Norwegian University of Life Sciences
Faculty of Food Safety and Infection Biology

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Streptococcus-like infections in farmed tilapia *(Oreochromis niloticus)* of Lake Kariba: Characterization of pathogenic species and host responses

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Contents

ACRONYMS/ABBREVIATIONS

ADB	-	African Development Bank
AF	-	Atrial Fibrillation
API	-	Analytical Profile Index
ATCC		American Type Culture Collection
DNA	-	Deoxyribonucleic Acid
DoF	-	Department of Fisheries
EIA	-	Environmental Impact Assessment
EUS	-	Epizootic Ulcerative Syndrome
FAO	-	Food and Agricultural Organisation
GATC		Glutamyl-TRNA Amidotransferase Subunit C
IMM	-	Immersion
IP	-	Intra Peritoneal
JFRO	-	Joint Fisheries Research Organisation
JMP	-	Java Memory Profiler
LD	-	Lethal Dose
MT	-	Metric Ton
NDP	-	National Development Plan
NORAD	-	Norwegian Agency for Development Cooperation
NORHED	-	Norwegian Programme for Capacity Building in Higher Education and Research for Development
PBS -	-	Phosphate Buffer Solution
PCR -	-	Polymerase Chain Reaction
RAP -	-	Rural Aquaculture Promotion
RNA -	-	Ribonucleic Acid
rRNA-	-	Ribosomal Ribonucleic Acid
SADC	-	Southern Africa Development Community
SAS -	-	Science Analysis System
TRAHESA	-	Training and Research in Aquatic and Environmental Health in Eastern and Southern Africa
USA -	-	United States of America
VWK-	-	Vacuum Waste Kit

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SUMMARY

Zambia is one of the largest aquaculture producers in sub-Saharan Africa, with tilapia production having increased exponentially during the last 20 years. Lake Kariba is the most active commercial fish farming site in the country at the moment, with several farms operating intensive cage farming of Nile tilapia (*Oreochromis niloticus*). Along with intensification of tilapia production over the last years, came diseases that have been reported since 2014 albeit without being well characterized. Increased mortalities and clinical signs suggestive of streptococcus like infections were reported in outbreaks. The overall objective of this study was to characterize streptococcus-like bacteria causing disease in farmed Nile tilapia on Lake Kariba with a view of developing a vaccine to protect the fish.

In Paper 1, a time-course study was conducted where streptococcus-like bacterial species associated with disease outbreaks were isolated, identified and characterized from two farms on Lake Kariba from 2014-2016. To do this, samples were purposively collected from diseased fish, subjected to standard methods of bacterial identification followed by confirmation using PCR and sequencing of the 16S RNA gene. The findings revealed the presence of *Streptococcus agalactiae* and *S. iniae* in 2014, and *Lactococcus garvieae* (*L. garvieae*) in 2015 and 2016. Due to the persistent re-isolation of *L. garvieae* from diseased fish at the farms with time, subsequent studies including fulfilling of Koch's postulates were focused on this bacterium.

In Paper 2, the pathogenesis of *L. garvieae* in Nile tilapia following administration by different routes (peritoneal and immersion) was examined. Differences between groups were assessed by number of mortalities, pathology/histopathology, bacterial re-isolation and *in situ* presence of the bacteria by immunohistochemistry. A significant difference ($p<0.0001$) was observed between *L. garvieae* re-isolation from tilapia following administration by intraperitoneal injection (IP) on one hand, and immersion (IMM) on the other. Similarly, more clinical signs and mortalities were observed in the IP compared to the IMM group where no mortalities due to infection occurred. These findings suggest that *L. garvieae* does not actively invade Nile tilapia but takes advantage of cuts or abrasions to cause disease.

In the final paper (Paper 3) the focus was assessment of the host response of Nile tilapia to vaccination by using an oil-based *L. garvieae* autovaccine. The *L. garvieae* autovaccine was produced in-house. Groups of naïve tilapia were injected intraperitoneally with either the autovaccine, adjuvant only or normal saline. After a period of immune induction, the fish were challenged with *L. garvieae*. Protection against infections was measured by lack of/reduced bacterial loads both by bacterial re-isolation and immunohistochemistry as well as absence of clinical signs/pathology. Significantly less *L. garvieae* ($p<0.03$) were re-isolated from the vaccinated group than the adjuvant only or control groups. Correspondingly, a significantly higher level ($p<0.001$) of anti-*L. garvieae* specific antibodies were observed in the vaccinated group compared to the adjuvant only or control groups at time of challenge. This coincided with protection against infection measured by absence/reduced *L. garvieae* re-isolation from internal organs, reduced clinical signs and lack of pathology in this group compared to the adjuvant only or control groups. The findings suggest that oil-based vaccines can protect tilapia against *L. garvieae* infection through an antibody mediated response.

NORSK SAMMENDRAG

Zambia er en av de største akvakulturnasjonene i Afrika sør for Sahara, og tilapia-produksjonen har økt kraftig de siste 20 årene. Lake Kariba er hovedsete for kommersielt oppdrett for øyeblikket, med flere anlegg som driver intensivt merdoppdrett av nil-tilapia (*Oreochromis niloticus*). Sammen med intensivering av tilapiproduksjon over de siste årene, kom sykdommer. De første tilfellene rapportert i 2014, men årsaken til dødeligheten ble ikke bestemt. Senere ble det påvist økt dødelighet og med kliniske tegn på streptokokk-infeksjon i forbindelse med sykdomsutbrudd.

På denne bakgrunnen, var det overordnede målet for denne studien å karakterisere streptokokk-lignende bakterier som årsak til sykdom i oppdrettet nil-tilapia i Karibasjøen med sikte på å utvikle en vaksine mot sykdommene.

I den første studien ble det gjennomført en tidsløpsstudie der streptokokk-lignende bakterier assosiert med sykdomsutbrudd ble isolert, identifisert og karakterisert fra to anlegg i Kariba-sjøen i 2014-2016. Prøvene ble målrettet samlet inn fra syk fisk, og prøvene ble analysert med standardmetoder for bakteriell identifikasjon etterfulgt av en bekreftelse basert på PCR og sekvensering av 16S RNA-genet. Funnene viste tilstedeværelsen av *Streptococcus agalactiae* og *S. iniae* i 2014, og *Lactococcus garvieae* (*L. garvieae*) i 2015 og 2016. På grunn av den vedvarende re-isolasjonen av *L. garvieae* fra syk fisk på gårdene med tiden, ble påfølgende studier gjennomført med den hensikt å oppfylle Kochs postulat.

I den andre studien ble patogenesen til *L. garvieae* studert i nil-tilapia etter smitte via ulike ruter, ved injeksjon eller pr badesmitte. Forskjeller mellom gruppene ble vurdert etter dødelighet, patologi/ histopatologi, bakteriell re-isolerign og *in situ* tilstedeværelse av bakteriene ved immunhistokjemi. En signifikant forskjell ($p < 0{,}0001$) ble observert mellom *L. garvieae* re-isolering etter administrering ved intraperitoneal injeksjon (IP) og badesmitte (IMM). Tilsvarende ble det påvist mer uttalte kliniske tegn på sykdom og dødelighet i undersøkelsesperioden i IP gruppen sammenlignet med IMM-gruppen, hvor det ikke oppstod dødelighet som følge av eksperimentell infeksjon. Disse funnene gir indikasjoner for at *L. garvieae* ikke er invasive i nil-tilapia, men utnytter kutt eller andre hudskader som inngangsport for sykdom.

I den siste studien ble vertsresponsen hos nil-tilapia studert etter vaksinasjon med en oljebasert *L. garvieae* autovaksine, produsert i laboratoriet. Grupper av ikke-vaksinert tilapia ble injisert intraperitonealt med enten en autovaksine inneholdende inaktiverte *L. garvieae* bakterier, bare adjuvans eller saltvann. Etter at immunitet var etablert ble fisken smittet eksperimentelt med *L. garvieae*. Beskyttelse mot infeksjon ble målt ved mangel på/redusert bakterienivå ved re-isolering av bakterien fra indre organer og in situ påvisning av bakterien i indre organer ved immunhistokjemi, samt fravær av

kliniske symptomer/patologi. *L. garvieae* ble re-isolert hyppigere fra adjuvans eller kontrollgruppene ($p<0,03$) sammenlignet med den vaksinerte gruppen. Videre ble det påvist et høyere nivå ($p <0,001$) av anti-*L. garvieae*-spesifikke antistoffer i plasma hos den vaksinerte gruppen sammenlignet med bare adjuvans eller kontrollgrupper på smittetidspunktet. Dette falt sammen med beskyttelse mot infeksjon målt ved fravær eller redusert hyppighet av re-isolering av L. *garvieae* fra indre organer, reduserte kliniske tegn og mangel på patologi i denne gruppen sammenlignet med kun adjuvans eller kontrollgrupper. Funnene viser at oljebaserte vaksiner kan beskytte tilapia mot *L. garvieae*-infeksjon og der antistoffer spiller en viktig rolle for beskyttelse.

PUBLICATIONS

Paper 1: Bwalya P, Simukoko C, Store CS, Store PC, Hang'ombe BM, Gamil A, Munang'andu HM, Evensen Ø, Mutoloki S. 2020. Characterization of Streptococcus-like Bacteria from diseased *Oreochromis niloticus* farmed on Lake Kariba in Zambia. Aquaculture 523: 735185. https://doi.org/10.1016

Paper 2: Bwalya P, Hang'ombe BM, Evensen Ø, Mutoloki S. 2021. *Lactococcus garvieae* isolated from Lake Kariba (Zambia) has low invasive potential in Nile tilapia *(Oreochromis niloticus).* Journal of Fish Diseases (accepted). DOI: 10.1111/jfd.13339

Paper 3: Bwalya P, Hang'ombe BM, Gamil AA, Munang'andu HM, Evensen Ø, Mutoloki S 2020. A whole cell *Lactococcus garvieae* auto vaccine protects Nile tilapia against infection PLoS One. 15(3): e0230739. doi: 10.1371/journal.pone.0230739.

1.0. INTRODUCTION

1.1. Fish farming in Zambia

Zambia has long been classified as a country with ideal conditions for fish farming based on temperatures, soil conditions and availability of water resources (Genschick et al., 2017; Musuka and Musonda, 2013). Aquaculture dates back to the 1950s (Albert and Simbotwe, 2013) with approximately 15 million hectares of water in form of lakes, rivers and swamps (Figure 1) (Albert and Simbotwe, 2013; Maulu et al., 2019). There is also an additional 8 million hectares of wetland (ADP, 2009; Albert and Simbotwe, 2013; Maulu et al., 2019; Shula and Mofya-Mukuka, 2015). In a nutshell Zambia has soil conditions, water availability and temperature range suitable for aquaculture, especially of tilapia species (Mudenda, 2009).

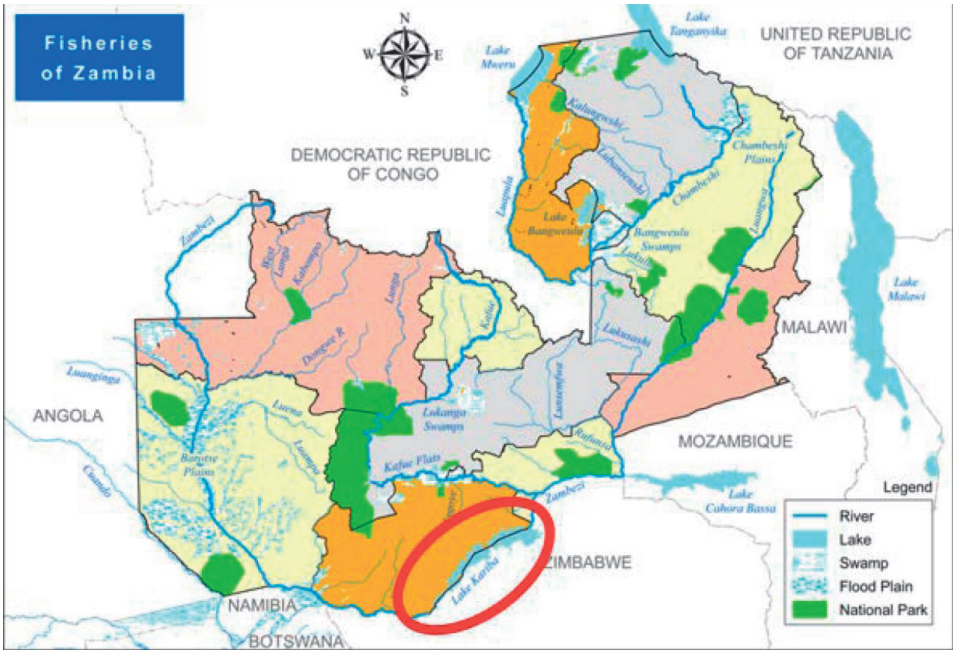


Figure 1. Map of Zambia showing locations of rivers, lakes, swamps and flood plains. Circle represents Lake Kariba where this study was undertaken.

1.2. History of fish farming in Zambia

The Joint Fisheries Research Organization (JFRO) undertook initial fish farming trials across the country dating back to 1950s (Albert and Simbotwe, 2013). Aquaculture development generated

some interest, but progress was slow because capture fisheries provided a cheap source of fish. The delays have also been due to limited markets, weak infrastructure, government policies and limited knowledge and skills to build the sector (Brummett et al., 2008; Genschick et al., 2017). There has also been little or no sharing of scientific discoveries and technologies both within and between countries Sub-Saharan African (Maulu et al., 2019). There is however evidence of some science and technology developments being reported in some African countries including Tanzania, South Africa, Kenya and Ghana (Satia, 2011).

Several large-scale undertakings in the country's aquaculture sector are having noticeable effects. Department of Fisheries in cooperation with non-governmental organizations (NGOs) and international assistance agencies did considerable work in promoting aquaculture practices that resulted in 6460 small-scale farmers operating over 13900 fish ponds (Mudenda et al., 2005; Simataa and Musuka, 2013). There are reports of the department of fisheries in Southern Province, under the Poverty Reduction Programme (PRO), undertaking a fish restocking program of Cichlids in 2010. A total of 36 reservoirs were stocked with 22,299 fingerlings (Musuka and Musonda, 2013). The aquaculture industry is now more diversified with fish being stocked in earthen ponds, tanks, pens and cages (Figure 2). Zambia has become the sixth largest producer of farmed fish in Africa and the first in the SADC region (Genschick et al., 2017) with some of the largest freshwater commercial farms in Africa operating in the country (Fisheries, 2016).

Aquaculture production is envisioned to play an important role not only in food and nutrition security but also in job creation, income generation and poverty reduction (Genschick et al., 2018; Maulu et al., 2019). The production has grown from 5,000 MT in 2006, to 30,285 MT in 2015 and 32,888 MT in 2017 (Fisheries, 2019). The increase in aquaculture production has been attributed to the emergence of commercial producers from the private sector (Shula and Mofya-Mukuka, 2015). Aquaculture is categorized as being either land based or cage culture (Albert and Simbotwe, 2013; Hasimuna et al., 2019; Maguswi, 1994). Land based include ponds, tanks, dams, weirs and pens with Lusaka, Northern and North Western Provinces having higher numbers in total compared to the other provinces. Cage culture is well established in Southern province on Lake Kariba by private companies that practice intensive farming, introduced in the 1990s. Lake Bangweulu in Luapula province has also seen some aquaculture cage farming.

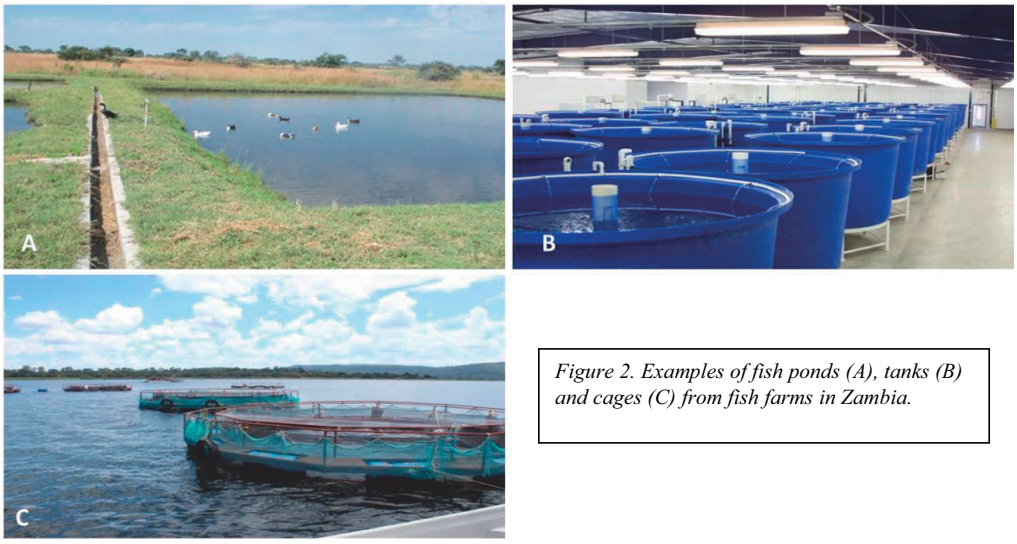


Figure 2. Examples of fish ponds (A), tanks (B) and cages (C) from fish farms in Zambia.

1.3. Production of Tilapia

Tilapia in Zambia was mostly cultured in ponds in the past but now there is an increasing number of floating net-cage farming with lake Kariba being the leading site. Major species farmed include *Oreochromis niloticus (O. niloticus) O. andersonii, O. rendalli,* and *O. macrochir*. Carp, crayfish (red claw, maron and yabbies') and catfish are also farmed to a lesser extent (Maulu et al., 2019; Mudenda, 2004; Musumali et al., 2009).

The initial challenge in expanding the aquaculture sector in Zambia was, in part, due to constraints in finding suitable cultivable indigenous species. Most of the indigenous species were not able to boost fish production to high levels as their performance was below par. Genetically Improved Farmed Tilapia (GIFT), a strain of *O. niloticus,* was introduced in Zambia in 1982 to improve the value of farmed fish and enhance productivity (Pillay, 1990) as is hardy, resilient and fast growing (Ponzoni et al., 2011). However, it escaped into the Kafue river (Deines et al., 2014; Howard, 2004; Schwanck, 1995; Thys van den Audenaerde, 1994) resulting in contamination of wild fish stocks. In subsequent years, *O. niloticus* has been widely distributed among small scale fish farmers in Solwezi district by the American Peace Corps under the Rural Aquaculture Promotion (RAP) project in collaboration with the Department of Fisheries, and has been accepted for its ability to thrive well on organic manure fertilized ponds and gave improved yields even under minimal pond management practices (Simataa and

Musuka, 2013; Trant, 2004).

Since *O. niloticus* reproduce at a fast rate, it tends to overcrowd and out-compete native species. Its production was discontinued in some areas due to some negative ecological and environmental concerns (Bbole et al., 2014; Deines et al., 2014; Kefi and Mwango, 2018). The invasive nature of the species was feared to lead to genetic homogenization and erosion resulting from the species ability to inbreed with its congeneric. The loss of biodiversity leads to genetic erosion and greater susceptibility to disease (Bbole et al., 2014; Deines et al., 2014). On Lake Kariba, *O. niloticus* was introduced as an aquaculture species in cage-culture and is now established in both middle and lower Zambezi basins (Howard, 2004). Fish farmed in Zambia by volume are shown in Figure 3.

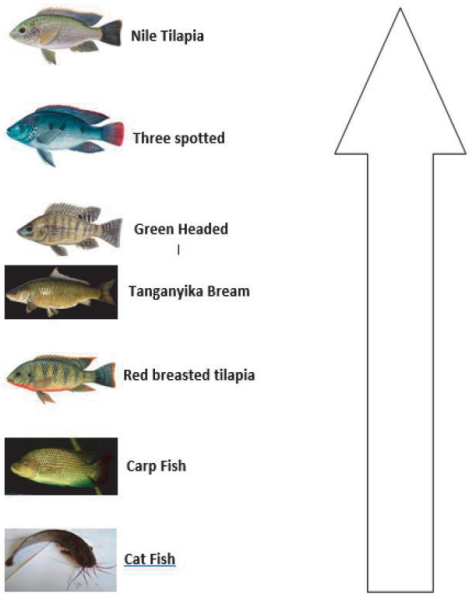


Figure 3: Fish species farmed in Zambia by volume. Catfish is the least farmed while Nile tilapia is produced the most.

1.4. Challenges associated with fish farming in Zambia

Constraints of aquaculture experienced in Zambia include the following: a) inadequate sources of quality fish seed, b) lack of quality fish feed, c) high cost of setting up fish farming enterprises, d) difficulty of accessing finances, e) inadequate extension services, f) inadequate marketing support and g) environmental contamination and diseases. These challenges are

discussed in-depth below.

