with temperature varying between 25°C and 30°C. The climate is generally warm and wet annually. The center is involved in the culture of Nile tilapia, Rufiji tilapia and hybrids obtained by cross-breeding Rufiji tilapia (*O. urolepisurolepis*) male and Nile tilapia (*O. niloticus)* female acclimatized to different salinities levels. The *O. urolepis urolepis* and *O. niloticus* brood stocks were collected from Rufiji River in Central Eastern Tanzania draining into Indian Ocean and Lake Victoria in Mwanza, respectively.

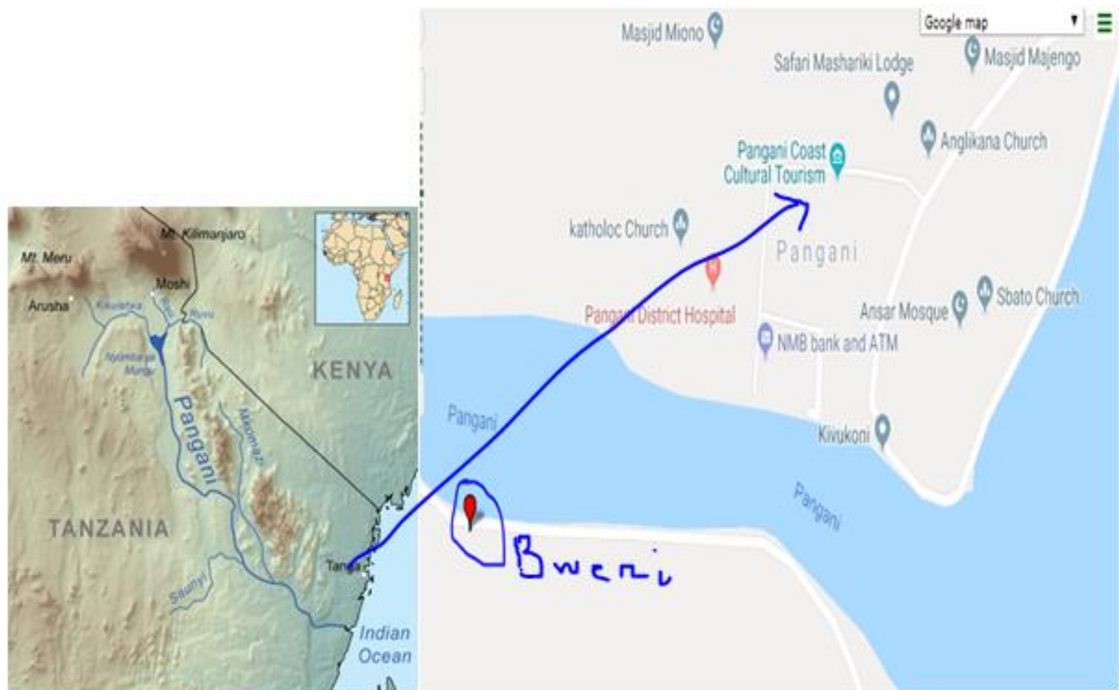


Figure 1: Map showing Pangani River and the study area: Source from Google map.

3.2 Study Design and Set Up

A complete randomized experimental study design was adopted in which a total of 12 tanks of about 31 m^3 were used per fish species with a set up as detailed in appendix one and two.

3.3 Study Setup and Sampling Strategy

The sampling of the whole fingerlings and water was done in three replicated tanks systems of about 31 m^3 set at salinity levels of 2, 15, 25, and 35 making a total of 12 tanks per fish species. A total of 120 fingerlings samples were collected where by 60 fingerlings of *O. urolepis urolepis* were collected from 12 tanks (five samples from each tank) and 60 fingerlings of hybrids were also collected from 12 tanks (five samples from each tank). Water samples were collected from each tank containing Rufiji tilapia and Hybrids fingerlings respectively.

3.3.1 Collection of fingerling samples

The whole fingerlings were collected out of different production units. From each tank five fingerlings were collected by using a cast net. Fingerlings were physically examined for the presence of gross lesions. Their weights and lengths were measured. Samples of fingerlings collected were packed in sterile universal bottles and put in a cool box with ice packs and transported to SUA for laboratory analysis.

3.3.2 Water sample collection

Water samples were collected from each tank and put into sterile 250 ml screw capped glass bottles. The bottle was submerged in water at a depth of about 15 cm to 20 cm below water surface at the middle from each tank. Samples collected were packed in a cool box with ice packs and transported to SUA for laboratory for analysis.

3.3.3 Measurement of physicochemical water parameters

In situ, water quality parameters such as temperature and pH were measured on site, using a thermometer and electronic pH meter (Metler Toledo 320 model). Dissolved Oxygen was measured using DO meter, as described by Ismail and co-workers (2016).

3.4 Bacteriological Analysis

3.4.1 Bacterial enumeration

Bacteria load (enumeration) was determined by total viable count and calculated as colony forming units per ml (CFU mL-1) as described by Shinkafi and Ukwaja (2010). Serial dilutions of the original stock sample of water and grounded fingerlings solution were prepared. Each dilution was plated on solidified freshly prepared plate count agar (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, India) and spread using a sterile glass rod and incubated at 37 ^{o}C for 24 hours after which the colonies that grown on the plates

were counted. Those counts within 30-300 colony forming units (cfu) were reported as total viable count (TVC).

3.4.1.1 Total viable bacterial count (TVC) from water sample

Serial dilutions of water samples (10-folds) were done aseptically starting with 1ml of water sample. Then, from 10^{-2}, 10^{-3} and 10^{-4}, 0.1ml was taken from the test tube using a sterile pipette and put into a sterile Petri dishes containing Plate count media (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, India). The surface spreading method of the inoculums was done using sterile swabs. All samples inoculated were incubated at 37 ^{o}C for 24 hours. The resulting colonies were counted, examined in terms of colour, size, dryness /wetness. Total viable count was calculated as colony forming unit (CFU mL-1).

3.4.1.2 Total viable bacterial count (TVC) from fingerlings

Sample of fish (fingerlings) was blended aseptically using sterile mortar and pestle and homogenized by mixing with normal saline. Serial dilutions (10-folds) were done starting with 1ml homogenate. Thereafter, 0.1 ml of samples was collected from dilutions 10^{-4}, 10^{-5} and 10^{-6} and spread aseptically onto sterile Petri dishes containing Plate count media (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, India). All samples inoculated were incubated at temperature ranges from 28 ^{o}C -37 ^{o}C for 24 hours. Assessment of colonies was done as described in 3.4.1.1 above.

3.5 Isolation and Identification of Bacteria from Water and Fish Samples

The distinct colonies from water and fish sample cultures were sub-cultured in order to get pure cultures. Aseptically, single colonies were streaked onto Blood Agar and MacConkey Agar (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, India). All inoculum were incubated over night at 37 ^{o}C. The grown colonies were identified according to

their difference in colony morphological characteristics of size, color and ability of hemolysing blood and fermenting lactose. The colonies were subjected to biochemical tests such as, Gram staining, oxidase, catalase, and coagulase, mannitol and IMVIC test.

3.5.1 Biochemical tests

Different biochemical tests (Catalase, Coagulase, Oxidase IMVIC, and Mannitol salt agar test) were performed to confirm the suspected isolates as shown below. This was done according to Markey *et al.* (2013) and Huicab-Pech *et al.* (2017).

3.5.1.1a Catalase test

The test was done to identify bacteria that contain catalase enzymes and differentiating aerobic and obligate anaerobic, gram positive bacteria. One drop of 3% hydrogen peroxide was put on microscopic slide and the bacteria colony from the bacterial culture incubated overnight by using plate count media was added by using sterile wooden loop. Quick Gas bubble or foam formation indicates positive result.

3.5.1.1b Coagulase test

The test was done to distinguish between *Staphylococcus aureus* from coagulase negative Staphylococcus spp.

i. **Slide coagulase test**

- A drop of normal saline was put on two separate slides. A colony of the test bacteria was emulsified in each of the drops to make suspension. A drop of rabbit plasma was then added and mixed gently with the suspension. Clumping (due to coagulation) of the organisms in 10 seconds indicate positive result.

ii. Test tube coagulase test

This test was done for the colony suspected to be *Staphylococcus aureus.* The dilution of one ml rabbit plasma in five ml of normal saline (1:5) was used in coagulase tube test. One ml of rabbit plasma was placed in test tubes. About 5 colonies of the test colonies was added and shaken. The sample was incubated for four hours at temperature 28-37 ^{o}C. The formation of clots indicated a positive result while where plasma remained wholly liquid indicated negative results.

3.5.1.1c Oxidase test

This test was done to identify the bacteria that produce Cytochrome C oxidase enzymes such as Streptococcus spp and *Micrococcus luteus.* A colony of bacteria culture was rubbed on the disc impregnated with oxidase reagents. The rapid change in colour was observed after 10-30seconds. The formation of deep blue color indicated positive results.

3.5.1.1d IMVIC Test

The test was done to distinguish members of family Enterobacteriaceae .

i. Citrate test

Colonies to be identified were inoculated into (5ml) sterile Simmon's Citrate media and incubated at 28-37 ^{o}C for 24 hrs and observed for the colour change.

ii: Indole reaction test

The micro-organisms to be identified were inoculated into universal bottles containing tryptone broth (5ml) and incubated for 24 hrs at temperature range 28-37 ^{o} C, and then five drops of Kovac's reagent was added. Then colour change was observed after 10 seconds.

iii: Methyl red test (MR)

The micro-organisms to be identified were inoculated into the universal bottle containing sterilized Glucose phosphate peptone water (5 ml). After 24 hrs of incubation at 37 ^{o} C about five drops of Methyl red solution was added. The color change was observed.

iv: Voges-Proskauer test (VP)

The micro-organisms to be identified were inoculated into the universal bottle containing sterilized Glucose phosphate peptone water (5 ml). After 24 hrs incubation at temperature range 28-37 ^{o} C about five drops alpha-napthol, was added followed by five drops of potassium hydroxide. The mixtures were allowed to stay for about 15 minutes and color change was observed.

