GENOMIC CHARACTERISATION AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* AND β-LACTAMASE-PRODUCING *ESCHERICHIA COLI* IN NILE PERCH (*LATES NILOTICUS*) IN LAKE VICTORIA-MWANZA, TANZANIA

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EXTENDED ABSTRACT

Nile perch (*Lates niloticus*) is one of the most important fish species in the Lake Victoria region due to its market potential and health benefits. Despite such potentials, reports on detention of exported fillet due to *Salmonella* have been reported, yet not much studied. A cross-sectional study design was employed to investigate the microbial safety of Nile perch and its salted sun-dried products, as well as their contamination with *Salmonella* spp. and *Escherichia coli* producing extended-spectrum beta-lactamases (ESBLs). The specific objectives of the study were to: i) Establish the prevalence and diversity of *Salmonella* serotypes in water and Nile perch from Lake Victoria, Tanzania, ii) Evaluate genetic diversity, antimicrobial resistance and pathogenic potential of *Salmonella* serovars isolated from water and Nile perch, iii) Determine the prevalence of ESBL-producing *E. coli* in water and Nile perch from Lake Victoria, Tanzania, iv) Establish genomic characteristics of ESBL-producing *E. coli* isolates from water and Nile perch; and, v) Assess microbial quality of frozen Nile perch and salted sun-dried products sold in domestic and regional markets.

Collected samples included Nile perch (from fishing grounds, landing sites, and domestic fish markets), lake water, swabs from surfaces of facilities used for fish transportation and salted sun-dried Nile perch products. Standard methods were used for analysis of bacteria, moisture content (MC) and water activity (A_w) in samples. Antimicrobial resistance in *Salmonella* spp. was determined by minimum inhibitory concentration (MIC) and for ESBL-producing *E. coli*, standard disc diffusion method was used. ESBL-producing *E. coli* were screened on MacConkey agar supplemented with 2 µg/mL cefotaxime and were confirmed by polymerase chain reaction (PCR). Fourteen *Salmonella* and 11 ESBL-producing *E. coli* isolates were selected for whole genome sequencing (WGS) using

Illumina MiSeq. The genomic characterisation and phylogenetic relatedness analysis of the isolates were established using WGS.

The study revealed 12 different *Salmonella* serovars, commonly *S. enterica* subsp. *salamae* 42:r:- and *S. waycross*. The prevalence of *S. enterica* subsp. *salamae* 42:r:- was 10% (6/60) in Nile perch from fishing grounds, 1.7% (1/60) from landing sites and 1.7% (1/60) from the markets. The magnitude of contamination in Nile perch from landing sites was significantly lower compared to other sources ($P<0.05$). The prevalence of *S. waycross* in the Nile perch from fishing grounds was 16.7% (10/60), 10% (6/60) at landing sites and 13.3% (8/60) in market settings. Prevalence between sites were comparable ($P>0.05$). Two of 12 ser. 42:r:- and six out of 30 *S. waycross* were resistant to sulfamethoxazole, and one out of 12 ser. 42:r:- and six out of 30 *S. waycross* were resistant to azithromycin. The results were not supported by the detection of gene *aac(6')-laa* encoding for aminoglycoside resistance in the sequenced isolates. Resistance to azithromycin could be associated with unknown chromosomal mutations in base pair in regions of 16S RNA and 23S RNA detected in sequenced isolates. The WGS also revealed ser. 42:r:- and ser. Fulica ST1208, ser. 42:r:- belonged to serogroup T. However, ser. Fulica had an unidentified serogroup. In addition, four *S. waycross* ST2460, one *S. waycross* ST3691, one *S. wien* ST2460 and *S. wien* ST3691 were reported. The *S. waycross* serovars belonged to serogroup S, while *S. wien* had unidentified serogroup. Moreover, *S. waycross* and *S. wien* had pathogenicity islands SPI-2 to SPI-5 with associated virulent genes, but lacked in ser. 42:r:- and ser. Fulica. Plasmid replicon type IncFII was only detected in *S. waycross* ST3691 and *S. wien* ST3691 and was not associated with resistance or virulent genes. Furthermore, serovars of subsp. *salamae* had unique *esp* and *ompT* genes for adaptation in aquatic environment, while *S. waycross* and *S. wien* had a cluster of specific *cit* genes encoding citrate utilisation and *oadAB* for

oxaloacetate decarboxylation in host cells. CSI phylogenetic analysis revealed a ser. 42:r:- clonal relationship to ser. Fulica. *Salmonella waycross* were clonally related to each other, while *S. wien* showed variations (Paper I and Manuscript II for SOBs I and II).

The overall prevalence of ESBL-producing *E. coli* in Nile perch from the Lake Victoria was 4.4% (8/180). The isolates were resistant to sulphamethoxazole-trimethoprim (100%), ampicillin/cloxacillin (100%), erythromycin 72.7% (8/11), tetracycline 90.9% (10/11) and nalidixic acid 63.6% (7/11). The isolates carried resistance genes for sulphonamides (*sul1* and *sul2*), trimethoprim (*dfrA* and *dfrB*), aminoglycosides (*aac(3)-IId*, *strA* and *strB*), tetracycline (*tet(B)* and *tet(D*) and fluoroquinolones (*qepA4*). In addition, the isolates harboured plasmid replicon types IncF, IncX, IncQ and Col and carried $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ genes as well as resistance encoding genes. ESBL-producing *E. coli* isolates formed three separate sequence type-phylogroup-serotype specific clades: C1, C2 and C3. Clade C1 was composed of five isolates (maximum of 13 single nucleotide polymorphisms [SNPs]) belonging to ST167, phylogroup A and serotype O9:H21. Two C2 isolates (max. 11 SNPs) belonged to ST156, phylogroup B1 and serotype (O-untypable) ONT:H28. Clade C3 was comprised of four isolates (max. 17 SNPs) of ST636, phylogroup B2 and serotype O45:H7. The virulence gene *gad* encoding glutamate decarboxylase was found in all isolates. In addition, C2 and C3 isolates harboured the following virurence genes; *iss* encoding for increased serum survival, *lpfA* for long polar fimbriae and *nfaE* encoding for diffuse adherence fimbrillar adhesin. The *vat* gene which play role in vacuolating autotransporter toxin was found only in C3, and was responsible for pathogenicity. A CSI phylogenetic analysis revealed ST167 were clonally related to corresponding public genome of *E. coli* strains obtained from humans, animals and the environment. The same scenario was reported for ST636 and ST156 of *E. coli* against their corresponding sequence types. In each sequence type, isolates showed the same

genotypic resistance and virulence profiles. The present study highlights the occurrence of low virulence, multidrug resistant ESBL-producing *E. coli* in a highly commercialised fish product obtained from Lake Victoria (Manuscript III for SOBs III and IV).

The study also revealed that salted sun-dried Nile perch products were not contaminated with *Salmonella* spp. and ESBL-producing *E. coli*. However, total viable counts (TVCs) of 4.5 log colony forming units (cfu)/g in fish heads with MCs of 38.0% and A_w of 0.682 were reported in products sampled during the rainy season and were significantly higher ($P<0.05$) than the corresponding samples collected during the dry season. The differences attributed by high humidity and rainy condition which lowerd the drying temperature. Fish chests collected during the rainy season had TVCs of 3.3 log cfu/g, MCs of 27.6% and A_w of 0.659, with no significant difference ($P>0.05$) to the values of samples dried during the dry season. Fish belly flaps that were sampled during the rainy season had TVCs of 3.3 log cfu/g at 26.4% MCs and 0.669 A_w, which were comparable ($P>0.05$) to those collected during the dry season. Bacteria identified from TVCs included *Staphylococcus* spp., *Enterobacter* spp., and *Psychrobacter* spp. (Paper IV SOB V).

The current study detected uncommon *Salmonella* serovars rarely associated with human diseases. The serovars are apparently not of public health importance because they are less virulent compared to other serovars frequently isolated from humans and animals. The reported serovars could be normal flora in Nile perch fish. The study also revealed the low prevalence of multidrug resistance ESBL-producing *E. coli* harbouring plasmids carrying β-lactamases and antimicrobial resistance genes. The plasmids and resistance genes reported in ESBL-producing *E. coli* showed no evidence of horizontal gene transfer (HGT) between the isolates. The salted sun-dried Nile perch products were safe for human consumption as the microbial parameters were within the acceptable limit set by the

Tanzanian standards. However, high TVCs and identified contaminated bacteria in dried products highlight the need to implement hygienic procedures during processing to ensure the improved quality and safety of the products for consumers.

DECLARATION

I, ZEBEDAYO BANIGA, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution for a degree award.

_____ _____

Zebedayo Baniga Date

(PhD. Candidate)

The above declaration is confirmed by:

_____ _____

Prof. Robinson H. Mdegela (Sokoine University of Agriculture) Date

(Supervisor)

_____ _____

Prof. Anders Dalsgaard (University of Copenhagen, Denmark) Date

(Supervisor)

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Finally, I am thankful to Almighty God for giving me courage, ability and guidance throughout my study.

DEDICATION

This work is dedicated to my beloved parents Mr. and Mrs Songelaeli Baniga Nkopi, my brothers and sisters, also my beloved wife Zena Ambros Mbilinyi, my sons Eli Zebedayo Baniga, Alfayo Zebedayo Baniga and Reuben Zebedayo Baniga for their invaluable assistance including praying for me throughout my study to completion of this work.

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DECLARATION

I, ZEBEDAYO BANIGA, do hereby declare to the Senate of Sokoine University of Agriculture that the listed papers above that make this thesis summarise my independent work efforts, it is my own original work and will not be part of another thesis in the "Published Paper" format in any other institution.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	American Type Culture Collection
A_w	Water activity
cgMLST	Core Genome Multi Locus Sequence Typing
CLSI	Clinical and Laboratory Standard Institute
CTX-M	Cefotaximase-Munchen
DANIDA	Danish International Development Agency
DNA	Deoxyribonucleic acids
ESBLs	Extended Spectrum Beta-Lactamases
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
HGT	Horizontal Gene Transfer
ISO	International Organization for Standardization
kb	kilobase
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight
MC	Moisture content
MIC	Minimum Inhibitory Concentration
MLST	Mult Locus Sequence Type
MS	Mass Spectrometry
OXA	Oxacillin
PCR	Polymerase Chain Reaction
QMS	Quality Management System

RASFF	Rapid Alert System for Foods and Feeds
RFLPs	Restriction Fragment Length Polymorphisms
SHV	Sulphydryl Variable
SISTR	*Salmonella In Silico* Typing Resource
SNPs	Single Nucleotide Polymorphisms
SPI	*Salmonela* Pathogenicity Island
SUA	Sokoine University of Agriculture
T3SS	Type Three Secretion System
T4SS	Type Four Secretion System
TEM	Temoneira
TZS	Tanzanian Standards
UK	United Kingdom
URT	United Republic of Tanzania
USA	United States of America
USFDA	Uinted States Food and Drug Administration
VBNC	Viable But Non-Culturable
WGS	Whole Genome Sequencing
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION AND BACKGROUND INFORMATION

1.1 Lake Vicroria: An overview

Lake Victoria is the largest tropical lake in the world. The lake occupies a surface area of approximately 68 800 km^2 and a catchment area of 229 800 km^2. It is the world's second largest fresh-water lake by surface area after Lake Superior in North America. The lake is shared by three riparian countries. Tanzania occupies the largest portion (approximately 51%), followed by Uganda (43%) and Kenya (6%). It is an important natural resource because it contributes to the national economy through fisheries activities. The productivity of Lake Victoria in terms of fishing, accounts for about 63% of total catch compared to other freshwater lakes in Tanzania (URT, 2013). Nile perch (*Lates niloticus*) is the most valuable and commercially leading fish species. *Lates niloticus* contribute to over 80% of the export market earning by value (URT, 2013).

1.1.1 Nile perch fishers and value chain

There are two types of Nile perch fishers in Lake Victoria. Low capital artisanal fishers mainly supply the domestic and regional markets, and middle income fishers mainly supply export markets, predominantly European countries (Kyangwa and Odongkara, 2005; Kirema-Mukasa, 2012). Nile perch value chain as described in this study is the chain through which the fish are conveyed from the point of capture to the marketplace as whole fish and/or fish products. The Nile perch value chain of Lake Victoria is conveyed into three branches of the chain (Appendix 1), according to the quality of fish, method of fishing and market demand. The first branch involves the high-quality Nile perch fish obtained for fish industries. These fish are processed mainly into fillets for the export market. The second branch involves Nile perch fish obtained mainly from artisanal fishers

who usually sell their fish at domestic fish markets for local consumers. In most cases, these fish are average and sometimes low quality (Kirema-Mukasa, 2012). The third branch involves traditionally processed fish and fish products, e.g. salted sun-dried products such as fish heads, chests and belly flaps, although other products like smoked fish are also processed. These are mainly for the regional market (Kyangwa and Odongkara, 2005). Raw materials used for processing salted sun-dried Nile perch bi-products (e.g. heads, chests and belly flaps) are from fish processing industries after other products have been obtained from fish, such as fillets and fish maws.

1.1.2 Nile perch handling and faecal bacterial alert notification

Nile perch processed for export markets are usually handled and transported in a cold chain system following the Quality Management System (QMS), from the point of capture to the fish processing industries (Kirema-Mukasa, 2012). The QMS includes Hazard Analysis Critical Control Point and other quality practices, such as Good Manufacturing Practices, and Good Hygiene Practices. Although the QMS for the Nile perch that are processed for export market is well established, exported fillets are occasionally contaminated by faecal bacteria (RASFF, 2017). The European Union(EU) food notification system reported *Salmonella* spp. and/or *Enterobacteriaceae* contaminated Nile perch fillets from Tanzania in 2003, 2004, 2009, 2014 and 2016 (RASFF, 2017). Although the alerts by the EU were insignificant as compared to the volume of fillets being exported, the presence of faecal bacteria may still indicate unhygienic fish handling practices along the value chain. Also, *Salmonella* spp. and *Enterobacteriaceae* suggest faecal pollution of the lake that may be associated with wastes from anthropogenic activities (David *et al.,* 2009; Mdegela *et al.,* 2015). The fish in domestic markets usually are displayed on open selling tables that can be prone to contamination. In addition, some fish vendors use beach sand to cover fish at the markets believing that the sand reduces

fish spoilage (Fig.1). This could be a potential source of the contamination of fish with faecal microbial pathogens, including *Salmonella* spp. and *Escherichia coli*.

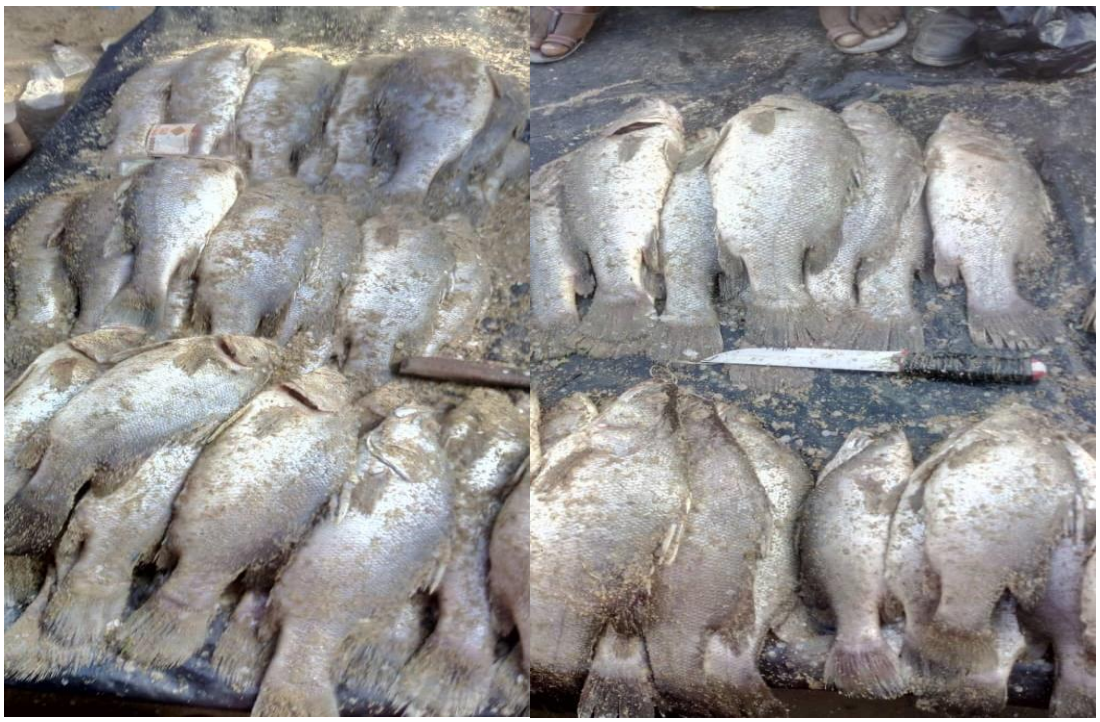

Figure 1: Nile perch displayed at domestic fish market

1.1.3 Lake Victoria pollution

Lake Victoria is prone to pollution from different pollutants due to anthropogenic activities, including discharge of sewage or effluent from households and hospitals that contain wastes from human and animal sources (David *et al.,* 2009; Mdegela *et al.,* 2015). The wastes may contain faecal bacteria, such as *Salmonella* spp., *E. coli* and other faecal associated pathogens of public health concern that may contaminate fish in their natural environment. The wastes may also contain antimicrobial residues that are important for the emergence of antimicrobial resistant bacteria and other pathogens in the aquatic environment. The presence of *Salmonella* spp. in the aquatic environment is not an absolute factor indicating faecal pollution. Several recent studies described that some *Salmonella* serovars, including *S. weltevreden*, can occur naturally in the aquatic

environment (Uddin *et al.,* 2015; Yang *et al.,* 2015; Li *et al.,* 2017; Liu *et al.,* 2018). Therefore, the contamination of Nile perch by *Salmonella* spp. may occur naturally in the aquatic environment since the bacteria may be present in water (Mdegela *et al.,* 2015; Uddin *et al.,* 2015).

1.2 Taxonomy, host range and reservoirs of *Salmonella*

1.2.1 History of *Salmonella* and taxonomy

Salmonella is a genus of rod-shaped, non-spore forming, facultatively anaerobic Gram-negative bacteria of the family *Enterobacteriaceae* (Kornacki, 2010). With only a few exceptions, they are motile and possess flagella (Acha *et al.,* 2006; Kornacki, 2010). *Salmonella* was first isolated by Theobald Smith in 1855 from the intestines of pigs infected with classical swine fever (Eng *et al.,* 2015). As described by Lamas *et al.* (2018), the genus *Salmonella* was named after Daniel Elmer Salmon, an American veterinary pathologist who discovered the pathogen while searching for the cause of common hog cholera (Salmon and Smith, 1886). *Salmonella* was subsequently classified according to the evolutionary changes (Ellermeier and Slauch, 2006). The genus *Salmonella* was later classified into two species, *S. enterica* and *S. bongori*. *Salmonella enterica* was further divided into six subspecies; *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI), being differentiated from each other based on biochemical reactions (Acha *et al.,* 2006; Ellermeier and Slauch, 2006; Tomastikova *et al.,* 2017) (Table 1.1).

Table 1: Differential characters of *Salmonella* species and subspecies

Character	*S.* enterica						*Salmonella bongori*
	Subsp. *enterica*	Subsp. *salamae*	Subsp. *arizonae*	Subsp. *diarizonae*	Subsp. *houtenae*	Subsp. *indica*	
Dulcitol	+	+	-	-	-	d	+
OPNG (2h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Culture with KCN	-	-	-	-	+	-	+
L(+)-tatrate	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
γ-Glutamyltransferase	+	+	-	+	+	+	+
β-Glucuronidase	-	D	-	+	-	d	-
Mucate	+	+	+	- (70%)	-	+	+
Salicin	-	-	-	-	+	-	-
Lactose	-	-	- (75 %)	+ (75%)	-	d	-
Lysis by phase 01	+	+	-	+	-	+	d
Natural habit	Warm blooded animals		Cold-blooded animals and the environment				

OPNG: ơ-nitropenyl-β-D-galactopyranoside; KCN, potassium cyanide; d, different reactions given by different serovars.

Source: (Bopp *et al.*, 1999; Jones *et al.*, 2000; Grimont and Weill, 2007; Abulreesh, 2012).

1.2.2 Hosts of *Salmonella* subspecies and reservoirs

Salmonella enterica subspecies e*nterica* are mainly responsible for over 95% of human infections associated with contaminated foods (Wiedmann and Zhang, 2011; Waldner *et al.*, 2012). Some *Salmonella* serovars in the subspecies *enterica* are host specific. These include *S. typhi* and *S. paratyphi* A and B, which are restricted to humans and are responsible for typhoid and paratyphoid fever; *S. pullorum* and *S. gallinarum*, which are specific to poultry and other serovars highly adapted to animals, such as *S. dublin* for bovines, *S. choleraesuis* and *S. typhisuis* for swine. All are responsible for animal paratyphoid (Tessari *et al.*, 2012; Waldner *et al.*, 2012; Ashton *et al.*, 2016). Other *Salmonella* serovars in subspecies *enterica* are hosts generalist and are mainly non-

typhoid. These include *S. typhimurium* and *S. enteritidis*, which are mostly involved in gastroenteritis and salmonellosis in humans and animals (Tessari *et al.,* 2012; Ashton *et al.,* 2016). Subspecies II to VI rarely cause infections in humans and their distributions differ significantly from other serovars in subspecies *enterica* (Brenner *et al.,* 2000; Tomastikova *et al.,* 2017). *Salmonella enterica* species are widely distributed as their serovars have been isolated from warm- and cold-blooded animals, soil, water and different environments, including aquatic environments with the main hosts being warm- and cold-blooded animals (Acha *et al.,* 2006; Wiedmann and Zhang, 2011; Zhou *et al.,* 2017). Aquatic environments and soil are also among the main reservoirs of *Salmonella* spp.

1.2.3 Naming of *Salmonella* serovars in subspecies

Naming of different serovars in the genus *Salmonella* was initially problematic and very confusing due to a number of serovars found in different subspecies (Agbaje *et al.,* 2011; Lamas *et al.,* 2018). Prior to 1966, all serovars in all subspecies except subspecies IIIa and IIIb were assigned names. In 1966, the World Health Organisation (WHO) Collaborating Centre began designating serovars by names only in subspecies I and dropped all by then existing serovar names in subspecies II, IV and VI and *S. bongori*, during the implementation of the harmonisation of the White-Kauffmann-Le Minor (WKL) scheme (Kelterborn, 1967; Brenner *et al.,* 2000; Agbaje *et al.,* 2011). Currently, all serovars in *S. enterica* subspecies, except *enterica,* are named based on their antigenic formula (Grimont and Weill, 2007; Nair *et al.,* 2014). Serovars of *S. enterica* subspecies *salamae* have consistently and commonly been found in cold-blooded animals, such as reptiles and amphibians, although they have also occasionally been found in warm-blooded animals like poultry (broilers), other birds, in environments, and seldom in humans as previously reported (Chandry *et al.,* 2012; Nair *et al.,* 2014; Sathyabama *et al.,* 2014; Krawiec *et al.,*

2015). Members of subspecies *salamae* are rarely reported to cause infections in humans and animals (Chandry *et al.,* 2012; Lamas *et al.,* 2018).

1.2.4 *Salmonella enterica* subspecies *salamae*

Salmonella enterica subspecies *salamae* (II) is one among the six subspecies of *enterica* species. Currently, there are approximately 505 reported serovars in subspecies *salamae*, which include serovar 42:r:-. This serovar was initially described as serovar Nairobi (Kelterborn, 1967; Nair *et al.,* 2014). Previously, serovar 42:r:- was reported in Nile perch fillets from Tanzania. The serovars were isolated by the United State Food and Drug Administration (USFDA) as part of a surveillance project for the rapid detection of outbreaks of food-borne illnesses under the Project PRJNA186035 (Wang *et al.,* 2015). In addition, serovar 42:r:- was also frequently reported from cold-blooded animals and other samples in Albania, Pakistan and the USA collected in the same project. In 2018, serovar 42:r:- was isolated from retail meat in Rwanda (Byukusenge *et al.,* 2019). This and other findings suggested that serovar 42:r:- can colonise different ecological niches, which include cold- and warm-blooded animals. Other serovars that are members of the subspecies *salamae* were previously reported from mouse stool in India (Sathyabama *et al.,* 2014), cold-blooded animals (e.g. reptiles, snakes and tortoises) in the USA (Desai *et al.,* 2013), poultry in Australia (Duffy *et al.,* 2012), and were also reported from humans in England and Wales (Nair *et al.,* 2014). Another serovar in subspecies *salamae* reported by the USFDA from different samples, including almond butter, is serovar Fulica. This serovar has also been rarely reported (Wang *et al.,* 2015).

1.2.5 *Salmonella enterica* subspecies *enterica*: *Salmonella waycross*

Salmonella enterica subspecies *enterica* constitutes the largest group of infectious strains of *Salmonella* (Acha *et al.,* 2006). Subspecies *enterica* has more than 1600 serovars and

consists of both host-specific and non-host specific *Salmonella* serovars of public health importance (Tessari *et al.,* 2012; Ashton *et al.,* 2016). Among the serovars in subspecies *enterica*, non-typhoid *Salmonella* are commonly isolated in different food chains, such as poultry, meat, pork and occasionally seafood. Currently there are evidences suggest that some serovars are naturally found in aquatic environments such as *S. weltevreden* reported in Vietnam and China (Uddin *et al.,* 2015; Li *et al.,* 2017). Other serovars in subspecies *enterica*, such as *S. waycross* and *S. wien*, have also been rarely reported to cause infections in humans. This does not imply these serovars are not pathogenic. Rather, their isolation in humans and food samples may not be frequently reported. *Salmonella waycross* was initially described by Seligmann and Saphra (1948) in the USA. Since its discovery, the serovar has been occasionally isolated from different foods, especially those of animal origin, such as poultry, beef and farmed animals and rarely from humans (Haddock *et al.,* 1991; Al-Nakhli *et al.,* 1999; Halatsi *et al.,* 2006; Stevens *et al.,* 2008; Kagambèga *et al.,* 2013). *Salmonella waycross* has also been isolated from Nile tilapia (*Oreochromis niloticus*) and water reservoirs in Burkina Faso (Traoré *et al.,* 2015).

1.2.6 *Salmonella* pathogenicity

Salmonella pathogenesis is defined as the ability of *Salmonella* to colonise hosts through virulence factors/genes located in plasmids or via different genes clusters located in bacterial chromosome, in the region called *Salmonella* Pathogenicity Islands (SPIs) (Fabrega and Vila, 2013; Pollard *et al.,* 2016). SPIs are commonly acquired by *Salmonella* during the evolutionary processes. There are five principal SPIs associated with pathogenicity processes, and the most studied being SPI-1 and SPI-2 (Fabrega and Vila, 2013). Two systems are located within SPIs. They are type three secretion system (T3SS) and type four secretion system (T4SS) which are normally found in *Salmonella* serovars, especially in subspecies *enterica*. In order for *Salmonella* to express virulence genes, both

the two T3SS and T4SS must be involved. These are the major factors in *Salmonella* and usually play an important role in the manipulation of the host cell physiology to cause disease (Silva *et al.,* 2017). One T3SS is encoded in SPI-1 and another in SPI-2. These are required in different stages of salmonellosis, as well as T4SS, which is located in SPI-6 and is usually responsible for macrophage survival and cell invasion (Gan *et al.,* 2010; Pollard *et al.,* 2016; Byndloss *et al.,* 2017). The key factors for the divergence of *Salmonella* subspecies and serovars are gains/acquisitions/insertions or deletions/losses of different gene clusters from SPIs, which occurred through the evolutionary process of the genus *Salmonella*. For example, the divergence of *S. enterica* subspecies *salamae* from other subspecies was due to the gain of the locus of enterocyte effacement (LEE) and the loss of SPI-5 (Chandry *et al.,* 2012; Desai *et al.,* 2013; Bale *et al.,* 2016). Different virulence genes that are carried or absent in *Salmonella* genomes determines the intensity of infectivity of one serovar over the other. Some virulence genes, including *spvB* and *pefA*, are serovar-specific. This means that they are usually found in *S. typhimurium* and *S. enteritidis*, while other genes are specific to *S. typhi* (e.g., *tcfABD* and *stgABD*) and *S. paratyphi* (e.g., *staA*) (Rotger and Casadesús, 1999; Skyberg *et al.,* 2006).

1.2.7 Plasmids and antimicrobial resistance genes in *Enterobacteriaceae*

Plasmids are small, circular double-stranded deoxyribonucleic acid (DNA) molecules that are extra-chromosomal and replicate independently within a host cell (Schleif, 1993; Allison, 2007). They often carry antimicrobial resistance and virulence-encoding genes that benefit the survival of an organism, especially under stressful conditions (Allison, 2007; Wiedmann and Zhang, 2011). Plasmids are classified into five categories based on functions they perform. These include resistant plasmids, which contain genes that provide resistance against antimicrobial compounds or poisons, and virulence plasmids that are responsible for infections (i.e., they convert non-pathogenic bacteria into pathogenic).

These plasmids are characterised by a high molecular weight that exceed 20 kb (Krzyzanowski *et al.,* 2014). Other plasmids, which are termed F-plasmids, contain *tra* genes for fertility. They are capable of conjugating and result in the expression of sex pilli. Degradative plasmids are another group of plasmids that enable digestion of unusual substances, such as toluene and salicyclic acid. The last group of plasmids, *col* plasmids which contain genes encoding bacteriocin, a protein that kills other bacteria (Wiedmann and Zhang, 2011). *Salmonella* spp. with or without plasmids cab be resistant to two or more antimicrobials. Thus, the presence of plasmids is not a mandatory for the occurrence of resistance in bacteria because such resistance genes can be carried in chromosomes (Singh *et al.,* 2010; Krzyzanowski *et al.,* 2014; Thung *et al.,* 2018). The plasmids can transfer their genes from one bacterium to another of the same or different species or strain in the process of horizontal gene transfer (HGT) through three mechanisms: transformation, transduction and conjugation (Schleif, 1993; Allison, 2007). Resistance and/or virulence genes in different plasmids are carried in *Enterobacteriaceae*, including non-typhoid *S. typhimurium*, *S. enteritidis* and *S. dublin* (Rychlik *et al.,* 2006; Sathyabama *et al.,* 2014; Silva *et al.,* 2017; Kagambèga *et al.,* 2018), but have rarely been reported in *S. waycross*, *S. wien*, serovar Nairobi/42:r:- as well as serovar Fulica of subspecies *salamae*. The HGT also were reported in *E. coli* isolates.