1.4.1. Inadequate sources of quality fish seed. Government institutions together with private companies have been set up in the country to offer quality seed. Earlier in the past only the 9 state hatcheries supplied the entire aquaculture sector. There is currently also five operating state-run hatcheries; Misanfu Aquaculture Research Station in Kasama Northern Province, Chinsali Aquaculture research station in Chinsali, Muchinga Province, National Aquaculture Research centre in Kitwe Copperbelt, Solwezi Aquaculture research station in Solwezi, Northwestern Province and Chipata Aquaculture research station in Chipata, Eastern Province in the country but production cannot meet the fingerling demand (Genschick et al., 2017; Kaminski et al., 2018). There are other government fish farms that also produce fingerlings, Chalata Government fish farm in Mkushi central Province, Kaoma Government fish farms in Kaoma Western Province, and Isoka Government fish farm in Isoka Northern Province. Several aquaculture producers, including Palabana Fisheries, Chirundu Bream, Savanna, Yalelo and Kafue fisheries have established hatcheries that provide fingerlings for sale and also for their use. These are located either in Southern Province or in major towns like Lusaka, Kitwe and Ndola. Despite this increase in fingerling producers, the demand still exceeds the supply, thus some commercial farmers have resorted to importing fingerlings.

1.4.2. Lack of quality fish feeds. At the start of this study project, there were no feed-producing companies specialized in fish feeds in Zambia. Farmers depended on millers producing the staple food and livestock feed for human consumption and terrestrial animals respectively. These millers are now producing feed for fish as well. Examples are Novatec Animal feed Limited, Savanna streams, Farm feed mills, Tiger Feeds and Olympic milling. One challenge was that raw materials for feed production were difficult to source, especially micro ingredients such as vitamins, fish meal and premixes. It was not until 2017 that reputable companies like Skretting came onto the market (Genschick et al., 2017; Hasimuna et al., 2019; Kaminski et al., 2018). Even with the present expertise in place, the cost of fish feed remain exorbitant and too costly for the average subsistence farmer, for which the feed accounts for 50% of the cost of production (Nyimbiri Beatrice, 2017). Lack of knowledge on how to estimate the actual biomass so as to identify feed requirements is also a contributing factor (Genschick et al., 2017; Kaminski et al., 2018; Namonje-Kapembwa and Samboko, 2020). Finally, the cost of transportation of feed from millers which included Siavonga, Lusaka and the copperbelt to fish farms pushes the cost of the

feed even higher.

1.4.3. High start-up costs. Before a fish farm is established, it is a requirement that an Environmental Impact Assessment (EIA) is done. The fees associated with these are prohibitive to many small-scale farmers. In addition, there are costs associated with Environmental Project Briefs (EPB), water rights and requirement before commencement of the enterprises (Hasimuna et al., 2019; Maulu et al., 2019; Musuka and Musonda, 2012). An estimate for a start-up capital for construction of a pond was found to be ZMK 24,750 (about $1000) (Namonje-Kapembwa and Samboko, 2020). Appropriate technologies required for fish grading, feeding and harvesting require huge financial investments hence small scale producers lack capacity to buy (Hasimuna et al., 2019).

1.4.4. Lack of access to capital. The level of productivity in aquaculture is partly dependent on availability of finances in terms of credit. Most financial institutions are reluctant to offer credit facilities for aquaculture investment as they consider it a risky venture. Inadequate awareness on the potential and viability of aquaculture is one reason attributed to this misconception (Albert and Simbotwe, 2013) Climate variability and unpredictability is another reason why financial institutions are reluctant to support such investments.

1.4.5. Inadequate extension services. Most farmers, especially at the small-scale level, lack basic husbandry and fish health knowledge, rather are dependent on indigenous knowledge passed on by parents or from peers. This information may lack scientific support and may be based on myths.

1.4.6. Environmental contamination and diseases. Heavy metal pollution is an issue of great environmental concern in Zambia (Nakayama et al., 2010). One of the country's major rivers, the Kafue, is a site for several fish farms and it runs through the Copperbelt region, a core mining area. Effluents from the mines that contain high levels of heavy metals are deposited in the Kafue River leading to high levels of contamination in fish in this river (Choongo et al., 2005; Nakayama et al., 2010). These heavy metals have been demonstrated to accumulate in several fish species including *O. niloticus*, *Serranochromis thumbergi* and *Cherax quadricarinarus* although no adverse effects have been were observed (Nakayama et al., 2010). The reported toxicity in most cases cannot conclusively be linked to pathological disorders of the

fish. Nonetheless, pathological changes such as reproductive abnormalities and granulomas in fish reared in cages containing sediments from the Copperbelt region of the Kafue river were observed (Mwase et al., 1998). Serranochromis fish from the Copperbelt region had lower coefficient of condition indicating poor health (lower plumpness and robustness) implied to be caused by accumulation of high copper and other heavy metal levels in sediments and fish. Fish were healthier towards downstream areas further from the mines (Choongo et al., 2005; Mbewe et al., 2016).

Diseases are an important constraint to the expansion of aquaculture (Krkošek, 2017; Salama and Murray, 2011; Ögüt, 2001). Intensification of fish farming inevitably leads to disease outbreaks and in countries where tilapia is produced on a large scale, bacteria and viruses represent the important causes of infectious diseases estimated to be represented by 55% and 23%, respectively (Kibenge et al., 2012). In Africa, Infectious spleen and kidney necrosis virus and Tilapia Lake virus are probably the most important viral pathogens responsible for high mortalities in farmed tilapia (Nicholson et al., 2017; Ramirez-Paredes et al., 2020). In Zambia however, there are no records to suggest the presence of these diseases. Tilapia lake virus has been tested in tilapia from several localities, both from wild and farmed fish but the virus has not been detected (Hang'ombe, pers. comm.). Bacterial diseases, on the other hand as stated above, are the most prevalent wherever tilapia is farmed. Streptococcus and aeromonads species are among the most important bacterial pathogens causing economic losses in tilapia (Citterio and Biavasco, 2015; Yang et al., 2014; Zhu et al., 2019). In Zambia, there were no records of infectious bacterial diseases in farmed tilapia before the commencement of these studies although outbreaks with reported clinical signs consistent with streptococcus-like infections had been reported (Hang'ombe, pers. Comm.). Characterization of these bacteria with a view of finding methods of mitigating losses the farmers were encountering was the motivation of these studies.

1.5. Streptococcus infections

Streptococcosis is a collective term used to describe a variety of diseases caused by Gram positive cocci bacteria of the genus *Streptococcus*, family *Streptococcaceae,* order *Lactobacillales* and phylum *Firmicutes* (Vos et al., 2011; Yanong and Francis-Floyd, 2002). In addition to these, there are several other closely related groups of bacteria that can cause similar diseases, including *Lactococcus*, *Enterococcus*, and *Vagococcus* (Yanong and Francis-Floyd, 2002). These infections are the so-called streptococcus-like infections. Most streptococcus-like

bacteria are part of the normal flora of hosts and therefore cause infection as opportunistic pathogens. Some fish have been known to have bacteria in a carrier state where they show no sign of disease but only succumb to disease when stressed (Ellis, 2001; Hiney et al., 1997). In aquaculture, streptococcus-like infections are a cause of high morbidity and mortalities in different fish species especially those reared in warm water such as tilapia (Yanong and Francis-Floyd, 2002). Three streptococcus-like species lead to great economic losses in tilapia farming wherever the fish are reared, and these include *S. agalactiae*, *S. iniae* and *Lactococcus garvieae* (Amal and Zamri-Saad, 2011; Anshary et al., 2014b; Liu et al., 2016).

1.5.1. *Streptococcus agalactiae* in Tilapia

Streptococcus agalactiae is a single streptococcal species Lancefield group B (GBS) organisms (Evans et al., 2002) that are subdivided into 10 serotypes according to polysaccharide composition of capsules Ia, Ib, II to IX (Chaffin et al., 2000; Persson et al., 2004; Slotved et al., 2007). Previously only Ib was associated with infections in fish (Vandamme et al., 1997) but currently, several serotypes of GBS are infective to tilapia (Suanyuk et al., 2008). There are two biotypes based on biochemical identification and haemolytic ability. Biotype I is beta-haemolytic while biotype II is γ-haemolytic (Soto et al., 2015). It is the most commonly encountered species in hot climate being associated with different freshwater, marine and estuary fish species (Evans et al., 2002). It causes significant morbidity and mortality in fish species with a world-wide distribution (Evans et al., 2002; Plumb et al., 1974; Robinson and Meyer, 1966) and a broad host range (Duremdez et al., 2004; Garcia et al., 2008; Salvador et al., 2005).

Predisposing factor to *S. agalactieae* infections include stress, for example due to high stocking density, low dissolved oxygen, high ammonium levels and changing or extreme water temperatures (Evans et al., 2009a; Mian et al., 2009).

Fish most affected include those weighing above 150 grams (Amal and Zamri-Saad, 2011) and the most common clinical signs include erratic swimming, unilateral or bilateral exophthalmia, corneal opacity, eye haemorrages, haemorrhages at the base of the fins and opercula, body curvature or vertebral deformities (Abuseliana et al.; Ali et al., 2010; Austin and Austin, 2012; Siti-Zahrah et al., 2008b). Sudden death with no clinical signs can also occur (Rodkhum et al., 2011; Ye et al., 2011). Postmortem findings include liquid in visceral cavities, liver and spleen

enlargement (Salvador et al., 2005; Zamri-Saad et al., 2010). Figure 4 shows some common lesions associated with *S. agalactiae* infections.

Figure 4. Common lesions observed in tilapia infected with streptococcus agalactiae. A) Ocular opacity B) Pale and distended liver C) Darkened skin and D) distended abdomen

1.5.2. *Streptococcus iniae* in Tilapia

There are two known serotypes of *S. iniae* that can be distinguished by enzyme activity, I and II (Goh et al., 1998; Pier and Madin, 1976). Serotype I is positive to Arginine dihydrolase activity while serotype II is not (Bachrach et al., 2001). Serotype II is hyper-capsulated causing the functional difference from serotype I (Barnes et al., 2003).

Predisposing factors to *S. iniae* in Nile tilapia are similar to those of *S. agalactiae*, including stress due to high stocking densities (Shoemaker et al., 2000). In fact, there are several reports where co-infections of *S. agalactiae* and *S. iniae* have been reported (Legario et al., 2020; Piamsomboon et al., 2020).

Most clinical signs are similar to those caused by *S. agalactiae* and include erratic swimming, lethargy, skin darkness and exophthalmia (Agnew and Barnes, 2007; Figueiredo et al., 2012). Enlarged spleen, ascites and discolored liver are seen postmortem (Salati, 2006). Disease outbreaks are sporadic with mortality rates of between 30% and 50 %. Asymptomatic infections can also occur (Rahmatullah et al., 2017).

1.5.3. *Lactococcus garvieae* in tilapia

First described as a Gram positive coccus causing septicemia in rainbow trout in Japan (Hoshina et al., 1958), *L. garvieae* was initially assigned to the genus *Streptococcus* based on phenotypic

similarities with other etiological agents. However, with development of genotyping methods, it was reclassified to a separate genus as *Lactococcus* (Domenech et al., 1993; Eldar et al., 1996). *Lactococcus garvieae* is a serious pathogen of both freshwater and marine fish (Collins et al., 1983; Kusuda et al., 1991). Since 1991, it has been recognized as a pathogen of fish in Japan and of trout throughout the Mediterranean region (European side). It has been isolated from a wide range of fish species (Meyburgh et al., 2017) and is an emerging pathogen of humans (Chan et al., 2011; Gibello et al., 2016) mainly due to handling and ingestion of raw fish (Gibello et al., 2016).

Predisposing factors for infections in fish include high water temperatures, low dissolved oxygen levels and increased ammonium levels (Anshary et al., 2014b; Liu et al., 2016). Clinical symptoms include melanosis, erratic swimming, ascites, exophthalmia (uni- and bilateral), rectal prolapse, haemorrhages in the periorbital and intraocular area, base of fins and perianal regions, opercular and the buccal region (Bastardo et al., 2012; Fukushima et al., 2017; Vendrell et al., 2006).

Serologically *L. garvieae* has been characterized by slide agglutination technique (Vendrell et al., 2006). This identifies an antigen, termed KG, in the cellular wall into KG+ and KG-. The KG+ type agglutinates with antiserum of KG 74409 strain while the KG- does not, as it possesses a specific envelop like substance which inhibits agglutination (Kitao, 1982). The KG- capsulated strain was found to be more virulent in causing infection than KG+ non capsulated in yellowtail (Kitao, 1983) and in rainbow trout (Barnes et al., 2002).

1.5.4. Differential diagnosis of streptococcus-like infections

Clinical signs caused by *S. agalactiae*, *S. iniae* and *L. garvieae* are similar and therefore difficult to distinguish (Bercovier et al., 1997; Mata et al., 2004; Siti-Zahrah et al., 2008a)}. Microscopically, tilapia infected with *S. iniae* have however been reported to show accumulation of eosinophilic material in cytoplasm of the tubular cells of the kidneys with nuclei displaced to the side (Chang and Plumb, 1996; Chen et al., 2007). Livers rarely show presence of bacterial cells although necrotic changes can be observed along hepatic arteries and near capsules. In contrast, kidneys of tilapia infected with *S. agalactiae* present dissolution of some tubules with cocci seen surrounding tubules, within interstitial cells and within glomeruli, necrotic foci with fibrin precipitation and lymphocyte infiltration. Bacterial cells can also be observed distributed

throughout the spleen with necrotic foci (Chen et al., 2007). In one study, there were more granuloma formation observed with *S. iniae* infections while ubiquitous presence of bacteria was observed with *S. agalactiae* infections (Perera et al., 1998).

In rainbow trout, *L. garvieae* produces a generalized disease with rapid onset of mortality. *S. iniae* infection results in a prolonged clinical course with specific lesions (Eldar and Ghittino, 1999).

1.5.5. Control of Streptococcus-like infections

The control of streptococcus infections mainly relies on the use of biosecurity strategies, antimicrobial compounds and vaccinations (Mishra et al., 2018). These measures are aimed at reducing the risk of introduction of pathogens into an aquaculture facility, limit spreading of pathogens if introduced and reduction of conditions of stress to the fish which enhance susceptibility to sub-clinical and clinical disease once pathogens are introduced (Bebak, 1996). These measures also seek to address the spread of disease beyond a facility by preventing a diseased animal or infectious agent leaving a facility.

1.5.5.1 Biosecurity/husbandry

Aquatic environment is an important factor in transmission of infections because there is a close relationship between fish and the environment in which they live. Suitable environmental conditions favor a functional immune system which leads to high disease resistance, growth rate and reproductive activity (Dominguez et al., 2005; Dominguez et al., 2004). Fluctuation of production of humoral defense substances are seen with water temperatures (Magnadóttir et al., 1999) and water salinity (Yada et al., 2002). In one study, aquatic environmental temperatures and salinity were observed to impact the concentration of blood IgM of Nile tilapia (Dominguez et al., 2004). IgM fluctuates with water in many other species (Alcorn et al., 2002; Caruso et al., 2002; Ebran et al., 2000). Biosecurity measures manipulate the environment so as to reduce risk of disease transmission, thus improving the welfare of fish (Holmer, 2010).

Biosecurity methods used in Zambia include obtaining healthy stock, routine collection of dead fish as these can be reservoir of disease pathogens, record keeping, quarantine which is isolation area for new arrivals with isolated water supply, manually cleaning of rearing units, water quality monitoring measuring water temperature, pH and dissolved oxygen, disinfection or use

of chemical for example salt, pest control. Good husbandry aimed at reducing stress of the fish or damage to the skin, fins, gills, or intestine are practiced and these include reducing overcrowding, the provision of good quality water, adequate and safe nutrition and good handling methods. The use of fish health specialists and laboratory disease diagnosis are measures inadequately used while vaccination, use of immunostimulants, probiotics, used in other countries (Yanong and Francis-Floyd, 2002) are yet to be introduced.

1.5.5.2. Antimicrobial compounds

Some antibiotics are known to be effective against streptococcus-like bacteria and have commonly been used. These include erythromycin, florfenicol and amoxicillin (Darwish and Hobbs, 2005; Treves and Brown, 2000). Oxytetracycline is also effective against *S. iniae* infection (Darwish and Hobbs, 2005). The use of antibiotics in the control of streptococcus-like bacterial infections have the following disadvantage: the withdrawal period is longer than it takes for the infections to return and the bacteria develop resistance to the antibiotics (Darwish and Hobbs, 2005). This resistance can be transferred to environmental bacteria and to human pathogens causing adverse effects ecologically and with negative public health implications (Cabello, 2006; Jones et al., 2004; Park et al., 2009). For *L. garvieae*, antibiotic sensitivity and resistance has been found to vary by geographical region (Diler et al., 2002; Klesius et al., 2000a; Ravelo et al., 2001; Vendrell et al., 2006). In Zambia, none of the feed manufacturers or fish farmers indicate that they use antibiotics as an additive or to cure infection though this fact remains to be proven by research that will look at the presence or absence of antibiotics.

1.5.5.3. Vaccination

Vaccination is among the most important measures to prevent fish diseases in aquaculture (Liu et al., 2016), and is believed to be the most environmentally friendly disease control strategy (Munang'andu et al., 2016). Vaccination is only effective if it provokes a systemic immune response (Li et al., 2015). Antimicrobial compound and vaccines are the main control methods for *S. iniae* in aquaculture (Cheng et al., 2010) with bacterins used on their own or as mixed vaccines together with extracellular products (Klesius et al., 2000b; Shoemaker et al., 2006). Live attenuated *S. iniae* strain defective in phosphoglucomutase and M-like protein vaccine were experimentally found to be effective in fish (Buchanan et al., 2005; Locke et al., 2008). Vaccination as a control of *S. agalactiae* in tilapia is effective and widely accepted and these vaccines include inactivated bacterial cells, live attenuated bacteria, recombinant antigens and

DNA vaccines (Liu et al., 2016). As already mentioned, vaccination is not yet practiced as a disease control procedure for fish diseases in Zambia.

1.5.5.3.1. Autovaccines

The use of an autogenous vaccines using a local bacteria is important as vaccine efficacy may be affected by the presence of different serotypes and strains (Adams, 2019). These vaccines have an advantage over commercial vaccines as they are not subjected to rigorous regulations (Attia et al., 2013; Von Hankó, 2009). They can rapidly be made available without comprehensive or complete characterization of the pathogens. They are based on the pathogen isolated from the target farm. Autogenous vaccines are therefore of great advantage in the face of an outbreak.

2.0. AIMS OF THE STUDY

The overall objective of this study was to characterize streptococcus-like bacteria causing disease in farmed Nile tilapia on Lake Kariba with the view of developing a vaccine to protect the fish against disease.

Specific objectives were as follows:

a. To identify and characterize streptococcus-like bacteria associated with diseases in farmed Nile tilapia.

b. To evaluate the pathogenesis of *L. garvieae* following administration by different routes.

c. To assess the host response and level of protection against infection of Nile tilapia using an *L. garvieae* autovaccine

3.0. MATERIALS AND METHODS

3.1. Study area and plan

The study area was two commercial fish farms on Lake Kariba in the Siavonga district located about 250 km south of Lusaka. The motivation for choosing this site was 1) the rapidly developing commercial fish farming on the Zambian side of Lake Kariba; 2) emergence of diseases as reported by the farmers; and 3) proximity to Lusaka where the UNZA veterinary school and laboratories are located. The **inclusion criteria for the fish farms were** a) reported incidences of disease outbreaks in fish and b) willingness to participate in the study.

The study plan was divided into 3 work packages (Figure 5)

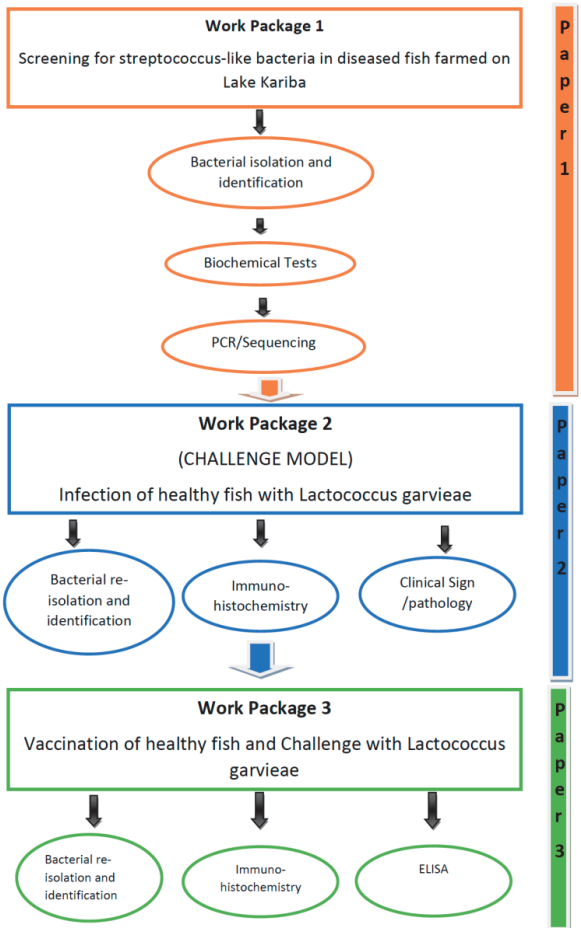


Figure 5: Schematic diagram showing work packages

3.2. Work package 1 entailed sample collections from diseased farmed fish, identification and characterization of the bacteria. As already stated, this was done from two commercial farms during 2014-2016. The motivation for focusing on streptococcus-like bacteria was based on the observed clinical symptoms and pathological findings of the diseased fish including the size of affected fish (>200g), skin ulcers and exophthalmia. Following sample collection, the bacteria were identified by standard techniques including Gram stain, biochemical tests, PCR and confirmed by sequencing. The results of these studies revealed the presence of *S. agalactiae* and *S. iniae* in 2014 and *L. garvieae* in 2015 and 2016, suggesting a shift in the dominant bacteria in the environment at the fish farms. Because of these findings, subsequent studies were focused on the latter.