3.5.1.1e Mannitol salt agar test

This test was done for Gram-positive bacteria and salt tolerant bacteria (*Staphylococcus* and Micrococcaceae). Also the test was done to differentiate between *Staphylococcus epidermids* from other members of *Staphylococcus spp*. A 24hrs grown colony was aseptically inoculated on a plate containing mannitol salt agar (MSA) (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, India) and then incubated overnight at 28 -37°C. Then grown colonies were observed for their fermentation and acid production indicated by color change of the media.

3.5.2 Molecular identification of selected isolates (*Staphylococcus spp and Bacillus spp*)

Molecular confirmation was done for presumptive selected *Staphylococcus* and *Bacillus spp* due to their high prevalence in the study area. Molecular identification of

Staphylococcus spp was done as described by Ismail *et al.* (2010), *Bacillus subtilis* according to Ashe *et al.* (2014) and *Bacillus cereus* according to Gdoura-Ben Amor *et al.* (2018).

3.5.2.1 DNA Extraction

Genomic (DNA) from pure bacterial culture of selected isolates were extracted by boiling method using water bath (Sila *et al.,* 2009). A 24hrs culture colony was taken and diluted with sterile deionized water into Eppendorf tubes and put into water bath and boiled at 100°C for 20 minutes. Purity of isolated DNA was qualitatively analyzed using 1.2% and 1.5% Agarose Gel electrophoresis for Staphylococcus and Bacillus spp respectively, with Gel red (4 µl) staining using 1X TBE buffer at 100V for 40 minutes. A 1000 base pair ladder for Staphylococcus spp and 50 bp- 1000 bp for (Bacillus spp) were used as molecular size marker. DNA bands were observed under UV light using Gel Doc System.

3.5.2.2 DNA amplification by PCR method

i. *Staphylococcus spp*

The genus and species specific primers used for speciation of Staphylococcus by PCR are described in Table 1. The PCR mixture contained each of the following per reaction: 6.25 µml PCR, buffer containing KCl and Tris–HCl (pH 8.4) salts, 25 mM $MgCl_2$ (Geneworks, Australia), 1.25 mM deoxyribonucleotide triphosphate (dNTPs) (Finnzymes, USA), 1.25 pmol/µl each primer (Geneworks, Australia), 10 mg/ml bovine serum albumin (BSA) (Sigma, Australia) and 1 U Taq (Geneworks, Australia). Deionized water was added to adjust the volume to 22.5 µl prior to the addition of DNA template. The polymerase chain reaction was conducted in TAKARA PCR thermal cycler machine for 35 cycles of denaturation at 94°C for 30s, annealing at 55 °C for 30 s,

extension at 72°C for 30 s and final extension step at 72°C for 7 min. The PCR products were separated on 1.2% agarose gels, stained with 4 µl Gel red including a 4 µl DNA marker of 1000bp in a gel electrophorences machine and visualized using UV transilluminator. Polymerase chain reaction was run with the control *S. epidermidis*.

ii. *Bacillus subtilis*

The PCR reaction mixtures (50 µl) contained, dNTPs 100 lmol; 1X PCR buffer (10 mM Tris Cl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01 % gelatin); each primer 10 pmol; Taq DNA polymerase (NEB) 0.75U and bacterial DNA 100 ng. The touch down PCR were run in TAKARA PCR Thermo cycler machine in a volume of 50 µl with initial denaturation of 94 ^{o}C for 5 min followed by ten cycles of touch down program (94 ^{o}C for 30 s, 70 ^{o}C for 20 s and 74 ^{o}C for 45 s, followed by a 1 ^{o}C decrease of the annealing temperature every cycle). After completion of the touchdown program, 25 cycles were subsequently performed (94 ^{o}C for 30 s, 60 ^{o}C for 20 s and 74 ^{o}C for 45 s) and ending with a 10 min extension at 74 ^{o}C. PCR reactions were run on a 1.5 % agarose gel in 1X TBE.

iii. *Bacillus cereus*

The amplification reactions were carried out in TAKARA PCR Thermo cycler machine as follows: 4min at 95°C, 30 cycles of 30s at 95 ^{o}C, 30s at 59 ^{o}C ^{0}C, and 1 min at 72 ^{o}C followed by a final extension step at 72 ^{o}C for 7min.

Table 1: Genus /species specific primer pairs used to identify staphylococcus and bacillus species

Primers	**Target**	**Bp**	**Primer sequence (5′–3′)**	**References**
TStaG422	16S rDNA gene	370	GGCCGTGTTGAACGTGGTCAAATCA	Ismail *et al.* (2010)
TStag765			TIACCATTTCAGTACCTTCTGGTAA	
Se705-1	*S. epidermidis*	124	ATCAAAAAGTTGGCGAACCTTTTCA	Ismail *et al.* (2010)
Se705-2			CAAAAGAGCGTGGAGAAAAGTATCA	
Sa442-1	*S. aureus*	108	AATCTTTGTCGGTACACGATATTCTTCACG	Ismail *et al.* (2010)
Sa442-2			CGTAATGAGATTTCAGTAGATAATACAACA	
Sap 1	*S. saprophyticus*	221	TCAAAAAGTTTTCTAAAAAATTTA	Ismail *et al.* (2010)
Sap 2			ACGGGCGTCCACAAAATCAATAGGA	
EN1F	*Bacillus subtilis*	1311	50-CCAGTAGCCAAGAATGGCCAGC-30,	Ashe *et al.* 2014
EN1R			50-GGAATAATCGCCGCTTTG TGC-30)	
F	*Bacillus cereus*	71	5′GAAAAAGATGAGTAAAAAACAACAA-3′	Gdoura-Ben Amor *et al.* (2018)
R			5′-CATTTGTGCTTTGAATGCTAG-3′	

3.6 Antibacterial Sensitivity Test

Antibacterial sensitivity testing of pathogenic isolates was performed by disc diffusion method on Muller Hinton (MH) Agar (Pronadisa, Spain) as described Ruangpan and Tendencia (2004) in the laboratory manual of standard methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environments. In this study the most commonly used antimicrobial agents in livestock and humans as described by Pezzoti *et al*. (2003) were tested for sensitivity. A panel of antimicrobial agents manufactured by Hamphire- England Oxoid company, Ltd, comprising 11 classes of antibiotics were tested (Table 2). The antibiotic reagents used and their concentrations are as shown in the (table 2). Colonies were picked and emulsified in 0.85% Sodium chloride to create a suspension matching 0.5 McFarland standard; at an approximate concentration of 1.5×10^8 CFU/ml.

About two hundred µl of the suspension was inoculated on the plates, spread using sterile swabs and allowed to dry for 2–5 min. The antibiotics were applied to the seeded agar plate. The plates were incubated at 35°C - 37°C for 48 hours under micro aerobic conditions. After 48 hours of incubation, the diameters of inhibition zones were measured by using a ruler. Interpretations and analysis on the response of isolates to antibiotics such as susceptible, intermediate and resistant was based on the standards of the Clinical and Laboratory Standards Institute (CLSI, 2010) and the Laboratory manual of standard methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environments.

Table 2: Antimicrobial agents used for sensitivity testing of the isolated bacteria

S/N	Antimicrobial agents	Concentration
1	Tetracycline (TE30),	30µg/ml
2	Chlorampenicol (C30),	30µg/ml
3	Gentamycine,(CN10),	10µg/ml
4	Amoxycilin (AMC30),	30µg/ml
5	Neomycine(N10),	10µg/ml
6	Ampicillin (AMP 10)	10µg/ml
7	Sulfamethoxazole (SMX 50),	50µg/ml
8	Cefataxime(CTX 30),	30µg/ml
9	Penicillin G (P 10).	10µg/ml
10	Erythromycine (E15)	15µg/ml
11	Ciproflaxcine (CIP 5),	5µg/ml

3.7 Data Analysis

In this study both categorical and continuous data were obtained. Data obtained were entered in Ms. Excel sheets version 2010, cleaned before being imported to SPSS software packages version 20. Descriptive statistics was used to estimates and compare means and standard deviation by SPSS version 20. Non parametric test (Kruskal Wallis test) was used to determine the difference in bacteria load and number of isolates between culture systems (all salinities). All values <0.05 were statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Water Quality Parameters and Fish Biodata

Measurements of physical-chemical parameters (pH, temperature, salinity), and DO (mg/l) were done in all tanks. The results (physical-chemical parameters, length and weight) obtained are shown in the table 3. Hybrids were observed to be bigger in weight and length as compared to Rufiji tilapia though all were of the same age and stocking density.

