

Finfish aquaculture in northern Nigeria: Characterisation of potential bacterial pathogens, disease management practices and steps towards improvement

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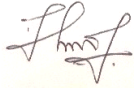
Dedication

To my beloved parents Alhaji Yakubu Jibrin Abubakar and Hajiya Maryam Yakubu

Declaration

I, Jibril Yakubu Jibril solely declare that this thesis in its entirety has been written by me except where references are specifically acknowledged by the contribution of others. The work from this thesis has not been submitted for any other higher degree or qualification at any other academic institution.

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Abbreviations

AIFP	Aquaculture and Inland Fisheries Project
AMR	Antimicrobial Resistance
AMX	Amoxicillin
ANI	Average Nucleotide Identity
AST	Antibiotic Susceptibility Testing
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
BLASTP	Protein BLAST
CARD	Comprehensive Antibiotic Resistance Database
CDS	Coding Sequences
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CAFAN	Catfish Farmers Association of Nigeria
CI	Confidence Interval
COL	Colistin
DNA	Deoxyribonucleic Acid
ECV	Epidemiologic Cut off Value
ENR	Enrofloxacin
ERY	Erythromycin
FAO	Food and Agriculture Organisation
FCT	Federal Capital Territory
FIP	Fishery Improvement Project
FLO	Florphenicol
FMARD	Federal Ministry of Agric and Rural Development
GCAT	Glycerophospholipid-cholesterol acyltransferase
GEN	Gentamicin
GUEP	General University Ethics Panel
IQR	Interquartile range

LGU	Local Government Units
LIFD	Low Income Food Deficit
MAFFT	Multiple Alignment using Fast Fourier Transform
MAS	Motile Aeromonas Septicaemia
MCA	Multiple Correspondence Analysis
MDR	Multiple Drug Resistance
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
MSC	Monitoring, Surveillance and Control
NADP	National Aquaculture Development Plan
NAFDAC	National Agency for Food, Drug administration and Control
NAQS	Nigeria Agriculture Quarantine Service
NBS	National Bureau of Statistics
NCBI	National Centre for Biotechnology Information
NSPFS	Nigeria's National Special Programme for Food Security
NIRSAL	Nigeria Incentive-Based Risk Sharing System for Agricultural Lending
NWT	Non-Wild Type
O-F	Oxidation fermentation test
OTC	Oxytetracycline
OXA	Oxolinic Acid
PABA	Para-aminobenzoic acid
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIA	Presidential Initiative on Aquaculture
QRDR	Quinolone Resistance Determining Region
RAS	Recirculatory Aquaculture Systems
RNA	Ribonucleic Acid
RND	Resistance-nodulation-cell division
SDS	Sodium Dodecyl Sulphate
SMX	Sulfamethoxazole

ST	Sequence Type
TSA	Tryptic Soy Agar
TSS	Type Secretion Systems
TSB	Tryptic Soy Broth
UNDP	United Nations Development Program
USAID	United States Agency for International Development
VCN	Veterinary Council of Nigeria
WECARD	West and Central African Council for Agricultural Research and Development
WGS	Whole genome sequencing
WHO	World Health Organisation
WT	Wild Type

Abstract

Nigeria is the largest aquaculture producer in Sub-Saharan Africa but has a fish demand-supply deficit of over 1 million tons. Aquaculture has been promoted as a viable pathway towards bridging this gap and achieving self-sufficiency in fish supply. The sector however, faces infectious disease challenges that threaten both food security and the socioeconomic well-being of the people especially in northern Nigeria, a region with a higher poverty rate and lower food security. Insufficient capacity for fish health management has led to many fish farmers using antibiotics sometimes inappropriately as the primary means of disease control and prevention. This poses a threat to both aquaculture sustainability and the One Health mantra. Despite these challenges, there is still a dearth of information on prevailing pathogenic bacterial strains as well as a poor understanding of general disease management and prevention strategies within the aquaculture sector especially in northern Nigeria. This thesis, therefore, aimed to address these knowledge gaps and characterise prevailing pathogenic bacteria of catfish, conduct a survey of infectious disease outbreaks and associated management practices and propose interventions towards improved biosecurity practice in aquaculture in northern Nigeria.

A bacteriological survey was conducted in the field of 55 catfish (*Clarias gariepinus*) displaying gross symptoms of infectious disease at nine sampling sites which included seven fish farms and two fish markets distributed across three states of northern Nigeria between June and August 2020. Forty-two bacterial isolates were successfully recovered in the UK from the original 100 isolated bacteria in Nigeria. Sixteen of the recovered bacterial isolates sampled from skin ulcers and kidney of catfish at six different sites were identified as *Aeromonas* spp. (including *A. hydrophila*, *A. veronii*, *A. dhakensis*, and *A. jandaei*) through a combination of morphological, biochemical, and molecular techniques (16S rRNA and whole genome sequencing and MLST typing). A further twelve suspected *Pseudomonas* spp. isolates were also cultured from catfish mainly associated with one market location.

The 16 *Aeromonas* spp. isolates displayed phenotypic virulence traits that included haemolysis, protease, DNase, gelatinase and lipase enzyme activities in addition to

bearing genes that code for these traits. Antibiotic resistance profiles of these 16 *Aeromonas* spp. against commonly used antibiotics in the study area determined using the microbroth dilution method of the Clinical and Laboratory Standards Institute (CLSI VET03) revealed that all 16 were wild type (WT) against gentamicin and erythromycin and non-wild type (NWT) against oxytetracycline and sulfamethoxazole, while the majority 15/16 (93.75%) were NWT against both amoxycillin and oxolinic acid. A smaller proportion of 12 (75%), 11 (68.75%) and 2 isolates (12.50%) were NWT against colistin, enrofloxacin and florfenicol respectively. Both chromosomal and plasmid-borne antibiotic resistance determinants were also detected in these *Aeromonas* spp. field isolates.

A structured questionnaire to inform on current aquaculture husbandry practices, disease occurrence, disease management, and biosecurity practices was administered via telephone interview to 45 catfish farmers across 10 of the 19 states that make up northern Nigeria between September 2021 and February 2022. The survey of fish farms revealed that 25 (56%) farms poorly implemented biosecurity on their farms and another 29 farms (64%) reported disease outbreaks. Only five (11.1%) farms consulted a veterinarian while self-medication with antibiotics was the most common (40/45; 88.89%) method of disease control and prevention. An online training workshop on biosecurity was designed, implemented and its impact on biosecurity practices of participating fish farmers assessed. A significant difference in biosecurity score ($p < 0.05$) was observed between the cohort that received online biosecurity training and the control group of fish farmers that did not attend the workshop.

This thesis revealed that biosecurity on participating fish farms was poorly implemented, however, an online training workshop on biosecurity significantly improved this outcome. Infectious disease outbreaks were common and disease management commonly involved self-prescribed antibiotics. The most frequently isolated bacteria from diseased catfish were *Aeromonas* spp. and found to carry multiple virulence and resistance genes. Therefore, the evidence presented here suggests the potential role of aquaculture in the emergence and dissemination of antibiotic resistance. The economic and public health (zoonotic) significance of *Aeromonas* spp. makes this a significant finding especially in the context of northern Nigeria, a region with an emerging aquaculture sector. A concerted effort is therefore

required to improve farms management practices and reduce inappropriate antibiotic use, to ensure sustainable aquaculture production and food security.

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Chapter 1

An overview of aquaculture production in Nigeria

1.1 Nigeria: an overview

Nigeria is comprised of 36 federating states and the Federal Capital Territory that are divided into six geopolitical zones (National Bureau of Statistics, 2020; Subasinghe et al., 2021) (Fig 1.1). The country has a total surface area of 923,770 km² that shares a land border with the Republic of Benin to the west, Niger Republic to the north, Chad, and Cameroon to the East and a southern coastline on the Gulf of Guinea in the Atlantic Ocean (FAO, 2016) (Fig 1.1).

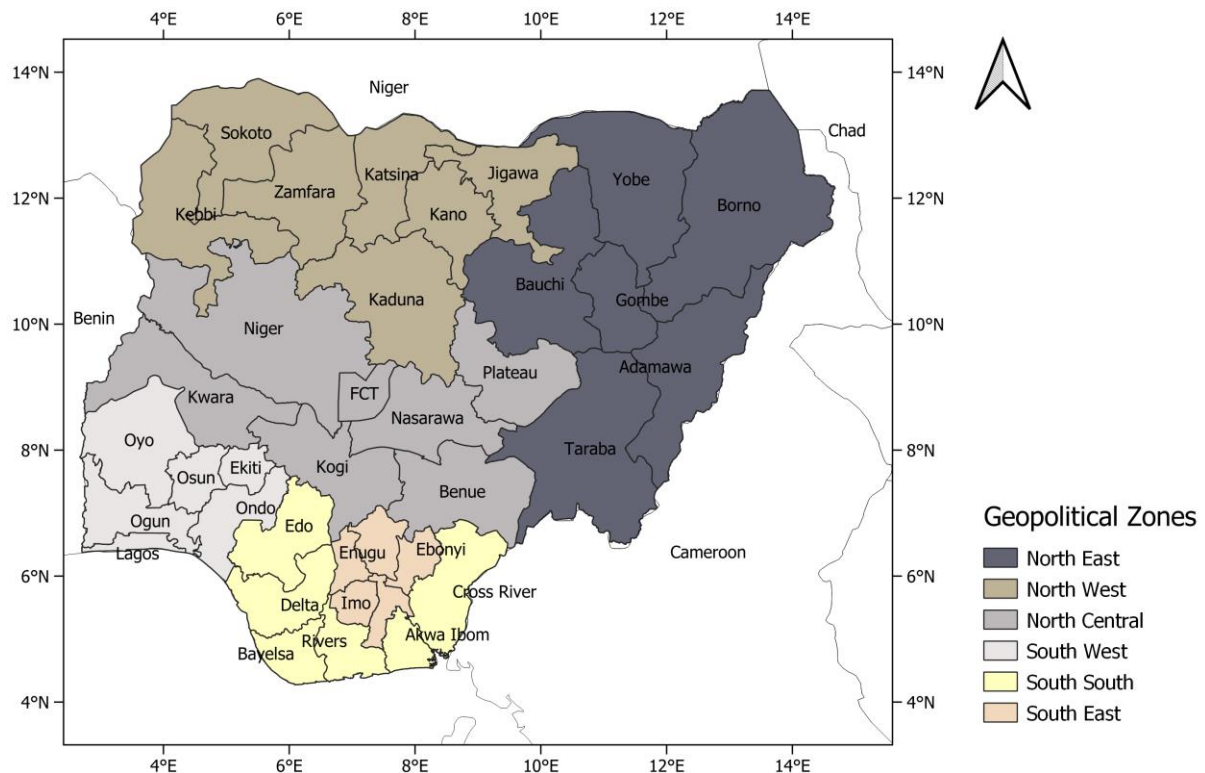


Fig 1. 1 Map of Nigeria showing the six geopolitical zones and their respective states

Nigeria has an estimated 209 million people, over 60% of which are under 25 years, making it the most populous African country and projected to be the third-most populous country in the world (440 million people) by 2050 (UN DESA, 2019; UN-HABITAT, 2023). Nigeria is one of the world's leading oil producers, but its economy, the largest in Africa (Akinsulie et al., 2023) is largely dependent on agriculture (Subasinghe et al., 2021).

Major rivers distributed across the country that can support fisheries and aquaculture represents 12% of the total surface area (an estimated 10,812,400 ha), in addition to lakes and reservoirs that make up another 1% of the total surface area (Subasinghe et al., 2021). Fish is a well-recognised affordable animal-source food in Nigeria and accounts for about 50% of total protein intake across all income strata of Nigerians that have an average annual per capita fish consumption of 11.2 kg, a value higher than Africa's average of 9.9 kg (Subasinghe et al., 2021).

1.2 Fish supply in Nigeria

Fish supply in Nigeria is achieved through both local production i.e., fisheries, aquaculture and importation (Subasinghe et al., 2021). Locally, the country produced 1.17 million tons of fish in 2018 mainly from small-scale fisheries which provides more than 70% of the Nigeria's domestic fish supply (Subasinghe et al., 2021). Artisanal marine and inland fisheries represent a major source of fish supply and contribute 2.09% to the country's agricultural economy (Subasinghe et al., 2021). In addition to provision of food, the fisheries sector contributes to poverty reduction by engaging an estimated 28.2 million people directly (8.6 million) and indirectly (19.6 million), thereby making a significant impact on the socioeconomic life of many Nigerians (Subasinghe et al., 2021; WorldFish, 2018). However, a reduction in industrial output likely from overfishing has resulted in a declining output from the fisheries sector since 2014 (National Bureau of Statistics (NBS), 2017; Subasinghe et al., 2021). Although the country has largely depended on fish importation to fill the supply gap, this has since proved to be unsustainable (Ewepu, 2019; Oboh, 2022). Attention has thus increasingly shifted towards enhancing aquaculture production to fill this gap.

1.3 Aquaculture production in Nigeria

Although Nigeria has an estimated 853-km stretch of coastal line and about 179,839 km² of exclusive economic zone, aquaculture production is still largely inland freshwater-based with a primary focus on fish culture that is dominated by catfish (*Clarias* spp. and *Heterobranchus* spp.), carp, tilapia and *Heterotis niloticus* (Slap water) (Amosu Albert Oluwatobi et al., 2017; Dauda et al., 2018; Elum & Mjimba, 2016; FAO, 2007; Kaleem & Bio Singou Sabi, 2020; Omeje et al., 2020). While interest in

tilapia production is gradually increasing across the country, it is unlikely this will significantly tilt the balance given the high consumer preference for catfish and the longer duration of tilapia production cycle.

Aquaculture in Nigeria started experimentally by the government around 1950 and the government remained the major aquaculture producer with support from international development partners like the United Nations Development Program (UNDP) and Food and Agriculture Organisation (FAO) that provided inputs and technical support for production, research and training (Anetekhai, 2013). Government dominance climaxed with the establishment of eleven River Basin Development Authorities whose mandates included running catfish farms to prove their commercial viability (Leadership, 2020).

By 1991, private participation had gained significance culminating with an estimated 5,000 fish farmers with a large concentration in the South – Western part of the country by 2009 (Anetekhai, 2013; Olanrewaju, 2019). Nigeria's rapid population growth and the consequent increased demand for fish, further encouraged intensification of aquaculture pushing the growth of small – to – medium scale farms and the establishment of large-scale intensively managed units (Olanrewaju, 2019). Consequently, the sector grew at a 12% percent annual rate, higher than the global average of 8% (Kaleem & Bio Singou Sabi, 2020; WorldFish, 2018). Presently, Nigeria is Africa's second largest aquaculture producer after Egypt (FAO, 2022). The country has an estimated 285,000 catfish grow-out farms that include 63% small scale, 30% homestead, 5% medium scale and 2% large scale (Oman et al., 2023a), mostly distributed across four production hubs that include South West, South South, South East and North Central geopolitical zones. Output-wise, approximately 1.1 million tons of catfish is produced annually mostly by small (< 5 tonnes/cycle; 65%) and large (> 10 tonnes/cycle; 22%) scale farms, while medium scale and homestead farms produce 10 and 3% respectively (Oman et al., 2023a).

1.4 Fish demand in Nigeria

Despite the progress made in aquaculture production and the country's huge potential for inland fisheries production, the demand-supply gap for fish is still unbridged,

making the country dependent on importation to meet 50% of its demand for fish (Obboh, 2022; Subasinghe et al., 2021). Nigeria's apex bank, the Central Bank of Nigeria revealed that the country's overall fish supply deficit stood at 1.9 million tons with an estimated \$1.2 billion spent annually on fish imports to meet the country's 2.7 million tons demand for fish (Ewepu, 2019). WorldFish estimated that a sustained annual increase in growth rate of about 22% is required to bridge the demand-supply gap which potentially stands to have a multiplier effect on youth employment, increased household income, improved nutrition and reduced child mortality (WorldFish, 2018).

A recent survey showed that despite cultured fish being more commonly consumed in the north than in the south of the country, access to fish by 50% households in this region was not adequate (Subasinghe et al., 2021). This suggests a higher demand for fish in northern Nigeria. Furthermore, while Nigeria is on the list of 55 Low Income Food Deficit (LIFD), both poverty and food insecurity are disproportionately higher in the north compared to the south of the country (Wudil et al., 2023). Only 16% of households in the north were reportedly food secure as against over 40% in the south (Wudil et al., 2023). However, aquaculture activity in the region is increasing at a significant proportion. For example, the number of fish farmers in a North-western state was reported to have increased from 683 to 1637 between the period 2008 to 2018, an increase of 200% (Gona et al., 2018). Despite this progress, fish supply in the region is still sourced hundreds of kilometres away mainly from Ibadan in South-western Nigeria often under poor welfare conditions (Grema et al., 2020).

1.5 Major cultured species in Nigeria

Catfish, predominantly *Clarias gariepinus*, represents over 90% of the farmed fish in Nigeria (Kaleem & Bio Singou Sabi, 2020; Obboh, 2022; Oman et al., 2023a; Subasinghe et al., 2021). While the world's total catfish output was 1.249 million tons (FAO, 2022), Nigeria's 1.1 million tons annual output makes Nigeria the world's largest producer of catfish. Despite being preceded by carp and tilapia, *C. gariepinus* has increasingly assumed a more dominant position (WorldFish, 2018). Better understanding of the species nutritional requirements and successful development of

methods for artificial propagation in the 1980s provided the initial impetus for its adoption, that was further influenced by its ability to better tolerate unfavourable culture conditions such as low levels of dissolved oxygen, low pH and varying salinity levels. The species rapid growth rate, efficient food conversion ratio and good palatability, has encouraged its widespread adoption and acceptance by farmers and consumers alike (Dauda et al., 2018; Olanrewaju, 2019). Furthermore, catfish's lack of scales which makes it easier to process might have influenced its widespread acceptance by consumers. Other cultured species in Nigeria include *Heterobranchus* spp., and *Heteroclarus* spp., common carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*) and African bony tongue (*Heterotis niloticus*) (Dauda et al., 2018; Omeje et al., 2020; Subasinghe et al., 2021).

1.6 Aquaculture production systems in Nigeria

Aquaculture in Nigeria is practised predominantly as an intensive catfish monoculture or polyculture involving catfish, tilapia and carp (Kaleem & Bio Singou Sabi, 2020; Subasinghe et al., 2021). Other systems including semi-intensive, extensive and integrated aquaculture production (Kaleem & Bio Singou Sabi, 2020).

Hatchery operations in Nigeria typically consists of overhead tanks, a series of flow-through troughs for incubation and hatching of fertilized eggs and flow-through tanks for raising fry/hatchlings to fingerlings or juveniles (Adewumi, 2015a). Grow out systems, 80% of which are operated by small-scale farmers, involve both brackish and freshwater cultivation and typically use earthen or concrete ponds, fibre tanks, pens, Recirculatory Aquaculture Systems (RAS) or other water tight containers that hold sufficient volumes of water to sustain fish production and can be used to raise fingerlings/juveniles to table-size fish (Adeleke et al., 2020a; Kaleem & Bio Singou Sabi, 2020). With numerous natural water bodies such as rivers, lakes and estuaries suitable for cage systems, there is potential to significantly increase annual aquaculture output. Cage aquaculture is emerging in Nigeria and has recently seen an increased inflow of investment mostly by large corporations for tilapia production (Adeleke et al., 2020a; Subasinghe et al., 2021).

Catfish production in Nigeria takes place either as table size production, which entails raising fish to table size from fingerlings/juvenile mostly by small-scale farmers or a combination of hatchery and table operations which are commonly practised by large commercial farms (Adeleke et al., 2020a). Another model involves public/private partnership or a cooperative. In this model, the government sets up a central farm that is managed by a private entity who provides satellite farms with inputs such as fingerlings/juveniles, feed and credit facilities. The satellite farms then pay back either in cash or with table size fish at the end of the production circle (Adeleke et al., 2020a; Anetekhai, 2013; Kaleem & Bio Singou Sabi, 2020). A third model is the less capital-intensive backyard/cottage farming, commonly practised by households to attain food sufficiency and improve income and integrated farming model promoted by the West and Central African Council for Agricultural Research and Development (WECARD) and the University of Ibadan (UI) to help small scale farmers improve income and productivity through the integration of fish, poultry/pig and rice farming/vegetable production (Adeleke et al., 2020a).

1.7 Socioeconomic significance of aquaculture in Nigeria

Fisheries and aquaculture contribute 3 – 4% to the country's annual GDP and account for about 50% of the country's animal-sourced food making the sector a key contributor to food and nutrition security (Subasinghe et al., 2021) in the country. The sector through its value chain also generates employment and income for a significant number of people (Subasinghe et al., 2021). According to one survey report, more than 50% of household income of 75% of surveyed small-scale fish farmers comes from aquaculture.

Data from the country's catfish farmers association (CAFAN) revealed that in 2016, aquaculture produced 34% (370,000 metric tonnes) of total fish production valued at over 1.3 billion dollars and employed an estimated 475,000 people (WorldFish, 2018). Although production is still largely dominated by small-to-medium scale operators in many parts of the country, there is a wide consensus that aquaculture has the potential to meet the growing demand for fish, contribute to economic growth and support the

sustainable livelihood of many communities, particularly in rural areas (Akinrotimi et al., 2011).

1.8 Aquaculture policy/regulatory framework

Although Nigeria's Federal Department of Fisheries and Aquaculture under the Ministry of Agriculture and Food Security is the competent authority saddled with the responsibility of management and policy formulation on fisheries development, the aquaculture sector lacks legislative backing. An attempt to change this was made in 2014 through draft legislation entitled the Fisheries Act (2014). This, however, is yet to pass the necessary legislative hurdles to become law. By implication, most of the development activities of the sector were unregulated, as there was neither a legal definition nor guidelines or code of conduct in place (FAO, 2005). While this act contains provisions for regulation of the activities of the sector, the 2001 New Nigeria Agricultural Policy (I.O. et al., 2016) should have provided a more enabling environment for aquaculture to be more productive. Unfortunately, the lack of adequate implementation of both provisions has left the sector largely unchanged. That notwithstanding, activities like the Aquaculture and Inland Fisheries Project (AIFP) under Nigeria's National Special Programme for Food Security (NSPFS) which lasted 35 months between July 2003 and June 2006 raised public awareness on aquaculture and fisheries making them national development priorities (Kamthorn & Jim, 2006). This project created an inventory of inland water bodies, fish farms and fish mills, provided technical assistance to private fish farms, coordinated a community-based management of inland waters and promoted increased fisheries production from lakes through linkage with private hatcheries and promoted reduced post-harvest loss through improved fish smoking demonstrations (Kamthorn & Jim, 2006). Similarly, the Presidential Initiative on Aquaculture (PIA) initiated in 2003 provided financial and technical assistance to government programmes supporting fish production further supported the growth of the sector, although not without challenges. These initiatives were public sector dominated and have since proved to be unproductive owing to inefficient deployment of resources, unsustainable funding and poor accountability (FMARD, 2008; Nzewi, Hope Ngozi, 2017). Therefore, this work did not stimulate the

sector's growth fast enough to meet growing domestic demand and achieve self-sufficiency.

More conscious efforts to stimulate further development of the aquaculture sector were put in place. For example, the National Aquaculture Strategy (FMARD, 2008) was introduced with the objective of bringing Nigeria to fish self-sufficiency in 5 to 25 years, through promotion of aquaculture by appropriate investment in a public–private partnership model, reduction of imports and the development and implementation of a national fish disease diagnosis and prevention scheme. Furthermore, the Nigerian Vision 2020 sought to increase domestic agricultural productivity, promoting the adoption of improved seed/fish broodstock (Subasinghe et al., 2021). The Agricultural Transformation Agenda (ATA) 2011 resulted in a moderate increase in production between 1999 and 2015 (Subasinghe et al., 2021). While the National Aquaculture Development Plan (NADP) 2011 attempted facilitating investment through identification of high potential aquaculture zones and an appropriate framework for aquaculture outreach to enhance adoption through awareness creation, its monitoring and evaluation was not implemented thus its performance could not be assessed (Subasinghe et al., 2021). The backward integration policy, an extension of previous policies which encouraged fish importers to re-channel their resources towards aquaculture, resulted in an estimated 20,000 tons addition to annual production (WorldFish, 2018). Although these policies have boosted aquaculture production, other factors have prevented the country from achieving self-sufficiency in fish production.

1.9 Challenges of aquaculture development in Nigeria

Nigeria has practised aquaculture on an commercial scale for more than three decades and has abundant natural resources favourable for aquaculture development, but it is yet to bridge the gap between demand and domestic production (Adeleke et al., 2020a; FAO, 2016; Oladimeji, 2017). This is despite joining the league of major aquaculture producers (countries with production over 100,000 tons), where the country currently ranks 18th globally and recorded a growth rate of 18% between 2006 and 2016, which is the second highest in Africa and third highest in the world

(FAO, 2020; Garlock et al., 2020). A combination of factors has impeded Nigeria's quest for self-sufficiency in fish production and recently forced the industry towards a downtrend as evidenced by the decrease in output between 2015 and 2018 (FAO, 2020).

At the macroeconomic level, the practice of changing policies by successive governments, which is common in Nigeria is inimical to long term investment plans as it diminishes investors' confidence and limits the flow of investment into the economy (Adedeji & Okocha, 2011; O. E. Ogbonna et al., 2022). Inflation and currency devaluation have made catfish production expensive because of high input cost (Oman et al., 2023a). This is exacerbated by lack/poor adoption of modern technologies, poor biosecurity and a lack of standardisation in farming practices. (Oman et al., 2023a). Similarly, access to finance is very difficult because of low profitability, low financial literacy, high interest rates (about 10% currently) and poor record keeping (Adedeji & Okocha, 2011; Oman et al., 2023a). Other factors include inefficient managerial skills, competition for suitable land and reliable water resources, quality seedlings and disease control (Adedeji & Okocha, 2011).

1.9.1 Human resources

Human capital is the only production factor on which all other factors are dependent upon for the success of the enterprise (Blaga & Jozsef, 2014). Losses in aquaculture could result from either the death of fish or reduced performance as a consequence of stress induced by factors like high population density, pH fluctuations, handling, oxygen depletion and water pollution, disease, predation, vandalism, flooding and many more which underpin the technical nature of aquaculture management (Adewumi, 2015a; C. Okere & K. Adeyemo, 2014). Technical challenges can arise in both hatcheries and grow out ponds and they include but are not limited to: poor quality brood stock management, poor record keeping of activities related to induced spawning, care of eggs, fries and fingerlings, feeding, mortalities and biosecurity and the failure to properly manage these factors. These issues have caused many enthusiastic farmers to suffer colossal losses of fish stocks (Adewumi, 2015a). Nigeria has sufficient capacity for aquaculture human resource development ranging from institutions with clear and specific aquaculture related mandates to the numerous

universities and polytechnics offering courses in fisheries and aquaculture. Despite the abundance of competent personnel that have been produced by these institutions, economic issues have prevented the aquaculture sector from benefitting from this pool of experts (Subasinghe et al., 2021).

Good husbandry practices like record keeping enhances a farm's integrity and highly valued by financial institutions for granting credit facility. The absence of such good practice has resulted in many farmers' inability to access bank loans (Oman et al., 2023a). To improve the efficiency and hence the productivity of fish farms, there is the need for the farmers to engage in regular training sessions to enhance their skills and encourage the adoption of technologies that will enhance productivity, as the current rate of technology adoption among farmers is still low (Adewumi, 2015a). Enhanced technical capacity through training is a requirement for a holistic biosecurity approach that aims to maximally reduce the risk of disease outbreaks in an aquaculture operation (Subasinghe, Alday-Sanz, et al., 2023). The lack of operational aquaculture extension service in Nigeria makes fulfilling this key requirement an uphill task. Thus, innovative and cost-effective approaches like the use of online medium might be a viable alternative.

1.9.2 Availability of quality fish seed

Supply of quality fish seed produced from high quality brood stock of proven genetic composition and performance is an important first step in the journey to a successful aquaculture enterprise (Adewumi, 2015a). While the industry produces around 55 million seedlings from all available sources annually, it is estimated that about one billion seedlings are required if the current demand is to be met (Adeleke et al., 2020a). Consequently, an ambitious plan was made to produce 1.25 billion seedlings through the government's Agricultural Transformation Agenda (Ogunji & Wuertz, 2023). However, just like many other government intervention programmes, this could not come to fruition due to poor implementation (Nwankwo et al., 2024). Additional factors that include lack of institutional mechanisms for ensuring quality control due to a lack of regulatory framework, incompetence resulting in inbreeding, and unsuitable facilities have resulted in compromised seed quality with an increased susceptibility to

infections, low survival and growth rate which results in reduced productivity and poor profitability (Adewumi, 2015a; Oman et al., 2023a). There is therefore the urgent need for a private sector driven initiatives to address the chronic shortage of quality seedlings as well as investment in research and technologies that ensure sustainability, for example cryopreservation of milt, that addresses the slaughtering of male brood stock for milt extraction and the perennial shortage of male catfish experienced during the dry season (Adewumi, 2015a).

1.9.3 Feed

Feed is the most important and critical input after good quality seed (Anetekhai, 2013). Feed accounts for over 60% of operational costs in intensive catfish aquaculture and, as such, the overall sustainability of the industry can be said to be largely dependent on the stability and quality of the feed subsector (Adeleke et al., 2020a). Fish feed in Nigeria is sourced either through on-farm milling, from small- and medium-scale feed mills or from corporate feed millers (Oman et al., 2023a). The prevailing economic situation has led to soaring feed costs forcing many farmers to rely on less effective feed as against the more expensive but proven corporate or imported feed (Oman et al., 2023a; Udo & Dickson, 2017) thereby posing a challenge to the stability and economic viability of the aquaculture sector (Udo & Dickson, 2017). Additionally, the feed subsector's is heavily dependent on importation of feed ingredients due to lack of sufficient local production for example over 80% of fish meal is imported (Udo & Dickson, 2017). This is further complicated by stiff competition from the livestock feed manufacturing sector, placing the small and medium-scale fish feed producers under difficult economic pressures (Adedeji & Okocha, 2011). This has left the subsector at the mercy of negative market forces such as currency devaluation, inflation and fluctuating foreign exchange with a consequent increase in the percentage of total operational costs attributable to feed increasing to about 80%, and thus increasing the overall cost of production (Adedeji & Okocha, 2011; Udo & Dickson, 2017). To meet the increasing demand for aquafeed and cushion this negative effect, interventions such as credit facilities, input subsidies, pricing policies and market liberalisation were introduced to stimulate an increase in production, but they have all failed to yield the desired result and domestic production output thus remains low (Udo & Dickson, 2017).

1.9.4 Access to land and water resources

Although Nigeria is estimated to have 14 million hectares of inland fresh water and another 1.7 million hectares of suitable land for aquaculture development (Adewumi, 2015a), access to these resources for aquaculture purposes has not been easy for most farmers. Generally, availability of land for agricultural purposes involves a complex interaction of variables such as the land tenure system, the population within an area and level of technology adoption, with the land tenure system putting serious limitation on the amount of land available for aquaculture in many parts of the country. The philosophy of the current land tenure system as enshrined in the Land Use Act of 1978, is that all lands belong to the state, held in trust by governors (who in practice have politicised its implementation) and that undeveloped land has no value (Emeka et al., 2017). Additionally, the land allocation system is reportedly cumbersome owing to bureaucracies associated with The Land Use Act and thus makes it difficult for investors to acquire land for aquaculture (Subasinghe et al., 2021). Technically, this locks up valuable land as “dead capital” and by implication citizens especially rural dwellers are left without title for their most prized asset. Land title is a key requirement for unlocking the potential of any land and the lack of this by most rural dwellers has significantly limited the amount of land available for agriculture and impedes farmers’ access to credit for critical investments and improved productivity (Emeka et al., 2017). This is more complicated in urban areas because of the population where there is a low level of technology adoption for land administration. The situation is made worse in the South-South (Fig 1) on both the land and coastal water, which has significantly impacted negatively on aquaculture development in the region (Adeleke et al., 2020a).

Rather than eliminate the uncertainties of land titles, the 1987 Act has in practice exacerbated it. The implementation of this act has never been complete and without controversy (Emeka et al., 2017; Makinde, 2019). There has been arbitrary and selective application of some portions of the law and in some instances outright revocation of the right of occupancy without recourse first to its authenticity. Similarly, there is loss of land by small scale farmers to non-agricultural acquisition and speculative large scale commercial agriculture (Emeka et al., 2017; Makinde, 2019). Consequently, there is reluctance by both courts and banks to accept statutory Certificate of Occupancy as conclusive evidence of the title of the holder or security

for loans (Emeka et al., 2017). Without a legislative framework in place that not only specifically guarantees seamless access to land and water resources for aquaculture but also ensures its security, achieving self-sufficiency in fish production may not be feasible.

1.9.5 Environmental impact

While striving for aquaculture self-sufficiency and sustainability in Nigeria, it is necessary to adopt practices that ensure its impact on the environment is negligible. The need to bridge the gap between fish demand and supply in Nigeria has been met with increase in intensively managed farms which currently stands at an estimated 285,000 catfish farmers (Oman et al., 2023a) compared to output from artisanal sources (Adewumi, 2015a). This has been accompanied by an increased waste production leading to eutrophication and destruction of natural ecosystem (Akinrotimi et al., 2011). Most of these aquaculture wastes, which often contain both organic and inorganic residues such as nitrogenous compounds, fertilizer, antibiotics, disinfectants and other chemicals, are often discharged directly without treatment either into water bodies or onto the streets in some urban areas as seen in Port Harcourt metropolis of Rivers State, South-South Nigeria (Akinrotimi et al., 2011). This results in the distortion of the aesthetic value of the environment, as well as a putrefying odour emanating from the discharge of effluents directly into the environment. These effluents are also known to induce stress and alter the balance of aquatic ecosystems through eutrophication, reduce dissolved oxygen, introduce new pathogens or resistance genes to the environment and, depending on the composition, can cause direct toxicity to the aquatic biota (Adewumi, 2015a; Akinrotimi et al., 2011; Martínez Cruz et al., 2012). Furthermore, activities down the value chain especially fish smoking that is mostly conducted with charcoal or firewood impacts the environment by contributing to deforestation and air pollution, that also has the potential to cause respiratory illness in humans (Oman et al., 2023a). There is therefore the need to properly regulate the sector to ensure proper planning and efficient waste management and fish processing so that while aquaculture is harnessed for economic development, it does not harm the environment.

1.9.6 Marketing and transportation

Apart from seasonality, scarcity and means of preservation, transportation is known to constitute a serious constraint to fish marketing in Nigeria (Adewumi, 2015a). Movement of goods generally and agricultural produce, relies heavily on road networks, many of which are either untarred or in a state of disrepair. Most fish farmers are therefore compelled to sell or distribute at farm gates to designated fish markets in towns or cities for onward uptake by processors, consumers and restaurant operators (Adeleke et al., 2020a; Adewumi, 2015a). This supply chain is largely controlled by wholesalers and middlemen who determine the price based on prevailing macroeconomic conditions (Cocker, 2014). This structure, however, often places the farmers in a disadvantaged position and thereby limits their profit. Considering the fact the aquaculture industry in Nigeria is dominated by small-to-medium scale farmers with limited capital base, such an arrangement has exacerbated their problems and discouraged potential new operators from entering the business.

1.9.7 Infectious diseases

Intensive aquaculture is associated with high stocking density. While high stocking density yields higher output/unit area, it can lead to reduced growth and survival rate, increasing the risk of disease outbreaks by inducing higher stress levels (Ezhilmathi et al., 2022; Lu et al., 2022). Although the innate immunity often succeeds in blocking the progress of an invading pathogen (Jeney, 2017), stressed fish have a compromised immune system that cannot function optimally (Yarahmadi et al., 2016). In addition to high density, intensively managed fish are exposed to other forms of stress that include poor water quality resulting from low dissolved oxygen, unfavourable temperature or pH, undesirable levels of carbon dioxide, ammonia, nitrite, hydrogen sulphide and organic matter; injury from handling; inadequate nutrition and poor sanitation that further contribute to suppression of fish immunity and promote infection (Agbede, 2012; Martínez Cruz et al., 2012). Furthermore, global warming because of climate change is also suggested to tilt the balance between pathogen, fish host and the environment in favour of the pathogen leading to emergence of novel pathogens as well as increased prevalence and spread of existing

pathogens (Rodger, 2016). This would be a complicating factor temperate aquaculture like that in Nigeria and especially given the challenges enumerated above.

Diseases, estimated to cost global aquaculture around six billion USD have been identified as the most serious constraint to aquaculture development (Assefa & Abunna, 2018; Dauda & H.I., 2015; Rodger, 2016). Diseases account for almost 50% of production losses, which is more pronounced in developing countries that produce about 90% of global aquaculture production (Assefa & Abunna, 2018; Dauda et al., 2015). Disease-associated losses occur in the form of investment lost due to fish mortality, cost of treatment, reduced growth rate during convalescence and the extended production cycle, which all results in increased production costs and a reduced profit margin or even complete loss (Agbede, 2012). Small scale catfish farmers in Nigeria lose an up to \$2,056.38 from diseases on a maximum net income of \$7,520.79 per cycle (Mukaila et al., 2023). Diseases in aquaculture thus damage livelihoods, cause loss of jobs, reduce income and threaten global food security (Assefa & Abunna, 2018).

Although the economic importance of fish diseases has been a major concern of the industry, the exact losses associated with diseases in Nigerian aquaculture are difficult to estimate due to poor record keeping, poorly equipped and non-accredited laboratories and the absence of a fish disease reporting mechanism (Adedeji & Okocha, 2011; Adeleke et al., 2020a; Dauda & H.I., 2015). That notwithstanding, some reports have shown that diseases have caused substantial monetary losses to farmers in the country (Adeyemo, 2013; C. Okere & K. Adeyemo, 2014; Dauda & H.I., 2015). In September 2013, reports emerged from across the country of mass mortality of fries at about 2-4 weeks with consequent failure to produce fingerlings and juveniles. The true reason behind this was, however, not fully diagnosed. While some believed this was due to nutritional causes, others blamed high temperatures triggered by climate change (Anetekhai, 2013). In any case, both represent risk factors that could predispose the fish to infectious diseases. A study in two fishing communities in the south of the country showed that 89.39% of fish farmers observed disease symptoms in their stocks, with haemorrhagic lesions being the most common symptom (Adeyemo, 2013). A similar study in a northern state also reported that 54.3% of farms

in the state had experienced disease outbreaks, 57.9% of which reported fin and tail rot as major disease symptoms (Dauda & H.I., 2015).

Whereas diseases of parasitic and fungal nature have been reported (Enyidi & Maduakor, 2017; Kawe et al., 2016; Solomon, 2017), there appears to be more research interest involving bacteria as the major cause of disease problems within Nigeria's aquaculture industry. This, however, does not in any way diminish the significance of parasites, viral or fungal agents as disease causing agents. Paucity of data on these other disease-causing agents from Nigeria makes it difficult to assess or establish their economic significance. Moreover, mortality from disease events in catfish aquaculture have been majorly associated with bacterial infections (Wise et al., 2021). Furthermore, the dominant interest in bacteria could also be said to have been propelled by public health concerns such as the growing interest in antimicrobial resistance and the need to elucidate the role of aquaculture in this phenomenon. Indeed a significant number of studies have presented relevant findings isolating bacteria such as *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, *Edwardsiella species*, *Flavobacterium columnare*, *Streptococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus* spp. and many more from both catfish and tilapia (Ashiru et al., 2011; Efuntoye et al., 2012; Enyidi & Maduakor, 2017; Ikpi & Offem, 2011; Imade & Akinnibosun, 2018; Saka, B. A., Adeyemo, 2017). While most of the studies relied on phenotypic traits and standard biochemical techniques for presumptive bacterial identification, Imade and Akinnibosun (2018) employed molecular techniques including 16S rRNA gene analysis and sequencing to identify their isolates. These researchers also went further to describe the virulence mechanisms of some of the isolates (Imade & Akinnibosun, 2018). Furthermore, an increasing number of studies have reported isolating similar bacteria associated with strictly morbid catfish (Anyanwu and Chah, 2014; Ikpi and Offem, 2011; Nkechi C. Okere, 2014; Ogbonna and Inana, 2018; V. O. Omeje, Chukwu, 2014). Other than isolating these organisms from overtly sick fish, no effort was made to describe the pathologies and, as such, no single organism can be said to be causing the symptoms as very few bacterial diseases are pathognomonic. Anyanwu et al., (2015) however, went on to reproduce similar lesions in a challenge study of catfish using *Aeromonas* isolates from a previous study in support of their virulence potential, rather than commensal role.

1.10 Infectious disease management practices

Aquaculture in Nigeria has grown at a significantly steady rate within the last few decades without a corresponding growth and standardisation in disease prevention and control practices. There is a lack of organised health management and biosecurity programme and insufficient qualified veterinarians with requisite skills and experience in fish disease management (Subasinghe et al., 2021). Similarly, both the understanding and application of biosecurity measures among fish farmers in Nigeria is poor (Grema et al., 2020; Nwabueze & Ofuoku, 2020; Oman et al., 2023a). Thus, disease management strategies within Nigeria's aquaculture have relied heavily on the use of antibiotics (Nkechi C. Okere, 2014; Saka, B. A., Adeyemo, 2017) and often by unsanctioned individuals.

Inadequacy of competent manpower in the field of fisheries and wildlife medicine within Nigeria's Veterinary Colleges has resulted in a chronic shortage of skilled and experienced aquatic veterinarians knowledgeable in fish disease prevention, treatment and control, which is further complicated by an insufficient number of diagnostic laboratories (Adedeji & Okocha, 2011). Although some of these institutions have begun recruiting and training staff in this field, the impact of this intervention remains grossly inadequate given the industry's size and potential. This is despite increased private sector investment. For example, Animal Care® - a foremost animal feed and diagnostic service provider has within the last five years extended its diagnostic services from its base in the South-West (the hub of aquaculture) of the country to Abuja, the nation's capital located in the centre of the country. It has also replicated the same in the north. This inadequacy has given room for unwholesome practices by farmers, such as indiscriminate use of drugs and chemicals (Adedeji & Okocha, 2011) and at times smoking sick fish; practices that not only reduce profitability of the enterprise but also endanger public health. Disease management and control, therefore, remains a major challenge to aquaculture development in the country.

While a farm's level of intensity might influence its infectious disease management strategies, certain elements are critical to infectious disease control irrespective of intensity level. These include adoption of good biosecurity practices (Khor et al., 2024),

good husbandry practices (Wanja et al., 2020), use of probiotics (Bondad-Reantaso et al., 2023), vaccination (Bondad-Reantaso et al., 2023; Desbois & Monaghan, 2023a), and selective breeding for resistant strains of catfish (J. Wang et al., 2024). Promotion of investment and adoption of these key elements will greatly enhance the economic viability of small, medium and large-scale fish farm enterprises in Nigeria and thus promote sustainability of the sector in the country.

1.10.1 Probiotics in Nigeria's aquaculture

Probiotics in aquaculture are living microbial additives that benefit the health of hydrobionts and therefore increase productivity (Martínez Cruz et al., 2012). To fit this definition, probiotics must possess certain characteristics that may include one or more of the following: antagonistic activity against pathogens, immunostimulation, exogenous enzyme production and other growth-promoting features, non-pathogenicity, ability to withstand acidic conditions and bile salts and freedom from plasmid-encoded resistance genes (Diyaolu et al., 2018; Merrifield et al., 2010). Therefore, probiotics represent an important tool for disease prevention and consequently reduced need for antibiotics especially in a country like Nigeria.

Dauda et al., (2013) have identified some of the challenges to encouraging the use of alternatives to antibiotics in Nigeria including insufficient robust data owing to inadequate research, inadequate specificity and possible introduction of exotic microbes with potential hazards. That notwithstanding, a number of studies have reported both in vitro antibacterial activities of strains *Lactobacillus* spp. isolated from indigenous fermented products, and their effect on improved growth performance of African catfish (*Clarias gariepinus*) as potential probiotic candidates for Nigeria's aquaculture (Diyaolu et al., 2018; Falaye et al., 2016; Ogunshe and Olabode, 2009; Oluyemi, 2019).