In order to fulfill Koch's postulates, one *L. garvieae* isolate from one of the farmed fish was injected into naive fish under experimental conditions. This study was also used to determine the LD_{50} of the bacteria in Nile tilapia (Paper 1) for use in subsequent work packages.

3.3. Work package 2. In this WP, the mode of infection of the fish (injection versus immersion) was tested under experimental conditions. The immersion route of infection, whilst natural, has not been previously documented for tilapia with *L. garvieae*. Furthermore, infection dynamics following infection of tilapia by *L. garvieae* were still not well understood at the start of these studies. Assessment of infection was done by observation of clinical signs and mortalities. Furthermore, bacterial re-isolation from internal organs and immunohistochemistry were also used as additional tools for assessing infection (Paper 2). In order for immunohistochemistry to be conducted, polyclonal antibodies against *L. garvieae* were produced in-house (see below).

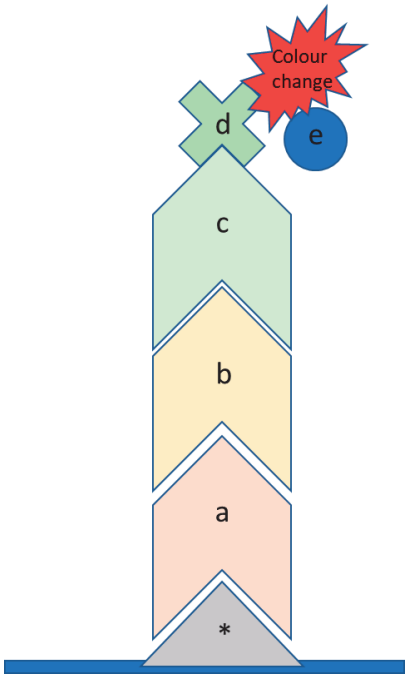
3.4. Work package 3. In this WP, the purpose was to examine host response after injection of Nile tilapia with an autovaccine. Assessment of the tilapia host response to *L. garvieae* involved the following components: a) autovaccine preparation; b) rabbit anti-*L. garvieae* polyclonal antibody production; c) experimental vaccination and challenge of the fish, and d) assessment of the host response and protection against infection.

For autovaccine preparation**,** the same bacterial isolate used in experiments in WP1&2 was used.

The bacteria were fermented in-house at NMBU, formulated in a water-in-oil formulation, and appropriate tests were done. Vaccination and challenge of the fish was done in the wet lab at the University of Zambia, School of Veterinary Medicine. The wet lab was not equipped with thermo-regulators, thus, experiments could only be done at ambient temperature. In experiment one (Paper 1) and two (Paper 2), the water temperature was about 24+/- 2 degrees. In experiment 3 (Paper 3) however, the water temperature was 20+/-2 degrees.

3.5. Immunoassays

Two immunoassays were used in the present study: immunohistochemistry and ELISA. Immunohistochemistry was used in addition to bacterial re-isolation as a method of detecting *L. garvieae* (antigen) *in situ*, in papers 2 and 3. This method involves selectively identifying antigens in a tissue by exploiting the principle of antibodies specifically binding to antigens (Coons and Kaplan, 1950). The antibody-antigen reaction is visualized by a colour-producing reaction (precipitate) evaluated in a light microscope.



Enzyme-Linked Immunosorbent Assay (ELISA), like immunohistochemistry, utilizes antibody-antigen interaction for the detection of specific antigens in liquid sample (Engvall and Perlmann, 1972). The principle here is that the antibody-bound antigen is detected by a reaction in which is an enzyme converts a colorless substrate (chromogen) to a colored product (Figure 6) that remains in solution (no precipitation). This assay was used in this study to detect antibodies produced by tilapia against *L. garvieae* in Paper 3.

*Figure 6. Schematic diagram of the Elisa assay used in the present study. Key: *immobilized L. garvieae antigen, a= test serum from tilapia, b= monoclonal anti tilapia antibodies, c= HRP-labelled rabbit anti-mouse IgG, d=HRP (Horse radish peroxide), e=substrate.*

4.0. SUMMARIES OF INDIVIDUAL PAPERS

4.1. Paper 1

Tilapia (*Oreochromis niloticus*) farming on Lake Kariba, Zambia has been practiced since early 2000s. In recent years, the industry has experienced disease outbreaks with clinical signs consistent with streptococcus-like infections. The objective of this study was to investigate the causative agents of these outbreaks. Affected fish were growers weighing approximately 200g or above from farms designated A and B, reared in cages on the lake. A total of eighty-six diseased fish were sampled between 2014 and 2016. Bacteria were cultured from internal organs and were characterized by biochemical tests, PCR and sequencing of the 16S rRNA gene or 16S-23S intergenic spacer region. The results showed the prevalence of *Lactococcus garvieae* at farm A in 2015 and 2016 outbreaks (15/20 and 23/40, respectively) and at farm B (6/20) in 2016. Infection of naïve fish with one isolate of *L. garvieae* recovered from the outbreaks resulted in clinical signs and mortalities akin of what had been observed during the outbreaks, confirming Koch's postulates. In the 2014 outbreaks, the low prevalence of *Streptococcus agalactiae* (4/15 and 2/11 respectively at farms A and B) and *S. iniae* (2/15) at farm A suggests that other pathogens were likely involved in the disease outbreak observed.

4.2. Paper 2

The purpose of this study was to examine the pathogenesis of *Lactococcus garvieae* (*L. garvieae*) following administration by different routes (intraperitoneal versus immersion) in Nile tilapia. 180 fish were divided into three groups consisting of 60 fish each. The fish in group 1 (IP group) were injected intraperitoneally with 3 x 10^5 cfu *of L. garvieae;* The fish in group 2 (IMM group) were infected with 3 x 10^5 cfu of *L. garvieae* by immersion while in group 3 (Control group), the fish were injected with PBS. Mortalities were recorded daily and on days 3, 5, 7, and 14, liver, kidney, spleen brain and eyes were sampled. Differences between groups were assessed by number of mortalities, pathology/histopathology, bacterial re-isolation and *in situ* presence of by immunohistochemistry. A significant difference ($p<0.0001$) was observed between *L. garvieae* re-isolation from tilapia following administration by intraperitoneal injection (IP) on one hand, and immersion (IMM) on the other. Similarly, more clinical signs and mortalities were observed in the IP compared to the IMM where no mortalities due to infection occurred. These findings suggest that *L. garvieae* does not actively invade Nile tilapia

but takes advantage of cuts or abrasions to cause disease.

4.3. Paper 3

The autovaccine was produced in-house using a bacterial isolate from a diseased fish from the target farm. Three groups of 150 fish each were injected with either 1) an oil-adjuvanted, inactivated whole cell autovaccine, 2) adjuvant only or 3) PBS (negative control). Approximately 660 degree days post vaccination, the fish were challenged with $9x10^5$ cfu bacteria/fish by intraperitoneal injection and monitored for a further 28 days. Protection against infections was measured by lack of/reduced bacterial loads both by bacterial re-isolation and immunohistochemistry as well as absence of clinical signs/pathology. Significantly less *L. garvieae* ($p<0.03$) was re-isolated from the vaccinated group either compared to the adjuvant only or control groups. Furthermore, a significantly higher level ($p<0.001$) of anti-*L. garvieae* specific antibodies were observed in the vaccinated group compared to the adjuvant only or control groups at time of challenge. This coincided with protection against infection measured by absence/reduced *L. garvieae* re-isolation from internal organs, reduced clinical signs and lack of pathology in this group. In the adjuvant only and control groups, bacteria were re-isolated from the kidney, liver, spleen, brain and eyes during the first 14 days. The findings suggest that oil-based vaccines can protect tilapia against *L. garvieae* infection through an antibody mediated response.

5.0. RESULTS AND DISCUSSION

5.1. Streptococcus-like bacteria are present and cause diseases in farmed Nile tilapia on Lake Kariba

The consistent isolation (2014-2016) of *S. agalactiae*, *S. iniae* and *L. garvieae* from diseased fish (Paper 1) affirms the presence of streptococcus-like bacteria in fish farms on Lake Kariba. Koch´s postulates (Paper 1) performed using one of the bacterial isolates from diseased fish obtained from one of the farms confirmed that these isolates were responsible for causing disease in the fish at the farms, consistent with observations elsewhere.

The source of the bacteria in the lake is unclear as the diversity of the isolates recovered from diseased fish (Paper 1) was very wide. One possibility is that they were introduced to the lake together with tilapia fingerling during fish farming operations. Indeed all (farmed) Nile tilapia in Lake Kariba were introduced for farming purposes. GIFT tilapia were imported from Thailand or through Zimbabwe at some point. It is also possible that the bacteria have been present in the lake as part of the microbial flora even before fish farming was initiated. With intensification of fish farming, increased availability of hosts and or immune incompetence of fish associated with stress as a result of intensification may have led to emergence of diseases outbreaks (Egan et al., 2014). This is well-known to happen wherever intensification of fish farming occurs (Pulkkinen et al., 2010). Whatever the source, the presence of streptococcus-like bacteria poses a threat to fish farming on Lake Kariba.

5.2. Streptococcus-like bacterial microflora in Lake Kariba is dynamic

Mixed infections with *S. agalactiae* and *iniae* were noted in 2014 with low prevalence and only *L. garvieae* caused primary infection from 2015 onwards. *L. garvieae* like some other bacteria have been known to dominate microbial communities in the environment through production of antimicrobial products (Ovchinnikov et al., 2016; Sudheesh et al., 2012; Tosukhowong et al., 2012). This was however not tested in this study. Mixed infections of *S. agalactiae, S iniae* and *L. garvieae* have been noted to occur (Anshary et al., 2014b; Karsidani et al., 2010). The sequences of *L. garvieae* isolates collected in 2015 were more variable that those in 2016 that were 100% identical to each other and to selected isolates from the Genbank. This suggest a narrowing in sequence space and loss in fitness from 2015 to 2016, akin to what has been described for quasi-species of RNA viruses (Lauring and Andino, 2010). Quasi-species in

bacteria have also been described (Bertels et al., 2017; Covacci and Rappuoli, 1998).

The *S. agalactiae* on phylogenetic analysis, were like those from different hosts and environments including humans and soil. This indicates a zoonotic concern that needs to be addressed (Evans et al., 2009a; Pereira et al., 2010). The *S. iniae* were closely identified with those from fish species including tilapia and rainbow trout, on phylogenetic analysis.

5.3. *L. garvieae* isolated from Lake Kariba has low invasive potential in Nile tilapia

There are several reports that describe experimental infections of Nile tilapia with *L. garvieae* in the literature (Evans et al., 2009b; Rattanachaikunsopon and Phumkhachorn, 2009; Tsai et al., 2013). In all these papers, *L. garvieae* induced >80% mortalities in naïve control fish if injected with $>10^6$ cfu bacteria. If however, the median lethal dose (LD_{50}) was calculated (approx. 10^3 cfu) and used (Rattanachaikunsopon and Phumkhachorn, 2009), not more than 50% mortalities were observed. These reports are consistent with what we found in the present study (Papers 1, 2&3), suggesting that although there may be differences in the virulence of *L. garvieae* infecting Nile tilapia, the mortalities induced by different isolates may not vary widely. When it comes to mortalities caused by the *L. garvieae* in field outbreaks, literature is scant as the bacteria can occur as a co-infection with *S. agalactiae* and *S. iniae* (Anshary et al., 2014a). In the present study, *L. garvieae* was observed to cause about 20% mortalities annually in 2015 and 2016 at one farm on Lake Kariba (Paper 1). This is comparable with what we observed in the lab when LD_{50} of bacteria was used to inject fish.

The findings that intraperitoneally injected *L. garvieae* induces significantly more infections ($p<0.0001$), clinical signs and pathology compared to immersion (Paper 2) points to the fact that the isolate used in these studies has low invasive potential in Nile tilapia. This explains, to a large extent, why cages with high stocking densities were affected more that those with low (Hang'ombe, person comm). Although not specifically examined in this study, fish stocked at high stocking density are more prone to injury (Stejskal et al., 2020) due, for example, to abrasion by rough surfaces, nutritional deficiencies, bacterial infection and aggressive interactions/predation among fish (Hoyle et al., 2007; Latremouille, 2003; Lellis, 2000).

5.4. Nile tilapia mount protective immune responses against *L. garvieae* infections

The vaccinated group showed no clinical signs or postmortem changes post challenge and bacteria were not re-isolated (except from spleen of one fish). More fish came out bacteria-positive by culture from the control group than the adjuvant only group, and bacteria was only re-isolated the first 7 days post infection. No bacteria were re-isolated from any group 14 days post infection. This is not the typical picture as *L. garvieae* is known to cause acute infection within 10 days post infection under experimental conditions (Tsai et al., 2012).

Protection is likely achieved by an antibody mediated response. The vaccinated group produced significantly higher antibodies ($p<0.001$) than the control or adjuvant groups. Correlation between circulating antibodies and protection against mortality has been shown for *Streptococcus iniae* in tilapia (Pasnik et al., 2005) and also *Aeromonas salmonicida* (Romstad et al., 2013).

In this study is suggests that the experimental challenge acted as a boost causing the increased antibody titers noted from 5 days post challenge. The high level of antibodies and lack of bacteria in the tissues at the time of sampling challenge suggest that antibodies play a part in the protection of the fish. The importance of antibodies for protection against extracellular bacteria is well known (Janeway et al., 2005; Schijns, 2001).

The grow-out cycle of Nile tilapia farmed in Zambia is 5 to 6 months and the duration of immunity for it to be successful should cover the entire economic cycle of the fish. It should be noted that fish are most vulnerable when water temperatures are above 18^0C, and on average fish are exposed to such temperatures over a period of 3 months in its lifetime. Field studies are needed to understand to what extent an oil-adjuvanted vaccine will protect the fish throughout the production cycle.

6.0. MAIN CONCLUSION

In conclusion the results of the present study confirm that streptococcus-/lactococcus-like bacteria were responsible for disease outbreaks in farmed fish on Lake Kariba, Zambia from 2014-2016. This is, to the best of our knowledge the first documentation of fish diseases affecting farmed fish on Lake Kariba in Zambia. The pathogens reported herein have zoonotic implications and thus require follow-up from relevant authorities. *S. agalactiae* and *S. iniae* in 2014 while *L. garvieae* from 2015 onwards. Furthermore, *Lactococcus garvieae* infection was successfully replicated in Nile tilapia. Clinical signs noted where those seen in the field and those noted by other studies. Infection was supported by bacterial re-isolation and demonstration of bacterial antigen in tissues. This work also showed that Nile tilapia can be protected against *L. garvieae* infection by using an inactivated oil-adjuvanted autovaccine. Bacterial re-isolation and demonstration of bacterial antigens from different organs while antibody response was evaluated by ELISA provided an alternative approach for testing vaccines that does not involve mortality.

7.0. RECOMMENDATIONS

Following the findings of this paper, it is being recommended that routine fish disease surveillance be conducted by relevant authority with the view of documenting and following up disease incidences. All vaccine and health development programs in aquaculture are to be documented. There is need for the strengthening of the policy that compels aquaculture farmers to adhere to biosecurity measures in order to prevent outbreak of fish diseases. Farmers should submit their plans for approval to relevant authorities periodically. Vaccines to protect fish from infections should be manufactured from bacteria isolated from the local environment (autogenous vaccines) in the absence of commercial vaccines. Field vaccine trials for *L. garvieae* should be conducted.

8.0. FUTURE PERSPECTIVES

Other projects to build on what has so far been done or follow up work would include testing if *L. garvieae* produced bacteriocins and caused the temporal evolution from *S. agalactieae*/*iniae* to *L. garvieae* that was seen in Lake Kariba from 2015 onwards. It would also be necessary to follow-up the suggested narrowing in sequence space and loss in fitness of *L. garvieae* isolates from 2015 to 2016 isolates. There is need to explore the importance of bacterial load (infection dose) by the immersion route and the differences between the strains of *L. garvieae* to better

understand the mode of infection. Additional studies to confirm with better certainty the autovaccine efficacy should be done, particularly from use under field conditions. There is need for the identification of fish species least susceptible to streptococcus infection in aquaculture and also for re-stocking programmes.

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Characterization of streptococcus-like bacteria from diseased *Oreochromis niloticus* farmed on Lake Kariba in Zambia

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ABSTRACT

Tilapia (*Oreochromis niloticus*) farming on Lake Kariba, Zambia has been practiced since early 2000s. In recent years, the industry has experienced disease outbreaks with clinical signs consistent with streptococcus-like infections. Affected fish were growers weighing approximately 200 g or above from farms designated A and B, reared in cages on the lake. The objective of this study was to identify and characterize the causative bacteria of these outbreaks. A total of eighty-six diseased fish were sampled between 2014 and 2016. Bacteria were cultured from internal organs and were characterized by biochemical tests, PCR and sequencing of the 16S rRNA gene or 16S−23S intergenic spacer region. The results showed the prevalence of *Lactococcus garvieae* at farm A in 2015 and 2016 outbreaks (15/20 and 23/40, respectively) and at farm B (6/20) in 2016. Infection of naïve fish with one isolate of *L. garvieae* recovered from the outbreaks resulted in clinical signs and mortalities akin of what had been observed during the outbreaks, confirming Koch's postulates. In the 2014 outbreaks, the low prevalence of *Streptococcus agalactiae* (4/15 and 2/11 respectively at farms A and B) and *S. iniae* (2/15) at farm A suggests that other pathogens were likely involved in the disease outbreak observed.

1. Background

Zambia is one of the largest aquaculture producers in sub-Saharan Africa focusing mainly on species from the *Cichlidae* family. Fish farming started in the 1950s with attempts to raise indengenous species in dams and earthen ponds (FAO, 2019). By 2014, fish production had reached 20,000 tons (ZambiaInvest, 2014), from 4501 tons in 2003 (FAO, 2019). Currently, the industry is expanding with many international players including Kariba Harvest and Yalelo fish farms as well as Skretting and Aller Aqua feed producers having established themselves on the market. Fish farming is practiced at two levels, land-based systems and floating cages. The former dominates among small scale farmers mainly in the central areas of the country, around Lusaka and on the Copperbelt as well as in Northern province (ACF/FSRP, 2009; Musuka and Musonda, 2013; Musuka and Felix, 2012). Cage farming on the other hand, is practised by commercial farmers mostly on Lake Kariba where it was introduced on the Zambian side in 2001.

The introduction of fish farming on Lake Kariba was followed by importation of *Oreochromis niloticus (O. niloticus)* breeding materials from Zimbabwe and Asia for management purposes and the desire for highly performing Genetically Improved Farmed Tilapia (GIFT). As with fish farming industries elsewhere, diseases started to emerge as farming intensified (Hastein et al., 2005; Pulkkinen et al., 2010; Sundberg et al., 2016). The first reports of disease outbreaks at one of the fish farms were in 2014. Nine out of 20 cages were affected with mortalities ranging between 0.3 and 15% in affected cages. The affected fish were those approaching harvest size (> 200 g) and mortalities were firstly reported to occur in the cooler months of the year (from May to August). In 2015, 33 of 37 cages were affected and this time mortalities ranged from 0.4% to 18% in affected cages while in 2016, almost all cages (46/48) were affected (mortalities ranging from 0.6 to 7.3%). The same size group of fish (> 200 g) were affected in 2015 and 2016 although disease outbreaks occurred during the hottest months (November to January). Clinical signs including erratic swimming, decreased uptake of food, lethargy, exophthalmia with intraocular haemorrhage and corneal opacity were observed suggesting streptococcus-like bacterial infections. No confirmed diagnosis was obtained prior to this study.