Table 3: The overall average of physical chemical water parameters measured at different salinity levels

	WQ			Rufiji tilapia fingerlings		Hybrids Fingerlings	
Salinity (ppt)	**pH**	**T°C**	**DO (mgL^{-1}**	**Wt (g)**	**Lgh (cm)**	**Wt (g)**	**Lgh (cm)**
2	8.28±0.91	26.37±0.153	6.58±0.21	3.49±2.08	5.5± 1.19	6.61±2.31	7.35±1.84
15	8.57±0.49	26.47±0.252	6.44±0.15	2.07±0.56	4.91±0.50	6.88±1.97	7.3±1.82
25	8.44±0.45	26.23±0.12	6.5±0.18	2.82±0.63	4.81±0.58	6.63±1.6	7.05±1.5
35	8.62±0.45	26.16±0.15	6.58±0.14	3.26±1.07	5.53±0.89	6.98±3.01	7.39±2.92

Abbreviations: NIL-Absent, Wt=Weight, Lgh=Length, WQ=Water quality parameters, Mean ± STD

4.2 Overall Bacteria Loads in Water and Fingerlings

The average bacterial load in hybrids fingerlings, Rufiji Tilapia fingerlings, in Water HB and in Water RF for all salinity levels were as presented in table 4. Results of KruskaWallis test indicated no significant difference (P>0.05) in bacteria loads in all

species of fish at different salinity levels shown in table 4. Bacteria load was observed to be high in hybrid fingerlings than in Rufiji tilapia fingerlings as shown in table 4. Salinity level of 2 was observed to have high bacterial load followed by 35, 15 and 25 respectively in both samples collected from hybrids and Rufiji tilapia fingerlings tanks (fig. 2). There are no statistical differences between environments (salinities) for the different categories (Kruskal-Wallis rank-sum test; $p>0.05$ for all comparison followed by Dunn's multiple comparison test

Table 4: Bacterial load, means ± standard deviation (CFUml-1) in both water and Fingerlings samples per salinity levels

Salinity (ppt)	Load log 10 CFUml-10 HB	Load log10 CFUml-1 RF	Load Log10 CFUml-1 RFW	Load Log 10 CFUml-1 HBW
2	6.34±0.12	5.27±0.02	5.62±0.53	5.55±0.65
15	5.00±0.38	4.57±0.44	4.56±0.50	5.35 ±26
25	4.79±0.94	4.44±0.45	4.28±0.16	5.07±0.53
35	6.04±0.83	5.99±0.70	4.93±0.75	6.42±0.44
Average	5.54±0.89	5.07±0.76	4.85±0.69	5.60±0.67

Abbreviation: HB- Hybrid fingerlings, RF-Rufiji tilapia fingerlings, RFW water from tanks containing Rufiji tilapia and HBW water from tanks containing Hybrids.

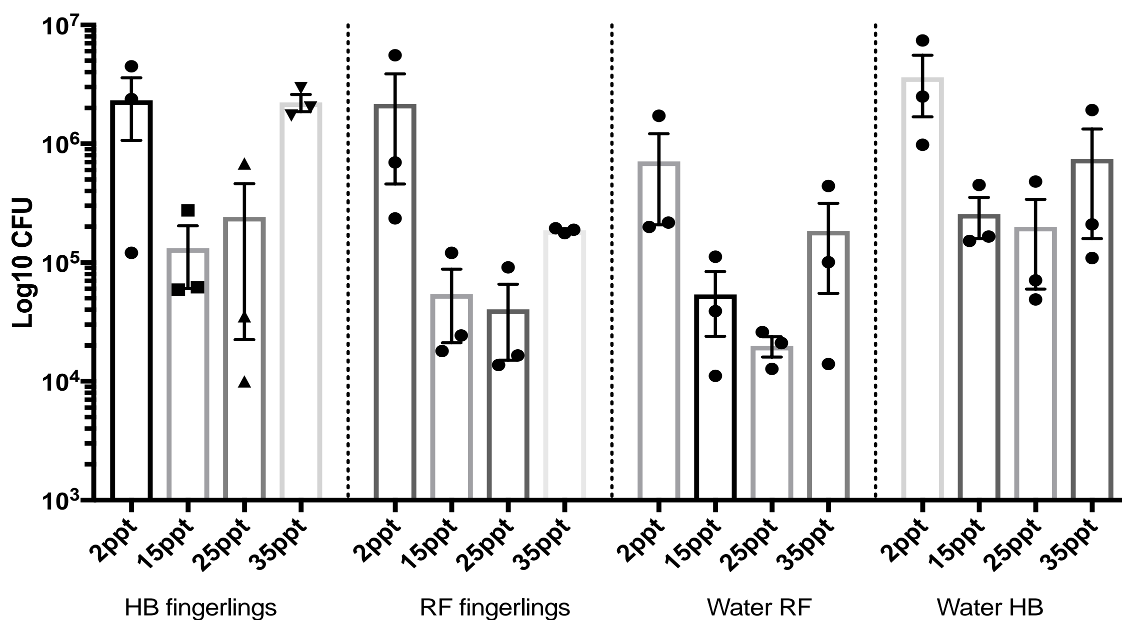


Figure 2: Bacterial load (CFUml-1) in all samples collected indicating similar trend in bacterial for all groups tested.

Table 5: Biochemical test

Bacteria isolate	Shape	Gram	Ose	Cata	Co	MSA	IMVIC			
							MR	**INDO**	**VP**	**C**
Staphylococcus spp	Cocci in Cluster	+	-	+	-	+	-	-	-	-
S. aureus	Cocci in Cluster	+	-	+	+	+	-	-	-	-
S.epidermidis	Cocci in Cluster	+	-	+	-	+	-	-	-	-
Bacillus spp	Rod	+	-	+	-	-	-	-	-	-
E.coli	Rod	-	-	+	-	-	+	+	-	-
M. luteus	Cocci in tetra pairs	+	+	+	-	-	-	-	-	-

Abbreviations: MSA=Mannitol salt agar, IMVC test includes (INDO= Indole test, MR= Methyl red,VP= Voges-proskauer test, C=Citrate test), +=positive, - =Negative , Gram= Gram staining, Co=Coagulase test, Cata=catalase test, Ose=Oxidase test

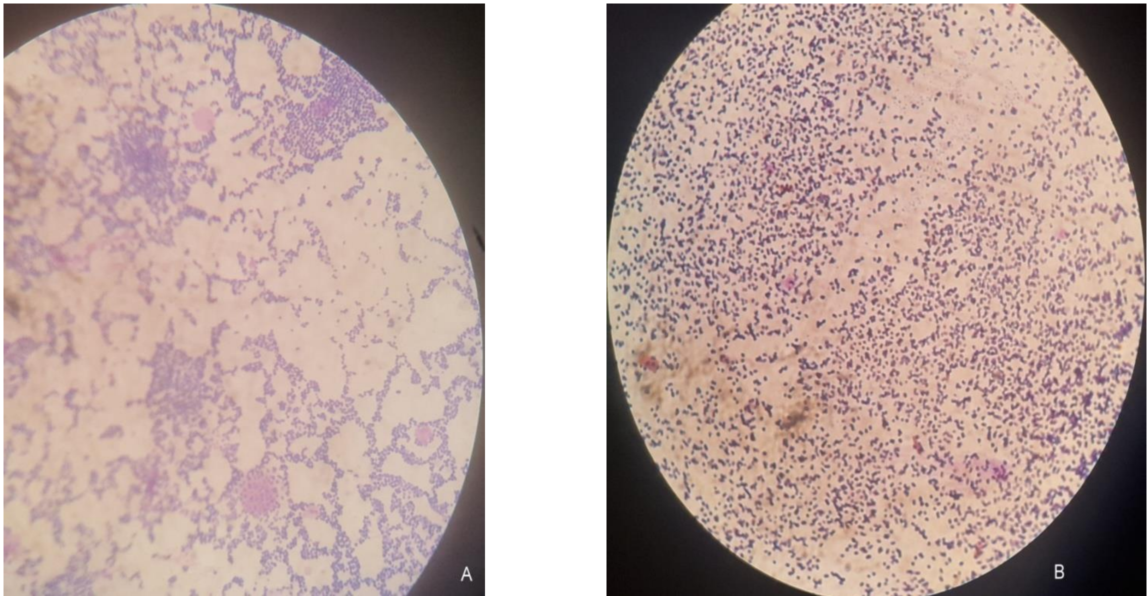


Figure 3: Gram positive *Staphylococcus aureus* (A) and *Staphylococcus epidermids* (B) isolated from hybrids tilapia as seen under light microscope showing a cluster of cocci shape

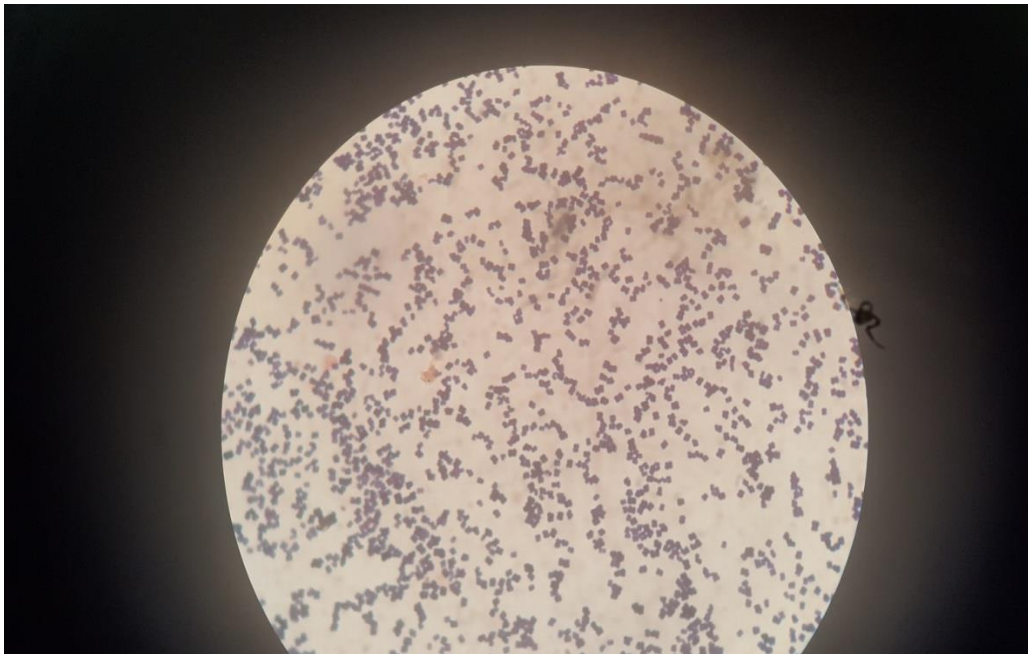


Figure 4: *Micrococcus luteus* showing gram positive cocci (in tetra pairs) as seen under light microscope