1.2.8 *Salmonella* in aquatic environments and their ecology

Salmonella spp. are the main food-borne pathogens responsible for salmonellosis in humans (Carrasco *et al.,* 2012; Abakpa *et al.,* 2015). Sources of salmonellosis in humans are contaminated foods, such as pork, poultry meat, eggs and rarely seafood (Antunes *et al.,* 2016; Fagbamila *et al.,* 2017). *Salmonella* in seafood, such as Nile perch, can occur either naturally due to bacteria present in the aquatic environment or due to subsequent poor fish handling along the value chain. There is increasing evidence to suggest that

Salmonella serovars like *S. weltevreden* can occur naturally in the aquatic environment as reported in Vietnam and China (Uddin *et al.,* 2015; Li *et al.,* 2017). These studies reported isolation of *Salmonella* serovars in fish and the aquatic environment with no link or association with faecal pollution. However, other serovars, including *S. typhimurium* and *S. enteritidis*, are directly associated with faecal pollution, and in most cases are isolated together with *E. coli*. *Escherichia coli* is typically used as an indicator of faecal pollution and faecal-related pathogens, such as non-typhoid *S. typhimurium* (Romich, 2008). The main sources of faecal pollution in aquatic environment include sewage, animal, human and agricultural wastes. The wastes may contain pathogens and antimicrobial residues resulting into cross-contamination of fish in its natural environment (David *et al.,* 2009; Kornacki, 2010; Onyuka *et al.,* 2011). Untreated household wastes deposited in the lake contribute significantly to the faecal pollution, which in turn serves as a reservoir for pathogens like *Salmonella* in fish (Prasad *et al.,* 2015). In addition, agricultural activities close to the lake are a source of faecal pollution in the lake because farmers apply animal manure to crop fields, which inturn get to the lake through rain water runoff (David *et al.,* 2009; Mdegela *et al.,* 2015). The persistence and survival of *Salmonella* in aquatic environments as well as its growth in water have been documented (Winfield and Groisman, 2003; Liu *et al.,* 2018). The survival of *Salmonella* in the aquatic environment was reported to be due to the ability of bacteria to exist in a viable but non-culturable (VBNC) state for a long time in water, as well as to live symbiotically within free living protozoa found in aquatic environment (Liu *et al.* (2018). The VBNC state is a state where the bacterial cells cannot be recovered with routine growth media, but are capable of growth when conditions become favourable (Roszak *et al.,* 1984; Oliver, 2005). Therefore, *Salmonella* spp. uses VBNC as a survival strategy to exist in harsh environments, include aquatic systems (Winfield and Groisman, 2003; Liu *et al.,* 2018). When bacteria enter another organism, such as fish, they can continue surviving and

replicate normally. Some *Salmonella* genes have been identified to play an important role in colonising aquatic plant surfaces and tissues to facilitate interaction of the bacteria with other microbial community in plants. These events can enhance survival of the bacteria in aquatic environments (Brandl *et al.,* 2013).

1.2.9 Detection of *Salmonella* species

There are different methods used for the detection of *Salmonella* spp. in food and water samples. These methods include biochemical and molecular techniques. After detection, *Salmonella* are serotyped using biochemical and genotyping approaches to identify different serovars that might be present in contaminated food and water samples.

1.2.9.1 *Salmonella* screening and biochemical confirmation

Salmonella spp. in food and water are cultured on Xylose Lysine Deoxycholate (XLD) agar and Bismuth Sulphate agar (BSA) after pre-enrichment in Buffered Peptone Water (BPW) at 37°C for 24 h, and enrichment in Rappaport Vassiliadis broth and Mueller-Kauffman Tetrathionate-novobiocin at 37°C for 24 h. XLD and BSA are selective media recommended by ISO 6579:2002/Amd.1: (2007) for screening of *Salmonella* spp. XLD is recommended as the primary selective medium and BSA as the secondary selective medium. Suspected *Salmonella* colonies that develop on XLD agar are pink with or without black centres. The colonies on BSA produce a characteristic brown to black colour with a metallic sheen. Suspect colonies from XLD and BSA are purified on nutrient agar medium, and biochemically confirmed on Triple Sugar Iron (TSI) agar slant with colour appearance (Fig. 2a) and subsequent agglutination in polyvalent *Salmonella* O- and H-antisera (Fig. 2b).

Fig.2a

Fig.2b

Figure 2: Appearance of *Salmonella* spp. in TSI and slide agglutination

1.2.9.2 *Salmonella* serotyping method

Serotyping is done according to the xMAP® *Salmonella* Serotyping Assay (Luminex, USA), which is a traditional serotyping based on standard method (ISO/TR 6579-3:2014). Results are interpreted according to the WKL scheme (Grimont and Weill, 2007). In addition, serotype prediction is done based on molecular technique using whole genome sequencing (WGS) based on the *Salmonella In Silico* Typing Resource (SISTR) tool. The method is sensitive compared to traditional serotyping based on WKL scheme approach, and it provides accurate results in a short time.

1.2.9.3 Confirmation of *Salmonella* using molecular techniques

Confirmation of *Salmonella* spp. using molecular technique provides accurate results in short period of time, although the method is relatively expensive. The method involves the use of PCR with specific set of primer sequences that target *Salmonella* spp. genes, e.g. ST11-ST15 primers sequence.

1.3 General antimicrobial resistance in *Enterobacteriaceae*

Most food-borne pathogens of public health importance belong to the family *Enterobacteriaceae*. They are Gram-negative intestinal flora that are responsible for animal and human infections. Members of *Enterobacteriaceae* worldwide have been reported to be resistant to different classes of antimicrobials, including aminoglycosides, sulphonamides, macrolides, fluoroquinolones and tetracycline (WHO, 2014; FSAI, 2015; Tadesse *et al.,* 2017). Resistance genes are mostly carried in bacterial plasmids, which enable them to be transmitted to different organisms, including humans and animals, through the HGT process. Members of *Enterobacteriaceae* include *Salmonella* spp. and *E. coli*, which are frequently associated with multidrug resistance and are responsible for different infections in animals and humans (Romich, 2008). The increase and dissemination of the antimicrobial resistance is associated with contaminated foods as well as different environments, including aquatic environments (WHO, 2014). Misuse and improper disposal of antibiotics, especially in developing countries like Tanzania, play a significant role in the occurrence of antimicrobial resistance (Okeke *et al.,* 2005; Sosa *et al.,* 2010; Tadesse *et al.,* 2017). In addition, the aquatic environment is reportedly a potential reservoir for antimicrobial resistance and may play an important role in dissemination and transfer of resistance genes to human via water and seafood (Egea *et al.,* 2012; Jiang *et al.,* 2012; Moremi *et al.,* 2016; Tekiner and Özpınar, 2016; Dib *et al.,* 2018).

1.4 *Escherichia coli* and ESBL-producing *Escherichia coli*

1.4.1 *Escherichia coli* and classification

Escherichia coli belongs to the family *Enterobacteriaceae*, which constitute part of normal intestinal flora of warm-blooded animals, including humans. They are Gram-negative, motile or nonmotile, facultatively anaerobic bacilli (Acha *et al.,* 2006). The

bacteria comprised both opportunistic commensal and pathogenic strains, which have ability to cause infection to humans and animals (Lupindu *et al.*, 2014; Madoshi *et al.*, 2016). The common infections caused by *E. coli* include both intestinal and extra-intestinal infections (Acha *et al.*, 2006; Lupindu *et al.*, 2014). Based on the nature of the infections, *E. coli* are classified into two main groups: extra-intestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (IPEC). The latter is subdivided into five variants: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) (Acha *et al.*, 2006; Köhler and Dobrindt, 2011; Donnenberg, 2013; Morabito, 2014). Other pathogenic strains are verotoxigenic *E. coli* (VREC) and avian pathogenic *E. coli* (APEC) (Iguchi *et al.*, 2009). Commensal *E. coli* are normally found as normal flora of intestines of all warm-blooded animals and exist as part of the indigenous flora performing vital roles like metabolism in animals (Donnenberg, 2013). Both pathogenic and commensal *E. coli* are grouped into four phylogroups (A, B1, B2, and D). All these phylogroups are able to cause intestinal and/or extra-intestinal infections (Carlos *et al.*, 2010; Donnenberg, 2013; Chakraborty *et al.*, 2015). Commensal *E. coli* have been described more as opportunistic, rarely cause infections and belong to phylogroups A and B1; ExPEC strains are mostly in phylogroups B2 and D (Clermont *et al.*, 2000; Derakhshandeh *et al.*, 2014). Some commensal and pathogenic strains of *E. coli* from all phylogroups have been reported to produce ESBLs (Branger *et al.*, 2005; Pallecchi *et al.*, 2007; Mshana, 2011).

1.4.2 *Escherichia coli* in aquatic environment and its ecology

Escherichia coli multiply in human and animals as its primary habitat, but it has a net negative rate of growth in the secondary habitat (aquatic environment), with half-lives of approximately 1 day in water, 1.5 days in sediment and 3 days in soil (Miyanaga *et al.*,

2006; Berthe *et al.,* 2013). This implies that *E. coli* does not live longer in non-host environments like water and, therefore, its presence in such an aquatic environment is due to the continuous excretion of waste from human and animal hosts. This is the reason for the use of *E. coli* as an indicator organism for recent faecal contamination in the environment like water (Miyanaga *et al.,* 2006). *Escherichia coli* rapidly (<2 to 14 days) lose their ability to grow in culture more frequently in secondary habitats (Miyanaga *et al.,* 2006; Berthe *et al.,* 2013). The persistence of *E. coli* in water from 4 to 14 days more often indicates that the water and sediment contain low levels of faecal pollution (Berthe *et al.,* 2013). Like *Salmonella* spp., *E. coli* can persist in aquatic environments in association with other biotic (algae and plants) or abiotic features that promote the survival of *E. coli* (Winfield and Groisman, 2003; Lyautey *et al.,* 2010; Berthe *et al.,* 2013). It has been hypothesised that once *E. coli* are released into aquatic environment from warm-blooded animals, the environment exerts selective pressure on bacterial population, leading to most of bacteria die within short period of time. The few surviving bacteria are able to persist due to their adaptation to the aquatic environment (Winfield and Groisman, 2003; Lyautey *et al.,* 2010).

1.4.3 *Escherichia coli* and resistance to β-lactams

Like other members of *Enterobacteriaceae*, *E. coli* can develop resistance to different antimicrobials, including first-, second- and third-generation cephalosporins. This resistance is attributed to the presence of bacterial genes encoding β-lactamase. This enzyme hydrolyses and inactivates β-lactam antibiotics (Pitout and Laupland, 2008; Shaikh *et al.,* 2015). The bacterial resistance can occur due to mutations in the genes coding for antibiotics as a result of changing the target specificity or the normal functions (Shaikh *et al.,* 2015; Munita and Arias, 2016). Resistance can also occur by alternate metabolic pathways, which by-pass the paths inhibited by antibiotics. Finally, resistance

can also be attributed to alterations of cell membrane permeability, which result in the active transport of antibiotics out of bacteria by efflux pumps (Munita and Arias, 2016). Therefore, the antimicrobial resistance in Gram-negative bacteria like *E. coli* can occur naturally, or can be acquired and often are composed of a combination of resistance mechanisms like β-lactamases, porin deletions and efflux pumps (Nikaido, 2009; Fernández and Hancock, 2012; Munita and Arias, 2016). Of all resistance mechanisms in Gram-negative bacteria, hydrolysis of β-lactam antibiotics by β-lactamases is the predominant mechanism (Sáenz *et al.,* 2004; Nikaido, 2009; Ruppé *et al.,* 2015). The ability of *E. coli* to produce β-lactamase enzyme is mainly due to genes that are acquired through plasmids. Plasmids that carry genes encoding resistance to cephalosporins including ceftazidime and ceftriazole contribute to the occurrence of multidrug resistance in pathogens (Pallecchi *et al.,* 2007; Pitout and Laupland, 2008; Shaikh *et al.,* 2015). These observations imply that plasmids can transfer the resistance genes among and between bacterial strains, and finally to humans and animals through HGT (Rossolini *et al.,* 2008). It is possible that the same types of ESBLs are present in clonally unrelated bacterial isolates or that different ESBLs have the same origin (Rossolini *et al.,* 2008; Rossi *et al.,* 2017). Different studies have documented *E. coli* as one of the organisms, which frequently harbours genes encoding various ESBLs, and cause a threat to the medical community in terms of treatment of enteric diseases (Pallecchi *et al.,* 2007; Rossolini *et al.,* 2008; Shaikh *et al.,* 2015).

1.4.4 Extended Spectrum Beta-Lactamases (ESBLs) and classification

Currently, there is no consensus on a precise definition of ESBLs. The commonly applied definition is that ESBLs are β-lactamases capable of conferring bacterial resistance to penicillin first-, second-, and third-generation cephalosporins and aztreonam (but not

cephamycins or carbapenems) by hydrolysing antibiotics inhibited by β-lactamase inhibitors, commonly clavulanic acid (Paterson and Bonomo, 2005; Rawat and Nair, 2010; Ghafourian *et al.*, 2015; Shaikh *et al.*, 2015; Sanjit *et al.*, 2017). It is known that β-lactams are the most frequently used class of antibiotics characterised by possession of a β-lactam ring as the chemical base (Rawat and Nair, 2010; Fernandes *et al.*, 2013; He *et al.*, 2014). The common mechanism for resistance toward antibiotic is the hydrolysis of β-lactams by opening its ring using β-lactamase enzymes carried in pathogens (Fernandes *et al.*, 2013). The β-lactamases are classified following the two schemes which are the Ambler scheme based on molecular classification and the Bush-Jacoby-Medeiros scheme according to functional classification system (Hall and Barlow, 2005; Mshana, 2011). The Ambler scheme classifies β-lactamases into four classes based on their amino acid sequence similarities and not on phenotypic characteristics. The four classes are A, C, and D enzymes, which utilise serine for β-lactam hydrolysis, and class B metallo-enzymes which require divalent zinc ions for substrate hydrolysis (Hall and Barlow, 2005; Wang *et al.*, 2013). By definition, a serine is an alpha (α)-amino acid used in the biosynthesis of proteins and contains an α-amino group, carboxyl group, and a side chain consisting of a hydroxymethyl group classified as a polar amino acid (Wang *et al.*, 2013). The Bush-Jacoby Medeiros scheme groups β-lactamase enzymes according to functional similarities, i.e. substrate and inhibitor profile group 1 to 4 (Bush and Jacoby, 2010).

1.4.5 Types and presence of ESBLs

There are four main types of ESBLs enzymes namely, Temoneira (TEM), Sulphydryl Variable (SHV), Cefotaximase-Munchen (CTX-M) and Oxacillin-hydrolysing capabilities (OXA) β-lactamases (Pitout and Laupland, 2008; Naseer and Sundsfjord, 2011). However, there are over 150 TEM β-lactamases which are point mutation derivatives of TEM-1 or

TEM-2; whereby, TEM-1 was first demonstrated in 1965 in *E. coli* from a patient in Greece named Temoneira, hence the name TEM (Datta and Kontomichalou, 1965). Within the TEM β-lactamases, there are exceptions in some of the enzymes i.e. TEM-1, TEM-2 and TEM-13, which are not ESBLs but have higher ability to hydrolyse penicillins than oxacillin and caphalothin, although cannot hydrolyse extended-spectrum cephalosporins, e.g. cefotaxime, ceftriaxone and ceftazidime (Smet *et al.,* 2010; Mshana, 2011). The TEM-1 and TEM-2 are inhibited by clavulanic acid and are predominantly found in *E. coli* and other members of *Enterobacteriaceae* such as *Klebsiella* spp. and *Enterobacter* spp. isolated from food, clinical and environmental samples (Smet *et al.,* 2010; Delgado *et al.,* 2016).

The SHV-type is another group of β-lactamase, where SHV-1 was first found in a *K. pneumoniae* strain and it confers resistance to broad spectrum penicillins. Moreover, SHV-2 was also first isolated in 1983 from *K. ozaenae* in Germany, and was able to hydrolyse cefotaxime, but to a lesser extent than the hydrolysis of ceftazidime (Brun-Buisson *et al.,* 1987; Heritage *et al.,* 1999). The same SHV-type β-lactamases were reported around the same period in 1983 in France and England (Heritage *et al.,*1999; Rupp and Fey, 2003; Paterson and Bonomo, 2005). Currently, there are more than 50 SHVs which are all derivatives of SHV-1 and SHV-2, majority of them are ESBLs (Paterson and Bonomo, 2005; Gupta and Datta, 2007). The most common β-lactamases which account for over 60% of *E. coli* resistance to ampicillin are TEM and SHV which are plasmid-mediated β-lactamases, though SHV are normally found in *Klebsiella* spp. (Livermore, 1995).

Another family of ESBLs is CTX-M type of β-lactamases enzymes discovered and described in Munich, Germany, in 1989. It confers resistance to cefotaxime more than to other oxyimino-beta-lactam substrates like ceftazidime, ceftriaxone, or cefepime, with the

enzymes thought to have originated from *Kluyvera* spp. (Decousser *et al.,* 2001; Humeniuk *et al.,* 2002; Paterson and Bonomo, 2005). The CTX-M enzymes are inhibited mostly by tazobactam as compared to sulbactam and clavulanate (Faheem *et al.,* 2013). CTX-M type ESBLs enzymes are divided into five main groups which are CTX-M group 1, 2, 8, 9, 25 and are further subdivided into several subgroups (Paterson and Bonomo, 2005). The emergence of different ESBLs CTX-M types in the early years of 2000s has resulted in the rapid change of the prevalence of ESBLs. The prevalence of the CTX-M ESBLs has surpassed that of SHV and TEM, which are mostly isolated from *Enterobacteriaceae*, especially *E. coli* (Faheem *et al.*, 2013; Matsumura *et al.,* 2016). Globally, ESBLs CTX-M type enzymes are the leading and mostly isolated from *E. coli* in humans and in food chains, including seafood (Machado *et al.*, 2008; Moremi *et al.*, 2016). Among the enzymes predominantly isolated from both aspects, i.e. clinical, foods, aquatic environment and in community settings is CTX-M-15 (Cha *et al.*, 2016; Delgado *et al.*, 2016; Moremi *et al.*, 2016; Atterby *et al.,* 2017). The genes encoding for CTX-M enzymes are often located in large plasmids ranging in size from 7 to 260 kb. The plasmids also harbour other genes, such as antimicrobial resistance genes, and are capable of transferring genes among and between bacteria to humans through HGT (Pallecchi *et al.*, 2007; Ghafourian *et al.*, 2015; Shaikh *et al.*, 2015). So far, more than 100 types of CTX-M have been described in members of the family *Enterobacteriaceae*, most prominently in *E. coli* (Zhao and Hu, 2013; Shaikh *et al.,* 2015).

The last group is OXA β-lactamases, capable of hydrolysing oxacillin and cloxacillin. This group of β-lactamases are commonly found in *Pseudomonas* spp., although have reported from other Gram-negative bacteria in *Enterobacteriaceae* (Shaikh *et al.,* 2015). The OXA-types β-lactamases do not normally hydrolyse extended-spectrum

cephalosporins, and hence are not considered ESBLs (Ghafourian *et al.,* 2015; Shaikh *et al.,* 2015). There are ten other members of OXA ESBLs derived from OXA-10 (Rawat and Nair, 2010). Other small groups of ESBLs include Serratia fonticola (SFO), Intergron-borne cephalosporinase (IBC), *Pseudomonas* extended-resistant (PER 1-2), Guiana extended-spectrum (GES), and Vietnam extended-spectrum (VEB 1-2), mostly being derived from the main ESBL types. The ESBLs PER shares about 25-27% homology to TEM and SHV, and was initially isolated from *Pseudomonas* spp. and subsequently from *Acinetobacter* spp. and *Salmonella* spp. (Vahaboglu *et al.,* 1998; Vahaboglu *et al.,* 2001; Neuhauser *et al.,* 2003). The VEB-1 is a plasmid-mediated enzyme first isolated from patient in France, and is about 38% homology to PER-1 and 2. It confers a higher level of resistance to cefotaxime, ceftazidime and aztreonam reversed by clavulanic acid (Rupp and Fey, 2003; Paterson and Bonomo, 2005; Shaikh *et al.,* 2015).

1.4.6 Detection of ESBL-producing *Enterobacteriaceae*

Different methods are used to screen for and confirm members of *Enterobacteriaceae* that produce ESBLs. These include disc diffusion, disc approximation (double disc synergy), molecular techniques, BD phoenix automated microbiology system and the combined disc test (Drieux *et al.,* 2008). All methods except molecular method, use the same principle as documented by the clinical laboratory and standard institute (CLSI, 2016), which is based on disc diffusion screening of ESBL-producing *E. coli, K. pneumoniae* and *Proteaus* spp. The CLSI proposes the use of either of the following discs, cefotaxime, ceftazidime, cefpodoxime, aztreonam and ceftriaxone for screening of ESBL-producing strains. The use of more than one disc increases the sensitivity of detection, which enhances the accuracy of the results. The zone diameter for confirmation of ESBL production should be ≤17 mm (CLSI, 2016). When broth dilution screening is used, the minimum inhibitory concentration (MIC) ≥2 μg/ml for aztreonam, cefotaxime, ceftazidime and cefpodoxime is a presumptive confirmation of ESBL production (Rawat and Nair, 2010; CLSI, 2016).

1.4.7 Confirmation of ESBL-producing *Enterobacteriaceae*

1.4.7.1 Disk approximation (double disc synergy) method

Disc approximation, also known as double disc synergy, is a recommended method of confirmation, because it is simple, reliable and inexpensive detection of ESBL production in bacteria (Gupta and Datta, 2007). The method involves three discs arranged on Muller Hinton agar (MHA) in such a way that appropriate distance is considered between discs; whereby, disc at the centre must contain amoxicillin-clavulanic acid with concentration of 30/10 µg and the other two discs contain an oxyimino β lactam (30 µg) i.e. ceftazidime 30 µg and cefotaxime 30 µg being placed in either at the centre disc (Drieux *et al.,* 2008; Rawat and Nair, 2010; Poulou *et al.,* 2014). The recommended distance between amoxicillin-clavulanate disc and the two other discs on MHA plate should be about 20 to 30 mm apart from centre to centre (Poulou *et al.,* 2014). After inoculation, MHA plates containing the discs are incubated at 37°C for 24 h. The clear extension of the edge of inhibition zone towards amoxicillin-clavulanate disc is interpreted as positive for ESBL production. During inoculation of suspension isolates on MHA, care should be taken, as a slit is cut on the agar plate in which the broth suspension of organisms being tested are sprayed, and the antibiotic discs should be placed about 3 to 4 mm from the slit (Drieux *et al.,* 2008; Poulou *et al.,* 2014; CLSI, 2016). Decrease of the distance between discs, e.g. from 30 to 20 mm, increases the sensitivity of the test. In addition, distortion of the circular inhibition zone after incubation period is also recorded as positive for ESBL production.

1.4.7.2 Molecular detection

Further confirmation of ESBL production in the *Enterobacteriaceae* group is by the use of molecular techniques. The PCR technique with specific set of primers sequences targeting the ESBL genes, e.g. CTX-M, TEM, SHV, OXA is applied. The PCR, Restriction

Fragment Length Polymorphisms (RFLPs) and DNA sequencing are other effective methods for detection and confirmation of ESBLs in bacteria, but they are more expensive compared to double disc synergy and standard disc diffusion method.

1. 5 Dissemination of antimicrobial resistance

Antimicrobial resistance is one of the main challenge in public health due to complications in treatment of the infections caused by resistant pathogens, such as *E. coli* and *Salmonella* spp. (WHO, 2014; Tadesse *et al.,* 2017). The prevalence of resistance is increasing, especially in Gram-negative bacteria in the family *Enterobacteriaceae*, which comprises the largest group of food-borne pathogens. The resistance of these bacteria has increased dramatically in susceptibility of most antibiotics by altering the efficacy of antibiotics (WHO, 2014). The vast spread of antimicrobial resistance in communities around the world is due to resistance genes that are mostly carried on plasmids in both pathogenic and commensal bacteria, and which can be transferred easily from one bacterium to another and then to animals and humans (Clewell, 2014; Fletcher, 2015; Thanner *et al.,* 2016). Predisposing factors contributing to dissemination of antimicrobial resistance include different food chains, environments, agricultural activities and poor waste disposal system, such as hospital waste and sewage (Fletcher, 2015; Thanner *et al.,* 2016). Resistance genes other than β-lactams that are frequently reported in *E. coli* and other enteric bacteria include sulphonamides, trimethoprim and ciprofloxacin (Clewell, 2014; WHO, 2014; Fletcher, 2015; Moremi *et al.,* 2016). Bacterial plasmids especially resistance plasmids play important role in dissemination of acquired resistance genes in different organisms through HGT (Rossolini *et al.,* 2008). On the one hand, aquatic ecosystems have a reservoir function, allowing the mixing of environmental organisms, human and animal pathogens such as of faecal origin, potentially promoting the gene transfer (Rossolini *et al.,* 2008). Moreover, the use of water bodies for irrigation, recreational and domestic

purposes, constitutes a potential pathway enabling transmission of antimicrobial resistance between hosts through the environment (Egea *et al.,* 2012; Tekiner and Özpınar, 2016).

1.6 Antimicrobial resistance in aquatic environments

Different studies have demonstrated that a variety of food stuffs, including seafoods, are contaminated by, and act as sources of multidrug resistant *E. coli* (WHO, 2014; FSAI, 2015). ESBL-producing *E. coli* have been reported in fish from aquaculture farms in China, as well as from water and fish guts in Switzerland (Jiang *et al.,* 2012; Abgottspon *et al.,* 2014). Common reservoirs of multidrug resistance *E. coli* are foods of animal origin (e.g. meat, poultry and pork), environment, seafood and aquatic environment (Ben Said *et al.,* 2015; Moremi *et al.,* 2016; Seni *et al.,* 2016). In Tanzania, pathogenic and commensal *E. coli* in animal manure, products of animal origin and humans have been reported as well (Moyo *et al.,* 2007; Lupindu *et al.,* 2014; Madoshi *et al.,* 2016; Katakweba *et al.,* 2018). Other studies have demonstrated the possible spread of ESBL-producing bacteria among *Enterobacteriaceae* through exposure to contaminated farmed fish, frozen mackerel, water and environmental sources (Ishida *et al.,* 2010; Jiang *et al.,* 2012; Nasreldin and Khaldoon, 2015).

1.7 Salted sun-dried Nile perch products

1.7.1 Importance of drying fish products, markets and microbial safety

Nile perch, like all fish, are the source of animal proteins and essential elements for human health (Immaculate *et al.,* 2013; Ikwebe *et al.,* 2017). High quality Nile perch fish are mostly processed as fillets for export markets, especially to EU countries. The remaining parts and rejects are processed and sold in domestic and regional markets for human consumption (Kirema-Mukasa, 2012). The domestic and regional markets for salted sun-dried products from Nile perch is growing rapidly. These products are exported to

countries like the Democratic Republic of Congo, Rwanda and Burundi (Kyangwa and Odongkara, 2005; Kirema-Mukasa, 2012). Although salted sun-dried Nile perch products are in high demand in the regional market, their handling and processing are not done in line with QMS. This may create uncertainties among consumers on the safety of products attributed to faecal contamination due to poor products handling (David *et al.,* 2009). Salting and sun-drying is an ancient method that has been applied as one of the most common means of food preservation (Majumdar *et al.*, 2017; Nagwekar *et al.,* 2017). Sun-drying is a simple, inexpensive and affordable method that is totally sun-dependent (Ikwebe *et al.,* 2017). Most of the products dried by this method are safe for human consumption and have a long shelf life, if post-processing handling is properly observed to avoid re-occurrence of bacteria contamination in the dried products (Nagwekar *et al.*, 2017).

1.7.2 Microbial, moisture content and water activity in salted sun-dried fish products

Previous studies demonstrated that salted sun-dried fish products are microbiologically safe for human consumption (Saritha *et al.,* 2012; Majumdar *et al.,* 2017). This is due to the mechanism of drying, in which the moisture content and water activity are decreased. Both are essential parameters for microbial growth in products (Chaijan, 2011; Sampels, 2015). Although this preservation method is affordable, drying, packaging and storage conditions post-processing may not be hygienically satisfactory to prevent microbial contamination of the dried products (Prakash *et al.,* 2011). Also, the fish products that are dried during the rainy season are likely to have high moisture content and water activity, which supports microbial growth including mesophilic, halotolerant and saprophytic opportunistic (Tsai *et al.,* 2005; Prakash *et al.*, 2011; Darvishi *et al.*, 2013; Koral *et al.*, 2013; Sampels, 2015; Sivaraman and Siva, 2015).

1.7.3 Bacterial flora identification using MALDI-TOF-MS

MALDI-TOF-MS (Matrix-assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) is a tool used for the identification of microorganisms that include bacteria, virus, and fungi. MALDI-TOF-MS identifies microbes based on the comparison to SuperSpectra containing sets of genus, species and subspecies biomarkers characterised for different groups of microorganisms. MALDI-TOF-MS works based on principles of spectrophotometry that enables identification and discrimination of bacteria into specific subspecies. It is a powerful and sensitive tool, which provides reliable and accurate results in a short period of time, and which is economical in terms of both labour and costs involved (Singhal *et al.,* 2015).

1.8 Problem Statement and Justification

Nile perch of the Lake Victoria and their products that are sold in domestic and regional markets are prone to contamination with multidrug resistance faecal pathogens like *Salmonella* spp. and *E. coli* (David *et al.*, 2009). This may lead to fish of poor quality and questionable safety. Unhygienic fish handling and selling environment in domestic fish markets may be additional predisposing factors contributing to poor microbial quality of fish due to the potential for cross-contamination with bacteria from wastes around the market (David *et al.,* 2009; Kirema-Mukasa, 2012; Baniga *et al.,* 2017). In domestic markets, fish are usually displayed on open tables with minimal control of insects and dust that may lead to cross-contamination with enteric bacteria, such as *Salmonella* spp. Salted sun-dried Nile perch products are important by-products marketed in the local and regional markets. However, the products may have poor quality and safety due to drying methods and storage that may contribute to microbial contamination. Moreover, weather conditions may affect the drying process and produce products that are insufficiently dried, which may favour survival and multiplication of halophylic and non-halophylic

bacteria, as well as pathogenic and non-pathogenic bacteria with public health implications (Mohamed *et al.,* 2017).

In Tanzania, there are limited studies that elucidated the types and the magnitude of *Salmonella* spp. and ESBL-producing *E. coli* and their sources in freshwater Nile perch from Lake Victoria as well as antimicrobial resistance that may be expressed by the two pathogens. In other parts of the world, various studies have described various foods, such as poultry, porks as well as seafood that contain different multidrug resistant *Salmonella* serovars and ESBL-producing *E. coli*, but, none of these studies have been reported in the wild Nile perch (Jiang *et al.,* 2012; Nasreldin and Khaldoon, 2015; Moremi *et al.,* 2016; Li *et al.,* 2017). Recent studies have shown that *Salmonella* spp. can exist and multiply in aquatic environments in association with other organisms such as fish and free-living protozoa (Liu *et al.,* 2018). The global spread of ESBL producers among *Enterobacteriaceae* that cause community and hospital-acquired infections have been associated with consumption of contaminated foods of animal origin. Poor food handling along the production chain has been documented to be an important predisposing factor for contamination by multidrug resistant microbials. The present study, therefore, investigated the potential of Nile perch as a reservoir of *Salmonella* serovars and multidrug resistant ESBL-producing *E. coli*. In addition, the relationship between the Nile perch handling and the presence of different *Salmonella* serovars and ESBL-producing *E. coli* in water and fish was explored. The information generated from this study provides more evidence concerning the public health implications of the bacteria isolated from Nile perch. The findings will help fisheries competent authorities to develop measures directed at minimising bacterial contamination due to fish handling and during fish products processing for preserving quality. In addition to *Salmonella* spp. and ESBL-producing *E.*

coli, other bacteria were identified in salted sun-dried Nile perch products that may have health implications in the safety of seafood for consumers.

The aim of this study therefore, was to investigate the microbialological safety of Nile perch and its salted sun-dried products, as well as their contamination with *Salmonella* spp. and *E. coli* producing ESBLs. Also, genomically characterise isolates to determine their ecology and adaptation to their aquatic environments. The main bacteria of interest in this study were *Salmonella* spp. and ESBL-producing *E. coli*. Different genomic features of *Salmonella* serovars and ESBL-producing *E. coli* were investigated including antimicrobial resistance, virulence potential, resistant genes, phylogenetic comparisons among and between isolates and plasmid profiles.

1.9 Objectives

1.9.1 The Overall Objective

To investigate the microbial quality and safety levels along the Nile perch value chain in Lake Victoria for market enhancement and safeguarding the public.

1.9.2 Specific Objectives

(i) To establish the prevalence and diversity of *Salmonella* serotypes in water and Nile perch from Lake Victoria, Tanzania.

(ii) To evaluate genetic diversity, antimicrobial resistance and pathogenic potentials of *Salmonella* serovars isolated from water and Nile perch.