1.11 Antibiotic resistance in Nigeria's aquaculture

Antimicrobial resistance is a global public and animal health threat (Organisation for Animal Health, 2022) driven by the use of antibiotics in aquaculture (Alhaji et al., 2021; Brunton et al., 2019; J. Chen et al., 2020a) and some of these concerns have indeed

been justified. Use of antibiotics under self-prescription and lack of observance of withdrawal periods is common among fish farmers in Nigeria (Ladan Adamu Aliyu, 2014; Olatoye & Basiru, 2013). Antibiotic resistance has thus been reported from aquaculture in Nigeria (Nkechi C. Okere, 2014; D. Ogbonna & Inana, 2018; Saka, B. A., Adeyemo, 2017). More worrying is the fact that the resistance reported in these studies from aquaculture in Nigeria is recorded in a significantly high proportion against medically important antibiotics, such as ofloxacin, whose use in animal production in developed nations like the USA is not only illegal but considered highly irresponsible (Saka, B. A., Adeyemo, 2017).

Reports from published literature indicates an exponential increase in resistance profiles among bacterial species. Ashiru et al. (2011) reported the resistance profile of *Aeromonas* species to include tetracycline, nitrofurantoin and augmentin out of ten antibiotics tested. By 2014, this has grown to include chloramphenicol, ceftriaxone, ampicillin, enrofloxacin, streptomycin, ciprofloxacin, ofloxacin, cefixime, gentamicin, cefuroxime and ceftazidime, at a multidrug resistance rate of 66% (Anyanwu & Chah, 2014; Nkechi C. Okere, 2014) and by 2017, Saka et al., 2017 reported a 100% multiple antibiotic resistance by all ten *A. hydrophila* strains isolated from catfish in their study.

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Although numerous bacterial species have been isolated and described from aquaculture in Nigeria, much of this information is either from apparently healthy fish or the culture water. There is thus a paucity of information on both prevailing pathogenic strains of bacteria and their distribution in Nigeria. This constitutes a limitation in designing an effective disease control strategy. Additionally, much of the information available on bacteria from aquaculture in Nigeria is from the southern part of the country. This is understandable given the fact that aquaculture is more widely practised in the south than in the north of Nigeria. Recently, however, north-central Nigeria (Fig 1) has joined the list of aquaculture hubs in the country (Oman et al., 2023a) and aquaculture activity is also rapidly increasing in other parts of northern Nigeria (Subasinghe et al., 2021). Given the significant gap in poverty level and food security between the north and the south (Subasinghe et al., 2021; Wudil et al., 2023) and the potential role of aquaculture in bridging this gap, it is important that infectious

disease status in the aquaculture sector of northern Nigeria is adequately understood. This will not only ensure sustainability and food security, but promote the socioeconomic well-being of the people. It will also inform a more targeted approach for the control of aquaculture infectious diseases in this region and the country at large.

This thesis, therefore, aims to describe the prevailing pathogenic bacteria of catfish, infectious disease outbreaks and associated management practices, and propose interventions towards improved biosecurity practice in aquaculture in northern Nigeria.

This aim will be achieved by executing the following set of objectives:

1. To describe disease outbreaks and associated management practices on fish farms in northern Nigeria.
2. To isolate and characterise bacteria from diseased catfish from farms and fish markets in northern Nigeria using conventional and molecular techniques.
3. To design and test the impact of an online workshop as a tool for improving biosecurity practice on fish farms in northern Nigeria.

Chapter 2

Disease outbreaks and associated management practices at fish farms in Northern
Nigeria

2.1 Introduction

Nigeria has an average annual per capita fish consumption of 11.2 kg and an estimated annual consumption of about 3.2 million tons making it the highest in Africa and among the highest in the world (Adeleke et al., 2020a; Kaleem & Bio Singou Sabi, 2020; Subasinghe et al., 2021). Much of the fish supply in the country comes from wild catch and importation, and domestic aquaculture is estimated to contribute only about one-third of national fish supply (Adeleke et al., 2020a; Amosu Albert Oluwatobi et al., 2017; Dauda et al., 2018; Oboh, 2022). This is, however, set to change as attention is increasingly shifting towards aquaculture as a sustainable source of meeting the country's growing demand for fish. Factors driving this change include stabilisation of supply from marine capture, a projected increase in the fish supply deficit within the coming decades, the high cost of fish imports (\$1 billion) due in part to currency devaluation, and a projected population of 229.8 million people with increased mean annual per capita fish consumption to 14.8 kg by 2050 (Adeleke et al., 2020a; Adewumi, 2015b; Amosu Albert Oluwatobi et al., 2017; Kaleem & Bio Singou Sabi, 2020; Subasinghe et al., 2021).

Aquaculture in Nigeria is predominantly inland freshwater (Subasinghe et al., 2021) and dominated by culture of African catfish (*Clarias* spp. and *Heterobranchus* spp.) which accounts for more than 80% total domestic output (Subasinghe et al., 2021). Other cultured species include tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*) and African arowana (*Heterotis niloticus*) (Adeleke et al., 2020a; Dauda et al., 2018). Although aquatic food production has grown steadily over recent decades to provide employment opportunities for about 450,000 people and to contribute 4.5% of GDP, it has not grown at a sufficient pace to meet the country's fish supply deficit, which stands at 2.5 million tonnes (Adeleke et al., 2020a; Adewumi, 2015b; Dauda et al., 2018; Oboh, 2022; Subasinghe et al., 2021). Low-level awareness of fish diseases, prevalent disease outbreaks and a lack of competence in disease management have been identified among other factors to be key limitations to aquaculture production in Nigeria (Adah et al., 2023a; Adeleke et al., 2020a; Kaleem & Bio Singou Sabi, 2020; Ogunleye et al., 2022).

Globally, diseases cost the aquaculture industry over \$6 billion in losses (Assefa & Abunna, 2018; Maldonado-Miranda et al., 2022a). In Nigeria, disease-associated losses on small scale catfish farms in a production cycle can reach more than \$2,000 per farm (Mukaila et al., 2023). Outbreaks of diseases represent a fundamental constraint to catfish farming with emerging and re-emerging infections principally responsible for the shortfall in Nigeria's aquaculture production (Ogunleye et al., 2022). Prevalences of suspected infectious disease outbreaks have been documented at both hatcheries and grow-out farms across the country (Adah et al., 2023; Dauda & H.I., 2015; Durojaiye et al., 2020; Ladan Adamu Aliyu, 2014; Omitoyin & Osakuade, 2021).

The last fish farm census held in 2004 showed that Nigeria had 2,600 fish farms and 215 feed mills, mostly located in the south of the country (Amosu Albert Oluwatobi et al., 2017). Recently however, aquaculture activity in the north of the country has increased significantly, with more frequent consumption of cultured fish compared to people living in the south (Subasinghe et al., 2021). Aquaculture is thus evolving and has the potential of gainfully employing millions of people in this most populous part of the country (Fig 2.1) where poverty is strikingly high (Jaiyeola & Choga, 2021). Despite this, farms in the north have received much less attention from scholars. Consequently, little information is available specifically for this area and there is a need for a better understanding of prevailing diseases and practices employed by fish farmers from across the region in the management of those disease challenges. It is also necessary to understand the organisational structure of the system components in terms of common production types, key players, governance structures and the flow of inputs and outputs along the value chain. Such information can guide the design and implementation of targeted interventions aimed at disease mitigation to help boost production.

Therefore, this present Chapter aims to investigate infectious disease outbreaks and associated management practices at fish farms located in the states of Northern Nigeria and the Federal Capital Territory (FCT) using a structured questionnaire administered through phone interviews with fish farmers and describe different components of the aquaculture system in Nigeria. This aim will be achieved through fulfilling the following objectives:

1. Generate a system map that captures the different components of Nigeria's aquaculture system, their connections, key players, flow of communication, inputs and outputs through focussed group discussion.
2. Describe the aquaculture husbandry practice in northern Nigeria using a structured questionnaire.
3. Determine the frequency of disease outbreaks and mortality events affecting aquaculture in the northern Nigeria through a structured questionnaire.
4. Identify practices used by catfish farmers for the prevention and management of suspected infectious diseases using a structured questionnaire approach
5. Identify possible risk factors associated with disease outbreaks and mortalities.

2.2 Materials and Methods

2.2.1 Study area

The study centred on the 19 states that make up the north of Nigeria and the Federal Capital Territory, Abuja (Figure 2.1). This region is located roughly between latitude 7 degrees and the northern (Niger Republic), western (Benin Republic) and eastern (Cameroon and Chad) borders of Nigeria (Fernando et al., 2020). It is characterised by a Sahelian hot, semi-arid and savannah climate (Gabriel et al., 2023).

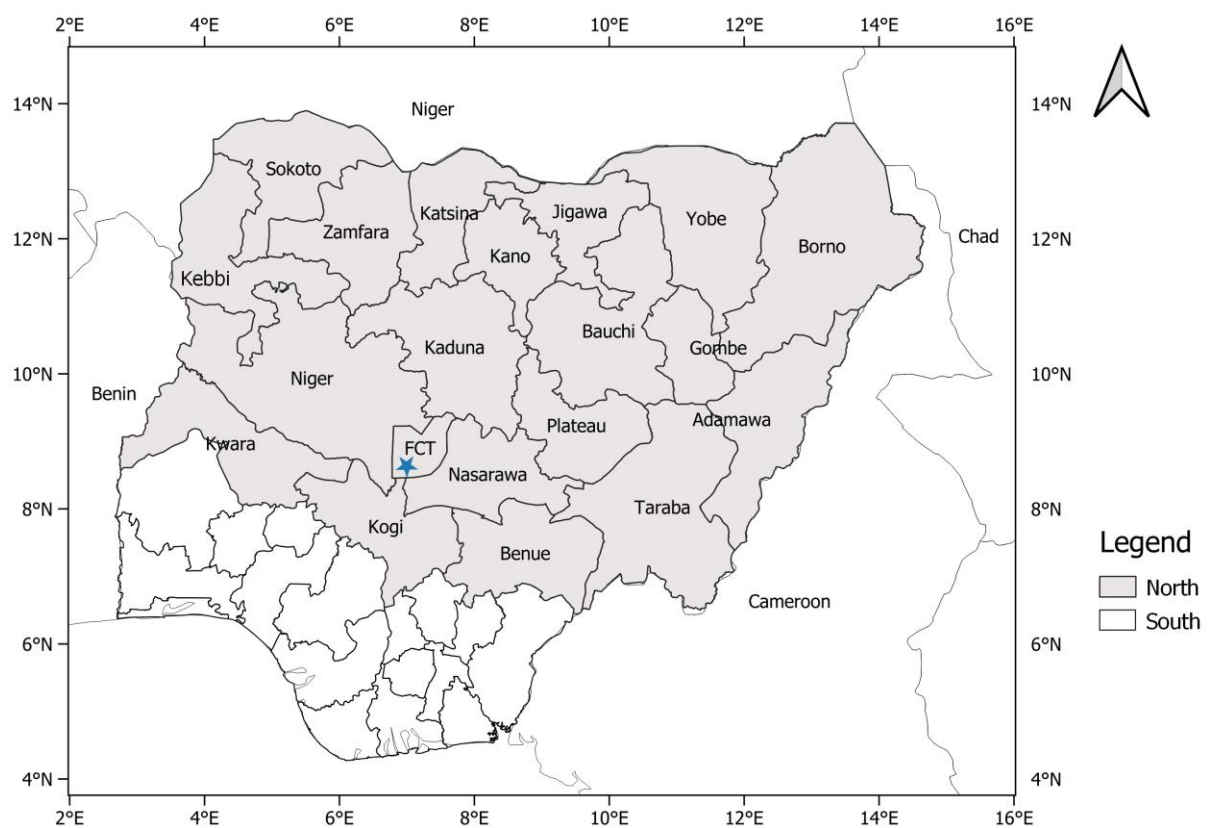


Fig 2. 1 Map of Nigeria showing the targeted survey location (shaded in grey) and the Federal Capital Territory (blue star)

2.2.2 Mapping the catfish aquaculture system in Nigeria

A modified method of Desbois et al. (2021) was used to create a system map from a combination of review of relevant literature and the researcher's field experience and contact with fish farmers. Three key dimensions i.e., governance, production and products were considered during the mapping exercise. Firstly, a proposed system

map was used to identify various components and actors involved in regulation, production and product distribution. The relationships between different components and actors were then suggested before tracing the flow of suspected inputs and outputs. Secondly, the system map was refined and validated through an online focussed group discussion with relevant stakeholders that consisted of two fish farmers and two veterinarians all identified through the researcher's personal contacts; the Director of the Department of Fisheries and Aquaculture at the Federal Ministry of Agriculture and Food Safety and a staff member of one of the State Ministries of Agriculture identified through one of the participating veterinarians, a representative of a commercial feed company and a veterinary drugs/agrochemical vendor identified through one of the participating farmers. Each participant was contacted via personal WhatsApp messaging to introduce the topic and aim of the exercise. A consensus date and time was agreed in consultation with all the participants. The exercise took place via the online meeting platform Zoom. During the exercise which lasted about 2 h, the proposed map was presented to the participants in a PowerPoint presentation before they were asked to verify the proposed map and identify additional actors not present on the initial map proposal. Amendments to the proposed map were suggested, additional actors identified and their roles and connections within the system established. The edited map was reviewed again and following the agreed consensus, a final digital version was created using the web-based software, Lucidchart (<https://www.lucidchart.com/>).

2.2.3 Survey design

2.2.3.1 Questionnaire design

To describe the various types of catfish production, as well as investigate infectious disease status and control in the region, a structured questionnaire consisting of both close and open-ended questions was designed. In the questionnaire, a total of 103 questions were grouped into three categories, i.e. farm structure/production and data on the participant as level of education and training on fish farming (51 questions); disease occurrence and management (22 questions) and disease prevention and biosecurity (30 questions) drawn from review of relevant literature.

Table 2. 1 Different types of questions under the three categories of the structured questionnaire.

Category	Open Ended	Close Ended	Total
Farm structure and production	15	36	51
Disease occurrence and Management	8	14	22
Disease prevention and biosecurity	7	23	30
Total	30	73	103

2.2.3.2 Recruitment of farmers

The absence of a register of fish farmers meant a random sampling technique could not be applied. The first set of three fish farmers were thus recruited using convenient sampling through personal contacts according to the following set of criteria:

1. An owner, manager, or farm worker conversant with the daily operations of a catfish/catfish and tilapia farm from any part of northern Nigeria.
2. The farmer must not be less than eighteen years of age.
3. The farmer must own/manage/work in a farm currently in operation.
4. Must willingly consent to participate in the survey.

Subsequent fish farmers were then recruited from across northern Nigeria using the snowballing technique (Parker et al., 2020). Enrolled farmers were asked to pass on contact details of the research team to other fish farmers so they could contact the research team. Once a new fish farmer made contact and was found to fulfil the above-mentioned criteria, the aim and objectives of the study was explained to the potential recruit. The farmer was then enrolled after granting verbal consent (Figure 2.2).

2.2.3.3 Questionnaire pre-testing

Pre-testing of the questionnaire was done via telephone call involving the first three farmers identified through personal contacts. Verbal consent was obtained after explaining the study aim, objectives and overall benefits to the farmer. Following this, the questionnaire was administered either at that point or by arranging for a more suitable date and time. Having been satisfied with the outcome of questionnaire pretesting, it was administered to other survey participants.

2.2.2.4 Survey delivery

The questionnaire was administered to consenting farmers at a date and time most suitable for the farmer. This was done either via telephone or social media platform (audio or video WhatsApp call) and each session lasted between 30 and 45 minutes. A total of 45 farmers completed the questionnaire and the whole exercise took six months in total (September 2021 – February 2022). Data generated were stored electronically on Microsoft OneDrive, on the University of Stirling's secure storage platform.

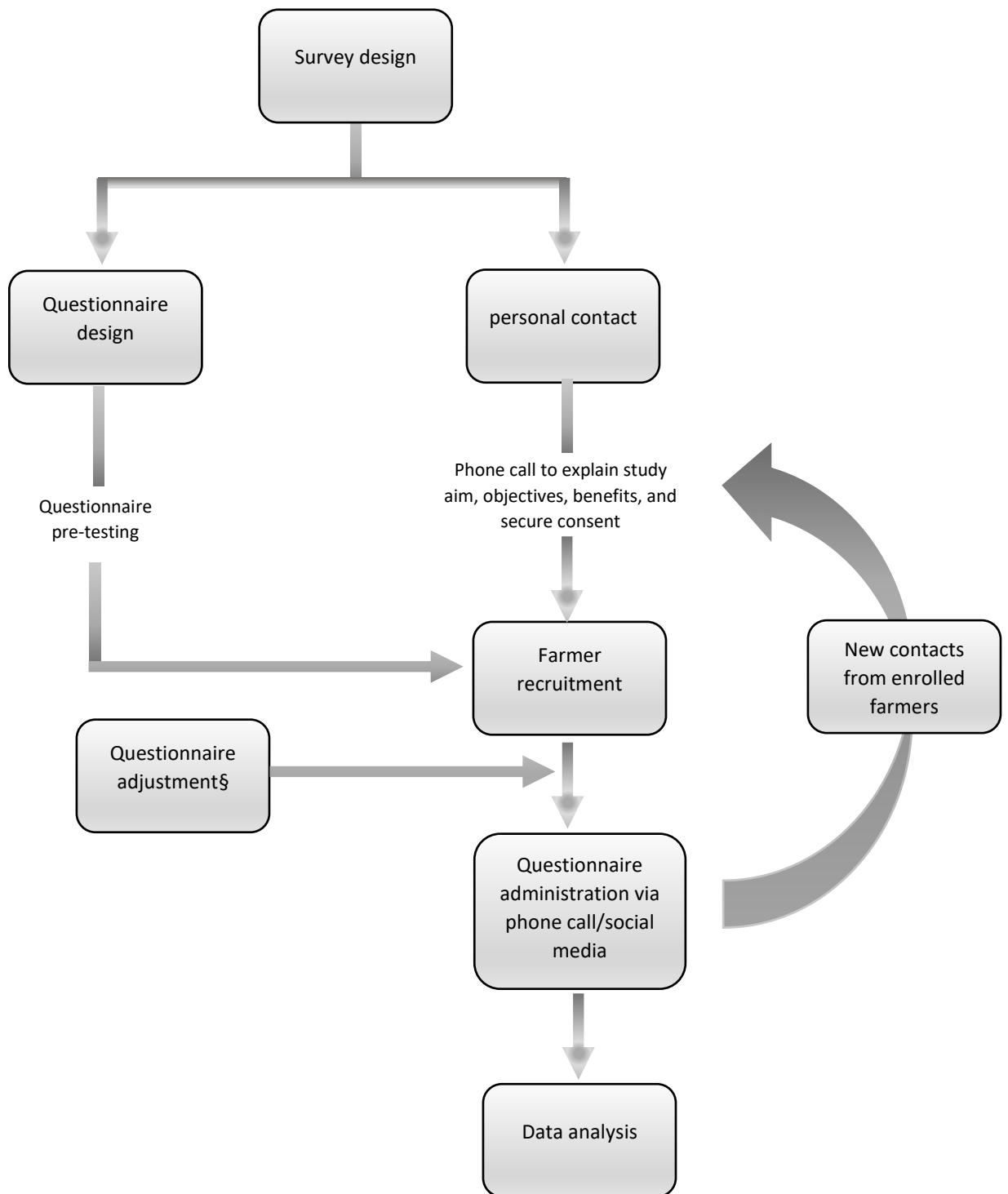


Fig 2. 2 Flow diagram showing the process of questionnaire design, farmer recruitment, and its administration.

2.2.3 Biosecurity Scoring

A scoring scheme to generate a single numeric value representing adherence of each farm to the general principles of good biosecurity was designed and applied to the questionnaire responses. Each participant (representing a single farm) was scored on 16 questions relating to biosecurity practices (Table 2.2) according to the method described by Adah et al. (2023). For each practice, a response demonstrating high adherence to the general practices of good biosecurity was awarded a score of 1 indicating good practice while a score of zero was awarded for poor practice (wrong response). A farm's score was obtained by summing the individual scores for the 16 variables. A mean biosecurity score for all the farms was then computed by adding each farm's score and dividing it by the total number of farms, and this value was used as a threshold to categorise a farm as having an overall good (scored higher than the mean) or poor (scored less than the mean) biosecurity practices.

Table 2. 2 Different biosecurity variables and their corresponding responses used in assessing a participant's farm biosecurity level.

Practice	Adherence to good biosecurity practice
Have different staff per unit	Yes
Keeping fish of different age groups/source in a single pond	No
Equipment sharing	No
Using dead animals/offal as feed	No
Using fish from river	No
Leaving dead fish in the pond	No
Post-harvest pond treatment	Yes
Disposal fish mortality	Yes
Dedicated farm clothing	Yes
Hand washing with soap before farm operations	Yes
Use of chemical foot bath	Yes
Equipment disinfection	Yes
Restricted pond access	Yes
Quarantine	Yes
Controlled workers movement to other farms	Yes
Monitoring water quality	Yes

2.2.4 Statistical Analysis

Data were recorded as values of 1 and 0 and stored in Microsoft Excel spreadsheet and exported to R for statistical analysis. Categorical variables were summarised as frequencies and proportions and presented as tables or bar charts while continuous variables were summarised as means and standard deviations (SD) or median with interquartile ranges. Additionally, percentage mortality >10% was categorised as high while $\leq 10\%$ was considered low (Mukaila et al., 2023). Multiple Correspondence Analysis (MCA) was also performed to determine the clustering of individual farms based on categorical variables. Chi square or Fisher's exact test at 5% significance level ($p \leq 0.05$) was then used to test the null hypothesis that mortality prevalence at a farm was the same irrespective of a participant's training on fish farming, observance of each of the 16 biosecurity practices and the farm's biosecurity category (Table 2.2). Similarly, odds of having a high mortality rate under a poor biosecurity condition or when not adhering to any of the elements of good biosecurity practice was also determined.

2.2.5 Ethical Approval

Ethical approval for this project was granted by the General University Ethics Panel (GUEP) of University of Stirling (Project ID 3688).

2.3 Results

2.3.1 Mapping the aquaculture system in Nigeria

Three dimensions that included governance, production, and products were considered for the construction of the aquaculture system map of Nigeria (Fig 2.3).

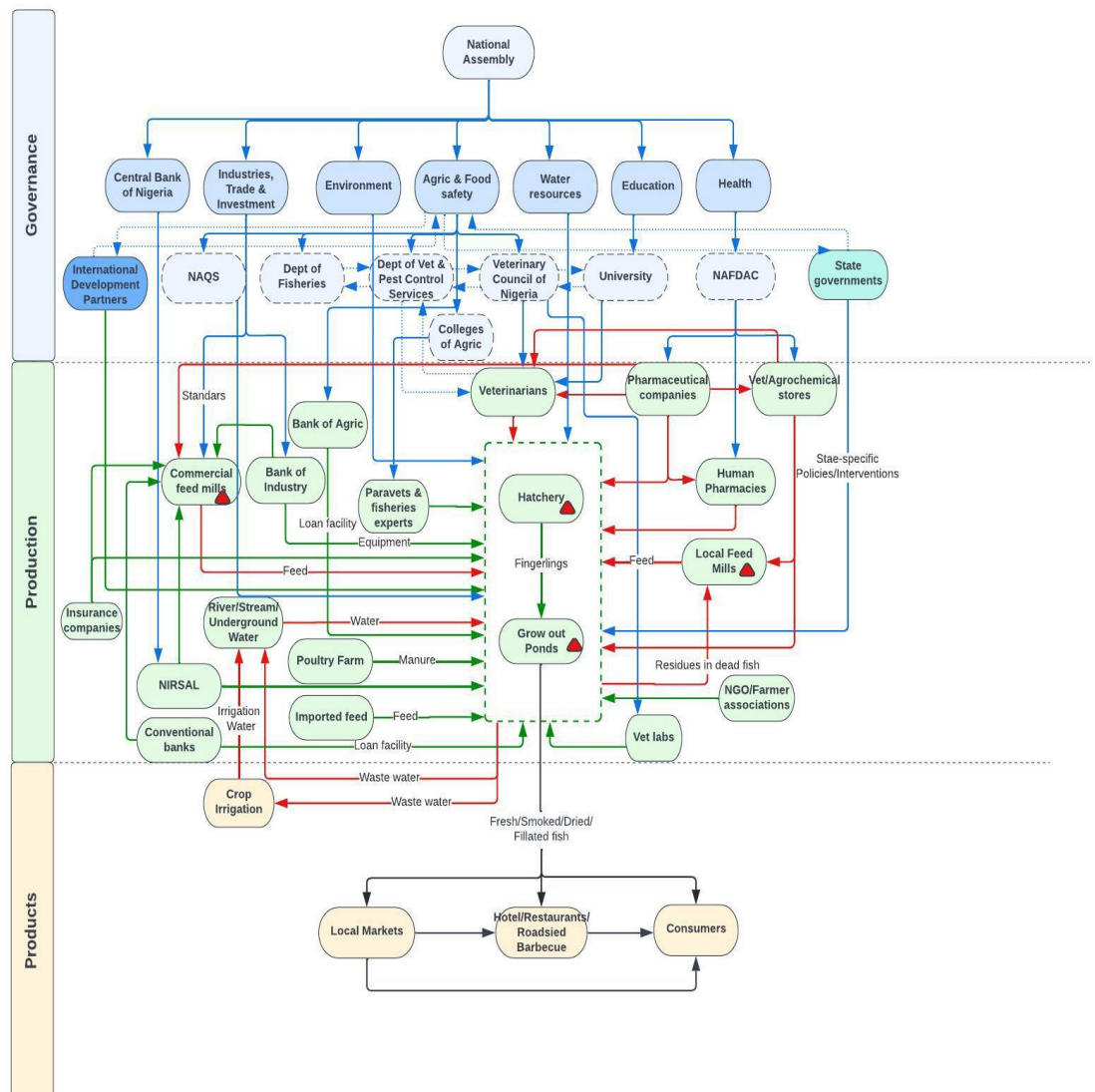


Fig 2. 3 Nigeria's aquaculture system map showing different actors and components, their connections and flow of communication i.e., regulatory in solid blue arrows, collaborative regulation in dotted blue arrows, flow of input in green, flow of output in black, flow of antibiotics in red and points where antibiotic are frequently used (red triangles). NAQS: Nigeria Agriculture Quarantine Service, NAFDAC: National Agency for Food, Drug administration and Control, NIRSAL: Nigeria Incentive-Based Risk Sharing System for Agricultural Lending.

Aquaculture governance in Nigeria is largely administered by the federal government in a multi-tier and collaborative manner (Fig 2.3). The National Assembly sits at the top of the structure and makes national laws that are implemented through departments and agencies of relevant ministries. While some ministries have an indirect relationship with the aquaculture sector, others relate directly with the sector. The *Federal Ministry of Agric and Food Safety* relate directly with the sector either through registration of fish farms and provision of extension services administered by the *Department of Fisheries and Aquaculture*; disease control and prevention through *Department of Veterinary and Pest Control Services*, and *Nigeria Agriculture Quarantine Service* (NAQS); regulation of veterinary practice through the *Veterinary Council of Nigeria* (VCN); provision of soft loans to fish farmer through the *Bank of Agric*; or training (*Colleges of Agric*) and research (*National Institute of Freshwater Fisheries Research*; *Nigeria Institute for Oceanography and Marine Research*). Similarly, *Ministry of Water Resources* regulates the use of natural water bodies for commercial activities including granting license for cage culture, while *Ministry of Environment* conducts environmental impact assessment on aquaculture establishments. The Central Bank of Nigeria also deals directly with the aquaculture sector through its de-risking scheme for agriculture and agribusinesses i.e., the Nigeria Incentive-Based Risk Sharing System for Agricultural Lending (NIRSAL).

The *Ministry of Education* has an indirect relationship with the sector through the universities that provide competent manpower in the form trained aquaculture experts and veterinarians just as the *Ministry of Health* engages indirectly by regulating pharmaceutical companies and vet/agrochemical stores through its agency, the *National Agency for Food, Drug administration and Control* (NAFDAC). The *Ministry of Trade and Investment* deals both directly through the *Bank of Industry* that supports the sector with equipment, and indirectly by ensuring compliance with standards in industries including feed mills through its agency the *Standards Organisation of Nigeria* (SON).

The federal government through the *Federal Ministry of Agric and Food Safety* also collaborates with state governments and internal development partners such WorldFish, United States Agency for International Development (USAID), Food and

Agriculture Organisation (FAO), the World Bank, and other governments and agencies in aquaculture-related policy formulation and /or implementation (Fig 2.3).

Operationally, the production unit consists of hatcheries and grow out ponds. Actors within or supporting the production unit include veterinarians, pharmaceutical companies, corporate commercial and local feed mills, pharmacies selling human medicines, veterinary diagnostic laboratories, banks, and vet/agrochemical shops, NGOs/fish farmer associations, poultry farms, banks, insurance companies and NIRSAL (Fig 2.3). Feed is sourced either directly from commercial feed mills or indirectly via local feed vendors, with most doubling roles as veterinary drugs/agrochemical vendors. Local feed mills are either on-farm or privately owned. Water for production is sourced either from rivers and streams or extracted from underground boreholes and wells.

Antibiotics have a complex route of getting to the production unit. Those involved in the antibiotic chain are veterinarians, human pharmaceutical shops, vet/agrochemical shops, local feed mills and standard commercial feed mills (Fig 2.3). Antibiotics are used in the system at hatcheries, grow-out ponds, local feed mills and commercial feed mills (Fig 2.3). Wastewater constitutes an important route through which antibiotics are recirculated in the system since there was no evidence of wastewater treatment. Wastewater was used either for crop irrigation or discharged directly into municipal wastewater channels or natural water bodies. There was also no evidence of antibiotics in imported fish feed at the time of mapping. Fish were sold by farmers either live as fresh fish, smoked, dried or filleted directly to consumers or hotels/restaurants/roadside barbecue or retailers/wholesalers.

Participants of the online focus group discussion agreed that the regulatory component of the map is not strictly implemented. Participants reported that certification, licensing, and environmental impact assessments are mostly implemented only at large-scale fish farms. The participants also mentioned challenges facing the sector which included poor quality seed, high cost of feed, difficulty in getting finance due to unfavourable loan conditions, poor marketing, diseases, poor competence of farmers, lack of extension services, high cost of logistics and flooding.

2.3.2 Survey Participants

Forty-five participants with a median (IQR) years of experience in fish farming of 7 (5-12) consented to taking part in the survey (Table 2.3). Four (8.9%) of these participants were educated to secondary/high school level while the other 41 (91%) had at least one tertiary level qualification. One (2.2%) participant played a supervisory role on a farm, another four (8.9%) were farm owners, 18 (40%) were farm managers, and the remaining 22 (48.9%) were both owner and manager of the farm. Twenty-four (53.3%) of the participants had no form of training before commencing fish farming, while the other 21 participants (47%) had various forms of training ranging from attendance at workshops and seminars, undertaking a farm internship or a completing post-secondary education in an aquaculture-related field before starting their farm (Fig 2.4).

Table 2. 3 Various features of survey participants including level of education, role on the farm, and training and experience relevant to fish farming.

Variable	Count (n)	Percent
Level of education		
Secondary	4	8.9
Tertiary	41	91.1
Role on the farm		
Supervisor	1	2.2
Owner	4	9.9
Manager	18	40
Owner/manager	22	48.9
Training on fish farming		
Yes	21	46.7
No	24	53.3
Total	45	100

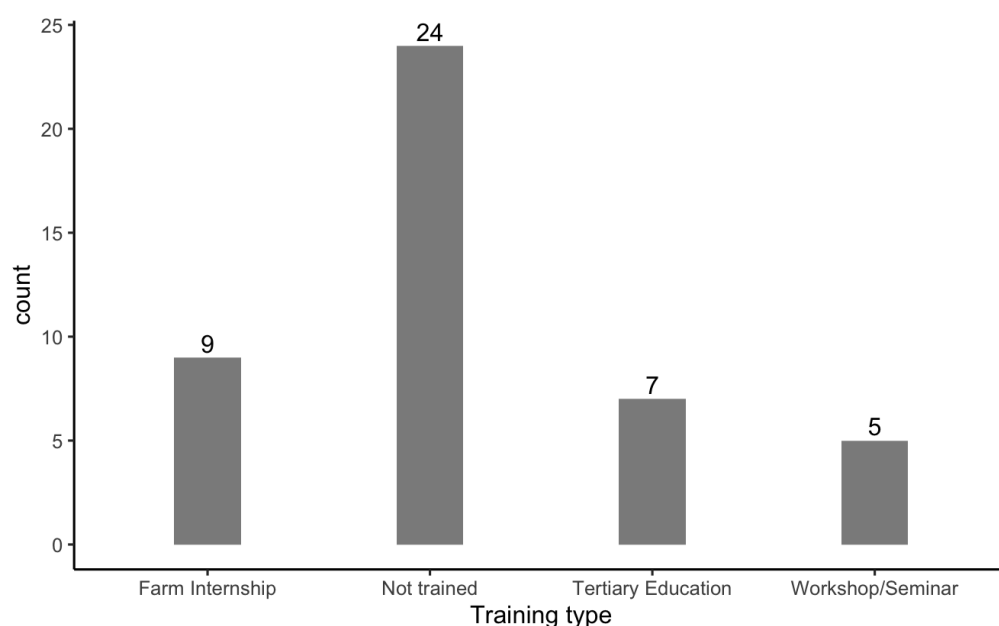


Fig 2. 4 A bar chart showing different kinds of training received by some survey participants before commencing fish farming.

2.3.3 Farm Location

Participants' farms were distributed across 10 of the 19 states that make up northern Nigeria (Fig 2.5). This distribution included 10 (22.2%) from Kano, eight (17.8%) from Katsina, six (13.3%) each from Yobe and Zamfara, three (6.7%) from the Federal Capital Territory (FCT), Bauchi, and Niger, and another two (4.4%) from Borno, Kaduna, and Nasarawa states. Five (11%) participants had changed farm location for expansion reasons since the start of their fish farming business while another two (4%) moved from a rented site to a site they owned.

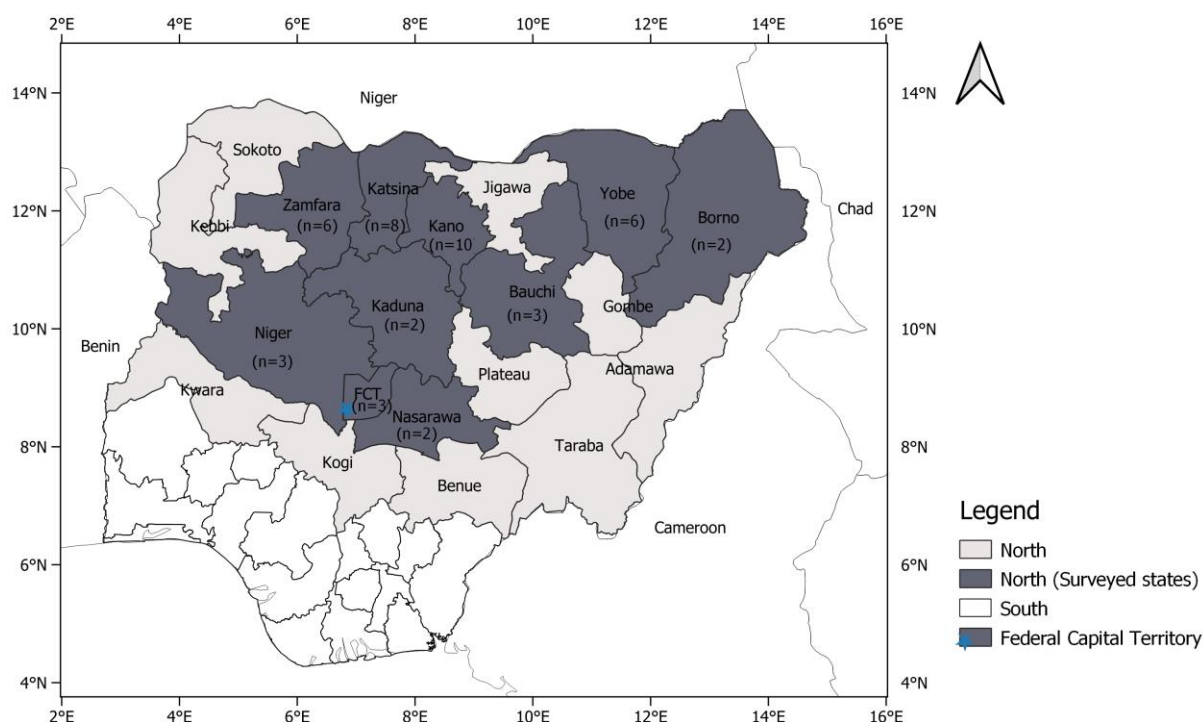


Fig 2. 5 A map of Nigeria showing number of participating farms (in bracket) and their state locations (states) in shaded in grey.

2.3.4 Production

All 45 survey participants operated a semi-closed aquaculture system using either a single pond type or a combination of more than one type. Individual pond types were concrete (29/45; 64.4%) featuring either partial recirculation (11/45; 24.4%) or periodic replacement of water (18/45; 40%), earthen (18/45; 40%) with periodic replacement of water, tarpaulin (16/45; 35.6%) with partial recirculation (4/45; 8.9%) and periodic replacement of water (12/45; 26.7%), plastic (10/45; 22.2%) operated as partial recirculation (2/45; 4.4%) or with periodic replacement of water (8/45; 17.8%), wooden (2/45; 4.4%) with periodic replacement of water, and metallic trough (1/45; 2.2%) with periodic replacement of water. Thirty-four participants (75.6%) practised only catfish culture while the other 11 (24.4%) practised both catfish and tilapia culture at the same site in different ponds (Table 2.4). The distribution of different production models including table size (live fish of about 500 g or above), smoked fish, table size and smoked fish, brood stock and table size, and a combination of brood stock, table size and smoked according to culture type (Figure 2.6).

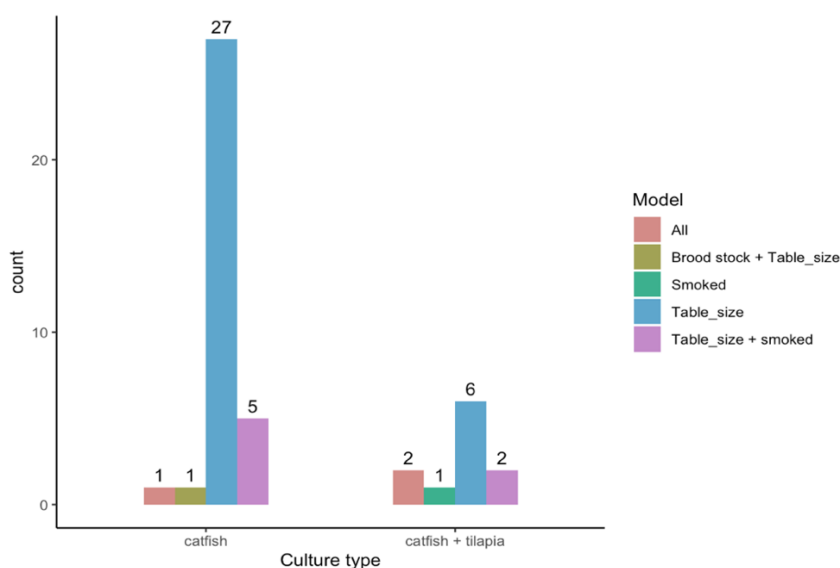


Fig 2. 6 Bar chart showing the distribution of different production models as practised by survey participants according to culture type.

Eighteen participants (40%) obtained fish stocks directly from hatcheries located away from their farms, whilst 12 (26.7%) obtained fish stocks through middlemen. Nine participants (20%) reported having an on-farm hatchery, four (8.9%) participants obtained stocks from their on-farm hatcheries and outside hatcheries, whilst the final two (4.4%) participants used a combination of on-farm hatcheries and middlemen to source their fish stock (Table 2.4). The distribution of these various sources of fish stocks according to culture type is shown in Figure 2.7. Reasons for alternating between sources of fish stocks included availability (4/6), quality (1/6) and a combination of these two factors (1/6).

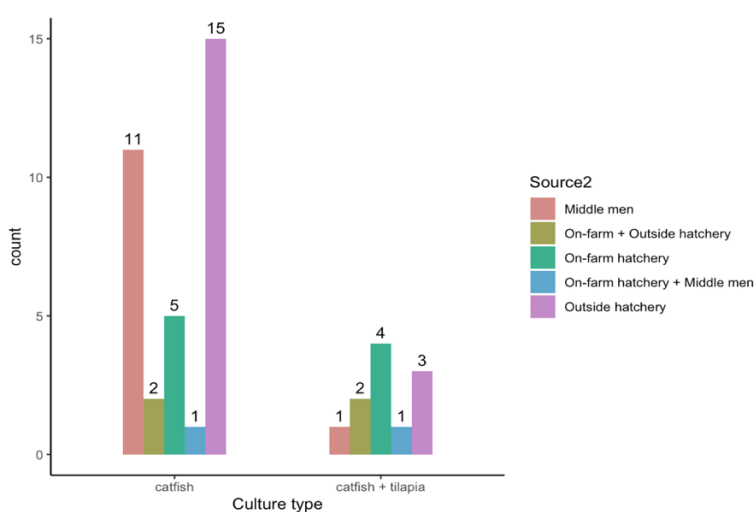


Fig 2. 7 Bar chart showing distribution of various sources of fish stock for survey participants according to culture type.

Table 2. 4 Different production variables with their respective counts and corresponding percentage among survey participants.

Variable	Count (n)	Percent (%)
Culture type		
Catfish	34	75.6
Tilapia	11	24.4
Total	45	100
Source of fish stock		
Outside hatchery	18	40.0
Middlemen	12	26.7
On-farm hatchery	9	20.0
On-farm + outside hatchery	4	8.90
On-farm hatchery + Middlemen	2	4.40
Total	45	100
Reason for using >1 fish source		
Availability	4/6	66.7
Quality	1/6	16.7
Availability + Quality	1/6	16.6
Total	6	100
Harvested biomass/cycle		
<1 ton	5	11.1
1 – 5 tons	21	46.7
6 – 10 tons	15	33.3
>10 tons	4	8.90
Total	45	100
Length of production cycle		
<4 months	1	2.20
4 – 6 months	43	95.6
>6 months	1	2.20
Total	45	100
Determining stocking density		
Pond dimension	24	53.3
Arbitrary estimate	21	46.7
Total	45	100
Source of water		
Borehole	36	80.0
River/stream	3	6.70
Borehole + well	2	4.40
Underground (high water table)	2	4.40
Hand-dug well	1	2.20
Dam	1	2.20
Total	45	100
Record keeping		
Yes (production only)	27	60.0
Yes (production + health)	7	15.6
No	11	24.4
Total	45	100

Participants reported stocking a minimum and maximum of 1,000 and 230,000 juveniles per farm respectively at a median stocking value of 5,000 juveniles and interquartile range of 7,000. Pond dimension was used to determine stocking density by 24 (53.3%) of survey participants while the remaining 21 (46.7%) participants estimated stocking density arbitrarily (Table 2.4).

Thirty-six participants (80%) reported using underground water from boreholes for production, three (6.7%) participants used river/stream, two (4.4%) used underground water (areas of high-water table) and a combination of borehole and hand-dug wells. The last two (4.4%) participants each used hand-dug wells and dams as sources of water respectively (Table 2.4).

Apart from locally produced commercial and self-compounded (produced on-site by the farmer) feeds that were exclusively used alone not in combination by 14 (31.1%) and 2 (4.4%) participants respectively, the other 29 participants used a combination of different feed types. These combinations ranged from imported and locally produced feed (10/45; 22.2%), imported, locally produced, and other feed type (7/45; 15.6%), locally produced and other feed type (7/45; 15.6%), locally produced and self-compounded (2/45; 4.4%), imported, locally produced, and self-compounded (1/45; 2.2%), imported, locally produced, self-compounded, and other feed type (1/45; 2.2%), and imported and self-compounded (1/45; 2.2%). Of the 15 participants that reported using other feed type, 11/15 used dead chicken/chicken offal, 2/15 used wild juvenile fish, 1/15 used a combination of chicken offal and dead sheep/goat, while the final participant (1/15) used a combination of dead chicken, lettuce, cabbage and duckweed. A total of 32/45 participants reported alternating between commercial feed brands. Factors influencing their choice were availability (12/32), cost (10/32), cost and availability (7/32), cost and quality (2/32) and quality and availability (1/32).