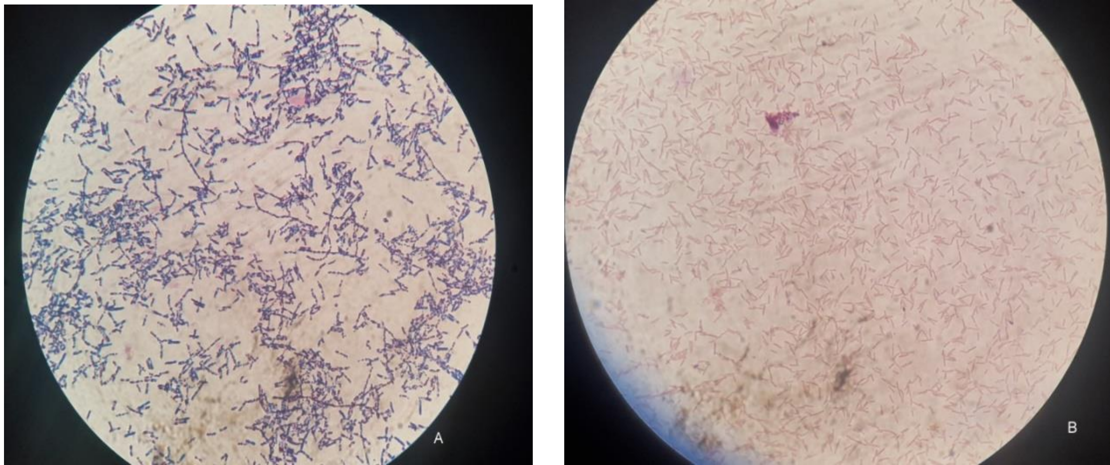


Figure 5: Gram positive members of *Bacillus spp* (A) and gram negative *E.coli* (B) isolated from hybrids tilapia as seen under light microscope.

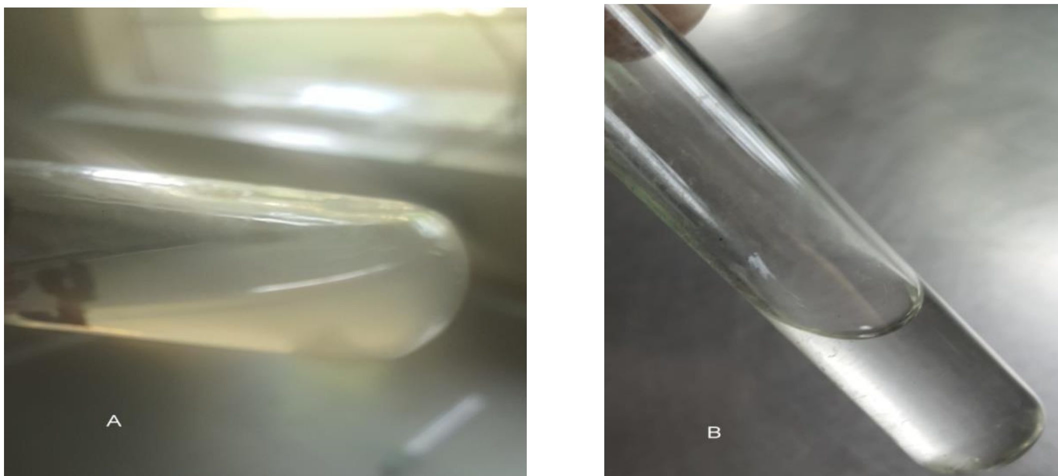


Figure 6: Test tube (A) coagulation due to enzymes (coagulase) produced by *S.aureus* in a rabbit plasma and test tube (B) showing the test tube containing *S.epidermids* a coagulase negative after four hours incubation at 28-37 ^{o}C

Figure 7: Bubbles produced by *Staphylococcus aureus* after adding 3% potassium hydroxide showing Positive catalase reaction (+ve)

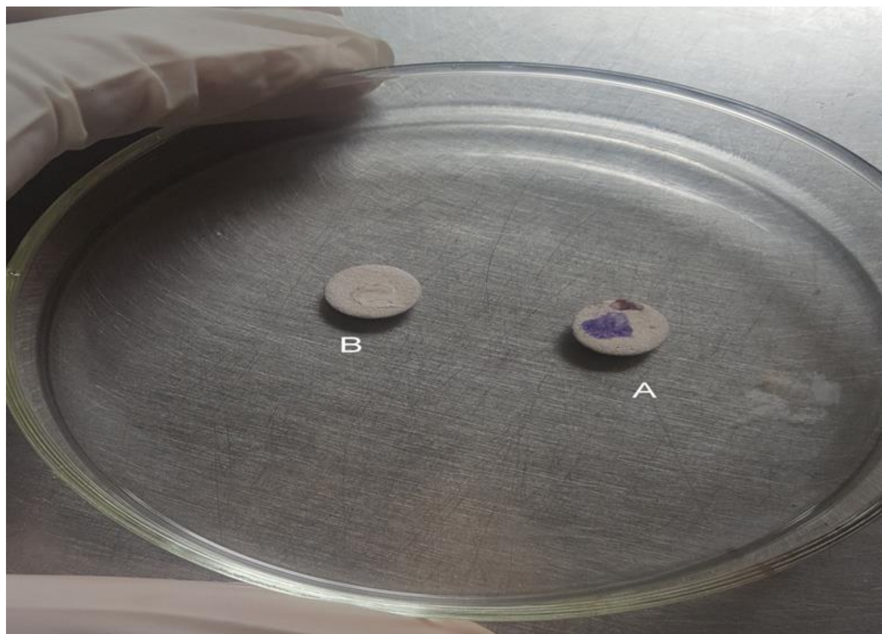


Figure 8: A positive reaction of *Micrococcus sp* (A) to oxidase test showing deep blue color after rubbing to the Oxidase disc test and negative reaction of *Staphylococcus sp* (B)

4.3 Occurrences of Bacteria from Water and Fingerlings per Salinity

A total of 132 bacteria isolates were isolated from both water and fingerlings from all salinity as shown in table 5. Salinity of 2 has the highest percentage of isolates followed

by 35, 15 and 25 has the lowest number of isolates as shown in table 6. The percentage of the isolates from water was significantly different in the percentage of isolates from fingerlings in all salinity at $p < 0.05$. There was no significant difference between the percentage of isolates from hybrids and Rufiji fingerlings respectively at $p > 0.05$(KruskaWallis test). The hybrids fingerlings had the highest isolates as compared to Rufiji tilapia, Water HB and Water RF respectively.

Table 6: Overall number of bacteria isolates per salinity in Fingerlings and Water sample (n=132)

Salinity (ppt)	**HB**	**RF**	**water HB**	**Water RF**	**Total**
2	20(15.2%)	18(13.6%)	6(4.6%)	5(3.8%)	49(37.1%)
15	12(9.1%)	10(7.6%)	4(3%)	2(1.5%)	28(21.2%)
25	7(5.3%)	6(4.6%)	2(1.5%)	2(1.5%)	17(12.9%)
35	19(14.4%)	12(9.1%)	3(2.3%)	4(3%)	38(28.8%)
Total	58(43.9%)	46(34.9%)	15(11.4%)	13(9.8%).	132(100%)

4.4 Bacteria Isolated from Water and Fingerlings per Salinity

A total of four genera were isolated namely Escherichia spp, Bacillus spp, Staphylococcus spp and Micrococcus sp. Six bacteria isolates were identified by biochemical and PCR method using species specific primers namely *S. aureus, S. epidermidis, Bacillus cereus, Bacillus subtilis E.coli, Micrococcus luteus.* The isolates obtained from water were similar to the isolates obtained in all fingerlings except Micrococcus spp. that was found only in hybrids fingerlings as shown in table 7. There was no significant difference in the frequencies of bacteria occurrences in all salinity at $p > 0.05$ (KruskaWallis test).

Table 7: Bacteria isolated in water and Fingerlings of Hybrids and Rufiji tilapia per genus showing high percentage in hybrids fingerlings (n=132)

Bacteria spp	RF	Hybrids	Water RF	Water HB	Total
E.coli	3(2.3%)	2(1.5%)	1(0.8%)	2(1.5%)	8(6.1%)
Bacillus spp	32(24.2%)	13(9.9%)	7(5.3%)	3(2.3%)	55(41.7%)
Staphylococcus sp	16(12.1%)	42(31.8%)	0	10(66.67%)	68(51.5%)
Micrococcus luteus	0	1(0.8%)	0	0	1(0.8%)
Total	51(38.6%)	58(43.9%)	8(6.1%)	15(11.4%)	132(100%)

4.5 Bacteria Isolated in Hybrids Fingerlings and Water Samples

Bacteria species isolated in Hybrids fingerlings and water per salinity levels are as shown in tables 8 and 9 respectively. There was no significant difference in the frequency of bacteria species isolated in hybrids fingerlings and water samples per salinity at $p > 0.05$ (Kruskal Wallis test). *Staphylococcus spp* was higher than all other species isolated from fingerlings followed by Bacillus spp. High number was isolated at salinity of 2 followed by 35, 15 respectively and the lowest was observed 25. Number of bacterial species isolated from fingerlings was higher than that from water samples.