(iii) To determine the prevalence of ESBL-producing *E. coli* in water and Nile perch from Lake Victoria, Tanzania.

(iv) To determine genomic characteristics of ESBL-producing *E. coli* isolates from water and Nile perch.

(v) To assess microbial quality of frozen Nile perch and salted sun-dried products sold in domestic and regional markets.

1.10 Sample size estimation

Sample size was estimated based on the formulae described previously (Daniel, 2009):

$$n = \frac{(Z_{\alpha})^2 \times P(1-P)}{d^2}$$

Where P; is the prevalence value, d^2 is the given standard statistical precision equal to 5%, Z_{α} is the statistical level of confidence at 95% = 1.96, n is the estimated sample number and 1 is a constant.

Salmonella prevalence values were considered based on a previous study conducted in other fish species from the same area as the present study; the prevalence of *Salmonella* was 19.5% in fish, 13.3% in water and 13.3% for ESBL-prducing *Enterobacteraceae* in fish (but samples were within what has calculated using 'P' of *Salmonella* spp.) (Mdegela *et al.,* 2015; Moremi *et al.,* 2016). After the given values were substituted in the equation, the calculated sample size was 248 for fish and 176 for water, yielding a total sample size of 424. Then, 48 more samples were added, which was equivalent to approximately 10% of the total calculated sample size which were aimed for swabs samples from facilities used for fish transportation. Therefore, after the sum up of the sample size, then total samples of this study were 472. These samples were only collected from artisanal fishers following Nile perch value chain, from point of capture to marketed fish and/or products. This study did not consider Nile perch or products processed in fish industries intended for export markets.

1.10.1 Sample distributions and sampling

Sampling points were located within the Lake Victoria along Mwanza basin, at different landing sites, domestic fish markets, and cold storage facilities in Ilemela and Nyamagana districts in Mwanza region. The details on sampling techniques, samples distribution and laboratory samples and sub-samples preparation and analysis are detailed in the respective attached articles and manuscripts.

1.11 Significance of study

This study has provided useful information that will benefit fisheries sector in Tanzania, in particular the Nile perch artisanal fishers. This is because, the findings reported will be important baseline for fisheries competent authority to raise awareness to fish vendors and processors on the improvement of hygienic fish handling along Nile perch value chain for improved quality and safety of fish and fish products. Also, this study finding will be a useful starting point to fish laboratory testing personnel to demand improved laboratory detection tools on *Salmonella* that will identify specific strains of public health importance from fish samples. This will help to reduce detention of fish and/ or fish products from *Salmonella* contamination claims. The findings also will be used as reference information to policy makers to facilitate on improving policies associated with fish quality control for fish and fish products for export markets.

1.12 Organisation of the Thesis

This thesis is prepared according to the “Published papers” format of Sokoine University of Agriculture. The thesis is organised into three chapters that are preceded by an extended abstract summarising the objectives of the study, materials and methods, main research findings and conclusions. Chapter One provides an introduction and relevant theoretical background information about Lake Victoria, Nile perch value chain and faecal pollution

of the lake. The background information about the genus *Salmonella* and its taxonomy, detailed literature on selected *Salmonella* serovars in subspecies *salamae* and subspecies *enterica* are also narrated in chapter, one. Furthermore, the means of detection and isolation of *Salmonella* in samples are also described. In addition to *Salmonella* spp., literature about ESBL-producing *E. coli*, including ESBL classification are included, as are the methods of detection and confirmation. Chapter One also contains the problem statement and justification, study objectives, sample size estimation and distributions, followed by references. Chapter Two contains the main findings and discussions presented in the form of published articles (I and IV), submitted manuscript III and manuscript II, which will be submitted to a peer reviewed scientific journal. The prepared manuscript has been written following the format of the target journal. Chapter Three provides summary of the general results and discussions, conclusions and recommendations. The list of appendices comes last in the thesis.

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CHAPTER TWO

PAPER ONE

Status of the Manuscript: Published in Food Control Journal

Prevalence and characterisation of *Salmonella waycross* and *Salmonella enterica* subsp. *salamae* in Nile perch (*Lates niloticus*) of Lake Victoria, Tanzania

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Abstract

A study was conducted to determine the prevalence, serotypes, antimicrobial resistance and plasmids profiles of *Salmonella* spp. in Nile perch (*Lates niloticus*) from Lake Victoria, Tanzania. Presence of *Salmonella* spp. and quantification of *Escherichia coli* in water and fish were investigated using standard bacteriological methods. Antimicrobial resistance was determined using minimum inhibitory concentration (MIC) method. A total of 352 samples were analysed including Nile perch, lake water and swabs from surfaces of facilities used for fish transportation. A total of 10 different *Salmonella* serovars were isolated. *Salmonella enterica* subsp. *salamae* with antigenic formula 42:r:- had a prevalence of 10.0% (n=60) in Nile perch from fishing grounds which were significant different (P<0.05) to the prevalence of this serovar at landing sites and domestic markets 1.7% (n=60 each). The prevalence of *Salmonella* Waycross (41:z4, z23:-) in Nile perch from fishing grounds was 18.3% (n=60) with no significant difference (P>0.05) compared to the ones at landing sites 10% (n=60) and markets 13.3% (n=60). The prevalence of *S. enterica* subsp. *salamae* 42:r:- in water from fishing grounds was 6.7% (n=60). Two out of 12 *S. enterica* subsp. *salamae* 42:r:- were resistant to sulfamethoxazole, while one was resistance to azithromycin. *S.* Waycross were resistant to sulfamethoxazole (6/30), azithromycin (6/30) and ampicillin (4/30). Seven *S.* Waycross carried plasmids, but none of *S. enterica* subsp. *salamae* 42:r:- carried plasmids. *E. coli* mean counts were 2.51 log cfu/g in fish intestines, 2.38 log cfu/g in gills, and 2.02 log cfu/ml in water. The frequent findings of *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross in Nile perch are surprising as these serovars are rarely associated with human diseases. Follow-up studies of their ecology in Nile perch and Lake Victoria are needed.

Keywords: Fisheries; *Salmonella*; food safety; plasmids; antimicrobial resistance

1. Introduction

The genus *Salmonella* is classified into two species which are *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further classified into subspecies I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*) and VI (*indica*) (Brenner et al., 2000; Ke et al., 2014). Subspecies I comprise most of pathogenic serovars especially non-typhoid responsible for foodborne salmonellosis (Puah, Chua, & Tan, 2016). Reservoirs of *Salmonella* spp. are diverse, depending on the type of serovars, and include warm and cold-blooded animals, but also water, plants and soil (Carrasco et al., 2012; Ke et al., 2014; Smith et al., 2015). Subspecies II-VI usually are found in poikilothermic animals including reptiles and amphibians, but are also isolated from environmental samples (Brenner et al., 2000; Larson & Spickler, 2013). Worldwide, human infections caused by non-typhoid *Salmonella*, are mainly associated with consumption of contaminated foods typically pork, poultry, eggs, fresh fruits and vegetables (Abakpa et al., 2015; Campioni et al., 2012; Zhou et al., 2017).

Although seafood seems rarely implicated as the source of salmonellosis (Elhadi et al., 2016; Rahimi et al., 2013), *Salmonella* spp. are occasionally isolated from fish, crustacean and shellfish products (Bujjamma et al, 2015; Raufu et al., 2014; Yang et al., 2015) where contamination may occur at any point along the value chain from capture or culture to marketed products (Amagliani, Brandi, & Schiavano, 2012). Some non-typhoid serovars such as *S.* Weltevreden, *S.* Agona, *S.* Newport and *S.* Senftenberg have been found in water (Li et al., 2014) and seafood such as shrimps (Uddin et al., 2015) and live molluscan shellfish (Martinez-Urtaza et al., 2003). Occurrence of *Salmonella* spp. in aquatic environments is usually associated with faecal pollutions from human and animal sources e.g. urban sewage and livestock manure (Raufu et al., 2014; Traoré et al., 2015). However, there are increasing evidences that certain *Salmonella* serovars, e.g. *S.* Weltevreden, may

occur naturally in aquatic environments (Kang et al., 2017; Nguyen et al., 2016; Uddin et al., 2015).

Artisanal fishery, i.e. small-scale, low technology and low capital of the Nile perch in Lake Victoria targets mostly the growing domestic and regional markets whereas the good quality Nile perch is processed into fillets products (chilled and frozen fillets) for export markets mainly to the European Union (EU) countries (Kirema-Mukasa, 2012). According to the national statistics in Tanzania (URT, 2013), the catch of 47,141 metric tons of Nile perch constituted 20.1% of the total fish landings valued about 200 million Tanzanian shillings. This makes Nile perch, the leading fish species by value, while the small freshwater carps (*Rastrineobola argentea*) made up 72.3% of the total fish landings. The fish supply chain for the export markets is well monitored and use best practices as opposed to the domestic market chain (Kussaga et al., 2014). Still, reports on rejection or detention of Nile perch products due to microbiological contaminations e.g. *Salmonella* spp. were reported by the EU member countries (RASFF, 2017). The most recent findings of *Salmonella* spp. contamination in Nile perch fillets from Tanzania were in 2016 (1), 2014 (1), 2009 (2), 2004 (1), and 2003 (1). The Nile perch in domestic markets are handled without complying with quality systems, e.g. fish are displayed on open tables with high risks of faecal contamination (Baniga et al., 2017; David et al., 2009). Little is known about hygiene conditions and faecal pollution levels of Nile perch sold on domestic markets along the chain from capture to the markets. This includes the sources, transmission routes and characteristics of *Salmonella* spp. that may be isolated from the Nile perch caught in Lake Victoria, Tanzania.

The aim of this study was to determine the prevalence, serovars, antimicrobial susceptibility and plasmids profiles of *Salmonella* spp. isolated from water and Nile perch from artisanal fishery in Lake Victoria, Mwanza, Tanzania.

2. Materials and Methods

2.1 Study site

The study was carried out along the Lake Victoria basin and sampling points were off-shore fishing grounds, landing sites and domestic fish markets located in Ilemela and Nyamagana districts in Mwanza, Tanzania (Fig.1). Other sampling points included water from rivers crossing urban areas and draining into the lake. The water temperature in the lake is between 24 to 26°C throughout the year.

2.2 Study design and sampling

A cross-sectional study was conducted from February to July 2017 where a total of 352 samples were collected and analysed for *Salmonella* spp. Samples included 180 Nile perch samples (60 from fishing grounds; 60 from landing sites and 60 from domestic fish markets), 124 water samples (fishing grounds, landing sites, river and wastewater treatment plant), and 48 swabs from contact surfaces of boats and buckets used to transport Nile perch. The size of each fish sample varied from 1 to 2 kg and water volume was 300 ml.

2.2.1 Samples from fishing grounds, landing sites and markets

Samples included 60 Nile perch and 60 water samples from six fishing areas in the lake (Fig. 1) and 24 swabs aseptically collected from boats contact surfaces after the fishermen cleaned the boat and before fish were stored. The samples were collected directly in the lake at locations where fishermen were fishing. At each sampling point visited, 5 to15 fish and water were collected with fish being placed into sterile plastic zip-lock bags. Water samples were collected using sterile 500 ml glass bottles. The bottle was immersed into the lake water at 20-cm depth while its screw cap was still capped. Thereafter, the cap was removed and water was collected. The bottle was again closed and removed out of the

water with some water poured out. Swabs were collected by swabbing a 100 cm^2 area using a cotton stick after which the swab was stored into a tube containing 10 ml of sterile Buffered Peptone Water (BPW) (Oxoid Ltd, Hampshire, England) as recommended in the ISO standard (ISO 18593:, 2004). Fish and water samples were preserved at chilling temperature about ±5ºC into an insulated box containing cooling elements and transported to the National Fish Quality Control Laboratory (NFQCL) in Mwanza. Samples were analysed within 4 hrs of sampling.

Additionally, 60 Nile perch and 36 water samples were collected from six different selected landing sites, and also 24 swabs were collected from buckets which were used by fish vendors to put fish after buying from fishers upon landing. The Nile perch were aseptically sampled from boats during the early morning before fish were off-loaded. This was done to determine any *Salmonella* contamination during transport of the Nile perch. The fish were put into sterile plastic zip-lock bags. Water samples were collected from onshore (<100 metres from the lake shore) and offshore (>200 metres). Moreover, water samples were collected from rivers at the course of entry into the lake and at a city wastewater treatment plant in Mwanza. Another 60 Nile perch were purchased from vendors at six domestic markets, placed into sterile plastic zip-lock bags and transported in an insulated box containing cooling elements to the laboratory for analysis.

2.3 Sample preparation and pre-enrichment

The Nile perch samples included intestines, gills, and flesh which were weighed into individual sterile stomacher bags in a ratio of 1:9 to sterile BPW, then homogenised for 60 sec using a Stomacher (Seward 400, UK). The fish mucus was collected by macerating by hand fish placed in bags with 225 ml of BPW; then the BPW were used for subsequent analysis. In order to obtain fish flesh samples, part of the fish surface was disinfected

using 70% methylated alcohol, then sterile scalpel blades were used to remove the skin from the disinfected area and to cut out 25-g of fish flesh. The fish flesh was transferred into 225 ml BPW and homogenised in a stomacher before analysis. Gill slats were removed by a sterile scissor, and then dissected into small pieces, weighed and 225 ml of BPW was added and mixed. For intestines, a sterile blade was used to open the fish carcass longitudinally, then the lower part of intestine was cut with a sterile scissor, weighed and BPW was added. For water samples, a 25-ml volume was filtered using 0.45µl filters then the filter was cut into two pieces and then mixed into 225 ml of BPW. All sample types added BPW were pre-enriched by incubation at 37°C for 24 hrs as per standard method.

2.3.1 Detection of Salmonella

Salmonella spp. was detected using standard bacteriological methods (ISO 6579:2002/Amd.1:2007). The presumptive strains were agglutinated on polyvalent *Salmonella* antisera (Rapid Lab Ltd, UK) and confirmed by Polymerase Chain Reaction (PCR) using ST11-ST15 primers in a multiplex reaction. The PCR reactions and cycling conditions followed the protocol described by Moussa et al. (2012). Certified Reference Material (CRMs) *S.* Typhimurium ATCC 13311 (Public Health, England) was used as positive control. The isolates were stored in 50% glycerol at -80ºC for further testing. Then, *Salmonella* isolates were shipped to the World Organization for Animal Health (OIE) *Salmonella* Reference laboratory in Padova, Italy (Istituto Zooprofilattico Sperimentale delle Venezie) for serotyping. Serovars were defined according to the xMAP® *Salmonella* Serotyping Assay (Luminex, USA) and when necessary, traditional serotyping was also carried out (ISO/TR 6579-3:2014). Results were interpreted according to the Kauffmann-White Scheme (Grimont & Weill, 2007).

2.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using Sensititre EUVSEC plates for *Salmonella* spp. (Trek Diagnostic System, East Grinstead, UK) based on guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). This included antimicrobials commonly used to treat human salmonellosis. *Salmonella* isolates were sub-cultured onto blood agar and incubated at 37°C for 24 hrs. Subsequently, loops full of bacterial cells were resuscitated into test tubes containing 5 ml sterile distilled water; bacterial turbidity was adjusted to match 0.5 McFarland standard. Afterward, 10 µl were transferred into 11 ml of Mueller Hinton broth II (Oxoid Ltd), vortexed and 50 µl were inoculated into each well of EUVSEC plate containing known concentration of antimicrobial agent and incubated at 37°C for 24 hrs. Plates were examined using SWIN software (Thermo Scientific, USA). Antimicrobials used and their epidemiological breakpoints (µg/ml) were: ampicillin (8), azithromycin (16), cefotaxime (0.5), ceftazidime (1), chloramphenicol (16), ciprofloxacin (0.064), colistin (2), gentamicin (2), meropenem (0.125), nalidixic acid (16), sulfamethoxazole (256), tetracycline (8), tigecycline (1) and trimethoprim (2).

2.3.3 Plasmids extraction and detection

Plasmids of *Salmonella* serotypes were isolated using bacteria pelleted from 1 ml of overnight culture in Luria Bertani broth (Sigma-Aldrich, Buchs, Switzerland) using the phenol-chloroform-isoamylalcohol (25:24:1) extraction method (Ausubel, 1994). Extracted plasmids were characterized by gel electrophoresis with 0.8% w/v agarose (SRL, India), parallel with *E. coli* strains 39R marker (147, 63, 36, 6.9 kb) and V517 as markers of different size (52-2.1 kb). The plasmids DNA bands were visualised and photographed under UV trans-illuminator using quantity one gel documentation system (UVP, England).

2.3.4 Enumeration of Escherichia coli

E. coli was analysed in 120 Nile perch samples (60 from fishing grounds and 60 from landing sites) and 60 water samples from fishing grounds. *E. coli* were enumerated to indicated level of faecal contamination using a pour plate technique with commercially available chromogenic agar, Brilliance^{TM} *E. coli*/Coliform selective agar (Oxoid Ltd) and incubated at 37ºC for 24 hrs as recommended by the manufacturer. Purple colonies of *E. coli* were counted using a colony counter. *E. coli* ATCC 8739 (Oxoid Ltd Remel Inc, UK) was used as a positive control.

2.4 Data Analysis

Data collected were entered in Microsoft Excel version 2010, (Microsoft Ltd, USA) then analysed using Stata version 14 (StataCorp LP). *E. coli* data were analysed using a negative binomial regression counting data model (Hilbe, 2011). Only samples with *E. coli* growth were considered to find the mean counts while samples with zero counts were not. All valid counts (<300 colony forming units) on each plate in dilutions were used as an offset in the analysis. The measure of data dispersions between the samples having *E. coli* counts were presented in boxplots prepared using Stata version 14. The detection limits for *E. coli* and *Salmonella* spp. were <10 cfu/g and absence in 25 g sample, respectively. Descriptive statistics was used to compute the prevalence of *Salmonella* spp. in different categories of samples analysed. The estimated prevalence obtained in samples were then compared using Chi-square in Epi Info ^{TM}, version 7.2 (CDC, Atlanta, USA). Antimicrobial resistance was interpreted based on epidemiological EUCAST breakpoints values (ECOFFs) (EUCAST, 2015). A difference was considered significant at $P < 0.05$.

3. Results

3.1 Prevalence of Salmonella in water, boat surfaces and Nile perch from fishing grounds

Table 1 shows the prevalence and distributions of *Salmonella* spp. and serovars in lake water, Nile perch and its intestines, gills and body surface. The results showed a significant difference ($P<0.05$) between the prevalence of *Salmonella* spp. in fish intestines and the ones found on fish surface and gills. *Salmonella* serovars predominantly recovered were *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross 41:z4z23:-. The prevalence of *S. enterica* subsp. *salamae* 42:r:- in Nile perch from fishing grounds was significantly higher ($P<0.05$) than those from landing sites and markets. *Salmonella enterica* subsp. *salamae* 42:r:- was detected in water samples which never at the same time contained *S.* Waycross. A significant higher prevalence of *S.* Waycross was observed in intestinal content from fish obtained at the fishing grounds as compared with intestines from fish at landing sites and markets ($P<0.05$). Only few samples of Nile perch intestines, gills, and lake water were positive for *E. coli*, with the highest mean counts of 2.51 log cfu/g found in intestines and the lowest in lake water 2.02 log cfu/ml (Fig. 2).

3.2 Prevalence of Salmonella in water, buckets and Nile perch at landing sites

The prevalence of *Salmonella* spp. in the Nile perch collected from boats at landing sites was 15.0% (n=60) which was lower than the prevalence in fish at point of capture and at markets. Fish intestines had a significantly higher *Salmonella* prevalence 10.0% (n=60) ($P<0.05$) compared to the fish surfaces 3.3% (n=60) and gills 1.7% (n=60) (Table 1). *Salmonella enterica* subsp. *salamae* 42:r:- and *S.* Waycross were also the most common serovars detected in the Nile perch at landing sites with *S.* Hvittinfoss and *S.* Typhimurium detected in a few intestinal samples. *Salmonella* spp. was also isolated in 16.7% (n=18) onshore water, while none was found in offshore. Serovars in onshore water were *S.* Senftenberg, *S.* Newport and *S. enterica* subsp *diarizonae* 61:l,v:z35. *Salmonella* spp. was

not detected in fish flesh. *Escherichia coli* mean counts in Nile perch at landing sites were 2.56 log cfu/g from 11/60 intestines, 1.97 log cfu/g from 10/60 gills and 2.10 log cfu/fish from 14/60 surface (Fig. 2). No significant difference (P>0.05) was observed between *E. coli* counts in intestines, gills and fish surfaces at fishing grounds and those counts in the same sample types at landing sites. However, fish flesh and swabs obtained at landing sites had *E. coli* counts below detection limit (<$1.0x10^1$ cfu/g/cm^2).

3.3 Prevalence of Salmonella in the Nile perch from domestic fish markets

The Nile perch at domestic markets had higher prevalence of *Salmonella* spp. 30.0% (n=60) compared to the ones from fishing grounds and landing sites. Fish surfaces had a higher prevalence 20.0% (n=60) compared to intestines 8.3% (n=60) and gills 5.0% (n=60) (P<0.05) (Table 1). Serovars detected on fish surfaces were *S.* Senftenberg (19:g,[s],t:-), *S.* Singapore (6,7:k :e,n,x), *S.* Newport (6,8 :e,h:1,2), *S.* Typhimurium (4:i:1,2), *S.* Hvittinfoss (16:b:e,n,x), *S. enterica* subsp. *enterica* (16:a:-) and *S.* Waycross, while in Nile perch intestines and gills were *S. enterica* subsp. *salamae* 42:r:-, *S.* Waycross and *S.* Typhimurium. None of *Salmonella* spp. was detected in fish flesh from markets.

3.4 Antimicrobial resistance and plasmids profiles of Salmonella serotypes

Table 2 shows *Salmonella* serovars isolated in water and Nile perch from the Lake Victoria, Tanzania which generally were susceptible to most of the antimicrobials tested. Few *Salmonella* serovars showed resistance to nalidixic acid 4.7% (n=64) at 64 µg/ml, ampicillin 7.8% (n=64) at 32-64 µg/ml, azithromycin 21.9% (n=64) at 64 µg/ml and sulfamethoxazole 34.4% (n=64) at 1024 µg/ml. For the main serovars detected in water and the Nile perch, only a few *S. enterica* subsp. *salamae* 42:r:- were resistance to sulfamethoxazole 2/12 and azithromycin 1/12 while few *S.* Waycross were resistant to sulfamethoxazole 6/30, azithromycin 6/30 and ampicillin 4/30. Seven *S.* Waycross carried

plasmids ranging from 2.0 to 63 kb sizes, but plasmids were not found in *S. enterica* subsp. *salamae* 42:r:- (Table 2). Other *Salmonella* serovars which carried plasmids included *S.* Senftenberg, (7.2, 4.4, 2.7 kb), *S.* Typhimurium (100, 3.0, 2.7kb) and *S. enterica* subsp. *diarizona* had 100, 3.0, 2.7 2.0 kb plasmids size.

4.0 Discussion

4.1 Prevalence of Salmonella in samples from fishing grounds

This study showed that Nile perch and water from fishing grounds commonly contained *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross. The *S. enterica* subsp. *salamae* 42:r:- initially was known as *S. enterica* subsp. *salamae* serovar Nairobi (Kelterborn, 1967; Nair et al., 2014). *Salmonella enterica* subsp. *salamae* has been commonly found in cold-blooded animals such as reptiles, amphibians, but has also occasionally been isolated from warm-blooded animals like poultry especially broilers (Chandry et al., 2012; Lamas et al., 2018). This subspecies rarely causes infections in humans and animals (Chandry et al., 2012; Lamas et al., 2018). Sources of *S. enterica* subsp. *salamae* 42:r:- in Nile perch and lake water may be faecal or other wastes from cold-blooded animals, e.g. reptiles, found along the shore of the lake and perhaps within the lake water (Ellermeier & Slauch, 2006; Larson & Spickler, 2013; Tomastikova et al., 2017). Since *S. enterica* subsp. *salamae* 42:r:- were not detected in river and onshore water, and at exit point of wastewater plant, this may further suggest that water and possibly Nile perch are reservoirs of *S. enterica* subsp. *salamae* 42:r:-.

Salmonella Waycross (41:z4,z23:-) was not only detected in the Nile perch from fishing grounds, but also in fish at landing sites, markets and in river water. *Salmonella* Waycross was described initially by Seligmann & Saphra, (1948) and has been found in poultry, beef, other farmed animals, and humans (Al-Nakhli et al., 1999; Haddock et al., 1991;

Halatsi et al., 2006; Kagambèga et al., 2013; Stevens et al., 2008). It has also been isolated from tilapia (*Oreochromis niloticus*) from water reservoirs in Burkina Faso (Traoré et al., 2015). The occurrence of *S.* Waycross in Nile perch and the lake environment may indicate as for *S. enterica* subsp. *salamae* 42:r:-. that this serovar show increased survival and possible ability to multiply in aquatic environments. It is uncertain if heavy rains and subsequent surface run-off water may introduce *S.* Waycross into the lake environment (Amagliani et al., 2012). Culture of swabs of boat surfaces did not yield any *Salmonella* spp. This indicates a low level of faecal contamination and that contact of the caught Nile perch to the boat surface is not an importance source of *Salmonella* spp. Such situation is further justifiable with the low levels of *E. coli* obtained in the same niches.

4.2 Prevalence of Salmonella in samples from landing sites

The lower prevalence of *Salmonella* spp. in the Nile perch at landing sites may indicate a die-off during handling and transport as compared with the higher prevalence seen in the fish at point of capture in the lake (Doyle, 2007). Since the Nile perch sampled at landing sites did not originate from the same catch as those at fishing grounds therefore, the *Salmonella* serovars in intestines and gills from Nile perch at landings could originate from water in fishing grounds which is supported by findings of similar serovars, i.e. *S. enterica* subsp. *salamae* 42:r and *S.* Waycross (Table 1).

Buckets used by fish vendors at landing site did not seem an important source of *Salmonella*, because *Salmonella* was not detected in swabs and *E. coli* contamination levels were low. There did not seem to be any correlation between the concentration of *E. coli* and occurrence of *S. enterica* subsp. *salamae* 42:r and *S.* Waycross found in fish and water (Fig. 2, Table 1).

4.3 Prevalence of Salmonella in samples from domestic fish markets

Poor fish handling could be the source of *Salmonella* in fish at the markets. The present study revealed that most of fish vendors used sand from the shore of the lake to cover fish, believing that it reduces spoilage. This practice is a potential source of microbiological contamination more importantly of *Salmonella* spp. The presence of different *Salmonella* serovars on fish surfaces from markets suggests bacterial cross-contamination from animals, poultry and environmental wastes seen around the markets. Since fish were displayed on open tables where flies and insects easily proliferate on fish, flies and poor waste disposal practices at nearby the markets are potential vehicles for transmission of *Salmonella* spp. to fish. The presence of *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross in intestines and gills of the Nile perch sampled at the domestic fish markets is likely associated with the serovars found in fish and/or water from fishing grounds. Our findings of *Salmonella* spp. in the Nile perch from domestic markets is supported by similar findings in a recent study on *Salmonella* in carp fish species sold in domestic markets in Mwanza (Baniga et al., 2017) indicating poor hygiene conditions at domestic fish markets (Begum et al., 2010; Budiati et al., 2013; David et al., 2009). Since fish, including the Nile perch are eviscerated and always cooked well before being consumed, their presence may not represent real public health risk. Still good hygiene practices should be done during fish processing and handling along the fish value chain (Amagliani et al., 2012).

4.4 Antimicrobial resistance and plasmids profiles of Salmonella serotypes

The antimicrobial resistance profiles of *Salmonella* spp. shown in Table 2 are in agreement with other studies from Tanzania, China, Burkina Faso and Vietnam that also reported *Salmonella* spp. from different fish species were relative susceptible to most antimicrobials tested (Baniga et al., 2017; Kuang et al., 2015; Traoré et al., 2015; Uddin et al., 2015). *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross showed low levels of

antimicrobial resistance as compared to other serovars, including those commonly found in animal and humans (Andoh et al., 2017; Chen, Wang, Su, & Chiu, 2013). Thus, if *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross emerge as cases of human salmonellosis they can be treated with standard antimicrobial therapy, e.g. ciprofloxacin. Antimicrobial resistance genes in *Salmonella* spp. are often carried on plasmids and can be transmitted to other bacteria via horizontal gene transfer (Szmolka et al., 2018). Most *Salmonella* serovars in our study did not contain plasmids and any role of the plasmids found remains to be elucidated.

5. Conclusions

The present study has demonstrated that Nile perch along the points of its value chain in the Lake Victoria basin are contaminated mainly with two uncommon non-typhoid *Salmonella* serovars, i.e. *S. enterica* subsp. *salamae* 42:r:- and *S.* Waycross. Further ecological studies of the sources and origin of these serovars are needed together with whole genome sequence analysis to determine their phylogeny and clonal status.

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Table 1: Prevalence and *Salmonella* serovars in water and Nile perch from different sampling points along the Lake Victoria, Tanzania

Location	Prevalence (%)	Sample type	Positives (%)	Sampling points	(Sampling point); serovar [no. of positive samples]
Fishing grounds	Fish 16/60 (26.7)	Intestines	12/60 (20.0)	1; 3; 4	(1) *S. enterica* subsp. *salamae* 42:r:- [2], (3) *S.* Waycross [3] and (4) *S.* Waycross [7]
		Gills	3/60 (5.0)	1; 4	(1) *S.* Hvittinfoss [1] (4) *S.* Waycross [2]
		Flesh	0/60 (0.0)	1-6	ND
		Surface of fish	6/60 (10.0)	1; 3	(1) *S. enterica* subsp. *salamae* 42:r:- [4], (3) *S.* Waycross [2]
	Water 5/60 (8.3)	Lake water	5/60 (8.3)	1; 4;	(1) S. *enterica* subsp. *salamae* 42:r:- [4]; (4) *S.* Typhimurium [1]
Landing sites	Fish 9/60 (15.0)	Intestines	6/60 (10.0)	7; 8; 9; 11; 12	(7, 9, 12) *S.* Waycross [3], (8) *S. enterica* subsp. *salamae* 42:r:- [1], *S.* Typhimurium [1], (11) *S.* Hvittinfoss [1]
		Gills	1/60 (1.7)	12	S. Waycross [1]
		Flesh	0/60 (0.0)	12	ND
		Surface of fish	2/60 (3.3)	12	S. Waycross [2]
	Water 3/36 (8.3)	Onshore water	3/18 (16.7)	8; 12	(8) *S.* Senftenberg [1]; (12) *S. enterica* subsp. *diarizona* [1] and *S.* Newport [1]
		Offshore water	0/18 (0.0)	7-12	ND
	Water 3/4	River outlet	N/A	A ; B ; D	(A) *S.* Singapore [1] and *S.* Tilene [1]; (B) and (D) *S.* Waycross [2]
	Swabs 0/48 (0.0)	Boats surface	0/24 (0.0)	1-6	ND
		Buckets surface	0/24 (0.0)	7-12	ND
Markets	Fish 18/60 (30.0)	Intestines	5/60 (8.3)	16; 17; 18	(16, 18) *S.* Waycross [4]; (17) *S.* Hvittinfoss [1]
		Gills	3/60 (5.0)	13, 14	(13) *S. enterica* subsp. *salamae* 42:r:- [1]; (14) *S.* Waycross [2]
		Flesh	0/60 (0.0)	13-18	ND
		Surface of fish	12/60 (20.0)	13, 14, 15, 16, 17	(13) *S.* Typhimurium [2]; (14) *S.* Singapore [1], (15) *S.* Enterica* [1]; (16, 17) *S.* Newport [3], *S.* Senftenberg [2], (17) *S.* Waycross [2], *S.* Hvittinfoss [1]
Wastewater	Water 2/4	Water inlets	N/A	E	(E) *S.* Typhimurium [1], *S.* Senftenberg [1]
		Water outlets	N/A	E	ND

Legend: Sampling points: Number 1-6 fishing grounds, 7-12 landing sites, 13-18 domestic fish markets; Letter ''A – D'' Rivers and 'E' is wastewater treatment plant;
N/A not applicable; ND not detected

*Serovar with only one flagellar antigen

Table 2: Antimicrobial resistance and plasmids profiles of *Salmonella* serovars isolated from water and Nile perch from Lake Victoria, Tanzania

Serovars	Plasmids "kb" (isolates)	Resistance pattern (number of isolates)
S. Waycross	63, 7.2, 5.1, 3.0 (1)	SMX, COL; GEN (1)
S. Waycross	3.0, 2.7, 2 (3)	Sensitive (3)
S. Waycross	63, 2 (2)	AZM; NAL; SMX (2)
S. Waycross	63 (1)	SMX (1)
S. Waycross	N/A (24)	AMP; NAL; SMX (1), AZM (2), SMX (1), AMP; AZM (2), AMP (1), Sensitive (16)
S. enterica subsp. *salamae* 42:r:-	N/A (12)	AZM; SMX (1), SMX (1), Sensitive (10)
S. Typhimurium	100, 3.0, 2.7 (1)	COL (1)
S. Typhimurium	36 (4)	AZM; SMX (3), Sensitive (1)
S. Hvittinfoss	N/A (4)	SMX (4)
S. Senftenberg	7.2, 4.4, 2.7 (3)	AZM; SMX (2), TET (1)
S. Senftenberg	N/A (1)	Sensitive (1)
S. Newport	N/A (4)	SMX (3), Sensitive (1)
S. Singapore	N/A (2)	Sensitive (2)
S. Enterica*	N/A (1)	Sensitive (1)
S. enterica subsp. *diarizona*	100, 3.0, 2.7, 2 (1)	AZM; SMX (1)
S. Tilene	N/A (1)	AMP; AZM; SMX (1)

Legend**:** AMP= Ampicillin; AZM= Azithromycin; COL= Colistin; GEN= Gentamicin; NAL= Nalidixic acid; SMX= Sulfamethoxazole; TET= Tetracycline. N/A not applicable. The number in brackets are showing the resistance and plasmids profile; *Serovar with only one flagellar antigen.