Participants reported harvesting biomass that ranged from <1 ton (11.1%), 1 – 5 tons 21 (46.7%), 6 – 10 tons (33.3%), and >10 tons (8.9%) in a production cycle that commonly lasted 4–6 month (95.6%) (Table 2.4) and employed between 2 - 24 people. Fish were predominantly harvested according to fish size i.e., larger ones first by 31

(68.9%) participants, whole crop at once by nine (20%) participants, randomly irrespective of size by four (8.9%) or a combination of whole crop and random harvest by one (2.2%). Fish harvest was achieved using either net (42.2%) or basket/net (57.8%) after draining pond water. Fish that have been sold are commonly transported alive (>95%) in plastic containers with some water (91.3%) and no oxygen supplementation (97.8%). Transportation of sold fish involved vehicles that included cars (46.8%), minibuses (14.5%), trucks (8.1%), and others (30.6%) including tricycles and motor bikes, over a minimum distance of 2 km and a maximum of 350 km. Calculated median travel distance between the farm and market/destination for sold fish was 10 km and interquartile range was 10 km. Twenty-three (51%) participants reported their customer base as strictly whole sellers who buy an entire crop at once, four used only retailers, and two other participants each had direct consumers and restaurants/hotels respectively as customers. The other participants sold their fish to more than one type of customer in different combinations and included four (8.9%) to whole sellers and consumers, three (6.7%) to whole sellers, restaurant/hotel and consumers, three (6.7%) to whole sellers and restaurants/hotel, two (4.4%) to whole sellers, retailers, and consumers, another two (4.4%) to whole sellers and retailers, and one (2.2%) participant sold fish to retailers and consumers. Individual fish weight was reported as the only factor determining price of harvested fish (45/45 participants) and final price was arrived at either by negotiation (77.8%) between the farmer and the buyer at the point of harvest, by the farmer (17.8%), or by the buyer (4.4%).

Whereas 11 (24.4%) participants stated that they do not keep any form of fish farm records, the other participants kept either only production records i.e., those related to stocking and feeding 27 (60%) or a combination of production and fish disease/health records (7; 15.6%). Fish farm records were mostly kept in paper format (29; 64.4%), electronically (4; 8.9%) or in both paper and electronic form (1; 2.2%) for a duration commonly not exceeding cycle length (19; 42.2%) and predominantly only examined at the end of production cycle for profit-loss analysis (31; 68.9%).

2.3.5 Disease Occurrence and Management

A total of 29 (64.4%) participants reported disease outbreaks on their farms either exclusively within the 12 months prior to the interview (12; 26.7%), from previous years

only (6; 13.3%), or from both previous years and the last 12 months prior to interview (11; 24.4%). Five (11.1%) of these outbreaks were reportedly observed within the first four weeks of stocking while 12 (26.7%) occurred after the first four weeks of stocking. These outbreaks were also reportedly distributed predominantly across the months of January to December (14; 31.1%). The other participants were not sure about the month of disease outbreak (4; 8.9%), could not remember (3; 6.7%), or stated that outbreak was not specific to any month (2; 4.4%). Twenty-three (51.1%) of the participants with reported disease outbreaks had either high (15; 33.3%) or low (8; 17.8%) mortality and reported a minimum and maximum estimated mortalities of 2 and 60% respectively with a median of 12% and interquartile range of 13%.

Table 2. 5 Proportions of disease occurrence at different times and how they are managed among survey participants.

Variable	Count (n)	Percent (%)
Disease outbreak		
12 months to interview	12	26.7
Previous years only	6	13.3
12 months to interview + previous years	11	24.4
Total	29	64.4
Abnormal observations		
Skin ulcer	15/23	65.2
Swollen abdomen	11/23	47.8
Fin/barbel rot	10/23	43.5
Hanging	6/23	26.0
Skin discoloration	6/23	26.0
Reduced activity	4/23	17.4
Skin congestion	3/23	13
Skin sloughing	2/23	8.7
Cracked head	2/23	8.7
Red mouth	1/23	4.3
Scratching against pond wall	1/23	4.3
Seeking help in disease management		
Yes, from another fish farmer	9	20.0
Yes, from a veterinarian	5	11.1
Yes, from an aquaculture/fisheries expert	5	11.1
No	22	48.9
NA	4	8.9
Total	45	100
Reason for not seeking help		

Experience from previous outbreaks	16	35.6
Lack of fish health expert	3	6.7
Use of online resources	2	4.4
Trained in fisheries	1	2.2
Total	22	100
Forms of intervention		
Treatment in water	32	71.1
Emergency harvest + treatment in water	4	8.90
Treatment in feed	2	4.40
In water and with feed	2	4.40
NA	5	11.1
Total	45	100
Treatment type		
chemicals/antibiotics	18	40.0
chemicals/antibiotics + salt	20	44.4
chemicals/antibiotics + salt + herbs	2	4.40
NA	5	11.1
Total	45	100
Mortality		
High mortality	15	33.3
Low mortality	8	17.8
No mortality	22	48.9
Total	45	100

Less than half i.e., 19/45 (42.2%) of participants reported seeking help in resolving disease problems when they arise (table 2.5). People commonly consulted included a fish farmer (9/19), a veterinarian (5/19), and a fish expert i.e., someone trained in aquaculture/fisheries related discipline at tertiary level (5/19) (Figure 2.8). The 22 participants that did not seek help in resolving disease challenges revealed experience (16/22), absence of a vet/expert nearby (3/22), use of online resources (2/22), and training in fisheries (1/22) as reasons (Figure 2.9). All these participants, however, reported positive outcomes despite not seeking expert help.

Skin ulcers 15/23, swollen abdomen (11/23), and fin/barbel rot (10/23) were the most common signs of disease reported (Table 2.5). Other signs were hanging (6/23), skin discoloration (6/23), reduced activity (4/23), skin congestion (3/23), skin sloughing (2/23), cracked head (2/23), and red mouth (1/23), and scratching against pond wall (1/23). When asked about perceived cause of disease, 13/23 participants mentioned poor water quality while another 3/23 were not sure. Other reported perceived causes

of disease included stress (2/23), crowding (1/23), poor feed quality (1/23), failure to treat pond before stocking (1/23), bacteria (1/23), and fungus (1/23).

Treatment in water (71.1%) was reported as the most common form of intervention in times of disease crisis. Another 8.9% of participants practised emergency harvest in addition to treatment in water. Treatment in feed as well as a combination of treatment in feed and water each accounted for 4.4% of interventions (Table 2.5). These treatments involved the use antibiotics/disinfectants (40%), combination of antibiotics/disinfectants with salt (44.4%) or combination of antibiotics/disinfectants, salt, and herbs (4.4%) (Table 2.5). Overall, antibiotics either singly (38) or as cocktail (30) were the most common item used for treatment (Fig 2.10). Other items used were disinfectants (11), herbs including bitter leaf (*Vernonia amygdala*, three), zogale' (*Moringa oleifera*, two), ararrabi (Frankincense tree, one) and Neem (*Azadirachta indica*, one). Additionally, drug/chemicals were used by 25 (55.6%) participants for disease prevention and 22/25 of them use antibiotics for this purpose. The other three participants used either blended moringa, chlorine, and salt respectively for disease prevention.

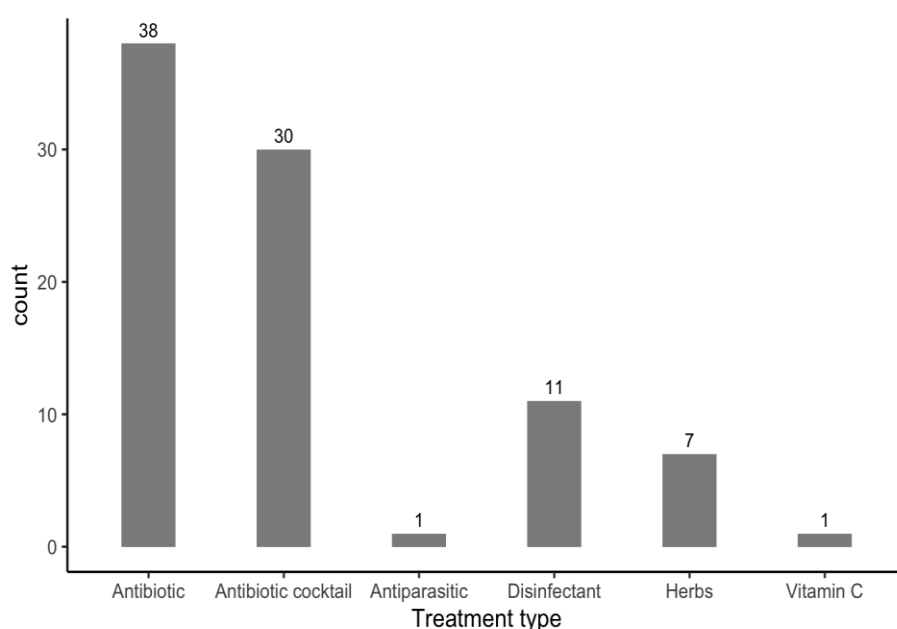


Fig 2. 8 Bar chart showing different items used by survey participants for treating fish diseases.

2.3.5.1 Awareness on probiotics and fish vaccine

Thirty-two (71.1%) participants were unaware of fish probiotics while another eight (17.8%) were aware but never tried using it either to save cost (5/8; 62.5%) or because they are not sure it would work (3/8; 37.5%). Another five (11.1%) participants were aware of fish probiotics and have tried using one. Four of these participants (4/5; 80%) said it worked while the other participant (1/5; 20%) said it did not. When those participants unaware of fish probiotics were asked about its potential adoption in the future, 25/32 (78.1%) of them were keen on doing so while others (7/32; 21.9%) were not or not sure.

While all (45/45) participants were not aware of a vaccine against any fish disease in the market, 25 (55.6%) of these participants were willing to try it when made available. Another 7 (15.6%) participants were not willing to use fish vaccine while the remaining 13 (28.9%) were not sure.

2.3.6 Biosecurity Assessment

Quarantine was reportedly practised by only 10 (22.2%) participants (Fig 2.10) that included 3/10 that did this for less than 2 weeks, 6/10 for 3 – 4 weeks and the other 1/10 for 8 weeks. Commonly, either salt and antibiotic combination (4/10), salt only (4/10) or antibiotics only (2/10) was routinely administered during this period.

Only six (13.3%) participants restrict access to their ponds (Fig 2.11). Among the 39 (86.7%) participants that allowed unrestricted access to ponds 38/39 do so without any form of biosecurity for reasons that included marketing (16/39) or both marketing and leisure purposes (23/39) the other participant (1/39) used a chemical foot bath before granting access to pond. Similarly, only two (4.4%) participants treated water for production using mechanical filtration while the other 43 (95.6%) participants did not practise any form of water treatment before use. Twenty-nine participants (64.4%) reported using organoleptic (appearance and smell) to routinely check for water quality. Another seven (15.6%) participants reportedly used water test kits, one participant (2.2%) used change in fish behaviour while the other 8 (17.8%) simply changed pond water every 24 h (3/8), 48 h (4/8) or 72 h (1/8).

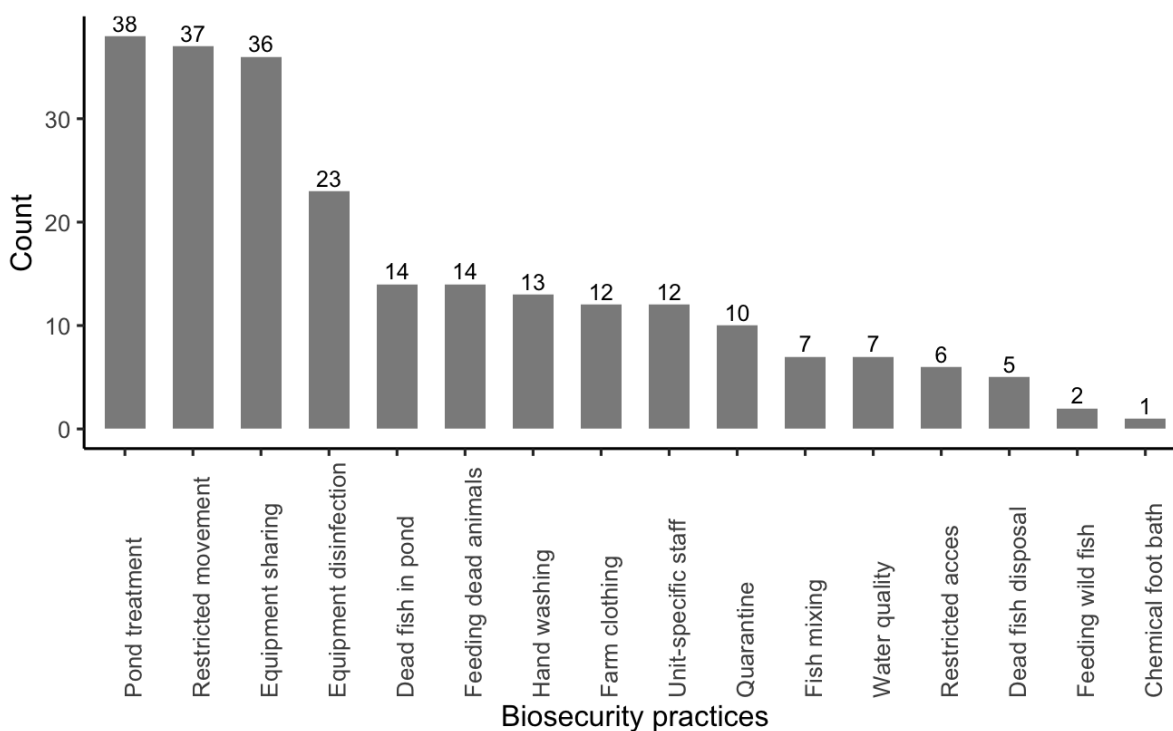


Fig 2. 9 Bar chart showing counts of different biosecurity practices as reported by survey participants.

Highly observed biosecurity practices included post-harvest pond treatment (84.4%) and restricting farm workers' movement to other farms (82.2%). While 36 (80%) participants shared equipment between ponds, 23 (51.1%) participants practiced equipment disinfection only one (2.2%) participant used chemical foot baths. From the 23 participants that disinfected farm equipment, 17/23 used salt while one participant each used bleach, chlorine, formalin, salt with formalin, salt with hydrogen peroxide, and salt with detergent. Additionally, 14 (31.1%) each left dead fish in the pond for other fish to eat or fed dead animal/offal to their fish just as fish from river were used to feed fish in the pond by another two (4.4%) participants. Thirteen participants (28.9%) reportedly wash their hands with soap and water before daily farm operation. Fish from different batches or of different age group were kept in the same pond by seven (15.6%) participants. Similarly, 12 (26.7%) participants each had unit-specific staff or dedicated farm clothing for their staff. Only five (11.1%) participants reported properly disposing dead fish by burying.

2.3.6.1 Biosecurity Scoring and Test of Associations

To permit investigating association between the mortality level and biosecurity status of a farm by means of odds ratio (OR), farms were categorised into those with high and low biosecurity level. To do this, both mean ($\pm$ SD) and median (IQR) biosecurity scores were first calculated and found to be 7.02 ($\pm$ 2.23) and 7.00 (5-8) respectively (Fig 2.10). Additionally, both density plot (Fig 2.10) and a Shapiro-Wilk test ($p = 0.008$) of normality confirmed the data to be not normally distributed. Thus, a biosecurity score of 7.00 was used as a threshold and 25 (56%) participants scored either at or below this value and their farms categorised as having low level of biosecurity. The other 20 (44%) participants scored above this value and their farms were categorised as having high biosecurity level.

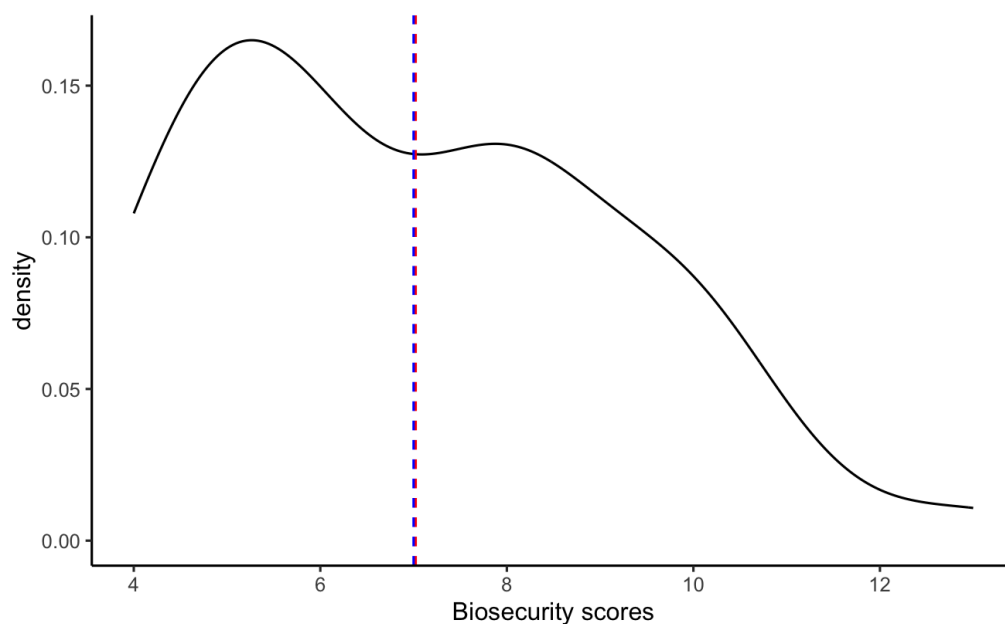


Fig 2. 10 Density plot showing the distribution of biosecurity scores of survey participants, mean (red dashed line) and median (blue dashed line) marks.

Multiple Correspondence Analysis (MCA) revealed two distinct clusters according to biosecurity level (BL) and that 24.18% of the total variability within the data was explained by the first two dimensions of the analysis (Fig 2.11). Farms characterised by positive coordinates on the first dimension (x-axis) shared high frequencies of

variables that included high biosecurity level, dedicated farm clothing, washing hands with soap and water before commencing daily farm operations, non-use of dead animals as feed and removal of dead fish from the pond. On the other hand, farms with negative coordinates on this dimension were mostly characterised by low biosecurity level, lack of dedicated farm clothing, hand washing with soap and water, and non-removal of dead fish from the pond. Similarly, farms with positive coordinates on the second dimension (y-axis), were characterised by high frequencies of using dead animals as feed, lack of pond treatment, and having unit-dedicated staff where applicable. Farms with negative coordinates on this axis, however, shared high frequencies of low biosecurity level, lack of dedicated farm clothing, not washing hands with soap and water, and not removing dead fish from the pond.

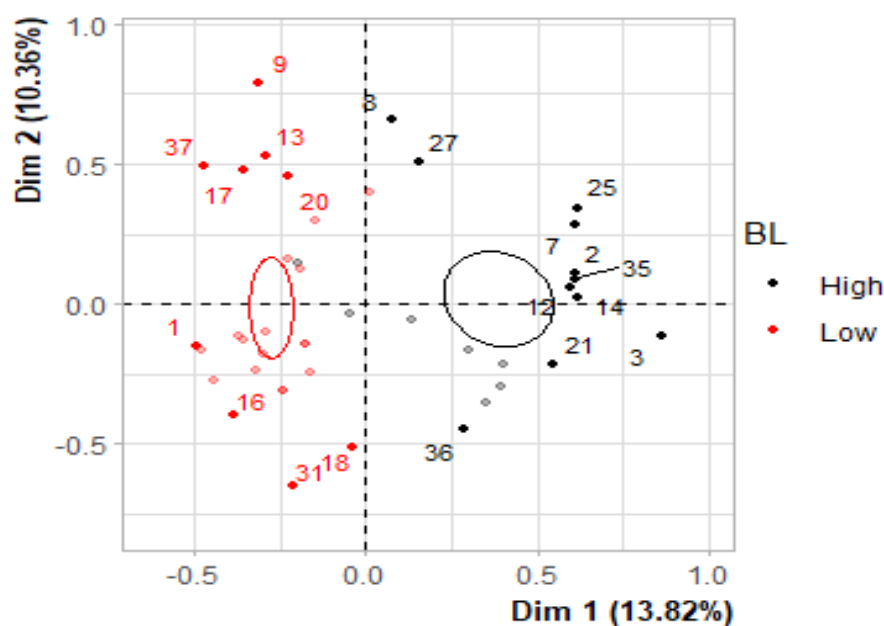


Fig 2. 11 Multiple Correspondence Analysis showing distribution of individual farms according to biosecurity level on the first two dimensions of the analysis

Except for using dead animals as feed where the odds of having high mortality was slightly reduced (OR = 0.11, 95% CI [0.01, 0.79], $p < 0.05$), there was no statistically significant difference in observing high mortality with respect to any of the test variables (Table 2.6). Although OR could not be calculated in the case of mixing fish from different sources or age group in the same pond because of a cell having a value

of zero, Fisher's exact test revealed a statistically significant association ($p < 0.05$) between this practice and high mortality on a farm (Table 2.6).

Table 2. 6 Odds of having high mortality with respect to training on fish farming, biosecurity score, and different elements of biosecurity as represented by odds ratio (OR), 95% confidence interval (95% CI), and statistical significance (p -value < 0.05).

Biosecurity element		Mortality			OR	95% CI	p-value
		High	Low	None			
Poor biosecurity	Low	8	5	12	0.70	0.10 - 4.21	0.71
	High	7	3	10			
Trained on fish farming	No	10	3	11	3.01	0.52- 22.33	0.22
	Yes	5	5	11			
Quarantine	No	14	6	15	4.12	0.28 – 146.35	0.30
	Yes	1	2	7			
Unrestricted pond access	No	12	8	19	NA	NA	0.54
	Yes	3	0	3			
Controlled workers movement to other farms	No	2	3	3	0.28	0.03 – 2.35	0.24
	Yes	13	5	19			
Unit-specific workers	No	11	5	15	2.11	0.28 – 16.70	0.50
	Yes	3	3	6			
Fish mixing	Yes	0	4	3	NA	NA	0.01*
	No	15	4	19			
Shared equipment	Yes	14	5	17	7.05	0.66 –234.69	0.11
	No	1	3	4			
Equipment disinfection	No	7	3	12	1.42	0.24 – 9.74	0.71
	Yes	8	5	10			
Using dead animals as feed	Yes	2	5	6	0.11	0.01 – 0.79	0.03*
	No	13	3	16			
Using fish from rivers as feed	Yes	1	0	1	NA	NA	1.00
	No	14	8	21			
Leaving dead fish in the pond	Yes	5	1	8	3.07	0.36 – 94.35	0.34
	No	10	7	14			
Mortality disposal	Improper	12	7	21	0.63	0.02 – 6.65	0.72
	Proper	3	1	1			
No post-harvest pond treatment	No	2	2	3	0.58	0.04 – 5.53	0.54
	Yes	13	6	19			
Dedicated farm clothing	No	9	7	17	0.25	0.01 – 2.05	0.22
	Yes	6	1	5			
Handwashing with soap and water	No	9	7	16	0.25	0.01 – 2.05	0.22
	Yes	6	1	6			
Chemical foot bath	No	14	8	22	NA	NA	0.51
	Yes	1	0	0			

2.4 Discussion

Aquaculture activity in the northern Nigeria is rapidly expanding and increasing in intensification. There are only pockets of studies describing the prevalence of disease challenges and the disease management practices employed by fish farmers in this region. To ensure sustainability, food security and secure the socioeconomic livelihoods of many people, it is imperative to have a broad understanding of the prevailing disease situation and associated management practices. This will inform the design and implementation of effective disease prevention and control strategies. In this study, data on farm structure/management, disease prevalence and management and disease prevention was collected from 45 fish farmers using a structured questionnaire delivered verbally by telephone.

This study revealed that around half of the participants scored at or below the mean biosecurity score. This suggests poor biosecurity practice among participating farms. Poor biosecurity adoption has been described as inimical to sustainable aquaculture (Nwabueze & Ofuoku, 2020). The fact that water represents an important route of infectious disease transmission for a fish farm and majority of survey participants use borehole water which is associated with negligible risk of infectious disease transmission shows achieving good biosecurity requires a more wholistic approach (Peeler, 2005). Farm level best practices that reduce stress and improve welfare were identified as an important step in disease control (World Bank, 2014) and this, in combination with good biosecurity minimises the risk of exposure of fish stock to the spread of infectious diseases within a facility, as well as their escape from such facility (Yanong & Erlacher-Reid, 2012). Ultimately, good biosecurity ensures improved health and welfare of aquatic animals which translates into increased productivity, reduced production cost from reduced antimicrobial use and consequently decreased emergence of antimicrobial resistance, and potentially improved access to market (World Organisation for Animal Health, 2022). Thus, farms operating under poor biosecurity conditions pose an economic risk to themselves through increased risk of mortalities, disease control and income loss. They also pose a risk to farmers in their immediate vicinity and even beyond in addition to safety risks to consumers and the environment. With antibiotics easily accessible over the counter without prescription owing to Nigeria's weak legislations around antibiotics (Okocha et al., 2021a) and the

fact that easy access to antibiotics and poor biosecurity are known drivers of antimicrobial misuse (Alhaji et al., 2021; Brunton et al., 2019), farms with poor biosecurity might be more likely to inappropriately deploy antibiotics for disease management. Poor biosecurity finding from this present study is consistent with other published literature from Nigeria (Nwabueze & Ofuoku, 2020; Omitoyin & Osakuade, 2021). Similar findings were also revealed by (Kyule-Muendo et al., 2022) who found less than 2% of farms in a county in Western Kenya use disinfection to prevent disease. Assefa & Abunna (2018) opined that stressful conditions resulting from poor biosecurity has the potential to cause treatment failure in diseased fish stock which could predispose such farms to frequent/repeated antibiotic use. They also suggested that any strategy for mitigating the effect of diseases in aquaculture should focus on prevention rather treatment (Assefa & Abunna, 2018). Other scholars further emphasised the critical role of biosecurity in ensuring food security for the rapidly growing human population as well as poverty alleviation and indicated that effective prevention and successful control and eradication of fish diseases when they occur depends upon the deployment and level of compliance with good biosecurity measures (Nwabueze & Ofuoku, 2020; Scarfe & Palić, 2020). Nigeria's lack of a legislative framework either for biosecurity (Obosi & Agbeja, 2015) or aquaculture practice in general (Nwabueze & Ofuoku, 2020), however, is a disincentive for the adoption of good biosecurity practice among fish farms and a threat to sustainable aquaculture and food security in the country.

Although biosecurity level was predominantly poor among participating farms from this present study, the finding was unexpectedly not significantly associated with high mortality level. This finding is consistent with that of Kyule-Muendo et al. (2022) from fish farms in Western Kenya. Others, however, found an association between biosecurity and mortality on fish farms from Kaduna State in northern Nigeria (Adah et al., 2023a). The unexpected lack of an association between poor biosecurity and high mortality level from this study might be attributed to both the study design and sample numbers. Non-probability sampling does not permit the determination of sampling error or data precision in relation to the target population and comes with the risk of failure to generate balanced information on the research question at hand (Stratton, 2021), which might have affected the outcome of this study. These limitations of the sampling method of this study might explain the difference between the finding of this

study and that of published by Adah et al. (2023). In their study, Adah et al. (2023) used a multistage random sampling technique to select 80 farms, while this study involved 45 farmers drawn using convenience and snowball sampling. This present study however, found a significant association ($p < 0.05$) between an element of poor biosecurity i.e., keeping fish of different source/age group in the same pond and level of mortality. Additionally, the odds of having high mortality when using dead animals as feed was slightly reduced at a statistically significant level (OR = 0.11, 95% CI [0.01, 0.79], $p < 0.05$). Again, this unexpected finding might be explained by the reasons provided earlier on the lack of association between biosecurity and mortality level.

The prevalence of disease outbreaks from this study (64.4%) is consistent with findings from the reports Georges et al. (2023) from Cameroon. It is however, less than 71.25% reported by Obosi & Agbeja (2015) from Ibadan South-Western Nigeria and 76.1% among fish farms with a low-level biosecurity in Western Kenya by Kyule-Muendo et al., (2022). These reports suggest that high disease prevalence is common across Nigeria and the threat to food security from fish disease is not limited to the country but also a challenge in other African countries. Poor biosecurity can precipitate disease outbreaks which often is accompanied by mortality (Adah et al., 2023a). The median mortality rate from this study that involved grow out farms was 12%. While a 12% median mortality rate might appear less than the median values of 22 and 15% for the Scottish and Norwegian Salmon industries respectively (Ellis et al., 2012), this would translate into a median loss of 600 tons of biomass at a median stocking capacity of 5,000 fish seeds and harvested weight of 1 kg per cycle of 4 – 6 mont 1,800 tons (36%). This would be a staggering amount of loss even self-sufficient countries let alone a country like Nigeria that is reliant on fish as a cheap protein source amid massive supply deficit.

More than half of participants from this study practised self-medication with antibiotics, predominantly oxytetracycline, which agrees with other reports from this region (Alhaji et al., 2021; Ladan et al., 2021) and suggests a low-level awareness and/or absence of or inaccessibility to fish health personnel within the fish farming community in addition to weak antibiotic regulations. Access to a fish health practitioner might have provided access to professional advice and thereby likely reduce the practice of self-medication. Low cost, ease of accessibility and a broad spectrum of activity of

oxytetracycline has made it the first drug of choice among farmers and thus most widely abused (Ladan et al., 2021). Perception of fish farmers from this region on the proper use of antimicrobials was reportedly poor (Alhaji et al., 2021). Increased level of fish farmers' awareness and compliance with guidelines on antimicrobial use will be in tandem with Nigeria's national action plan on antimicrobial resistance through an One Health approach and will facilitate good antimicrobial stewardship which is a key component of the global action plan on antimicrobial resistance (Alhaji et al., 2021; Federal Government of Nigeria, 2017). Lack of such awareness potentially predisposes the farmers to treatment failure owing to poor treatment regime or choice of wrong antibiotic which may warrant either repeat treatment with another antibiotic or the concurrent use of multiple antibiotics. This constitutes a risk for both emergence and spread of antimicrobial resistance as well as antibiotic residues and might explain why antibiotic cocktails represent the next common drug of choice after oxytetracycline by participants in this study. There is thus the potential for the development antibiotic resistance among bacterial species within the system and increased financial loss to these farmers. Antibiotic use in aquaculture in Nigeria and around the world is suggested to pose serious economic and health risks (Bondad-Reantaso et al., 2023; Lulijwa et al., 2020; Okocha et al., 2021b). In addition to exerting selection pressure on aquatic microbiota leading to the emergence of antibiotic resistance in pathogenic and non-pathogenic microbes, antibiotics use in aquaculture predisposes consumers to subtherapeutic doses of antibiotics in the form of antibiotic residues and thus raising concerns about the safety of aquaculture products (Bondad-Reantaso et al., 2023; Durojaiye et al., 2020; Okocha et al., 2021b).

This present study also revealed the use of herbs including bitter leaf (*Vernonia amygdala*), zogale' (*Moringa oleifera*), ararrabi (Frankincense tree), and Neem (*Azadirachta indica*). While the efficacy of other plant-based compounds as immunostimulants and therapeutics in aquaculture has been demonstrated (W. Zhang et al., 2022), this has yet to be shown for the plant-based materials reported in this present study.

The majority (91%) of participants in this study were educated at tertiary level which implies considerable level of literacy among fish farmers sampled and may also suggest that these group of farmers may be easier to work with and train on effective

disease prevention strategies. This finding of high literacy level among fish farmers is consistent with those of other authors (Alhaji et al., 2021; Georges et al., 2023a; Nwabueze & Ofuoku, 2020; Okocha et al., 2021a; Omitoyin & Osakuade, 2021; Umar et al., 2019). Literacy facilitates effective communication and could serve as an incentive for innovation adoption (Okocha et al., 2021) or improved practices (Omitoyin & Osakuade, 2021). Less than half (47%) of the participants from this study, however, received any form of training before starting a fish farm which is comparable to 46% reported by Omitoyin & Osakuade, (2021). This suggests many participants may not have been fully aware of the challenges ahead or adequately equipped with the necessary skills on fish farming before embarking on the business which could increase the risk of losses for this set of farmers. This is evident in the high proportion (75.6%) of participants that do not keep any kind of record in this study emphasising the need for proper record keeping which constitutes a key component of an effective biosecurity programme. While this finding agrees with that of Adah et al. (2023), it is in sharp contrast to the 100% record keeping by farmers in the report of Okocha et al. (2021a). This might suggest a higher level of awareness among fish farmers from southern Nigeria. Training/workshops provide farmers with opportunity to learn skills and innovations for an efficient and profitable venture from either experienced farmers or experts.

This study further revealed that the majority of farmers (57.8%) are small scale producers with an output of less than 5 tonnes per cycle commonly in concrete ponds. This is more than the 38.7% reported by Okocha et al., (2021) as small-scale producers from South-Western states of Nigeria. Similarly, these finding contrasts those of (Adah et al., 2023a) and Okocha et al., (2021) both of whom reported the use earthen ponds as more common. Although aquaculture was first experimented in the north, it was more widely adopted and consequently more established in the south particularly in South-Western region which eventually became the hub of aquaculture activity in the country. The same reason could explain why the years of experience as observed in this study is lower than that reported by Okocha et al., (2021).

Catfish monoculture is the most common culture type (76%) operated by participants in this study which closely compares to 74.1% by Okocha et al. (2021a) from South-western Nigeria but higher than 68.75% reported by Adah et al. (2023) from Northern

Nigeria and less than 100% by Durojaiye et al. (2020) from South-western Nigeria. Despite the difference in figures between these studies, each represents a majority operating catfish monoculture. This is consistent with the report of Oman et al. (2023) and suggests a strong affinity for catfish production by fish farmers from both northern and southern Nigeria. This might be explained by consumer preference for this species over tilapia and other cultured fish species across Nigeria (Byrd et al., 2021). This study also revealed considerable use of either external sources or third party (middlemen) by participants to source for fish stock as only 20% use on-farm hatcheries. This constitute potential risk to the farmer because there would be no guarantee on both the genetic performance and the health status of fish received. Additionally, a middleman might source from different places to supply a single farm which increases the risk of disease transmission. A similar trend was reported among fish farmers in Maiduguri North-Eastern Nigeria (Umar et al., 2019).

The majority of participants use borehole (underground water) as source of water for production. The use of borehole for water supply in northern Nigeria is common and might be due to the collapse/inefficiency of the municipal water supply. Given that most of these farmers are small scale producers, some operating in their backyard, adopting a borehole can be easily understood. Using a borehole in Nigeria is associated with additional energy cost because of epileptic power supply. Therefore, having the same borehole serve a dual-purpose could be seen as an efficient way of offsetting that cost in addition to generating additional family income. While Umar et al. (2019) and Adah et al. (2023) reported a similar finding on the water source, Okocha et al., (2021a) on the other hand reported the use of river/stream as more common than boreholes. The presence of water bodies has been shown to influence fish farm location (Nwabueze & Ofuoku, 2020). Thus, a farm's proximity or otherwise to a water body might influence their choice of water source.

Determination of pond capacity was found to be done using arbitrary estimates by close to half of participants. This could potentially result in wrong stocking densities imposing avoidable stress on the fish which can result in disease outbreaks because of stress-induced lowered immunity. Assefa & Abunna (2018) described appropriate stocking densities as an important early-stage disease control strategy in aquaculture especially when dealing with ectoparasites.

Whereas all participants in this study were completely unaware of any fish vaccines in the market, a minority were aware of fish probiotics and most of them are hesitant on using it either for wanting to keep production cost low or are doubtful about its efficacy. This suggests poor awareness on these proven disease control and prevention strategies which might be attributed to lack of such within the Nigerian market.

This study has attempted to describe the aquaculture system in Nigeria as well as disease prevalence and associated management practices from different states of northern Nigeria. Evidence here suggests that aquaculture in Northern Nigeria is similar in many respects to that in Southern Nigeria. Governance is administered centrally by the Federal Government while production system and outputs are common in both regions (Fig 2.3). Thus, aquaculture practice could be said to be the same across the country. However, the present study is limited by two important factors. First, convenience and snowballing sampling used in this study are non-probability sampling techniques which means conclusions from this study should be cautiously generalised to the wider aquaculture sector of northern Nigeria. The lack/inaccessibility of a comprehensive register of fish farmers from this region ruled out probability sampling as an option and thus the first set of participants had to be recruited by convenience sampling. These first set of participants were then relied upon to introduce more participants and because of this, it took longer than anticipated and invariably affected the final number of participants because of time constraints. Secondly, this study assumed the information provided by participants is accurate because it relied solely on the participants' recollection of events on their farms without any means of verification. There is thus a chance for recall bias which has shown to influence research outcomes (Crump, 2020). This can potentially limit the strength of any conclusion. Nonetheless, it has provided an useful insight into the sector and added to existing information on the topic that could direct future work.

For effective disease prevention and control, it is recommended that fish farmers are adequately trained on fish farm biosecurity and have access to extension services. It is also recommended that more investment is made in enhancing the capacity of veterinarians and para-veterinarians in the field of fish health management as well as provision of diagnostic services for fish diseases. Finally, we recommend that a

regulatory framework on aquaculture and access to and use of antibiotics in Nigeria should be revisited and tightened so that unauthorised/unsupervised prescription and administration is strictly controlled. As rightly pointed out by Subasinghe et al. (2023), without proper guidelines and regulatory framework, controlling stocking density and maximum allowed biomass will be difficult to implement among farmers. It will also be difficult for farmers to perform regular health checks, conduct disease surveillance and report diseases to relevant authorities where such regulatory frameworks are non-existent (Subasinghe, Alday-Sanz, et al., 2023).

Subsequent phases of this work will investigate bacterial diseases of fish using apparently sick fish from farms and markets. Attempt will be made to identify and characterise incriminating bacteria. A further intervention will be adopted to address the gaps/inadequacies identified from this study. Specifically, fish farmers will be trained on effective fish farm biosecurity to assess the impact this might have on improving biosecurity and consequently disease and mortality.

2.5 Conclusion

This study has established that a section of fish farms from some states of northern Nigeria are small scale producers operating under inadequate level of biosecurity and have little or no awareness of other disease prevention strategies like the use of vaccines and probiotics. Some elements of biosecurity were also significantly associated with high mortality. Furthermore, mortality from disease outbreaks was found to be a common occurrence and self-medication commonly with antibiotics was the most widely practised means of disease prevention and control. While access to social media platforms has facilitated information sharing among fish farmers and likely contributed to the problem in Nigeria, information sharing has been shown to significantly influence better yield among fish farmers in other places like Uganda (Mutuyaba et al., 2023). Aquaculture in Nigeria like in many parts of the world is thus a potential hotspot for the emergence of antimicrobial resistance and must therefore be given comparable amount of attention as other sectors under the national action plan on antimicrobial resistance.

Chapter 3

Characterisation of bacteria isolated from cultured catfish (*Clarias gariepinus*) exhibiting macroscopic signs of disease from selected states of northern Nigeria

3.1 Introduction

Nigeria's growing demand for fish has driven a steady increase in finfish aquaculture dominated by catfish (*Clarias gariepinus*) since the start of the millennium making Nigeria the largest aquaculture producer in sub-Saharan Africa and the largest catfish producer in the world (Alhaji et al., 2021; Mukaila et al., 2023). However, the industry is threatened by many factors including infectious diseases accompanied by the uncontrolled application of antimicrobials which is forecasted to increase with increasing intensification (Alhaji et al., 2021). This trend is fuelled in part by the lack of prerequisite skills and knowledge of fish farming by some farmers as indicated by findings from Chapter 2. More than half of the participants in that survey ventured into aquaculture without any form of training. This has implications on the efficiency of husbandry practices like proper stocking density that directly impacts the health status of fish stocks. High stocking density is a common feature of intensive aquaculture. In addition to fast-tracking disease onset through stress induced immunosuppression, high stocking density greatly enhances the transmission rate of infectious disease and increases the likelihood of mortality especially when variables like water quality is suboptimal (Behringer et al., 2020a; Lieke et al., 2020; Pérez-Sánchez et al., 2018). Small & Bilodeau, (2005) demonstrated the effect stress can have on disease outcomes when they subjected a group of catfish to 30 minutes of low-water stress before challenging both experimental and control groups with *Edwardsiella ictaluri*, the causative agent of enteric septicaemia of catfish (ESC). The result was a 16.6% increase in mortality in the stressed group compared to the control group.

Although the work in Chapter 2 did not quantify losses by farmers in monetary terms, high disease prevalence was observed. This supports the report of Mukaila et al., (2023) that small scale farmers who make up about 80% of fish farmers in Nigeria suffer losses as high as \$2,000 USD per cycle. Increased mortality is a direct form of disease-related loss that significantly impacts production (Adah et al., 2023b). This type of loss was reported by more than 60% of participants in the previous chapter. Other forms of losses due to diseases in aquaculture occur in the form of reduced efficiency in feed conversion with attendant decline in growth rate and overall productivity, reduced fecundity, increased production cost due to disease management, reduced marketability and lost income due to low market value or

reduced production (Adah et al., 2023a; Behringer et al., 2020b; Maldonado-Miranda et al., 2022b).

Whereas all fish disease symptoms reported by participants in Chapter 2 (Table 2.2) are consistent with infectious disease, bacteria are a common cause of diseases of fish as well as disease-associated deaths in aquaculture (Kaleem & Bio Singou Sabi, 2020; Maldonado-Miranda et al., 2022a). They can thrive even in the absence of susceptible hosts and their activity is enhanced under warm aquatic conditions (Maldonado-Miranda et al., 2022a). This is a problem particularly for tropical aquaculture in Nigeria where the climate is warm and aquaculture biosecurity is poor which might explain the significantly high mortality levels reported from fish farms in the country (Adah et al., 2023a). A similar trend was observed in our survey where various components of biosecurity (Figure 2.7) were reportedly lacking on many farms. The apparent lack of regulation of both the aquaculture sector and access to antibiotics by relevant authorities and incompetence in fish disease management has facilitated the adoption of antibiotics as a common means of disease control by farmers with little or no idea of prudent antibiotic use leading to misuse of these critical compounds (Imade & Akinnibosun, 2018). This was also evident in Chapter 2 where less than 20% of participants consulted a veterinarian and use of antibiotics either singly or in combination was the most common means of disease control. Antibiotic use in aquaculture induces the development of resistance in both fish-associated bacterial communities with a potential for horizontal transfer to the environment and humans which directly poses a risk to public health through product contamination with bacteria bearing such genes and indirectly through their contamination of aquatic environments either as naked DNA from dead bacteria or contained in whole living bacteria (Pepi & Focardi, 2021). This phenomenon is further exacerbated by the presence of antibiotic residues in fish tissues and the environment as fish are not efficient at metabolising antimicrobial agents and many antibiotics are discharged in water bodies through farm effluent (Alhaji et al., 2021). It is estimated that up to 80% of antibiotics ingested by fish reportedly reach the environment unabsorbed through the faeces (Pepi & Focardi, 2021).

Antimicrobial resistance has been recognised as a global public and animal health concern and known to be influenced by antimicrobial use in humans, animals and the

environment (Organisation for Animal Health, 2022). Therapeutic and non-therapeutic antibiotic application as demonstrated in Chapter 2 (Section 2.3.4) is a common feature of intensive aquaculture as also reported by numerous authors (Alhaji et al., 2021; Desbois et al., 2021; Durojaiye et al., 2020; Ladan et al., 2021; Pepi & Focardi, 2021) and described as a driver of antibiotic use (Alhaji et al., 2021; Brunton et al., 2019; J. Chen et al., 2020a). Consequently, there is a potential for the selection and dissemination of antimicrobial resistant microbes since legislation around antimicrobial use and aquaculture operations – another driver of antibiotic use – in most of these countries are either non-existent or not strictly enforced (Brunton et al., 2019; Desbois et al., 2021; Imade & Akinnibosun, 2018). Published literature indicates that climate change will worsen the issue as fish hosts become stressed and diseased which will attract antibiotic use (Pepi & Focardi, 2021). A positive correlation between increase in temperature and mortality rate in aquaculture has been reported with a 1°C rise in water temperature resulting in up to 6 and 8% rise in mortality rate for bacterial (Reverter et al., 2020) and viral infections (Combe et al., 2023) respectively. Similarly, a country's average annual temperature was shown to positively correlate its Multiple Antibiotic Resistance (MAR) indices (Reverter et al., 2020). This is particularly disturbing for tropical countries like Nigeria with warmer climates that is at risk of increased global warming due to climate change, highlighting the need for an urgent and concerted effort to tackle this problem. Empirical evidence of the relationship between antibiotic use in aquaculture and increased resistant bacteria and genes has been established with extra-label antibiotic use a major cause of resistance (Imade & Akinnibosun, 2018).