Streptococcus-like infections in tilapia are caused by Gram-positive, cocci bacteria belonging to the Family Streptococcaceae, Phylum

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Firmicutes and Order *Lactobacilles. The genus Streptococci is large, with over 50 species and among them, Streptococcus iniae (S. iniae), S. agalactiae (*syn *S. difficile), S. dysagalactiae and S. parauberis* have been documented to infect fish (Curras et al., 2002; Eldar and Ghittino, 1999; Soltani et al., 2012; Zamri-Saad et al., 2010). Other closely related species infecting fish include *Lactococcus garvieae* (*L. garvieae)*, *L. piscium, Vagococcus salmoninarum* and *Carnobacterium piscicola* (Bercovier et al., 1997). Not all, however, have been shown to cause disease in tilapia. *Streptococcus agalactiae* and *S. iniae* have been well-known for some time, causing typically exophthalmia, ulcers or haemorrhages on the body and fins, eye opacity and mortalities (Chen et al., 2007). Predisposing factors are high temperatures (between 24° and 35 °C), over stocking and poor water quality, to name some. Differentiation between the two infections is difficult, although *S. agalactiae* is believed to cause higher mortalities compared to *S. iniae*. *Lactococcus garvieae* is another disease-causing bacteria in tilapia. It has been well characterized in rainbow trout where it causes similar clinical signs as *S. iniae* although with different histopathological changes (Eldar and Ghittino, 1999). In tilapia, *L. garvieae* has only been reported to cause disease during the last decade and there are not many reports to this effect besides (Evans et al., 2009b; Rattanachaikunsopon and Phumkhachorn, 2009; Tsai et al., 2013).

The purpose of the present study was to identify bacteria associated with diseases in farmed tilapia on Lake Kariba from 2014 to 2016 with a view of developing an intervention program. The bacteria was collected from diseased fish during outbreaks and was identified by standard biochemical methods followed by PCR and sequencing. Of the *L. garvieae* isolated from the farms, one was randomly selected and used to confirm Koch's postulates by infecting naïve fish.

2. Materials and methods

This study was conducted in two parts, the first involved isolation and characterization of bacteria from diseased fish from farms. The second experiment entailed fulfilment of Koch's postulates by infecting naïve fish using one of the bacterial isolates from the first part.

2.1. Ethical consideration

This study was approved by the Excellence in Research Ethics and Science (ERES) Converge, a private Research Ethics Board of Zambia (IRB No. 00005948, Protocol Number: 2016/JUNE/028).

2.2. Bacterial isolation and characterization

2.2.1. Fish and study design

Farmed Nile tilapia from two fish farms designated A and B where disease outbreaks had been reported during the period 2014–2016 were targeted. Affected fish were reared in grow-out cages on lake Kariba, weighing about 200 g or more. Sample collections were undertaken three times, during the cool season (June to August 2014) and twice during the hot season (November to January 2015&2016).

2.2.2. Sample collection

Fish with clinical signs including erratic swimming, exophthalmia, ocular opacity and body haemorrhages/ulcers were purposively sampled. The fish were caught by dip-netting and sacrificed by stunning with a blow to the head followed by severing of the dorsal vein. Clinical signs were recorded. In 2014, 15 fish were sampled from farm A and 11 fish from farm B. In 2015, 20 fish were sampled farm A only and in 2016, 20 fish each were sampled from farms A and B (86 fish in total for the whole study).

From each fish, the following organs were collected: eye, brain, kidney, liver and spleen. From each of these organs, swabs were collected for bacteriology during post-mortem and stored in Brain Heart Infusion (BHI) broth. In addition, a section of each organs was collected and stored in formalin for histopathology. All samples were transported overnight cooled (4 °C) to the University of Zambia, School of Veterinary Medicine (UNZA) in Lusaka for further processing.

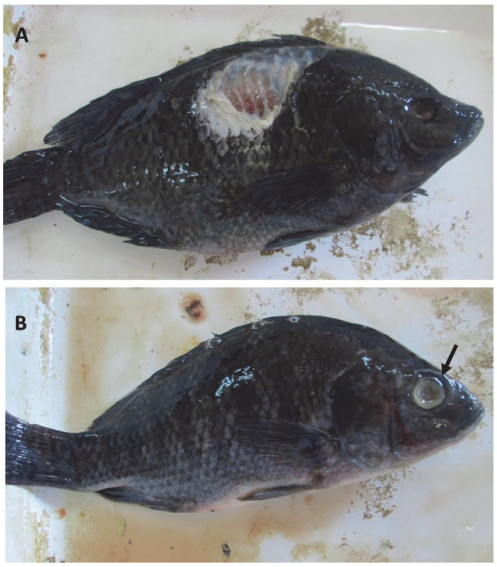

Fig. 1. Photograph showing a skin ulcer (A) and corneal opacity (B) from a field outbreak of streptococcosis in tilapia (*Oreochromis niloticus*) in the present study. Arrow points to a sunken eye, another common finding.

Table 1
Clinical signs and post-mortem findings observed in fish sampled from different farms in this study.

Year of sampling		2014	2015	2016	*p* value
A. Clinical findings observed in individual fish					
Total number of fish sampled		26	20	40	
Number fish observed	skin ulcers	15	5	2	0.0001
	Ocular opacity	10	24	30	0.0001
	Exophthalmia	10	9	6	0.3
	Abdominal distension	5	4	2	0.2
	Circling	5	2	4	0.2
	Endophthalmia	4	5	1	0.2
B. Post mortem findings in individual fish					
Total number of fish sampled		26	20	40	
Number fish observed	Pale livers	9	15	13	0,349
	Distended gallbladders	6	6	12	0,6063
	Enlarged spleens	12	11	8	0,2275
	Congested brains	11	1	3	0,0002

2.2.3. Bacterial isolation

Bacterial samples collected from the field were aseptically streaked on Brain Heart Infusion Agar (BHIA: Unipath, England), Tryptic Soya Agar (TSA) (HiMedia, India), Blood Agar (HiMedia, India) and Nutrient Agar (NA: HiMedia, India). The plates were then incubated at 24 °C for 48 h. To obtain pure colonies, single colonies from these plates were harvested and plated on new NA plates.

2.2.4. Biochemical characterization

Conventional biochemical tests were used to characterize the bacteria. A loop-full of bacteria was aseptically added to 5mls of Phenol red broth containing 1% sugar and incubated at 37 °C for 24 h to test fermentation of different sugars. Sulphur reduction, Indole production and

Table 2
Biochemical characteristics of bacterial isolates from diseased tilapia *Oreochromis niloticus* farmed on Lake Kariba compared to referenced strains.

Test conducted	Year of isolation							Reference strains		
	2014	2015/16	2015/16	2015/16	2015/16	2015/16	2015/16	*L. garvieae*[a,*]	*S. iniae*[b]	*S. agalactiae*[c]
Isolate identity	Z15, 2Z, Z8, Z10, 8Z, 10Z	LGZM11	LGZM44	LGZM14, LGZM5, LGZM13, LGZM2	LGZM12, LGZM1	LGZM39 LGZM41 LGZM29 LGZM10 LGZM4 LGZM6 LGZM8 LGZM6	LGZM7			
Tolerance at 4% NaCl	ND	+	+	+	+	+	+	+	+	−
Tolerance at 6.5% NaCl	ND	+	+	+	+	+	+	+		−
Gram stain	+	+	+	+	+	+	+	+	+	+
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Hemolysis	β	α	α	α	α	α	α	α	α β	α β
Camp test	ND	−	−	−	−	−	−	−	+(93%)	−
Catalase	−	−	−	−	−	−	−	−	−	−
Oxidase	−	−	−	−	−	−	−	−	−	−
Esculin	ND	+	+	+	+	+	+	+	+	−
Galactose	ND	+	+	+	+		+	+	+(56%)	+
Raffinose	−	−	−	+	−	+	−	−	−	−
Salicin	ND	+	+	+	+	+	+	+	+	+(11–95%)
Maltose monohydrate	ND	+	+	+	+	+	−	+	+	+
Xylose	ND	−	+	+	+	+	+	−	−	−
Mannitol	ND	+	+	+	+	+	+	+(80–90%)	+	−
Trehalose	+	+	+	+	+	+	+	+	+(95%)	+
Inulin	−	−	−	+	+	+	+	−	−	−
Sorbitol	−	−	−	−	−	+	+	−	-(3%+)	−
Lactose monohydrate	−	+	+	+	+	+	+	+	V (11%+)	−
Urease	ND	−	−	−	−	−	−	−	−	−
Sucrose	ND	+	+	+	+	+	+	V (11%+)	+(99%)	+
Glucose	ND	+	+	+	+	+	+	+	+	+
Lactose	−	+	+	+	+	+	+	+	V (11%+)	−
Gas production	ND	−	−	−	−	−	−	−	−	−
Citrate	ND	−	−	−	−	−	−	−	−	−
Sulphur	ND	−	−	−	−	−	−	−	−	−
Indo	ND	−	−	−	−	−	−	−	−	−
Motility	−	−	−	−	−	−	−	−	−	−
Vancomycin sensitivity	+	ND	ND	ND	ND	ND	ND	−	−	−

V = variable.
[a] Chen et al., 2001.
[b] Agnew and Barns, 2007.
[c] Baya et al., 1990.
[*] ATCC 43921.

motility tests were determined by using Sulphide Indole Motility (SIM) media, again by adding a loop-full of bacteria and incubating at 37 °C for 24 h.

2.2.5. Bacteria DNA isolation

The Cetyl Trimethyl Ammonium Bromide (CTAB) (William et al., 2012) was used to extract bacterial DNA. Briefly, 1.5 mls of bacteria in BHIB culture was centrifuged at 9300 ×*g* for 10 min. The supernatant was carefully discarded and the pellet re-suspended in 567 µl of 1×TE buffer. To this, 30 µl of 10% SDS containing 3 µl proteinase K was added and the mixture incubated at 37 °C for 1 h. 100 µl of 5 M NaCl containing 80 µl CTAB-NaCl was then added, mixed and incubated at 65 °C for 10 min. Thereafter, an equal volume of Chloroform-Isoamylalcohol (24:1) was added, mixed gently and centrifuged at 9300 ×*g* for 10 min. The aqueous layer was transferred to a fresh microcentrifuge tube. An equal volume of Phenol-Chloroform-Isoamylalcohol (25:24:1) was added and centrifuged at 9300 ×*g* for 10 min. The supernatant was removed and placed in a fresh microcentrifuge tube. To this, bacterial DNA was precipitated by adding 0.6 volumes of isopropanol and centrifuging at 13,400×*g* for 10 min. The supernatant was discarded and the pellet washed with 70% ethanol. This was followed by centrifugation at 13,400×*g* for 10 min. After carefully removing the supernatant, the pellet was air-dried and redissolved in 50 µl 1×TE buffer prior to stored at −20 °C until required.

2.2.6. Polymerase chain reaction

To detect streptococcal species, specific primers for the detection of Group B *Streptoccocus* (GBS) flanking the variable regions V8 (DSF1- TGCTAGGTGTTAGGCCCTTT) and V9 (DSR1- CTTGCGACTCGTTGTAC CAA) of the 16S rRNA (Ahmet et al., 1999) were used. The PCR was performed in 25 µl reaction mixtures containing 50 to 100 ng bacterial DNA, 1× buffer (New England biolab, USA), 0.5 µl dNTP, 0.5 µl of each primer (10 pMol) and 0.02 U/µl Phusion DNA polymerase (New England biolab, USA). PCR was performed in a T100™ Thermal cycler (Bio-Rad, USA) with the following steps: initial denaturation 98 °C for 30 s; 35 cycles of [denaturation (98 °C for 10 s), annealing (55 °C for 30 s) and extention (72 °C for 30 s)]; and a final extension step of 72 °C for 10 min. The PCR products were separated by using 1 or 2% agarose gel electrophoresis containing Syber safe® DNA gel stain (Invitrogen, USA). DNA bands were visualised using a UV illuminator.

2.2.7. Gel extraction and purification

Gel purification of PCR products was done using the QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer' instructions. Bands of the expected size were excised from 1 or 2% agarose gels.

2.2.8. Sequencing, alignment and phylogenetic analysis

All PCR products from 2014 streptococcus-like isolates were processed for sequencing while a selection was picked from 2015 and 2016

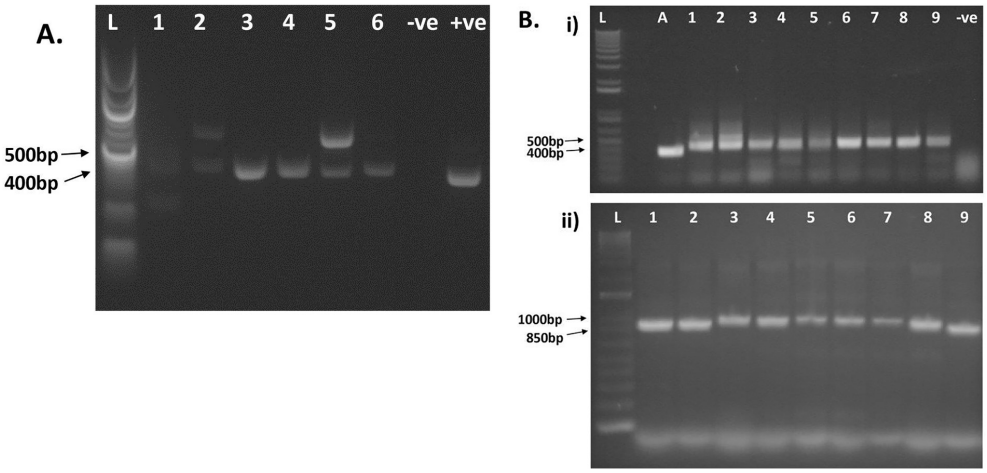

Fig. 2. Gel electrophoresis of PCR products of bacterial isolates in the present study. A) Bacterial isolates from A) 2014 sampling. Key: L = ladder (100mb DNA ladder (New England Biolabs®)); 1–6 = samples; −ve & +ve = non-template and positive control (known *Streptococcus agalactiae*), respectively. Note top band (500 bp) in samples 2 and 5 (*Streptococcus iniae*); otherwise all bands equal to 400 bp were *S. agalactiae*. B) 2015/2016 sampling. (i) Key: L = ladder (1 kb plus DNA ladder (Invitrogen®)), Lanes A = *Streptococcus agalactiae* control (400 bp band); 1–9 = samples giving band size = 450 bp; −ve = non-template control. (ii) PCR products of same samples as in (i) performed with *Lactococcus garviae*-specific primers. Key: L = ladder (1 kb plus DNA ladder (Invitrogen®)) Lanes 1–9 = samples giving band size (928 bp); non-template control not shown.

samples for economic reasons. Purified PCR products were sequenced commercially at GATC Biotec now Eurofins (www.eurofins.no) using the same primers as for PCR. The sequence data was trimmed, assembled and aligned using CLC Main Workbench 6.0 (www.clcbio.com). Blasting against known sequences in the Genbank was done using NCBI Blast algorithim. Additionally, Mega7 software (Kumar et al., 2016) was used for alignment and evolutionary analyses of the *L. garvieae* 16S rRNA sequences. The phylogenetic tree was inferred by the Maximum Likelihood method, bootstrapped 1000 times on the basis of the Jukes-Cantor (Jukes, 1969).

2.3. Infection experiment

2.3.1. Fish

One hundred Nile tilapia averaging 10 ± 2 g were purchased from Palabana fisheries, a local commercial fish farm located east of Lusaka where no outbreaks of Streptocossus had been reported. The Fish were transported by road in oxygenated plastic bags to the University of Zambia, School of Veterinary Medicine. They were then allowed to acclimatize for 10 days in 60 l glass tanks. The fish were kept at a temperature of 24.4 ± 1.9 °C. Feeding was done once daily with commercial fish mash (Juvenile crumble 45% (Novatek, Zambia)). Dissolved oxygen (6.7 ± 0.12 mg/l) and pH (7.8 ± 0.12) were measured daily.

Proir to commencement of the challenge experiment, 10 fish were sacrificed and samples including eyes, brain, liver, kidney and spleen were collected for bacterial culture and inoculated directly on Nutrient Agar. The plates were incubated at 24 °C for 48 h.

2.3.2. Preparation of Lactococcus garvieae for fish infections

After identification of the bacteria (Section 2.2), one isolate of *L. garvieae* (LGMZ8, Table 2) was randomly selected and used to infect naïve fish. To do this, a pure colony was first inoculated on BHIB and incubated for 48 h at 25 °C. The bacteria was then washed thrice by suspension in sterile normal saline, centrifuged and discarding the supernatant. The turbidity of the bacteria was adjusted to McFarland turbidity No 4.0 with saline, equivalent to 12×10^8 colony forming units (cfu), and then diluted to 3×10^8 cfu ml^{-1}.

2.3.3. Study design and infection of fish

The fish were subdivided into 7 groups by dip netting and sequential allocation into separate tanks (A–G). Each group was allocated 10 fish except for the control group (7 fish).

The bacterial inoculum was prepared by 10-fold serial dilutions to yield six concentrations of bacteria (3×10^8 cfu ml^{-1} to 3×10^3 cfu ml^{-1}). Each group (A to F) was intraperitoneally (IP) injected with one bacterial concentration, 0.1 ml per fish. Fish in group G were injected with 0.1 ml of normal saline. Monitoring was done daily for 20 days and clinical signs as well as mortalities were recorded.

2.3.4. Bacterial re-isolation from infected fish

Dead or moribund fish were collected and dissected. Swabs were taken from the liver, spleen, kidney, brain and eye and inoculated onto nutrient agar. The plates were incubated aerobically at 24 °C for 48 h. Confirmation of the presence of *L. garvieae* was done as already described in Section 2.2.

3. Results

3.1. Clinical signs and post-mortem findings of fish at farms A and B

During the 2014 disease outbreaks at Farms A and B, the affected fish weighing ≤200 g and reared in cages were sampled during the cooler months of the year. Skin ulcers (Fig. 1a) were observed in some of the fish sampled over the whole period (2014–2016). However, significantly more ulcers ($p < .0001$) were observed in those sampled in 2014 compared to 2015 and 2016 combined (Table 1). In contrast, significantly more fish ($p < .0001$) with ocular opacity (Fig. 1b) were observed in 2015 and 2016 than in 2014 (Table 1a). On the other hand, no difference in terms of exophthalmia, endophthalmia or circling between diseased fish sampled were observed in 2014, 2015 or 2016. The commonest post mortem changes observed were pale livers, distended gall bladders, enlarged spleen and congested brains (Table 1b). However, no significant difference in terms of these changes were observed

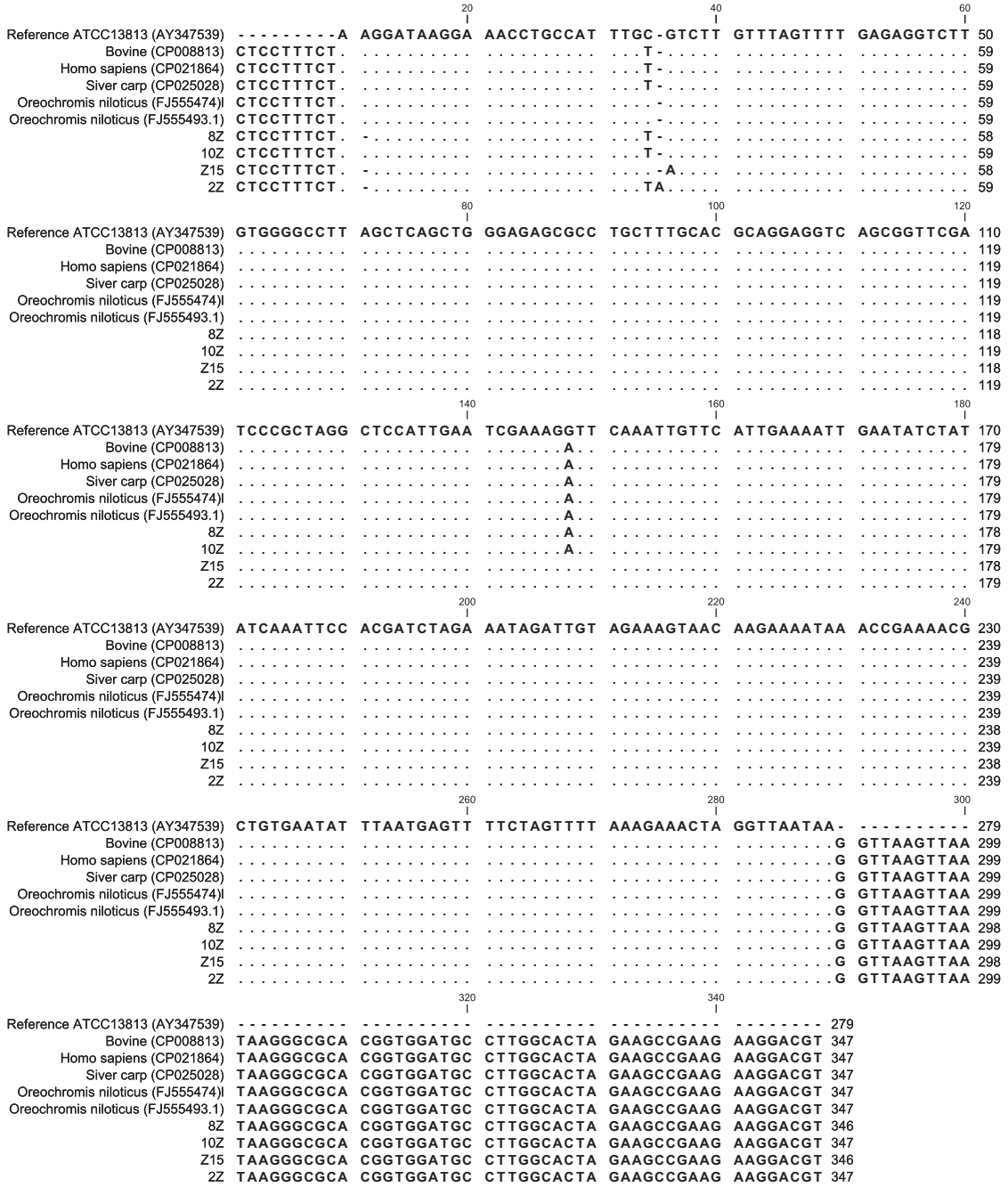

Fig. 3. Alignment of intergenic spacer of *Streptococcus agalactiae* isolated from diseased fish in this study compared to selected isolates from the Genbank. – represents identical nucleotides.