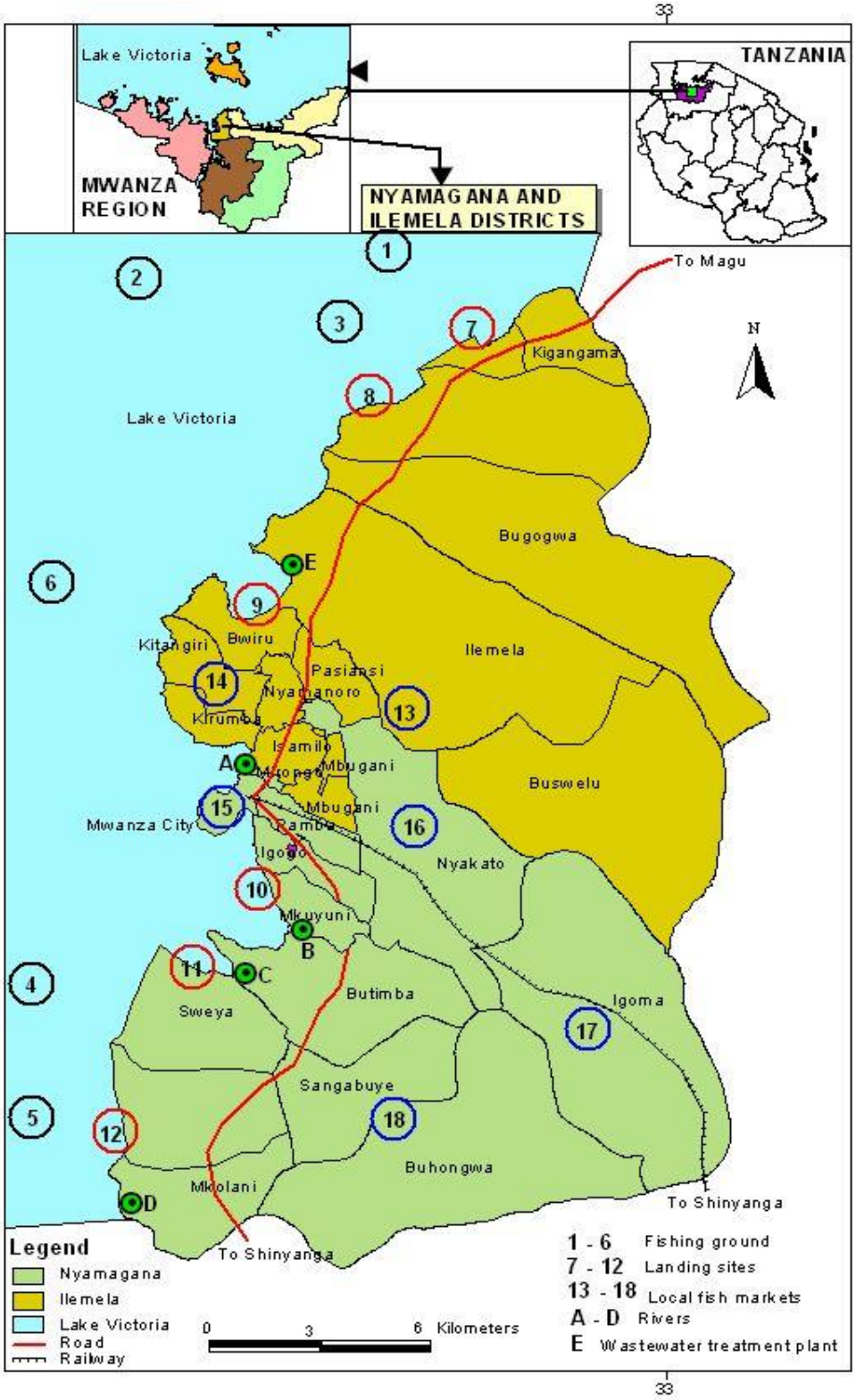

Figure 1: Sketch map of Lake Victoria, Tanzania showing sampling points

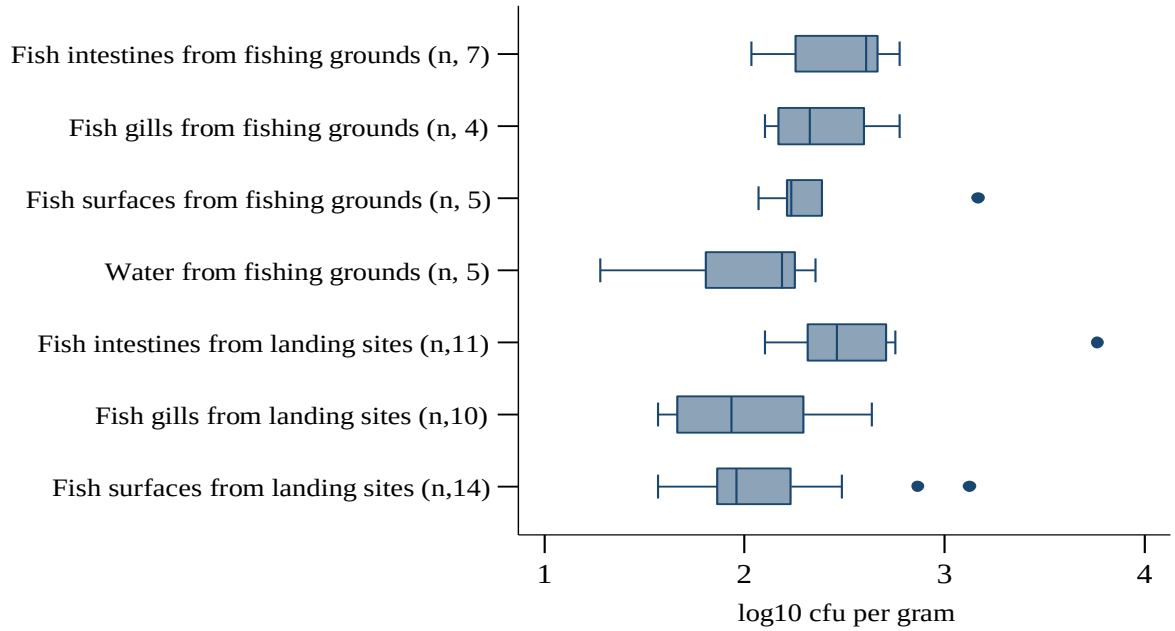

Figure 2: Box plot of *Escherichia coli* counts in water and Nile perch from Lake Victoria, Tanzania

PAPER TWO

Status of the Manuscript: To be Submitted to the Journal of Applied Environmental Microbiology

Genomic characterisation of *Salmonella* isolates from Nile perch (*Lates niloticus*) in Lake Victoria, Tanzania

Running Title: Genomics of rarely isolated *Salmonella* serovars in Lake Victoria

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ABSTRACT

Salmonella enterica species comprises >2600 serovars grouped into host specific, host adapted and host generalist strains. Some serovars are potentially pathogenic, while others are not. We used whole genome sequencing for molecular characterisation and genomic comparisons of *S. enterica* subsp. *salamae* (serovar 42:r:- and serovar Fulica), *S.* Waycross and *S.* Wien previously isolated in water and Nile perch. The selected isolates involved six of serovar 42:r:- and one of serovar Fulica, five of *S.* Waycross and two of *S.* Wien. Serovar 42:r:- and Fulica were ST1208, and serovar 42:r:- belonged to serogroup T. Four *S.* Waycross and one *S.* Wien were ST2460, while one *S.* Waycross and one *S.* Wien were ST3691. *S.* Waycross was serogrouped as S. *Salmonella* Waycross and *S.* Wien contained *Salmonella* Pathogenicity Islands (SPIs) SPI-2 to SPI-5 with associated virulence genes. These SPIs was absent in serovar 42:r:-, serovar Fulica. One resistance gene was shared by all strains (*aac(6')-laa*) encoding for resistance to aminoglycosides. The plasmid replicon type IncFII was detected in *S.* Waycross ST3691 and *S.* Wien ST3691, and did not contain antimicrobial or virulence genes. It was further revealed that serovars of subspecies *salamae* had unique *esp* and *ompT* genes essential for adaptation in an aquatic environment, while *S.* Waycross and *S.* Wien had *cit* and *oadAB* genes that are important for adaptation in harsh condiions. Phylogenetic analysis revealed relatedness among serovar 42:r:- (<17 single nucleotide polymorphisms, SNPs), and between serovar Fulica and serovar 42:r:- (<78 SNPs). *Salmonella* Waycross was clonal (<20 SNPs), but *S.* Wien presented high variations. The findings indicate that our strains are apparently not a public health concern and that they have unique genomic features supporting their adaptation to the aquatic environment.

IMPORTANCE

Salmonella enterica has six recognised subspecies (I-VI) with >2600 serovars. Some are potentially pathogenic while others are not. These serovars are grouped into host specific, host adapted and host generalist groups. Overall, and mostly for subspecies I, *Salmonella* hosts are warm blooded animals, while the hosts for subspecies II-VI are cold-blooded animals. Some *Salmonella* serovars may be naturally resident in aquatic environments. We carried out whole genome sequencing of selected serovars of subspecies *enterica* and *salamae* previously isolated from aquatic environment. The specific genomic features that are important for adaptation and survival in the aquatic environment in comparison to host specific and host generalist serovars was determined. We also assessed the potential virulence of isolates. The results support our hypothesis that the examined strains originated from aquatic environments and are apparently not a public health concern.

Keywords: *Salmonella*, host-adaptation, virulence, resistance genes, clonal relatedness

INTRODUCTION

The genus *Salmonella* is classified into two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* with >2600 serovars is further divided into six subspecies; *enterica* I, *salamae* II, *arizonae* IIIa, *diarizonae* IIIb, *houtenae* IV, and *indica* VI (1). The subspecies *enterica* contains more than 1600 serovars, are mainly non-typhoid *Salmonella* associated with gastroenteritis and salmonellosis in humans and animals (1, 2). The hosts of *S. enterica* subsp. *enterica* are warm blooded animals. Serovars in this subspecies are widely distributed globally as have been reported in warm blooded animals (3), soil (4), aquatic environments and seafood, such as shrimps (5, 6). The products commonly associated with *Salmonella* spp. contamination originated from poultry (2, 7), meat from domestic ruminants (8–10) and pork (11) and have been occasionally reported in seafood (5, 12). The hosts of subspecies II-VI are commonly cold-blooded animals, such as reptiles, amphibians and most fish species. The serovars in these subspecies rarely cause infections in animals and humans (13–15). The occurrence of *Salmonella* spp. in aquatic environments normally is associated with faecal pollution due to influx of wastes of human, animal and agricultural origins (16–18).

Although *Salmonella* spp. are generally associated with faecal pollution, there are evidences suggesting that some serovars such as *S.* Weltevreden, occurring naturally in aquatic environment especially from Asian countries including China (19), Vietnam (6) and Thailand (20). *S.* Weltevreden is one of the aquatic serovars reported to be of public health importance in Asian countries (21). The prevalence of this serovar in humans was reported to be 13.5% and 3.1% in Thailand and China, respectively (20, 21). Moreover, *S.* Waycross subspecies *enterica* and serovar 42:r:- of subspecies *salamae* have been isolated from Nile perch and aquatic environment of Lake Victoria, Tanzania (22). The persistence and survival of *Salmonella* in aquatic environment is associated with existence in the

viable but non-culturable state and in a symbiotic co-existence with free living protozoan (4, 23). The public health importance of *S.* Waycross and *salamae* serovar 42:r:- remain uncertain (22).

Genomic evolutionary reports suggest that subspecies *salamae* was initially related to *Escherichia coli* 0157:H7 and *E. coli* 0103:H25, before a divergence associated with genomic changes like acquisitions and deletions of nucleotides in chromosome (14, 24). The divergence includes the gain of the locus of enterocyte effacement and the loss of *Salmonella* pathogenicity island-5 (SPI-5) in subspecies *salamae* (25–27). The genomic physiognomies in serovars of *S. enterica* subsp. *enterica* have been widely studied, but limited information is available about subspecies *salamae* (28, 29). On the other hand, *S.* Waycross is a member of *enterica* group that has been rarely isolated from warm-blooded animals (8, 30) and has been isolated from aquatic environments and seafood (22). Although *S.* Waycross belongs to the same subspecies with other frequently isolated serovars, such as *S.* Typhimurium and *S.* Enteritidis, it has seldom been reported in human infections (31). Limited information is available describing the genomic characteristics of *S.* Waycross in relation to the aquatic environment.

The current study aimed to investigate the potential pathogenicity and antimicrobial resistance genes of *Salmonella* serovars previously isolated from water and Nile perch in Lake Victoria, Tanzania. In addition, genomic specificities and diversity within selected aquatic serovars of *Salmonella* along with their phylogenetic relatedness to serovars of clinical importance were also studied.

RESULTS AND DISCUSSION

Whole genome sequencing of fourteen isolates of *Salmonella* previously isolated from water and Nile perch fish from Lake Victoria was done. Analysis of the sequences identified six of seven subspecies *salamae* as serovar 42:r:-, of serogroup T. The

remaining one was identified as serovar Fulica but had unidentified serogroup, all of multilocus sequence type (MLST) ST-1208 (Table 1). Within the subspecies *salamae*, strains of serovar 42:r:- formed a closely related phylogenetic cluster of less than 17 SNPs with a slight variation from serovar Fulica with 78 SNPs (Fig. 1 and see Table S3a in the Supplementary Material). Placed in a global context, our isolates of subspecies *salamae* were closely related to other public strains from database of serovars 42:r:- and Fulica of the same ST-1208, but were genetically distant from other serovars within the subspecies *salamae*. This was supported by previous description that bacterial phylogeny presented by MLST usually show sequence type-based clustering, where strains of the same sequence type clustered together but different sequence types do not (32, 33). The clonal relationship between serovar 42:r:- and serovar Fulica of subspecies *salamae* could be attributed to their common ST type, but may also suggest that the two serovars might have evolved from the same lineage (33). Moreover, five of the sequenced isolates were identified as *S.* Waycross, of which four were ST-2460 and one was ST-3691. They were all of serogroup S which is in agreement with previous findings (34, 35). The four *S.* Waycross ST-2460 were clonal (20 SNPs apart), but differed in up to 26065 SNPs from *S.* Waycross ST3691 (Fig. 2, and see Table S3b in the Supplementary Material). When compared to other publicly available *S.* Waycross, we noticed that our strains were closely related to their corresponding serovars from elsewhere although an ST-based clustering was observed, where strains of ST-2460 formed a separate clade from those of ST3691. In addition, unidentified serogroup *S.* Wien ST2460 and *S.* Wien ST3691 were also reported in this study and were determined to be genetically distant both from each other and also from other *S.* Wien from the public domain, probably because of their differences in sequence types (Fig. 3, and see Table S3c in the Supplementary Material). Genetic variations observed among strains of the same sequence type might be due to point

mutation in terms of nucleotide insertions or deletions, and also differences in genome content leading to SNP differences (36, 37).

Salmonella enterica subsp. *salamae* are considered to rarely cause infections in humans (14, 26). The ability of *Salmonella* to become pathogenic requires different genes clusters (virulence genes) located in the region of pathogenicity island, which are involved in cellular infection. Most of these genes are located in chromosomes, although others can be carried in plasmids (38). There are five principal SPIs (SPI-1 to SPI-5) found in the *Salmonella* spp. that containing virulence factors important for the pathogenicity (25, 38). The genes in SPI-3 are usually involved in intestinal colonisation and intracellular survival due to the presence of *mgtBC* genes, which encode for magnesium transport into cells (39). The SPI-4, which encodes a protein involved in non-fimbrial adhesion, is responsible for the intestinal phase of the disease. SPI-5 carries genes that are usually co-regulated with either genes from SPI-1 or SPI-2 (such as *avrA*, which is a putative inner membrane protein, and *sopB*, which encodes inositol phosphatase) that have been implicated with the ability to invade *HeLa* cells (26, 39, 40). Presently, SPI-2, SPI-3, SPI-4 and SPI-5 were detected in *S.* Waycross ST2460, *S.* Waycross ST3691 and also (with the exception of SPI-3) in *S.* Wien ST2460.However, these SPIs were not present in *S.* Wien ST3691 as well as in serovar 42:r:- and serovar Fulica of subspecies *salamae* (Table 1). The presence of principal pathogenicity islands with associated virulence genes in *S.* Waycross and *S.* Wien is an indication of their potential pathogenic compared with serovars in subspecies *salamae*; SPI-5, which usually carries virulent genes like *pipABC* that encode effector proteins, has been previously reported in *Salmonella* strains (27, 41). Therefore, the present results from this study provide an evidence that most of virulent genes with public health implications are found in *Salmonella* serovars of subspecies *enterica*, but not in serovars of subspecies *salamae*. The common pathogenicity island found in all strains was

C63PI which normally encoded by virulent genes *sitABCD* responsible for the iron uptake system in *Salmonella* spp. (42, 43). The lack of the most virulent genes in serovars of subspecies *salamae* in the present study is the reason why the serovars in this subspecies should not be considered as potential non-typhoid *Salmonella* of public health importance.

The only antimicrobial resistance encoding gene that was detected in the strains we examined was *aac(6')-laa*, which encodes resistance for aminoglycoside antibiotics e.g. gentamicin. This gene did not express phenotypic resistance to *S. enterica* subsp. *salamae* ser. 42:r:- and *S.* Waycross. However, the isolates showed resistance to macrolide antibiotics i.e. azithromycin (21). The reason for the lack of phenotypic resistance of the isolates could be due to various factors including the concentration and quality of the antimicrobial discs used as previously described (44) or due to intrinsic factors e.g. nutrient contents, A_w and pH that inhibited the expression of the resistance genes (45). The resistance to macrolide antibiotics reported in this study could be associated with unknown chromosomal mutations detected in our strains. Mutation in base pair in the region of 16S rRNA, and a macrolide-lincosamide-streptogramin type B resistance mutation in the region of 23S rRNA were detected, similar to a prior study described by Sigmund et al. (46). The plasmid replicon type IncFII (SARC14) detected in *S.* Waycross ST3691 and *S.* Wien ST3691 was not linked to either antimicrobial resistance or virulence encoding genes, because none of these genes were present in this plasmid.

The genome-wide comparisons of our isolates against *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis revealed that *S. enterica* subsp. *salamae* possessed specific genetic traits of *esp* genes cluster, *ompT* and lysine methyltransferase (Fig. 4). The *esp* genes region encodes an autotransporter, while the *ompT* protease is associated with substrate specificity as it encodes the omptin outer membrane serine, which cleaves host cationic antimicrobial

peptides and is play essential roles in immune defences against pathogens (47). The reported genes (*esp* and *ompT* as well as lysine methyltransferase) in subspecies *salamae* have been described in *E. coli* and other Gram-negative bacteria, where they are involved in biofilm formation, which is for protection harsh conditiona and toxic compounds (47, 48). Therefore, the genes found in serovar 42:r:- and serovar Fulica are involved in host adaptation and survival in the aquatic environment. In addition, we performed a genomic comparison between serovars of subspecies *enterica* (*S.* Typhi, *S.* Typhimurium, *S.* Enteritidis, *S.* Weltevreden including *S.* Waycross and *S.* Wien) and serovars of subspecies *salamae* i.e. 42:r:- and Fulica (Fig. 5). The serovars from subspecies *enterica* shared common genes not found in subspecies *salamae*. These include the *cit* genes cluster encoding for citrate uptake, utilisation and transport across the cell membrane of the host cells, *oadAB* genes encoding oxaloacetate decarboxylation and iron uptake that are also essential in adaptations to environmental conditions and *saf* genes encoding putative fimbrial operons in typhoid and non-typhoid *Salmonella* strains (49, 50). The *saf* genes cluster have also been described in *Salmonella* spp. that form biofilms; as with other biofilm producing bacteria, the increased survival of *Salmonella* spp. in a variety of environments including the aquatic environment and enhanced resistance to multiple antimicrobials has been reported by Zeng et al. (51). The genes represented the difference between *Salmonella* serovars in subspecies *enterica* to those of subspecies *salamae*. These genetic specificities that differentiated our strains within subspecies, and also from the host generalist and host specific *Salmonella*, were reflected when they were plotted together in a core genome MLST tree (Fig. 6). We observed a serovar based clustering where *S.* Typhi serovars clustered together close to *S.* Paratyphi A. The explaination could be that both cause related diseases in humans and have the same clinical signs of infection (i.e. typhoid and paratyphoid) (52, 53). *S.* Waycross was closely related to *S.* Wien and both were closely related to *S.* Weltevreden. The clonal relatedness between *S.* Wien and

S. Waycross to the hypothesised aquatic environmental serovar *S.* Weltevreden (6, 19) provides support for our suggestion that *S.* Waycross and *S.* Wien could reside in the aquatic environment. On the other hand, the clustering of host specific *S.* Gallinarum and the host generalist *S.* Enteritidis could possibly reflect that both serovars infects the same host animal (poultry) due to the fact may share the specific genomic features.

Conclusion

We report *S.* Waycross ST 2460 and *S.* Waycross ST3691 of serogroup S, and also *S.* Wien ST240 and *S.* Wien ST3691 with unidentified serogroup. *Salmonella* Waycross strains were clonally related to *S.* Wien. All *S.* Waycross and *S.* Wien harboured SPI-2 to SPI-5 with associated virulence genes, which were not found in serovar 42:r:- and serovar Fulica of subspecies *salamae*. Both serovars 42:r:- and Fulica were ST1208. They were clonally related and clustered together in a tree of SNPs, but 42:r:- belonged to serogroup T, while Fulica belonged to an unidentified serogroup. *S. enterica* subspecies *salamae* carried specific genes essential for their adaptation to the aquatic environment. Public genomic comparisons of our strains and host specific as well as generalist *Salmonella* established serovar based clustering. The antimicrobial resistance gene *aac(6')-laa*, which confers resistance to aminoglycosides, was present in all isolates. *S.* Waycross ST3691 and *S.* Wien ST3691 revealed similar plasmid replicon type IncFII, which was not associated with either resistance or virulence genes. In general, the presently-examined strains do not pose public health concern, compared to other frequently isolated non-typhoid *Salmonella* serovars, such as *S.* Typhimurium and *S.* Enteritidis. The characterised strains lacked principal pathogenicity islands that usually carry potential virulence genes.

MATERIALS AND METHODS

Salmonella isolates, DNA extraction and whole genome sequencing (WGS)

Source of samples

We analysed fourteen *Salmonella* isolates including seven *S.* Waycross and seven *S. enterica* subsp. *salamae* serovar 42:r:- previously obtained from water and Nile perch samples collected from Lake Victoria during February to July 2017. The isolates had been previously serotyped based on the White-Kauffmann-Le Minor (WKL) scheme (54) and tested for antimicrobial susceptibility against fourteen antimicrobial agents (22).

DNA extraction

Overnight *Salmonella* cultures (395 µl) were mixed with 20 µl of Proteinase K (AM2546; Thermo Fisher Scientific, Waltham, MA, USA), heated at 56ºC for 1 hr, and treated with RNase (5 µl) for 10 min at room temperature. The DNA extraction was done using the Maxwell RSC culture cell DNA kit (Promega, Madison, WI, USA) following the manufacturer's protocol. The concentration and purity of the DNA were determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). The obtained DNA was resolved by 1% (w/v) agarose gel electrophoresis in parallel with a 1 kb ladder standard. DNA preparations were stored at -20 °C until further processing. Genomes of selected *Salmonella* isolates were sequenced using a 250 bp paired-end-read format using a Nextera XT kit and the MiSeq instrument (Illumina, Inc, San Diego, CA, USA). Raw reads were submitted to the European Nucleotide Archive.

Genomic data analysis

Genomic characterisation of Salmonella sequences

The sequenced reads were *de novo* assembled using SPAdes assembler v3.9.0 in EnteroBase (55). Serotyping prediction was done using the *Salmonella In Silico* Typing

Resource (SISTR) pipeline (56). The mult locus sequence type (MLST) was determined from EnteroBase based on the Achtman MLST scheme of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) (57). The assembled genomes were submitted to the Center for Genomic Epidemiology (Jyngby, Denmark) for different genomic characterisation using different tools, such as SPIFinder v1.0, https://cge.cbs.dtu.dk/services/SPIFinder/ for the detection of the SPIs, PlasmidFinder v2.0 to determine plasmids as previously described (58), and ResFinder v2.2 to detect antimicrobial resistance genes (59). Selected virulence genes were detected using MyDBFinder v1.2 against a self-gathered database of *Salmonella* virulence genes (https://cge.cbs.dtu.dk/services/MyDbFinder/). In addition, chromosomal point mutations were detected using PointFinder. An identification threshold of 95% and minimum length coverage of 60% were selected for all categories analysed.

Phylogenetic relatedness of Salmonella strains

We investigated the genetic diversities among and between the sequenced *Salmonella* serovars of subspecies *salamae* and subspecies *enterica*. Serovar 42:r:- of subspecies *salamae* (accession number SAMN02845238) was used as a reference to infer SNPs calling for serovar 42:r:- and serovar Fulica. The phylogenetic SNPs tree for *S.* Waycross strains was constructed using *S.* Waycross (accession number SAMN04160804) as the reference. *Salmonella* Wien (accession number SAMN01933185) was used as the reference for phylogenetic tree comparison of *S.* Wien strains. The public genomes for each serovar used in CSI phylogeny trees were selected based on subspecies, serovars, and sequence type similarities corresponding to the strains examined in the present study. *Salmonella* strains selected for the phylogeny trees and their accession numbers are indicated in Supplementary Table S1a, S1b, and S1c.The SNP phylogeny tree was inferred using the CSI Phylogeny v1.4 online tool available at the Center for Genomic

Epidemiology with the use of previously described default options (60). The newick files of the tree were downloaded and edited using the iTOL v4 online tool for the display and annotation of phylogeny tree as previously described (61). The SNPs data are provided in Supplementary Table S2a, S2b and S2c. Moreover, the global genomic comparison scale of our strains was determined. For this, the study strains were subjected to the global phylogenetic context consistent with the selected host generalist *S.* Typhimurium and *S.* Enteritidis, host adapted *S.* Dublin and *S.* Choleraesuis and host specific *S.* Typhi, *S.* Paratyphi A and B, and *S.* Gallinarum. This phylogeny was constructed using core genome (cg) MLST in EnteroBase with the use of *S. bongori* (accession number SAMN02603391) as the reference. Our strains were also compared to host generalist *S.* Typhimurium and *S.* Enteritidis, and host specific *S.* Typhi on a genome-wide scale to identify specific genetic features that characterised them. In a BLAST Atlas analysis, the strains were annotated using Prokka (62), and BLAST Atlas was used in GView to compare genomic features in subspecies *enterica* and subspecies *salamae*. In this comparison, *S.* Typhi was first used as the reference to identify genomic features found in serovars of subspecies *enterica* including *S.* Waycross and *S.* Wien, but not in serovars of subspecies *salamae*. Then, *S. enterica* subsp. *salamae* serovar 42:r:- was used as a reference against all other strains to identify putative genetic elements specific to serovar 42:r:- and serovar Fulica of subspecies *salamae*.