Table 8: Overall bacteria isolated in Hybrids fingerlings per salinity (n=58)

		Salinity levels			
Bacteria species	**2ppt**	**15ppt**	**25ppt**	**35ppt**	**Total**
S *aureus*	1(1.7%)	0	0	1(1.7%)	2(3.45%)
S. *epidermidis*	1(1.7%)	0	0	1(1.7%)	2(3.45%)
Staphylococcus spp	14(24.14%)	8(13.9%)	5(8.6%)	10(17.2%)	38(65.5%)
M. luteus	0	1(1.7%)	0	0	1(1.7%)
B. cereus	1(1.7%)	0	0	1(1.7%)	2(3.45%)
B. subtilis	0	0	0	0	0
Bacillus sp	5(8.6%)	2(3.45%)	1(1.7%)	2(3.45%)	11(18.97%)
E. coli	1(1.7%)	0	0	1(1.7%)	2(3.45%)
Total	23(39.7%)	11(18.97%)	8(13.9%)	16(27.6%)	58(100%)

Table 9: Percentages of bacteria isolated in water collected from Hybrids tanks per salinity (n =15)

	Salinity levels				
Bacteria species	**2ppt**	**15ppt**	**25ppt**	**35ppt**	**Total**
S. *aureus*	0	0	0	0	0
S. *epidermidis*	1(6.67%)	0	0	0	1(6.67%)
Staphylococcus spp	3(20%)	2(13.3%)	1(6.67%)	2(13.3%)	9(60%)
M. luteus	0	0	0	0	0
Bacillus cereus	1(6.67%)	0	0	0	1(6.67%)
Bacillus subtilis	0	0	0	1(6.67%)	1(6.67%)
Bacillus sp	1(6.67%)	0	0	1(6.67%)	1(6.67%)
E. coli	1(6.67%)	0	0	1(6.67%)	2(13.3%)
Total	7(46.677%)	2(13.3%)	1(6.67%)	5(33.3%)	15(100%)

4.6 Bacteria species isolated in Rufiji tilapia fingerlings and water per salinity

Bacteria species isolated in the samples of Rufiji tilapia fingerlings and water were as shown in table 10 and 11 respectively. There was no significant difference in the bacteria species isolated in Rufiji tilapia fingerlings and water samples per salinity at $p>0.05$(Kruskal wallis test).

Table 10: Bacteria isolated from Rufiji Tilapia fingerlings per salinity (n=51)

	Salinity level (ppt)				
Bacteria species	**2**	**15**	**25**	**35**	**Total**
S. aureus	0	0	0	0	0
S. epidermidis	1(1.96%)	0	0	2(3.92%)	3(5.88%)
Staphylococcus spp	5(9.8%)	2(3.92%)	2(3.92%)	2(3.92%)	11(21.57%)
M. luteus	0	0	0	0	0
B. cereus	2(3.92%)	1(1.96%)	1(1.96%)	4(7.84%)	8(15.69%)
B. subtilis	1(1.96%)	0	0	1(1.96%)	2(3.92%)
Bacillus sp	10(19.61%)	4(7.84%)	3(5.88%)	7(13.73%)	24(47.06%)
E. coli	2(3.92%)	0	0	1(1.96%)	3(5.88%)
Total	21(41.18%)	7(13.73%)	6(11.77%)	17(33.33%)	51(100%)

Table 11: Percentage of bacteria species isolated from water collected in Rufiji tilapia tanks (n=8)

	Salinity level(ppt)				
Bacteria species	**2**	**15**	**25**	**35**	**Total**
S. aureus	0	0	0	0	0
S. epidermidis	0	0	0	0	0
Staphylococcus spp	0	0	0	0	0
Micrococcus luteus	0	0	0	0	0
Bacillus cereus	0	0	1(12.5%)	1(12.5%)	2(25%)
Bacillus subtilis	0	0	0	0	0
Bacillus sp	3	1(12.5%)	0	1(12.5%)	5(62.5%)
E. coli	0	1(12.5%)	0	0	1(12.5%)
Total	3(37.5%)	2(25%)	1(12.5%)	2(25%)	8(100%)

4.7 Molecular Analysis

A total of 36 samples out of the Gram positive cocci bacteria isolates were run in TAKARA PCR Thermo cycler machine using a pair of Genus- species specific primers in order to confirm the presence of S*taphylococcus* spp as shown in table 1. In proportion, 69.4% of DNA samples amplified for the 1F-2R primers in a PCR were positive for *Staphylococcus spp*, 5.6% *S. aureus*, 16.7% *S. epidermidis* and the remaining 30.6% were negative (Table 12).

A total of 20 samples out of the isolated Gram positive rod (*bacillus spp*) were also confirmed by PCR methods using species specific primers for the amplification of *Bacillus subtilis* and *Bacillus cereus* genes (Table 1). Out of those 20 isolates 60% were positive for Bacillus *cereus* and 10% were *B. subtilis* (Table 13).

In comparison, the results obtained by biochemical tests and PCR, out of 56 isolates tested by both methods 67.9% were identified and confirmed by both the biochemical

tests and PCR though there were some isolates (*Staphylococcus saprophyticus*) that were confirmed by biochemical test but not PCR method (table 12).

Table 12: Results of the samples confirmed for *Staphylococcus spp* by PCR methods

S/N	Sample ID	Fish sp	*Staphy. Spp*	*S. aureus*	*S.epdermids*	*S.saplophyticus*
1	35T2-1	HB	+	-	-	-
2	2W	HB	+	-	+	-
3	2T2-L2	RF	+	-	-	-
4	2T2-2	RF	+	-	-	-
5	15T2-3	RF	+	-	-	-
6	2T2-L3	RF	+	-	-	-
7	25T2-2	RF	+	-	+	-
8	2T2-5	HB	+	-	-	-
9	35T2-2	HB	+	-	-	-
10	35T3-1	HB	+	-	-	-
11	15T1-1	HB	-	-	-	-
12	35T2-4	HB	-	-	-	-
13	25T2-3	RF	+	-	-	-
14	2T2-L	RF	+	-	-	-
15	25W-1	RF	-	-	-	-
16	15W-1	HB	+	-	+	-
17	2T1-2	HB	+	-	-	-
18	25T2-L	RF	-	-	-	-
19	2T2-1	RF	+	-	-	-
20	15W-3	HB	-	-	-	-
21	15T2-2	HB	+	-	+	-
22	2T2-1	HB	+	-	-	-
23	2T1-2-1	HB	+	+	-	-
24	2T2-6	RF	+	-	-	-
25	25T2-5-1	HB	-	-	-	-
26	35T3-1-2	HB	+	-	+	-
27	15T2-3	HB	-	-	-	-
28	35T1-3	HB	+	-	+	-
29	25T2-5-1	RF	+	+	-	-
30	2T2-1	HB	+	-	-	-
31	25T2-3	RF	-	-	+	-
32	2T2-1-1	HB	-	-	-	-
33	15W	HB	-	-	-	-
34	2W	HB	+	-	-	-
35	35W	HB	+	-	-	-
36	25T2-1	HB	+		+	-
	CONTROL	*S.epidermis*	+	-	+	-

Table 13: Results of the samples confirmed for *Bacillus spp* (using species specific primers) by PCR methods

S/N	Sample ID	Fish sp	*Bacillus cereus*	*Bacillus subtilis*
1	35T2-4HB	HB	-	+
2	35T3-5RF	RF	+	-
3	35T2-4RF	RF	-	+
4	2T2-6-3RF	RF	+	-
5	35T3-3HB	HB	+	-
6	35T1-2RF	RF	-	-
7	2T1-4RF	RF	-	-
8	15T1-2RF	RF	+	-
9	15T2-1 HB	HB	+	-
10	35T2-5RF	HB	+	-
11	2T2-5RF	RF	+	-
12	25T2-1RF	RF	+	-
13	35T1-2HB	HB	+	-
14	35T1-6RF	RF	+	-
15	35T1-3RF	RF	+	-
16	25T1-1RF	RF	+	-
17	35T2-3RF	RF	-	-
18	15T1-2HB	HB	-	-
19	25T1-3RF	RF	-	-
20	15T3-1HB	HB	-	-
21	CONTROL	B.cereus	+	-

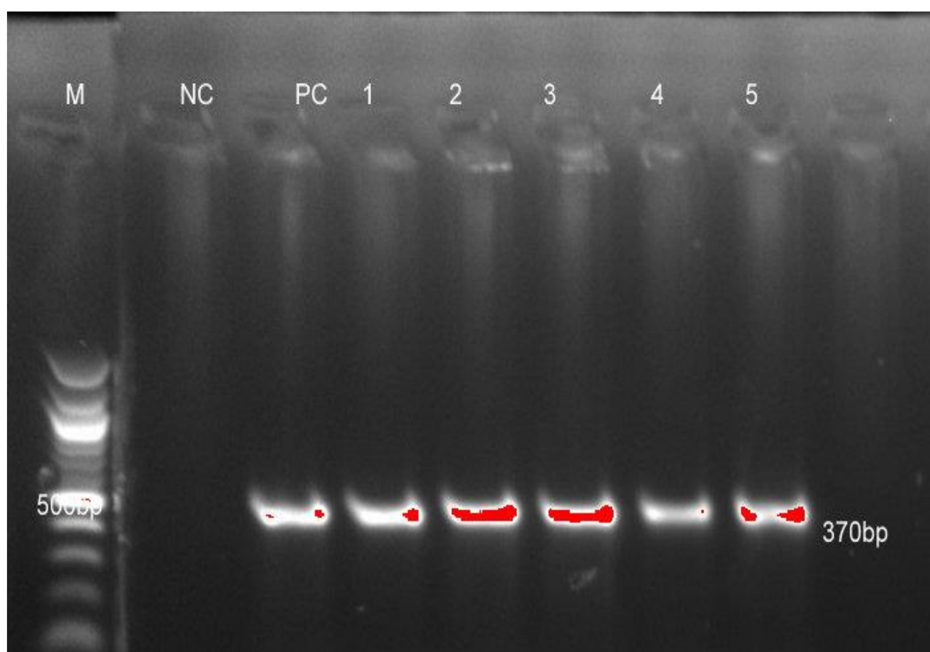
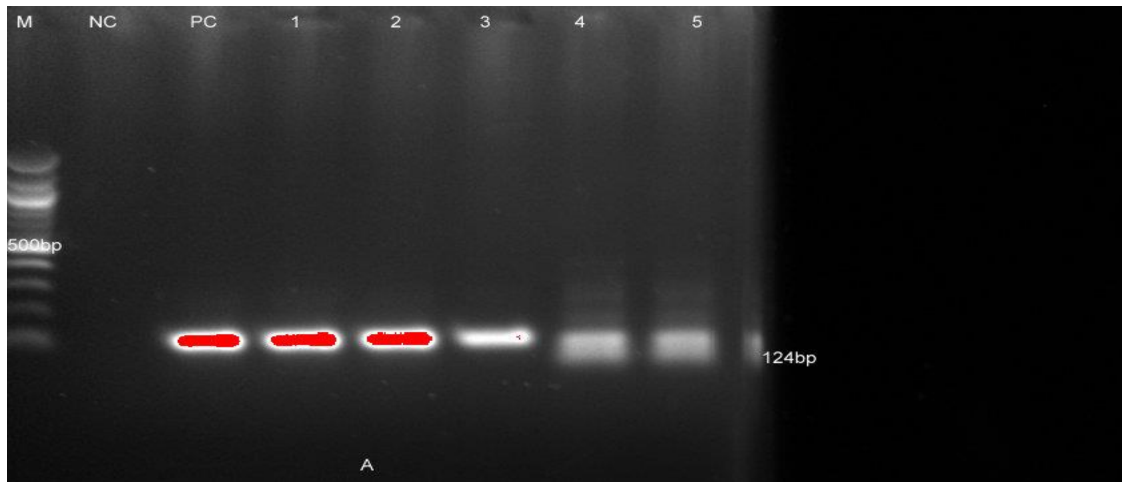