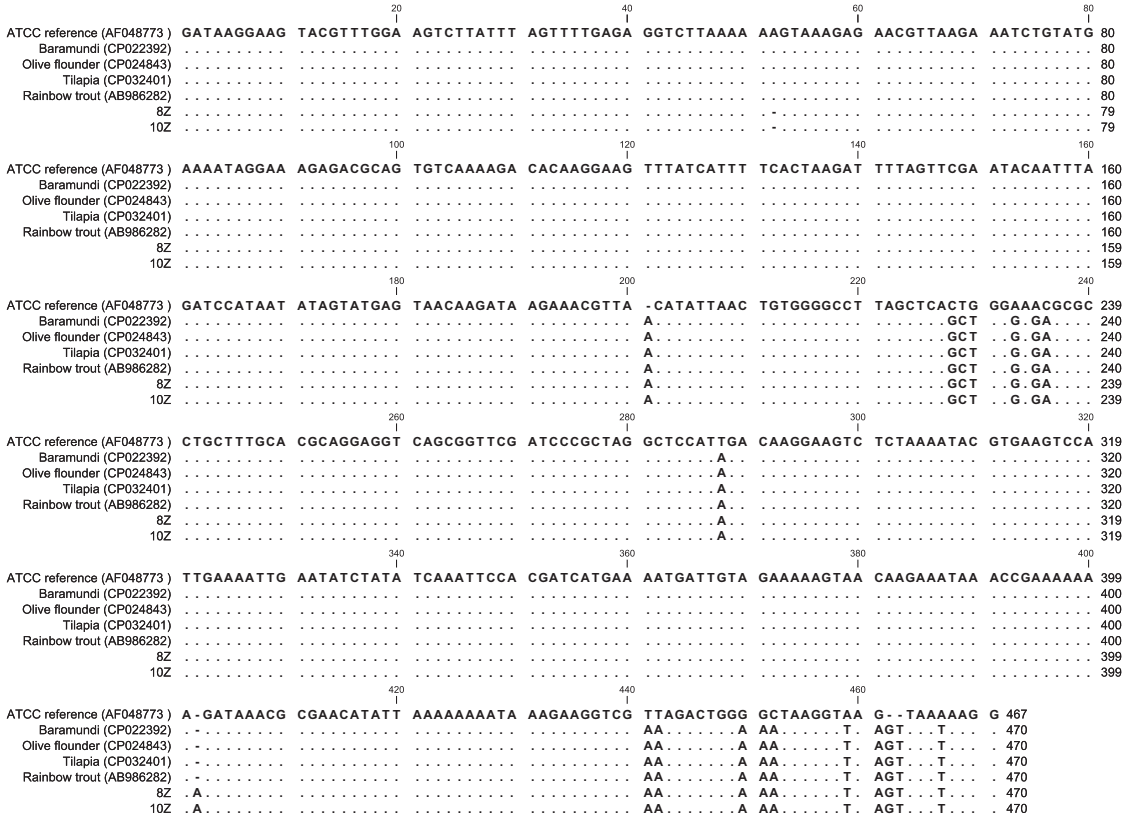

Fig. 4. Alignment of intergenic spacer of *Streptococcus iniae* isolated from diseased fish in this study compared to selected isolates from the Genbank. – represents identical nucleotides.

between fish sampled in different years except for congestion in the brain where the fish sampled in 2014 dominated ($p < .0002$).

3.2. Gram stain and biochemical characterization of bacteria

In 2014, *Streptococcus*-like bacteria characterized by Gram positive cocci, oxidase and catalase negative, non-motile with beta hemolysis on 5% sheep's blood agar (Table 2) were identified from 4/15 fish sampled from farm A. From farm B, streptococcus-like bacteria were isolated from 2/11 fish.

In 2015, 15/20 fish from farm A showed *Streptococcus*-like bacteria characteristics. In 2016, 17/20 fish from farm A and 6/20 from farm B showed similar characteristics to those observed at Farm A in 2015 (Table 2). The most striking difference between these (2015 and 2016 isolates) and the 2014 isolates is that isolates from 2015 and 2016 produced α hemolysis on 5% Sheep blood agar, in contrast to β as was the case with those isolated from 2014 (Table 2). As already mentioned, only isolates identified as streptococcus-like by biochemical methods were retained and characterized further.

3.3. Confirmation of S. agalactiae, S. iniae and L. garvieae by PCR and sequencing

Bacterial isolates ($n = 4$) identified as *Streptococcus*-like by biochemical methods were subjected to PCR using primer set (DSF1 and DSR1). The resulting products from 2014 samples produced bands of either 400 bp or 500 bp on 1% agarose gel (Fig. 2a). When purified and sequenced followed by blasting them against known sequences in the NCBI database, the products matched the 16S−23S rRNA intergenic spacer of *S. agalactiae* (400 bp) and *S. iniae* (500 bp). Two samples were positive for both *S. agalactiae* and *S. iniae* (Fig. 2a).

Alignment of the 16S−23S intergenic spacer sequences of the 400 bp products of *S. agalactiae* from the present study with selected sequences from bovine, *Homo sapiens*, silver carp and *Oreochromis niloticus* including the reference strain (ATCC 13813 (Accession number AY347539)) showed high similarities, with only one nucleotide difference (Z2 and Z15) or two nucleotides (8Z and 10Z) variations from the reference strain (Fig. 3). Two of the isolates from this study (8Z and 10Z) had 100% similarity with sequences from other sources.

For the 16S−23S intergenic spacer sequences of *S. iniae* (500 bp products) aligned with reference strain ATCC 29178 (AF048773) and other selected sequences from other fish species, several differences were observed with the reference strain (Fig. 4). Interestingly the sequences of isolates from this study (8Z and 10Z) were 100% identical to all other sequences (except the reference) randomly selected from the gene bank except for one insertion at position 402 (Fig. 4).

Primer sets DSF1 and DSR1 yielded products of 450 bp on 1% Agarose gel when used to prime PCR reactions for isolates from 2015 and 2016 (Fig. 2i). After purification, sequencing and blasting against known sequences, these products yielded a match with 16S rRNA of *L. garvieae* but also several unidentified bacteria with equally as high identity (99–100%) (not shown). This prompted us to design new

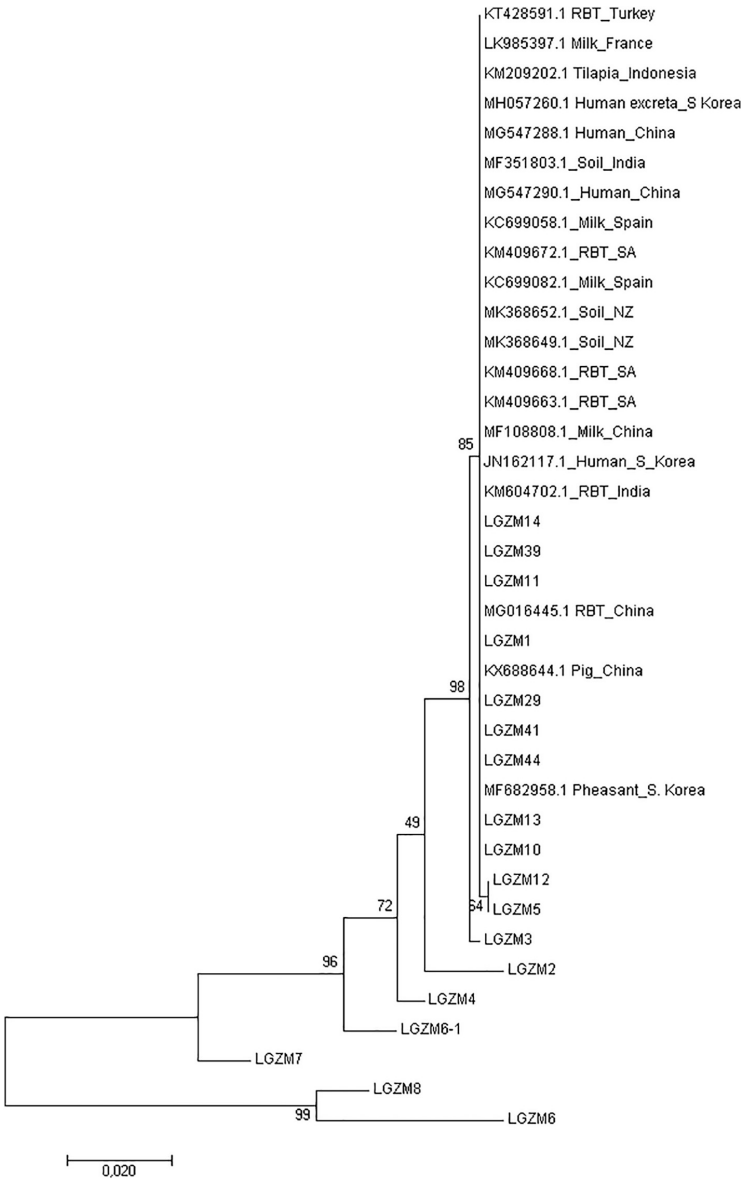

Fig. 5. Phylogenetic tree of the 16S rRNA of *Lactococcus garvieae* isolated from diseased fish in the present study compared to selected isolates from the Genbank. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes, 1969). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016), bootstrapped 1000 times. Key: Zambian isolates are abbreviated LGZM; sequences with accession numbers are from the Genbank and show host source and country of origin.

primers (LG-167F-TACCGCATAACAATGAGAATC and LG-1095-CTTAA CCCAACATCTCACGAC) to enable us to identify *L. garvieae* with greater certainty. These new primers flank the 16S rRNA gene from 167 bp to 1095 bp yielding a product of 928 bp. Thus PCR was repeated for all isolates collected in 2015 (n = 15) and 2016 (n = 23) and all samples yielded expected bands (Fig. 2ii). When purified, sequenced and blasted against known sequences in the NCBI database, 99–100% match with *L. garvieae*, including the reference strain ATCC 43921 was observed.

3.4. Phylogenetic analyses

The 16S−23S rRNA intergenic spacer sequences for *S. agalactiae* and *S. iniae* obtained in the present study were short and conserved, therefore no attempt to construct phylogenetic trees was done. However for *L. garvieae*, the sequences for the 16S rRNA gene were long and variable thus a best fit model was first performed using the Mega7 software to determine the most appropriate algorithm for use with the Maximum Likelihood method, followed by phylogenetic tree construction based on the Jukes-Cantor model (Jukes, 1969).

The results show that there was a wide variation in the sequences of isolates obtained in 2015 (Fig. 5, Table 3). About half (7 isolates) of these sequences were 100% homologous and clustered together with the reference strain (AF 283499) and other selected isolates from the Genbank, representing isolates from different geographical locations

Table 3
Bacteria isolated in the present study and corresponding accession numbers in the Genbank.

Isolate	Accession number	Bacteria	Year isolated
Z15	MK364799	*Streptococcus agalactiae*	2014
2Z	MK364800	*S. agalactiae*	2014
10Z	MK364801	*S. agalactiae*	2014
8Z	MK364802	*S. agalactiae*	2014
Z8	MK364803	*S. iniae*	2014
Z10	MK364804	*S. iniae*	2014
LGZM11	MK346123	*Lactococcus garvieae*	2015
LGZM39	MK346124	*L. garvieae*	2016
LGZM14	MK346125	*L. garvieae*	2015
LGZM5	MK346126	*L. garvieae*	2015
LGZM12	MK346127	*L. garvieae*	2015
LGZM1	MK346128	*L. garvieae*	2015
LGZM41	MK346129	*L. garvieae*	2016
LGZM29	MK346130	*L. garvieae*	2016
LGZM44	MK346131	*L. garvieae*	2016
LGZM13	MK346132	*L. garvieae*	2015
LGZM10	MK346133	*L. garvieae*	2015
LGZM7	MK346134	*L. garvieae*	2015
LGZM4	MK346135	*L. garvieae*	2015
LGZM6	MK346136	*L. garvieae*	2015
LGZM8	MK346137	*L. garvieae*	2015
LGZM2	MK346138	*L. garvieae*	2015
LGZM6-1	MK346139	*L. garvieae*	2015

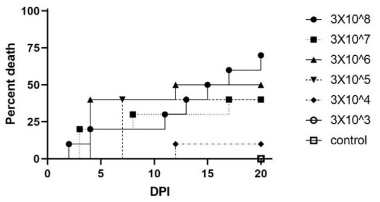

Fig. 6. Cumulative mortalities of fish following infection with different concentrations of *Lactococcus garvieae* in the present study. $n = 10$ except for the control group where $n = 7$.

and hosts (fish, humans, soil, milk etc). All these isolated clustered together into 1 large clade. Interestingly, the 4 sequences from the 2016 isolates were 100% identical to each other and also to the reference strain as well as other selected sequences from the Genbank (Fig. 5, Table 3).

3.5. Disease reproduction in naïve fish

Naïve fish that were infected with *L. garvieae* in order to fulfill Kock's postulates exhibited clinical signs in the form of darkening of the skin, lethargy, exophthalmia and corneal opacity followed by death. Other signs observed included erratic, uncoordinated swimming. Post mortem of sick fish frequently revealed enlarged spleen and distended gall bladder.

As a general trend, dose-dependent mortalities were observed in injected fish where 7/10 fish that received the highest concentration of bacteria (3×10^8 cfu ml^{-1}) died, while only one from the group injected with 3×10^4 cfu ml^{-1} died (Fig. 6). In contrast, none of those that received 3×10^3 cfu ml^{-1} or PBS only died. Similarly, the onset of mortalities was also dose-dependent, with the first mortality occurring at 2 dpi from the group that received 3×10^8 cfu ml^{-1} while the first and only fish from the 3×10^4 cfu ml^{-1} group died on 11 dpi (Fig. 6). *L. garvieae* was re-isolated from all internal organs of all moribund or dead fish.

4. Discussion

The findings of the present study demonstrate that Streptococcus-like bacteria are present in the fish farming environment of Lake Kariba in Siavonga district, Zambia and that they are important disease-causing agents in farmed fish. In 2015 and 2016, high prevalences of *L. garvieae* (15/20 in 2015; 17/20 in 2016) were isolated from diseased fish from farm A. One isolate from this collection was used to infect naïve fish under experimental conditions and this resulted in the reproduction of disease with clinical signs and mortalities (Fig. 6) akin to that observed in disease outbreaks at the farm, and as previously reported by others (Evans et al., 2009b), pursuant to Kock's postulates (Brock, 1999). The low prevalences of *Streptococcus agalactiae* (4/15), *S. iniae* (2/11) and no *L. garvieae* in 2014 is curious and suggests that other pathogens were important in causing disease that year. However, since this study focussed on bacteria with Streptococcus-like properties, no other bacteria isolated from the fish were characterized further. This is the first study that systematically characterizes bacterial infections causing disease in fish on lake Kariba.

The increasing trend in the number of cages with elevated mortalities at farm A from 2014 (9/20), 2015 (33/37) and 2016 (46/48) side by side with increasing prevalence of *L. garvieae* (none in 2014; 15/20 in 2015 and 17/20 in 2016 at farm A; and prevalences of 0/11 in 2014 and 6/20 in 2016 at farm B (mortality data not available) is interesting. Bacteria are well known to dominate microbial communities in their environments through the production of antimicrobial products and *L. garvieae* has been shown do this through production of bacteriocins (Ovchinnikov et al., 2016; Sudheesh et al., 2012; Tosukhowong et al., 2012). Although this was not tested in the present study, it is not unlikely that this may have been one mechanism that the temporal evolution from *S. agalactiae/ S. iniae* to *L. garvieae* took effect.

Streptococcus agalactiae/S. iniae and *L. garvieae* infections in fish are believed to induce similar clinical signs (Eldar and Ghittino, 1999; Sudheesh et al., 2012) and may be difficult to differentiate. Consistent with this, similar clinical signs including exophthalmia, skin ulcers and corneal opacity were observed during outbreaks from 2014 to 2016. While *L. garvieae* was not detected in 2014, it was the dominant cause of infections in 2015 and 2016 and during this time, significantly more ocular opacity ($p < .0001$) than the previous year were observed suggesting that this bacterium induces more ocular opacity compared to skin ulcers.

In the present study, alignment of the 16S−23S rRNA intergenic spacer region of isolates obtained in this study compared with those in the Genbank revealed high identity between *S. agalactiae* isolates of this study and those from different hosts and environments including homosapiens and soil (Fig. 3). These findings are consist with, and in agreement with the view of others that *S. agalactiae* are of zoonotic concern and should be addressed (Evans et al., 2009a; Pereira et al., 2010). On the other hand, alignment of the 16S−23S rRNA intergenic space region of *S. iniae* isolates obtained in the present study with known ones from the Genbank showed close identity with only isolates from other fish species including tilapia and rainbow trout (Fig. 4). While *S. iniae* is also zoonotic (CDC, 1996; Weinstein et al., 1997) the findings of the present study suggest that the isolates obtained in this study are more host specific compared to *S. agalactiae*.

Phylogenetic analysis of the 16S rRNA gene of *L. garvieae* isolates obtained in the present study show that in general, *L. garvieae* isolates are identical to each other and to the reference strain (accession number AF 283499) used in this study. The sequences of the present study clustered together with isolates from different geographical locations (including Asia, middle East and Europe) and hosts (humans, bovine milk, soil etc) (Fig. 5, Table 3). As with *S. agalactiae*, these findings suggest that *L. garvieae* has a weak host specificity and that the bacteria isolated in the present study are potentially zoonotic as reported elsewhere (Eraclio et al., 2018; Kim et al., 2011). The sequences of isolates collected in 2015 were more variable that those in 2016 that

were 100% identical to each other and also to selected isolates from the Genbank (Fig. 5, Table 3). These findings are interesting and should be followed-up as they suggest a narrowing in sequence space and loss in fitness from 2015 to 2016, akin to what has been described for quasi-species of RNA viruses (Lauring and Andino, 2010). Quasi-species in bacteria have also been described (Bertels et al., 2017; Covacci and Rappuoli, 1998).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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RESEARCH ARTICLE

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Lactococcus garvieae isolated from Lake Kariba (Zambia) has low invasive potential in Nile tilapia (*Oreochromis niloticus*)

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Abstract

The pathogenesis of *Lactococcus garvieae* (*L. garvieae*) was assessed in Nile tilapia (*Oreochromis niloticus*) following administration by two different routes of infection (intraperitoneal versus immersion), using 180 fish divided into three groups. The first group of fish was injected intraperitoneally (IP) with 3×10^5 colony-forming units (cfu) *of L. garvieae;* the second group was infected by immersion (IMM) into water containing 9.6×10^5 cfu/ml *L. garvieae*, and in group 3 (Control), the fish were injected IP with sterile normal saline. Mortalities were recorded daily, and on 3, 5, 7, and 13 days post-infection (dpi), liver, kidney, spleen, brain and eyes were sampled. The level of infection between groups was assessed by number of mortalities that occurred, pathology/histopathology of internal organs, bacterial re-isolation and presence of bacteria in situ determined using immunohistochemistry. A significant difference ($p < .0001$) was observed between *L. garvieae* re-isolation from tilapia following administration by IP injection and IMM. Similarly, more clinical signs and mortalities ($p < .001$) were observed in the IP group compared to the IMM group where no mortalities were observed. These findings suggest that *L. garvieae* has a low invasive potential in Nile tilapia with intact skin/external barriers and highlights the importance of maintaining fish without cuts or abrasions under field conditions.