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Table 1: Genomic characteristics of *S. enterica* subsp. *enterica* (*S.* Waycross and *S.* Wien) and *S. enterica* subsp. *salamae* (42:r:- and Fulica)

Strain ID	Serovar	ST	Group	*Salmonella* Pathogenicity Island (SPIs)	Virulence genes corresponding to SPIs
A8	*S.* 42:r:-	1208	T	SPI-9, C63PI	*sitABCD*
A9	*S.* 42:r:-	1208	T	C63PI	*sitABCD*
B6	*S.* 42:r:-	1208	T	C63PI	*sitABCD*
D5	*S.* 42:r:-	1208	T	SPI-9, C63PI	*sitABCD*
D6	*S.* 42:r:-	1208	T	C63PI	*sitABCD*
I1	*S.* 42:r:-	1208	T	C63PI	*sitABCD*
A7	*S.* Fulica	1208	-	SPI-9, C63PI	*sitABCD*
C2	*S.* Waycross	2460	S	SPI-2, SPI-3, SPI-4, SPI-5, SPI-13, C63PI	*spiABC; mgtBC; avrA; pipABCD; sopB; siiABCDEF; gtrAB; gacD; sitABCD*
D3	*S.* Waycross	2460	S	SPI-2, SPI-3, SPI-4, SPI-5, SPI-13, C63PI	*spiABC; mgtBC; avrA;pipABCD; sopB; siiABCDEF; gtrAB; gacD; sitABCD*
E5	*S.* Waycross	2460	S	SPI-2, SPI-3, SPI-4, SPI-5, SPI-13, C63PI	*spiABC; mgtBC; avrA; pipABCD; sopB; siiABCDEF; gtrAB; gacD; sitABCD*
E9	*S.* Waycross	2460	S	SPI-2, SPI-3, SPI-4, SPI-5, SPI-13, C63PI	*spiABC; mgtBC; avrA; pipABCD; sopB; siiABCDEF; gtrAB; gacD; sitABCD*
G5	*S.* Waycross	3691	S	SPI-2, SPI-3, SPI-4, SPI-5, SPI-13, C63PI	*spiABC; mgtBC; avrA; pipABCD; sopB; siiABCDEF; gtrAB; gacD; sitABCD*
H6	*S.* Wien	3691	-	SPI-13, C63PI	*gtrAB; gacD; sitABCD*
A1	*S.* Wien	2460	-	SPI-2, SPI-4, SPI-5, SPI-13, C63PI	*spiABC; avrA; pipABCD; sopB; siiABCDEF; gtrAB; gacD; sitABCD*

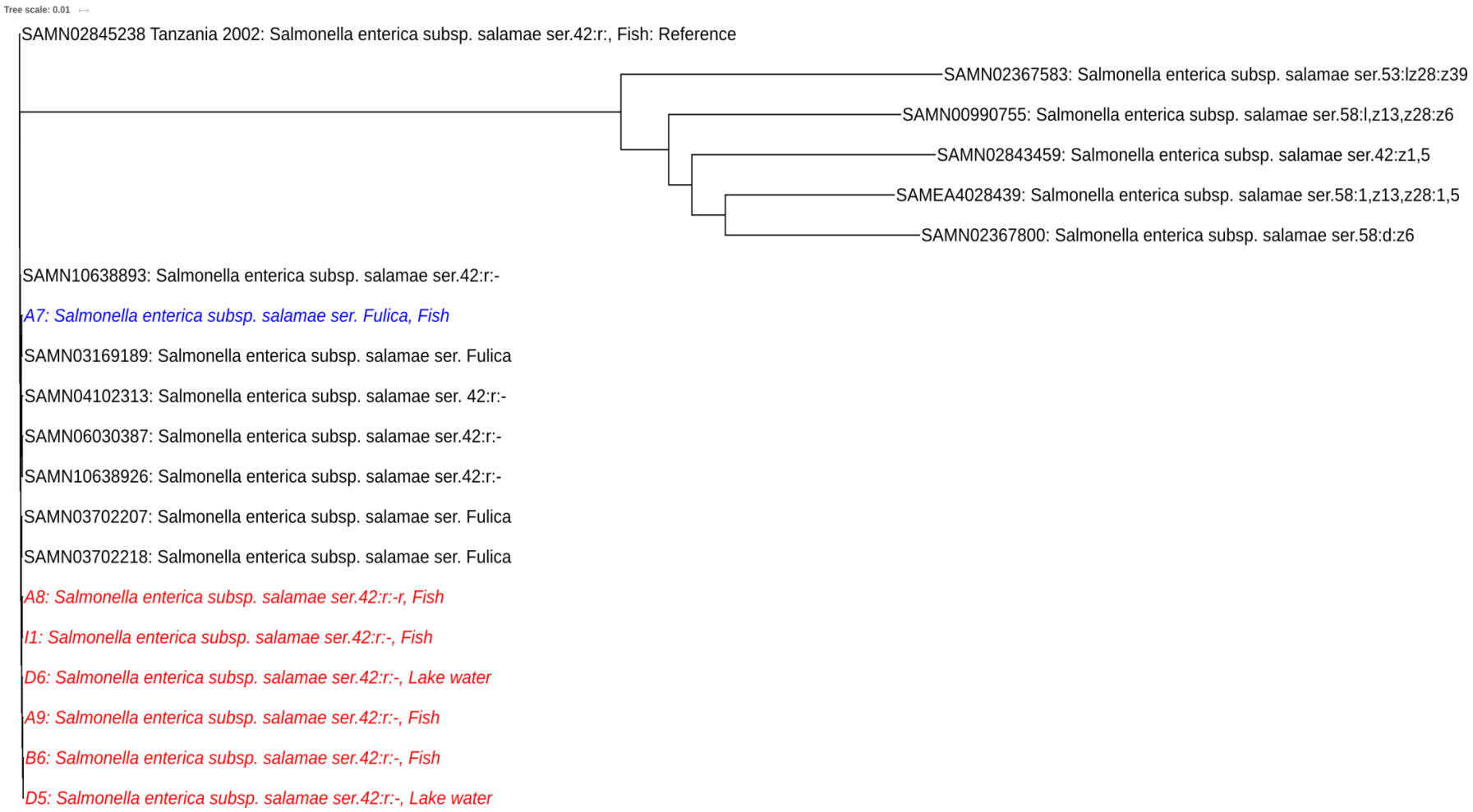

Figure 1: CSI Phylogenetic SNPs tree indicating the genetic relatedness among and between *S. enterica* subsp. *salamae* ser.42:r:- and ser. Fulica

Legend: The coloured indicates the isolates from our study

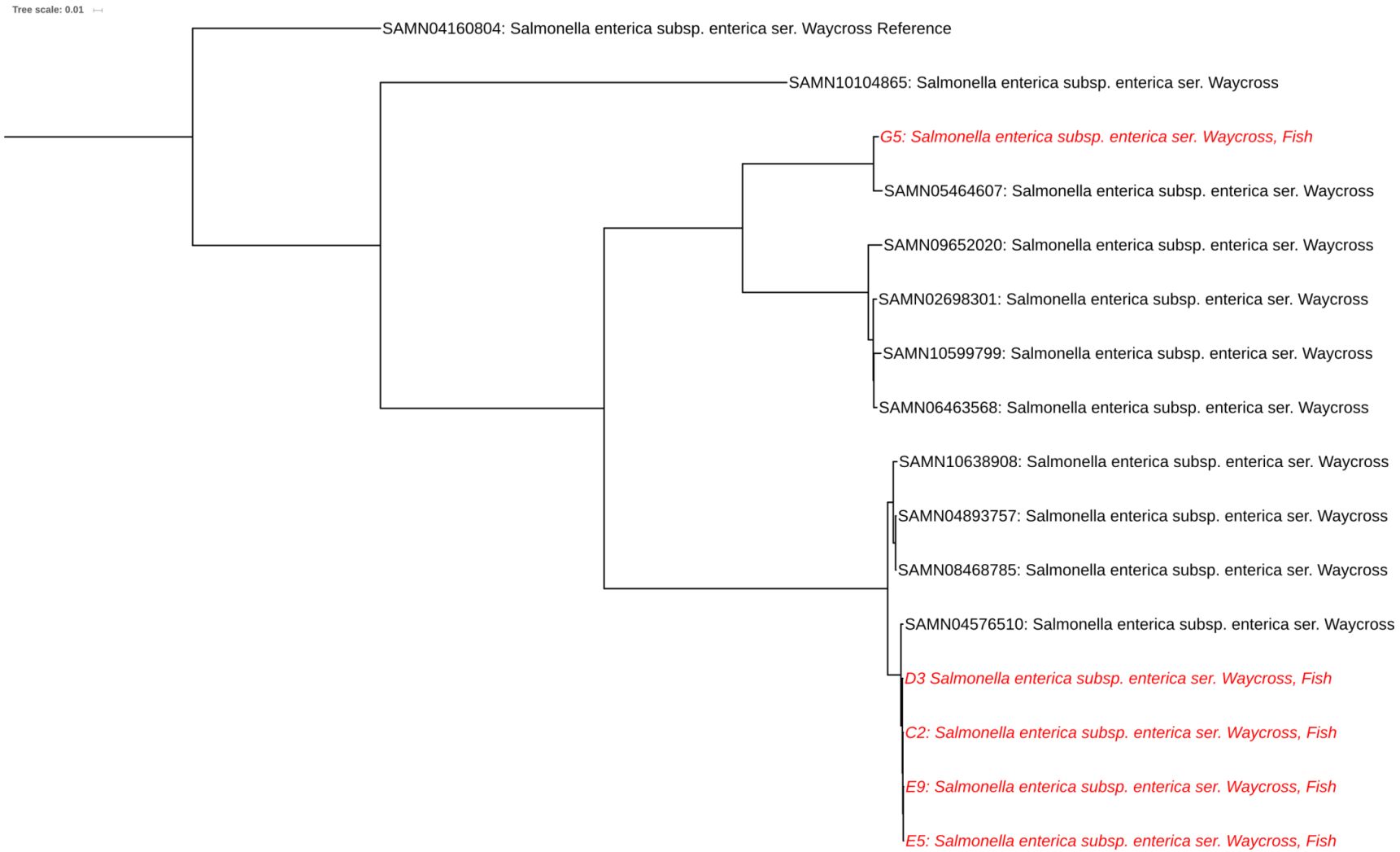

Figure 2: CSI Phylogenetic SNPs tree indicating the genetic relatedness among and between *S.* Waycross

Legend: The coloured indicates the isolates from our study

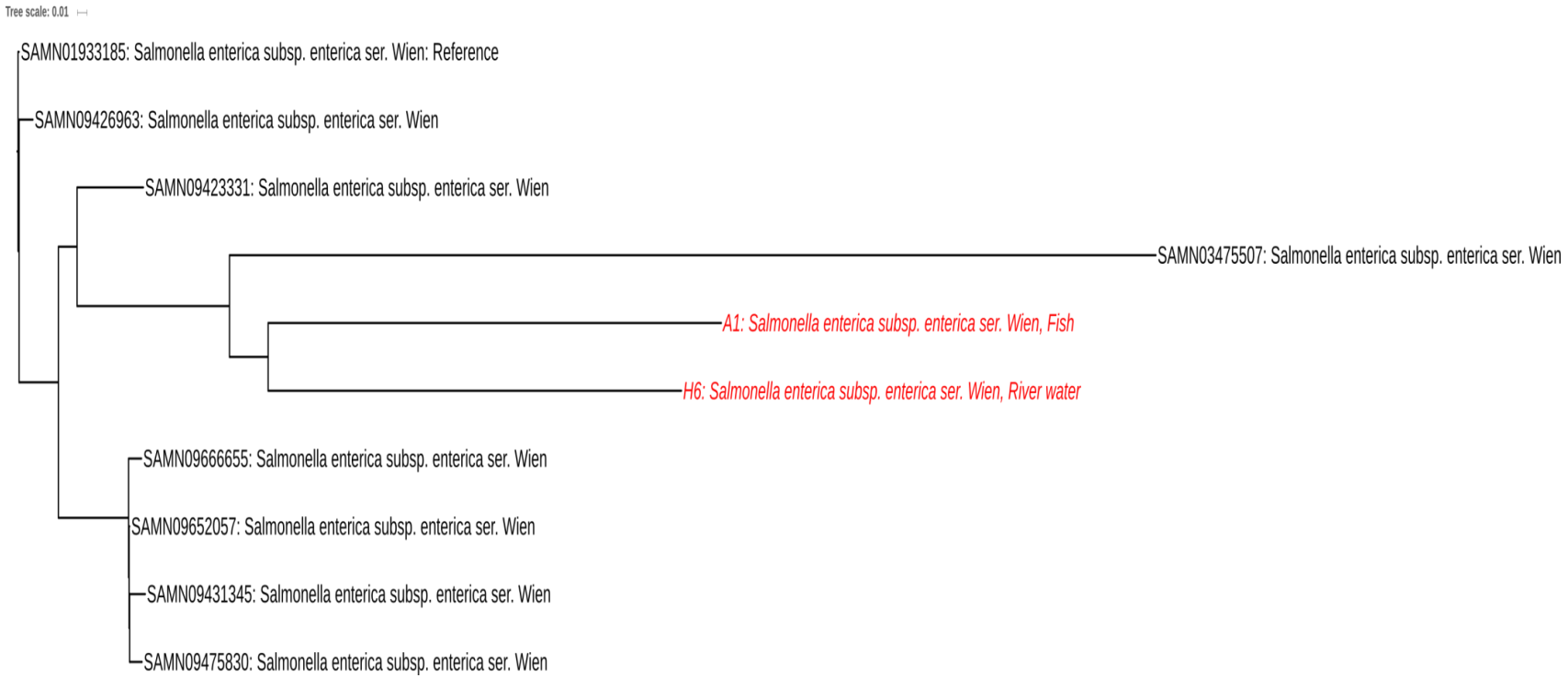

Figure 3: CSI Phylogenetic SNPs tree indicating the genetic relatedness among and between *S.* Wien

Legend: The coloured indicates the isolates from our study

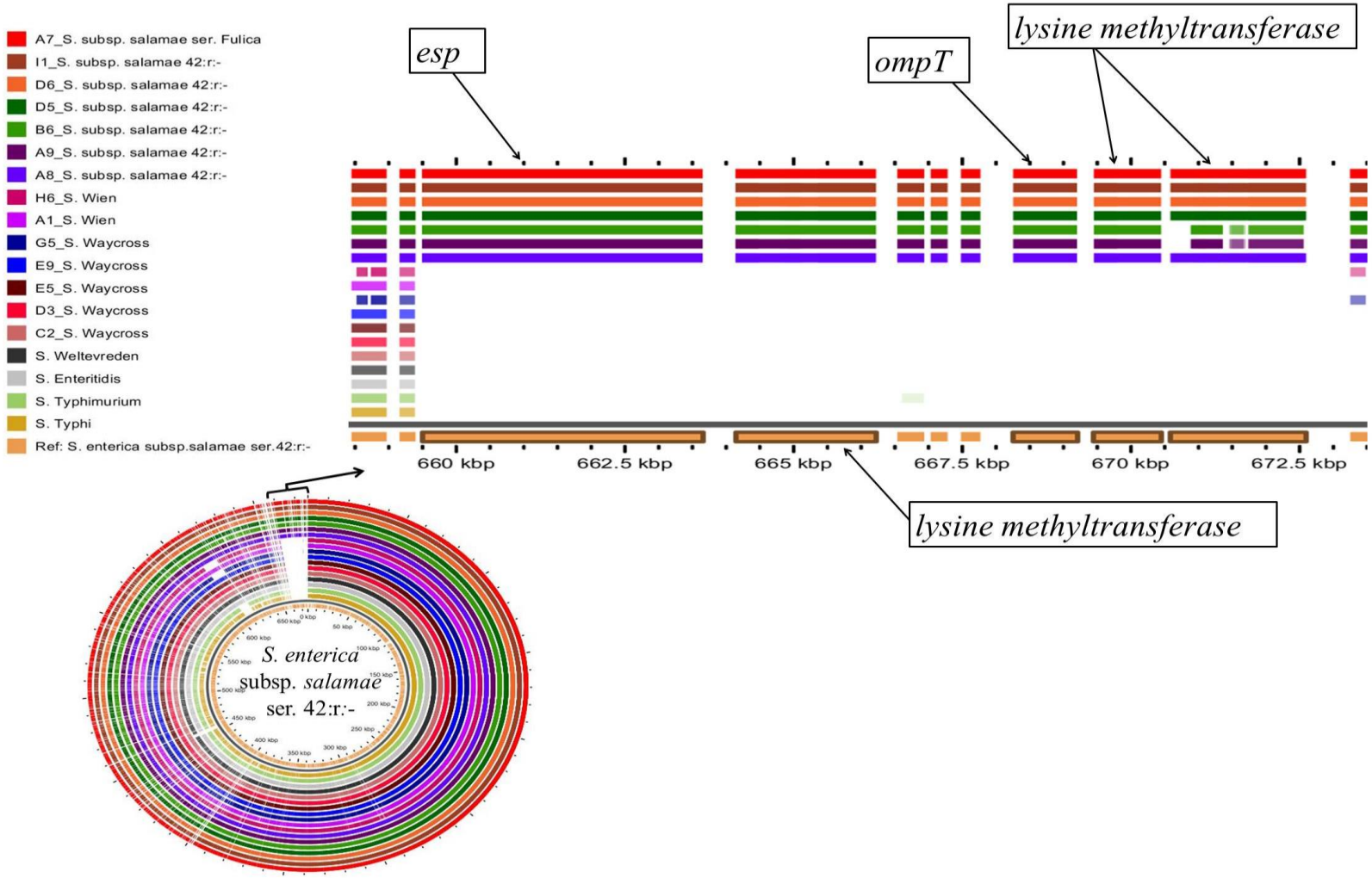

Figure 4: Genomic comparison of our strains showing genes specific for *S. enterica* subsp. *salamae*

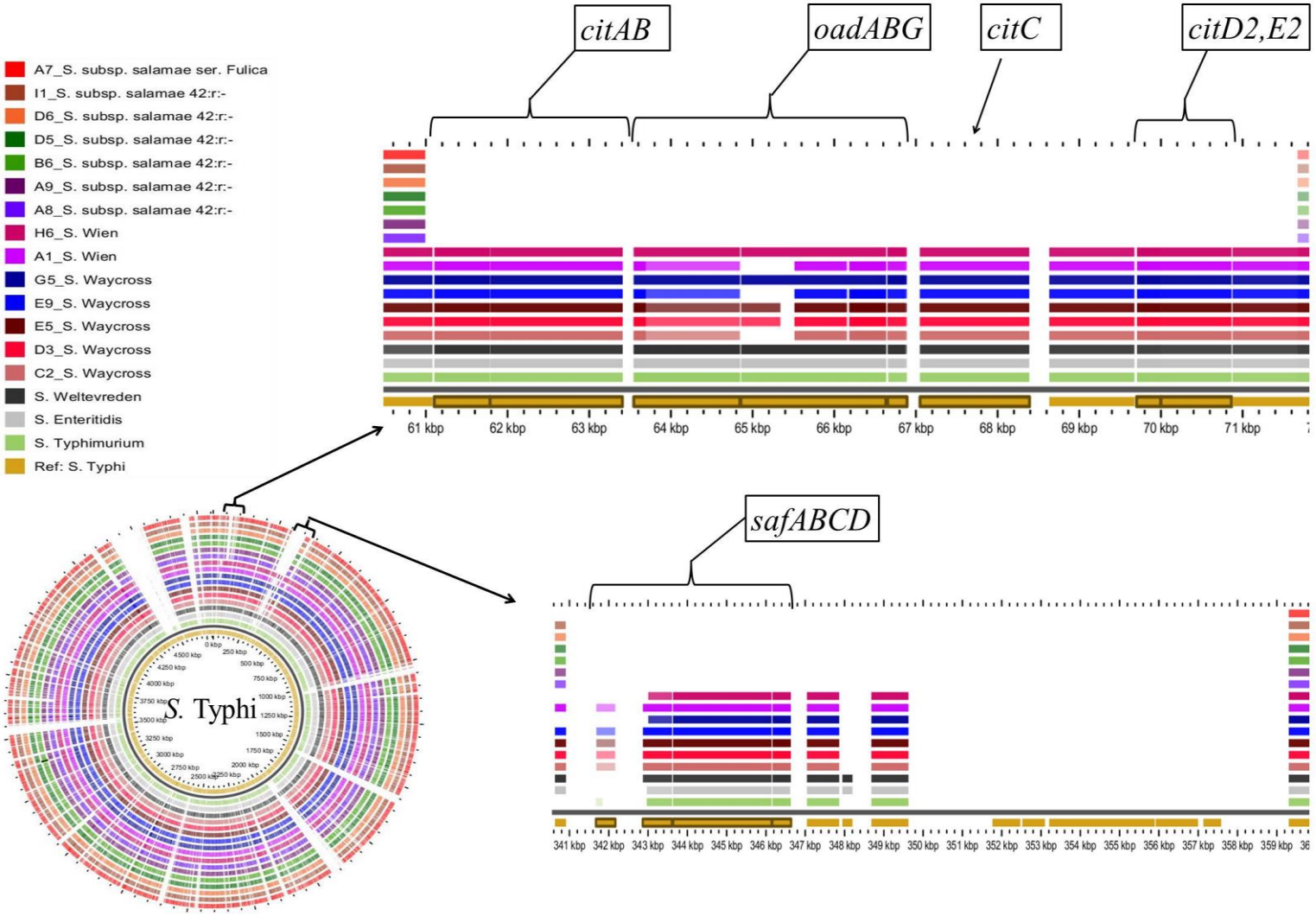

Figure 5: Genomic comparison of our strains showing genes specific for *S. enterica* subsp. *enterica* (*S.* Waycross and *S.* Wien)

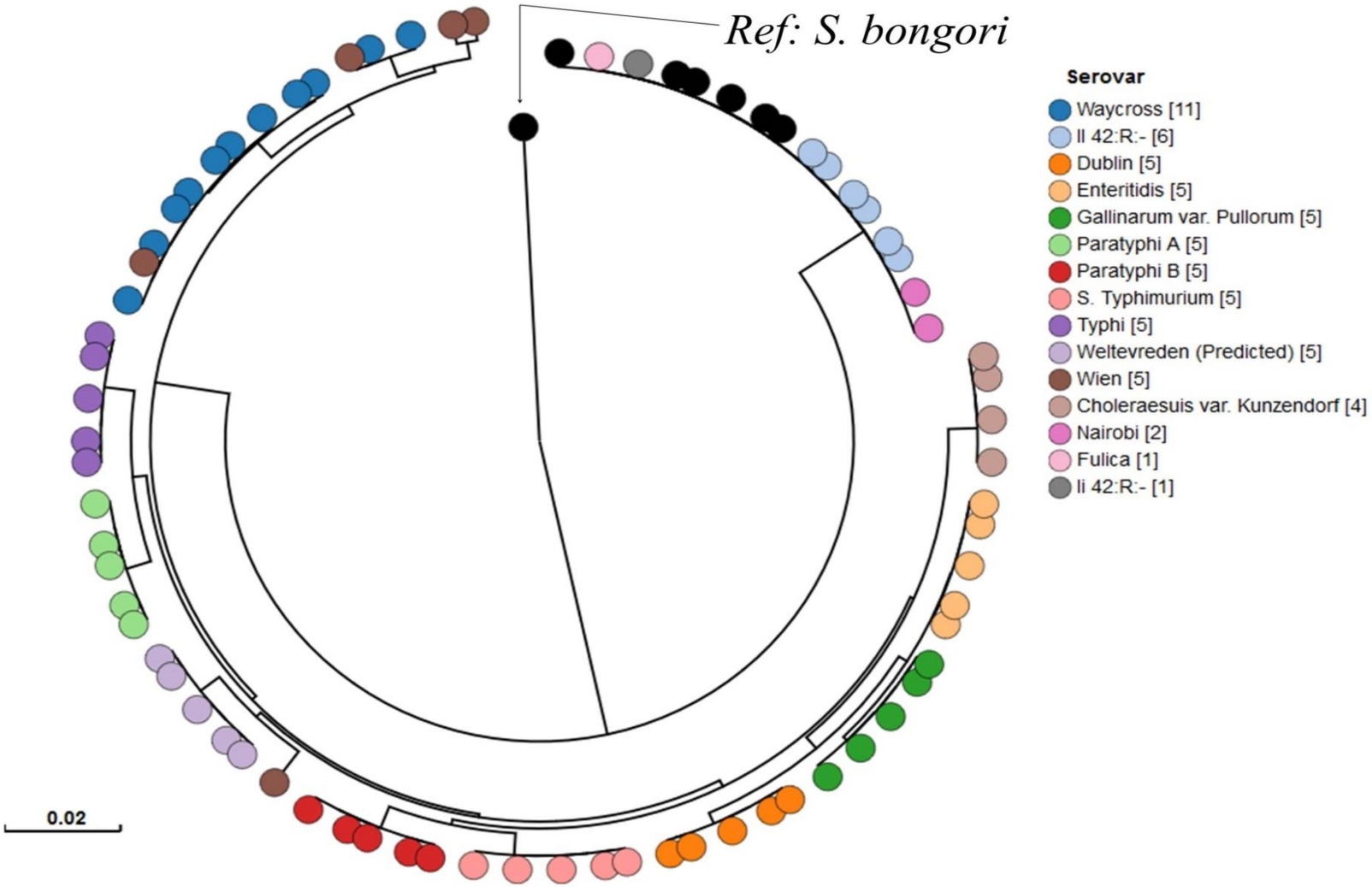

Figure 6: Public genomic comparisons of our strains, host specific, host adapted and generalist *Salmonella* serovars based on cgMLST

PAPER THREE

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Genomics of ESBL-producing *Escherichia coli* in the aquatic environment and Nile perch (*Lates niloticus*) of Lake Victoria, Tanzania

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Running Title: Genomics of ESBL-*E. coli* in Lake Victoria

ABSTRACT

Extended Spectrum Beta-Lactamase (ESBL) producing bacteria constitute an emerging global health issue with food products being vehicles of transmission and the aquatic environments serving as potential reservoirs. This study aimed to characterize ESBL-producing *E. coli* in Nile perch and water from Lake Victoria in Tanzania. A total of 180 samples of Nile perch and 60 water samples were screened for ESBL-producing *E. coli* on MacConkey agar supplemented with 2 µg/ml of cefotaxime and confirmed by $bla_{\text{CTX-M}}$ and bla_{TEM} PCR. Antimicrobial resistance was determined by the disc diffusion method and the ESBL-producing isolates were whole genome sequenced (WGS). ESBL-producing *E. coli* were detected in eight of the 180 analysed Nile perch samples and only one water sample was positive (1.7%, n=60). Isolates were resistant to sulphamethoxazole-trimethoprim (100%), ampicillin/cloxacillin (100%), erythromycin 72.7% (8/11), tetracycline 90.9% (10/11) and nalidixic acid 63.6% (7/11). This mostly corroborates the resistance genes that they carried for sulphonamides (*sul1* and *sul2*), trimethoprim (*dfrA* and *dfrB*), aminoglycosides (*aac(3)-IId*, *strA* and *strB*), tetracycline (*tet(B)* and *tet(D*) and fluoroquinolones (*qepA4*). They harboured plasmid replicon types IncF, IncX, IncQ, Col and carried $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ genes generally found on the same contigs as the IncF plasmid replicon. Although epidemiologically unrelated, the strains formed three separate sequence type-phylogroup-serotype specific clades: C1, C2 and C3. Clade C1 included five strains (13 SNPs maximum) belonging to ST167, phylogroup A, and serotype O9:H21; the two C2 strains (11 SNPs maximum) belong to ST156, phylogroup B1, and serotype ONT:H28, and C3 was made of four strains (17 SNPs maximum) of ST636, phylogroup B2, and serotype O45:H7. The common virulence gene *gad* was reported in all strains. In addition, strains in C2 and C3 possessed *iss*, *lpfA* and *nfaE* virulence genes and the *vat* gene was found only in C3. The present study reports

the occurrence of multidrug resistant ESBL-producing *E. coli* carrying plasmid-mediated ESBL genes in off-shore water and Nile perch in Lake Victoria. Strains formed three clonal clusters of unknown origin. This study reveals that the Lake may serve as reservoir for ESBL-producing bacteria that can be transmitted by fish as a food chain hazard of One-Health concern.

Keywords: Nile perch; food safety; ESBL; antimicrobial resistance; *Escherichia coli*

INTRODUCTION

E. coli and other related bacteria can produce extended-spectrum β-lactamase (ESBL) enzymes that hydrolyse a broad spectrum of β-lactam drugs such as cephalosporins (e.g. ceftazidime, cefotaxime) and monobactams (e.g. aztreonam, nocardicin); classes of antimicrobials which are critical in human medicine (Adelowo et al., 2018; Lavilla et al., 2008; Shaikh et al., 2015). ESBL-producing Gram-negative bacteria especially those producing CTX-M enzymes, have emerged as important pathogens causing healthcare as well as community-associated infections worldwide.

Studies in many different countries increasingly document how foods may be important sources of ESBL-producing *E. coli*, such as vegetables, poultry, pork and other animal foods. In particular poultry meat has been reported commonly to be associated with ESBL-producing *E. coli* globally (Chishimba et al., 2016; Falgenhauer et al., 2019; Nguyen et al., 2016; Overdevest, 2011; Projahn et al., 2019). Possible horizontal transfer of resistance genes from urban sewage and clinical isolates to bacteria associated with fish and aquatic recipients of wastewater have been documented (Blaak et al., 2014; Jiang et al., 2013; Kümmerer, 2009; Martinez, 2009).

The β-lactamase enzymes are derived from mutations in temoneira (TEM), sulphydryl variable (SHV) and cefotaximase-Munchen (CTX-M) genes located on bacterial plasmids or chromosomes (Adelowo et al., 2018; Ben Said et al., 2015; Legese et al., 2017). These genes can easily be horizontally transferred from one bacterial strain to another including across bacterial species (Lavilla et al., 2008). The SHV enzyme seems more dominant in *Klebsiella pneumoniae* among the *Enterobacteriaceae* (Rupp and Fey, 2003). The CTX-M enzymes are often the most common ESBLs produced by *E. coli*, and have been isolated in human clinical, animal food and environmental samples (Boonyasiri et al., 2014; Dib et al., 2018; Egea et al., 2012). On the other hand, the TEM-enzymes especially of TEM-1 and TEM-2 are predominantly reported in *E. coli* and other members of *Enterobacteriaceae* such as *Klebsiella* spp. and *Enterobacter* spp. isolated in both food, clinical and environmental samples around the world as previously described (Smet et al., 2010; Delgado et al., 2016).

The reservoirs of ESBL-producing *E. coli* are warm blooded animals including humans which can transmit the bacteria to different environments, e.g. aquatic environments, through faecal pollution (Jiang et al., 2012). Recently, studies have reported ESBL-producing *E. coli* in seafood such as shrimps, sardines, farmed fish, Nile tilapia (*Oreochromis niloticus*) as well as in frozen mackerel (Dib et al., 2018; Jiang et al., 2012; Moremi et al., 2016b; Nasreldin and Khaldoon, 2015). It has been documented that urban sewage, e.g. from hospitals is an important source of ESBL-producing enteric bacteria (Abgottspon et al., 2014; Ojer-Usoz et al., 2013). Thus, ESBL-producing bacteria in sewage and in run-off water from agricultural soil fertilised with livestock manure can enter the aquatic environment where antimicrobial resistance genes may be transferred

horizontally to the ubiquitous bacterial flora although the rate and health importance of such transfer is unknown.

In Tanzania, ESBL-producing *E. coli* have been isolated from different sources such as human specimens, foods, and aquatic environments. The ESBL-producing *E. coli* have been reported in humans in different regions of Tanzania (Katakweba et al., 2018; Moyo et al., 2010; Seni et al., 2016) and also in livestock and poultry (Katakweba et al., 2018), the importance of livestock and their meats as sources of ESBL-producing *E. coli* has also been reported (Mwanyika et al., 2016). ESBL-producing *Enterobacteriaceae* recovered from the aquatic environments and tilapia from Lake Victoria were previsouly characterized (Moremi et al., 2016b). This study was carried out with tilapia, which is a fish species commonly found in shallow water which often is polluted by discharged human wastes of different sources mostly of faecal origin. Thus, a variety of ESBL-producing bacteria was found in the samples analysed by Moremi et al. (2016b). Our study investigated the occurrence of ESBL-producing *Enterobacteriaceae* spp. along the chain from capture to market including water and Nile perch at offshore deep water fishing areas.We further applied whole genome sequencing (WGS) which provides important information about antimicrobial resistance and virulence genes in bacterial pathogens and is a powerful molecular tool in investigations of disease outbreaks.

The aim of this study was to determine the occurrence and genomic characteristics of ESBL-producing *E. coli* isolated in water at off-shore fishing grounds and in Nile perch (*Lates niloticus*) from Lake Victoria, Tanzania.

MATERIALS AND METHODS

Study design, sample collection and processing

The study employed a cross-sectional design and was conducted from February to July, 2017. A total of 240 samples of water and Nile perch were collected and analysed for ESBL-producing *Enterobacteriaceae* and the isolates were tested for antimicrobial resistance. This sample size was calculated based on the estimated prevalence of ESBL-producing *Enterobacteriaceae* spp. detected in other fish spp. from Lake Victoria reported by Moremi et al. (2016b) using the following formula:

$$n = \frac{(Z_{\alpha})^2 \times P(1-P)}{d^2}$$

Out of 240 samples, 180 were Nile perch; and were collected from fishing grounds (60), landing sites (60) and domestic fish markets (60). The size of fish sampled were ranging between 1 to 2 kg by weight. The selection of Nile perch samples at markets was based on the availability of fish from vendors. At each market ten samples were collected from five retailers, with two fish purchased from each vendor. We did not register how many Nile perch were on sale at each market. Details on sample collection, transport, and processing are provided by Baniga et al. (2019). Nile perch samples were prepared by weighing intestines, gills, flesh and fish surface mucus in a ratio of 1:9 to Buffered Peptone Water (BPW) (Oxoid Ltd, Hampshire, England). Fish intestines, gills and flesh (15 g to 25 g volumes) were homogenised for 60 sec in a stomacher (Seward 400, UK) while fish mucus were obtained and analysed by massaging the fish surface in a sterile stomacher bag containing 225 ml BPW. Stomacher bags used to obtain the surface mucus of fish were of 16063/0606 size i.e. Stomacher® Bags, Seward Genuine 16063/0606, (www.seward.co.uk). The collection of fish mucus sample was done while fish was still intact before other sub-samples i.e. flesh, gills and intestines were collected. All fish samples were processed following standard method (ISO 6887-3, 2003). Twenty-five ml

of each water sample were filtered using a standard filter membrane of 0.45 µl pore size (Thermo Fisher Scientific, Waltham, USA). After filtration, the filter membrane was torn into pieces which were placed into 225 ml of BPW which were shaken rigorously and then incubated at 37°C overnight for enrichment before plating out on the MacConkey agar. Thus, the limit of detection was 4 cfu ESBL-producing *E. coli* per 100 ml. Water samples handling and processing was done according to ISO and Tanzanian standards incorporated with some modifications (ISO 9308-1, 2000; TZS 117:1981).