Applicable biological strategies for disease control in aquaculture including vaccination, phage therapy, use of probiotics/prebiotics/synbiotics/postbiotics, phytobiotics and quorum sensing interference have been extensively reviewed by Pérez-Sánchez et al., (2018). Antimicrobial susceptibility testing (AST) is used to determine the suitability of an antibiotic for the treatment of a given bacterial infection by establishing a minimum inhibitory concentration (MIC). It is thus, an effective strategy for controlling the emergence of resistant pathogens that has been deployed in both clinical and terrestrial animal practice as well as in aquatic veterinary practice. However, differences in growth conditions between bacteria involved in aquatic animal diseases and those routinely isolated from terrestrial animals/clinical setting,

necessitated separate guidelines for AST and the interpretation of results obtained for bacteria of aquatic animal origin. This has been achieved for a select number of bacteria as described in Clinical and Laboratory Standards Institute (CLSI) VET03 (CLSI, 2020a) and VET04 (CLSI, 2020). Additionally, issues of host range and variations in environmental factors like salinity and temperature that can affect the antibiotic concentration to which a pathogen is exposed to, and thus clinical outcome, have constituted a limitation in generation of sufficient pharmacokinetic data to permit establishment of clinical break points for most bacteria of aquatic animal origin (CLSI, 2020a). This restricts the application of the interpretive categories of sensitive, intermediate or resistant on most bacteria from aquatic animals except for *Aeromonas salmonicida* (CLSI, 2020b). Thus, an epidemiological cut off value (ECV) that relies solely on microbiological data is used instead and interpretive criteria has been successfully developed for *Aeromonas hydrophila*, *Flavobacterium columnare* and *Flavobacterium psychrophilum* (CLSI, 2020b). ECV categorises bacteria as wild type (WT) and non-wild type (NWT) based on phenotypic evidence of acquired and/or mutational resistance (CLSI, 2020a).

Surveys on supposedly pathogenic bacteria and their resistance profiles from cultured catfish (*Clarias gariepinus*) in Nigeria have focused largely on healthy fish and the surrounding water (Danba et al., 2015; Effiong & Isaac, 2020; Igbinosa et al., 2017; Imade & Akinnibosun, 2018; Mailafia et al., 2021). While this significantly contributes to our understanding of the microbial diversity within the system and its role in the emergence and dissemination of resistance, there has been little investigation of, and characterisation of pathogenic bacteria from aquaculture in Nigeria. Although Ogbonna & Inana, (2018) isolated both Gram-positive and Gram-negative bacteria from skin swabs of infected catfish (*Clarias gariepinus*) in southern Nigeria, more publications have focussed on apparently healthy catfish or the culture water (Danba et al., 2015; Effiong & Isaac, 2020; Igbinosa et al., 2017; Mailafia et al., 2021; Ogbukagu et al., 2021). There is thus still an important knowledge gap on prevailing pathogenic bacterial strains in the country and in particular, northern Nigeria. This is a limitation towards the design and implementation of targeted control strategies like diagnostic testing and vaccine development, a strategy proven to reduce the need for antibiotic use in aquaculture in some circumstances or even disease eradication as shown in the case of the rinderpest virus (Desbois & Monaghan, 2023). Bartie et al.

(2023) emphasised regular surveillance to identify prevalent strains of pathogenic organisms in circulation to ensure that vaccines are working or for vaccine formulation adjustment. Similarly, Wamala et al. (2018) opined that proper understanding of the epidemiology of a disease's aetiologic agent underpins better treatment and control strategies. Additionally, the World Health Organisation (WHO) global action plan on antimicrobial resistance emphasises the need to strengthen our knowledge and evidence base of resistant microbes through surveillance and research (Pepi & Focardi, 2021). Aquaculture is known to provide employment opportunity, improve income generation, and provide low-cost high-quality protein thereby promoting food and nutrition security (Obiero et al., 2019). Thus, to achieve sustainability in aquaculture production and ensure food and nutrition security in Nigeria, especially in the north where poverty rates are higher and food security lower as compared to the south (Liverpool-Tasie et al., 2021; Subasinghe et al., 2021), disease control strategies must be effective, and this relies on accurate identification of the pathogen.

This chapter will use conventional biochemical methods and molecular techniques to accurately identify and characterise bacterial strains from diseased catfish collected from two northern states (Niger and Nasarawa) of Nigeria and the Federal Capital Territory Abuja. This will be accomplished by undertaking the following objectives:

1. Isolate and identify bacteria from diseased catfish (*Clarias gariepinus*) from selected farms and markets using conventional biochemical methods, 16S *rRNA* sequencing and whole genome sequencing.
2. Determine the profiles of potential virulence factors expressed by the identified bacteria using phenotypic assays.
3. Determine the antibiotic resistance profiles of the identified bacteria using the CLSI microbroth dilution method.
4. Determine the genetic basis of virulence and resistance profiles using whole genome sequence data and bioinformatic tools.

3.2 Materials and Methods

3.2.1 Sampling of catfish exhibiting signs of infectious disease

A total of 55 catfish (*Clarias gariepinus*) weighing between 200g and 800g displaying symptoms consistent with infectious disease (skin ulcers, fin and barbel rot, bloated abdomen, haemorrhages) were collected from nine sites which included seven fish farms and two fish markets between June and August 2020. All sampled farms practiced catfish monoculture either as grow out only (n = 3) or a combination of hatchery and grow out (n = 4). The first farm was enlisted based on report of disease occurrence through personal contacts and subsequent farms were enlisted through either personal contacts or previously enrolled farms. Four of these farms were from Niger state, two from Nasarawa state and one from Abuja. Kado fish market, the only fish market in the city of Abuja was used as one of the sampling sites while the second, Suleja fish market in Niger state was selected because of its proximity to the researcher. This was to limit travel distance due to Covid-19 restrictions in 2020. Farms were visited once a report of disease occurrence/mortality was made available, while each market was visited three times at two-week intervals in the morning to coincide with the arrival of fish stock. A minimum of three fish samples either taken from recently dead fish on ice or alive in a clean transparent plastic container containing pond water were collected per visit. These fish samples were then transported to the laboratory within 1 h of collection.

3.2.2 Collection of samples from catfish and processing

Immediately on arrival at the laboratory, live fish samples were euthanised using MS-222 (tricaine methanesulfonate) at 300mg/l for 20 – 30 min. Samples were taken from skin lesions using sterile cotton swabs and immediately streaked on Tryptic Soy Agar (TSA) plates. Each fish was then dissected through a ventral incision between the anal opening and interbranchial membrane. Visceral organs were carefully excised to expose the posterior kidney. Kidney swab samples were then collected as described for skin lesions. The TSA plates were incubated at 28°C for 24 h. Kidney and spleen tissue samples for histological examination were also collected and placed in 10% neutral buffered formalin.

3.2.3 Preliminary bacterial identification

Distinct colonies that had formed on the TSA plates were collected and subjected to Gram staining, motility and oxidase tests to inform a preliminary identification. Each colony was then subcultured in Tryptic Soy Broth (TSB) at 28°C for 24 h and stored in 15% glycerol at -4°C.

3.2.4 Recovery, validation and presumptive identification of bacterial isolates

Following transportation to the UK, each bacterial isolate was recovered on TSA plates at an incubation temperature of 28°C for 24 h. Isolated colonies morphologically consistent with those from Section 3.2.3 were sub-cultured on TSA under the same culture conditions. Gram staining, motility and oxidase tests were also conducted for validation. Validated isolates were then subjected to further tests that would permit a presumptive species identification including Oxidative-Fermentative (OF) test, 2,4-diamino-6,7-diisopropylpteridine (O/129) vibriostatic test and API 20E test (Lim & Hong, 2020).

3.2.5 Histological Processing of Tissue Samples

Kidney and spleen tissue samples preserved in 10% neutral buffered formalin (Section 3.2.2) were processed and embedded in paraffin wax at the histology laboratory, University of Abuja Teaching Hospital Gwagwalada, Nigeria before transporting them to the UK. Upon arrival at the Histology laboratory, Institute of Aquaculture, University of Stirling, sections 5 µ thick were cut on RM2255 microtome (LEICA Biosystems), placed on a glass slide, stained with haematoxylin and eosin (H&E) and cover slipped. Digital images were captured using ZEISS scanner (Huff et al., 2017) and slides were viewed using QuPath (Morriss et al., 2020), an open-source software for digital pathology and whole slide image analysis.

3.2.6 DNA extraction

Genomic DNA was extracted from bacterial colonies incubated under the same conditions in Section 3.2.2 using the modified salt precipitation method (Bartie et al., 2020) with modifications. Briefly, bacterial cell pellets were obtained by transferring 1 mL actively growing TSB culture into a 1.5 mL Eppendorf tube and centrifuged (21000 x g for 1 min at room temperature). The supernatant was then discarded. Cells were lysed by adding 220 µL of lysis solution (20 µL 10% SDS + 200 µL SSTNE buffer) and 3 µL proteinase K (10 mg/mL) to the cell pellet. This was mixed by inversion and incubated overnight at 55°C at 3 rpm in a rotator incubator – Techne Hybridiser HB (Techne Cambridge Ltd). Each lysed sample was heated for 15 min at 70°C to inactivate proteinase K, allowed to cool down to room temperature and then treated with 5 µL RNase A (2mg/mL) at 37°C and 3 rpm for 1 h in the same rotator incubator described above. A 0.7 × volume of 5 M NaCl was then added and incubated on ice for 10 min to precipitate the protein in each sample tube. Following this, each sample was centrifuged twice at 21,000 x g for 10 min and the supernatant carefully pipetted into a new centrifuge tube. An equal volume of room temperature isopropanol was added to the supernatant to precipitate DNA. This was thoroughly mixed by gentle inversion (5-6 times) and then incubated on ice for 10 min. The pellet was centrifuged (21000 x g, 10 min at room temperature) and the supernatant carefully discarded. The tube containing precipitated DNA was briefly centrifuged again and trace amounts of isopropanol removed by pipetting. The DNA pellet was washed twice by adding 1 mL 70% ethanol and incubating (22 rpm, overnight at room temperature) on a sample rotator – Rotator Drive STR4 (Stuart Scientific). The second washing step was performed for 1 h. The DNA was dried at 50°C for 2 – 3 min and dissolved in 15 – 50 µL 5 mM pH 8.0 Tris buffer for 1 h. Both the quantity and quality of the final DNA sample was determined using a Nanodrop spectrophotometer (Labtech International Ltd, UK) and then viewed on 1% agarose gel (Meridian bioscience, UK). Genomic DNA samples were stored long term at -20°C.

3.2.7 16S ribosomal RNA gene amplification and sequencing

The 16S *rRNA* gene was amplified using the genomic DNA template with universal primers 27F and 1492R (De Lillo et al., 2006) in 30 cycles of enzyme activation at 95°C for 1 min, denaturation at 95°C for 15 s, primer annealing at 56°C for 15 s, extension at 72°C for 1 min and final extension at 72°C for 2 min. The amplification reaction consisted of a 30 µL total volume containing 50 ng genomic DNA, 1 x MyTaq HS Mix (Meridian Bioscience) and 0.3 µM of each primer. Amplified gene products were electrophoresed in 1% agarose gel (Meridian bioscience, UK) containing 0.1 mg/mL ethidium bromide in 0.5 x TAE at 50 V for 10 min and another 20 min at 80 V. The amplicon image was captured using InGenius gel imaging system (Syngene, UK) under UV light.

PCR products were purified using the GeneJET (ThermoScientific) PCR purification kit according to manufacturer's instructions. Purified PCR amplicons were then sequenced (Eurofins Genomics) and sequence reads assembled into contigs using SeqMan 5.08 software and blasted on the NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast>) to check for similarity with closest matching sequences in the database.

3.2.8 Phenotypic virulence assays

Bacterial isolates were recovered on TSA plates from the glycerol stocks under the same conditions described in Section 3.2.4. An overnight TSB broth culture at 28°C and 150 rpm was then washed twice by transferring 1 mL TSB culture into 1.5 mL Eppendorf tube and centrifuging (2000 x g for 1 min at room temperature) in 1 mL phosphate buffered saline (PBS). Optical density (OD) of washed cells was adjusted to 1.0 at A600 and then used to inoculate five different assay plates that can detect phenotypes associated with the production of virulence factors specifically haemolysis (Chacon et al., 2003), serine protease (Lee et al., 2010), DNase (Scoaris et al., 2008; modified as lacked toluidine blue), lipase (Carissimi et al., 2007) and gelatinase (Dela Cruz et al., 2016) followed by overnight incubation at 28°C. A halo zone around the spot of inoculation indicated a positive result while the absence of this zone was indicative of a negative result.

3.2.9 Antibiotic susceptibility testing

3.2.9.1 Growth curve determination

To enable accurate determination of the active growth phase of the bacteria and determination of a standard growth curve, two isolates i.e., *A. hydrophila* (strain DC1K) and *Aeromonas veronii* (strain JC1U) were randomly selected using. Each isolate was recovered from -70°C as previously described (Section 3.2.4) and subcultured in TSB under the same condition described in Section 3.2.6 and adjusted to OD 1.0 at A600 before being inoculated in quadruplicate in a conical flask of sterile TSB at a ratio of 1:100 and incubated at 28°C and 150 rpm. The OD at A600 was recorded over a period of 24 h at 1 h intervals and mean absorbance for every hour recorded.

3.2.9.2 Determination of standard curve for control and representative bacterial strains

The control strain used for antibiotic susceptibility testing (*A. salmonicida* NCIMB 1102) was recovered from -70°C under the same condition earlier described (Section 3.2.4). This strain and one of the representative bacterial strains (DC1K), were individually subcultured in TSB in triplicate and incubated under the same condition for growth curve determination (Section 3.2.9.1) to their respective log phase i.e., 24 h and 7 h from the start of incubation. Each broth culture was adjusted to OD 0.2, 0.4, 0.6, 0.8 and 1.0 at A600. Each bacterial suspension was then serially diluted 1:10 six times. From the last three dilutions (10^{-4} – 10^{-6}), a 10 µL aliquot was spread-plated onto a TSA plate and incubated at 28°C overnight. Following this, the number of colony forming units (cfu) was counted for each dilution of every replicate and the cfu/mL averaged. The relationship between the OD and cfu/mL was established by means of a standard curve.

3.2.9.3 Minimum inhibitory concentration (MIC) test

MIC testing was done using the microbroth dilution method according to Clinical and Laboratory Standards Institute's (CLSI) Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated from Aquatic Animals (CLSI

VET03) guidelines using *A. salmonicida* NCIMB 1102 as the control strain. Similarly, MIC values were interpreted according to the guidelines contained in Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated from Aquatic Animals (CLSI VET04). Published literature breakpoints were used as guidelines for interpretation of MIC data of antibiotics not covered in CLSI VET04; notably amoxicillin (Scarano et al., 2018), colistin sulphate (Baron et al., 2017) and sulfamethoxazole (Duman et al., 2020).

Bacterial isolates that showed a positive result for at least one phenotypic virulence assay and known to be a common fish pathogen was selected for the MIC test. In total, 16 isolates, all *Aeromonas* species, were selected. Antibiotics used for this test included those identified as commonly used in the management of fish diseases in the study area from the previous chapter (Section 2.3.4). These antibiotics and their respective highest concentrations tested were gentamicin (64 µg/mL), erythromycin (512 µg/mL), florfenicol (32 µg/mL), oxytetracycline (8 µg/mL), enrofloxacin (1 µg/mL), oxolinic acid (1 µg/mL), amoxicillin (512 µg/mL), colistin sulphate (512 µg/mL) and sulfamethoxazole (512 µg/mL).

3.2.10 Whole genome sequencing (WGS)

Genomic DNA of each of the sixteen *Aeromonas* isolates used for MIC testing in Section 3.2.7.3 was recovered from -20°C and the nucleic acid concentration and purity estimated using a Nanodrop spectrophotometer (Labtech International Ltd, UK). The DNA concentration was determined using a Qubit® ds DNA Broad Range Assay kit according to the manufacturer's instructions. Each genomic DNA sample was also electrophoresed in 0.8% agarose gel (Meridian bioscience, UK) with a Lambda HindIII marker (ThermoFisher) and viewed under UV light using the InGenius gel imaging system (Syngene, UK) to check for any evidence of degradation. Finally, DNA library preparation and microbial whole genome sequencing was performed using two 150 bp paired end reads (PE150) to a depth of 100 on an Illumina Novaseq 6000 platform by Novogene (Cambridge, UK) Ltd, a sequencing provider.

3.2.11 Analysis of WGS data

3.2.11.1 Genome assembly and annotation

Raw reads from Section 3.2.8 were assembled into circular DNA and their functional genes annotated. Reads from the sequencing libraries were used separately during the assembly process. Overall read quality was optimised using fastp (S. Chen et al., 2018). This tool was used to filter out reads with error rates of 1 in 100 or more i.e., accuracy of more than 99% (QC > 20), ensure desired read length (150 nt), trim primers/adapters and set a minimum complexity (entropy > 15). Spades v3.14.0 (Bankevich et al., 2012) and the Unicycler-pipeline v0.4.8 (Wick et al., 2017) were then used to assemble reads that exceeded the defined quality thresholds. Average nucleotide identity (ANI), a widely recognised genome-based species delineation method (Livingstone et al., 2018), was applied to compare the initial de novo assembly output using FastANI v1.33 (Jain et al., 2018). All *Aeromonas* spp. available in the NCBI database at the time of analysis were included to determine the species identification and identify the closest genome sequence. Each assembly was then re-aligned against the closest related genome using CONTIGuator v2.7.5 (Galardini et al., 2011) to order the contigs when continuity was incomplete. Assembled genomes from this study and their respective accession numbers are listed in Table 3.1. Subsequently, all genomes and circular plasmids were annotated for functional genes by Prokka v1.14.5 (Seemann, 2014).

3.2.11.2 Pangenome analysis

To explore the pangenome in detail, Roary v3.13.0 (Page et al., 2015) was used to cluster orthologous genes into four main categories; core genome comprising hard core (genes shared by all sixteen genomes), soft core (genes shared by most of the sixteen genomes), accessory genome comprising shell (genes found shared by a majority of the sixteen genomes) and cloud (genes unique to a minimal subset of these sixteen genomes) genomes. Phandango v1.3.0 (Hadfield et al., 2018) was used to visualise the phylogenetic relationship between the genomes based on the accessory genome.

3.2.11.3 MLST typing

MLST types were determined for the 16 field isolates with mlst v2.19.0 (Seemann T, 2020b) and PubMLST (Jolley & Maiden, 2010) using the six housekeeping genes namely *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA* of the *Aeromonas* MLST scheme (Martino et al., 2011).

Table 3. 1 GenBank accession numbers of the sixteen assembled whole genome sequences of *Aeromonas* isolates from diseased catfish (*Clarias gariepinus*) in Nigeria

Accession number	Species	Isolate ID	State	Origin
GCA_963425835	<i>A. hydrophila</i>	DC1K	Niger	Farm
GCA_963425665	<i>A. hydrophila</i>	KMC2U	FCT	Market
GCA_963425795	<i>A. hydrophila</i>	KMC6U	FCT	Market
GCA_963425555	<i>A. hydrophila</i>	SMC11U	Niger	Market
GCA_963425545	<i>A. hydrophila</i>	SMC7K	Niger	Market
GCA_963425605	<i>A. hydrophila</i>	SUC4U	FCT	Farm
GCA_963425685	<i>A. dhakensis</i>	JC1K	Niger	Farm
GCA_963425535	<i>A. dhakensis</i>	SMC10U	Niger	Market
GCA_963425595	<i>A. jandaei</i>	KMC5U	FCT	Market
GCA_963425525	<i>A. jandaei</i>	KMC7K	FCT	Market
GCA_963425655	<i>A. jandaei</i>	KMC10U	FCT	Market
GCA_963425635	<i>A. veronii</i>	DC2K	Niger	Farm
GCA_963425735	<i>A. veroni</i>	JC1U	Niger	Farm
GCA_963425485	<i>A. veronii</i>	SC5K	Niger	Farm
GCA_963425575	<i>A. veronii</i>	SMC8U	Niger	Market
GCA_963425475	<i>A. veronii</i>	SMC9U	Niger	Market

3.2.11.4 Screening for antibiotic resistance and virulence genes

Screening for the presence of antibiotic resistance and virulence genes in the field isolate genomes was initially conducted through the ABRicate v1.0.0 pipeline (Seemann T, 2020a) using multiple databases: NCBI (Feldgarden et al., 2019) and PlasmidFinder (Carattoli et al., 2014). For genetic determinants of resistance

phenotypes not detected via ABRicate, the gene presence-absence file, an output file of the pangenome analysis pipeline, Roary (Section 3.2.9.2) was searched manually for any relevant genes. In the case of quinolones, where point mutation in the quinolone resistance determining region (QRDR) is important for resistance (Opal & Pop-Vicas, 2020a), relevant genes, namely *gyrA*, *gyrB*, *parC* and *parE*, were manually screened for point mutations generally known to be consistent with phenotypic resistance.

To account for genetic determinants of phenotypes associated with virulence traits (i.e., haemolytic, proteinase, DNase, lipase and gelatinase activity), not detected via the ABRicate-associated database i.e., virulence factor database (vfdb), sequence alignment of relevant genes via the ABRicate pipeline was performed. This was achieved in two steps; first, a mini-database of sequences of genes of interest was created by collecting the sequences directly in FASTA format if readily available on NCBI, or indirectly using published primers (Table 3.1) of the genes via the BLASTN tool (Torkian et al., 2020). Second, the FASTA file from step one above was then used through the ABRicate pipeline to align each sequence against all sixteen annotated genomes (Section 3.2.9.1).

Table 3. 2 Ten primer sets used for the BLASTN extraction of gene sequences from NCBI for the ABRicate mini-database.

Gene	Function	Primer sequence ((5'→3'))	Amplicon size (bp)	Reference
<i>gelE</i>	Gelatinase	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	419	(Lopes et al., 2006)
<i>act</i>	Cytotoxic enterotoxin	F: ATCGTCAGCGACAGCTTCTT R: CTCATCCCTTGGCTTGTGT	500	(Azzam-Sayuti et al., 2021)
<i>alt</i>	Heat-labile cytotoxic toxin	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	(Azzam-Sayuti et al., 2021)
<i>ast</i>	Heat-stable enterotoxin	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331	(Nhin et al., 2021)
<i>hly</i>	Haemolysin	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGG	597	(Azzam-Sayuti et al., 2021)
<i>aer</i>	Aerolysin	F: CAAGAACAAGTTCAAGTGGCCA R: ACGAAGGTGTGGTTCCAGT	309	(Azzam-Sayuti et al., 2021)

<i>ahp</i>	Serine protease	F: ATTGGATCCCTGCCTATCGCTTCAGTTCA R: GCTAAGCTTGCATCCGTGCCGTATTCC	911	(Azzam-Sayuti et al., 2021)
<i>exu</i>	DNase	F: AGACATGCACAACCTCTTCC R: GATTGGTATTGCCTTGCAAC	323	(Gao et al., 2020)
<i>lip</i>	Lipase	F: ATCTTCTCCGACTGGTTCGG R: CCGTGCCAGGACTGGGTCTT	382	(Azzam-Sayuti et al., 2021)
<i>fla</i>	Flagella	F: TCCAACCGTYTGACCTC R: GMYTGTTGCGRATGGT	608	(Azzam-Sayuti et al., 2021)

3.2.11.5 Manual curation of resistance-associated genes

Because of variations in the identities of resistance genes specifically from the beta-lactamase (*bla*) and tetracycline (*tet*) group between ABRicate and Roary pipelines (Section 3.2.9.4), all resistance genes detected from both pipelines were manually curated. Relevant sequences, based on either Prokka annotation or identification through ABRicate using the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023), were manually recovered using their gene IDs from their respective output files. These were translated into proteins before individually aligning each protein sequence against UniProtKB/Swiss-prot database using BLASTP (<https://www.uniprot.org/blast>) for sequence homology and identity. Each of these protein sequences were then analysed using the pFam tool (Mistry et al., 2021) to identify protein motifs and obtain insight into their functional annotation. Furthermore, each protein sequence was aligned using MAFFT (Yamada et al., 2016) to identify sequence clusters i.e., note when sequences cluster together and thereby confirm the consistency of the annotations.

A customised reference file of these curated genes was created for ABRicate, which was then used to annotate the original sequences. Following a single iteration, both the automatic and manual annotations were found to be consistent.

3.2.11.6 PCR amplification of manually curated resistance genes

To enable PCR amplification of manually curated genes, pairs of discriminating primers (Table 3.2) were designed from conserved regions of genes of interest (Section 3.2.9.5). Unipro UGENE v45.1 (Kolchenko et al., 2023) was used to validate these primer sets against respective genomes from the collection of 16 *Aeromonas* spp. genomes. Validated PCR primer sets i.e., those able to amplify the gene of interest, were then used to amplify target genes from respective genomes of interest, i.e. those with a positive prediction for the gene of interest as well as a number of those with a predicted negative outcome, to confirm primer specificity. Each PCR reaction contained 15 ng genomic DNA in a 15 uL total volume containing 1 x MyTaq HS mix (Meridian Bioscience) and 0.2 mM of each primer. To determine the optimum annealing temperature and PCR condition for each primer set, a gradient PCR between 52°C and 60°C was performed. Final PCR conditions were as follows; initial denaturation at 95°C for 3 min; followed by 30 cycles of 95°C for 15 s, 58°C for 15 s, (except *blaOXA-12* at 60°C) 72°C for 30 s; and a final extension at 72°C for 2 min. To confirm amplification was successful, amplicons were electrophoresed and viewed as described in Section 3.2.5.

Table 3. 3 Fourteen primer sets designed for amplification of manually curated genes encoding resistance determinants.

Gene	Resistance determinant	Primer sequence (5'→3')	Amplicon size (bp)
<i>tetA(A)</i>	Tetracycline	F: CTGACGGGCTGTTTCCTTTTG R: CCGAAAATGACCCAAAGCGC	188
<i>tetA(C)</i>		F: CTCCTTG CATGCACCATTC R: AGACAGTCATAAGTGCGGCG	185
<i>tetA(E)</i>		F: CAGGCACCGTTTATGTTCGC R: GGGAGATCATCTCGCGTATGG	157
<i>tetR(A)</i>		F: TCAGCTACTTCACTGTTGGGG R: GACAACGAGCCTCCTTTTCG	191
<i>tetR(C)</i>		F: TGGAAGGTCTAACGACGCG R: TTGCCCTTCAGGAACGAACG	181
<i>tetR(E)</i>		F: ACGACTAAGCTTGGACGACG	195

		R: GAACGGGTGTGATGCTTCAC	
<i>blaFOX-7</i>	Beta-lactam	F: GGATCTCTCCGGCAATCCTG	179
		R: CGATCTCGCTCACCTTGAGG	
<i>blaOXA-724</i>		F: ACAGCGGCTTTCTGGTAGAC	192
		R: AGTCGAAACGGTCGACGTAC	
<i>blaOXA-12</i>		F: TCTCTATGCTGACGGCAACG	162
		R: TAGCTCGGCTTGTAAGGCAG	
<i>sul1</i>	Sulfur drug	F: ATATCGGGATAGAGCGCAGG	171
		R: GGCCGATGAGATCAGACGTA	
<i>sul2</i>		F: ATCACATTGCGGCGTTCTTT	197
		R: TTTGCGCGAAACAGACAGAA	
<i>mcr-7.1</i>	Colistin	F: GCGACATTGAAAAGTGCAGC	186
		R: CGTGCAGGTAAAGTCCCATG	
<i>mcr-3.2</i>		F: TCAGGATTACGTCTCGGTCG	186
		R: AGGAACATCAGGGTCGGTTT	
<i>mcr-3.12</i>		F:	
		ATGGGCCGACCTATTACAAGC	
		R:	
		GTATTTGTCCTCGTACTTTTTCA	

3.2.11.7 PCR screening with *A. dhakensis* and vAh ST251 specific primers

To screen for hypervirulent strains among the six study *A. hydrophila* genomes, primers specific for the *intA_5* gene which codes for an integrase protein of hypervirulent *A. hydrophila* (vAh) ST251 were used (Bartie et al., 2023). Additionally, primers that target the *yjcS* gene which codes for a metallo b-hydrolase protein of *A. dhakensis* were chosen using the published PCR conditions (Bartie et al., 2023) to further distinguishing *A. dhakensis* from ST 251 *A. hydrophila* in this present study.

3.3 Results

3.3.1 Fish sampling and preliminary bacterial identification

A breakdown of sampling site, number of fish samples collected, gross lesions and distinct bacterial colonies identified from those fish is shown in Table 3.4. A total of 55 catfish (*Clarias gariepinus*) samples weighing between 100 and 800 g showing various signs of disease (Figure 3.1) including skin ulcers (67.3%), fin and barbel rot (70.9%), skin pallor (27.3%), congested skin (12.7%), petechial haemorrhages (9.1%) and distended abdomen (3.6%) were collected from nine different sampling locations that included seven farms and two fish markets.



Fig 3. 1 Diseased catfish showing various signs including A: skin pallor (yellow arrow), barbel rot (blue arrow) congestion (black arrow) and petechial haemorrhages (circle), B: deep ulcer (red arrow), C: fin rot (blue arrow) and D: superficial ulcers (green arrow)

Table 3. 4 Number of catfish samples, gross pathological lesions and number of distinct bacterial colonies obtained according to nine sampling locations (site)

Site	Site code	No of fish collected	Gross lesions	colonies
Farm	ALF	3	Skin ulcers, fin rot	6
Farm	DHA	4	Skin ulcers, barbel rot	4
Farm	JAV	3	Skin ulcers, discoloration, barbel rot	6
Farm	UMA	3	Fin and barbel rot, skin ulcers	6
Farm	FLE	3	Distended abdomen	2
Market	KMT	16	Hyperaemic skin regions, skin ulcers, barbel rot, liver paleness	33
Market	SMT	13	Skin ulcers and discoloration, fin and barbel rot	21
Farm	SHI	5	Hyperaemic skin regions and ulcers	10
Farm	SUL	5	Hyperaemic skin regions and ulcers, liver paleness	12
		55		100

Following culturing and preliminary identification techniques (Gram staining, motility and oxidase testing), a total of 100 distinct colonies (Table 3.5) were identified. These consisted of a single oxidase-positive, non-motile Gram-negative coccus; four oxidase-negative, non-motile Gram-negative cocci; twenty-two oxidase-negative, non-motile Gram-positive cocci; one oxidase negative, non-motile, Gram-positive rod; four oxidase negative, motile, Gram-negative short rods; three oxidase negative, non-motile, Gram-negative short rods; and sixty-five oxidase positive, motile, Gram-negative short rods. These isolates were stored in glycerol and transported to the UK.

Table 3. 5 Preliminary field characterisation of 100 isolates in Nigeria

Isolate No	Site code	Swab Type	Sample Strain ID	Colonial morphology	Gram Stain	Oxidase	Motility
1	ALF	Kidney	AC1Ka	Yellow, smooth and raised	+ cocci	-	-
2	ALF	Kidney	AC1Kb	Cream, smooth and raised	- short rods	+	-
3	ALF	Kidney	AC2Ka	Yellow, smooth and raised	+ cocci	-	-
4	ALF	Kidney	AC2Kb	Cream, smooth and raised	- short rods	+	+
5	ALF	Kidney	AC3Ka	Yellow, smooth and raised	+ cocci	-	-
6	ALF	Kidney	AC3Kb	Cream, smooth and raised	- short rods	+	+
7	DHA	Kidney	DC1K	Cream, smooth and raised	- short rods	+	+
8	DHA	Kidney	DC2K	Cream, smooth and raised	- short rods	+	+
9	DHA	Kidney	DC3K	Yellow, smooth and raised	+ cocci	-	-
10	DHA	Kidney	DC4K	Brown, smooth and raised	- short rods	+	+
11	JAV	Kidney	JC1K	Cream, smooth and raised	- short rods	+	+
12	JAV	Skin Ulcer	JC1U	Cream, smooth and raised	- short rods	+	+
13	JAV	Kidney	JC2K	Cream, smooth and raised	- short rods	+	+
14	JAV	Skin Ulcer	JC2U	Yellow, smooth and raised	+ rods	-	-
15	JAV	Kidney	JC3K	Cream, smooth and raised	- short rods	+	+
16	JAV	Skin Ulcer	JC3U	Yellow, smooth and raised	+ cocci	-	-
17	UMA	Kidney	UC1K	Cream, smooth and raised	- short rods	+	+
18	UMA	Skin Ulcer	UC1U	Cream, smooth and raised	- short rods	+	+
19	UMA	Kidney	UC2K	Yellow, smooth and raised	+ cocci	-	-
20	UMA	Skin Ulcer	UC2U	Yellow, smooth and raised	+ cocci	-	-
21	UMA	Kidney	UC3K	Yellow, smooth and raised	+ cocci	-	-
22	UMA	Skin Ulcer	UC3U	Cream, smooth and raised	- short rods	+	+
23	FLE	Kidney	FC1K	Cream, smooth and raised	- short rods	-	+
24	FLE	Kidney	FC2K	Cream, smooth and raised	- short rods	+	+
25	KMT	Kidney	KMC1K	Cream, smooth and raised	- short rods	+	+
26	KMT	Skin Ulcer	KMC1U	Cream, smooth and raised	- short rods	+	+
27	KMT	Kidney	KMC2K	Cream, smooth and raised	- short rods	+	+
28	KMT	Skin Ulcer	KMC2U	Cream, smooth and raised	- short rods	+	+
29	KMT	Kidney	KMC3Ka	Cream, smooth and raised	- short rods	-	+

30	KMT	Kidney	KMC3Kb	Cream, smooth and raised	- short rods	+	+
31	SMT	Kidney	SMC1K	Cream, smooth and raised	- short rods	+	+
32	SMT	Skin Ulcer	SMC1U	Cream, smooth and raised	- short rods	+	+
33	SMT	Kidney	SMC2K	Cream, smooth and raised	- short rods	+	+
34	SHI	Kidney	SC1K	Cream, smooth and raised	- short rods	+	+
35	SHI	Kidney	SC2K	Cream, smooth and raised	- short rods	+	+
36	SHI	Skin Ulcer	SC2U	Cream, smooth and raised	- short rods	+	+
37	SHI	Kidney	SC3Ka	Yellow, smooth and raised	+ cocci	-	-
38	SHI	Kidney	SC3Kb	White, smooth, and raised	+ cocci	-	-
39	SHI	Skin Ulcer	SC3U	Cream, smooth and raised	+ cocci	-	-
40	SHI	Kidney	SC4K	Cream, smooth and raised	- cocci	-	-
41	SHI	Skin Ulcer	SC4U	Cream, smooth and raised	- short rods	-	-
42	SHI	Kidney	SC5K	Cream, smooth and raised	- short rods	+	+
43	SHI	Skin Ulcer	SC5U	Yellow, smooth and raised	+ cocci	-	-
44	SUL	Kidney	SUC1K	Cream, smooth and raised	- short rods	-	-
45	SUL	Skin Ulcer	SUC1U	Cream, smooth and raised	- cocci	+	-
46	SUL	Kidney	SUC2K	Cream, smooth and raised	- cocci	-	-
47	SUL	Skin Ulcer	SUC2U	Cream, smooth and raised	- short rods	+	+
48	SUL	Kidney	SUC3K	Yellow, smooth and raised	+ cocci	-	-
49	SUL	Skin Ulcer	SUC3U	Cream, smooth and raised	- short rods	+	+
50	SUL	Liver	SUC3L	Cream, smooth and raised	- short rods	+	+
51	SUL	Kidney	SUC4K	Cream, smooth and raised	- short rods	+	+
52	SUL	Skin Ulcer	SUC4U	Cream, smooth and raised	- short rods	+	+
53	SUL	Kidney	SUC5K	Cream, smooth and raised	- short rods	+	+
54	SUL	Skin Ulcer	SUC5U	Cream, smooth and raised	- short rods	+	+
55	SUL	Liver	SUC5L	Cream, smooth and raised	- cocci	-	-
56	KMT	Kidney	KMC4K	Yellow, smooth and raised	+ cocci	-	-
57	KMT	Skin Ulcer	KMC4U	Cream, smooth and raised	- short rods	+	+
58	KMT	Kidney	KMC5K	Cream, smooth and raised	+ cocci	-	-
59	KMT	Skin Ulcer	KMC5U	Cream, smooth and raised	- short rods	+	+
60	KMT	Kidney	KMC6K	Cream, smooth and raised	- short rods	+	+
61	KMT	Skin Ulcer	KMC6U	Cream, smooth and raised	- short rods	+	+

62	KMT	Kidney	KMC7K	Cream, smooth and raised	- short rods	+	+
63	KMT	Skin Ulcer	KMC7U	Cream, smooth and raised	- cocci	-	-
64	KMT	Kidney	KMC8K	Yellow, smooth and raised	+ cocci	-	-
65	KMT	Skin Ulcer	KMC8U	Yellow, smooth and raised	+ cocci	-	-
66	KMT	Kidney	KMC9K	Yellow, smooth and raised	+ cocci	-	-
67	KMT	Skin Ulcer	KMC9U	Cream, smooth and raised	- short rods	+	+
68	KMT	Kidney	KMC10K	Yellow, smooth and raised	+ cocci	-	-
69	KMT	Skin Ulcer	KMC10U	Cream, smooth and raised	- short rods	+	+
70	KMT	Kidney	KMC11K	Yellow, smooth and raised	- short rods	-	-
71	KMT	Skin Ulcer	KMC11U	Pink, smooth and raised	+ cocci	-	-
72	SMT	Kidney	SMC3K	Cream, smooth and raised	+ cocci	-	-
73	SMT	Skin Ulcer	SMC3U	Cream, smooth and raised	- short rods	+	+
74	SMT	Kidney	SMC4K	Cream, smooth and raised	- short rods	+	+
75	SMT	Kidney	SMC5K	Cream, smooth and raised	- short rods	+	+
76	SMT	Kidney	SMC6K	Cream, smooth and raised	- short rods	+	+
77	SMT	Kidney	SMC7K	Cream, smooth and raised	- short rods	+	+
78	SMT	Kidney	SMC8K	Cream, smooth and raised	+ cocci	-	-
79	SMT	Skin Ulcer	SMC8U	Cream, smooth and raised	- short rods	+	+
80	SMT	Kidney	SMC9K	Cream, smooth and raised	- short rods	+	+
81	SMT	Skin Ulcer	SMC9U	Cream, smooth and raised	- short rods	+	+
82	SMT	Kidney	SMC10K	Cream, smooth and raised	- short rods	-	+
83	SMT	Skin Ulcer	SMC10U	Cream, smooth and raised	- short rods	+	+
84	SMT	Kidney	SMC11K	Cream, smooth and raised	- short rods	-	+
85	SMT	Skin Ulcer	SMC11U	Cream, smooth and raised	- short rods	+	+
86	SMT	Kidney	SMC12K	Cream, smooth and raised	- short rods	+	+
87	SMT	Skin Ulcer	SMC12U	Cream, smooth and raised	- short rods	+	+
88	SMT	Kidney	SMC13K	Cream, smooth and raised	- short rods	+	+
89	SMT	Skin Ulcer	SMC13U	Cream, smooth and raised	- short rods	+	+
90	KMT	Kidney	KMC12K	Cream, smooth and raised	- short rods	+	+
91	KMT	Skin Ulcer	KMC12U	Cream, smooth and raised	- short rods	+	+
92	KMT	Kidney	KMC13K	Cream, smooth and raised	- short rods	+	+
93	KMT	Skin Ulcer	KMC13U	Cream, smooth and raised	- short rods	+	+

94	KMT	Kidney	KMC14K	Cream, smooth and raised	- short rods	+	+
95	KMT	Skin Ulcer	KMC14U	Cream, smooth and raised	- short rods	+	+
96	KMT	Kidney	KMC15K	Cream, smooth and raised	- short rods	+	+
97	SMT	Skin Ulcer	SMC14U	Cream, smooth and raised	- short rods	+	+
98	KMT	Kidney	KMC16K	Cream, smooth and raised	- short rods	+	+
99	KMT	Skin Ulcer	KMC16U	Cream, smooth and raised	- short rods	+	+
100	KMT	Liver	KMC16L	Cream, smooth and raised	- short rods	+	+

3.3.2 Recovery, validation and primary bacterial identification in the UK

Forty-two of the 100 isolates that were initially collected and stored in glycerol were successfully revived. Phenotypic traits of these isolates were confirmed to be the same as before. After primary bacterial identification tests (Figure 3.2) and 16S *rRNA* sequence alignment, 16 and 12 isolates were tentatively identified as *Aeromonas* spp. and *Pseudomonas* spp. respectively (Table 3.6). The remaining isolates were also tentatively identified as two isolates of *Macrococcus* spp., *Micrococcus* spp., *Plesiomonas* spp., and one isolate each of *Serratia*, *Psychrobacter*, *Acinetobacter*, *Elizabethkingia*, *Citrobacter*, *Exiguobacterium*, and *Staphylococcus* spp.

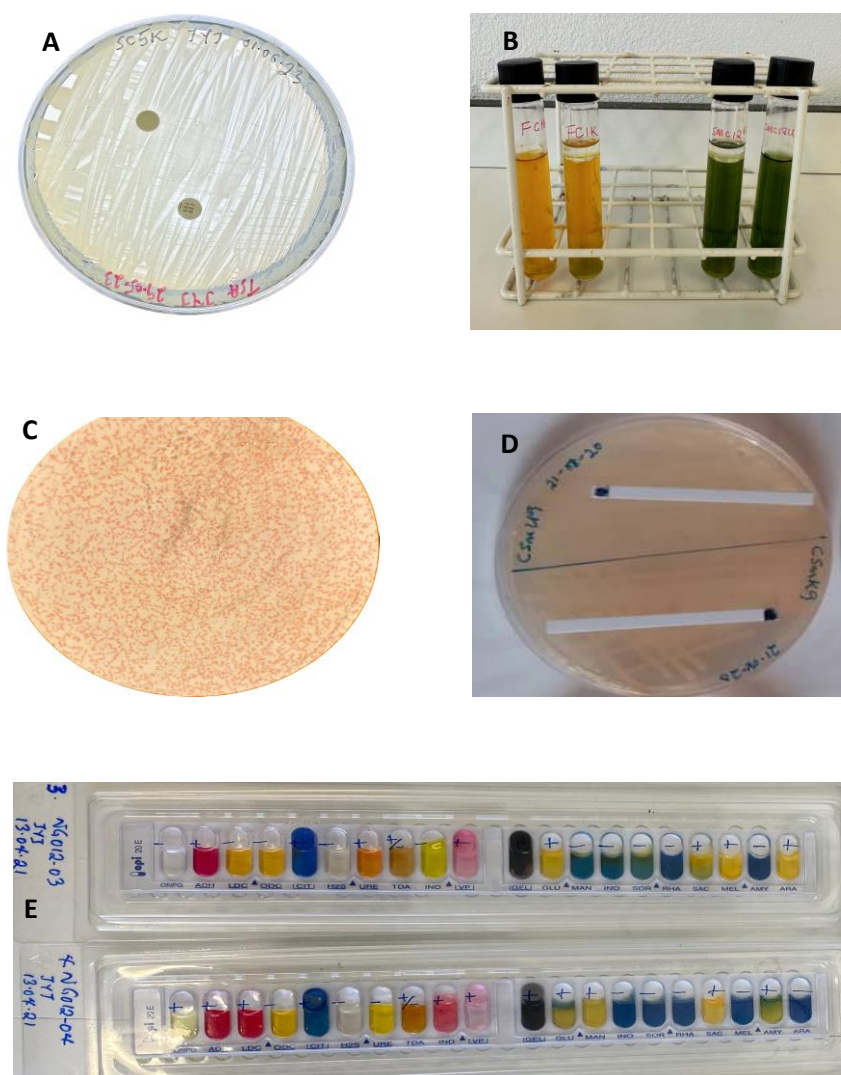


Fig 3. 2 Primary bacterial identification tests showing A, Resistant O/129 vibriostatic agent; B, Oxidative fermentative test (O-F); C, Gram-negative rod shaped cells; D, Oxidase positive strip test; E, API 20E

Table 3. 6 Eight different tests used for tentative identification of bacterial isolates from diseased catfish from Nigeria

Recovered Isolate No	Strain ID	Site	Gram Stain	Oxidase	Motility	O-F	O/129	API 20E	16S ID	Tentative ID
11	JC1K	Farm	- short rods	+	+	F	R	-	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
12	JC1U	Farm	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
42	SC5K	Farm	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
52	SUC4U	Farm	- short rods	+	+	F	R	<i>Aeromonas hydrophila</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
7	DC1K	Farm	- short rods	+	+	F	R	-	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
8	DC2K	Farm	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
28	KMC2U	Market	- short rods	+	+	F	R	-	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
59	KMC5U	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
61	KMC6U	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
62	KMC7K	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
69	KMC10U	Market	- short rods	+	+	F	R	-	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
77	SMC7K	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
79	SMC8U	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
81	SMC9U	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.