KEYWORDS
immunohistochemistry, infection, invasiveness, *Lactococcus garvieae*, tilapia

1 | INTRODUCTION

Lactococcus garvieae (*L. garvieae*) is a Gram positive, cocci bacteria causing lactococcosis in a wide range of vertebrate and invertebrate hosts (Aguirre and Collins, 1993; Facklam and Elliott, 1995) with a worldwide distribution (Vendrell et al., 2006). It is the only species in the genus *Lactococcus* that is a major pathogen of fish (Miyauchi et al., 2012). It is characterized by septicaemia with high morbidity and mortality in several fish species (Chen et al., 2002; Eldar et al., 1996; Kang et al., 2004).

Lactococcus garvieae has been shown to cause disease in fish at water temperatures above 15°C (Sharifiyazdi et al., 2010). It affects rainbow trout (Eldar and Ghittino, 1999; Ravelo et al., 2001), Tilapia (Evans et al., 2009; Vendrell et al., 2006), yellow tail (Zlotkin et al., 1998) and several other fish species. In rainbow trout, it is a source of great economic losses especially in the Mediterranean region (Pastorino et al., 2019).

Infections of Nile tilapia (*Oreochromis niloticus*) with *L. garvieae* have gained prominence during the last decade (Evans et al., 2009; Tsai et al., 2012). In Zambia, the first reports were in 2015, affecting farmed

tilapia on Lake Kariba, south of the capital city of Lusaka. Fish approaching market size (>200 g) were affected and outbreaks occurred mostly during the hottest months (November to January). Clinical signs, including erratic swimming, lethargy, exophthalmia and corneal opacity, were observed. In general, mortalities were low, typically below 20% with differences between affected cages (Hang'ombe, pers comm.).

As a first step towards the understanding of this disease, the purpose of this study was to assess the pathogenesis of *L. garvieae* following administration by two different routes (intraperitoneal versus immersion) in Nile tilapia. There are several reports of intraperitoneal injection of *L. garvieae* in tilapia (Bwalya, Hang'ombe, et al., 2020; Evans et al., 2009; Tsai et al., 2012), while, in contrast, no documentation exists of infection by immersion. This information is necessary not only for the effective biosecurity procedures but also for the development of challenge models for use in vaccine development.

2 | MATERIALS AND METHODS

This study was undertaken according to the recommendations of the Care and Use of Laboratory Animals of the National Health Research Ethics Committee of Zambia. The protocol was approved by the Excellence in Research Ethics and Science (ERES) Converge, a private Research Ethics Board (IRB. No 00005948, Protocol Number: 2016/JUNE/028). Prior to handling, all fish were treated with Benzocaine. All efforts were made to minimize suffering and stress of the fish.

2.1 | Endpoint *L. garvieae* dose (LD_{50}) determination

The experiment to determine the 50% endpoint (LD_{50}) is described elsewhere (Bwalya, Simukoko, et al., 2020). Briefly, 100 Nile tilapia (*Oreochromis niloticus*) were divided into 7 groups of 10 fish each. Six of these groups were each injected with 0.1 ml containing a log titration (from 3×10^8 colony-forming units (cfu) ml^{-1} to 3×10^3 cfu/ml of *L. garvieae*). The seventh group (control) was injected with 0.1 ml of normal saline. The fish were then monitored for 20 days during which time clinical signs and mortalities were recorded.

The following mortalities were observed: 70% in the group injected with 3×10^8 $cfuml^{-1}$; 40% in 3×10^7 cfu/ml; 50% in 3×10^6 cfu/ml; 40% in 3×10^5 cfu/ml; and 10% in 3×10^4 cfu/ml. No mortalities were observed in the group injected with 3×10^3 cfu/ml or normal saline only. The LD_{50} of *L. garvieae* was calculated by using a modified arithmetical method of Reed and Muench (Saganuwan, 2011), and this was determined to be equal to 9.6×10^5 cfu.

2.2 | Fish

One hundred and ninety Nile tilapia with average weight of 10 ± 2 g were purchased from Palabana fisheries, a hatchery located east of Lusaka, Zambia. The hatchery had no previous history of disease outbreaks. The fish were acclimatized for 10 days in 60-L glass tanks supplied with 50 L of dechlorinated flow-through water at the School of Veterinary Medicine, University of Zambia. Constant aeration was supplied through air stones, and the water temperature ranged between 24.4 ± 2°C. Fish were fed daily on commercial dry pellets, equivalent to approximately 3% of their body weight.

To confirm the absence of *L. garvieae* infection, 10 fish were sampled and killed. Bacterial cultures of swabs from eyes, brain, liver, kidney and spleen from each fish were used to examine the presence of bacteria in the fish. The swabs were cultured on nutrient agar (Oxoid, UK), and blood agar (Oxoid, UK) plates incubated at 24°C for 48 hr.

2.3 | Preparation of *Lactococcus garvieae* for challenge

Lactococcus garvieae previously isolated from diseased Nile tilapia on Lake Kariba in Siavonga district (Bwalya, Simukoko, et al., 2020) was used. The isolate had been stored in Brain Heart Infusion Broth (BHIB [Unipath, England]) with 20% glycerol at −20°C until used. After thawing, the bacteria were inoculated onto BHIB and incubated at 25°C for 48 hr. The bacterial cells were then washed three times with sterile normal saline, collected by centrifugation (3,000 × *g* for 5 min) from the broth and re-suspended in fresh sterile saline. The turbidity was adjusted to McFarland turbidity No 4.0, equivalent to 12×10^8 cfu. This was further diluted down to 3×10^6 cfu/ml that was used to inject fish.

2.4 | Experimental challenge with *L. garvieae*

Before initiating the experiment, 10 fish were sampled for prescreening of *L. garvieae*. None of the fish was found to be infected with the bacteria in any of the tissues examined (spleen, liver, kidney, brain and eyes).

One hundred and eighty fish were divided into 3 groups of 60 fish each. Each group was further divided into three replicates of 20 fish. The groups were treated as follows: Group 1 was intraperitoneally (IP) injected with 0.1 ml of *L. garvieae* (3×10^5 cfu/fish); fish in group 2 were infected with *L. garvieae* by immersion (IMM), while in Group 3, fish were injected IP with 0.1 ml normal saline (control group). Prior to injection, the fish in groups 1 and 3 were sedated using Benzocaine (Sigma-Aldrich, Germany) using 5 ml/L. For Group 2 (IMM), the water flow was reduced to 10 L in each tank containing 20 fish with additional aeration. The water flow was then stopped, and *L. garvieae* added to a final concentration of 9.6×10^5 cfu/ml of water. The fish were kept in the bacterial solution for 30 min after which normal water flow was restored.

2.5 | Sample collection and processing

The fish to be sampled were killed by firstly sedating them with Benzocaine (as described above) followed by stunning them with a

blow to the head before decapitation. On 3, 5, 7 and 13 days post-infection (dpi), 9, 6, 6 and 6 fish, respectively, were sampled from each group. A swab was collected from each of the spleen, liver, kidney, brain and eyes excised from each individual and then streaking directly on nutrient agar plates. The tissues were then preserved in 10% phosphate-buffered formalin for Hematoxylin and Eosin (H&E) and immunohistochemistry staining. The H&E staining was carried out according to standard procedures for histological staining, and the slides examined using a Zeiss light microscope.

For immunohistochemistry, the procedure was done as previously described (Bwalya, Hang'ombe, et al., 2020).

2.6 | Statistical analysis

Fisher's exact test with the help of the JMP statistical software (SAS institute Inc.) was used to compare differences between proportions of fish expressing *L. garvieae* antigens in tissues versus those without. Only two outcomes, the presence or absence of antigens with a confidence level of 95% was used for these analyses. The chi-square test ($\alpha = 0.05$) was used to compare mortalities between groups.

3 | RESULTS

3.1 | Comparison of infection resulting from intraperitoneal infections or immersion administration

More fish were observed with clinical signs in the IP-injected group compared to the IMM group, both in terms of numbers and severity of infection. Although the first clinical signs were observed on 3 dpi in both groups (Table 1), exophthalmia, erratic swimming and uni- or bilateral corneal opacity were observed in the IP as opposed to IMM group where only uni- or bilateral corneal opacity was observed (Table 1).

Consistent with the severity of clinical signs, mortalities were only observed in the IP group where 20% of the fish died following challenge (Figure 1), representing a significant difference ($p < .001$) between the IP-infected and the IMM or untreated controls. All mortalities occurred between 3 and 7 dpi (Figure 1).

3.2 | *L. garvieae* re-isolation from different organs and groups of fish

Screening for infection with *L. garvieae* from sampled fish was performed by inoculating swabs from different tissues on nutrient agar plates. *Lactococcus garvieae* was only re-isolated from the IP group and not from the IMM or uninfected control fish, representing a significant difference of $p < .0001$. The bacteria were re-isolated from almost all fish in the IP group sampled on 3 to 7 dpi (Table 2). At 13 dpi, the number of infected fish decreased to 50%. No *L. garvieae* was re-isolated from the uninfected control or the IMM group despite clinical and gross lesion of corneal opacity observed in the IMM group.

TABLE 1 Summary of clinical signs observed in groups of Nile tilapia infected with *Lactococcus garvieae* in the present study

DPI	Group	No affected	Clinical signs
3	IP	2	Unilateral exophthalmia, corneal opacity
3	Immersion	4	Unilateral/bilateral corneal opacity
3	Control	0	–
5	IP	1	Skin haemorrhage
5	Immersion	0	–
5	Control	0	–
7	IP	1	Skin haemorrhage
7	Immersion	0	–
7	Control	0	–
13	IP	0	–
13	Immersion	0	–
13	Control	0	–

Note: Nine fish per group were sampled on day 3; 6 fish per group were sampled at each of the remaining sampling times.

Abbreviations: DPI, days post-infection; IP, intraperitoneally injected group.

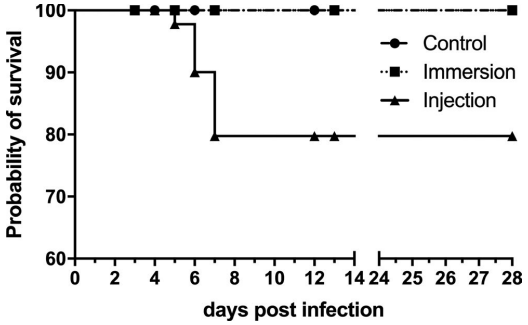

FIGURE 1 Survival plot for injection, immersion and control groups. No mortalities were observed in immersed or control groups, and lines are therefore superimposed. Symbols in the plot show sampling and events (IP injection group) time points

3.3 | Distribution of *L. garvieae* antigens in different organs by immunohistochemistry

Lactococcus garvieae antigens were observed in different tissues as positive immunolabelling in and around blood vessels as well as surrounding connective tissues (Figure 2).

Consistent with clinical signs and mortalities, *L. garvieae* was observed in significantly more fish ($p < .001$) infected by IP compared

TABLE 2 Percentage of *Lactococcus garvieae* re-isolated from individual fish from different groups of tilapia

	Percentage of infected fish at each sampling point (%)		
DPI	Injection	Immersion	control
3	89 (8/9	0	0
5	83 (5/6)	0	0
7	100 (6/6)	0	0
13	50 (3/6)	0	0

Note: Swabs from spleen, liver, kidney, brain and eyes were inoculated on nutrient agar. When *L. garvieae* was isolated from at least one organ, then that fish was considered infected.

Abbreviation: DPI, days post-infection.

of this bacterium is not well understood. In the present study, we observed a significant difference ($p < .0001$) between *L. garvieae* re-isolation from tilapia following administration by IP injection on one hand, and IMM on the other (Table 2). Similarly, more clinical signs were observed in the IP compared to the IMM group, while no mortalities were observed following immersion challenge, and the IP group suffered 20% cumulative mortality. In line with this, no bacteria were re-isolated from the IMM group despite bacterial antigens being present in situ by immunohistochemistry, likely representing remnants of bacteria/bacterial components. These findings suggest that *L. garvieae* has a mild or low invasive potential in tilapia with intact external barriers (skin/gills) and that the number of bacteria penetrating the primary barriers is few or too few to establish a life-threatening infection. We did not explore the importance of bacte-

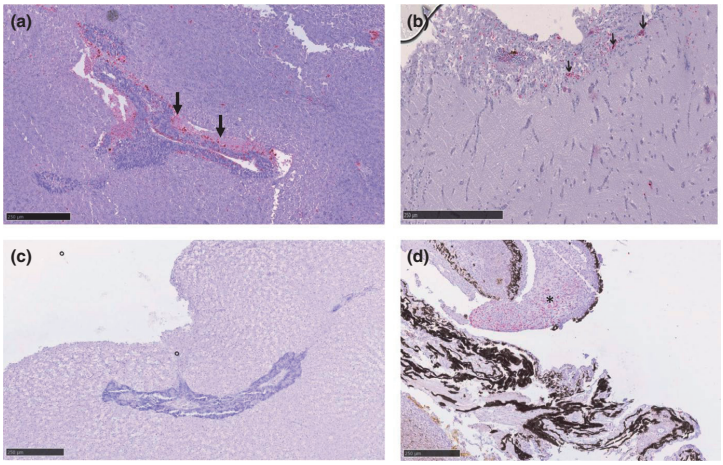

FIGURE 2 Immunohistochemistry staining of *Lactococcus garvieae* in the liver (a), brain (b) and eye (d) in tilapia at 3 days post-intraperitoneal injection. c is liver (uninfected control). Bacterial antigens are observed as red stains (arrows) in or around blood vessels as well as surrounding tissue stroma (a&b) as well as the sclera (*) of eyes (d)

to IMM with immunohistochemistry staining. At any time-point sampled, fish with positive staining in at least one organ were significantly higher ($p < .008$) in the IP group compared to the immersion group (Figure 3). At 3 dpi, the highest proportions of organs with *L. garvieae*-positive staining, irrespective of group, were the kidney and liver, followed by the spleen and eyes, and, lastly, the brain. The number of fish with positive reactions in different organs remained relatively constant, in the IP group throughout the sampling period. In contrast, the proportion of fish with positive reactions in different organs declined over time from 3 to 13 dpi in the IMM group.

As with the clinical signs, more *L. garvieae* was detected in fish infected by IP injection compared to IMM (Figure 3), while none were observed in the uninfected controls.

4 | DISCUSSION

There are presently no reports that compare intraperitoneal (IP) injection with immersion (IMM) as routes to artificially infect tilapia with *L. garvieae*, and the mode of transmission or portals of entry

rial load (infection dose) by the immersion route, and this should be established in follow-up studies as it is likely that the invasiveness is also dependent on number of bacteria in the water.

Differences between strains of *L. garvieae* have not been the subject of this study, but it should be explored in future studies. The contrasting infection patterns and clinical pictures based on the route of administration in the present study are consistent with a previous report in rainbow trout (Shahi et al., 2018) and as a consequence underlines the importance of husbandry procedures that limit injury to the skin/surface of the fish as a means of reducing infection and mortalities from *L. garvieae* in the field. In another study in which rainbow trout were infected through immersion administration compared to injection, no difference in clinical signs or mortalities was observed (Avci et al., 2014), although the IP injection group succumbed much earlier than the immersion group, supporting the view that the former induces more severe reactions. Notably, in this same study (Avci et al., 2014), the dose for immersion groups was 2 logs higher than that of those injected, which probably explains or has an impact on the mortalities in the immersion group. The reason

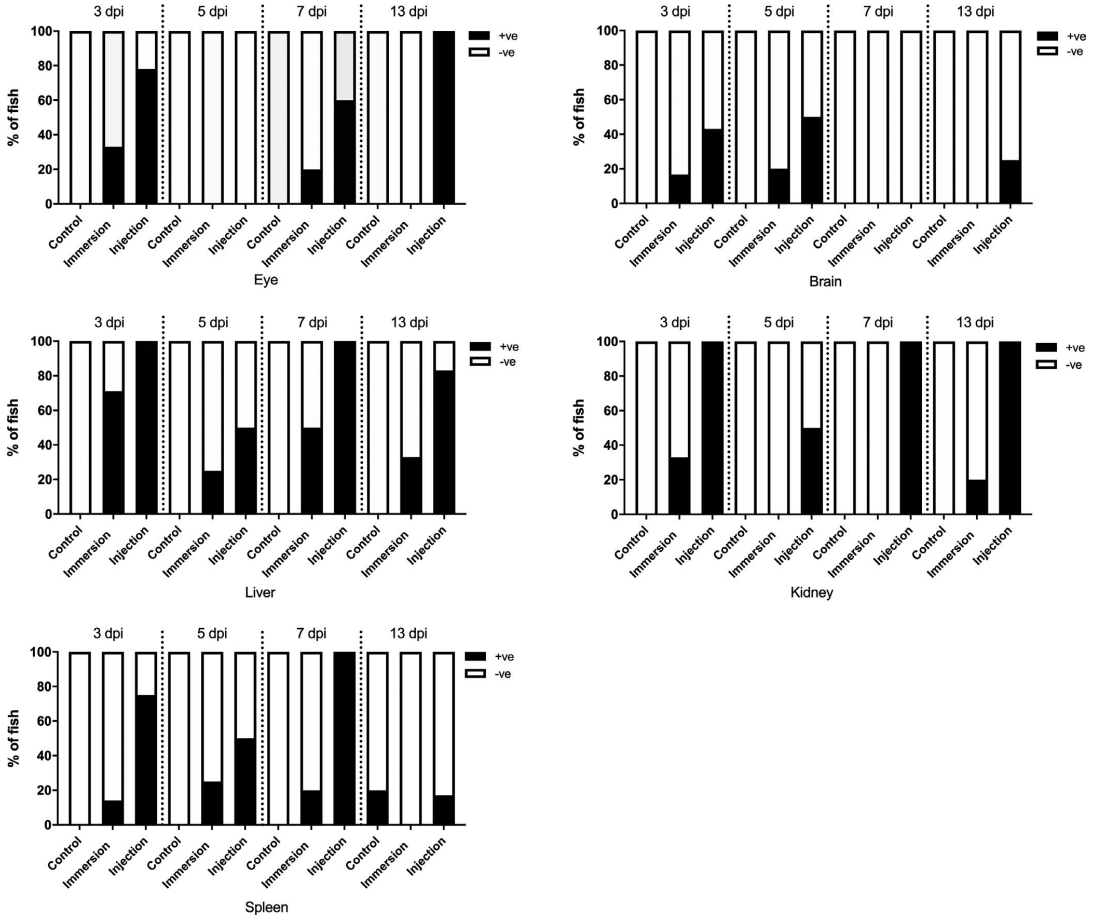

FIGURE 3 Immunohistochemistry staining of different tissues of Nile tilapia against *Lactococcus garvieae*. DPI = days post-infection; n at 3, 5, 7 and 13 is equal to 9, 6, 6 and 6, respectively

for this difference is unclear although species resistance of tilapia to *L. garvieae* infection compared to rainbow trout could be a contributing factor (Algoet et al., 2009).

The cumulated level of mortality observed in the present study is low compared with what others have found (Evans et al., 2009; Tsai et al., 2012, 2013). We used a low dose in this experiment (10^5 cfu) compared to that used by others (10^8 cfu) (Evans et al., 2009; Tsai et al., 2012, 2013). As mentioned, we did not explore differences between *L. garvieae* isolates. Previously, *L. garvieae* has been grouped into non-agglutinating (virulent) and agglutinating (avirulent) phenotypes, KG- and KG+, respectively (Yoshida et al., 1996). The *L. garvieae* used in the present study was not typed with regard to phenotype. However, when the strain used here was administered at a dose of 10^8 cfu for LD_{50} titration, a mortality of 70% was achieved (Bwalya, Simukoko, et al., 2020), which suggests that the isolate is comparable in virulence to what other have reported (Tsai et al., 2012). The low dose of 10^5 cfu used in the present study was in accordance with the end point calculation, with an aim not to overwhelm the immune system of the fish under an experimental setting.

The finding that fish in the IMM group only presented uni- or bilateral ocular opacity (Table 1) in this study is interesting and suggests that eyes may be a route of entry for the bacteria or that the eye is affected during early stages of infection. It is somewhat surprising, though, that no *L. garvieae* was re-isolated from the eyes of these fish albeit detection by immunohistochemistry.

Lactococcus garvieae was detected in different organs by immunohistochemistry and not re-isolated by culture, especially in fish infected by immersion (Table 2). These findings are consistent with previous reports (Bwalya, Hang'ombe, et al., 2020; McNeilly et al., 2002) and suggest that bacterial remnants can be detected by immunohistochemistry even when they are no longer viable. This has been observed also for bacterial antigens in vaccines where positive staining for *Aeromonas salmonicida* was

shown in macrophages/melanomacrophages months after vaccination (Grove et al., 2003). On the other hand, these results differ to the findings of others (Martinson et al., 2008; Munang'andu et al., 2012), pointing to the fact that the sensitivity of immunohistochemistry compared to pathogen re-isolation depends on several factors including the type of the pathogen in question. The demonstration of *L. garvieae* antigens by immunohistochemistry in significantly more internal organs of fish ($p < .0001$) infected by IP compared to IMM was expected and points to the fact that once systemic, the bacteria readily spreads between internal organs as shown by others (Avci et al., 2014).