Figure 9: Representative of 68% positive results for the first batch PCR amplification of staphylococcus spp at 370bp.



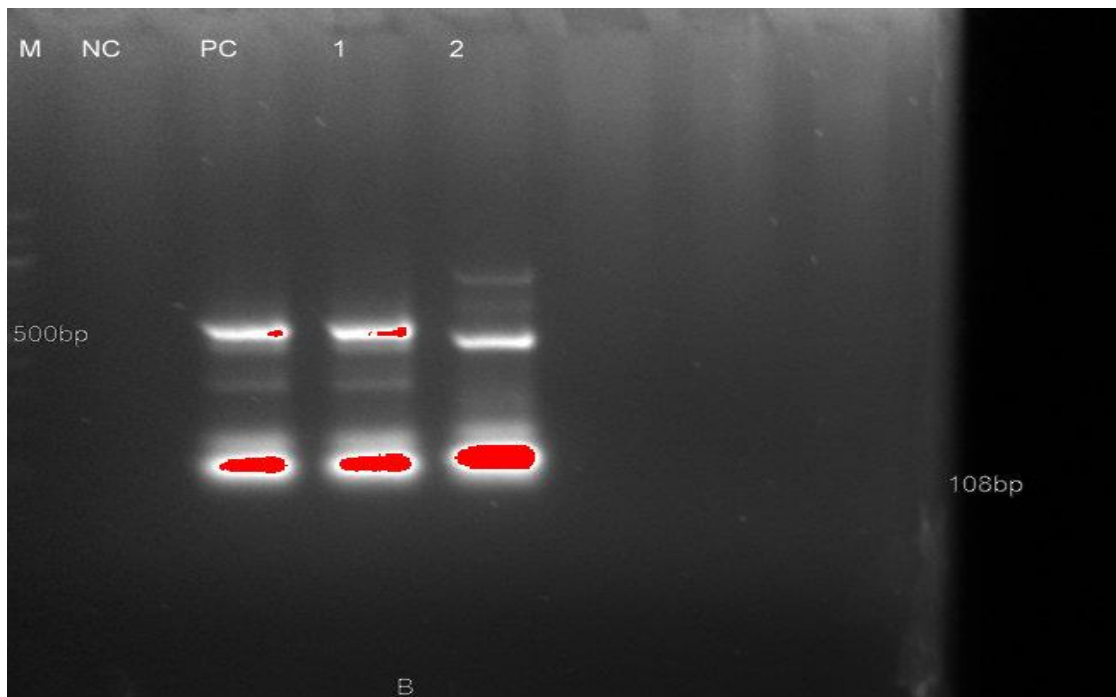


Figure 10: Represents positive results of PCR amplification of (A)*Staphylococcus epidermidis*, and (B) *S. aureus*.

Abbreviation: M- marker 100bp, NC -negative control, PC -positive control

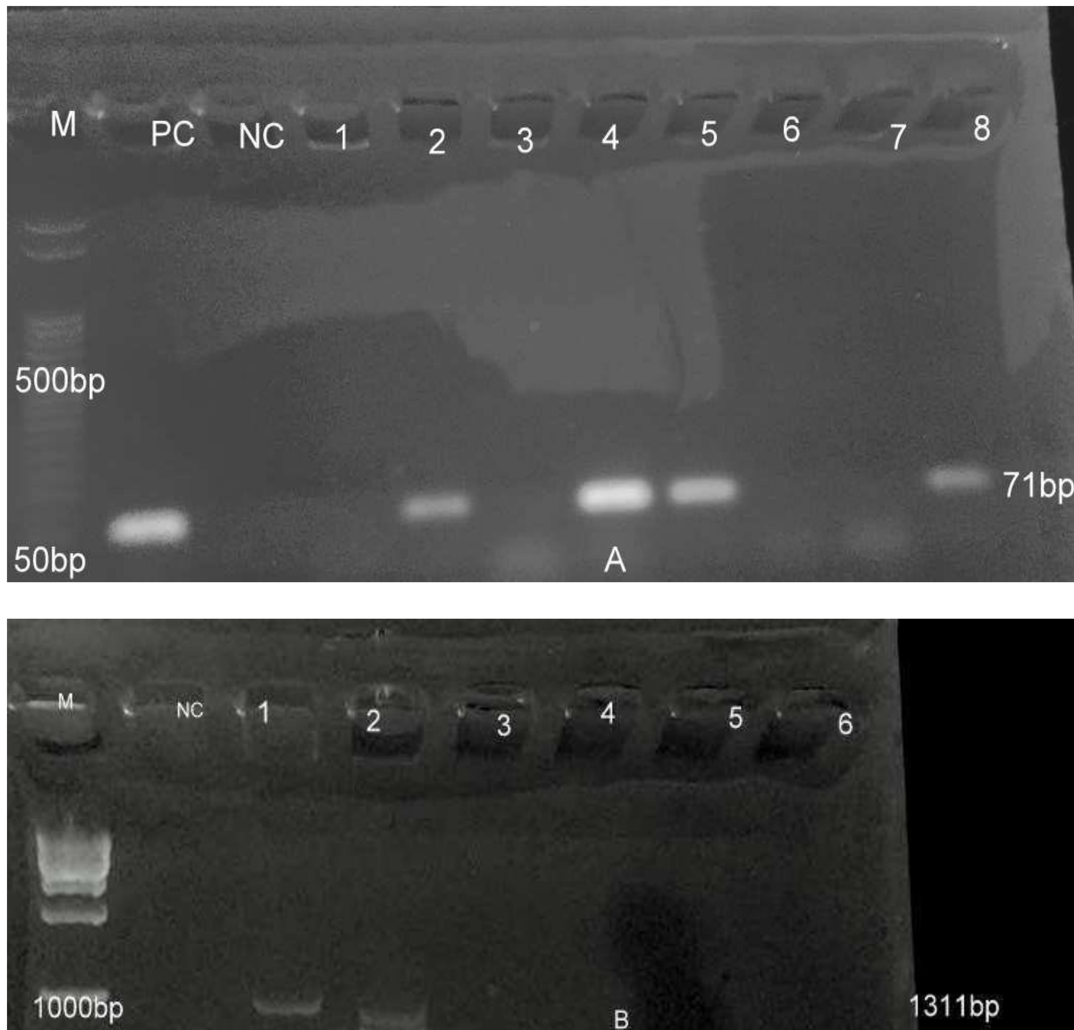


Figure 11: Represents of positive results of PCR amplification of a positive products (A) *Bacillus cereus* at 71bp first batch and (B) *Bacillus subtilis*.

4.8 Antibacterial Sensitivity Test

A total of fifteen bacteria isolates were tested for different antibacterial agents namely two *S. aureus* isolates, four *S. epidermidis*, two B*acillus spp* (*B. cereus* and *B.subtilis*) one *Micrococcus sp,* one *E. coli* and five *Staphylococcus* spp. The results of sensitivity test were interpreted and are presented in Table 14. The results show that bacteria tested was sensitive at least to one antibiotic as shown in figure 12.

The antimicrobial susceptibility test showed that all isolates were highly sensitive to Ciproflaxcine (100%) and Tetracycline (86.7%), highly intermediate to Chloramphenicol

(93.3%) of the tested isolates and highly resistance (100%) to Sulfamethoxazole and Penicillin G. The cumulative effectiveness of the antibiotics to all bacteria isolates tested obtained in this study was Ciproflaxcine 100% > Tetracycline (86.7%) > Erythromycine (33.3%) > Cefataxime (33.3%) > Neomycine (26.7%) > Amoxicillin (13.3%) > Chloramphenicol (6.7%), > Gentamycine (6.7%) > Ampicillin (6.7%) > Sulfamethoxazole and Penicillin G (0%) as shown in Table 15.

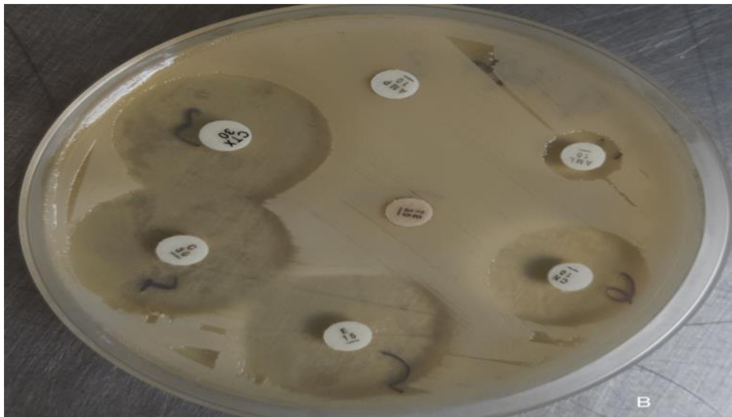


Figure 12: Antibacterial sensitivity test performed on *S. aureus* isolate (B), on Muller Hinton agar showing zones of inhibition after 24hrs.