83	SMC10U	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
85	SMC11U	Market	- short rods	+	+	F	R	<i>Aeromonas sobria</i>	<i>Aeromonas</i> spp.	<i>Aeromonas</i> spp.
44	SUC1K	Farm	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
10	DC4K	Farm	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
15	JC3K	Farm	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
22	UC3U	Farm	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
29	KMC3Ka	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
31	SMC1K	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
33	SMC2K	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
74	SMC4K	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
80	SMC9K	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
86	SMC12K	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
87	SMC12U	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
89	SMC13U	Market	- short rods	+	+	NF	R	-	<i>Pseudomonas</i> spp.	<i>Pseudomonas</i> spp.
23	FC1K	Farm	- short rods	-	+	NF	R	-	<i>Serratia</i> spp.	<i>Serratia</i> spp.
41	SC4U	Farm	- short rods	-	-			-	<i>Citrobacter</i> spp.	<i>Citrobacter</i> spp.

6	AC3Kb	Farm	- short rods	+	+	NF	S	-	<i>Elizabethkingia</i> spp.	<i>Elizabethkingia</i> spp.
2	AC1Kb	Farm	- short rods	+	-	NF	R	<i>Phocoenobacter uteri</i>	<i>Psychrobacter</i> spp.	<i>Psychrobacter</i> spp.
30	KMC3Kb	Market	- short rods	+	+	F	S	-	<i>Plesiomonas</i> spp.	<i>Plesiomonas</i> spp.
60	KMC6K	Market	- short rods	+	+	F	S	-	<i>Plesiomonas</i> spp.	<i>Plesiomonas</i> spp.
97	SMC14U	Market	- short rods	+	+	NF	R	-	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i> spp.
19	UC2K	Farm	+ cocci	-	-	NA	NA	NA	<i>Micrococcus</i> spp.	<i>Micrococcus</i> spp.
21	UC3K	Farm	+ cocci	-	-	NA	NA	NA	<i>Micrococcus</i> spp.	<i>Micrococcus</i> spp.
37	SC3Ka	Farm	+ cocci	-	-	NA	NA	NA	<i>Exiguobacterium</i> spp.	<i>Exiguobacterium</i> spp.
38	SC3Kb	Farm	+ cocci	-	-	NA	NA	NA	<i>Staphylococcus</i> spp.	<i>Staphylococcus</i> spp.
39	SC3U	Farm	+ cocci	-	-	NA	NA	NA	<i>Macrococcus</i> spp.	<i>Macrococcus</i> spp.
43	SC5U	Farm	+ cocci	-	-	NA	NA	NA	<i>Macrococcus</i> spp.	<i>Macrococcus</i> spp.
48	SUC3K	Farm	+ cocci	-	-	NA	NA	NA	<i>Macrococcus</i> spp.	<i>Macrococcus</i> spp.

O-F: Oxidative fermentative test, O/129: vibriostatic test, R: resistant, S: sensitive, F: fermentative, NF: non-fermentative, NA: not applicable, 16S ID: 16S *rRNA* closest species alignment, -: inconclusive

3.3.3 Histologic presentation

Although there were significant fixation artefacts, nothing striking was identified on the histology slides other than suggestions of tubular degenerative changes in some cases and moderate congestion in others.

3.3.4 Phenotypic virulence

Sixteen out of the 42 isolates comprising those tentatively identified as *Aeromonas* spp. displayed positive results for all five phenotypic virulence assays including haemolysis (Chacon et al., 2003), protease (Lee et al., 2010), DNase (Scoaris et al., 2008; modified as lacked toluidine blue), lipase (Carissimi et al., 2007) and gelatinase (Dela Cruz et al., 2016) (Table 3.5). While none of the 12 isolates tentatively identified as *Pseudomonas* spp. were positive for haemolysis and DNase assays, ten, five and one of these isolates were respectively positive for gelatinase, lipase, and protease assays (Table 3.7). Among other tentatively identified bacterial species, *Serratia* spp. was positive for protease, gelatinase, DNase, and lipase activities; *Exiguobacterium* spp. for protease, gelatinase, and DNase; *Elizabethkingia* and one *Micrococcus* spp. isolate for gelatinase and DNase while the second *Micrococcus* isolate and one *Macrococcus* spp. were respectively positive for DNase and Protease. The remaining eight isolates were negative for all the assays. Overall, the total number of positive results by assay type and prevalence was 30, 22, 21, 20, and 16 isolates out of the 42 isolates tested for gelatinase, lipase, DNase, protease, and haemolysis respectively.

Fig 3. 3 Positive phenotypic virulence assays including A, haemolysis, B, lipase; C, protease and D, DNase.

Table 3. 7 Result of five different phenotypic virulence attributes including haemolysis (H), (Chacon et al., 2003), protease (P) (Lee et al., 2010), DNase (D) (Scoaris et al., 2008), lipase (L) (Carissimi et al., 2007) and gelatinase (G) (Dela Cruz et al., 2016) activity performed on 42 recovered bacterial isolates collected from diseased catfish in Nigeria

Recovered Isolate No	Strain ID	Tentative ID	H	P	G	D	L
11	JC1K	<i>Aeromonas</i> spp.	+	+	+	+	+
12	JC1U	<i>Aeromonas</i> spp.	+	+	+	+	+
28	KMC2U	<i>Aeromonas</i> spp.	+	+	+	+	+
42	SC5K	<i>Aeromonas</i> spp.	+	+	+	+	+
59	KMC5U	<i>Aeromonas</i> spp.	+	+	+	+	+
61	KMC6U	<i>Aeromonas</i> spp.	+	+	+	+	+
52	SUC4U	<i>Aeromonas</i> spp.	+	+	+	+	+
62	KMC7K	<i>Aeromonas</i> spp.	+	+	+	+	+
69	KMC10U	<i>Aeromonas</i> spp.	+	+	+	+	+
77	SMC7K	<i>Aeromonas</i> spp.	+	+	+	+	+
79	SMC8U	<i>Aeromonas</i> spp.	+	+	+	+	+
81	SMC9U	<i>Aeromonas</i> spp.	+	+	+	+	+
83	SMC10U	<i>Aeromonas</i> spp.	+	+	+	+	+
85	SMC11U	<i>Aeromonas</i> spp.	+	+	+	+	+
7	DC1K	<i>Aeromonas</i> spp.	+	+	+	+	+
8	DC2K	<i>Aeromonas</i> spp.	+	+	+	+	+
15	JC3K	<i>Pseudomonas</i> spp.	-	+	+	-	+
22	UC3U	<i>Pseudomonas</i> spp.	-	-	-	-	-
29	KMC3Ka	<i>Pseudomonas</i> spp.	-	-	+	-	-
31	SMC1K	<i>Pseudomonas</i> spp.	-	-	+	-	-
33	SMC2K	<i>Pseudomonas</i> spp.	-	-	+	-	-
74	SMC4K	<i>Pseudomonas</i> spp.	-	-	+	-	-
80	SMC9K	<i>Pseudomonas</i> spp.	-	-	+	-	+
86	SMC12K	<i>Pseudomonas</i> spp.	-	-	+	-	+
87	SMC12U	<i>Pseudomonas</i> spp.	-	-	+	-	+
89	SMC13U	<i>Pseudomonas</i> spp.	-	-	+	-	+
44	SUC1K	<i>Pseudomonas</i> spp.	-	-	+	-	-
10	DC4K	<i>Pseudomonas</i> spp.	-	-	-	-	-
23	FC1K	<i>Serratia</i> spp.	-	+	+	+	+
2	AC1K	<i>Psychrobacter</i> spp.	-	-	-	-	-
30	KMC3Kb	<i>Plesiomonas</i> spp.	-	-	-	-	-
60	KMC6K	<i>Plesiomonas</i> spp.	-	-	-	-	-
97	SMC14U	<i>Acinetobacter</i> spp.	-	-	-	-	-
6	AC3Kb	<i>Elizabethkingia</i> spp.	-	-	+	+	-
19	UC2K	<i>Micrococcus</i> spp.	-	-	-	+	-
21	UC3K	<i>Micrococcu</i> spp.	-	-	+	+	-

41	SC4U	<i>Citrobacter</i> spp.	-	-	-	-	-
37	SC3Ka	<i>Exiguobacterium</i> spp.	-	+	+	+	-
38	SC3Kb	<i>Staphylococcus</i> spp.	-	-	-	-	-
39	SC3U	<i>Macrococcus</i> spp.	-	+	-	-	-
43	SC5U	<i>Macrococcus</i> spp.	-	-	-	-	-
48	SUC3K	<i>Macrococcus</i> spp.	-	-	-	-	-

+: positive result, -: negative result

3.3.5 Minimum inhibitory concentration (MIC)

3.3.5.1 MICs of field isolates

The MIC test results are presented in Table 3.6. All 16 isolates were designated wild type (WT) against gentamicin (GEN) and erythromycin (ERY) and non-wild type (NWT) against oxytetracycline (OTC) and sulphamethoxazole (SMX), while 15/16 were NWT against amoxycillin (AMX) and oxolinic acid (OXA). For colistin (COL), enrofloxacin (ENR), and florfenicol (FLO), 12/16, 11/16, and 2/16 were NWT respectively.

The highest NWT profile involving seven different antibiotic classes was displayed by *A. dhakensis* JC1K, while the least involving only three antibiotic classes was displayed by strain *A. veronii* DC2K. The remaining 14 isolates had a profile either involving five antibiotic classes as displayed by *A. hydrophila* DC1K, *A. hydrophila* SMC7K, *A. hydrophila* SMC11U, *A. veronii* SC5K, *A. veronii* SMC8U, *A. veronii* SMC9U, and *A. jandaei* KMC7K or six antibiotic classes as displayed by *A. hydrophila* KMC2U, *A. hydrophila* KMC6U, *A. hydrophila* SUC4U, *A. veronii* JC1U, *A. jandaei* KMC5U, *A. jandaei* KMC10U and *A. dhakensis* SMC10U.

Table 3. 8 Minimum inhibitory concentration (MIC) in µg/mL and epidemiological cut-off values (ECV) categories i.e., wild type (WT) and non-wild type (NWT) of 16 *Aeromonas* spp. isolated from diseased catfish in northern Nigeria against oxytetracycline (OTC), sulfamethoxazole (SMX), amoxicillin (AMX), oxolinic acid (OXA), erythromycin (ERY), gentamicin (GEN), florfenicol (FLO), enrofloxacin (ENR), and colistin sulphate (COL).

Species	Strain ID	OTC		SMX		AMX		OXA		COL		ENR		FLO		GEN		ERY		NWT Profile
		Type	MIC	Type	MIC	Type	MIC	Type	MIC	Type	MIC	Type	MIC	Type	MIC	Type	MIC	Type		
A. hydrophila	DC1K	8	NWT	> 512	NWT	> 512	NWT	0.062 5	NWT	64	NWT	0.0156	WT	1	WT	1	WT	16	WT	5
A. hydrophila	KMC2U	8	NWT	> 513	NWT	> 512	NWT	> 1.0	NWT	256	NWT	0.5	NWT	2	WT	0.5	WT	16	WT	6
A. hydrophila	KMC6U	> 8.0	NWT	> 514	NWT	> 512	NWT	> 1.0	NWT	64	NWT	0.25	NWT	2	WT	1	WT	16	WT	6
A. hydrophila	SMC7K	1	NWT	> 515	NWT	> 512	NWT	0.062 5	NWT	128	NWT	0.0156	WT	1	WT	0.25	WT	8	WT	5
A. hydrophila	SMC11U	1	NWT	> 516	NWT	> 512	NWT	0.125	NWT	128	NWT	0.0078	WT	1	WT	0.5	WT	16	WT	5
A. hydrophila	SUC4U	> 8.0	NWT	> 517	NWT	> 512	NWT	> 1.0	NWT	256	NWT	0.25	NWT	2	WT	0.5	WT	32	WT	6
A. veronii	JC1U	> 8.0	NWT	> 518	NWT	> 512	NWT	> 1.0	NWT	32	NWT	1	NWT	1	WT	2	WT	8	WT	6
A. veronii	SC5K	> 8.0	NWT	> 519	NWT	> 512	NWT	0.062 5	NWT	2	WT	0.0625	NWT	2	WT	0.5	WT	8	WT	5
A. veronii	DC2K	> 8.0	NWT	> 520	NWT	> 512	NWT	0.031 3	WT	2	WT	0.0156	WT	1	WT	4	WT	4	WT	3

								0.062												5
<i>A. veronii</i>	SMC8U	0.5	NWT	> 521	NWT	512	NWT	5	NWT	16	NWT	0.0156	WT	1	WT	1	WT	4	WT	5
<i>A. veronii</i>	SMC9U	> 8.0	NWT	> 522	NWT	> 512	NWT	> 1.0	NWT	4	WT	> 1.0	NWT	1	WT	2	WT	8	WT	5
<i>A. jandaei</i>	KMC5U	> 8.0	NWT	> 523	NWT	256	NWT	> 1.0	NWT	8	NWT	> 1.0	NWT	1	WT	0.5	WT	4	WT	6
<i>A. jandaei</i>	KMC7K	> 8.0	NWT	> 524	NWT	512	NWT	> 1.0	NWT	4	WT	> 1.0	NWT	1	WT	1	WT	8	WT	5
<i>A. jandaei</i>	KMC10U	> 8.0	NWT	> 525	NWT	16	WT	> 1.0	NWT	256	NWT	> 1.0	NWT	32	NWT	1	WT	8	WT	6
<i>A. dhakensis</i>	JC1K	> 8.0	NWT	> 526	NWT	> 512	NWT	> 1.0	NWT	256	NWT	1	NWT	4	NWT	1	WT	16	WT	7
<i>A. dhakensis</i>	SMC10U	> 8.0	NWT	> 527	NWT	> 512	NWT	1	NWT	> 512	NWT	1	NWT	2	WT	1	WT	32	WT	6
NWT(n)		16		16		15		15		12		11		2		0		0		

3.3.5.2 MIC of control strain

Recorded MIC values of six antibiotics covered in CLSI against the control strain (*Aeromonas salmonicida* NCIMB 1102) alongside the expected CLSI range for each antibiotic are shown in Table 3.9. These results show that all were within the CLSI recommended range.

Table 3. 9 Comparison of MIC values ($\mu\text{g/mL}$) of control strain *Aeromonas almonicida* NCIMB 1102 for six antibiotics tested and published CLSI MIC ranges

S/No	Antibiotic	MIC	CLSI MIC Range
1	Gentamicin	0.5	0.25 – 1.0
2	Oxytetracycline	0.125	0.12 – 1.0
3	Erythromycin	4.0	4.0 – 32
4	Florfenicol	1.00	0.5 – 2.0
5	Enrofloxacin	0.004	0.004 – 0.03
6	Oxolinic Acid	0.03	0.008 – 0.03

3.3.6 Genome analysis

Assembled genome sizes of the *Aeromonas* spp isolates ranged from 4.13 to 4.88 Mb with a minimum and maximum coding sequences of 3,767 and 4,420 respectively coding for 3,900 and 4,570 genes (Table 3.10). Identities of these genomes were verified by ANI values ranging from 96.4 to 99.7% (Table 3.9) to the closest published genome. An ANI threshold of above 95% is required for species delineation (Livingstone et al., 2018). Additionally, between one and four plasmids were detected per genome in nine of these sixteen genomes, with sizes ranging from 2.53 to 9.17 Kb. Pangenome analysis revealed a total of 17,785 genes broken down into core genes (726) found in all sixteen genomes, soft core genes (37) found in 95 – 99% of the genomes, shell genes (7,941) found in < 95 – 99% of the genomes, and cloud genes (9,081) found in < 15% of the genomes (Table 3.11).

Table 3. 10 Average Nucleotide Identity (ANI) and Prokka annotation of *Aeromonas* spp. strains from diseased catfish in Nigeria showing total number of bases, number of coding sequences (CDS), number of genes, number of plasmids and the respective plasmid sizes

Species	Strain ID	ANI (%)	Bases	CDS	Gene	Plasmid (n)	Plasmid size (kb)
<i>A. hydrophila</i>	DC1K	97.1	4766411	4271	4413	0	0
<i>A. hydrophila</i>	KMC2U	97.0	4701013	4215	4357	0	0
<i>A. hydrophila</i>	KMC6U	99.7	4831453	4378	4529	0	0
<i>A. hydrophila</i>	SMC7K	97.1	4763694	4333	4480	2	6.49/5.91
<i>A. hydrophila</i>	SMC11U	97.1	4728006	4303	4448	3	7.54/5.91/5.64
<i>A. hydrophila</i>	SUC4U	99.7	4878631	4420	4570	0	0
<i>A. dhakensis</i>	JC1K	97.5	4688190	4203	4358	0	0
<i>A. dhakensis</i>	SMC10U	98.8	4705356	4186	4341	1	5.42
<i>A. jandaei</i>	KMC5U	96.6	4384542	4006	4142	2	9.17/7.79
<i>A. jandaei</i>	KMC7K	96.6	4379836	4000	4137	2	9.17/7.79
<i>A. jandaei</i>	KMC10U	96.9	4503071	4071	4212	1	6.98
<i>A. veronii</i>	JC1U	96.8	4406299	4014	4164	1	9.17
<i>A. veronii</i>	SC5K	96.4	4279483	3886	4022	0	0
<i>A. veronii</i>	DC2K	96.5	4586420	4177	4321	0	0
<i>A. veronii</i>	SMC8U	96.4	4436069	4043	4176	2	4.80/2.53
<i>A. veroni</i>	SMC9U	96.5	4134763	3767	3900	4	7.77/5.77/5.10/2.66

Table 3. 11 Number of genes according to each pangenome component using Roary for the 16 study genomes

S/No	Component	No of genes	Strain (%)
1	Core	726	100
2	Soft core	37	95 – 99
3	Shell	7,941	< 95 – 15
4	Cloud	9,081	< 15
Total		17,785	

3.3.7 MLST Typing

The MLST typing result obtained using the scheme for the genus *Aeromonas* is presented in Table 3.11. MLST could only assign sequence types to three of the sixteen isolates, specifically *A. veronii* JC1U (ST425), *A. dhakensis* JC1K (ST557) and *A. dhakensis* SMC10U (ST696). The remaining thirteen isolates presented with

sequences that did not match exactly with the alleles at most of the loci and were returned as unknown sequence types.

Table 3. 12 MLST typing of sequenced *Aeromonas* spp. strains from diseased catfish from northern Nigeria listing exact matching alleles (whole numbers), closely matching allele (whole number preceded by ~) from the PubMLST database at each of the six gene loci and the sequence type (ST) or unknown (U1 – 10).

Species	Strain ID	<i>gyrB</i>	<i>groL</i>	<i>gltA</i>	<i>metG</i>	<i>ppsA</i>	<i>recA</i>	ST
<i>A. hydrophila</i>	DC1K	~769	~653	~410	~123	~269	~470	U1
<i>A. hydrophila</i>	KMC2U	713	645	571	548	779	~606	U2
<i>A. hydrophila</i>	KMC6U	276?	~473	~1	474	~404	527	U3
<i>A. hydrophila</i>	SMC7K	355	~473	~121	~350	~292	~437	U4
<i>A. hydrophila</i>	SMC11U	355	~473	~121	~350	~292	~437	U4
<i>A. hydrophila</i>	SUC4U	276?	~473	~1	474	~404	527	U3
<i>A. dhakensis</i>	JC1K	241	294	88	400	105	436	557
<i>A. dhakensis</i>	SMC10U	415	469	431	267	457	160	696
<i>A. jandaei</i>	KMC5U	~433	~416	~671	~586	~380	~451	U5
<i>A. jandaei</i>	KMC7K	~433	~416	~671	~586	~380	~451	U5
<i>A. jandaei</i>	KMC10U	~433	~607	~641	~466	452?	~451	U6
<i>A. veronii</i>	JC1U	319	154	311	148	341	330	425
<i>A. veronii</i>	SC5K	~633	227	717	~488	~395	~323	U7
<i>A. veronii</i>	DC2K	46	687	762	~111	826	~805	U8
<i>A. veronii</i>	SMC8U	40	574	80	640	682	~694	U9
<i>A. veronii</i>	SMC9U	~225	551	471	351	~810	~371	U10

MLST loci: *gyrB*, β subunit of DNA gyrase; *groL*, type I chaperon; *gltA*: citrate synthase I, *metG*: methionyl-tRNA synthetase, *ppsA*: phosphoenolpyruvate synthase, *recA*: recombinase A

3.3.8 Genetic determinants of virulence

A total of 18 genes associated with three different secretory systems including Type 1 Secretion System (T1SS), Type 2 Secretion System (T2SS) and Type 3 Secretion System (T3SS) were detected at varying frequencies from all sixteen genomes (Table 3.13). While gene sequences related to T1SS (1) and T3SS (4) were restricted to only *A. veronii* and *A. jandaei* strains, T2SS sequences (13) were detected in all 16 isolates. The cytotoxic enterotoxin gene *act*, serine protease gene *ser*, and DNase gene *exu* were detected in all sixteen *Aeromonas* spp. strains, while the aerolysin gene *aerA* was present in all isolates except the *A. jandaei* group (Table 3.10). Similarly, the lipase gene *lip*, was detected in all *A. hydrophila* isolates, two *A. veronii* isolates (JC1U

and DC2K), one *A. jandaei* (KMC10U) and the two *A. dhakensis* isolates (JC1K and SMC10U). The gelatinase gene, *gel* was present in none of the 16 *Aeromonas* spp. isolates.

Table 3. 13 Potential virulence genes detected in *Aeromonas* spp. isolates from diseased catfish from Nigeria.

Species	ID	T1SS	T2SS	T3SS	<i>aerA</i>	<i>hemolysin</i>	<i>act</i>	<i>ser</i>	<i>exu</i>	<i>lip</i>	<i>gel</i>
<i>A. hydrophila</i>	DC1K	-	+	-	+	+	+	+	+	+	-
<i>A. hydrophila</i>	KMC2U	-	+	-	+	+	+	++	+	+	-
<i>A. hydrophila</i>	KMC6U	-	+	-	+	+	+	+	+	+	-
<i>A. hydrophila</i>	SMC7K	-	+	-	+	+	+	+	+	+	-
<i>A. hydrophila</i>	SMC11U	-	+	-	+	+	+	+	+	+	-
<i>A. hydrophila</i>	SUC4U	-	+	-	+	+	+	+	+	+	-
<i>A. veroni</i>	JC1U	+	+	+	+	-	+	+	+	+	-
<i>A. veroni</i>	SC5K	+	+	-	+	-	+	+	+	-	-
<i>A. veroni</i>	DC2K	+	+	+	+	-	+	+	+	+	-
<i>A. veroni</i>	SMC8U	+	+	-	+	-	+	+	+	-	-
<i>A. veroni</i>	SMC9U	+	+	-	+	-	+	+	+	-	-
<i>A. jandaei</i>	KMC5U	+	+	+	-	-	+	+	+	-	-
<i>A. jandaei</i>	KMC7K	+	+	+	-	-	+	+	+	-	-
<i>A. jandaei</i>	KMC10U	+	+	+	-	-	+	+	+	+	-
<i>A. dhakensis</i>	JC1K	-	+	-	+	+	+	+	+	+	-
<i>A. dhakensis</i>	SMC10U	-	+	-	+	+	+	+	+	+	-

Virulence genes: T1SS, type 1 secretion system protein coding genes; T2SS: type 2 secretion system protein coding genes, T3SS: type 3 secretion system protein coding genes, *aerA*: aerolysin A gene, *hem*: haemolysin gene, *act*: cytotoxic enterotoxin gene, *ser*: serine protease gene, *exu*: DNase gene, *lip*: lipase gene, *gel*: gelatinase gene

3.3.9 Genetic determinants of resistance

3.3.9.1 Resistance genes detected via ABRicate and Roary pipelines

Detection of resistance genes within the genomes of the 16 sequenced *Aeromonas* species via ABRicate pipeline revealed the presence of genes mediating resistance to nine different antibiotic classes specifically beta-lactams, tetracyclines (Table 3.14), carbapenems, cephalosporins, sulfamethoxazole, trimethoprim, colistin, streptomycin and chloramphenicol (Table 3.15).

Two beta-lactam resistance genes, *bla* in all sixteen *Aeromonas* spp. genomes, and *bla1* in *A. jandaei* KMC10U genome, were detected via Roary. Through ABRicate, beta-lactam resistance gene *blaOXA-726* was detected in all *A. hydrophila* and *A. dhakensis* genomes and not in any other genome sequenced. Similarly, *blaOXA-12* was detected in one *A. veronii* strain SMC9U and all *A. jandaei* genomes. The presence of other genes conferring resistance to beta-lactams, including *blaFOX-7* and *blaOXA-725*, were restricted to only the *A. veronii* genomes, just as *blaAQU* and *blaCMY-FOX* were respectively detected only in *A. dhakensis* and *A. jandaei* genomes.

The only two cephalosporin resistance genes detected i.e., *cepS* and *cepH* were associated with only *A. hydrophila* genomes and not any other genome. Genes conferring resistance against carbapenems on the other hand were more widely distributed. These genes including *imiH* and *cphA3* that were associated only with *A. hydrophila* and *A. veronii* respectively. The other three genes including *cphA2* was detected in *A. hydrophila* and *A. dhakensis*, *cphA4* in *A. veronii* and *A. jandaei*, the last one, *cphA7*, in *A. hydrophila*, *A. veroni*, and *A. jandaei*.

Two tetracycline resistance genes including *tetA* and *tetA* were respectively restricted to one *A. jandaei* and one *A. hydrophila* isolate while *tetA* was detected in two *A. hydrophila*, two *A. veronii*, and two *A. jandaei* isolates. Sulfonamide drug resistance determinant, *sul1* was detected in one *A. hydrophila* and one *A. jandaei* isolates while *sul2* was found in *A. dhakensis* JC1K. The trimethoprim resistance gene *dfrA1* was detected in the same *A. jandaei* isolate carrying *sul1*, while two copies of the mobile

colistin resistance gene *mcr-7.1* was found in all three *A. jandaei* isolates. Streptomycin resistance genes *aadA1* and *aph(3')-Ib* were respectively detected in *A. hydrophila* KMC2U and *A. dhakensis* JC1K, while the chloramphenicol resistance genes *catB2* and *catA2* were also detected in *A. hydrophila* KMC2U and *A. veronii* DC2K respectively.

Table 3. 14 Beta-lactam and tetracycline resistance genes detected on chromosomal and plasmid DNA (presence, +; absence, -) of *Aeromonas* isolates from diseased catfish in Nigeria using ABRicate and Roary (in square brackets) pipelines before curation and after resolving gene identities (in bold italics) by manual curation (BLASTp + pFAM + Tree)

	Species	Strain ID	Beta-lactam												Tetracycline											
			Before								After				Before						After					
			[bla]	[bla1]	blaAQU	blaCMY-FOX	blaFOX-7	blaOXA-12	blaOXA-725	blaOXA-726	blaAQU-3	blaFOX-7	blaOXA-12	blaOXA-724	[tetA]	[tetA1]	[tetA2]	[tetR]	tetC	tetE	tetA(A)	tetA(C)	tetA(E)	tetR(A)	tetR(C)	tetR(E)
1	<i>A. hydrophila</i>	DC1K	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
2	<i>A. hydrophila</i>	KMC2U	+	-	-	-	-	-	+	-	-	-	+	-	+	-	-	+	-	-	+	-	-	+	-	
3	<i>A. hydrophila</i>	KMC6U	+	-	-	-	-	-	+	-	-	-	+	-	-	+	+	-	+	-	-	+	-	-	+	
4	<i>A. hydrophila</i>	SMC7K	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
5	<i>A. hydrophila</i>	SMC11U	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
6	<i>A. hydrophila</i>	SUC4U	+	-	-	-	-	-	+	-	-	-	+	-	-	+	+	-	+	-	-	+	-	-	+	
7	<i>A. veroni</i>	JC1U	+	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	
8	<i>A. veroni</i>	SC5K	+	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	
9	<i>A. veroni</i>	DC2K	+	-	-	-	-	+	-	-	-	+	-	+	-	+	+	-	+	-	-	+	-	-	+	
10	<i>A. veroni</i>	SMC8U	+	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	
11	<i>A. veroni</i>	SMC9U	+	-	-	-	+	+	-	-	+	+	-	+	-	+	+	-	+	-	-	+	-	-	+	
12	<i>A. jandaei</i>	KMC5U	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	(+)	-	-	(+)	-	-	+	
13	<i>A. jandaei</i>	KMC7K	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	(+)	-	-	(+)	-	-	+	
14	<i>A. jandaei</i>	KMC10U	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	
15	<i>A. dhakensis</i>	JC1K	+	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
16	<i>A. dhakensis</i>	SMC10U	+	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	
No of isolates (out of 16)			16	1	2	3	1	4	4	8	2	1	8	8	5	1	4	4	1	4	1	1	6	1	1	6

Table 3. 15 Resistance genes against carbapenems, cephalosporins (CEP), sulfonamides (SUL), trimethoprim (TMP), colistin (COL), streptomycin (STR), and chloramphenicol (CHL) detected in *Aeromonas* isolates from diseased catfish in Nigeria via ABRicate and Roary (in square brackets) pipeline. Presence, +; absence, -, two gene copies present, ++.

S/No	Species	ID	Carbapenems					CEP		SUL		TMP	COL			STR		CHL	
			<i>imiH</i>	<i>cphA2</i>	<i>cphA3</i>	<i>cphA4</i>	<i>cphA7</i>	<i>cepS</i>	<i>cepH</i>	<i>sul1</i>	<i>sul2</i>	<i>dfrA1</i>	<i>mcr-7.1</i>	<i>[mcr-3.2]</i>	<i>[Mcr-3.12]</i>	<i>aadA1</i>	<i>aph(3')-Ib</i>	<i>catB3</i>	<i>catA2</i>
1	<i>A. hydrophila</i>	DC1K	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
2	<i>A. hydrophila</i>	KMC2U	-	-	-	-	+	-	+	+	-	-	-	-	+	+	-	+	-
3	<i>A. hydrophila</i>	KMC6U	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
4	<i>A. hydrophila</i>	SMC7K	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
5	<i>A. hydrophila</i>	SMC11U	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
6	<i>A. hydrophila</i>	SUC4U	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
7	<i>A. veroni</i>	JC1U	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
8	<i>A. veroni</i>	SC5K	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
9	<i>A. veroni</i>	DC2K	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
10	<i>A. veroni</i>	SMC8U	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
11	<i>A. veroni</i>	SMC9U	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	<i>A. jandaei</i>	KMC5U	-	-	-	-	+	-	-	-	-	-	++	+	-	-	-	-	-
13	<i>A. jandaei</i>	KMC7K	-	-	-	-	+	-	-	-	-	-	++	+	-	-	-	-	-
14	<i>A. jandaei</i>	KMC10U	-	-	-	+	-	-	-	+	-	+	++	+	-	-	-	-	-
15	<i>A. dhakensis</i>	JC1K	-	+	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-
16	<i>A. dhakensis</i>	SMC10U	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
No of isolates (out of 16)			3	4	1	4	4	4	2	2	1	1	3	3	8	1	1	1	1

3.3.9.2 Manual curation of b-lactam, tetracycline and colistin resistance genes

To resolve the differences in gene identities specifically from the *bla* and *tet* group of genes between ABRicate and Roary pipelines (Table 3.14), all resistance genes detected from both pipelines were manually curated. Following this, genes identified as *blaOXA-726* by ABRicate were confirmed as *blaOXA-724*. Similarly, *blaOXA-725* from ABRicate was resolved as *blaOXA-12* increasing the number of genes in this class to eight. The gene *blaAQU* was also resolved as *blaAQU-3* while *blaCMY-FOX* was found to share no sequence homology with the *bla* group of genes. The genes *blaFOX-7* and *blaOXA-12* were found to be consistent after manual curation.

Manual curation of the *tet* genes (table 3.14) revealed that all three genes are different alleles of the *tetA* gene. Additionally, these genes exist alongside their respective regulatory genes in the same genomes. Colistin resistance determinants (Table 3.15), however, remained as predicted by both ABRicate and Roary following manual curation.

Resistance phenotype, corresponding number of resistance genes/mutation and the total number of resistance phenotypes accounted for by the presence of resistance genes/mutation are shown in Table 3.16. Only in the case of amoxycillin was at least one resistance gene detected in every NWT strain (15/15). The other six antibiotics with NWT strains including colistin, enrofloxacin, oxolinic acid, oxytetracycline, sulfamethoxazole, and florfenicol respectively had 10/12, 9/11, 9/15, 8/16, 3/16, and 0/2 resistance genes present. Additionally, resistance genes were detected in two wild type strains i.e., *A. jandaei* KMC10U (WT against amoxicillin) and *A. jandaei* KMC7K (WT against colistin).

Table 3. 16 Resistance phenotype (Type) and corresponding number of genes (n) or mutation (N) mediating resistance against oxytetracycline (OTC), sulfamethoxazole (SMX), amoxicillin (AMX), oxolinic acid (OXA), colistin (COL), enrofloxacin (ENR), florfenicol (FLO), gentamicin (GEN), erythromycin (ERY) and resistance phenotype accounted for by presence of resistance gene(s)/mutation (Genotype/phenotype) in *Aeromonas* species isolated from diseased catfish in Nigeria

Species	Strain ID	OTC		SMX		AMX		OXA		COL		ENR		FLO		GEN		ERY	
		Type	n	Type	n	Type	n	Type	N	Type	n	Type	N	Type	n	Type	n	Type	n
<i>A. hydrophila</i>	DC1K	NWT	0	NWT	0	NWT	1	NWT	0	NWT	1	WT	0	WT	0	WT	0	WT	0
<i>A. hydrophila</i>	KMC2U	NWT	2	NWT	1	NWT	1	NWT	1	NWT	1	NWT	1	WT	0	WT	0	WT	0
<i>A. hydrophila</i>	KMC6U	NWT	2	NWT	0	NWT	1	NWT	1	NWT	1	NWT	1	WT	0	WT	0	WT	0
<i>A. hydrophila</i>	SMC7K	NWT	0	NWT	0	NWT	1	NWT	0	NWT	1	WT	0	WT	0	WT	0	WT	0
<i>A. hydrophila</i>	SMC11U	NWT	0	NWT	0	NWT	1	NWT	0	NWT	1	WT	0	WT	0	WT	0	WT	0
<i>A. hydrophila</i>	SUC4U	NWT	2	NWT	0	NWT	1	NWT	1	NWT	1	NWT	1	WT	0	WT	0	WT	0
<i>A. veronii</i>	JC1U	NWT	0	NWT	0	NWT	1	NWT	1	NWT	0	NWT	1	WT	0	WT	0	WT	0
<i>A. veronii</i>	SC5K	NWT	0	NWT	0	NWT	1	NWT	0	WT	0	NWT	0	WT	0	WT	0	WT	0
<i>A. veronii</i>	DC2K	NWT	2	NWT	0	NWT	1	WT	0	WT	0	WT	0	WT	0	WT	0	WT	0
<i>A. veronii</i>	SMC8U	NWT	0	NWT	0	NWT	1	NWT	0	NWT	0	WT	0	WT	0	WT	0	WT	0
<i>A. veronii</i>	SMC9U	NWT	2	NWT	0	NWT	2	NWT	1	WT	0	NWT	1	WT	0	WT	0	WT	0
<i>A. jandaei</i>	KMC5U	NWT	2	NWT	0	NWT	1	NWT	1	NWT	2	NWT	1	WT	0	WT	0	WT	0
<i>A. jandaei</i>	KMC7K	NWT	2	NWT	0	NWT	1	NWT	1	WT	2	NWT	1	WT	0	WT	0	WT	0
<i>A. jandaei</i>	KMC10U	NWT	2	NWT	1	WT	1	NWT	1	NWT	2	NWT	1	NWT	0	WT	0	WT	0
<i>A. dhakensis</i>	JC1K	NWT	0	NWT	1	NWT	2	NWT	1	NWT	1	NWT	1	NWT	0	WT	0	WT	0
<i>A. dhakensis</i>	SMC10U	NWT	0	NWT	0	NWT	2	NWT	0	NWT	1	NWT	0	WT	0	WT	0	WT	0
Genotype/phenotype		8/16		3/16		15/15		9/15		10/12		9/11		0/2		0/0		0/0	

3.3.9.3 Resistance determinants of quinolone antibiotics

A single point mutation involving serine to isoleucine (Table 3.17) was observed within the quinolone resistance determining region of *gyrA* in 9/11 and 9/15 enrofloxacin and oxolinic acid NWT isolates respectively.

Table 3. 17 Mutations in the quinolone resistance determining region of *gyrA* from 16 *Aeromonas* spp. isolates sampled from diseased catfish in Nigeria and resistant phenotype.

S/No	ID	Species	ENR	Amino acid loci	Mutation	OXA
1	DC1K	<i>A. hydrophila</i>	WT	-	-	NWT
2	KMC2U	<i>A. hydrophila</i>	NWT	83	Ser → Ile	NWT
3	KMC6U	<i>A. hydrophila</i>	NWT	83	Ser → Ile	NWT
4	SMC7K	<i>A. hydrophila</i>	WT	-	-	NWT
5	SMC11U	<i>A. hydrophila</i>	WT	-	-	NWT
6	SUC4U	<i>A. hydrophila</i>	NWT	83	Ser → Ile	NWT
7	JC1U	<i>A. veronii</i>	NWT	83	Ser → Ile	NWT
8	SC5K	<i>A. veronii</i>	NWT	-	-	NWT
9	DC2K	<i>A. veronii</i>	WT	-	-	WT
10	SMC8U	<i>A. veronii</i>	WT	-	-	NWT
11	SMC9U	<i>A. veronii</i>	NWT	83	Ser → Ile	NWT
12	KMC5U	<i>A. jandaei</i>	NWT	83	Ser → Ile	NWT
13	KMC7K	<i>A. jandaei</i>	NWT	83	Ser → Ile	NWT
14	KMC10U	<i>A. jandaei</i>	NWT	83	Ser → Ile	NWT
15	JC1K	<i>A. dhakensis</i>	NWT	83	Ser → Ile	NWT
16	SMC10U	<i>A. dhakensis</i>	NWT	-	-	NWT
Total			11 (NWT)			15(NWT)

ENR: Enrofloxacin, OXA: Oxolinic acid

3.3.9.4 Multidrug resistance efflux pumps

Genes coding for multidrug resistance efflux pumps detected within the genomes of isolated *Aeromonas* spp. is presented in Table 3.18. Genes including *emrA*, *emrB*, *emrD*, *mdtL_1*, *mdtA_3*, *acrA*, and *acrB* were each detected in all sixteen isolates. Another gene, *emrE* was detected only in *A. hydrophila* strain KMC2U and *A. dhakensis* JC1K. Others including *mexA* and *mexB* were distributed between *A. hydrophila* and *A. jandaei*, and *A. hydrophila* and *A. veronii* respectively.

Table 3. 18 Genes coding multidrug resistance efflux pumps identified from the genomes of *Aeromonas* spp. isolated from diseased catfish in Nigeria.

Strain ID	Species	<i>emrA</i>	<i>emrB</i>	<i>emrD</i>	<i>emrE</i>	<i>mexA</i>	<i>mexB</i>	<i>mdtL_1</i>	<i>mdtA_3</i>	<i>acrA</i>	<i>acrB</i>
DC1K	<i>A. hydrophila</i>	+	+	+	-	-	-	+	+	+	+
KMC2U	<i>A. hydrophila</i>	+	+	+	+	+	-	+	+	+	+
KMC6U	<i>A. hydrophila</i>	+	+	+	-	-	+	+	+	+	+
SMC7K	<i>A. hydrophila</i>	+	+	+	-	-	-	+	+	+	+
SMC11U	<i>A. hydrophila</i>	+	+	+	-	-	-	+	+	+	+
SUC4U	<i>A. hydrophila</i>	+	+	+	-	-	-	+	+	+	+
JC1U	<i>A. veronii</i>	+	+	+	-	-	+	+	+	+	+
SC5K	<i>A. veronii</i>	+	+	+	-	-	+	+	+	+	+
DC2K	<i>A. veronii</i>	+	+	+	-	-	+	+	+	+	+
SMC8U	<i>A. veronii</i>	+	+	+	-	-	+	+	+	+	+
SMC9U	<i>A. veronii</i>	+	+	+	-	-	+	+	+	+	+
KMC5U	<i>A. jandaei</i>	+	+	+	-	+	-	+	+	+	+
KMC7K	<i>A. jandaei</i>	+	+	+	-	+	-	+	+	+	+
KMC10U	<i>A. jandaei</i>	+	+	+	-	+	-	+	+	+	+
JC1K	<i>A. dhakensis</i>	+	+	+	+	-	-	+	+	+	+
SMC10U	<i>A. dhakensis</i>	+	+	+	-	-	-	+	+	+	+

3.3.9.5 PCR amplification of manually curated resistance genes

Primers designed for the amplification of manually curated *bla* resistance (Table 3.2) genes produced expected amplicons between 100 and 200 bp marker bands (Figure 3.4). However, *blaFOX-7* primer produced amplicons of expected sizes in two of the three negative controls used. Similarly, a higher molecular weight amplicon than expected was produced in the negative control for *blaOXA-12*.

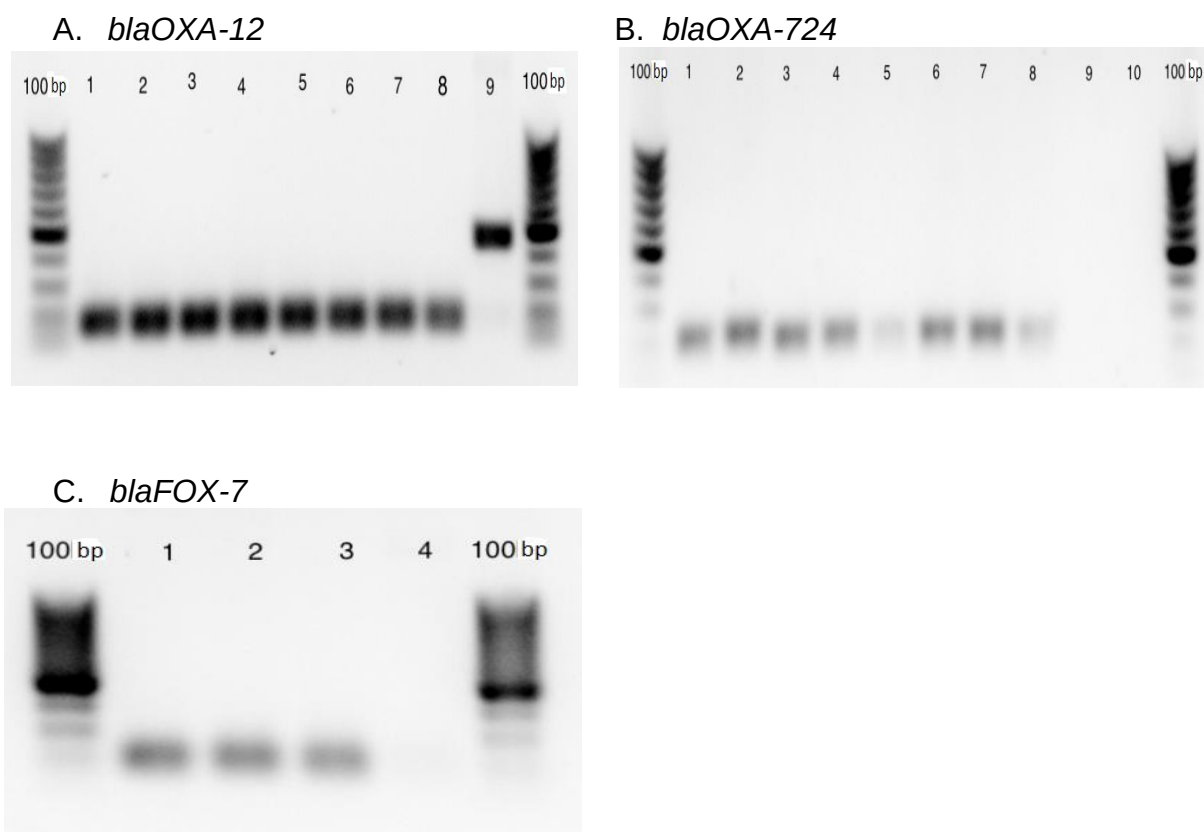


Fig 3. 4 PCR amplifcons of *bla* resistance genes. A: 1, DC1K; 2, JC1U; 3, SC5K; 4, SMC8U; 5, SMC9U; 6, KMC5U; 7, KMC7K; 8, KMC10U; 9, JC1K (-ve control); B: 1, SMC10U; 2, JC1K; 3, DC1K; 4, KMC2U; 5, KMC6U; 6, SMC7K; 7, SMC11U; 8, SUC4U; 9, JC1U (-ve control); 10, KMC5U (-ve control); C: 1, SMC9U; 2, DC1K; 3, KMC5U; 4, JC1K

Tetracycline resistance genes (Figure 3.5) including *tetA(C)* and *tetA(E)* and their respective regulatory genes, *tetR(C)* and *tetR(E)* produced amplicons of expected sizes and from only the predicted strains. The other tetracycline resistance gene, *tetA(A)* and its regulatory gene, *tetR(A)*, on the other hand, were amplified in both predicted strains and some control strains (WT).