Study site

The study was conducted along the Tanzanian basin of Lake Victoria in Mwanza. Sampling points included fishing grounds (open water), landing sites and domestic fish markets located in Ilemela and Nyamagana districts in the Mwanza region. At the fishing grounds, there were no other activities besides fishing and there were no toilets facilities on board. At some of the landing sites, there were households nearby and a range of different human activities including agriculture and scavenging animals, e.g. cattle, goats, dogs around the landing sites. Also, there were several migratory fish-eating birds around the shore of the lake. At every landing site there were toilet facilities, but it is uncertain whether these discharged into the lake. In the domestic markets, a variety of goods were sold and toilets were present at all markets visited during sampling. However, there was poor waste management around the markets due to accumulation and access of wastes in the garbage bins and also lack of proper wastewater discharge. Processing of samples and bacteriological analysis were done at the National Fish Quality Control Laboratory, Mwanza and at the laboratory of the Department of Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture (SUA). The WGS of ESBL-producing

E. coli was done at the Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark.

Identification of ESBL-producing *Enterobacteriaceae*

ESBL-producing *Enterobacteriaceae* in Nile perch and water were screened on MacConkey agar (Oxoid Ltd) supplemented with 2 mg/L cefotaxime (Moremi et al., 2016b). Briefly, 1 ml of an overnight enriched sample in BPW at 37°C, was added to the MacConkey agar plates by the pour plate method and incubated overnight at 37°C (CLSI, 2016). Then, colonies with characteristics of *Enterobacteriaceae* were selected for ESBL confirmation using the double disc synergy method (CLSI, 2016; Drieux et al., 2008). The discs used were ceftazidime (30 µg) and cefotaxime (30 µg) (HiMedia Laboratories Pvt. Ltd Mumbai, India) which were placed 20 mm apart from the centre of a Muller Hinton agar (MHA) plate (Oxoid Ltd) where a amoxicillin/clavulanic acid (20 µg/10 µg) disc was placed. Plates were incubated at 37°C for 24 hrs. An inhibition zone around any of the discs under test increasing towards amoxicillin/clavulanic acid disc was interpreted as a presumptive ESBL-producing isolate and the bacterial species were subsequently identified using API 20E (bioMe´rieux, France) based on the analytical profile index. The confirmed ESBL-producing *E. coli* isolates were further analysed by PCR targeting β-lactamases genes: $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} in a single multiplex reactions using primer sequences described by Nasreldin and Khaldoon (2015) to confirm their ESBL-producing status. The confirmed ESBL-producing isolates were used for further analyses.

Antimicrobial disc susceptibility testing

The ESBL-producing *E. coli* isolates were subjected to antimicrobial susceptibility testing using standard disc diffusion method on MHA following the protocol and recommended

antimicrobials by CLSI (2016). The following antimicrobials were selected: ampicillin/cloxacillin (AX; 10 µg), amoxicillin/clavulanic acid (AMC; 30 µg), gentamicin (GEN; 10 µg), sulphamethoxazole-trimethoprim (SXT; 25 µg), ciprofloxacin (CIP; 5 µg), cefotaxime (CTX; 30 µg), chloramphenicol (CHL; 30 µg), nalidixic acid (NAL; 30 µg), ceftazidime (CAZ; 30 µg), erythromycin (ERY; 15 µg), imipenem (IMP; 10 µg), and tetracycline (TET; 30 µg) (HiMedia Laboratories Pvt.). Although CLSI does not recommend the inclusion of erythromycin and ampicillin/cloxacillin, these antibiotics are comonly prescribed in Tanzania and were therefore included in the testing. *E. coli* ATCC 25922 was used as a quality control.

DNA extraction and whole genome sequencing

DNA was extracted from the eleven ESBL-producing *E. coli* isolates using Maxwell RSC culture cell's DNA kit following the manufacturer's protocol in the automated Maxwell RSC instrument (Promega, Wisconsin, USA). Genomes were sequenced at the 250 bp paired-end-read format using Nextera XT kit and the MiSeq instrument (Illumina, Inc, San Diego, CA, USA). Raw sequence reads have been submitted to the European Nucleotide Archive (ENA) under the project number PRJEB34642 with the accession numbers of each sample available in **Supplementary material Table S1 and S3**.

Data analysis

Data collected were entered and stored into Microsoft Excel version 2010 (Microsoft Ltd, USA) and the prevalence of ESBL-producing *E. coli* in water and Nile perch at each sampling point were determined. *E. coli* genomes were *de novo* assembled using SPAdes 3.9.0 (Bankevich et al., 2012). Further analyses were performed using various web bioinformatics tools from the Centre for Genomic Epidemiology (CGE)

(https://cge.cbs.dtu.dk/services/), Enterobase (http://enterobase.warwick.ac.uk/), and BLASTn at National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Plasmid replicons were detected using PlasmidFinder v2.0 (Carattoli et al., 2014) while virulence genes were determined using VirulenceFinder v.2.0 (Joensen et al., 2014), and *In-Silico* analysis of resistance genes using ResFinder v2.2 (Zankari et al., 2012) from CGE using default settings.

Resistance to heavy metals and detergents was assessed using MyDbFinder 1.2 where our genomes were analysed against plasmid-mediated genes such as the tellurite resistance gene *tehA* (NC_000913.3), the detergent-resistant phospholipase A, *pldA* (NC_003198.1) and the quaternary ammonium compound efflux *qacEdelta* (NG_048042.1) (Moremi et al., 2016b). Further heavy metal resistance operons encoding resistance to copper, cobalt, zinc, cadmium, magnesium, mercury and chromium were analysed through the subsystem annotation in RAST (http://rast.nmpdr.org/rast.cgi) according to Brettin et al. (2015). The contigs harbouring these genes and their positions on the contigs were compared to contigs harbouring plasmids in the strains to confirm their location in the genomes. The location of β-lactamases genes on plasmids or chromosomes were determined by analysing the contigs harbouring the $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ using BLASTn in NCBI (Zhang et al., 2000).

Sequence types of the isolates were detected using *In Silico* MLST typing tool based on the seven housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* for *E. coli* in Enterobase v.1.1.2 (Achtman et al., 2012). The phylogenetic analysis was performed using CSI Phylogeny v1.4 (Kaas et al., 2014) with the default options. The phylogenetic analysis included strains from this study and publicly available genomes of *E. coli* isolated from

humans, foods and the environments of the same sequence types as those identified for our strains. In addition to sequence type similarities, strains with the same serotype as our isolates was an added criterion in selection of public genomes for phylogeny analysis. *E. coli* strain K12 sub-strain MG1655 (accession number SAMN02604091) was used as reference to root the tree. The list of *E. coli* strains used in phylogenetic analysis with their accession numbers are indicated in the table (**Supplementary material Table S1**). The newick file of the tree were downloaded and edited using iTOL v4 (https://itol.embl.de/) (Letunic and Bork, 2016). The pairwise SNPs difference data can be found in a table (**Supplementary material Table S2**).

RESULTS

Prevalence of ESBL-producing *E. coli* in Nile perch and water

The overall prevalence of ESBL-producing *E. coli* in Nile perch was 4.4% (8/180), while only one water sample collected off-shore in the lake contained ESBL-producing *E. coli*. One Nile perch sample 1.7% (1/60) caught at fishing grounds off-shore contained ESBL-producing *E. coli* whereas two fish samples from landing sites contained ESBL-producing *E. coli* 3.3% (2/60) as did five fish samples purchased in domestic fish markets 8.3% (5/60) (**Table 1**).

Genomic characteristics and phylogenetic analysis of ESBL-producing *E. coli* from Nile perch

Analysis of the genome sequences from the eleven ESBL-producing *E. coli* revealed three clades (C1, C2 and C3) separated by unique sequence type, serotype, phylogroup and also virulence and resistance genes patterns (**Tables 2** and **3**). The clade C1 included five clonal related strains (Z1 to Z5) with a maximum of 13 SNPs difference, all belonging to ST167, phylogroup A, and having serotype O9:H21. These strains possessed only one

virulence gene (*gad)* and were isolated from fish and water samples collected at off-shore fishing grounds, as well as from fish purchased at the landing sites.

Two clonal strains (Z8 and Z9) formed clade C2 with 11 SNPs apart. They belong to ST156, phylogroup B1, and showed serotype ONT:H28 and were both isolated from Nile perch obtained at local fish markets. These two strains contained additional virulence genes including *gad, iss, lpfA* and *nfaE*.

Strains Z6, Z7, Z10 and Z11 isolated from Nile perch from fish markets formed the phylogenetic clade C3 and had a maximum of 17 SNPs difference. These four strains belong to ST636, phylogroup B2, and have serotype O45:H7 and harbour the *vat* gene in addition to all the virulence genes present in C2 strains.

In a comparison with *E. coli* isolated from humans, animals and the environment globally, our strains showed a sequence type-based clustering where the public available ST167 genomes clustered with our C1 strains, as did the public available ST156 and ST636 genomes with our C2 and C3 strains, respectively. However, we recorded some wide within-clade variations between our strains and the public genomes of the same STs with up to 5104, 2440 and 1391 SNPs difference in C1, C2 and C3, respectively (**Figure 1** and **Supplementary material Table S2**).

Antimicrobial resistance, plasmid replicon profiles, heavy metal and detergent resistance

The phenotypic resistance patterns, as well as occurrence of resistance genes and plasmid replicon types in the eleven ESBL-producing *E. coli* showed a similar clustering pattern as shown above (**Tables 3** and **4**; **Supplementary material Table S3**). C1 strains showed

phenotypic and genotypic resistances to aminoglycosides (*aadA2*, *aac(3)-IId*), sulphonamide-trimethoprim (*sul1*, *sul2*, *dfrA12*), floroquinolone (*qep*A4), tetracycline (*tetB*, *tetD*), macrolides (*mphA*, *mdfA*), chloramphenicol (*catA1*) and the beta-lactamases ampicillin/cloxacillin and cefotaxime (CTX-M-15, TEM-1B). However, strains Z1, Z2 and Z4 of C1 did not show phenotypic resistance to erythromycin despite the presence of *mphA* and *mdfA* conferring resistance to macrolides. All C1 strains have the plasmid replicon types IncFIA, IncFIB, IncFII, IncX1, Col8282 and Col156 with the pMLST of the IncF plasmid hosting the $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ genes being IncF [F48: A1: B49]. Strains Z8 and Z9 of clade C2 showed resistance to sulphonamide-trimethoprim (*sul1*, *dfrB4*), floroquinolone (*qepA4*), tetracycline (*tetB*), macrolides (*mphA*, *mdfA*), chloramphenicol (*catA1*) and the beta-lactamases ampicillin/cloxacillin and cefotaxime (CTX-M-15, TEM-1B). They were susceptible to aminoglycosides and only possessed one plasmid replicon type Col440I harboring the beta-lactam genes.

The C3 strains were susceptible to floroquinolones and chloramphenicol but showed resistance to aminoglycosides (*aadA1*, *strA*, *strB*), sulphonamide-trimethoprim (*sul2*, *dfrA1*), tetracycline (*tetB*), macrolides (*mphA*, *mdfA*), and the beta-lactamases ampicillin/cloxacillin and cefotaxime (CTX-M-15, TEM-1B). Despite the presence of *tetB*, strain Z6 did not show phenotypic resistance to tetracycline. All C3 strains have plasmid replicon types IncFIA, IncFIB, IncFII, IncQ1, ColRNAI and Col (BS512) with the pMLST of the IncF harbouring the $bla_{\text{TEM-1B}}$ gene being IncF [F1:A1:B1] and they carried the $bla_{\text{CTX-M-15}}$ on their chromosome. Strains in all three clades were sensitive to imipenem.

All clade C1 and C2 strains harboured the plasmid-mediated detergent resistance gene, *qacEdelta*, encoding resistance to quaternary ammonium compounds. All the strains

contained resistance genes to copper, cobalt, zinc, cadmium, magnesium, mercury and tellurite which were chromosomally located (**Supplementary material Table S4**). However, chromium resistance was only recorded in strains of clade C1 isolated from fishing grounds and landing sites.

DISCUSSION

The occurrence of ESBL-producing *E. coli* was low in Nile perch and water samples collected at off-shore fishing grounds and at landing sites of Lake Victoria, but higher in fish from local fish markets in Mwanza. ESBL-producing *E. coli* have been reported in different wild-caught fish species in Switzerland, Algeria and India (Abgottspon et al., 2014; Dib et al., 2018; Singh et al., 2017), as well as in farmed fish in China (Jiang et al., 2012) and Egypt (Ishida et al., 2010). Moremi et al. (2016b) reported a slightly higher prevalence of ESBL-producing bacteria in wild-caught tilapia sold at markets in Mwanza (13.3%) as compared to our findings in Nile perch (8.3%). Diverse bacterial species were found in the tilapia (Moremi et al., 2016b) where as only *E. coli* was isolated in our Nile perch. This is probably because tilapia is usually found in shallow water with relative high levels of faecal pollution and hence higher possibility of finding a variety of *Enterobacteriaceae* than finding these bacteria in the Nile perch which are caught in deep-water with lower pollution levels. Thus, these studies suggest that the aquatic environment and fish are likely sources and dissemination routes for ESBL-producing bacterial species and their resistance genes although their relative importance from a public health perspective is uncertain.

The low prevalence of ESBL-producing *E. coli* in Nile perch from fishing grounds is likely due to the fact, that these fish are commonly found and caught in deep water far

away from the lake shore where faecal pollution is low compared to the shallow water near the shore of the lake (Baniga et al., 2019). Any occurrence of ESBL-producing *E. coli* in Nile perch caught off-shore could be attributed to their eating habits feeding on other fish species, e.g. tilapia and sardines which are found in shallow water with relatively higher faecal contamination levels. This hypothesis is corroborated by the findings of Moremi et al. (2016b) where gut intestinal samples from tilapia contained ESBL-producing bacteria. Since boats of artisanal small-scale fishers do not have toilet facilities, the fishermen have to defecate into the lake with a potential introduction of ESBL-producing *E. coli* into the lake environment.

ESBL-producing *E. coli* isolated in Nile perch from markets belonged to clades C2 and C3 and seem to harbour more virulence genes than isolates originating from fish caught off-shore. This higher pathogenicity indicates a human origin and their occurrence in fish sold at markets is likely caused by faecal cross-contamination from fish handlers and vendors. This is further supported by the fact that most of the market strains of C3 belong to phylogroup B2 which is usually comprised of extra-intestinal pathogenic *E. coli* strains (Abgottspon et al., 2014; Huang et al., 2016; Sánchez-Benito et al., 2017). It is important to note that our prevalences of ESBL-producting *E. coli* are generally very low not allowing for any statistical comparison between fish of different origin. Moreover, possible associations between occurrence of ESBL-producing *E. coli* in fish and human handling activities can be better studied through comparative genomic investigations including clinical human isolates and our environmental strains.

The virulence gene *gad* harboured by C1 strains is an important gene for both commensal and pathogenic enteric bacteria especially *E. coli* as the reaction products of *gad* are

essential for survival in an acidic environment and for successful colonization of the host cell (Tramonti et al., 2002). The additional virulence genes shown by C2 and C3 strains included *nfaE* which is essential for diffuse adherence fimbrillar adhesion, *iss* plays a significant role in increased serum survival, and the *vat* gene which is important for vacuolating autotransporter toxin in host cells for pathogenicity processes. These virulence genes were previously reported from *E. coli* in humans as well as from pigs and bovine (Ahmed et al., 2017; Madoshi et al., 2016; Szmolka et al., 2012). The *lpfA* gene is important for long polar fimbriae and has been commonly associated with the ability to invade epithelial cells in animals and humans (Dogan et al., 2012).

Our strains were epidemiologically unrelated but their grouping into three clades with further clustering with other *E. coli* strains of the same STs isolated from human, food and environmental samples around the globe underlines the transmission potential of the ESBL genes across various niches and locations favoured by plasmids that they harbour. Our strains of clade C1 (ST167) belong to the phylogroup A known to contain commensal strains (Abgottspon et al., 2014; Huang et al., 2016; Sánchez-Benito et al., 2017) and this is supported by the presence of only one virulence gene (*gad*). Moremi et al. (2016b) reported a single environmental sample from Mwanza containing an ESBL-producting *E. coli* of ST167, belonging to the newly defined phylogroup E harbouring $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$. This ST167 strain of phylogroup E differed from our strains of ST167 of phylogroup A with up to 5104 SNPs. The variation among strains of the same STs can be attributed to the differences in serotype as well as their genetic content such as the differences in the ESBL genes that they contained. ESBL-producing *E. coli* ST167 have been previously reported isolated in humans in China and Spain carrying $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes (Huang et al., 2016; Sánchez-Benito et al., 2017). This substanciates the

possibilities of horizontal gene transfer of the ESBL genes from food products or the environment to humans where bacterial pathogens can aquire them. Such transmission are favoured by plasmids on which the resistance genes are located (Anjum et al., 2019) as is the case for the *bla*-$_{\text{CTXM-15}}$ and *bla*$_{\text{TEM-1}}$ genes which were located on the IncF plasmid (**Table 4**). We observed the plasmid IncF [F1:A1:B1] within all four C3 strains which had the same ST636 and the same pMLST and showed a maximum of 17 SNPs difference. The C3 strains all came from intestinal samples and we cannot explain their clonal nature as the origin and previous exposures of the fish purchased at the markets are unknown. The IncF [F48:A1:B49] was shown in all five C1 strains originating from intestinal and surface mucus samples of fish from one landing site as well as from fish (gills) and water collected off-shore. Similarily to C3 strains, the C1 strains were epidemiologically unrelated and we are not able to explain the clonal nature of the C1 strains. In contrast to our study, the ESBL-producing bacteria reported in tilapia from the Mwanza region by Moremi et al. (2016b) included a much more diverse population of bacterial species, a variety of ESBL genes and no indication of a clonal relationship. The occurrence of genes encoding for resistance to metals such as copper, cobalt, zinc, cadmium, and mercury, and also genes encoding resistance to detergents, is similar to previous findings in fish and aquatic environment of the Lake Victoria; the genes have been reported to play role in environmental persistence support in bacterial survival (Hounmanou et al., 2019; Moremi et al., 2016b). Most of metal resistance genes were located on the chromosome while ESBL-genes were located on the plasmids, and it is uncertain to what extent exposure to metals, e.g. used as livestock feed additives and as pollutants in aquatic environments may play a role as co-selectors of ESBL resistance.

In addition to the ESBL genes, our strains are all multidrug resistant despite their relatively low virulence. The resistance genes found have been frequently reported in Gram-negative bacteria and since the genes are often plasmid-mediated, they may be circulated horizontally among different bacterial species (van Hoek et al., 2011). These additional resistances shown by the ESBL-producing *E. coli* may also reflect the frequent use of antimicrobials reported in human and veterinary medicine in the Mwanza region (Moremi et al., 2016a; Seni et al., 2016). The location of the additional resistance genes requires further analysis and depiction of the genetical environment of $bla_{\text{CTX-M15}}$ genes in the $bla_{\text{CTX-M-15}}$ encoding isolates (Moremi et al., 2016b).

Some strains in C1 and C3 did not show phenotypic resistance to antimicrobials that they harboured resistance genes for i.e. erythromycin and tetracycline. This is an increasing observation in genomic studies and could be due to various factors including the concentration and quality of the antimicrobial discs (Eze et al., 2014) or intrinsinc factors inhibiting the expression of the resistance genes (Weill et al., 2017). This has also been associated with random mutations which could be accumulated in gene sequences encoding resistance to some antimicrobials (Davis et al., 2011; Hussain et al., 2014).

In conclusion, we report a very low prevalence of ESBL-producing *E. coli* in Nile perch from Lake Victoria. Our data suggest that as far as ESBL-producing enteric bacteria are concerned, the consumption of Nile perch represent limited food safety risks compared with other human exposures to ESBL-producing *E. coli* e.g. through direct human-to-human faecal transmission and consumption of livestock meat products. The grouping of the eleven ESBL-producing *E. coli* into three clades each showing identical characteristics, e.g. STs, phylogroup, antimicrobial resistance and virulence genes, is

surprising and we are not able to explain the clonal nature of these clades as the *E. coli* strains were epidemiologically unrelated. All isolates harboured $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes together with additional antimicrobial and detergent resistance genes carried by the common plasmids replicon types IncF, IncX, IncQ, and Col. Further studies are needed to determine the role of fish and aquatic environments as sources of ESBL-producing bacteria and resistance genes including the importance of faecal pollution sources, e.g. discharge of sewage and run-off water from fertilised agricultural soil, as well as the ecology of such resistant bacteria in aquatic environments.

Failure to obtain other *Enterobacteriaceae* spp. than *E. coli* is likely because of the selective isolation procedure used with supplement of cefotaxime to obtain ESBL-producing strains; thus, most of other bacteria present both in deep water and fish from markets were likely sensitive to cefotaxime and did therefore not grow on the MacConkey agar. Although *E. coli* isolated from Nile perch at fish markets were of different sequence types and harboured more virulence genes compared to isolates from deep water, a comparison study with ESBL-producing *E. coli* from clinical specimens with environmental strains may provide further information about transmission with the aquatic environment, fish and human compartments.

ETHICS STATEMENT

The present study required no ethical approval because we did not manipulate any live fish in the study. Fish samples used in the study were fished by fishermen and constitute dead but fresh fish products ready for marketing and consumption. We purchased these fish from the fishermen, in boats at landing sites and in the markets from fish retailers as

indicated in the manuscript like any other fish consumer, then we brought them to the laboratory for analyses.

AUTHOR CONTRIBUTIONS

ZB collected samples, carried out laboratory and data analysis and wrote the draft manuscript. YH participated in laboratory analysis and performed genomic data analysis, editing and critical reviewing of the manuscript. EK participated in genomic data analysis and reviewing the manuscript. LK and RH supervised the study and were involved in reviewing the manuscript and facilitation of resources for the study. AD supervised the study and provided guidance and resources, contributed in genomic data analysis, revised the manuscript and final approval of the manuscript. All authors read and approved the final manuscript for submission.

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SUPPLEMENTARY MATERIAL

Table S1: List of *Escherichia coli* genomes used in the phylogenetic analysis (XLSX)

Table S2: Pairwise SNPs value between strains (XLSX).

Table S3: Genomic characteristics of the eleven ESBL-producing *E. coli* of the study (XLSX)

Table S4: Heavy metals and toxic compounds detected in ESBL-producing *E. coli* (XLSX)

CONFLICT OF INTEREST

No conflict of interest declared among the authors of this paper.

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Table 1. Prevalence of ESBL-producing *E. coli* in water and Nile perch from Lake Victoria, Tanzania

Origin	Sample type	Sub-sample type
Fishing grounds	Nile perch 1/60 (1.7%)	Fish gills 1/60 (1.7%)
	Water 1/60 (1.7%)	N/A[a]
Landing sites	Nile perch 2/60 (3.3%)	Fish intestines 1/60 (1.7%)
		Fish surface mucus 2/60 (3.3%)
Markets	Nile perch 5/60 (8.3%)	Fish intestines 4/60 (6.7%)
		Fish gills 2/60 (3.3%)

[a] N/A = Not applicable

Table 2. Genomic characterisation of ESBL-producing *E. coli* isolated in Nile perch

Code	Origin	Sample type	MLST	Serotype	Phylogroup	Virulence genes[a]
Z1	Landing site	Fish surface	167	O9:H21	A	*gad*
Z2	Landing site	Fish surface	167	O9:H21	A	*gad*
Z3	Landing site	Fish intestines	167	O9:H21	A	*gad*
Z4	Fishing grounds	Water	167	O9:H21	A	*gad*
Z5	Fishing grounds	Fish gills	167	O9:H21	A	*gad*
Z6	Markets	Fish intestines	636	O45:H7	B2	*gad*, *nfaE*, *iss*, *vat*
Z7	Markets	Fish intestines	636	O45:H7	B2	*gad*, *nfaE*, *iss*, *vat*
Z8	Markets	Fish gills	156	ONT:H28	B1	*gad*, *iss*, *lpfA*
Z9	Markets	Fish gills	156	ONT:H28	B1	*gad*, *iss*, *lpfA*
Z10	Markets	Fish intestines	636	O45:H7	B2	*gad*, *nfaE*, *iss*, *vat*
Z11	Markets	Fish intestines	636	O45:H7	B2	*gad*, *nfaE*, *iss*, *vat*

[a] *gad*, glutamate decarboxylase; *nfaE*, diffuse adherence fimbrillar adhesin gene; *iss*, increased serum survival; *vat*, vacuolating autotransporter toxin; and *lpfA*, long polar fimbriae.

Table 3. Phenotypic and genotypic antimicrobial resistance of ESBL-producing *E. coli* isolates[a]

Code	Aminoglycoside	Sulphonamide-Trimethoprim	Fluoroquinolone	Tetracycline	Macrolide	B-lactamases	Chloramphenicol
Z1	GEN/+; *aadA2*, *aac(3)-IId*	SXT/+; *sul1*, *sul2*, *dfrA12*	CIP,NAL/+; *qep*A4	TET/+; *tetB*, *tetD*	-/; *mphA*, *mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z2	-/; *aadA2*, *aac(3)-IId*	SXT/+; *sul1*, *sul2*, *dfrA12*	CIP,NAL/+; *qepA*4	TET/+; *tetB*, *tetD*	-/; *mphA*, *mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z3	-/; *aad*A2, *aac(3)-IId*	SXT/+; *sul1*, *sul2*, *dfrA12*	CIP,NAL/+; *qep*A4	TET/+; *tetB*, *tetD*	ERY/+; *mphA*,*mdfA*	AX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z4	-/; *aadA2*, *aac(3)-IId*	SXT/+; *sul1*, *sul2*, *dfrA12*	CIP,NAL/+; *qep*A4	TET/+; *tetB*, *tetD*	-/; *mphA*; *mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z5	-/; *aadA2*, *aac(3)-IId*	SXT/+; *sul1*, *sul2*, *dfrA12*	CIP,NAL/+; *qep*A4	TET/+; *tetB*, *tetD*	ERY/+; *mphA*,*mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z6	GEN/+; *aadA1*, *strA*, *strB*	SXT/+; *sul2*, *dfrA1*	ND	-/; *tetB*	ERY/+; *mphA*,*mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	ND
Z7	GEN/+; *aadA1*, *strA*, *strB*	SXT/+; *sul2*, *dfrA1*	ND	TET/+; *tetB*	ERY/+; *mphA*,*mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	ND
Z8	ND	SXT/+; *sul1*, *dfrB4*	CIP,NAL/+; *qep*A4	TET/+; *tetB*	ERY/+; *mphA*,*mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z9	ND	SXT/+; *sul1*, *dfrB4*	CIP,NAL/+; *qep*A4	TET/+; *tetB*	ERY/+; *mphA*,*mdfA*	AX/+; CTX-M-15, TEM-1B	CHL/+; *cat*A1
Z10	GEN/+; *aadA1*, *strA*, *strB*	SXT/+; *sul2*, *dfrA1*	ND	TET/+; *tetB*	ERY/+; *mphA*,*mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	ND
Z11	GEN/+; *aadA1*, *strA*, *strB*	SXT/+; *sul2*, *dfrA1*	ND	TET/+; *tetB*	ERY/+; *mphA*,*mdfA*	AX,CTX/+; CTX-M-15, TEM-1B	ND

[a] AX, ampicillin/cloxacillin; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CHL, chloramphenicol; CTX, cefotaxime; ERY, erythromycin; GEN, gentamicin; IMP, imipenem; NAL, nalidixic acid; SXT, sulphamethoxazole-trimethoprim; and TET, tetracycline.
ND, no phenotypic and genotypic resistance; +, phenotypic resistance; -, no phenotypic resistance.