Finally, in the present study, the livers and kidneys were, on average, the two organs with the highest frequency of bacterial re-isolation and presence of bacterial antigens in situ; *that is*, bacteria persisted the most in these organs. If this represents a state of deposition of bacterial components undergoing breakdown (in macrophages and similar cell types) or are targets of internalized *L. garvieae* from where the bacteria spreads to other internal organs including the spleen, eyes and finally the brain, remains to be proven.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon request.

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III

PLOS ONE

RESEARCH ARTICLE

A whole-cell *Lactococcus garvieae* autovaccine protects Nile tilapia against infection

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Abstract

The autovaccine was produced in-house using a bacterial isolate from a diseased fish from the target farm. Three groups of 150 fish each were injected with either 1) an oil-adjuvanted, inactivated whole cell autovaccine, 2) adjuvant only or 3) PBS (negative control). Approximately 660 degree days post vaccination, the fish were challenged with $9x10^5$ cfu bacteria/fish by intraperitoneal injection and monitored for a further 28 days. Protection against infections was measured by lack of/reduced bacterial loads both by bacterial re-isolation and immunohistochemistry as well as absence of clinical signs/pathology. Significantly less *L. garvieae* ($p<0.03$) was re-isolated from either the adjuvant only or control groups compared to the vaccinated group. Furthermore, a significantly high amount ($p<0.001$) of anti-*L. garvieae* specific antibodies were observed in the vaccinated group compared to the adjuvant only or control groups at time of challenge. This coincided with protection against infection measured by absence/reduced *L. garvieae* re-isolation from internal organs, reduced clinical signs and lack of pathology in this group. In the adjuvant only and control groups, bacteria were re-isolated from the kidney, liver, spleen, brain and eyes during the first 14 days. The findings suggest that oil-based vaccines can protect tilapia against *L. garvieae* infection through an antibody mediated response.

Introduction

Tilapia farming in Zambia is a relatively young but rapidly growing industry. Although commercialization started as far back as the 1990s, the surge in production was not until 2010 that cage-based commercial farming intensified on Lake Kariba [1]. Zambia's annual aquaculture production presently stands around 30,000 metric tons [2].

As with intensive fish farming elsewhere that is affected by fish diseases [3], the increase in the fish production on Lake Kariba soon faced the same problem. Lactococcosis outbreaks caused by *Lactococcus garvieae (L. garvieae)* have been experienced since early this decade [4].

Lactococcus garvieae is a facultatively anaerobic, non-motile, non-spore forming, Gram-positive, ovoid cocci bacteria belonging to the Phylum *Firmicutes*, Family *Streptococcaceae*, Order *Lactobacilles* and genus Lactococcus. It is well-known for infecting and causing disease in rainbow trout [5, 6] and yellowtail (*Seriola quinqueradiata*) [7]. Clinical signs include

exophthalmia, conjunctivitis, melanosis, erratic swimming, anorexia, internal hemorrhage and congestion of blood vessels, peritonitis, meningoencephalitis and septicaemia [8–12].

In tilapia, *L. garvieae* infections are a cause of an emerging disease that became of major importance during the last decade [13, 14]. Infections are most severe when water temperatures are above 20˚C [14, 15]. Economic losses occur as a result of mortalities (high or low), downgrading of carcasses due to unsightly skin lesions and reduced growth rate [8, 13, 14]. No protective commercial vaccines for tilapia are available on the market at the moment.

The objective of this study was to develop a whole bacterial cell autogenous oil-based vaccine for the protection of tilapia against *L. garvieae* infections. Autogenous vaccines are custom-made that are produced on a small to medium scale; and are based on pathogens isolated from a farm on which they are to be used. They have the advantage of being less amenable to rigorous regulations applicable to commercial vaccines [16] and allow for more rapid availability without complete and comprehensive characterization in the face of an outbreak [17].

To evaluate the effect of the vaccine, protection against infection was done by bacterial re-isolation and immunohistochemistry supported by clinical signs/ pathology. Infection is a prerequisite of disease and therefore a good proxy for total protection.

Materials and methods

This study was undertaken in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Health Research Ethics Committee of Zambia. The protocol was approved by the Excellence in Research Ethics and Science (ERES) Converge, a private Research Ethics Board (Protocol Number: 2016/JUNE/028). All efforts were made to minimize suffering and stress of the fish, both during handling and sampling. As infection was one of the humane endpoints, subjects were withdrawn from the experiment (euthanized and sampled) before clinical signs appeared. Where signs of disease or abnormal behaviour (lethargy, disorientation, aberrant swimming etc) were observed, the fish were euthanised by stunning with a blow to the head followed by dislocation of the cervical vertebra.

Fish

A total of 460 healthy Nile tilapia (*Oreochromis niloticus*) with mean weight of 41.5 ± 16.5g were purchased from Palabana fisheries, a commercial fish farm located in Chirundu district, South-East of Zambia. The fish farm had no previous history of disease outbreaks and the subjects were transported by road in oxygenated bags to the University of Zambia, School of Veterinary Medicine wet-lab. The fish were kept in 500 L tanks supplied with flow-through dechlorinated water and aerated using stone bubblers. They were allowed to acclimatize for 10 days prior to commencement of the experiment. The fish were fed daily on commercial pellets at a rate of 3% body weight. Daily water temperature averaged 20 ±2˚C, mean daily dissolved oxygen was 7.9 ± 2 mg/L and pH was 7 ± 0.2.

Antigens and vaccine formulation

Lactococcus garvieae previously isolated from a diseased fish at a farm on Lake Kariba with a partial 16S RNA sequence (Genbank accession number MK346137) and stored in Tryptic Soy Broth (TSB, HiMedia, India) and 20% glycerol was used. The bacteria was propagated in TSB and incubated at 37˚C on a shaker at 180 rpm for 72 hours. Thereafter, it was centrifuged at 800 x g for 19 min at room temperature to pellet the cells. The bacteria was then inactivated with 4% formalin for 72h followed by dialysis using PBS for 48h. The inactivation process was confirmed by inoculation of the antigen on nutrient agar followed by incubation at 37˚C for

72h to demonstrate the absence of bacterial growth. The vaccine was formulated using 10^9 cfu/mL as a water-in-oil emulsion using the ISA 763 VG adjuvant (Seppic, France) according to manufacturer's guidelines. The adjuvant only group was prepared in the same way but without bacteria. The preparations were then stored at 4˚C until used. The vaccine and adjuvant only were determined to be sterile by lack of bacterial growth on nutrient and Sheep's Blood Agar after 72h incubation at 37˚C.

Vaccination of fish

Prior to the start of the study, 10 fish were sacrificed, sampled and tested for the presence or absence of bacterial infections by bacteria re-isolation (described below). Furthermore, ELISA was also done on serum from these fish to confirm that the fish had not recently been in contact with *L. garvieae*.

The fish (n = 450) were divided into in 3 groups (Control, Adjuvant and Vaccine) by dip-netting and sequential allocation. The control fish were injected with PBS: Adjuvant group were injected with adjuvant only and the Vaccine group with *L. garvieae* vaccine. The total number of fish per group was 150 individuals. Each group was further split into two replicates, one for observation (surveillance) and the other for sampling (Fig 1). For sampling, each group was placed in a separate tank (A-C), each containing 90 individuals. The rest of the fish were pooled together in tank D (surveillance), containing 60 control, 60 vaccinated and 60 adjuvant-only groups all mixed together. The fish in tank D were marked by clipping of the dorsal fin, caudal fin or left unclipped to differentiate between groups. All fish were injected intraperitoneally with 0.1ml of vaccine, adjuvant-only or PBS.

Challenge experiment

Following vaccination, all fish in the four tanks (A, B, C and D) were allowed a period of 6 weeks for immune induction (Fig 1). On day 43 (approximately 660 degree days) post vaccination (dpv), the fish were challenged by intraperitoneal injection of 0.1 ml of *L. garvieae* suspension (9.6 x10^5 cfu bacteria/fish). Monitoring was done for 28 dpc during which clinical signs were recorded and sampling for bacterial re-isolation was done.

Sample collection

At each sampling time point on days 21 and 42 post vaccination; and 3, 5, 7, 14, and 28 post challenge, 10 fish per group from tanks A, B and C were sampled by dip-netting and anaesthetized by using Benzocain (Sigma-Aldrich, Germany) at a dosage of 5 ml/L. Blood was first collected from the caudal vein into non-heparinised tubes. Thereafter, bacteriological samples were collected by aseptically inserting a sterile loop into each of the liver, kidney, spleen, eyes and brain and then streaking directly on nutrient agar plates (HiMedia, India). The plates were then incubated aerobically at 24˚C for 48 hours. Parallel samples were also collected in 10% phosphate-buffered formalin.

Clinical signs of disease post-challenge

Fish were observed twice daily for clinical signs. Furthermore, the fish were inspected during sampling for any clinical signs. Surveillance for clinical signs or mortalities was done primarily in tank D although attention to other tanks was also paid.

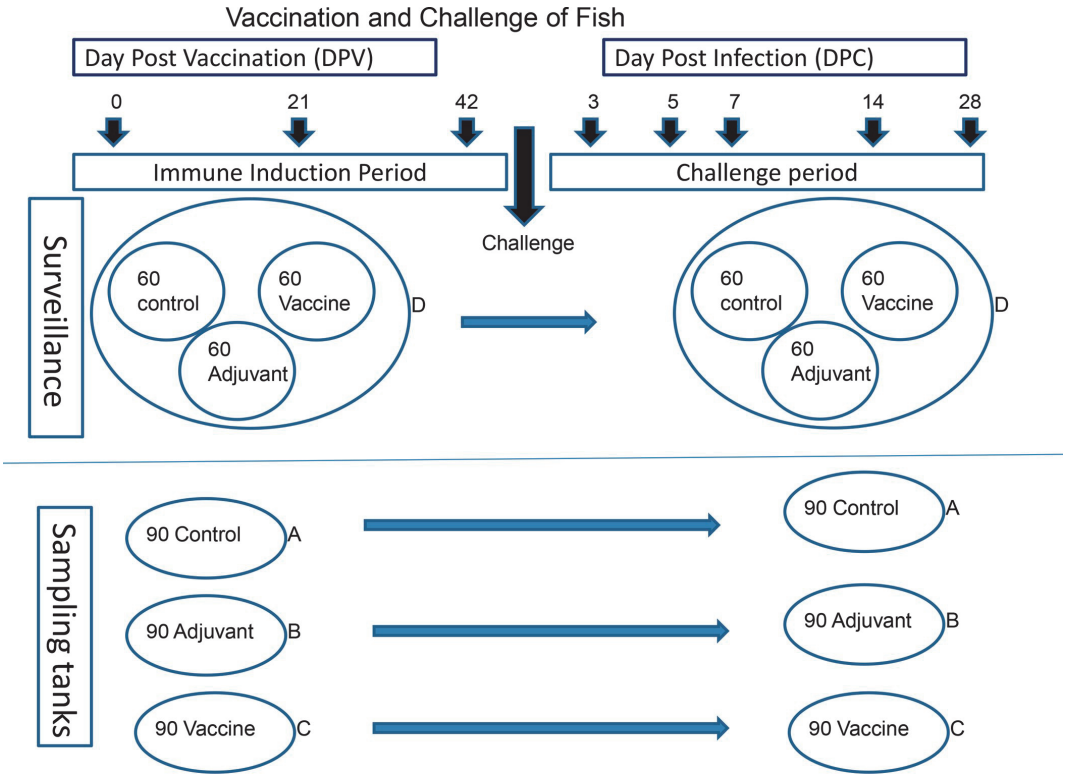

Fig 1. Schematic diagram showing the experimental design of the present study. Different groups of fish were injected intraperitoneally with the indicated preparations and allowed a period of 42 days to mount an immune response. Thereafter, the fish were challenged with 9 x 10^5 cfu of bacteria per fish. Key: Cont = control (PBS) group; Vac = vaccinated group; Adj = adjuvant only group; DPV = days post vaccination: DPC = Days post challenge.

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Production of hyperimmune serum

Production of hyperimmune serum against *L. garvieae* was outsourced to the Section for Experimental Biomedicine at the Norwegian University of Life Sciences in Oslo, Norway. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (https://www.aaalac.org/). The animal experiment was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and the Food Safety Authority (application ID: FOTS 13566) and executed in compliance with the local and national regulations associated with laboratory animal experiments. The rodent and rabbit section of the facility is a Specific Pathogen Free (SPF) unit and follows a health monitoring program recommended by Federation of European Laboratory Animal Science Associations/FELASA (http://www.felasa.eu/). The care of the animals was carried out by two veterinary nurses with FELASA B certification and the study was performed by a veterinarian with FELASA C certification.

Lactococcus garvieae previously isolated from a diseased fish at one farm on Lake Kariba with partial 16S RNA sequence (Genbank accession number MK346137) was used. The bacteria was propagated in 300 ml of TSB in a fermenter at 37˚C until an optical density (OD) of 0.6 at 595 nm was reached. Bacterial cells were collected by centrifugation at 800 x g for 19 min at room temperature and then inactivated with 4% formalin for 72h. Bacterial cells were collected again by centrifugation and washed in PBS three times. Finally, the cells were resuspended in PBS to OD_{595} 0.6.

A rabbit reared according to standard procedures was used to produce hyperimmune serum. The rabbit was first allowed to acclimatize for 2 weeks prior to the first (primary) injection of *L. garvieae* emulsified in Freund's complete adjuvant. The rabbit was injected with 10^8 cfu bacteria in 0.5 ml. All booster vaccines were formulated in Freund's incomplete adjuvant and were administered on days 14 (1^{st} booster), 21 (second booster) and 28 (third booster). Blood samples were collected before the first injection and on day 28 before the third booster. The terminal bleeding was done on day 42.

Specificity and reactivity of the hyperimmune serum against other bacteria

To measure the titres of immunoglobulins rabbit serum prior- and post-harvest, ELISA plates (NUNC, Maxisorp) were first coated with 100 ul per well of *L. garvieae* (0.6 OD_{595}). Prior to this, the bacteria was diluted in 0.1 M coating buffer (0.1 M Carbonate buffer, pH 9.6) in 5mL suspension and then homogenized in a mixer mill (Anders Pihl As) at 30s per min. Plates coated with two of these bacterial dilutions (1:100 and 1:1000) were incubated overnight at 4˚C. Following incubation, the plates were washed three times with 250ul washing buffer (1 L PBS plus 0.5 mls Tween 20 (PBST)) per well to remove unbound antigen. 250ul of blocking buffer (5% fat-free dry milk in PBS) was added to each well and plates incubated at room temperature for 2 hours after which the they were washed as described above. 100ul of rabbit *L. garvieae* serially diluted antiserum (1:100, 1:1000, 1:10000, and 1:20000) was added and plates incubated at room temperature for 1 hour. Plates were then washed and 100ul of goat anti-rabbit polyclonal antibody conjugated with Horse Radish Peroxidase (HRP) (GE Healthcare, UK) diluted to 1:1000 in 1% fat-free dry milk was added to each well and incubated at room temperature for 1 hour. After washing, 100ul of O-Phenylenediamine Dihydrochloride (OPD) substrate containing H_20_2 (DAKO, Denmark) prepared according to manufacturer's recommendations was added. The plates were incubated at room temperature in the dark and the reaction was stopped by adding 50 ul of (55mls of 1M H_2SO_4 in 945mls water) after 10 min. A spectrophotometer (Tecan GENios) was used to read the absorbance at 492nm.

To test the specificity of the hyperimmune serum, Elisa was used. First, the serum was adsorbed by dilution 1:1000 in PBS containing 0.57 OD_{595} of *L. garvieae* followed by adsorbed for 2 hours at 37˚C. The mixture was centrifuged at 8000 x g for 5 min to pellet bacterial cells and recover the serum. The serum was then used in an Elisa (as described above) as a primary antibody. Un-adsorbed antibodies were also used in parallel as positive controls.

For non-reactivity of the hyperimmune serum against other bacteria, *Streptococcus garvieae*, *Yersinia ruckeri*, *Aeromonas hydrophila* and *L. garvieae* were tested by Elisa. The different bacteria were coated on Elisa plates and the procedure performed as described above.

Histopathology and immunohistochemistry

Formalin fixed tissues were used for histopathology and were processed according to standard procedures for haematoxylin and eosin staining.

For immunohistochemistry, paraffin-embedded sections were cut into ultra-thin slices of 3–4 μm onto poly-L-lysine coated glass slides. Dewaxing was done by incubating the slides at

58˚C for 25 min followed by two incubation steps in xylene. The sections were rehydrated by graded alcohol incubations of 5 min each.

A ring was then drawn around the sections using a PAP-pen to prevent the solutions from flowing off the specimens. For antigen retrieval, the tissues were pretreated with 10 nmol of citrate buffer, pH 6.0 containing 10% of trypsin at 37˚C for 10 min. All incubations were performed in humidified chambers at room temperature and about 150 μL of different reagents were added to each specimen. After pre-treatment, the slides were washed in ice-cold PBS twice for 10 min. 5% (w/v) bovine serum albumin (BSA) in TBST for 2 hours was used to block non-specific binding. Thereafter, the slides were incubated with rabbit anti-*L. garvieae* serum (diluted 1: 500 in 2.5% BSA in TBST, see below). Non-immune serum was also included as a negative control. This was followed by incubation overnight at 4˚C. Washing was done twice in TBST for 10 min and unless otherwise stated, all washing steps were done this way. Next, biotinylated goat anti-rabbit immunoglobulin (1:300 in 2.5% BSA in TBST) was added and incubated for 30 min. After washing, the sections were covered with Streptavidin-Alkaline Phosphatase Conjugate (Roche, Germany) and incubated for 30 min. After another wash, the sections were treated with Diaminobenzidine (DAB) (Sigma, USA) prepared as per manufacturers containing 3% H_2O_2 for 5 min. After this, washing was done in running water for 5 min, followed by counter-staining with Mayer's modified hematoxylin for 30 seconds. The slides were then washed in running water for 5 min and mounted using Aquamount. For negative controls, rabbit anti-*L. garvieae* antibody was replaced with non-immune rabbit serum.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples collected from fish as described in the sample collection section were used. The blood was allowed to clot at 4˚C. The serum was separated by centrifugation at 1204 x g, 4˚C for 10 min and then stored in separate tubes at -20˚C until required.

Preparation of the bacteria and coating of plates was done as described in the section of the production of hyperimmune serum. ELISA plates (NUNC, Maxisorp) were coated with 100 μL of homogenized *L. garvieae* (0.6 OD_{595}) in PBS and incubated overnight at 4˚C. Unbound antigens were removed by washing with 250 μL per well with PBS and 0.5% Tween20 (PBST), 3 times. Thereafter, 250 μL per well of blocking buffer (5% (w/v) Bovine Serum Albumin (BSA in PBST) was added and incubated at room temperature for 2 hours. The plates were washed 3 times as described above. Diluted serum (from the experimental fish) in PBS (1/40 or 1/80) was added (100 μL per well) to the ELISA plates and incubated overnight at 4˚C. The plates were then washed followed by incubation with 100 μL per well of monoclonal anti-tilapia IgM antibody ((1:30 dilution) Aquatic Diagnostics, Britain) and incubation for 60 min at room temperature. After another washing step, 100 μL per well of rabbit anti-mouse IgG-HRP diluted 1/1000 in PBST was added and incubated at room temperature for an hour. Following washing, 100 μL per well of O-Phenylenediamine Dihydrochloride (OPD) substrate containing H_2O_2 (DAKO, Denmark) prepared as per manufacturers recommendations was added. The plates were incubated for 10 min at room temperature after which the reaction was stopped by adding 50 μL per well of 2M Sulphuric acid. Reading of results was done using a spectrophotometer (Tecan GENios) at 492 nm.

Statistical analysis

Fischer's exact and Chi square tests were used to determine independence or association between the treatment groups and outcomes of infection measured either by bacterial re-isolation or immunohistochemistry with the help of the JMP program (SAS Institute, USA) or Graphpad Prism 5.0 (www.graphpad.com). Regression analysis was used to examine statistical

significance between groups in the antibody responses. All statistical tests were done with 95% confidence level.

Results

Clinical signs and autopsy findings

No *L. garvieae* was detected in the 10 fish that were sacrificed and tested before the start of the experiment.

In experimental groups, clinical signs were observed mostly in the control (PBS) and adjuvant only groups (Table 1). The most common sign was ocular opacity, uni- or bilateral with or without exophthalmia. The frequency was highest in the control followed by the adjuvant only group. In the former, fish with clinical signs were first observed at 3 days post challenge (dpc) and culminated on 5 dpc. In the adjuvant group, the first onset of clinical signs was on 5 dpc followed by 7 dpc (Table 1). In the vaccinated group, only two fish showed clinical signs, one at 3 dpc, likely due to physical injury unrelated to challenge and another one at 14 dpc, this time with corneal opacity (Table 1). No mortalities were observed in any of the groups.