The primer for the amplification of the mobile colistin resistance gene, *mcr-3.12*, successfully amplified the gene only from *A. dhakensis* strains and not from *A. hydrophila* as predicted from the bioinformatic analyses or from any of the WT control strains (Figure 3.6). The primer set for the other colistin resistance genes, *mcr-3.2* and *mcr-3.7*, however, amplified these genes in both predicted and some control strains.

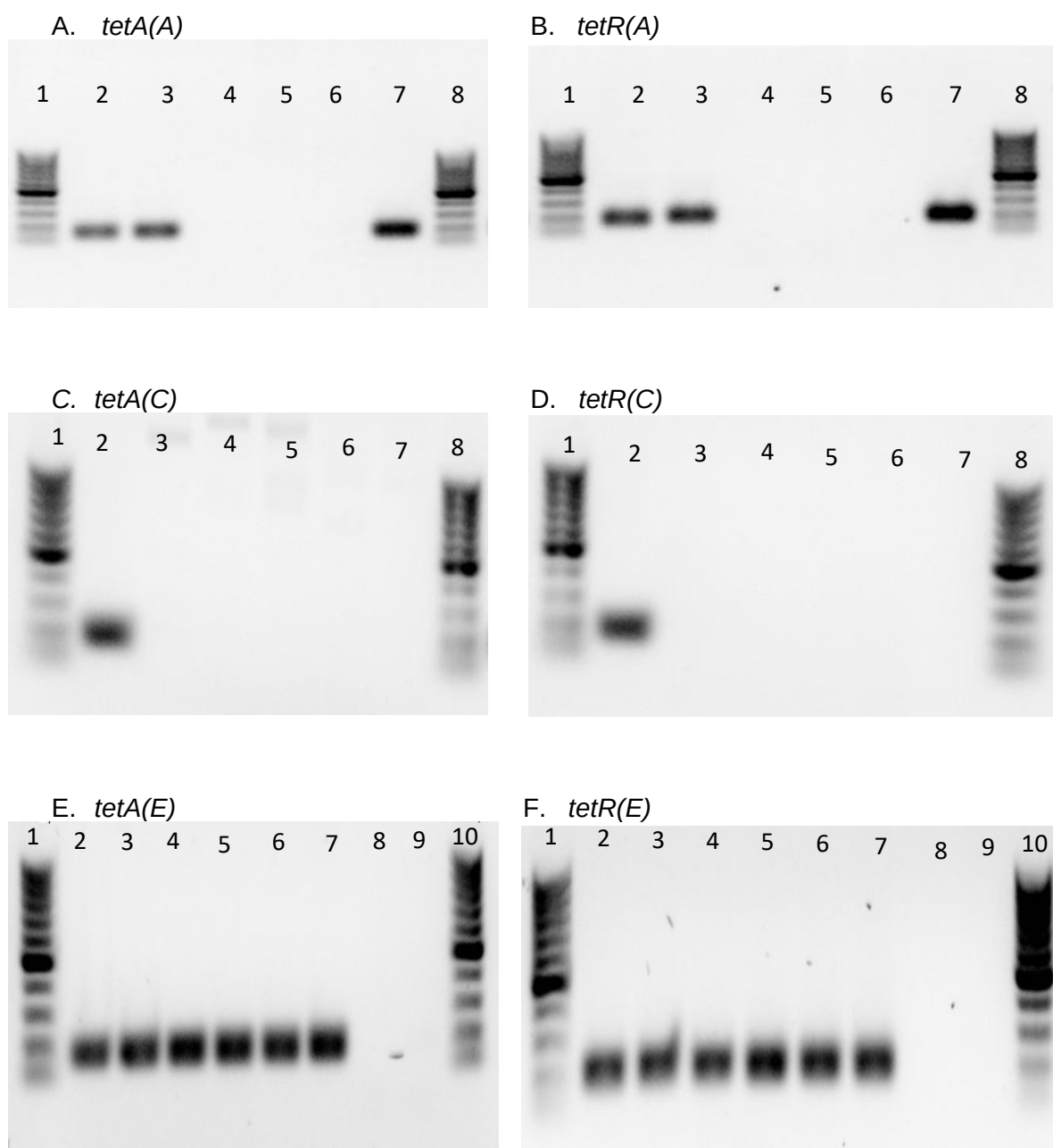


Fig 3. 5 PCR amplicons of *tet* resistance genes. A/B: 1, 100 bp ladder; 2, KMC10U; 3, KMC6U (-ve control); 4, KMC2U (-ve control); 5, SMC7K (-ve control); 6, SMC8U (-ve control); 7, JC1K (-ve control); 8, 100 bp ladder; C/D: 1, 100 bp ladder; 2, KMC2U; 3, KMC6U (-ve control); 4, SMC7K (-ve control); 5, SC5K (-ve control); 6, JC1K (-ve control); 7, SMC10U (-ve control); 8, 100 bp ladder; E/F: 1, 100 bp ladder; 2, KMC6U; 3, SUC4U; 4, DC2K; 5, SMC9U; 6, KMC5U; 7, KMC7K; 8, KMC2U (-ve control); 9, KMC10U (-ve control); 10; 100 bp ladder.

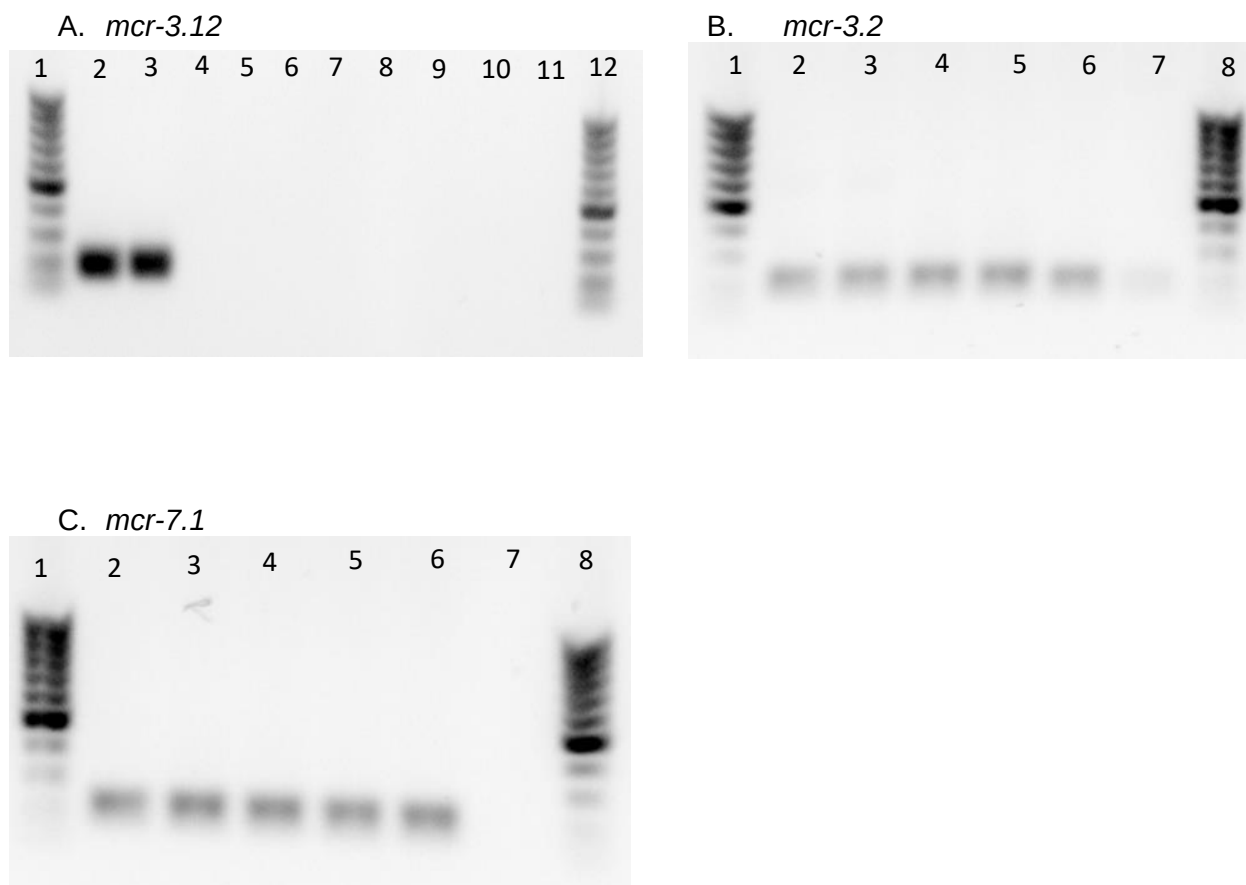


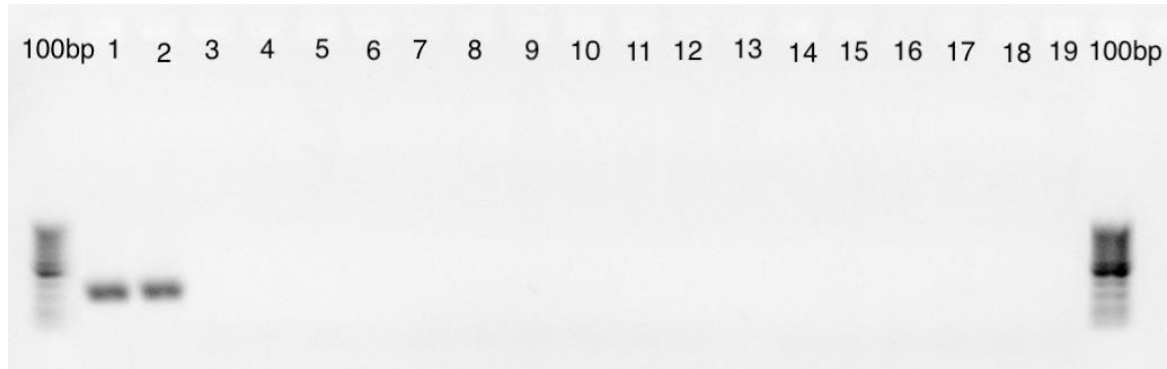
Fig 3. 6 PCR amplicons of *mcr* resistance genes. A: 1, 100 bp ladder; 2, SMC10U; 3, JC1K; 4, DC1K; 5, KMC2U; 6, KMC6U; 7, SMC7K; 8, SMC11U; 9, SUC4U; 10, JC1U (-ve control); 11, KMC5U (-ve control); 12, 100 bp ladder; B: 1, 100 bp ladder; 2, KMC5U; 3, KMC7K; 4, KMC10U; 5, JC1K; 6, DC1K; 7, SC5K; 8, 100 bp ladder; C: 1, 100 bp ladder; 2, KMC5U; 3, KMC7K; 4, KMC10U; 5, JC1K; 6, DC1K; 7, SC5K; 8, 100 bp ladder

3.3.10 Identification with *A. dhakensis* and vAh ST251 specific primers

As expected, the *intA_5* primer for detection of hypervirulent *A. hydrophila* (vAh) did not produce an amplicon from any of the *A. hydrophila* strains or from other *Aeromonas* spp. tested other than with the two control strains (*A. hydrophila* TN27 vAh ST251, *A. hydrophila* TN1 vAh ST251) (Figure 3.7). The *yjcS* primer for detection of *A. dhakensis* produced expected amplicons for both *A. dhakensis* strains from this study as well as the four positive *A. dhakensis* control isolates (*A. dhakensis* TN5, *A.*

dhakensis TN49, *A. dhakensis* CIP 107500T, *A. dhakensis* CECT 7289) and not from any other *Aeromonas* spp. (Figure 3.7).

A. *intA_5*



B. *yjcS*



Fig 3. 7 PCR amplification of the *A. dhakensis int_5* gene and *A. hydrophila yjcS* genes. A: 1-2, vAh ST251 positive controls; 3-4, *A. hydrophila* negative controls from Kerry et al., 2023; 5-9, *A. hydrophila* from this study; 10-11, *A. dhakensis* from this study; 12-16, *A. veroni* from this study; 17-19, *A. jandaei* from this study. B: 1-4, *A. dhakensis* positive controls; 5-6, *A. dhakensis* from this study; 7-20, negative controls.

3.4 Discussion

Aquaculture plays an important role in food security and socioeconomic life of the Nigerian population (Okocha et al., 2021a). However, diseases, complicated by the absence of an effective disease control framework including legislative, human and infrastructure pose a serious challenge to the sustainability of the industry (Imade & Akinnibosun, 2018). This has facilitated the indiscriminate use of antibiotics by farmers for disease control. Comprehensive information on pathogenic bacteria responsible for specific disease outbreaks of fish within aquaculture facilities is still lacking from published literature in this country. Identifying strains of pathogenic bacteria in circulation within the system and elucidating the level of antibiotic resistance in these strains will guide targeted control strategies aimed at reducing loss and ensuring sustainability.

Bacteria were isolated from diseased catfish (*C. gariepinus*) samples drawn from farms and markets, identified, and characterised for virulence and antibiotic resistance using biochemical methods, phenotypic assays and molecular techniques. A combination of primary identification tests (Gram staining and biochemical tests), 16S *rRNA* gene sequencing and ANI score of genomes, identified 16 isolates as *Aeromonas* spp. (six *A. hydrophila* isolates, five *A. veronii*, three *A. jandaei* and two *A. dhakensis*). Additionally, MLST typing revealed three isolates i.e., *A. dhakensis* strain JC1K, *A. dhakensis* SMC10U, and *A. veronii* JC1U as ST557, ST696, and ST425 respectively, the remaining isolates being uncategorised. Members of the genus *Aeromonas* represent an important group of pathogens with a diverse range of hosts primarily aquatic species causing septicaemia and furunculosis (Lim & Hong, 2020; Ünver & Bakıcı, 2021). They are also implicated in cases of gastrointestinal infections and septicaemia in humans where they are considered emerging pathogens (Gonçalves Pessoa et al., 2019; Li et al., 2022; Wamala et al., 2018).

Isolation of multiple *Aeromonas* spp. from fish including those reported from this study is consistent with a recent report from the study area (Adah et al., 2024) and another study from Southern China (Li et al., 2022). While *A. hydrophila* is widely incriminated as the main cause of Motile *Aeromonas* Septicaemia (Abdelhamed et al., 2019; Barger et al., 2020; D. Zhang et al., 2020), other species such as strains of *A. veronii* have

been identified as the etiologic agent of this condition in diseased rohu (*Labeo rohita*), a species of carp (Tyagi et al., 2022). Similarly, *A. veronii* has been isolated at a higher prevalence compared to other *Aeromonas* spp. from diseased ornamental fish from Sri Lanka (Tyagi et al., 2022). These findings in addition to the comparatively high number of *A. veronii* strains isolated in this study might suggest a growing significance of *A. veronii* as a fish pathogen in *Clarias* catfish.

Overall, *Aeromonas* spp. were recovered at a higher rate (16/42) than other genera and were widespread in distribution. *A. hydrophila* strains were isolated from both sampling sites i.e., farm and market in two of the three sampled states. Although each of the *A. dhakensis* isolates came from different sites, both originated from the same state. Similarly, all five *A. veronii* strains were isolated from the same state but included two strains from the market and the other three isolates from different farms. The three *A. jandaei* strains also originated from the same market in one state. The fact that two of these *A. jandaei* strains came from fish samples collected on the same day from the same retailer might suggest they originate from the same farm. These findings suggest that *A. hydrophila* may be more widely distributed than other *Aeromonas* spp. in northern Nigeria and might be more important in bacterial diseases of catfish (*C. gariepinus*) in this region. The fact that samples were collected within a three-month window in a single year did not permit the evaluation of seasonality on the distribution of *Aeromonas* spp. from this study. A further study involving samples from different times of the year will be necessary to evaluate this. This finding contradicts another report from the same region where a higher number of *A. caviae* and *A. veronii* were isolated from diseased catfish (*C. gariepinus*) compared to *A. hydrophila* (Adah et al., 2024). The fact that this present study involved a smaller number of samples and the failure to recover all bacterial isolates while that of Adah et al., (2024) involved higher number of samples collected over a 12 months period might explain the contradiction between the two studies (Adah et al., 2024). While time constraint might have hindered evaluation of seasonal distribution of *Aeromonas* spp. from the study area, this study has nonetheless, highlighted the role of this important group of pathogens in bacterial diseases of catfish in the study area.

Genome sequencing permitted identification of the STs by MLST and individual strain characterisation with an insight into the molecular epidemiology. Whereas *A.*

dhakensis ST557 was incriminated in a fatal case of coinfection with hepatitis B virus in a twenty-nine-year-old patient following consumption of snakehead (*Channa argus*) in mainland China (Huang et al., 2020), this is the first report of either ST557 or the other two STs from catfish in Nigeria. Both ST557 and ST425 (isolated from a case of enteritis in China) have been isolated from human cases of infection which has obvious zoonotic implications for these strain types. The remaining STs were uncategorised and largely variable in nature, However, for three unknown STs, identical STs were found in isolates sampled from different fish associated within the same market sampling location (*A. hydrophila* unknown ST U4 and *A. jandaei* unknown ST U5) and shared between different sites for unknown strain type U3 *A. hydrophila*.

Between one and four plasmids ranging in size from 2.7 to 9.2 kb were detected in nine isolates distributed across all four *Aeromonas* spp. from this study. Plasmids and transposons are known to facilitate conjugative transfer of resistance genes (Pepi & Focardi, 2021). Given the role of plasmids in horizontal gene transfer, this finding further highlights the role of aquaculture in the emergence and dissemination of antibiotic resistance. In addition to facilitating antibiotic resistance, plasmids may encode other traits such as virulence, metabolic capacities, and functions that enable bacteria to persist in the environment by resisting noxious agents like heavy metals (Opal & Pop-Vicas, 2020b). Two plasmids from this study were found to contain *tetE* gene that mediates resistance against tetracycline antibiotics. This finding is consistent with the findings of Pérez-García et al. (2021), who detected these sequences encoding resistance proteins and other factors in multiple plasmids from mesophilic aeromonads from fish, water and sediment samples in Mexico.

Bacterial secretion systems play an important role in determining bacterial survivability, colonisation and virulence (Horna & Ruiz, 2021; Korotkov & Sandkvist, 2019) including within the *Aeromonas* genus (Barger et al., 2020). Three different type secretion systems (TSS) including TSS1, TSS2, and TSS3 were evident by the presence of genes encoding various components of these systems within the study genomes. The T3SS has been described as the most prevalent secretion system among members of the genus *Aeromonas* (Gonçalves Pessoa et al., 2019). This study, however, found the T2SS to be the most common among the four *Aeromonas*

spp. identified. This finding is consistent with that of Tyagi et al., (2022) who reported T2SS in 86.2% of *A. veronii* isolates as compared to 27.6% for T3SS from rohu (*Labeo rohita*), in India. Additionally, T1SS and T3SS were restricted to *A. veronii* and *A. jandaei* isolates in this present study. This might suggest that these two secretion systems are more relevant in the pathogenesis of *A. veronii* and *A. jandaei*.

The *prfE* gene (Appendix 3.2) found in *A. veronii* and *A. jandaei* field isolates codes for the membrane fusion protein, PrfE, of the T1SS (Lucke et al., 2020). In addition to connecting the other two components of T1SS i.e., ABC transporter of the inner membrane and the outer membrane protein, this protein, through its N-terminal cytoplasmic part, is involved in substrate recognition thus making it a critical component of the system (Kanonenberg et al., 2013). Substrates associated with this secretion system such as haemolysins, proteases, lipase for which phenotypic evidence of their activity was demonstrated in this study, are important in bacterial pathogenesis (Kanonenberg et al., 2013) by mediating red blood lysis and host tissue damage.

Thirteen genes coding for different components of T2SS were identified (Appendix 3.2), some present in all isolates and others restricted to some species. Unlike T1SS and T3SS that transport substrates in a one step process, T2SS is a two-step translocation system that involves substrate modification within the periplasmic space after crossing the cytoplasmic membrane before being translocated across the outer membrane in the second step (Korotkov & Sandkvist, 2019). This system confers survival advantage to pathogenic and environmental bacteria by mediating the extracellular transport of various toxins, lipase and enzymes that digest complex carbohydrates, nucleic acids, proteins facilitating adherence and biofilm formation, and notoriously recognised for its prolific secretion of the protease enzyme (Korotkov & Sandkvist, 2019). Although the structural relationship between various components of the system has received significant attention and are well understood (Korotkov & Sandkvist, 2019), the mechanism behind the successful translocation of both soluble and insoluble proteins across the outer membrane is still yet to be elucidated (Korotkov & Sandkvist, 2019).

Type three secretion system (T3SS), also known as the injectisome, has been shown to be the most important virulence factor in *A. salmonicida* (Vanden Bergh & Frey, 2014). It forms a needle-like apparatus for direct delivery of effector proteins into host cytoplasm (Tyagi et al., 2022). The systems involvement in suppression of the fish immune system, in addition to secretion of extracellular virulence factors has been extensively reviewed by Gonçalves Pessoa et al. (2019) and Vanden Bergh & Frey, (2014). It acts as a delivery channel for the injection of effectors that undermine the host immune response and alter critical host pathways such as the actin cytoskeletal signal transduction pathway important in maintaining structural integrity of eukaryotic cells, cell division and many other important functions (Balta et al., 2021), leading to bacterial colonisation, survival, and eventual replication (Horna & Ruiz, 2021). Similar T3SS structural protein coding genes of *Pseudomonas aeruginosa*, *pscS* and *pscR* (Horna & Ruiz, 2021), were identified in *A. veronii* and *A. jandaei* isolates in this present study. While homologous genes namely *ascS* and *ascR* have been reported in *A. salmonicida* (Vanden Bergh & Frey, 2014), this is the first study reporting the *pscS* and *pscR* genes in *Aeromonas* spp. These two genes together with a third gene, *pscT* – not found in this study – encode the inner membrane proteins PscS, PscR, and PscT which along with their secretory regulator, PscU, and another protein PcrD make up the export apparatus or the cup for the secretion of the T3SS components. This along with the basal body, constitute the membrane component of T3SS in *Pseudomonas aeruginosa* (Horna & Ruiz, 2021). This finding suggests a possible horizontal transfer from *Pseudomonas* spp. or a strong homology at least in the genes coding for the export apparatus of the T3SS in both *P. aeruginosa* and these *Aeromonas* spp. This may not be surprising since both are members of the ‘Ycs’ family of T3SS, whose members share high level of T3SS structural protein conservation – particularly in the membrane components (Vanden Bergh & Frey, 2014). A further elucidation of this structure in motile *Aeromonas* spp. might reveal the exact functional relationship of this protein with other components of the system and its role in the virulence of motile *Aeromonas* spp..

Lipase enzyme (*lip*) and aerolysin (*aerA*) genes were detected in all species except *A. jandaei*, while the haemolysin gene (*hly*) was restricted to *A. hydrophila* and *A. dhakensis* isolates. Genes encoding cytotoxic enterotoxin (*act*), serine protease (*ser*), and DNase (*exu*), however, were detected in all four species. No specific genetic

determinant of gelatinase activity was found in any of these isolates. Cytotoxic enterotoxin encoded by *act*, mediates degenerative damage on intestinal villi and mucus-producing cells – associated with bloody diarrhoea in humans – as well as induces haemolytic activity (Gonçalves Pessoa et al., 2019). Aerolysin is an extracellular protein, which mediates osmotic lysis of blood cells after binding a membrane-specific glycoprotein resulting in altered cell permeability while lipase is involved in the hydrolysis of membrane lipid and causes intestinal damage (Gonçalves Pessoa et al., 2019). The absence of specific genetic determinants in isolates with evidence of *in vitro* phenotypic activity of these enzymes might be explained by the presence genetic determinants of other virulence factors in these isolates. For example, in addition to its primary caseinolytic effect resulting in host tissue damage, bacterial invasion, haemorrhages and skin ulcers, serine protease also acts on host nutrient sequestration and immune response as well as the activation of glycerophospholipid-cholesterol acyltransferase (GCAT), a lipase enzyme described as a very lethal virulence factor in *A. salmonicida* capable of damaging erythrocyte membrane (Gonçalves Pessoa et al., 2019; Tyagi et al., 2022). Additionally, the varying distribution of virulence factor genes from this study corroborate the assertion of Tyagi et al. (2022) on the wide expression of extracellular toxins/enzymes among aeromonads.

Antibiotics exert their effect on bacteria through the inhibition of critical bacterial processes including cell wall, protein, and nucleic acid synthesis or through antimetabolite activity, alteration of cell membrane, or competitive antagonism (Pepi & Focardi, 2021). Development of resistance confers selective advantage to bacteria allowing them to evade the antibiotic effect that ensures their survival and propagation to the detriment of susceptible members of the bacterial community (Pepi & Focardi, 2021). Antibiotic resistant bacteria have been described as a global threat to health and livelihoods (Gaurav et al., 2023a). This study found evidence of acquired resistance and/or mutations to amoxicillin, oxytetracycline, sulfamethoxazole, colistin, oxolinic acid, enrofloxacin, and florfenicol in all *Aeromonas* spp. isolated. The highest levels were recorded with oxytetracycline, sulphamethoxazole, amoxicillin, and oxolinic acid. This finding agrees with that of (Gaurav et al., 2023a) who noted a high level of resistance in *Aeromonas* spp. of zebra fish origin against the same classes of antibiotics as tested in this study. Additionally, presumptive multiple antibiotic

resistance (MDR) as demonstrated by an NWT profile of a minimum of three and a maximum of seven antibiotics was observed in all isolates. This likely indicates widespread antibiotic usage by fish farmers in this part of Nigeria (Ladan et al., 2021). A similar MDR pattern of concern was reported in catfish associated *Aeromonas* spp. by Adeyemi et al., (2022) host from south-western Nigeria, Dhanapala et al., (2021) from fish host in Sri Lanka and Hossain et al., (2019) from fish host Korea. However, Hayatgheib et al., (2021) reported only 36% presumptive multi-drug resistant *Aeromonas* spp. in fish from France. The difference between this study and that of Hayatgheib et al., (2021) might be explained by the strict regulation of antibiotic use for animal production in Europe compared to Nigeria (Ferri et al., 2022).

Antibiotic use in aquaculture is associated with selection and development of antibiotic-resistant pathogens (Brunton et al., 2019; Desbois et al., 2021). Chen et al., (2020) describes the occurrence of antibiotic-resistant bacteria and antibiotic resistance genes in aquaculture as a threat to public health because of the potential of resistance transfer from aquatic organisms to humans. Furthermore, such therapeutic applications have been shown to result in antibiotic residues in fish tissues (Ladan et al., 2021; Okocha et al., 2021a) especially in countries where access to antibiotics is loosely regulated, thereby exposing consumers to sub-therapeutic doses of these compounds. The fact that evidence of acquired resistance in this study relates to antibiotics that have been classified as either critically important (amoxicillin, oxolinic acid, colistin) or highly important (oxytetracycline, sulfamethoxazole) for human medicine by WHO, makes this finding even more disturbing. While the idea of banning the use of certain antibiotics in aquaculture is an important and plausible step which has indeed been implemented in the European Union, North America and other developed nations (Lulijwa et al., 2020), the concept of cross resistance i.e., exposure to one class of antibiotic leading to resistance to an additional unrelated class of antibiotic, makes this approach alone insufficient in addressing the emergence and spread of antibiotic resistance especially in developing nations like Nigeria. Hence, regulating access to, as well as prudent use of antibiotics are complementary strategies for success.

Beta-lactam antibiotics acylate the enzyme transpeptidase involved in the cross-linking of the peptide bond thereby inhibiting this final step in the production of

peptidoglycan component of the bacterial cell wall resulting in loss of viability and lysis of the bacteria (Pepi & Focardi, 2021). Several beta-lactamase and metallo-beta-lactamase genes were found in this study which could explain the NWT phenotype observed in fifteen out of the sixteen isolates tested. Additionally, aeromonads are known to be intrinsically resistant to penicillins (Dhanapala et al., 2021a). A similarly high level of resistance against beta-lactam antibiotics has been reported in numerous studies (Dhanapala et al., 2021a; Hossain et al., 2019a). The detection of beta-lactamase resistance genes in this study including *blaAQU-2*, *blaAQU-3*, *blaFOX-7* of class C in *A. veronii* and *A. jandaei* isolates including the WT strain and the OXA-type (*blaOXA-12* and *blaOXA-724*) of class D – implicated in much of the resistance cases involving beta-lactam antibiotics (Wang et al., 2021) from *A. hydrophila* and *A. dhakensis* isolates, and *cepS* of class C in *A. hydrophila* alone is consistent with the report of Dubey et al., (2022). who found similar genes with the exception of *blaAQU-3* and *Aeromonas* spp. distribution from different hosts including catfish, swordtail fish, Indian carp, insect, sardine and humans. Similarly, Ramsamy et al., (2021) found *blaOXA-724* in *A. hydrophila* strains from a pig slaughterhouse in South Africa, while *blaOXA-12* and *blaFOX-7* were reported in *A. veronii* of a diseased Indian carp origin (Tyagi et al., 2022) and in catfish pond sediment (Dubey et al., 2023). While this distribution might suggest a degree of species specificity of some beta-lactamase genes among aeromonads, other reports point to the contrary. As an example, *blaOXA-12* and *blaOXA-724* have detected in beta-lactam resistant *A. veronii* and in *A. jandaei* from a septicemic patient in Thailand, catfish, catfish pond sediment and chicken from USA (Dubey et al., 2023; Hatrongjit et al., 2020; Wang et al., 2021). These enzymes hydrolyse almost all beta-lactam antibiotics including those considered as drugs of last resort which can have serious consequences in healthcare settings. Finding these genes suggest the involvement of beta-lactamase genes in emergence and dissemination of resistance in different animal hosts and the environment. A gene might be present in its active form but not expressed for several reasons or could exist in pseudo form containing a stop codon which could be the case with the WT strain in this study.

This study found multiple efflux pumps in *Aeromonas* spp. which is consistent with the findings of (Dubey et al., 2022). Because of their ability to pump out a broad range of structurally and chemically diverse substrates, efflux pumps play an important role in

the emergence of multidrug resistant bacteria, in addition to virulence and many bacterial survival/adaptive mechanisms (Gaurav et al., 2023a). While six major families of efflux pumps have been described in bacteria, the resistance-nodulation-cell division (RND) superfamily is the most potent and crucial for the development of antibiotic resistance, virulence, and biofilm formation in Gram-negative bacteria (Athar et al., 2023; Gaurav et al., 2023a). Genes including *acrA*, *acrB*, *acrE*, and *mexB* that code for various components of the RND superfamily were found in this study. This efflux pump is involved in the extrusion of and thus mediates bacterial resistance against quinolones, tetracyclines, penicillins, phenicols, rifamycins, macrolides and other antibiotics (Athar et al., 2023; Tyagi et al., 2022).

This study found all isolated *Aeromonas* spp. displaying the NWT phenotype against oxytetracycline. High level of oxytetracycline NWT aeromonads and non-aeromonads isolated from fish were also reported previously (Saticioglu et al., 2019; Woo et al., 2022). However, Hayatgheib et al., (2021) reported a lesser level of oxytetracycline of NWT aeromonads from fish in France. While the difference in sample size between this study and that of Hayatgheib et al., (2021) might offer an explanation for the difference in NWT level, better control over antibiotic use for fish production in France sounds more plausible. Specific resistance genes attributable to such phenotype were, however, found in 50% (8/16) of the isolates from this study. Two of these isolates had the gene (*tetE*) located on plasmids. Absence of specific resistance genes in some of these isolates is consistent with the findings of Saticioglu et al., (2019) who found no evidence of any of the *tet* genes screened in NWT *F. psychrophilum* isolates. This absence could be explained by the presence of efflux pumps as has been discussed previously or may suggest the involvement of other resistance mechanisms against oxytetracycline in these isolates. The three different tetracycline resistance genes detected in this study (*tetA*, *tetC* and *tetE*) agree with earlier reports where multiple *tet* genes were identified in *Aeromonas* spp. (Hayatgheib et al., 2021; Woo et al., 2022).

All 16 isolates displayed an NWT phenotype against sulfamethoxazole. In a different study, however, most motile *Aeromonas* spp. from rainbow trout (*Oncorhynchus mykiss*), trout (*Salmo trutta macrostigma*) and common carp (*Cyprinus carpio*) Turkey were found to be susceptible to sulfamethoxazole (Duman et al., 2020) by MIC test. This suggests a more widespread use of sulfamethoxazole in Nigerian aquaculture

than in Turkey. Genes mediating resistance against sulphonamides including *sul1* and *sul2* detected in this study is consistent with findings in published literature (Duman et al., 2020; Thaotumpitak et al., 2023). The absence of these genes in most of the isolates suggests involvement of other resistance mechanisms. Efflux pumps indicated above could be involved in the ejection of multiple unrelated compounds (Gaurav et al., 2023a). Additionally, over production of para-aminobenzoic acid (PABA) or point mutations in the *folP* gene encoding structural changes in dihydropteroate synthetase resulting in an enzyme with reduced affinity for sulphonamide have been shown to produce resistance (Zinner & Mayer, 2020).

Quinolones exert broad spectrum activity against both Gram-positive and Gram-negative bacteria through disruption of the enzymes DNA topoisomerase IV and gyrase, thereby inhibiting nucleic acid synthesis and causing breakage of the bacterial chromosome (Pepi & Focardi, 2021). More NWT phenotypes for oxolinic acid (15/16) than for enrofloxacin (11/16) were observed in this study. While a *gyrA* point mutation (at amino acid locus 83) within the quinolone resistance determining region (QRDR) of DNA gyrase might explain the NWT phenotype against both antibiotics in nine out of the sixteen isolates, two and another four isolates without mutations in this region of DNA gyrase or topoisomerase IV were NWT against enrofloxacin and oxolinic acid respectively. This suggests the involvement of other resistance pathways in these six isolates that might not necessarily be present in the other isolates. Quinolones are known to be among the substrates of the RND superfamily of efflux pumps (Athar et al., 2023; Tyagi et al., 2022). Genes coding for components of this RND pump in addition to other efflux pumps were detected in these isolates. Phenotypic resistance to quinolones and the genetic basis of such phenotype from this study contradicts the report of Thaotumpitak et al. (2023) from Thailand who found a low resistance level (20%) against oxolinic acid and *qnrS* from 15 *A. hydrophila* strains. This might indicate a lower application of quinolones in tilapia production in the farms in Thailand Thaotumpitak et al. (2023).

Except for the two *A. veronii* strains, the presence of three mobile colistin resistance genes including *mcr-3.12*, *mcr-3.2*, and *mcr-3.7* found in nine of the eleven colistin NWT isolates likely explains their reduced susceptibility to this antibiotic. The presence of this gene in a WT *A. jandaei* and its absence from the two NWT *A. veronii* strains in

this study suggests a non-functional *mcr* gene and the role of other resistance mechanisms respectively. Similar genes including *mcr-3.27*, *mcr-3-like*, and *mcr-7-like* genes were identified in *A. jandaei*, *A. veronii*, and *A. hydrophila* by Li et al., (2022) while *mcr-3* was reported in an *A. veronii* isolate from Nile tilapia, *A. hydrophila* from hybrid red tilapia and a patient with septicaemia in Thailand (Dubey et al., 2022; Hatrongjit et al., 2020), and *Aeromonas* spp. from rainbow trout in France (Hayatgheib et al., 2021).

3.5 Conclusion

This study detected four key motile *Aeromonas* spp. including *A. hydrophila*, *A. veronii*, *A. jandaei* and *A. dhakensis* implicated in MAS from diseased *Clarias* spp. catfish in northern Nigeria and to a lesser extent other bacterial genera predominated by suspected *Pseudomonas* species. *Aeromonas* spp. from this study displayed *in vitro* phenotypic virulence properties and was found to bear genes encoding such traits which suggest they might be responsible for the symptoms observed in diseased catfish samples they were isolated from. Additionally, all *Aeromonas* spp. displayed presumptive multidrug resistance against three to six different antibiotic classes including those classified as critically important by WHO (J. Chen et al., 2020a). Genes determining such resistance phenotypes were also detected on both chromosomal and plasmid DNA. The evidence here highlights the critical role that aquaculture and the misuse of antibiotics play in fostering the emergence and dissemination of antibiotic resistance, and the risk of rapid spread. This study thus emphasises the significant economic and public health ramifications of *Aeromonas* spp., particularly in Nigeria's rapidly growing aquaculture sector. We therefore recommend the implementation of simple and cost-effective disease prevention strategies like training fish farmers on effective biosecurity to reduce losses, antibiotic use to ensure sustainable aquaculture production.

Chapter 4

Development and evaluation of an online workshop as an intervention to improve biosecurity implementation and awareness of fish farms in Northern Nigeria and the Federal Capital Territory, Abuja

4.1 Introduction

Increased aquaculture intensification has been associated with increased disease prevalence which in some instances almost resulted in the collapse of the sector (Kyule-Muendo et al., 2022). Deployment of adequate biosecurity is thus necessary if disease-associated losses are to be avoided, since this prevents emerging health challenges, curbs disease impacts and enhances profitability (Georges et al., 2023a), thereby mitigating the economic consequences associated with disease losses (Kyule-Muendo et al., 2022) and ensuring sustainability.

Biosecurity in aquaculture is a conscious effort that encompasses physical and management measures that cumulatively serve to reduce infection risk within the system/establishment (World Organisation for Animal Health, 2022). Biosecurity is thus the cornerstone of preventive medicine, and its successful implementation can result in reduced antibiotic use, improved productivity and animal health and welfare (Damiaans et al., 2020a; Scarfe & Palić, 2020). It represents an efficient and cost-effective way of both minimising stress and eliminating sources of infection that ultimately results in the production of healthy stock and a safe product (Adah et al., 2023; Behringer et al., 2020). Biosecurity consists of internal measures, taken to prevent the entry and establishment of infectious organisms, and external components, which are measures taken to prevent the spread and exit of such pathogens from a group of animals or a farm site (Damiaans et al., 2020a; Yanong & Erlacher-Reid, 2012).

Mortality on fish farms has been linked to breaches in biosecurity measures (Ali et al., 2020) and subsequently promoting the use of antibiotics for both prophylactic and therapeutic purposes with the consequential emergence and spread of antibiotic resistance (Adah et al., 2023a; Alhaji et al., 2021). In Chapter 2 of this thesis, biosecurity measures that included quarantine, restricted pond access, water quality assessment, dedicated farm clothing and proper disposal of fish mortalities were not commonly implemented. Commonly practised breaches in biosecurity included equipment sharing and failure to wash hands with soap and water before daily operations. Over 60% of survey participants reported they had disease episodes on their farms before the survey (Table 2.5). Similarly, (Khor et al., 2024) reported that

biosecurity was not implemented by most fish farms surveyed from Ogun and Delta states of southern Nigeria at the point of stocking. Furthermore, a significant association between high mortality and breach in biosecurity was also detected. Reducing the economic impact caused by such mortalities and the associated morbidities will thus require the implementation of appropriate biosecurity measures (Adah et al., 2023a). Both knowledge and practice of biosecurity among fish farmers in Nigeria has been reported as poor (Adah et al., 2023a; Alhaji et al., 2021; Khor et al., 2024; Nwabueze & Ofuoku, 2020; Omitoyin & Osakuade, 2021).

Interventions and education that serve to improve biosecurity practices among fish farmers will be a critical first step towards aquaculture disease prevention, control and eradication for sustainable aquaculture production in Nigeria. The introduction of legislations that addressed biosecurity risk factors contributed to the sharp fall in number of new cases of infectious salmon anaemia in Norway (Scarfe & Palić, 2020). Raising farmers awareness on biosecurity through training and education has been shown to positively improve both farmers' attitude and their implementation of biosecurity both in aquaculture and terrestrial animal production (BASAK et al., 2022a; Dione et al., 2020; Georges et al., 2023b) and therefore recommended as an important element of a holistic and progressive biosecurity management approach for aquaculture (Bjørndal et al., 2024; Subasinghe, Alday-Sanz, et al., 2023). For such approach to be truly productive in a place Nigeria, issues such as poor-quality broodstock and access to extension service will need to be tackled in addition to putting in place mechanisms that encourage adoption of good biosecurity practice post-training.

This work is, therefore, aimed at developing an intervention in the form of online workshop and investigating its impact on improving biosecurity through the deployment of a weighted scoring scheme to assign biosecurity scores to participating farms. This will be achieved through the following set of objectives:

1. Train fish farmers from northern Nigeria on effective fish farm biosecurity practices through the delivery of an online workshop.

2. Periodically collect data on biosecurity from workshop participants twice at two monthly intervals using a structured questionnaire and score biosecurity practices using a weighted scoring scheme.
3. Determine if lessons learnt from the workshop beneficially impacts biosecurity as practised by workshop participants on their farms using appropriate statistical methods.

4.2 Materials and Methods

4.2.1 Study area

The study involved fish farmers drawn from 13 states of northern Nigeria and the Federal Capital Territory Abuja. The study lasted for six months between the period of June and October 2023.

4.2.2 Training of fish farmers

4.2.2.1 Development of training material

A PowerPoint presentation was put together and used as the core training material. In developing the material, findings on poor biosecurity from the previous survey in Chapter 2 of this present thesis contributed to the content of the training workshop. Hence relevant literature was consulted on the concept of disease, biosecurity and how knowledge can be applied to prevent disease occurrence on fish farms. Images were also included in the presentation to aid understanding. The relevance of record keeping and how to improve on record keeping by fish farmers was also incorporated.

4.2.2.2 Participant recruitment

A schematic representation of participant recruitment is presented in Figure 4.1. Forty-four participants involved in an initial survey on fish diseases and management from Chapter 2 of this present thesis (Table 2.3) were randomly assigned into two equally sized intervention groups. A third control group comprising 22 participants was recruited by snowball sampling. The lack of a comprehensive fish farm register and the fact the intervention cohort in this present study were already drawn using snowball sampling in Chapter 2 of this present thesis meant that random sampling could not be used. A similar limitation has prevented other researchers from adopting a random sampling method (Georges et al., 2023a). Participants of the first survey were thus asked to pass on our contact details to other farmers with a request for them to contact us via WhatsApp, if they were interested in participating in the study. After making contact, the aim and objectives of this present study were explained to the prospective

participant. When a verbal consent was obtained, the participant was enrolled according to the scheme shown in Figure 4.1.