Table 14: Results of antibiotic sensitivity tests showing fifteen bacteria species and the responses antibiotic.

Bacteria sp	**AMC 30**	**ZOI (mm)**	**AMP 10**	**ZOI (mm)**	**CIP 5**	**ZOI (mm)**	**CN 10**	**ZOI (mm)**	**N 10**	**ZOI (mm)**	**E 15**	**Z OI**	**TE 30**	**ZOI (mm)**	**P 10**	**CTX 30**	**ZOI (mm)**	**C 30**	**ZOI**	**SMX 50**
M. luteus	S	25	S	20	S	13	R	6	S	16	S	26	S	24	R	S	17	I	15	R
S.epi HB	R		R		S	15	R	8	R		R		S	19	R	R		I	16	R
S.epi RF	R		R		S	15	R		R		R		S	22	R	R		I	14	R
S.epiHB	R		R		S	19	S	23	R	9	S	21	S	22	R	R		I	14	R
S.epiHB	R		R		S	11	I	13	R	9	R	10	S	21	R	R		I	14	R
S.aureHB	R		R		S	20	I	13	R	8	S	25	R		R	S	21	S	20	R
S.aureHB	R		R		S	20	I	13	R	8	S	25	R		R	S	21	S	20	R
E.coli RF	R		R		S	19	I	13	R		R		S	21	R	R		I	15	R
Staph.HB	R	14	R		S	19	I	13	R	9	R		S	19	R	S	7	I	15	R
Staph.HB	R	12	R		S	13	R		R		R		S	20	R	S	8	I	16	R
Staph.HB	R		R		S	12	R		S	16	R		S	19	R	R		I	14	R
E.coliHB	S	26	R		S	16	I	13	R		R	7	S	20	R	R		I	15	R
B.subtilis	R		R		S	18	R		S	16	S	25	S	21	R	R		I	14	R
B.cereus	R		R		S	20	R		S	16	R		S	22	R	R		I	16	R
Staph RF	R		R		S	19	R		R	7	R		S	21	R	R		I	16	R

Abbreviations: S- sensitive, R- resistance, I-intermediate, HB- Hybrids, RF- Rufiji tilapia, *S. epi- Staphylococcus epidermidis, S. aureStaphylococcus aureus, Staph- Staphylococcus spp*, Micrococcus-*Micrococcus luteus*, *Escherichia coli,* Tetracycline-TE30, Chloramphenicol-(C30), Gentamycine,-(CN10), Amoxycilin-(AMC30), Neomycine (N10), Ciproflaxcine- (CIP 5), Cefataxime-(CTX 30), Sulfamethoxazole -(SMX 50), Ampicillin- (AMP 10) Erythromycine- (E15) and Penicillin G- (P 10).

Table 15: Overall percentages of the bacterial isolates tested shown as sensitivity, intermediate and resistance per antibiotic agents used (n=15 isolates)

Antibiotic agent	**Sensitive (% isolates)**	**Intermediate(% isolate)**	**Resistance (%isolates)**
Tetracycline (TE30)	13 (86.7%)	-	2(13.3%)
Chloramphenicol (C30)	1(6.7%)	14(93.3%)	-
Gentamycine (CN10)	1(6.7%)	6(40%)	8(53.3%)
Amoxycilin (AMC30)	2(13.3%)	-	13(86.7%)
Neomycine (N10)	4(26.7%)	-	11(73.3%)
Ciproflaxcine (CIP 5)	15(100%)	-	-
Cefataxime (CTX 30)	5(33.3%)	-	10(66.7%)
Sulfamethoxazole(SMX 50)	-	-	15(100%)
Ampicillin (AMP 10)	1(6.7%)	-	14(93.3%)
Erythromycine (E15)	5(33.3%)	-	10(66.7%)
Penicillin G (P 10)	-	-	15(100%)

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Efforts to increase fingerlings production in Tanzania to meet the demand is undermined by occurrence of diseases and mortalities at the hatchery. This study was conducted to evaluate the effect of salinity levels on bacterial infections to Rufiji tilapia and its hybrid with Nile tilapia fingerlings. Physico-chemical water parameters (PH, Temperature, Salinity (ppt), and dissolved oxygen (DO) were found to be in a recommended range in all tanks according to the standards set for water quality management of pond culture as described by Bhatnagar and Devi (2013) (Table 3).

The occurrence of bacterial infections in fry and fingerlings could be an indication of presence of certain predisposing factors exposing them to the infections. It might be associated with the feeds that were used to feed fries and fingerlings after hatching as it was locally formulated without assessing its quality (Mmochi, A. J. personal communication, 2016). According to Silva (2010), type of feeds used also plays important role in determining the occurrences of microbial community in the fish as well as the culture systems. Furthermore, water used to drain the culture systems might also be a source of occurrences of bacteria in hatcheries (Karimi, 2015).

The observed bacteria load was not statistically significant different between Hybrids and Rufiji tilapia fingerlings as well as in water at different salinity levels. The bacteria load was higher in hybrid fingerlings (average load 10^7CFU^{ml-1}) compared to what recovered in Rufiji tilapia fingerings (average load 10^6 CFU^{ml-1}) as shown in table 4.

This might indicate that the hybrids though reported to have high salinity tolerance with good growth performance in both freshwater and marine water as described by Mapenzi and Mmochi (2016), are more susceptible to bacterial infections. The recorded bacterial load in this study was above the acceptable limit of approximately 10^4-10^8CFU^{ml-1} bacterial load (Hardi *et al.*, 2011), hence might lead to mortalities if not intervened. This was similar to the findings obtained by Hardi *et al.* (2018) who reported high mortality in *O. niloticus* in Samarinda, East Kalimantan, Indonesia at bacterial load above 10^3CFU^{ml-1}.

In reference to salinity levels, the bacteria load was not statistically different in all salinity levels (Table 4). This indicated lack of salinity influences on bacteria load. This is similar to the findings reported by Silva (2010) in her experiment in Wageningen, Netherlands. However bacteria load was observed to be high at salinity levels of 2 and decreased at salinity of 15 and 25 respectively (Table 4). This was similar to the findings by Bolivar *et al.* (2001) and Silva *et al.* (2014) who reported increased bacterial load at low salinity concentration and decreased at high salinity level. This might be because as salinity level increases, the survival and multiplication of bacteria might be inhibited as salt is known to have antimicrobial activity. Salinity level of 35 was observed to have the highest load after 2 this might be due to adaptation of extreme halophilic bacteria to high salinity as described by Melissa (2018). Therefore salinities between 15 and 25 could be the best salinity level for farming these fish species as they were observed to have lowest load of bacteria. Also highest growth performances were reported to be in those salinity levels as compared to 2 and 35 (Nehemia *et al.*, 2013; Mapenzi and Mmochi, 2016).

It would be expected that the difference in salinity levels had different effects on bacteria community (species) (Ringo *et al.,* 1995). However, in this study there was no significant

difference in the bacteria community isolated in all salinity levels. Again there is the statistical difference between the intermediate salinities on one hand and 2 and 35 on the other hand. This indicates that the salinity levels had no influences on bacterial communities. This observation is similar to the findings by Silva (2010), who reported similarity in bacterial communities of tilapia fish in both fresh and salt water treatments. The same author also explained that this could be due to an identical genetic composition in the bacterial flora in fresh and salt water systems and the proliferation of bacterial communities being common to both groups.

The ratio of positive results obtained by biochemical versus PCR methods out of 56 isolates tested indicated that, majority of bacteria (67.9%) identified and confirmed with biochemical testing were also identified with PCR testing though there are some isolates (*S. saprophyticus*) that were confirmed by biochemical test and were not detected by PCR method (Table 12). This is an indication that biochemical results some time appears to be less sensitive than PCR methods (Rhoads *et al.,* 2012). According to Rhoads *et al.* (2012), PCR method is highly sensitive but requires specialized laboratory to test bacteria of interest. Therefore, DNA sequencing should be done to confirm the isolated bacteria.

In this study, the dominant bacteria belong to the members of genus Staphylococcus and Bacillus (Table 5). This is because members of this species can be found everywhere in the environment, in water, skin, intestine, and in soil as well as parasites of other organisms like plants (Karimi, 2015). Some normal floral of humans such as *Staphylococcus sp.* was found predominantly in fingerlings and water and could be due to handling processes that could have introduced these bacteria species while some may be found naturally. *Staphylococcus spp* was highly isolated in hybrid fingerlings being

low in Rufiji tilapia *(O. Urolepis uroleps)* fingerlings. While in Rufiji tilapia fingerlings Bacillus spp was observed to dominate (Table 7). This observation implies that, these species could be antagonistic to each other when co-infecting the fish. It has been documented that, high load of Bacillus species could inhibit the growth of other pathogenic bacteria species (Mohsen *et al.,* 2016). Some strain of *B. subtilis* reported to exhibit an antimicrobial activity against *Vibrio spp*, *Streptococcus sp* and *Staphylococcus spp* and some strain of *B. amyloliquefaciens* inhibits growth of *Vibrio spp, Edwardsiella tarda* and *Streptococcus sp* (Zhao *et al.,* 2012; Chen *at el.,* 2016). According to Silva (2010), Bacillus species naturally secrete several antibiotic compounds and enzymes that can penetrate and degrade the biofilms. Also, has the ability to compete for nutrients and space avoiding the fast growth and reproduction of other bacteria species.