Table 4. Plasmid profiles and location of the β-lactam genes in ESBL-producing *E. coli*

Node	Plasmid replicon types	pMLST	Location of ESBL genes
Z1	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF[F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z2	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF[F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z3	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF[F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z4	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF[F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z5	IncFIA; IncFIB; IncFII; IncX1; Col8282; Col156	IncF[F48:A1:B49]	Plasmid (CTX-M-15: TEM-1B)
Z6	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF[F1:A1:B1]	Chromosome (CTX-M-15)
			Plasmid (TEM-1B)
Z7	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF[F1:A1:B1]	Chromosome (CTX-M-15)
			Plasmid (TEM-1B
Z8	Col440I	N/A	Plasmid (CTX-M-15: TEM-1B)
Z9	Col440I	N/A	Plasmid (CTX-M-15: TEM-1B)
Z10	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF[F1:A1:B1]	Chromosome (CTX-M-15)
			Plasmid (TEM-1B)
Z11	IncFIA; IncFIB; IncFII; IncQ1; ColRNAI; Col (BS512)	IncF[F1:A1:B1]	Chromosome (CTX-M-15)
			Plasmid (TEM-1B)

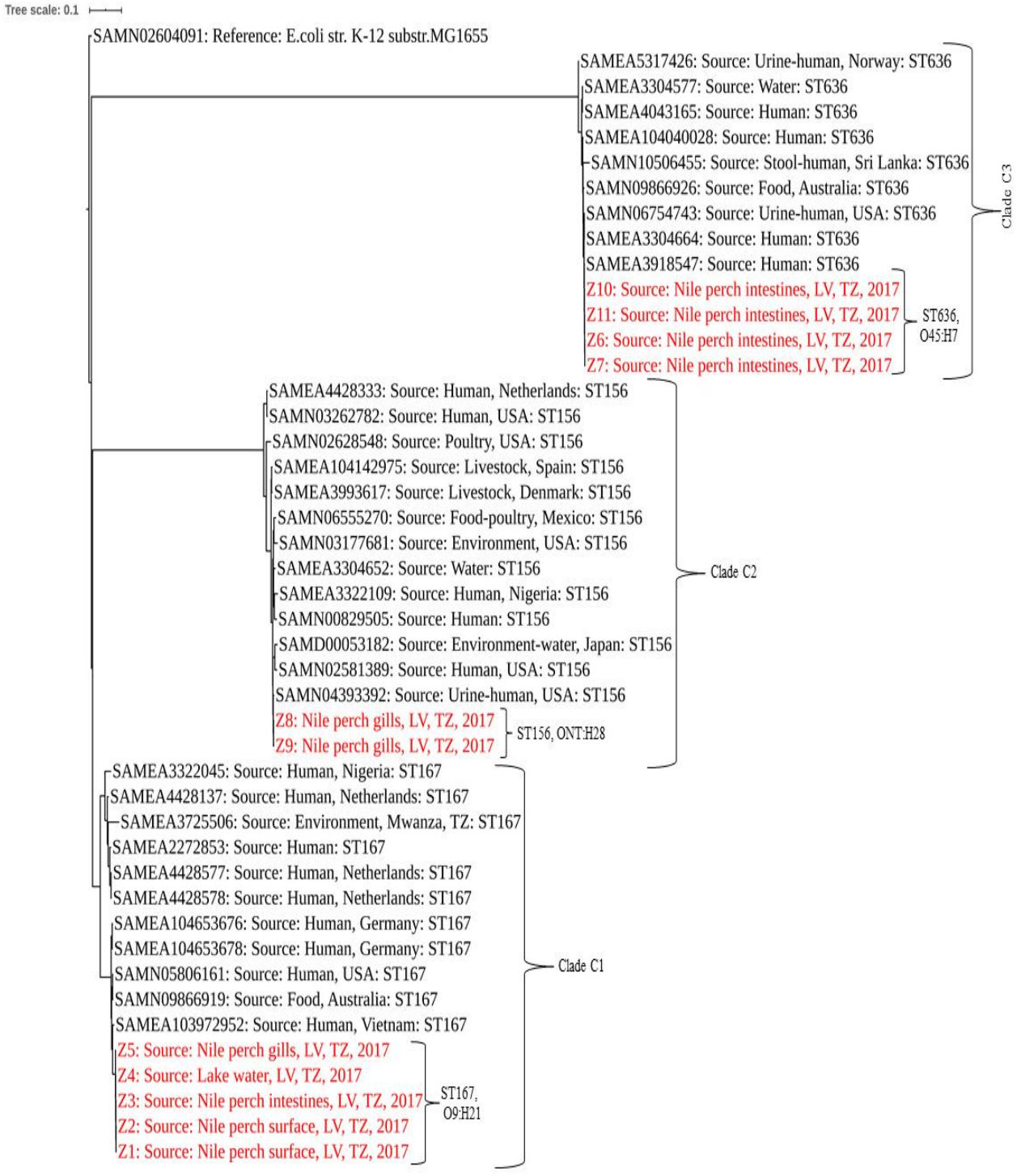

Figure 1: Phylogenetic tree based on SNPs showing clonal relationship among ESBL-producing *E. coli* isolated from Nile perch and global public genomes of *E. coli* strains from enterobase. Our samples have highlighten in red colour

Legend: LV-Lake Victoria, TZ-Tanzania

PAPER FOUR

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Microbial quality of Nile perch (*Lates niloticus*) and physico-chemical properties of salted sun-dried products sold at regional markets, Tanzania

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Abstract

A study was conducted to investigate the microbial and physico-chemical qualities of salted sun-dried Nile perch (*Lates niloticus*) products and frozen fish from various storage facilities in Mwanza, Tanzania. The bacterial flora, moisture contents (MCs), and water activity (A_w) were investigated using standard methods. A total of 120 samples were collected for microbiological analysis, 90 of the samples were additionally analysed for MC and A_w. Findings showed that the mean Total Viable Counts (TVCs) in salted sun-dried products sampled during rainy season was 4.5 log cfu/g in fish heads with MCs of 38.0% and A_w of 0.682. This was significantly higher (P<0.05) than what we recorded during the dry season with mean TVCs of 3.0 log cfu/g at MCs of 24.6% and A_w of 0.625. Fish chests had TVCs of 3.3 log cfu/g and MCs of 27.6% and A_w of 0.659 in rainy season, with no significant seasonal difference (P>0.05). Fish belly flaps had TVCs of 3.3 log cfu/g at 26.4% MCs and 0.669 A_w in rainy season which were comparable (P>0.05) to those dried in the dry season. The microbial species recovered were *Staphylococcus* spp., *Enterobacter* spp., *Psychrobacter* spp., and *Bacillus* spp. Neither *Escherichia coli* nor extended-spectrum beta-lactamase producing *Enterobacteriaceae* were detected. Frozen Nile perch had TVCs of 5.7 log cfu/g on skin, 5.4 log cfu/g gills and 2.9 log cfu/g in flesh and were within acceptable limit set by Tanzanian standards. These results revealed that dried Nile perch products are generally safe for human consumption; however, the recovered bacteria indicate a need of implementing hygienic procedures during processing of products for improved quality and safety.

Keywords: Salted sun-dried fish; microbial quality; food safety; physico-chemical parameters

INTRODUCTION

Nile perch (*Lates niloticus*) from Lake Victoria is one of the most important fish species for fisher folks in Tanzania as well as for the nation due to its economic and nutritional health benefits (Kirema-Mukasa, 2012). Fish are an important source of animal protein and other essential elements to sustain human health (Ikwebe et al., 2017; Immaculate et al., 2013; Majumdar et al., 2017). Nile perch of good quality are processed as fillets for export markets especially to the European countries and Asia while other fish parts are processed for domestic and regional African markets (Kabahenda and Hüsken, 2009; Kirema-Mukasa, 2012). Currently, the Nile perch market is growing due to product diversification including salted sun-dried bi-products such as heads, chests, belly-flaps and whole fish which are sold for human consumption. The salted sun-dried Nile perch products are mostly exported to countries such as the Democratic Republic of Congo, Rwanda and Burundi (Kirema-Mukasa, 2012).

Salting and sun-drying is an ancient preservation method which has been applied to different foods such as fish, meat, and vegetables (Immaculate et al., 2013; Nagwekar et al., 2017). Sun-drying of fish is simple, cheap, and affordable, but an adequate dried product requires enough sun (Ikwebe et al., 2017). The method can improve the shelf life of products if post-processing handling is properly done to avoid bacterial contamination (Nagwekar et al., 2017). Although the salted sun-dried Nile perch products have been widely marketed in East and Central African regional markets, limited information is available on their microbiological quality and safety aspects.

Moisture content (MC) and water activity (A_w) are important factors in food quality, preservation and shelf life of food stuffs. Also, they are used to predict microbial growth and determine the microbiological stability of food products (Bevilacqua et al., 2017;

Nielsen, 2010). Previous studies have described how the MCs and A_w can influence microbial growth on salted sun-dried fish and fish products (Nagwekar et al., 2017; Sampels, 2015). This includes the mechanism of products drying process which reduces the moisture content and water activity to minimise microbial proliferation in food. Although the preservation method is affordable, the drying condition, packaging, and storage may not be hygienically satisfactory to maintain the quality of the dried products. Nile perch are caught in deep waters usually with low levels of microbial contamination (Immaculate et al., 2013; Koral et al., 2013). However, during subsequent handling along the value chain from capture to market, different bacteria of public health implications may come in contact with the fish, causing a decline in its safety (Immaculate et al., 2013). Therefore, the determination of microbiological quality of frozen Nile perch from cold storage facilities is very important as a strategy for safeguarding consumer's health.

The aim of this study was therefore to investigate the microbial quality and safety of frozen Nile perch and its bi-products in line with physico-chemical qualities of processed sun-dried products marketed in the Lake Victoria region.

MATERIALS AND METHODS

Sampling, laboratory sample preparation and analysis

A total of 120 samples were collected from March to July 2018. The samples included frozen Nile perch from cold storage facilities and salted sun-dried bi-products (heads, chests and belly flaps). Sampling locations were located in Ilemela and Nyamagana districts of Mwanza region, Tanzania. Microbiological and MC analysis were done at the National Fish Quality Control Laboratory (NFQCL), Mwanza and A_w analysis at the Department of Food Technology, Nutrition and Consumer Sciences laboratory, Sokoine

University of Agriculture (SUA) in Morogoro. Identification of bacterial isolates was done at the Department of Veterinary and Animal Sciences, University of Copenhagen. Size of each sample was about 2 kg for frozen fish and 400 g of salted sun-dried products.

Ninety processed salted sun-dried Nile perch products were collected from different processors at the Kirumba market in Mwanza. All samples were analysed for *Salmonella* spp., *Escherichia coli*, total coliform counts (TCCs), total viable counts (TVCs), and Extended Spectrum Beta-Lactamase (ESBL) producing *Enterobacteriaceae* using standard methods as described below. Samples were collected both in the rainy season (March-May) and dry season (June-July). Forty-five dried products (15 samples of each type) were collected during the rainy season and forty-five in the dry season (15 each sample type). Samples were collected using sterile rubber gloves and placed into sterile plastic zip-lock bags and transported to NFQCL for analysis. In the laboratory, each salted sun-dried sample was divided into three portions with the first portion used for microbiological analysis; the second portion was used for MCs analysis, and the third portion was packed and transported to SUA for the A_w analysis. For microbiological analysis; a 25 g sample was chopped and mixed into Buffered Peptone Water (BPW) (Oxoid Ltd, Hampshire, England) in sterile stomacher bags and homogenised in a stomacher (Seward 400, UK) before analysis.

A total of 30 frozen Nile perch were collected using sterile rubber gloves from storage facilities for microbiological analysis. Samples were placed in sterile plastic zip-lock bags, preserved in an insulated box containing cooling elements and transported to NFQCL for analysis. In the laboratory, frozen fish were thawed at room temperature for 2-3 hrs, and then by using sterile scissors and surgical blades; gills, skin and flesh were removed and

chopped into a 25 g sample which was mixed with sterile 225 ml of BPW and analysed as per protocols stated in each method based on parameter analysed.

Detection of *Salmonella* species

Salmonella spp. was detected using the International Organisation Standard (ISO) method (ISO 6579:2002/Amd.1:2007). Briefly, pre-enrichment was done in BPW at 37°C for 24 hrs followed by enrichment in Rappaport Vassiliadis broth (Oxoid Ltd) at 42°C for 24 hrs and Mueller-Kauffman Tetrathionate-novobiocin broth (Oxoid Ltd) at 37°C for 24 hrs. Presumptive *Salmonella* colonies were biochemically confirmed on Triple Sugar Iron agar (Oxoid Ltd) and isolates were tested for agglutination with polyvalent *Salmonella* antisera (Rapid Lab Ltd, UK) with strain *S.* Typhimurium ATCC 13311 (Public Health, England) used as a positive control.

Enumeration of *Escherichia coli,* total coliforms (TCCs) and ESBL producing *Enterobacteriaceae*

Enumeration of *E. coli* and TCCs were done on Brilliance *E. coli*/coliforms selective agar medium (BE/C) (Oxoid Ltd) by the spreading technique following the manufacturer's instructions. Serial dilutions were made according to ISO method (ISO 6887-1, 2017). From each dilution, 0.1 ml was drawn and inoculated onto prepared petri dishes containing sterile BE/C medium; the inoculum was spread, left to solidify, and then incubated at 37°C for 24 hrs. After incubation, bacteria were counted with the aid of a colony counter where colonies with purple colour were identified as *E. coli* while coliform bacteria were pink. *E. coli* ATCC 25922 was used as positive control. Enumeration of ESBL-producing *Enterobacteriaceae* was done on MacConkey agar (Oxoid Ltd), supplemented with 2 µg/ml of cefotaxime as described by Moremi et al. (2016).

Enumeration of Total Viable Counts (TVCs)

TVCs were enumerated on Plate Counts agar (PCA) (Oxoid Ltd) at 30ºC (ISO 4833-1, 2013). Serial dilutions were made as per ISO 6887-1. (2017) and from each dilution, 1 ml was drawn and inoculated into a sterile petri dish. The molten PCA was poured, mixed, and left to solidify and the plate incubated at 30°C for 72 hrs. Colonies i.e. <300 colony forming unit (cfu), were counted with the aid of a colony counter. Colonies representing different morphological types were selected from the PCA plates and stored in 50% glycerol in liquid nitrogen for further analysis.

Moisture content analysis

The MC in salted sun-dried Nile perch products was determined according to the Association of Official Analytical Chemists Standard (AOAC) method number 950.46 (B) (AOAC, 2006). Briefly, 2 g of the sample (in duplicate) was weighed and evenly distributed into pre-heated petri dishes, then heated in an oven set at 102°C for 16 hrs parallel to an equal weight of pure pentahydrate copper sulphate ($CuSO_4.5H_2O$) as a control. Afterward, the sample was cooled in desiccator for 30 min then reweighed. The average MCs (from the duplicates) were calculated and reported in percentage as per AOAC requirements.

Water activity analysis

The A_w was analysed according to the standard method (ISO 21807, 2004) using Novasina water activity meter (Pfaffikon, Switzerland). Briefly, a duplicate 2 g of grinded salted sun-dried sample was measured and placed into the water activity meter, left to stabilise for 20 to 30 min before the reading was recorded. The average of the duplicates was calculated and recorded as the final reading. Figures were reported with three decimals.

Microbial identification on salted sun-dried Nile perch products

The selected isolates from PCA were transported to Denmark for identification using Matrix Assisted Laser Desorption Ionization-Time-of-Flight (MALDI-TOF) technology. The isolates were checked for purity following subculture onto blood agar and incubation at 37ºC for 24 hrs, and then a single colony was selected and placed on glass slide for identification in Vitek MS MALDI-TOF Mass Spectrometer (bioMérieux, Inc., France). The identification of isolates were interpreted based on a comparison to SuperSpetra containing sets of genus, species and strains biomarkers characteristic for respective groups of microorganisms as stated in the instructions of the MALDI-TOF. Only isolates with an identification of 80% or more confidence was trusted.

Data analysis

Analysis of data was performed using Stata version 14 (StataCorp LP) descriptive statistics to obtain mean, standard deviation, and to show data variability in different parameters analysed. Also, the frequencies distributions of *Salmonella* spp. in different sample categories were determined. Seasonal variation in the different parameters analysed was analysed using single factor ANOVA. Results were presented in box-plots figures. The significance was defined at $P<0.05$.

RESULTS AND DISCUSSIONS

Overall, high TVCs were reported in salted sun-dried Nile perch products collected during the rainy season with mean counts ranging from 3.3 to 4.5 log cfu/g while those collected during the dry season had lower mean counts ranging from 2.9 to 3.1 log cfu/g ($P<0.05$) (Fig. 1a). These results are still however, within the acceptable limit set by the Tanzanian standard i.e. 1.0 x 10^6 cfu/g (TZS, 1988). The seasonal difference in TVCs was likely

attributed to observed unhygienic products handling during, and after processing as well as drying conditions which attracted insects like flies on the dried products as also reported in previous studies (Immaculate et al., 2013; Nagwekar et al., 2017). The study shows the relationship between TVCs, MC, and A_w obtained in salted sun-dried Nile perch products collected in the rainy season and dry season, in that, the increase of MCs and A_w was proportional to the increase of TVCs in samples. The TVCs in Nile perch products were supported by the MC and A_w results obtained. High MC values ranging from 26.4% to 38.0% and A_w of 0.659 to 0.682 were recorded in products sampled during the rainy season when compared to products sampled during the dry season in which, MCs ranged from 18.3% to 24.6% and A_w 0.619 to 0.643, respectively (Fig. 1b-1c). The average results of MCs and A_w were within the limits range required in salted dried fish and fish products (MCs 15-30%) and (A_w 0.60-0.75); but, were higher than the minimum limits for prevention of bacterial growth (MCs <15%; A_w <0.6) as specified in standards (IS, 2001; ISO, 1999). High TVCs, MCs and A_w values as seen in our products sampled during the rainy season are normally associated with high humidity, rainy weather conditions and low drying temperature (Logesh et al., 2012; Patterson and Ranjitha, 2009). Thus the drying time during the rainy season is longer than in the dry season which together with poor hygienic conditions of processing premises might contribute to the products being more exposed to insect infestation and bacterial contamination (Ikwebe et al., 2017; Sivaraman and Siva, 2015). Our results are in agreement with other studies reporting high TVCs in salted sun-dried fish products (Nagwekar et al., 2017; Saritha et al., 2012; Sulieman and Mustafa, 2012). Also, MC values found in the current study were lower than the one reported for different salted dried fish by Nuwanthi et al. (2016), but they were in agreement with the studies reported by Majumdar et al. (2017). The A_w values in this study, were lower than 0.8 described by Koral et al. (2013), and 0.77 reported by Lin et al.

(2012); irrespective of the season of sampling; however, they were higher than 0.5 reported by Geetha et al. (2014). Salt and drying process are key factors contributing significantly to reduction of TVCs, MCs, and A_w in products (Ginigaddarage et al., 2018; Majumdar et al., 2017). The MCs and A_w values reported in the different salted sun-dried Nile perch products can be expected to support microbial growth during the rainy season so that products undergo microbial spoilage faster than those processed in the dry season. Although the concentrations of bacteria suggest that the salted sun-dried Nile perch products are safe for human consumption, some of the identified bacteria especially *Staphylococcus xylosus*, *Bacillus megaterium*, *Klebsiella oxyota,* and *Enterobacter cloacae* might affect the products safety. These bacteria have been reported in other studies to be responsible for histamine formation in salted fish and therefore, pose a risk of histamine toxicity to humans consuming the products (Koral et al., 2013; Lin et al., 2012; Tsai et al., 2005). Histamine is a toxin formed by microbial decarboxylation of histidines as a result of time-temperature irregularity/abuse during storage of salted fish and/or fish products (Koral et al., 2013; Nagwekar et al., 2017; Tsai et al., 2005). The current study did not analyse histamine in salted sun-dried products, however, it is an area worthy further studies in order to quantify the potential food safety risks to humans.

Our findings also showed that, only three out of 15 samples of belly flaps collected in rainy season had mean TCCs of 4.4 x 10^1 cfu/g and fish heads (1/15) had 1.4 x 10^1 cfu/g and *Salmonella* spp. was detected on the fish heads (6.7%, n=15) and, belly flaps (6.7%, n=15) samples collected during the rainy season whereas samples did not contain *Salmonella* spp. in the dry season. These findings are similar to those reported by Gabriel and Alano-Budiao. (2015). Presence of TCCs and *Salmonella* spp. in dried products could be an indication of the poor products handling after processing leading to cross-

contamination with bacteria from the environment. The absence of *E. coli* and ESBL-producing *Enterobacteriaceae* in the tested samples suggests that salted sun-dried Nile perch products were not contaminated with faecal bacteria.

Microbial load in fresh fish are an important determinant of the storage time of the products. The results of TVCs in frozen Nile perch showed high counts in skin and low in flesh (Fig. 2). The high TVCs indicate that the fish can rapidly decompose and undergo spoilage when exposed to ambient temperature, as a result of metabolic activities. Moreover, *E. coli* concentrations were low where; 3/30 of fish gills had mean counts of 2.4 log cfu/g and skin (2/30) with counts of 2.1 log cfu/g while *E. coli* was not detected in flesh samples. The TCCs showed (13/30) that fish gills had mean counts of 2.8 log cfu/g, skin (7/30) had mean counts of 3.0 log cfu/g. The presence of *E. coli* and TCCs in samples albeit in low concentrations may imply poor fish handling as also described in other studies (Saritha et al., 2012; Sulieman and Mustafa, 2012); despite of the fact that, they were within acceptable limits as stated in Tanzania standard (TZS, 1988). The reported *Salmonella* spp. in fish gills (13.3%, n=30) and skin (6.7%, n=30) may suggest that the contamination may have occurred in the aquatic environment where fish were caught rather than from storage facilities.

Conclusions

The results of the present study provide important baseline information on the status of microbial quality in Nile perch products, which is essential for policy decisions geared towards safeguarding the quality and safety of these products to consumers. The different bacteria species recovered from salted sun-dried products provide an indication that there is a need for public authorities in the fisheries sector to recommend hygienic procedures to

fit in salted sun-drying processing method. Adoption of other drying methods that minimise contamination such as solar conduction dryers needs to be considered in order to preserve the quality and safety of Nile perch products in the study area.

CONFLICT OF INTERESTS

There is no conflict of interest among authors of this paper in relation to this work.

ACKNOWLEDGEMENTS

The authors acknowledge the support from Danish International Development Agency (DANIDA) through the Innovations and Markets for Lake Victoria Fisheries (IMLAF) project (DFC file no. 14-P01-TAN) for supporting the research work. The authors also acknowledge the technical assistance rendered by of staff at the NFQCL, Mwanza, Food Technology, Nutrition and Consumer Sciences Laboratory at SUA and at the Department of Veterinary and Animal Sciences, University of Copenhagen.

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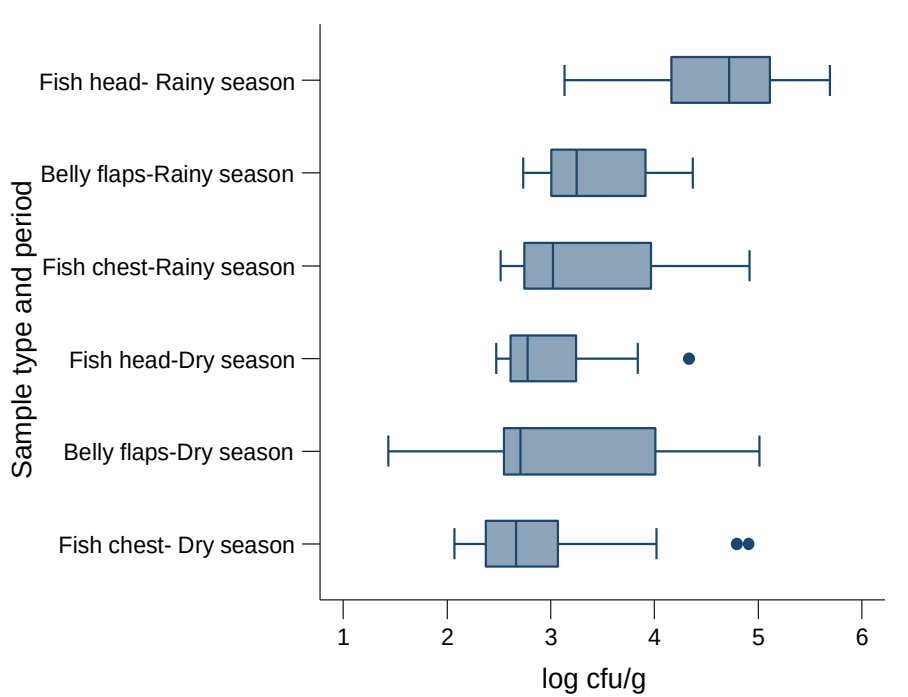

Figure 1a: Box plot of total viable bacterial counts in salted sun-dried Nile perch products

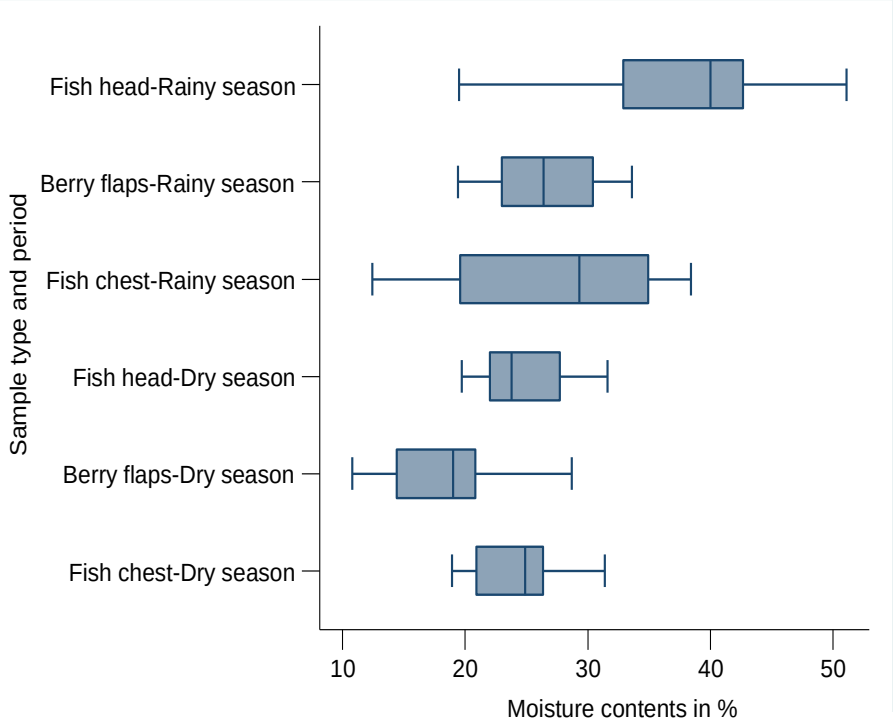

Figure 1b: Box plot of moisture contents (%) in salted sun-dried Nile perch products

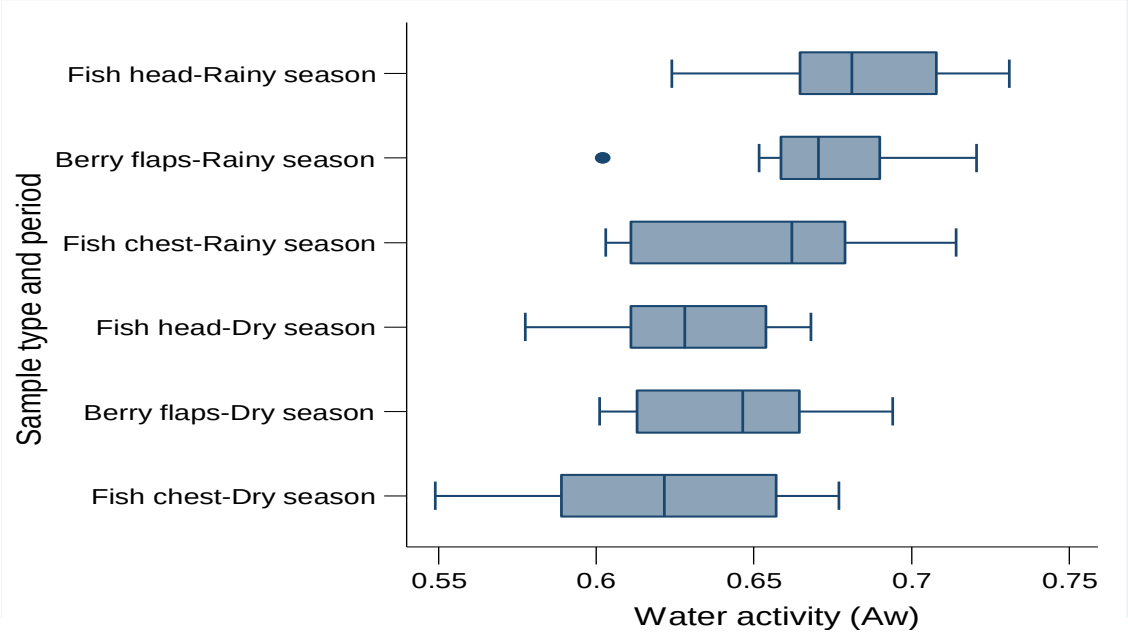

Figure 1c: Box plot of water activity (A_w) in salted sun-dried Nile perch products

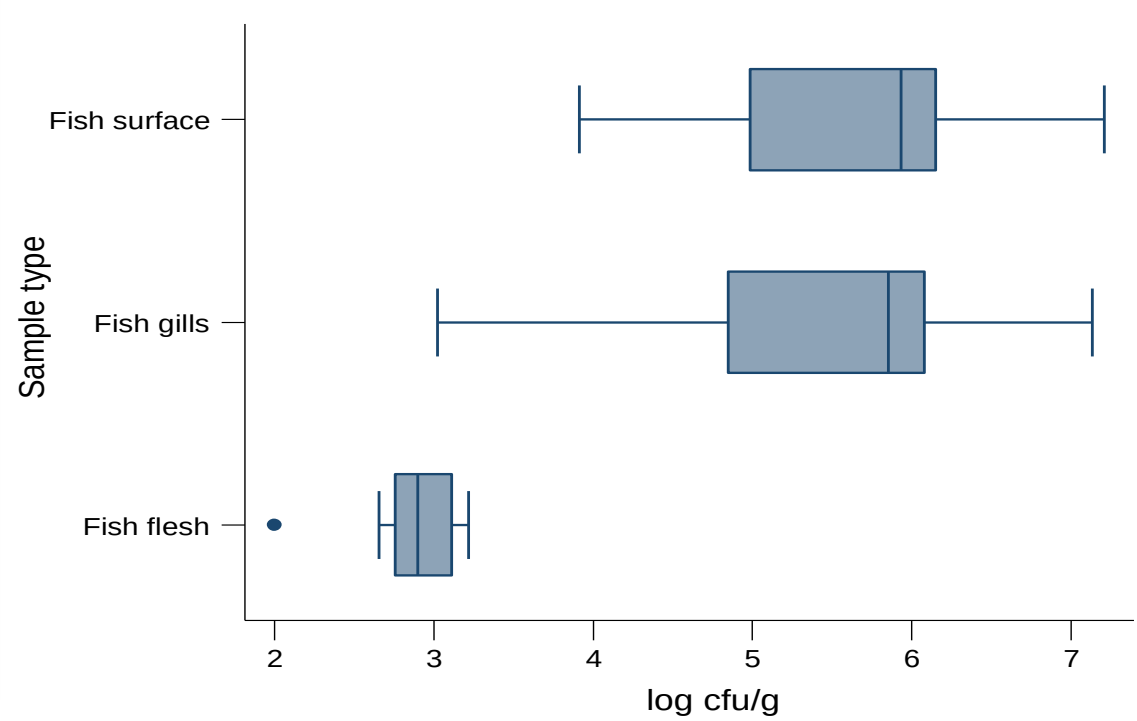

Figure 2: Box plot of total viable counts in frozen Nile perch samples

CHAPTER THREE

3.0 GENERAL RESULTS AND DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

3.1 General results and discussion

The present study reports an overall prevalence of *S. enterica* subsp. *salamae* serovar 42:r:- in Nile perch from Lake Victoria of 3.9% (n=180) and *S. enterica* subsp. *enterica* serovar *waycross* of 13.9% (n=180). Other serovars identified were Fulica of subspecies *salamae* and *S. wien* of subspecies *enterica*. These serovars rarely cause disease in humans and animals, and are less virulent than frequently isolated non-typhoid serovars, such as *S. typhimurium* and *S. enteritidis* (Lamas *et al.*, 2018; Baniga *et al.*, 2019). The sequenced serovars of subspecies *salamae* had no principal SPI-1 to SPI-5, while SPI-2 to SPI-5 were detected in *S. waycross* and *S. wien*. The presence or absent of the SPIs in *Salmonella* spp. is attributable to the genomic changes during evolutionary process of bacteria (Manuscript II). The SPIs are important for virulence potential in *Salmonella* spp., especially of subspecies *enterica*. Therefore, I conclude that the isolates reported in this study apparently have no potential public health importance. Furthermore, these *Salmonella* serovars were not related to faecal pollution because samples had very low counts of *E. coli* (Paper I and Manuscript II). The isolates contained few plasmids and the antimicrobial resistance profiles as most of the isolates indicated they were susceptible to most of the antimicrobials tested. The only resistance gene detected in all sequenced isolates was *aac(6')-laa* encoding resistance to aminoglycosides. The gene was not among acquired gene because was located in chromosomes not in plasmids, therefore could be associated with natural resistance in *Salmonella* spp. The plasmid replicon type IncFII was detected in *S. waycross* ST3691 and *S. wien* ST3691, which did not contain antimicrobial resistance or virulence genes. Phylogenetic analysis showed that serovar 42:r:- and serovar

Fulica were clonal related to each other. Both had the same ST1208, although 42:r:- belonged to serogroup T while Fulica belonged to an unidentified serogroup. Likewise, *S. waycross*, which belonged to serogroup S, was clonally related to *S. wien* of an unidentified serogroup as the two serovars clustered together in the phylogenic tree, and also shared a sequence type.