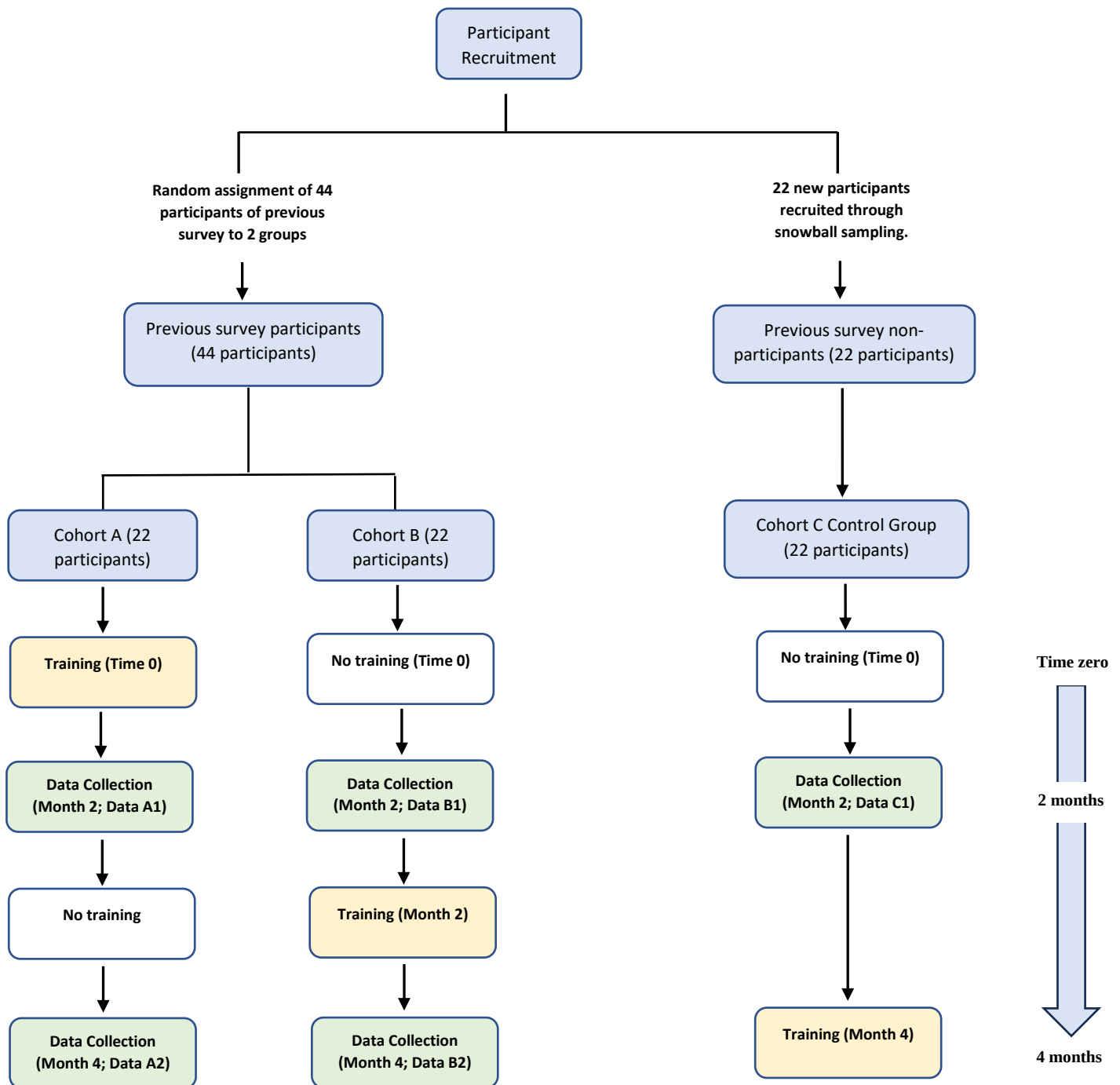


Fig 4. 1 Schematic representation of the process of participant recruitment and data collection

4.2.2.3 Training workshop delivery

The dates, times and durations for the online training workshops were agreed in consultation with all participants. Participants were individually contacted via telephone call and asked for their preferred days and times. A date and time preferred by majority of participants was chosen for the workshop. The training workshop was delivered to each cohort online via a Zoom meeting. Each training workshop was divided into three sessions i.e., introduction/presentation of findings from Chapter 2 of the current thesis, biosecurity and a question-and-answer session. The first workshop involved participants from cohort A and was delivered at time 0 (Figure 4.1). The second workshop was delivered after the first data collection at two months after the first workshop and involved members cohort B members (Figure 4.1). Although training for the control cohort (cohort C) was not a requirement for this experiment, members of this cohort were nonetheless trained. This was done to avoid members of this cohort feeling disadvantaged since communication between farmers from different cohorts was beyond the control of the researchers. This training workshop was administered after the last data collection at 4 months following the first workshop (Figure 4.1).

4.2.2.4 Participant assessment of the workshop

Before the end of each Zoom session, an anonymous evaluation of the workshop by the participants was requested via Jisc online surveys (<https://admin.onlinesurveys.ac.uk>). Links to the evaluation were shared with participants via personal WhatsApp messages. Participants were posed seven statements and asked to agree, disagree or strongly disagree; they could also indicate whether they were not sure (Table 4.1). Details of the assessment are shown in below.

Table 4. 1 Template for the assessment of online workshop on fish farm biosecurity by participants.

Question No.	Question	Agree	Not Sure	Disagree	Strongly Disagree
1	I have learnt something useful about biosecurity from this workshop				
2	I will change my fish farm biosecurity practices as a result of what I have learnt				
3	The workshop met my expectations				
4	The workshop was well organised				
5	I would attend a similar workshop in future				
6	Is there anything more that you would you like us to help you with? (Free text answer)				
7	Any recommendations on how to improve the workshop? (Free text answer)				

4.2.3 Data collection

4.2.3.1 Questionnaire design

A structured questionnaire was designed that contained 47 closed ended questions, some conditional i.e., answer depends on the response to a preceding question, divided into three sections specifically: farm demographics, internal biosecurity and external biosecurity (Table 4.2). Data on farm demographics were farm location, culture type, role of participant on the farm, number of dead fish in the preceding two months and number of times medication was used on the farm. The two sections devoted to biosecurity practices, i.e., internal and external biosecurity, were further divided into ten subsections specifically; traffic management, source of fish stock, feed management, presence of pets and rodents unique to external biosecurity: while internal biosecurity contained husbandry management and health management as unique subsections; water management and cleaning and disinfection were common to both biosecurity sections.

Table 4. 2 Number of questions in the categories of the structured biosecurity questionnaire

Section	Subsection	No. of questions
Farm demographics	-	6
External biosecurity	Traffic management	2
	Source of fish stock	6
	Water management	3
	Feed management	3
	Pets and rodents	1
	Cleaning and disinfection	6
	Sub-total	21
Internal biosecurity	Husbandry management	5
	Water management	3
	Health management	6
	Cleaning and disinfection	6
	Sub-total	20
Total		47

4.2.3.2 Scoring biosecurity questions

In order to have a score indicative of a farm's biosecurity level, the method of Damiaans et al. (2020) was used with modifications in assigning weighted scores to questions. Instead of awarding equivalent to 100 to each section on biosecurity i.e., external and internal biosecurity as followed by Damiaans et al. (2020), a score of 0.5 was assigned in this present study. This was done so that a farm could score a maximum of 1 or 100%. The weight for each section was then distributed across all sub-sections of that section according to the significance in disease transmission (Oidtmann et al., 2013), and the likely frequency of fish contact with a pathogen via such route. Infected live fish is the most important pathway for pathogen introduction onto a farm (Oidtmann et al., 2013) and thus, the source of fish stock received the highest weighting under external biosecurity (Table 4.6). This was closely followed by

water and feed management respectively. Weights were more evenly distributed in internal biosecurity. Water management and cleaning and disinfection were each scored 0.15 while the other two subsections scored 0.1 each (Table 4.6). A response that represents the optimum biosecurity situation received full points for a particular question, which in this case was a score of 1, while any other response received no points. Weighted scores were then computed according to the corresponding weighting of each section i.e., dividing the score for a question by the sum of the number of questions in that subsection multiplied by the weighting of the subsection. For conditional questions following the choice of a non-optimal option, however, the sum of the scores of the non-optimal option and the conditional questions were less than the optimal choice. For example, the score for choosing the option “borehole” as source of water is greater than the combined scores of any other option for the same question and the conditional questions that follow including water treatment and the type of treatment.

4.2.3.3 Questionnaire pretesting

To ensure clarity of questions and avoid incorrect responses by participants, the survey was pretested on three participants. This was necessary to maximally reduce errors associated with responses to questions (Nanda et al., 2013). The questionnaire was administered via a telephone call. Clues including delayed response, incorrect/unrelated responses to the questions and requests by the participant for further explanation of a question were used to assess the clarity of a question. Questions with issues were adjusted accordingly to enhance clarity (Table 4.3) and the questionnaire was retested with a different three randomly selected participants. Having confirmed the questionnaire was understandable and would elicit the expected response, it was deemed ready for administration to all the study participants.

4.2.4 Data collection on biosecurity practice

Because time constraint did not permit a long term follow up on participating farms, data were collected twice at two months interval to at least cover the minimum length of catfish production cycle in Nigeria which is typically 4 - 6 months. Data were thus

collected at two and four months respectively after the training workshop from cohort A participants while cohort B participants data was collected at two months before and two months after the training workshop (Figure 4.1). From cohort C, however, data was collected only once at the beginning of the study and before their training workshop (Figure 4.1). This was conducted either via telephone call at a time convenient for the participant. To account for the possible influence of participation in the previous survey in Chapter 2 of this thesis on the outcome of this present study, only cohort A members were trained before the first data collection leaving cohort B as an internal control. Thus, the first data set from cohorts A and B (A1 and B1) and the only data set for cohort C (C1) were collected two months after cohort A training workshop. Two months later, the second data set (A2 and B2) were then collected following the Cohort B training workshop.

4.2.5 Data analysis

Data were initially stored in an Excel spreadsheet and then exported to the programming language R for analysis. Demographic data were analysed using descriptive statistics and mean scores were computed for each subsection under the two components of biosecurity. To test the effect of training workshop on biosecurity practices at various time points, the Wilcoxon signed-rank test was used to compare mean biosecurity scores at these time points where the data to be compared at such time points were paired/dependent i.e., A1 v A2 and B1 v B2; while the Mann Whitney U test was used where such data were unpaired/independent. Consequently, the mean biosecurity score at two months after the training workshop was compared within each intervention group (A1 v B1, B1 v B2, and A1 v B2) and between the intervention and control cohort (A1 v C1 and B2 v C1). Similarly, mean biosecurity scores were compared at four months after the training workshop within intervention cohort (A2 v B1) and between the intervention and control cohorts (A2 v C1). Additionally, mean biosecurity scores at two months following the training workshop were compared with mean biosecurity scores at four months post training within (A1 v A2) and between (A2 v B2) cohorts. Furthermore, mean biosecurity scores were compared between B1 and C1 to determine the effect of enrolment in the previous survey on the outcome of this present study. Percent mortality was computed as the number of dead fish divided by total number of fish stocked multiplied by 100.

Spearman correlation coefficient was also used to test for a linear association between biosecurity scores, mortality and antibiotic use at the different time points. A farm's overall level of biosecurity compliance (compliance rate) as described by (Georges et al., 2023) was then computed for the different time points and used to classify a farm as low (0-25), intermediate (26-75) and high (76-100).

4.2.6 Ethics approval

Ethical approval for this project was granted by the General University Ethics Panel (GUEP) of University of Stirling with project ID 13754.

4.3 Results

4.3.1 The training material

A PowerPoint presentation (Appendix 4.2) used as workshop training material had 37 slides including the title slide. Introduction of the researcher, agenda and key findings from the previous survey in Chapter 2 of this present thesis were each presented in a single slide. The concept of disease and how it can be introduced and established on a fish farm were also described in two slides. The next 28 slides described biosecurity, its relevance to disease prevention in aquaculture, its various components, and how it can be implemented. Then a final slide described the template fish farm record sheet (Appendix 4.3) that captured basic details about the farm, husbandry practices, disease and medication.

4.3.2 Training workshop participants

A total of 50 participants were involved in the study. This included 29 participants of the previous survey from Chapter 2 of this present thesis divided into 15 and 14 participants that respectively formed cohorts A and B (Figure 4.2). The other 21 participants were those recruited as the control group/cohort C (Figure 4.2).

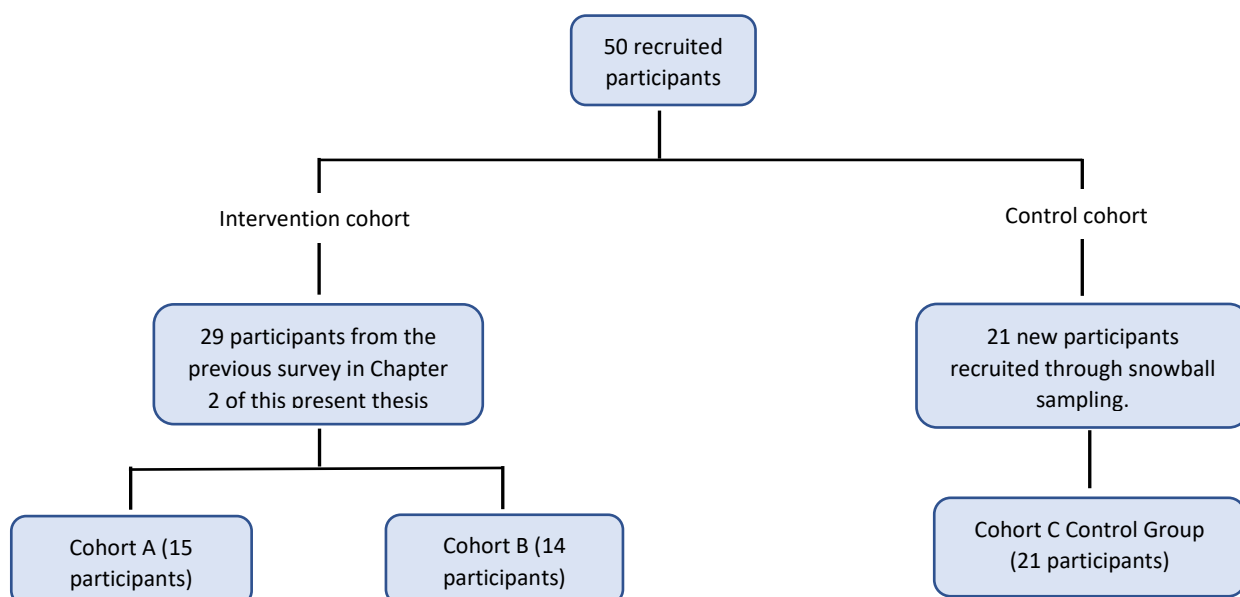


Fig 4. 2 Schematic representation of the final participants involved in the study

These participants were located across 13 out of the 19 states within northern Nigeria (Figure 2.3). Twenty-four of the participants own their farms but do not run them, another 18 own and run their farms while the other eight were only farm managers. Catfish culture only was the dominant culture type (40/50), while seven participants practised catfish alongside tilapia culture, with the remaining three participants practising catfish and tilapia polyculture (Table 4.3).

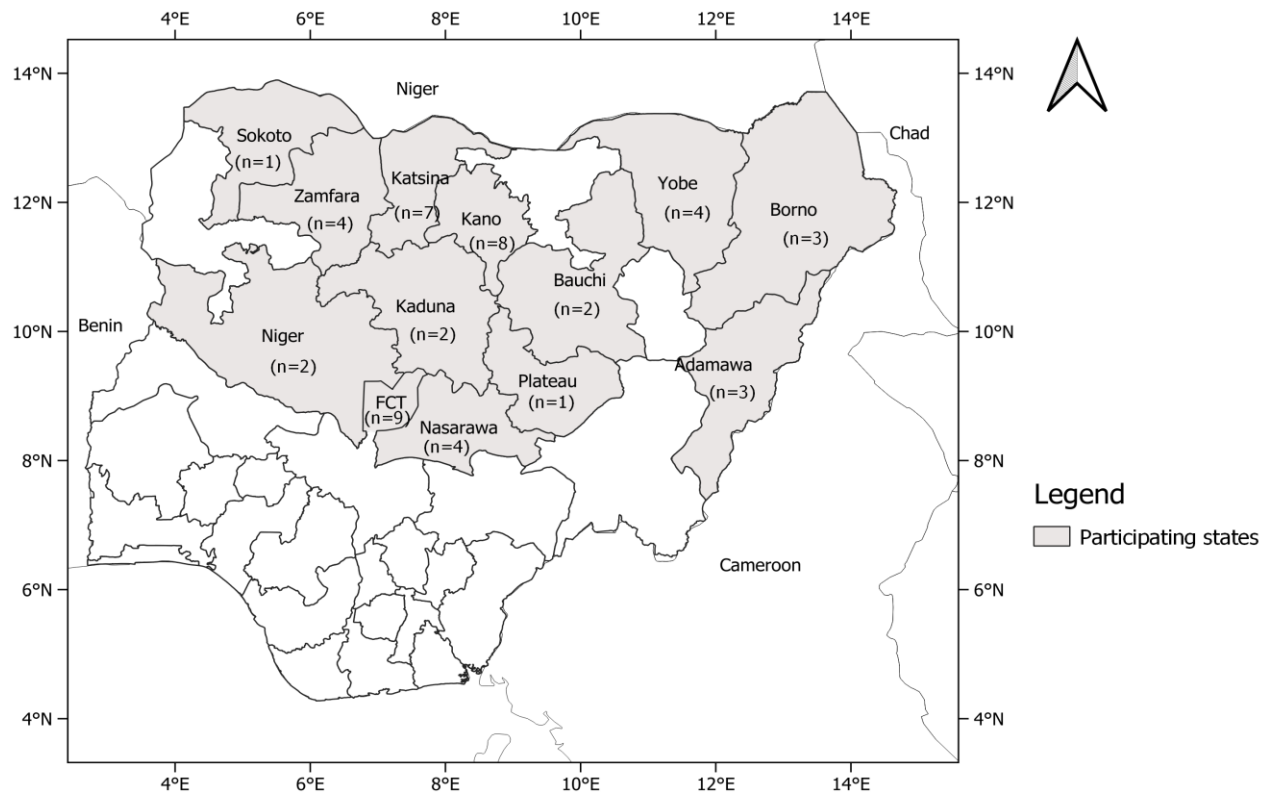


Fig 4. 3 A map of Nigeria showing the number of training workshop participants (in brackets) and their locations (states) shaded in grey.

Table 4. 3 Different culture types practised by training workshop participants and their roles (O – farm owners only, O&M – owners and managers of their farms, M – farm managers only) according to location (state) in northern Nigeria.

State No	State	Catfish only			Catfish & tilapia			Polyculture			Total
		O	O&M	M	O	O&M	M	O	O&M	M	
1	Abuja	4	2	1	-	1	1	-	-	-	9
2	Adamawa	1	-	-	-	-	1	-	1	-	3
3	Bauchi	2	-	-	-	-	-	-	-	-	2
4	Borno	1	1	-	-	-	-	1	-	-	3
5	Kano	4	2	-	1	1	-	-	-	-	8
6	Katsina	4	3	-	-	-	-	-	-	-	7
7	Nasarawa	2	1	1	-	-	-	-	-	-	4
8	Niger	-	1	1	-	-	-	-	-	-	2
9	Plateau	-	1	-	-	-	-	-	-	-	1
10	Sokoto	1	-	-	-	-	-	-	-	-	1
11	Yobe	1	1	1	-	-	1	-	-	-	4
12	Zamfara	2	-	-	-	-	1	-	1	-	4
13	Zaria	-	2	-	-	-	-	-	-	-	2
Total		22	14	4	1	2	4	1	2	0	50

4.3.3 Online training workshop

Workshops were held on Saturdays at 8:00 pm Nigerian local time and lasted approximately 1 h each. Each session involved an introduction on the survey and its relevance to the aquaculture sector. This was followed by a brief presentation of key findings of the previous survey in Chapter 2 of this present thesis. Presentation on fish farm biosecurity lasted about 20 minutes in each session. The last section of the workshop was dedicated to a question-and-answer session. Additionally, a fish farm record sheet template was shared with the participants before the end of every training workshop.

4.3.4 Assessment of online training workshop by participants

Participants' assessment of the workshop is presented in Table 4.4. All participants from the three cohorts agreed they learnt something useful on biosecurity from the workshop. Except for cohort A, the participants from cohorts B and C also agreed the workshop met their expectations, it would change their practices based on what they

had learnt, and they would attend a similar workshop in the future. In terms of workshop organisation, the majority of participants from all the cohorts i.e., 13/15 from cohort A, 13/14 from cohort B, and 18/21 from cohort C agreed the workshop was well organised. Even though a minority disagreed, they did not provide recommendations on how to improve on future workshops.

Table 4. 4 Participants' assessment of the online biosecurity workshop, suggestions and recommendations

Question	Cohort A %(n)				Cohort B %(n)				Cohort C %(n)				Overall agreement (%)
	Agree	Not Sure	Disagree	Strongly disagree	Agree	Not Sure	Disagree	Strongly disagree	Agree	Not Sure	Disagree	Strongly disagree	
I have learnt something useful about biosecurity from this workshop	100(15)	0	0	0	100(14)	0	0	0	100(21)	0	0	0	100
I will change my fish farm biosecurity practices because of what I have learnt	86.7(13)	6.7(1)	0	6.7(1)	100(14)	0	0	0	100(21)	0	0	0	96
The workshop met my expectations	86.7(13)	6.7(1)	0	6.7(1)	100(14)	0	0	0	90.5(19)	9.5(2)	0	0	92
The workshop was well organised	86.7(13)	6.7(1)	0	6.7(1)	92.9(13)	7.1(1)	0	0	85.7(18)	9.5(2)	4.8(1)	0	88
I would attend a similar workshop in future	93.3(14)	0	6.7(1)	0	100(14)	0	0	0	100(21)	0	0	0	98
Is there anything more that you would you like us to help you with? (Free text answer)	More about other culturable spp. in Nigeria Feeding regime that can help prevent disease How to identify a good fish variety More on quarantine Soft copy of the presentation												
Any recommendations on how to improve the workshop? (Free text answer)	The workshop time should be longer next time												

4.3.5 Questionnaire pretesting

Pretesting of the biosecurity identified three issues that negatively affected the question responses; specifically, clarity, specificity and certain questions that needed to be divided into more than one question. In other cases, either new questions or new response choice were added (Table 4.5). In total, seven different changes were made from both the internal and external biosecurity sections.

Table 4. 5 Questions of concern from questionnaire pretesting, issues identified with those questions and actions taken to fix the problem.

Question No	Item	Status	Issue	Comment
External biosecurity				
1	Access to farm/pond	Old question	Clarity	Rephrased
2	Stocking pond from rivers	New question	-	Addition
3	“Other” form of water treatment	New response choice	-	Addition
4	Cleaning and disinfection	Old question	Compressed	Separated into three questions
Internal biosecurity				
5	Daily operational workflow	Old question	Clarity	Rephrased both question and answer options
6	Water management	Old question	Non-specificity	Rephrased one question and changed response choices of another question
7	Cleaning and disinfection	Old question	Non-specificity	Rephrased one question and changed response choices of another question

4.3.6 Weighted scoring of biosecurity components

Table 4.6 shows the weights assigned to each subsection of the biosecurity questionnaire and their respective mean biosecurity score at different time points. The

sum of mean biosecurity scores was consistently higher at each time point for every intervention cohort (A1, A2, B1, and B2) compared to the control cohort, C1 (Table 4.6). Within the intervention group, however, the sum of mean biosecurity scores at four months (A2) was higher than that at two months post training (A1) workshop for cohort A while for cohort B, the sum of mean biosecurity scores post training workshop (B2) was higher than that before the training workshop (B1) (Table 4.6).

Table 4. 6 Weighting and mean biosecurity scores of each subsection of both external and internal biosecurity before the training workshop (B1 and C1) and after the training workshop (A1, A2, and B2)

	Category	Weight	Mean score				
			A1	A2	B1	B2	C1
A	External biosecurity						
1	Traffic management	0.05	0.04	0.04	0.03	0.05	0.03
2	Source of fish stock	0.15	0.11	0.12	0.09	0.13	0.10
3	Water management	0.13	0.12	0.12	0.12	0.13	0.10
4	Feed management	0.10	0.08	0.09	0.08	0.10	0.06
5	Animals, pets, and rodents	0.02	0.01	0.01	0.01	0.01	0.01
6	Cleaning and disinfection	0.05	0.03	0.04	0.03	0.04	0.03
Subtotal		0.50	0.39	0.42	0.36	0.46	0.33
B	Internal biosecurity						
1	Husbandry	0.10	0.09	0.09	0.08	0.09	0.06
2	Water management	0.15	0.06	0.08	0.07	0.10	0.06
3	Health management	0.10	0.06	0.05	0.04	0.06	0.04
4	Cleaning and disinfection	0.15	0.05	0.07	0.07	0.09	0.05
Subtotal		0.50	0.26	0.29	0.26	0.34	0.21
Total		1.00	0.65	0.71	0.62	0.80	0.54

4.3.7 Effect of workshop on biosecurity practice

4.3.7.1 Classification of farms according to level of biosecurity compliance

The distribution of farms according to the level of biosecurity compliance before and after the training is shown in Table 4.7. In cohort B where data were collected before and after the training workshop, only 7.1% (1/14) of participating farms was

categorised as “High” biosecurity compliant before the training workshop (B1). After the training workshop, however, this figure rose to 71.4% (10/14) (Table 4.7). In the case of cohort A where both time points of data collection i.e., A1 and A2 were respectively two- and four-months post training workshop, less farms (20% - 3/15) were categorised as “High” compliant at A1 compared to A2 (46.7% - 7/15) (Table 4.7). Although data were collected only once i.e., before the training workshop from cohort C, the majority (95.2% - 20/21) of farms in this group were classed as “Intermediate” compliant (Table 4.7).

Table 4. 7 Distribution of farms from different cohorts according to biosecurity compliance rate before the training workshop (B1 and C1) and after the training workshop (A1, A2, and B2)

Classification	A1	A2	B1	B2	C1
Low	0	0	0	0	0
Intermediate	12	8	13	4	20
High	3	7	1	10	1
Total	15	15	14	14	21

A1: Cohort A data at two months post training, A2: Cohort A data at four months post Training, B1: Cohort B data before training, B2: data at two months post training, C1: Cohort C data

4.3.7.2 Comparison of biosecurity scores

When the mean biosecurity score was compared before and after the training workshop in cohort B i.e., B1 v B2, there was a significant difference ($p < 0.05$) between the data from these two time points (Table 4.8). Similarly, even though both data collections in cohort A i.e., A1 and A2 were after training workshop, there was a significant difference ($p < 0.05$) in biosecurity scores from these two points (Table 4.8). When the mean biosecurity scores were compared between the different cohorts, there was also a significant difference ($p < 0.05$) both at two months post training workshop i.e., A1 v C1, B2 v C1, A1 v B2 and at four months post training workshop i.e., A2 v B1 and A2 v C1 (Table 4.8). There was, however, no significant difference ($p > 0.05$) between cohorts A1 and B1, and A2 and B2 (Table 4.8)

Table 4. 8 Comparison of mean biosecurity scores of participating farms within and between cohorts, before and after the training workshop and significance by Wilcoxon signed-rank test Mann Whitney U test.

Question	Variable	Statistic	p-value
A. Training had no effect on biosecurity practices at two months after training:			
i. within previous survey cohort	A1 v B1	129.5	0.293
	B1 v B2	0.000	0.001*
ii. previous survey with new cohort	A1 v C1	230.5	0.020*
	B2 v C1	276.5	0.000*
iii. between the intervention cohort replicates at two months post-training:	A1 v B2	20.00	0.000*
B. Training had no effect on biosecurity practices at four months after training:			
iv. within previous survey cohort:	A2 v B1	155.5	0.029*
v. compared with non-survey cohort:	A2 v C1	258.5	0.001*
C. Changes in practice were not influenced by time after training (two months versus four months after training):			
vi. within Cohort A	A1 v A2	12.50	0.013*
	A2 v B2	66.00	0.092
vii. between the cohorts			
D. Previous enrolment in survey has no effect on biosecurity practices	B1 v C1	204.0	0.060

A1: Cohort A data collected at two months post training, A2: Cohort A data collected at four months post training, B1: cohort B data collected before training, B2: Cohort B data collected at two months post training, C1: cohort C data collected at two months before training. *significant, p-value < 0.05.

4.3.8 Association biosecurity scores with mortality and antibiotic use

The Spearman correlation coefficient revealed a weak negative correlation between biosecurity score and mortality in all cohorts, except at two months following training in cohort B (B2) where it was weakly and positively correlated (Table 4.9). Similarly,

use of antibiotics was negatively correlated with the biosecurity score albeit weakly in all cohorts.

Table 4. 9 Correlation between biosecurity score, mortality and antibiotic use for the three survey cohorts at different data points by Spearman correlation coefficient

Data point	Mortality	Antibiotic use
A1	- 0.22	- 0.26
A2	- 0.10	- 0.28
B1	- 0.04	- 0.08
B2	0.25	- 0.31
C1	- 0.05	- 0.09

A1: cohort A data at two months post training, A2: cohort A data at four months post training, B1: cohort B data before training, B2: cohort B data at two months post training, C1: cohort C data

4.4 Discussion

Aquaculture is proven to play an important role in the socioeconomic sustenance of the population and in achieving food and nutrition security both from this present study and in published literature (Adah et al., 2023a; Kyule-Muendo et al., 2022). However, evidence from our previous study in Chapter 2 and from published literature (Assefa & Abunna, 2018; Maldonado-Miranda et al., 2022b; Mukaila et al., 2023) show that infectious disease is a problem. Similarly, evidence from available literature (Khor et al., 2024; Nwabueze & Ofuoku, 2020; Omitoyin & Osakuade, 2021) and Chapter 2 (Fig 2.11) shows that biosecurity is poorly implemented on fish farms in Nigeria. Because inadequate biosecurity underpins many disease outbreak episodes with consequent economic losses (Subasinghe & Shinn, 2023), this present study was designed to train fish farmers in biosecurity and assess the impact this will have on improving biosecurity practices implemented on fish farms in northern Nigeria, thus potentially help to reduce disease outbreaks, subsequent mortality and the use of antibiotics.

In this Chapter, farmers from the intervention cohort (A and B) were trained on biosecurity and then biosecurity practices from both intervention and control cohorts (cohort C) were subsequently assessed using a structured questionnaire to assess the impact of the training on their implementation of biosecurity. A significant difference ($p < 0.05$) in biosecurity scores both at two and four months after training between the control (C) and intervention cohorts (A and B) (Table 4.8) was observed, indicating better biosecurity implementation after training. Additionally, a greater number of farms from the intervention cohort (A and B) were categorised as “High” biosecurity compliant compared to the control cohort (C) (Table 4.7). These findings suggest that the training received by the intervention group (A and B) had a positive influence on improved implementation of biosecurity practices. Furthermore, this emphasises the role of fish farmer education/training as a critical component of any fish disease control/eradication strategy. Subasinghe et al. (2023) suggested people management, which involves educating and managing relevant stakeholders, should be considered as a main component of a holistic and progressive aquaculture biosecurity management strategy. In their study, BASAK et al. (2022) found better biosecurity in Jessore, a district where hatchery operators were more educated and trained in

biosecurity compared to Mymensingh district of Bangladesh. Similarly, a lack of training and consequently inadequate biosecurity implementation was suggested as a possible cause of high mortality on fish farms by Georges et al. (2023). The study also reported a strong and significant positive linear relationship between the fish farmer's level of education/training on fish farming and the rate of compliance with biosecurity in some districts in Cameroon. The fact that no farm was classified as low compliant, even from control group where a farm was found to be "High" compliant in this study, as noted by Georges et al. (2023), this might suggest a higher level of biosecurity awareness in this part of Nigeria than in Cameroon.

When assessing the effect of time after training on changes in biosecurity practice i.e., two months versus four months within the intervention group, a significant difference ($p < 0.05$) was observed in cohort A. Between cohorts A and B, however, this was not the case ($p > 0.05$). Even though a definitive explanation for this difference might be difficult given that we only had one data collection point post training from cohort B, factors beyond the scope of this study might have affected the level of biosecurity compliance of participants from cohort A, particularly at two months post training which resulted in a difference between the first and second data points. This is further indicated by the higher number of "High" compliant farms in cohort B at two months post training compared to cohort A within the same period after training (table 4.7) and the insignificant difference in mean biosecurity scores ($p > 0.05$) between the first data point for both cohorts i.e., A1 (data collection point at two months after training) and B1 (data collection point before training), despite the training received by cohort A (Table 4.8).

Unexpectedly, enrolment of the intervention group i.e., cohorts A and B in the previous survey was found not to significantly influence biosecurity practice ($P > 0.05$) in this study. Although the previous survey did not involve training on biosecurity, questions on biosecurity were, however, discussed with the respondents during the questionnaire administration. It was expected that this might have primed and influenced biosecurity practice in farms that participated.

Spearman correlation coefficient revealed a weak negative linear relationship at all data points between a farm's biosecurity score and mortality except at two months

post training in cohort B, as well as between the biosecurity score and antibiotic use. The weak nature of this relationship might be explained by the small number of samples in each cohort since a wider range of data points tends to give higher correlation values (Schober et al., 2018) or the sampling method used in this study. Nonetheless, this finding suggests that both mortality and use of antibiotics on fish farms might reduce with higher biosecurity scores. The absence of a register for fish farmers made it impossible to use a random sampling approach for this study and hence the choice of convenience as a sampling method. Convenience sampling is a non-random type of sampling, and this constitutes a violation of one of the assumptions of the correlation coefficient (Schober et al., 2018). This report is consistent with those of higher mortality in farms lacking in some components of internal biosecurity (Adah et al., 2023a) or high mortalities where awareness on biosecurity was low (Kyule-Muendo et al., 2022).

The present study is further limited by time as follow up data were collected for only four months which is about the length of one production cycle in Nigeria's aquaculture. Reliance on a farmer's account was another limitation since data were collected via telephone call and not on site. These limitations particularly the sampling method makes it impossible to make inference to the wider population of fish farmers in this region based on findings from this study. A database of fish farmers would have provided a sampling frame that would enable a more representative study involving a larger number of farmers. By this way, an inference could be made to the wider population and critical questions like the relationship between biosecurity level of a farm and fish mortality or use antibiotics might be answered definitively.

Further studies involving a more representative sample and a longer follow up of data collection will be needed to better understand the relationship between biosecurity score and mortality or the use of antibiotics. We also recommend that relevant authorities and not-for-profit organisations like WorldFish Centre operating in Nigeria consider training fish farmers on biosecurity as part of their strategy for sustainable aquaculture development and to take advantage of the online medium in doing so. This would be an effective way of reaching a wider range of farmers since the internet is now accessible in almost every part of the country. Training the trainers will also

encourage more widespread good practice and form a farmer's network including access to expertise to contact if there are any biosecurity issues.

4.5 Conclusion

This present study was able to establish that training fish farmers from northern Nigeria on biosecurity via an online platform significantly improved the biosecurity practice on the farms of participants. Furthermore, evidence here suggests that fish mortality and use of antibiotics on these farms might reduce with higher biosecurity scores. Thus, online training on biosecurity might improve biosecurity in the wider fish farming population, reduce fish mortality and the use of antibiotics on fish farms. This has implications in terms of addressing sustainability, food security and future food safety.

Chapter 5

General discussion

5.1 General discussion

Nigeria is the largest aquaculture producer in Sub-Saharan Africa (FAO, 2022) and the largest fish consumer on the continent. The country is, however, unable to meet its demand for fish due to numerous challenges (Adewumi, 2015b), that is made worse by the lack of a sufficient regulatory framework and insufficient capacity for disease prevention and control (Adeleke et al., 2020b; Oman et al., 2023b). Consequently, fish farmers frequently suffer disease losses and often resort to self-medicating stocks with antibiotics, sometimes inappropriately. There is also a poor understanding of the prevailing disease situation and disease management practices employed by fish farmers in northern Nigeria, a region with both a higher population and higher poverty rate compared to southern Nigeria but with an emerging aquaculture sector (Jaiyeola & Choga, 2021). Aquaculture in this region could thus constitute a source of environmental contamination, selection for antibiotic-resistance genes, and potential of human infections with antibiotic-resistant pathogens, thereby posing a One Health challenge. This thesis aimed to: determine disease occurrence and associated management on fish farms in northern Nigeria (Chapter 2); isolate and characterise bacteria from fish showing signs of disease (Chapter 3); and design and evaluate an online intervention towards improving biosecurity (Chapter 4).

The majority of fish farmers engaged in this present study were small-scale producers rearing predominantly catfish (Table 2.4), which is consistent with the report of Oman et al. (2023) who found 63% of catfish farmers in Nigeria are small scale farmers and produce 65% of the country's catfish output. Small-scale catfish farmers thus constitute an important segment of the catfish value chain that needs attention to ensure sustainability, food security and secure the socioeconomic livelihood of many Nigerians. This set of farmers should be the primary target for interventions that seek to prevent high levels of disease and inappropriate antibiotic use (Table 2.5) and promote food security through sustainable aquaculture production.

This thesis found that Nigeria has a range of institutions concerned with fish disease and antibiotic use to aid the promotion of sustainable aquaculture in the country (Fig 2.3). However, specific legislation that will coordinate these institutions for successful and profitable aquaculture production is lacking (Adeleke et al., 2020a). Additionally,

implementation of the existing policy regulations on aquaculture which largely lies with the federal government is minimally conducted and focuses mainly on large-scale fish farms (Oman et al., 2023a). By implication, the aquaculture sector in Nigeria is largely unregulated since the large-scale fish farms constitute only ca. 2% of the fish farms in the country and produce only about 22% of the country's total output (Oman et al., 2023a). Furthermore, it was noted that some departments and agencies of government concerned with aquaculture regulation might move or change owing to creation and/or collapse of government ministries, a situation that is commonplace in Nigeria with changing governments. This inconsistent governance structure might result in frequent alteration of the aquaculture system map (Fig 2.3) and might affect implementation of relevant policies and regulations. Lack of proper guidelines and a regulatory framework predispose the sector to practices that undermine productivity and increases the risk of disease outbreaks because of non-adherence to standard health management practices including regular fish health monitoring, disease surveillance and reporting (Subasinghe et al., 2023).

Biosecurity was found to be poorly implemented among participating fish farms in northern Nigeria (Fig 2.11). This finding is consistent with published evidence of the poor state of biosecurity across the aquaculture sector in Nigeria (Nwabueze & Ofuoku, 2020; Omitoyin & Osakuade, 2021) and other parts of Africa (Kyule-Muendo et al., 2022). Poor biosecurity is a factor driving underperformance and low productivity in Nigeria's catfish production that has already suffered a decline because of inflation and currency devaluation and made production more expensive (Oman et al., 2023a). In addition to predisposing a farm to infectious disease outbreaks that might extend to other farms especially in an epidemiological cluster, poor biosecurity increases the likelihood of antibiotic misuse which has consequences for food safety if withdrawal periods are not adhered to, the efficacy of antibiotics if antibiotic resistance is selected, and the environment which may become polluted with antimicrobial residues (Alhaji et al., 2021; Okocha et al., 2021a; Subasinghe et al., 2023). Although this present study did not find an association between poor biosecurity and high mortality, perhaps due to the relatively limited size and scope of the work, it did find a significant association ($p < 0.05$) between an element of poor biosecurity (keeping fish of different source/age group in the same pond) and farm-level mortalities. This finding is consistent with similar reports from Nigeria and beyond (Adah et al., 2023a; Ali et al., 2020; Kyule-

Muendo et al., 2022), and poor biosecurity, at least in part, thus constitutes a threat to sustainable aquaculture.

The high level of disease outbreaks (64.4%; Table 2.5) may partly reflect the poor state of biosecurity in the Nigeria's aquaculture sector and this is consistent with published work from other parts of the country and around Africa (Georges et al., 2023a; Kyule-Muendo et al., 2022; Obosi & Agbeja, 2015). Infectious diseases in aquaculture are associated with deleterious impacts that range from decreased growth rate, decreased fecundity, increased mortality and decreased marketability that cost the industry significant economic losses (Behringer et al., 2020b). As capture fisheries have levelled off, increasing aquaculture production has been advocated to sustain the status of fish as an important contributor to global nutrition and food security especially in developing countries (Bjørndal et al., 2024). This is more pertinent for sub-Saharan Africa, a region with both the greatest need for increased aquaculture production and a greater projected negative impacts of climate change on aquaculture (Bjørndal et al., 2024). Food insecurity is already a major challenge in Africa and aquaculture has been identified as a potentially major contributor in tackling this problem (Akegbejo-Samsons, 2022). Thus, reports of high disease prevalence in aquaculture across sub-Saharan Africa which can be traced back to poor biosecurity is a threat to food security in the region with a potential to get worse if not adequately addressed.

Four different motile *Aeromonas* spp. that included six *A. hydrophila* isolates, five *A. veronii*, three *A. jandaei*, and two *A. dhakensis* were identified from apparently diseased catfish (Table 3.9). This finding is consistent with published works involving diseased catfish from a state in northern Nigeria (Adah et al., 2024) and other food and ornamental fish from around the world (Dhanapala et al., 2021a; Li et al., 2022; Tyagi et al., 2022). Motile *Aeromonas* spp. particularly *A. hydrophila* are associated with outbreaks of motile *Aeromonas* septicaemia (MAS) that can result in a high mortality rate (Bartie et al., 2023a; Baumgartner et al., 2017). They are also implicated in cases of gastrointestinal infections and septicaemia in humans where they are considered to be emerging zoonotic pathogens (Gonçalves Pessoa et al., 2019; Li et al., 2022; Wamala et al., 2018). Infection involving *Aeromonas* spp. especially the hypervirulent *A. hydrophila* (vAh) ST251 is a source of concern for aquaculture

establishments around the world and poses a challenge from both an aquaculture and One Health perspective (Bartie et al., 2023b).

Motile *Aeromonas* spp. from this present study displayed phenotypic virulence traits and possessed genetic determinants underpinning such virulence phenotypes (Fig 3.3; Table 3.10). These *Aeromonas* spp. also carried genetic determinants of secretion systems that are known to enhance bacterial virulence, survivability and host colonisation (Horna & Ruiz, 2021; Korotkov & Sandkvist, 2019). This finding is consistent with the report of (Tyagi et al., 2022). Secretory systems serve as delivery channels for effector molecules essential for bacterial pathogenesis (Kanonenberg et al., 2013). Substrates of these secretory systems include toxins and enzymes that mediate digestion of complex molecules and proteins that facilitate adherence and biofilm formation (Korotkov & Sandkvist, 2019). They are also involved in host immunosuppression by acting as delivery channel for the injection of effectors that undermine the host immune response and alter critical host pathways such as the actin cytoskeletal signal transduction pathway important in maintaining structural integrity of eukaryotic cells, cell division and many other important functions (Balta et al., 2021) leading to bacterial colonisation, survival and eventual replication (Horna & Ruiz, 2021).

In terms of infectious disease management, this present study revealed that self-medication with antibiotics, predominantly oxytetracycline was practised by more than half of survey participants, which is consistent with other reports on the use of antibiotics in aquaculture from across Nigeria (Alhaji et al., 2021; Ladan et al., 2021; Okocha et al., 2021a). Oxytetracycline's low cost, broad spectrum of activity, and its ease of access has made it a first line antibiotic among fish farmers and thus most likely used widely (Fig 2.10) (Ladan et al., 2021). Furthermore, the antibiotic cycle within Nigeria's aquaculture system was revealed to be a complex one that involved numerous sources, multiple actors and delivery channels, and had multiple hotspots where antibiotics are frequently used and hence critical points for the potential emergence of antibiotic resistance (Fig 2.3). This is an indication of lack of proper control over both access to and the application of antibiotics which supports the assertion of Okocha et al. (2021a) that, unlike developed nations like in the USA and

Europe, regulations on antimicrobial use in animal production are not adequately enforced in Nigeria.

In addition to weakly enforced antibiotic regulations, poor antibiotic stewardship as further demonstrated by the finding that antibiotic cocktails were the next common drug of choice after oxytetracycline, is also an indication of a poor level of awareness of fish farmers from northern Nigeria on the proper use of antimicrobials (Alhaji et al., 2021), as well as the absence of or inaccessibility to fish health personnel within the fish farming community. Poor farmers' perception and awareness on necessary guidelines on antimicrobial use will be an impediment in the achievement of good antimicrobial stewardship as stipulated in Nigeria's National Action Plan on Antimicrobial Resistance through a One Health approach (Alhaji et al., 2021; Federal Government of Nigeria, 2017). Additionally, the potential for treatment failure because of improper antibiotic regimen and therefore repeat medication may increase the losses incurred by farmers. Hence, indiscriminate use of antibiotics (Fig 2.3) has been identified as a challenge undermining catfish production in Nigeria (Oman et al., 2023a). Antibiotic use in aquaculture in Nigeria and around the world is suggested to pose serious economic and health risks (Bondad-Reantaso et al., 2023; Lulijwa et al., 2020; Okocha et al., 2021b). Until the factors including regulation of antibiotic usage and poor level of farmers' awareness as enumerated above are properly addressed, misapplication of antibiotics by fish farmers can be expected to remain commonplace. Consequently, these antibiotics will continue to exert selection pressure on resident microbes including pathogens leading to the emergence and spread of antibiotic resistance, potentially predisposing consumers to antibiotic residues and thus raising concerns about the safety of aquaculture products (Bondad-Reantaso et al., 2023; Durojaiye et al., 2020; Okocha et al., 2021b).