In this study the presence of antimicrobial resistance by some of bacterial isolates (Table 15) although no antibiotic applied might be due to the accumulation of surplus antimicrobials and antimicrobial residues from other sources such as water effluent as reported by Karimi (2015). Also another possible source could be pharmaceutical effluent discharged into natural water bodies that were used to drain the culture systems (Tiamiyu *et al.,* 2015).

Those surplus and residues could accumulate and establish selective pressure favoring selection and growth of antimicrobial-resistant bacteria as well as resistant gene (Done and Halden, 2015). The resistance may also occur naturally due to spontaneous mutation of the bacterial gene on chromosomes causing changes on the drug binding's sites of pathogenic bacteria (Watts *et al.,* 2017). According to Tiamiyu *et al.* (2015) and Watts *et al.* (2017), the introduced residues of antimicrobial may affect microbial community present and introduce antibiotic resistance in the bacteria. The resistant gene could also

be introduced through terrestrial bacteria carrying resistant genes entering the culture systems (Subramanian *et al.,* 2008). Furthermore, the potential transfer of resistant bacteria and resistance genes from aquaculture environments to humans may occur through direct consumption of antimicrobial-resistant bacteria present in fish and associated products (Wamala *et al.,* 2018). The resistance gene transferred to human results in the reduction of drug efficacy to bacteria treatment (Done and Halden, 2015; Tiamiyu *et al.,* 2015).

The isolates tested were observed to be more sensitive to ciproflaxcine (100%) and tetracycline (86.7%) and resistant to penicillin (100%) and sulfamethoxazole (100%) (Table15). This observation was similar to the findings reported by Austin and Austin (2012). This implies that those isolates might have acquired the resistances gene or naturally (normally) resists to penicillin and sulfamethoxazole as described by Watts *et al.* (2017). Penicillin was reported to be ineffective to some *E. coli* and other Gram negative bacteria naturally (Levy and Marshall, 2004).

There were some variations in sensitivity among the same group of bacteria species as shown in table 15. The causes of those variations in resistances and susceptibility among the same group of bacteria to same antibacterial agent are unclear (Rose *et al*., 2009).

5.2 Conclusions

From this study the following conclusions can be drawn:

- Salinity differences have no significant influence on the bacterial load in water, Rufiji tilapia and Hybrids fingerlings.

- Salinity of 15 and 25 was observed to have low bacteria load as compared to 2 and 35.
- There was no significance difference in bacteria species isolated in water and fingerlings in all water salinity levels;
- Different bacteria species have been isolated in water and fingerlings of which some are potentially reported to be pathogenic to fish, thus if the fingerlings are improperly managed and environmental stress increases it may cause diseases and mortalities;
- Antibacterial susceptibility test indicated all isolates to be 100% sensitive to Ciproflaxcine and different sensitivity levels to some of the antibiotics.

5.3 Recommendations

- Biosecurity measures should be taken into account in order to protect fingerlings as well as human since the presence of pathogenic bacteria imply that if fingerlings are improperly managed and environmental stress increases it may cause diseases and mortalities and some of them are zoonotic for example *S. aureus.*
- It is also recommended that the brooding stocks (parents) should be checked if they are not infected before using for breeding.
- The sanitary conditions under which fishes are reared in tanks should be improved by following good standards of aquaculture practices such as use of good quality water free of contamination, the use of feed free of contaminants, and regular exchange of water after specific period of time.

- It is also recommended that salinity of 15 and 25 should be used for the farming of hybrids and Rufiji tilapia in order to increase their productivity as it was observed to have low bacteria loads.
- Despite of antibiotics being discouraged in treatment of fish diseases, tested antimicrobial indicated some isolates to be not sensitive to some of the antibiotics. Therefore, though the sources of antibiotics in a culture system are unknown, more studies that seeks to identify and characterize antibiotic susceptibility in an environment and fish community are recommended to safeguard fish as well as consumers.
- Furthermore, identification by biochemical and PCR method used might be less accuracy to all bacteria present in the culture system, therefore DNA sequencing are more recommended in order to increase the sensitivity and specificity of the identified isolates.

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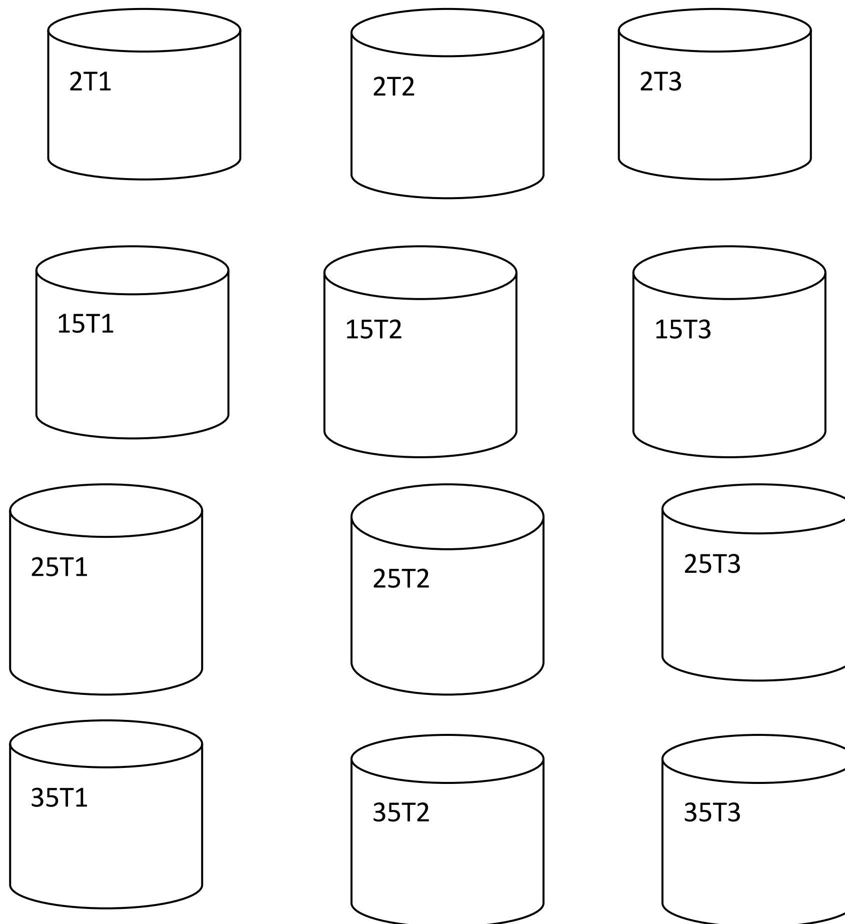
APPENDICES

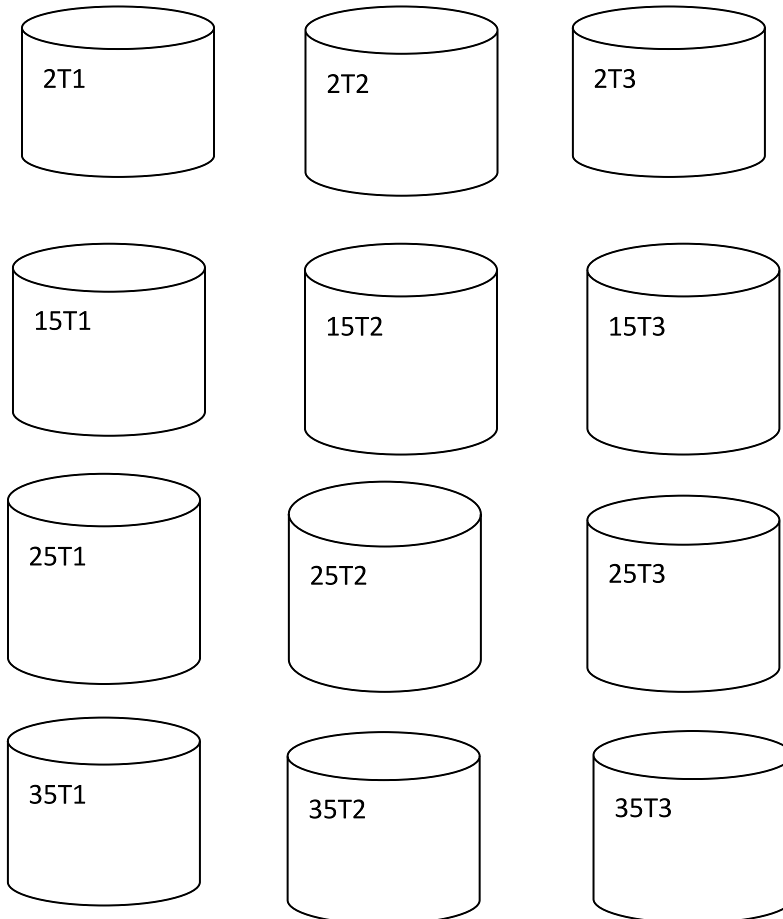
Appendix 1: Experimental set up for Rufiji tilapia fingerlings at IMS –Mariculture Center –Pangani, Tanga



Description for the experimental setup

Out of 12 tanks three replicates was used for each salinity level per fish species. A total 20 fry were stocked per tank. The stocking of fish was done through randomization processes. The first randomization involved catching and stocking the same number of fish serially from 2T1 to 35TI. Second from 15T2, 25T2, 35T2 and 2T2 and finally from 25T3, 35T3, 2T3 and 15T3

Appendix 2: Experimental set up for hybrids fingerlings at IMS –Mariculture Center – Pangani, Tanga



Description for the experimental setup

Out of 12 tanks three replicates was used for each salinity level per fish species. . A total 20 fry were stocked per tank. The stocking of fish was done through randomization processes. The first randomization involved catching and stocking the same number of fish serially from 2T1 to 35T1. Second from 15T2, 25T2, 35T2 and 2T2 and finally from 25T3, 35T3, 2T3 and 15T3