During autopsy, changes were observed in the control and adjuvant groups only (Table 2). As with clinical signs, lesions were first observed in the control groups, before onset in the adjuvant only group. No changes were observed in the vaccinated group at any of the sampling time points.

Bacterial re-isolation from tissues of fish from different treatment groups

Assessment of fish infected with *L. garvieae* was done by bacterial re-isolation from the kidney, spleen, liver, brain and eyes (n = 10) in each group per time point post challenge. Where *L. garvieae* was re-isolated from at least one organ, the fish was considered infected. In general, the number of infected fish was relatively low, with overall 20% of the control and 28% of the adjuvant only groups against 6% of the vaccinated group (Table 3). There was, however, a significant difference ($p<0.02$) in the number of fish infected by the bacteria between groups. Significantly less bacteria ($p<0.03$) was re-isolated from the vaccinated group compared to either the adjuvant only or the control groups. The results also showed that control fish were infected first (3 dpc) followed by the adjuvant only and vaccinated groups from which bacteria was re-isolated at 5 dpi. No *L. garvieae* was re-isolated from any fish from 14 to 28 dpc.

Distribution of *L. garvieae* in different organs

In the vaccinated group, *L. garvieae* was only re-isolated from one organ (spleen) and this was on 5 dpc. In contrast, the bacteria was isolated from all organs in both the control and adjuvant groups. Furthermore, the trends of bacterial re-isolation from different organs from these groups suggested that the liver, kidney and spleen were infected first, followed by the eyes and finally the brain (Table 4). The control groups had the highest number of *L. garvieae* isolation on day 3, with less re-isolations at 5 dpc.

A Kruskal-Wallis test of these observations shows that there was a significant difference ($p<0.03$) between treatment groups with the vaccinated fish being lower. This was further analysed by a multiple logistic regression where the dependent variable was 'positive bacterial identification (>0)' and independent variables are treatment (control, adjuvant, or vaccinated), organs and days post challenge. The overall model was statistically significant ($p = 0.000$), $chi2 = 30.7$, with vaccination significantly lowering ($p = 0.013$) the odds ratio of positive bacterial growth from the defined organs (20-fold less likely than adjuvant and 15-fold compared to controls). Bacterial re-isolation also decreased with increasing days post challenge ($p<0.01$).

Table 1. Frequency of clinical signs observed in the different treatment groups in the present study. DPC = days post challenge.

DPC	Clinical finding	Treatment group (n = 10/time point/group)		
		Vaccination	Adjuvant	Control
3	Body wound /skin ulcer	0.1	0	0
	Bilateral ocular opacity plus exophthalmia	0	0	0.1
	Unilateral ocular opacity plus exophthalmia	0	0	0.1
5	Bilateral ocular opacity plus exophthalmia	0	0	0.1
	Bilateral ocular opacity	0	0.1	0
	Unilateral ocular opacity plus exophthalmia	0	0.2	0.3
7	Unilateral ocular opacity plus exophthalmia	0	0.2	0
14	Unilateral ocular opacity	0.1	0	0
	Bilateral ocular opacity plus exophthalmia	0	0.1	0
28	Swimming upside down plus wound below mouth	0	0.1	0

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Lactococcus garvieae hyperimmune serum

Rabbit hyperimmune serum was harvested on day 42, after *L. garvieae*-specific immunoglobulins reached an OD_{595} value of 1.55 against background pre-immunization OD of 0.4. The best bacterial concentration for coating the plates was 1/100 while the best antiserum working concentration was 1:1000. A lower concentration resulted in suppression of the assay while dilutions above 1:1000 resulted in weak signals.

No reactivity was observed when the hyperimmune serum was first adsorbed with *L. garvieae* (results not shown). Similarly, the serum did not cross-react with any of the bacteria (*S. agalactiae*, *A. hydrophila or Y. ruckeri*) tested.

Immunohistochemistry

Immunohistochemistry was done as an additional method to demonstrate the presence of *L. garvieae* in different tissues in the present study, immunohistochemistry was utilized. By this method, *L. garvieae* antigens were observed firstly around blood vessels and surrounding areas in tissues examined (hepato-pancreas (liver), kidney, spleen, brain and eyes, and also in interstitial areas (Fig 2). The most optimal titration of rabbit hyperimmune serum for use in immunohistochemistry assays was 1:500.

A significantly lower number of fish ($p<0.01$) with *L. garvieae* antigens was observed in the vaccinated group compared to the adjuvant only or controls. On the other hand, no difference ($p = 0.05$) in the number of fish with antigens was observed between the control and the adjuvant only groups.

Unlike bacterial re-isolation, *L. garvieae* was demonstrated in all groups in the liver, kidneys and spleen starting with 3, 5, 7 and 14 dpc. No antigens were observed in any of the groups at 28 dpc. In the liver, at least half of the fish had antigens in the control and adjuvant only groups

Table 2. Frequency of changes in fish observed during autopsy. DPC = days post challenge.

DPC	Autopsy finding	Treatment group (n = 10)		
		Vaccination	Adjuvant	Control
3	Pale liver plus enlarged spleen	0	0	0.3
	Pale liver	0	0	0.1
5	Enlarged spleen plus distended gall bladder	0	0.1	0
14	Pale liver plus enlarged spleen	0	0.2	0

https://doi.org/10.1371/journal.pone.0230739.t002

Table 3. Percentage fish infected with L. garvieae in different groups following challenge. Infection was assessed by bacterial re-isolation from internal organs of 10 fish per group at each time point.

Days post infection	Vaccinated	Adjuvant only	Control
3	0	30%	70%
5	30%	50%	30%
7	0	60%	0
14	0	0	0
28	0	0	0
Overall	6%	28%	20%

https://doi.org/10.1371/journal.pone.0230739.t003

while fewer fish were positive in vaccinated group (Fig 3). A similar trend was observed in the kidneys and spleen for all groups. *L. garvieae* antigens was consistently with lower prevalence in vaccinated groups in all organs and over the entire course of the challenge period (Fig 3). Relative to the distribution of bacteria in different organs, the brain had the least, with few fish showing positive reaction at 5 dpi and 7dpi in controls and adjuvant groups, as well as at 14 dpi in the latter (Fig 3).

Enzyme linked immunosorbent assay (ELISA)

For the assessment of fish responses to vaccination, fish sera diluted at 1/40 or 1/80 yielded similar results in this study and so for clarity, only results of 1/40 are presented. Fish were negative for *L. garvieae* specific antibodies prior to vaccination.

Following vaccination however, anti-*L. garvieae* specific antibody titres increased significantly ($p<0.001$) compared to non- vaccinated and adjuvant only groups at both days 21 and 42 post vaccination (Fig 4).

At 3 dpi, a marked drop in antibody levels was observed in the vaccinated group but this was followed by a steady increase from 5dpc onwards until the end of the study. In the control

Table 4. Summary of the frequencies of *L. garvieae* re-isolation from different tissues. A sterile inoculation loop was pierced into each of the indicated organs during sampling and inoculated on Nutrient Agar (HiMedia, India) plates which, were then incubated at 24˚C for 48 hours. No bacteria were re-isolated from any group on days 14 and 28 post challenge. n = 10.

Tissue	Treatment	Day post challenge		
		3	5	7
	Vaccinated	0	0	0
Kidney	Adjuvant	0.1	0.3	0
	Control	0.5	0	0
	Vaccinated	0	0	0
Liver	Adjuvant	0	0.2	0
	Control	0.3	0	0
	Vaccinated	0	0.3	0
Spleen	Adjuvant	0.2	0.4	0.1
	Control	0.5	0.2	0
	Vaccinated	0	0	0
Eyes	Adjuvant	0.2	0.4	0.2
	Control	0.3	0.3	0
	Vaccinated	0	0	0
Brain	Adjuvant	0	0	0.1
	Control	0.3	0.1	0

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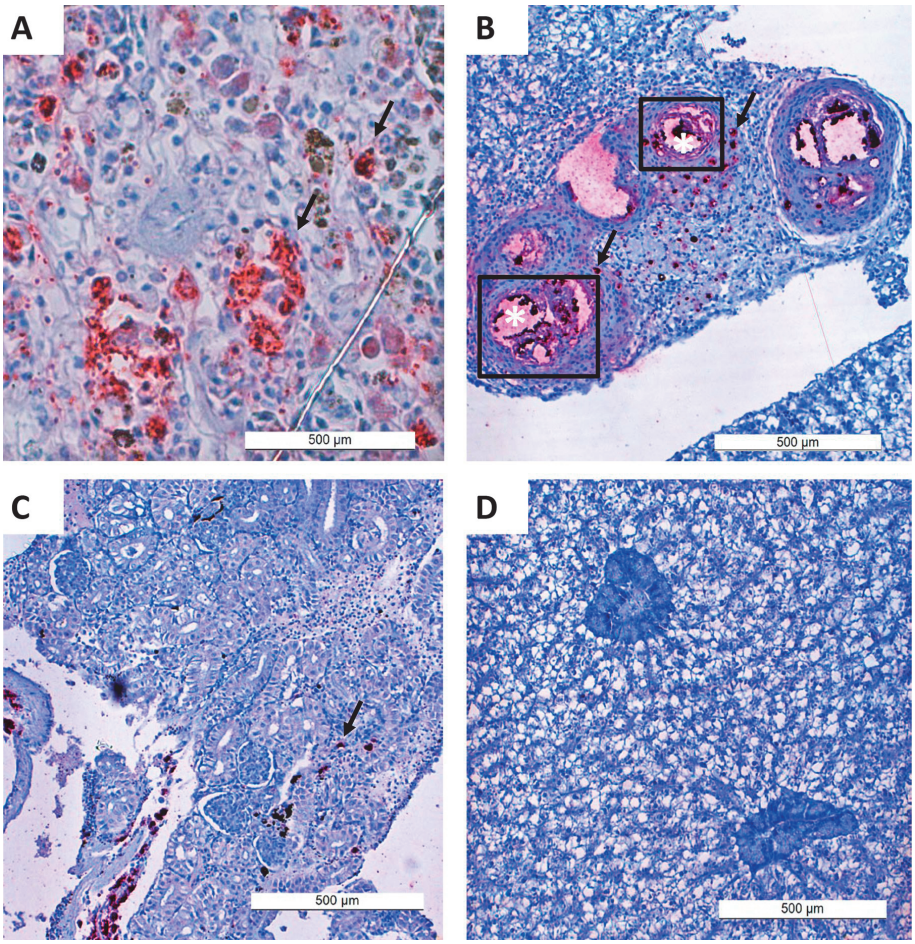

Fig 2. Immunohistochemistry staining of *L. garvieae* in different tissues of Nile tilapia following intraperitoneal injection. Bacterial antigens were observed as red stains (arrows) in the spleen (A), hepato-pancreas (liver) (B), the and kidney (C). In the liver (B), the bacteria is concentrated in and around blood vessels (*) which are likely portals of entry. Image (D) is a negative control (Liver), showing no positive stain.

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group, anti-*L. garvieae* remained at background levels throughout the priming period of 42 days. 3 days after challenge however, a slight increase was observed followed by a rapid return to background levels from 5 dpc (Fig 4). The response of fish in the adjuvant group was in-between, with a slight pre-challenge increase followed by a drop at 3 dpi and a slight but non-significant increase in antibody titres following challenge.

Discussion

Lactococcus garvieae infections in farmed tilapia became a problem in the industry during the last decade [8, 18, 19]. There are very few reports on studies of efficacious vaccines against this

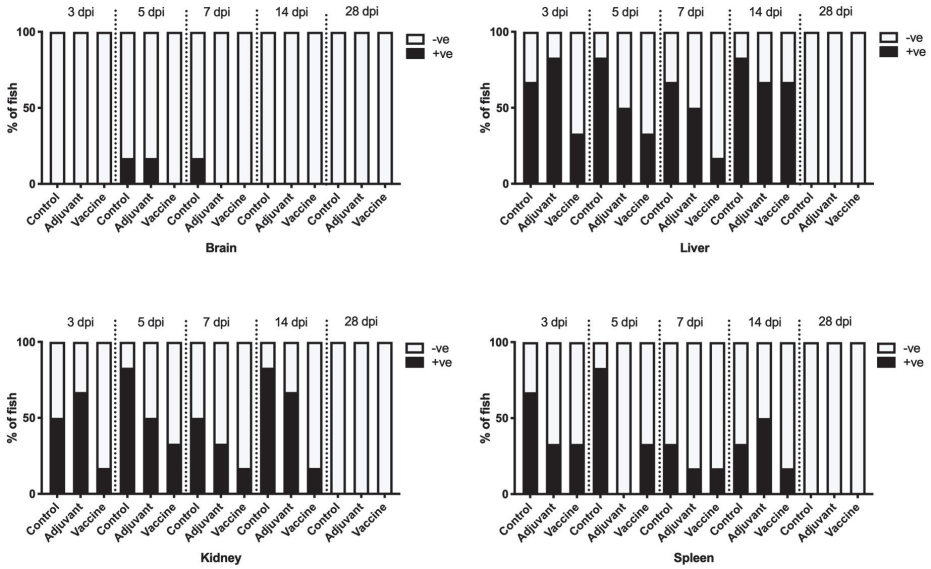

Fig 3. Percentage of fish organs positive for *L. garvieae* per group and organs (brain, liver, kidney and spleen) 3–28 days post challenge. At each sampling point, n = 10.

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bacterial infection in tilapia besides Tsai and co-workers [19] who reported full protection with inactivated whole bacteria combined with recombinant GADPH. The primary objective of the present study was to develop a protective oil-based, autogenous vaccine for the protection of tilapia on Lake Kariba. Our findings suggest that a whole bacterial cell autovaccine can protects Nile tilapia against *L. garvieae* infection. The vaccinated group in the present study was significantly more protected than the adjuvant only ($p = 0.004$) or the control ($p = 0.03$) groups. These findings support the reports of Tsai and others [19] that oil-adjuvanted vaccines can induce protection of tilapia against infection with *L. garvieae*. Our findings further suggest that the mechanism by which this is achieved is likely via an antibody mediated response. No clinical signs or post-mortem changes were observed in the vaccinated group nor was *L. garvieae* re-isolated from any of the tissues (except spleen of one fish) at any time point following challenge (Table 4). In contrast, clinical signs and post-mortem changes were observed in the adjuvant only and unvaccinated control groups (Tables 1 and 2) during the first 14 days following challenge. Furthermore, significantly more *L. garvieae* was re-isolated from the control ($p = 0.04$) and adjuvant only ($p = 0.004$) groups in this study during the first 7 days. No bacteria was re-isolated from any fish in any group from 14 dpc. The reason for this is yet to be investigated but *L. garvieae* under experimental conditions has been shown to induce acute infections [20], typically within 10 days post infection. Fish that do not succumb within this time recover from the infection and it is not unlikely that they clear the bacteria with time.

The low prevalence of infection in the present study contrast reports of others [19, 20] where significant mortalities were observed following challenge of tilapia with *L. garvieae*. A major difference between this and the two studies by Tsai and colleagues was the dosage used (10^5 CFU in the present study versus 10^8 CFU in the other studies). 9.6×10^5 CFU/fish was used in the present study as determined by LD50 in an earlier study [21] according to standard procedures of

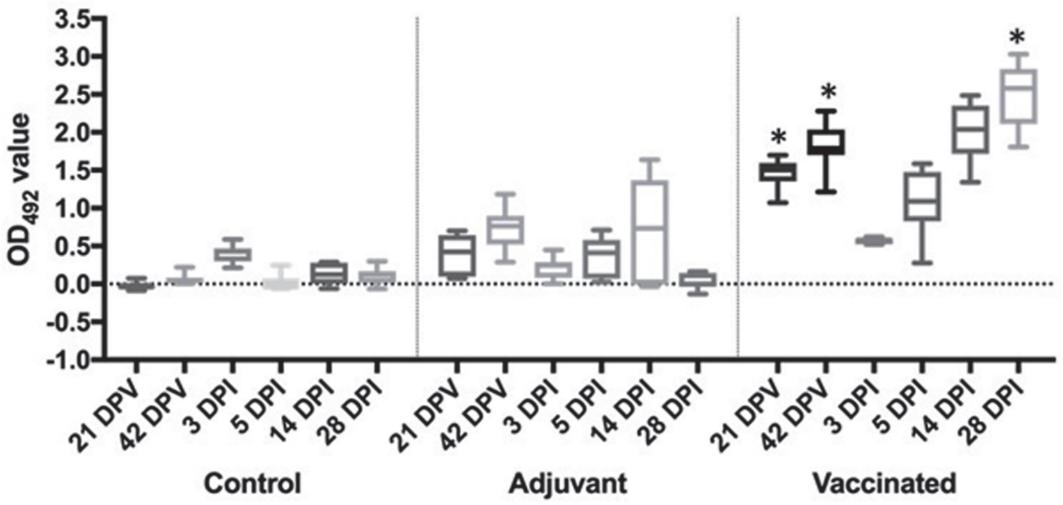

Fig 4. Antibody levels measured by ELISA shown as box-plots over time post vaccination (dpv) and days post challenge (dpc), expressed as OD_{492}. Variations between groups were analysed by Regression analysis with 95% confidence. Note a non-specific increase in the adjuvant group and the drop in antibody levels by 3 dpi in vaccinated fish (and in adjuvant group). Key: *significantly different (at least $p<0.05$).

https://doi.org/10.1371/journal.pone.0230739.g004

determining infectious dosages for bacteria [22]. It is not explained how the dosage of 10^8 was arrived at in the other two studies [19, 20]. The difficulty associated with challenge models is that huge investments both in terms of time and resources are required to optimize them through pathogen characterization, host susceptibility, optimal environmental conditions etc. [23]. Hence alternative, equally effective methods that can rapidly provide estimates for protection and are in line with animal welfare for fish and 3Rs [24, 25] are required.

Immunohistochemistry was employed as an additional method to demonstrate, *in situ*, the presence of *L. garvieae* in different organs in the present study. This method detects both viable bacteria at the time of sampling, and also unviable/inactivated bacteria including antigens in the vaccine used. It is therefore not surprising that *L. garvieae* was demonstrated in the vaccinated group where no corresponding bacteria was re-isolated., consistent with previous reports [26, 27]. The demonstration of bacteria by immunohistochemistry in the present study was more sensitive than bacterial re-isolation. This is in contrast with previous reports where the opposite was reported [28, 29], suggesting that the sensitivity of immunohistochemistry relative to pathogen re-isolation depends on several factors, including the type of pathogen in question. Nevertheless, apart from the vaccinated group where vaccine-associated inactivated antigens were present, estimation of bacterial loads in different tissues by immunohistochemistry and bacterial re-isolation were on average comparable.

In the present study, *L. garvieae* infection establishment in tilapia progressed very rapidly, peaking within 3–5 days. This is consistent with findings of others [18]. The distribution of *L. garvieae* in different tissues (based on the trends and frequency of infected organs over time) as observed from bacterial re-isolation (Table 3) and immunohistochemistry in the adjuvant and control groups in the present study (Table 4) suggests that bacterial localization following internationalization occurs firstly in the liver, kidney and spleen before spreading to the brain. These results are similar to the findings in other studies in rainbow trout following infection by different routes [30], although no chronological order of infection development was suggested in that study.

Nile tilapia immunised with oil-based *L. garvieae* vaccine in the present study produced antibodies significantly higher ($p<0.001$) than the controls or adjuvant only groups by 21 days post vaccination (Fig 4). This trend continued until 3 days post challenge when the antibody titres dropped sharply. The high antibody titres in the vaccinated group at the point of challenge and the absence of bacteria in tissues of this group as demonstrated by lack of bacterial re-isolation (Table 3) suggests that antibodies play a significant role in the protection of the fish against infection. This agrees with the mechanism of action of oil-adjuvanted vaccines and also known protective mechanisms against extracellular pathogens [31, 32]. The drop in antibody titres shortly after challenge is consistent with previous observations in Atlantic salmon [33] which reflected virus neutralization or the "consumption" of antibodies through antibody-antigen complex formation. Interestingly, the antibody titres in the vaccinated group in the present study increased from 5 dpc until the end of the experiment suggesting that following challenge, the bacteria acted as a boost. This was however not the case in the control or adjuvant only groups where no antibodies were detected.

Conclusion

The results of the present study suggests that tilapia can be vaccinated and protected against L. garvieae by using inactivated oil adjuvanted autovaccines. However, because of the low challenge pressure used for challenge and the relatively small number per group of the fish, additional studies are required to confirm with greater certainty the findings of the present study. Bacteria were re-isolated from different organs and also demonstrated by immunohistochemistry while the antibody response was evaluated by elisa. This combination of findings provides an alterntive approach for testing vaccines that does not involve mortality. This is in line with fish welfare and the reduction in fish suffering (3Rs).

Supporting information

S1 Data.
(XLSX)

S2 Data.
(XLSX)

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