Received: 30 October 2020 | Revised: 6 January 2021 | Accepted: 7 January 2021

DOI: 10.1111/jfd.13339

RESEARCH ARTICLE

Journal of Fish Diseases WILEY

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

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Funding information
Direktoratet for Utviklingssamarbeid, Grant/Award Number: TAN-13/0027 and QZA-0485 RAF-19/0018

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.

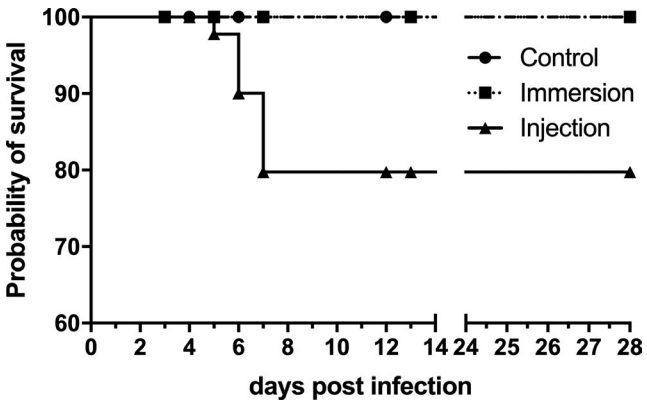

FIGURE 1 Survival plot for injection, immersion and control groups. No mortalities were observed in immersed or control groups, and lines are therefore superimposed. Symbols in the plot show sampling and events (IP injection group) time points

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 bacterial 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.

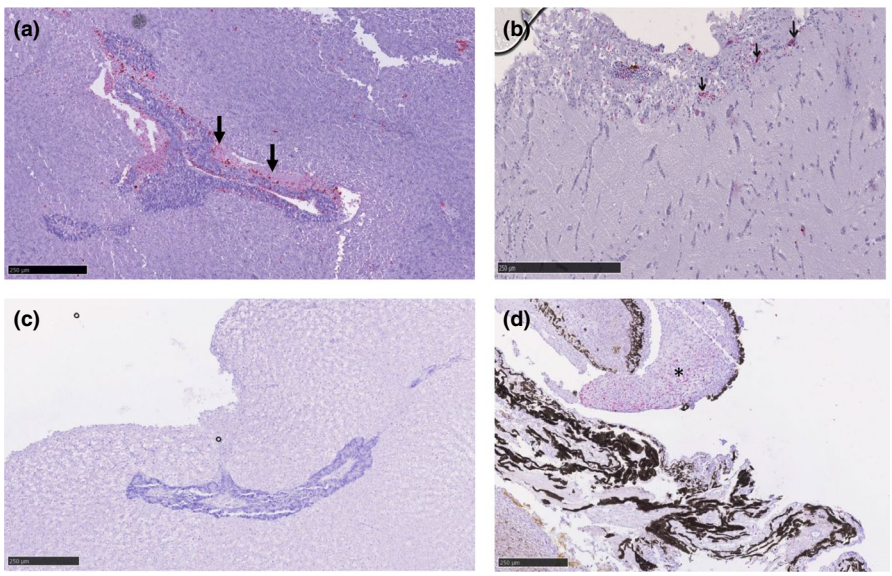

FIGURE 2 Immunohistochemistry staining of *Lactococcus garvieae* in the liver (a), brain (b) and eye (d) in tilapia at 3 days post-intraperitoneal injection. c is liver (uninfected control). Bacterial antigens are observed as red stains (arrows) in or around blood vessels as well as surrounding tissue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