This study also revealed a lack of awareness among survey participants on vaccination as an effective fish disease prevention strategy. This might be explained by the lack of any fish vaccine in Nigeria indicating a need for investment in this critical fish disease control tool for the country's aquaculture industry. Although antimicrobials have proved useful in control of fish diseases, their repeated use has not been without detrimental effect, primarily the development of resistance necessitating the deployment of effective preventive strategies (Rathor & Swain, 2024). Vaccination has

thus been advocated and indeed proven to reduce the need for antibiotic use in aquaculture in some circumstances or even disease eradication as shown in the case of the rinderpest virus (Desbois & Monaghan, 2023b). Since its introduction to aquaculture in the 1940s, vaccination has played a key role in the effective control of both bacterial and viral diseases in aquaculture and the sector presently has over 50 vaccines for more than 30 fish species (Rathor & Swain, 2024).

Evidence of acquired resistance and/or mutations against amoxicillin, oxytetracycline, sulfamethoxazole, colistin, oxolinic acid, enrofloxacin, and florfenicol was demonstrated in the *Aeromonas* spp. (Table 3.7). The highest number of number of isolates with evidence of acquired resistance were recorded with oxytetracycline, sulphamethoxazole, amoxicillin and oxolinic acid (Table 3.7). Additionally, all *Aeromonas* spp. isolated in this present study demonstrated presumptive resistance to multiple antibiotics. These findings corroborate the earlier mentioned poor antibiotic stewardship and predominant misuse of oxytetracycline among fish farmers from the study area and agrees with the reports of Adah et al. (2024) involving *Aeromonas* spp. from the same study area and Hossain et al., (2019) who recorded a high level of resistance in *Aeromonas* spp. of zebra fish origin against the same set of antibiotics classes from Seoul, Korea. Similar antibiotic resistance patterns were reported in fish-associated *Aeromonas* spp. from south-western Nigeria (Adeyemi et al., 2022) and elsewhere including Sri Lanka and Korea (Dhanapala et al., 2021b; Hossain et al., 2019b). Hayatgheib et al. (2021) however, reported only 36% presumptive multi-drug resistant *Aeromonas* spp. from France. This low-level multi-drug resistance might be explained by the strict regulation of antibiotic use for animal production in France compared to Nigeria.

The use of antibiotics in aquaculture promotes the selection and development of antibiotic resistant pathogens (Brunton et al., 2019; Desbois et al., 2021) and ensures their propagation to the detriment of susceptible members of the bacterial community (Pepi & Focardi, 2021). Additionally, such applications have also been shown to result in antibiotic residues in fish tissues (Ladan et al., 2021; Okocha et al., 2021a) especially in places where access to antibiotics is loosely regulated thereby exposing consumers to subtherapeutic doses of these compounds. Therefore, antimicrobial resistance in aquaculture which is primarily driven by the use of antibiotics in the

system is concerning because of the potential negative impacts on both aquaculture and consumers as well as its contribution to total environmental resistance through mobile resistance elements (Subasinghe et al., 2023). Furthermore, the fact that evidence of acquired resistance against antibiotics that have been classified as either critically important (amoxicillin, oxolinic acid, colistin) or highly important (oxytetracycline, sulfamethoxazole) for humans by WHO (J. Chen et al., 2020a) was demonstrated in this present study makes this finding more concerning. Consequently, antibiotic resistance in aquaculture is a threat to public health and livelihoods because of the potential for the transfer of resistance genes from aquaculture to humans (J. Chen et al., 2020b; Gaurav et al., 2023b).

The threat of resistance gene transfer from aquaculture to humans was demonstrated by finding at least one plasmid in nine *Aeromonas* spp. isolates from this present study. Two of those plasmids contained genes that mediate resistance against tetracycline antibiotics. This finding is consistent with that of Pérez-García et al. (2021) who also detected sequences encoding resistance proteins and other factors in multiple plasmids from mesophilic aeromonads. The extremely high genome plasticity of members of the genus *Aeromonas* and the fact that they sit at the interface of the three components of One Health i.e., humans, animals and the environment, make them a classical One Health challenge (Lamy et al., 2022). Plasmids and transposons to facilitate conjugative transfer of resistance genes (Pepi & Focardi, 2021). In addition to facilitating antibiotic resistance, plasmids may encode other traits such as virulence, metabolic capacities and functions that enable bacteria to persist in the environment by resisting noxious agents like heavy metals (Opal & Pop-Vicas, 2020b). This makes plasmids significantly important in both horizontal gene transfer and bacterial pathogenesis which highlights a role for aquaculture in the emergence and dissemination of antibiotic resistance.

The fact that pathogens are often widespread can remain unidentified and their host range undetermined, means disease control and health management plans need to incorporate holistic biosecurity if they are to be successful (Subasinghe et al., 2023). The poor level of fish farmers' awareness on biosecurity in Nigeria as demonstrated in this present study and other published literature (Khor et al., 2024; Nwabueze & Ofuoku, 2020; Omitoyin & Osakuade, 2021) is thus a source of concern that warrants

urgent intervention. This present study revealed a higher level of biosecurity compliance (Table 4.7) among fish farmers trained on fish farm biosecurity compared to control cohort and consequently a significant difference ($p < 0.05$) in biosecurity score between the two cohorts (Table 4.8). This indicates that the training received by the intervention cohort had a positive influence on improved implementation of biosecurity practices and further emphasises the role of fish farmer education/training as a critical component of any fish disease control/eradication strategy. Basak et al. (2022) reported a similar finding where biosecurity was enhanced in a district of Bangladesh where hatchery operators were more educated and trained in biosecurity compared to another district with less trained farm hands. Similarly, Georges et al. (2023) reported a strong and significant positive linear relationship between fish farmer's level of education/training on fish farming and rate of compliance with biosecurity in some districts in Cameroon. Lack of training and consequently inadequate biosecurity implementation was suggested as a possible cause of high mortality on fish farms (Georges et al., 2023a). Training on biosecurity has also been shown to have a positive impact in the livestock sector on both attitude of farmers towards biosecurity and its implementation in Uganda (Dione et al., 2020). Subasinghe et al. (2023) suggested people management, which involves educating and managing relevant stakeholders, should be considered as a main component of a holistic and progressive aquaculture biosecurity management strategy. Training fish farmers via the online medium can be an effective way of achieving this since the internet is now available even in remote areas and can provide easy access to farmers. Participants of the online training workshop on biosecurity overwhelmingly agreed (100%) that they learnt something useful from the workshop and would change their biosecurity practices as result (Table 4.4). Furthermore, the dire need for increased aquaculture production in Nigeria and indeed sub-Saharan Africa, and the threat that disease, as a consequence of poor biosecurity pose to this, means a holistic approach that incorporates improving the education of fish farmers and farm other stakeholders should be vigorously encouraged (Bjørndal et al., 2024).

This present study evaluated the aquaculture system in Nigeria particularly tracing the channel of antibiotic distribution and usage within the system, infectious disease prevalence from aquaculture in northern Nigeria, and trained fish farmers from the region on how to improve fish farm biosecurity. The study was, however, limited by the

number of samples involved and lack of a structured sampling frame that would have permitted the use of random sampling. This significantly limits the extent to which conclusions from the study can be inferred to the wider population of fish farmers from northern Nigeria. Time constraints did not permit for a follow up long enough in Chapter 4 of this thesis to evaluate some of the objectives of that chapter with high level of confidence. A 12 month follow up period which covers at least two production cycles would have produced more confident results as well as provide insights on the long term effect of the intervention. Facilities slated for sample processing and storage were shot down due to the Covid-19 restrictions and a make shift arrangement had to be made with a small private laboratory. This laboratory, however, didn't have a good microscope and another laboratory had to be used simultaneously. Consequently, processed bacterial samples could only be stored in a household refrigerator (- 4°C) while tissue sections were processed through a third party. This lack of access to standard laboratory facilities for fish tissue processing and storage of collected bacteria affected both the quantity and quality of the data generated in Chapter 3 of this thesis. Hence, clear evidence to demonstrate the involvement of bacterial infection could not be demonstrated.

Therefore, we propose the following recommendations:

1. Nigeria's aquaculture regulatory framework should be strengthened through legislation that will enable better coordination of relevant institutions for improved and sustainable aquaculture production.
2. Implementation of existing policies and regulations on aquaculture in general and especially those relating to disease prevention and control, as well as use of antibiotics, should be enhanced through provision of more resources for relevant institutions. This should facilitate generation of a database of fish farmers through better extension services and disease monitoring. Although governments are usually faced with competing demands amid scarce resources, aquaculture's role in ensuring food and nutrition security through the provision of a cheaper source of protein, its significance in the socioeconomic life of many Nigerians, and a potential source of foreign exchange through exportation should make the industry high on the government's scale of priorities. The lack of such standards were responsible for the suspension of catfish export to the US and UK from Nigeria. Additionally, compliance with

relevant regulations by fish farmers could be incentivised by way of recognised certifications, subsidised inputs, better access to finance, improved access to markets or schemes that facilitate easy access to quality feed. Similar strategies proved useful in accelerating Monitoring, Surveillance and Control (MSC) certification; a scheme under the Fishery Improvement Project (FIP) aimed at enhancing sustainable yellowfin tuna production in Filipin (Tolentino-Zondervan et al., 2016). Participating fishers were prioritised by for livelihood assistance and training by Local Government Units (LGU), granted preferential market access and higher than local market price for tuna (Tolentino-Zondervan et al., 2016).

3. Training fish farmers on biosecurity will significantly improve implementation of biosecurity practices on fish farms, given the evidence collected in this present study (Table 4.8). This should reduce infectious disease outbreaks, reduce the emergence of antibiotic resistance through reduced need for antibiotic use and promote a safe and sustainable aquaculture production in northern Nigeria and the country.
4. Other relevant actors in the aquaculture value chain such as feed millers and vet/agrochemical shop owners should be included in future training on biosecurity. This will raise more awareness on the collective responsibility towards better antibiotic stewardship.
5. With respect to Chapter 3 of this thesis, further studies to demonstrate evidence of bacterial involvement in infectious disease of catfish as well as other drivers of disease from the study area will be required. Additionally, the *in vivo* pathogenic potentials of isolated *Aeromonas* spp. will need to be demonstrated in challenge studies. Furthermore, identified plasmids could be characterised further especially those bearing resistance genes by curing and bacterial transformation to establish their role in resistance transfer.

Following on the outcomes of this thesis, future work could focus on enhancing fish farmers' capacity in implementing biosecurity through increased awareness. Strategies that could facilitate easy access to easily comprehensible resources by the fish farmers and relevant stakeholders that could translate into measurable improvement in biosecurity and antimicrobial stewardship in line with Nigeria's National Action Plan on AMR could be explored. Additionally, studies to further

investigate the epidemiology of bacterial infection particularly of *Aeromonas spp.* in both the study area and other regions of the country and thereby aiding our understanding of the importance of bacterial disease in aquaculture in Nigeria could be initiated.

5.2 Final conclusions

This present thesis established that reports of disease incidences were high among fish farms surveyed and that self-medication with antibiotic either singly or as a cocktail was the preferred means of disease control. Multidrug resistant and potentially pathogenic *Aeromonas spp.* bearing both virulence and resistance genes either on chromosomal or plasmid DNA were isolated from apparently diseased catfish samples. Furthermore, a system map that captured relevant actors in Nigeria's aquaculture sector, particularly those involved in antibiotic supply, and points where antibiotic are commonly used was established. Finally, this thesis established that online training workshop on biosecurity significantly improved implementation of biosecurity on farms of participating fish farmers. Aquaculture in northern Nigeria is thus faced with disease challenges and lack of proper control and prevention strategies that has facilitated the uncontrolled and inappropriate use of antibiotics which threatens not only sustainability and food security but also One Health which this study aimed to address and offer sustainable solutions.

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Appendices

Chapter 2 Appendices

Appendix 2.1 Farmer interview guide

A. Farm Background Information

1. Respondent's ID
2. Level of education: ☐Primary ☐Secondary ☐Tertiary
3. Role of respondent on the farm.....
4. For how long the farm has been in operation: ☐ < 1 year ☐ 1 – 5yrs ☐ > 5yrs
5. Years of Manager's experience: ☐ < 1 year ☐ 1 – 5yrs ☐ > 5yrs
6. Any fish production/management training: ☐Yes ☐No
7. If yes, what kind of training have you had? ☐Tertiary level education in aquaculture/fisheries ☐Workshop/Seminar on fish farming ☐Trained as an intern on a farm
8. Number of staff:
9. Farm location:
10. Culture Type: ☐Catfish ☐Tilapia ☐Polyculture (Both Catfish & Tilapia)
11. Have you always farmed your fish from your current location? ☐Yes
☐No
12. If no, when and why did you change and where did you move from?
.....
.....
13. Production type: ☐Outdoor ☐Indoor
14. System type: ☐Standard earthen pond ☐Flow-through earthen pond
☐Indoor flow-through ☐Outdoor standard flow-through
☐Outdoor partial flow-through ☐RAS ☐Other
.....
15. Pond Type: ☐Earthen ☐Concrete tanks ☐Plastic tanks ☐Fibre glass tanks
☐Wooden vats ☐Metallic tanks (bathtubs) ☐Other
.....
16. Stocking density at start of cycle
17. Stocking density at harvest

18. Can you explain how you determine your stocking density?
.....
.....
.....
19. Source of Fingerling/Juveniles: ☐ Own hatchery ☐ Other Hatcheries ☐ Wild
☐ Other (Middlemen)
20. Do you always use the same source? ☐ Yes ☐ I alternate based on
availability ☐ I alternate based on price ☐ I alternate based on quality of the
stock
21. Production model: ☐ Brood stock ☐ Table fish ($\geq 1\text{kg}$) ☐ Smoked fish ☐ All
22. What is the duration of your production cycle? ☐ <4 months ☐ 4-6 months
☐ >6 months
23. Output per production cycle: ☐ <1 Tonne ☐ 1-5 Tonnes ☐ 6-10
Tonnes ☐ >10 Tonnes
24. Source of Water: ☐ Borehole ☐ Well ☐ River/Stream
☐ Municipal
25. If you use stream, are there other fish farms upstream along the banks of the river?
☐ Yes ☐ No ☐ I don't know
26. Type of Feed: ☐ Imported Commercial ☐ Local Commercial ☐ Self-
compounded (On-farm) ☐ Other
.....(Trash food and waste including dead
animals like poultry or ruminants)
27. Do you always use the same type of feed? ☐ Yes ☐ No
28. If no, could you tell what makes you change
.....
.....
29. If you use commercial feed can you tell the product/manufacturer?
.....
30. Have you always used this manufacturer? ☐ Yes ☐ No
31. If no why did you change? ☐ Cost ☐ Quality ☐ Availability ☐ Other
.....
32. Do you keep farm records? ☐ Yes ☐ No
33. If yes, can you select all that apply: ☐ Production records ☐ Health/Disease records
☐ Both

34. In what format do you keep these records? ☐Paper ☐Electronic
35. For how long do you keep these records?
36. Do you at any point examine these records ☐Yes ☐No
37. If so, when? ☐At the end of production cycle ☐When I have disease problems
☐Other

Product Harvest Sales, and Transport

38. How are the fish harvested?

.....

39. Do you harvest whole crop at once or just what is needed and the larger fish?

☐Whole crop at once i.e. whole farm ☐Just what is needed according
 to size i.e. larger fish first ☐Just what is needed randomly

40. Who do you sell your product to? ☐Whole sellers ☐Retailers

☐Restaurants/Hotels ☐Consumers ☐Middlemen

41. If you sell to more than one buyer, could you give an estimated percentage for each buyer

Whole sellers Retailers Restaurants/Hotels Consumers
 Middlemen

42. Do different buyers demand for different quality or size of fish? Yes No

43. If yes, can you explain further

.....

44. Who transports the fish and what type of vehicle is used? ☐Mini bus ☐car

☐Truck ☐Other

45. How far away do the fish travel typically (in km)? How long in time are the journeys?

.....

46. Are the fish always transported alive? ☐Yes ☐No

47. How are the fish collected? ☐Polythene bags ☐Plastic containers with water

☐Plastic containers without water ☐Other

48. Any idea if these containers have provision for oxygenation? ☐Yes ☐No ☐I'm not
 sure

49. Apart from weight, what else affects their sales price?

.....
.....
.....(Visible lesions?)

50. When is price agreed? ☐ At the start of production ☐ At the point of collection

51. Who mostly determine the final price for your fish? ☐ I do ☐ The off taker ☐ We negotiate ☐ Middle men

B. Disease Occurrence and Management

1. Have you experienced any significant disease incidence within the last one year? ☐ Yes ☐ No

2. If yes, was there any mortalities from the incidence? ☐ Yes ☐ No

3. If yes, can you give an estimate in percentage losses

4. What do you think or believe was responsible for the disease outbreak?
.....
.....
.....

5. Which month did this problem start?

6. At what point during the production cycle did the problem start? ☐ Within 1 month of stocking ☐ After one month of stocking

7. In most years, do you have disease incidence? ☐ Yes ☐ No ☐ Not applicable

8. What season of the year do you have most diseases/mortalities ☐ Rainy ☐ Dry

9. Do you seek any expert help to resolve the problem? ☐ Yes ☐ No ☐ Yes, after a failed trial

10. If yes, was the problem resolved? ☐ Yes ☐ No

11. If yes/yes after failed trial, from whom? ☐ Another farmer ☐ Veterinarian ☐ Fisheries Department ☐ Veterinary drugs vendor ☐ Other
.....

12. If no, why not?

13. During disease events, what measure(s) have you taken to mitigate the problem?

☐ Emergency harvest ☐ Change water ☐ Treatment ☐ Change water and Treat

14. In case of treatment, who provides the service? ☐ Myself ☐ Someone else ☐ Not applicable

15. If someone else, who was it?

16. Which of the following was used for the treatment?
☐ Chemical agent/drug ☐ Salt ☐ Traditional medicine
17. Have you tried other approaches? ☐ Yes ☐ No
18. If yes, can you tell what approach that was and why did you change to your option in question
.....
.....
19. If chemical/drug was used can you tell which one(s) and how it was administered?
.....
20. If you used traditional medicine can you tell which one and how it was delivered?
.....
21. If you didn't do emergency harvest, did your chosen intervention work? ☐ Yes ☐ No
22. When you have disease problems do you treat all the fish on the farm? ☐ Yes ☐ No

C. Prevention of Infection

1. Do you quarantine new stock (isolate them for a period before transferring them to rearing ponds)? ☐ Yes ☐ No
2. If yes, can you describe what you do and for how long?
.....
.....
.....
3. Do you use any chemicals to prevent disease (e.g., to clean the pond walls when preparing, or added to the feed)? ☐ Yes ☐ No
4. If yes, what chemicals do you use?
.....
5. Access to pond (Tick all that apply):
☐ Only farm workers have access to pond
☐ Customers and visitors may also access the farm. If so, how often does this happen and what precautions do you take? Select all that apply.

Circumstance	Precautionary Measures
☐ Essential Visits e.g., Marketing purposes, maintenance work	☐ Visitors register ☐ Use of PPE e.g., disposable coveralls ☐ Use of chemical foot bath

☐ Leisure Visits	☐ None of the above
---	--

6. Do you treat the water used for production? ☐Yes ☐No
7. If so, with what and how do you do it?

8. How do you monitor water quality? ☐Change in water colour/clarity
☐Use of water testing kits ☐I send the water for lab analysis ☐
 Change in fish behaviour ☐Mortalities
9. How frequently do you monitor the water quality?
10. Does this frequency change? ☐Yes ☐No
11. If so, why does it change?

12. Do the same farm workers operate in different sections of the farm e.g., hatchery and grow-out ponds or ponds containing fish of different ages? ☐Yes ☐No
13. Do you keep fish of different ages or batches together? ☐Yes ☐No
14. Is farm worker's movement to other farms checked? ☐Yes ☐No
15. Are there provisions for chemical foot bath, hand washing with soap and changing of clothes for farm workers? ☐Yes ☐No
16. Do you share equipment like nets, hose, plastic containers between ponds/units?
☐Yes ☐No
17. Do you disinfect these equipment either between ponds or between use?
☐Yes ☐No
18. If yes, can you mention which disinfectant you use

19. Do you treat your ponds post-harvest? ☐Yes ☐No
20. If yes, which of the following do you use? Tick all that apply. ☐Salt
☐Agricultural lime ☐formaldehyde ☐Chlorine ☐Potash
☐Hydrogen Peroxide ☐Other

21. Can you describe how you go about the cleaning and preparation of the pond for a new cycle

22. How do you dispose of dead fish? (Tick all that apply)
☐ I bury dead fish
☐ I incinerate dead fish
☐ I feed them to the other fish
☐ I discard them to compost away from the fish but within the farm
☐ Other
23. Have you used probiotics on your farm?
☐ Not aware of any fish probiotic in the market ☐ Yes ☐ No
24. If yes, did it work? ☐ Yes ☐ No
25. If no, why have you not done so?

26. If no, would you consider using probiotics in the future? ☐ Yes
☐ No
27. Have you ever vaccinated your fish? ☐ Yes ☐ No
28. If yes, was it beneficial? ☐ Yes ☐ No
29. If no, could you tell why? ☐ I am not aware of any available fish vaccine ☐
 They do not work ☐ Too expensive ☐ Other

30. If no, would you consider using them when made available or improved?
☐ Yes ☐ No

Appendix 2.2 Participant information sheet

A SURVEY OF DISEASES AFFECTING THE AQUACULTURE INDUSTRY OF NORTHERN NIGERIA

This study is designed to investigate fish diseases and their possible causes that are commonly found in fish farms in northern Nigeria. This will help to assess the scale of problems and the selection of effective prevention and control measures. This work is also intended to support the growth of the aquaculture sector and individual farmers to enhance their livelihoods.

This work forms part of my PhD which is fully funded by the Commonwealth Scholarship Commission at the Institute of Aquaculture, University of Stirling, Scotland, UK.

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Do you consent to participate in this survey ☐Yes ☐No

Would you like me to send you the combined anonymised results of the survey once the study is complete?
☐Yes ☐No

Name of Respondent:

Do you agree that anonymised data from your answers can be included in my PhD thesis and published in any medium, for example as peer-reviewed articles, conference presentations and project funding applications?

☐Yes ☐No

Respondent's coded ID:

Date:

Chapter 3 Appendices

Appendix 3.1 Export permit for bacterial isolates from Nigeria

FEDERAL MINISTRY OF AGRICULTURE AND RURAL DEVELOPMENT
DEPARTMENT OF VETERINARY AND PEST CONTROL SERVICES
New Secretariat Area 11, P.M.B. 135, Garki, Abuja

Telegrams
Telephone



VSD/269/S.2/1622
Ref. No. 17th November, 2020
.....20.....

To whom it may concern

PERMISSION TO EXPORT BIOLOGICS OUT OF NIGERIA

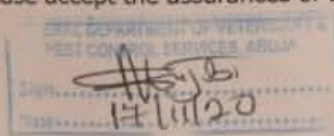
The Department hereby grants permission to **Dr Jibril Yakubu** of Dept of Agric Biotechnology, National Biotechnology Development Agency, Lugbe Abuja Nigeria, to Export biologics with details below to the United Kingdom.

Type	Quantity	Date of Production	Exporting country
Aeromonas Bacterial Isolates	150	09-11-2020	Nigeria

Validity: 31st November, 2020

This permission is written in lieu of an export permit as we are currently experiencing some technical problems with the database software for the processing of import/ export permits.

Please accept the assurances of the Chief Veterinary Officer's regards.


17/11/20

Dr. Chinyere Akujobi
For: Chief Veterinary Officer Nigeria

Appendix 3.2 Secretory systems

	Gene	Annotation	n	DC1K	DC2K	JC1K	JC1U	KMC10U	KMC2U	KMC5U	KMC6U	KMC7K	SC5K	SMC10U	SMC11U	SMC7K	SMC8U	SMC9U	SUC4U
1	prsE	Type I secretion system membrane fusion protein PrsE	8		+		+	+		+		+	+				+	+	
2	epsG	Type II secretion system protein G	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	epsF_1	Type II secretion system protein F	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	epsE	Type II secretion system protein E	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	xcpW	Type II secretion system protein J	8	+		+			+		+			+	+	+			+
6	epsF_3	Type II secretion system protein F	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	epsH	Type II secretion system protein H	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	epsC	Type II secretion system protein C	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	epsF_2	Type II secretion system protein F	9	+		+			+		+			+	+	+			+
10	epsL	Type II secretion system protein L	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	epsM	Type II secretion system protein M	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	outN	Type II secretion system protein N	15	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
13	gspK_1	Putative type II secretion system protein K	3					+		+		+							
14	xcpT	Type II secretion system protein G	1				+												
15	pscE	Type III export protein PscE	5		+		+		+		+								
16	pscF	Type III export protein PscF	5		+		+		+		+								
17	pscR	Type III export protein PscR	5		+		+		+		+								
18	pcsS	Type III export protein PscS	2					+		+									

Appendix 3.3 Multidrug efflux pumps

	Gene	Annotation	n	DC1K	DC2K	JC1K	JC1U	KMC10U	KMC2U	KMC5U	KMC6U	KMC7K	SC5K	SMC10U	SMC11U	SMC7K	SMC8U	SMC9U	SUC4U
1	mdlB	Multidrug resistance-like ATP-binding protein MdlB	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	norM_1	Multidrug resistance protein NorM	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	norM_2	Multidrug resistance protein NorM	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	ybhS	putative multidrug ABC transporter permease YbhS	13	+		+	+	+	+	+	+	+	+	+	+	+			+
5	yheI	putative multidrug resistance ABC transporter ATP-binding/permease protein YheI	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	emrD	Multidrug resistance protein D	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	mdtG	Multidrug resistance protein MdtG	8	+		+			+		+			+	+	+			+
8	bmr3	Multidrug resistance protein 3	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	mdtH	Multidrug resistance protein MdtH	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	mdtL_2	Multidrug resistance protein MdtL	16	+	+	+	+	++	+	++	+	++	+	+	+	+	+	+	+
11	mdtC	Multidrug resistance protein MdtC	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	mdtA_2	Multidrug resistance protein MdtA	16	+	+	+	+	++	+	++	+	++	+	+	+	+	+	+	+
13	mepA_1	Multidrug export protein MepA	16	+	+	++	+	+	+	+	+	+	+	++	+	+	+	+	+
14	mdtA_1	Multidrug resistance protein MdtA	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	mdtN	Multidrug resistance protein MdtN	16	++	+	++	+	++	+	+	+	+	+	++	+	+	+	+	+
16	acrB	Multidrug efflux pump subunit AcrB	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	mdtA_3	Multidrug resistance protein MdtA	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	mdtE	Multidrug resistance protein MdtE	8	+		+			+		+			+	+	+			+
19	acrA	Multidrug efflux pump subunit AcrA	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	mdtL_1	Multidrug resistance protein MdtL	12	+	+	+	+		+		+		+		+	+	+	+	+
21	mepA_3	Multidrug export protein MepA	14	+	+		+	+	+	+	+	+	+			+	+	+	+
22	acrE	Multidrug export protein AcrE	11	+		+		+	+	+	+	+		+	+	+			+
23	mdtA_6	Multidrug resistance protein MdtA	5		+		+						+				+	+	
24	ybhR	putative multidrug ABC transporter permease YbhR	5				+	+		+		+	+						
25	mdtE_1	Multidrug resistance protein MdtE	5	+							+				+	+			+
26	mexB	Multidrug resistance protein MexB	7		+		+				+		+				+	+	+
27	mdtA_5	Multidrug resistance protein MdtA	5		+		+						+				+	+	
28	ybhF	putative multidrug ABC transporter ATP-binding protein YbhF	5				+	+		+		+	+						
29	acrE_2	Multidrug export protein AcrE	6		+		+				+		+					+	+
30	mdtA_4	Multidrug resistance protein MdtA	5		+		+						+				+	+	
31	acrE_1	Multidrug export protein AcrE	4		+								+				+	+	
32	mdtE_2	Multidrug resistance protein MdtE	2			+								+					
33	acrA_1	Multidrug efflux pump subunit AcrA	2			+								+					
34	acrB_2	Multidrug export protein AcrF	2			+								+			+	+	
35	emrE	Multidrug transporter EmrE	2					+	+										
36	mdtH_2	Multidrug resistance protein MdtH	2															+	+
37	mdtL_3	Multidrug resistance protein MdtL	1			+													
38	mexA	Multidrug resistance protein MexA	1						+										
39	mdtN_1	Multidrug resistance protein MdtN	1															+	
40	acrA_2	Multidrug efflux pump subunit AcrA	1															+	

Chapter 4 Appendices

Appendix 4.1 Participant information sheet/Consent form

Consent Form for conducting an online survey

1. Research Project Title

Evaluation of the Impact of Online Workshop as an Intervention on Biosecurity as Practiced by Fish Farms in Northern Nigeria and The Federal Capital Territory

2. Opening text:

You are invited to participate in a web-based online workshop on the above topic. You have been invited because you are a fish farmer/fish farm manager from this region, and you are 18 years or older. This survey is part of a PhD research project being conducted by myself (Jibril Yakubu Jibril), a research postgraduate student at the University of Stirling. You will receive online training in the form of a workshop which should last approximately an hour. You will then be requested to answer a questionnaire up to two times. Each should take approximately 30 minutes to complete.

Please read through these terms before agreeing to participate below.

3. Background, aims of project

Biosecurity on fish farms in this region has been found to be relatively poor, which can lead to fish disease outbreaks and need for control such as with antibiotics. This has consequences on the farmer's income, public health, and the environment. Therefore, this project aims to determine if an online workshop has beneficial influence on improved farm biosecurity practices. Objectives of this project include:

1. To train fish farmers in northern Nigeria on effective fish farm biosecurity practices through an online workshop.
2. To determine if lessons learnt from the workshop beneficially impacts biosecurity as practiced by workshop participants on their farms.
3. To determine if there is an association between mortality and/or the need for antibiotic treatment of fish with biosecurity level.

4. Do I have to take part?

No. Your participation in this survey is voluntary. You may refuse to take part in the research or exit the survey at any time without penalty by pressing the 'leave' button during the training/closing the browser or by declining to participate in the follow up questionnaire sessions. You are free to decline to answer any question for any reason – we have included a 'prefer not to say' option.

5. Are there any potential risks in taking part?

There are no foreseeable risks involved in participating.

6. Are there any benefits in taking part?

You will receive training on biosecurity practices and will receive a water quality testing tool or a £10 phone voucher for taking part in this survey.

7. What happens to the data I provide?

Your answers (research data) will be completely anonymous. This research data will be stored in a file accessible only to the researcher and will be kept for a maximum period of 10 years on One Drive – a secure data storage platform used by the University of Stirling.

Contact information will be retained to enable your participation in the training and for the follow up survey(s) only and destroyed immediately thereafter. The information will be stored in a file accessible only to the researcher on One Drive. Your IP address will not be required or stored.

At the end of the training workshop, you will be required to participate in an additional interview by phone. If you choose to provide contact information such as your phone number your survey responses may no longer be anonymous to the researcher. However, no names or identifying information would be included in any publications or presentations based on these data and your responses to this survey will remain confidential.

We will ask all participants for their permission to use direct quotes.

8. Will the research be published?

The research may be published in the form of thesis/journal article/workshop/conference presentation. The University of Stirling is committed to making the outputs of research publically accessible and supports this commitment through our online open access repository STORRE. Unless funder/publisher requirements prevent us this research will be publicly disseminated through our open access repository.

9. This project is funded as part of my PhD research programme by the Commonwealth Scholarship Commission in London.

10. Who has reviewed this research project?

The ethical approaches of this project have been approved via The University of Stirling General University Ethics Panel (GUEP)

11. Your rights

You have the right to request to see a copy of the information we hold about you and to request corrections or deletions of the information that is no longer required.

You have the right to withdraw from this project at any time without giving reasons and without consequences to you. You also have the right to object to us processing relevant personal data however, please note that once the data are being analysed and/or results published it may not be possible to remove your anonymised data from the study.

12. Whom do I contact if I have concerns about this study or I wish to complain?

If you would like to discuss the research further, you can reach me via my email address j.y.jibril@stir.ac.uk or my supervisor, Dr Andrew Desbois (andrew.desbois@stir.ac.uk).

You have the right to lodge a complaint against the University regarding data protection issues with the Information Commissioner's Office (<https://ico.org.uk/concerns/>). The University's Data Protection Officer is Joanna Morrow, Deputy Secretary. If you have any questions relating to data protection these can be addressed to data.protection@stir.ac.uk in the first instance.

Thank you for your participation.

13. Electronic Consent

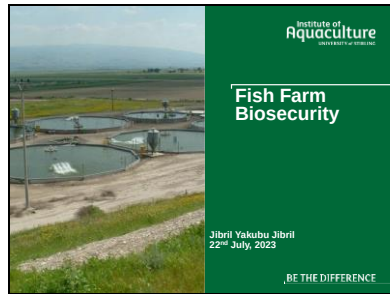
Please select your choice below. Clicking on the "Agree" button indicates that:

- You have read and understood the above information
- You are 18 years of age or older
- You voluntarily agree to participate

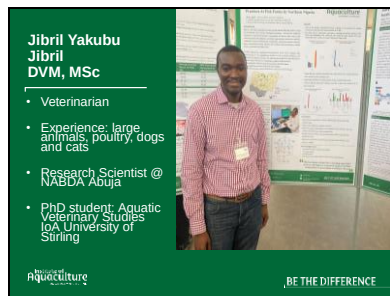
Agree	☐
Disagree	☐

Appendix 4.2 Biosecurity workshop PowerPoint presentation

Slide 1



Slide 2



Slide 3



Slide 4



Slide 5



Slide 6

What is Biosecurity?

Measures taken to prevent, control, and eradicate disease/infectious agent from a farm.

The key objectives of biosecurity are to reduce the risk of disease:

- > *introduction* into a farm
- > *spread* within a farm
- > *escape* from a farm



Slide 7

Why is Biosecurity Important?

Disease directly impacts profitability

- reduced growth rate
- cost of medication
- mortalities
- loss of market



Slide 8

Why is Biosecurity Important?

Main Benefits

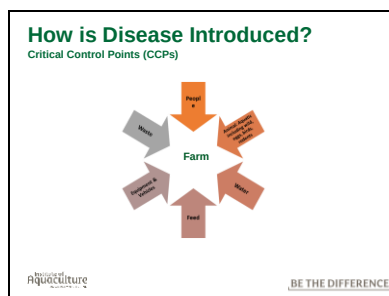
- Improved productivity
- Increased economic returns
- Improved farm reputation
- Fulfill regulatory requirements
- Meet international standards to access overseas markets



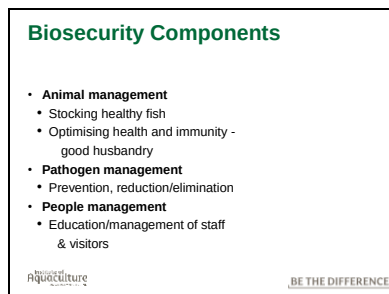
Slide 9



Slide 10



Slide 11



Slide 12

Biosecurity Components

- **Animal management**
 - Stocking healthy fish
 - Optimising health and immunity - good husbandry
- **Pathogen management**
 - Prevention, reduction/elimination
- **People management**
 - Education/management of staff & visitors

External

Internal

Aquaculture

BE THE DIFFERENCE

Slide 13

External Biosecurity Components

- Perimeter fencing



Aquaculture

BE THE DIFFERENCE

Slide 14

External Biosecurity Components

- Perimeter fencing
- Use reputable source of disease-free stock – wild stock can be risky



Aquaculture

BE THE DIFFERENCE

Slide 15

External Biosecurity Components

- Perimeter fencing
- Use reputable source of disease-free stock – wild stock can be risky
- Restricted access to the farm



Roadside Aquaculture
BE THE DIFFERENCE

Slide 16

External Biosecurity Components

- Perimeter fencing
- Use reputable source of disease-free stock – wild stock can be risky
- Restricted access to the farm
- Quarantine, stock assessment & testing



Roadside Aquaculture
BE THE DIFFERENCE

Slide 17

External Biosecurity Components

- High quality water sources
- Foot dips & strict sanitary measures for staff and visitors – farm-dedicated clothing & hand hygiene
- Pest control
- Vehicle and equipment disinfection



Roadside Aquaculture
BE THE DIFFERENCE

Slide 18

Internal Biosecurity Components

- Strict sanitary & personal hygiene measures
- Dedicated staff to specific units where possible
 - Otherwise, in daily routine start with highest health status
- Parallel water flow system



Slide 19

Internal Biosecurity Components

- Avoid equipment sharing where possible
- Cleaning and disinfection of equipment
- Effective post harvest pond treatment
- Prompt removal of sick/dead animals



Slide 20

CCP: People

- Restrict Access





Slide 21



Slide 22



Slide 23



Slide 24

CCP: Vehicles & Equipment

- Designated delivery and loading areas and provisions for cleaning and disinfection





Slide 25

CCP: Vehicles & Equipment

- Designated delivery and loading areas and provisions for cleaning and disinfection
- Dedicated farm equipment





Slide 26

CCP: Vehicles & Equipment

- Designated delivery and loading areas and provisions for cleaning and disinfection
- Dedicated farm equipment
- Where not possible, ensure Effective cleaning and disinfection between use





Slide 27

CCP: Vehicles & Equipment

- Designated delivery and loading areas and provisions for cleaning and disinfection
- Dedicated farm equipment
- Where not possible, ensure Effective cleaning and disinfection between use
- Cleaning & disinfection should be done within a dedicated area in each unit



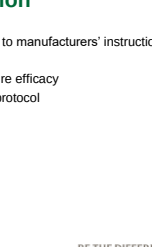
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Slide 28

Effective Disinfection

- Prepare disinfectant according to manufacturers' instruction
- Record date of replenishment
- Regular replenishment to ensure efficacy
- Follow the 6-step disinfection protocol



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6-Step Disinfection Protocol



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Extension

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CCP: Water, Feed & Waste

- Water
 - Use good quality water
 - Never add water used to ship fish to your system
 - Separate flow channel for each pond & proper discharge
 - Regular monitoring of water quality parameters



Slide 31

CCP: Water, Feed & Waste

- Water
 - Use good quality water
 - Never add water used to ship fish to your system
 - Separate flow channel for each pond & proper discharge
 - Regular monitoring of water quality parameters
- Feed
 - Use manufactured commercial feed where possible
 - Store feed in well ventilated, secure, cool room to avoid fungal growth/nutrient breakdown/pest contamination
 - Avoid using wild catch & animal offal as feed
 - Never use dead fish & animals



Slide 32

CCP: Water, Feed & Waste

- Water
 - Use good quality water
 - Never add water used to ship fish to your system
 - Separate flow channel for each pond & proper discharge
 - Regular monitoring of water quality parameters
- Feed
 - Use manufactured commercial feed where possible
 - Store feed in well ventilated, secure, cool room to avoid fungal growth/nutrient breakdown/pest contamination
 - Avoid using wild catch & animal offal as feed
 - Never use dead fish & animals
- Waste
 - Proper disposal of dead animals, water and effluents
 - Clean & disinfect any equipment used for disposal before returning it to any unit



Slide 33

Emergency Procedure

- Clearly defined emergency triggers e.g., certain level of mortality
- Written plan of necessary actions such as cessation of any activity, who to notify, securing the area etc.
- Contact details of a veterinarian/fish health expert or appropriate authority
- Contingency plans for safe destruction/disposal of large volumes of diseased or dead stock and decontamination of ponds/equipment




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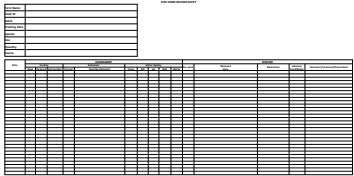
Record Keeping

- Detailed production & health/disease records
- Detailed fish movement into, within and off the farm
- Keep record of all visitors
- Ensure staff & visitors are aware of biosecurity measures applicable to them



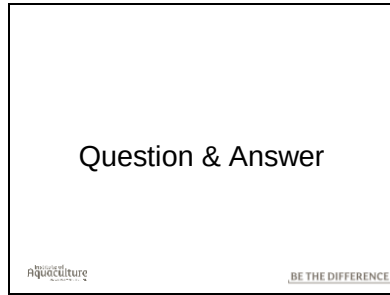

Slide 35

Template Record Sheet






Slide 36



Slide 37



Appendix 4.3 Template fish farm record sheet

E. EXTERNAL BIOSECURITY MANAGEMENT

Traffic Management

31. Who has access to your farm/Pond ☐ Only farm workers ☐ Other people as well.
32. Do you/your workers work in other farms? ☐ Yes ☐ Sometimes ☐ No

Source of Fish Stock

1. Do you stock your pond with fish from the river? ☐ Yes ☐ No
2. Do you operate a hatchery on your farm? ☐ Yes ☐ No
3. If yes, do you also get fish from other hatcheries? ☐ Yes ☐ No
4. If no (Question 2), do you always use new/dedicated container for bringing fish?
☐ Yes ☐ No, the supplier use his containers
5. If no (Question 2), do you quarantine new fish stock (isolate them in a separate pond for a period before transferring them to rearing ponds)? ☐ Yes ☐ No
6. If yes, for how long? ☐ < 2 weeks ☐ 2 – 4 weeks ☐ > 4 weeks

Water Management

1. What is your source of water ☐ Borehole ☐ River/Stream
☐ Dam ☐ Well
2. If you don't use borehole, do you treat the water before using? ☐ Yes
☐ Sometimes ☐ No
3. If yes, how do you treat it? ☐ Filtration ☐ UV radiation
☐ Ozonation ☐ Other

Feed Management

1. Do you protect your feed from pests and rodent e.g., mice/rats, cats etc.? ☐ Always
☐ Sometimes ☐ No
2. Do you use dead animals e.g., dead chicken to feed your fish? ☐ Yes
☐ Sometimes ☐ No
3. Do you use fish from the river to feed your fish? ☐ Yes ☐ Sometimes
☐ No

Pets and Rodents

1. Do you keep out rodents, birds, and other animals from the farm? ☐Yes ☐No

Cleaning and Disinfection

1. Do you have chemical footbaths at entry and exit points of your farm? ☐Yes
☐No
2. Do you have dedicated farm clothing & shoes? ☐Yes ☐No
3. Do you practice handwashing with soap and water before daily farm operations? ☐Yes ☐No
4. Do you borrow equipment from outside the farm? ☐Yes ☐Sometimes ☐No
5. If yes/sometimes, do you disinfect such equipment before bringing them to your farm? ☐Yes ☐Sometimes ☐No
6. Where applicable, do you disinfect any vehicles coming into your farm? ☐Yes
☐No ☐Not applicable

F. INTERNAL BIOSECURITY MANAGEMENT

Husbandry Management

1. How do you calculate/determine the number of fish to stock in a pond? ☐ I use pond dimensions ☐ I use rough estimate
2. If you operate more than one unit, e.g., hatchery and grow out, do you have dedicated staff for every unit? ☐ Yes ☐ No ☐ I have only one unit
3. If you answered no above, how do you operate daily? ☐ I start from the hatchery ☐ I start from the grow out unit ☐ I operate randomly
4. Do the same farm workers operate in all farm units e.g., hatchery and grow out?
☐ Yes ☐ No
5. Do you keep fish of different ages or batches in the same pond? ☐ Yes ☐ No

Water Management

1. Is the water outlet of one pond connected to another pond? ☐ Yes ☐ No
2. Do you regularly check water quality?? ☐ Yes ☐ Sometimes ☐ No
3. If you answered yes, what parameter(s) do you check? ☐ Temperature
☐ pH ☐ Dissolved oxygen ☐ Ammonia

Health Management

1. Do you isolate sick fish in a separate pond? ☐ Yes ☐ No
2. Do you consult a veterinarian/fish health expert when disease occurs on your farm?
☐ Yes ☐ Sometimes ☐ No
3. Do you promptly remove dead fish from the pond? ☐ Yes ☐ Sometimes
☐ No
4. How do you dispose of the dead fish? ☐ Incineration(burning) ☐ Burial
☐ On-farm dump site ☐ Other
5. Do you use probiotics? ☐ Yes ☐ No
6. Do you vaccinate your fish? ☐ Yes ☐ No

Cleaning and Disinfection

1. Do you share equipment between ponds/units? ☐Yes ☐Sometimes
☐No
2. If yes/sometimes, do you disinfect such equipment after every use? ☐Yes
☐Sometimes ☐No
3. If you have more than one unit, do you have foot bath at the entry point of every unit? ☐Yes ☐No
4. Do you treat your pond after harvest i.e., before restocking? ☐Yes
☐Sometimes ☐No
5. do you treat wastewater from your farm before discharging it? ☐Yes
☐Sometimes ☐No
6. If you answered "Yes/sometimes" to the last question, how do you do that?
.....