The overall prevalence of ESBL-producing *E. coli* in Nile perch was 4.4% (n=180). More specifically, the prevalence in Nile perch was 1.7% (n=60) fish from fishing grounds, 3.3% (n=60) fish sampled at the landing site, and 8.3% (n= 60) Nile perch from domestic fish markets. Generally, the overall prevalence of ESBL-producing *E. coli* reported in Nile perch in this study was very low and it is the first to be documented in this fish species. The low prevalence in Nile perch in general reflects the fact that Nile perch are deep freshwater fish. They live in water that is much less polluted that the habitat of fish like sardines and Nile tilapia, which live in shallow water that is highly polluted due to the influx of humans and animals waste (Mdegela *et al.,* 2015). In addition, the finding of ESBL-producing *E. coli* in Nile perch is due to its eating habit; where the Nile perch consume other fish, including the shallow water dwelling sardines and Nile tilapia. Therefore, Nile perch can travel across water bodies while already harbouring faecal bacteria that could include multidrug resistant bacteria. In the present study, the ESBL-producing *E. coli* isolates were ST156-serotype ONT:H28 belonging to commensal phylogroup B1, ST167-serotype O9:H21 that belongs to commensal phylogroup A, and ESBL-producing *E. coli* ST636-serotype O45:H7 belonging to pathogenic phylogroup B2 (Manuscript III). Commensal *E. coli* rarely cause infections in animals and humans because are opportunistic bacteria (Atterby *et al.*, 2017). However, infections due to *E. coli* of pathogenic phylogroup B2 have been reported in humans, especially urinary tract

infections (Sánchez-Benito *et al.,* 2017). The results were supported by the detection of virulence encoding genes in ESBL-producing *E. coli*. ST167 harbours the *gad* gene encoding glutamate decarboxylation and ST156 harbours *gad*, *lpfA*, and *iss*, which are involved in human and animal infections (Tramonti *et al.,* 2002). ST636 harbours four virulence genes (*gad*, *iss*, *nfaE* and *vat*) which have also been reported in other extra-intestinal pathogenic *E. coli* strains (Szmolka *et al.,* 2012; Ahmed *et al.,* 2017). All ESBL-producing *E. coli* isolates showed phenotypic resistance to sulphamethoxazole-trimethoprim (100%), ampicillin/cloxacillin, erythromycin, chloramphenicol, tetracycline, and nalidixic acid, which correlated with genotypic resistance shown by isolates possessing β-lactamases and resistance genes *sul1*, *sul2*, *dfrA1*, *dfrA12*, and *dfrB*, which code for sulphonamides-trimethoprim resistance; *qepA*, encoding resistance to fluoroquinolones; *tetB* and *tetD*, encoding tetracycline resistance; and *catA*, encoding resistance to chloramphenicol. These presently-reported antimicrobial resistance profiles have also been frequently reported in other commensal and pathogenic strains of *E. coli* in foods, environmental samples and clinical samples (Abgottspon *et al.,* 2014; Atterby *et al.,* 2017).

Salted sun-dried Nile perch products are important, especially in regional markets around Lake Victoria, due to its market potential and consumption. In the current study, the salted sun-dried Nile perch products were not contaminated with *Salmonella* spp. and ESBL-producing *E. coli*. The bacteriological parameters were within the criteria for acceptance set by the Tanzanian Bureau of Standard (TZS, 1988). However, some bacteria were recovered from salted sun-dried Nile perch products collected during the rainy season. These included *Staphylococcus xylosus*, *Bacillus megaterium*, *Klebsiella oxyota*, and *Enterobacter cloacae*. These bacteria could have public health implications due to their

involvement in the production of histamine, which can pose a health risk to consumers (Koral *et al.,* 2013). Samples collected during the rainy season had higher moisture contents and water activity than the dried products sampled during the dry season (Paper IV).

3.2 Conclusions

The present study has demonstrated that Nile perch along its value chain in Lake Victoria were contaminated mainly with uncommon serovars of non-typhoid *Salmonella*. The serovars were less virulent compared to commonly isolated *Salmonella* serovars and also were susceptible to most antimicrobials tested. Aquatic *Salmonella* serovars reported in this study are not associated with faecal pollution, and could be normal flora in Nile perch. The low prevalence of ESBL-producing *E. coli* reported can represent limited food safety risks. In addition, antimicrobial resistance shown by the isolates could imply health risks due to the presence of possible gene transfer between commensal and pathogenic bacteria, then to humans and animals. Both high prevalence of *Salmonella* spp. and ESBL-producing *E. coli* were reported in Nile perch collected from domestic markets. This implies that the fish in these markets are handled poorly; hence improvement of hygienic fish handling to fish vendors is inevitable. On the other hand consuming salted sun-dried products represent none of health risk as the products are safe for human consumption. This is due to concentrations of microbial contamination found in this study being within acceptable limits set by Tanzanian standards. However, improvement of hygienic fish handling in artisanal fishers along the value chain and also during and after processing of products is highly recommended to avoid any possibility of occurrence of bacteria of public health interest.

3.3 Recommendations

The findings reported for the first time aquatic *Salmonella* serovars which could be normal flora in Nile perch. Their presence in Nile perch was not associated with faecal pollution. Based on knowledge gained through my study, the finding provides important information to policy makers and control import authorities to consider improving laboratory testing capacity by including DNA based typing techniques in addition to, the current phenotypic method for detection of *Salmonella* spp. at species level. Tests should be improved to the level of identifying specific serovars of public health importance. Currently, *Salmonella* alerts reported by EU more likely are associated with these rare serovars which have no public health implications. This is because the authority does not have improved *Salmonella* testing scheme with ability to identify serovars of public health importance. Strengthening testing capacity, likely will reduce rejection or detention of exported fish products because of *Salmonella* contamination. Since Nile perch are usually found in deep fresh-water areas with very limited and/or no faecal pollution, consequently the likelihood of detecting *Salmonella* serovars of public health concern will be very low. Therefore, it is high time now for policy makers and testing authorities to consider allocating funds to improve laboratory testing capacity for *Salmonella* spp., which will help to reduce unnecessary rejection or detention fish and fish products. Moreover, more studies are proposed to link up the reported aquatic *Salmonella* serovars and ESBL-producing *E. coli* from this study and clinical isolates. Improving hygienic fish handling is highly encouraged especially fish in domestic markets; this will reduce the risk of cross-contamination of fish with pathogens from waste around markets. Since the isolates, in particular the multidrug resistance ESBL-producing *E. coli* were reported with resistance genes carried in plasmids, improving hygienic handling will reduce possibility of HGT among and between the isolates and hence to humans.

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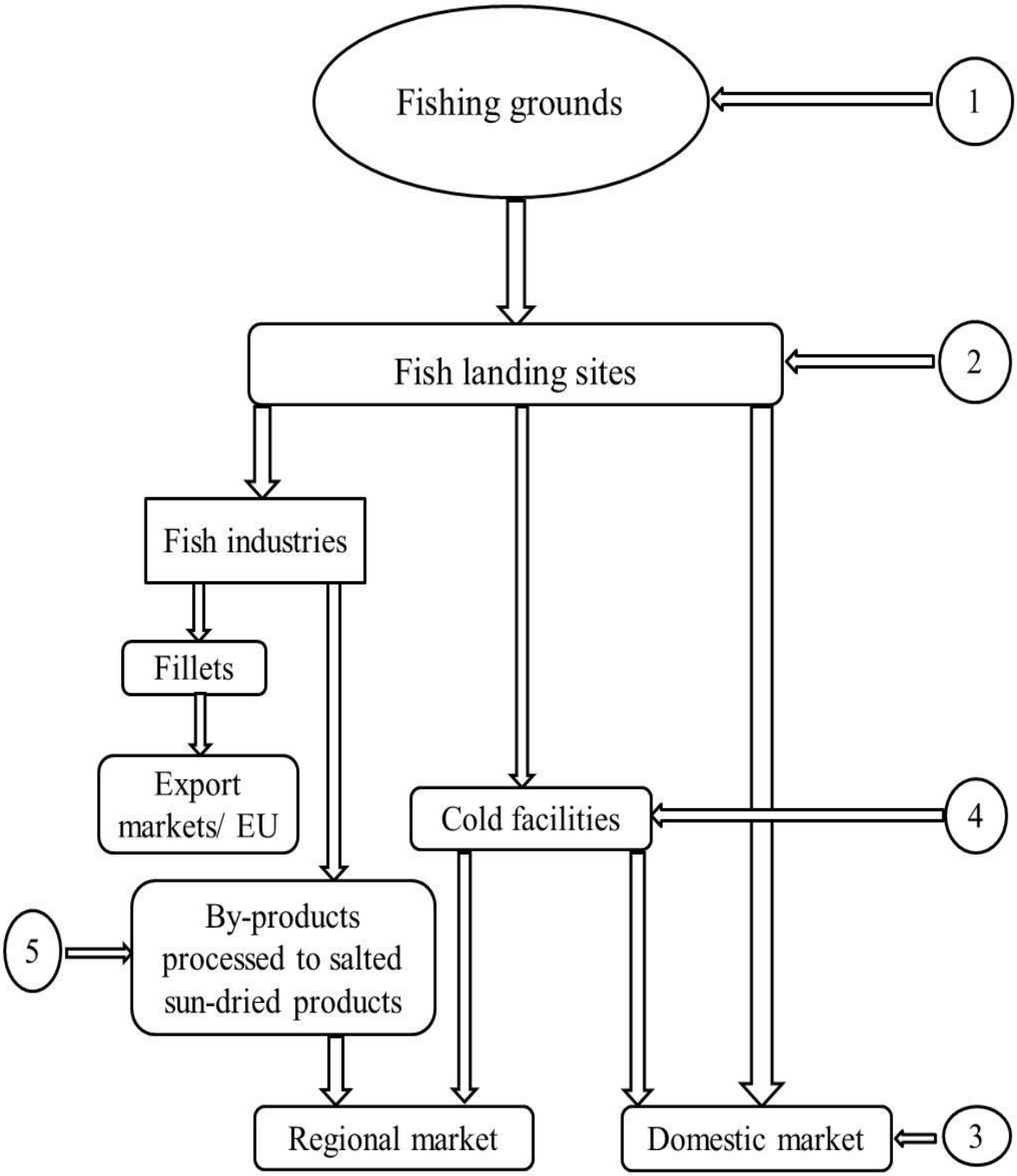
APPENDICES

Appendix 1: Diagrammatical Nile perch value chain showing main sampling points



Appendix 2a: Supplementary Table S1a for Manuscript II: List of *Salmonella enterica* subsp. *salamae* serovar 42:r:- and Fulica used in phylogenetic analysis

S/N	Accession No.	Genus	spp	subsp.	serovar	Source	Year	Country
1	SAMN10638926	*Salmonella*	*enterica*	*salamae*	42:r:-	meat retail	2018	Rwanda
2	SAMN10638893	*Salmonella*	*enterica*	*salamae*	42:r:-	Farm/Oris aries	2018	Rwanda
3	SAMN06030387	*Salmonella*	*enterica*	*salamae*	42:r:-	human	N/A	Canada
4	SAMN04102313	*Salmonella*	*enterica*	*salamae*	42:r:-	Raw Macadamia Nuts	2015	USA
5	SAMN02845238	*Salmonella*	*enterica*	*salamae*	42:r:-	Frozen Nile perch	2002	Tanzania
6	Sample A8	*Salmonella*	*enterica*	*salamae*	42:r:-	Nile perch fish	2017	Tanzania
7	Sample A9	*Salmonella*	*enterica*	*salamae*	42:r:-	Nile perch fish	2017	Tanzania
8	Sample B6	*Salmonella*	*enterica*	*salamae*	42:r:-	Nile perch fish	2017	Tanzania
9	Sample D5	*Salmonella*	*enterica*	*salamae*	42:r:-	Lake water	2017	Tanzania
10	Sample D6	*Salmonella*	*enterica*	*salamae*	42:r:-	Lake water	2017	Tanzania
11	Sample I1	*Salmonella*	*enterica*	*salamae*	42:r:-	Nile perch fish	2017	Tanzania
12	SAMN02843459	*Salmonella*	*enterica*	*salamae*	42:z:l,5	mullen leaves	2001	Albania
13	SAMN00990755	*Salmonella*	*enterica*	*salamae*	58:l,z13,z28:z6	N/A	N/A	N/A
14	SAMEA4028439	*Salmonella*	*enterica*	*salamae*	58:l,z13,z28:l,5	human	1884/1962	
15	SAMN02367800	*Salmonella*	*enterica*	*salamae*	58:d:z6	Iguana	1985	USA
16	SAMN02367583	*Salmonella*	*enterica*	*salamae*	53:lz28:z39	N/A	N/A	N/A
17	SAMN03169189	*Salmonella*	*enterica*	*salamae*	Fulica	Human	2012	UK
18	SAMN03702207	*Salmonella*	*enterica*	*salamae*	Fulica	almond butter	2014	USA
19	SAMN03702218	*Salmonella*	*enterica*	*salamae*	Fulica	almond butter	2014	USA
20	Sample A7	*Salmonella*	*enterica*	*salamae*	Fulica	Nile perch fish	2017	Tanzania

Appendix 2b: Supplementary Table S1b & S1c for Manuscript II: List of *Salmonella* Waycross and *S.* Wien used in phylogenetic analysis

S/N	Accession No.	Genus	spp	subsp.	serovar	Source	Year	Country
1	SAMN02698301	*Salmonella*	*enterica*	*enterica*	Waycross	octopus	2013	Philippines
2	SAMN04160804	*Salmonella*	*enterica*	*enterica*	Waycross	stool	2004	Canada
3	SAMN04576510	*Salmonella*	*enterica*	*enterica*	Waycross	human	2015	UK
4	SAMN04893757	*Salmonella*	*enterica*	*enterica*	Waycross	stool	2013	USA
5	SAMN05464607	*Salmonella*	*enterica*	*enterica*	Waycross	stool	2011	USA
6	SAMN06463568	*Salmonella*	*enterica*	*enterica*	Waycross	stool	2016	USA
7	SAMN08468785	*Salmonella*	*enterica*	*enterica*	Waycross	Human	2017	USA
8	SAMN09652020	*Salmonella*	*enterica*	*enterica*	Waycross	N/A	2016	UK
9	SAMN10104865	*Salmonella*	*enterica*	*enterica*	Waycross	Human	2017	UK
10	SAMN10599799	*Salmonella*	*enterica*	*enterica*	Waycross	Human	2018	UK
11	SAMN10638908	*Salmonella*	*enterica*	*enterica*	Waycross	human	2018	Rwanda
12	Sample C2	*Salmonella*	*enterica*	*enterica*	Waycross	Nile perch fish	2017	Tanzania
13	Sample D3	*Salmonella*	*enterica*	*enterica*	Waycross	Nile perch fish	2017	Tanzania
14	Sample E5	*Salmonella*	*enterica*	*enterica*	Waycross	Nile perch fish	2017	Tanzania
15	Sample E9	*Salmonella*	*enterica*	*enterica*	Waycross	Nile perch fish	2017	Tanzania
16	Sample G5	*Salmonella*	*enterica*	*enterica*	Waycross	Nile perch fish	2017	Tanzania
17	SAMN01933185	*Salmonella*	*enterica*	*enterica*	Wien	human	1988	France
18	SAMN03475507	*Salmonella*	*enterica*	*enterica*	Wien	human	2015	UK
19	SAMN09423331	*Salmonella*	*enterica*	*enterica*	Wien	human	2015	UK
20	SAMN09426963	*Salmonella*	*enterica*	*enterica*	Wien	human	2018	UK
21	SAMN09431345	*Salmonella*	*enterica*	*enterica*	Wien	human	2017	UK
22	SAMN09475830	*Salmonella*	*enterica*	*enterica*	Wien	human	2018	UK
23	SAMN09652057	*Salmonella*	*enterica*	*enterica*	Wien	human	2017	UK
24	SAMN09666655	*Salmonella*	*enterica*	*enterica*	Wien	human	2018	UK
25	Sample A1	*Salmonella*	*enterica*	*enterica*	Wien	Nile perch fish	2017	Tanzania
26	Sample H6	*Salmonella*	*enterica*	*enterica*	Wien	Water	2017	Tanzania

Appendix 3: Supplementary Table S3 for Manuscript II: Genomic sequencing data, virulence profile and occurrence of antimicrobial resistance genes in different *Salmonella* strains

Sample and code number			A7	A8	A9	B6	D5	D6	I1	A1	C2	D3	E5	E9	G5	H6
Sample			NP fish	NP fish	NP fish	NP fish	L. water	L.water	NP fish	NP fish	NP fish	NP fish	NP fish	NP fish	NP fish	R. water
Date of collection (MM/Year)			2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017
Region/Country			MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ
Sample type			Intestine	Intestine	Intestine	Intestine	water	water	Intestine	Surface	Intestine	Intestine	Intestine	Intestine	Intestine	water
Sampling Location			LV	LV	LV	LV	LV	LV	LV	LV	LV	LV	LV	LV	LV	LV
Number of contigs			150	118	172	148	94	123	79	116	67	82	70	83	78	93
N50			78403	133671	73381	91347	175704	109609	226978	148056	312058	168771	227892	187155	140504	138919
Genome size (bp)			4775198	4792107	4797164	4763962	4800001	4788828	4799696	4737395	4742170	4742542	4741533	4741231	4711795	4700978
Coverage			52	64	46	62	91	56	86	42	61	79	86	79	70	71
Accession No.																
Phylogeny group			-	T	T	T	T	T	T	-	S	S	S	S	S	-
Salamonella subspecies			*salamae*	*salamae*	*salamae*	*salamae*	*salamae*	*salamae*	*salamae*	*enterica*	*enterica*	*enterica*	*enterica*	*enterica*	*enterica*	*enterica*
Serovars			Fulica	II 42:r:-	II 42:r:-	II 42:r:-	II 42:r:-	II 42:r:-	II 42:r:-	Wien	Waycross	Waycross	Waycross	Waycross	Waycross	Wien
H1-phase-1 flagella			r	r	r	r	r	r	r	z4,z23	z4,z23	z4,z23	z4,z23	z4,z23	z4,z23	z4,z23
H2-phase-2 flagella			-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sequence Type	MLST		1208	1208	1208	1208	1208	1208	1208	2460	2460	2460	2460	2460	3691	3691
	eBG		276	276	276	276	276	276	276	ND	ND	ND	ND	ND	ND	ND
	wgMLST/ST		188682	188684	188687	188683	188690	188689	188692	188688	188691	196047	196048	196050	196049	196054
	rMLST (rST)		1384	1384	1384	1384	1384	1384	1384	28644	28645	28644	28644	28644	42224	42224
	RMLST eBG		276	276	276	276	276	276	276	-	-	-	-	-	339	339
	cgMLST + Hier cc V2/ST		153296	153636	153637	153635	153636	153639	153636	153638	153640	156666	156667	156669	156668	156673
Housekeeping genes	*aro*C	Chorismate synthase protein	293	293	293	293	293	293	293	545	545	545	545	545	61	61
	*dna*N	DNA polymerase III subunit beta	264	264	264	264	264	264	264	503	503	503	503	503	117	117

	*hem*D	uroporphyrinogen-III synthase	80	80	80	80	80	80	80	68	68	68	68	68	25	25
	*his*D	histidinol dehydrogenase	257	257	257	257	257	257	257	656	656	656	656	656	832	832
	*pur*E	5-(carboxyamino)imidazole ribonucleotide mutase	294	294	294	294	294	294	294	157	157	157	157	157	35	35
	*suc*A	2-oxoglutarate dehydrogenase E1 component	271	271	271	271	271	271	271	101	101	101	101	101	320	320
	*thr*A	bifunctional aspartate kinase/homoserine dehydrogenase I	236	236	236	236	236	236	236	3	3	3	3	3	102	102
Salmonella Pathogenicity Islands (SPIs)	SPI-2	*Salmonella* Pathogenicity Islands 2	-	-	-	-	-	-	-	+	+	+	+	+	+	-
	SPI-3	*Salmonella* Pathogenicity Islands 3	-	-	-	-	-	-	-	-	+	+	+	+	+	-
	SPI-4	*Salmonella* Pathogenicity Islands 4	-	-	-	-	-	-	-	+	+	+	+	+	+	-
	SPI-5	*Salmonella* Pathogenicity Islands 5	-	-	-	-	-	-	-	+	+	+	+	+	+	-
	SPI-9	*Salmonella* Pathogenicity Islands 9	+	+	-	-	+	-	-	-	-	-	-	-	-	-
	SPI-13	*Salmonella* Pathogenicity Islands 13	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	C63PI	Iron uptake system, sit operon	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Virulence genes SPI-2	*spiA*		-	-	-	-	-	-	-	+	+	+	+	+	+	-
	spiB		-	-	-	-	-	-	-	+	+	+	+	+	+	-
	spiC		-	-	-	-	-	-	-	+	+	+	+	+	+	-
	spiR		-	-	-	-	-	-	-	+	+	+	+	+	+	-
	ttr		-	-	-	-	-	-	-	+	+	+	+	+	+	-

SPI-3	*mgtB*		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	mgtC		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	slsA		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	cigR		-	-	-	-	-	-	-	+	+	+	+	+	+	+
SPI-4	*siiA*		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	siiB		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	siiC		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	siiD		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	siiF		-	-	-	-	-	-	-	+	+	+	+	+	+	+
SPI-5	*pipA*		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	pipB		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	pipC		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	pipD		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	sopB		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	sigD		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	sigE		-	-	-	-	-	-	-	+	+	+	+	+	+	+
SPI-9	*N/A*		-	-	-	-	-	-	-	-	-	-	-	-	+	+
SPI-13	*gtrA*		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	gtrB		-	-	-	-	-	-	-	+	+	+	+	+	+	+
	gacD		-	-	-	-	-	-	-	+	+	+	+	+	+	+
C63PI	*sitA*		+	+	+	+	+	+	+	+	+	+	+	+	+	+
	sitB		+	+	+	+	+	+	+	+	+	+	+	+	+	+
	sitC		+	+	+	+	+	+	+	+	+	+	+	+	+	+
	sitD		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Plasmid	*IncFII*	*SARC14*	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Antibiotic resistance genes	Aminoglycoside	*aac(6')-Iaa*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Quinolone		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Macrolide		-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Phenicol		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Trimethoprim		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sulphonamide		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Fluoroquinolone		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tetracyclines		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Rifampicin		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Colistin		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Beta-lactam		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unkown chromosomal mutations	16S rRNA	Nucleotides changes	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	23S rRNA	Nucleotides changes	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Legend: NP: Nile perch, MZ: Mwanza, TZ: Tanzania, LV: Lake Victoria

Appendix 4: Supplementary Table S1 for Manuscript III: List of *Escherichia coli* genomes used in the phylogenetic analysis

S/N	ST	Accession	Source	Country
1	156	SAMD00053182	Environment/water	Japan
2	156	SAMEA3993617	Livestock	Denmark
3	156	SAMEA4428333	Human	Netherland
4	156	SAMEA104142975	Livestock	Spain
5	156	SAMN02628548	Poultry	USA
6	156	SAMN03177681	Environment	USA
7	156	SAMN03262782	Human	USA
8	156	SAMN02581389	Human	USA
9	156	SAMEA3304652	Water	
10	156	SAMEA3322109	Human	Nigeria
11	156	SAMN00829505	Human	
12	156	SAMN04393392	Urine-human	USA
13	156	SAMN06555270	Food-poutry	Mexico
14	167	SAMEA104653676	Human	Germany
15	167	SAMEA104653678	Human	Germany
16	167	SAMN05806161	Human	USA
17	167	SAMN09866919	Food	Australia
18	167	SAMEA103972952	Human	Vietnam
19	167	SAMEA2272853	Human	
20	167	SAMEA3322045	Human	Nigeria
21	167	SAMEA4428137	Human	Netherlands
22	167	SAMEA4428577	Human	Netherlands
23	167	SAMEA4428578	Human	
24	636	SAMEA3304664	Human	
25	636	SAMN06754743	Human	USA
26	636	SAMN09866926	Food	Australia
27	636	SAMEA5317426	Urine-human	Norway
28	636	SAMEA3304577	Water	
29	636	SAMEA3918547	Human	
30	636	SAMEA4043165	Human	
31	636	SAMEA104040028	Human	
32	636	SAMN10506455	Stool-human	Sri Lanka

Appendix 5: Supplementary Table S3 for Manuscript III: General genomic characteristics of the eleven ESBL-producing *Escherichia coli* of the study

Sample and code number			Z1	Z2	Z3	Z4	Z5	Z6	Z7	Z8	Z9	Z10	Z11
Sample			NP fish	NP fish	NP fish	L. water	NP fish	NP fish	NP fish	NP fish	NP fish	NP fish	NP fish
Date of collection (MM/Year)			2017	2017	2017	2017	2017	2017	2017	2017	2017	2017	2017
Region/Country			MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ	MZ-TZ
Sample type			Surface	Surface	Intestines	water	Gills	Intestines	Intestines	Gills	Gills	Intestines	Intestines
Sampling location			LV	LV	LV	LV	LV	LV	LV	LV	LV	LV	LV
Number of contigs			160	268	151	154	163	132	158	121	131	179	149
N50			85434	47355	81058	94091	82461	157796	118210	116139	113371	84202	139072
Genome size (bp)			4861037	4860864	4861222	4868050	4862865	4968287	4962853	4811065	4807583	4950195	4959374
Coverage			57	36	57	65	78	84	47	72	69	51	60
Accession No.													
Phylogeny group			A	A	A	A	A	B2	B2	B1	B1	B2	B2
Serovars			O9:H21	O9:H21	O9:H21	O9:H21	O9:H21	O45:H7	O45:H7	H28	H28	O45:H7	O45:H7
O-Antigen			O9	O9	O9	O9	O9	O45	O45	-	-	O45	O45
H-Antigen			H21	H21	H21	H21	H21	H7	H7	H28	H28	H7	H7
Sequence type	MLST		167	167	167	167	167	636	636	156	156	636	636
	cgMLST/ST		88792	91352	88791	88794	88793	88798	88795	88804	88797	88796	88802
	rMLST (rST)		101352	101352	101352	101352	101352	15675	15675	2238	2238	15675	15675
Housekeeping genes	*adk*	Adenylate kinase	10	10	10	10	10	13	13	6	6	13	13
	fumC	Fumarate hydratase class II	11	11	11	11	11	108	108	29	29	108	108
	gyrB	DNA gyrase subunit B	4	4	4	4	4	10	10	32	32	10	10
	icd	Isocitrate dehydrogenase	8	8	8	8	8	97	97	16	16	97	97
	mdh	Malate dehydrogenase	8	8	8	8	8	18	18	11	11	18	18
	purA	Adenylosuccinate synthetase	13	13	13	13	13	68	68	8	8	68	68
	recA	Recombinase A	2	2	2	2	2	93	93	4	4	93	93
Virulence genes	*gad*	Glutamate decarboxylase	+	+	+	+	+	+	+	+	+	+	+
	iss	Increased serum survival	-	-	-	-	-	+	+	+	+	+	+

	nfaE	Diffuse adherence fimbrillar adhesin gene	-	-	-	-	-	+	+	-	-	+	+
	vat	Vacuolating autotransporter toxin	-	-	-	-	-	+	+	-	-	+	+
	lpfA	Long polar fimbriae	-	-	-	-	-	-	-	+	+	-	-
Plasmid	*IncFIA*		+	+	+	+	+	+	+	-	-	+	+
	IncFIB		+	+	+	+	+	+	+	-	-	+	+
	IncFII		+	+	+	+	+	+	+	-	-	+	+
	IncQ1		-	-	-	-	-	+	+	-	-	+	+
	IncX1		+	+	+	+	+	-	-	-	-	-	-
	Col156		+	+	+	+	+	-	-	-	-	-	-
	Col8282		+	+	+	+	+	-	-	-	-	-	-
	ColRNAI		-	-	-	-	-	+	+	-	-	+	+
	Col(BS512)		-	-	-	-	-	+	+	-	-	+	+
	Col440I		-	-	-	-	-	-	-	+	+	-	-
Antibiotic resistance genes	Aminoglycoside	*aadA2; aac(3)-IId*	+	+	+	+	+	-	-	-	-	-	-
		aadA1; strA; strB	-	-	-	-	-	+	+	-	-	+	+
	Fluoroquinolone	*qepA4*	+	+	+	+	+	-	-	+	+	-	-
	Macrolide	*mphA; mdfA*	+	+	+	+	+	+	+	+	+	+	+
	Chloramphenicol	*catA1*	+	+	+	+	+	-	-	+	+	-	-
	Trimethoprim	*dfrA12*	+	+	+	+	+	-	-	-	-	-	-
		dfrA1	-	-	-	-	-	+	+	-	-	+	+
		dfrB4	-	-	-	-	-	-	-	+	+	-	-
	Sulphonamide	*sul1*	+	+	+	+	+	-	-	+	+	-	-
		sul2	+	+	+	+	+	+	+	-	-	+	+
	Tetracyclines	*tetB*	+	+	+	+	+	+	+	+	+	+	+
		tetD	+	+	+	+	+	-	-	-	-	-	-
	Beta-lactamases	*CTX-M-15*	+	+	+	+	+	+	+	+	+	+	+
		TEM-1B	+	+	+	+	+	+	+	+	+	+	+

Legend: NP: Nile perch, MZ: Mwanza, TZ: Tanzania, LV: Lake Victoria

Appendix 6: Supplementary Table S4 for Manuscript III: Heavy metals and toxic compounds detected in ESBL-producing *Escherichia coli*

		Heavy metals and toxic compounds detected in ESBL-producing *E. coli*							Detergent resistance	
Code	Host/Location on chromosome	Copper resistance protein *CopC/CopD*	Cobalt-zinc-cadmium resistance (Cation efflux system protein *CusA*)	Zinc resistance-associated protein	Mercury resistance Operon (*MerC/MerT/MerE*)	Magnesium and cobalt efflux protein *CorC*	Tellurite resistance gene *tehA*	Chromate transport protein *ChrA*	Detergent-resistant phospholipase A (*pldA*)	Quaternary ammonium compound efflux *qacEdelta*
Z1	Hosting contig	19	33	40	3	26	27	64	Negative	64
	Position in contig (bp)	52211 - 53211	3810 - 6953	29670 - 30095	10590 - 11915	33375 - 34253	32636 - 33628	14412 - 15617	Negative	12715 - 13192
Z2	Hosting contig	29	73	52	71	46	100	75	Negative	75
	Position in contig (bp)	16447 - 16821	13567 - 16710	6282 - 6707	7975 - 9327	24925 - 25803	2503 - 3495	4721 - 5926	Negative	7146 - 7623
Z3	Hosting contig	18	35	4	23	24	32	67	Negative	67
	Position in contig (bp)	53215 - 53589	48593 - 51736	6861 - 7286	63638 - 64963	28251 - 29129	32636 - 33628	4721 - 5926	Negative	7146 - 7623
Z4	Hosting contig	31	29	50	3	24	27	64	Negative	64
	Position in contig (bp)	31916 - 32290	50819 - 53962	24281 - 24706	200638 - 201963	37553 - 38430	28698 - 29690	14412 - 15617	Negative	12715 - 13192
Z5	Hosting contig	20	35	54	25	9	32	68	Negative	68
	Position in contig (bp)	53215 - 53589	4100 - 7243	6282 - 6707	10591 - 11916	29032 - 29910	32636 - 33628	14412 - 15617	Negative	12715 - 13192
Z6	Hosting contig	10	22	36	31	24	26	Negative	Negative	Negative
	Position in contig (bp)	74350 - 74724	13470 - 16613	6421 - 6840	32113 - 33438	26977 - 27855	22389 - 23381	Negative	Negative	Negative
Z7	Hosting contig	1	34	48	5	27	28	Negative	Negative	Negative
	Position in contig (bp)	344033 - 344407	26510 - 29653	6480 - 6899	16365 - 17690	26839 - 27717	22389 - 23381	Negative	Negative	Negative
Z8	Hosting contig	26	22	40	48	32	15	Negative	Negative	48
	Position in contig (bp)	45553 - 45927	6217 - 9360	6421 - 6846	1399 - 1821	35592 - 36470	1769 - 2761	Negative	Negative	13051 - 13528
Z9	Hosting contig	16	9	42	49	9	19	Negative	Negative	49
	Position in contig (bp)	19193 - 19567	6217 - 9360	24281 - 24706	24201 - 24623	98051 - 98929	1769 - 2761	Negative	Negative	12494 - 12971
Z10	Hosting contig	32	23	51	44	29	31	Negative	Negative	Negative
	Position in contig (bp)	30509 - 30883	62916 - 66059	23365 - 23784	8010 - 9335	36411 - 37289	22389 - 23381	Negative	Negative	Negative
Z11	Hosting contig	3	10	39	35	10	25	Negative	Negative	Negative
	Position in contig (bp)	271546 - 271920	13470 - 16613	23365 - 23784	8010 - 9335	106516 - 107394	27446 - 28438	Negative	Negative	Negative

Legend: bp: basepair