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

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

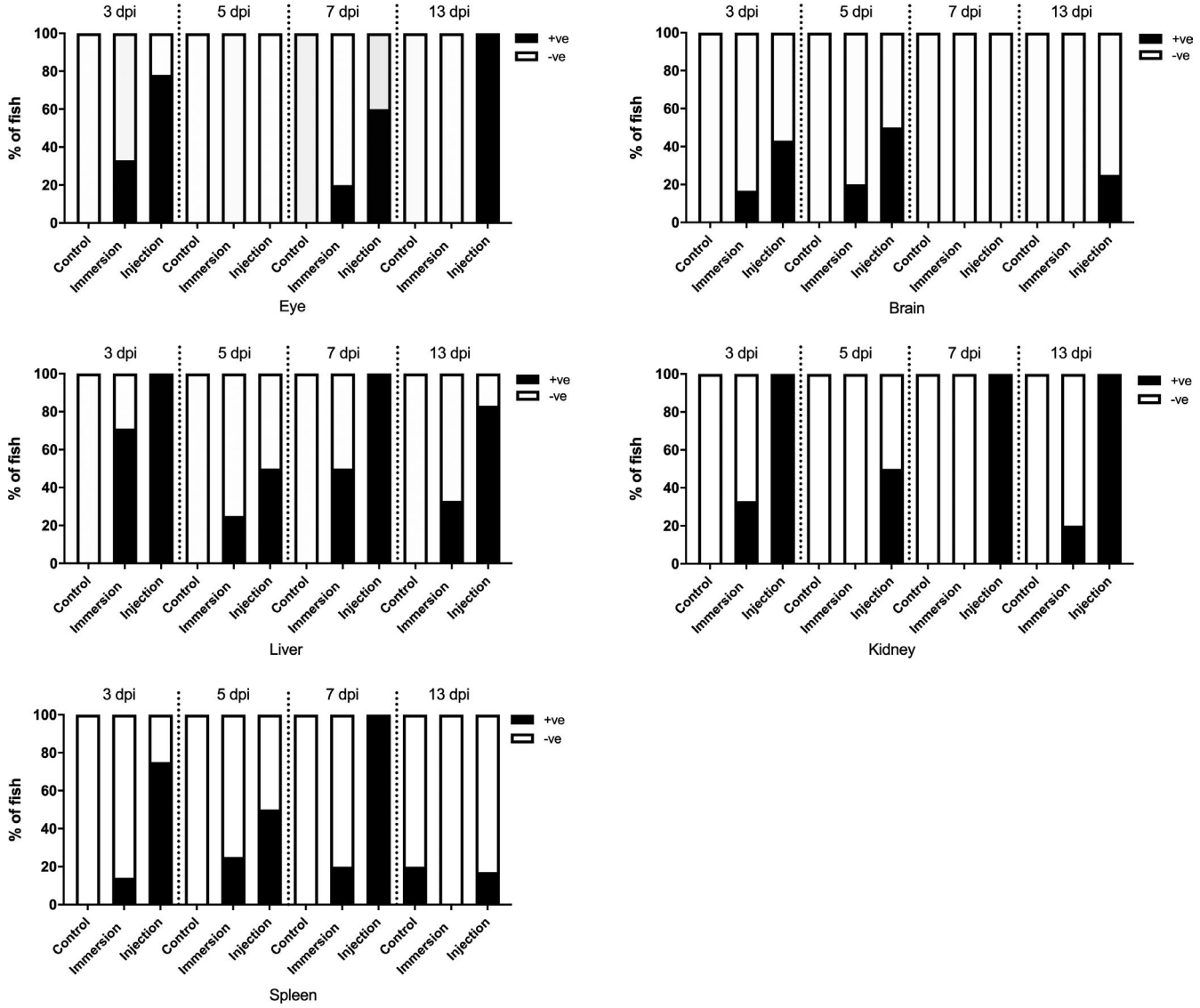

FIGURE 3 Immunohistochemistry staining of different tissues of Nile tilapia against *Lactococcus garvieae*. DPI = days post-infection; n at 3, 5, 7 and 13 is equal to 9, 6, 6 and 6, respectively

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.

ACKNOWLEDGEMENTS

The work was funded by NORAD through the Norhed program and Trahesa project numbers TAN-13/0027 and QZA-0485 RAF-19/0018.

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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How to cite this article: Bwalya P, Hang'ombe BM, Evensen Ø, Mutoloki S. *Lactococcus garvieae* isolated from Lake Kariba (Zambia) has low invasive potential in Nile tilapia (*Oreochromis niloticus*). *J Fish Dis*. 2021;44:721–727. https://doi.org/10.1111/jfd.13339